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Testing congruency of geographic and genetic population structure for a freshwater mussel (Bivalvia: Unionoida) and its host fish

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The macrogeographic dispersal of unionoid mussels is largely dependent on movement by their host fish. The snuffbox mussel *Epioblasma triquetra* (Unionoida) and other congeners use a novel trapping behaviour to parasitize potential host fish with their larvae (glochidia). Common logperch (*Percina caprodes*) trapped by *E. triquetra* survive the trapping behaviour, whereas other darter species (*Etheostoma* and *Percina*) do not, thus, making the *P. caprodes–E. triquetra* relationship a good candidate system for a coevolutionary study. We hypothesized that the geographic genetic structure of *E. triquetra* should closely match that of its host, albeit with greater interpopulation divergences as a result of its dependency on the host for dispersal. Mantel tests of parallel pairwise matrices of population divergence (Jost's *D*) and genetic assignment tests based on microsatellite DNA data showed that the genetic population structures of both species were broadly, but not perfectly, congruent. Therefore, it appears that *P. caprodes* are not solely responsible for the genetic population structure observed for snuffbox and may not necessarily be the mussel's only host across its entire range. This suggests the potential for a geographic mosaic for coevolution in unionoids and darters. The findings of the present study reinforce the need for a joint study and conservation of unionoids and host fish aiming to protect these coevolved taxa.

ADDITIONAL KEYWORDS: *Epioblasma* – host–parasite coevolution – geographic structure – *Percina* – post-glacial recolonization.

INTRODUCTION

The adaptive interplay between species across space and time is a central tenet of evolutionary biology (Thompson, 1994). The central basin of North America, with its vast network of highly dendritic rivers, is an ideal location for studying evolutionary relationships within its diverse freshwater fauna at both recent and ancient timescales. The effects of ancient features such as the Appalachian and Ozark mountain ranges and recent glaciations have influenced the biogeography and biodiversity of the entire region (Benke & Cushing, 2005; Soltis *et al*., 2006). Two particularly speciose aquatic groups with a high degree of endemism are the darters (Euteleostei: Percidae) and freshwater mussels (Bivalvia: Unionoida) (Bănărescu, 1991; Near & Benard, 2004; Bogan & Roe, 2008). Both groups date back to the Tertiary epoch and show substantial diversification related to Pleistocene glacial events (Bănărescu, 1991; Wiley, 1992; Bogan & Roe, 2008). Despite this wealth of evolutionary diversification, mussels have the unfortunate distinction of being among the most endangered organisms on Earth (Ricciardi & Rasmussen, 1999; Lydeard *et al*., 2004).

It is likely the declines of freshwater mussels are due in part to their specialized life cycle involving a

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parasitic stage on fish. Fish are critical to the survival of unionoid mussels because reproductive failure will occur without the presence of an appropriate host fish (Bogan, 1993). Unionoids appear intrinsically linked to the evolution of their hosts because speciation in unionoids often appears to be in lock-step to speciation and/or changes in hosts (Graf, 1997; Zanatta & Murphy, 2006; Bogan & Roe, 2008). Thompson (1994) argued that parasitism was a special case of coevolution and organisms with a parasitic life stage provide fertile opportunities for investigating coevolution. For members of the unionid genus *Epioblasma* and darter hosts, research by Jones *et al*. (2006) demonstrated close coevolutionary relationships with regards to genetics and host usage and geographic variation in parasite–host relationships. The obligate parasitic larval stage and potential host specificity makes mussel–host pairs very interesting for phylogeographic and coevolutionary studies (Jones *et al*., 2006; Zanatta & Murphy, 2006; Bogan & Roe, 2008). Understanding the biology, ecology, and geographic genetic structure of host fish species is critically important for the conservation of imperiled unionoid mussels (Bogan, 1993; Bogan & Roe, 2008).

Of all the North American freshwater mussel groups, the genus *Epioblasma* (Rafinesque, 1831) is the most endangered (Bogan, 1993). Of the approximately 20 species in the genus, 14 are considered to be extinct and the status of the remaining taxa are threatened or uncertain (Williams *et al*., 1993; Graf & Cummings, 2007). Because the members of this genus prefer small to medium size, silt-free streams, they are considered to be more sensitive to human mediated disturbances than other mussels native to the central basin of North America (Peacock, Haag & Melvin, 2005). The snuffbox mussel, *Epioblasma triquetra* (Rafinesque, 1820) is the most widely distributed member of the genus (Johnson, 1978), which may in part be responsible for its persistence. Extant populations of *E. triquetra* are generally small and fragmented and the potential for their loss as a result of catastrophic accidents is possible (Butler, 2007). If efforts are not made to preserve this species and its congeners, it is conceivable *Epioblasma* may be the first genus of North American unionoid mussel to go extinct.

Host fish suitability testing for *E. triquetra* has been undertaken at several laboratories. These tests have identified several potential host fish species for use in artificial propagation, including common logperch, *Percina caprodes* (Rafinesque, 1818). *Percina caprodes* was confirmed as a host for *E. triquetra* populations in the Ohio River basin (OSU Division of Molluscs, 2009), the Tennessee River basin, the Upper Mississippi (Hillegass & Hove, 1997), the Ozarks (North and South slope) (Barnhart, Riusech & Baird, 1998), and the Great Lakes (McNichols, Mackie & Ackerman, 2004). The *P. caprodes* and *E. triquetra* species pair are often found in close association with one another (Butler, 2007; Barnhart, Haag & Roston, 2008). It has been reported that *E. triquetra* and other congeners use a fascinating behaviour to infest a potential host fish (Barnhart *et al*., 2008). Gravid female *E. triquetra* gape with their valves open waiting for a fish to insert its rostrum into the gap. The mussel's valves rapidly close upon rostrum insertion, trapping the fish. The mussel forms a 'gasket' with its mantle flesh around the mouth of the fish and pumps glochidial larvae into the fish's buccal cavity, enabling the glochidia to attach to the fish's gills (video clips of this behaviour and logperch foraging are accessible at: http://unionid.missouristate.edu/gallery/Epioblasma/default.htm; Barnhart *et al*., 2008). The majority of darter species examined by Barnhart *et al*. (2008) were unsuitable hosts, with glochidial infestation being unsuccessful or fish dying of crushed skulls. By contrast, the long conical snout and strong skull of *P. caprodes* enable this species to survive capture and infestation (Barnhart *et al*., 2008). In addition, the foraging behaviour of *P. caprodes* (rooting in the substrate with its rostrum) renders them particularly susceptible to snuffbox parasitism (Scott & Crossman, 1973; Barnhart *et al*., 2008).

This co-adapted relationship between *E. triquetra* and *P. caprodes* make an excellent system for studying coevolution, and has the potential to influence rates of speciation in both genera (Thompson, 1994; Near & Benard, 2004; Bogan & Roe, 2008). On the basis of distributional and genetic data, *E. triquetra* dispersed from a Mississippian refugium via host fish following Pleistocene deglaciation (Zanatta & Murphy, 2008). *Percina caprodes* (*sensu lato*) was previously thought to be a single species (Scott & Crossman, 1973) but now appears to be a species complex with *P. caprodes s.s.* similarly dispersing from a Mississippian refugium (Near, 2008). A phylogeographic study of *E. triquetra* documented significant population structure and genetic differentiation across the species range, with a population in the Ozark Highlands (St Francis River, Missouri) qualifying as a distinct species (Zanatta & Murphy, 2008).

The present study seeks to test the congruency of genetic population structure found in *E. triquetra* (Zanatta & Murphy, 2008) with the genetic population structure of its putative natural host, *P. caprodes*. Coevolutionary theory and empirical studies show that the phylogeny, population structure, and local adaptations of a parasitic species are highly correlated (sometimes inversely) to that of its host species (Dybdahl & Lively, 1996; Nuismer, Thompson &

Gomulkiewicz, 2003; Noel, Angers & Lapointe, 2005; Whiteman & Parker, 2005; Criscione & Blouin, 2007; Gomez-Diaz *et al*., 2007; Whiteman, Kimball & Parker, 2007; Geist & Kuehn, 2008). Because unionoid mussels are parasitic in their larval stage (via host-vector), it is logical to study and understand the movement and genetic population structure of their assumed hosts.

