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RESEARCH ARTICLE

Conservation genetics of eastern hellbenders *Cryptobranchus alleganiensis alleganiensis* in the Tennessee Valley

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Abstract

The eastern hellbender has declined across much of its range and is a candidate for listing under the Endangered Species Act. Some of the most viable remaining populations exist in the Southern Appalachian Region in the Tennessee Valley watershed; however these populations are highly isolated and fragmented, and occupy several physiographic provinces, suggesting they may exhibit significant levels of genetic differentiation. We investigated genetic and phylogeographic relationships among eastern hellbender populations across the Tennessee Valley, using nuclear microsatellite markers and mitochondrial sequence data. Our population genetic analyses of microsatellite data revealed a strong pattern of isolation by stream distance, and 4 genetically distinct populations. These four populations were mainly associated with major watersheds, although middle Tennessee samples were difficult to assign to any particular population. Our phylogeographic analysis of mtDNA resulted in a strongly supported monophyletic ingroup containing nine largely allopatric clades, which also largely corresponded to major watersheds. Our findings suggest that hellbenders from different watersheds in the Tennessee Valley should be recognized as genetically distinct populations, and care should be taken to balance the needs of rescuing declining populations with translocation or headstart programs, while also preserving genetic diversity across the region.

Keywords Hellbender salamander · Genetic structure · Phylogeography · Conservation · Tennessee River

Introduction

A declining species with a large but highly fragmented range can pose challenges to wildlife conservation managers as they attempt to strike a balance between preserving important genetic variation across the landscape while restoring declining or extirpated populations through translocation and captive breeding programs (Hedrick 2001; Bouzat et al. 2009; Weeks et al. 2011). Isolated populations may be locally differentiated, and treating all populations as equivalent could miss important genetic diversity. Moreover, captive breeding/translocation programs using different source populations may be ineffective if the translocated individuals are poorly adapted to the target location, and may exacerbate declines by promoting outbreeding depression (Weeks et al. 2011). On the other hand, as populations become smaller and more isolated the effects of drift and inbreeding become more significant: the loss of allelic diversity may reduce a population's ability to adapt to changing selective pressures, and reduced heterozygosity can expose deleterious alleles with a consequent decline in average fitness of the population, (Gilpin and Soule 1986; Templeton et al. 1990).

These concerns are pertinent in efforts to conserve declining populations of North America's largest salamander, the hellbender, *Cryptobranchus alleganiensis* (Cryptobranchidae). The hellbender is one of three extant giant salamander

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species and had an extensive historic distribution in the USA (Petranka 1998). The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is found in the Tennessee, Ohio, New, Allegheny, and Susquehanna drainages ranging from southern New York to northern Georgia, with disjunct Midwest populations in the Missouri and Meramac drainages in Missouri. Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) have a much smaller range, limited to the Ozark region of Arkansas and Missouri. Although they have a relatively wide distribution, hellbenders are also habitat specialists preferring cool, clear rocky streams with large rock cover objects or crevices that provide shelter, feeding, and nest sites. Hellbenders take at least 5–6 years to reach reproductive maturity, can live over 25 years (Nickerson and Mays 1973a; Peterson et al. 1983), and tend to show high site fidelity (Nickerson and Mays 1973b; Bodinof et al. 2012). This combination of life history, habitat specialism, and low rates of dispersal all contribute to a tendency for populations to be isolated and increase the risk of local extinction.

Comparisons between historic and contemporary field surveys have documented extensive declines in the distribution and abundance of hellbender populations, with dramatic demographic shifts to older individuals indicating a lack of recruitment (Nickerson and Mays 1973a, b; Peterson et al. 1983, 1988; Bothner and Gottlieb 1991; Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011b). Numerous factors have been implicated including river impoundment, poor water quality and siltation, persecution, illegal collection and disease (Nickerson and Mays 1973a; Williams et al. 1981; Briggler et al. 2007; Burgmeier et al. 2011a; Bodinof et al. 2011). The limited historic range, and evidence of dramatic declines (Trauth et al. 1992; Wheeler et al. 2003), led to listing of the Ozark hellbender, *Cryptobranchus alleganiensis bishopi* as endangered under the Endangered Species Act (ESA) in 2011 (USFWS 2011a). Furthermore, concerns over range-wide declines in the eastern hellbender *Cryptobranchus alleganiensis alleganiensis* resulted in their nomination as a candidate for listing as threatened under the ESA in 2013. Both subspecies are included in appendix III of the Convention on International Trade (CITES) in Endangered Species of Wild Fauna (USFWS 2011b).

