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)

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ABSTRACT

Species in the genus *Epioblasma* have specialized life history requirements and represent the most endangered genus of freshwater mussels (Unionidae) in the world. A genetic characterization of extant populations of the oyster mussel *E. capsaeformis* and tan riffleshell *E. florentina walkeri sensu late* was conducted to assess taxonomic validity and to resolve conservation issues for recovery planning. These mussel species exhibit pronounced phenotypic variation, but were difficult to characterize phylogenetically using DNA sequences. Monophyletic lineages, congruent with phenotypic variation among species, were obtained only after extensive analysis of combined mitochondrial (1396 bp of 16S, cytochrome-*b*, and ND1) and nuclear (515 bp of ITS-1) DNA sequences. In contrast, analysis of variation at 10 hypervariable DNA microsatellite loci showed moderately to highly diverged populations based on F_{ST} and R_{ST} values, which ranged from 0.12 to 0.39 and 0.15 to 0.71, respectively. Quantitative variation between species 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 *Etheostoma flabellare*. Lengths of glochidia differed significantly ($P < 0.001$) among species and populations, with mean sizes ranging from 241 to 272 μm. The texture and colour of the mantle-pad of *E. capsaeformis sensu stricto* is smooth and bluish-white, whereas that of *E. f. walkeri* is pustuled and brown, with tan mottling. Based on extensive molecular, morphological and life history data, the population of *E. capsaeformis* from the Duck River, Tennessee, USA is proposed as a separate species, and the population of *E. f. walkeri* from Indian Creek, upper Clinch River, Virginia, USA is proposed as a distinct subspecies.

INTRODUCTION

Genetic characterization of closely related species provides the opportunity to understand the formation and maintenance of sympatric forms, and to reveal cryptic species. Such opportunities are uncommon in nature; however, model systems are beginning to emerge. For example, pupfishes (*Cyprinodon* spp.) living in Death Valley in southeastern California and southwestern Nevada are distinct morphologically and behaviourally, but exhibit low levels of genetic divergence at mitochondrial DNA (mtDNA) markers; e.g. 0.32–0.49% between *C. diabolis* and *C. nevadensis* (Echelle & Dowling, 1992). Other model systems include cichlid fishes in East African rift lakes (Stauffer *et al*., 1995), sturgeons in the Mobile River basin (Avise, 2000), and coral reef fishes (Serranidae: *Hypoplectus*) (McCartney *et al*., 2003). These species are morphologically and behaviourally distinct, but exhibit low levels of divergence at molecular markers. Such species are of great interest to biologists, but are inherently difficult to define, because their genetic characterization typically requires analyses of a large number of molecular and phenotypic characters.

Here, we describe the phenotypic variation, molecular genetic variation, fish-host specificity and historical levels of sympatry of two closely related freshwater mussel species, the oyster mussel *Epioblasma capsaeformis* (Lea, 1834) and tan riffleshell *E. florentina walkeri* (Wilson & Clark, 1914), endemic to the southeastern United States. Species in the genus *Epioblasma* have specialized life history characteristics and likely represent the most endangered genus of freshwater mussels in world. Ten of the 17 recognized species are extinct, and all but one species (*E. triquetra*) is listed as federally endangered. Species descriptions for the genus can be found in Johnson's (1978) monograph on *Epioblasma*, which discusses conchology and systematics and divides the group into five distinct subgenera. The species of interest in this study belong to the subgenus *Torulosa* (commonly known as riffleshells), which includes *E. biemarginata*, *E. capsaeformis*, *E. florentina*, *E. phillipsi*, *E. propinqua*, *E. sampsoni*, *E. torulosa*, and *E. turgidula*. However, only *E. capsaeformis*, *E. florentina*, and *E. torulosa* have extant populations; the other five species are presumed extinct (Williams *et al*., 1993). Extant species are characterized by relatively small sizes

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(30–70 mm) and extreme sexual dimorphism between the female and male shells. The posterior ends of female shells in this subgenus are expanded and inflated, an area of the shell called the marsupial expansion. This shell enlargement partially houses the swollen gills of gravid females and accommodates 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 in order to metamorphose to the juvenile stage. Because of these seemingly derived characters, species in this genus are considered advanced members of the Unionidae [U.S. Fish and Wildlife Service (USFWS), 1984].

The severe global decline of freshwater mussels has been well documented (Neves *et al.*, 1997; Lydeard *et al.*, 2004). For example, of North America's approximately 300 mussel species, 213 (72%) are listed as endangered, threatened or of special concern, and approximately 35 species (12%) have become extinct in the last 100 years (Williams *et al.*, 1993; Neves *et al.*, 1997; Neves, 1999). Most of the endangerment is caused by habitat loss and degradation 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 working to protect and restore rivers, to release propagated juvenile mussels for population augmentation and range expansion, and to relocate adult mussels to more protected habitats. These recovery actions are needed to help save many species from extinction. However, as these mussel conservation efforts increase, 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, King & Starliper, 1998). Determining genetic relationships among donor and recipient populations will require phylogenetic and taxonomic analyses (Avise, 2000). Holistic analyses should examine a suite of multiple independent genotypic and phenotypic characters, to include traits expressed in molecular markers, anatomy, morphology and life history (Davis, 1983; Mayden & Wood, 1995). Furthermore, differences of opinion on mussel taxonomy are persistent, stemming from an incomplete understanding of variation in morphology, anatomy, life history and molecular genetics (Heard & Guckert, 1971; Davis, 1984; Stiven & Alderman, 1992; Hoeh & Gordon, 1996; Berg & Berg, 2000). Disagreements are especially acute when closely related or morphologically ambiguous species or populations are assessed. Hence, various genetic studies have been conducted to help clarify taxonomic and phylogenetic uncertainity among mussel taxa (Lydeard, Mulvey & Davis, 1996; Mulvey *et al.*, 1997; Roe & Lydeard, 1998; King *et al.*, 1999; Roe, Hartfield & Lydeard, 2001; Serb, Buhay & Lydeard, 2003).

Early taxonomic uncertainty regarding *E. capsaeformis* can be traced to questions concerning the population in the Duck River, Tennessee (TN), USA. Bryant Walker, an early 20th century malacologist, noted in an unpublished letter that the large marsupial expansion of the female shell for this population was different from that of individuals in the Clinch River (Jones, 2004). More recently, field biologists have also questioned the taxonomic affinity of the Duck River population because of obvious differences in shell morphology and coloration of the mantle-pad. 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. florentina walkeri* were the same species. Because of these taxonomic uncertainties and their potential effect on recovery plans and status of these two endangered species (USFWS, 1984, 2004), a comprehensive taxonomic analysis was needed. The objectives of this study were (1) to determine the taxonomic validity of *E. capsaeformis* and *E. florentina walkeri*, and (2) to demonstate the utility of a holistic approach to resolving taxonomic designations at the species level.

METHODS

Type specimens and species sympatry

Type specimens, shell material and collection records for *Epioblasma capsaeformis*, *E. florentina walkeri* and *E. florentina florentina*, as well as subspecies of *E. torulosa* were examined at the following museums in the USA: 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 University, Museum of Biological Diversity, Columbus, Ohio (OSM); and National Museum of Natural History, Washington, D.C. (USNM). Collection records from Johnson (1978), Parmalee and Bogan (1998), and the U.S. Fish and Wildlife Service (1984, 2004) also were examined. A total of 11 type specimens and 421 collection lots of shell material were examined at the six museums (Jones, 2004). Type specimens provided standard references for comparing shells from various rivers, and collection records were used to determine historical levels of sympatry among taxa.

