A Holistic Approach to Taxonomic Evaluation of Two Closely Related Endangered Freshwater Mussel Species, the Oyster Mussel (*Epioblasma capsaeformis*) and Tan Riffleshell (*Epioblasma florentina walkeri*) (Bivalvia: Unionidae)

by

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(ABSTRACT)

Primers for 10 polymorphic DNA microsatellite loci were developed and characterized for the endangered oyster mussel *Epioblasma capsaeformis* from the Clinch River, TN. Microsatellite loci also were amplified for individuals collected from the following additional populations or species: (1) *E. capsaeformis* from Duck River, TN; (2) *E. florentina walkeri* from Indian Creek, upper Clinch River, VA; (3) *E. florentina walkeri* from Big South Fork Cumberland River, TN; and (4) *E. torulosa rangiana* from Allegheny River, PA. Allelic diversity ranged from 9-20 alleles/locus, and averaged 13.6/locus for all 5 populations investigated. Average expected heterozygosity (H_E) per locus ranged from 0.78-0.92, and averaged 0.86.

A genetic characterization of extant populations of *E. capsaeformis* and *E. florentina walkeri* was conducted to assess taxonomic validity and to resolve conservation issues related to recovery planning. These mussel species exhibit pronounced phenotypic variation, and are difficult to characterize phylogenetically using DNA sequences. Monophyletic lineages, congruent with phenotypic variation among

species, were obtained only after extensive analysis of combined mitochondrial (1378 bp of *16S, cytochrome-b, ND1*) and nuclear (515 bp of *ITS-1*) DNA sequences. In contrast, analysis of variation at 10 hyper variable DNA microsatellite loci showed moderate to highly divergent populations based on F_{ST} values, which ranged from 0.12-0.39. Quantitative genetic variation was observed in fish host specificity, with transformation success of glochidia of *E. capsaeformis* significantly greater ($p<0.05$) on the greenside darter *Etheostoma blennioides*, and that of *E. f. walkeri* significantly greater ($p<0.05$) on the fantail darter *E. flabellare*. Lengths of glochidia differed significantly ($p<0.001$) between species, with sizes ranging from 241-272 µm. Underwater photographs of mantle-pads and micro-lures of female mussels documented fixed phenotypic variation between species. The texture and color of the mantle-pad of *E. capsaeformis* is smooth and bluish-white, while that of *E. f. walkeri* is pustuled and brown, with tan mottling. Based on extensive molecular, morphological, and life history data, a population of *E. capsaeformis* from the Duck River, TN is described and proposed as a separate species, and a population of *E. f. walkeri* from the upper Clinch River, VA is described and proposed as a separate subspecies.

Genetic management guidelines were developed to assess taxonomic status, genetic variation of donor-recipient populations targeted for augmentation, and field and laboratory protocols to maximize genetically effective population size, minimize genetic changes in captive-reared progeny, and prevent the release of juvenile mussels into non-native drainages. A pragmatic approach to species recovery is advocated; one that incorporates the principles of conservation genetics into breeding programs, but prioritizes the immediate demographic needs of critically endangered mussel species.

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CHAPTER 1

Development and Characterization of Microsatellite Loci in the Endangered Oyster Mussel *Epioblasma capsaeformis* (Bivalvia:Unionidae).

ABSTRACT

Primers for 10 polymorphic microsatellite loci were developed and characterized for the endangered oyster mussel *Epioblasma capsaeformis* from the Clinch River, TN. Microsatellite loci also were amplified for individuals collected from the following additional populations or species: (1) *E. capsaeformis* from Duck River, TN; (2) *E. florentina walkeri* from Indian Creek, upper Clinch River, VA; (3) *E. florentina walkeri* from Big South Fork Cumberland River, TN; and (4) *E. torulosa rangiana* from Allegheny River, PA. Amplification occurred for most loci in these closely-related endangered species or populations; therefore, a high level of flanking sequence similarity was inferred for this group of species and populations. Allelic diversity ranged from 9-20 alleles/locus, and averaged 13.6/locus for all 5 populations investigated. Average expected heterozygosity (H_E) per locus ranged from 0.78-0.92, and averaged 0.86. This study demonstrated the feasibility of using PCR primers to amplify microsatellite loci across freshwater mussel species, and that the loci investigated contained adequate variation to conduct population genetic studies.

INTRODUCTION

North America contains the greatest diversity of freshwater mussels in the world, including nearly 300 species. However, the mollusk superfamily (Unionoida) is the most imperiled group of animals in the United States, with 213 species (72%) listed as endangered, threatened, or of special concern (Williams et al. 1993; Neves et al. 1997). Most of the endangerment is caused by habitat loss or destruction affecting the natural structure and function of free-flowing rivers (Neves et al. 1997). Without immediate efforts to recover imperiled species in U.S. watersheds, the extinction of additional species is likely. To address the threat of species losses, biologists have developed techniques to propagate and culture endangered freshwater mussels for release of juveniles into rivers to augment or restore populations. However, recovery activities of many species will require genetic analysis of source and recipient populations to help manage species recovery (Miller and Kapuscinski 2003).

Microsatellites or simple sequence repeats (SSRs) are tandemly-repeated motifs of 1-6 base pairs and are found in the genomes of most organisms. Microsatellites are codominantly-inherited nuclear DNA markers that can be isolated in abundance for most species (Zane et al. 2002). Because microsatellites are highly variable, they can be very informative for inferring population genetic structure and dynamics (Balloux and Lugon-Moulin 2002, Zhang and Hewitt 2003). The popularity and usefulness of these markers in population genetic studies has spurred recent reviews on their advantages, putative functions, mutational mechanisms and homoplasy (see Balloux and Lugon-Moulin 2002, Estoup et al. 2002, Li et al. 2002, Zhang and Hewitt 2003). For endangered species, these

markers are particularly useful because they can be amplified using polymerase chain reaction (PCR). This method allows non-lethal sampling of organisms, because only a small amount of tissue is needed as starting material to isolate DNA and to observe amplification products. The goal of this study was to demonstrate the feasibility of using PCR primers to amplify microsatellite loci across freshwater mussel species and their utility in population genetic studies.

METHODS

Primer development and screening of allelic diversity

Samples of mantle tissue were collected from the following species and locations: (1) *Epioblasma capsaeformis* in the Clinch River, Hancock Co., TN, and Duck River, Maury, Co., TN; (2) *Epioblasma florentina walkeri*, in the upper Clinch River, Tazewell Co., VA, and Big South Fork Cumberland River, Scott County, TN; and (3) *Epioblasma torulosa rangiana* from the Allegheny River, Venango County, PA. A small piece of mantle tissue (20-30 mg) was collected non-lethally from 6-20 live mussels from each population (Naimo et al. 1998). Tissue was preserved in 95% ethanol and stored at -20°C prior to DNA extraction. Total genomic DNA was isolated from ~20 mg of fresh mantle tissue using the Purgene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA concentration was determined by flourescence assay (Hoefer TKO 1000 Flourometer, Hoefer Scientific Instruments, San Fransisco, CA), and its quality was visually inspected in a 0.8% agarose gel.

Microsatellite loci were isolated using a modified non-radioactive capture-hybridization method at the National Cancer Institute, Laboratory of Genomic Diversity, Frederick, MD (Refseth et al. 1997, Sarno et al. 2000). PCR amplification conditions followed those of Eackles and King (2002) and consisted of 100 ng of genomic DNA, 1x PCR buffer (Perkin Elmer), 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM each primer, and 1.0 U Ampli*Taq* DNA polymerase (Perkin-Elmer Applied Biosystems (ABI), Perkin-Elmer Corp., Foster City, CA, USA) in a total volume of 20 µL. PCR thermal cycling conditions were as follows: 94 °C for 2 min; followed by 35 cycles of 94 °C for 40 sec, 58 °C for 40 sec, and 72 °C for 1 min; followed by a final extension at 72 °C for 1 min; and a hold at 4 °C (Eackles and King 2002). Microsatellite loci initially were examined for polymorphism using a 7% polyacrylamide gel, followed by further analysis on an ABI3100 automated sequencer. The GENOTYPER (ABI) software determined allele size and POPGENE32 was used to determine heterozygosity values for each locus.

RESULTS AND DISCUSSION

The name of each locus, primer sequences, primer melting temperature, repeat motif of each locus, base-pair size range of alleles/locus, number of alleles/locus, observed heterozygosity (H_O), and expected heterozygosity (H_E) for a combined analysis of all 5 populations are reported in Table 1. Allelic diversity ranged from 9-20 alleles/locus, and averaged 13.6 alleles/locus, while average expected heterozygosity (H_E) per locus ranged from 0.7807-0.9215, and averaged 0.8566.

In this study, I have described the development and characteristics of 10 microsatellite primer pairs designed from DNA of the endangered oyster mussel *Epioblasma capsaeformis*, collected from the Clinch River, TN. These primer pairs have been used in a population genetic study (Chapter 2) of mussel species belonging to the genus *Epioblasma*, and represent only the second set of microsatellite primers to be published for freshwater mussels. The first set of microsatellite primers was developed by Eackles and King (2002) for the endangered pink mucket *Lampsilis abrupta.* These authors developed primer pairs to amplify 15 loci in *L. abrupta.* I recommend that both sets of microsatellite primers be screened using DNA from additional species in the freshwater mussel subfamily Lampsilinae to determine the applicability of these DNA loci for intraspecific population genetic studies.

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Table 1. Characteristics of 10 microsatellite DNA loci developed using DNA from the endangered oyster mussel (*Epioblasma capsaeformis*). The size range of alleles/locus, number of alleles/locus, observed heterozygosity (H_O), and expected heterozygosity (H_E) are from the analysis of the following species and populations: (1) *E. capsaeformis*, Clinch River, TN, (2) *E. capsaeformis*, Duck River, TN, (3) *E. florentina walkeri*, Indian Creek, upper Clinch River, VA, (4) *E. florentina walkeri*, Big South Fork Cumberland River, TN, and (5) *E. torulosa rangiana*, Allegheny River, PA.

Locus	Primer Sequence (5'-3')	Melting Temp. °C	Repeat Motif	Size Range (bp)	No. of Alleles	H_0	H_E
*Ecap*1	F: TGCATCATATGAAATGTGTTCG R: TCAGCATATTTCAAAGCAAACA	59.4 58.5	$(GA)_{17}(GT)_{17}$	146-190	20	0.5439	0.8856
*Ecap*2	F: ATCCTCAGGTTGGTGGTCAG R: TTTGAAAACCTTGTGATTGGC	60.0 60.0	$(GT)_{14}$	107-109	9	0.3276	0.7985
*Ecap*3	F: GGATGATGGGGAAAAGTAGATG R: TGCAACATTACCTGCCTTCA	59.7 60.3	$(GT)_{15}$	236-286	17	0.4615	0.8984
*Ecap*4	F: GTGCCCCAGTGCTAGACATT R: AGAACAAAACACCCGTGTCC	60.1 59.9	$(CA)_{10}$	98-120	10	0.4545	0.8264
*Ecap*5	F: TTTGAACACATTCGCCTCAG R: GAATTTGCCTCATCAGCCAC	59.8 60.6	$(GT)_{29}$	176-224	20	0.5472	0.9215
*Ecap*6	F: GATTTTGATTTTACGCTCCTGG R: GGTTAGTGTTAGGAGTGACCGG	60.0 59.9	$(GT)_{22}$	186-240	13	0.3091	0.7807
*Ecap*7	F: ACGAAAAATGTTGTCATCCATT R: GCCTAGACGACAAGCAAACC	58.4 59.9	$(CA)_{25}$	106-130	12	0.5870	0.8679
*Ecap*8	F: TGCAGACATCGTAGCGATATG	59.9	$(CA)_{15}$	127-159	11	0.3469	0.8849

	R: ATTTCCAGTTGCAAGTCTCATT	57.9					
*Ecap*9	F: AAAAAGGTGTGGAGAGAGATGG R: CCACTCTGCAGATATCGTATCG	59.6 59.8	$(GT)_{15}TT(GT)_2$	130-162	12	0.5472	0.8350
*Ecap*10	F: ACACTGCAGACATCGTAGCG R: TCACATACTTTGGGGACTTTCA	60.1 59.5	$(AC)_{20}$	115-143	12	0.7241	0.8675

CHAPTER 2

Taxonomic Evaluation of Two Closely Related Endangered Freshwater Mussel Species, the Oyster Mussel (*Epioblasma capsaeformis*) and Tan Riffleshell (*Epioblasma florentina walkeri*) (Bivalvia: Unionidae)

ABSTRACT

Species in the genus *Epioblasma* have specialized life history requirements and represent the most endangered genus of freshwater mussels (family Unionidae) in the world; 10 of the recognized 17 species already are extinct. A genetic characterization of extant populations of *E. capsaeformis* and *E. florentina walkeri* was conducted to assess taxonomic validity and to resolve conservation issues related to recovery planning. These mussel species exhibit pronounced phenotypic variation, but are difficult to characterize phylogenetically using DNA sequences. Monophyletic lineages, congruent with phenotypic variation among species, were obtained only after extensive analysis of combined mitochondrial (1378 bp of *16S, cytochrome-b, ND1*) and nuclear (515 bp of *ITS-1*) DNA sequences. In contrast, analysis of variation at 10 hyper variable DNA microsatellite loci showed moderate to highly diverged populations based on F_{ST} values, which ranged from 0.12-0.39. Quantitative genetic variation was observed in fish host specificity, with transformation success of glochidia of *E. capsaeformis* significantly greater ($p<0.05$) on greenside darter *Etheostoma blennioides*, and that of *E. f. walkeri* significantly greater ($p<0.05$) on fantail darter *E. flabellare*. Lengths of glochidia differed significantly ($p<0.001$) between species, with sizes ranging from 241-272 µm. Underwater photographs of mantle-pads and micro-lures of female mussels documented fixed phenotypic variation between species. The texture and color of the mantle-pad of *E. capsaeformis* is smooth and bluish-white, while that of *E. f. walkeri* is pustuled and brown, with tan mottling. Based on extensive molecular, morphological, and life history data, a population of *E. capsaeformis* from the Duck River, TN is described and proposed

as a separate species**,** and a population of *E. f. walkeri* from the upper Clinch River, VA is desribed and proposed as a distinct subspecies.

INTRODUCTION

North America contains the greatest diversity of freshwater mussels in the world, including nearly 300 species. However, this superfamily (Unionoida) of mollusks is the most imperiled group of animals in the United States, with 213 species (72%) listed as endangered, threatened, or of special concern (Williams et al. 1993; Neves et al. 1997, Neves 1999). Already, approximately 35 species, or 12% of the North American mussel fauna, have become extinct in the last 100 years, an extinction rate comparable to estimated faunal losses in tropical rainforests (Ricciardi and Rasmussen 1999). Most of the endangerment is caused by habitat loss and destruction due to impoundment, sedimentation, water pollution, dredging, and other anthropogenic factors that affect the natural structure and function of free-flowing rivers (Neves et al. 1997; Neves 1999). Without immediate efforts to recover this mussel fauna, the extinction of additional species is likely.

To help minimize future species losses, biologists are attempting to protect and restore natal rivers, propagate and release juvenile mussels for population augmentation and range expansion, and relocate adult mussels to more protected habitats. These recovery actions are desperately needed to help save many mussel species from extinction. As freshwater mussel conservation efforts increase in the 21st century, it is imperative that the most appropriate source populations are used to restore extirpated or augment waning populations in order to protect the genetic resources of species (Villella et al. 1998). Determining genetic relationships among donor and recipient populations will require phylogenetic and taxonomic analyses, especially between closely related

species or populations (Avise 2000). Genetic analyses should include multiple independent genotypic and phenotypic characters, to include traits expressed as molecular markers, anatomy, morphology and life history (Kat 1983, Davis 1983, Hillis 1987, Nei 1987, Avise 1994, Williams and Mulvey 1994, Hoeh et al. 2001). This need for a multi-character approach was best expressed by Mayden and Wood (1995): "*There is no inherent bias as to what types of attributes are informative for the discovery of descent of natural groups. Traits may include any of those detected in various types of data sets from morphology, physiology, ecology, genetics, behavior, etc. Hence, all discoverable and heritable types of traits are equally informative towards the discovery, description, and justification of naturally occurring biological diversity*".

Disagreements on mussel taxonomy and phylogenetics are persistent, stemming from an incomplete understanding of variation in morphology, anatomy, life history and genetics (Heard and Guckert 1971, Davis 1983, Stiven and Alderman 1992, Hoeh and Gordon 1996, Lydeard et al. 1996, Berg and Berg 2000). Traditional taxonomy has focused on morphological characters associated with shell and soft-part anatomy, such as shell shape, ray patterns and coloration of the periostracum, tooth structure, coloration of foot and gills, and number and placement of marsupial gills in gravid females (Lea 1834, Conrad 1853; Simpson 1896, 1900, 1914; Sterki 1898; 1903, Ortmann 1910, 1912; Heard and Guckert 1971). Early molecular genetic studies provided classifications based on allozyme variation in conjunction with morphological data (Davis 1981, 1983, 1984; Davis et al. 1981). Recent genetic analyses have compared nuclear and mitochondrial DNA sequences to infer phylogenetic relationships among various mussel taxa (Lydeard et al. 1996, Mulvey et al. 1997, Roe and Lydeard 1998a, King et al. 1999, Roe et al.

2001, Serb et al. 2003). Generally, classifications based on genetic data have supported traditional taxonomic analyses from morphology, especially at taxonomic designations above the species level (Davis 1981, 1984, Lydeard et al. 1996, Avise 2000). Disagreements between genetic and morphological data usually involve closely related or morphologically ambiguous species or populations (Hartl 2000).

Mussel species in the genus *Epioblasma* have specialized life history traits and represent the most endangered genus of freshwater mussels in world; already, 10 of the recognized 17 species are extinct. Species descriptions can be found in Johnson's (1978) monograph of the *Epioblasma* taxon, which discusses systematics and divides the group into five distinct subgenera. The primary species of interest in this study belong to the *Torulosa* subgenus, which includes *E. biemarginata, E. capsaeformis*, *E. florentina*, *E. phillipsi, E. propinqua*, *E. sampsoni*, *E. torulosa,* and *E. turgidula*. Based on morphological similarity, these species are considered to be closely related. Only *E. capsaeformis, E. florentina,* and *E. torulosa* have extant populations; the remaining five species are presumed extinct. Extant species are characterized by relatively small size (30-70 mm) and extreme sexual dimorphism of the male and female shell. The posterior end of the female shell of species in this subgenus is expanded and swollen, an area of the shell called the marsupial expansion. The marsupial expansion houses the swollen gills of gravid females and the mantle-pad, a modified portion of the mantle that functions to attract host fish. Freshwater mussels are unique among bivalves because their parasitic larvae (glochidia) must attach to a fish host to metamorphose to the juvenile stage. Because of these seemingly derived features, species in this genus are considered advanced members among the Unionidae (USFWS 1984).

The main objective of this study was to determine the taxonomic validity of two endangered mussel species, the oyster mussel *Epioblasma capsaeformis* (Lea 1834) and tan riffleshell *Epioblasma florentina walkeri* (Wilson and Clark 1914). The taxonomic uncertainty of the *E. capsaeformis* population in the Duck River, TN can be traced to Bryant Walker, an early 20th century malacologist. In an unpublished letter, he noted that the large marsupial expansion of the female shell for the population in the Duck River was different from that of individuals in the Clinch River, creating uncertainty in the taxonomic placement of this population (see letter in Appendix I). In the last two decades, field biologists also have questioned the taxonomic affinity of this population because of obvious differences in shell morphology and coloration of the mantle-pad (S. Ahlstedt, United States Geological Survey, Knoxville, TN, pers. comm.). However, a recent molecular genetic study by Buhay et al. (2002) using DNA sequences from the *ND1* region of the mitochondrial genome suggested that extant populations of *E. capsaeformis* and *E. f. walkeri* were the same species. Because of these taxonomic uncertainties and their potential effect on recovery plans and status of these two species (USFWS 1984, 2003), a comprehensive taxonomic analysis was needed.

METHODS

Type specimens and species distributions

Type specimens, shell material, and collection records for *Epioblasma capsaeformis* (Lea 1834), *Epioblasma florentina florentina* (Lea 1857) and *Epioblasma florentina walkeri* (Wilson and Clark 1914) were examined at the following museums: Academy of Natural Sciences of Philadelphia, Pennsylvania (ANSP); Carnegie Museum,

Pittsburgh, Pennsylvania (CM); Florida Museum of Natural History, Tallahassee, Florida (FLMNH); Museum of Comparative Zoology, Cambridge, Massachusetts (MCZ); Ohio State Museum, Columbus, Ohio (OSM); and National Museum of Natural History (USNM), Washington, D.C. Collection records from Johnson (1978), Parmalee and Bogan (1998), and U.S. Fish and Wildlife Service (1984, 2003) also were examined. Type specimens provided standard references for comparing shell material from various rivers, and the collection records were used to construct species distribution maps. Museum specimens of *Epioblasma capsaeformis* from the Duck River were identified using only the female shell. The female shell of this population was distinguishable from females of other populations of *Epioblasma capsaeformis* using the following criteria: (1) length of the base of the marsupial expansion of young individuals (3-5 y), ranging in size from 35-45 mm, is shorter than other females of *E. capsaeformis* of similar age and size, and (2) height of the marsupial expansion of adult females from the Duck River is greater than that of *E. capsaeformis* females in other rivers. Shell characters of males are not readily distinguishable among populations.

Sample collection

Samples of mantle tissue from live female mussels were collected from various river locations throughout the ranges of these species: (1) *Epioblasma capsaeformis*, Clinch River (CR) between Horton Ford (CRKM 321) and Swan Island (CRKM 277), Hancock Co., TN; (2) *E. capsaeformis*, Duck River (DR) at Lillard Mill (DRKM 287.7), Maury, Co., TN; (3) *E. florentina walkeri*, Indian Creek (IC), a tributary to the upper Clinch River (CRKM 518.2), Tazewell Co., VA; (4) *E. florentina walkeri*, Big South

Fork Cumberland River (BSF) from Station Camp Creek, Scott County, TN, downstream to Bear Creek, McCreary County, KY; and (5) *E. torulosa rangiana* from the Allegheny River (AR), Venango County, PA. Sample sizes were limited and commensurate with the endangered status of each species. A small piece of mantle tissue (20-30 mg) was collected non-lethally from 8-20 live mussels from each population (Naimo et al. 1998). Tissue was preserved in 95% ethanol and stored at -20°C prior to DNA extraction. Total genomic DNA was isolated from ~20 mg of fresh mantle tissue using the Purgene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA). DNA concentration was determined by flourescence assay (Hoefer TKO 1000 Flourometer, Hoefer Scientific Instruments, San Fransisco, CA), and its quality visually inspected in a 0.7% agarose gel.

DNA sequences

Sequences of three regions of mitochondrial DNA (mtDNA) and one region of nuclear DNA (nDNA) were amplified by polymerase chain reaction (PCR) in a PTC-200 Thermal Cycler (MJ Research) using primers and conditions reported in the following sources: (1) *16S*, ribosomal RNA (Lydeard et al. 1996) (2) *ND1*, first subunit of NADH dehydrogenase (Buhay et al. 2002, Serb et al. 2003), (3) *cytochrome-b* (Merritt et al. 1998, Bowen and Richardson 2000), and (4) *ITS-1* (nDNA), first internal transcribed spacer region between the *5.8S* and *18S* ribosomal DNA genes (King et al. 1999). Primer sequences are reported in Table 1.

The PCR amplification conditions for *16S* consisted of 25 ng of genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, and 1.5 U Ampli*Taq* DNA polymerase (Perkin-Elmer Applied Biosystems (ABI), Perkin-Elmer

Corp.; Foster City, CA, USA) in a total volume of 25 µL. PCR thermal cycling conditions were: 94 °C for 2 min; followed by 5 cycles of 94 °C for 20 sec, 42 °C for 20 sec, 72 °C for 1 min; followed by 35 cycles of: 94 °C for 20 sec, 45 °C for 20 sec, 72 °C for 1 min; a final extension at 72 °C for 2 min; and a final hold at 4 °C.

The PCR amplification conditions for *ND1* consisted of 100 ng of genomic DNA, 1x PCR buffer, 4.0 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 µM each primer, and 1.5 U Ampli*Taq* DNA polymerase in a total volume of 20 µL. PCR thermal cycling conditions were: 95 °C for 7 min; followed by 35 cycles of: 94 °C for 40 sec, 47 °C for 60 sec, 72 °C for 90 sec; a final extension at 72 °C for 2 min; and a final hold at 4 °C.

The PCR amplification conditions for *cytochrome-b* consisted of 25 ng of genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, and 1.5 U Ampli*Taq* DNA polymerase in a total volume of 20 µL. PCR thermal cycling conditions were: 94 °C for 2 min; followed by 40 cycles of: 94 °C for 1 min., 50 °C for 1 min., 72 °C for 2 min.; a final extension at 72 °C for 6 min; and a final hold at 4 °C.

The PCR amplification conditions for *ITS-1* consisted of 100 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM each primer, and 1.0 U Ampli*Taq* DNA polymerase in a total volume of 20 µL. PCR thermal cycling conditions were: 94 °C for 2 min; followed by 35 cycles of: 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 90 sec; and a final extension at 72 °C for 5 min; and a 4 °C hold.

All PCR products were sequenced with a Big Dye Terminator Cycle Sequencing kit with Ampli*Taq* DNA Polymerase (Applied Biosystems, Foster City, CA). Cycle sequence reactions were purified using a Qiagen DNA Purification kit (Qiagene,

Carlsbad, CA), and subjected to electrophoresis and sequencing using an Applied Biosystems 3100 automated sequencer.

DNA Microsatellites

Microsatellites are highly variable codominant nuclear markers, and often are informative for inferring genetic structure between closely related populations or species (Balloux and Lugon-Moulin 2002, Zhang and Hewitt 2003). Microsatellite loci and primers (Chapter 1) were isolated using a modified non-radioactive capture-hybridization method at the National Cancer Institute, Laboratory of Genomic Diversity, Frederick Maryland (Refseth et al. 1997, Sarno et al. 2000). The PCR amplification protocols follow Eackles and King (2002) and consisted of 100 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM each primer, and 1.0 U Ampli*Taq* DNA polymerase (Perkin-Elmer Applied Biosystems (ABI), Perkin-Elmer Corp.; Foster City, CA) in a total volume of 20 µL. PCR thermal cycling conditions were: 94 °C for 2 min; followed by 35 cycles of 94 °C for 40 sec, 58 °C for 40 sec, 72 °C for 1 min; followed by a final extension at 72 °C for 1 min; and a hold at 4 °C (Eackles and King 2002).

Shell morphology, length of glochidia and fecundity

The lengths of 20 glochidia of 5 female mussels from each population sample location were measured using an ocular micrometer and dissecting microscope. To assess differences in the marsupial swelling among populations, simple linear regression equations of total length (x-axis) of adult females versus the height (y-axis) of the

marsupial expansion were computed for each population. Fecundity was obtained by counting the number of glochidia from each of 6-10 females per population.

Mantle-pad phenotypes and micro-lures

Photographs of the mantle-pad and micro-lures of live female mussels were taken using a Nikonos V underwater camera with macro-lenses (28 or 35 mm) and Kodak 200 Ektachrome film. Female mussels were held in temperature-controlled water recirculating artificial streams with gravel-filled bottoms. This set-up allowed females to display their mantle-pad while photographs could be taken, and behavioral observations of micro-lure movements were made under controlled conditions. A hand-held video recorder was used to document micro-lure movements; digital recordings are stored at the Virginia Cooperative Fish and Wildlife Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Tech. Observations of micro-lure movements, and coloration and texture of the mantle-pad were made for *Epioblasma capsaeformis* (CR) (N>50), *E. capsaeformis* (DR) (N=12), *E. florentina walkeri* (IC) (N=12), *E. florentina walkeri* (BSF) (N=14), and *E. torulosa rangiana* (AR) (N=10).

Fish host specificity

Gravid females of *E. capsaeformis* and *E. florentina walkeri* were collected from the Clinch, Duck and Big South Fork Cumberland rivers in the above-mentioned reaches. No gravid females of *E. florentina walkeri* from Indian Creek were used for fish host analyses in this study because the population is very small and critically endangered. Fish host specificity was determined using 3 species of darters; greenside darter *Etheostoma*

blennioides, fantail darter *Etheostoma flabellare*, and redline darter *Etheostoma rufilineatum*, which had been identified as natural hosts for *E. capsaeformis* and *E. florentina walkeri* (Yeager and Saylor 1995, Rogers et al. 2001). Each fish species represents a particular darter subgenus (clade); *Etheostoma*, *Catonotus*, and *Nothonotus*, respectively (Jenkins and Burkhead 1993). Fish hosts were collected from the upper North Fork Holston River, near Saltville, Virginia, where no populations of *Epioblasma* spp. reside. Common and scientific names follow Robins et al. (1991) for fishes, and Turgeon et al. (1998) for mussels.

Methods for infesting fish with mussel glochidia followed those of Zale and Neves (1982). A plastic container 29 cm long, 19 cm wide, and 12 cm deep was filled with 1500 mL of water to hold fish (1 hr) during infestations; water was aerated and agitated with an airstone. Thirty fish each of *E. blennioides*, *E. flabellare*, and *E. rufilineatum* were infested together with glochidia from 2 female mussels added to the container. Three (N=3) infestations were conducted for each mussel population. After infestation, fish were separated by species and placed in 38 L aquaria at low densities, i.e., 5-10 per aquarium, to allow transformation of glochidia to juveniles. Contents from the bottoms of aquaria were siphoned every 2 to 3 d until juvenile mussels were collected, then siphoned every 1 to 2 d until juveniles completed excystment from fish.

DATA ANALYSIS

DNA sequences

Phylogenetic analysis was conducted primarily to determine genetic distinctiveness of DNA sequence haplotypes among remaining populations of

Epioblasma capsaeformis and *Epioblasma florentina walkeri*. Variable nucleotide sites were used to infer ancestral genealogical relationships among haplotypes and to provide statistical support for any inferred taxonomic groups. DNA sequences were edited and aligned using the program SEQUENCHER (version 3.0, Gene Codes Corporation, Ann Arbor, MI). Phylogenetic analyses were performed using PAUP*, version 4.0b2 (Sinauer Associates, Sunderland, MA). Genetic distances among haplotypes were estimated by Jukes-Cantor model of nucleotide substitution using PAUP* (Swofford 1998). Phylogenetic trees were constructed by maximum parsimony (MP) and minimum evolution (ME). The MP tree was constructed using a branch-and-bound search with ACCTRAN and TBR options; insertions and deletions were treated as missing data. The ME tree was constructed using Jukes-Cantor genetic distances and neighbour-joining algorithm followed by tree-bisection-reconnection branch swapping. Characters were treated as unordered and of equal weight for the analysis due to ingroup taxa being closely related (Nei and Kumar 2000). Bootstrap analyses (100 replicates) were conducted using the FAST step-wise addition option of PAUP* to assess support for the individual nodes of each phylogenetic tree (Felsenstein 1985). Sequences from mtDNA and nDNA were combined for analysis in a total evidence approach (Kluge 1989). This approach combines the sequence data from all four genes to enhance resolution of phylogenetic relationships; separate analyses of each gene sequence also were conducted. The in-group taxa were *Epioblasma capsaeformis* (CR), *E. capsaeformis* (DR), *E. florentina walkeri* (IC), *E. florentina walkeri* (BSF), and *E. torulosa rangiana* (AR). Sequences of the Cumberland combshell *Epioblasma brevidens* and snuffbox *Epioblasma triquetra* from the Clinch River were designated as out-groups.

