Phylogeography of the freshwater mussel species Lasmigona costata: testing post-glacial colonization hypotheses

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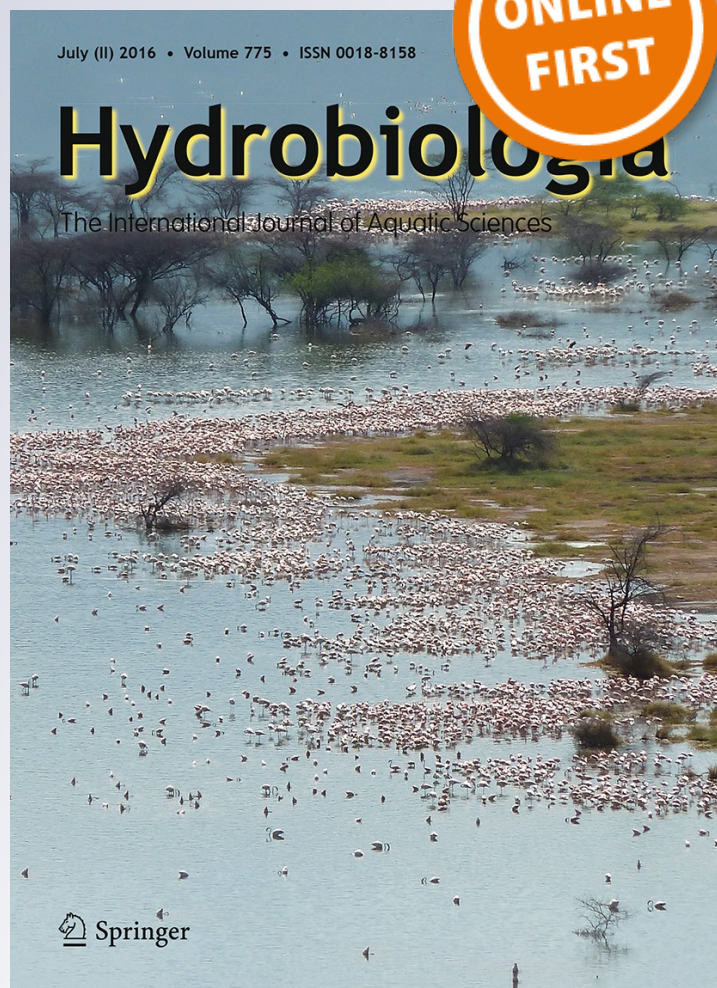Daelyn A. Woolnough · David T. Zanatta

Abstract Understanding genetic diversity across large spatial scales helps to reveal patterns of population structure. Mitochondrial DNA sequences and microsatellite loci were used to analyze the phylogeography of a common unionid species (*Lasmigona costata*) from the Laurentian Great Lakes and historically connected river drainages. Phylogeographic patterns were assessed to determine colonization routes into the Great Lakes following glacial recession. A suite of seven microsatellite loci were genotyped and a fragment of the mitochondrial gene COI was sequenced. Multiple analyses using microsatellite allele frequencies suggest at least two distinct genetic populations for *L. costata*. A total of seven hypothesized post-glacial dispersal scenarios were compared using isolation by distance to test the various dispersal models. Evidence was strongest for two post-glacial dispersal routes into the Great Lakes: one utilizing a connection between the Wabash and Maumee River watersheds, and one utilizing a connection between the Wisconsin River and Green Bay watersheds. A highly differentiated and monophyletic population of *L. costata* was identified in the Ozark Highlands, which may constitute a unique taxonomic entity.

Keywords Freshwater mussels · mtDNA · Microsatellites · Glacial refugia · Population genetics

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Introduction

Gaining an understanding of phylogeography and population structure is important for the conservation of imperiled species. Conservation and restoration projects involving relocation and captive propagation should be limited to areas with similar genetic profiles when possible, and priority for conservation should be assigned to populations and geographic regions with higher and/or unique genetic diversity (Jones et al., 2006; Fraser, 2008). Recent glaciation events have

influenced the current distribution patterns and population genetic structure of North American biodiversity (Pielou, 1991; Hewitt, 1996; Soltis et al., 2006). Phylogeographic studies on aquatic organisms have led to increased understanding of shifting drainage patterns following the last Pleistocene glacial recession (e.g., Roe et al., 2001; Soltis et al., 2006; Inoue et al., 2013; Zanatta & Harris, 2013). Using the population, genetic structure of common and widespread species assists in the understanding of how drainage patterns have changed over time, which will help make inferences regarding the population genetic structure of rare species (Berg et al., 1998; Galbraith et al., 2015).

The Pleistocene epoch lasted from approximately 2.5 million years ago to 10,000 years ago (Pielou, 1991). Throughout this period, ice sheets advanced and retreated across much of northern North America with the Laurentide ice sheet covering much of eastern and central North America and the Cordilleran ice sheet in the west (Pielou, 1991). The scouring force of the Laurentide ice sheet was responsible for creating the Great Lakes basins. Glaciers covered the Laurentian Great Lakes region at least six times in the last million years (Larson & Schaetzl, 2001). After the final glacial recession, approximately 10,000 years ago, glacial meltwater filled these basins producing large proglacial lakes. Isostatic rebound, channel erosion, and shifting lake outlets caused dramatic variations in lake size, water levels, and watershed configuration before reaching their current state (Pielou, 1991; Larson & Schaetzl, 2001; Calkin & Feenstra, 1985; Hansel et al., 1985).

The Great Lakes and St. Lawrence River watersheds currently support 47 species of freshwater mussel and the genetic structure among populations should largely be influenced by historic range expansion and contraction (Hewitt, 1996, 2000; Excoffier, 2004; Haag, 2010). During peak glaciations, the ranges of aquatic organisms were reduced to smaller refugia (Soltis et al., 2006). In North America, river systems in the Ozarks Highlands of southeastern Missouri and northern Arkansas and the Ohio, Tennessee, and Cumberland river systems have been identified as potential refugia for mussels in the glaciated portions of the Mississippi River watershed, as well as the Great Lakes region (Johnson, 1978; Zanatta & Murphy, 2008). Multiple dispersal routes for colonization of the Great Lakes

have been suggested (van der Schalie, 1945; Mandrak & Crossman, 1992; Graf, 2002; Elderkin et al., 2007), however, understanding these pathways and the genetic consequences that have resulted from post-Pleistocene colonization often requires species-specific data. The genetic diversity of mussel populations found in previously glaciated areas is typically lower than genetic diversity in non-glaciated regions, presumably due to founder effects (Hewitt, 1996; Zanatta & Murphy, 2007, 2008; Inoue et al., 2013; Zanatta & Harris, 2013; Scott et al., 2014). However, if a recently exposed watershed is colonized via dispersal routes from multiple refugia, genetic diversity may be higher due to the mixing of divergent lineages.

