Phylogeography and Genetic Variability of the Freshwater Mussels (Bivalvia: Unionidae) Ellipse, *Venustaconcha ellipsiformis* (Conrad 1836), and Bleeding Tooth, *V. Pleasii* (Marsh 1891)

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Phylogeography and genetic variability of the freshwater mussels (Bivalvia: Unionidae) Ellipse, *Venustaconcha ellipsiformis* (Conrad 1836), and Bleeding Tooth, *V. pleasii* (Marsh 1891)

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Abstract: Following the retreat of the last Pleistocene glaciers ~10,000 years before present, aquatic organisms re-colonized previously uninhabitable regions from various glacial refuges. Glaciations had major impacts shaping patterns of genetic diversity and population structure for organisms throughout North America. Knowledge of genetic population structure is critical for successful conservation programs involving an increasingly threatened freshwater fauna. Due to variations in life history and ecology, species-specific planning may be the most effective method for preserving rare or threatened species. The Ellipse mussel (*Venustaconcha ellipsiformis*) and its congener the Bleeding Tooth mussel (*V. pleasii*) are species of conservation concern through much of their respective ranges in the Midwestern United States. The Ellipse is found in small to medium rivers from the northern Ozark highlands north to the Upper Mississippi River drainage and into tributaries of Lake Michigan and Lake Huron. Mitochondrial DNA from the COI and ND1 regions was amplified to assess the genetic diversity and structure of these species. Phylogenetic analyses confirmed that *V. ellipsiformis* and *V. pleasii* are distinct species. Little variation was recovered in the Ellipse with a single common haplotype dominating throughout its range. For Ellipse, only limited genetic differentiation was found among the geographic regions sampled, with consistently significant differentiation only found between populations in the Illinois River drainage and populations in the northern Ozarks. The general low to moderate genetic structure among various geographically distant Ellipse populations suggests this species dispersed rapidly from unglaciated refugia with little time for genetic isolation to occur. The data suggest that *V. ellipsiformis* populations should be treated as three separate management units: northern Ozark highlands, Upper Mississippi River drainage, and the Illinois River/ Great Lakes drainages.

Key words: population genetics, Lampsilini, mtDNA, Ozark highlands, post-glacial colonization

The United States is home to the most diverse freshwater mussel fauna in the world (Lydeard *et al.* 2004, Graf and Cummings 2007, Bogan and Roe 2008). High levels of endemism occur in central and southeastern North America, making the region an important contributor to worldwide mussel diversity. However, due to a combination of habitat destruction and degradation associated with anthropogenic events, over 70% of the North American mussel fauna is now listed as endangered, threatened, or of special concern (Williams *et al.* 1993).

The Ellipse, *Venustaconcha ellipsiformis* (Conrad 1836), and Bleeding Tooth or Plea's Mussel, *V. pleasii* (Marsh 1891) are small (up to 75 mm), elliptical mussels that are generally found in swift currents of small to medium streams (van der Schalie and van der Schalie 1963, Oesch 1995). The Ellipse has a broad historical distribution in the central United States, ranging from Indiana and Michigan, west to Minnesota, and southwest to Oklahoma and Arkansas (Watters *et al.* 2009). Conversely, the Bleeding Tooth is a narrow range endemic of the White River drainage of the central Ozarks in Missouri and Arkansas (Oesch 1995, Riusech and Barnhart 2000). The Ellipse and Bleeding Tooth are considered species of conservation concern in many parts of their respective ranges (Williams *et al.* 1993).

Glochidia larvae of most freshwater mussels are obligate parasites on fish, and rely on this life stage for dispersal (Barnhart *et al.* 2008). Identified host fish for the Ellipse and Bleeding Tooth include several species of darters, sculpins, and the brook stickleback (Riusech and Barnhart 2000, Allen *et al.* 2007). These hosts all have relatively low vagility, which likely limits the dispersal abilities of both the host and parasite to relatively short distances (Woolnough *et al.* 2009). The inability to disperse long distances coupled with patchy distribution patterns likely reduces gene flow between populations, effectively isolating populations between drainages (Allen *et al.* 2007). Genetic drift may increase genetic differentiation between these populations (Hartl and Clark 2007). The level of genetic differentiation can, therefore, be viewed as a result of the competing forces of genetic drift increasing divergence and gene flow promoting homozygosity. Assessment of dispersal abilities and amount of gene flow between populations is then critical when attempting to preserve genetic diversity through conservation efforts.

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In addition to intrinsic effects related to individual dispersal abilities of a species and its host fish, historic geological changes have greatly affected genetic population structure in North American mussels. The primary geological event shaping modern day populations in the central and northern parts of North America was the Pleistocene glaciation (Larson and Schaetzl 2001). During this period, northern populations of aquatic organisms resided in multiple glacial refugia, isolating populations from one another (Soltis *et al.* 2006). Following the retreat of the last Pleistocene glaciers ~10,000 years before present, aquatic organisms used multiple dispersal routes to recolonize previously glaciated areas (Mandrak and Crossman 1992, Graf 2002). If individuals from a single glacial refuge colonized these newly colonized habitats, they likely experienced bottleneck effects and show reduced levels of genetic variation (Hewitt 1996). Therefore, higher genetic diversity can be expected in southern Ellipse populations or populations in unglaciated regions. Variation in population genetic structure between glaciated and unglaciated regions has been previously documented in several North American unionid species (Elderkin *et al.* 2007, 2008, Zanatta *et al.* 2007, Zanatta and Murphy 2008).

The factors influencing genetic variation in the North American mussel fauna makes it inadvisable to propose generalized propagation and conservation programs for unionids without *a priori* knowledge of evolutionary history and population genetics for the species in question (*e.g.*, Neves 2004). This is evidenced by the variation in genetic structure found among populations in the same watershed (*e.g.*, Grobler *et al.* 2006, Jones *et al.* 2006). Variation in dispersal abilities, life history, behaviors, and local adaptations to environmental conditions can significantly alter patterns of genetic diversity even in species with similar distributions (Elderkin *et al.* 2008).