Host–parasite relationships can also be indicative of potential conservation units (Whiteman & Parker, 2005; Criscione & Blouin, 2007; Geist & Kuehn, 2008). On the basis of coevolutionary theory and a study on the interactions of genetic structure of the European Pearl Mussel (*Margaritifera margaritifera L.*) and its host brown trout (*Salmo trutta L.*; Geist & Kuehn, 2008), we expect the genetic population structure (using microsatellite genotyping) of *P. caprodes* to closely match *E. triquetra* (Zanatta & Murphy, 2008). Logperch populations, the mobile host (independent) species, are predicted to show reduced geographic structure and higher levels of gene flow. Despite its widespread use for phylogeographic studies, mitochondrial DNA sequence analysis was not used to compare the comparative phylogeographic structure of *E. triquetra* and *P. caprodes* because both species show limited mitochondrial geographic structure or diversity (Turner *et al*., 1996; Zanatta & Murphy, 2008). If the two species show congruent or covarying population structure, geographic genetic patterns in *P. caprodes* can be used to predict and guide the identification and management of distinct conservation units within *E. triquetra* (Zanatta & Murphy, 2008). For example, if populations of *P. caprodes* in the St Francis River (Missouri) were significantly divergent from other populations, it would support the taxonomic distinctiveness of *E. triquetra* in the St Francis River (Zanatta & Murphy, 2008). Because logperch are still common in many places snuffbox populations have been extirpated, the genetic structure of *P. caprodes* can be used to assist in informing reintroductions of snuffbox mussels.

MATERIAL AND METHODS

Sample localities and tissue collection

Nonlethal tissue samples were collected from both species at six localities in the USA and one in Canada (Fig. 1) comprising the most robust populations of *E. triquetra*. Tissue collection and genetic methods and data for *E. triquetra* are provided in Zanatta & Murphy (2008). To parallel the data for *E. triquetra* (Zanatta & Murphy, 2008), 310 specimens of *P. caprodes* were collected from the seven sites: 21 individuals from the Bourbeuse River (BR) at Reiker Ford, Missouri; 57 individuals from the Clinch River (CR) at Brooks Island near Sneedville, Tennessee; 29 individuals from Davis Creek/Huron River (HR) near Ann Arbor, Michigan; 22 individuals from French Creek (FC), upstream of Cambridge Springs, Venango County, Pennsylvania; 77 individuals from the St Croix River (SC) at Interstate Park, Wisconsin; 20 individuals from the St Francis River (SF), near Patterson, Missouri; and 84 individuals from the Sydenham River (SYD) near Florence, Ontario (Fig. 1). Fin tissue was nonlethally excised using a small (2 mm diameter) caudal fin punch and preserved in 95% ethanol. Vouchers from each sampling locality are available from the authors.

DNA extraction and genetic analysis

Genomic DNA was extracted from *P. caprodes* fin samples using a simple lysis extraction method. Approximately 10 mg of fin tissue per fish were individually lysed in 96-well plates using 250 μL lysis buffer per well (50 mM Tris, pH 8, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulphate, and 100 μg proteinase K), and incubated 8–12 h at 37 °C. Genomic DNA and degraded proteins were precipitated using 500 μL of 80% isopropanol per well and centrifuged at 13 000 g for 45 min. After removing the supernatant, DNA pellets were rinsed with 1 mL of 70% ethanol and re-centrifuged, air-dried at room temperature for 20 min, and resuspended in 50–200 μL of TE buffer (10 mM Tris pH 8, 1 mM EDTA). Extraction yields and quality were assessed using horizontal electrophoresis in 1% agarose gels alongside a Fermentas™ molecular mass ladder.

Microsatellites developed for the Roanoke logperch, *Percina rex* (Jordan and Jenkins, 1889) (Dutton *et al*., 2008) were used because no species-specific microsatellite markers have been developed for *P. caprodes*. The 16 loci developed for *P. rex* were tested on the *P. caprodes* samples collected from the seven localities using polymerase chain reaction (PCR) and thermocycler conditions *sensu* Dutton *et al*. (2008). Primers for optimized loci were labeled with either 5′-HEX or -NED fluorescent labels and amplified using Eppendorf Mastercycler and MJ Research PTC-100 thermocyclers. Double-stranded PCR products were stained with SYBR Green and visualized in 1% agarose gels alongside a 1KB+ ladder to estimate product quality and size. Non-overlapping amplified microsatellite loci were pooled by size class and dye colour and genotyped using an Applied Biosystems (ABI) 3730 automated sequencer and ROX-500 size standard (ABI dye set DS-32), and scored using SoftGenetics GENEMARKER® software.

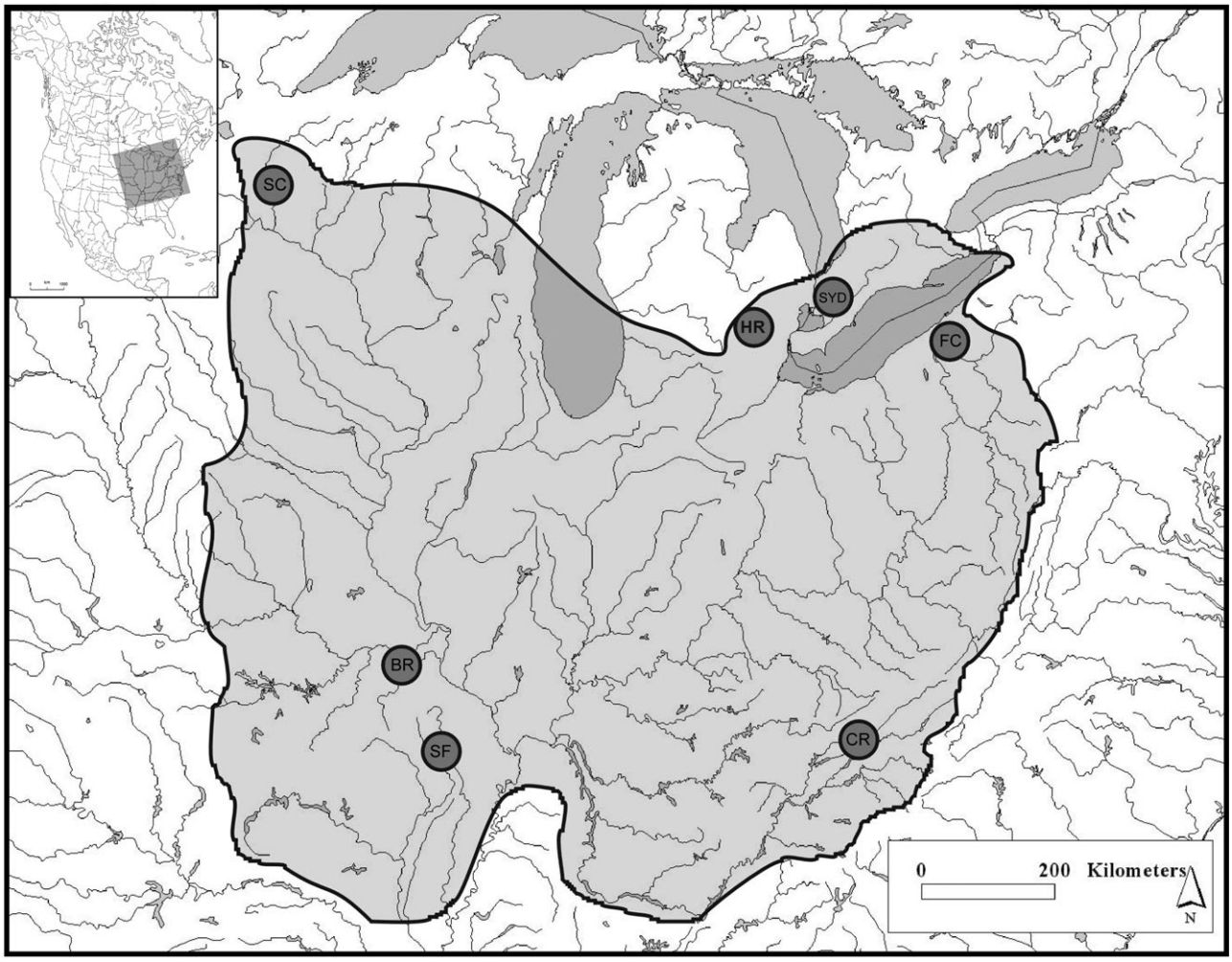

Figure 1. The distribution of populations where tissue collections were made for *Epioblasma triquetra* (historical range shaded in light grey) and *Percina caprodes* (not shown, although much more widely distributed; Scott & Crossman, 1973). Sample site localities: Bourbeuse River (BR), at Reiker Ford 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); Huron River (HR), 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, 90.4885 °W); Sydenham River (SYD), near Florence, ON (42.6912 °N, 82.9892 °W). Inset shows key map of North America.