Significant areas of Tennessee, North Carolina and Georgia form the Tennessee Valley, made up of numerous watersheds draining into the mainstem Tennessee river. These watersheds have historically provided suitable habitat for hellbenders (Mayasich et al. 2003). In many cases the headwaters of these southern watersheds are located within USDA Forest Service and National Park Service public lands, which may provide protection from the declining water quality found elsewhere across the range (Briggler et al. 2007; Pugh et al. 2015; Freake and DePerno 2017). However, the process of making informed decisions about managing hellbender populations in the southeast part of their range has been hindered by a relative paucity of contemporary demographic and population genetics studies in this region. Surveys in the Blue Ridge physiographic province identified healthy populations with successful recruitment within the Cherokee National Forest and Great Smoky Mountains National Park (Nickerson et al. 2002; Hecht-Kardasz et al. 2012; Pugh et al. 2013; Freake and DePerno 2017), and extensive survey efforts over the last decade in North Carolina and Georgia (M. Freake, Lee University; L. Williams and J. Humphries, North Carolina Wildlife Resource Commission; J. Groves, Curator Emeritus, NC Zoo; T. Floyd, Georgia Department of Natural Resources; unpublished data) indicate the presence of several populations with relatively high densities of hellbenders and consistent reproduction. Thus, the southern Appalachians may represent one of the most important regions for preserving eastern hellbenders in the United States. However, the Tennessee River watershed has experienced extensive habitat loss and alteration from land use changes, mining, deforestation and hydroelectric dam construction, contributing to extensive isolation and fragmentation of hellbender populations, with declines observed in all regions of the Tennessee watershed. For example, Miller and Miller (2005) detected only large adults with high rates of physical abnormalities in four Cumberland and Tennessee River populations in the Highland Rim physiographic province of middle Tennessee, and recent surveys indicate virtual extirpation of three of these populations (Miller and McGinnity, Middle Tennessee State University, Nashville Zoo, unpublished data). Even in protected watersheds there has been extensive habitat loss and isolation caused by hydroelectric dams. Consequently, eastern hellbenders in Tennessee, North Carolina and Georgia are a Species of Greatest Conservation Need (GADNR 2015; NCWRC 2015; TWRA 2015).

Initial range-wide studies of hellbender genetic structure relied on mitochondrial markers and found low variation within drainages, but significant variation between drainages (Routman et al. 1994; Sabatino and Routman 2009). They concluded that hellbenders comprise at least 8 major populations, which could be considered separate management units. Tonione et al. (2011) augmented the mitochondrial data with nuclear microsatellite data and again identified at least eight distinct populations. Routman et al. (1994) and Sabatino and Routman (2009) suggested that the results from mitochondrial data are consistent with a dramatic Pleistocene range contraction to a southern refugium (possibly Ozark or Tennessee drainage) followed by a more recent range expansion.

Using microsatellite markers, Crowhurst et al. (2011) investigated genetic relationships of hellbenders in Missouri and identified three genetic clusters. Subsequently, Unger et al. (2013) conducted the most extensive range-wide study of genetic variation using microsatellite

markers. They were able to sample many more populations within each watershed, allowing assessment of genetic structure at range-wide and watershed levels. In addition, they conducted a fine scale analysis of populations in the Tennessee River watershed allowing assessment of structure at the level of the entire Tennessee River drainage, within and between basins, within and among sub-basins, and within and among stream reaches within sub-basins. Their analysis indicated two distinct genetic populations at the range-wide scale, consisting of a northern Ohio River drainage group, and a southern group dominated by Tennessee River drainage populations. They also found weak evidence for secondary structure of two populations in the Tennessee drainage. Partitioning of genetic variation was highest within streams (~ 93–98%), with lower but still significant levels (< 4%) at higher hierarchical levels. They found a strong fit to an isolation by stream distance (IBSD) model for genetic variation.

Some discordance in these previous studies is apparent, with some indicating numerous genetic groups, with others rather few. A missing element in these studies is the combination of fine scale nuclear microsatellite and mitochondrial data. So the purpose of this study was to explore the possibility of additional genetic structure within the Tennessee Valley in the southeastern Appalachian region, using a fine scale mitochondrial and microsatellite data set. Rivers in this area are characterized by greater spatial complexity in terms of elevation, geology, land use and hierarchical structure than in many other regions (Unger et al. 2013), which may facilitate divergence among hellbender populations; thus, if the Pleistocene refugium hypothesis is correct, there may be considerable inter-population variation in genetic diversity even prior to contemporary anthropogenic impacts. This variation should be reflected when assigning hellbender populations to specific management units.

Methods

Compliance with ethical standards

This study was performed with the approval of the Institutional Review Board of Lee University. We received permission from the USDA Forest Service and National Park Service to conduct fieldwork in the Cherokee National Forest, and Great Smoky Mountains National Park. Animals were collected under permit from the Tennessee Wildlife Resource Agency (# 1505) and National Park Service (#GRSM-2012-SCI-0307) and released unharmed at the capture site. To protect hellbenders from illegal collection and disturbance, publication of detailed location data is prohibited by the state and federal permitting agencies. However these locations are on file with the Tennessee Wildlife Resource Agency (Pandy.English@tn.gov), North Carolina Wildlife Resource Commission (Lori.Williams@ncwildlife.org); and Georgia Department of Natural Resources (Thomas.Floyd@dnr.ga.gov).