Mitochondrial DNA (mtDNA) has long been used to assess patterns of phylogeography (Avise *et al.* 1987) and genetic structure in threatened aquatic species with respect to patterns of recent glacial history (Stepien and Faber 1998, Soltis *et al.* 2006, Elderkin *et al.* 2007, 2008, Zanatta and Murphy 2007, 2008). The main reason is that the mutation rate of mtDNA is much higher than that of nuclear DNA, allowing haplotype frequencies to drift and create genetic differences between populations in short periods of time (Beebee and Rowe 2008). This process produces significant genetic variation in most populations that are not extremely bottlenecked.

While information on the ecology, life history, and distribution of *Venustaconcha ellipsiformis* and *V. pleasii* are well established (van der Shalie and van der Schalie 1963, Riusech and Barnhart 2000, Allen *et al.* 2007), no research has documented the genetic diversity and population structure over their range. This study attempts to: (i) confirm species distinctions of *V. ellipsiformis* and its purported congener *V. pleasii*; (ii) resolve genetic relationships among populations across the range of the *V. ellipsiformis*; (iii) assess genetic diversity and differentiation among areas colonized following the Pleistocene glaciation and areas of probable glacial refuge; and (iv) put patterns of genetic variation and divergence in the context of conservation of these species.

MATERIALS AND METHODS

Sampling locations and collection

Specimens of *Venustaconcha ellipsiformis* and *V. pleasii* were collected from 16 waterbodies across the Midwestern U.S.A. *Venustaconcha ellipsiformis* specimens were collected from the following locations: Looking Glass River (Grand River/ Great Lakes drainage, Michigan (MI); 42.82228N, 84.69944W), Thornapple River (Grand River/ Great Lakes drainage, MI; 42.6114N, 85.0248W), Pine River (Saginaw River/ Great Lakes drainage, MI; 43.3212N, 84.7406W), Bourbeuse River (Meramec River drainage, Missouri (MO); 38.3799N, 91.0732W), Gasconade River, (MO; 38.3757N, 91.8272W), Buffalo Creek (Wapsipinicon River/ Upper Mississippi River drainage, Iowa (IA); 42.2061N, 91.4467W), Zumbro River (Upper Mississippi River drainage, Minnesota (MN); 44.0745N, 92.6634W), Salem Creek (Zumbro River/ Upper Mississippi River drainage, MN; 44.0705N, 92.8213W), Sugar Creek (Illinois River drainage, Indiana (IN); 40.6972N, 87.3817W), Mackinaw River (Illinois River drainage, Illinois (IL); 40.5711N, 88.6751W and 40.5542N, 88.5739W), Horse Creek (Illinois River drainage, IN; 41.1773N, 88.1468W), Ferson Creek (Fox River/ Illinois River drainage, IL; 41.9332N, 88.3411W), Forked Creek (Illinois River drainage, IL; 41.2552N, 88.1056W), Bray Creek (Illinois River drainage, IL; 40.5433N, 88.6267W) (Fig. 1). *Venustaconcha pleasii* specimens were collected from two locations: James River (White River drainage, MO; 37.2659N, 92.9501W) and Flat Creek (James River/ White River drainage, MO; 36.8022N, 93.7377W). Visual searches were conducted in riffle habitat from several tributary streams in each system, with 5–10 adult individuals being chosen for genetic analysis. Mantle tissue samples from each mussel were excised (Berg *et al.* 1995), therefore, only maternal mitotypes were recovered (Breton *et al.* 2007). Tissue samples were preserved in 95% ethanol in the field and stored at -80 °C upon return to the lab.

DNA extraction and genetic analysis

DNA was extracted from the individual samples using an overnight Proteinase K digestion of mantle tissue in cell lysis buffer followed by an alcohol extraction method (Sambrook

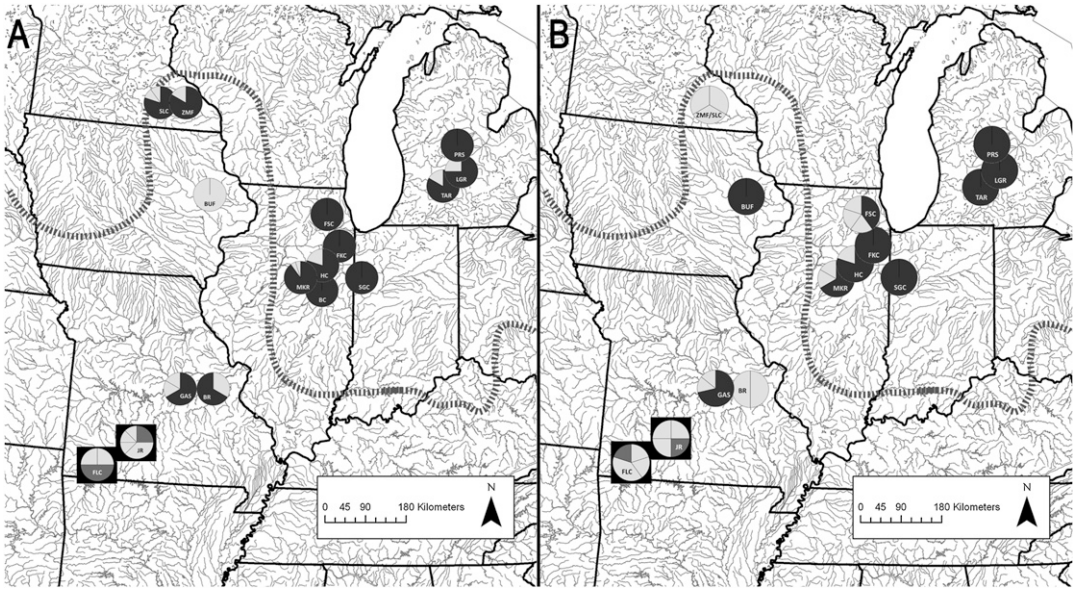

Figure 1. Sampling locations for *Venustaconcha ellipsiformis* (circles) and *V. pleasii* (squares) in the midwestern United States. The dashed line represents the approximate Wisconsin glacial maximum. Pie charts represent the frequency of (**A**) COI and (**B**) ND1 haplotypes encountered at each sampling location (Table 1). Common haplotypes (COI and ND1 haplotype 3 for *V. ellipsiformis* and COI haplotype 2 and ND1 haplotype 1 for *V. pleasii*) are shaded in dark grey. Uncommon and haplotypes unique to single populations (COI and ND1 haplotypes 1, 2, 4–13 for *V. ellipsiformis* and COI haplotypes 1, 3–7 and ND1 haplotypes 2–7) are shaded in light grey.