Lasmigona costata (Rafinesque, 1820), fluted-shell, is a relatively widespread and common mussel found throughout the Mississippi and Ohio rivers extending into the Great Lakes watershed (draining to the Atlantic Ocean) and the Red River of the North that drains into the Arctic Ocean (Watters et al., 2009; Haag, 2010). Unionid populations in the Great Lakes region have declined in large part due to the introduction of dreissenid mussels [*Dreissena polymorpha* (Pallas, 1771) and *Dreissena rostriformis bugensis* (Andrusov, 1897)] beginning in the late 1980s (Ricciardi & MacIsaac, 2000; Carlton, 2008). *Lasmigona costata* is so named due to the ridges found along the posterior of its dorsal margin. This fast-growing unionid belongs to the tribe Anodontini and can attain a large size, with a maximum length of 150 mm, but is relatively thin shelled and laterally compressed. *Lasmigona costata* is known to have over 25 potential host fish species from multiple different families, making this species a host-fish generalist (Watters et al., 2009; Cummings & Watters, 2010; Haag, 2012). The large range and relatively high abundance make *L. costata* an ideal species for understanding freshwater mussel genetic structure. Captive propagation programs are an integral part of freshwater mussel recovery plans, highlighting the need for large-scale assessment of freshwater mussel genetic structure (Haag & Williams, 2014).

Using microsatellite genotypes and mitochondrial sequence data, this study analyzes the phylogeography of *L. costata* to determine genetic structure and test hypotheses of post-glacial colonization into the Great Lakes.

Methods

Sampling locations, species, and protocol

Lasmigona costata specimens were collected from 14 sites across eight major regions (Fig. 1): Lake Erie tributaries (MI) [Black R. (BL) and Belle R. (BEL)], Maumee River drainage [Auglaize R. (AU) and St. Joe R. (EFWB)], Wabash River drainage [White R. (WH) and Eel R. (EEL)], Lake Michigan and Lake Huron tributaries (MI) [Grand R. (GR) and Saginaw R. (SA)], Illinois River drainage [Kankakee River (IL)], Green Bay drainage [(Red R. (RE) and Wolf R. (WO)], Wisconsin River drainage [Upper Wisconsin R. (UW)], and the Ozark highlands [Bourbeuse R. (BO) and Gasconade R. (GA)]. These drainages were chosen specifically to test three hypothesized post-glacial colonization routes: (1) between the Maumee and Wabash river watersheds (Hypothesis A), (2) between the Illinois river and Lake Michigan watersheds (Hypothesis B), and (3) between the Wisconsin River and Green Bay watersheds (Hypothesis C). Mussels were sampled snorkeling or using tactile search methods for at least 2.5 person hours. Each individual was measured for length (mm) using calipers and age was estimated using length-at-age parameters ($K = 0.25$, $L_{inf} = 133.9$, $t_0 = 0.049$) found in Haag & Rypel (2011).

DNA extraction

A non-lethal biopsy technique developed by Berg et al. (1995) was performed to obtain a small (~ 1 cm^2) sample of mantle tissue. Biopsied tissue samples were then stored in 95% ethanol until they could be processed in the lab. Total genomic DNA was extracted from tissue samples using an alcohol extraction method similar to Sambrook et al. (1989).
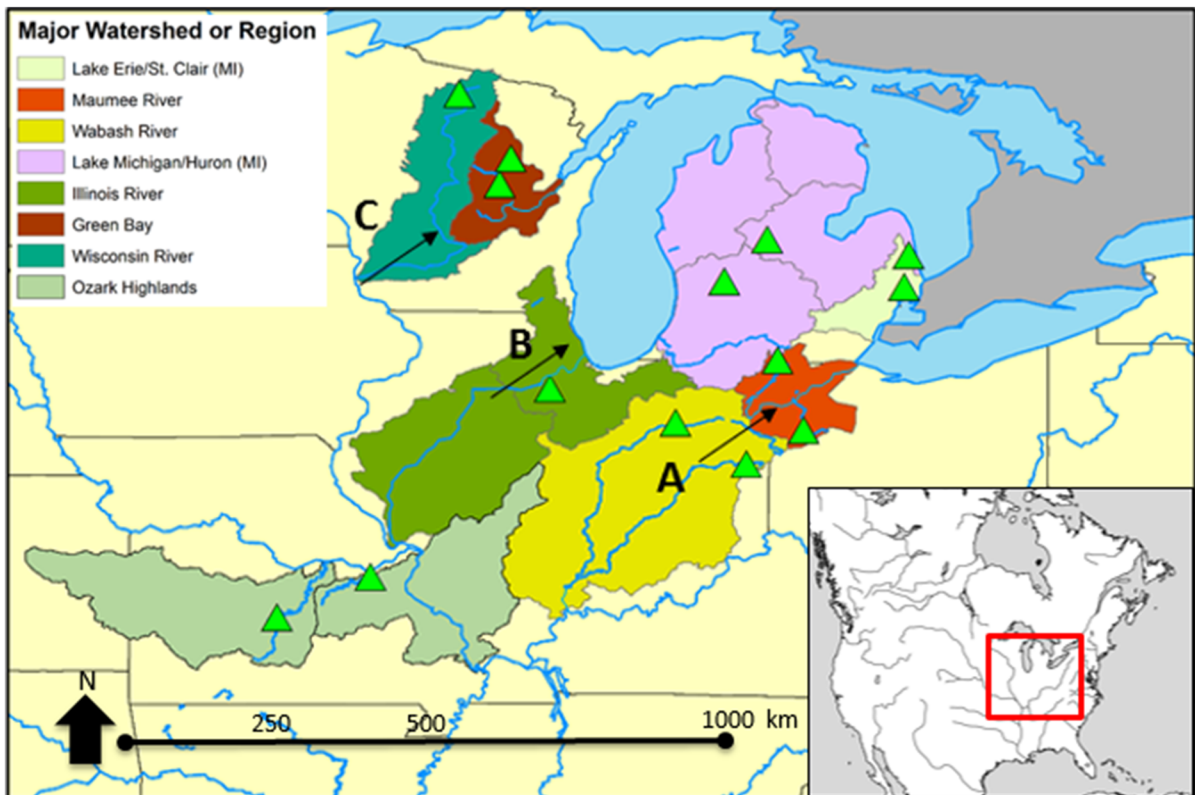
Fig. 1 Collection locations for unionids (green triangles) and major watershed or region denoted by color. Approximate location of the hypothesized connection between the Wabash and Maumee River watersheds (*A*), approximate location of the hypothesized connection between the Illinois River watershed and Lake Michigan (*B*), and approximate location of the hypothesized connection between the Wisconsin River and the Green Bay watersheds (*C*). *Inset* is a map of North America showing the location of the study area

Tissue samples were placed in a 1.5-ml tube along with 250 μl of 1 × lysis buffer and 15 μl of proteinase K (20 mg/ml). The samples were incubated for at least 16 h at 37°C. Following incubation, 500 μl of 80% isopropanol and 10 μl of 5 M NaCl were added and each sample was centrifuged for 45 min at 13,300 rpm. The supernatant was discarded and a second alcohol wash was performed with 1,000 μl of 70% ethanol, centrifuging for 45 min, to further purify the genomic DNA. The supernatant was discarded and the DNA pellet was re-suspended in 150 μl ddH$_2$0.