Microsatellite study design

For this study, we collected 712 individual hellbender tissue samples during 2006–2014 from 45 locations in 40 rivers across the Tennessee River watershed in Tennessee, North Carolina, Georgia and Virginia, and in the one known extant Cumberland population in Tennessee (Fig. 1). The majority

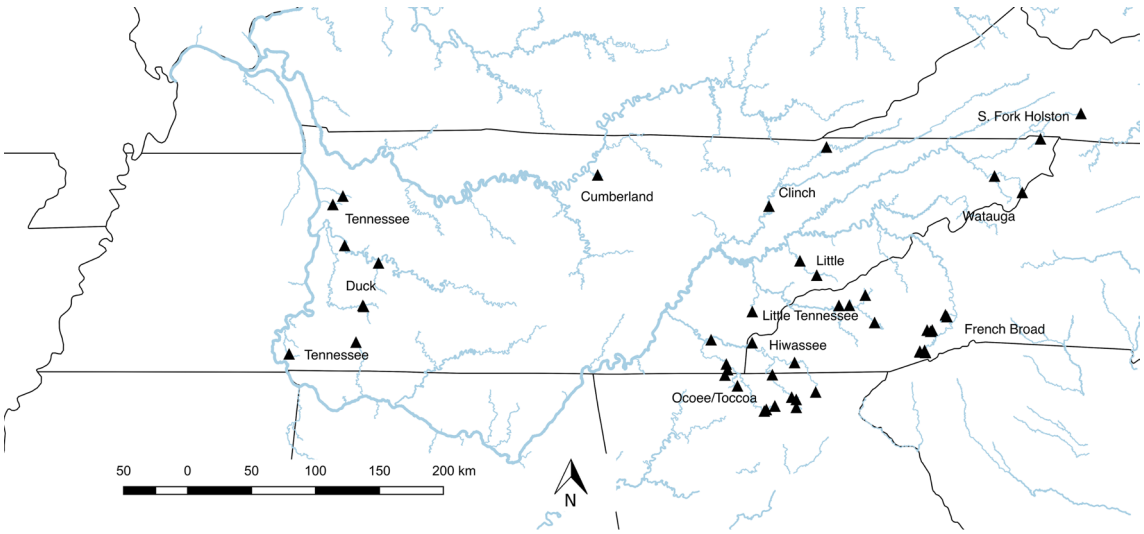

Fig. 1 Map of the Tennessee Valley showing microsatellite sample locations (triangles) and major watersheds draining into the Tennessee River

of these samples (n = 581) were included in the study by Unger et al. (2013). The additional 131 samples included in this study extend the range through middle Tennessee, and fill gaps within the Blue Ridge province of Tennessee and North Carolina. For each hellbender capture we recorded GPS coordinates and biometrics including total length and mass. During the breeding season we also sexed hellbenders based on cloacal swelling. After processing we released each hellbender at its capture site. Tissue samples consisted of 2–5 mm tail or toe clips stored in 95% ethanol, or blood samples stored in lysis buffer (1 M Tris, 0.5 M EDTA pH 8.0, 5 M NaCl, 20% SDS). All new samples were extracted with Qiagen DNeasy® Blood and Tissue kit following the animal tissue protocol.

Genotyping

Salamanders were genotyped at 12 microsatellite loci following methods described in Unger et al. (2010). The PCR products were either analyzed on an ABI 3130xl automatic sequencer and genotyped using GENEMAPPER version 3.7, or on a Licor 4300 automated sequencer and genotyped using SAGA 2™ version 3.3.0. To ensure equivalent genotyping across all platforms, we ran a subset of samples with each system, and we reamplified samples with low intensity or ambiguous signals. Extraction and analysis of samples from Unger et al. (2013) are described there. We checked all loci for null alleles, allelic drop out, or linkage disequilibrium for each sample location (including those from Unger et al. 2013) using MICROCHECKER (Van Oosterhout et al. 2004). A few loci showed evidence of null alleles for 1 or 2 populations, but there was no evidence of systematic problems with null alleles, therefore no data were excluded.

Genetic variation

We estimated standard metrics of genetic variation for each sampling site including number of observed alleles per locus (A_P), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (G_{IS}), and deviation from Hardy–Weinberg equilibrium using GenoDive version 2.0b27 (Meirmans and Van Tienderen 2004).

Population genetic structure

To assess global population differentiation, we estimated both F_{ST} and G''_{ST} (Meirmans and Hedrick 2011) for populations with $N \geq 10$ and assessed significance for each using a permutation test with 999 permutations using GenoDive. We also estimated all pairwise distances using both F_{ST} and G''_{ST} for populations with $N \geq 10$ (Online Resource 1).

For all populations with $N \geq 10$, we tested for IBSD with a Mantel test (Mantel 1967) using GenoDive (statistic = Spearman's r; permutations = 1000). Following (Rousset 1997) we used $F_{ST}/1 - F_{ST}$ and $\log_{10}$ geographic distance (km). Because many loci had many alleles (up to 63), we repeated the IBSD analysis with G''_{ST}, a standardized measure of genetic differentiation which corrects for markers with more than two alleles (Meirmans and Hedrick 2011).