Statistical analysis

Multilocus genotypic data collected for *E. triquetra* (15 microsatellite loci) and *P. caprodes* (eight microsatellite loci) were analyzed as described in Zanatta & Murphy (2008). Some of the genotypic data for *E. triquetra* from Zanatta & Murphy (2008) had additional statistical analyses conducted to assist in comparisons with the *P. caprodes* dataset. The statistical approaches for both species are summarizesd below.

Microsatellite data were tested for potential genotyping errors as a result of stuttering, short allele dominance, and null alleles (Pompanon *et al*., 2005) using a Monte Carlo simulation of expected allele size differences using MICROCHECKER (Van Oosterhout *et al*., 2004). Allele 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 *sensu* Brookfield (1996). Genetic diversity within each geographic sample was summarized as allelic richness (A) (measured as the mean number of alleles per locus after correcting for sample size) and expected and observed heterozygosity (H_E, H_O). Allelic richness was calculated in FSTAT, version 2.9.3.2 (Goudet, 1995). Data were standardized for sample size using a process of rarefaction

(Petit, El-Mousadik & Pons, 1998). Detection of deviations from Hardy–Weinberg equilibrium (HWE) and randomization tests for linkage disequilibria were conducted using GENEPOP, version 3.4 (Raymond & Rousset, 1995).

Genetic variance partitioning within and among populations was assessed using analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) in GENALEX software (Peakall & Smouse, 2006). Pairwise divergences among conspecific populations were estimated using F_{ST} (Weir & Cockerham, 1984) and D (Jost, 2008; Jost, 2009; Crawford, 2010). The corresponding P-values for F_{ST} and D 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} or D by chance distribution of genotypes. The D measure is analogous to a standardized F_{ST}, although it is better suited for interspecific comparisons as partitioning variance into α and β diversity components (Jost, 2008, 2009). F_{ST} estimates were calculated using FSTAT (Goudet, 1995); values of D were calculated using the Internet-based software SMOGD (Crawford, 2010; http://www.ngcrawford.com/django/jost/).

Individual-based analysis using STRUCTURE 2.2 (Pritchard, Stephens & Donnelly, 2000) assessed relationships among conspecific populations of both *E. triquetra* and *P. caprodes*. The number of distinct populations (K) represented in the set of samples was estimated for each species, using model conditions (1) of no admixture among genetic groups as a result of the substantial geographic separation between sampling sites and (2) uncorrelated allele frequencies (assumed independent loci). Ten replicate trials were run for values of K (number of genetic groups) from one to ten. This allowed for a range of hypotheses from panmixia to substructure within sampling locations (Pritchard *et al*., 2000). This procedure calculates $Pr(X|K)$ (the posterior probability of K) that the observed set of genotypes (X) would occur across a designated range of hypotheses or possible values of K (Pritchard *et al*., 2000) and for determining the optimal solution for the number of genetic groups present within each dataset. Model outcomes include the inferred proportional membership (q) of individual genotypes to each identified group (Pritchard *et al*., 2000). Each trial used an initial burn-in of 50 000 iterations followed by 50 000 additional iterations. To further evaluate the geographic genetic structure among sampling localities, principal coordinate analysis (PCA) (GENALEX; Peakall & Smouse, 2006) was used to ordinate genetic distance estimates (Nei, 1972, 1978) calculated for the genotypic data of both individuals and sampling localities of *E. triquetra* and *P. caprodes*. The use of multivariate ordination rather than phonetic clustering of genetic distances enables greater retention of information and patterns, and avoids potential phylogenetic misinterpretations (Lessa, 1990).

Genetic isolation by distance within each species was measured by comparing genetic divergence $[F_{ST}/(1 - F_{ST})]$ (or D) to geographic distances measured in river kilometers for pairs of populations. Geographic distances among populations were measured in ARCVIEW GIS, version 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 connected at the end of the last Pleistocene glaciation (Calkin & 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 within each species were tested using a Mantel test (Mantel, 1967) in GENEPOP, version 3.4 (Raymond & Rousset, 1995).

Correlations between the geographic and genetic population structures of *E. triquetra* and *P. caprodes* were assessed via Mantel tests (Mantel, 1967) in GENEPOP, version 3.4 (Raymond & Rousset, 1995). The statistical significance of correlations between pairwise matrices of genetic divergence (D) for *E. triquetra* and *P. caprodes* were tested using 10 000 permutation Markov chains in GENEPOP. The prediction that genetic structuring would be more pronounced in the parasitic (*E. triquetra*) versus host species (*P. caprodes*) was tested using a Wilcoxon paired-sample test (Zar, 1996). Population-level genetic diversity (allelic diversity and heterozygosity) for *E. triquetra* and *P. caprodes* were also regressed.

RESULTS

E. TRIQUETRA

Genetic and statistical analyses for geographically representative healthy populations of *E. triquetra* were previously reported by Zanatta & Murphy (2008). Summary statistics for the *E. triquetra* microsatellite dataset remain identical to those reported by Zanatta & Murphy (2008) and are not repeated here.

Significant structuring was evident among populations of *E. triquetra*. Of the total variation, 18.3% was the result of differences among populations (AMOVA: $\Phi_{ST} = 0.284$). All pairs of populations exhibited significant F_{ST} differences after correcting for multiple

Table 1. Pairwise F_{ST} (Weir & Cockerham, 1984; above diagonal) and D (Jost, 2008) for seven populations of *Epioblasma triquetra* and *Percina caprodes* from the same geographic locations

	BR	CR	HR	FC	SC	SF	SYD
Epioblasma triquetra							
BR	–	0.146	0.163	0.164	0.193	0.336	0.172
CR	0.420	–	0.125	0.084	0.170	0.189	0.078
HR	0.400	0.445	–	0.109	0.150	0.289	0.066
FC	0.407	0.326	0.359	–	0.191	0.226	0.071
SC	0.331	0.490	0.359	0.538	–	0.322	0.138
SF	0.661	0.505	0.649	0.589	0.675	–	0.290
SYD	0.373	0.299	0.190	0.253	0.408	0.690	–
Percina caprodes							
BR	–	0.054	0.079	0.049	0.125	0.073	0.090
CR	0.271	–	0.065	0.033	0.145	0.080	0.085
HR	0.366	0.364	–	0.038	0.116	0.084	0.025
FC	0.257	0.218	0.232	–	0.127	0.056	0.045
SC	0.366	0.522	0.437	0.452	–	0.124	0.123
SF	0.400	0.378	0.400	0.295	0.465	–	0.101
SYD	0.339	0.365	0.065	0.198	0.435	0.425	–

Sample sites and abbreviations are shown in Fig. 1.

comparisons (Table 1). Pairwise values of D (Jost, 2008, 2009) similarly showed substantial pairwise divergence among populations, with values typically two- to four-fold higher than F_{ST} estimates (Table 1). These differences were most pronounced for population pairs with relatively low F_{ST} estimates (e.g. CR–SYD pair).

As previously reported by Zanatta & Murphy (2008), the branching pattern formed in the Neighbour-joining network of Nei's D_A genetic distances (Fig. 2A) closely resembled the branching pattern of the rivers of origin among the sampled populations (Fig. 1).