Microsatellites

Seven microsatellite loci were amplified for *L. costata* (LcoD10, LcoD48, LcoD50, LcoB114, LcoC75, LcoD162, and LcoD158; Galbraith et al., 2011) with reagent concentrations as in Galbraith et al. (2015). Amplifications were run with the following conditions: 94°C for 10 min followed by 40 cycles of 94°C for 45 s, annealing temperature (48°C for C75; 50°C for LcoD162; 52°C for LcoD10, LcoD50, LcoB114, and LcoD158; and 52.8°C for LcoD48) for 1 min, and 72°C for 1 min. The samples were held at 72°C for 20 min for a final extension.

Microsatellite PCR fragments were stained with SYBR Green and visualized to verify PCR success and fragment length using gel electrophoresis with a 1.5% agarose gel. Using an Applied Biosystems (ABI) 3730 DNA analyzer, all microsatellite loci were genotyped and alleles were scored using algorithms in GENE-MARKER software (2010 SoftGenetics LLC, Pennsylvania) and subsequently proofread and confirmed by eye.

Each microsatellite locus was assessed for likelihood of null alleles using the method developed by Brookfield (1996) and executed in MICRO-CHECKER v 2.2.3 (Oosterhout et al., 2004). The number of alleles, the number of private alleles, observed and expected heterozygosity, and deviations from Hardy–Weinberg equilibrium were calculated for each locus-population combination using GENA-LEX v.6.5 (Peakall & Smouse, 2012) and mean allelic richness was calculated using FSTAT v. 2.9.3.2 (Goudet, 1995). One locus (LcoD48) was out of HWE at 4 sampling locations. Analyses were run both with and without this locus. Linkage disequilibrium was calculated using GENEPOP v.4.2 (Raymond & Rousset, 1995). Linkage disequilibrium was analyzed using the log-likelihood ratio statistic with a dememorization number of 1,000 with 100 batches and 1,000 iterations per batch.

Microsatellite genotype data were compared at multiple loci using a Bayesian clustering analysis, implemented in STRUCTURE v.2.3.3 (Pritchard et al., 2000). This analysis assessed the most likely number of populations (K) ranging from 1 to 15 (i.e., for the 14 sampling locations) and using 200,000 burn-in steps and an additional 200,000 Markov chain Monte Carlo (MCMC) repeats. This analysis assumed no a priori sampling location information, allowed for admixture, and was iterated 7 times for each assumed K. STRUCTURE HARVESTER (Earl & vonHoldt, 2012) was used to group individuals into the most likely number of populations (*K*) using the Evanno et al. (2005) ΔK method.

Analysis of Molecular Variance (AMOVA) was implemented using GENALEX to test the significance of genetic structure among sampling locations. Differentiation among sampling locations was estimated by calculating pairwise F_{ST} with P values obtained using 9,999 permutations of the dataset.

A neighbor-joining (NJ) tree was created with the program TREEFIT (Kalinowski, 2009) using Nei's genetic distance matrices (Nei, 1972). Genetic distances between populations were compared to genetic distances in the tree to calculate an R^2 value, assessing overall fit of the distances to the topology.

Mantel tests were performed using GENALEX to test for isolation by distance (Mantel, 1967). Linearized F_{ST} [$F_{ST}/(1 - F_{ST})$] was used as a measure of genetic distance between sites. Geographic river distances (km) between sites were calculated using Google Earth v. 7.1 (Google Inc., 2009). Straight-line distance was used when measuring across lakes. Mantel tests were conducted under a variety of scenarios utilizing different hypotheses regarding post-glacial colonization routes (Fig. 1 and permutations among these routes; $n = 7$ hypotheses). Each Mantel test was evaluated and compared based on strength of regression and P value to determine which model best explained the data.

Mitochondrial DNA

A fragment of the female lineage mitochondrial gene cytochrome c oxidase subunit I (COI) was amplified for a subset of individuals per site using the COI

primers and polymerase chain reaction (PCR) conditions described in Campbell et al. (2005). To confirm amplifications, mitochondrial PCR fragments were stained with SYBR Green and visualized using gel electrophoresis with a 1.5% agarose gel. After verification of amplification, reactions were purified using Exonuclease I (Amersham Biosciences cat# E70073X, 10 U/ml) and Shrimp Alkaline Phosphatase (Amersham Biosciences cat# E70092X 1 U/ml). A solution was created with 78 µl ddH$_2$O, 2 µl ExoI, and 20 µl SAP and then 1.5 µl of this mixture was added to each PCR product. Reaction products were incubated at 37°C for 40 min then 80°C for 20 min to denature enzymes. The 5′ end of the amplified COI region was cycle sequenced using a 'Big Dye' Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Inc.) with the forward COI primer. The reaction was visualized on an ABI 3100 automated DNA sequencer.

Mitochondrial DNA sequences were proofread using 4PEAKS v.1.7.1 (Griekspoor & Groothuis, 2006) and edited using MESQUITE v.3.0 (Maddison & Maddison, 2008). Sequences were aligned using CLUSTAL W v.2.1 (Larkin et al., 2007). Unique haplotypes were identified for each species using COLLAPSE v.1.2 (Posada, 2004). Missing nucleotides and gaps were defined as missing data. The number of polymorphic sites and nucleotide diversity (π) were calculated using ARLEQUIN v. 2.0 (Schneider et al., 2000). Mean uncorrected (*p*) genetic distances between groups were calculated using MEGA 6 (Tamura et al., 2013). TCS v.1.21 (Clement et al., 2000) was used to create a haplotype network using a 95% connection limit with gaps defined as missing data. Haplotypes were categorized into haplogroups based on haplotype clusters (Elderkin et al., 2008). Loops and reticulations in the haplotype network were resolved using the method described in Fetzner & Crandall (2003).

A phylogenetic analysis using Bayesian inference was performed using MRBAYES v.3.2.2 (Ronquist et al., 2012). The initial model of evolution (HKY + G) was determined by comparing 24 models of evolution in MRMODELTEST v.2.2 (Nylander, 2004). MRBAYES was run using 1,000,000 generations and six concurrent Markov Chains and 2 hot chains sampled at intervals of every 100 generations for a total of 60,000 trees. A 25% burn-in (15,000 trees) was used to ensure stationary of the log-likelihood values. Additional COI sequences were obtained from GenBank for use as outgroups and additional ingroups (Online Resource 1).

