

# The phylogeographical and management implications of genetic population structure in the imperiled snuffbox mussel, *Epioblasma triquetra* (Bivalvia: Unionidae)

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Seven populations of the imperiled snuffbox mussel, *Epioblasma triquetra*, were sampled from across the central basin of North America. Samples were genotyped using 15 microsatellite DNA loci, and maternal history was inferred using mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit-I (COI) sequences. Populations in the Clinch and St Francis rivers were quite distinct in their mtDNA. The population in the St Francis River had a unique, fixed haplotype. Among a suite of haplotypes, the population in the Clinch River had two unique haplotypes of common ancestry. The other populations were dominated by a common haplotype, which also occurred in the Clinch River population. Analysis of DNA microsatellites revealed much greater divergences and showed significant genetic structure between populations in the formerly glaciated regions. Divergence has occurred between the populations, as evidenced by moderate to high fixation indices ( $F_{ST}$  and  $R_{ST}$  values) and nearly perfect assignment tests. These results indicate the occurrence of three glacial refugia for *E. triquetra*: the Tennessee River, rivers south of the Ozark Crest, and the lower Ohio River drainage near the confluence with the Mississippi. Populations in the lower Ohio River were likely to be responsible for the postglacial reinvasion into formerly glaciated regions, and into the upper Tennessee River drainage. The population of the St Francis River may constitute a distinct taxonomic entity. Conservation efforts, if necessary for this imperiled species, should not mix populations. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, 93, 371–384.

ADDITIONAL KEYWORDS: conservation genetics – freshwater mussels – microsatellite DNA – mtDNA – population genetics – postglacial redistribution.

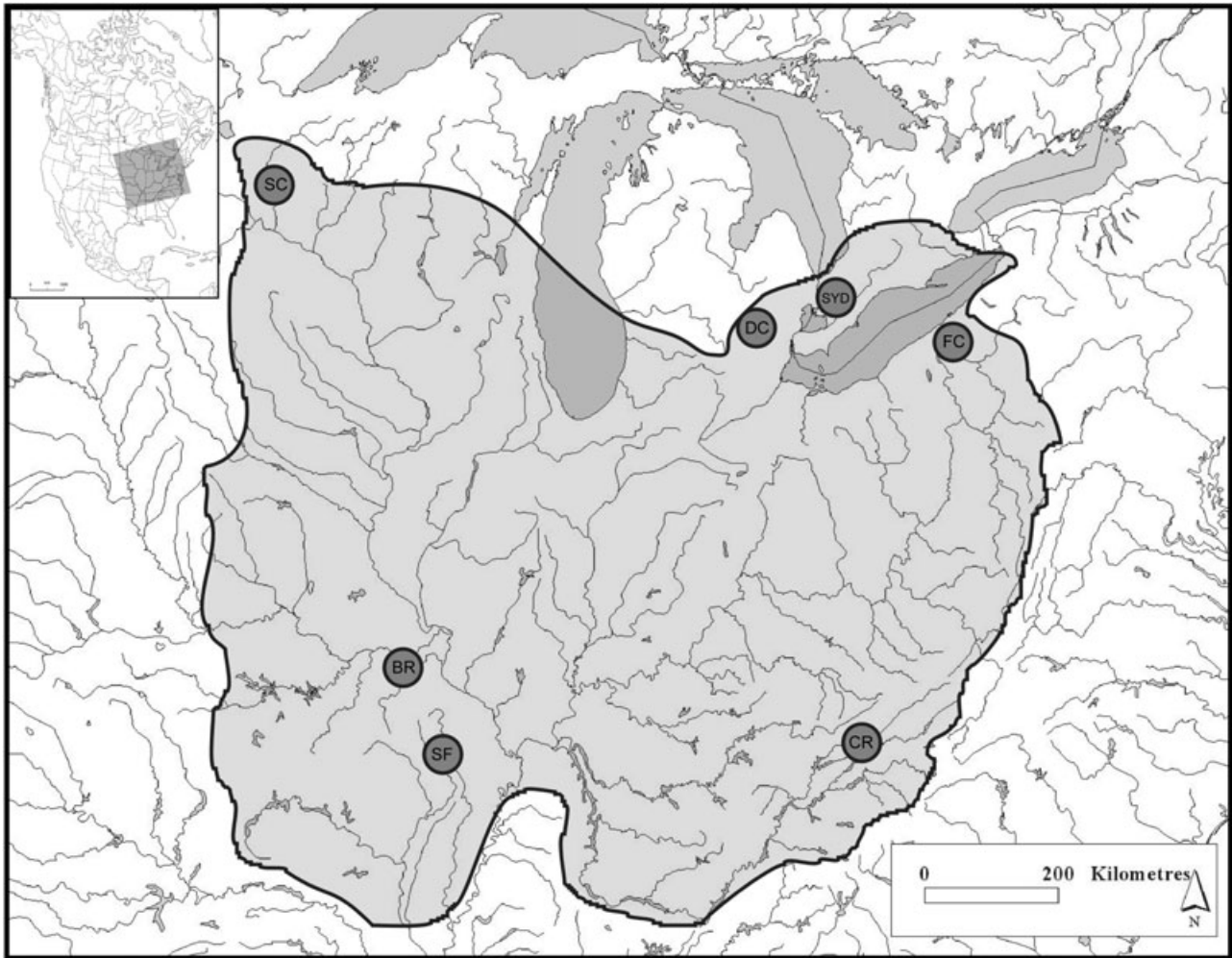
## INTRODUCTION

Of the generally declining freshwater mussels (Lydeard *et al.*, 2004; Strayer *et al.*, 2004), members of the genus *Epioblasma* (Simpson, 1900) have suffered particularly severe declines over the last 100 years. Of 20 recognized species, 13 became extinct in the twentieth century. Apparently, members of this genus are especially intolerant of human-induced changes (Peacock, Haag & Melvin, 2005). The decline of *Epioblasma* can be traced to the advent of land clearing for row-crop maize agriculture by Native Americans,

and the concomitant increases in run-off and turbidity in streams and rivers (Peacock *et al.*, 2005). However, the rate of decline has intensified over the past century. The construction of impoundments, habitat alteration, and industrial and urban pollution are significant contributors to the demise of unionids (Bogan, 1993, 2006).