The optimal solution for number of populations predicted in the simulation implemented in STRUCTURE was $K = 7$, with a probability of $\mathrm{Ln}[Pr(X|K)] = -5432.8$. The seven predicted populations exactly matched the seven sampling localities (Fig. 3A), with strong concordance between individual assignments (group membership) and their sampling locations.

Nei's genetic distances (Nei, 1972, 1978) among individuals and populations for the 15 microsatellite loci were ordinated using PCA. Axis 1 and 2 accounted for 52.4% (34.5% and 17.9%, respectively) of the total variation (Fig. 4A). Axis 1 of the PCA separated individuals from the St Francis River from the other six populations and Axis 2 secondarily grouped individuals from the St Croix River apart from the other populations (Fig. 4A). A population-level ordination for *E. triquetra* revealed similar results, with the first two axes accounting for 65.8% of the total variation, with the St Francis and St Croix River populations again showing as distinct for Axis 1 and Axis 2, respectively (Fig. 4B).

P. caprodes

Eight of 16 published loci for *P. rex* (Dutton *et al*., 2008) amplified consistently for *P. caprodes* across all localities. All eight loci were polymorphic, with a total of 174 alleles observed (6–35 alleles/locus, mean = 22). Genetic diversity, as measured by allelic richness (A), varied somewhat by population (6.9–10.9 alleles/locus), with the lowest allelic richness occurring in the St Francis River and the highest in the Sydenham River (Table 2). Expected heterozygosities were in the range 0.67–0.76 and varied somewhat between populations (Table 2). Analysis of the microsatellite dataset with MICROCHECKER showed no evidence for genotyping errors as a result of stuttering or large-allele dropout, suggesting the potential for non-amplifying (null) alleles in the dataset. All of the loci exhibited significant deviation from HWE expectations, although only two loci showed any signs of null alleles (Table 2). Only one locus (Prex44) consistently showed substantial heterozygosity deficits across all sampled populations, with significant deficits at five of the seven locales (Table 2). For the remaining loci, deviation from HWE largely occurred within individual sites, suggesting local substructure rather than systemic null alleles. Randomization tests for linkage disequilibrium by locus and population did not indicate any significant linkage disequilibria for any of the 196 di-locus combinations in *P. caprodes*.

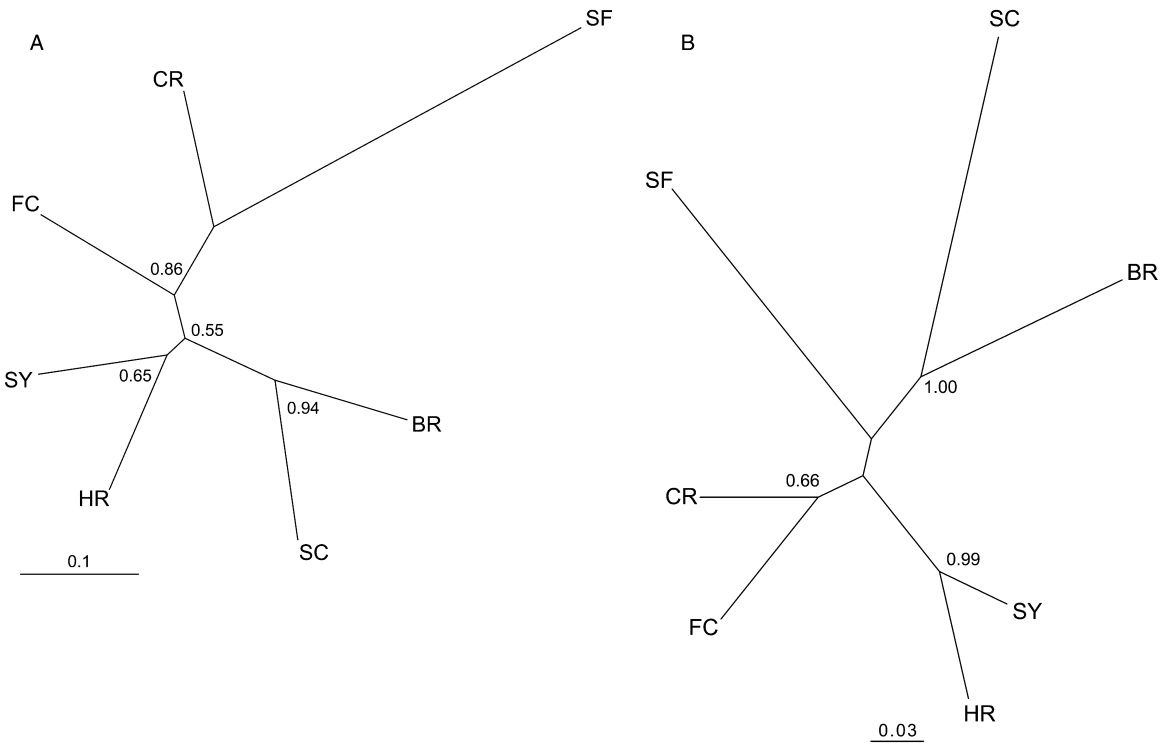

Figure 2. Unrooted Neighbour-joining network based on Nei's D_A (Nei, Tajima & Tateno, 1983) genetic distance for (A) seven populations of *Epioblasma triquetra* (Zanatta & Murphy, 2008) and (B) seven populations *Percina caprodes*. Numbers indicate nodes with bootstrap support of more than 50% for 1000 replications. Location abbreviations are defined in Fig. 1.

Significant population structuring was evident for *P. caprodes*. This significant structure was found despite the limited sampling of *P. caprodes* associated with the need to match populations with sampling of *E. triquetra* (see above). Of the total variation, 9.5% was the result of differences among populations ($\Phi_{ST} = 0.153$). Similar to the results obtained for *E. triquetra*, all pairwise F_{ST} and D divergence estimates among *P. caprodes* populations were significant after correcting for multiple comparisons (Table 1). D-values were comparable to F_{ST}, although generally higher (Table 1).

As with the dendrogram of genetic distances for *E. triquetra* (Fig. 2A), the branching pattern formed in the NJ network of Nei's D_A genetic distances for *P. caprodes* (Fig. 2B) also closely resembles the branching pattern of the rivers of origin among the sample populations (Fig. 1).

The most probable number of genetic groups within the *P. caprodes* sample set predicted using STRUCTURE was $K = 5$ with a $\ln[Pr(X|K)] = -9710.0$. The five predicted populations showed varying degrees of congruence with the seven sampling localities (Fig. 3B). Individuals from the St Croix and St Francis rivers were predicted to have high membership coefficients to their sampling locations as a result of their geographical isolation (Fig. 1). All individuals from the St Croix River and all but one from the St Francis River were assigned to their sampling locations; the mis-assigned individual from the St Francis River was more similar to the Ohio/Tennessee/Northern Ozark populations (Fig. 3B). Individuals from the Great Lakes (Sydenham River and Huron River) showed evidence of two mixed or indeterminate populations and showed varying membership probabilities to either or both. Individuals from the Clinch River and Bourbeuse River overlapped in their predicted population of origin. Finally, individuals from French Creek were assigned to the Great Lakes, the Bourbeuse/Clinch River and the St Francis populations.