DNA microsatellites

Amplification products containing microsatellite loci were initially examined for size polymorphism using a 7% polyacrylamide gel, followed by further analysis on an Applied Biosystems 3100 automated sequencer and GENOTYPER (ABI) software to determine allele size. Significance of any deviations from Hardy-Weinberg equilibrium (HWE) and genotypic linkage equilibrium (LE) were tested for each locus and each pair of loci per population, respectively. Variability across 10 microsatellite loci for each mussel population was quantified in terms of allele frequencies/locus, percentage of polymorphic loci, observed heterozygosity, average expected heterozygosity, mean number of alleles per locus, mean allele size range, maximum range, total number of alleles, number of unique alleles, population differentiation (F_{ST}) and gene flow (N_M); population genetic statistics were calculated using POPGENE32 software.

Shell morphology, length of glochidia and fecundity

Lengths of glochidia and estimated fecundity from population samples were compared using analysis of variance (ANOVA) (SAS Institute, 2001). To test for differences in total length versus the height of marsupial expansion of adult females, the slopes of fitted lines of each regression equation were compared among populations using the homogeneity of regression coefficients test statistic (SAS Institute 2001).

Fish host specificity

The degree of fish host specificity among populations was quantified as mean number of juvenile mussels transformed per fish for each darter species. Means were

transformed into mean percentages using the total number of juveniles transformed per infestation and compared using ANOVA. Mean percentages were normally distributed according to the Kolmogorov-Smirnov goodness-of-fit test. Arc-sine transformations were performed on proportion data prior to statistical analysis.

Designation of conservation units

The Phylogenetic Species Concept of Cracraft (1983), defined as the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent, was primarily used to define species in this study because of the current allopatric distributions of the in-group taxa. However, species-level designations were based on the presence of multiple diagnostic characters (fixed in every individual in a population) or unique characters to populations of each putative species. Designation of taxonomic status was based on examination of a suite of characters from the following data sets: (1) molecular genetics, (2) shell and mantle-pad morphology of female mussels, (3) length of glochidia (4) degree of fish host specificity, and (5) population distribution and other relevant ecological and life history information. The case for species-level designations was strengthened if concordance among multiple independent characters occurred within and between populations of each putative species (Avise 2000). Furthermore, the 'Biological Species Concept' of Mayr and Ashlock (1991), defined as a group of interbreeding natural populations that is reproductively isolated from other such groups, was applied indirectly. In populations that were recently sympatric, evidence for lack of gene flow (exchange of genes) was established using molecular genetic and morphological data. Populations were considered to deserve subspecies designation if

they met the definition of Frankham et al. (2002): namely, groupings of populations, within a species, that share a unique geographic range or habitat and are distinguishable from other subdivisions of the species by multiple, independent, genetically-based traits. Populations were considered 'Evolutionarily Significant Units' if they met the criteria of Waples (1991): a population (or group of populations) that (1) is substantially reproductively isolated from other conspecific population units, and (2) represents an important component in the evolutionary legacy of the species.

RESULTS

Type specimens

A total of 11 type specimens and 421 collection lots of shell material representing the in-group *Epioblasma* spp. were examined at the 6 museums (Appendix I and II). *Epioblasma capsaeformis* was described by Lea (1834) from specimens collected from the Cumberland River around Nashville, TN; type specimens examined were FLMNH64249 (Topotype), MCZ178568 (Idiotype), MCZ178570 (Holotype), and USNM84939 (Holotype). The shell material of *E. capsaeformis* from the Cumberland River drainage was nearly identical to those examined from the Tennessee River drainage; no discernable differences were observed. The marsupial expansion of the female shell was consistently colored green, the background color of the periostracum was yellowish-green, and the ray pattern not as fine and evenly spaced as that of the subspecies of *Epioblasma florentina*.

Epioblasma florentina florentina was described by Lea (1857) from specimens collected from the Tennessee River around Florence, AL. Type specimens examined

were ANSP56403 (Paratype), MCZ161897 (Paratype), MCZ161898 (Holotype), MCZ178569 (Cotype), MCZ178929 (Idiotype)], and USNM84948 (Holotype). *Epioblasma florentina walkeri* was described by Wilson and Clark (1914) from specimens collected from the East Fork Stones River in the Cumberland River drainage. The type specimen examined was MCZ293010 (Paralectotype). Shells of *E. f. florentina* and *E. f. walkeri* varied in color and size throughout their ranges in the Cumberland and Tennessee drainages, but no consistent differences between these two major drainages were observed. However, consistent variation in periostracum color, yellow vs. tan, between *Epioblasma florentina florentina* and *E. f. walkeri* was observed in shells collected in mainstem river channels vs headwaters, respectively. For example, in the Tennessee River drainage, shells collected from the upper Holston River watershed, Middle Fork and South Fork Holston rivers, are typical *E. f. walkeri*, but shells collected further downstream in the mainstem Holston River from Kingsport to Knoxville, TN are *E. f. florentina*. This longitudinal separation situation was mimicked in the Cumberland River drainage for the Stones River watershed. No shell character intergrades among *E. capsaeformis* (CR), *E. capsaeformis* (DR), *E. florentina*, and *E. torulosa* were observed.

Species distributions and sympatry

Presumably the Clinch River (CR) form of *Epioblasma capsaeformis* was once widely distributed throughout the Tennessee River system in Virginia, Tennessee and northern Alabama, and in the Cumberland River system in Kentucky and Tennessee; collectively known as the Cumberlandian Region (Figure 1). Currently, extant populations occur in the Clinch River, TN and VA, where it is common and reproducing,

and the Nolichucky River, TN, where it is rare. It may persist in the Powell River, TN, a major tributary to the Clinch, where it was collected live in the early 1990s. However, because no live individuals or relict shells of *E. capsaeformis* (CR) have been collected in almost 10 years, it is presumed extirpated. The population of *E. capsaeformis* (DR) is now restricted to the Duck River, TN; however, based on examination of shell material and collection records, it probably occurred historically in the Buffalo River, TN (Parmalee and Bogan 1998), and in the Tennessee River at Muscle Shoals and lower Shoal Creek near Florence, AL (Appendices I and II). No shell material and collection records were found for occurrences of this morphologically distinct population in the Cumberland River drainage.

Epioblasma florentina florentina and *E. florentina walkeri* also occurred throughout the Cumberlandian Region. Populations of the former subspecies now are extinct, whereas the latter subspecies occurs in the Cumberland River drainage in the Big South Fork Cumberland River, TN and KY, where it is uncommon but reproducing, and in the Tennessee River drainage in Indian Creek, a tributary of the upper Clinch River, VA, where it is uncommon but reproducing. Additional populations may persist in the Hiwassee River, Polk County, TN; Middle Fork Holston River, VA, where one male was collected live in 1997; and the Duck River, where a fresh dead female shell (Appendix I; OSM #29072) was collected in 1988. However, the viability of these populations is tenuous (Parmalee and Hughes 1994, Winston and Neves 1997, Henley et al. 2000, Jones et al. 2001, Rogers et al. 2001, S. Ahlstedt, U.S. Geological Survey, Knoxville, TN, pers. comm. 2003). The taxonomic identity of historical populations of *E. f. walkeri* in the Tennessee River drainage is unknown; however, based on geographic data and shell

material, it is likely that they were closely related to *E. f. walkeri* (IC) (Fig. 1). Various subspecies of *Epioblasma torulosa,* such *E. t .torulosa* and *E. t. gubernaculum*, were widespread historically in the Cumberlandian region; however, these populations are now extirpated. The only remaining viable populations of this species are those of *E. t. rangiana* in the Ohio River and Great Lakes drainages.

Populations of *E. capsaeformis* and subspecies of *E. florentina* and *E. torulosa* were sympatric historically in many rivers (Parmalee and Bogan 1998). These species were sympatric in parts of their ranges in the Cumberland River drainage in the following rivers: Cumberland River, KY; Big South Fork Cumberland River, KY; Beaver Creek, KY; Obey River, TN; Harpeth River, TN and Red River, TN; and in the Tennessee River drainage in following rivers: Clinch River, VA; Holston River, TN; Middle Fork Holston River, VA; South Fork Holston River, VA; French Broad River, NC; Little Tennessee River, TN; Hiwassee River, TN; Limestone Creek, AL; Elk River, TN; Richland Creek, TN; Hurricane Creek, AL; and Flint River, AL. For example, *E. capsaeformis* (CR) and *E. f. walkeri* (IC) were sympatric in the upper Clinch River near Richlands, VA. Populations of *E. capsaeformis* (DR) and *E. f. walkeri* occurred together in the Duck River, TN, while *E. capsaeformis*, *E. f. florentina*, *E. torulosa torulosa* all occurred in the Tennessee River at Muscle Shoals, AL. Populations of *E. torulosa* were widespread throughout the region and therefore commonly co-occurred with those of *E. capsaeformis* and *E. f. florentina* in many rivers. For example, *E. t. gubernaculum* and *E. capsaeformis* were sympatric historically throughout the Clinch River, and both species were abundant in the river at Pendleton Island, VA.

Phylogenetic analysis of DNA sequences

DNA sequence data from combined mitochondrial DNA regions of *16S* (468 bp), *cytochrome-b* (360 bp), and *ND1* (550 bp) and from the nuclear DNA region *ITS-1* (515 bp), revealed 156 variable sites, 70 of which were phylogenetically informative under maximum parsimony analysis (Table 4). Variable sites were most frequent in *cytochrome-b* (0.16), followed by *ND1* (0.11), *16S* (0.06) and *ITS-1* (0.02). Two indels occurred in *16S* and 12 in *ITS-1*. Even though indels were not included in the phylogenetic analysis of this study, they provided additional evidence for phylogenetic distinctiveness among DNA haplotypes, several of which were unique among populations. For example, in *ITS-1*, a thymine insert exists at bp 153 in *E. capsaeformis* (DR), and a deletion at bp 511 in *E. t. rangiana*; additional indels of interest also are present in the in-group at this sequence region. Observed nucleotide site variation defined 16 haplotypes in the mussel species examined (Table 4). The greatest number of observed DNA haplotypes was 6 in the population of *E. capsaeformis* (CR), with haplotypes *Ec*CR3 and *Ec*CR4 the most distinct. The least number of haplotypes observed was 1 (*Efw*BSF1) in the population of *E. f. walkeri* (BSF). All haplotypes of combined sequences were unique to each population. However, many of the haplotypes from the *16S* region were identical among taxa, to include, *Ec*CR1, *Ec*CR5, *Ec*CR6, *Efw*IC1, *Efw*IC2, *Efw*BSF1, and *Etr*AR1, indicating a low level of nucleotide variation at this region. Furthermore, none of the mtDNA or nDNA sequence regions showed any of the in-group taxa to be monophyletic when analyzed alone. Interestingly, DNA sequences of *ITS-1* did not differentiate *Epioblasma brevidens* from the in-group taxa. The Jukes-Cantor genetic distance values among DNA haplotypes are reported in Table 3. The in-

group taxa were characterized by low genetic distances ranging from 0.00053-0.00795, while the out-group taxa were characterized by greater distances ranging from 0.04844-0.05868.

The phylogenetic analysis of haplotypes using ME and MP produced nearly identical tree topologies, with the exception that haplotypes *E. capsaeformis* 2 (CR), *E. capsaeformis* 5 (CR), and *E. capsaeformis* 6 (CR) collapsed to form a polytomy in the MP tree (Figure 2). Only one ME tree was retained, which scored 0.09395. The MP analyses of the combined sequence data resulted in 31 equally parsimonious trees of 180 length (CI = 0.917, RI = 0.853). All putative species groups were recovered as monophyletic lineages in both the ME and MP trees and are well supported by bootstrap values (Figure 2). The tree topology places *E. capsaeformis* (DR) as basal to other members of the in-group. However, this node and other internal nodes are not well supported by bootstrap values and are collapsed in the strict consensus tree.

Population genetic analysis using DNA microsatellites

All 10 microsatellite loci were unambiguously scored across all 5 in-group mussel populations. Significant deviations from HWE (α=0.05), showing deficiency of heterozygotes, were observed in *E. capsaeformis* (CR) at *Ecap2-7*; in *E. capsaeformis* (DR) at *Ecap1, 5, 6, 8*; in *E. f. walkeri* (BSF) at *Ecap1, 4, 6, 8, 9*; in *E. f. walkeri* (IC) at *Ecap1, 6*; and in *E. t. rangiana* (AR) at *Ecap1, 10*. Significant deviations from LE (α=0.05) were observed at 15 pairs of alleles in *E. capsaeformis* (CR); 0 pairs in *E. capsaeformis* (DR); 1 in *E. f. walkeri* (BSF); 1 in *E. f. walkeri* (IC); and 1 in *E. t. rangiana* (AR). Deviations from HWE and LE could be the result of recent population

bottlenecks (see Discussion) or significant levels of close inbreeding, perhaps due to hermaphroditic reproduction, which is know to occur in populations of unionids (van der Schalie 1966, 1970).

Allele frequencies at each locus for each population are reported in Table 5 and variability across microsatellite loci in Table 6. All 10 microsatellite loci amplified for samples taken from *E. capsaeformis* (CR), *E. capsaeformis* (DR) and *E. f. walkeri* (BSF); however, *Ecap3* did not amplify in *E. f. walkeri* (IC) and *Ecap7* in *E. t. rangiana* (AR), despite repeated PCR trials using varying conditions. Lack of amplification at these loci may indicate the presence of null alleles; e.g., alleles are present in the genome but do not amplify because of nucleotide site mutations in the primer flanking regions (Culver et al. 2001, Zhang and Hewitt 2003). Overall genetic variation was greatest in *E. capsaeformis* (CR) and lower in *E. f. walkeri* (IC), *E. f. walkeri* (BSF) and *E. capsaeformis* (DR) as quantified by heterozygosity and total number of alleles. Genetic variation was moderate in *E. t. rangiana*; however, sample size was low (N=6) for this population. Therefore, it is likely that observed genetic variation under-represents what actually occurs in this population. The population of *E. t. rangiana* in the Allegheny River probably exceeds 1 million animals and occurs over many river kilometers (R. Villella, USGS-BRD, Shepherdstown, WV, personnel communication); therefore, it is possible that actual genetic variation is high. Differences in allele frequencies among populations were especially evident at *Ecap1*, *Ecap3*, *Ecap5*, *Ecap6*, and *Ecap8*. Fixed alleles occurred in *E. f. walkeri* (BSF) at *Ecap6* (allele 234) and *Ecap8* (allele 137).

Many unique alleles were observed in all 5 populations (Table 12) and at every locus (Table 5). On average, 48% of the alleles observed at a locus were unique to a

population. A noteworthy locus was *Ecap3*, where more than 76% of the alleles are unique to a species or a population. For example, 66% of the alleles observed in *E. capsaeformis* (CR) at this locus (alleles 268, 270, 274, 276, 282, 286) are unique, 55% in *E. f. walkeri* (BSF) (alleles 242, 256, and 262), and 75% in *E. t. rangiana* (alleles 236, 238, 250 and 252), and again *Ecap3* did not amplify in *E. f. walkeri* (IC). Interestingly, the pattern of allele frequencies and numbers of alleles are very different for *E. capsaeformis* (CR) compared to that of *E. f. walkeri* (IC). Both species occur in the Clinch River and were sympatric historically at the periphery of their respective ranges near Richlands, VA (CRKM 510-515). However, these populations overlap in allele frequency only by 25% based on shared alleles, and share only 21% of same sized alleles.

Overall, the level of allele fequency divergence among populations was high based on F_{ST} estimates. Pair-wise comparisons ranged from 0.1164-0.3864, with the most closely related taxa being *E. capsaeformis* (CR) and *E. capsaeformis* (DR), and the most distantly related being *E. f. walkeri* (IC) and *E. f. walkeri* (BSF) based on microsatellite data (Table 7). Values for F_{ST} can range from 0 (no differentiation) to 1 (complete differentiation); values between 0.05-0.15 generally reflect moderate to high levels of genetic divergence, values >0.15 reflect high levels and values >0.25 are considered great (Wright 1978, Hartl 2000, Balloux and Lugon-Moulin 2002). The F_{ST} measures the probability that two alleles at a locus in an individual are identical by descent (Wright 1978, Hartl 2000). Correspondingly, the levels of gene flow, N_m (number of migrants between populations/generation), between populations were low, ranging from 0.3971-1.8976 (Table 7). Values for N_m <1 indicate low levels of gene flow (0 = no gene flow); values >1 reflect progressively higher levels of gene flow. For clarification, the terms

"migrants" and "gene flow" should not be interpreted as the actual transmission of genes; in this context, these are statistical coefficients reflecting proportions of shared alleles.

Shell morphology, length of glochidia and fecundity

Regression equations derived from the height of the marsupial expansion of adult females varied significantly among populations ($p<0.001$, ANOVA). All pair-wise comparisons are significant (Table 8), except that of *E. capsaeformis* (DR) vs. *E. florentina walkeri* (BSF), and *E. florentina walkeri* (IC) vs. *E. florentina walkeri* (BSF). The maximum height of the marsupial expansion of female mussels was greatest in *E. capsaeformis* (DR), followed by *E. florentina walkeri* (BSF), *E. capsaeformis* (CR), and *E. florentina walkeri* (IC) (Table 8).

Mean lengths of glochidia of female mussels varied among populations and species, and were significantly different ($p<0.001$); all pair-wise comparisons were significant (Table 9). Glochidia of *E. florentina walkeri* (IC) were the longest, averaging 271.9 µm, while those of *E. t. rangiana* (AR) were shortest at 241.3 µm. Significant differences also were observed in the variances. For example, length varied considerably (SE = 11.5) for glochidia of *E. f. walkeri* (BSF); ranging in length from 231-282 µm, and many appeared somewhat asymmetrical (Table 9). In contrast, glochidia of *E. capsaeformis* (CR) appeared very symmetrical and varied little (SE = 5.2).

A mean fecundity of 18,757 glochidia/female mussel ($p<0.05$) was significantly greater for *E. capsaeformis* (DR), which ranged from 6,668-38,716 (Table 10). No significant differences were observed in fecundity among *E. capsaeformis* (CR), *E. f. walkeri* (IC), and *E. f. walkeri* (BSF).

Mantle-pad phenotypes and micro-lures

The mantle-pads and micro-lures of female mussels were distinct for each species and varied little among populations, with the exception of subtle color differences in the pads. The color of the mantle-pad of female *E. capsaeformis* (CR) is bluish-white (Figure 3A+B), which has been reported previously (Ortmann 1924, USFWS 2003). The surface texture of the pad is smooth, and the dorsal margins of both left and right mantle-pads are black; i.e., the margin of the pad is colored black, forming a ~2-3 mm band. This black band cannot be seen while the female is displaying her mantle-pad, but can be observed by gently prying the valves apart and separating the right and left mantle-pads. In Figure 3B, a horizontal line can be seen running vertical in the middle of the mantle-pad. This line demarcates where the dorsal margins of the left and right pads meet and press together to form the entire mantle-pad display; just below this line is the location of the black bands.

Attached to the posterior end of each mantle-pad is a micro-lure that seemingly mimics the cerci of insect larvae (Figure 3B). The color of the micro-lures was bluish to light gray with black fringes near the tips. The lures are modified papillae of the incurrent aperture (siphon), located on the posterior region of the mantle-pad. In Figure 3B, the two micro-lures can be seen attached between the brown-colored incurrent aperture (above) and the bluish-white colored mantle-pad (below). In *E. capsaeformis* (CR), this region is not invaginated, but rather the attachment points of the micro-lures can be seen on the mantle-pad when the female is displaying. The movement of the micro-lures is also distinct in *E. capsaeformis* (CR). The micro-lure attached to the left mantle-pad rotates clockwise in a circular pattern, whereas the micro-lure in the right pad rotates

counterclockwise, and both are prominently displayed together. The micro-lures of all the in-group *Epioblasma* spp. in this study, except that of *Epioblasma torulosa rangiana* (see species account below), moved in a rhythmical manner, indicating that it is an innervated structure. This is the first description of the presence, morphology and movement of micro-lures in mussel species of the genus *Epioblasma*.

The mantle-pad of female *E. capsaeformis* from the Duck River (DR) is dark-purple to slate-gray (Figure 3C) (Ortmann 1924). The surface texture of the pad is spongy, and the dorsal margins of both left and right mantle-pads are dark-purple to black. The color of the micro-lure was tan. In this population, the posterior portion of the mantle-pad is invaginated where it meets the incurrent aperture; therefore, the attachment points of the micro-lures were concealed and could not be seen when the female was displaying. The micro-lure protrudes out of this invaginated region as if to mimic the larva of a caddis-fly. The movement of the micro-lures of these females is different than that of *E. capsaeformis* (CR). Only one micro-lure is prominently displayed, and it moved in a side-to-side sweeping motion.

The mantle-pad of female *E. f. walkeri* from Indian Creek (IC) is gray with a mottled black background (Figure 3E+F). The surface texture of the pad is pustuled, and the dorsal margins of both left and right mantle-pads were tan. The color of the micro-lures was dark brown to black, and the posterior portion of the mantle-pad was invaginated. Only one micro-lure was prominently displayed, and it moved in a side-to-side sweeping motion.

The mantle-pad of female *E. f. walkeri* in the Big South Fork Cumberland River (BSF) is brown with a mottled tan background (Figure 3G-H). The surface texture of the

pad is pustuled, but the pustules tend to be finer and pointed when compared to those of *E. f. walkeri* (IC). However, the dorsal margins of both left and right mantle-pads, also is tan. The color of the micro-lures was brown, and the posterior portion of the mantle-pad was invaginated. Only one micro-lure was prominently displayed, and it moved in a side-to-side sweeping motion. However, the micro-lure was more bulbous, and the side-to-side movement tended to be slower than that of *E. f. walkeri* (IC).

The mantle-pad of female *E. torulosa rangiana* in the Allegheny River is white, and the surface texture of the pad is smooth (see photographs in Appendix II, Fig. 9). The dorsal margins of both left and right mantle-pads also are white. This species apparently does not have a true micro-lure. Field and laboratory observations of 10 females indicate that this species only has a vestigial "nub" of tissue where the micro-lure is located in the other ingroup species. Thus, no protrusion or movement of a micro-lure was observed for females of this species. The posterior portion of the mantle-pad is not invaginated and appears incomplete; i.e., a gap is present between the upper portion of the mantle-pad and the incurrent aperture. The shells of this subspecies and others of *Epioblasma torulosa* are relatively thick for its size and typically contain 1 or 2 knobs located on the center of the shell. In addition, the female shell does not have denticulations along the margin of the marsupial expansion, as determined by examination of numerous shells in museum collections. In contrast, the shells of the other in-group taxa are thin, have denticulations, and do not have knobs on the mid-portion of the shell. Because females of *E. torulosa rangiana* lack a micro-lure, have a thicker shell with knobs, and do not have denticulations on the shell, which perhaps is indicative of this species being a more primitive member of the *torulosa* clade.

Fish host specificity

Fish host specificity varied significantly ($p<0.001$) among *E. capsaeformis* (CR), *E. capsaeformis* (DR) and *E. f. walkeri* (BSF) (Table 11). Glochidia of *E. capsaeformis* (CR) transformed in greatest numbers on the greenside darter *Etheostoma blennioides*, which produced an average of 44% of the juveniles obtained from the three host fish species. Glochidia of *E. capsaeformis* (DR) and *E. f. walkeri* (BSF) transformed in greatest numbers on the fantail darter *Etheostoma flabellare*, which produced an average of 59% and 73% of the juveniles, respectively. In contrast both of these latter mussel species transformed infrequently on *E. blennioides*. These findings corroborate those of Rogers et al. (2001), who also found *E. flabellare* to be the most suitable host for *E. f. walkeri* in Indian Creek.

DISCUSSION

Type specimens and species distributions

Examination of type specimens and shell material from various rivers yielded several conclusions about geographic variation in shell morphology among in-group taxa. First, there was an overall lack of clinal and geographic variation in periostracum color and ray pattern in shells of *E. capsaeformis* throughout the Cumberlandian Region, an observation supporting the view that these characters are heritable. It is unlikely that these shell characters could be maintained over a wide geographic range, of varying environmental conditions, without a strong genetic basis. Second, variation in periostracum color, yellow vs. tan, between *E. florentina florentina* and *E. f. walkeri* may represent clinal variation, large river vs. headwater forms, subspecies, or separate species.

A.E. Ortmann considered the two forms as merely clinal variants of *E. florentina*, a claim supported by his observation that the big river form appeared to grade into the headwater form as one progressed upstream (Ortmann 1918, 1924, and 1925). However, it is unclear whether these color variants of *E. florentina* merely represent clinal variation, for the following reasons: (1) large distances of seemingly unoccupied habitat commonly occurred between mainstem and headwater populations (e.g., Clinch and Holston rivers >200 RKM), and ecological conditions between mainstem and headwater locations are substantial; (2) a transitional series of shells representing a continuously distributed population of this species from mainstem to headwaters does not exist; and (3) because most populations are extirpated, additional genetic, morphological and life history data are absent and unobtainable. For example, specimens of *E. f. florentina* collected from the lower Clinch River near its mouth are short (~30-35 mm), solid, and thick-shelled, and do not resemble the larger-sized headwater form *E. f. walkeri* (IC) (Appendix II, Fig. 6). Therefore, because these subspecies were known to occur in distinct geographic regions and habitats, we believe *E. florentina* minimally was a polytypic species complex and best categorized by a trinomial system of nomenclature. Third, the lack of shell intergrades among historically sympatric populations of *E. capsaeformis*, *E. florentina*, and *E. torulosa* supports their recognition as separate species.

For many mussel species, their distributions throughout the Cumberlandian Region are not continuous or random, but rather occur in discrete and predictable river reaches. Species endemic to the region, such as most species in the genus *Epioblasma*, primarily occurred in the following four geographic areas: (1) middle and upper Cumberland River drainage, (2) middle and upper Duck River, (3) middle Tennessee

River drainage from Bear Creek upstream to the Paint Rock River, and (4) upper Tennessee River drainage, which is from Walden Gorge near Chattanooga to upstream (Wilson and Clark 1914, Ortmann 1918, 1924, and 1925, Neel and Allen 1964). The lower reaches of the Tennessee and Cumberland rivers and Walden Gorge appear to be barriers to dispersal for some mussel species, especially those with limited dispersal abilities, e.g., species that use darters, minnows and sculpins as their hosts. The in-group taxa of *Epioblasma* spp. exhibited genetic variation concordant with these geographic areas. Therefore, it is likely that patterns of genetic variation of mussel species endemic to this region are partitioned accordingly, and should be considered in the conservation management plans of each species.

Phylogenetic analysis of DNA sequences

Phylogenetic analysis of the combined mtDNA and nDNA sequences revealed that the in-group taxa are closely related but distinct. From the suite of diagnostic molecular and phenotypic characters, it is apparent that the combined sequences were unique to their respective populations, suggesting absence of gene flow among populations, and concordant with observed phenotypic variation (Table 12). The tree topology (Figure 2) places *E. capsaeformis* (DR) as basal to the other ingroup taxa, and *E. t. rangiana* (AR), *E. f. walkeri* (IC) and *E. f. walkeri* (BSF) together in the same group. However, the phylogenetic relationships implied by this topology likely are incorrect. The absence of a micro-lure and thicker nodulus shell of *E. t. rangiana* suggests that this species is the more primitive, basal member of the group. Lack of agreement between a species-tree and gene-tree is not uncommon for closely related species (Avise 2000, Hartl

2000, Nei and Kumar 2000), and discordance between the two is possible under various scenarios (Sites and Crandall 1997 and references therein). For example, species can hybridize with one another, resulting in mtDNA haplotypes that are introgressed and shared between species, which otherwise are distinct morphologically and genetically at nuclear loci (Sites and Crandall 1997).

DNA sequence divergence of 3-6% is typical of inter-specific comparisons in unionids (Lydeard et al. 1996, Roe and Lydeard 1998a, Roe et al. 2001, Serb et al. 2003). However for recently diverged taxa, estimates of genetic distance can be low, and are dependent on the amount of time elapsed since reproductive or geographic isolation of populations (Nei and Kumar 2000). "If an average rate of mtDNA evolution of 2% per million years is assumed, then 330 base pair (bp) substitutions would be expected per million years in a 16,500 bp long mtDNA molecule. If the entire molecule is surveyed, this corresponds to 3.3 substitutions per 10,000 yr. Therefore, even with complete isolation, populations that have colonized habitats since the end of the Pleistocene will show little divergence" (Billington 2003). For example, Pupfishes (*Cyprinodon* spp.) living in Death Valley in southeastern California and southwestern Nevada are distinct morphologically and behaviorally, but exhibit low levels of genetic divergence at mtDNA markers; e.g., 0.32-0.49% between *C. diabolis* and *C. nevadensis* (Echelle and Dowling 1992). In an earlier study, Miller (1981) suggested that *C. diabolis* is perhaps only 10,000-20,000 years old. Other excellent examples of closely related species that have been difficult to characterize genetically include African cichlids in Lake Malawi, which are morphologically and behaviorally distinct, but contain nearly identical mtDNA

haplotypes (Stauffer et al. 1995), finches in the Galapagos (Nei 1987), and sturgeons in the Mobile River basin (Avise 2000).

A limited molecular survey of the mtDNA genome may not contain sufficient genetic variation to discriminate "species-level differences" among recently-diverged taxa, such as certain groups of freshwater fishes and mussels. Therefore, several technical aspects of our phylogenetic analysis are worthy of further discussion. First, coalescence of the in-group taxa into their respective monophyletic lineages only was achieved by sequencing ~1900 bp of DNA sequences. As shown, use of only one DNA sequence region was insufficient to discriminate among in-group species with high statistical support. Failure to sequence an adequate number of nucleotide base-pairs can result in an unresolved paraphyletic tree, such as in Buhay et al. (2002). Thus, for closely-related mussel species, a robust analysis is necessary to discern phylogenetic relationships and to reduce the level of paraphyly among populations. For example, haplotypes *Ec*CR3 and *Ec*CR4 were difficult to render monophyletic within the *E. capsaeformis* (CR) clade, which was achieved only after sequencing the full 1900 bp. In many cases, it is likely that the level of paraphyly is an artifact of how much of the genome is investigated. Second, certain DNA sequence regions contained more genetic variation than others in this study, such as *cytochrome-b* and *ND1*. This finding highlights the need to better understand DNA sequence variation among unionids, especially in the mtDNA genome. Analysis of the complete mtDNA sequence regions of *cytochrome-b* and *ND1* and other regions with potentially higher rates of nucleotide substitution, such as the control region, is technically feasible and should be targeted in future analyses (see Serb and Lydeard 2003). Combining mtDNA sequences of up to 1000-2000 bp from these regions should

provide sufficient polymorphic nucleotides to make stronger phylogenetic inferences among closely related matrilineal lineages. At least three or four diagnostic characters, uncompromised by homoplasy, are recommended for robust statistical recognition of a putative gene-tree clade in most phylogenetic appraisals (Avise 2000).