Among the extant species of *Epioblasma*, the snuffbox mussel, *Epioblasma triquetra*, has the largest range (Johnson, 1978; Williams *et al.*, 1993; Watson, Metcalfe-Smith & Di Maio, 2000). Zoogeographically, *E. triquetra* is one of the few unionids in the Tennessee and Cumberland river systems, as well as in rivers south of the Ozark Crest in Missouri and Arkansas. Johnson (1978) suggested populations of *E. triquetra* might have persisted since before the Cretaceous uplift of these highland regions. The Cumberlandian and Ozarkian refugia have been assumed

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**Figure 1.** The historical range (shaded in light grey) and distribution of populations where tissue collections were made for *Epioblasma triquetra*. Sample site localities: Bourbeuse River (BR), at Reiker Ford near Union, MO (38.3856°N, 91.0729°W); Clinch River (CR), at Upper Brooks Island near Sneedville, TN (36.5378°N, 83.1152°W); Davis Creek (DC), near Ann Arbor, MI (42.4678°N, 83.7444°W); French Creek (FC), upstream of Cambridge Springs, PA (41.9689°N, 79.8653°W); St Croix River (SC), at Interstate Park, MN/WI (45.3932°N, 92.6646°W); St Francis River (SF), upstream of Wappapello Reservoir, MO (37.2369°N, 904885°W); Sydenham River (SYD), near Florence, ON (42.6912°N, 82.9892°W).

to be the source of postglacial recolonization in the Ohio and Mississippi River drainages, respectively (Johnson, 1978). Other possible refugia for *E. triquetra* include the Meramec River system in Missouri, and the Allegheny and Monongahela river drainages in Western Pennsylvania and West Virginia (Johnson, 1978). Larvae (glochidia) of freshwater mussels are parasites on fish, which serve to complete their life cycle and disperse progeny. Species of *Epioblasma* primarily use darters (Percidae: *Etheostoma* and *Percina*) as host fish (Jones *et al.*, 2006). *Epioblasma* have evolved a unique host attraction strategy of luring then capturing a potential host in order to infest the host with their glochidia (Jones *et al.*, 2006). The passage for mussels and their associated

host fish into the Lake Erie drainage is believed to be a historical connection between the drainages of the Wabash and Maumee rivers (Bailey & Smith, 1981; Calkin & Feenstra, 1985; Graf, 2002).

*Epioblasma triquetra* is the only species of *Epioblasma* not listed under the United States (US) Endangered Species Act. Today, many populations have been greatly reduced or have disappeared completely (Fig. 1; R. Butler, pers. comm.; Watson *et al.*, 2000). The American Malacological Society and American Fisheries Society consider it to be a threatened species (Williams *et al.*, 1993). It is state listed in many parts of its range, and is federally endangered in Canada. It is currently under status review for potential listing under the US Endangered Species

Act (R. Butler, pers. comm.). The historical range in the US and Canada includes the following areas: the upper White River system, Missouri; Missouri River drainage, Kansas and Missouri; Mississippi River system, Wisconsin, Minnesota, and Iowa; Illinois River drainage, Illinois; Tennessee and Cumberland River systems; Green River drainage, Kentucky; Ohio River system, from Indiana to Pennsylvania; the St Lawrence River system in the drainages of Lakes Michigan, Erie, and Huron (Johnson, 1978; Watson *et al.*, 2000).

Microsatellite markers for unionids are now being developed at a fast pace, and include five published sets (Eackles & King, 2002; Geist *et al.*, 2003; Jones *et al.*, 2004; Shaw *et al.*, 2006; Zanatta & Murphy, 2006). Using microsatellite markers, significant population structure was found in the European pearl mussel, *Margaritifera margaritifera*. Many populations both within and among river drainages had unique alleles, and high pairwise fixation indices ( $F_{ST}$  values) and genetic distances (Nei, 1972; Geist & Kuehn, 2005). Similarly, populations of *Lampilis cariosa* along the Atlantic coast of Maine showed significant population structure (Kelly & Rhymer, 2005). Genetic variation between mussel populations in rivers draining into salt water would be expected to be high, as there would be less opportunity for the transport of glochidia by host fish between populations.

Molecular data are critical for the conservation management of imperiled freshwater mussels. Ecology, captive care, and propagation have been emphasized in the planning for the recovery of endangered freshwater mussels (NNMCC, 1998). Animals should be relocated in areas that contain similar genetic profiles in order to augment populations. Data on the genetic characteristics of mussel populations are needed to make informed decisions regarding the numbers, localities, and logistical concerns of potential relocations or population augmentation through artificial propagation (Villella, King & Starliper, 1998). Herein, we use both multilocus microsatellite genotypes and mitochondrial DNA (mtDNA) sequences to assess population structure and to measure gene flow in *E. triquetra* across several faunal provinces of the central basin of North America.

## MATERIAL AND METHODS

### SAMPLE LOCALITIES AND TISSUE COLLECTION

Literature searches and consultation with local conservation managers and malacologists were conducted to locate extant populations of *E. triquetra* (Johnson, 1978; Oesch, 1995; Watson *et al.*, 2000; Hornbach, 2001; R. Butler, pers. comm.; T. Smith,

pers. comm.; S. McMurray, pers. comm.; B. Sietman, pers. comm.; J. Jones, pers. comm.; R. Sherman, pers. comm.). Specimens of *E. triquetra* (131) were collected from six localities in the US and from one locality in Canada. From each of the following locations 20 individuals were collected: the Bourbeuse River (BR) at Reiker Ford, Missouri; the Clinch River (CR) at Brooks Island near Sneedville, Tennessee; French Creek (FC), upstream of Cambridge Springs, Venango County, Pennsylvania; the St Croix River (SC) at Interstate Park, Wisconsin; Davis Creek (DC), Huron River drainage near Ann Arbor, Michigan; the St Francis River (SF), near Patterson, Missouri. Eleven individuals were also collected from the Sydenham River (SYD) near Florence, Ontario (Fig. 1). Photographic vouchers are available from the authors.

Mantle tissue (~30 mg) was nondestructively excised following the protocols of Berg *et al.* (1995). Tissues were placed in cryovials, frozen on dry ice, and subsequently stored at -80 °C.