Sample collection

Samples of mantle tissue from live mussels were collected from various river locations throughout the ranges of these species: (1) *E. capsaeformis*, Clinch River (CR) between Horton Ford (CRKM 321) and Swan Island (CRKM 277), Hancock, 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., Virginia (VA); (4) *E. florentina walkeri*, Big South Fork Cumberland River (BSF) from Station Camp Creek, Scott Co., TN, downstream to Bear Creek, McCreary Co., Kentucky (KY); and (5) *E. torulosa rangiana* from Allegheny River (AR), Venango Co., Pennsylvania (PA). Sample sizes were limited because of the endangered status of each species (Table 1). Some subspecies were not included in the study because they are presumed extinct, i.e. *E. florentina florentina*, *E. florentina curtisi* and *E. torulosa torulosa*. A small piece of mantle tissue (20–30 mg) was collected non-lethally from 8 to 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 about 20 mg of fresh mantle tissue using the Purgene DNA extraction kit (Gentra Systems). DNA concentration was determined by flourescence assay (Hoefer TKO 1000 Flourometer), 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) 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 & Richardson, 2000), and (4) ITS-1 (King *et al.*, 1999). The PCR conditions described earlier are also detailed in Jones (2004).

Table 1. Collection locations and sample sizes for DNA sequences and DNA microsatellite loci investigated for five mussel species in the genus *Epioblasma*. Collection locations are described in the Methods section.

Species	Collection location	Total sample size	mtDNA			nDNA	Microsatellite loci									
			16S	cytochrome-*b*	ND1	ITS-1	*Ecap1*	*Ecap2*	*Ecap3*	*Ecap4*	*Ecap5*	*Ecap6*	*Ecap7*	*Ecap8*	*Ecap9*	*Ecap10*
In-group taxa																
E. capsaeformis	Clinch River (CR)	20	10	10	8	10	18	20	12	19	19	19	20	19	18	10
E. florentina walkeri	Indian Creek, Clinch River (IC)	8	6	5	2	5	8	6	8	6	5	7	4	6	8	3
E. florentina walkeri	Big South Fork Cumberland River (BSF)	14	10	10	2	10	13	12	11	12	11	13	11	10	12	6
E. capsaeformis	Duck River (DR)	12	10	10	8	10	12	12	12	12	12	12	12	10	10	7
E. torulosa rangiana	Allegheny River (AR)	6	6	6	1	6	6	6	4	5	6	6	6	5	5	3
Out-group taxa																
E. triquetra	Clinch River (CR)	1	1	1	1	1	–	–	–	–	–	–	–	–	–	–
E. brevidens	Clinch River (CR)	1	1	1	1	1	–	–	–	–	–	–	–	–	–	–

All PCR products were sequenced with a Big Dye Terminator Cycle Sequencing kit with Ampli*Taq* DNA Polymerase (Applied Biosystems). Cycle sequence reactions were purified using a Qiagen DNA purification kit, and subjected to electrophoresis and sequencing using an Applied Biosystems 3100 automated sequencer.

Phylogenetic analyses were conducted primarily to determine genetic distinctiveness of DNA sequence haplotypes among populations of *E. capsaeformis* and *E. 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). Phylogenetic analyses were performed using PAUP* (version 4.0b2, Swofford, 1998). Pairwise genetic distances among haplotypes were calculated using uncorrected *p*-distance in PAUP*. Phylogenetic trees were constructed by both maximum parsimony (MP) and minimum evolution (ME) methods. However, because the extent of sequence divergence was low among in-group taxa, MP was designated as the primary tree-building method (Nei & Kumar, 2000; Felsenstein, 2004). Characters were treated as unordered and of equal weight for the analysis because of the in-group taxa being closely related (Nei & Kumar, 2000). The MP tree was constructed by a branch-and-bound search with ACCTRAN and TBR options; insertions and deletions were treated as missing data. The ME tree was constructed by a neighbour-joining algorithm followed by TBR. The model for sequence evolution for ME analysis was determined by the program MODELTEST 3.6 (Posada & Crandall, 1998); searches for all gene portions were conducted by the HKY 85 model (Hasegawa *et al.*, 1985). Bootstrap analyses (10,000 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 were also conducted. In-group taxa were *E. capsaeformis* (CR), *E. capsaeformis* (DR), *E. florentina walkeri* (IC), *E. florentina walkeri* (BSF), and *E. torulosa rangiana* (AR). Because of obvious morphological differences (see Results), the latter species was used informally as an out-group taxon to compare phenotypic and molecular genetic variation within the subgenus *Torulosa*. However, DNA sequences of the Cumberland combshell *Epioblasma brevidens* and snuffbox *Epioblasma triquetra* from the Clinch River were designated as the primary out-group taxa. These latter two species are substantially diverged at a suite of phenotypic and molecular genetic characters from the in-group taxa, and classified in different subgenera, *Plagiola* and *Truncillopsis*, respectively (Johnson, 1978).

DNA microsatellites

Microsatellite loci and primers were isolated by a modified non-radioactive capture-hybridization method, and developed and characterized using DNA of *E. capsaeformis* (Jones *et al.*, 2004). The PCR amplification protocols (Eackles & King, 2002) consisted of 100 ng of genomic DNA, $1 \times$ PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM each primer and 1.0 U Ampli*Taq* DNA polymerase (ABI) in a total volume of 20 μl. PCR thermal cycling conditions were those of Eackles & King (2002).

Amplification products containing microsatellite loci initially were examined for size polymorphism using a 7% polyacrylamide silver stained gel, followed by further analysis using 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) was 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 allele size range, total number of alleles, number of unique alleles and population differentiation (F_{ST}) were calculated using POPGENE32 software (Yeh, Yang & Boyle, 1999). Population differentiation also was measured using the R_{ST} test (Slatkin, 1985) using RST CALC software (Goodman, 1997). R_{ST} assumes a stepwise mutation model (Kimura & Otha, 1978), whereas F_{ST} assumes an infinite allele model (Kimura & Crow, 1964; see Balloux & Lugon-Moulin, 2002 for a review of the putative advantages and disadvantages of each statistic). Values for F_{ST} and R_{ST} can range from zero (no differentiation) to one (complete differentiation); values from 0.05 to 0.15 reflect moderate to high levels of genetic differentiation, values >0.15 reflect very high levels and values >0.25 are considered great (Wright, 1978; Balloux & Lugon-Moulin, 2002).

Phenotypic variation of mantle-pads, micro-lures and glochidia

Photographs of the mantle-pad and micro-lures of live female mussels were taken using a Nikonos V underwater camera with 28 or 35 mm macro lenses and Kodak 200 Ektachrome film. Female mussels were held in temperature-controlled water in recirculating artificial streams with gravel-filled bottoms. This set-up allowed females to display their mantle-pad and behavioural observations of micro-lure movements to be recorded under controlled conditions. A hand-held video recorder was used to document micro-lure movements; digital recordings are stored at the Department of Fisheries and Wildlife Sciences, Virginia Tech. Observations of micro-lure movements and coloration and texture of the mantle-pad were made for *E. 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) ($N = 10$). Lengths of 20 glochidia from five female mussels of each population were measured using an ocular micrometer and dissecting microscope. Lengths of glochidia from population samples were compared using analysis of variance (ANOVA) (SAS Institute, 2001).