The resolving power of modern molecular DNA techniques allows for discrimination between individuals, demes, populations, species and higher taxonomic categories. Many geographically and demographically independent populations, and even groups of individuals within populations, potentially can be rendered monophyletic or identifiable with sufficient molecular markers. Therefore, the criteria of monophyly or diagnosable units, currently used to define phylogenetic species, are relative and simplistic concepts that should be interpreted cautiously. Sequence data are useful for evaluating phylogenetic relationships and evolutionary states of populations (Zhang and Hewitt 2003); however, for delineating closely-related species, additional analyses of multi-locus nuclear markers, such as microsatellites, are usually necessary. Most taxonomic questions typically involve closely-related species or populations; thus, traditional genetic analyses that employ large sample sizes and a suite of co-dominant nuclear markers to assess levels of gene-flow between populations are strongly recommended.

DNA microsatellites and population history

In contrast to the DNA sequence loci, hyper-variable DNA microsatellites portrayed highly diverged mussel populations. Data obtained from DNA microsatellites provided additional evidence to demonstrate how genetically distinct the in-group taxa

actually are from each other. The presence of unique alleles, fixed alleles, potential null alleles, and high F_{ST} values corroborated the results of other data sets used in this study; i.e., that actual gene-flow between populations is infrequent to non-existent. These data support the inference that the in-group populations are reproductively isolated from each other. Interestingly, the results of this study suggest that these once sympatric populations, such as *E. capsaeformis* and *E. t. gubernaculum* in the Clinch River, were nearly indistinguishable at certain DNA sequence regions (e.g., the *16S* region of the mtDNA genome), but divergent at a suite of nuclear (DNA microsatellites) and quantitative loci (e.g., shell morphology, length of glochidia and fish host specificity). Of course, allele frequency differentiation could have arisen in part by random genetic drift and varying selection regimes during geographic isolation of populations. With the exception of *E. capsaeformis* (CR) and *E. f. walkeri* (IC) in the Clinch River, study populations are demographically separated by several hundred to more than a thousand river kilometers. Therefore, only reproductive isolation adequately explains how variation at both mtDNA and nDNA molecular markers, and complex morphological and life history traits, were maintained by these once-sympatric populations throughout various rivers in the Cumberlandian Region.

Analysis of allele frequency variation at microsatellite loci also provided insights into the different population histories of each mussel species. For example, the lower observed heterozygosities and average number of alleles/locus for *E. capsaeformis* (DR), *E. f. walkeri* (IC) and *E. f. walkeri* (BSF) suggest that these populations were bottle-necked (Table 6). Recent impacts to populations resulting in severe population declines include hydropower operations on the Duck River, toxic spills in the upper Clinch River,

and coal, gas and oil exploration in the Big South Fork Cumberland River (S. Ahlstedt, USGS pers. com., Jones et al. 2001). In contrast, the population of *E. capsaeformis* (CR) in the lower Clinch River is large, relatively undisturbed, and has not been bottle-necked by anthropogenic factors. Thus, reduction of allelic diversity through anthropogenic impacts may help to explain the low overlap in alleles and high F_{ST} values among some of the studied populations.

Shell morphology, length of glochidia and fecundity

The greater height of the marsupial expansion of adult female *E. capsaeformis* from the Duck River appears to be a quantitative genetic difference between this population and that of *E. capsaeformis* in the Clinch River, and other in-group species. Even though the slope of the regression equation of *E. capsaeformis* (DR) was not significantly different from that of *E. florentina walkeri* (BSF), the maximum height of the marsupial expansion of the former species was much greater than that of the latter species (Table 8). Furthermore, even large females (>50 mm) of *E. capsaeformis* collected from the Tennessee River at Muscle Shoals, AL, and the Holston River below Rogersville, TN, presumably productive reaches of river, do not have the extremely protruded marsupial expansion of similar-sized female *E. capsaeformis* (DR) (Appendix II, Fig. 4). Therefore, increased shell length alone does not account for the increased height of the marsupial expansion of female *E. capsaeformis* (DR). In general, the marsupial expansion of the female shell of *E. capsaeformis* (DR) is higher and more protruded as females grow in size, compared to females of other in-group *Epioblasma* spp.

Variation in mean lengths of glochidia of female mussels also appears to be a significant quantitative genetic difference among in-group taxa. It is unlikely that developmental characteristics of glochidia are influenced by environmental factors.

No fecundity estimates of the largest specimens of *E. capsaeformis* (DR) were included in the 6 samples taken for this species (Table 10). Based on direct observations of the gravid condition of large females, such as in Figure 3C, it is likely that maximum fecundity for this species exceeds 50,000 glochidia. Greater fecundity in this species is probably related to its greater maximum size (>70 mm), relative to the other ingroup species. Maximum fecundity in *E. capsaeformis* (CR), *E. f. walkeri* (BSF) and *E. f. walkeri* (IC) is probably around 20,000 glochidia, which already has been reported for *E. f. walkeri* in Indian Creek (Rogers et al. 2001).

Mantle-pad phenotypes and micro-lures

Discrete phenotypic variation in color and texture of mantle-pads and movement of micro-lures likely represents classical Mendelian trait differences among in-group taxa. Historic populations of the in-group taxa likely contained more variation in mantle-pad coloration than exists today. A.E. Ortmann (1924, 1925) reported that the mantle-pad of *E. capsaeformis* from the Duck River and middle reaches of the Tennessee River was dark grey to black, whereas the color of the mantle-pad of this species in rivers throughout the upper Tennessee River drainage was white to blue. Interestingly, based on hand-written notes presumably by him on the inside of female shells deposited in the Carnegie Museum, at least a few individuals of *E. capsaeformis* from the lower North Fork Holston River, VA had a dark-colored mantle-pad. However, his notes indicated

that the vast majority of females from this river had the bluish-white pad. Thus, females with the dark-colored mantle-pad apparently were rare in this river and perhaps other rivers in the upper Tennessee River drainage at the time of his surveys (early 1900's). Because this particular mantle-pad color variant of *E. capsaeformis* has been extirpated from the middle and upper reaches of the Tennessee River drainage, we can only speculate on its taxonomic significance. Historically, color seemingly was a polymorphic character controlled by alleles occurring at different frequencies among populations. If so, the near fixation of different colored mantle-pad phenotypes in the middle (gray-black) and upper reaches (bluish-white) of the Tennessee River drainage suggests adaptively significant directional selection. Furthermore, according to notes written on shells of *E. torulosa gubernaculum* from the Clinch and Holston rivers, this subspecies possessed a dark gray to blackish mantle-pad, in contrast to the white mantle-pad of *E. torulosa rangiana* in the Great Lakes and Ohio River drainages. These additional observations on mantle-pad color variation for these two species were not reported in Ortmann's (1918) mussel survey paper of the upper Tennessee River drainage.

The coloration of the mantle-pad of *Epioblasma capsaeformis* that inhabited the Cumberland River system is unknown. This species now is extirpated from the drainage, and the color of the mantle-pad was not described by Wilson and Clark (1914) during their survey of the river basin. However, local residents that worked on the Cumberland River prior to its impoundment reported seeing the display of the bluish-white mantle-pad presumably of this species on the river bottom (B. Butler, USFWS, Asheville, NC, pers. comm.).

Fish host specificity

The different colored mantle-pads of the in-group taxa may be adaptively significant and indicate how species persist in certain environments and attract fish hosts. The cryptically-colored mantle-pads of *E. f. walkeri* (IC) and *E. f. walkeri* (BSF) appear better adapted to head-water habitats, where displaying females are camouflaged in shallow, small-stream habitats. In contrast, females of *E. capsaeformis* (CR) with their bright bluish-white mantle-pads may be more vulnerable to predation in headwaters, and therefore less likely to persist in small stream habitats. This latter species seems better adapted to larger rivers where increased depth and width can provide protection to displaying females.

The bluish-white pad of *E. capsaeformis* (CR) may be better at attracting brightly-colored darters, such as *Etheostoma blennioides* and *E. rufilineatum*. These darters, as well as other-closely related fish species belonging to the subgenera *Etheostoma* and *Nothonotus* respectively, co-occur in abundance with *E. capsaeformis* (CR) in the Clinch River. In the spring of the year, male darters become brightly colored to serve as a mating cue for females. The bright mantle-pad of *E. capsaeformis* (CR) may attract these darters and illicit reproductive responses. We speculate that color of the mantle-pad acts to initially attract a fish host to the displaying female mussel, and then, the movement of the micro-lure to resemble insect larvae brings the fish into close contact with the female mussel and her glochidia. A unique behavior of mussel species in the *Torulosa* subgenus is that displaying females quickly close their shells when touched. This snapping behavior can actually capture host fish, similar to how a Venus flytrap captures insects. Such behavior likely facilitates infestation of glochidia on the fish host. We have

collected gravid *E. capsaeformis* (CR) with darters trapped between the shells, inside the mantle-pad cavity. In addition, biologists have observed darters probing inside the mantle pad of *E. capsaeformis* (CR) and seeing the female mussel snapshut and capture a host fish (T. Brady, USFWS, Genoa, Wisconsin, pers. comm.).

One of the main fish hosts for *E. f. walkeri* (BSF) and *E. f. walkeri* (IC) is *E. flabellare*, which also prefers headwater environments. Spawning males of *E. flabellare* become darkly colored, as well as other fish species in the subgenus *Catonotus*, and may be attracted to similar colors, such as the darker pads of *E. capsaeformis* (DR), *E. f. walkeri* (BSF) and *E. f. walkeri* (IC). Resident hosts for *E. capsaeformis* (DR) are unknown because life history studies have not been conducted for this population. However, the fish-host specificity data developed in this study suggest that darter species in the subgenus *Catonotus* are candidates. Incidentally, the Duck River fish fauna is one of the richest in the Southeast and may contain many additional hosts for *E. capsaeformis* (DR).

The fish host specificity data support the hypothesis that certain species of darters are quantitatively better hosts for particular in-group mussel taxa (Table 10). These results indicate that fish host specificity is a major source of quantitative genetic variation for freshwater mussels. One weakness of this study is that fish host tests were conducted only using fish species from one river drainage. Additional trials using sympatric fish species and stocks could reveal additional quantitative genetic relationships. Such variation is more likely to be fitness-related and may serve to isolate mussel populations geographically, ecologically and ultimately, reproductively. Other potential sources of

quantitative genetic variation in need of study include spawning temperatures and glochidial release periods.

The major histo-compatibility complex (MHC) of fishes has been implicated in controlling fish host specificity of glochidia and parasite resistance in general (Lefevre and Curtis 1910, Howard and Anson 1922, Hedrick and Kim 2000). However, it is not known to what degree mussel glochidia can recognize suitable fish hosts; i.e., whether the fish is rejecting glochidia or the reverse is true. Variation at MHC genes may serve as an important source of adaptively significant molecular markers in freshwater mussels, and already is being used in freshwater fish (see Hedrick et al. 2001). Other sources of adaptively significant molecular markers used in mollusks include gamete recognition proteins (lysin) in Pacific abalone and blue mussels (Swanson and Vacquier 1995, Vacquier 1998, Riginos and McDonald 2003). Data gathered on these protein markers could elucidate how communities of co-occurring mussel species maintain reproductive isolation.

Designation of conservation units and management implications

The results of this study provide evidence of a new species and subspecies of freshwater mussel in the Tennessee River drainage. The population of *E. capsaeformis* in the Duck River should be recognized as a separate species from *E. capsaeformis* in the Clinch River because of the following differences: (1) coloration and texture of the mantle-pad, (2) greater height of marsupial expansion of female shell, (3) smaller size of glochidia, (4) differing fish host specificity, (5) greater fecundity, and (6) behavioral differences in the movement of micro-lures. Furthermore, the populations of *E.*

capsaeformis (DR) and *E. capsaeformis* (CR) were not closely related genealogically relative to the other in-group taxa, and lacked a suite of shared characters to unite them together as a species. We propose that the common and scientific names of *E. capsaeformis* in the Duck River be the darter-snapper pearlymussel *Epioblasma ahlstedti*; however, these names will be officially designated in peer reviewed publications. The scientific name is given in honor of biologist Stephen A. Ahlstedt, U.S. Geological Survey, Knoxville, TN, who has dedicated 30 years of service to freshwater mussel conservation in the United States.

The population of *E. f. walkeri* (IC) in Indian Creek should be designated as a separate subspecies from *E .f. walkeri* (BSF) because of genetic distinctiveness and allopatric geographic ranges. Differences in coloration of mantle-pads, size of glochidia, molecular markers, and ranges in Tennessee River and Cumberland River drainages, respectively, allowed us to reliably identify and classify each population as a taxonomically separate entity. These populations are not deserving of separate species designation because they shared the following traits: (1) honey-yellow to brown-colored periostracum, (2) similar fish host specificity, (3) pustuled mantle-pads, and (4) preference for headwater stream habitats. In addition, the two populations had different, but similar-sized glochidia and were closely related genealogically based on the molecular phylogeny. We propose that the population of *E. f. walkeri* (IC) in the upper Clinch, VA, be named the golden riffleshell *Epioblasma florentina aureola*.

Because in-group populations appeared geographically, demographically, and genetically independent, the criterion of reproductive isolation is met with a reasonable level of confidence and therefore should qualify each population as a biological species

under the biological species concept (BSC). However, because of important shared traits discussed above, we recommend that populations of *E. f. walkeri* (IC) and *E .f. walkeri* (BSF) be considered as evolutionary significant units (ESU's). Again the level of historical sympatry and lack of intergrades among these disparate populations supports this conclusion. Due to the current level of allopatry and complex modes of reproduction of unionids, direct tests of reproductive isolation are unlikely in the near future. As propagation and culture technology advance, crossing and heritability studies could be conducted to substantiate the genetic basis of phenotypic and quantitative traits. However, lack of direct data on reproductive isolation should not prevent a reasonable and prudent diagnosis of biological species using the best scientific data, such as those presented in this study. We recommend that management agencies recognize the proposed taxonomic changes when implementing recovery plans of *E. capsaeformis* and *E .f. walkeri*, and manage these species based on appropriate geographic and genetic data.

Conclusions

Molecular genetic methods typically do not measure variation at loci that are adaptively significant (Hard 1995, Hallerman 2003). Most molecular markers are considered selectively neutral, or nearly neutral, because environmentally mediated selection generally does not control their variability, but rather non-selective forces, such as random genetic drift and mutation, determine changes in allele frequency (Kimura 1983, Ohta 1992, Hard 1995, Hallerman 2003). Moreover, the relationship between molecular genetic variation (e.g., allelic diversity and heterozygosity) and an animal's fitness is poorly understood (Hansson and Westerberg 2002). It has been assumed that

genetic variation at neutral loci provides an adequate surrogate measure for variation at fitness-related loci; however, this assumption is rarely questioned and not well supported by empirical data (Hard 1995). Nature provides many examples of animal populations that contain little detectable genetic variation, but seemingly thrive in the wild (see Hard 1995). Therefore, molecular genetic variation may not be an adequate surrogate for genetic variation at fitness-related loci. In this study, variation at phenotypic and quantitative markers among in-group mussel taxa was incongruent with the low level of variation observed at DNA sequences. In fact, it was the phenotypic and quantitative characters that allowed us to put the DNA sequence data into perspective, and to conclude that the in-group taxa were valid, but closely related species or ESUs.

Modern taxonomic and phylogenetic studies should combine complementary information from molecular markers, morphology, life history and biogeography whenever possible. Holistic analyses allow biologists to seek concordance among multiple independent data sets, and to minimize errors interpreting ambiguous or misleading characters (Avise 2000). Such comprehensive analyses are especially justified for endangered species, if study results may alter or jeopardize the status of a species. We strongly recommend that molecular genetic studies, aimed at delineating freshwater mussel species, be augmented by biologically meaningful data from an animal's distribution, phenotype, life history traits and important functional protein markers.

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Table 1. Primer sequences used to amplify mussel mitochondrial and nuclear DNA sequences using polymerase chain reaction.

	Primers (5'-3')	
Locus	Forward	Reverse
ITS-1	AAAAAGCTTCCGTAGGTGAACCTG	AGCTTGCTGCGTTCTTCATCG
16S	CCGGTCTGAACTCAGATCACGT	CGCCTGTTTATCAAAAACAT
ND-1	TGGCAGAAAAGTGCATCAGATTAAAGC	TCGGAATTCTCCTTCTGCAAAGTC
cyt-b	AAGAAGTATCATTGCGGTTG	TGTGGGGCGACTGGTATTACTAA

Table 2. Sample locations and sample sizes for DNA sequences and microsatellites investigated for five mussel species in the genus *Epioblasma*. *Specific sites are described in methods section.

			mtDNA			nDNA										
		Total		*cyto-*			Microsatellite Loci									
Species	Collection* location	sample size	*16S*	*chrome-b*	*ND1*	*ITS-1*	*Ecap1*	*Ecap2*	*Ecap3*	*Ecap4*	*Ecap5*	*Ecap6*	*Ecap7*	*Ecap8*	*Ecap9*	*Ecap10*
In-group taxa																
Epioblasma capsaeformis	Clinch River	20	10	10	8	10	18	20	12	19	19	19	20	19	18	10
Epioblasma florentina walkeri	Indian Creek, Upper Clinch River	8	6	5	2	5	8	6	8	6	5	7	4	6	8	3
Epioblasma florentina walkeri	Big South Fork Cumberland River	14	10	10	2	10	13	12	11	12	11	13	11	10	12	6
Epioblasma capsaeformis	Duck River	12	10	10	8	10	12	12	12	12	12	12	12	10	10	7
Epioblasma torulosa rangiana	Allegheny River	6	6	6	1	6	6	6	4	5	6	6	6	5	5	3
Out-group taxa																
Epioblasma triquetra	Clinch River	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---
Epioblasma brevidens	Clinch River	1	1	1	1	1	---	---	---	---	---	---	---	---	---	---

Table 3. Pair-wise comparisons of genetic distances among combined mitochondrial (*16S, cytochrome-b, ND1*) and nuclear (*ITS-1*) DNA haplotypes using a Jukes-Cantor model of nucleotide substitution. Abbreviations for species are the following: *Epioblasma capsaeformis*, Clinch River (*Ec*CR), *Epioblasma florentina walkeri*, Indian Creek (*Efw*IC), *Epioblasma florentina walkeri*, Big South Fork Cumberland River (*Efw*BSF), *Epioblasma torulosa rangiana*, Allegheny River (*Etr*AR), *Epioblasma triquetra* (*Et*), and *Epioblasma brevidens* (*Eb*).

Haplo-types	*Ec*CR1	*Ec*CR2	*Ec*CR3	*Ec*CR4	*Ec*CR5	*Ec*CR6	*Efw*IC1	*Efw*IC2	*Efw*BSF1	*Etr*AR1	*Etr*AR2	*Etr*AR3	*Ec*DR1	*Ec*DR2	*Et*1	*Eb*1
*Ec*CR1	-------															
*Ec*CR2	0.00158	-------														
*Ec*CR3	0.00370	0.00422	-------													
*Ec*CR4	0.00317	0.00264	0.00158	-------												
*Ec*CR5	0.00105	0.00053	0.00370	0.00211	-------											
*Ec*CR6	0.00158	0.00105	0.00423	0.00264	0.00053	-------										
*Efw*IC1	0.00264	0.00422	0.00317	0.00264	0.00369	0.00422	-------									
*Efw*IC2	0.00316	0.00475	0.00370	0.00316	0.00422	0.00475	0.00053	-------								
*Efw*BSF1	0.00528	0.00581	0.00581	0.00422	0.00528	0.00581	0.00369	0.00422	-------							
*Etr*AR1	0.00423	0.00476	0.00370	0.00317	0.00423	0.00476	0.00264	0.00317	0.00529	-------						
*Etr*AR2	0.00634	0.00688	0.00582	0.00529	0.00635	0.00688	0.00476	0.00529	0.00741	0.00159	-------					
*Etr*AR3	0.00529	0.00582	0.00476	0.00423	0.00529	0.00582	0.00370	0.00423	0.00635	0.00106	0.00159	-------				
*Ec*DR1	0.00635	0.00742	0.00635	0.00582	0.00688	0.00742	0.00476	0.00529	0.00741	0.00583	0.00795	0.00689	-------			
*Ec*DR2	0.00582	0.00688	0.00582	0.00529	0.00635	0.00689	0.00423	0.00476	0.00688	0.00530	0.00742	0.00636	0.00053	-------		
*Et*1	0.04903	0.04956	0.04844	0.04788	0.04900	0.04956	0.04844	0.04900	0.04903	0.04911	0.05069	0.04908	0.05072	0.05015	-------	
*Eb*1	0.05584	0.05581	0.05525	0.05638	0.05524	0.05581	0.05525	0.05582	0.05868	0.05708	0.05809	0.05704	0.05755	0.05698	0.04952	-------

Table 4. Haplotypes and variable sites (**Bold**) in combined analysis of *16S*, *cytochrome-b*, *ND1* and *ITS-1* DNA sequences of ingroup taxa *Epioblasma capsaeformis*, Clinch River (*Ec*CR); *E. florentina walkeri*, Indian Creek (*Efw*IC); *E. f. walkeri*, Big South Fork Cumberland River, (*Efw*BSF); *E. torulosa rangiana*, Allegheny River (*Etr*AR); *E. capsaeformis*, Duck River (*Ea*DR); and outgroup taxa *Epioblasma triquetra* (*Et*) and *Epioblasma brevidens* (*Eb*).

		16S (468 bp)	*cytochrome-b* (360 bp)
		1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 4 4	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3
		1 3 2 4 6 7 2 3 3 4 5 5 5 5 8 8 0 1 1 1 2 3 4 5 6 5 5	1 1 2 4 5 6 7 7 8 9 9 9 9 9 0 0 0 0 1 1 1 3 4 5 5 6 6 7 8 8 8 9 0 0 0 1 1 4 4 4 4 5 7 8 8 9 0 1 1
Haplotype	*N*	2 7 8 9 4 9 8 5 7 9 9 0 1 9 0 2 4 5 6 7 6 2 3 7 6 1 4 1 5 1 2	0 3 2 3 2 4 0 5 7 2 4 5 6 9 3 6 8 9 2 7 9 3 8 1 3 3 9 2 1 4 7 0 1 2 8 1 4 1 2 3 7 2 7 6 9 5 7 0 5
*Ec*CR1	4	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A **T** A T A C A G G C G T C G A G A T A G G A **G** G A C G A T G G G C T G A C G C T C A
*Ec*CR2	1	T T T A G : T A T C C T A A A A : G A C T A G T A A C **A** A C G	A A G C G A A A **T** A T A C A G G C G T C G A G A T A G G A **G** G A C G A T G G G C T G A C G C T C A
*Ec*CR3	2	T T T A G : T A T **A** C T A A A A : G A C T A G T A A C G A C G	A **G** G C G A A A C A T A C A G G C G T C G A **T** A T A G G A A G A C G A T G G G C T G A C G C T C A
*Ec*CR4	1	T T T A G : T A T **A** C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A C A T A C A G G C G T C G A G A T A G G A A G A C G A T G G G C T G A C G C T C A
*Ec*CR5	1	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A **T** A T A C A G G C G T C G A G A T A G G A **G** G A C G A T G G G C T G A C G C T C A
*Ec*CR6	1	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A **T** A T A C A G G C G T C G A G A T A G G A **G** G A C G A T G G G C T G A C G C T C A
*Efw*IC1	5	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A C A T A C A G G C G T C G A G A T A G G A A G A C G A T G G G C T G A C G C T C A
*Efw*IC2	1	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A C A T A C A G G C G T C G A G A T A G G A A G A C G A T G G G C T **A** A C G C T C A
*Efw*BSF1	10	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C **A** A A A C A T A **T** A G G C G T C G A G A T A G G A A G A C G A T G G G C T G A C G C T C A
*Etr*AR1	1	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G C G A A A C A T A C **C** G G C G T C G A G A T A G **A** A A G A C G A T G G G C T G A C G C T C A
*Etr*AR2	3	T T T A G : T A T C C T A A A A : G A C T A G T A A C G A C **A**	A A G C G A A A C A T A C **C** G G C G T C G A G A T A G **A** A A G A C G A T G G G C T G A C G C T C A
*Etr*AR3	2	T T T A G : T A **C** C C T A A A A : G A C T A G T A A C G A C **A**	A A G C G A A A C A T A C **C** G G C G T C G A G A T A G **A** A A G A C G A T G G G C T G A C G C T C A
*Ec*DR1	9	**C** T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G **T** G A A A C A T A C A G G C G T C G A G A T A G G A A G A C G **G C A** G G C T G A C G C T C A
*Ec*DR2	1	**C** T T A G : T A T C C T A A A A : G A C T A G T A A C G A C G	A A G **T** G A A A C A T A C A G G C G T C G A G A T A G G A A G A C G **G C A** G G C T G A C G C T C A
*Et*1	1	**C C C** A A : T **G** T C C **C C T G** A **A** G A T T A **A** T **G G T** G A C **A**	**T** A **T** C G A **G G** C **G** T **C** C A **A A T A C T** G **G** G **G C G A** G **T** A G **G G A** A T G **A A T G** G **G T A T A** C A
*Eb*1	1	T **C C G A A C G** T C **T C C** A **G G A : G T C T T A G G T** G **T T A**	**G G** G **T** G **G G** A C A **C C** C A **A A T A C T A** A **T** A T **G** G G A **G A** A **G A** A **C** G **A** G C T G **G T A T** T **T G**

Table 4. Extended.

Haplotype	*N*	*Cytochrome-B* (33333333 / 12233445 / 87847067)	*ND-1* (550 bp) (111111111122222222222222233333333444444444444444555555555555555 / 123346690244556789002233445885889913333600022344668011134446666 / 210950638912035205585901398128452036963583951425810391036147)	*ITS-1* (515 bp) (111111111 / 422255556 / 478912356 7)
*Ec*CR1	3	G**A**ACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAGT**G**:G::
*Ec*CR2	2	G**A**ACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAGT**G**:G::
*Ec*CR3	2	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAG**G**A:G**C**T
*Ec*CR4	1	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAG**G**A:G**C**T
*Ec*CR5	1	G**A**ACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAG**G**A:G**C**T
*Ec*CR6	1	G**A**ACGGGA	CCACTTAATTAACTGCTAGCAATCTAGT**C**TGTTCACCTCGAGGCT**A**TCTACTCCTCCTTG	CTAGTA:G::
*Efw*IC1	5	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTC**A**AGGCTGTCTACTCCTCCTTG	CTAGT**G**:G::
*Efw*IC2	1	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTC**A**AGGCTGTCTACTCCTCCTTG	CTAGT**G**:G::
*Efw*BSF1	10	GGACGGGA	CCACTTAATTAACTGCTAGCAATCT**G**GTTTGTTCACCTCGA**A**GCTGTCTACTCCTCCTTG	CTAG**G**G:G**C**T
*Etr*AR1	1	GGACGGGA	CC**T**CTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCTGTCTACTCCTCCTTG	CTAGTA:G::
*Etr*AR2	3	GGACGGGA	CC**T**CTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCTGTCTACTCCTCCTTG	**TG**AGTA:G::
*Etr*AR3	2	GGACGGGA	CC**T**CTTAATTAACTGCTAGCAATCTAGTTTGTTCACCTCGAGGCTGTCTACTCCTCCTTG	CTAGTA:G::
*Ec*DR1	9	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAG**A**TTGTTCACCTCGAGGCTGTCTACTCCTCCTTG	CTAGT**TT**G::
*Ec*DR2	1	GGACGGGA	CCACTTAATTAACTGCTAGCAATCTAG**A**TTGTTCACCTCGAGGCTGTCTACTCCTCCT**C**G	CTAGT**TT**G::
*Et*1	1	**A**G**GT**GGG**G**	**A**T**A**T**T**A**T**AT**C**A**GTCTT**TA**A**C**C**ATC**CG**A**TT**C**GT**CTGT**CT**T**GG**AA**T**C**A**TT**GG**C**CT**A**C**C**T**TT**A**	CTAGTA:G::
*Eb*1	1	G**A**G**T**A**A**A**A**	C**T**AC**C**A**T**G**C**T**C**A**T**A**G**C**CG**G**A**T**G**C**T**T**A**A**T**TT**A**C**T**C**GTTA**T**AG**G**A**C**T**G**C**T**T**G**T**T**T**A**C**T**A**C**T**A**	C**A**G**C**C**A**:**A**::

Table 4. Extended.

		ITS-1												
		1	2	2	3	3	3	3	4	4	4	5		
		9	1	3	7	8	8	8	3	6	6	1		
Haplotype	*N*	6	5	9	7	5	6	7	8	7	7	8	1	
*Ec*CR1	3	:	G	:	A	:	:	:	:	C	**T**	**C**	T	
*Ec*CR2	2	:	G	:	A	:	:	:	:	C	**T**	**C**	T	
*Ec*CR3	2	:	G	:	A	:	:	:	:	C	:	:	T	
*Ec*CR4	1	:	G	:	A	:	:	:	:	C	:	:	T	
*Ec*CR5	1	:	G	:	A	:	:	:	:	C	:	:	T	
*Ec*CR6	1	:	G	:	A	:	:	:	:	C	:	:	T	
*Efw*IC1	5	:	G	:	A	:	:	:	:	C	:	:	T	
*Efw*IC2	1	:	G	:	A	:	:	:	:	C	:	:	T	
*Efw*BSF1	10	:	**A**	:	A	:	:	:	:	C	:	:	T	
*Etr*AR1	1	:	G	:	A	:	:	:	:	C	:	:	;	
*Etr*AR2	3	:	G	:	A	**G**	**T**	**T**	**T**	C	:	:	;	
*Etr*AR3	2	:	G	:	A	:	:	:	:	C	:	:	;	
*Ec*DR1	9	:	G	:	A	:	:	:	:	C	:	:	T	
*Ec*DR2	1	:	G	:	A	:	:	:	:	C	:	:	T	
*Et*1	1	**T**	G	:	A	:	:	:	:	**T**	:	:	T	
*Eb*1	1	**T**	G	**Δ**	**C**	:	:	:	:	C	:	:	T	

Table 5. Allele frequencies of DNA microsatellites examined in the in-group *Epioblasma* species. Allele sizes are given in number of base pairs to include the primer flanking regions. *Locus did not amplify for all mussel species.