### DNA EXTRACTION AND GENETIC ANALYSES

Total genomic DNA was extracted from ~15 mg of frozen preserved mantle tissue samples by standard phenol extraction (Hillis *et al.*, 1996). A 630-bp sequence of cytochrome *c* oxidase subunit-I (COI) mtDNA was amplified using primers described by Campbell *et al.* (2005) to determine variation among populations. The polymerase chain reaction (PCR) mixture for COI consisted of 1.0 µL of genomic DNA, 1.0 µM of each primer, 0.4 mM deoxyribonucleotide triphosphate (dNTP), 1 × PCR buffer [2.0 mM Tris-HCl, 10 mM KCl, 0.01 mM EDTA, and 0.1 mM dithiothreitol (DTT)], 2.0 mM MgCl<sub>2</sub>, and 1.5 U Taq (Promega™ or Fisher™). Each PCR was amplified at a 25-µL reaction volume in a PTC-200 thermocycler (MJ Research). Conditions for the amplification of COI were as follows: 92 °C for 2 min; five cycles of 92 °C for 40 s, 40 °C for 40 s, 72 °C for 90 s; 25 cycles of 92 °C for 40 s, 50 °C for 40 s, 72 °C for 90 s; 72 °C for 10 min; and a final hold at 4 °C.

Double-stranded PCR products were visualized using 1.0% agarose gels stained with ethidium bromide. PCR products were purified using Quiaquick DNA (Quiagen, Inc.) purification kits. The 5' end of the amplified products was cycle-sequenced using 'Big Dye' Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Inc.) with the forward COI primer (50 °C annealing temperature), and were visualized on an ABI 3100 automated DNA sequencer.

A suite of 15 microsatellite loci (GenBank accession numbers: AY650390, AY650393–650397, AF512384–512386, AF512395–512398, and DQ396404–396406) developed for other unionid species [*Epioblasma capsaeformis* (Jones *et al.*, 2004), *Lampsilis abrupta*

(Eackles & King, 2002), and *E. torulosa rangiana* (Zanatta & Murphy, 2006)] were used to characterize the genetic population structure of *E. triquetra* in this study.

The amplification of these loci followed methods slightly modified from Zanatta & Murphy (2006). Forward PCR primers were ordered with an M13 tail on the 5' end, following the protocol of Schuelke (2000). PCR reactions were performed in a 25 µL solution containing 1.0 µL of genomic DNA, 8 pmol of each of the forward primer with an M13 tail, the reverse primer, and a 6-FAM or VIC-labelled M13 primer, 0.3 µM dNTP, 1× PCR buffer (2.0 mM Tris-HCl, 10 mM KCl, 0.01 mM EDTA, and 0.1 mM DTT), 2.0 µM MgCl<sub>2</sub>, and 1.5 U Taq (Promega™ or Fisher™). Each PCR run (94 °C for 2 min; 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 1 min; a final extension step of 72 °C for 1 min and a hold at 4 °C – Eppendorf Mastercycler 534X thermocycler) included a negative control. Double-stranded PCR products were visualized with a 1 kb + ladder to estimate the fragment length in a 1.0% agarose gel stained with ethidium bromide. Non-overlapping amplified microsatellite loci were multiplexed and genotyped with an LIZ size standard (dye set DS-33) using an Applied Biosystems (ABI) 3100 automated sequencer and scored using ABI's GeneMapper software.

#### STATISTICAL ANALYSES OF MTDNA

DNA sequences were first aligned using ClustalX (Thompson *et al.*, 1997) and MacClade (Maddison & Maddison, 1997). Using TCS v. 1.21 (Clement, Posada & Crandall, 2000), the sequences were summarized by identifying unique haplotypes. TCS v. 1.21 was also used to construct a haplotype network based on the number of nucleotide mutations between different haplotypes. A maximum parsimony analysis was performed via a heuristic search with 1000 replications of random stepwise additions using PAUP\* v. 4.0b10 (Swofford, 1998). To gauge the robustness of nodes within the resulting trees, bootstrap values were calculated. Bootstrapping used 1000 replications and heuristic searching with ten random stepwise additions. Sequences of COI from *Epioblasma brevidens* (GenBank no. AF156527), *E. capsaeformis* (GenBank no. AY654996), *Epioblasma florentina walkeri* (GenBank no. AY094374), and *E. torulosa rangiana* (GenBank no. AY094374) were used as out-group taxa to root trees.

A second phylogenetic analysis used Bayesian inference implemented in MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2001). The initial model of evolution was determined by comparing 24 models of evolution in MrModeltest 2.2 (Nylander, 2004). MrBayes was run using 1 000 000 generations, sampling every 100 gen-

erations (10 000 trees total), and the most likely tree was calculated using posterior probabilities with a burn-in of 50 000 generations (500 trees). A 50% majority-rule consensus tree was constructed from the remaining 9500 trees.

Hierarchical analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) was used to estimate the partitioning of COI haplotypes within and among populations. The AMOVA was conducted using ARLEQUIN v. 2.0 (Schneider, Roessli & Excoffier, 2000).

#### STATISTICAL ANALYSES OF MICROSATELLITE DNA

Genetic diversity in each sample (for the microsatellite DNA dataset) was summarized as allelic richness (*A*), which was measured as the mean number of alleles per locus after correcting for sample size and expected heterozygosity (*H<sub>E</sub>*). Allelic richness was calculated in FSTAT v. 2.9.3.2 (Goudet, 1995). These data were standardized for sample size using a process of rarefaction (Petit, El-Mousadik & Pons, 1998). Detection of deviations from the Hardy-Weinberg equilibrium and randomization tests for linkage disequilibria were conducted using GENEPOP v. 3.4 (Raymond & Rousset, 1995).

The microsatellite dataset was tested for genotyping errors caused by stuttering, short allele dominance, and null alleles using a Monte Carlo simulation of expected allele size differences using MICROCHECKER (Van Oosterhout *et al.*, 2004). Allele size-difference frequencies were determined to deviate from expectations if they fell outside the Bonferroni-corrected 95% confidence interval generated by the simulation. Predicted frequencies of null alleles were calculated according to the method developed by Brookfield (1996).

An AMOVA (Excoffier *et al.*, 1992) was used to estimate partitioning of genetic variance within and among populations (Schneider *et al.*, 2000) using data pooled across loci. *F<sub>ST</sub>* (Weir & Cockerham, 1984) and stepwise fixation indices *R<sub>ST</sub>* (Slatkin, 1995) values were calculated for all pairs of populations, along with corresponding *P* values, in order to test the null hypothesis of panmictic populations by permuting genotypes among populations to calculate the probability of obtaining equal or greater *F<sub>ST</sub>* (or *R<sub>ST</sub>*) by chance distribution of genotypes. Corresponding to the *F<sub>ST</sub>* values, geneflow was inferred using *N<sub>m</sub>* (number of migrants per generation), calculated using  $N_m = (1/F_{ST} - 1)/4$ .