Results

A total of 451 *L. costata* were collected at 14 sites from 14 rivers in 8 major watersheds (Table 1). Based on shell lengths ($\bar{x} = 104.5 \pm 16.8$ mm S.E.), all *L. costata* specimens were estimated to be between 2 and 20 years old, with a mean age of 6.8 years.

Microsatellite DNA Genotypes

Genotypes from 444 *L. costata* were obtained, of which 401 individuals amplified at more than 5 loci and were used in all of the analyses (Table 1). MICROCHECKER was implemented to evaluate the probability of null alleles and scoring errors at each locus using the Brookfield (1996) method. All loci had low estimated probabilities ($P < 0.1$) of null alleles. No scoring errors due to stutter or large allele dropout were found at any of the microsatellite loci. The number of alleles, number of private alleles, mean allelic richness, and the observed and expected heterozygosity for each locus-population combination were calculated (Table 2). Deviations from HWE for *L. costata* using a Bonferroni adjusted α of 0.0005 showed that one locus, LcoD48, was out of HWE at 4 sampling locations. All analyses were performed both with and without this locus and results were congruent, therefore LcoD48 was included in the results reported. No significantly linked loci were found at any sampling locations.

Using the data generated from the STRUCTURE analysis, the Evanno et al. (2005) ΔK method indicated the most likely number of populations is 4, with a probability of Ln$P(K) = -13525.1875$ (ΔK = 3.45, Fig. 2). STRUCTURE clustered most individuals from the Wabash and Lake Erie drainages together. The Grand R. and Saginaw R. were also clustered together, as well as another strongly differentiated group comprised of the Ozark individuals. The Wisconsin R., Illinois R., and Green Bay samples showed high admixture between these putative populations.

AMOVA was performed using all sample sites with $n > 10$. The global F_{ST} values for *L. costata* was 0.037

Table 1 Number of *Lasmigona costata* collected at each sampling site with corresponding site codes

Watershed/region	Sites	Site code	Latitude	Longitude	*n*	Number genotyped
Lake Erie/St. Clair (MI)*	Black R.	BL	43°18′55.78″N	82°38′13.22″W	29	25
	Belle R.	BEL	42°52′22.50″N	82°42′30.20″W	31	24
Maumee River	Auglaize R.	AU	40°41′0.00″N	84°16′8.50″W	18	18
	St. Joe R.	EFWB	41°45′9.99″N	84°39′56.90″W	30	29
Wabash River	White R.	WH	40°10′12.18″N	85° 8′57.30″W	30	30
	Eel. R.	EEL	40°47′49.63″N	86°13′53.54″W	30	22
Lake Michigan/Huron (MI)[a]	Grand R.	GR	42°56′39.48″N	85°29′32.49″W	98	79
	Saginaw R.	SA	43°36′3.71″N	84°49′16.86″W	28	15
Illinois River	Kankakee R.	IL	41°19′19.70″N	88° 9′25.39″W	27	27
Green Bay	Red R.	RE	44°50′29.33″N	88°45′36.54″W	30	29
	Wolf R.	WO	44°27′0.00″N	88°55′58.26″W	30	29
Wisconsin River	Upper Wisconsin R.	UW	45°49′58.49″N	89°32′46.95″W	30	29
Ozark Highlands	Bourbeuse R.	BO	38°26′41.03″N	90°54′48.62″W	24	24
	Gasconade R.	GA	37°49′17.41″N	92°20′40.51″W	24	21

Sites are grouped together based on major watershed or region and GPS position of each site is displayed. Number genotyped displays the number of *L. costata* genotyped at 5 or more loci per site

[a] Collected by Bergner (2013) in 2010

($P < 0.001$). Pairwise F_{ST} values for all site combinations were calculated (Table 3). The majority of pairwise F_{ST} values were highly significant ($P < 0.001$), however some notable exceptions were between the St. Joseph R. and the Auglaize R. in the Maumee R. watershed, and also between Wolf R. and the Red R. in the Green Bay watershed. The NJ network for *L. costata* explained 95.5% of the pairwise genetic distance data used to calculate the NJ network (Fig. 3). The results from the NJ network, AMOVA, and STRUCTURE analyses were congruent.

Various hypotheses of post-glacial colonization were tested using linear regression and Mantel tests (Table 4). The model explaining the most genetic differentiation by geographic distance was the model including the Wabash R.-Maumee R. and Wisconsin R.-Green Bay colonization routes (Hypothesis A + C; Fig. 4; $R^2 = 0.19$; $P = 0.001$). However, the model that included all hypothesized colonization routes (Hypothesis A + B + C) also showed significant isolation by distance ($R^2 = 0.17$; $P = 0.002$), but did not differ strongly from the results generated with only hypothesis A + C. No other models of isolation by distance tested were statistically significant ($P > 0.05$).

Mitochondrial DNA sequences

Sequencing of the mitochondrial DNA (mtDNA) COI gene produced consistent fragments of 481 bp for 59 *L. costata* (Table 5). Seventeen unique haplotypes were found (Genbank Accession #KU985185-KU985201). The number of individuals sequenced (range 2–25), the number of haplotypes for each population (range 1–5), the average number of pairwise differences (range: 0 to 4), the number of unique haplotypes for each major watershed (range 0–3), and the average nucleotide diversity (π) for each major watershed or region (range: 0 to 0.00858) are found in Table 5.

Phylogenetic analysis for *L. costata* (Fig. 5) showed strong support for this species forming a monophyletic clade (posterior probability = 0.999) and three haplotypes found in the Ozark Highlands (Hap 15, Hap 16, and Hap 17) formed a monophyletic clade (posterior probability = 0.886). Mean uncorrected (*P*) genetic distance between the Ozark highland clade and all other *L. costata* COI haplotypes was estimated at 2.82%. Three haplogroups were identified (Fig. 6). The Ozark Highland region formed one unique haplogroup and the other two haplogroups are

Table 2 The number of alleles (private alleles in parentheses), mean allelic richness (based on min $n = 11$) observed heterozygosity (H_o), expected heterozygosity (H_e), and number of individuals for each locus-population combination for *Lasmigona costata*

