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RESEARCH ARTICLE

Range-wide population genetic analysis of the endangered northern riffleshell mussel, *Epioblasma torulosa rangiana* (Bivalvia: Unionoida)

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Abstract The northern riffleshell (*Epioblasma torulosa rangiana*) is a critically endangered unionoid species in need of conservation throughout its range. It is the only unionoid to be federally protected in both Canada and the U.S. We use sequences from two mtDNA genes and 15 microsatellite loci to assess genetic variation among 86 individuals from the four populations in the three remaining drainages in which *E. t. rangiana* is known to be reproducing. All of these populations are in formerly glaciated landscapes that emerged <10 kya. The mtDNA sequence data do not indicate significant geographic structure among the populations. However, allelic data from the microsatellite loci reveal highly significant population structuring. Individuals of *E. t. rangiana* can be assigned to their own river of origin with 98.8% accuracy. Significant isolation-by-distance occurs. This analysis will be useful to conservation managers in documenting the genetic structure, patterns of isolation, and genetic variability within and among populations of *E. t. rangiana*.

Keywords Freshwater mussels · Unionoida · Population structure · Microsatellites · mtDNA

Introduction

Freshwater mussels (also known as unionids, naiads, or clams) are bivalves from the superfamily Unionoida. Most unionoids require a host fish for their glochidium larvae (Nedeau et al. 2000; Parmalee and Bogan 1998). Unionoids are often considered the most endangered group of animals in the world (Lydeard et al. 2004; Strayer et al. 2004). Over 70% of the approximately 300 species in North America are considered imperiled (Bogan 1993; Williams et al. 1993). A combination of largely human-induced impacts such as habitat degradation, destruction of freshwater environments, and the introduction of exotic species have caused catastrophic declines in many unionoids over the past 150 years.

The northern riffleshell (*Epioblasma torulosa rangiana*, NRS) is a critically endangered, stream-dwelling, freshwater mussel (Staton et al. 1998; USFWS 1994; Williams et al. 1993). Several subspecies are described for *E. torulosa* (Johnson 1978). All of these subspecies except NRS are presumed to be extinct. The other subspecies of *E. torulosa* once existed in the Cumberland, Tennessee, and Ohio River systems, but individuals have not been found alive in decades (Parmalee and Bogan 1998). Unionoids that presumably had an origin in the Mississippi basin, including the NRS, appear to have invaded the lower Great Lakes following the last retreat of the Wisconsin glaciation in the late Pleistocene, as recently as 9,000 ybp (Graf 2002). The vector for the unionoids and their associated host fish into the Lake Erie drainage is believed to be an historical connection between the drainages of the Wabash and Maumee rivers (Bailey and Smith 1981; Calkin and Feenstra 1985; Graf 2002). Much of the historic range of the NRS occurs in recently glaciated areas.

The NRS is the only North American unionoid to be federally listed as endangered in both the U.S. and Canada. The historical range includes the Ohio River drainage and the drainage of western Lake Erie and

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Lake St. Clair. Historically, the NRS was found in Illinois, Indiana, Kentucky, Michigan, Ohio, Pennsylvania, West Virginia, and Ontario. Today, it is extremely rare and most populations are greatly reduced or have disappeared completely (Fig 1; USFWS 1994). The only reproducing populations occur in Allegheny River and French Creek in Pennsylvania and the Sydenham River in Ontario (Fig. 1). All remaining populations occur in glaciated regions that emerged <10 kya.

The decline of mussels in the genus *Epioblasma* is associated with the advent of land clearing for row-cropping and the associated increases in runoff 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, industrial and urban pollution are all possible contributors to the demise of unionoids (Bogan 1993). Since the invasion of the zebra mussel in the late 1980's, populations of the NRS have been extirpated from the Detroit River and western Lake Erie (Schloesser et al. 1998; Schloesser and Nalepa 1994). The zebra mussel remains a major threat to populations in both the Allegheny River and French Creek where dams provide habitat for a seed population from which zebra mussel veligers can spread (R. Villella, USGS Leetown Science Center; D. Crabtree, TNC, personal communications). The population of the NRS in the Sydenham River is less threatened by zebra mussels because of the lack of large impoundments on the river (Staton et al. 2003).

Five published sets of microsatellite loci have been developed for five unionoid species (Eackles and King 2002; Geist et al. 2003; Jones et al. 2004; Shaw et al. 2006; Zanatta and Murphy 2006). Significant population structure was found in the European pearl mussel (*Margaritifera margaritifera*) using microsatellite markers (Geist and Kuehn 2005). Many populations of *M. margaritifera* both within and among river drainages had unique alleles and high pairwise F_{ST} values and genetic distances (Geist and Kuehn 2005; Nei 1972; Weir and Cockerham 1984). Similarly, using microsatellites developed by Eackles and King (2002), populations of *Lampilis cariosa* along the Atlantic coast of Maine showed significant population structure (Kelly and Rhymer 2005). Genetic variation between unionoid populations draining into salt water would be expected to be high as there would be much less opportunity for the transport of glochidia by host fish between populations.