et al. 1989). Extracted genomic DNA was stained with SYBR Green™ and electrophoresed in a 1.5% agarose gel to confirm that genomic DNA had been properly extracted. Two mtDNA regions were amplified: cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1) using primers described in Campbell *et al.* (2005). Each reaction included 1 μL extracted DNA, 1 μL 10X PCR buffer, 1 μL bovine serum albumin, 0.3 μL of forward primer, 0.3 μL of reverse primer, 0.2 μL of dNTP, 0.05 μL *Taq* polymerase (Qiagen, Inc.), and deionized H_2O for a total reaction volume of 10 μL per sample. The thermocycler amplification conditions for both mtDNA regions were as follows: An initial heating of the sample to 92°C for 2 minutes; five cycles of 92°C for 40 seconds, 40°C for 40 seconds, and 72°C for 90 seconds; 25 cycles of 92°C for 40 seconds, 50°C for 40 seconds, and 72°C for 90 seconds; with a final elongation step 72°C for ten minutes and held at 4°C until they were placed in the freezer. Amplified PCR product was stained in SYBR Green and run on a 1.5% agarose gel electrophoresis to confirm successful amplification of the correct fragment length of DNA. The amplified samples were then purified using a QIAquick© PCR Purification Kit (Qiagen, Inc.). Amplified DNA was quantified using an ABI Nanodrop (Applied Biosystems, Inc.). The 5′ end of the amplified products were cycle-sequenced using 'Big Dye' Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Inc.) with the respective COI or ND1 forward primers (50 °C annealing temperature) and visualized on an ABI 3100 automated DNA sequencer (Applied Biosystems, Inc.) at the Michigan State University Research Technology Support Facility (RTSF) in East Lansing, Michigan.

Data analysis

Datasets for the sequenced genes were aligned using BIOEDIT (Hall 1999) and MACCLADE (Maddison and Maddison 1997) software. COLLAPSE v.1.2 (Posada 2004) was used to identify unique haplotypes from both *Venustaconcha ellipsiformis* and *V. pleasii* samples. Metrics for genetic diversity—number of haplotypes, number of polymorphic sites, and nucleotide diversity (π)—were calculated using ARLEQUIN v. 2.0 (Schneider *et al.* 2000) for each population sampled.

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 and ND1 sequences from *Epioblasma torulosa rangiana* (Lea 1838; COI-DQ479946,

ND1-DQ220720), *Lampsilis cardium* (Rafinesque 1820; COI-AF120653, ND1-GU085346), and *Pyganodon grandis* (Say 1829; COI-AF156504, ND1-GU085370) were used as outgroup taxa to root trees. PAUP* v. 4.0b10 was also used to calculate pairwise genetic distances among the haplotypes of *Venustaconcha ellipsiformis* and *V. pleasii.*

A second phylogenetic analysis used Bayesian inference implemented in MrBayes v3.0b4 (Huelsenbeck and 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 with 6 concurrently running Markov Chains and 2 hot chains, sampling every 100 generations.

TCS v. 1.21 (Clement *et al.* 2000) was used to construct a haplotype network based on the number of nucleotide mutations between different haplotypes. This program uses statistical parsimony to connect haplotypes based on a 95% confidence interval.

Hierarchical analyses of molecular variance (AMOVA) (Excoffier *et al.* 1992) was used to estimate the partitioning of haplotypes within and among populations. Because of limited numbers of samples taken from each sampling location, samples were pooled by major watershed/region (Great Lakes drainage, Northern Ozarks drainages, Illinois River drainage, and Upper Mississippi River drainage, and *Venustaconcha pleasii* in the White River drainage). An additional analysis was conducted to test genetic differentiation between glaciated and unglaciated sampling locations of *V. ellipsiformis*. AMOVAs for both COI and ND1 datasets were carried out in ARLEQUIN v. 2.0 by estimating Φ_{ST} from the absolute number of nucleotide differences and 16,000 permutations. To determine pooled sampling location differentiation, pairwise F_{ST} values were calculated using uncorrected *p*-distance (number of nucleotide substitutions). Finally, the number of haplotypes, number of polymorphic sites, and nucleotide diversity (π) were regressed against latitude of *V. ellipsiformis* sampling locations (of both *V. ellipsiformis* and *V. pleasii*) to test if geography and glaciation patterns could be determinate in assessing patterns of genetic diversity for these closely related species.

RESULTS

DNA sequencing consistently resulted in a 569 bp fragment for COI and 549 bp fragment for ND1. Because of the lack of sufficient overlap between samples successfully amplified, the COI and ND1 datasets could not be combined and were thus run separately in each analysis. A total of 13 distinct haplotypes were found from 101 *Venustaconcha ellipsiformis* COI sequences (Table 1; GenBank Accession numbers DQ220725 and KC537292–KC537304) and seven distinct haplotypes were found from nine COI sequences of *V. pleasii* (Table 2; GenBank Accession numbers KC537305–KC537311). A total of 13 distinct haplotypes were found from 56 *V. ellipsiformis* ND1 sequences, (Table 3; GenBank Accession numbers DQ220722 and KC537312–KC537323) and seven distinct haplotypes were found from 12 ND1 sequences of *V. pleasii* (Table 4; GenBank Accession numbers KC527324–KC537330). Metrics of genetic diversity by locus and population are in Tables 1 (COI) and 3 (ND1) for *V. ellipsiformis* and Tables 2 (COI) and 4 (ND1) for *V. pleasii.*

Phylogenetic analysis of the COI (Fig. 2) and ND1 loci (not shown) clearly distinguish *Venustaconcha ellipsiformis* and *V. pleasii* as distinct, monophyletic clades with high bootstrap support and Bayesian posterior probabilities confirming the status as two separate species. Average pairwise genetic distance between *V. ellipsiformis* and *V. pleasii* COI haplotypes was 4.31% (± 0.04% *SE*) and between ND1 haplotypes was 5.38% (± 0.08% *SE*). Haplotype networks were confirmed by the findings of the phylogenetic analysis (Fig. 3).