To further assess population genetic structure across the entire study area and begin to better understand the geographic distribution of genetically isolated populations, we used STRUCTURE 2.3 to assign individuals to population genetic clusters (K) based on Hardy–Weinberg equilibrium within populations and linkage equilibrium between loci within populations (Pritchard et al. 2000; Hubisz et al. 2009). We analyzed our complete data set (712 samples) by running a series of analyses for $K = 1$–10; with 4 replicate analyses for each K, burn-in = 100,000 MCMC generations, and the posterior distribution was estimated using an additional 1,000,000 MCMC generations. We used the admixture model that incorporates the possibility for some individuals to have mixed cluster ancestry. To prevent overestimation of K, which can occur when sampling is not uniform, we used the uncorrelated alleles model (Pritchard et al. 2000; Hubisz et al. 2009; Unger et al. 2013). The default setting was used for the F_k prior, and a uniform prior was used for the α parameter. Convergence was assessed by monitoring plots of the log probability of the data and α across each individual run and by comparing mean values of log probability of the data and α across replicate runs of the same value of K. Similar mean estimates of these parameters were interpreted as a sign of convergence on the posterior distribution. To automate these STRUCTURE analyses, we used the python utility StrAuto v0.3.1 (Chhatre and Emerson 2017). To estimate the best number of clusters, we used the ΔK method of (Evanno et al. 2005) implemented with STRUCTURE HARVESTER (Earl and vonHoldt 2012). The ΔK method tends to favour smaller values of K that represent more highly differentiated sets of populations in systems that deviate from an island model (Evanno et al. 2005). The run with the highest log-likelihood for a given K was used to assign q values, the proportion of an individual's genome assigned to each population. Additional population genetic structure was assessed by rerunning STRUCTURE, as above, on each watershed that formed a cluster in the original STRUCTURE analysis (see results). To better understand how genetic variation is partitioned at different hierarchical levels, we used the results of the STRUCTURE analyses to group sample sites into populations and used this grouping in an Analysis of Molecular Variance (AMOVA) analysis (stepwise mutation model and 2000 permutations) in GenoDive. This allowed us to estimate within and between population genetic variation

for populations that are determined by genetic data rather than a priori by geographic location (Pritchard et al. 2000).

Phylogeographic structure

To further explore genetic structure within this region, we analyzed mitochondrial sequence data from two genes—cytochrome oxidase I (*COI*) and cytochrome B (*CytB*). This data set included 116 individual hellbenders collected from 19 sites across the Tennessee Valley, and from the one known extant Cumberland population in Tennessee, along with previously published sequence data from Sabatino and Routman (2009), which sampled broadly across the range of both hellbenders subspecies. We used their *COI* and *CytB* primers to amplify the target loci, and the PCR products were cleaned up with Qiagen QIAquick® PCR cleanup kits. We used a Licor 4300 automated sequencer with Epicentre Sequitherm Excel™ II or USB Thermo Sequenase Cycle™ chemistry to conduct bidirectional sequencing, and we aligned the sequences using Licor e-Seq™ (version 3.1) and AlignIR™ (version 2.1) software. The *COI* and *CytB* sequences were then concatenated in GENEIOUS R8 v8.05 (Kearse et al. 2012). We performed a Bayesian phylogenetic analysis on the concatenated mtDNA dataset using MrBayes v3.2.6 (Ronquist et al. 2012). Following Sabatino and Routman (2009) we used samples from New, Current, and Eleven Point Rivers as the outgroup. The best-fit model for each locus and each codon partition was chosen using the Bayesian Information Criterion implemented in PartitionFinder v1.01 (Lanfear et al. 2012). The codon partition models chosen for both loci were K80 + I, HKY + I, and GTR + G, for the first, second, and third codon positions respectively. For the MrBayes analyses, two independent runs, each with four Markov chains, were used with the default temperature parameter of 0.2. Default priors were used with random trees to start each Markov chain. Chains were run for 1 million generations with topology and model parameter estimates sampled every 200 generations. The first 100K sampled trees from each of the two runs were discarded as burn-in. Convergence was assessed using the standard deviation of split frequencies and the potential scale reduction factors (see MrBayes v3.2 manual available from: http://mrbayes.sourceforge.net/ mb3.2_manual.pdf). MrBayes analyses were executed using the GENEIOUS MrBayes Plugin v2.2.2 and a custom command block. We used DnaSP version 5.10.1 (Librado and Rozas 2009) to assess haplotype and nucleotide diversity for the concatenated sequences.

Results

Microsatellites

Genetic variation

From the 712 individuals genotyped at 12 microsatellite loci, we found 249 alleles (Table 1). The total numbers of alleles for each locus across all samples ranged from 13 to 62 (median = 16), and the mean observed number of alleles per locus varied from 2.7 (Ocoee1) to 13.7 (Hiwassee1). There was strong similarity between observed (H_O) and expected (H_E) heterozygosity, and we did not observe significant evidence of strong inbreeding (G_{IS}) in any of the populations with $N \geq 10$. Only 2 sites (Duck2 and FB4) differed significantly from Hardy–Weinberg equilibrium (heterozygosity test, Bonferroni correction critical $P = 0.0011$), and their values of G_{IS} were close to zero.