PCA ordination of Nei's genetic distances (Nei, 1972, 1978) among *P. caprodes* individuals and populations each resulted in two significant axes (Fig. 5). For the individual-based ordination, Axis 1 and Axis 2 accounted for 51.9% (27.8% and 24.0%, respectively) of the total variation. Axis 1 of the PCA separated individual *P. caprodes* from the St Croix River apart from the other populations (Fig. 5A). Individuals from populations other than

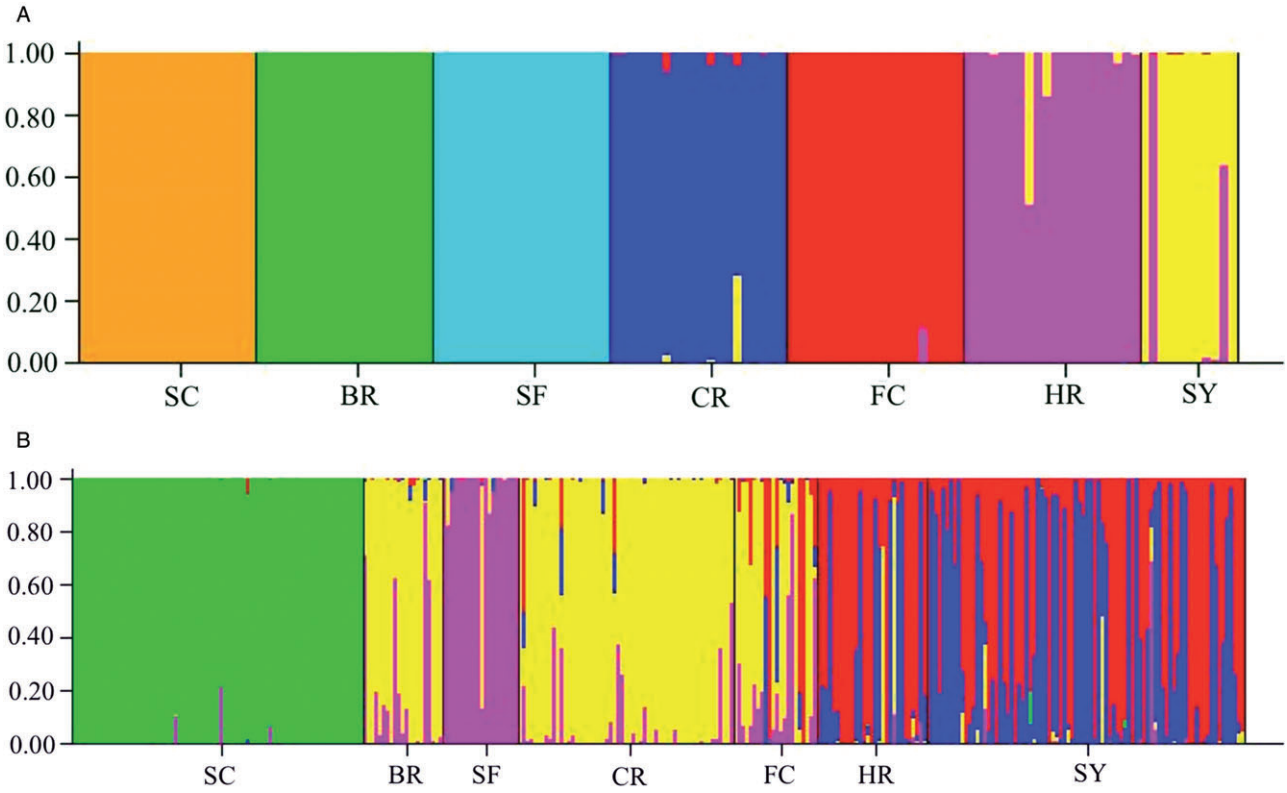

Figure 3. Graphical output from STRUCTURE of individual assignment to populations for (A) *Epioblasma triquetra* [K = 7; ln(*Pr*(*X*|*K*) = –5432.8)] and (B) *Percina caprodes* [K = 5; ln(*Pr*(*X*|*K*) = –9710.0)]. Conditions for both simulations were 100 000 iterations with a burn-in of 50 000 iterations, with no admixture and uncorrelated allele frequencies. *x*-axis, individual samples; *y*-axis, posterior-probabilities for membership to a group.

the St Croix River had a great deal of overlap, and did not form distinct groupings using this analysis. The population-level ordination (Fig. 5B) explained 65.3% of the total variation and showed a clear separation of the St Croix River population along Axis 1 and the Great Lakes populations (Huron and Sydenham Rivers) along Axis 2. Similar to the STRUCTURE analysis, the ordination had individuals from the French Creek population showing affinities for both the Upper Tennessee and the Great Lakes populations.

Genetic distance and geographic distance (following approximate post-glacial routes of invasion) were not significantly correlated for either *P. caprodes* (for F_{ST} $P = 0.435$ and Jost D $P = 0.410$) or *E. triquetra* (for F_{ST} $P = 0.733$ and Jost D $P = 0.602$).

Dataset comparison

The Neighbour-joining dendrograms estimated for *E. triquetra* (Fig. 2A) and *P. caprodes* (Fig. 2B) exhibited several similarities and some marked differences. Both network diagrams closely resemble historical post-glacial patterns of river confluence, with the network for *P. caprodes* making a slightly better approximation. The marked differences were the groupings at the deeper nodes of each dendrogram, which also had substantially lower bootstrap support than those connecting geographical subsets within each species.

Comparisons of the pairwise interpopulation *D*-values (Jost, 2008, 2009) between the *E. triquetra* and *P. caprodes* datasets (paired testing of intraspecific divergence values between sampling locations for the two species) were made using a Mantel test. The comparison of pairwise site *D*-values for *E. triquetra* versus *D*-values for *P. caprodes* showed a significant correlation ($r = 0.397$, $P = 0.031$) based on 10 000 permutations (Fig. 6). Paired testing of interpopulation divergence values confirmed that *D* for *E. triquetra* were significantly greater than *D*-values for *P. caprodes* based on a nonparametric one-tailed Wilcoxon test ($P < 0.01$) (Zar, 1996). No population-level correlations of genetic diversity (allelic diversity and heterozygosity) between *E. triquetra* and *P. caprodes* were found ($P > 0.05$).

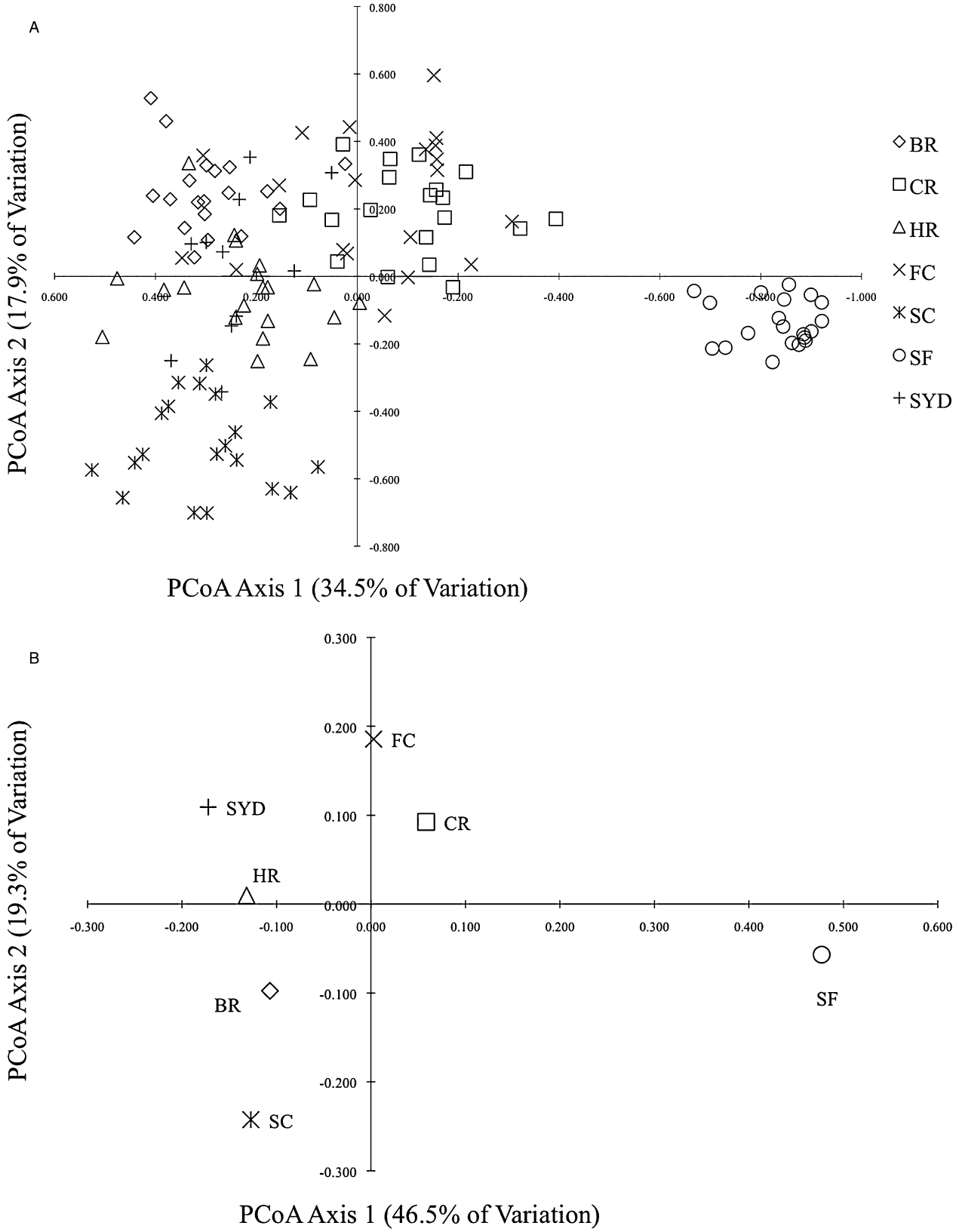

Figure 4. Results of a principal coordinate analysis based on multilocus genotypes of individuals of *Epioblasma triquetra* from seven populations (Zanatta & Murphy, 2008), showing ordination results for (A) individuals and (B) populations.