Herein, we use both multilocus microsatellite genotypes and mtDNA sequences to assess population structure and measure gene flow in the central basin of North America. Molecular data are critical for the perpetuation of imperiled freshwater mussels. Ecology, captive care, and propagation have been emphasized in the planning for the recovery of endangered freshwater mussels (NNMCC 1998). Villella et al. (1998) proposed that animals should be relocated in areas that contain similar genetic profiles in order to augment populations. Data on the genetic characteristics of mussel

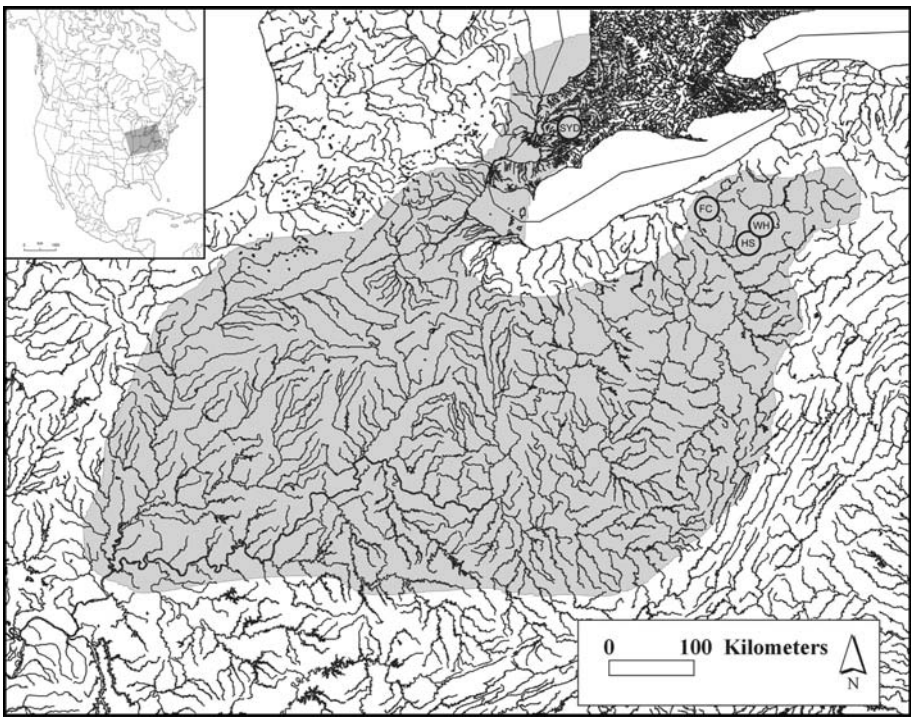

Fig. 1 The historical range (shaded) and distribution of remaining populations where tissue collections were made during the current study for *Epioblasma torulosa rangiana*. Sample site localities: SYD = Sydenham River, Florence ON Canada (42.6912N, 82.9892W); FC = French Creek, Venango Co. PA (41.7969N, 80.0939W); WH = West Hickory, Allegheny River, Forest County PA (41.5694N, 79.4049W); and HS = Hunter Station, Allegheny River, Forest County PA (41.4722N, 79.4992W)

populations are needed to make informed decisions regarding the numbers, localities, and logistical concerns of potential relocations or population augmentation through artificial propagation.

Methods

Mantle tissue (~30 mg) was non-destructively excised following protocols by Berg et al. (1995). Sampling localities were selected by searching the literature for the remaining significant populations of NRS (Staton et al. 1998; USFWS 1994). Eighty-six specimens of NRS were collected from four localities: in Pennsylvania USA, 23 individuals from the Allegheny River at West Hickory, Forest County; 23 individuals from the Allegheny River at Hunter Station, Forest County; 20 individuals from French Creek, Venango County; and 20 individuals from the Sydenham River near Florence, Chatham-Kent Region, Ontario Canada (Fig. 1). Tissues were initially frozen in dry ice and subsequently stored at –80 °C or preserved in 95% ethanol.

Total genomic DNA was extracted from ~15 mg of frozen or ethanol preserved mantle tissue samples by standard phenol extraction (Hillis et al. 1996) or using the Purgene DNA extraction kit (Gentra Systems).

Two mitochondrial DNA (mtDNA) regions were sequenced to determine variation among populations. The following primers and sources were used: (1) a 630 bp sequence of cytochrome c oxidase subunit-I (COI) using primers LCO1490 and HCO2198 (Folmer et al. 1994) and (2) a 360 bp sequence of the cytochrome *b* (Cyt *b*) gene using forward primer 5′-AAGAAGTATCATTGCGGTTG-3′ and reverse primer 5′-TGTGGGGCGACTGGTATTACTAA-3′ (Bowen and Richardson 2000; Merritt et al. 1998). The PCR reaction mixture for both COI and Cyt *b* consisted of 1.0 μL of genomic DNA, 1.0 μM of each primer, 0.4 mM dNTP, 1 × PCR buffer (2.0 mM Tris–HCl, 10 mM KCl, 0.01 mM EDTA, and 0.1 mM DTT), 2.0 mM $MgCl_2$, 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: 92°C for 2 min; 5 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. Conditions for the amplification of Cyt *b* were: 94°C for 2 min; followed by 40 cycles of: 94°C for 1 min., 50°C for 1 min., 72°C for 2 min.; a final extension at 72°C for 6 min; and a final hold at 4°C.