Fish-host specificity

Gravid females of *E. capsaeformis* and *E. florentina walkeri* were collected from the Clinch, Duck and Big South Fork Cumberland Rivers. No gravid females of *E. florentina walkeri* from Indian Creek were used for fish-host analyses in this study, because the population is critically endangered. Fish host specificity was determined using three species of darters—greenside darter (*Etheostoma blennioides*), fantail darter (*Etheostoma flabellare*) and redline darter (*Etheostoma rufilineatum*)—which had been identified previously as natural hosts for both species (Yeager & Saylor, 1995; Rogers *et al*., 2001). Each fish species represents a particular darter subgenus (clade); *Etheostoma*, *Catonotus* and *Nothonotus*, respectively (Jenkins & Burkhead, 1993). Fish hosts were collected from the upper North Fork Holston River, near Saltville, VA, where no populations of *Epioblasma* currently reside. Methods for infesting fish with mussel glochidia were standardized and similar to those of Zale & 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 h) during infestations; water was aerated and agitated with an airstone. Thirty fish each of *Etheostoma blennioides*, *Etheostoma flabellare* and *Etheostoma rufilineatum* were infested together using glochidia from two female mussels added to the container. Three replicate ($N = 3$) 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 three days until juvenile mussels were collected, then every day thereafter until juveniles completed excystment from fish.

The degree of fish host specificity among mussel 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.

RESULTS

Type specimens

Epioblasma capsaeformis was described by Lea (1834) from specimens collected from the Cumberland River around Nashville, TN. Shell material of *E. capsaeformis* from the Cumberland River drainage was nearly identical to that examined from the Tennessee river drainage; no consistently discernable differences were observed. The marsupial expansion of the female shell was coloured green, background colour of periostracum was yellowish-green, and the green ray pattern was not as fine and evenly spaced as that of *E. florentina walkeri*. The latter subspecies was described by Wilson & Clark (1914) from specimens collected from the East Fork Stones River in the Cumberland River drainage. Shells of *E. florentina walkeri* were brown to tan coloured with fine rays covering the periostracum. No shell character intergrades between *E. capsaeformis* and *E. florentina walkeri* were observed. In addition, shells of *E. capsaeformis* from the Duck River were morphologically distinct from those in the Clinch River; namely, the marsupial expansion of females was significantly larger ($P < 0.05$) (see Jones, 2004 for descriptions). The female shell of the Duck River population was distinguishable from those of females of other populations of *E. capsaeformis* using the following criteria: (1) length of the base of the marsupial expansion of young individuals (i.e. 3–5 years and ranging in size from 35–45 mm), was shorter than those of females of *E. capsaeformis* from other rivers of similar age and size and (2) height of the marsupial expansion of adult females was greater than those of *E. capsaeformis* females in other rivers (Fig. 3D). Shell characters of males were not readily distinguishable among populations.

Species distributions and historical levels of sympatry

Based on shell material examined in museums, the Clinch River (CR) form of *E. capsaeformis* was distributed throughout the Tennessee River system in Virginia, Tennessee, North Carolina, Georgia and Alabama and in the Cumberland River system in Kentucky and Tennessee (see collection records in Jones, 2004). The more upland portions of the Tennessee and Cumberland Rivers are known as the Cumberlandian region (Fig. 1). Extant populations occur in the Clinch River, TN and VA, where the species is common and reproducing, and in the Nolichucky River, TN, where it is rare. The population of *E. capsaeformis* (DR) is now restricted to the Duck River, TN;

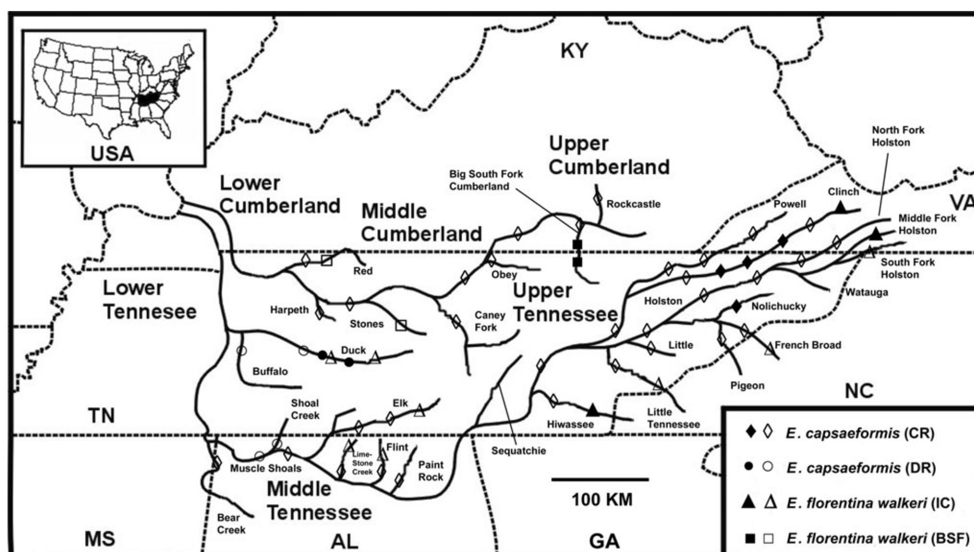

Figure 1. Distribution of *Epioblasma capsaeformis* (DR) (current ●, historic ○), *E. capsaeformis* (CR) (current ◆, historic ◇), *E. florentina walkeri* (IC) (current ▲, historic △) and *E. florentina walkeri* (BSF) (current ■, historic □) throughout the Cumberlandian physiographic region, USA.

however, based on examination of shell material and collection records, it occurred in other rivers of the Tennessee River system, including the Buffalo River, TN (Parmalee & Bogan, 1998) and the Tennessee River at Muscle Shoals and lower Shoal Creek near Florence, AL (Jones, 2004). Historically, *E. florentina walkeri* also occurred throughout the Cumberlandian Region. Extant populations occur in the Cumberland River drainage in the Big South Fork Cumberland River, TN and KY, where the species is uncommon but reproducing and in the Tennessee River drainage in the upper Clinch River and its tributary Indian Creek, VA, where it is uncommon but reproducing (Rogers, Watson & Neves, 2001).

Populations of *E. capsaeformis* and *E. florentina walkeri* were sympatric historically in many rivers throughout parts of their ranges in the Cumberland and Tennessee River drainages, including: Cumberland River, KY; Big South Fork Cumberland River, KY; Beaver Creek, KY; Obey River, TN; Harpeth River, TN, Red River, TN; 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 (Parmalee & Bogan, 1998; Jones, 2004). For example, *E. capsaeformis* (CR) and *E. florentina walkeri* (IC) were sympatric in the upper Clinch River near Richlands, VA. Populations of *E. capsaeformis* (DR) and *E. florentina walkeri* occurred together in the Duck River, TN, whereas both forms of *E. capsaeformis* (CR and DR) occurred in the Tennessee River at Muscle Shoals, AL. Furthermore, other taxa belonging to the subgenus *Torulosa*, such as *E. biemarginata*, *E. florentina florentina*, *E. propinqua* and *E. torulosa torulosa*, also occurred at Muscle Shoals (Parmalee & Bogan, 1998).