Population genetic structure

We found a significant IBSD pattern (Fig. 2) across populations with $N \geq 10$ using both F_{ST} ($P < 0.001$; $R^2 = 0.170$) and G''_{ST} ($P < 0.001$; $R^2 = 0.183$).

Pairwise genetic differentiation values (see Online Resource 1) were significantly higher from those expected under panmixia; however, F_{ST} values (median = 0.057; range −0.010 to 0.140; $P < 0.001$) were lower than pairwise G''_{ST} values (median = 0.346; range −0.059 to 0.645; $P < 0.001$), indicating that the large number of alleles at each locus downwardly biased values for F_{ST}; therefore, G''_{ST} is reported for all further analyses. The lowest pairwise G''_{ST} values were found within French Broad (median = 0.073; range −0.059 to 0.176). The highest pairwise G''_{ST} values were between SF Holston and all other sites (median = 0.543; range 0.376 to 0.645).

For the complete data set, the ΔK method indicated highest support for grouping individuals into 4 genetic populations (Table 2; Figs. 3, 4; $K = 4$), although a secondary peak was observed at $K = 5$ (Fig. 4). For $K = 4$, all sites from within French Broad were assigned to one population (PP ≥ 0.93). All sites from within the Ocoee/Toccoa were assigned to a second population (PP ≥ 0.89). All Hiwassee sites were assigned to a third population (PP = 0.61–0.91), which also included all Little (PP = 0.91–0.93), and Little Tennessee sites (PP = 0.73–0.95) as well as the sites Tenn3 (PP = 0.96) and Clinch1 (PP = 0.94). The fourth population included Holston2 (PP = 0.98), Tenn1 (PP = 0.80) and Clinch2 (PP = 0.87). All other sites were assigned to one or more of these populations, but typically with lower (< 0.65) posterior probabilities. We further tested for the

Table 1 Genetic diversity of hellbender populations in the Tennessee Valley based on 12 microsatellite loci

Population no.	Location, State	Watershed	N	A_P	H_O	H_E	G_{IS}	p
1	Cumb1, TN	Cumberland	3	3.8	0.75	0.81	0.077	0.2
2	Tenn1, TN	Tennessee	1	–	–	–	–	–
3	Tenn2, TN		2	3.3	0.64	0.77	0.171	0.086
4	Tenn3, TN		1	–	–	–	–	–
5	Duck1, TN	Duck	1	–	–	–	–	–
6	Duck2, TN		5	4.0	0.72	0.82	0.077	0.001
7	Duck3, TN		2	2.9	0.63	0.90	0.259	0.002
8	Duck4, TN		11	6.9	0.82	0.82	− 0.007	0.84
9	Hiwassee1, TN	Hiwassee	51	13.7	0.86	0.89	0.025	0.006
10	Hiwassee2, NC		1	–	–	–	–	–
11	Hiwassee3, GA		24	9.7	0.89	0.85	− 0.053	0.017
12	Hiwassee4, NC		2	2.9	0.80	0.79	− 0.067	0.789
13	Hiwassee5, GA		6	5.9	0.82	0.86	0.041	0.07
14	Hiwassee6, GA		20	9.4	0.86	0.84	− 0.028	0.152
15	Hiwassee7, GA		20	9.4	0.87	0.84	− 0.028	0.167
16	Hiwassee8, GA		27	9.8	0.83	0.83	0.002	0.452
17	Ocoee1, TN	Ocoee/Toccoa	5	2.7	0.51	0.49	− 0.077	0.394
18	Ocoee2, TN		10	7.4	0.87	0.84	− 0.034	0.264
19	Ocoee3, GA		34	7.0	0.77	0.76	− 0.017	0.338
20	Toccoa1, GA		15	7.7	0.86	0.82	− 0.049	0.097
21	Toccoa2, GA		20	7.7	0.78	0.79	0.013	0.336
22	Toccoa3, GA		20	8.8	0.84	0.82	− 0.023	0.235
23	Toccoa4, GA		3	3.8	0.83	0.81	− 0.053	0.445
24	Toccoa5, GA		30	9.3	0.86	0.82	− 0.050	0.018
25	Clinch1, TN	Clinch	1	–	–	–	–	–
26	Clinch2, TN		1	–	–	–	–	–
27	LittleTenn1, TN	Little Tennessee	9	7.5	0.86	0.84	− 0.028	0.299
28	LittleTenn2, NC		3	3.7	0.75	0.79	0.044	0.38
29	LittleTenn3, NC		24	9.1	0.84	0.84	0.007	0.361
30	LittleTenn4, NC		3	3.8	0.89	0.85	− 0.049	0.366
31	LittleTenn5, NC		19	8.8	0.86	0.84	− 0.025	0.201
32	Little, TN	Little	63	9.9	0.82	0.83	− 0.002	0.536
33	Little2, TN		1	–	–	–	–	–
34	FB1, NC	French Broad	1	–	–	–	–	–
35	FB2, NC		27	10.4	0.86	0.82	− 0.054	0.017
36	FB3, NC		12	8.0	0.84	0.83	− 0.017	0.365
37	FB4, NC		31	11.0	0.88	0.83	− 0.066	0.001
38	FB5, NC		26	10.1	0.87	0.82	− 0.060	0.006
39	FB6, NC		14	8.6	0.89	0.84	− 0.066	0.02
40	FB7, NC		21	9.4	0.84	0.82	− 0.027	0.209
41	FB8, NC		20	9.8	0.84	0.84	0.001	0.521
42	FB9, NC		21	9.8	0.90	0.84	− 0.069	0.013
43	Watauga1, TN	Watauga	3	4.3	0.92	0.90	− 0.048	0.519
44	Watauga2, TN		13	7.3	0.81	0.82	0.012	0.401
45	Holston1, TN	Holston	7	6.3	0.85	0.82	− 0.027	0.354
46	Holston2, VA		77	9.3	0.79	0.79	0.001	0.481