Locus	Allele	*Epioblasma capsaeformis* (CR)	*Epioblasma f. walkeri* (IC)	*Epioblasma f. walkeri* (BSF)	*Epioblasma capsaeformis* (DR)	*Epioblasma t. rangiana* (AR)
Ecap1	146	0.1111	--------	--------	--------	--------
	148	0.0278	--------	--------	--------	--------
	150	--------	--------	--------	--------	0.1667
	152	0.0278	--------	--------	--------	0.2500
	154	0.0278	--------	0.2692	--------	0.1667
	156	--------	--------	--------	0.2083	0.1667
	158	0.1667	--------	0.2692	0.6667	0.0833
	160	0.0278	--------	0.2308	0.1250	0.1667
	162	0.1111	--------	0.0769	--------	--------
	164	0.0556	--------	0.0769	--------	--------
	166	--------	--------	0.0769	--------	--------
	168	0.0556	--------	--------	--------	--------
	170	0.0833	--------	--------	--------	--------
	172	0.0278	--------	--------	--------	--------
	174	0.1111	0.7500	--------	--------	--------
	176	0.0278	0.2500	--------	--------	--------
	178	0.0556	--------	--------	--------	--------
	180	0.0278	--------	--------	--------	--------
	184	0.0278	--------	--------	--------	--------
	190	0.0278	--------	--------	--------	--------
Ecap2	107	0.1500	--------	--------	--------	--------
	111	--------	--------	--------	--------	0.1677
	115	--------	--------	0.9286	0.0417	--------
	119	0.1000	--------	0.0714	--------	0.0833
	121	0.2250	0.3333	--------	0.1250	0.4167
	123	0.5000	0.0833	--------	0.5833	0.2500
	125	--------	--------	--------	0.1667	--------
	127	--------	--------	--------	0.0417	0.0833
	129	0.0250	0.5833	--------	0.0417	--------
*Ecap3**	236	--------	--------	--------	--------	0.1250
	238	--------	--------	--------	--------	0.1250
	242	--------	--------	0.2273	--------	--------
	250	--------	--------	--------	--------	0.2500
	252	--------	--------	--------	--------	0.2500
	256	--------	--------	0.0909	--------	--------
	260	--------	--------	0.3182	--------	0.2500
	262	--------	--------	0.2273	--------	--------
	264	0.0833	--------	0.1364	--------	--------
	268	0.2083	--------	--------	--------	--------

	270	0.2083	--------	--------	---------	--------
	274	0.0833	--------	--------	---------	--------
	276	0.0417	--------	--------	---------	--------
	278	0.0833	--------	--------	0.4583	--------
	280	0.1667	--------	--------	0.5417	--------
	282	0.0833	--------	--------	--------	--------
	286	0.0417	--------	--------	--------	--------
Ecap4	98	--------	--------	0.7500	--------	--------
	100	0.0263	--------	--------	--------	--------
	102	0.0526	0.5833	0.0417	0.1250	0.5833
	104	0.1316	0.1667	0.0417	0.4167	0.1667
	106	0.4737	0.1667	--------	0.3750	0.1667
	108	0.0526	--------	--------	0.0417	--------
	110	0.0789	--------	0.1667	0.0417	--------
	112	--------	0.0833	--------	--------	0.0833
	114	0.0526	--------	--------	--------	--------
	120	0.1316	--------	--------	--------	--------
Ecap5	176	0.0526	--------	--------	--------	--------
	184	0.1316	--------	--------	--------	--------
	186	--------	--------	--------	0.5417	--------
	188	0.1053	--------	--------	0.1667	0.0833
	190	0.2632	--------	--------	--------	0.1667
	192	0.0526	0.4000	0.0909	--------	--------
	194	0.0263	--------	--------	--------	--------
	196	0.0526	--------	--------	--------	0.1667
	198	0.0526	--------	0.6818	--------	0.0833
	200	--------	--------	0.1818	--------	0.2500
	202	0.0526	--------	0.0455	--------	--------
	204	0.0789	--------	--------	--------	--------
	208	0.0789	--------	--------	0.0417	--------
	210	--------	--------	--------	--------	0.0833
	212	--------	0.1000	--------	0.2500	--------
	214	0.0263	--------	--------	--------	0.0833
	216	--------	0.2000	--------	--------	--------
	220	0.0263	--------	--------	--------	--------
	222	--------	0.3000	--------	--------	--------
	224	--------	--------	--------	--------	0.0833
Ecap6	216	0.0526	--------	--------	--------	--------
	218	0.1316	--------	--------	--------	--------
	224	0.0263	--------	--------	--------	--------
	226	--------	--------	--------	--------	0.4167
	228	0.0263	--------	--------	--------	0.2500
	230	--------	--------	--------	0.0833	0.2500
	232	--------	0.2000	--------	0.1667	--------
	234	0.3158	0.8000	1.0000	--------	--------
	236	--------	--------	--------	0.7500	--------
	238	0.3684	--------	--------	--------	0.0833
	240	0.0789	--------	--------	--------	--------

*Ecap7**	106	0.0250	--------	--------	--------	--------
	108	0.0250	--------	--------	--------	--------
	110	0.0500	0.6250	--------	--------	--------
	114	0.1250	--------	0.1000	0.5417	--------
	116	--------	--------	0.6500	0.0833	--------
	118	0.0500	--------	0.1000	--------	--------
	120	0.0750	--------	0.0500	--------	--------
	122	0.1750	0.3750	0.0500	0.2083	--------
	124	0.3000	--------	--------	0.0833	--------
	126	0.1000	--------	--------	0.0417	--------
	128	0.0500	--------	0.0500	--------	--------
	130	0.0250	--------	--------	0.0417	--------
Ecap8	127	0.2105	--------	--------	--------	--------
	131	--------	--------	--------	0.1000	0.6000
	133	0.0789	0.1667	--------	0.3500	--------
	137	0.0526	--------	1.0000	--------	--------
	141	0.0526	0.8333	--------	--------	--------
	143	0.3947	--------	--------	--------	--------
	145	0.1842	--------	--------	--------	--------
	147	--------	--------	--------	0.1000	--------
	149	--------	--------	--------	--------	0.4000
	155	0.0263	--------	--------	0.4000	--------
	159	--------	--------	--------	0.0500	--------
Ecap9	130	--------	--------	--------	--------	0.1000
	134	0.0833	--------	--------	--------	0.3000
	136	0.0833	0.1250	0.8750	0.1000	--------
	138	0.1944	0.8750	0.1250	--------	0.2000
	140	0.0556	--------	--------	0.1500	--------
	142	0.1111	--------	--------	0.2000	0.3000
	144	0.2500	--------	--------	0.3500	--------
	148	--------	--------	--------	0.2000	--------
	150	0.0833	--------	--------	--------	0.1000
	152	0.0833	--------	--------	--------	--------
	156	0.0278	--------	--------	--------	--------
	162	0.0278	--------	--------	--------	--------
Ecap10	115	0.0500	--------	--------	--------	--------
	119	--------	--------	--------	--------	0.1667
	123	0.1500	0.1667	--------	0.8571	--------
	125	0.1000	--------	--------	--------	0.8333
	127	--------	0.1667	--------	--------	--------
	129	0.0500	0.3333	--------	--------	--------
	131	0.0500	0.3333	--------	0.1429	--------
	133	0.1500	--------	0.4167	--------	--------
	135	0.2000	--------	0.2500	--------	--------
	137	0.1000	--------	0.3333	--------	--------
	139	0.0500	--------	--------	--------	--------
	143	0.1000	--------	--------	--------	--------

Table 6. Summary of genetic variation across 10 microsatellite loci examined for the in-group species of *Epioblasma*.

Species	% Polymorphic loci	Observed hetero-zygosity	Expected hetero-zygosity	Mean number of alleles/locus	Mean allele size range (bp)	Maximum range (bp)	Total alleles	No. of unique alleles
Epioblasma capsaeformis (CR)	100	0.6333	0.8347	9.7	28.4	44	97	39
Epioblasma f. walkeri (IC)	90	0.3593	0.5238	2.5	9.1	30	25	2
Epioblasma f. walkeri (BSF)	80	0.3850	0.4217	3.4	6.0	22	34	5
Epioblasma capsaeformis (DR)	100	0.4236	0.6025	4.1	13.4	28	41	6
Epioblasma t. rangiana (AR)	90	0.4778	0.7355	4.1	16.9	36	41	12

Table 7. Pair-wise F_{st} estimates (below diagonal) and N_m (above diagonal) using data from 10 microsatellite loci.

All pairwise comparisons of F_{st} estimates are significant (p<0.05).

Species	*Epioblasma capsaeformis* (CR)	*Epioblasma f. walkeri* (IC)	*Epioblasma f. walkeri* (BSF)	*Epioblasma capsaeformis* (DR)	*Epioblasma t. rangiana* (AR)
Epioblasma capsaeformis (CR)	------	0.9595	0.9359	1.8976	1.3082
Epioblasma f. walkeri (IC)	0.2067	------	0.3971	0.5687	0.5599
Epioblasma f. walkeri (BSF)	0.2108	0.3864	------	0.5273	0.5390
Epioblasma capsaeformis (DR)	0.1164	0.3053	0.3216	------	0.8008
Epioblasma t. rangiana (AR)	0.1604	0.3087	0.3169	0.2379	------

Table 8. Mean shell length, mean height of the marsupial expansion, and linear regression equations of height of the marsupial expansion (*y*-axis) to total length (*x*-axis) of adult female mussels are reported. Most pairwise comparisons of regression equation slopes were significantly different ($p<0.001$), except equations B vs D, and C vs D. *The *p*-value indicates level of significance for the fitted-line of regression equations A-D.

Mussel species	*N*	Mean length of shell (mm) (range)	Mean height of marsupial expansion (mm) (range)	Regression equations A-D	R-squared	*p*-value*
Epioblasma capsaeformis (CR)	63	40.7 (30.4-58.4)	8.3 (2.8-14.6)	A. $y = -4.92 + 0.324x$	0.54	$p<0.003$
Epioblasma capsaeformis (DR)	62	41.9 (31.0-68.9)	9.6 (3.1-24.6)	B. $y = -19.8 + 0.701x$	0.76	$p<0.001$
Epioblasma f. walkeri (IC)	55	40.0 (30.7-46.1)	5.7 (1.0-10.2)	C. $y = -16.7 + 0.561x$	0.85	$p<0.001$
Epioblasma f. walkeri (BSF)	20	40.5 (30.3-45.2)	8.5 (2.0-14.1)	D. $y = -19.7 + 0.696x$	0.51	$p<0.007$

Table 9. Mean (SD) lengths of glochidia measured for the in-group mussel species. All pairwise comparisons are significantly different (p<0.001).

Mussel species	*N*	Mean length (μm)
Epioblasma f. walkeri (IC)	100	271.9 (9.5)
Epioblasma f. walkeri (BSF)	100	264.7 (11.5)
Epioblasma capsaeformis (CR)	100	255.7 (5.2)
Epioblasma capsaeformis (DR)	100	248.0 (9.2)
Epioblasma t. rangiana (AR)	20	241.3 (8.5)

Table 10. Fecundity estimates for female mussels of *Epioblasma* species.

Mussel species	Number of females (*N*)	Mean Length (mm)	Range (mm)	Mean number of glochidia/ female	Range
Epioblasma capsaeformis (CR)	10	41.5	36.7-46.4	13,008	7,780-16,876
Epioblasma f. walkeri (IC)	7	42.2	40.6-45.7	7,602	3,261-12,558
Epioblasma f. walkeri (BSF)	6	42.8	40.5-45.2	9,606	1,828-16,921
Epioblasma capsaeformis (DR)	6	45.4	35.8-56.44	18,757*	6,668-38,716

Mean fecundity for *Epioblasma capsaeformis* (DR) is significantly different ($p<0.05$).*

Table 11. Mean (SE) percentages of juvenile mussels transformed per fish host species.

	Mean # juveniles/fish sp.		
Mussel species	*Etheostoma blennioides*	*Etheostoma flabellare*	*Etheostoma rufilineatum*
Epioblasma capsaeformis (CR)	1. 44 (±5)	2. 24 (±2)	3. 32 (±6)
Epioblasma capsaeformis (DR)	4. 17 (±11)	5. 59 (±8)	6. 23 (±4)
Epioblasma f. walkeri (BSF)	7. 10 (±3)	8. 73 (±7)	9. 17 (±6)

Pairwise comparisons (p<0.001), S=significant, N=not significant:

	1.	2.	3.	4.	5.	6.	7.	8.
2.	S							
3.	N	N						
4.	S	N	N					
5.	N	S	S	S				
6.	S	N	N	N	S			
7.	S	N	S	N	S	N		
8.	S	S	S	S	N	S	S	
9.	S	N	N	N	S	N	N	S

Table 12. Diagnostic or unique morphological and molecular genetic characters examined for the in-group species of *Epioblasma*.

Species	Morphological characters	DNA haplotypes	Polymorphic DNA sequence sites	Microsatellite alleles
Epioblasma capsaeformis (CR)	• periostracum: yellow & green • mantle-pad color: blue to bluish-white • mantle-pad texture: smooth • micro-lure display: 2 together; the left lure rotates clockwise, while the right lure rotates counter-clockwise	*Ec*CR 1-6	• *16S*: 179 • *cytochrome-b*: 13, 87, 148, 184, 327 • *ND1*: 282, 444 • *ITS*-1: 467, 468	*Ecap1*: 146, 148, 168, 170, 172, 178, 180, 184, 190; *Ecap2*: 107; *Ecap3*: 268, 270, 274, 276, 282, 286; *Ecap4*: 100, 114, 120; *Ecap5*: 176, 184, 194, 204, 220; *Ecap6*: 216, 218, 224, 240; *Ecap7*: 106, 108; *Ecap8*: 127, 143 145; *Ecap9*: 152, 156, 162; *Ecap10*: 115, 139, 143
Epioblasma f. walkeri (IC)	• periostracum: honey-yellow to brown, with fine evenly-spaced rays • mantle-pad color: light grey with mottled black background • mantle-pad texture: pustulated • micro-lures: 1 lure prominent; rotates clockwise sweeping side-to-side	*Efw*IC 1-2	• *cytochrome-b*: 252 • *ND1*: 405	*Ecap5*: 222; *Ecap10*: 127
Epioblasma	• periostracum: honey-yellow to brown	*Efw*BSF 1	• *cytochrome-b*: 52,	*Ecap1*: 166; *Ecap3*: 242, 256, 262;

f. walkeri (BSF)	with fine evenly-spaced rays • mantle-pad color: brown with mottled tan background • mantle-pad texture: pustulated • micro-lures: : 1 lure prominent; rotates clockwise sweeping side-to-side		96 • *ND1*: 249, 423 • *ITS*-1: 215	*Ecap4*: 98
Epioblasma capsaeformis (DR)	• periostracum: yellow & dark-green • mantle-pad color: dark purple to slate-grey • mantle-pad texture: spongy • micro-lures: 1 lure prominent; rotates clockwise sweeping side-to-side	*Ec*DR 1-2	• *16S*: 2 • *cytochrome-b*: 43, 208, 211, 214 • *ND1*: 281, 564 • *ITS*-1: 152, 153	*Ecap2*: 125; *Ecap5*: 186; *Ecap6*: 236; *Ecap8*: 147, 159; *Ecap9*: 148
Epioblasma t. rangiana (AR)	• periostracum: brown and green • mantle-pad color: white • mantle-pad texture: smooth • micro-lures: absent	*Etr*AR 1-3	• *16S*: 167, 452 • *cytochrome-b*: 99, 172 • *ND1*: 30, • *ITS*-1: 44, 127, 385, 386, 387, 388, 511	*Ecap1*: 150; *Ecap2*: 111; *Ecap3*: 236, 238, 250, 252; *Ecap5*: 210, 224; *Ecap6*: 226; *Ecap8*: 148; *Ecap9*: 130; *Ecap10*: 119

Figure Headings

Figure 1. The distribution of *Epioblasma capsaeformis* (DR) (current ●, historic ○), *Epioblasma capsaeformis* (CR) (current ♦, historic ◊), *Epioblasma florentina walkeri* (IC) (current ▲, historic △), and *Epioblasma florentina walkeri* (BSF) (current ■, historic □).

Figure 2. Inferred phylogenetic relationships among the examined species of *Epioblasma* are shown. DNA haplotypes were described using minimum evolution (ME) and maximum parsimony (MP) analysis (tree length = 180, CI = 0.917, RI = 0.853). The ME tree is shown; the MP tree is identical with the exception that haplotypes *E. capsaeformis* CR2, *E. capsaeformis* CR5, and *E. capsaeformis* CR6 collapse to form a polytomy. The numbers above the branches (ME/MP) represent bootstrap support (100 replicates); only values >50% are shown. The tree was generated using DNA sequences from the combined mitochondrial DNA regions of *16S* (468 bp), *cytochrome-b* (360 bp), *ND1* (550 bp) and the nuclear DNA region *ITS-1* (515 bp). The out-group taxa are *Epioblasma triquetra* and *Epioblasma brevidens*.

Figure 3. Mantle-pad displays of mussel species in the genus *Epioblasma* are: (A) female oyster mussel *Epioblasma capsaeformis* (CR) displaying at Wallen Bend in the Clinch River, Hancock County, TN, (B) micro-lures of *E. capsaeformis* (CR), (C) mantle-pad display and micro-lure of *E. capsaeformis* (DR), Duck River, Maury County, TN, (D) marsupial shell expansion of female *E. capsaeformis* (DR) (E) mantle-pad display of tan riffleshell pearlymussel *E. florentina walkeri* (IC), Clinch River, Tazewell County, VA,

(F) micro-lure of *E. f. walkeri*, (IC), (G-H) mantle-pad display of *E. f. walkeri* (BSF), Big South Fork Cumberland River, Scott County, TN.

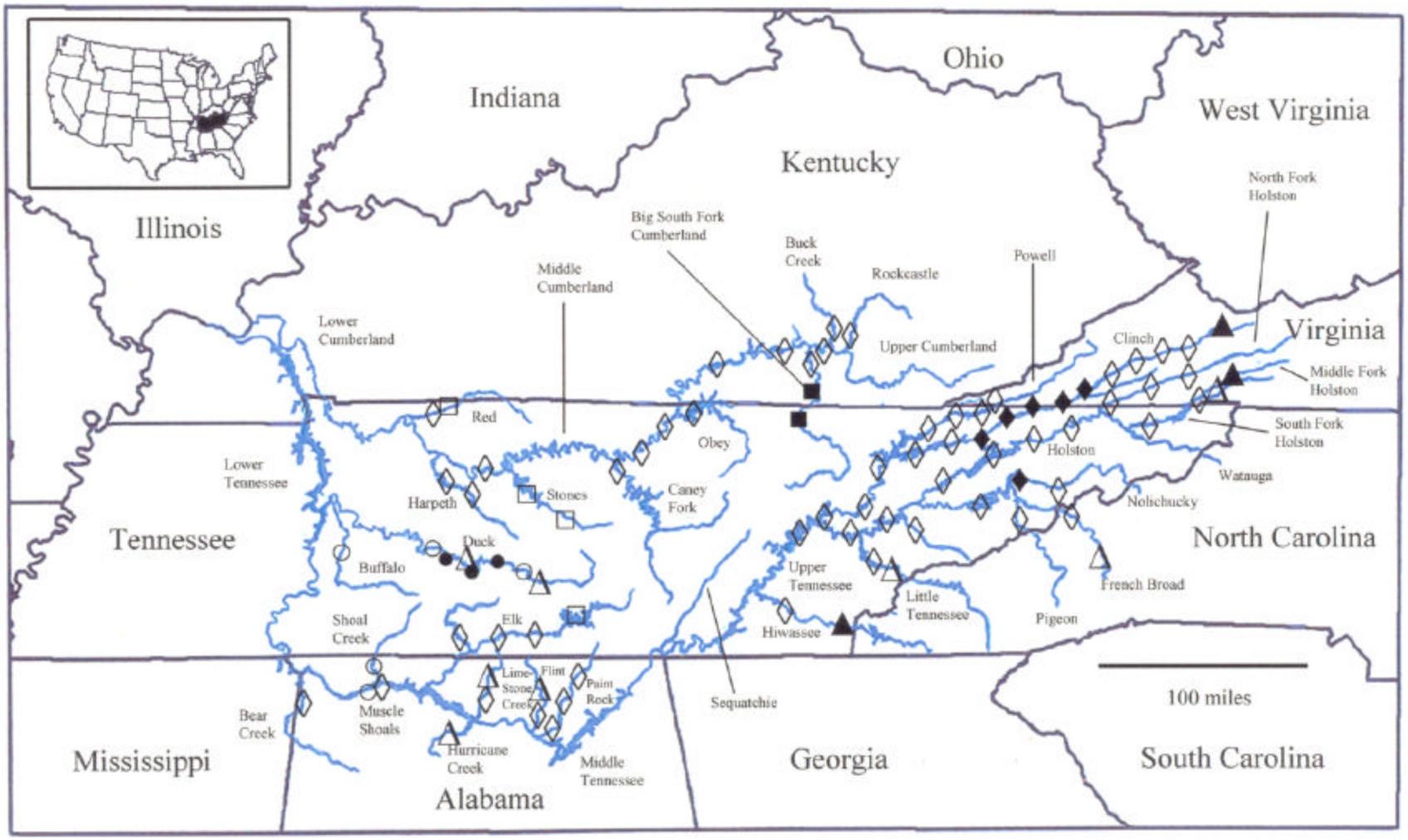

Ohio
Indiana
West Virginia
Kentucky
Illinois
North Fork Holston
Big South Fork Cumberland
Buck Creek
Rockcastle
Powell
Middle Cumberland
Lower Cumberland
Upper Cumberland
Clinch
Virginia
Middle Fork Holston
South Fork Holston
Red
Obey
Holston
Lower Tennessee
Watauga
Harpeth
Stones
Caney Fork
Nolichucky
Tennessee
North Carolina
Duck
Buffalo
Upper Tennessee
French Broad
Little Tennessee
Shoal Creek
Elk
Pigeon
Hiwassee
Lime Stone Creek
Flint
Paint Rock
Sequatchie
100 miles
Bear Creek
Muscle Shoals
Hurricane Creek
Middle Tennessee
Mississippi
Georgia
South Carolina
Alabama

Figure 1.

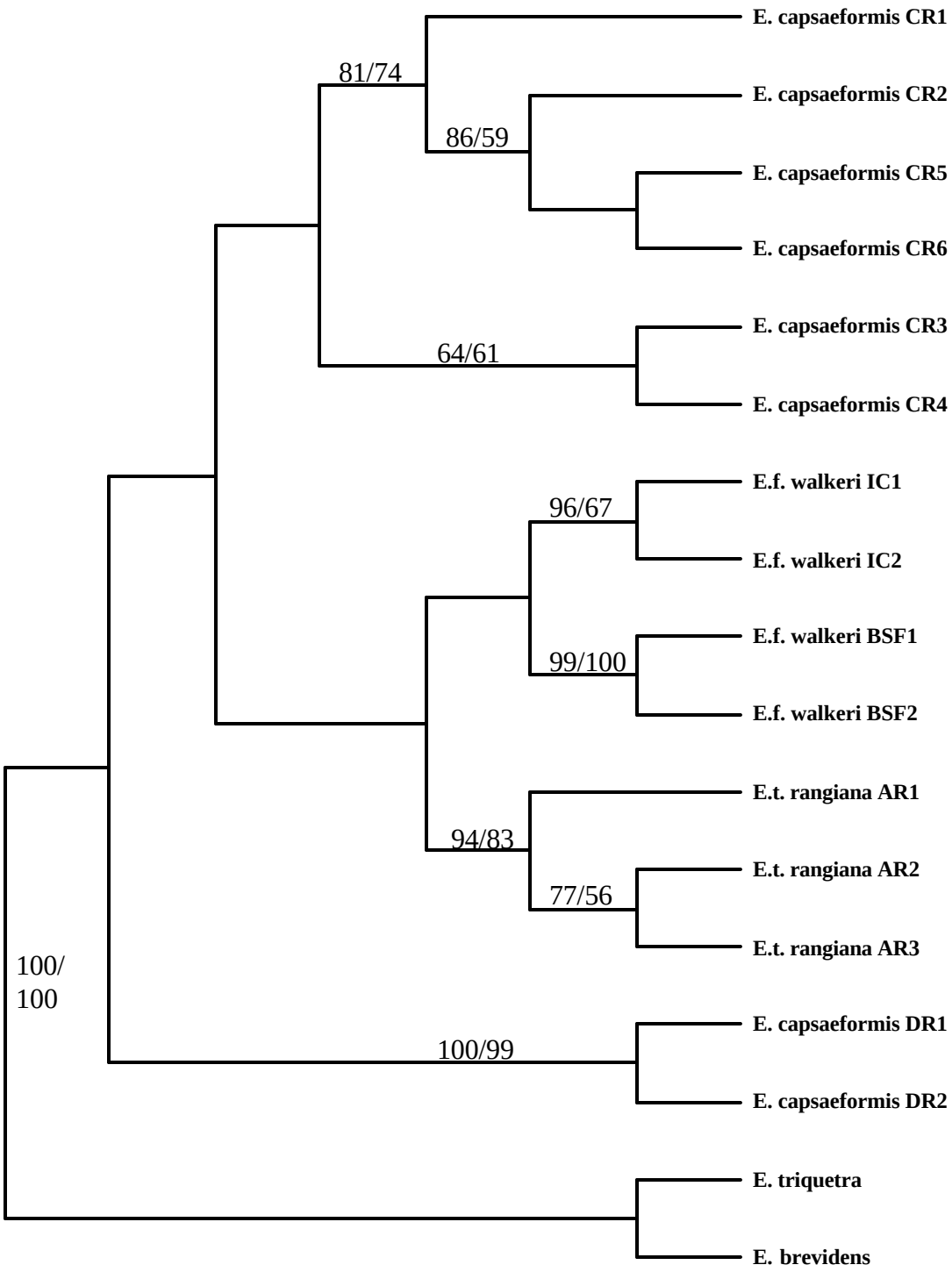

E. capsaeformis CR1
E. capsaeformis CR2
E. capsaeformis CR5
E. capsaeformis CR6
E. capsaeformis CR3
E. capsaeformis CR4
E.f. walkeri IC1
E.f. walkeri IC2
E.f. walkeri BSF1
E.f. walkeri BSF2
E.t. rangiana AR1
E.t. rangiana AR2
E.t. rangiana AR3
E. capsaeformis DR1
E. capsaeformis DR2
E. triquetra
E. brevidens
81/74
86/59
64/61
96/67
99/100
94/83
77/56
100/
100
100/99

Figure 2.

Figure 3.

APPENDIX I

COLLECTION RECORDS

Abbreviations: ANSP=Academy of Natural Sciences of Philadelphia, Pennsylvania; CM=Carnegie Museum, Pittsburgh, Pennsylvania; FLMNH=Florida Museum of Natural History, Tallahassee, Florida; MCZ=Museum of Comparative Zoology, Cambridge, Massachusetts; OSM=Ohio State Museum, Columbus, Ohio; USNM=National Museum of Natural History; NA=Not Available; cf. = to be compared with *Epioblasma capsaeformis*.

Species	Museum #	Location	Collector	Date
1. *Epioblasma capsaeformis* cf.	ANSP 100504 **Cotype**	Duck River, TN	H.H. Smith	NA
2. *Epioblasma capsaeformis* cf.	ANSP 100538	Shoal Creek Lauderdale Co., AL	H.H. Smith	NA

The following letter by Bryant Walker was found in a small box in this collection lot (ANSP 100538), where it still remains. The letter was a hand-written note to a physician, and to my knowledge has never been published. The hand-writing was difficult to read and was deciphered exactly as is by Jess Jones, with assistance from Paul Calloman, Elana Benamy, and Earle Spamer of ANSP, 8 January 2003:

"I also send some *Truncillas* that may be of interest. The Shoal Creek form is typical *capsaeformis* as I understand it. The male of the Duck R. form is very similar, but the females have invariably the enormous expansion of the specimen sent. In the Clinch, on the other hand, the females are quite typical in form, but the males are usually decidedly more elongated. The Duck R. form has been generally called "*turgidulus*" but it is not. Lea's *turgidulus* is the male of *deviata* as I proved to my own satisfaction, at least, while I was in Washington."

"P.S. The element of uncertainty in *capsaeformis* matter is the fact that we don't know what the ? form of the Cumberland is. The ? shell I had at Phila. & which agreed best with Lea's figure of the type was from the Duck R., variety *expansa*. The only Cumberland R. ? I have seen is an immature shell belonging to Ferris, and it is apparently like the Tenn. R. & Shoal Crk. form. If the ? Cumberland R. form is *expansa* like the Duck R. shell, that would be typical *capsaeformis* & *expansa* could not be used".