Phylogenetic analysis of DNA sequences

Including out-group taxa, DNA sequence data from combined mtDNA regions of 16S (468 bp), cytochrome-*b* (360 bp) and ND1 (568 bp), and from the nDNA region ITS-1 (515 bp), (1911 bp total), revealed 156 variable nucleotide sites, 70 of which were phylogenetically informative under MP analysis. A total of 10 phylogenetically informative sites were observed at 16S, 31 at cytochrome-*b*, 26 at ND1 and 3 at ITS-1. [The mtDNA and nDNA sequences of this study have been deposited in GenBank under accession numbers: 16S (DQ208503–DQ208546), cytochrome-*b* (DQ208547–DQ208590), ND1 (DQ208591–DQ208613), and ITS-1 (DQ208614–DQ208657)]. However, only variable sites ($N = 41$) from in-group taxa are reported in Table 2; the entire site matrix including out-group taxa is available in Jones (2004). Variable sites were most frequent in the complete site matrix at cytochrome-*b* (0.16), followed by ND1 (0.11), 16S (0.06) and ITS-1 (0.02). As observed in the aligned DNA sequences, 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 to populations. For example, in ITS-1, a thymine insert was observed at bp 153 in all haplotypes of *E. capsaeformis* (DR), and a deletion at bp 511 in *E. torulosa rangiana*; additional indels of interest were also present in the in-group at this sequence region (Table 2). Observed nucleotide site variation defined 14 haplotypes in the in-group species examined (Table 2). The greatest number of observed DNA haplotypes was six in the population of *E. capsaeformis* (CR), with haplotypes *Ec*CR3 and *Ec*CR4 the most distinct. The smallest number of haplotypes observed was one (*Efw*BSF1) in the population of *E. florentina 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, including *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. None of the mtDNA or nDNA sequence regions showed any of the in-group taxa to be monophyletic when analysed alone. Interestingly, DNA sequences of ITS-1 did not differentiate *E. brevidens* from the in-group taxa. The uncorrected *p*-distance values among DNA haplotypes are reported in Table 3. In-group taxa were characterized by low genetic distances ranging from 0.00053 to 0.00795, while out-group taxa were characterized by greater distances ranging from 0.04844 to 0.05868.

The phylogenetic analysis of haplotypes using MP and ME optimality criteria produced nearly identical tree topologies, with the exception that haplotypes *E. capsaeformis* CR5, and *E. capsaeformis* CR6 form a bifurcating interior node in the ME

Table 2. Haplotypes and variable sites (underlined) in combined analysis of 16S, cytochrome-*b*, *ND1* and ITS-1 DNA sequences of in-group 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); and *E. capsaeformis*, Duck River (*Ec* DR).

Haplotype	*N*	16S (468 bp)					cytochrome-*b* (360 bp)														ND-1 (568 bp)								ITS-1 (515 bp)														
		2	167	179	351	452	13	43	52	87	96	99	148	172	184	208	211	214	252	327	30	249	281	282	405	423	444	564	44	127	151	152	153	166	167	215	385	386	387	388	467	468	511
*Ec*CR1	4	T	T	C	G	G	A	C	G	T	C	A	G	G	G	A	T	G	G	A	A	A	T	T	G	G	A	T	C	T	T	G	:	:	:	G	:	:	:	:	T	C	T
*Ec*CR2	1	T	T	C	A	G	A	C	G	T	C	A	G	G	G	A	T	G	G	A	A	A	T	T	G	G	A	T	C	T	T	G	:	:	:	G	:	:	:	:	T	C	T
*Ec*CR3	2	T	T	A	G	G	G	C	G	C	C	A	T	G	A	A	T	G	G	G	A	A	T	T	G	G	A	T	C	T	G	A	:	C	T	G	:	:	:	:	:	:	T
*Ec*CR4	1	T	T	A	G	G	A	C	G	C	C	A	G	G	A	A	T	G	G	G	A	A	T	T	G	G	A	T	C	T	G	A	:	C	T	G	:	:	:	:	:	:	T
*Ec*CR5	1	T	T	C	G	G	A	C	G	T	C	A	G	G	G	A	T	G	G	A	A	A	T	T	G	G	A	T	C	T	G	A	:	C	T	G	:	:	:	:	:	:	T
*Ec*CR6	1	T	T	C	G	G	A	C	G	T	C	A	G	G	G	A	T	G	G	A	A	A	T	C	G	G	A	T	C	T	T	A	:	:	:	G	:	:	:	:	:	:	T
*Efw*IC1	5	T	T	C	G	G	A	C	G	C	C	A	G	G	A	A	T	G	G	G	A	A	T	T	A	G	G	T	C	T	T	G	:	:	:	G	:	:	:	:	:	:	T
*Efw*IC2	1	T	T	C	G	G	A	C	G	C	C	A	G	G	A	A	T	G	A	G	A	A	T	T	A	G	G	T	C	T	T	G	:	:	:	G	:	:	:	:	:	:	T
*Efw*BSF1	10	T	T	C	G	G	A	C	A	C	T	A	G	G	A	A	T	G	G	G	A	G	T	T	G	A	G	T	C	T	G	G	:	C	T	A	:	:	:	:	:	:	T
*Etr*AR1	1	T	T	C	G	G	A	C	G	C	C	C	G	A	A	A	T	G	G	G	T	A	T	T	G	G	G	T	C	T	T	A	:	:	:	G	:	:	:	:	:	:	:
*Etr*AR2	3	T	T	C	G	A	A	C	G	C	C	C	G	A	A	A	T	G	G	G	T	A	T	T	G	G	G	T	T	G	T	A	:	:	:	G	G	T	T	T	:	:	:
*Etr*AR3	2	T	C	C	G	A	A	C	G	C	C	C	G	A	A	A	T	G	G	G	T	A	T	T	G	G	G	T	C	T	T	A	:	:	:	G	:	:	:	:	:	:	:
*Ec*DR1	9	C	T	C	G	G	A	T	G	C	C	A	G	G	A	G	C	A	G	G	A	A	A	T	G	G	G	C	C	T	T	T	T	:	:	G	:	:	:	:	:	:	T
*Ec*DR2	1	C	T	C	G	G	A	T	G	C	C	A	G	G	A	G	C	A	G	G	A	A	A	T	G	G	G	T	C	T	T	T	T	:	:	G	:	:	:	:	:	:	T

Table 3. Pairwise genetic distances (uncorrected *p*-distance) among combined mitochondrial (16S, cytochrome-*b*, ND1) and nuclear (ITS-1) DNA haplotypes. Abbreviations: *Ec*CR, *Epioblasma capsaeformis*, Clinch River; *Efw*IC, *E. florentina walkeri*, Indian Creek; *Efw*BSF, *E. florentina walkeri*, Big South Fork Cumberland River; *Etr*AR, *E. torulosa rangiana*, Allegheny River; *Ec*DR, *E. capsaeformis*, Duck River; *Et*, *E. triquetra*; *Eb*, *E. brevidens*.

Haplotypes	*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	0.00369	0.00421	–	–	–	–	–	–	–	–	–	–	–	–	–	–
*Ec*CR4	0.00316	0.00263	0.00158	–	–	–	–	–	–	–	–	–	–	–	–	–
*Ec*CR5	0.00105	0.00053	0.00369	0.00211	–	–	–	–	–	–	–	–	–	–	–	–
*Ec*CR6	0.00158	0.00105	0.00422	0.00263	0.00053	–	–	–	–	–	–	–	–	–	–	–
*Efw*IC1	0.00263	0.00421	0.00316	0.00263	0.00369	0.00421	–	–	–	–	–	–	–	–	–	–
*Efw*IC2	0.00316	0.00474	0.00369	0.00316	0.00421	0.00474	0.00053	–	–	–	–	–	–	–	–	–
*Efw*BSF1	0.00527	0.00579	0.00580	0.00421	0.00527	0.00579	0.00369	0.00421	–	–	–	–	–	–	–	–
*Etr*AR1	0.00421	0.00474	0.00369	0.00316	0.00421	0.00474	0.00263	0.00316	0.00527	–	–	–	–	–	–	–
*Etr*AR2	0.00632	0.00685	0.00579	0.00527	0.00632	0.00685	0.00474	0.00527	0.00685	0.00211	–	–	–	–	–	–
*Etr*AR3	0.00527	0.00579	0.00474	0.00421	0.00527	0.00579	0.00369	0.00422	0.00633	0.00105	0.00211	–	–	–	–	–
*Ec*DR1	0.00632	0.00738	0.00632	0.00580	0.00685	0.00738	0.00475	0.00527	0.00738	0.00580	0.00791	0.00686	–	–	–	–
*Ec*DR2	0.00579	0.00685	0.00579	0.00527	0.00633	0.00685	0.00422	0.00474	0.00685	0.00527	0.00738	0.00633	0.00053	–	–	–
*Et*1	0.04746	0.04796	0.04693	0.04638	0.04743	0.04796	0.04691	0.04744	0.04748	0.04747	0.04902	0.04747	0.04904	0.04851	–	–
*Eb*1	0.05381	0.05378	0.05328	0.05431	0.05326	0.05379	0.05326	0.05379	0.05648	0.05488	0.05590	0.05487	0.05540	0.05487	0.04792	–