Genetic distances between populations were estimated using Nei's *D<sub>A</sub>* genetic distance (Nei, Tajima & Tatenno, 1983), as implemented in the DISPAN program (Ota, 1993). The resulting distance matrix was used to construct a neighbour-joining (NJ)

network in MEGA version 3.1 (Kumar, Tamura & Nei, 2004). Bootstrapping was performed by first generating 1000 distance matrices, which were then used to generate 1000 NJ trees in DISPAN (Ota, 1993).

Assignment tests were used to estimate the probability of each individual originating from a given population (Paetkau *et al.*, 1995; Rannala & Mountain, 1997). GeneClass2 (Piry *et al.*, 2004) was used to implement the assignment tests (Rannala & Mountain, 1997). This technique estimated the posterior probabilities of allele frequencies given the observed allele frequencies from that population, and then estimated the probability for each individual of belonging to any of the populations. For each population,  $\chi^2$  was calculated to test whether the level of assignment was greater than could be expected by chance.

A test to detect recent population bottlenecks using infinite-allele, two-phased, and stepwise mutation models was implemented in BOTTLENECK (Cornuet & Luikart, 1996).

RESULTS

RESULTS FROM MTDNA

Sequencing consistently resulted in 614 bp from COI. Eight distinct haplotypes were identified among the populations (GenBank accession numbers EF507804–507816). Haplotypes were resolved and the frequencies of each haplotype are presented in Table 1. The same single haplotype (Etri1) was most frequent in all of the populations except for the St Francis River population. This indicated that *E. triquetra* may have been bottlenecked prior to postglacial reinvasion into the previously glaciated regions.

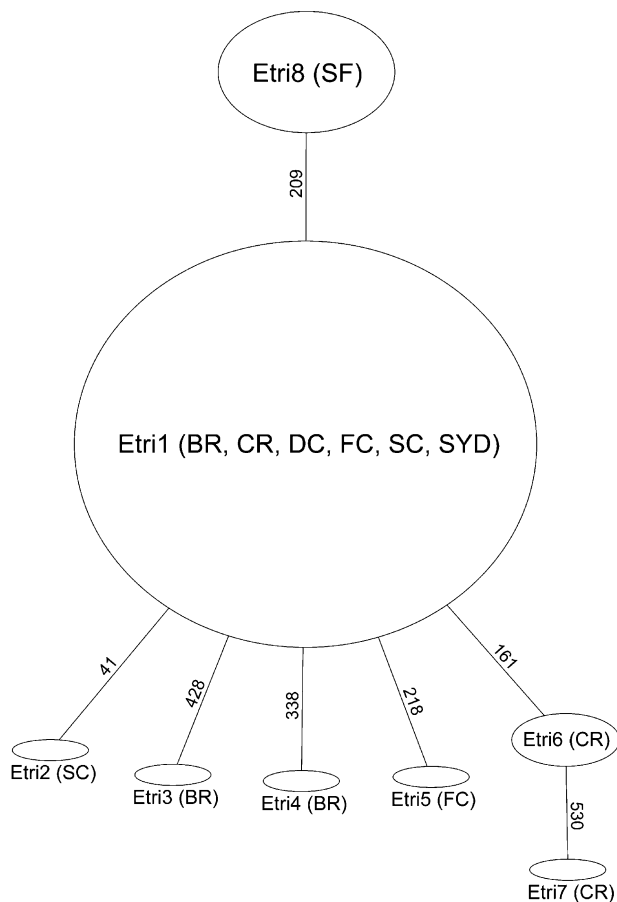
Haplotype networks constructed for COI (Fig. 2) grouped individuals of *E. triquetra* from the St Francis River along unambiguous population boundaries. Bayesian inference and maximum parsimony analysis yielded nearly identical and compatible topologies for the COI gene sequences. When forced into a phylogenetic comparison (e.g. parsimony or Bayesian analyses), *E. triquetra* exhibited a shallow gene genealogy at COI that would be expected among recently diverged groups.

Nucleotide diversity for COI within populations (Table 1) consisted of only one or two base pairs varying between haplotypes. No diversity was observed in the Davis Creek, the St Francis or the Sydenham River populations. Nucleotide diversity ranged from 0.000% to 0.326%. The St Francis River population was fixed for a unique haplotype (Etri8).

Significant population structure was evident using COI sequences in *E. triquetra* ( $P < 0.001$ ). The AMOVA for COI indicated that only 32.73% of variation in *E. triquetra* resided within populations, whereas 67.27% occurred among populations (Table 2).

Table 1. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes, and indices of population diversity for cytochrome *c* oxidase subunit-I (COI) in seven populations of *Epioblasma triquetra*

Haplotypes and polymorphic nucleotide sites	Populations												
	BR ( <i>n</i> = 18)	CR ( <i>n</i> = 18)	DC ( <i>n</i> = 18)	FC ( <i>n</i> = 13)	SC ( <i>n</i> = 16)	SF ( <i>n</i> = 20)	SYD ( <i>n</i> = 10)						
1	A	T	A	A	G	A	A	0.888	0.611	1.000	0.923	0.937	1.000
2	T											0.063	
3								0.056					
4								0.056					
5											0.077		
6													
7													
8													
Number of haplotypes:	3	3	1	2	2	2	1	3	3	1	2	2	1.000
Number of polymorphic sites:	2	2	0	1	1	1	0	2	2	0	1	1	1
Nucleotide diversity per site ( $\pi$ ) within population (%):	0.326 ± 0.036	0.326 ± 0.036	0.000	0.163 ± 0.012	0.163 ± 0.009	0.163 ± 0.009	0.000	0.326 ± 0.036	0.326 ± 0.036	0.000	0.163 ± 0.012	0.163 ± 0.009	0.000



**Figure 2.** Haplotype network for eight cytochrome *c* oxidase subunit-I (COI) haplotypes observed in *Epioblasma triquetra*. Each node represents a haplotype, and the size of the node is relative to the number of individuals sharing that haplotype. Location abbreviations are defined in Figure 1.

#### RESULTS FROM MICROSATELLITES

Of the 15 microsatellite loci amplified for *E. triquetra*, 14 were highly polymorphic, with a total of 228 alleles observed (3–32 alleles/locus, mean = 15.2). Genetic diversity as measured by *A* varied somewhat between populations (3.5–6.6 alleles/locus), with the lowest allelic richness occurring in the St Croix River, and the highest allelic richness occurring in the Clinch

River (Table 3). Expected heterozygosities ranged from 0.56 to 0.79 and varied somewhat between populations (Table 3).