3. *Epioblasma capsaeformis* cf.	ANSP 391133	Duck River, Hardison Mill, Maury Co., TN	S. Ahlstedt	1/16/86
4. *Epioblasma capsaeformis* cf.	CM 61.646	NA	Hartman Collection	NA
5. *Epioblasma capsaeformis* cf.	CM 61.4491	Shoal Creek, Lauderdale Co., AL	B. Walker	11/2/09
6. *Epioblasma capsaeformis* cf.	CM 61.6498	Shoal Creek, Lauderdale Co., AL	A.E. Ortmann	NA

7. *Epioblasma capsaeformis* cf.	CM 61.7696	Tennessee River, Florence, Lauderdale Co., AL	A.E. Ortmann	NA
8. *Epioblasma capsaeformis* cf.	CM 61.7697	Shoal Creek, Lauderdale Co., AL	A.E. Ortmann	NA
9. *Epioblasma capsaeformis* cf.	CM 61.11267	Duck River, Maury Co., TN	A.E. Ortmann	8/26/21
10. *Epioblasma capsaeformis* cf.	CM 61.11497	Duck River, Maury Co., TN	A.E. Ortmann	9/6/22
11. *Epioblasma capsaeformis* cf.	CM 61.11669	Duck River, Lillards Mill, Marshall Co., TN	A.E. Ortmann	8/25/23
12. *Epioblasma capsaeformis* cf.	CM 61.11670	Duck River, Wilhoite, Marshall Co., TN	A.E. Ortmann	8/27/23
13. *Epioblasma capsaeformis* cf.	CM 61.11672	Duck River, Leftwich, Maury Co., TN	A.E. Ortmann	9/3/23
14. *Epioblasma capsaeformis* cf.	FLMNH 64238	Tennessee River, Florence, Lauderdale Co., AL	A.A. Hinkley	1904
15. *Epioblasma capsaeformis* cf.	FLMNH 64241	Duck River, Wilhoite, Marshall Co., TN	NA	NA
16. *Epioblasma capsaeformis* cf.	FLMNH 64242	Duck River, Hardison Mill, Marshall Co., TN	NA	NA
17. *Epioblasma capsaeformis* cf.	FLMNH 64245	Tennessee River, Muscle Shoals, Lauderdale Co., AL	H.H. Smith	11/1/09
18. *Epioblasma capsaeformis* cf.	FLMNH 64250	Shoal Creek, Lauderdale Co., AL	H.H. Smith	10/1/09
19. *Epioblasma capsaeformis* cf.	FLMNH 226003	Duck River, TN	NA	NA
20. *Epioblasma capsaeformis* cf.	FLMNH 269045	Duck River, Columbia, Maury Co., TN	A.A. Hinkley	NA
21. *Epioblasma capsaeformis* cf.	FLMNH 269049	Duck River, TN	Marsh	NA
22. *Epioblasma capsaeformis* cf.	FLMNH 269058	Duck River, Columbia, Maury Co., TN	B. Walker	NA
23. *Epioblasma capsaeformis* cf.	MCZ 6210	Duck River, Columbia, Maury Co., TN	R.E. Call	NA
24. *Epioblasma capsaeformis* cf.	MCZ 29828	Shoal Creek, Lauderdale Co., AL	B. Walker Collection	NA
25. *Epioblasma*	MCZ	Duck River, Wilhoite,	Goodrich	NA

capsaeformis cf.	83995	Marshall Co., TN		
26. *Epioblasma capsaeformis* cf.	MCZ 89441	Shoal Creek, Lauderdale Co., AL	H.H. Smith	NA
27. *Epioblasma capsaeformis* cf.	MCZ 236214	Duck River, Lilliard Mill, Marshall Co., TN	H.D. Athearn	9/30/56
28. *Epioblasma capsaeformis* cf.	MCZ 236718	Tennessee River, Muscle Shoals, AL	B. Walker Collection	NA
29. *Epioblasma capsaeformis* cf.	MCZ 272794	Duck River, Maury Co., TN	B. Isom P. Yokley	9/3/65
30. *Epioblasma capsaeformis* cf.	OSM 8628	Duck River, TN	NA	1800's
31. *Epioblasma capsaeformis* cf.	OSM 12246	Duck River, 431 Bridge, Maury Co., TN	C.B. Stein	7/19/64
32. *Epioblasma capsaeformis* cf.	OSM 14496	Duck River, Sowell Ford, Maury Co., TN	P. Yokely	7/7/65
33. *Epioblasma capsaeformis* cf.	OSM 14864	Duck River, Milltown, Marshall Co., TN	D.H. Stansbery	9/5/64
34. *Epioblasma capsaeformis* cf.	OSM 15149	Duck River, Wilhoite Mill, Marshall Co., TN	D.H. Stansbery	9/8/64
35. *Epioblasma capsaeformis* cf.	OSM 16229	Duck River, Sowell Ford, Maury Co., TN	P. Yokely B. Isom	9/2/65
36. *Epioblasma capsaeformis* cf.	OSM 16238	Duck River, Columbia, Maury Co., TN	P. Yokely B. Isom	9/1/65
37. *Epioblasma capsaeformis* cf.	OSM 33341	Duck River, Milltown, Marshall Co., TN	D.H. Stansbery W.J. Clench	10/14/72
38. *Epioblasma capsaeformis* cf.	OSM 33922	Duck River, Wilhoite Mill, Marshall Co., TN	B.G. Isom P. Yokley	9/3/65
39. *Epioblasma capsaeformis* cf.	OSM 33959	Duck River, Milltown dam, Marshall Co., TN	B.G. Isom P. Yokley	9/3/65
40. *Epioblasma capsaeformis* cf.	OSM 34074	Duck River, Leftwich, Maury Co., TN	P. Yokely B. Isom	9/3/65
41. *Epioblasma capsaeformis* cf.	OSM 50107	Duck River, TN	NA	prior to 1931
42. *Epioblasma capsaeformis* cf.	OSM 50108	Duck River, TN	NA	prior to 1928
43. *Epioblasma capsaeformis* cf.	OSM 52509	Duck River, Lillard Mill, Marshall Co., TN	S. Ahlstedt	10/1/82

44. *Epioblasma capsaeformis* cf.	OSM 57291	Duck River, TN	Wheatley	prior to 1882
45. *Epioblasma capsaeformis* cf.	OSM 67899	Duck River, Shelbyville, TN	Call	1885
46. *Epioblasma capsaeformis* cf.	OSM 68523	Duck River, Lillard Mill, Marshall Co., TN	H.D. Athearn	9/30/56
47. *Epioblasma capsaeformis* cf.	USNM 510913	Duck River, Columbia, TN	NA	NA
48. *Epioblasma capsaeformis* cf.	USNM 521353	Duck River, Columbia, TN	NA	NA
49. *Epioblasma capsaeformis*	ANSP 48394	Paint Rock River, Woodville, AL	B. Walker	NA
50. *Epioblasma capsaeformis*	ANSP 48395	Clinch River, Clinchport, Scott Co., VA	B. Walker	NA
51. *Epioblasma capsaeformis*	ANSP 48396	Clinch River, Black Fox Ford, TN	B. Walker	NA
52. *Epioblasma capsaeformis*	ANSP 48397	Powell River, Greens Ford, TN	B. Walker	NA
53. *Epioblasma capsaeformis*	ANSP 56398	Tennessee River	J.G. Anthony	NA
54. *Epioblasma capsaeformis*	ANSP 56582	Tennessee River	Swift Collection	NA
55. *Epioblasma capsaeformis*	ANSP 68369	Harpeth River near Bellevue	S.N. Rhoads	20 May 1895

Notes: Female mussels from this lot were some of the largest observed in this study; the following measurements (mm) were taken:

Length	Height	Marsupial expansion (length)	Marsupial expansion (height)
58.4	39.9	38.4	11.4
49.9	32.3	30.5	11.6
46.7	32.0	27.3	7.9
40.7	25.8	23.5	8.1
35.6	24.3	17.2	4.3

56. *Epioblasma capsaeformis*	ANSP 68374	Harpeth River, Bellevue, Davidson Co., TN	S.N. Rhoads	NA
57. *Epioblasma capsaeformis*	ANSP 100539	Clinch River	H.H. Smith	NA
58. *Epioblasma capsaeformis*	ANSP 103847	Elk River, Fayetteville, TN	H.H. Smith	NA

Notes: The shells of specimens from the Elk River have a dullish hue and significant amount of umbonal erosion; they tend to be small in size (~35-40 mm long).

59. *Epioblasma capsaeformis*	ANSP 103933	Paint Rock River, Trenton, AL	H.H. Smith	NA
60. *Epioblasma capsaeformis*	ANSP 103959	Paint Rock River, Princeton, AL	H.H. Smith	NA
61. *Epioblasma capsaeformis*	ANSP 103960	Paint Rock River, Princeton, AL	H.H. Smith	NA
62. *Epioblasma capsaeformis*	ANSP 103968	Paint Rock River, New Hope, AL	H.H. Smith	NA
63. *Epioblasma capsaeformis*	ANSP 103973	Paint Rock River, Paint Rock, AL	H.H. Smith	NA
64. *Epioblasma capsaeformis*	ANSP 103991	Paint Rock River, Holly Tree, AL	H.H. Smith	NA

Notes: Female mussels from the Paint Rock River appear distinctive because the marsupial shell expansion is broad and long relative to the remainder of the shell, and the denticulations are numerous, very fine, but stout. Also, the coloration of the shell appears enhanced, including the ray pattern. The following measurements from 5 females (mm) were taken:

Length	Height	Marsupial expansion (length)	Marsupial expansion (height)
42.5	27.9	28.4	11.2
37.3	23.8	24.8	8.9
37.2	23.9	22.4	8.4
35.0	22.7	20.2	6.8
41.1	27.8	29.1	11.2

65. *Epioblasma capsaeformis*	ANSP 125979	Tennessee River	C.M. Wheatley Collection	NA
66. *Epioblasma capsaeformis*	ANSP 125980	Duck River, TN	C.M. Wheatley Collection	NA
67. *Epioblasma capsaeformis*	ANSP 125981	Tennessee River, Muscle Shoals, AL	C.M. Wheatley Collection	NA

Notes: Several individuals from this lot were some of the largest observed in this study; the following measurements (mm) were taken:

Length	Height	Marsupial expansion (length)	Marsupial expansion (height)
? 52.1	32.8	31.0	10.5
? 52.1	31.7	33.5	11.1
? 57.0	37.0		
? 53.8	35.7		
? 47.2	29.6		

68. *Epioblasma*	ANSP	Cumberland River, TN	NA	NA

capsaeformis	125983			
69. *Epioblasma capsaeformis*	ANSP 125984	Tennessee River, AL	C.M. Wheatley Collection	NA
70. *Epioblasma capsaeformis*	ANSP 150819	Clinch River, Clinchport, Scott Co., VA	R.W. Jackson	1930
71. *Epioblasma capsaeformis*	ANSP 177860	Obey River, Pickett Co., TN	W.G. Parris	NA

Notes: Female mussels from this lot appeared small and slow growing; the following measurements (mm) were taken:

Length	Height	Marsupial expansion (length)	Marsupial expansion (height)
33.1	22.2	16.3	3.7
33.3	22.1	18.1	4.1

72. *Epioblasma capsaeformis*	ANSP 218968	Shoal Creek, Lauderdale Co., AL	W.J. Clench	NA
73. *Epioblasma capsaeformis*	ANSP 218989	Clinch River, Clinchport, Scott Co., VA	R.W. Jackson	1930
74. *Epioblasma capsaeformis*	ANSP 219008	Clinch River, Clinchport, Scott Co., VA	W.J. Clench	NA
75. *Epioblasma capsaeformis*	ANSP 226964	Clinch River, Scott Co., VA	W.W. Robinette	NA
76. *Epioblasma capsaeformis*	ANSP 312234	Clinch River, Kyles Ford, Hancock Co., TN	D. Stansbery J. Jenkinson	10/12/66
77. *Epioblasma capsaeformis*	ANSP 335043	Clinch River, Kyles Ford, Hancock Co., TN	Fuller & Bereza	8/30/74
78. *Epioblasma capsaeformis*	ANSP 358955	Copper Creek, creek mile 1.4, Scott Co., VA	S. Ahlstedt	5/12/80
79. *Epioblasma capsaeformis*	ANSP 358960	Copper Creek, creek mile 2.1, Scott Co., VA	S. Ahlstedt	5/12/80
80. *Epioblasma capsaeformis*	ANSP 360020	Clinch River, Kyles Ford, Hancock Co., TN	S. Ahlstedt	7/31/74
81. *Epioblasma capsaeformis*	ANSP 360040	Clinch River, river mile 205.2, Scott Co., VA	S. Ahlstedt	8/13/72
82. *Epioblasma capsaeformis*	ANSP 360521	Clinch River, 5 miles downstream of Kyles Ford Hancock Co., TN	S. Ahlstedt	6/17/76
83. *Epioblasma capsaeformis*	ANSP 361204	Clinch River, Frost Ford, Hancock Co., TN	TVA	10/15/83

84. *Epioblasma capsaeformis*	ANSP 361253	North Fork Holston River, river mile 6.3, Scott Co., VA	TVA	11/16/78
85. *Epioblasma capsaeformis*	ANSP 361324	North Fork Holston River, Saltville, Scott Co., VA	TVA	NA
86. *Epioblasma capsaeformis*	ANSP 372637	West Prong Little Pigeon River, Sevierville, Sevier Co., TN	A. Bogan K. Seevers	11/25/88
87. *Epioblasma capsaeformis*	ANSP 372651	Little Pigeon River, Rt. 66 bridge, Sevierville, Sevier Co., TN	A. Bogan K. Seevers	11/25/88
88. *Epioblasma capsaeformis*	ANSP 376902	Clinch River, Clinchport Scott Co., VA	D. Tanner	8/12/70
89. *Epioblasma capsaeformis*	ANSP 376926	Clinch River, Wallen Bend, Hancock Co., TN	D. Tanner	8/8/70
90. *Epioblasma capsaeformis*	ANSP 376947	Clinch River, Wallen Bend, Hancock Co., TN	D. Tanner	NA
91. *Epioblasma capsaeformis*	ANSP 376992	Clinch River, Sneedville, Hancock Co., TN	D. Tanner	6/30/71
92. *Epioblasma capsaeformis*	ANSP 377073	Clinch River, Sneedville, Hancock Co., TN	D. Tanner	8/12/71
93. *Epioblasma capsaeformis*	ANSP 389176	Clinch River, 1 mile upstream of Kyles Ford, Hancock Co., TN	R. Dillon	July 75
94. *Epioblasma capsaeformis*	ANSP 389177	Powell River, McDowell Shoal, Hancock Co., TN	R. Dillon	August 1975
95. *Epioblasma capsaeformis*	ANSP 389178	Clinch River, Speers Ferry, Scott Co., VA	R. Dillon	7/27/75
96. *Epioblasma capsaeformis*	ANSP 397430	Elk River, Kelso, Lincoln Co., TN	D. Tanner	7/12/70
97. *Epioblasma capsaeformis*	ANSP 397431	Shoal Creek, AL	D. Tanner	NA
98. *Epioblasma capsaeformis*	CM 61.309	Big Moccasin Creek, Moccasin Gap, Scott Co., VA	A.E. Ortmann	5/16/13
99. *Epioblasma capsaeformis*	CM 61.645	Tennessee River, Knoxville, Knox Co., TN	Hartmann Collection	August 1947
100. *Epioblasma capsaeformis*	CM 61.921	Tennessee River, Knoxville, Knox Co., TN	Hartmann Collection	August 1947
101. *Epioblasma capsaeformis*	CM 61.1300	Tennessee River, Knox Co., TN	A.E. Ortmann	NA
102. *Epioblasma*	CM	Clinch River, Richlands,	A.E. Ortmann	9/20/13

	capsaeformis	61.6106	Tazewell Co., VA		
103.	*Epioblasma capsaeformis*	CM 61.6107	Clinch River, Raven, Tazewell Co., VA	A.E. Ortmann	9/21/12
104.	*Epioblasma capsaeformis*	CM 61.6303	Tennessee River, Tuscumbia, Colbert Co., AL	Smith Collection	August 1947
105.	*Epioblasma capsaeformis*	CM 61.6305	Clinch River, Cedar Bluff, Tazewell Co., VA	A.E. Ortmann	5/11/13
106.	*Epioblasma capsaeformis*	CM 61.6306	Clinch River, Cleveland, Russell Co., VA	A.E. Ortmann	5/13/13
107.	*Epioblasma capsaeformis*	CM 61.6308	Clinch River, St. Paul, Wise Co., VA	A.E. Ortmann	5/14/13
108.	*Epioblasma capsaeformis*	CM 61.6367	Clinch River, Fink, Russell Co., VA	A.E. Ortmann	5/12/13
109.	*Epioblasma capsaeformis*	CM 61.6461	North Fork Holston River, Rotherwood, Hawkins Co., TN	A.E. Ortmann	7/13/13
110.	*Epioblasma capsaeformis*	CM 61.6459	North Fork Holston River, Hilton, Scott Co., VA	A.E. Ortmann	7/7/13
111.	*Epioblasma capsaeformis*	CM 61.6460	Clinch River, Speers Ferry, Scott Co., VA	A.E. Ortmann	7/8/13
112.	*Epioblasma capsaeformis*	CM 61.6756	North Fork Holston River, Rotherwood, Hawkins Co., TN	A.E. Ortmann	9/5/13
113.	*Epioblasma capsaeformis*	CM 61.6757	Clinch River, Clinchport, Scott Co., TN	A.E. Ortmann	9/8/13
114.	*Epioblasma capsaeformis*	CM 61.6758	Clinch River, Clinch River Station, Claiborne Co., TN	A.E. Ortmann	9/11/13
115.	*Epioblasma capsaeformis*	CM 61.6759	Powell River, Combs, Claiborne Co., TN	A.E. Ortmann	9/12/13
116.	*Epioblasma capsaeformis*	CM 61.6760	Holston River, Holston Station, Grainger Co., TN	A.E. Ortmann	9/15/13
117.	*Epioblasma capsaeformis*	CM 61.6761	Holston River, McMillan Knox Co., TN	A.E. Ortmann	9/16/13
118.	*Epioblasma capsaeformis*	CM 61.6762	Nolichucky River, Chunns Shoals, Hamblen Co., TN	A.E. Ortmann	9/17/13
119.	*Epioblasma capsaeformis*	CM 61.701	Powell River, Cumberland Gap, TN	Hartman Collection	1947
120.	*Epioblasma capsaeformis*	CM 61.7052	Paint Rock River, Paint Rock, Jackson Co., AL	A.E. Ortmann	NA

121. *Epioblasma capsaeformis*	CM 61.7053	Paint Rock River, Trenton, Jackson Co., AL	A.E. Ortmann	NA
122. *Epioblasma capsaeformis*	CM 61.7054	Paint Rock River, Holly Tree, Jackson Co., AL	A.E. Ortmann	NA
123. *Epioblasma capsaeformis*	CM 61.7199	South Fork Holston River, Pactolus, Sullivan Co., TN	A.E. Ortmann	5/20/14
124. *Epioblasma capsaeformis*	CM 61.7200	Holston River, Nocton, Grainger Co., TN	A.E. Ortmann	5/22/14
125. *Epioblasma capsaeformis*	CM 61.7201	Holston River, Turley Mill, Jefferson Co., TN	A.E. Ortmann	5/23/14
126. *Epioblasma capsaeformis*	CM 61.7202	Holston River, McBee Ford, Hodger, Jefferson Co., TN	A.E. Ortmann	5/25/14
127. *Epioblasma capsaeformis*	CM 61.7506	Elk River, Fayetteville, Lincoln Co., TN	A.E. Ortmann	NA
128. *Epioblasma capsaeformis*	CM 61.7621	Clinch River, Clinton, Anderson Co., TN	A.E. Ortmann	9/7/14
129. *Epioblasma capsaeformis*	CM 61.7622	Clinch River, Edgemoor, Anderson Co., TN	A.E. Ortmann	9/8/14
130. *Epioblasma capsaeformis*	CM 61.7623	Clinch River, Salway, Knox Co., TN	A.E. Ortmann	9/12/14
131. *Epioblasma capsaeformis*	CM 61.7624	Holston River, Churchhill, Hawkins Co., TN	A.E. Ortmann	8/25/15
132. *Epioblasma capsaeformis*	CM 61.7625	Holston River, Austin Mill, Hawkins Co., TN	A.E. Ortmann	8/24/14
133. *Epioblasma capsaeformis*	CM 61.7626	Holston River, Mascot, Knox Co., TN	A.E. Ortmann	9/6/14
134. *Epioblasma capsaeformis*	CM 61.7627	Little Pigeon River, Sevierville, Sevier Co., TN	A.E. Ortmann	8/31/14
135. *Epioblasma capsaeformis*	CM 61.7697	Shoal Creek, Lauderdale Co., AL	A.E. Ortmann	NA
136. *Epioblasma capsaeformis*	CM 61.7698	Bear Creek, Franklin Co., AL	A.E. Ortmann	NA
137. *Epioblasma capsaeformis*	CM 61.8809	Big Moccasin Creek, Moccasin Gap, Scott Co., VA	A.E. Ortmann	9/9/15
138. *Epioblasma capsaeformis*	CM 61.8810	Powell River, Combs, Claiborne Co., TN	A.E. Ortmann	9/13/15
139. *Epioblasma capsaeformis*	CM 61.8811	Clinch River, Oakman, Grainger Co., TN	A.E. Ortmann	9/14/15

140. *Epioblasma capsaeformis*	CM 61.8812	Clinch River, Black Fox Ford, Union Co., TN	A.E. Ortmann	9/15/15
141. *Epioblasma capsaeformis*	CM 61.8813	Holston River, Mascot, Knox, Co., TN	A.E. Ortmann	9/16/15
142. *Epioblasma capsaeformis*	CM 61.8814	Clinch River, Edgemoor, Anderson Co., TN	A.E. Ortmann	9/17/15
143. *Epioblasma capsaeformis*	CM 61.11671	Richland Creek, Wales, Giles Co., TN	A.E. Ortmann	8/31/23
144. *Epioblasma capsaeformis*	FLMNH 41504	Clinch River, Kyles Ford, Hancock Co., TN	NA	NA
145. *Epioblasma capsaeformis*	FLMNH 41529	Clinch River, Clinchport, Scott Co., VA	NA	NA
146. *Epioblasma capsaeformis*	FLMNH 64240	Tennessee River, AL	NA	NA
147. *Epioblasma capsaeformis*	FLMNH 64244	Tennessee River, AL	NA	NA
148. *Epioblasma capsaeformis*	FLMNH 64247	Bear Creek, Burelson, Franklin Co., AL	H.H. Smith	8/1/09
149. *Epioblasma capsaeformis*	FLMNH 64248	Holston River, Sullivan Co., TN	G. Andrews	NA
150. *Epioblasma capsaeformis*	FLMNH 64249 **Topotype**	Cumberland River, Nashville, Davidson Co., TN	NA	NA
151. *Epioblasma capsaeformis*	FLMNH 64251	Clinch River, Clinchport, Scott Co., VA	NA	NA
152. *Epioblasma capsaeformis*	FLMNH 64252	North Fork Holston River, Hilton, Scott Co., VA	A.E. Ortmann	7/7/13
153. *Epioblasma capsaeformis*	FLMNH 64338	Tennessee River, Florence, Lauderdale Co., AL	H.H. Smith	11/1/09
154. *Epioblasma capsaeformis*	FLMNH 64340	Tennessee River, AL	NA	NA
155. *Epioblasma capsaeformis*	FLMNH 195047	Clinch River, Pendleton Island, Scott Co., VA	J.Jenkinson	11/17/81
156. *Epioblasma capsaeformis*	FLMNH 225999	Obey River, Byrdstown, Picket Co., TN	NA	NA
157. *Epioblasma capsaeformis*	FLMNH 226000	Clinch River, Olney	NA	NA

158. *Epioblasma capsaeformis*	FLMNH 226001	Powell River, Lee Co., VA	NA	NA
159. *Epioblasma capsaeformis*	FLMNH 226002	Holston River, TN	NA	NA
160. *Epioblasma capsaeformis*	FLMNH 229368	Clinch River, Kyles Ford, Hancock Co., TN	NA	NA
161. *Epioblasma capsaeformis*	FLMNH 269044	Holston River, TN	G. Andrews	NA
162. *Epioblasma capsaeformis*	FLMNH 269048	Clinch River, Hancock Co., TN	B. Walker	NA
163. *Epioblasma capsaeformis*	FLMNH 269051	Tennessee River, Florence, Lauderdale Co., AL	W. Simpson	NA
164. *Epioblasma capsaeformis*	MCZ 16402	Holston River, Knoxville, Knox Co., TN	R.E. Call	NA
165. *Epioblasma capsaeformis*	MCZ 16772	North Fork Holston River, Cloud Ford, Sullivan Co., TN	A.H. Clarke	NA
166. *Epioblasma capsaeformis*	MCZ 16773	North Fork Holston River, Cloud Ford, Sullivan Co., TN	A.H. Clarke	NA
167. *Epioblasma capsaeformis*	MCZ 29828	Shoal Creek, Lauderdale Co., AL	B. Walker Collection	NA
168. *Epioblasma capsaeformis*	MCZ 46714	Clinch River, Clinton, Anderson Co., TN	NA	NA
169. *Epioblasma capsaeformis*	MCZ 46731	Holston River, Austin's Mill, Knox Co., TN	M.D. Burber	NA
170. *Epioblasma capsaeformis*	MCZ 63005	North Fork Holston River, Hilton, Scott Co., VA	B. Walker	NA
171. *Epioblasma capsaeformis*	MCZ 72352	Clinch River, Clinton, Anderson Co., TN	NA	NA
172. *Epioblasma capsaeformis*	MCZ 79759	Clinch River, Clinchport, Scott Co., VA	R.W. Jackson	NA
173. *Epioblasma capsaeformis*	MCZ 83037	Powell River, Jonesville, Lee Co., VA	W.J. Clench & Archer	1932
174. *Epioblasma capsaeformis*	MCZ 89441	Shoal Creek, Lauderdale Co., AL	H.H. Smith	NA
175. *Epioblasma capsaeformis*	MCZ 98537	Duck River, Hardinson's Mill, TN	W.J. Clench H. Van Der Schalie	1933
176. *Epioblasma*	MCZ	Clinch River, Kyles Ford,	B. Walker	NA

	capsaeformis	152217	Hancock Co., TN	Collection	
177.	*Epioblasma capsaeformis*	MCZ 159742	Cumberland River/Beaver Creek, Rowena Ferry, Russell Co., KY	H. van der Schalie	NA
178.	*Epioblasma capsaeformis*	MCZ 178568 **Idiotype**	Cumberland River	J.G. Anthony	NA
179.	*Epioblasma capsaeformis*	MCZ 178570 **Holotype**	Cumberland River	J.G. Anthony	NA
180.	*Epioblasma capsaeformis*	MCZ 190469	Powell River, Tazewell, Claiborne Co., TN	W.J. Clench & Turner	August 1954
181.	*Epioblasma capsaeformis*	MCZ 192902	North Fork Holston River, Morrison City, Sullivan Co., TN	H.D. Athearn	NA
182.	*Epioblasma capsaeformis*	MCZ 198413	Elk River, Fayetteville, Lincoln Co., TN	A. Clarke	NA
183.	*Epioblasma capsaeformis*	MCZ 200345	Clinch River, Fort Blackmore, Scott Co., VA	H.D. Athearn	10/4/53
184.	*Epioblasma capsaeformis*	MCZ 236078	Buck Creek, Rt. 192 Bridge, Pulaski Co., KY	H.D. Athearn	5/17/59
185.	*Epioblasma capsaeformis*	MCZ 236708	Obey River, Pryor Bend, Pickett Co., TN	W.G. Parris	NA
186.	*Epioblasma capsaeformis*	MCZ 236709	Paint Rock River, Holly Tree, Jackson Co., AL	B. Walker Collection	NA
187.	*Epioblasma capsaeformis*	MCZ 236710	Flint River, Gurley, Madison Co., AL	H.H. Smith	NA
188.	*Epioblasma capsaeformis*	MCZ 236712	Paint Rock River, Princeton, Jackson Co., AL	H.H. Smith	NA
189.	*Epioblasma capsaeformis*	MCZ 236714	Powell River, Greens Ford, Long Hollow, Union Co., TN	NA	NA
190.	*Epioblasma capsaeformis*	MCZ 236715	Powell River, Lee Co., VA	W.W. Robinette	11/28/30
191.	*Epioblasma capsaeformis*	MCZ 236716	Paint Rock River, Trenton, Jackson Co., AL	B. Walker Collection	NA
192.	*Epioblasma capsaeformis*	MCZ 236717	Paint Rock River, Jackson Co., AL	NA	NA
193.	*Epioblasma capsaeformis*	MCZ 236718	Tennessee River, Muscle Shoals, AL	B. Walker Collection	NA
194.	*Epioblasma*	MCZ	Clinch River, “Rounds”,	J.R. Brotherton	October

	capsaeformis	236719	Hancock Co., TN		1930
195.	*Epioblasma capsaeformis*	MCZ 250877	Clinch River, Kyles Ford, Hancock Co., TN	R.I. Johnson	7/8/64
196.	*Epioblasma capsaeformis*	MCZ 263196	Nolichucky River, Warrensburg, Greene Co., TN	D.H. Stansbery W.J. Clench	9/19/6
197.	*Epioblasma capsaeformis*	MCZ 267435	Mouth of Copper Creek, Scott Co., VA	NA	May 1968
198.	*Epioblasma capsaeformis*	MCZ 268725	Clinch River, Clinchport, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/9/63
199.	*Epioblasma capsaeformis*	MCZ 268767	Clinch River, Nash Ford, Russell Co., VA	D.H. Stansbery J.J. Jenkinson	10/7/65
200.	*Epioblasma capsaeformis*	MCZ 268800	Clinch River, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/9/65
201.	*Epioblasma capsaeformis*	MCZ 268841	Clinch River, Rt. 25 E Bridge, Grainger Co., TN	D.H. Stansbery J.J. Jenkinson	10/13/65
202.	*Epioblasma capsaeformis*	MCZ 268868	Clinch River, Rt. 80 Bridge, Russell Co., VA	D.H. Stansbery J.J. Jenkinson	10/6/65
203.	*Epioblasma capsaeformis*	MCZ 268920	Clinch River, Speers Ferry, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/7/65
204.	*Epioblasma capsaeformis*	MCZ 268927	Clinch River, Speers Ferry, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/9/65
205.	*Epioblasma capsaeformis*	MCZ 269018	Clinch River, Cleveland, Russell Co., VA	D.H. Stansbery J.J. Jenkinson	10/7/65
206.	*Epioblasma capsaeformis*	MCZ 269121	Mouth of Copper Creek, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/10/65
207.	*Epioblasma capsaeformis*	MCZ 274943	Elk River, U.S. Rt. 64 Bridge, Fayetteville Co., TN	W.J. Clench D.H. Stansbery	10/3/67
208.	*Epioblasma capsaeformis*	MCZ 276402	Harpeth River, Franklin, William Co., TN	H.D. Athearn	9/6/54
209.	*Epioblasma capsaeformis*	MCZ 288386	Powell River, Tazewell, Claiborne Co., TN	W.J. Clench D.H. Stansbery	1968
210.	*Epioblasma capsaeformis*	MCZ 288387	Obey River, Duncan Ford, Lilydale, Pickett Co., TN	NA	NA
211.	*Epioblasma capsaeformis*	MCZ 289436	Red River, Adams, Robertson Co., TN	H.D. Athearn	5/29/66
212.	*Epioblasma capsaeformis*	MCZ 293618	Clinch River, Clinchport, Scott Co., VA	R.E. Winters	9/23/78

213. *Epioblasma capsaeformis*	MCZ 294156	Clinch River, Fort Blackmore, Pendelton Island, Scott Co., VA	R.J. Neves	8/4/83
214. *Epioblasma capsaeformis*	MCZ 295377	Harpeth River, Davidson Co., TN	S.T. Dillon	12/26/55
215. *Epioblasma capsaeformis*	OSM 4147	NA	NA	NA
216. *Epioblasma capsaeformis*	OSM 8546	Clinch River, St. Paul, Russell Co., VA	C.B. Stein	7/16/63
217. *Epioblasma capsaeformis*	OSM 8680	Clinch River, St. Paul, Wise Co., VA	D.H. Stansbery	8/29/63
218. *Epioblasma capsaeformis*	OSM 9525	NA	NA	NA
219. *Epioblasma capsaeformis*	OSM 10378	Tennessee River	NA	1800's
220. *Epioblasma capsaeformis*	OSM 10757	Clinch River, 1.1 upstream of Speers Ferry, Scott Co., VA	C.B. Stein	7/17/63
221. *Epioblasma capsaeformis*	OSM 11273	Clinch River, Speers Ferry, Scott Co., VA	C.B. Stein	7/17/63
222. *Epioblasma capsaeformis*	OSM 11360	Clinch River, Rt. 65 Bridge, Scott Co., VA	C.B. Stein	7/16/63
223. *Epioblasma capsaeformis*	OSM 11399	Clinch River, Rt. 619 Bridge, Scott Co., VA	C.B. Stein	7/15/63
224. *Epioblasma capsaeformis*	OSM 12132	Clinch River, Rt. 23 Bridge, Speers Ferry, Scott Co., VA	F.J. Moore	8/1/64
225. *Epioblasma capsaeformis*	OSM 16036	Clinch River, Fort Blackmore, Scott Co., VA	Moore Matanzo	3/26/66
226. *Epioblasma capsaeformis*	OSM 16165	Elk River, Rt. 50 Bridge, Franklin Co., TN	P. Yokley B. Isom	9/17/65
227. *Epioblasma capsaeformis*	OSM 16184	Elk River, Rt. 64 Bridge, Lincoln Co., TN	B.G. Isom P. Yokley	9/24/65
228. *Epioblasma capsaeformis*	OSM 16360	Clinch River, Nash Ford, Russell Co., VA	D.H. Stansbery J.J. Jenkinson	10/7/65
229. *Epioblasma capsaeformis*	OSM 16381	Clinch River, Cleveland, Russell Co., VA	D.H. Stansbery J.J. Jenkinson	10/7/65
230. *Epioblasma capsaeformis*	OSM 16415	Clinch River, St. Paul, Wise Co., VA	D.H. Stansbery J.J. Jenkinson	10/8/65
231. *Epioblasma capsaeformis*	OSM 16574	Clinch River, Dungannon, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/9/65