Venustaconcha ellipsiformis and *V. pleasii* formed separate networks of closely related haplotypes. *Venustaconcha ellipsiformis* populations had on average 1.8 haplotypes per population for COI and 1.9 haplotypes per population for ND1. Populations were mostly dominated by a single common haplotype (h3 for both COI and ND1, Tables 1 and 3, Fig. 3). These two common haplotypes comprised 85.1% of all COI sequences and 69.6% of all ND1 sequences (Table 1 and 3). These haplotypes were recovered from 92.8% (COI) and 83.3% (ND1) of sampling locations (Table 1 and 3, Fig. 1). The more limited sampling of *V. pleasii*, resulted in one shared haplotype for both COI and ND1 between the two sampling locations, however these haplotypes did not dominate either population (Table 2 and 4, Fig. 1).

Significant population structure was evident using COI and ND1 sequences in *Venustaconcha ellipsiformis.* The AMOVA for COI and ND1 respectively indicated that 7.53% (COI; $\Phi_{ST} = 0.075$, $p = 0.028$) and 6.88% (ND1, $\Phi_{ST} = 0.069$, $p < 0.001$) of the variation resided within *V. ellipsiformis* populations, whereas 92.47% (COI) and 93.12% (ND1) occurred among populations. Based on F_{ST} for COI, significant differences ($p < 0.05$) were only observed between pooled population samples in the Illinois River drainage and the Upper Mississippi River and the Illinois River drainage and the Northern Ozarks (Table 5). A somewhat different pattern among pooled populations in pairwise F_{ST} was apparent for ND1 with significant differences ($p < 0.05$) were observed between the Great Lakes drainage and the Upper Mississippi River and between the Illinois River drainage and the Northern

Table 1. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for COI in 14 populations of *Venustaconcha ellipsiformis*. Sampling locations are: Ferson Creek (FSC), Forked Creek (FKC), Horse Creek (HC), Mackinaw (MKR), and Bray Creek (BRC), Illinois; Sugar Creek (SGC), Indiana; Buffalo Creek (BFC), Iowa; Pine River (Saginaw River drainage; PRS), Looking Glass River (LGR), and Thornapple River (TAR), Michigan; Zumbro River (ZMF) and Salem Creek (SLC), Minnesota; Gasconade River (GAS), Bourbeuse River (BR), Missouri.

Haplotypes and polymorphic nucleotide sites																											Populations													
																											Great Lakes			Northern Ozarks		Upper Mississippi		Illinois River drainage						
																											TAR	PRS	LGR	BAS	GAS	BUF	ZMF	SLC	SGC	MKR	HC	FSC	FKC	BRC
	6	9	11	12	13	28	108	160	169	171	212	213	239	246	261	299	331	421	458	465	543	570					*n*=6	*n*=6	*n*=4	*n*=6	*n*=6	*n*=2	*n*=6	*n*=10	*n*=6	*n*=20	*n*=10	*n*=10	*n*=4	*n*=5
1	C	G	T	G	T	T	T	T	T	G	G	C	G	G	A	T	T	T	T	A	A	C					-	-	-	-	-	2	-	-	-	-	-	-	-	-
2	*	*	*	*	*	*	*	*	*	A	*	*	*	*	G	*	*	*	*	*	G	T					-	-	-	2	-	-	-	-	-	-	-	-	-	-
3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*					5	6	3	4	4	-	5	8	6	18	8	10	4	5
4	*	*	*	*	G	*	*	A	A	*	T	*	T	*	*	C	A	A	*	*	G	*					-	-	-	-	1	-	-	-	-	-	-	-	-	-
5	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	G	*					-	-	-	-	1	-	-	-	-	-	-	-	-	-
6	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*					-	-	-	-	-	-	-	-	-	-	2	-	-	-
7	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	C	*	G	*					-	-	1	-	-	-	-	-	-	-	-	-	-	-
8	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	G	*					-	-	-	-	-	-	-	-	-	1	-	-	-	-
9	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*					-	-	-	-	-	-	-	-	-	1	-	-	-	-
10	*	T	G	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*					-	-	-	-	-	-	-	1	-	-	-	-	-	-
11	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	G	*					-	-	-	-	-	-	-	1	-	-	-	-	-	-
12	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	G	*					1	-	-	-	-	-	-	-	-	-	-	-	-	-
13	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*					-	-	-	-	-	-	1	-	-	-	-	-	-	-
																																								-
Number of haplotypes:																											2	1	2	2	3	1	2	3	1	3	2	1	1	1
Number of polymorphic sites:																											1	0	2	3	9	0	1	4	0	2	1	0	0	0
Nucleotide diversity (π) per population:																											0.0006	0	0.0018	0.0035	0.0053	0	0.0006	0.0004	0	0.0004	0.0006	0	0	0
± Standard deviation:																											0.0008		0.0017	0.0029	0.0037		0.0008	0.0005		0.0005	0.0007			

Table 2. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for COI in 2 populations of *Venustaconcha pleasii*. Sampling locations are: Flat Creek (FLC) and James River (JR), Missouri.

Haplotypes and polymorphic nucleotide sites									Populations	
									F	
			1	2	2	3	4	5	L	J
	3	3	1	2	6	0	9	5	C	R
	3	9	4	6	1	0	6	9	$n = 4$	$n = 8$
1	T	A	G	G	G	A	T	T	1	-
2	*	*	*	*	*	G	*	*	2	2
3	C	*	*	*	*	G	C	*	1	-
4	*	*	*	*	A	G	*	*	-	3
5	*	G	*	*	*	G	*	*	-	1
6	*	*	*	A	*	G	C	*	-	1
7	*	*	A	*	*	G	*	C	-	1
Number of haplotypes:									3	5
Number of polymorphic sites:									3	6
Nucleotide diversity (π) per population:									0.0026	0.0031
± Standard deviation:									0.0024	0.0023

Ozarks drainages (Table 5). For both loci, *V. pleasii* was strongly divergent from all *V. ellipsiformis* populations (Table 5).