Dashes correspond to populations where sample size $N = 1$ and no diversity metrics can be calculated

N sample size, A_P observed number of alleles per locus, H_O observed heterozygosity, H_E expected heterozygosity, G_{IS} inbreeding coefficient, p heterozygosity test for G_{IS}, averaged across loci (critical $p = 0.0011$ with Bonferonni correction for multiple samples)

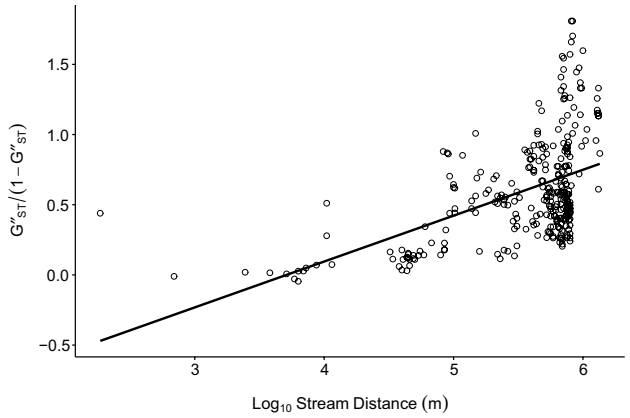

Fig. 2 Scatter plot of genetic differentiation $G''_{ST}/(1-G''_{ST})$ and LOG_{10} stream distance for all sampling sites with $N \geq 10$ in the Tennessee Valley

possibility for additional structure within the Hiwassee/Little Tennessee/Little watersheds by excluding all other populations from the analysis; we found evidence for two groups with Little River clustering separately from the other populations (Table 3), and this clustering of Little River was also evident for $K = 5$ (see Online Resource 2).

These four clusters were mainly associated with major watersheds such as the French Broad and Holston (Fig. 5). The Ocoee watershed drains into the Hiwassee River, and these two drainages were genetically divergent. Samples from the Cumberland, Tennessee, Duck, and Watauga Rivers were assigned to one or more of these populations, but with relatively low posterior probabilities. Some of these sites were assigned to a single cluster with high posterior probabilities (e.g. Tenn3; PP = 0.961), however this assignment should be interpreted cautiously because of the small sample size (N = 1).

Based on the AMOVA analysis, most of the variation (66%) is found among populations identified by STRUCTURE (Table 4).

Mitochondrial phylogeographic structure

The phylogeographic analysis of mtDNA resulted in a strongly supported monophyletic ingroup containing nine largely allopatric clades; however, the relationships among these clades are generally not well resolved (Fig. 6). The nine clades largely correspond to major watersheds. In the eastern part of the Tennessee Valley, the Hiwassee and Ocoee samples formed two well-supported clades (although one Hiwassee haplotype was associated with the Ocoee clade), while samples from Tellico, Little Tennessee, French Broad, Holston and Watauga watersheds formed a large single clade. In the western part of the Tennessee Valley, samples from the Duck watershed formed a clade with Tenn2 (a Tennessee River tributary), while two other Tennessee tributaries (Tenn1 and Tenn3) formed another clade. Powell and Clinch tributary samples formed another clade, while the Cumberland samples grouped with Ohio and Susquehanna watershed rivers. For rivers with more than 3 samples, haplotype and nucleotide diversity was highest in Hiwassee1 and Ocoee2 populations (Table 5).