Double-stranded PCR products were visualized using 1.0% agarose gels stained with ethidium bromide or Sybr Green II. PCR products were purified using a Microcon YM-100 (Millipore, Inc.) or Quiaquick DNA purification kits. The 5′end of the amplified products were cycle-sequenced using 'Big Dye' Terminator Cycle Sequencing Ready Reaction (Perkin Elmer Applied Biosystems, Inc.) with the primers LCO1490 or Cyt *b* forward primer (50°C annealing temperature) and visualized on an ABI 377 or ABI 3100 automated DNA sequencer.

The characterization of 15 microsatellite loci followed methods described in Zanatta and Murphy (2006): GenBank accession numbers DQ396403-DQ396408, AF512384, AF512386, AF512395, AF512397, AF51298, AY650392, AY650394, AY650396, AY650397. Nine of these loci were developed by Eackles and King (2002) and Jones et al. (2004) for other unionoid species.

Statistical analyses

Mitochondrial DNA sequences

Since the entire mtDNA genome is effectively inherited as one locus, the COI and Cyt *b* datasets for each specimen were combined. DNA sequences first were aligned using CLUSTALX (Thompson et al. 1997) and MACCLADE (Maddison and Maddison 1997). MACCLADE was used to identify unique 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 the resulting trees, Bootstrap values were calculated. Bootstrapping used 1000 replications and heuristic searching with 10 random stepwise additions. In addition, sequences of COI and Cyt *b* genes from *Venustaconcha ellipsiformis* (DQ220725, DQ479937), sequenced concurrently with *E. t. rangiana*, were used as the outgroup taxon to root trees.

A second phylogenetic analysis used Bayesian inference (Huelsenbeck and Ronquist 2001), using MRBAYES v3.0b4. The initial model of evolution for MRBAYES (Huelsenbeck and Ronquist 2001) 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 generations (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 9,500 trees.

Hierarchical analyses of molecular variance (AMOVA) (Excoffier et al. 1992) was used to estimate the partitioning of haplotypes within and among populations. AMOVAs were carried out in ARLEQUIN v.

2.0 (Schneider et al. 2000). F_{ST} (Weir and Cockerham 1984) was calculated for all pairs of populations. *P*-values for *F*-statistics were calculated to test the null hypothesis of panmictic populations by permuting haplotypes among populations to calculate the probability of obtaining equal or greater F_{ST} by chance distribution of genotypes. Sequential Bonferroni correction, after Rice (1989), was used to correct *P*-values for multiple comparisons.

Microsatellites

Genetic diversity in each sample was summarized as allelic richness (A), measured as the mean number of alleles per locus after correcting for sample size and expected heterozygosity (H_E). 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 et al. 1998). Detection of deviations from Hardy-Weinberg equilibrium and randomization tests for linkage disequilibria were conducted using GENEPOP v. 3.4 (Raymond and Rousset 1995).

To approximate effective population size (N_e), the maximum likelihood method of Beerli and Felsenstein (1999) implemented in MIGRATE v. 2.0 (Beerli 1997–2004) to estimate θ, where $\theta = 4\ N_e\mu$. Using an infinite allele model (because many of the repeat motifs were complex), ten short chains (1000 trees) and three long chains (10,000 trees) were used to calculate the posterior probabilities for this analysis. Calculation of N_e from θ depended on knowing mutation rates, which were not well understood for complex microsatellites. Because there were no direct estimated of microsatellite mutation rates for bivalves, data were relied upon from other taxa. The estimated mutation rates ranged from 10^{-6} to 10^{-3} for tetra- and trinucleotides and 10^{-6} to 10^{-2} for dinucleotides when repeat numbers range from 5 to 19, 5 to 17, and 5 to 31, respectively (Lai and Sun 2003). The number of repeats for the loci used in our study fell at the medium to high end of the range. Therefore, 10^{-4} was used as an approximate estimate of mutation rates.

A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used to estimate the partitioning of genetic variance within and among populations. An AMOVA was carried out in ARLEQUIN v. 2.0 (Schneider et al. 2000) using data pooled across loci. F_{ST} (Weir and Cockerham 1984) was calculated for all pairs of populations. *P*-values for *F*-statistics were calculated to test the null hypothesis of panmictic populations by permuting genotypes among populations to calculate the probability of obtaining equal or greater F_{ST} by chance distribution of genotypes. Sequential Bonferroni correction, after Rice (1989), was used to correct *P*-values for multiple comparisons.

The microsatellite dataset was tested for genotyping errors due to 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)

Assignment tests were used to estimate the probability of each individual originating from a given population (Paetkau et al. 1995; Rannala and Mountain 1997). GENECLASS2 (Piry et al. 2004) was used to implement the assignment tests as described by Rannala and Mountain (1997). This technique estimated the posterior probabilities of actual 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 χ^2 was calculated to test whether the level of assignment was greater than could be expected by chance.