	Lake Erie/St. Clair (MI)		Maumee R.		Wabash R.		Lake Michigan/Huron		Illinois R.	Green Bay		Wisconsin R.	Ozark Highlands	
	BL	BEL	AU	EFWB	WH	EEL	GR	SA	IL	RE	WO	UW	BO	GA
LcoC75														
# of alleles	22 (4)	19 (0)	19 (0)	20 (1)	27 (1)	24 (0)	28 (5)	13 (0)	23 (2)	22 (0)	22 (0)	20 (0)	18 (1)	18 (2)
Ho	0.960	0.875	1.000	0.862	0.933	0.909	0.844	0.929	0.769	0.897	0.931	0.929	0.304[a]	0.263[a]
He	0.930	0.921	0.930	0.920	0.954	0.950	0.895	0.875	0.945	0.890	0.924	0.923	0.922	0.924
Allelic richness	14.054	13.320	15.214	12.672	16.189	16.411	12.140	11.239	15.071	11.921	13.251	12.894	13.346	13.885
n	25	24	16	29	30	22	77	14	26	29	29	28	23	19
LcoD48														
# of alleles	2 (0)	2 (0)	2 (0)	3 (0)	3 (1)	3 (1)	4 (0)	2 (0)	2 (0)	4 (0)	2 (0)	2 (0)	3 (0)	1 (0)
Ho	0.520	0.417	0.647	0.250	0.333*	0.619	0.487[a]	0.571	0.538	0.385*	0.600	0.481	0.042[a]	0.000
He	0.449	0.444	0.438	0.418	0.331	0.441	0.508	0.490	0.497	0.534	0.487	0.489	0.260	0.000
Allelic richness	2.000	2.000	2.000	2.393	2.597	2.524	2.404	2.000	2.000	2.846	2.000	2.000	2.773	1.000
n	25	24	17	28	30	21	78	14	26	26	25	27	24	18
LcoD158														
# of alleles	10 (1)	14 (0)	11 (0)	13 (0)	12 (1)	13 (1)	16 (1)	8 (0)	14 (0)	12 (0)	11 (0)	14 (0)	19 (1)	20 (2)
Ho	0.619	0.789	0.611	0.828	0.857	0.571	0.736[a]	0.667	0.769	0.679	0.750	0.759	0.870	0.889
He	0.840	0.891	0.838	0.880	0.870	0.771	0.811	0.760	0.865	0.832	0.825	0.825	0.911	0.915
Allelic richness	8.305	11.302	8.598	9.816	9.303	8.876	8.168	7.071	10.137	8.443	8.151	8.938	12.671	14.241
n	21	19	18	29	28	21	72	15	26	28	28	29	23	18
LcoB114														
# of alleles	10 (0)	15 (0)	10 (0)	13 (0)	15 (1)	13 (0)	15 (0)	11 (0)	16 (0)	13 (0)	14 (1)	15 (0)	16 (1)	18 (2)
Ho	0.840	0.913	0.941	0.857	0.828	0.909	0.730	0.923	0.885	0.862	0.679	0.708	0.625	0.778
He	0.858	0.899	0.877	0.885	0.889	0.883	0.863	0.873	0.896	0.863	0.864	0.883	0.919	0.921
Allelic richness	8.242	11.281	9.277	9.016	10.238	9.776	8.817	10.326	11.009	9.588	9.321	10.646	12.327	13.63
n	25	23	18	29	29	22	74	13	26	28	28	24	24	18
LcoD10														
# of alleles	11 (0)	11 (0)	14 (0)	13 (0)	18 (0)	15 (1)	14 (0)	11 (1)	27 (4)	16 (0)	22 (0)	16 (0)	20 (2)	18 (1)
Ho	0.800	0.889	0.889	0.857	0.963	0.857	0.852[a]	1.000	1.000	0.897	0.857	0.826	0.957	0.944
He	0.807	0.819	0.866	0.870	0.932	0.912	0.889	0.897	0.944	0.916	0.943	0.905	0.936	0.934
Allelic richness	9.308	9.393	10.576	9.770	13.293	11.884	9.759	11.000	15.499	11.913	14.716	11.659	14.255	14.435
n	15	18	18	28	27	21	54	11	27	29	28	23	23	18

Table 2 continued

	Lake Erie/St. Clair (MI)		Maumee R.		Wabash R.		Lake Michigan/Huron		Illinois R.	Green Bay		Wisconsin R.	Ozark Highlands	
	BL	BEL	AU	EFWB	WH	EEL	GR	SA	IL	RE	WO	UW	BO	GA
LcoD50														
# of alleles	15 (2)	17 (1)	12 (0)	10 (0)	18 (1)	14 (0)	21 (0)	13 (0)	22 (1)	21 (0)	21 (1)	19 (0)	15 (1)	17 (1)
Ho	0.870	0.913	0.882	0.720	0.963	0.733	0.775	0.667	0.923	0.897	0.893	0.731	0.833	0.905
He	0.853	0.898	0.870	0.780	0.863	0.893	0.851	0.838	0.940	0.917	0.856	0.854	0.906	0.920
Allelic richness	10.499	11.921	9.794	7.574	10.356	11.771	10.563	10.772	14.680	12.992	12.148	11.093	11.363	12.73
n	23	23	17	25	27	15	71	15	26	29	28	26	24	21
LcoD162														
# of alleles	13 (1)	11 (0)	8 (0)	16 (1)	15 (0)	9 (0)	22 (4)	12 (0)	20 (0)	24 (3)	19 (1)	14 (0)	18 (3)	17 (3)
Ho	0.783	0.833	0.875	0.769	0.643	0.833	0.872	1.000	0.846	0.931	0.786	0.889	0.792	0.810
He	0.861	0.844	0.822	0.855	0.897	0.826	0.914	0.882	0.918	0.933	0.908	0.895	0.930	0.908
Allelic richness	9.266	9.199	7.054	9.901	10.861	8.659	11.073	11.489	12.982	14.08	11.956	10.380	13.304	12.634
n	23	18	16	26	28	12	78	12	26	29	28	27	24	21
Mean He	0.80	0.82	0.81	0.80	0.82	0.81	0.82	0.80	0.86	0.84	0.83	0.82	0.83	0.79
Average allelic richness	8.81	9.77	8.93	8.73	10.41	9.99	8.99	9.13	11.63	10.25	10.22	9.66	11.43	11.79

This table also displays average H_e and allelic richness across all loci for each sampling location. Sampling locations coded as in Table 1