Discussion

Population genetic structure

Genetic diversity varied among rivers, and even though there were many populations with small sample sizes (often associated with declining populations), levels of expected heterozygosity were generally high. Among populations with $N \geq 10$, Hiwassee1 in Tennessee exhibited the highest allelic richness and expected heterozygosity. We did not find compelling evidence of strong inbreeding in any of our populations; however hellbenders can live 20–30 years (Nickerson and Mays 1973a; Peterson et al. 1983) and it may take several generations for genetic markers to show a significant excess of homozygotes. Our data set is also limited by high variation in sample size and stratification. An ideal design would comprise ~ 50 individuals per watershed, with consistent sub-sampling of 15–20 individuals per individual stream (e.g. Unger et al. 2013). However, significant population declines in parts of the Tennessee Valley (Miller and Miller 2005; Freake and DePerno 2017) have resulted in some watersheds being represented by just 1–5 individuals, while productive watersheds often exhibited high variation in individual stream sample sizes. These factors make it difficult to reliably test for evidence of inbreeding or population bottlenecks. Nevertheless, we did note that the lowest average number of alleles per locus and lowest level of expected heterozygosity was found in Ocoee1, a small highly isolated tributary of the Ocoee where the population comprises just a few large adults, with no evidence of reproduction (Freake and DePerno 2017). It is possible that a combination of demographic stochasticity and inbreeding depression have contributed to a failure to currently reproduce in this population. It seems likely that these small tributaries may historically have been the terminal tips of larger populations and thus acted somewhat as sink patches requiring rescue by mainstem or neighboring tributary populations. The extensive fragmentation across the Tennessee Valley caused by dam construction and land use changes have left the small higher elevation populations entirely isolated from other populations with no chance of natural rescue; yet these are the populations with highest water quality thanks to their location within

Table 2 Proportions of each hellbender population assigned to each cluster by the STRUCTURE analysis

Population no.	River, State	Watershed	$K=4$ Clusters				N
			1	2	3	4	
1	Cumb1, TN	Cumberland	0.076	0.017	0.292	**0.615**	3
2	Tenn1, TN	Tennessee	**0.795**	0.071	0.079	0.054	1
3	Tenn2, TN		**0.649**	0.079	0.228	0.044	3
4	Tenn3, TN		0.017	0.013	0.009	**0.961**	1
5	Duck1, TN	Duck	**0.341**	0.075	**0.405**	0.179	1
6	Duck2, TN		**0.456**	0.01	**0.484**	0.050	5
7	Duck3, TN		0.069	0.029	**0.638**	0.263	2
8	Duck4, TN		**0.541**	0.037	0.300	0.122	11
9	Hiwassee1, TN	Hiwassee	0.052	0.043	0.055	**0.849**	51
10	Hiwassee2, NC		0.041	0.037	0.019	**0.903**	1
11	Hiwassee3, GA		0.012	0.058	0.049	**0.881**	24
12	Hiwassee4, NC		0.025	0.165	0.197	**0.613**	2
13	Hiwassee5, GA		0.026	0.08	0.246	**0.648**	6
14	Hiwassee6, GA		0.011	0.046	0.029	**0.914**	20
15	Hiwassee7, GA		0.009	0.055	0.051	**0.885**	20
16	Hiwassee8, GA		0.017	0.02	0.059	**0.905**	27
17	Ocoee1, TN	Ocoee	0.045	0.004	**0.921**	0.030	5
18	Ocoee2, TN		0.047	0.01	**0.896**	0.048	10
19	Ocoee3, GA		0.009	0.009	**0.971**	0.011	34
20	Toccoa1, GA		0.006	0.031	**0.893**	0.071	15
21	Toccoa2, GA		0.007	0.012	**0.969**	0.012	20
22	Toccoa3, GA		0.006	0.018	**0.962**	0.015	20
23	Toccoa4, GA		0.013	0.011	**0.959**	0.018	3
24	Toccoa5, GA		0.006	0.016	**0.928**	0.050	30
25	Clinch1, TN	Clinch	0.025	0.017	0.019	**0.939**	1
26	Clinch2, TN		**0.871**	0.024	0.081	0.023	1
27	LittleTenn1, TN	Little Tennessee	0.111	0.069	0.093	**0.728**	9
28	LittleTenn2, NC		0.073	0.040	0.030	**0.857**	3
29	LittleTenn3, NC		0.012	0.014	0.031	**0.943**	24
30	LittleTenn4, NC		0.017	0.013	0.020	**0.950**	3
31	LittleTenn5, NC		0.046	0.048	0.037	**0.868**	19
32	Little, TN	Little	0.033	0.011	0.024	**0.932**	63
33	Little2, TN		0.066	0.014	0.012	**0.909**	1
34	FB1, NC	French Broad	0.006	**0.985**	0.004	0.005	1
35	FB2, NC		0.013	**0.930**	0.010	0.047	27
36	FB3, NC		0.006	**0.971**	0.007	0.016	12
37	FB4, NC		0.006	**0.961**	0.009	0.024	31
38	FB5, NC		0.005	**0.974**	0.009	0.013	26
39	FB6, NC		0.006	**0.969**	0.009	0.015	14
40	FB7, NC		0.005	**0.960**	0.011	0.025	21
41	FB8, NC		0.013	**0.966**	0.008	0.013	20
42	FB9, NC		0.006	**0.964**	0.012	0.018	21
43	Watauga1, TN	Watauga	0.232	0.096	0.059	**0.612**	3
44	Watauga2, TN		0.263	0.089	0.128	**0.520**	13
45	Holston1, TN	Holston	0.175	0.019	0.138	**0.668**	7
46	Holston2, VA		**0.975**	0.007	0.009	0.008	77