Significant deviations from Hardy–Weinberg expectations occurred at four out of 105 locus–population combinations, after a Bonferroni correction (Table 3). These deviations indicated the possible presence of null alleles or other locus-specific genotyping errors. Additionally, the deviations could have resulted from insufficient sample sizes in the presence of a relatively large number of alleles at several loci. Analysis of the microsatellite dataset showed no evidence for genotyping errors caused by stuttering or large-allele dropout, suggesting the presence of non-amplifying alleles as a probable source of genotyping error. Estimated null frequencies varied by population and locus, but ranged as high as 0.197 for one locus–population combination, with a mean frequency of 0.069. Randomization tests for linkage disequilibrium by locus and population did not indicate any significant linkage disequilibria for any of the 735 di-locus combinations in *E. triquetra*.

Significant population structuring was evident for *E. triquetra*. Of the total variation, 20.6% was resulted from differences among populations (Table 4). Based on  $F_{ST}$ , significant differences were observed among all pairs of populations after correcting for multiple comparisons (Table 5).  $R_{ST}$  values were comparable with  $F_{ST}$  and were significant between all populations (Table 6). Of note,  $R_{ST}$  showed consistently higher divergence than  $F_{ST}$  between the St Francis River population and the populations in Davis Creek, French Creek, the St Croix River, and the Sydenham River.

The levels of gene flow, inferred from  $N_m$ , between populations were low to moderate, ranging from 0.399 to 3.321 (Table 5). Values of  $N_m < 1$  indicate low levels of gene flow (0 = no gene flow); values of  $N_m > 1$  reflect progressively higher levels of gene flow.

The branching pattern formed in the NJ network of Nei's  $D_A$  genetic distances (Fig. 3) closely resembles the branching pattern of the rivers of origin among the sample populations (Fig. 1).

In the assignment to populations, only one of the 131 individuals was incorrectly assigned the wrong

**Table 2.** Results of analysis of molecular variance (AMOVA) for *Epioblasma triquetra* based on cytochrome *c* oxidase subunit-I (COI) sequence data

Source of variation	d.f.	Sum of squares	Percentage of variation	$P \leq$
Among populations	6	19.0	67.27	0.001
Within populations	106	9.9	32.73	0.001
Total	112	29.0		

**Table 3.** Locus name, allele size range, and GenBank accession number, with number of alleles (private alleles in parentheses) and observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities for *Epioblasma triquetra* by locus and population

Locus, allele size range & Genbank accession	Populations†								All <i>E. triquetra</i>
	BR	CR	DC	FC	SC	SF	SYD		
Ecap2	# of alleles	1 (0)	3 (1)	3 (0)	3 (0)	2 (0)	2 (0)	3 (0)	5
133–154 bp	$H_E$	Only 1 Allele	0.54	0.14	0.50	0.05	0.11	0.33	0.54
AY650390	$H_O$		0.63	0.15	0.25*	0.05	0.11	0.36	0.24*
	$n$	19	19	20	20	20	18	11	127
Ecap5	# of alleles	9 (0)	13 (3)	9 (1)	10 (0)	7 (1)	9 (1)	8 (0)	26
190–253 bp	$H_E$	0.80	0.91	0.67	0.87	0.56	0.84	0.83	0.93
AY650393	$H_O$	0.85	0.94	0.70	0.75	0.40	0.89	1.00	0.77*
	$n$	20	16	20	20	20	19	11	126
Ecap6	# of alleles	3 (0)	4 (2)	2 (0)	3 (1)	2 (0)	3 (0)	2 (0)	6
247–255 bp	$H_E$	0.48	0.46	0.51	0.67	0.41	0.27	0.37	0.57
AY650394	$H_O$	0.70	0.41	0.30	0.70	0.45	0.30	0.27	0.46*
	$n$	20	17	20	20	20	20	11	128
Ecap7	# of alleles	4 (0)	13 (1)	8 (2)	13 (2)	3 (0)	7 (1)	9 (1)	21
126–162 bp	$H_E$	0.32	0.93	0.77	0.89	0.50	0.76	0.91	0.87
AY650395	$H_O$	0.26	0.94	0.94	0.94	0.33	0.78	0.90	0.72*
	$n$	19	18	17	17	15	18	10	114
Ecap8	# of alleles	2 (0)	8 (3)	6 (1)	2 (0)	3 (0)	4 (1)	2 (0)	12
161–189 bp	$H_E$	0.33	0.77	0.69	0.51	0.10	0.76	0.37	0.92
AY650396	$H_O$	0.40	0.83	0.60	0.40	0.10	0.80	0.27	0.83*
	$n$	20	18	20	20	20	20	11	129
Ecap9	# of alleles	3 (0)	7 (1)	1 (0)	3 (0)	3 (0)	2 (0)	3 (1)	8
137–159 bp	$H_E$	0.28	0.77	Only 1 Allele	0.25	0.53	0.05	0.42	0.57
AY650397	$H_O$	0.25	0.63*		0.28	0.53	0.05	0.30	0.29*
	$n$	20	19	18	18	17	20	10	122
LabC2	# of alleles	2 (0)	3 (1)	1 (0)	2 (0)	1 (0)	1 (0)	1 (0)	3
150–160 bp	$H_E$	0.19	0.12	Only 1 Allele	0.11	Only 1 Allele	Only 1 Allele	Only 1 Allele	0.07
AF512384	$H_O$	0.21	0.12		0.12				0.07
	$n$	19	17	18	17	14	20	7	112
LabC23	# of alleles	3 (1)	9 (1)	4 (0)	5 (1)	5 (0)	6 (2)	7 (1)	16
210–254 bp	$H_E$	0.44	0.90	0.45	0.75	0.68	0.77	0.79	0.85
AF512385	$H_O$	0.47	0.85	0.44	0.94	0.71	0.58	0.80	0.67*
	$n$	19	13	18	18	17	19	10	114
LabC24	# of alleles	1 (0)	5 (1)	2 (0)	4 (0)	2 (0)	2 (0)	2 (0)	5
152–173 bp	$H_E$	Only 1 Allele	0.68	0.51	0.58	0.51	0.06	0.42	0.51
AF512386	$H_O$		0.94	0.29	0.95*	1.00*	0.06	0.56	0.56
	$n$	17	18	17	20	19	17	9	117
LabD111	# of alleles	5 (1)	20 (6)	5 (0)	14 (2)	8 (1)	6 (1)	10 (0)	32
262–357 bp	$H_E$	0.76	0.93	0.73	0.92	0.81	0.76	0.91	0.87
AF512395	$H_O$	0.82	0.95	0.89	0.95	0.80	0.71	1.00	0.72*
	$n$	17	20	19	20	20	17	11	124
LabD206	# of alleles	11 (4)	10 (3)	7 (0)	6 (0)	6 (0)	6 (0)	7 (0)	17
219–283 bp	$H_E$	0.81	0.85	0.78	0.80	0.79	0.75	0.84	0.93
AF512397	$H_O$	0.74	0.78	0.83	0.74	0.70	0.71	0.82	0.87
	$n$	19	18	19	19	20	17	11	123
LabD213	# of alleles	8 (1)	17 (0)	13 (3)	11 (0)	5 (0)	9 (0)	9 (0)	23
169–259 bp	$H_E$	0.84	0.94	0.92	0.89	0.58	0.87	0.87	0.87
AF512398	$H_O$	0.79	0.95	0.95	0.90	0.65	0.76	0.82	0.76
	$n$	19	20	20	20	20	17	11	127
Etr114	# of alleles	4 (0)	14 (1)	9 (1)	8 (1)	5 (0)	2 (0)	9 (1)	19
138–186 bp	$H_E$	0.62	0.92	0.89	0.76	0.70	0.33	0.88	0.90
DQ396404	$H_O$	0.68	1.00	0.95	0.74	0.80	0.30	0.82	0.74*
	$n$	19	19	19	19	20	20	11	127
Etr124	# of alleles	12 (1)	16 (2)	13 (1)	11 (0)	10 (0)	12 (0)	11 (0)	21
118–160 bp	$H_E$	0.90	0.94	0.88	0.90	0.87	0.84	0.92	0.93
DQ396405	$H_O$	0.95	0.95	0.89	0.95	0.90	0.74	0.82	0.89
	$n$	19	20	19	20	20	19	11	128
Etr140	# of alleles	5 (0)	11 (2)	6 (0)	9 (0)	5 (0)	8 (1)	6 (0)	14
157–183 bp	$H_E$	0.67	0.85	0.73	0.88	0.78	0.84	0.81	0.84
DQ396406	$H_O$	0.65	0.89	0.74	0.95	0.94	0.89	0.75	0.83
	$n$	20	19	19	19	17	18	8	120
Mean $H_E$		0.49	0.77	0.58	0.69	0.52	0.53	0.65	
Allelic richness		3.61	6.60	4.24	5.04	3.49	4.02	5.09	