Evidence of effects of glaciation on genetic diversity was observed. AMOVA recovered significant F_{ST} values between glaciated and unglaciated regions, however F_{ST} values were in the low to moderate range (Wright 1978; Table 6). Only *Venustaconcha ellipsiformis* ND1 nucleotide diversity showed a significant correlation with latitude ($R^2 = 0.405$, $p = 0.014$).

Discussion

Within-population structure

Most of the genetic variation in *Venustaconcha ellipsiformis* and *V. pleasii*, is found within populations. Average haplotype diversity of these species is but similar to sympatrically occurring unionids found in small to medium rivers (*e.g.*, *Epioblasma triquetra* (Rafinesque, 1820) (Zanatta and Murphy 2008) and *Elliptio dilatata* (Rafinesque 1820) (Elderkin *et al.* 2008)).

The pattern of population genetic variation found in the Ellipse and Bleeding Tooth mussels is largely congruent with sympatrically occurring unionids and fishes. The Pleistocene glaciations had major impacts on the genetic structure of organisms in North America (Soltis *et al.* 2006), including unionids and their hosts (*e.g.*, Elderkin *et al.* 2007, 2008, Zanatta and Murphy 2007, 2008). During the repeated advances of Pleistocene glaciers, aquatic species were forced to retreat into one or more glacial refugia causing the loss of within-population diversity due to genetic drift and possible bottlenecks (Hewitt 1996). As the glaciers receded, fishes and their associated unionid parasites dispersed from these refugia and recolonized suitable habitats (Mayden 1988, Mandrak and Crossman 1992, Graf 2002). The newly founded populations tended to have reduced genetic diversity as they were most probably colonized by a small number of individuals (Hewitt 1996). *Venustaconcha ellipsiformis* and *V. pleasii* follow the expected pattern with populations in unglaciated regions showing more genetic diversity than more northerly regions unaffected by the Pleistocene glaciers. A strong pattern of fewer haplotypes per population, lower within-population nucleotide diversity, and fewer polymorphic sites per population as latitude increases was observed in the analyses of *V. ellipsiformis* and *V. pleasii* populations. Furthermore, *V. ellipsiformis* populations in formerly glaciated habitats showed low to moderate, but statistically significant genetic divergence from populations in unglaciated regions. As expected, populations of *V. ellipsiformis* in recently glaciated Great Lakes and Illinois River drainages show markedly lower genetic diversity than *V. ellipsiformis* and *V. pleasii* in unglaciated Ozark highlands. This indicates that repeated glaciations in the modern range of *V. ellipsiformis* may have resulted in a genetic bottleneck, whereas *V. pleasii* was not bottlenecked as populations were not directly affected by glaciations. Furthermore, the more northerly populations in recently glaciated watersheds may have further reduced genetic diversity because of a founder effect following the most recent glacial retreat.

Among-population structure

Phylogenetic analyses gave strong support for *Venustaconcha ellipsiformis* and *V. pleasii* as distinct sibling species. Pairwise genetic distance between these species is similar to values observed among species in the genus *Epioblasma* (Rafinesque 1831; Jones *et al.* 2006). Genetic data are further supported by the range disjunctions between the two species, differential host usage (Riusech and Barnhart 2000), and differing shell morphologies (Oesch 1995). The existence of distinct clades for species and species complexes in the various drainages of the central highlands (Ozark and Ouachita mountains) has been shown for fish species (Near *et al.* 2001, Berendzen *et al.* 2010) including confirmed hosts for *V. ellipsiformis* and *V. pleasii* (Ray *et al.* 2006). Based on the phylogeographic pattern revealed in other unionids in central North America (Grobler *et al.* 2006, Elderkin *et al.* 2007, Elderkin *et al.* 2008), additional specimens from the *Venustaconcha* Thiele, 1934 species

Table 3. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for ND1 in 12 populations of *Venustaconcha ellipsiformis*. Sampling locations are as in Table 1.

Haplotypes and polymorphic nucleotide sites																			Populations									
																			Great Lakes			Northern Ozarks		Upper Mississippi		Illinois River drainage			
	3 3	5 6	5 7	8 1	1 6 0	2 0 5	2 0 7	2 4 7	2 5 2	3 1 8	4 1 0	4 7 2	4 8 0	4 8 3	4 9 4	5 1 6	5 2 5	5 3 0	T A R *n*=6	P R S *n*=4	L G R *n*=2	B R *n*=6	G A S *n*=7	B U F *n*=2	Z M F *n*=3	S G C *n*=6	M K R *n*=6	H C *n*=5	F S C *n*=5	F K C *n*=4
1	G	G	T	G	G	T	T	T	A	G	A	T	G	G	A	G	G	A	-	-	-	3	-	-	-	-	-	-	-	-
2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	3	-	-	-	-	-	-	-	-
3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	6	4	2	-	5	2	-	6	4	4	2	4
4	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	G	-	-	-	-	-	-	-	-	-	-	1	-
5	T	*	*	*	*	*	*	*	*	*	T	*	A	*	*	*	C	G	-	-	-	-	-	-	-	-	-	-	1	-
6	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	T	G	-	-	-	-	-	-	-	-	1	-	1	-
7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	-	1	-	-	-	-	-	-	-
8	*	T	A	*	T	A	A	A	T	A	*	A	*	*	*	*	*	G	-	-	-	-	1	-	-	-	-	-	-	-
9	*	*	*	A	*	*	*	*	*	*	*	*	*	A	*	*	*	G	-	-	-	-	-	-	-	-	-	1	-	-
10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	G	-	-	-	-	-	-	-	-	1	-	-	-
11	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	-	-	-	-	-	-	1	-	-	-	-	-
12	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	-	-	-	-	-	-	1	-	-	-	-	-
13	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	G	-	-	-	-	-	-	1	-	-	-	-	-
Number of haplotypes:																			1	1	1	2	3	1	3	1	3	2	4	1
Number of polymorphic sites:																			0	0	0	1	9	0	3	0	2	2	4	0
Nucleotide diversity (π) per population:																			0	0	0	0.0033	0.0063	0	0.0049	0	0.0012	0.0007	0.0031	0
± Standard deviation:																						0.0025	0.0042		0.0044		0.0012	0.0009	0.0025	

Table 4. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indicies of population diversity for ND1 in 2 populations of *Venustaconcha pleasii*. Sampling locations are as in Table 1.