232. *Epioblasma capsaeformis*	OSM 16628	Clinch River, Rt. 619 Bridge, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/9/65
233. *Epioblasma capsaeformis*	OSM 16700	Clinch River, 1.5 m below Speers Ferry, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/10/65
234. *Epioblasma capsaeformis*	OSM 16735	Copper Creek, at mouth, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/10/65
235. *Epioblasma capsaeformis*	OSM 16799	Clinch River, Rt. 25 Bridge, Grainger Co., TN	D.H. Stansbery J.J. Jenkinson	10/13/65
236. *Epioblasma capsaeformis*	OSM 17268	Copper Creek, at mouth, Scott Co., VA	D.H. Stansbery J.J. Jenkinson	10/10/65
237. *Epioblasma capsaeformis*	OSM 18725	Paint Rock River, Rocky Hollow Ford, Jackson Co., AL	D.H. Stansbery	9/30/66
238. *Epioblasma capsaeformis*	OSM 18742	Estill Fork Paint Rock River, Jackson Co., AL	D.H. Stansbery	9/29/66
239. *Epioblasma capsaeformis*	OSM 19074	Elk River, Rt. 64 Bridge, Lincoln Co., TN	D.H. Stansbery	10/4/66
240. *Epioblasma capsaeformis*	OSM 19401	Powell River, near Hoop, Claiborne Co., TN	D.H. Stansbery	9/22/67
241. *Epioblasma capsaeformis*	OSM 19562	Powell River, near Hoop, Claiborne Co., TN	D.H. Stansbery	10/23/67
242. *Epioblasma capsaeformis*	OSM 19854	Elk River, Rt. 50 bridge	B.G. Isom	9/19/67
243. *Epioblasma capsaeformis*	OSM 20636	Paint Rock River, 0.5 m below Swain, Jackson Co., AL	D.H. Stansbery	9/29/66
244. *Epioblasma capsaeformis*	OSM 20803	Powell River, near Hoop, Brooks Bridge, Claiborne Co., TN	D.H. Stansbery	9/20/68
245. *Epioblasma capsaeformis*	OSM 20869	NA	NA	1800's
246. *Epioblasma capsaeformis*	OSM 22425	Powell River, Rt. 25 Bridge, Claiborne Co., TN	D.H. Stansbery F. McMurray	9/16/67
247. *Epioblasma capsaeformis*	OSM 23212	Powell River, near Hoop, Brooks Bridge, Claiborne Co., TN	D.H. Stansbery	10/19/69
248. *Epioblasma capsaeformis*	OSM 23397	Nolichucky River, Rt. 2486 Bridge, Green Co., TN	D.H. Stansbery W.J. Clench	9/19/68
249. *Epioblasma capsaeformis*	OSM 23423	Nolichucky River, Rt. 160 Bridge, Hamblen Co., TN	D.H. Stansbery et al.	10/17/69
250. *Epioblasma*	OSM	Powell River, 1 m from	W.W. Robinette	1900's

	capsaeformis	23886	Jonesville, Lee Co., VA		
251.	*Epioblasma capsaeformis*	OSM 24378	Clinch River, below Rt. 25 Bridge, Claiborne Co., TN	D.H. Stansbery W.J. Clench	9/19/68
252.	*Epioblasma capsaeformis*	OSM 24871	Harpeth River, Snead Rd. Bridge, Williamson Co., TN	H.D. Athearn	9/6/64
253.	*Epioblasma capsaeformis*	OSM 24997	Elk River, Rt. 64 Bridge, Lincoln Co., TN	H.D. Athearn	8/10/57
254.	*Epioblasma capsaeformis*	OSM 25461	Copper Creek, at mouth, Scott Co., VA	D.H. Stansbery W.J. Clench	10/7/70
255.	*Epioblasma capsaeformis*	OSM 25623	Clinch River, Clinchport, Scott Co., VA	D.P. Taner	8/11/70
256.	*Epioblasma capsaeformis*	OSM 26251	Clinch River, 2 m upstream of Kyles Ford, Hancock Co., TN	D.P. Tanner	8/8/70
257.	*Epioblasma capsaeformis*	OSM 26619	Clinch River, Kyles Ford, Hancock Co., TN	D.H. Stansbery	9/21/67
258.	*Epioblasma capsaeformis*	OSM 26968	Clinch River, Kyles Ford, Hancock Co., TN	D.H. Stansbery W.J. Clench	10/1/71
259.	*Epioblasma capsaeformis*	OSM 27028	Buck Creek, at Stab, Pulaski Co., KY	J.J. Jenkinson	9/23/71
260.	*Epioblasma capsaeformis*	OSM 28137	Clinch River, at mouth of Possumtrot Creek, Hancock Co., TN	D.H. Stansbery et al.	10/18/69
261.	*Epioblasma capsaeformis*	OSM 28285	NA	Dr. Troost	1800's
262.	*Epioblasma capsaeformis*	OSM 29722	Caney Fork River, Dekalb Co., TN	W.R. Haag R.M. Anderson	5/28/88
263.	*Epioblasma capsaeformis*	OSM 33222	Clinch River, 4.2 m below Horton Ford, Hancock Co., TN	D.H. Stansbery W.J. Clench	10/16/72
264.	*Epioblasma capsaeformis*	OSM 33506	Clinch River, Sneedville, Hancock Co., TN	D.H. Stansbery W.J. Clench	10/16/72
265.	*Epioblasma capsaeformis*	OSM 34812	Paint Rock River, Woodville, AL	NA	prior to 1973
266.	*Epioblasma capsaeformis*	OSM 34813	Shoal Creek, Lauderdale, Co. TN	NA	prior to 1973
267.	*Epioblasma capsaeformis*	OSM 35021	Holston River, Three Springs, Hamblen Co., TN	J.F. Boepple	10/8/09
268.	*Epioblasma capsaeformis*	OSM 35081	Clinch River, Clinchport, Scott Co., VA	J.F. Boepple	9/21/09

269. *Epioblasma capsaeformis*	OSM 35118	Clinch River, Grainger Co., TN	J.F. Boepple	10/25/09
270. *Epioblasma capsaeformis*	OSM 35138	Clinch River, Union Co., TN	J.F. Boepple	10/27/09
271. *Epioblasma capsaeformis*	OSM 35151	North Fork Holston River, Wilhelm, Scott Co., VA	J.F. Boepple	9/30/09
272. *Epioblasma capsaeformis*	OSM 36885	Clinch River, Kyles Ford, Hancock Co., TN	NA	prior to 1974
273. *Epioblasma capsaeformis*	OSM 37870	Clinch River, TN	NA	1800's
274. *Epioblasma capsaeformis*	OSM 37871	Clinch River, TN	NA	1800's
275. *Epioblasma capsaeformis*	OSM 37872	Clinch River, TN	J. Lewis	1800's
276. *Epioblasma capsaeformis*	OSM 37945	Clinch River, at mouth of Possumtrot Creek, Hancock Co., TN	M.E. St. John	6/14/71
277. *Epioblasma capsaeformis*	OSM 38071	Buck Creek, at Rt. 1003, Pulaski Co., KY	D.H. Stansbery	10/31/75
278. *Epioblasma capsaeformis*	OSM 38123	Tennessee River	NA	1800's
279. *Epioblasma capsaeformis*	OSM 38124	Tennessee River	NA	1800's
280. *Epioblasma capsaeformis*	OSM 38236	Paint Rock River, Little Nashville bridge, Jackson Co., AL	D.H. Stansbery W.J. Clench	10/4/67
281. *Epioblasma capsaeformis*	OSM 38295	Estill Fork Paint Rock River, Jackson Co., AL	C. B. Stein J. Frederick	10/31/73
282. *Epioblasma capsaeformis*	OSM 38313	Paint Rock River, along Rt. 65, 0.8 m S of Princeton, Jackson Co., AL	C. B. Stein J. Frederick	10/31/73
283. *Epioblasma capsaeformis*	OSM 38522	Paint Rock River, Rocky Hollow Ford, Jackson Co., AL	B.G. Isom	9/21/65
284. *Epioblasma capsaeformis*	OSM 38533	Paint Rock River, bridge 1 m S of Princeton, Jackson Co., AL	B.G. Isom	9/21/65
285. *Epioblasma capsaeformis*	OSM 39144	Estill Fork Paint Rock River, Jackson Co., AL	D.H. Stansbery K.G. Borror	10/1/76
286. *Epioblasma capsaeformis*	OSM 39464	Paint Rock River, along Rt. 65, 0.8 m S of Princeton, Jackson	D.H. Stansbery	10/2/76

		Co., AL		
287. *Epioblasma capsaeformis*	OSM 39533	Larkin Fork Paint Rock River, at mouth, Jackson Co., AL	D.H. Stansbery	10/2/67
288. *Epioblasma capsaeformis*	OSM 39696	Estill Fork Paint Rock River, Jackson Co., AL	D.H. Stansbery K.G. Borror	9/28/76
289. *Epioblasma capsaeformis*	OSM 40236	Clinch River, Horton Ford, Hancock Co., TN	D.H. Stansbery W.J. Clench	7/29/77
290. *Epioblasma capsaeformis*	OSM 43193	Clinch River, Kyles Ford, Hancock Co., TN	D.H. Stansbery K.G. Borror	10/22/78
291. *Epioblasma capsaeformis*	OSM 42007	Clinch River, Clinchport, Scott Co., VA	C.R. Ciola K.L. Ciola	7/2/78
292. *Epioblasma capsaeformis*	OSM 43166	Copper Creek, at mouth, Scott Co., VA	D.H. Stansbery K.G. Borror	10/21/78
293. *Epioblasma capsaeformis*	OSM 43193	Clinch River, Kyles Ford, Hancock Co., TN	D.H. Stansbery K.G. Borror	10/22/78
294. *Epioblasma capsaeformis*	OSM 43364	Clinch River, the Rounds, Hancock Co., TN	D.H. Stansbery K.G. Borror	10/22/78
295. *Epioblasma capsaeformis*	OSM 43401	Clinch River, Clinchport, Scott Co., VA	J.M. Condit C.R. Ciola	7/16/78
296. *Epioblasma capsaeformis*	OSM 44080	Clinch River, Brooks Island, Hancock Co., TN	D.H. Stansbery	8/17/68
297. *Epioblasma capsaeformis*	OSM 44155	Clinch River, Kyles Ford, Hancock Co., TN	D.H. Stansbery	8/17/68
298. *Epioblasma capsaeformis*	OSM 45468	Big South Fork Cumberland River, mouth of Troublesome Creek, McCreary Co., KY	S. Call M. Warren	8/29/79
299. *Epioblasma capsaeformis*	OSM 47879	Copper Creek, CM 2.1, Scott Co., VA	S. Ahlstedt	5/12/80
300. *Epioblasma capsaeformis*	OSM 49328	Clinch River, Slant, Scott Co., VA	C. Coney	1/5/81
301. *Epioblasma capsaeformis*	OSM 49422	Buck Creek, at Stab, Pulaski Co., KY	Fallo	10/11/80
302. *Epioblasma capsaeformis*	OSM 49461	Clinch River, Slant, Scott Co., VA	C. Coney	12/15/80
303. *Epioblasma capsaeformis*	OSM 49787	Buck Creek, between Rt. 461 & Rt. 80, Pulaski Co., KY	Fallo	5/25/81
304. *Epioblasma capsaeformis*	OSM 50053	Caney Fork River, WSW of Stonewall, Smith Co., TN	Schmidt	7/12/81

305. *Epioblasma capsaeformis*	OSM 50361	Clinch River, Pendleton Island, Scott Co., VA	S. Ahlstedt	11/25/79
306. *Epioblasma capsaeformis*	OSM 50508	Little River, Davis Ford, Blount Co., TN	J. Webb via S. Ahlstedt	1980
307. *Epioblasma capsaeformis*	OSM 53895	Clinch River, Pendleton Island, Scott Co., VA	R.J. Neves	8/4/83
308. *Epioblasma capsaeformis*	OSM 53909	Copper Creek, CM 1.8, Scott Co., VA	S. Ahlstedt	5/13/80
309. *Epioblasma capsaeformis*	OSM 54842	Clinch River, Clinchport, Scott Co., VA	D.H. Stansbery	10/21/73
310. *Epioblasma capsaeformis*	OSM 54895	Clinch River, Cleveland, Russell Co., VA	D.H. Stansbery	10/20/73
311. *Epioblasma capsaeformis*	OSM 54929	Clinch River, 7.8 m above Horton Ford, Scott Co., VA	D.H. Stansbery W.J. Clench	8/12/74
312. *Epioblasma capsaeformis*	OSM 54956	Clinch River, Clinchport, Scott Co., VA	J.M. Condit C.R. Ciola	7/9/81
313. *Epioblasma capsaeformis*	OSM 54976	Clinch River, Pendleton Island, Scott Co., VA	S. Ahlstedt	9/22/84
314. *Epioblasma capsaeformis*	OSM 55225	Buck Creek, at Stab, Pulaski Co., KY	D.H. Stansbery W.J. Clench	8/15/74
315. *Epioblasma capsaeformis*	OSM 55425	Clinch River, 0.3 m N of Cleveland, Russell Co., VA	D.H. Stansbery G.T. Watters	10/7/85
316. *Epioblasma capsaeformis*	OSM 57038	Clinch River, Walkers Ford, Union Co., AL	NA	1800's
317. *Epioblasma capsaeformis*	OSM 57325	Cumberland River, Nashville, Davidson Co., TN	Lindsley	prior to 1886
318. *Epioblasma capsaeformis*	OSM 57336	French Broad River, TN	Edgar	prior to 1886
319. *Epioblasma capsaeformis*	OSM 57295	Tennessee River, Muscle Shoals, Lauderdale Co., AL	B. Pylas	prior to 1886
320. *Epioblasma capsaeformis*	OSM 57341	Tennessee River, Florence, Lauderdale Co., AL	G. White	prior to 1886
321. *Epioblasma capsaeformis*	OSM 67903	Clinch River, Union Co., TN	Wetherby	1876
322. *Epioblasma capsaeformis*	OSM 68059	Cumberland River, Nashville, Davidson Co., TN	Downie	prior to 1886
323. *Epioblasma*	OSM	Clinch River, Anderson Co., TN	Boepple	10/30/09

capsaeformis	68070			
324. *Epioblasma capsaeformis*	USNM 25719	Tennessee River	NA	NA
325. *Epioblasma capsaeformis*	USNM 592118	Beaver Creek, Russell Co., KY, E. of Rowena Ferry	van der Schallie	1947
326. *Epioblasma capsaeformis*	USNM 84939 **Holotype**	Cumberland River, TN	NA	NA
327. *Epioblasma capsaeformis*	USNM 84940	French Broad River, TN	NA	NA
328. *Epioblasma capsaeformis*	USNM 84941	Tennessee River, Florence, Lauderdale Co., AL	NA	NA
329. *Epioblasma capsaeformis*	USNM 126955	Clinch River, Walkers Ford, TN	NA	NA
330. *Epioblasma capsaeformis*	USNM 133474	Waldens Creek, Lee Co., VA	NA	NA
331. *Epioblasma capsaeformis*	USNM 133491	Clinch River, Robinette, Scott Co., VA	NA	NA
332. *Epioblasma capsaeformis*	USNM 133516	Clinch River, Robinette, Scott Co., VA	NA	NA
333. *Epioblasma capsaeformis*	USNM 150071	Powell River, Jonesville, Lee Co., VA	NA	NA
334. *Epioblasma capsaeformis*	USNM 218049	Elk River, Fayetteville, TN	NA	NA
335. *Epioblasma capsaeformis*	USNM 464761	Clinch River, Robinette, Scott Co., VA	NA	NA
336. *Epioblasma capsaeformis*	USNM 656580	Shoal Creek, Lauderdale Co., AL	NA	NA
337. *Epioblasma capsaeformis*	USNM 656721	Clinch River, Clinchport, Scott Co., VA	NA	NA
338. *Epioblasma capsaeformis*	USNM 656742	Clinch River, Clinchport, Scott Co., VA	NA	NA
339. *Epioblasma capsaeformis*	USNM 853932	Red River, Robertson Co., TN	NA	NA
340. *Epioblasma f. florentina*	ANSP 48399	Holston River, Knox Co., TN	B. Walker	1919
341. *Epioblasma f. florentina*	ANSP 56403	Tennessee River, AL	NA	NA

	Paratype			
342. *Epioblasma f. florentina*	ANSP 100541	Tennessee River, Muscle Shoals, Lauderdale Co., AL	H.H. Smith	1910
343. *Epioblasma f. florentina*	ANSP 125986	Tennessee River, Muscle Shoals, AL	Wheatley Collection	NA
344. *Epioblasma f. florentina*	ANSP 125989	Tennessee River, Muscle Shoals, AL	Wheatley Collection	NA
345. *Epioblasma f. florentina*	ANSP 365637	Tennessee River, Florence, Lauderdale Co., AL	Alfred University Collection	NA
346. *Epioblasma florentina*	ANSP 397429	Red River, TN	D. Tanner	NA
347. *Epioblasma florentina*	CM 61.4491 soft-part collection	Shoal Creek, Lauderdale Co., AL	A.E. Ortmann	NA
348. *Epioblasma florentina*	CM 61.6763 soft-part collection	Holston River, Holston Station, Grainger Co., TN	A.E. Ortmann	NA
349. *Epioblasma f. florentina*	FMNH 269060	Holston River, Knoxville, Knox Co., TN	G. Andrews	NA
350. *Epioblasma f. florentina*	FMNH 269061	Duck River, Columbia, Maury Co., TN	A.A Hinkley	NA
351. *Epioblasma f. florentina*	FMNH 269062	Tennessee River, Florence, Lauderdale Co., AL	W. Simpson	NA
352. *Epioblasma f. florentina*	FMNH 270274	Tennessee River, Tuscumbia, Colbert Co., AL	H.H. Smith	NA
353. *Epioblasma f. florentina*	MCZ 16831	Tennessee	J.G. Anthony	NA
354. *Epioblasma f. florentina*	MCZ 37392	Paint Rock River, Poplar Bluff Madison Co., AL	E.W. Roper	NA
355. *Epioblasma f. florentina*	MCZ 161897 **Paratype**	Tennessee	J.G. Anthony	NA
356. *Epioblasma f. florentina*	MCZ 161898 **Holotype**	Tennessee	J.G. Anthony	NA
357. *Epioblasma f. florentina*	MCZ 178569 **Cotype**	Tennessee	J.G. Anthony	NA

358. *Epioblasma f. florentina*	MCZ 178929 **Idiotype**	Alabama	J.G. Anthony	NA
359. *Epioblasma f. florentina*	MCZ 236720	Tennessee River, Florence, Lauderdale Co., AL	B. Walker	NA
360. *Epioblasma florentina*	MCZ 236721	Obey River, Duncan Ford, Pickett Co., TN	W.G. Parris	NA
361. *Epioblasma f. florentina*	MCZ 236722	Duck River, Columbia, Maury Co., TN	B. Walker	NA
362. *Epioblasma f. florentina*	MCZ 288092	Harpeth River, Davidson Co., TN	S.T. Dillon	12/22/55
363. *Epioblasma f. florentina*	OSM 10376	Clinch River, TN	NA	NA
364. *Epioblasma f. florentina*	OSM 10377	Clinch River, TN	NA	NA
365. *Epioblasma f. florentina*	OSM 10449	Clinch River, TN	NA	NA
366. *Epioblasma f. florentina*	OSM 37873	Clinch River, TN	NA	NA
367. *Epioblasma f. florentina*	OSM 56948	Clinch River, TN	NA	NA
368. *Epioblasma f. florentina*	OSM 57244	Tennessee River, Muscle Shoals, Lauderdale Co., AL	L.B. Thorton B. Pybas	1857-1886
369. *Epioblasma f. florentina*	OSM 67870	Tennessee River, Muscle Shoals, Lauderdale Co., AL	H. Moores	1886
370. *Epioblasma f. florentina*	USNM 84948 **Holotype**	Tennessee River, Florence, AL	G. White	NA
371. *Epioblasma f. walkeri*	ANSP 103740	Flint River, Maysville, AL	H.H. Smith	NA
372. *Epioblasma f. walkeri*	ANSP 103903	Hurricane Creek, Gurley, AL	H.H. Smith	NA
373. *Epioblasma f. walkeri*	ANSP 103997	Flint River, Gurley, AL	H.H. Smith	NA
374. *Epioblasma f. walkeri*	CM 61.6765 soft-part collection	South Fork Holston River, Barren, Washington Co., VA	A.E. Ortmann	NA

375. *Epioblasma f. walkeri*	CM 61.6767 soft-part collection	South Fork Holston River, Emmitt, Sullivan Co., TN	A.E. Ortmann	NA
376. *Epioblasma f. walkeri*	CM 61.11668 soft-part collection	Duck River, Wilhoite, Marshall Co., TN	A.E. Ortmann	NA
377. *Epioblasma f. walkeri*	FMNH 64260	Stones River, Walter Hill, Rutherford Co., AL	NA	NA
378. *Epioblasma f. walkeri*	FMNH 269050	Flint River, Madison Co., AL	B. Walker	NA
379. *Epioblasma f. walkeri*	FMNH 269057	Tennessee	C.T. Simpson	NA
380. *Epioblasma f. walkeri*	FMNH 269059	Tennessee	NA	NA
381. *Epioblasma f. walkeri*	MCZ 98461	Stones River, Murfreesboro, Rutherford Co., TN	W.J. Clench van der Schalie	1933
382. *Epioblasma f. walkeri*	MCZ 268686	Stones River, Couchville, Davidson Co., TN	D.H. Stansbery J. Jenkinson	10/14/65
383. *Epioblasma f. walkeri*	MCZ 274910	East Fork Stones River, Walterhill, Rutherford Co., TN	D.H. Stansbery	April 1968
384. *Epioblasma f. walkeri*	MCZ 276026	Middle Fork Holston River, Rt. 91 bridge, Smyth Co., VA	D.H. Stansbery W.J. Clench	9/16/68
385. *Epioblasma f. walkeri*	MCZ 293010 **Paralecotype**	East Fork Stones River, Walterhill, Rutherford Co., TN	Wilson & Clark	8/21/11
386. *Epioblasma f. walkeri*	MCZ 293653	Middle Fork Holston River, Chilhowie, Smyth Co., VA	R.E. Winters	9/2/77
387. *Epioblasma f. walkeri*	OSM 14223	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	B.G. Isom R.M. Sinclair	9/23/64
388. *Epioblasma f. walkeri*	OSM 14295	Stones River, above Couchville, Pike Bridge, Davidson Co., TN	B.G. Isom R.M. Sinclair	9/24/64
389. *Epioblasma f. walkeri*	OSM 14371	Stones River, above mouth of Stewart Creek, Rutherford Co., TN	B.G. Isom R.M. Sinclair	9/24/64
390. *Epioblasma f. walkeri*	OSM 14487	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	C.B. Stein	7/19/64

391. *Epioblasma f. walkeri*	OSM 14580	East Fork Stones River, 1.5 m above W. Fork confluence, Rutherford Co., TN	B.G. Isom R.M. Sinclair	9/24/64
392. *Epioblasma f. walkeri*	OSM 15150	Duck River, Wilhoite Mills, Marshall Co., TN	D.H. Stansbery	9/8/64
393. *Epioblasma f. walkeri*	OSM 15595	Stones River, above Couchville, Pike Bridge, Davidson Co., TN	D.H. Stansbery J.J. Jenkinson	10/14/65
394. *Epioblasma f. walkeri*	OSM 16266	Clinch River, Cedar Bluff, Rt. 460 Bridge, Tazewell Co., VA	D.H. Stansbery J.J. Jenkinson	10/6/65
395. *Epioblasma f. walkeri*	OSM 16344	Clinch River, Rt. 80 Bridge, Russel Co., VA	D.H. Stansbery J.J. Jenkinson	10/6/65
396. *Epioblasma f. walkeri*	OSM 16998	Red River, Rt. 41 Bridge, Robertson Co., TN	D.H. Stansbery	10/8/66
397. *Epioblasma f. walkeri*	OSM 22029	Red River, mouth of Sulphur Fork, Montgomery Co., TN	D.H. Stansbery	10/8/66
398. *Epioblasma f. walkeri*	OSM 23078	Red River, 4.5 m NE of Adams, Robertson Co., TN	D.H. Stansbery	10/11/69
399. *Epioblasma f. walkeri*	OSM 23157	Red River, Rt. 161 Bridge, Robertson Co., TN	D.H. Stansbery	10/11/69
400. *Epioblasma f. walkeri*	OSM 24342	Middle Fork Holston River, Rt. 91 bridge, Smyth Co., VA	D.H. Stansbery W.J. Clench	9/16/68
401. *Epioblasma f. walkeri*	OSM 25330	Middle Fork Holston River, Rt. 638 bridge, Smyth Co., VA	D.H. Stansbery	8/28/70
402. *Epioblasma f. walkeri*	OSM 29072	Duck River, Rt. 65 Bridge, Maury Co., TN	S.A. Ahlstedt	4/26/88
403. *Epioblasma f. walkeri*	OSM 34943	Middle Fork Holston River, Chilhowie, Smyth Co., VA	D.H. Stansbery	10/16/73
404. *Epioblasma f. walkeri*	OSM 42198	Middle Fork Holston River, Rt. 638 Bridge, Smyth Co., VA	D.H. Stansbery F.L. Kokai	7/24/78
405. *Epioblasma f. walkeri*	OSM 42231	Clinch River, Cedar Bluff, Rt. 460 Bridge, Tazewell Co., VA	C.R. Ciola	7/1/78
406. *Epioblasma f. walkeri*	OSM 42434	Clinch River, Cedar Bluff, below R&R bridge, Tazewell Co., VA	C.R. Ciola G.M. Wargowsky	10/8/78
407. *Epioblasma f. walkeri*	OSM 43294	Clinch River, Cedar Bluff, below R&R bridge, Tazewell Co., VA	J.M. Condit C.R. Ciola	7/15/78

408. *Epioblasma f. walkeri*	OSM 44731	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	D.H. Stansbery C. Boone	8/11/76
409. *Epioblasma f. walkeri*	OSM 50299	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	D.H. Stansbery	9/20/81
410. *Epioblasma f. walkeri*	OSM 52059	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	D.H. Stansbery W.J. Clench	10/2/67
411. *Epioblasma f. walkeri*	OSM 52069	East Fork Stones River, Walterhill, Rt. 231 Bridge, Rutherford Co., TN	J.D. Putnam	11/7/70
412. *Epioblasma f. walkeri*	OSM 53252	Clinch River, Cedar Bluff, Tazewell Co., VA	R. Taylor	7/10/83
413. *Epioblasma f. walkeri*	OSM 57118	French Broad River, Asheville, Buncombe Co., NC	J.F. Hardy	NA
414. *Epioblasma f. walkeri*	USNM 29898	French Broad River, Asheville, NC	NA	NA
415. *Epioblasma torulosa gubernaculum*	MCZ 5751	Clinch River, Union Co., TN	R.E. Call	1908
416. *Epioblasma torulosa gubernaculum*	MCZ 16795	Clinch River, Clinchport, Scott, Co., VA	A.H. Clarke	NA
417. *Epioblasma torulosa gubernaculum*	MCZ 29824	Stone Creek, Lee Co., VA	B. Walker	NA
418. *Epioblasma torulosa gubernaculum*	MCZ 55501	Clinch River, Clinton, Anderson Co., TN	W.J. Clench M.D. Barber	NA
419. *Epioblasma torulosa gubernaculum*	MCZ 236726	Powell River, Jonesville, Lee Co., VA	B. Walker	NA
420. *Epioblasma torulosa gubernaculum*	MCZ 236768	Clinch River, TN	NA	NA
421. *Epioblasma t. gubernaculum*	MCZ 294151	Clinch River, Pendleton Island, Scott Co., VA	R.J. Neves	NA

APPENDIX II

DESCRIPTIONS OF SPECIES AND POPULATIONS

***Epioblasma capsaeformis* cf., Duck River**. Type Locality: Duck River, TN. Type Specimen: None dedicated.

DESCRIPTION: The shell of adult ***Epioblasma capsaeformis*** from the Duck River is of medium length (40-70 mm), and on average greater in length than *E. capsaeformis* from the Clinch River. The key characteristics of the Duck River form of *E. capsaeformis* are the following: (1) distinctly expanded marsupial swelling of the female shell, (2) slate-gray to dark purple mantle-pad, (3) display of a single micro-lure that moves slowly side to side, and (4) a spongy texture of the mantle-pad. The posterior portion of the mantle-pad is invaginated where it meets the incurrent siphon; therefore, the attachment points of the micro-lures are concealed and cannot be seen when the female is displaying. Shell characteristics of this species differ from the Clinch River form of *E. capsaeformis* (CR) mainly in the female shell. In young individuals, the base of the marsupial swelling is constricted, appearing narrow and projected. However, as the female ages and grows in size, the marsupial swelling becomes extremely protruded and enlarged, compared to the main body of the shell. The marsupial swelling is dark green, sometimes appearing almost black. The denticulations along the margin of the marsupial swelling are typically large and widely spaced. The male shell is short and high and contains a shallow sulcus, whereas the males of *E. capsaeformis* (CR) are more elongated. The periostracum of adults is yellow and green, becoming more yellowish at the anterior-end. The shell surface contains distinct broad to fine green rays that typically are irregularly spaced. The periostracum color and ray pattern are very similar to *E.*

capsaeformis (CR); the males of each species are nearly indistinguishable. The periostracum is dull to waxy when cleaned.

Figure 1. Female *Epioblasma capsaeformis* from the Duck River.

A. Tan colored, single micro-lure display.

B. Spongy surface texture of mantle-pad.

C. Marsupial expansion of female shell.

D. Mantle-pad and micro-lure display.

Figure 2. Shells of *Epioblasma capsaeformis*, Duck River.