Table 2. Locus name with number of alleles (private alleles in parentheses), observed (H_O) and expected (H_E) heterozygosities, and estimated frequency of a null allele (for each locus across all populations), for *Percina caprodes* by locus and population. Italicized numbers in parentheses indicate the number of private alleles

Locus		BR	CR	HR	FC	SC	SF	SYD
Prex31	Number of alleles	3 (*0*)	7 (*2*)	4 (*0*)	3 (*0*)	3 (*0*)	4 (*0*)	3 (*0*)
Estimated null allele	H_O	0.67	0.65	0.19*	0.32*	0.46	0.55	0.10
Frequency = 0.015	H_E	0.62	0.61	0.43	0.56	0.48	0.70	0.12
	N	21	54	21	22	70	20	83
Prex33	Number of alleles	6 (*1*)	5 (*0*)	5 (*0*)	5 (*0*)	7 (*2*)	5 (*0*)	7 (*0*)
Estimated null allele	H_O	0.48	0.23*	0.36*	0.55	0.29	0.55*	0.75
Frequency = 0.000	H_E	0.48	0.63	0.77	0.68	0.28	0.66	0.79
	N	21	52	28	22	77	20	84
Prex35	Number of alleles	3 (*0*)	5 (*0*)	4 (*0*)	4 (*0*)	4 (*0*)	3 (*0*)	5 (*0*)
Estimated null allele	H_O	0.29	0.14*	0.34	0.23	0.06	0.05*	0.39
Frequency = 0.000	H_E	0.38	0.31	0.31	0.29	0.06	0.14	0.40
	N	21	50	29	22	77	20	84
Prex36	Number of alleles	7 (*0*)	9 (*0*)	10 (*0*)	7 (*0*)	15 (*1*)	7 (*0*)	12 (*1*)
Estimated null allele	H_O	0.62	0.80	0.93	0.68	0.70*	0.45*	0.79
Frequency = 0.087	H_E	0.79	0.81	0.82	0.80	0.87	0.75	0.81
	N	21	45	28	22	77	20	84
Prex41	Number of alleles	16 (*0*)	23 (*1*)	19 (*0*)	18 (*1*)	17 (*0*)	10 (*0*)	27 (*4*)
Estimated null allele	H_O	0.90	0.93	0.93	0.95	0.94	1.00	0.94
Frequency = 0.000	H_E	0.94	0.94	0.94	0.92	0.92	0.84	0.95
	N	21	54	27	22	77	20	84
Prex42	Number of alleles	15 (*1*)	16 (*1*)	13 (*0*)	14 (*0*)	23 (*12*)	8 (*0*)	22 (*0*)
Estimated null allele	H_O	1.00	0.91	0.86	0.77	0.92	0.70	0.71*
Freq = 0.004	H_E	0.92	0.92	0.87	0.92	0.94	0.82	0.93
	N	21	56	28	22	77	20	84
Prex44	Number of alleles	14 (*0*)	17 (*1*)	13 (*0*)	15 (*1*)	22 (*1*)	8 (*0*)	273 (*2*)
Estimated null allele	H_O	0.53*	0.49*	0.44*	0.43*	0.82	0.70	0.59*
Frequency = 0.041	H_E	0.91	0.82	0.87	0.93	0.91	0.81	0.94
	N	19	51	27	21	74	20	83
Prex46	Number of alleles	14 (*1*)	18 (*0*)	15 (*1*)	18 (*1*)	26 (*8*)	11 (*0*)	223 (*1*)
Estimated null allele	H_O	0.57*	0.82	0.86	1	0.84*	0.75	0.89
Frequency = 0.038	H_E	0.90	0.88	0.93	0.95	0.92	0.85	0.94
	N	21	57	28	21	76	20	84
Mean H_E		0.74	0.74	0.74	0.76	0.67	0.70	0.73
Allelic richness		9.52	9.32	9.40	10.18	10.10	6.90	10.90

Sample sites and abbreviations are shown in Fig. 1.
*Indicates locus–population combinations with H_o significantly different from H_E after sequential Bonferroni correction (Rice, 1989), experiment-wide $\alpha = 0.05$.

DISCUSSION

The results obtained in the present study conform to expectations for closely coevolved species (Thompson, 1994). Both the mussel and its host show strong geographic structuring, with marginally concordant geographic patterns of genetic structure and diversity. The results have important conservation implications because they uncover additional population structuring for an imperiled species (*E. triquetra*), which was previously revealed when analyzing the mussel alone (Zanatta & Murphy, 2008).

This is the first genetic study to investigate coevolution in an obligate parasitic unionoid mussel and its host fish in North America. A recent study (Geist & Kuehn, 2008) on European Pearl Mussel (*M. margaritifera*) and its host, brown trout (*S. trutta*), in Central Europe also found broad congruence in genetic differentiation among populations with higher differentiation in the parasitic mussel. By contrast