\*Indicates locus–population combinations with  $H_O$  significantly different from  $H_E$ , after a sequential Bonferroni correction (Rice, 1989), experiment-wide  $\alpha = 0.05$ .

†Location abbreviations are defined in Figure 1.

**Table 4.** Results of analysis of molecular variance (AMOVA) for *Epioblasma triquetra* based on 15 variable microsatellite loci

Source of variation	d.f.	Sum of squares	Percentage of variation	$P \leq$
Among populations	6	256.1	20.58	0.001
Within populations	255	1021.8	79.42	0.001
Total	261	1277.9		

**Table 5.** Pairwise fixation index  $F_{ST}$  (Weir & Cockerham, 1984) values (below diagonal) – all values were significant after sequential Bonferroni correction ( $\alpha = 0.05$ ); and  $N_M$  (number of migrants per generation, above diagonal) for all populations of *Epioblasma triquetra*

Population*	BR	CR	DC	FC	SC	SF	SYD
BR	–	1.313	1.080	1.147	0.940	0.399	1.109
CR	0.160	–	1.523	2.559	1.045	0.913	2.915
DC	0.188	0.141	–	1.766	1.203	0.505	3.321
FC	0.179	0.089	0.124	–	0.913	0.746	3.222
SC	0.210	0.193	0.172	0.215	–	0.439	1.284
SF	0.385	0.215	0.331	0.251	0.363	–	0.519
SYD	0.184	0.079	0.070	0.072	0.163	0.325	–

\*Location abbreviations are defined in Figure 1.

**Table 6.** Pairwise stepwise fixation index  $R_{ST}$  (Slatkin, 1995) for all populations of *Epioblasma triquetra*

Population*	BR	CR	DC	FC	SC	SF	SYD
BR	–						
CR	0.200	–					
DC	0.607	0.386	–				
FC	0.198	0.106	0.482	–			
SC	0.468	0.225	0.136	0.335	–		
SF	0.254	0.221	0.736	0.441	0.572	–	
SYD	0.380	0.108	0.210	0.170	0.104	0.570	–

\*Location abbreviations are defined in Figure 1.

All values were significant after sequential Bonferroni correction ( $\alpha = 0.05$ ).

population (Table 7).  $\chi^2$  tests revealed that assignments to populations were greater than would be expected by chance for all populations ( $P < 0.01$ , 1 d.f.).

No evidence for recent bottlenecks occurred in any of the populations using an infinite-allele, two-phase, or stepwise mutation model ( $P > 0.05$ ).

## DISCUSSION

### POPULATION STRUCTURE AND ZOOGEOGRAPHY

The COI sequence data revealed significant population structure, characterized by closely related mtDNA sequences; hence, minimal nucleotide variation was observed among populations of *E. triquetra*. Most of the populations in formerly glaciated regions were dominated by a single haplotype (Etri1). The popula-

tion in the Clinch River had several individuals with haplotypes Etri6 and Etri7, and these lineages shared a common ancestor. In addition, this population had the most common haplotype (Etri1). These unique and common haplotypes indicate that the population in the Clinch River may have been isolated for a long period of time. If true, then it seems reasonable that the population was invaded recently by individuals with the common haplotype found in all of the northern populations, or alternatively the population retained the presumably pleisiotypic variant. A single, unique haplotype (Etri8) was fixed in the population of St Francis River. This population occurs in the geologically ancient Ozark Highlands of Missouri, and is likely to have been isolated since preglacial times. These findings suggest a single origin and common