[a] Indicates significant deviation from Hardy–Weinberg Equilibrium using a Bonferroni adjusted α
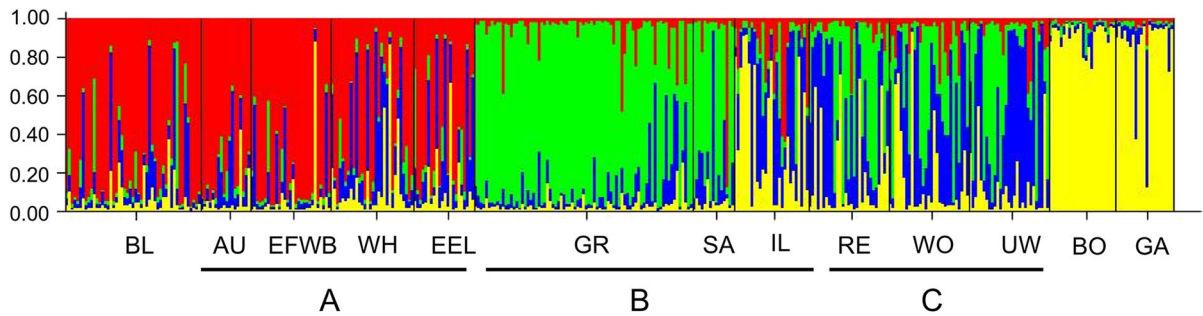
Fig. 2 Output from STRUCTURE for *Lasmigona costata* showing individual assignment to populations at $K = 4$ (Ln$P(K) = -13,525.1875$; $\Delta K = 2.70$). Simulation was performed with 200,000 iterations and 200,000 burn-in iterations while allowing for admixture. Individuals are along the *x*-axis and posterior probabilities of assignment to populations are along the *y*-axis. *A*, *B*, and *C* along the *x*-axis refer to the three hypothesized post-glacial dispersal routes (Fig. 1)

Table 3 Pairwise F_{ST} values (below diagonal) and associated *p* values (above diagonal) for *Lasmigona costata* derived from 7 microsatellite loci (Galbraith et al., 2011)

|	BL	AU	EFWB	WH	EEL	GR	IL	RE	WO	UW	BO	GA
Black R.	–	0.003	*	*	*	*	*	*	*	*	*	*
Auglaize R.	0.014	–	0.462	0.001	0.008	*	0.002	*	0.001	*	*	*
St. Joe R.	0.020	0.000	–	0.087	*	*	*	*	*	*	*	*
White R.	0.020	0.011	0.005	–	*	*	*	*	*	*	*	*
Eel R.	0.025	0.016	0.022	0.020	–	*	*	*	*	*	*	*
Grand R.	0.041	0.050	0.059	0.060	0.065	–	*	*	*	*	*	*
Kankakee R.	0.030	0.018	0.028	0.028	0.033	0.028	–	0.005	0.036	0.001	*	*
Red R.	0.044	0.028	0.041	0.038	0.042	0.026	0.011	–	0.442	0.023	*	*
Wolf R.	0.039	0.024	0.041	0.044	0.041	0.018	0.007	0.000	–	0.365	*	*
Upper Wisconsin R.	0.029	0.024	0.036	0.030	0.033	0.019	0.015	0.008	0.001	–	*	*
Bourbeuse R.	0.050	0.045	0.050	0.030	0.060	0.079	0.046	0.050	0.063	0.051	–	0.071
Gasconade R.	0.051	0.047	0.056	0.032	0.061	0.084	0.052	0.058	0.069	0.051	0.005	–

* $P < 0.001$

found throughout the Great Lakes and Mississippi drainages.

Discussion

Analyses of nuclear microsatellites for *Lasmigona costata* revealed evidence for at least two post-glacial dispersal routes into the Laurentian Great Lakes and the mtDNA sequence data identified a unique population in the Ozark Highlands. The Ozarks are well known as a region of high endemism for aquatic organisms (Mayden, 1988; Crandall & Templeton, 1999). Three similar haplotypes were found in the Ozark Highlands for *L. costata*, constituting one haplogroup. These haplotypes are on average 2.82% divergent from other *L. costata* haplotypes found in this study or those included in the analyses from GenBank. The three unique haplotypes in the Ozarks form a strongly supported monophyletic clade (Fig. 5). This intraspecific divergence at COI is much higher than has been observed for the related species *Lasmigona subviridis* (0.17–0.35%; King et al., 1999). These unique and divergent mtDNA haplotypes, combined with genetic divergence found in the microsatellite genotypes suggests that *L. costata* in the Ozark Highlands may represent a distinct lineage and merits further investigation.
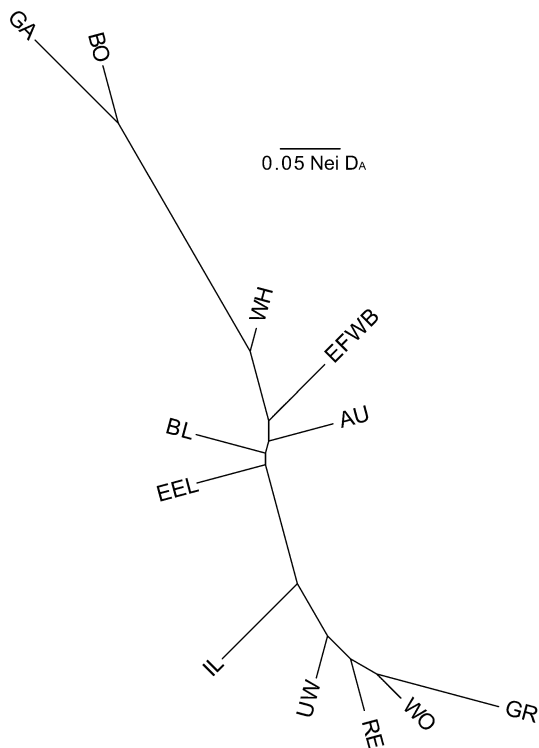
Fig. 3 Neighbor-joining (NJ) network of pairwise genetic distances (Nei's D) for *Lasmigona costata* collection locations created using allele frequency data from seven microsatellite loci (Galbraith et al., 2011). The R^2 value of the NJ network is 0.96. Site codes as used in Table 1

Table 4 R^2 values derived from the linear regression model testing genetic distance (Linearized F_{ST} [$F_{ST}/(1-F_{ST})$], calculated using microsatellite genotypes) and geographic river distance (km) associated with all combinations of hypothesized post-glacial dispersal routes (Fig. 1)

Dispersal routes	P	R^2
Hypothesis A	0.123	0.027
Hypothesis B	0.489	0.000
Hypothesis C	0.199	0.018
Hypothesis A + B	0.122	0.039
Hypothesis B + C	0.198	0.016
Hypothesis A + C	0.001	0.190
Hypothesis A + B + C	0.002	0.171

Associated P values calculated using Mantel test implemented in GenAlEx

Isolation by distance (IBD) of *L. costata* in the Great Lakes region was statistically significant when assuming connections between the Wabash and