Isolation by distance was measured by comparing genetic distance $[F_{ST}/\ (1–F_{ST})]$ to geographic distance measured in river kilometers for pairs of populations. Geographic distances between populations were measured in ARCVIEW GIS v. 3.2 (Environmental Systems Research Institute 2001). The distance between the Great Lakes drainage and the Ohio River drainage was estimated by measuring modern land distance between the Maumee and Wabash Rivers. These rivers were connect at the end of the last Pleistocene glaciation (Calkin and Feenstra 1985) and were the hypothesized vector for the post-glacial reinvasion of the Lake Erie drainage for freshwater mussel species originating from the Mississippi basin (Graf 2002). The statistical significance of the correlation between geographic and genetic distance matrices were tested using a Mantel test in GENEPOP v. 3.4 (Raymond and Rousset 1995).

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

Results

Sequencing consistently resulted in 604 bp from COI and 317 bp from Cyt *b*. Ten distinct haplotypes were

identified for the combined COI and Cyt *b* datasets (GenBank accession numbers DQ479944–DQ479949 and DQ479938–DQ479943). Haplotypes resolved and the frequencies of each haplotype are presented in Table 1. A single haplotype dominated in all of the populations indicating that NRS may have been bottlenecked prior to post-glacial reinvasion into the Great Lakes and Ohio River drainages.

The phylogram constructed for combined COI and Cyt *b* dataset (Fig. 2) did not group NRS individuals along unambiguous population boundaries. The Maximum parsimony analysis of the COI and Cyt *b* gene sequences yielded five equally parsimonious trees of 104 steps and with consistency indices (CI) of 0.99. A 50% majority-rule consensus tree created through Bayesian inference (initial model: HKY+G) produced a very similar topology to that of maximum parsimony (9500 trees, mean log likelihood = –1743.4). Where branches between the haplotypes were resolved, bootstrap support and Bayesian posterior probabilities were >50% (Fig. 2).

Nucleotide diversity within populations (Table 1) was reduced in French Creek and in the Sydenham River. Only two haplotypes were observed in both the Sydenham River and French Creek populations. Values of nucleotide diversity in the Allegheny River's populations were from 0.761–1.739%.

The AMOVA for the mtDNA sequence data indicated that 96.6% of variation in the NRS resided within populations, with 3.8% occurring among populations. The AMOVA indicated significant population structure in the mtDNA sequence data ($P = 0.017$). However, pairwise F_{ST} values (Table 2) did not indicate significant differentiation between any of the populations after a sequential Bonferroni correction. One of the pairwise F_{ST} values was negative; when the true F_{ST} value is close to zero, bias or sampling variation could lead to negative estimates (Weir and Cockerham 1984).

Thirteen of the 15 microsatellite loci amplified for *E. t. rangiana* were highly polymorphic, with a total of 206 alleles observed (2–28 alleles/locus, mean = 13.7). Allele size ranges of the nine loci used from the literature overlapped with those observed for *Lampsilis abrupta* (Eackles and King 2002) and *Epioblasma capsaeformis* (Jones et al. 2004). Genetic diversity, as measured by allelic richness (A), did not vary greatly by population (5.4–6.1 alleles/locus) with the lowest allelic richness occurring in the Sydenham River and the highest in the Allegheny River at West Hickory (Table 3). Expected heterozygosities were high (0.70–0.72) and were very similar among populations (Table 3). Estimates of θ ranged from 0.025 in French

Table 1 Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for COI in four populations of *Epioblasma torulosa rangiana*

Haplotypes and polymorphic nucleotide sites																									Population			
COI														Cyt *b*														
	5	5	7		1	1	2	2	3	5	5	5	5			1	4	4	4	5	9		1	1	2			
	6	7	1	3	6	0	4	6	8	9	5	4	7	6	5	0	1	8	5	3	4	9	8	9	7	West Hickory (Allegheny River; $n = 21$)	Hunter Station (Allegheny River; n =22)	French Creek ($n = 16$)	Sydenham River ($n = 17$)
1	G	T	G	T	G	C	G	G	A	T	T	G	A	T	A	C	G	T	A	A	T	A				0.762	0.636	0.938	0.824
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G				–	–	0.062	–
3	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				–	0.045	–	–
4	A	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				0.095	0.227	–	–
5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.				–	0.045	–	–
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.				–	0.045	–	–
7	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.				–	–	–	0.176
8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.				0.048	–	–	–
9	A	.	A	.	.	T	A	.	G	C	.	T	G	C	.	A	A	C	G	G	.	.				0.048	–	–	–
10	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.				0.048	–	–	–
Number of haplotypes:																										5	5	2	2
Number of polymorphic sites:																										16	7	1	1
Nucleotide diversity per site within population (%):																										1.739 ±0.388	0.761 ±0.173	0.109 ±0.014	0.109 ±0.013