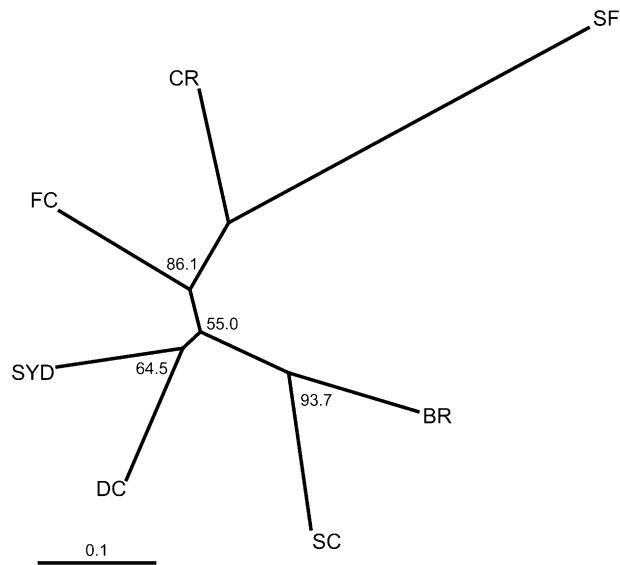


**Table 7.** Results of maximum likelihood assignment tests (Cornuet *et al.*, 1999) by population and by drainage for *Epioblasma triquetra*

	BR	CR	DC	FC	SC	SF	SYD
Bourbeuse River, MO (BR)	<b>20</b>	–	–	–	–	–	–
Clinch River, TN (CR)	–	<b>20</b>	–	–	–	–	–
Davis Creek, MI (DC)	–	–	<b>20</b>	–	–	–	1
French Creek, PA (FC)	–	–	–	<b>20</b>	–	–	–
St. Croix River, MN/WI (SC)	–	–	–	–	<b>20</b>	–	–
St. Francis River, MO (SF)	–	–	–	–	–	<b>20</b>	–
Sydenham River, ON (SYD)	–	–	–	–	–	–	<b>10</b>
Obs. correctly classified	20	20	20	20	20	20	10
Exp. correctly classified	2.86	2.86	2.86	2.86	2.86	2.86	1.57
$\chi^2$ (1 d.f)	30.01	30.01	30.01	30.01	30.01	30.01	14.12
Percent correctly classified	<b>100.0*</b>	<b>100.0*</b>	<b>100.0*</b>	<b>100.0*</b>	<b>100.0*</b>	<b>100.0*</b>	<b>90.9*</b>

\*Represents significance at  $\alpha = 0.05$ .

$\chi^2$  values indicate whether individuals are assigned more frequently to their own population than would be expected by chance if there were no differences among populations (1 d.f). Values in bold indicate the number of individuals that were correctly assigned to their own population.



**Figure 3.** An unrooted neighbour-joining network based on Nei's  $D_A$  (Nei *et al.*, 1983) genetic distance for seven populations of *Epioblasma triquetra*. Numbers indicate nodes with bootstrap support of more than 50% for 1000 replications. Location abbreviations are defined in Figure 1.

ancestry for all of the populations now occupying formerly glaciated regions.

There appears to be only low levels of mtDNA divergence present in the many studies of unionid phylogeography published to date. The results of this study conform to this emerging pattern. No haplotypic population structure was observed in *E. torulosa rangiana* in recently glaciated landscapes (Zanatta &

Murphy, 2007). In contrast, the mtDNA sequence data revealed population structure of unionids in older nonglaciated landscapes (*Lexingtonia dollabelloides*; Grobler *et al.*, 2006), and between the central basin and along the Atlantic coast (*Lasmigona subviridis*; King *et al.*, 1999). Unlike the unionids, mtDNA haplotypes were found to have significant population structure in walleye (*Sander vitreus*; a host fish for many unionids) between various tributaries of Lake Erie, Lake Superior, and the Mississippi basin (Stepien & Faber, 1998).

For *E. triquetra*, the results from the microsatellite genotyping were more informative for inferring population structure in recently glaciated areas than the mtDNA sequence data. The microsatellite analyses indicated that the sampled populations of *E. triquetra* were not panmictic. However, the lack of significant nucleotide divergence in the mtDNA data indicated a recent common ancestry. Many of the common alleles were broadly distributed. Moderate gene flow appears to have recently occurred between some of the populations in the Ohio and Tennessee rivers, and in the Great Lakes drainage. Gene flow was most evident between relatively young populations, which occupied regions that emerged from glaciation < 10 000 years ago. Many of the populations north of the glacial extent are now effectively isolated because of dams along the Ohio and Mississippi rivers, and because zebra mussels largely eradicated unionid populations and have destroyed potential habitats in the Great Lakes (Schloesser & Nalepa, 1994; Zanatta *et al.*, 2002; Schloesser *et al.*, 2006). The pattern of genetic distance in *E. triquetra* closely paralleled the branching patterns of the rivers systems in the central basin

of North America. Unionid mussels may be ideal organisms for a stepping stone model because they are found in fragmented, patchy populations (mussel beds) along interconnected freshwater river systems. Their difficulty of dispersion is a function of riverine linear geography.

The central highlands of North America have long been recognized as regions of high endemism for aquatic organisms (Crandall & Templeton, 1999; Mayden, 1988; Turner *et al.*, 1996). Many unionid species are endemic to the Cumberland Plateau and Ozark Highland regions (Johnson, 1978; Oesch, 1995; Parmalee & Bogan, 1998). *E. triquetra* has unique haplotypes in each of these ancient faunal provinces, and is a good model species for biogeographical studies of unionids in the central basin of North America. Although our data suggest that *E. triquetra* may have been able to invade these highland regions at the end of the last glacial period, one question remains: why are this and other endemic species (and unique haplotypes of *E. triquetra*) unable to disperse out of these areas?

Our data test many of the zoogeographical hypotheses of Johnson (1978). According to Johnson (1978) and Ortmann (1925) the centre of origin of *E. triquetra* was obscure. Johnson (1978) proposed that the genus *Epioblasma* originated in the Cumberlandian Region, which historically consisted of two of the world's most ancient rivers, the Cumberland and Tennessee rivers. This region has the greatest diversity of species of *Epioblasma*, with 20 of the 28 currently recognized taxa including 14 endemics (Johnson, 1978; Parmalee & Bogan, 1998; Jones *et al.*, 2006). Johnson's (1978) hypothesis that *E. triquetra* postglacially spread from the Cumberlandian Region into the Ohio River system is rejected by the results from both the mtDNA and the microsatellites. The results imply that a Pleistocene glacial refuge for *E. triquetra* existed outside of the Tennessee River drainage, possibly in the lower Ohio or in the Meramec drainage of the middle Mississippi River. *E. triquetra* is the only member of the genus to occur in the upper Mississippi River sub-basin (i.e. the St Croix River). The genetic data suggest that a single glacial refuge was responsible for the invasion, either through the lower Ohio River drainage or via a refuge in the Meramec River (i.e. the Bourbeuse River) in the northern portion of the Ozark Plateau (Johnson, 1978). The presence of the same genetic signature (identical haplotype, similar microsatellite genotypes, and recent gene flow) in the Great Lakes and Upper Ohio River drainages (Davis Creek, Sydenham River, and French Creek populations) suggests a common ancestry of *E. triquetra* populations in the Great Lakes and upper Ohio drainages, and for all of the formerly glaciated populations (Ortmann, 1924; van der Schalie, 1945; Graf, 2002).