Maumee R. watersheds and the Wisconsin R. and Green Bay watersheds. Historical connections have been suspected between the Wabash R. and Maumee R. watersheds due to changes induced by Isostatic rebound, glacial spillways, and headwater stream capture event enabling dispersal for aquatic organisms. Headwater capture likely played an important role in the biogeographic patterns of aquatic organisms present in Maumee R. and Lake Erie watersheds (Krebs et al., 2013). The St. Joseph R. of the Maumee and the St. Mary's R., once headwaters of the Wabash R., were captured by the Maumee R. following the draining of the proglacial lakes (Van der Schalie, 1945; Calkin & Feenstra, 1985; Sunderman, 1987; Pielou, 1991). In the 1830s, the Erie Canal was created connecting the Wabash R. to the Lake Erie drainage (Sunderman, 1987). This canal could have facilitated dispersal between the Wabash and Maumee R. watersheds at a much more recent timescale than post-glacial connections or stream capture events. Post-glacial connections between the Wisconsin R. and the Green Bay watershed have also been hypothesized (van der Schalie, 1945; Hansel et al., 1985; Pielou, 1991; Clark et al., 2008). Proglacial Lake Oshkosh formed over present day Green Bay and the surrounding watershed around 13,600 years ago and after the proglacial lake drained about 12,000 years ago, the Green Bay watershed likely continued to drain southwest into the Wisconsin R. for some time, enabling dispersal of aquatic organisms into the Great Lakes (Clark et al., 2008). The configuration of the Wisconsin and Wolf-Fox watersheds (i.e., adjacent to) also suggests that the Wolf and Fox rivers were captured into the Green Bay drainage as a result of isostatic rebound following the draining of the proglacial lakes.

The genetic structure observed for *L. costata* in this study suggests northward range expansion from at least two separate and isolated glacial refugia. These patterns of genetic structure are similar to suggestions by Elderkin et al. (2007) for *Amblema plicata* (Say, 1817). Evidence for multiple glacial refugia has been suggested for other species of mussel, however post-glacial colonization patterns often differ (Elderkin et al., 2007, 2008; Zanatta & Murphy, 2008; Inoue et al., 2013). Multiple species of fish, which are known hosts of *L. costata,* also show similar phylogeographic patterns, including darters (e.g., *Etheostoma* and *Percina*), northern
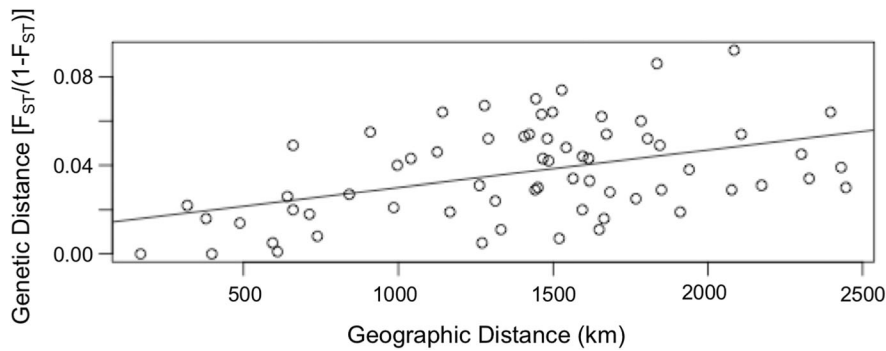
Fig. 4 Linear regressions of pairwise genetic distance [$F_{ST}/(1 - F_{ST})$] and geographic (by water) distance (km) between sites for *Lasmigona costata*. The geographic distance matrix assumes a connection between the Wabash River and the Maumee River, and the Wisconsin River and the Wolf River ($R^2 = 0.190$, $P = 0.001$), calculated using a Mantel (1967) test

Table 5 Number of *Lasmigona costata* sequenced at each major watershed or region, the number of COI haplotypes found at each sampling site, the mean number of pairwise differences among haplotypes, and the average nucleotide diversity (π)

Major watershed/region	n	No. of haplotypes	No. of unique haplotypes	Mean N of pairwise differences	π
Lake Erie/St. Clair (MI)	8	3	0	1.54	0.006022
Maumee R.	7	5	1	4.00	0.008584
Wabash R.	4	4	3	2.83	0.006159
Lake Michigan/Huron (MI)	25	5	0	3.91	0.008398
Illinois R.	2	2	1	2.00	0.004292
Green Bay	3	3	2	4.00	0.008333
Wisconsin R.	3	1	0	0.00	0.000000
Ozark Highlands	7	3	3	0.57	0.001190

The specific site locations included in each defined major watershed or region can be found in Table 1

hogsucker (*Hypentelium nigricans*; Lesuer, 1817), and smallmouth bass (*Micropterus dolomieu*; Lacépède, 1802) (Strange & Burr, 1997; Near et al., 2001; Berendzen et al., 2003; Borden & Krebs, 2009; Cummings & Watters, 2010).