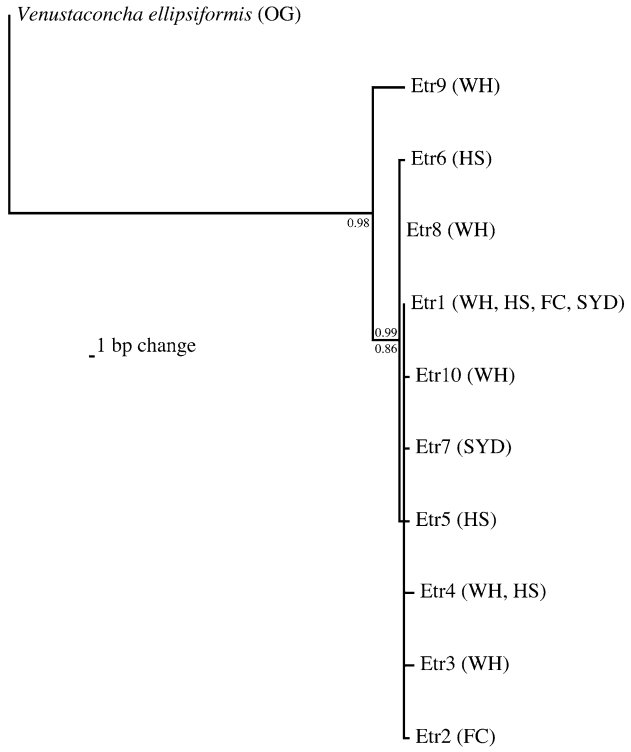

Fig. 2 A 50% majority-rule consensus tree created through Bayesian inference (10,000 trees, burn-in = 500 trees, mean log likelihood = –1743.4) constructed from the COI and Cyt *b* sequence data for four populations of *Epioblasma torulosa rangiana*. The strict consensus of five equally parsimonious trees (104 steps, CI = 0.99, RI = 0.90, RC = 0.89) is identical except that the haplotypes 1, 2, 3, 4, 5, 6, 7, 8, and 10 formed a polytomy. Numbers above the branches indicate the percentage of bootstrap replicates where the clade was found under a maximum parsimony framework. The numbers below the nodes are the calculated posterior probabilities (greater than 50%), indicating the proportion of trees that these nodes appeared. The population(s) where each haplotype occurred are in brackets

Creek to 0.291 in the Sydenham River. Using a mutation rate of 10^{-4}, estimates of N_e were 62 in French Creek, 573 in the Allegheny River at West Hickory, 719 in the Allegheny River at Hunter Station, and 727 in the Sydenham River.

Table 2 Pairwise F_{ST} values among four populations of *Epioblasma torulosa rangiana* for the combined mitochondrial DNA sequences of COI and Cyt *b*. F_{ST} values were not significant after adjusting for multiple comparisons via a sequential Bonferroni correction (Rice 1989), experiment-wide $\alpha = 0.05$

	Allegheny River		French Creek	Sydenham River
	West Hickory	Hunter Station		
Allegheny River – West Hickory	–			
Allegheny River – Hunter Station	–0.004	–		
French Creek	0.009	0.094	–	
Sydenham River	0.024	0.113	0.090	–

Significant deviations from Hardy-Weinberg expectations occurred at 7 out of 60 locus-population combinations after a Bonferroni correction (Table 3). These deviations indicated the possible presence of null alleles or other locus-specific genotyping errors. Analysis of the microsatellite dataset showed no evidence for genotyping errors due to stuttering of 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 0.21 for some locus-population combinations, with a mean frequency of 0.076.

Randomization tests for linkage disequilibrium by locus and population did not indicate any significant linkage disequilibrium for any of the 405 di-locus combinations in the NRS.

Significant population structuring was evident from analysis of the microsatellite dataset for the NRS. Of the total variation, 8.2% was due to 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). F_{ST} values were highest between the Sydenham River and all populations and lowest between the two populations in the Allegheny River.

Tests of the level of assignment to population followed the criteria described by Rannala and Mountain (1997). Most individuals were correctly assigned to their own populations (Table 6). Chi-squared tests revealed that assignments to populations were greater than would be expected by chance for all populations ($P < 0.01$, 1 d.f.). All but one of the ''misclassified'' individuals were assigned to the next-nearest population geographically, suggesting that misclassified individuals could be the result of recent gene flow from nearby populations.

A Mantel test revealed a significant correlation between geographic and genetic distance for the NRS ($r = 0.99$, $P = 0.042$, Fig. 3).

There was no evidence for recent bottlenecks in any of the populations using, infinite allele, two-phased, or stepwise mutation models ($P > 0.05$).