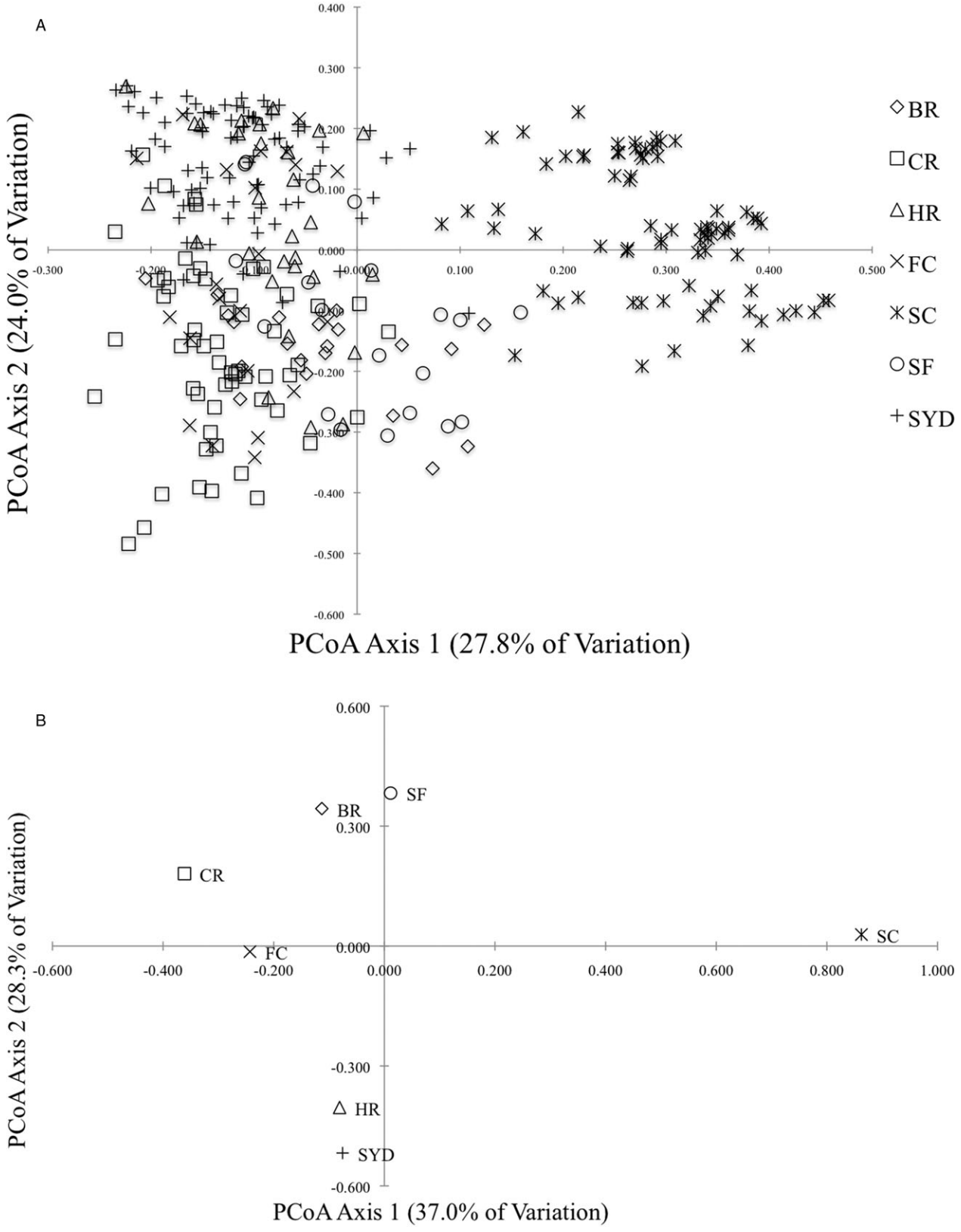

Figure 5. Results of a principal coordinates analysis based on multilocus genotypes of individuals of *Percina caprodes* from seven populations, showing ordination results for (A) individuals and (B) populations.

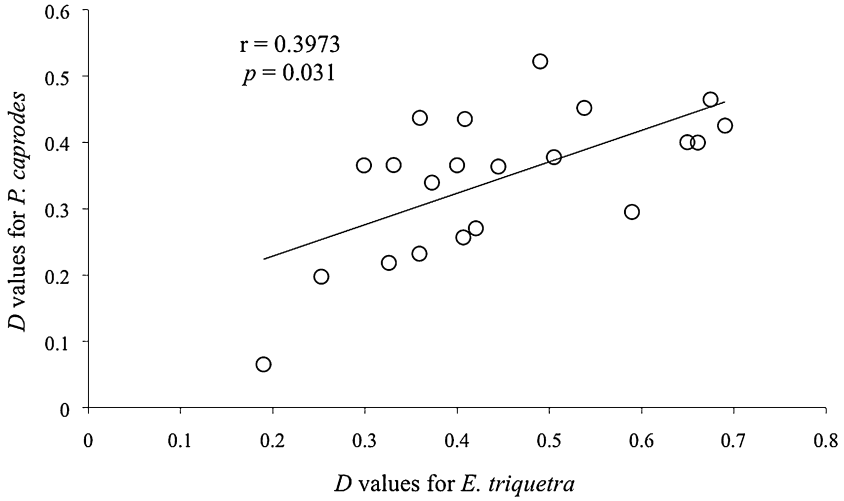

Figure 6. Covariation of pairwise intraspecific population divergence values (*D*-values; Jost, 2008) for *Epioblasma triquetra* and *Percina caprodes* based on 15 and eight microsatellite loci, respectively. Correlation and *P*-values were calculated using a Mantel test (10 000 permutations).

to the *M. margaritifera–S. trutta* study (Geist & Kuehn, 2008), significant population-level correlations between genetic diversity metrics (allelic richness and observed heterozygosities) were not found in the present study. Although both obligate parasitic systems are between a freshwater mussel and a host fish, they are vastly different in ecology and behaviour. *Margaritifera margaritifera* can reach densities in the tens to hundreds per square meter in highly oligotrophic systems and are often the only unionoid present, whereas *E. triquetra* are found at low densities (usually less than $0.1/m^2$), in highly productive, and highly diverse systems with > 20 unionoid species in the community (Butler, 2007; Metcalfe-Smith *et al*., 2007). The host infection strategies of the two mussels also are extremely different; *M. margaritifera* broadcasts its glochidia, whereas *E. triquetra* uses host-capture (Barnhart *et al*., 2008). The hosts also differ in their ecology and behaviour: *P. caprodes* is a warm-water benthic omnivore and *S. trutta* is a cold-water predator (Scott & Crossman, 1973). Geist & Kuehn (2008) found an interesting relationship between population-level genetic diversity for *M. margaritifera* and densities of *S. trutta*. Unfortunately, these data for *E. triquetra* and *P. caprodes* are not available, although they may be interesting to investigate in future studies. It appears *S. trutta* may be the only host for *M. margaritifera* in the Central European streams sampled by Geist & Kuehn (2008). *Salmo trutta* is probably solely responsible for the genetic structure of the mussel in the region studied by Geist & Kuehn (2008) [Atlantic salmon (*Salmo salar L.*), is known to act as a host in Atlantic coastal drainages; Machordom *et al*., 2003; Bouza *et al*., 2007; OSU Division of Molluscs, 2009. By contrast, *E. triquetra* is likely to have additional hosts (discussed further below); thus, their genetic structure may not be completely dependant on *P. caprodes*. Contrasting the study on *M. margaritifera–S. trutta* by Geist & Kuehn (2008) and the present study on the *E. triquetra–P. caprodes* relationship reveals the need for additional research on these unique relationships.

Genetic structure in *P. caprodes*

The analysis of the microsatellite DNA dataset produced some interesting population structure results for *P. caprodes*. Significant structure was found despite the limited sampling of *P. caprodes*. The St Croix River population was clearly distinct from all other logperch populations and may represent an as-yet undescribed taxon (Near, 2008). Recent phylogenetic analysis using sequence data from two mitochondrial genes (cytochrome *b* and NADH subunit 2) and nuclear DNA (S7 ribosomal protein intron 1) found that Upper Mississippi *P. cf. caprodes*, including samples from St Croix River, were a distinct species from *P. caprodes s.s.* Thus, *P. caprodes s.s.* is in need of description (Near, 2008). In support of this hypothesis, the results from the individual- and population-based analyses provide evidence of a divergent genetic lineage concordant with the phylogeny of Near (2008).

Other populations of *P. caprodes* showed substantial geographic structure with all analytical approaches. As noted above, the St Croix River *P. cf. caprodes* population was most distinct in all of the analyses. The St Francis River population showed the

next greatest degree of divergence in terms of genetic distance, and showed substantial differentiation from all other study populations. These findings are congruent with other studies reporting that distinct, drainage-specific genetic patterns for fish and unionoid species in the geologically ancient southern Ozarks (Turner *et al*., 1996; Turner & Trexler, 1998; Serb, 2006; Serb & Barnhart, 2008). In particular, the results of the present study are consistent with those observed for other species of darter (Percidae: Etheostomatinae). For example, the greenside darter (*Etheostoma blennoides*) complex (Piller, Bart & Hurley, 2008); rainbow darter, *Etheostoma caeruleum* (Ray, Wood & Simons, 2006); and crystal darter, *Crystallaria asprella* (Morrison *et al*., 2006) showed similar population structuring in the Upper Mississippi and Ozark Mountains.

The remaining *P. caprodes* populations showed varying amounts of distinctiveness. The Bourbeuse River (Meramec River drainage) and Clinch River (Tennessee River drainage) populations appear to form a single population in the STRUCTURE analysis (Fig. 3B). This pattern is indicative that these populations, connected by the Tennessee River, Ohio River, and Mississippi River (several hundred river kilometers) have maintained a relatively high level of gene flow for millennia. The Huron River and Sydenham River (Great Lakes) populations show similar patterns in the STRUCTURE analysis, although individuals are predicted to belong to two distinct populations. This result is harder to explain but could be the signature of two glacial refuges for *P. caprodes* in the Great Lakes: one via the Maumee River–Wabash River spillway and the other via the Illinois River–Chicago River spillway (Bailey & Smith, 1981). Conversely, the observed pattern could reflect the more recent post-glacial separation and lower founding diversity of these populations (Bernatchez & Wilson, 1998). Individuals from French Creek (Upper Ohio River drainage) show affinities to the Bourbeuse/Clinch and the Huron/Sydenham populations. This result is indicative of recent gene flow between the Bourbeuse/Clinch and the Great Lakes populations.