Our findings were similar to those for three species of *Epioblasma* investigated by Jones *et al.* (2006). They reported little variation in sequence data at three mtDNA (16S, ND1, cytochrome *b*) and one nuclear DNA (ITS-1) loci between their taxa. However, large genetic distances, using microsatellite DNA, indicated little gene flow between species and populations of *E. capsaeformis*, *E. florentina walkeri*, and *E. torulosa rangiana*. Jones *et al.* (2006) proposed that an undescribed species of *Epioblasma* exists in the Duck River, Tennessee, and that the populations of *E. florentina walkeri* in the Tennessee River drainage constituted a subspecies different from those in the Cumberland River drainage. Similarly, our findings may support the description of a new species in the St Francis River, pending further analysis including the recording of morphological characters.

The phylogeographical linkage between a unionid and its parasitized host has yet to be made, but attempts to confirm this relationship should be carried out. Population structure in the host fish for *E. triquetra* is not well known. Laboratory testing of host fish for *E. triquetra* show consistent metamorphoses of glochidia on the logperch (*Percina caprodes*), blackside darter (*Percina maculata*), mottled sculpin (*Cottus bairdi*), banded sculpin (*Cottus carolinae*), Ozark sculpin (*Cottus hypselurus*), and blackspotted topminnow (*Fundulus olivaceus*) (Yeager & Saylor, 1995; Hillegass & Hove, 1997; Barnhart, Riusech & Baird, 1998; McNichols & Mackie, 2002; McNichols & Mackie, 2003; McNichols, Mackie & Ackerman, 2004; Watters *et al.*, 2005). The most likely natural hosts are logperch and blackside darter, as these species have been observed being captured and infested with glochidia by female *E. triquetra* (see <http://unionid.missouristate.edu/gallery/Epioblasma/default.htm> for a video of this host-capture behaviour). Sculpins (*Cottus* sp.) are not likely to be hosts, because *E. triquetra* is generally not found to be closely associated with them in some habitats. Moreover, the host-capture behaviour observed in *Epioblasma* does not appear to be effective on the wide-bodied sculpins (C. Barnhart, pers. comm.). A study of logperch in the Ozark and Ouachita highlands showed significant population structure using allozymes, but little mtDNA divergence (Turner *et al.*, 1996); mirroring our findings for *E. triquetra*. The population structure found in this study also closely reflected the structure recovered by Near, Page & Mayden (2001) for *Percina evides*. They found that three maternal lineages occurred in the upper Mississippi and Missouri River drainage; the Ohio, Tennessee, and Cumberland River drainages; and the White River drainage (southern Ozark highlands). Our findings differed. Evidence for significant divergence occurred between the Ozark and Tennessee

River drainages, and the Meramec and the Upper Mississippi only.

#### GENETIC DIVERSITY

Within-population genetic diversity (allelic richness and expected heterozygosities) was estimated to investigate the possibility of loss of variation. Allelic richness was highest in the population of Clinch River. This could be attributed to the addition of alleles into this population from the hypothesized refuge in the lower Ohio River. Most of the populations in the formerly glaciated regions had somewhat lower allelic richness, possibly related to a founder effect of relatively few individuals. There was no evidence for recent population bottlenecks in any of the populations.

Deviations from the Hardy–Weinberg equilibrium are not unusual. Heterozygote deficiencies in allozyme and microsatellite loci have been reported as causes for Hardy–Weinberg disequilibrium in other freshwater mussels (Nagel, Badino & Alessandria, 1996; Johnson *et al.*, 1998; Jones *et al.*, 2006) and in other bivalves (Zouros & Foltz, 1984; Raymond *et al.*, 1997). A likely source of disequilibrium in this study is the limited sample sizes in the presence of high allelic diversity. Analyses indicated that excess of homozygotes at several loci may also have resulted from non-amplifying alleles, possibly as a consequence of motif anomalies or mutations in the flanking region. Non-amplifying alleles may be quite common in bivalves, even in species for which the microsatellite primers were designed (McGoldrick *et al.*, 2000). The low level (6.9%) of estimated null-allele frequency is likely to make this factor irrelevant for the microsatellite data used in this study. A recent study showed that mean null-allele frequencies as high as 20% did not significantly change the results in a simulated dataset (Dakin & Avise, 2004).

#### CONSERVATION IMPLICATIONS

Moderate gene flow appeared to have been recently occurring between sampling localities north of the glacial extent and the Clinch River population. However, there was little evidence for recent gene flow between the St Francis River population and other populations of *E. triquetra*. None of the sites sampled were in close proximity to each other, making it difficult to determine the threshold of riverine distance at which gene flow occurs. The specialized habitat preference of *E. triquetra* for shallow riffle areas of small to medium sized rivers (Oesch, 1995; Parmalee & Bogan, 1998; Watson *et al.*, 2000) generally excludes it from larger systems, i.e. the mainstems of the Mississippi or Ohio Rivers. The relatively recent construction of impoundments and

the introduction of dreissenid mussels have further isolated these remaining populations. Many of the intervening riverine and lacustrine habitats are now inhospitable to *E. triquetra*.

The importance of maintaining genetic diversity is well recognized. Although many of the populations of *E. triquetra* have experienced decline, it is still relatively healthy in several drainages. However, based on private alleles, high  $F_{ST}$  values, and nearly no misclassification in the assignment test, we recommend that the populations sampled in this study be treated as separate management units (MU; Moritz, 1994). In addition, the population in the St Francis River, Missouri, is particularly distinctive. This population meets the criteria of Moritz (1994) for being considered as an evolutionary significant unit (ESU); significant population structure occurs at both mitochondrial and nuclear loci. The St Francis River population requires further investigation into its taxonomic status. Regardless, this population should receive special consideration if the decision is made to elevate *E. triquetra* to a species of concern under the US Endangered Species Act. Efforts in artificial propagation and possible translocations to reintroduce or augment populations should be made to maintain the significant levels of genetic variation, while maintaining distinctiveness. If the population from a particular region has been extirpated or is too small to propagate, collections of brood stock from the nearest viable population, in riverine distance and known genetic profile, is recommended.

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