tree (ME tree not shown). The MP analysis of the combined sequence data resulted in 31 equally parsimonious trees of 180 steps in length (CI = 0.917, RI = 0.853) (Fig. 2). The ME tree score was 0.09395. All five population groups were recovered as monophyletic lineages in both the MP and ME trees, and most were well supported by bootstrap values (Fig. 2). The tree topology placed *E. capsaeformis* (DR) as basal to other members of the in-group. However, this node and other internal nodes were not well supported by bootstrap values and were collapsed in the strict consensus tree.

Population genetic analysis using DNA microsatellites

Allele frequencies at each locus for each population are reported in the Appendix and summary statistics of variation across microsatellite loci are reported in Table 4. All ten microsatellite loci amplified in samples taken from *E. capsaeformis* (CR), *E. capsaeformis* (DR) and *E. florentina walkeri* (BSF); however, *Ecap3* did not amplify in *E. florentina walkeri* (IC) and *Ecap7* in *E. torulosa rangiana* (AR), despite repeated PCR trials using varying conditions. Lack of amplification at these loci may indicate the presence of null alleles; e.g. allelic variation may be present at these loci but do not amplify because of nucleotide sequence variation in the primer-annealing regions (Culver, Menotti-Raymond & O'Brien, 2001; Zhang & Hewitt 2003). All ten microsatellite loci were unambiguously scored across all five in-group mussel populations.

Significant deviations from HWE ($\alpha = 0.05$), showing deficiency of heterozygotes, were observed in *E. capsaeformis* (CR) at *Ecap2–7*; in *E. capsaeformis* (DR) at *Ecap1, 5, 6* and *8*; in *E. florentina walkeri* (BSF) at *Ecap1, 4, 6, 8* and *9*; in *E. florentina walkeri* (IC) at *Ecap1* and *6*; and in *E. torulosa rangiana* (AR) at *Ecap1* and *10*. Significant deviations from LE ($\alpha = 0.05$) were observed at 15 pairs of alleles in *E. capsaeformis* (CR); 0 pairs in *E. capsaeformis* (DR); one in *E. florentina walkeri* (BSF); one in *E. florentina walkeri* (IC); and one in *E. torulosa rangiana* (AR).

Overall genetic variation was greatest in *E. capsaeformis* (CR) and lower in *E. florentina walkeri* (IC), *E. florentina walkeri* (BSF) and *E. capsaeformis* (DR) as quantified by heterozygosity and total number of alleles observed. Genetic variation was

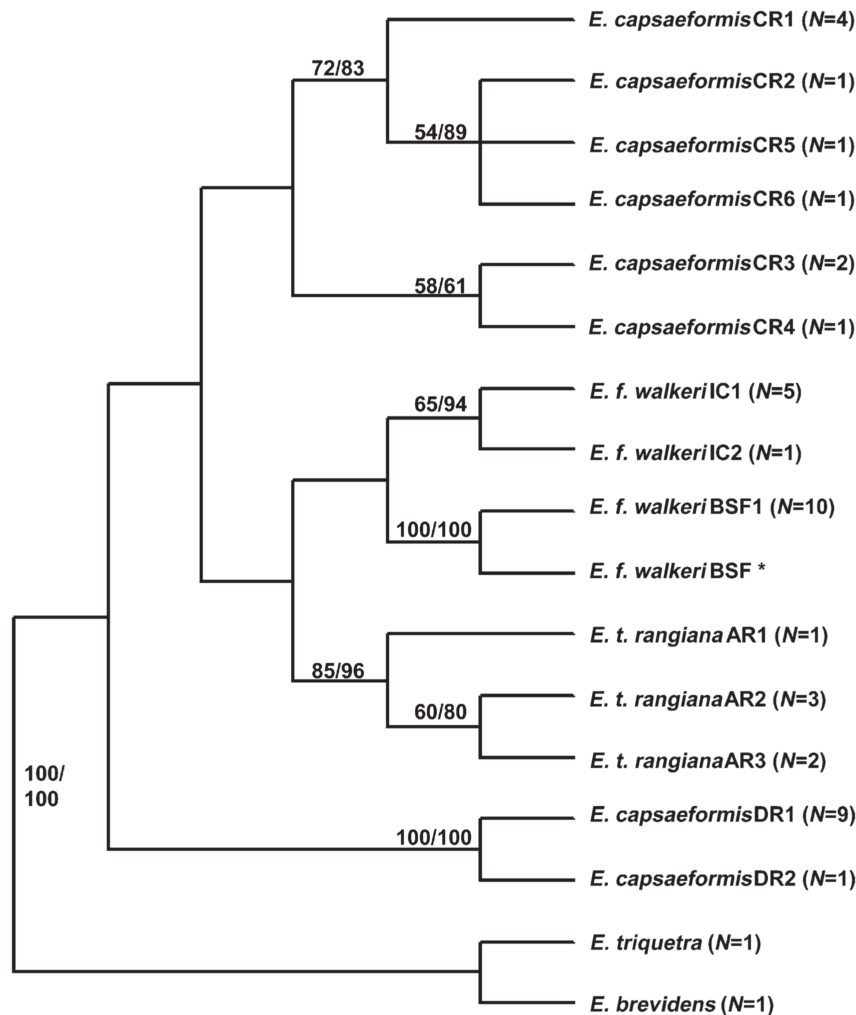

Figure 2. Phylogenetic relationships among the examined *Epioblasma* spp. were inferred from the combined mitochondrial DNA regions of 16S (468 bp), cytochrome-*b* (360 bp), ND1 (568 bp) and the nuclear DNA region ITS-1 (515 bp) using maximum parsimony (MP) (31 equally parsimonious trees were resolved; length = 180 steps; CI = 0.917; RI = 0.853). Numbers above the branches (MP/Minimum Evolution) represent bootstrap support (10,000 replicates); only values >50% are shown. Numbers in parentheses at the end of each taxonomic name represent the number of observed haplotypes. *All BSF haplotypes were identical; however, to clearly demonstrate the monophyly of this population, an additional sequence was added to the analysis. Out-group taxa are *E. triquetra* and *E. brevidens*.

Figure 3. Mantle-pad displays of mussel species in the genus *Epioblasma*. **A.** Female oyster mussel *E. capsaeformis* (CR) Clinch River, Hancock County, Tennessee (TN), USA. **B.** Micro-lures of *E. capsaeformis* (CR). **C.** Mantle-pad and micro-lure of *E. capsaeformis* (DR), Duck River, Maury County, TN. **D.** Marsupial shell expansion of female *E. capsaeformis* (DR). **E.** Mantle-pad of tan riffleshell *E. florentina walkeri* (IC), Clinch River, Tazewell County, Virginia. **F.** Micro-lure of *E. florentina walkeri* (IC). **G, H.** Mantle-pad of *E. florentina walkeri* (BSF), Big South Fork Cumberland River, Scott County, TN. The gaps between shell valves of displaying female mussels are approximately 2 cm. The arrows indicate micro-lures.

moderate in *E. torulosa rangiana*; however, sample size was low ($N = 6$) for this population. Therefore, it is likely that observed genetic variation under-represented true variation actually residing in this population. The population of *E. torulosa rangiana* in the Allegheny River probably exceeds one million individuals and occurs over many river kilometers (R. Villella, USGS, personal communication, 2002); therefore, it is possible that actual genetic variation is high. Differences in allele

Table 4. Summary of genetic variation among 10 microsatellite loci examined for in-group species of *Epioblasma*.