Comparison of *P. caprodes* and *E. triquetra* genetic structures

The covarying genetic patterning between *P. caprodes* and *E. triquetra* suggests shared history and potential coevolutionary relationship between the two species. The use of more variable and faster-evolving genetic microsatellite DNA markers was essential for elucidating patterns of geographic structure to test for covariation as both species show limited phylogeographic structure based on mitochondrial DNA (Turner *et al*., 1996; Zanatta & Murphy, 2008). Although the two species exhibited similar geographical patterns, their population structures were weakly correlated and not perfectly congruent. Although the geographic genetic structure of the two species might show chance similarity as a result of a shared hydrographic history within major drainages (regional phylogeography; Avise, 1992), the biological relationship between the two species (Barnhart *et al*., 1998) strongly suggests that *P. caprodes* is an important, if not exclusive, dispersal host for *E. triquetra*. The imperfect congruency of host and parasite supports the geographic mosaic concept of coevolution (Thompson, 1994), indicating that coevolutionary dynamics occur at a geographic scale greater than local populations but below the species level. Thus, although *P. caprodes* may be the primary host for *E. triquetra* across most of their sympatric range, historical events related to Pleistocene disturbances may have resulted in alternate adaptive trajectories in the western portions of both species' distribution.

Although *P. caprodes* is a known host for *E. triquetra* (Hillegass & Hove, 1997; Barnhart *et al*., 1998, 2008; Butler, 2007), differences in the basic biology and ecological tolerances of *E. triquetra* and *P. caprodes* may be responsible for the imperfect fit of their respective genetic population structures. Comparative studies of geographic genetic structure and gene flow show that *P. caprodes* has limited phylogeographic structure and high levels of gene flow compared to other darters (Turner *et al*., 1996; Turner & Trexler, 1998). By contrast, *E. triquetra* has extremely restricted mobility for most of its life cycle, and dispersal via glochidial transport by host fish is dependent on successful infection of a suitable host (Barnhart *et al*., 1998). As such, dispersal of *E. triquetra* is limited to successfully encountering and infecting a fish host within a finite seasonal window, whereas *P. caprodes* is not limited to life stage or season for movement and dispersal. In addition, *E. triquetra*, similar to other members of its genus, is largely restricted to riffles and other highly oxygenated, high-flow areas of streams and rivers (Parmalee & Bogan, 1998; Williams, Bogan & Garner, 2008), whereas *P. caprodes* is a habitat generalist (Scott & Crossman, 1973). Extant populations of *E. triquetra* are often separate by large areas of unsuitable habitats, whereas *P. caprodes* may be able to disperse and survive through these habitats unsuitable for *E. triquetra*. *Percina caprodes* infected with glochidia will not disperse entirely across large areas of unsuitable habitat and newly metamorphosed juvenile *E. triquetra* will not survive. These pronounced ecological differences may account for much of the observed higher F_{ST} and *D*-values and greater geographic subdivision for *E. triquetra*, and highlight the importance of *P.*

caprodes as a dispersal vector and coevolutionary host (Turner *et al*., 1996; Turner & Trexler, 1998).

Another factor leading to incomplete congruency of the genetic population structures may be the use of host(s) by *E. triquetra*. For example, *Percina maculata* (blackside darter [Girard, 1859]) and several sculpins (*Cottus* spp.) are confirmed as a hosts (Hillegass & Hove, 1997; Barnhart *et al*., 1998; OSU Division of Molluscs, 2009). Cottids are not a probable natural host for *E. triquetra* and other rifflehsells (genus *Epioblasma*) because they are not found in same habitats (Rogers, Watson & Neves, 2001; Jones *et al*., 2006). Other darters are similarly not hosts because Barnhart *et al*. (2008) reported that the heads of darters from the genus *Etheostoma* were crushed upon capture by *E. triquetra*, whereas most *P. caprodes* survived. However, testing the survival of *P. maculata* or other congeners after *E. triquetra* parasitism (capture) was not mentioned (Barnhart *et al*., 2008). Geographic analysis of the genetic population structure for *P. maculata* (in addition to that of *P. caprodes* reported in the present study) may be informative for further resolving historical dispersal and gene flow of *E. triquetra*.

Implications for evolution and conservation

The combined datasets for *E. triquetra* and *P. caprodes* provide evidence for their coevolutionary history. The broadly congruent phylogeographic patterns of *P. caprodes* and *E. triquetra* suggest additional conservation units in the latter that were not considered fully by Zanatta & Murphy (2008). As the sole member of its genus not yet threatened with imminent extinction, *E. triquetra* is on the verge of Federal listing in the USA (Butler, 2007) and is already listed under Canada's Species at Risk Act (Environment Canada, 2007). Ecologically and evolutionarily sound decisions need to be made not only to manage, but also to restore populations across the range of *E. triquetra*. The conservation units identified by Zanatta & Murphy (2008) are consistent with the results of the present study; however, the distinct taxonomic unit of *P. cf. caprodes* in the St Croix River (Upper Mississippi drainage) suggests that the St Croix *E. triquetra* deserve similar consideration to St Francis River *E. triquetra* (southern Ozarks) as a potentially distinct taxonomic unit (Zanatta & Murphy, 2008). Other populations of *E. triquetra* should be managed separately, although it appears that *P. caprodes* hosts could be used for propagation from other drainages within the Great Lakes and Ohio/Tennessee/Middle Mississippi drainages. The results of this molecular study should be further tested using a reciprocal host testing experiment (Zale & Neves, 1982) to determine whether hosts from genetically distinct drainages act as similarly effective hosts across drainages (Jones *et al*., 2006). The present study reinforces the finding of Geist & Kuehn (2008) proposing that an understanding of the genetic structure of both mussel and host is critical in conservation planning and that single species analyses are not sufficient when considering co-adapted species.

Conclusions

The study of host–parasite relationships can be a powerful tool in evolutionary studies and the conservation of biodiversity (Thompson, 1994). The genetic population structure relationships between host and parasite have been investigated in other systems (Dybdahl & Lively, 1996; Nuismer *et al*., 2003; Noel *et al*., 2005; Whiteman & Parker, 2005; Criscione & Blouin, 2007; Gomez-Diaz *et al*., 2007; Whiteman *et al*., 2007). By contrast to the studies listed above, which used parasites to delineate genetic population structure in their host species, we have assessed the geographic genetic structure of the host fish (*P. caprodes*) to help resolve its shared history with *E. triquetra*. This is the first genetic study of its kind in North America involving a unionoid mussel and its host, and has reinforced the findings that diversity is not recognized when investigating the genetic diversity and structure of the mussel alone (Geist & Kuehn, 2008). Studying the relationships in genetic population structure of other mussel–host relationships will be extremely valuable for enhancing our understanding of the coevolutionary history and dynamics between these parallel species flocks, as well as aiding unionoid conservation in better determining which are the best natural hosts (Geist & Kuehn, 2008). Comparative phylogeographic and genetic studies should be complemented by behavioural and ecological studies to assess the extent of host specificity within and among unionoid mussels, as well as to experimentally evaluate coevolutionary relationships between mussel species and host fish (Jones *et al*., 2006).

The present study demonstrates and reinforces that unionoids and their host fish make a unique model system for the study of host–parasite coevolution and comparative phylogeography, and that further investigation is warranted. Understanding the genetic population structure of unionoids and their host fish species will shed light on their coevolutionary history. This will also assist in the management and restoration of populations of unionoids where the original population has been lost but the host remains and also will assist managers in the determination of which source populations to use to augment or reintroduce extirpated populations. As such, understanding the coevolutionary history of mussels and their

hosts should prove to be a valuable tool for helping to ensure their evolutionary legacy.

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