Haplotypes and polymorphic nucleotide sites															Populations	
															F	
		1	1	1	1	2	3	3	3	3	3	4	5	5	L	J
	6	2	4	4	4	8	0	4	5	6	8	9	1	3	C	R
	9	5	0	4	9	1	5	1	9	8	9	3	5	6	$n = 5$	$n = 4$
1	C	T	T	T	T	T	T	T	T	C	A	G	A	C	1	1
2	*	C	*	C	*	*	*	*	*	*	*	*	*	*	1	-
3	T	C	*	C	*	C	*	C	*	T	G	*	G	*	2	-
4	T	C	*	C	*	C	C	C	*	*	G	*	G	*	1	-
5	T	C	C	C	*	C	*	C	*	T	G	*	G	A	-	1
6	T	C	C	C	*	C	*	C	*	T	G	*	G	A	-	1
7	T	C	*	C	C	C	*	C	C	T	G	T	G	*	-	1
Number of haplotypes:															4	4
Number of polymorphic sites:															9	14
Nucleotide diversity (π) per population:															0.0088	0.0135
± Standard deviation:															0.0060	0.0095

complex should be considered in a phylogeographic context. These include probable *V. pleasii* from the Neosho/Arkansas River drainage and newly revealed putative members of the *Venustaconcha* clade: *Villosa constricta* (Conrad, 1838; mid-Atlantic drainages), *Villosa perpurpurea* (Lea, 1861; Upper Tennessee River drainages), and *Villosa trabalis* (Conrad, 1834; Upper Tennessee and Cumberland River drainages) (Kuehnl 2009).

Significant regional genetic structure was found among *Venustaconcha ellipsformis* populations. The differences between the analyses of the COI and ND1 datasets likely resulted from limited sample sizes in the ND1 dataset. Low to moderate (and statistically significant) levels of genetic divergence were found among populations in the Great Lakes/Illinois River (glaciated) and the northern Ozarks and the Upper Mississippi populations (non-glaciated). Low levels of divergence were also found between the Upper Mississippi and the Northern Ozarks (significant only using ND1); all non-glaciated regions. The divergence found between the unglaciated Upper Mississippi and Northern Ozarks populations of *V. ellipsiformis* is congruent with a hypothesized northern glacial refuge in the "Driftless Area" of the upper Midwest (Rowe *et al.* 2004). Furthermore, as in Rowe *et al.* (2004), our data largely fail to meet expectations for a northward expansion out of a southern refugium, with genetic diversity showing little to no gradient from south to north. Divergence was not significant between the Great Lakes and the Illinois River; all in formerly glaciated regions, giving further support for these populations arising from a single glacial refuge. However, limited evidence of a south-to-north pattern of decreasing genetic diversity moving out of glacial refuges is consistent with multiple glacial refugia and rapid colonization of newly deglaciated waterways. The low to moderate levels of genetic divergence are also expected given the limited time for isolation and genetic drift to have an effect on structure (Freeland 2005). Furthermore, our results are consistent with the pattern of post-glacial colonization into the central Great Lakes region hypothesized for *V. ellipsiformis* by van der Schalie and van der Schalie (1963).

The results of this study are broadly congruent with other recent phylogeographic studies on unionids. The patterns of genetic structure observed in *Elliptio dilatata* (Elderkin *et al.* 2008) and *Epioblasma triquetra* (Zanatta and Murphy 2008) are the best for direct comparison to that of *Venustaconcha ellipsiformis* because these species share similar small-medium river habitats and have somewhat overlapping distributions. For *E. dilatata*, low to moderate levels of differentiation were found among populations in previously glaciated regions (Great Lakes and Ohio basins), but unglaciated regions were highly divergent from populations in the central highlands in the White River and Ouachita River drainages (Elderkin *et al.* 2008). Similarly in *E. triquetra*, using COI sequence data, high levels of divergence were found between populations from the southern slope of the Ozarks (St. Francis River) and previously glaciated regions, but low levels among the northern slope of the Ozarks (Bourbeuse River) and previously glaciated regions (Zanatta and Murphy 2008). The patterns observed for *V. ellipsiformis* and *V. pleasii* are somewhat puzzling in that they are not known from the Ohio River drainage (Watters *et al.* 2009). Furthermore, specimens from the Illinois River and Lake Michigan drainages were not considered in studies on *E. dilatata* or *E. triquetra*.

The relatively low levels of genetic differentiation observed among Ellipse populations can be linked to the phylogeography and population structure of sympatrically occurring fish species – some of which are probable glochidial hosts. The phylogeographic pattern of a parasitic organism and its host should be broadly congruent and has been shown to be so in unionids (Zanatta and Wilson 2011). Several darter species of *Etheostoma* and *Percina* and sculpins (*Cottus*) are confirmed hosts for the Ellipse and Bleeding Tooth (Ruisech and Barnhart 2000, Allen *et al.* 2007). The distribution of the rainbow darter, *Etheostoma caeruleum* (Storer 1835), a confirmed host for both Ellipse and Bleeding Tooth (Ruisech and Barnhart 2000, Allen *et al.* 2007), is much broader that of the Ellipse and Bleeding Tooth, but the patterns of phylogeography and population structure