Species	% Polymorphic loci	Observed heterozygosity	Expected heterozygosity	Mean number of alleles/locus	Mean allele size range (bp)	Maximum range (bp)	Total number of alleles	Number of unique alleles
E. capsaeformis (CR)	100	0.6333	0.8347	9.7	28.4	44	97	39
E. f. walkeri (IC)	90	0.3593	0.5238	2.5	9.1	30	25	2
E. f. walkeri (BSF)	80	0.3850	0.4217	3.4	6.0	22	34	5
E. capsaeformis (DR)	100	0.4236	0.6025	4.1	13.4	28	41	6
E. t. rangiana (AR)	90	0.4778	0.7355	4.1	16.9	36	41	12

Locality abbreviation is given in parenthesis (see Table 1).

frequencies among populations were especially evident at loci *Ecap1*, *Ecap3*, *Ecap5*, *Ecap6* and *Ecap8*. Fixed alleles were observed in *E. florentina walkeri* (BSF) at *Ecap6* (allele 234) and *Ecap8* (allele 137).

Many unique alleles were observed in all five population groups and at every locus (see Appendix). 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 were unique, 55% in *E. florentina walkeri* (BSF) and 75% in *E. torulosa rangiana*; as previously stated, *Ecap3* did not amplify in *E. florentina walkeri* (IC). Interestingly, the patterns of allele frequencies and numbers of alleles were very different in *E. capsaeformis* (CR) from those of *E. florentina walkeri* (IC). Both species occur in the Clinch River and historically were sympatric at the periphery of their respective ranges near Richlands, VA (CRKM 510–515). However, these populations share only 21% of equal-sized alleles and overlapped in allele frequency only by 25% on equal-sized alleles. Overall, the level of allele frequency divergence among populations was high based on F_{ST} and R_{ST} estimates (Table 5). Pairwise F_{ST} comparisons ranged from 0.1164 to 0.3864, with the most similar taxa being *E. capsaeformis* (CR) and *E. capsaeformis* (DR), and the most dissimilar being *E. florentina walkeri* (IC) and *E. florentina walkeri* (BSF) based on microsatellite data. Pairwise R_{ST} comparisons ranged from 0.1458 to 0.7065, with the most closely related taxa also being *E. capsaeformis* (CR) and *E. capsaeformis* (DR); however, the most distantly related were *E. capsaeformis* (DR) and *E. florentina walkeri* (BSF).

Phenotypic variation of mantle-pads, micro-lures and glochidia

The mantle-pads and micro-lures of female mussels were distinct for each taxon and varied little within populations, with the exception of subtle colour differences in the pads. The mantle-pad of female *E. capsaeformis* (CR) was bluish-white (Fig. 3A, B), as reported previously (Ortmann, 1924; USFWS, 2004). Attached to the posterior end of each mantle-pad was a micro-lure, a cylindrical projection about 5 mm in length, that seemingly mimics the cercae of some insect larvae (Fig. 3B). The micro-lures were bluish to light grey with black fringes near the tips. The lures were 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-coloured incurrent aperture (above) and the bluish-white coloured mantle-pad (below). In *E. capsaeformis* (CR), this region was not invaginated, but rather the attachment points of the micro-lures could be seen on the mantle-pad when the female was displaying. In contrast, the posterior portions of the mantle-pad of *E. capsaeformis* (DR), *E. florentina walkeri* (BSF) and *E. florentina walkeri* (IC) were invaginated where they met the incurrent aperture (Fig. 3C, F, G); therefore, the attachment points of the micro-lures of females from these populations were concealed and not visible when the female was displaying. Thus, the micro-lure protruded out of this invaginated region in females from these latter populations.

The movement of the micro-lures was also distinct in *E. capsaeformis* (CR). The micro-lure attached to the left mantle-pad rotated clockwise in a circular pattern, while the micro-lure in the right pad rotated counterclockwise, and both were prominently displayed together. The micro-lures of all the in-group *Epioblasma* spp. in this study, except that of *E. torulosa rangiana*, which lacks a micro-lure (Jones, 2004), moved in a rhythmical manner, indicating that they are innervated structures. This is the first description of the presence and movements of micro-lures in mussel species of the genus *Epioblasma*.

The mantle-pad of female *E. capsaeformis* from the Duck River (DR) was dark-purple to slate-grey (Fig. 3C) (Ortmann, 1924). The surface texture of the pad was spongy, and the colour of the micro-lure was tan. Movement of the micro-lures of these females was different from that of *E. capsaeformis* (CR). Only one micro-lure was prominently displayed, and it moved in a side-to-side sweeping motion.

The mantle-pad of female *E. florentina walkeri* from Indian Creek (IC) was grey with a mottled black background (Fig. 3E, F). The surface texture of the pad was pustuled, and

Table 5. Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) estimates among populations of *Epioblasma* using data from 10 microsatellite loci.

Species	*E. capsaeformis* (CR)	*E. f. walkeri* (IC)	*E. f. walkeri* (BSF)	*E. capsaeformis* (DR)	*E. t. rangiana* (AR)
E. capsaeformis (CR)	–	0.4153	0.5205	0.1458	0.5710
E. f. walkeri (IC)	0.2067	–	0.5781	0.5946	0.5269
E. f. walkeri (BSF)	0.2108	0.3864	–	0.7065	0.2989*
E. capsaeformis (DR)	0.1164	0.3053	0.3216	–	0.6657
E. t. rangiana (AR)	0.1604	0.3087	0.3169	0.2379	–

All pairwise comparisons are significant ($P < 0.05$) unless marked with an asterisk (*).

the colour of the micro-lures was dark brown to black. Only one micro-lure was prominently displayed, and it moved in a side-to-side sweeping motion.

The mantle-pad of female *E. florentina walkeri* in the Big South Fork Cumberland River (BSF) was brown with a mottled tan background (Fig. 3G, H). The surface texture of the pad was pustuled, but the pustules tended to be finer and pointed as compared to those of *E. florentina walkeri* (IC). The micro-lures were brown, only one 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 was slower than that of *E. florentina walkeri* (IC).

The mantle-pad of female *E. torulosa rangiana* in the Allegheny River was white, and the surface texture was smooth (Jones, 2004). This species apparently does not have a true micro-lure. Field and laboratory observations of numerous females indicated that this species only has a vestigial 'nub' of tissue where the micro-lure is located in the other in-group species. The shells of this subspecies and others of *E. torulosa* were relatively thick for their size and typically possessed one or two knobs located on the centre of the shell. In addition, the female shell did not have denticulations along the margin of the marsupial expansion. In contrast, the shells of the other in-group taxa were thin, had denticulations and did not have knobs on the mid portion of the shell.

Mean lengths of glochidia of female mussels varied among populations and species and were significantly different in all pairwise comparisons ($P < 0.001$) (Table 6). Glochidia of *E. florentina walkeri* (IC) were the longest, averaging 271.9 μm, whereas those of *E. torulosa rangiana* (AR) were shortest at 241.3 μm. Significant differences were also observed in the variances. For example, length varied considerably ($SE = 11.5$) for glochidia of *E. florentina walkeri* (BSF), ranging from 231 to 282 μm, and many appeared somewhat asymmetrical (Table 6). In contrast, glochidia of *E. capsaeformis* (CR) were symmetrical and varied little in length ($SE = 5.2$).