We found weak evidence in support of a link between Illinois R. and Lake Michigan populations of *L. costata*. This lack of support may be due in part to high amounts of intermixing in the Illinois R. Another possible reason for this lack of evidence is that the number of pairwise distances that are different when comparing IBD in the hypotheses A + B+C model compared to only hypotheses A + C is small (14 out of 105) relative to the number of pairwise distances that change when comparing other models. The Illinois R. watershed and Lake Michigan have had multiple historic connections. During the end of the Pleistocene, proglacial Lake Chicago formed over the southern basin of present day Lake Michigan (Hansel et al., 1985; Larsen, 1987; Pielou, 1991). This lake drained to the southwest through the Chicago outlet into the Illinois River valley. The Chicago outlet remained an important outflow for proglacial lakes until approximately 4,000 years ago (Hansel et al., 1985; Larsen, 1987). Given the known shared connections between Lake Michigan and the Illinois R., it seems plausible that this outlet may have facilitated dispersal for some aquatic organisms. A canal constructed between Lake Michigan and the Illinois R. in 1892 (Melching et al., 2015) continues to exist as the Chicago Sanitary and Shipping Canal. This dispersal route remains a pathway facilitating dispersal between the Mississippi R. and Great Lakes watersheds for native and invasive species (Irons et al., 2006; Melching et al., 2015).
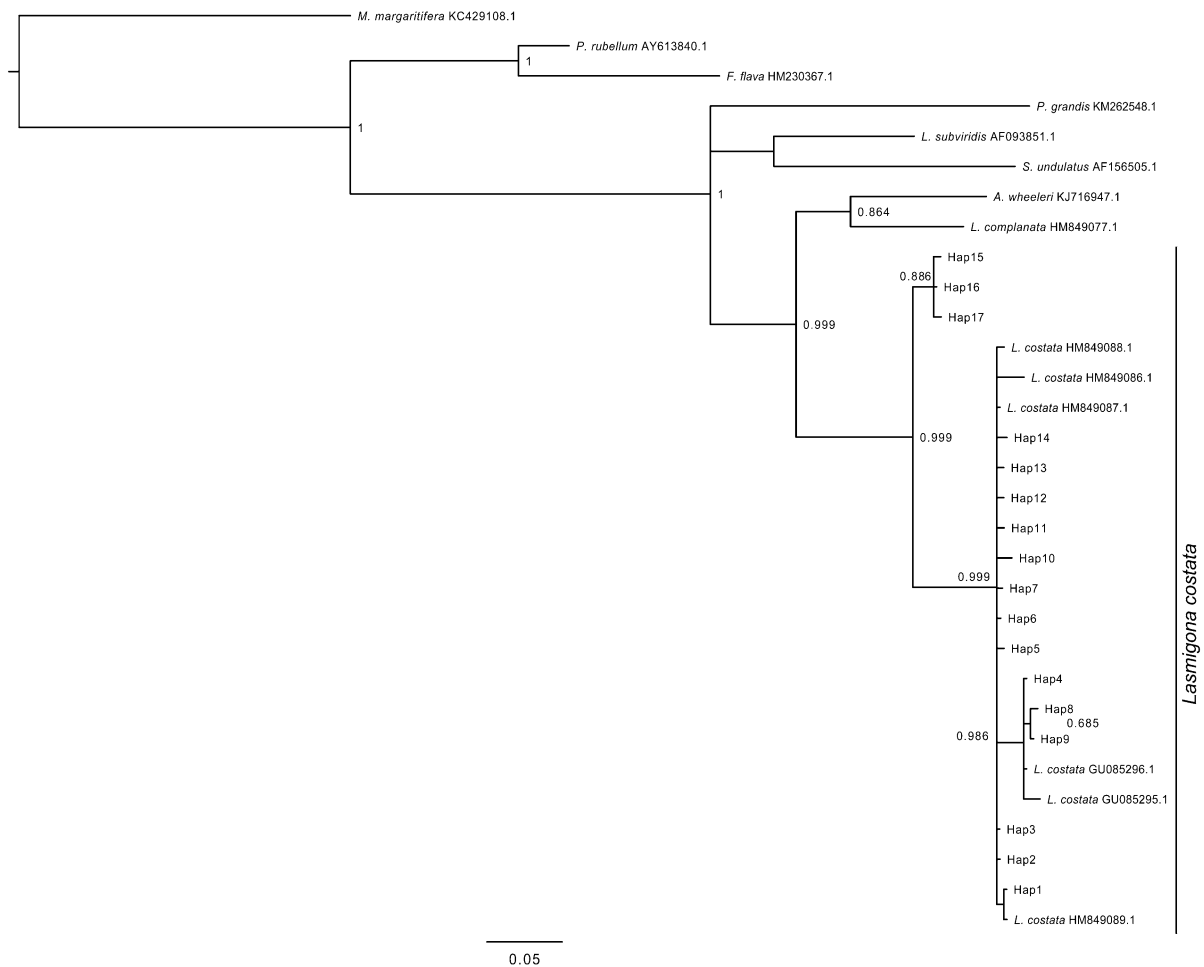
Fig. 5 Bayesian phylogram of *Lasmigona costata* from 8 major watersheds or regions (Table 1) resolved using mitochondrial gene COI sequences. *Pyganodon grandis, Fusconaia flava, Pleurobema rubellum, Arcidens wheeleri, Strophitus undulatus, Lasmigona subviridis,* and *Lasmigona complanata* sequences were used as outgroups and the tree was rooted with *Margaritifera margaritifera.* Accession numbers for sequences obtained from Genbank are shown. Posterior probabilities, indicating the proportion of trees created with the same topology, are adjacent to nodes and were created using all sequenced *L. costata* individuals. However, for visualization, only collapsed haplotypes are shown in phylogram. Scale bar represents the mean number of base pair substitutions per site

Conservation and management implications

This study expands on some previous studies (Elderkin et al., 2007; Galbraith et al., 2015), however large-scale patterns of genetic structure occasionally differed among unionid species (Elderkin et al., 2008; Zanatta & Murphy, 2008; Scott et al., 2014). Therefore, elucidating the phylogeographic commonalities among species may be necessary for conservation. This requires more research to determine dispersal capabilities during all life stages (e.g., Ferguson et al. 2013) and a clear understanding of the complex phylogeographic patterns displayed by a variety of freshwater mussel species.

Declines in freshwater mussel populations continue in North America (Neves, 1999; Haag, 2012). Populations in the Great Lakes are especially vulnerable and with drastic reductions in abundance and diversity following the invasion of dreissenid mussels in the late 1980s (Metcalfe-Smith et al., 1998; Schloesser et al., 2006; Lucy et al., 2014). Approximately 40% of freshwater mussel species in the Great Lakes would likely fall into extirpated, endangered, or threatened status (Metcalfe-Smith et al., 1998). Conservation
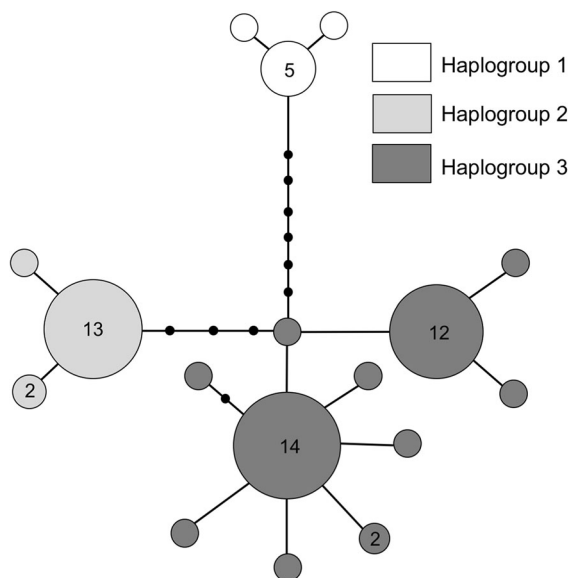
Fig. 6 Haplotype network for *Lasmigona costata* derived from COI mtDNA sequences. Each circle represents a unique haplotype. Loops and reticulations were removed using the process outlined in Fetzner & Crandall (2003). Haplotypes are categorized into haplogroups based on network structure. The number of individuals with each haplotype is displayed inside each *circle*. Unlabeled *colored circles* had only individual with that haplotype and the small *black circles* represent intermediate haplotypes. Each *line* connecting two circles represents 1 base pair difference between adjacent haplotypes

efforts should attempt to keep individuals with similar genetic profiles together (Morris & Burridge, 2006; Hoftyzer et al., 2008; Galbraith et al., 2015). Relocation and propagation efforts in the Great Lakes should avoid mixing stock from the Lake Erie watershed with those from Lakes Huron or Michigan watersheds. Galbraith et al. (2015) found similar patterns of genetic structure in freshwater mussels in the Great Lakes region independent of differences in host use or life history or conservation status, suggesting that genetic structure of common species could be useful surrogates for predicting genetic structure of rare species. This study further demonstrates the genetic structure among the Great Lakes drainages reflect divergent glacial refugia and post-glacial dispersal routes. Cryptic genetic structure should be understood to avoid erroneously mixing individuals from separate populations.

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