Discussion

Maternal History

Very little mtDNA variation was observed in the NRS. The sequence data did not reveal any significant population structure. In the combined mtDNA dataset all

Table 3 Number of alleles (private alleles in parentheses) and observed (H_o) and expected (H_E) heterozygosities for *Epioblasma torulosa rangiana* by locus and population

Locus		Allegheny River		French Creek	Sydenham River	All *E. t. rangiana*
		West Hickory	Hunter Station			
Etr90	# of alleles	14 (0)	16 (4)	11 (3)	13 (7)	28
	H_o	0.96	0.83	0.68	0.95	0.89
	H_E	0.90	0.93	0.84	0.83	0.93
	n	23	23	19	20	85
Etr114	# of alleles	16 (2)	9 (0)	13 (0)	9 (2)	19
	H_o	0.75	0.61	0.95	0.80	0.78
	H_E	0.91	0.83	0.88	0.82	0.91
	n	20	18	19	20	77
Etr124	# of alleles	12 (3)	11 (1)	11 (1)	9 (2)	19
	H_o	0.61	0.65	0.53[a]	0.89	0.67
	H_E	0.57	0.67	0.88	0.85	0.76
	n	23	23	19	19	84
Etr140	# of alleles	7 (1)	8 (1)	6 (1)	9 (4)	15
	H_o	0.65	0.65	0.80	0.85	0.74
	H_E	0.68	0.63	0.70	0.79	0.76
	n	23	23	20	20	86
Etr145	# of alleles	7 (0)	7 (0)	6 (2)	7 (2)	12
	H_o	0.64	0.35	0.50	0.85	0.59[a]
	H_E	0.73	0.77	0.83	0.74	0.84
	n	22	23	8	20	73
Etr187	# of alleles	14 (1)	13 (1)	13 (3)	11 (3)	23
	H_o	0.85	0.52[a]	0.85	0.85	0.73[a]
	H_E	0.90	0.85	0.90	0.75	0.92
	n	23	23	20	20	86
LabC2	# of alleles	3 (0)	2 (0)	3 (0)	2 (0)	3
	H_o	0.45	0.30	0.06	0.60	0.37
	H_E	0.41	0.26	0.18	0.51	0.41
	n	22	23	16	20	81
LabC24	# of alleles	2 (0)	2 (0)	2 (0)	2 (0)	2
	H_o	0.09	0.04	0.06	0.20	0.10
	H_E	0.09	0.04	0.06	0.18	0.09
	n	23	23	18	20	84
LabD111	# of alleles	5 (0)	8 (0)	7 (0)	5 (0)	9
	H_o	0.61	0.65	0.47	0.65	0.60
	H_E	0.76	0.73	0.74	0.75	0.76
	n	23	23	19	20	85
LabD206	# of alleles	9 (1)	10 (0)	8 (0)	8 (0)	11
	H_o	0.60	0.55	0.75	0.85	0.68
	H_E	0.78	0.75	0.80	0.78	0.83
	n	20	22	20	20	81
LabD213	# of alleles	15 (1)	9 (0)	10 (0)	12 (3)	18
	H_o	0.91	0.78	0.72	0.95	0.84
	H_E	0.92	0.84	0.87	0.91	0.91
	n	23	23	18	19	83
Ecap4	# of alleles	7 (1)	7 (1)	3 (0)	6 (2)	10
	H_o	0.33[a]	0.13[a]	0.65	0.60	0.42[a]
	H_E	0.80	0.80	0.54	0.69	0.80
	n	23	23	20	20	84
Ecap6	# of alleles	6 (0)	7 (0)	6 (0)	3 (0)	7
	H_o	0.59	0.70	0.75	0.70	0.68
	H_E	0.68	0.78	0.71	0.64	0.79
	n	22	23	20	20	85
Ecap8	# of alleles	7 (0)	6 (0)	7 (1)	7 (1)	11
	H_o	0.78	0.65	0.60	0.35[a]	0.60[a]
	H_E	0.77	0.73	0.59	0.74	0.74
	n	23	23	20	20	86

Table 3 continued

Locus		Allegheny River		French Creek	Sydenham River	All *E. t. rangiana*
		West Hickory	Hunter Station			
Ecap9	# of alleles	10 (1)	13 (1)	11 (3)	7 (0)	19
	H_o	0.57	0.43[a]	0.53[a]	0.60	0.53[a]
	H_E	0.84	0.89	0.90	0.63	0.91
	n	23	21	17	20	81
Mean H_E		0.72	0.70	0.71	0.70	
Allelic richness		6.11	5.81	5.80	5.41	

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

Table 4 Results of analysis of molecular variance (AMOVA) for *Epioblasma torulosa rangiana*

Source of variation	d.f.	Sum of squares	Percentage of variation	*P*≤
Among populations	3	71.2	8.17	0.00
Within populations	168	827.2	91.83	0.00
Total	171	898.4		

of the populations were dominated by a single haplotype. No data described within-species haplotype diversity for any other unionoid species found in recently glaciated landscapes. In contrast, the mtDNA sequence data were useful in determining the population structure of unionoids in older, non-glaciated landscapes (*Lexingtonia dollabelloides*; Grobler et al. 2006) and between the central basin and the Atlantic coast (*Lasmigona subviridis*; King et al. 1999). Mitochondrial DNA haplotypes were found to have significant population structure in walleye (*Sander vitreus*) between various tributaries of Lake Erie, Lake Superior, and the Mississippi basin (Stepien and Faber 1998).