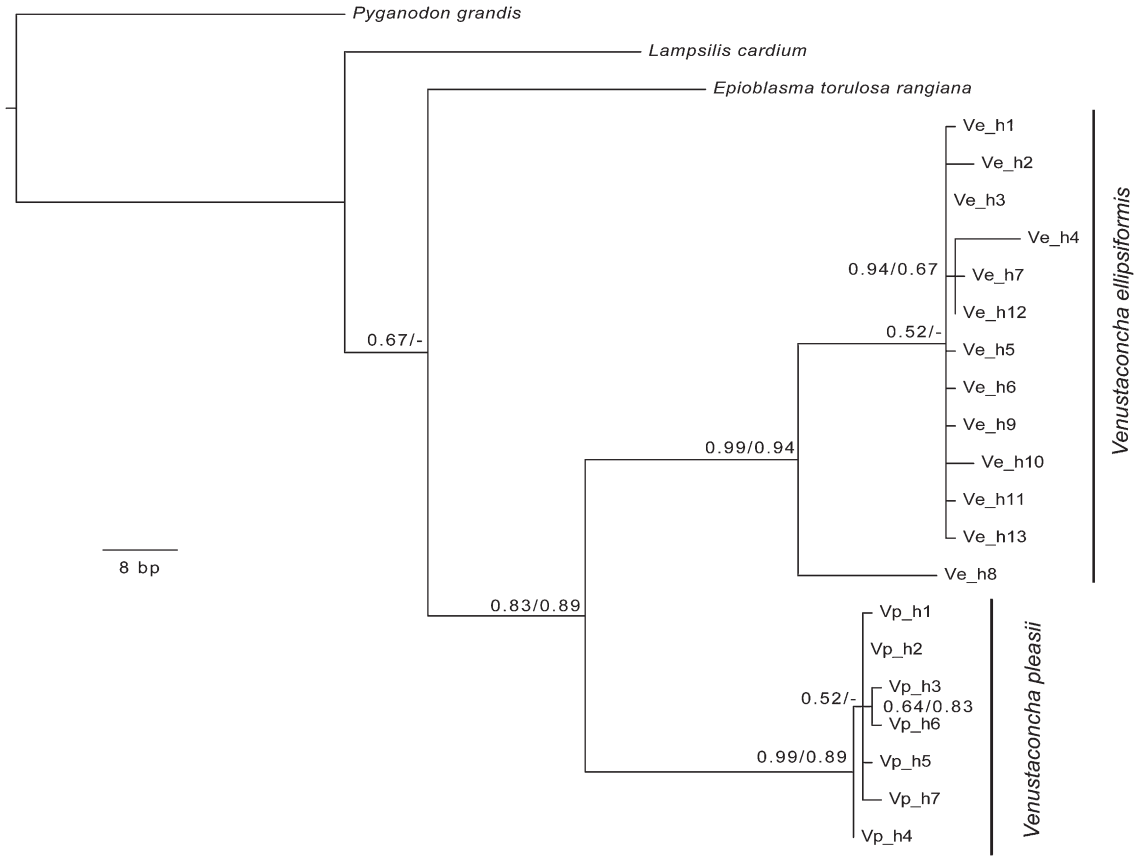

Figure 2. Phylogram of *Venustaconcha ellipsiformis* (Ve) and *V. pleasii* (Vp) from 16 populations in the United States Midwest. *Epioblasma torulosa rangiana*, *Lampsilis cardium*, and *Pyganodon grandis* were used as outgroups and the tree was rooted with *Pyganodon grandis*. The numbers above the branches and before the slash indicate the proportion of bootstrap replicates where the clade was found under a maximum parsimony framework. The numbers after the slash are the calculated posterior probabilities (greater than 50%), indicating the proportion of trees that these nodes appeared in the Bayesian topology.

of the rainbow darter in the Ozark highlands and Upper Mississippi River appears to closely match that of the Ellipse and Bleeding Tooth (Ray *et al.* 2006). The congruency of pattern breaks down in the Illinois River and Great Lakes drainages, as rainbow darters in this region belong to a clade that appears to have invaded the region via the Ohio and Wabash River systems, where the Ellipse and Bleeding Tooth are not present. Two other sympatrically occurring species with Ellipse and Bleeding Tooth, the gilt darter, *Percina evides* (Jordan and Copeland 1877), and Ozark minnow, *Notropis nubilus* (Forbes 1878), also show a similar phylogeographic patterns between the Ozarks and Upper Mississippi systems (Near *et al.* 2001, Berendzen *et al.* 2010), but neither of these species have distributions that extend into the Illinois River or Great Lakes drainages. Each of these fish species showed low to moderate levels of genetic differentiation among populations in northern Ozark streams (*i.e.*, Gasconade, Osage, Meramec river systems) and the Upper Mississippi drainage, similar to the patterns observed for the Ellipse mussel.

Genotyping of microsatellite DNA markers have proven useful in determining fine-scale genetic structure and recent differentiation among unionid populations (*e.g.*, Kelly and Rhymer 2005, Jones *et al.* 2006, Zanatta *et al.* 2007), typically revealing more resolution than mtDNA sequence data. Additional study of these Ellipse and Bleeding Tooth populations using microsatellite markers is recommended. Amplifying and optimizing published microsatellite loci for other lampsiline mussels has been attempted for *Venustaconcha ellipsiformis* (Eackles and King 2002, Jones *et al.* 2004, Zanatta and Murphy 2006), but only two of these loci (LabD111 and LabC23; Eackles and King 2002) consistently amplified and were polymorphic in populations from Michigan (Zanatta, unpublished data). Additional