A. Young *Epioblasma capsaeformis* (42 mm long), Duck River, Maury Co., TN.

B. *Epioblasma capsaeformis* (Duck River form), Shoal Creek, Lauderdale Co., AL, CM 61.7697.

C. *Epioblasma capsaeformis*, Duck River, TN, FMNH 226003.

D. *Epioblasma capsaeformis*, (Duck River form), Tennessee River, Florence AL., FMNH 64238.

***Epioblasma capsaeformis* (Lea 1834), oyster mussel**. Synonymy by Johnson (1978), and Parmalee and Bogan (1998). Type Locality: Cumberland River. Type Specimen: U.S. National Museum of Natural History, Washington, D.C.

DESCRIPTION: The shell of adult *Epioblasma capsaeformis* is of small to medium length (30-50 mm). The key characteristics of the typical form of *E. capsaeformis* are the following: (1) yellow and green colored periostracum, (2) bluish-white colored mantle-pad, (3) smooth texture of the mantle-pad, and (4) simultaneous display of two micro-lures that move synchronously in a circular motion; the left micro-lure moves clockwise, and the right micro-lure moves counterclockwise. The dorsal margin of the mantle-pad in both left and right valves is black, forming a discrete uniform band ~2-3 mm wide. The posterior portion of the mantle-pad is not invaginated where it meets the incurrent aperture; therefore, the attachment points of the micro-lures can be seen when the female is displaying. The denticulations along the margin of the marsupial swelling are typically finer and more closely spaced than those of females of *E. capsaeformis* (DR) from the Duck River. The periostracum of adults is yellow and green, becoming more yellowish at the anterior end. The marsupial swelling of the posterior end of the female is distinctly darkened green, a characteristic diagnostic of the species. The shell surface contains distinct broad to fine green rays that typically are irregularly spaced. The male shell of *E. capsaeformis* from the Clinch River is typically more elongate than the male shells of *E. capsaeformis* (DR) and *E. florentina*.

Figure 3. Female oyster mussel *Epioblasma capsaeformis* from the Clinch River, TN.

A. Bluish-white mantle-pad display.

B. Mantle-pad display.

C. Mantle-pad and micro-lure display.

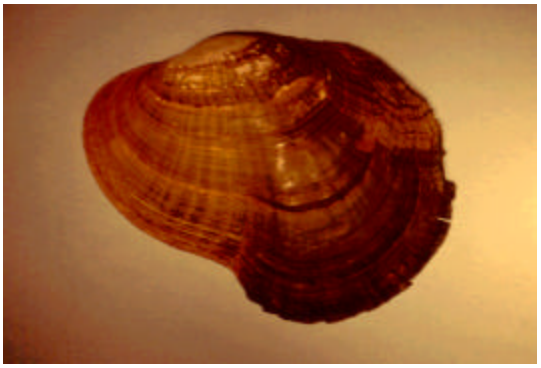
D. Two micro-lures rotating synchronously.

Figure 4. Shells of the oyster mussel *Epioblasma capsaeformis.*

A. *E. capsaeformis*, Tennessee River at Muscle Shoals, AL.

B. *E. capsaeformis*, Duck River (top); *E. capsaeformis*, Tenn. River, Muscle Shoals, AL (bottom). Shells are ~50 mm long.

C. Fresh dead shells of *E. capsaeformis*, Horton Ford, Clinch River, TN.

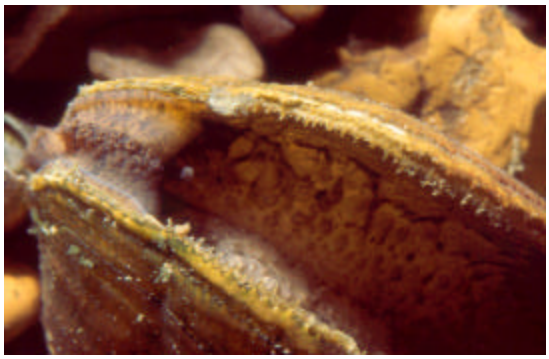


D. *E. capsaeformis* Cumberland River, holotype USNM 84939.

***Epioblasma florentina walkeri* cf.** Type Locality: Indian Creek, upper Clinch River, VA. Type Specimen: None dedicated.

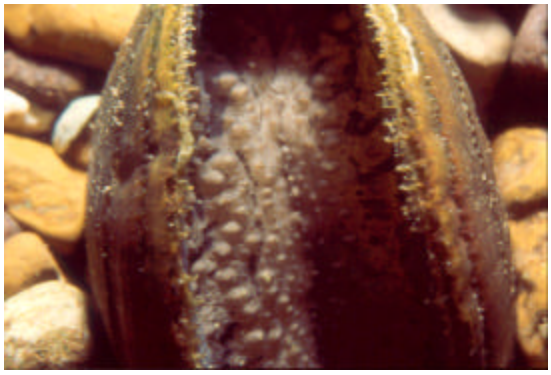
DESCRIPTION: The shell of adult *Epioblasma florentina walkeri* from Indian Creek is of small to medium length (30-50 mm long). The key characteristics of this form of *E. florentina walkeri* are the following: (1) honey-yellow to brown colored periostracum, with fine, evenly spaced rays covering the entire surface of shell, (2) mantle-pad colored gray with a black mottled background, (3) mantle-pad is pustuled, and (4) only a single micro-lure displayed, which moves slowly side to side in a sweeping movement. The posterior portion of the mantle-pad is invaginated where it meets the incurrent aperture; therefore, the attachment points of the micro-lures are concealed and cannot be seen when the female is displaying. The dorsal margin of the mantle-pad in both left and right valves is tan, forming a discrete uniform band ~2-3 mm wide. The denticulations along the margin of the marsupial swelling of the female shell are fine and closely spaced. The shape and coloration of the male shell of *E. f. walkeri* from Indian Creek are very similar to that of the male shell of *E. florentina walkeri* from the Cumberland River drainage, both typically have a shallow sulcus.

Figure 5. Female *Epioblasma florentina walkeri* from the Clinch River, VA.



A. Mantle-pad (invaginated) and micro-lure display.

B. Gray colored mantle-pad display.



C. Pustuled mantle-pad and fine denticulations along shell margin.

D. Mantle-pad and micro-lure display.

Figure 6. Shells of *Epioblasma florentina walkeri* and yellow blossom *E. florentina florentina*.

A. *E. f. walkeri*, Clinch River, Tazewell Co., VA.

B. *E. f. walkeri*, Middle Fork Holston River, Smyth Co., VA, OSM 24342.

C. *E. f. florentina*, Clinch River, Anderson Co., TN, OSM 10377.

D. *E. f. florentina*, Tennessee River, Lauderdale Co., AL, FMNH 269062.

***Epioblasma florentina walkeri* (Wilson and Clark 1914), tan riffleshell**. Synonymy by Johnson (1978), and Parmalee and Bogan (1998). Type Locality: East Fork Stones River, Rutherford County, Tennessee. Type Specimen: Museum of Zoology, University of Michigan, Ann Arbor, Michigan.

DESCRIPTION: The shell of adult *Epioblasma florentina walkeri* is of small to medium length (30-50 mm long). The key characteristics of the typical form of *E. florentina walkeri* are the following: (1) honey-yellow to brown colored periostracum, with fine, evenly spaced rays covering the entire surface of the shell, (2) mantle-pad colored brown with a tan mottled background, (3) mantle-pad pustuled, and (4) only a single micro-lure displayed, which moves slowly side to side in a sweeping movement. The posterior portion of the mantle-pad is invaginated where it meets the incurrent aperture; therefore, the attachment points of the micro-lures are concealed and cannot be seen when the female is displaying. The dorsal margin of the mantle-pad in both left and right valves is tan, forming a discrete uniform band ~2-3 mm wide. The denticulations along the margin of the marsupial swelling of the female shell are large and widely spaced.

Figure 7. Females of tan riffleshell *Epioblasma florentina walkeri* from the Big South Fork Cumberland River, TN.

A. Mantle-pad and micro-lure display.

B. Pustules on mantle-pad.

C. Brown colored mantle-pad.

D. Live *E. f. walkeri*, Parchcorn Creek Shoal, Scott Co., TN.

Figure 8. Shells and river habitat of the tan riffleshell *Epioblasma florentina walkeri*.

A. Female shell of *E. f. walkeri*, Parchcorn Creek Shoal, Scott Co., TN.

B. Female shell of *E. f. walkeri*, paralecotype, MCZ 293010, East Fork Stones River, Rutherford Co., TN.

C. Female *E. f. walkeri*, Parchcorn Creek Shoal.

E. Big South Fork Cumberland River, Parchcorn Creek Shoal, Scott Co., TN; March 2002.

***Epioblasma torulosa rangiana* (Lea 1838), northern rifflesheII.** Type Locality: Ohio River. Type Specimen: U.S. National Museum of Natural History, Washington, D.C.

DESCRIPTION: The shell of adult *Epioblasma torulosa rangiana* is medium in length (40-70 mm). The key characteristics of *E. torulosa rangiana* are the following: (1) chestnut brown-colored periostracum, with evenly spaced green rays covering the entire surface of the shell, (2) shell with 1-2 knobs located on the center of the shell, (3) mantle-pad colored white, (4) smooth surface texture of mantle-pad, and (5) micro-lures absent. The posterior portion of the mantle-pad is incomplete where it meets the incurrent aperture; therefore, when the female mussel is displaying a small gap is present between the mantle-pads at the posterior end. The dorsal margin of the mantle-pad in both left and right valves is white. There are no denticulations along the margin of the marsupial swelling of the female shell. The male shell of *E. t. rangiana* also has knobs and a shallow to prominent sulcus.

Figure 9. Female northern riffleshell *Epioblasma torulosa rangiana* from the Allegheny River, PA.

A. Mantle-pad display; micro-lures are absent.

B. Denticulations on shell are absent.

C. Female shell of *E. torulosa rangiana,* Allegheny River, PA.

D. Male shell of *E. torulosa gubernaculum* with large knobs (center), Clinch River, Union Co., TN, MCZ 5751.

CHAPTER 3

Genetic Management Guidelines for Captive Propagation of Freshwater Mussels (Unionoida)

ABSTRACT

North America contains the greatest diversity of freshwater mussels in the world, roughly 300 species. However, this superfamily of mollusks is the most imperiled group of animals in the United States, with 35 species extinct and 70 species listed as endangered or threatened. To prevent additional species losses, biologists recently have developed methods to propagate juvenile mussels for release to the wild to restore and augment populations. From 1997-2002, mussel propagation facilities in the United States have released > 1 million juveniles of more than a dozen endangered species, and survival of juveniles 1-3 years of age in the wild already has been documented. Based on this success and expectations for the continued growth of these programs, agencies and facilities involved with mussel propagation now must seriously consider the genetic implications of releasing captive-reared progeny. Preservation of genetic diversity will require sufficient genetic analysis of source populations to confirm and conserve the existence of valid species, subspecies and unique populations. Hatchery protocols must be established to prevent artificial selection and other genetic risks affecting adaptive traits of progeny released to the wild. In this paper, I discuss and propose guidelines to assess taxonomic status, genetic variation of donor and recipient populations targeted for augmentation, and laboratory and field protocols to maximize genetically effective population size, minimize genetic changes in captive-reared progeny, and prevent the release of juvenile mussels into non-native drainages. I advocate a pragmatic approach to species recovery that incorporates the principles of conservation genetics into breeding programs, and prioritizes the immediate demographic needs of critically endangered mussel species.

INTRODUCTION

North America contains the greatest diversity of freshwater mussels in the world, nearly 300 species. However, this superfamily (Unionoida) of mollusks is the most imperiled group of animals in the United States, with 213 species (72%) listed as endangered, threatened, or of special concern (Williams et al. 1993; Neves 1999). Already, approximately 35 species, or 12% of the North American mussel fauna, have become extinct in the last 100 years, an extinction rate comparable to estimated faunal losses in tropical rainforests (Ricciardi and Rasmussen 1999). For example, the Tennessee River basin was home historically to 102 species of mussels, and hence is considered the center of mussel diversity in North America (Parmalee and Bogan 1998). Of those original 102 species, 12 are extinct, 26 are endangered, 20 are extirpated from the basin, and only about 30 species have stable populations (Parmalee and Bogan 1998). Most of the endangerment is caused by habitat loss and degradation due to dams, sedimentation, water pollution, dredging, and other anthropogenic factors (Neves et al. 1997; Neves 1999). Without immediate efforts to recover the 70 federally protected species in U.S. watersheds, the extinction of additional species is likely.

Propagation and culture of endangered mussel species has been recommended in recovery plans as a primary conservation strategy to increase population size, and to reintroduce species to sites within their historic ranges (USFWS 2003). A joint policy concerning controlled propagation has been developed by the U.S. Fish and Wildlife Service and the National Marine Fisheries Service to provide guidance and consistency in implementation of recovery activities by captive propagation programs (USFWS 2000).

This policy states that controlled propagation is a useful tool for establishing new, self-sustaining populations, supplementing or enhancing wild populations and holding offspring of listed species for part of their development if suitable natural conditions do not exist (USFWS 2000). Over the last 10 years, propagation technology has been developed at the Freshwater Mollusk Conservation Center at Virginia Polytechnic Institute and State University, Blacksburg, Virginia, and at other facilities in the United States to produce endangered juvenile mussels for this purpose. Currently, 11 federal and state facilities propagate mussels in the Southeast and Midwest, including: University of Minnesota, Mammoth Cave National Park (Kentucky), Kentucky Department of Fish and Wildlife at Frankfort, Southwest Missouri State University, Southeast Aquarium Research Institute (Georgia), Tennessee Tech University, USFWS Genoa National Fish Hatchery (Wisconsin), USFWS White Sulphur Springs National Fish Hatchery (West Virginia), USFWS Warm Springs National Fish Hatchery (Georgia), Virginia Department of Game and Inland Fisheries, and Virginia Polytechnic Institute and State University. From 1997-2003, these facilities have conducted critical life history studies on freshwater mussels and released over 1 million juveniles of more than a dozen endangered species into rivers throughout the midwestern and southeastern United States. Already, survival of laboratory-reared juveniles 1-3 years of age after release has been documented in some rivers. For example, researchers at Southwest Missouri State University produced thousands of juvenile Neosho mucket *Lampsilis rafinesque* and released them in the Fall and Verdigris Rivers, Kansas, in 2000. Biologists already have recovered 28 live individuals of this species at their respective release sites in 2002 (Christopher Barnhart, Southwest Missouri State University, personnel communication,

2003). The endangered Higgin's-eye pearlymussel *Lampsilis higginsi* and endangered oyster mussel *Epioblasma capsaeformis* have been successfully propagated and recovered at release sites in the upper Mississippi River, Wisconsin, and Clinch River, Tennessee, respectively (Roger Gordon, USFWS, Genoa National Fish Hatchery, personal communication, 2002; Jones and Neves, unpublished data, 2004). Propagation of native aquatic species, such as freshwater mussels, now offers state and federal hatcheries an opportunity to expand their mission and play an important role in conservation of biological diversity in the United States.

Federal and state biologists are optimistic about using propagation technology as a recovery tool for endangered mussels, and as mitigation for mussel populations killed by toxic spills or other anthropogenic impacts. However, as these programs mature and become more successful, the genetic implications of releasing captive-reared progeny to natal or other rivers must be considered. Propagation programs will be challenged to increase population sizes while simultaneously trying to avoid negative consequences of altering the genetic resources of populations (Hallerman 2003). Because little is known about conservation genetics of freshwater mussels, scientists and resource managers will have to apply science developed by fisheries professionals involved with conservation of fish and marine bivalve genomes and other organisms (Lannan 1980, Meffe 1986, Gaffney et al. 1993, Waples 1999, Hallerman 2003).

As biologist attempt to recover mussel populations in the United States, situations will arise where efforts to propagate critically endangered species conflict with fundamental principles of conservation genetics. Populations of some mussel species now contain so few individuals that applying the principles and practices of conservation

genetics will be difficult or impractical in the initial stages of recovery. Geneticists, field biologists, and aqua-culturists will be required to decide whether the propagation of a mussel species can alleviate the immediate threat of its extinction, and whether these recovery actions are more compelling than any potential genetic hazards that could be incurred. We are likely to discover cryptic taxa and genetic population structure within species that warrant protection; thus, a collaborative effort will be critical to guide recovery goals for endangered freshwater mussels.

In this paper, I discuss how the principles of population genetics can be applied to protect the genetic resources of mussel populations. My intent is to identify and justify practical genetic guidelines for recovery activities directly associated with captive propagation of freshwater mussels. Readers should be aware that the current state-of-knowledge concerning mussel propagation technology is still in its infancy. Hence, many of the population genetic concerns discussed are based upon theoretical principles. Key biological information is lacking; e.g., population genetic structure, degree and distribution of adaptive genetic variation, numbers of juveniles needed to demographically boost and effectively restore populations, robust estimates of juvenile mortality in the laboratory and field, and minimal viable population sizes. Questions concerning effects of artificial propagation technology on variation of phenotypic and quantitative genetic traits are just now being asked. Thus, propagation programs will need to take an adaptive approach to management of mussel resources, one that readily learns from results and is able to apply current science.

Life History of Freshwater Mussels

Freshwater mussels are filter-feeders that live most of their lives embedded in gravel, sand or mud substrates in rivers or lakes. Freshwater mussels are generally long-lived animals that exhibit slow to moderate population recruitment rates. Many mussel species commonly live for more than 20 years, with some living more than 150 years (Ziuganov et al. 1998). Mussels have a unique life history, requiring that their larvae (glochidia) parasitize a fish host to complete their life cycle. Eggs of female mussels are fertilized internally by sperm released by males into the water and taken in during siphoning. The sexes are separate in most species, but hermaphroditism among unionids is not uncommon (van der Schalie 1966, 1970). The embryos then develop in the gills of the female until becoming mature glochidia. Depending on the species, mussel glochidia are brooded in the gills of females during either the winter (bradytictic) or summer (tachytictic). Winter brooders typically release their glochidia in late winter, spring and summer, while summer brooders release glochidia only in summer. Once mature, the female then releases the glochidia into the water, where they must attach and encyst on the gills, fins or epidermis of a suitable host fish for metamorphosis to the juvenile stage. Metamorphosis typically requires 2-3 weeks, depending on seasonal water temperatures. Once this parasitic transformation is complete, juveniles excyst and drop from the fish host to begin their lives on the bottom of a river or lake. Most mussel species require specific fish hosts to transform to juveniles and to disperse into new habitats. To maximize attachment of glochidia to host fish, some mussel species produce glochidia in packets (termed conglutinates) or have modified mantle-lures that closely resemble prey items. Female mantle tissue and conglutinates can mimic insect larvae and pupae,

leeches, and even other fish, all of which seem to attract host fish closer for possible infestation by glochidia (Parmalee and Bogan 1998).

Causes of Decline and Extinction

The decline of mussel species and abundance throughout North America in the 20th century is attributed to the degradation of habitat from damming, pollution, and silting of rivers, and now more recently, from competition with the exotic zebra mussel (*Dreissena polymorpha*). Dams change the flow, temperature and dissolved oxygen regimes of free-flowing rivers such that the reproductive cycle of freshwater mussels is disrupted; gametogenesis is inhibited and fish hosts that prefer shallow free-flowing river habitat are extirpated from impounded reaches. Thus, dams prevent or inhibit dispersal of mussels, limiting their ability to colonize habitats and expand their ranges. Pollution and siltation of rivers degrades benthic habitats and interferes with osmoregulation, feeding, and survival of adults and juveniles. Zebra mussels attach to the shell of native mussels and directly interfere with feeding, respiration and reproduction, causing a decline in physiological condition and eventually death (Neves 1999). Therefore, both habitat degradation and zebra mussels accelerate mussel population declines by negatively effecting vital rates, notably reproduction, survival and dispersal. Threats associated with habitat degradation and invasive species generally fit into the declining-population paradigm, which identifies factors that cause populations to become small (Caughley 1994). Once populations become small, they are threatened by a suite of biological problems, such as demographic and environmental stochasticity, genetic drift, and inbreeding depression (Caughley 1994, Meffe 1996). These problems fit into the small-

population paradigm, which deals with the effect of small size on the likelihood of persistence of a population (Caughley 1994). Both paradigms are considered primary themes of conservation biology and are useful for identifying, prioritizing, and alleviating threats to imperiled species. Identifying threats to population persistence for species targeted for recovery is an important step in determining the feasibility and necessity of captive propagation. Factors that have been implicated in declines of freshwater mussels have created a suite of small-population problems for many species, as well as the complete loss of populations throughout large portions of species' ranges. Thus, once the causes of decline are identified and corrected, conservationists can implement augmentations and reintroductions to remedy small population problems and to re-establish populations in their historic ranges. Propagation programs should be viewed as a recovery tool that is integrated within larger ecosystem management programs involving habitat protection and restoration. Propagation of endangered mussel species is not a substitute for addressing factors responsible for their decline.

Guideline: Threats to population persistence should be identified and corrected prior to implementing captive propagation for a species.

Subsequent topics and guidelines discussed in this paper are divided into two parts: (1) genetic characterization of mussel populations; which primarily involves defining conservation units such as species, evolutionarily significant units (ESUs), and management units (MUs), and (2) field and laboratory protocols that identify genetic hazards and minimize genetic risks to species and populations targeted for recovery. My

discussion begins with a review of species concepts and their relevance to genetic characterization of populations.

PART I: Genetic Characterization of Mussel Populations; Defining the Units of Conservation

Species Concepts

Well supported biological classifications provide a basis for effective management decisions (Avise 1994, Villella et al 1998). A key first step in a scientifically sound recovery program requires that populations of a species are characterized genetically, and when appropriate, classified taxonomically. Genetic analyses are needed to resolve genetic similarity or taxonomic uncertainty among populations, and to determine which populations are most appropriate to use for restoration or augmentation. Therefore, if populations are significantly diverged genetically, those qualifying as distinct conservation units can be identified and prioritized for recovery and propagation. Genetically based characters from an animal's morphology, life history, behavior, and genes can be used to identify and define conservation units (Avise 1994). However, defining a distinct population at the appropriate taxonomic level is not always a clear-cut endeavor. Agreement among researchers over species concepts, methodologies and the criteria used to delineate populations can be contentious, and has yet to reach consensus (see Mayden and Wood 1995, Bowen 1999, and Moritz 2002 for reviews).

Currently, no universally accepted definition of *species* exists, but such a definition is needed to provide a conceptual framework for testing species-level boundaries among populations (Frankham et al. 2002). The Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC) are the definitions used most frequently by biologists, and offer testable criteria to delineate taxa; however, other species concepts also have been proposed (see Mayden and Wood 1995). Here, I discuss the basic advantages and disadvantages of each species concept; in depth discussions can be found in Mayr and Ashlock (1991) and Mayden and Wood (1995).

Under the Biological Species Concept (BSC), "A species is a group of interbreeding natural populations that is reproductively isolated from other such groups" (Simpson 1961, Mayr and Ashlock 1991). The BSC has been the most influential definition of species in population genetics and conservation biology because it emphasizes reproductive compatibility among individuals within and among populations to define a species. Species are viewed typically as organisms which reproduce sexually by exchange of genes, while gene exchange is limited or prevented by reproductive isolating mechanisms between populations of different species (Dobzhansky et al. 1977). An isolating mechanism is defined as any genetically conditioned impediment to gene exchange between populations (Dobzhansky et al. 1977). Isolating mechanisms can be behavioral, physiological, morphological or genetic in nature. The BSC is particularly useful for delineating taxa that are sympatric. According to the BSC, sympatric populations of the same species should exchange alleles, whereas distinct species within the same geographic region should not. Consequently, if any genetic marker shows lack of gene exchange, then two sympatric populations belonging to different species have

been identified (Frankham et al. 2002). The BSC has been criticized because the use of reproductive isolation as the criterion to identify species has two main limitations: (1) applying the test to allopatric populations is very difficult, and (2) some species are not completely isolated reproductively from other species (Waples 1995). Furthermore, the reproductive biology of many invertebrate species, such as freshwater mussels, is poorly understood; therefore, crossing experiments or heritability studies currently cannot be conducted to help determine their status as biological species.

The Phylogenetic Species Concept defines a species as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft 1983). Species are viewed as monophyletic lineages of organisms, distinguished by a set of genetically based characters that are unique and fixed in a population of a putative species. A *monophyletic* lineage is defined as a taxon in which all of the individuals are descended from a single common ancestor, and are identified when every individual in a population shares one or more unique characters; i.e., they can be unequivocally distinguished from individuals in other populations (Frankham et al. 2002). In contrast, a *polyphyletic* lineage is a taxon containing individuals from multiple ancestral lineages and without a set of characters to uniquely define it (Frankham et al. 2002). The PSC is appropriate for delineating and classifying taxa that are allopatric. However, the PSC suffers from not knowing how many genetically-based character-differences are adequate to determine whether two or more populations are significantly different. Modern molecular DNA methodologies allow discrimination between individuals, demes, populations, and species, suggesting that many geographically and demographically independent populations could be rendered monophyletic with an

appropriate set of molecular markers. Therefore, caution is warranted when evaluating monophyletic lineages as species. For example, DNA sequences at the *cytochrome-b* region of the mitochondrial DNA (mtDNA) genome are highly-divergent (9%) among some individuals within locally contiguous populations of the endangered Higgins eye pearlymussel *Lampsilis higginsii* (Bowen and Richardson 2000). It is likely that individuals containing these unique, monophyletic mtDNA lineages do not constitute distinct species. These individuals are morphologically indistinguishable and share many important biological traits. The presence of these divergent mtDNA lineages in one local population is explained by the life history and zoogeography of the species. Historic populations were probably isolated for long enough time periods to allow divergence of mtDNA, which was followed by a recent period of population mixing (Bowen and Richardson 2000). Therefore, the criterion of phylogenetic distinctiveness or monophyly may not inherently diagnose species-level differences. Hence, the PSC should be tested using a suite of genetic characters, to be discussed in the following sections.

Guideline: Species boundaries should be investigated using appropriate character sets and species concept(s) as a testable hypothesis.

Species Designations

Taxonomic uncertainty is prevalent for freshwater mussels, and species binomials may not represent actual biological species. Many unionid genera, such as *Elliptio*, *Epioblasma*, *Fusconaia* and *Pleurobema*, contain populations of uncertain taxonomic validity. Thus, if the existence of closely related species or sub-species is suspected, then

determining their distinctiveness generally requires a comprehensive analysis, to include examining both quantitative and molecular genetic characters (Davis 1983, Hillis 1987, Nei 1987, Avise 1994, Hoeh et al. 2001). Such comprehensive analyses are justified for endangered species, especially if the findings of such studies can affect the status of a species.

Freshwater mussels have been classified primarily on differences in shell morphology and the soft anatomy of the body. Other traits, such as fish host specificity, mantle-lure displays, morphology of glochidia, fecundity, reproductive periods and molecular genetic characters also can help identify species. Variation in morphological and life history characters can represent quantitative genetic differences among populations. Quantitative genetic traits and their expression are considered complex because they are typically controlled by many genes and can be affected by environmental conditions (Hard 1995, Hartl 2000, Hallerman 2003). Furthermore, phenotypic and quantitative characters can have adaptive significance; i.e., they define structures and functions related to an animal's fitness (Hard 1995). Thus, measuring variation of phenotypic and quantitative traits is more likely to assess biologically meaningful genetic differences among populations. In addition, phenotypic and quantitative traits are easier for biologists to measure in the field and laboratory. If phenotypic or quantitative genetic characters appear fixed, or contain predictable variation within and among populations, a genetic basis can be inferred. However, phenotypic and quantitative genetic characters can vary in response to environmental conditions, or due to other genes (alleles) that individuals carry (Hallerman 2003). For example, if individuals in a mussel population have different phenotypic traits, such as

shape and color of the mantle-lure display, then the trait may be polymorphic or may indicate a different species. In such cases, molecular genetic techniques can be used to infer whether gene flow is occurring among populations with different phenotypes or quantitative traits. If a gene is fixed in one phenotype compared to the other, then a distinct species may have been identified.

Genetic variation at molecular loci is useful for inferring population genetic structures and processes, including: (1) historical isolation of populations, (2) evolutionary divergence of species and populations, (3) genealogical relationships among species or populations, (4) phylogeographic patterns, and (5) population genetic processes, such as gene flow and genetic differentiation (Avise 1994, 2000; Nei and Kumar 2000). For example, if the number of nucleotides in a DNA sequence differs by only a few nucleotides among populations, then the evolutionary divergence of the populations is inferred to be recent, and the populations closely related. The populations then can be analyzed to reflect genealogical (phylogenetic) and geographical (phylogeographic) relationships. Molecular data then can be used to infer which population lineages are unique and to prioritize the most important for conservation. Phylogenetic analyses are primarily historical in their approach and are effective for determining the tree-branching pattern of population lineages or the genealogical relationships of the taxa. In contrast, taxonomic analyses are primarily concerned with how populations are categorized; whether they are species, subspecies, etc. The distinction between these related disciplines is fundamental. Phylogenetic analyses typically are performed after a suite of taxa has been delineated taxonomically.

A main disadvantage of measuring genetic variation with molecular markers is that these markers generally do not measure variation at loci that are adaptively significant (Hard 1995, Hallerman 2003). Molecular markers are considered selectively neutral because selection does not control their variability; rather, non-selective forces, such as random genetic drift and mutation, determine changes in allele frequency. Thus, the utility of molecular markers for serving as surrogates for fitness-related genes is questionable (Hard 1995). Furthermore, data obtained from molecular markers must be interpreted in the context of the taxa and markers investigated. The amount of genetic variation at molecular marker loci can vary considerably between taxonomic groups (clades), and is dependent on the marker being investigated. For example, amplification of mtDNA sequences in a region of the genome that has a high rate of nucleotide substitution, such as in the transcription control-region (*D-loop*), may reveal significant genetic variation between closely related species, whereas amplification of a region that has a slower rate of nucleotide substitution, such as *16S* rDNA, may show little variation. Therefore, the choice of molecular marker targeted can significantly influence the results of a study. Furthermore, mtDNA has limitations. In most species, it is maternally inherited as a single unit, and its variation is prone to founder effects, bottleneck effects and drift, which can lead to inference of erroneous phylogenies (Avise 2000, Frankham et al. 2000, Nei and Kumar 2000). This is especially true for many populations of freshwater mussels, which have experienced severe population reductions and are known to have complex reproductive patterns, such as hermaphroditic self-fertilization by female mussels. Hermaphroditism essentially is an extreme form of inbreeding, and has the potential to greatly skew patterns of genetic variation among species and populations.

In addition, bi-parental inheritance of mitochondrial DNA has been documented in at least one freshwater mussel species, *Pyganodon grandis* (Liu et al 1996). This study showed that females inherit mtDNA from the mother, whereas males from both the mother and father. The degree of genetic divergence between the male and female lineage of this species can be substantial (Liu et al. 1996). It is currently unknown to what degree bi-parental inheritance patterns exist in other freshwater mussel species. Therefore, it is critical that a robust genetic analysis be used to delineate taxa. Thorough analyses should include a suite of molecular markers (i.e., both mtDNA and nuclear DNA) and quantitative genetic traits. Studies that attempt to delineate species based on a limited amount of molecular genetic data, such as mtDNA alone, risk trivializing species concepts.