Doubly Uniparental Inheritance (DUI; Hoeh et al. 2002; Zouros et al. 1992) was not an issue with the mtDNA sequence data used in this study. This mode of mitochondrial inheritance causes males to be heteroplasmic for two highly divergent mtDNA genomes (an M and an F genome; partly showing different evolutionary history) and females to be homoplasmic (F genome only). DUI has not been reported, to date, in any species of *Epioblasma*. NCBI-BLAST (/www.ncbi.nlm.nih.gov/BLAST) was used to determine if the sequences were from the expected taxon and mitochondrial genome (i.e. F-type). The results of the BLAST search revealed that all sequences were of F-type mtDNA from the unionid genus *Epioblamsa*. Also of note, all animals collected from the two Allegheny River sites (including most of the unique haplotypes, i.e. haplotype 9) were females and therefore could not have been from the M-type genome.

Table 5 Pair-wise *F*-statistics (Weir and Cockerham 1984) for all *Epioblasma torulosa rangiana* populations

	Allegheny River		French Creek	Sydenham River
	West Hickory	Hunter Station		
Allegheny River – West Hickory	–			
Allegheny River – Hunter Station	0.019[a]	–		
French Creek	0.054[a]	0.042[a]	–	
Sydenham River	0.125[a]	0.121[a]	0.126[a]	–

[a] Indicates significance after adjusting for multiple comparisons via a sequential Bonferroni correction (Rice 1989), experiment-wide $\alpha = 0.05$

Table 6 Results of maximum likelihood assignment tests (Cornuet et al. 1999) by population and by drainage for *Epioblasma torulosa rangiana*

	Allegheny River		French Creek	Sydenham River
	West Hickory	Hunter Station		
Allegheny River – West Hickory	**18**	7	–	–
Allegheny River – Hunter Station	5	**15**	–	–
French Creek	–	1	**20**	–
Sydenham River	–	–	–	**20**
Obs. correctly classified	18	15	20	20
Exp. correctly classified	5.75	5.75	5	5
χ^2 (1 d.f.)	17.9	13.3	24.0	24.0
Percent correctly classified	**78.3**[a]	**65.2**[a]	**100.0**[a]	**100.0**[a]

χ^2 values are for the test of whether individuals assign 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 which correctly assigned to their own population. [a] represents significance at $\alpha = 0.01$

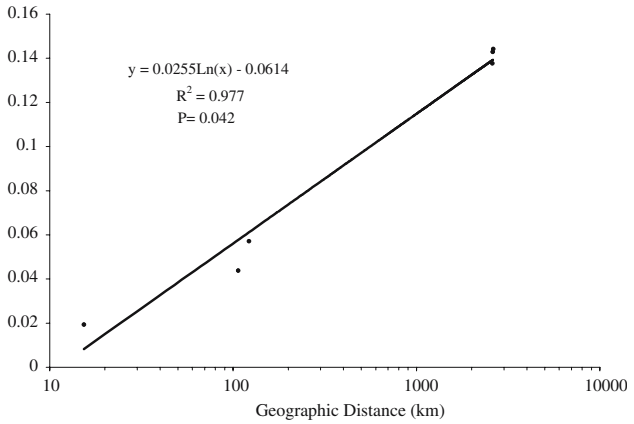

Fig. 3 Regression of geographic (river) distance (km) versus genetic distance $[F_{ST}/(1–F_{ST})]$ for microsatellite data from *Epioblasma torulosa rangiana*. *P*-value calculated using a Mantel test

Population structure

For the NRS, the results from the microsatellite genotyping were much more informative than the mtDNA sequence data. The results of the microsatellite analyses indicated that the remaining populations of the NRS are not panmictic. The lack of structure in mtDNA and the presence of microsatellite structure could be an indication of recent common ancestry between the populations. Genetic structuring owed, in part, to isolation by distance of dispersal. Microsatellite variability was greater within than among sample groups, suggesting that the NRS metapopulation was once relatively homogeneous. Many of the common alleles were broadly distributed. Gene flow was indicated to have occurred throughout the range of the NRS, at least until the very recent proliferation of anthropogenic barriers (e.g., dams and habitat alteration along the Ohio River) and relatively older natural barriers (i.e., rerouting of the Wabash and Maumee drainages following the glacial retreat and isostatic rebound that separated the Great Lakes drainage from the Ohio River drainage). The distribution of low frequency, unique microsatellite alleles supported the hypothesis that the genetic structure resulted from gene flow and not common ancestry. Thus, genetic structuring was strongly associated with geography (Slatkin and Maddison 1990), in particular isolation by distance (Kimura and Weiss 1964). Kelly and Rhymer (2005) also showed strong correlations between genetic distance and geographic distance in the unionoid *Lampsilis cariosa*. Unionoid 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. This makes difficulty-of-dispersion a function of riverine linear geography.