Fish-host specificity

Fish-host specificity varied significantly ($P < 0.001$) among *E. capsaeformis* (CR), *E. capsaeformis* (DR) and *E. florentina walkeri* (BSF) (Table 7). Glochidia of *E. capsaeformis* (CR) transformed in greatest numbers on 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. florentina walkeri* (BSF) transformed in greatest numbers on fantail darter *Etheostoma flabellare*, which produced an average of 59% and 73% of the juveniles, respectively; these mussel species transformed infrequently on *Etheostoma blennioides*. Our results corroborate those of Rogers *et al.* (2001), who also reported *Etheostoma flabellare* as the most suitable host for *E. florentina walkeri* in Indian Creek.

Table 6. Mean (SE) lengths of glochidia measured for the *Epioblasma* species.

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

All pairwise comparisons were significantly different ($P < 0.001$). Locality abbreviation is given in parentheses (see Table 1).

Table 7. Mean (±SE) percentages of juvenile mussels transformed per fish host species.

Mussel species	Mean number of juveniles per darter		
	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

	(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

Abbreviations: S, significant; N, not significant.
Pairwise comparisons ($P < 0.001$).

DISCUSSION

Type specimens and species sympatry

Examination of type specimens and shell material for *E. capsaeformis* and *E. florentina walkeri* from various rivers yielded several conclusions about geographic variation in shell morphology among in-group taxa. There was little clinal or geographic variation in periostracum colour and ray pattern in shells of *E. capsaeformis* throughout the Cumberlandian Region—an observation supporting the view that these characters are genetically determined. It is unlikely that these shell characters could be maintained over a wide geographic range, of varying environmental conditions, without a strong genetic basis. The lack of shell intergrades among historically sympatric populations of *E. capsaeformis* and *E. florentina walkeri*, as well as with other members of the *Torulosa* subgenus, supports their continued recognition as separate species. Further, their geographic co-occurrence and similarity in molecular and morphological characters suggest that species in this subgenus represent a recent radiation of sympatric forms within the Unionidae, perhaps the first to be recognized for freshwater bivalve molluscs.

The distributions of many mussel species throughout the Cumberlandian Region are neither continuous nor random, but rather occur in discrete and predictable river reaches. Species endemic to the region, as with 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, including the Buffalo River, (3) middle Tennessee River drainage from Bear Creek upstream to the Paint Rock River and (4) upper Tennessee River drainage, from Walden Gorge near Chattanooga to all upstream major tributaries (Wilson & Clark, 1914; Ortmann, 1918, 1924, 1925; Neel & Allen, 1964). The lower reaches of the Tennessee and Cumberland Rivers and Walden Gorge appeared 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 patterns of genetic variation concordant with these geographic areas.

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. The suite of diagnostic nucleotides demonstrates that the combined sequences were unique to their respective populations, suggesting the absence of contemporary gene flow among populations, which was concordant with observed phenotypic variation. The MP tree topology (Fig. 2) placed *E. capsaeformis* (DR) basal to the other in-group taxa, and *E. torulosa rangiana* (AR), *E. florentina walkeri* (IC) and *E. florentina walkeri* (BSF) together in the same group. However, the phylogenetic relationships implied by this topology received no bootstrap support, and hence may not be correct. The absence of a micro-lure and thicker, noduled shell of *E. torulosa rangiana* suggests that this species may be a more basal member of the *Torulosa* clade. Lack of agreement between a species-tree and gene-tree is not uncommon for closely related species (Avise, 2000; Hartl, 2000; Nei & Kumar, 2000), and discordance between the two is possible under various scenarios (Sites & Crandall, 1997 and references therein).

DNA sequence divergence of 3–6% is typical of interspecific comparisons in unionids (Lydeard *et al*., 1996; Roe & Lydeard, 1998; Roe *et al*., 2001; Serb *et al*., 2003). However, estimates of genetic distance can be low for recently diverged taxa, and likely are dependent on the amount of time elapsed since the reproductive or geographic isolation of populations (Nei & Kumar, 2000). Hence, a limited molecular survey of the mtDNA genome may not contain sufficient genetic variation to discriminate 'species-level differences' among recently diverged taxa. In our study, coalescence of the in-group taxa into their respective monophyletic lineages was achieved only by sequencing about 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, as in Buhay *et al*. (2002). Thus, in some cases, the level of paraphyly may be an artefact of how much of the genome is investigated. Furthermore, certain DNA sequence regions contained more nucleotide variation than others in this study, such as cytochrome-*b* and ND1. This finding highlights the need better to 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 & Lydeard, 2003). Combining mtDNA sequences of up to 1000–2000 bp or greater 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).

DNA microsatellites and population history

In contrast to the DNA sequence markers, hypervariable DNA microsatellite markers portrayed highly diverged mussel populations based on F_{ST} and R_{ST} analyses. 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} and R_{ST} values corroborated inference for other data sets, i.e. that gene flow between populations is infrequent or absent. These data supported the inference that the in-group populations are reproductively isolated from each other. Interestingly, our results suggest that these historically sympatric populations, such as *E. capsaeformis* and *E. florentina walkeri* in the Clinch River, were nearly indistinguishable at certain DNA sequence regions (e.g. the 16S region of the mtDNA genome), but divergent for a suite of nuclear loci (DNA microsatellites) and quantitative traits (e.g. shell and mantle-pad morphology, length of glochidia, fish-host specificity). However, with the exception of *E. capsaeformis* (CR) and *E. florentina walkeri* (IC) in the Clinch River, study populations are geographically separated by several hundred to more than a thousand river kilometers. Thus, allelic divergence likely arose in part by mutation, random genetic drift and varying selective regimes during the geographic isolation of populations.

Analysis of allele frequency variation at microsatellite loci also provided insights into the different population histories of each mussel species. For example, the lower heterozygosities and average number of alleles per locus for *E. capsaeformis* (DR), *E. florentina walkeri* (IC) and *E. florentina walkeri* (BSF) suggest that these populations have been demographically bottlenecked (Table 4). Recent impacts on populations contributing to 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 basin (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 bottlenecked by known anthropogenic factors. Thus, reduction of allelic diversity through anthropogenic impacts may help to explain the low overlap in alleles and the high F_{ST} and R_{ST} values among some of the studied populations. Deviations from HWE and LE at some loci could be the result of recent population bottlenecks or high levels of close inbreeding, the latter perhaps due to facultative hermaphroditism in unionids (van der Schalie, 1970). As many taxonomic questions typically involve closely related species or populations, traditional genetic analyses that employ larger sample sizes and a suite of co-dominant, multi-locus nuclear markers to assess the levels of genetic divergence between populations are recommended.

Mantle-pad phenotypes and length of glochidia

Historical populations of the in-group taxa may have contained more variation in mantle-pad coloration than exists today. 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 that in rivers throughout the upper Tennessee River drainage was white to blue (Ortmann, 1918). Thus, historically, colour was seemingly a polymorphic character. If so, the fixation of differently coloured mantle-pad phenotypes in the middle (grey-black) and upper reaches (bluish-white) of the Tennessee River drainage may suggest adaptively significant directional selection, or the effects of random genetic drift as populations declined. The coloration of the mantle-pad of *E. capsaeformis* that inhabited the Cumberland River system is unknown. This species is extirpated now from the drainage, and the colour of the mantle-pad was not described by Wilson & Clark (1914) during their survey of the river basin. However, local residents that recreated on the BSF Cumberland River reported seeing the display of the bluish-white mantle-pad, presumably of this species, on the river, bottom (R.S. Butler, USFWS, personal communication, 2003).