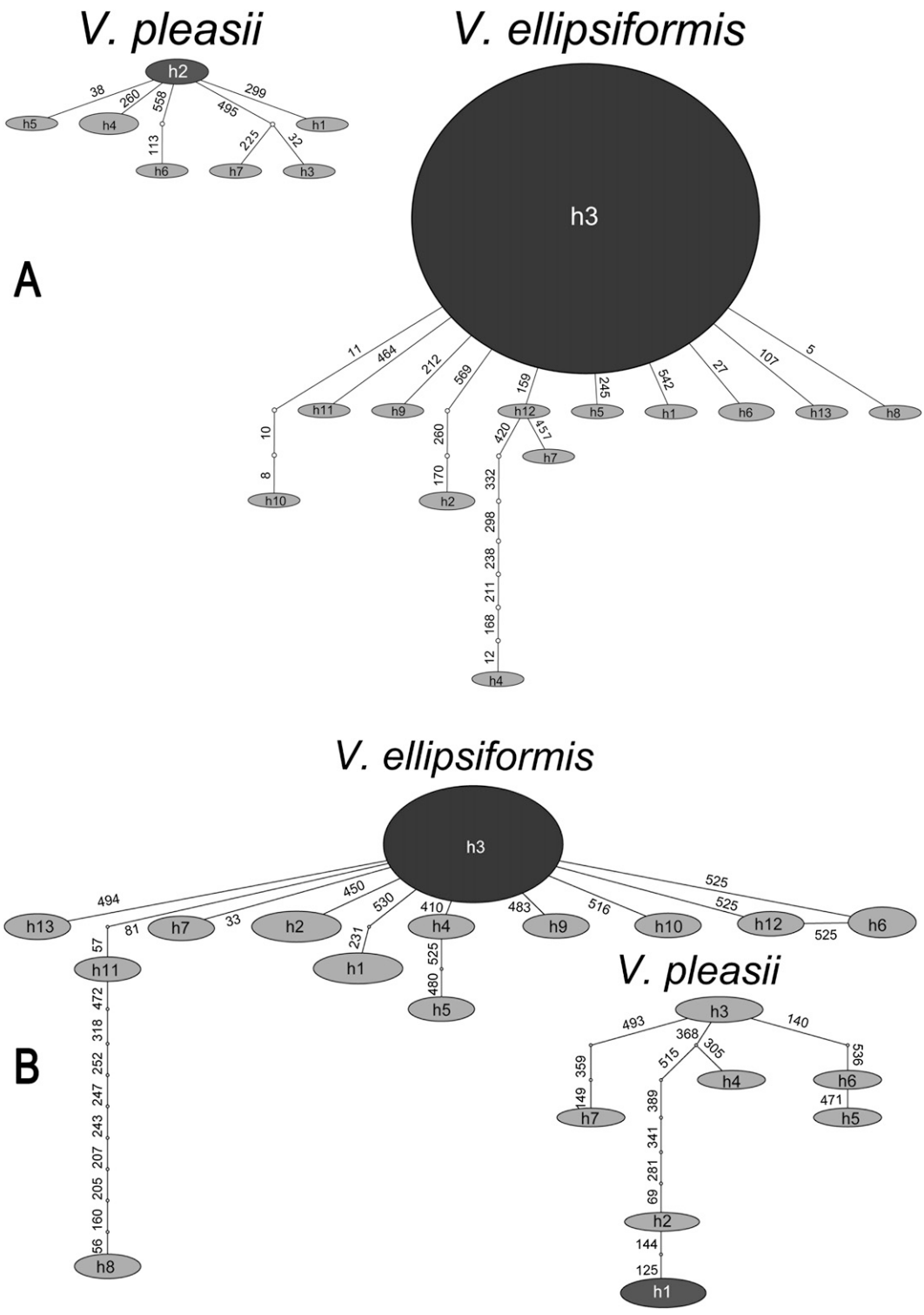

Figure 3. Spanning networks of mtDNA haplotypes at the (**A**) COI and (**B**) ND1 locus for *Venustaconcha ellipsiformis* and *V. pleasii*. Connecting lines represent a single base pair difference between two haplotypes and numbers between each node represents location of the base pair change. Sizes of the circles correspond to the frequency of haplotypes encountered, and small nodes represent intermediate haplotypes not encountered in sampling. Dark grey circles represent common shared haplotypes.

Table 5. Pairwise F_{ST} values among four pooled populations of *Venustaconcha ellipsiformis* and *V. pleasii* from ND1 (below diagonal) and COI (above diagonal). Values in bold are statistically significant ($\alpha = 0.05$).

	Great Lakes drainage	Northern Ozarks drainages	Upper Mississippi drainage	Illinois River drainage	*V. pleasii* White R. drainage
Great Lakes drainage	-	0.043 ($p = 0.113$)	0.023 ($p = 0.172$)	0.068 ($p = 0.068$)	**0.959 ($p < 0.001$)**
Northern Ozarks drainages	0.063 ($p = 0.053$)	-	**0.051 ($p = 0.031$)**	**0.187 ($p = 0.002$)**	**0.912 ($p < 0.001$)**
Upper Mississippi drainage	**0.189 ($p = 0.015$)**	-0.014 ($p = 0.490$)	-	**0.061 ($p = 0.006$)**	**0.950 ($p < 0.001$)**
Illinois River drainage	-0.011 ($p = 0.663$)	**0.111 ($p < 0.001$)**	0.087 ($p = 0.124$)	-	**0.981 ($p < 0.001$)**
V. pleasii White R. drainage	**0.910 ($p < 0.001$)**	**0.851 ($p < 0.001$)**	**0.846 ($p < 0.001$)**	**0.930 ($p < 0.001$)**	-

markers specifically designed for *Venustaconcha* will need development before future fine-scale genetic analyses can be conducted.

Conservation implications and summary

This study confirms *Venustaconcha ellipsiformis* and *V. pleasii* are distinct species. Low to moderate levels of genetic structure was found among populations in Upper Mississippi and across the Chicago outlet between Great Lakes and Illinois River. In the interest of conservation and recovery of populations, *V. ellipsiformis* populations appear to be generally compatible within recently glaciated regions. Based on the evidence presented herein and using a cautionary approach, there is partial support (mostly from COI dataset) for three management units (Moritz 1994, Fraser and Bernatchez 2001) in *V. ellipsiformis*: (1) northern Ozarks (2) Upper Mississippi (3) Illinois River and Great Lakes. Following previously published guidelines, these management units should be taken into consideration when planning future conservation, propagation and population augmentation programs for *V. ellipsiformis* (Neves 2004, Jones *et al.* 2006). It is recommended that additional sampling and fine-scale population genetic analysis be conducted with additional individuals from the southern part of the range of *V. ellipsiformis*, including the upper Arkansas River drainage. The findings of this study should be further tested at a finer scale with microsatellite DNA markers.

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Table 6. Pairwise F_{ST} values among four pooled populations of *Venustaconcha ellipsiformis* and *V. pleasii* from ND1 (below diagonal) and COI (above diagonal) in glaciated and unglaciated regions. Values in bold are statistically significant ($\alpha = 0.05$).

	Glaciated Sites: Great Lakes + Illinois R. drainages	Unglaciated Sites: Northern Ozarks + Upper Mississippi R. drainages	*V. pleasii* White R. drainage
Glaciated Sites: Great Lakes + Illinois R. drainages	-	**0.035 ($p = 0.002$)**	**0.982 ($p < 0.001$)**
Unglaciated Sites: Northern Ozarks + Upper Mississippi R. drainages	**0.086 ($p < 0.001$)**	-	**0.934 ($p < 0.001$)**
V. pleasii White R. drainage	**0.947 ($p < 0.001$)**	**0.867 ($p < 0.001$)**	-

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