The values estimated for θ and N_e in the NRS do not match with recent measured densities and census estimates for the NRS. Densities and population size estimations from recent surveys in French Creek are reported to be 6.51 m^{-2} with an estimated 500,000 individuals (D. Crabtree, TNC, personal communication). In the Allegheny River at West Hickory and Hunter Station, the estimates are 2.09 m^{-2} and 7.57 m^{-2} in the, respectively, and the overall population estimated at > 1,000,000 individuals (R. Villella, USGS Leetown Science Center, personal communication). At the low end of the scale, densities in the Sydenham River are estimated at 0.19 m^{-2} with a population size of only 10,000–30,000 individuals (J. Metcalfe-Smith, Environment Canada, personal communication). The Sydenham population seems to be the anomaly in terms of N_e. However, the explanation may be that the observed diversity is a relic of a much larger population. Although no density or census estimates exist for the now extirpated Detroit River's population, relative catch per unit effort estimates indicate the population was once quite large (Schloesser et al. 1998; Schloesser et al. 2006). The high N_e in the Sydenham River may be a reflection of a large amount of gene flow between the populations in the Sydenham River and the historically large population in the Detroit River. Similarly, some large populations of the unionoid *Margaritifera margaritifera* in Europe are not necessarily those with the highest genetic diversity and/or effective population sizes (Geist and Kuehn 2005). Although plausible, this theory is not supported by analysis of the genetic data, as there was no evidence of recent population bottlenecking in the Sydenham River's population.

There appears to be a high degree of gene flow between the two sampling locations on the Allegheny River. One individual from the Hunter Station sampling locality on the Allegheny River was assigned to French Creek indicating the possibility of gene flow between these populations. Hunter Station's population on the Allegheny River is the closest sampling locale to French Creek's population (107 km, river distance). Individuals from both French Creek's and the Sydenham River's populations were always correctly assigned. The accuracy of assignments documents genetic divergence of the sampled populations.

Population structure in the host fishes for the NRS is not known. Laboratory testing of host fish for the NRS show consistent metamorphoses of glochidia (larval mussels) on the banded darter (*Etheostoma zonale*), bluebreast darter (*Etheostoma camurum*), Iowa darter

(*Etheostoma exile*), blackside darter (*Percina maculata*), mottled sculpin (*Cottus bairdi*), and banded sculpin (*Cottus carolinae*) (McNichols and Mackie 2002; McNichols and Mackie 2003; McNichols et al. 2004; O'Dee and Watters 1998). However, the natural host or hosts are still unknown. It is likely a combination of several species of *Etheostoma* and *Percina* as sculpins (*Cottus* spp.) are generally not found closely associated with the NRS. Primers amplifying microsatellite loci for several species of *Etheostoma* have recently been published (Tonnis 2006). Investigations of the population genetics of host fish and the associated mussels should be conducted concurrently to determine if the population structure of the host closely parallels that of the parasite. This would further clarify the population structure of imperiled mussels and assist in the management of remaining populations by filling gaps where the mussels may have historically occurred, but only host fishes remain.

Genetic diversity

Within-population genetic diversity was estimated in terms of allelic richness and expected heterozygosities to determine whether some populations of NRS had low levels of diversity that might suggest loss of variation, or not. Allelic richness was similar in all populations. The Sydenham River (with the lowest value of allelic richness), the most isolated of the remaining populations.

The deviations from Hardy-Weinberg equilibrium observed in this study were not unusual. Heterozygote deficiencies in allozyme loci have been reported from other unionoids (Johnson et al. 1998; Nagel et al. 1996) and other bivalves (Raymond et al. 1997; Zouros and Foltz 1984). Our findings could have had several sources. Analysis indicated that the excess of homozygotes at some loci (Ecap4, Ecap8, Ecap9, Etr145, Etr187) likely 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 loci were designed (McGoldrick et al. 2000). The level of estimated null-allele frequency has made it null allele a non-issue for 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 of a simulated population genetic dataset (Dakin and Avise 2004). The mean null allele frequency for the microsatellite loci used in this study was only 7.6%, far below the threshold value described.

Conservation and management implications

Natural gene flow appeared to occur between sampling localities in close proximity to each other. We obtained an extremely good fit to the isolation-by-distance model (Fig. 3). All but one of the misclassified individuals in the assignment test (Table 6) were assigned to the nearest population. The two localities sampled in the Allegheny River were only 15 km apart. Consequently, we recommend that recovery efforts using artificial propagation and translocations should be made to reestablish or augment populations from the geographically closest (in river distance) remaining population.

The importance of maintaining population-level genetic diversity is well recognized. Although significant populations of NRS remain in only three drainages, based on private alleles, high F_{ST} values and nearly no misclassification in the assignment test, we recommend that the populations in the Sydenham River, the Allegheny River, and French Creek be treated as separate management units (MU). Efforts in artificial propagation and possible translocations to reintroduce or augment populations should be made to maintain the significant levels of genetic variation in the populations. Because the population in the Sydenham River occurs in Canada, effectively it is a separate MU. This population is protected under Canada's Species at Risk Act (SARA). All of the other populations of the NRS described herein are found in the U.S.A. and are protected under the U.S. Endangered Species Act.

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