Phenetic measures of genetic distance, such as percent sequence divergence among populations, are inappropriate for delimiting taxa (Frost and Hillis 1990, Hoeh and Gordon 1996), especially for recently diverged taxa such as unionids (Davis et al. 1981). There is no absolute threshold level, (such as 5%) of DNA sequence divergence among populations that indicates species-level differences. Hence, species concepts should not be based on genetic distance alone, and a simplistic reliance on genetic distance-based taxonomic concepts should be abandoned (Davis 1983, Hoeh and Gordon 1996). Criteria for species or subspecies-level differentiation using phenetic measures are arbitrary by nature (Frost and Hillis 1990), and usually relevant only when estimated empirically within the clade of interest. As indicated earlier, the degree of genetic variation of molecular markers can vary considerably among taxa of freshwater mussels. Species in certain clades are characterized by low levels of genetic divergence (<1-2%),

while for others, levels of divergence are much greater (>3-5%). For example, Serb et al. (2003) investigated phylogenetic relationships among freshwater mussel species belonging to the genus *Quadrula* by analyzing variation in the *ND1* region of the mtDNA genome. These authors reported that most interspecific pairwise uncorrected p-distance values ranged from 3.65-15.35% (Serb et al. 2003). However, even within the genus, certain groups contained species that are seemingly closely related and defined by lower levels of DNA sequence divergence (e.g. 0.15-3.29%), such as the *pustulosa* species group (e.g., *Quadrula aurea* vs. *Quadrula pustulosa*) (Serb et al. 2003). The *Epioblasma* spp. studied in Chapter 2 of this thesis also are a good example of closely related species characterized by low levels of DNA sequence divergence (~0.5-1.0%). Differences in the levels of divergence among faunal groups are partially explained by the age of the lineages. Older lineages generally are characterized by greater levels of divergence, whereas younger lineages are not (Nei 1987).

Phenotypic and molecular genetic traits that are diagnostic of a species should be fixed in populations. Hence, for sympatric populations, I advocate using the BSC to discriminate taxa by testing for gene exchange among populations; and for allopatric populations, I advocate using the PSC to test for monophyletic lineages by seeking concordance among multiple independent data sets. Ideally, differences between species should be evident for phenotypic traits, quantitative traits and molecular markers.

Scientific rigor is important in taxonomic studies of rare species because results can affect a species' status, recovery activities taken on its behalf, and the future viability of remaining populations (Roe and Lydeard 1998a, Villella et al. 1998). Additionally,

only recognized species of invertebrates are afforded protection under the U.S. Endangered Species Act.

Guideline: Species designations should be assessed using multiple independent characters from a mussel's molecular and quantitative genetics, to include: genes, morphology, life history, behavior and distribution.

Population-level Concepts

Several concepts and definitions have been proposed to define taxonomic or phylogenetic units below the level of species that are meaningful for conservation purposes. An *Evolutionarily Significant Unit* (ESU) is "a population (or group of populations) that: (1) is substantially reproductively isolated from other conspecific population units, and (2) represents an important component in the evolutionary legacy of the species" (Waples 1991). The concept originally was proposed by Ryder (1986) and further developed by Waples (1991) for use in conservation of distinct populations of Pacific salmonids. Because the ESU concept emphasizes the criterion of reproductive isolation, it is technically similar to "species" as defined by the BSC. The criterion of reproductive isolation is satisfied if reproductive isolating barriers (RIBs) exist between populations. For example, an RIB may be a behavioral mechanism, such as differences in seasonal timing (fall vs. spring) of spawning runs that prevent gene exchange between populations of salmon. The criterion of evolutionary legacy is met if the population: (1) occupies unique habitat, (2) exhibits unique adaptation(s) to its environment, (3) is genetically distinct, or (4) if it went extinct, poses a significant loss to the ecological or

genetic diversity of the species (Waples 1991). Many traits have been used to identify ESUs in Pacific salmonids and other aquatic species (Nielsen 1995), to include differences in morphology, life history differences (e.g., seasonal timing and location of spawning runs, age at maturation), distribution, habitat occupied by a population, and gene frequencies (e.g., lack of shared alleles between populations). The application of the ESU concept to populations of a freshwater mussel species is untested. However, mussel populations that show differentiation of morphological, ecological (e.g., habitat preferences), and life history (e.g., fish host specificity, spawning and larval release periods) traits, coupled with significant divergence at molecular markers, may qualify as an ESU in the sense defined by Waples (1991). Populations that exhibit divergence through a range of phenotypic or genetic characters are likely to be an important component in the evolutionary legacy of a species, with some divergent traits indicating substantial reproductive isolation. Populations that fit this definition of an ESU may lack diagnostic morphological traits that clearly define them as species, but exhibit quantitative genetic traits that serve as reproductive isolating mechanisms. Furthermore, the remaining populations of many endangered mussels species are few and generally isolated from each other geographically; therefore, even if one population was extirpated, it is likely to pose a significant loss to the ecological or genetic diversity of the species. Thus the remaining populations of many endangered mussel species may qualify as ESU's as defined by Waples (1991).

As proposed by Moritz (1994, 2002), a population is an ESU if: (1) the population exhibits unique forms of mtDNA not shared with other populations of a species (reciprocal monophyly), and (2) the population is characterized by significant divergence

of allele frequencies in the nuclear genome. Hence, Moritz's definition is based solely on molecular genetic data and lacks the criterion of reproductive isolation proposed by Waples (1991). It also does not address the issue of evolutionary significance of differentiation. This distinction is substantial, and empirical testing is needed to determine the relevance of Moritz's criteria for mussel populations. It is possible, based on his criteria and reasons discussed earlier, that geographically separated populations of a mussel species that are fragmented due to an anthropogenic factor such as dams, could experience changes in allele frequencies that qualify them as ESUs sensu Moritz, but diverged by unnatural causes, and do not show differentiation of characters that might be judged evolutionarily significant.

Subspecies are defined as "groupings of populations, within a species, that share a unique geographic range or habitat and are distinguishable from other subdivisions of the species by multiple, independent, genetically based traits" (Frankham et al. 2002). Subspecies typically are not characterized by a high degree of reproductive isolation; therefore, individuals from different subspecies populations can mate and produce viable offspring.

Management Units (MUs) are populations that are genetically distinct, but not as divergent as ESUs or subspecies, and that warrant conservation status. Management Units are identified as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, even if alleles are not completely diverged or monophyletic (Moritz 1994). Management units are usually smaller, geographically separated populations nested within the entire population of an ESU or species. For example, in Chapter 2 of my thesis, the subspecies *Epioblasma florentina walkeri* in the Big South

Fork Cumberland River was proposed for designation as an ESU. Historically, this ESU was comprised of multiple, geographically separated populations throughout the Cumberland River watershed. These historic populations likely were divergent at nuclear DNA loci and probably would have qualified as MUs. Likewise, the population of *Epioblasma florentina walkeri* in the upper Clinch River is the Tennessee River watershed ESU-equivalent, which historically may have been comprised of multiple MUs distributed throughout the watershed.

Geminate Evolutionary Units (GEU) were proposed by Bowen (1998) to identify progenitors of future biodiversity. Species or populations that fit into the GEU category are characterized by: (1) recent separation in geological time from sibling populations, (2) existence of behavioral barriers to gene flow (e.g., different spawning and glochidial release periods), (3) high diversity in morphological, ecological, or genetic traits, and (4) morphological differentiation coupled with homogeneity at molecular markers (Bowen 1998). Certain mussel species in the genus *Epioblasma* (subgenus *Torulosa*), commonly called riffleshells, fit into the GEU category (see thesis Chapter 2). These species are characterized by a high degree of morphological differentiation and low levels of DNA sequence divergence (<1-2%).

The criteria used to define ESUs are different from those used to define species under the BSC, PSC, and from those used to define subspecies. Therefore, I suggest that data sets on phenotypes, life history characters, and genetic variation, as well as professional judgment and experience all play an important role in designating populations as ESUs.

Guideline: Unique populations of a species should be identified using data on phenotypic, life history and genetic characters and analyzed using an appropriate population-level concept as a testable hypothesis.

Genetic Characterization of Mussel Populations

If multiple populations of an endangered species exist, then genetic characterization of the remaining populations is necessary. Studies should compare genetic structure of species within and between major rivers systems. In order to identify genetically distinct populations and to develop management recommendations based on characterization of genetic differentiation (Bowen and Richardson 2000). When populations are isolated from each other geographically or reproductively, patterns of genetic distinctiveness are likely to emerge when a suite of genetic markers is used. Interpretation of the value of genetic data such as fixed or unique alleles, allele frequency differences, percentage of sequence divergence, or phenotypic and quantitative trait differences between populations, should be done on a case-by-case basis, and involve biologists familiar with the species and population genetics. Many mussel species have suffered drastic population reductions in the last 100 years, and the few populations of some species may be genetically "bottlenecked". Consequently, genetic variation within and among populations can be low or skewed; therefore, the alleles that remain may not accurately reflect population history, complicating phylogeographic analysis (Avise 1994). Designating conservation units based on a limited number of genetic markers can prove arbitrary and is not advisable; therefore, it is critical that genetic characterizations be based on multiple concordant data sets (Avise 2000). Genetic data obtained from the

mitochondrial genome should be supported by data from the nuclear genome, and molecular marker data should be substantiated by data from phenotypic and quantitative traits whenever possible.

Guideline: Genetic characterization of mussel populations should assess phenotypic and genetic variation within and among populations using multiple independent genetic markers.

Designation of conservation units requires assimilation of data from molecular markers, species life history, and zoogeography. For example, Berg et al. (1998) found low genetic variation across large distances (1000 km) among populations of the mapleleaf *Quadrula quadrula* in the Ohio, Tennessee and Tensas rivers, but high genetic variation within populations. These authors attributed this genetic population structure to high levels of gene flow among populations, presumably from the high dispersal ability of glochidial *Q. quadrula* through mobile host fishes. In contrast, Roe and Lydeard (1998a) found high genetic variation and distinctiveness among populations of the federally threatened inflated heelsplitter *Potamilus inflatus*, one occurring in Amite River (Mississippi River drainage) in Louisiana, and the other in the Black Warrior River (Mobile River drainage) in Alabama. These populations of *P. inflatus* are geographically separated, and it is unlikely that they have interacted in millennia. The management implications for these mussel species are quite different; whereas the population genetics of *Q. quadrula* suggests maintenance of population connectivity, those of *P. inflatus* do not. Thus, management actions for a species with high gene flow and little among-

population variation will differ substantially from that for a species with restricted gene flow and large among-population variation. Although preservation of a few populations of a species that have high gene flow will conserve most of its genetic diversity, protection of numerous populations within a geographic region will be necessary for taxa that have low gene flow (Berg et al. 1998).

Guideline: Populations that qualify as an ESUs or subspecies should be managed as separate conservation units whenever possible. Populations that qualify as MUs should be managed to maximize retention of genetic diversity throughout the range of a species.

PART II: Implementation of Hatchery Supplementation Programs and Assessment of Genetic Hazards

Propagation and Recovery Goals

As conservation units are identified, the focus of recovery of efforts for some species will shift to implementation of artificial propagation to produce and release sufficient numbers of juvenile mussels of suitable physiological and genetic quality to alleviate the immediate threat of extinction for an endangered mussel species, and to demographically boost its population(s) to the point where it is self-sustaining. Accomplishing this goal will require restoration, augmentation and protection of viable populations of targeted species and their habitats. Restoration requires the re-establishment of populations in historic habitats from which the species has been extirpated, while augmentation requires rehabilitating demographically depressed

populations with hatchery-reared progeny. However, to achieve these goals, propagation programs will need to adopt straightforward guidelines to help protect and maintain genetic diversity within and among populations of a species, prior to initiating artificial propagation activities.

Criteria for down-listing endangered mussel species are stated in federal recovery plans (e.g., USFWS 1984, 2003) and are useful for developing propagation goals. These plans provide basic biological information pertinent to the recovery of a species. For example, recovery plans typically require 3-6 distinct viable populations of a mussel species for down-listing a species from endangered to threatened. Plans define a viable population as a wild, naturally reproducing population that is large enough to maintain sufficient genetic variation to enable the species to evolve and respond to natural habitat changes without further intervention (USFWS 2003). Populations are considered distinct when they are separated from others to the extent that a single event would not likely eliminate or significantly reduce more than one population.

Guideline: Each mussel species targeted for recovery using propagation technology should have a recovery plan that defines: (1) necessity of genetic characterization of remaining populations, (2) number of populations to be augmented or reintroduced to effectively recover the species, (3) appropriate locations for release of juvenile mussels, (4) number of juveniles to be released per year at a site, (5) number of gravid females to be collected per year for broodstock, and (6) field and laboratory protocols to minimize genetic risks incurred by recovery activities.

Genetic Hazards and Risks

Hatchery and field activities associated with captive propagation programs pose four types of genetic hazards for a targeted population (Busack and Currens 1995). Therefore, personnel involved with the design and implementation of hatchery supplementation programs need to recognize genetic *hazards*, and understand how to avoid or minimize *risks* associated with propagation activities of targeted species. A *hazard* is an adverse genetic consequence of hatchery activities on a population, whereas a *risk* is the probability that a hazard will occur. The four types of genetic hazards are: (1) extinction, (2) loss of within-population genetic variation, (3) loss of between-population variation, and (4) domestication selection (Busack and Currens 1995). The risk is generally low for causing the extinction of a species (Type 1 Hazard) by recovery activities of a hatchery program; however, the over-collection of broodstock warrants further consideration, which will be discussed in the next section. The loss of within-population genetic variation (Type 2 Hazard) is generally caused by propagation of progeny from a limited number of parental broodstock. Random genetic drift occurs at a rate inversely proportional to the genetically effective population size (N_e); therefore, it is accelerated when only a few adults are used as broodstock to produce progeny for release back into the natal population (Hallerman 2003). The loss of between-population variation (Type 3 Hazard) is caused when genetic distinctiveness is reduced or lost due to mixing populations that otherwise would not interact naturally (through migration). Mixing distinct populations can result in outbreeding depression, a loss of fitness due to disruption of local adaptation or of coadapted gene complexes (Hallerman 2003). Because scientists are still uncertain about the effects of losing genetic variation on

population fitness, prudent hatchery personnel will try to minimize human-caused losses of genetic variation (Hard 1995, Waples 1999). Domestication selection (Type 4 Hazard) is the consequence of any change in the selection regime experienced by a cultured population relative to what it would have experienced in the wild (Waples 1999). Hatcheries can alter selection regimes in several ways, which will be discussed in detail below.

Selection of Broodstock Source Populations

Gravid female mussels typically are collected directly from their natal river to use as hatchery broodstock to propagate juvenile mussels. Therefore, elaborate factorial mating designs for males and females to increase genetically effective population size of hatchery produced progeny currently are not necessary or technically feasible in freshwater mussel propagation. Further, viable populations of many endangered mussel species are few, and some species are now reduced to a single population. In these cases, the need for among population genetic analysis required will be limited or none, and selection of source populations for translocation or captive propagation generally can be based on geography alone. Populations in close proximity to one another within a river basin are typically best suited for use as broodstock to restore or augment adjacent populations with propagated juveniles. When possible, collection of gravid females for augmenting a population should come from the natal river, or from the closest viable population. Restoration of a species into a historic river of occurrence should utilize broodstock from the closest adjacent watershed with the most similar ecological characteristics. Source populations should be similar to the recipient population based on:

(1) genetic lineage, (2) life history patterns, and (3) ecology of originating environment (Miller and Kapuscinski, 2003). However, the close proximity of populations may not necessarily preclude the need for genetic analysis, especially for mussel species that have limited dispersal capabilities. Fine-scale geographic patterns of genetic variation may exist for these species. In such cases, the desire to preserve native population genetic structure (to avoid Type 3 Hazard) must be carefully balanced with the need to augment the population with progeny from a population in another stream.

Guideline: Collection of gravid female mussels for an augmentation ideally should come from the natal river, or from the closest viable population, and that of restoring species into historic river habitat from the closest adjacent river or watershed.

Collection of an excessive number of adult female mussels for broodstock from a population can effectively "mine" natural populations by removing reproductive individuals from their source population and potentially contribute to declines (Type 1 Hazard) (Miller and Kapuscinski 2003). This can happen when the survival of hatchery-reared progeny is less than those produced naturally. For critically endangered species comprised of a single small population, it may be necessary to establish a target number of females to be collected each year for use as broodstock. This practice can help prevent over-collection of gravid females from a population and allow for some level of annual *in situ* reproduction to occur. For example, the main population of endangered tan riffleshell (*Epioblasma florentina walkeri*) in the upper Clinch River watershed occurs in a short reach (~400 m) of a small tributary stream. The size of the population has been estimated

at N=2000 (Rogers et al. 2001). However, based on my field observations of the number of gravid females releasing glochidia per year in the spring, the effective population size N_e is much smaller. In such cases, establishing an appropriate number of gravid females to be collected per year for propagation of juveniles from a small population is a prudent measure to ensure annual *in situ* population reproduction. Therefore, it is important that the success of propagation efforts be carefully monitored to determine whether recruitment of hatchery-reared juveniles exceeds that of naturally-produced juveniles and that artificial propagation actually contributes to the growth of the targeted population.

Guideline: Establish an appropriate number of gravid females to be collected each year from a small population for propagation, and protocols to monitor survival and recruitment of artificially propagated juveniles.

Management of Effective Population Size

Populations of endangered mussel species are often small and susceptible to extirpation, precipitous declines, and loss of genetic variation from ecological, demographic and anthropogenic disturbances. Once populations become small, genetic variation can be further eroded by non-selective forces, such as inbreeding and genetic drift (Hallerman 2003). Loss of within-population genetic variation (Type 2 Hazard) can result in a reduced capacity of populations to adapt to changing environments, which is manifested as a decrease in fitness of the individuals within a population (Meffe 1986). Therefore, consideration of genetically effective population size (N_e) has been incorporated into conservation programs for managing captive-reared vertebrates and

invertebrates (Neves 1997). Effective population size is defined as "the size of an idealized population that would have the same amount of inbreeding or random gene frequency drift as the population under consideration" (Kimura and Crow 1963). An "idealized population" is one that has an equal number of breeders per generation, 1:1 sex ratio, equal probability of reproductive success among breeding pairs within the population, and a population size that does not fluctuate from generation to generation. Departures from these ideal conditions will reduce N_e. Further, the actual mean population size (N) is almost always larger than N_e, as many individuals in a population are immature, too old to reproduce, and reproductive success varies more than under idealized conditions. Obviously, the conditions that define an idealized population do not exist in natural populations. However, these concepts are useful for estimating mathematically how a reduction in population size can decrease N_e, increase inbreeding and genetic drift, and increase the probability of expression of deleterious alleles and loss of genetic variation, which can ultimately decrease fitness within the population and its ability to adapt to environmental change.

Currently, no empirical data are available to relate a decrease in N_e with a decrease in fitness or adaptability of a freshwater mussel population. Moreover, documenting a causal relationship between molecular genetic variation (heterozygosity) within a population and its fitness is difficult. Avise (1994) stated: *"In general, there are several reasons for exercising caution in interpreting the low molecular heterozygosities reported for rare species: (a) most of the reductions in genetic variation presumably have been the outcomes rather than the causes of population bottlenecks; (b) at least a few widespread and successful species also appear to have low heterozygosities, as estimated*

by the same molecular methods; (c) in some endangered species such as the northern elephant seal, low genetic variation appears not to have seriously inhibited population recovery from dangerously low levels (at least to this point in time); and (d) the fitness cost of inbreeding is known to differ widely among species, with some taxa highly susceptible but others relatively immune to fitness depression accompanying inbreeding". For example, significant decreases in genetic heterozygosity were documented in elephant seals *Mirounga angustirostris* as a result of severe population declines due to over-hunting in the 18th-19th centuries. These populations have recovered and appear to be thriving, but contain little detectable genetic variation (Hoelzel 1993). Many examples are known of small founder populations colonizing new habitats with no evidence of demographic failure due to inbreeding, low heterozygosity, or other bottleneck effects (Brown 1994), especially for fish and mollusks. In contrast, a review by O"Brien et al. (1987) reported diminished sperm count, morphological abnormalities, and decreases in other fitness-related traits in populations of felids, such as African cheetah *Acinonyx jubatus* and Florida panther *Felis concolor*, which exhibited low molecular genetic variation. Thus, molecular genetic variation may be an adequate surrogate for measuring genetic variation at fitness-related loci for some species, but not for others (Hard 1995). In addition, self-fertilization or cross-fertilization through hermaphroditism is well documented in freshwater mussels, but the effect on N_e is unknown. This reproductive strategy likely decreases N_e dramatically; however, in theory, its occurrence over an evolutionary time scale may also confer resistance to inbreeding depression within populations. That is, in the long history of mussel populations and species, hermaphroditism may have increased the probability and rate of pairing and expression of

deleterious alleles within populations, allowing for environmentally-mediated selection to eliminate individuals expressing maladaptive phenotypes. Once these unfit individuals and genes are "purged" from populations, the effects of inbreeding depression on the viability of extant populations may be minimal.

Although untested in unionids, outbreeding depression, a decrease in fitness of progeny from the mating of distantly related individuals, may pose a threat to population viability in some species of marine mollusks (Lannan 1980 a & b; Boudry et al. 2002; Gaffney et al. 2002). If freshwater mussel populations are small, have limited dispersal capabilities, and are subject to intense selection, they may have developed coadapted gene complexes by adaptation to local environments, to include local host fish communities, or by intrinsic genic coadaptation (Hallerman 2003). Hence, freshwater mussels may be vulnerable to outbreeding depression, a hypothesis that needs to be tested.

Guideline: Increasing population density to alleviate the immediate demographic and environmental threats to small populations of endangered mussel species in the initial stages of recovery will generally be of higher priority than managing for increasing genetically effective population size or genetic diversity.

Management of effective population size and genetic variation for mussel species should be of concern to biologists. However, technical constraints confronting propagation of many endangered species may dictate that some of these genetic concerns will be difficult to accommodate. Consideration of other factors, such as differences in

life history traits, population demographics, habitat preferences, distribution, and other adaptive traits will be of more immediate concern. Some species are now so rare or difficult to collect that obtaining only a few gravid females per year for propagation is difficult. However, the high fecundity and output of glochidia by individual females provides an opportunity to propagate many more juveniles than what would have been naturally produced, and such recovery opportunities should be exploited to alleviate threats to small populations.

Assuming that management for a large N_e is necessary to avoid inbreeding and loss of genetic variation, what then, are a few simple field and laboratory guidelines that biologists can follow to accomplish these goals? Popular management guidelines - such as the "50/500 rule", which recommends an N_e of 50 to prevent inbreeding depression and 500 to prevent long-term erosion of genetic variability by genetic drift (Frankel and Soule, 1981) - are helpful guidelines, but generally are not feasible goals for critically endangered mussel species. Therefore, a long-term perspective is needed to increase N_e over many generations, especially for small populations. In addition, since little is known about mussel reproductive biology, equal sex ratios have to be assumed. For example, if 10 gravid females are collected as broodstock, it might be assumed that each female was fertilized by one male, and therefore, N_e=20. However, it is likely that N_e is naturally low in most mussel populations due to hermaphroditic reproduction and low cross-fertilization success between males and females. Effective population size for equal sex ratios can be calculated using the formula: $N_e = 4N_mN_f/(N_m + N_f)$, where N_m and N_f are the number of breeding males and breeding females, respectively (Kimura and Crow 1963). If the objective is to restore a population into historical habitat, then multiple (at least 3-

6) gravid female mussels should be collected annually from various sites to represent a range of river locations, habitats and sub-populations within the source population. A target sample of 20-25 randomly collected animals contains ~98% of the expected heterozygosity of a wild population (Lacy 1994), and could be achieved for most populations in 1-5 years. If the objective is to augment a population, then multiple gravid female mussels should be collected, ideally from the population under consideration, or from the nearest adjacent population. All females should be tagged prior to their release back to the river or if held in a hatchery as captive broodstock. This will prevent excessive use and over-representation of genetic resources of a limited number of females (see discussion of Ryman and Laikre effect below). In addition, tagged mussels can be tracked in the field and hatchery for survival.

Guideline: Maintain largest feasible genetically effective population size N_e of captive-reared, juvenile mussels by collecting an appropriate number of gravid adult females each year to use as broodstock, and when feasible, rotate broodstock year by year.

Domestication Selection

Domestication selection (Type 4 Hazard) causes genetic changes in captive-held populations. Domestication selection alters allele frequencies in a captive gene pool, over a run of generations, and can result in a domesticated strain. Such genetic changes have been well documented in fishes and other groups (Meffe 1986 and references therein). In the captive rearing environment, artificial selective forces can replace those of natural selection. Domestication selection occurs because a different set of progeny survive in

the hatchery than would have survived in the wild. Genetic changes can affect morphological, physiological, or behavioral traits, and lead to decreased performance and survival of captive-reared progeny in natural environments. Because mussel propagation is still in its infancy, domestication selection has not been documented in the rearing of a mussel species, but has been documented in the rearing of fishes in hatcheries (Miller and Kapuscinski 2003). For example, many salmon hatcheries that produce fish to augment wild populations are careful to collect breeders from different time-periods during the spawning run of a particular stock (Hallerman 2003). This field-collection practice allows genetic representation of breeders that collectively spawn from early to late in the run. Similar practices may be necessary for some species of mussels to prevent artificial selection. For example, females of the endangered oyster mussel *Epioblasma capsaeformis* in the Clinch River, Tennessee, typically begin displaying their mantle-pad lure and releasing glochidia to host fish in April and continue into early June. Some females display early in the spring, while others display much later. These differences in timing of release of glochidia by *E. capsaeformis* may be genetically controlled, and suggest that gravid females should be collected at different times throughout the glochidial release period. For example, if time of glochidial release is under genetic control, the excessive propagation and release of juvenile mussels from females collected in the early spring could shift forward the glochidial release-period of a targeted population relative to that of the wild population.

Research is needed to determine how domestication selection could alter the genetics of captive-reared juvenile mussels at several stages in the propagation process, to include investigation of the effects of: (1) the most appropriate time of year to remove

glochidia from the parental mussel to maximize maturity of glochidia, (2) marginally-suited host fish to transform glochidia to the juvenile stage, (3) inappropriate diet, substratum, exposure to disease, and rearing temperatures, and (4) length of culture period in captivity before release to the wild. A conservative strategy is to minimize domestication selection by mimicking natural regimes for temperature, diet, growth rates and size of juveniles at release relative to naturally-produced juveniles, and mimicking habitat of a species as possible throughout the propagation process. This can be achieved by thoroughly understanding mussel-host fish relationships, growth rates, dietary and habitat requirements of each species.

Guideline: Reduce artificial selection during propagation and culture of juvenile mussels by mimicking life history processes, diet, and habitat of a targeted species as closely as possible in the hatchery.

Laboratory Protocols to Prevent Mixing of Mussel Species

The establishment of laboratory protocols to prevent the inadvertent mixing of species or stocks is important to protect the integrity of genetic resources. Most propagation facilities rearing juvenile mussels for augmentation or restoration are cultivating multiple species and stocks from different drainages. For example, at the Freshwater Mollusk Conservation Center at Virginia Tech University, juveniles of 6-9 endangered mussel species are produced per year, representing species from several major river drainages. In these situations, separate tank systems are required for holding host fish and grow-out of juveniles from different drainages. Because juvenile mussels

are small in size (~200-1000 μm) for the first 60 days of life, and can easily attach to laboratory equipment used for handling juveniles, such as sieves, siphons and Petri dishes, these items also should be kept separate and disinfected regularly. All hatchery personnel should be trained in field and laboratory protocols to reduce the risk of unintentional mixing of cultured populations.

Guideline: Protocols to prevent mixing of species or stocks through inadvertent exchanges of juveniles on laboratory equipment is of high priority to protect genetic resources of freshwater mussel populations.

Release of Propagated Juveniles

A suite of factors should be considered before juvenile mussels are released to the river. Such planning is especially important for critically endangered populations with small effective population (N_e) sizes. Small populations (e.g. N=500-2000 and N_e is <50-100) warrant special attention if they are serving as a source population for augmentation or restoration, or if they are being augmented themselves. Production and release of thousands of juveniles from a small number of adult females into a small (<1000) recipient population can significantly decrease N_e, due to unequal contributions of progeny from only a few progenitors (Ryman and Laikre, 1991). Therefore, a target number of offspring should be established for release into a small population prior to augmentation. Excess progeny could be released at nearby adjacent shoals or at other acceptable sites. Second, selection of suitable release sites should be based on at least the following criteria: (1) biological requirements of species such as presence of fish hosts,

(2) habitat quality, and (3) a thorough assessment of localized and upstream threats. Third, juveniles should be released at the earliest life-stage possible that will maximize survival in the wild. There is a trade-off between how long juveniles are reared in the hatchery to increase survival rate relative those reared naturally, and continued exposure to the hatchery environment and the extent of domestication selection (Hallerman 2003). Exposure to natural environmental patterns and selective forces at an early life stage may prove most beneficial to ensure fitness in the wild of hatchery-reared juveniles. Fourth, juveniles should be released under moderate-to-low flow conditions to allow settlement on the river bottom, and at the appropriate time of year (spring-summer). Fifth, release methods and sites should be selected to increase the range and connectivity of localized demes and populations. For example, juveniles could be released at suitable sites between known locations of upstream and downstream demes. Furthermore, release of fish hosts infested with glochidia allows for natural dispersal and colonization of habitats otherwise excluded by only releasing hatchery-reared juveniles, spreads-out risk of mortality at localized river reaches, and may help minimize future inbreeding. This practice, however, risks dispersal and loss of juveniles after settlement in unfavorable areas, hence posing a tradeoff.

Guideline: Release an appropriate number of juvenile mussels at release sites to maximize effective population size (N_e), and at an early life stage to maximize survival in the wild and minimize the effects of domestication selection in the hatchery.

Conclusions

I advocate application of the principles of conservation genetics in species recovery efforts for freshwater mussels. However, these principles should be recognized as guidelines, and not as goals (Neves 1997). Propagation will continue to develop as a recovery tool to hopefully prevent further species extinctions. Programs will require flexibility in adapting and implementing genetic principles. Propagation may effectively alleviate problems associated with small populations, and can re-establish populations extirpated by known and ameliorated causes. Although propagation offers benefits for conservation and restoration, managers of propagation facilities must recognize how each stage in the propagation process can affect the genetic integrity of mussel populations targeted for recovery. Because of the many unknowns in mussel biology and uncertainties in long-term effects, hatchery programs should be treated as adaptive management experiments, with careful attention to monitoring and re-evaluation of goals and protocols.

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VITA

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