Variation in mean lengths of glochidia of female mussels is seemingly a quantitative genetic difference among in-group taxa. It seems unlikely that the developmental size characteristics of glochidia are strongly influenced by environmental factors.

Fish-host specificity

The differently coloured mantle-pads of the in-group taxa may be adaptively significant and indicate how species persist in certain environments and attract different fish hosts. The cryptically coloured mantle-pads of *E. florentina walkeri* (IC) and *E. florentina walkeri* (BSF) appear better adapted to headwater 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 such habitats. This latter species seems better adapted to larger rivers where increased depth and width can provide greater protection to displaying females from predators.

The bluish-white pad of *E. capsaeformis* (CR) may be better at attracting brightly coloured darters, such as *Etheostoma blennioides* and *Etheostoma 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 coloured to serve as a mating cue for females. The bright mantle-pad of *E. capsaeformis* (CR) may attract these darters and elicit reproductive or aggressive responses. We hypothesize that initially the colour of the mantle-pad acts to attract a fish host to the displaying female mussel, and then the movement of the micro-lures to resemble a prey item brings the fish into close contact with the female mussel and her glochidia. A unique behaviour of mussel species in the subgenus *Torulosa* is that displaying females quickly close their shells when touched. This snapping behaviour can actually capture host fish. Such behaviour and comb-like denticulations along the margin of the shell likely facilitate the capture and subsequent infestation of the fish host by glochidia. We have observed gravid *E. capsaeformis* (CR) and *E. florentina walkeri* (IC) with darters trapped between the shells, inside the mantle cavity. In addition, biologists have observed darters probing inside the mantle-pad of *E. capsaeformis* (CR) and observed that the female mussel snapped shut and captured the darter host (T. Brady, USFWS, personal communication, 2001).

One of the primary fish hosts for *E. florentina walkeri* (BSF) and *E. florentina walkeri* (IC) is *Etheostoma flabellare*, which also prefers headwater environments. Spawning males of *Etheostoma flabellare* become darkly coloured, as in other fish species in the subgenus *Catonotus*, and may be attracted to similar colours, such as the darker pads of *E. capsaeformis* (DR), *E. florentina walkeri* (BSF) and *E. florentina walkeri* (IC). Resident hosts for *E. capsaeformis* (DR) are unknown because a life history study has not been conducted on this population. However, our fish-host specificity data suggest that darter species in the subgenus *Catonotus* are candidates. We note that the Duck River fish fauna is one of the richest in the southeastern United States and may contain additional host fishes for *E. capsaeformis* (DR).

The fish-host specificity data indicate that certain species of darters are quantitatively better hosts for particular in-group mussel taxa (Table 7). These results support the hypothesis that fish host specificity is a factor driving expression of quantitative genetic characters for freshwater mussels. One weakness of our study is that fish-host tests were conducted using fish species from only one river drainage. Additional trials using both sympatric and allopatric populations of fish species could reveal additional mussel-fish host relationships. Fish-host use is likely fitness-related and may isolate mussel populations geographically, ecologically and, ultimately, reproductively. Other potential sources of quantitative genetic variation needing study in mussels include spawning temperatures, spawning seasonality and glochidial release periods. Gamete recognition proteins (lysins) in species of Pacific abalone have been implicated as reproductive isolation mechanisms in these molluscs (Swanson & Vacquier, 1995; Vacquier, 1998). Therefore, comparable studies on these and other protein markers in mussels may elucidate how highly diverse communities of co-occurring species maintain reproductive isolation.

Taxonomic implications

The results of this study provide evidence of a new species and subspecies of mussel in the Tennessee River drainage. We propose that the population of *E. capsaeformis* in the Duck River be recognized as a new species separate from *E. capsaeformis* in the Clinch River because of (1) distinctiveness of molecular genetic markers, (2) differences in coloration and texture of the mantle-pad, (3) greater height of marsupial expansion of the female shell, (4) smaller size of glochidia, (5) differing fish-host specificity and (6) behavioural differences in the movement of micro-lures. Furthermore, the populations of *E. capsaeformis* (DR) and *E. capsaeformis* (CR) were not closely related phylogenetically relative to other in-group taxa based on the molecular phylogeny, and lacked a suite of shared characters to unite them as a species; therefore, both populations qualify as phylogenetic species under the Phylogenetic Species Concept of Cracraft (1983).

We propose that the population of *E. florentina walkeri* (IC) in Indian Creek be designated as a separate subspecies from *E. florentina walkeri* (BSF) because of the following differences: (1) distinctiveness of molecular genetic markers, (2) coloration of mantle-pad, (3) size of glochidia and (4) allopatric ranges in the Cumberlandian Region. This suite of differences allowed us to identify reliably and classify each population as a taxonomically separate entity. We believe these populations are not deserving of a separate species designation because they shared (1) honey-yellow to brown-coloured periostracum, (2) similar fish-host specificity, (3) pustuled mantle-pad and (4) preference for headwater stream habitats. In addition, the two populations had different, but similar-sized glochidia and were closely related phylogenetically based on the molecular phylogeny.

Because populations of *E. capsaeformis* (CR), *E. capsaeformis* (DR) and *E. florentina walkeri* (IC and BSF) were geographically, demographically and genetically independent, the criterion of reproductive isolation was met with a reasonable level of confidence and therefore should qualify each population as a biological species under the Biological Species Concept (Mayr & Ashlock, 1991). However, because of the important shared traits discussed earlier, we recommend that populations of *E. f. walkeri* (IC) and *E. f. walkeri* (BSF) be considered as subspecies. The level of historical sympatry and lack of intergrades between these disparate populations support these conclusions. 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 further substantiate the genetic basis of phenotypic and quantitative traits. However, lack of direct data on reproductive isolation should not prevent the reasonable and prudent designation of biological species using the best available data, such as those presented in this study.

CONCLUSIONS

Variation at phenotypic and quantitative markers among in-group mussel taxa in this study 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 assess DNA sequence data, and to conclude that the in-group taxa were valid, but closely related, species or subspecies. Furthermore, only reproductive isolation adequately explains how variation at both mtDNA and nDNA molecular markers, as well as complex morphological and life history traits, were maintained by these historically sympatric populations throughout various rivers in the Cumberlandian Region. We strongly recommend that future taxonomic and phylogenetic studies combine complementary information from molecular markers, functional protein markers, morphology, life history traits, behaviour and biogeography whenever possible. Holistic analyses using a suite of characters 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 imperiled species, where study results could alter or jeopardize the protective status of a species. Finally, our holistic analysis of populations of *E. capsaeformis* and *E. florentina walkeri* clearly shows that these taxa are biologically and ecologically distinct and should be managed accordingly.

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APPENDIX

Allele frequencies of DNA microsatellites examined for in-group *Epioblasma* species. Allele sizes are given in number of base pairs including the primer flanking regions.

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

Continued

Continued

Locus	Allele	*Epioblasma capsaeformis* (CR)	*Epioblasma f. walkeri* (IC)	*Epioblasma f. walkeri* (BSF)	*Epioblasma capsaeformis* (DR)	*Epioblasma t. rangiana* (AR)
	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	–	–	–

Continued

Continued

Locus	Allele	*Epioblasma capsaeformis* (CR)	*Epioblasma f. walkeri* (IC)	*Epioblasma f. walkeri* (BSF)	*Epioblasma capsaeformis* (DR)	*Epioblasma t. rangiana* (AR)
	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**	–	–	–	–

*Locus did not amplify for all mussel species. Alleles unique to a population are shown in bold.