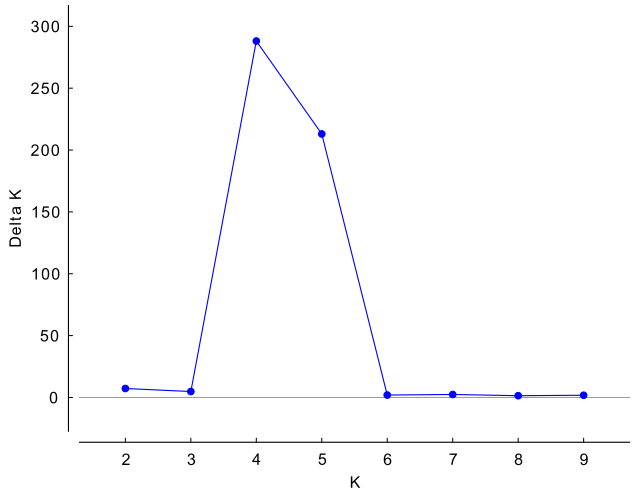

Fig. 3 Plot of ΔK values for eastern hellbender putative clusters (K) across the Tennessee Valley, obtained from STRUCTURE HARVESTER

Table 3 Proportions of Hiwassee, Little Tennessee, and Little populations assigned to each cluster by the STRUCTURE analysis

Population no.	Location, State	$K=2$		N
		1	2	
9	Hiwassee1, TN	0.153	**0.847**	51
10	Hiwassee2, NC	0.023	**0.977**	1
11	Hiwassee3, GA	0.063	**0.937**	24
12	Hiwassee4, NC	0.434	**0.566**	2
13	Hiwassee5, GA	0.226	**0.774**	6
14	Hiwassee6, GA	0.022	**0.978**	20
15	Hiwassee7, GA	0.029	**0.971**	20
16	Hiwassee8, GA	0.033	**0.967**	27
27	LittleTenn1, TN	**0.682**	0.318	9
28	LittleTenn2, NC	**0.503**	0.497	3
29	LittleTenn3, NC	0.313	**0.687**	24
30	LittleTenn4, NC	0.320	**0.680**	3
31	LittleTenn5, NC	0.214	**0.786**	19
32	Little, TN	**0.968**	0.032	63
33	Little2, TN	**0.946**	0.054	1

the protected watersheds of public USDA Forest Service and National Park Service lands (Mast and Turk 1999; Quinn et al. 2013; Pugh et al. 2015; Freake and DePerno 2017). Future management options could involve translocations from stable source populations to reduce the risk of local extinction of declining patches.

Consistent with our expectations, hellbender microsatellite markers exhibited strong patterns of IBSD, with nearby watersheds being more closely related than distant watersheds. Previous studies have observed similar patterns, and is likely attributable to the habitat specialization of hellbenders, requiring large shelter rocks and low levels of sedimentation. As a result, hellbender populations are typically found in upland river systems, with large mainstem rivers (such as the Tennessee River itself) offering significant barriers to migration due to high levels of sedimentation. The current distribution of hellbenders is likely to be the product of geological and climatological events, which greatly altered the hydrology of contemporary rivers, producing a complex spatial and temporal pattern of refugia and invasions over multiple glaciation cycles. Thus in the context of the contemporary landscape, distant populations are likely to have been isolated from each other for longer periods, and thus likely to accumulate more differences.

Therefore, in fully aquatic species with high levels of philopatry and strong IBSD, genetic structure is likely to partition strongly within historically connected river systems, and show significant differentiation between watersheds separated by unsuitable mainstem segments. Our analysis of microsatellite markers revealed strong patterns of genetic variation differentiation between watersheds. While Unger et al. (2013) found only weak support for at most two clusters in the Tennessee River watershed, our analysis found four strongly supported clusters which corresponded to specific watersheds. The Hiwassee, Little Tennessee and Little River watersheds formed one large cluster even though their respective confluences with Tennessee River mainstem are relatively distant (50–100 km). We did find evidence for additional structure within the Hiwassee/

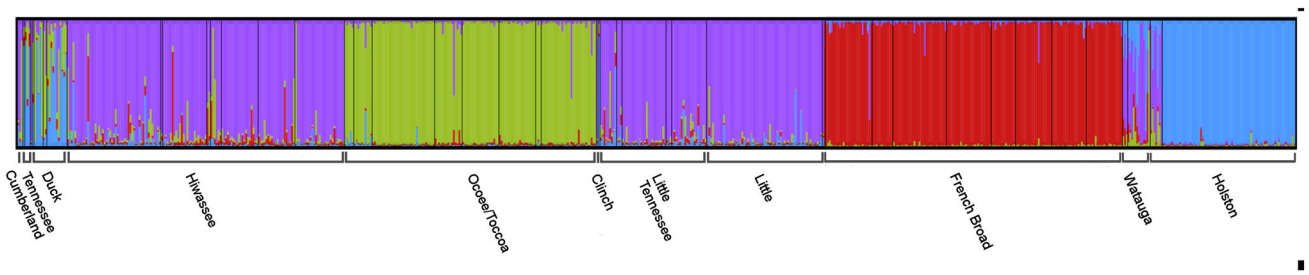

Fig. 4 Assignment of Tennessee Valley hellbenders to genetic clusters estimated with STRUCTURE for $K=4$

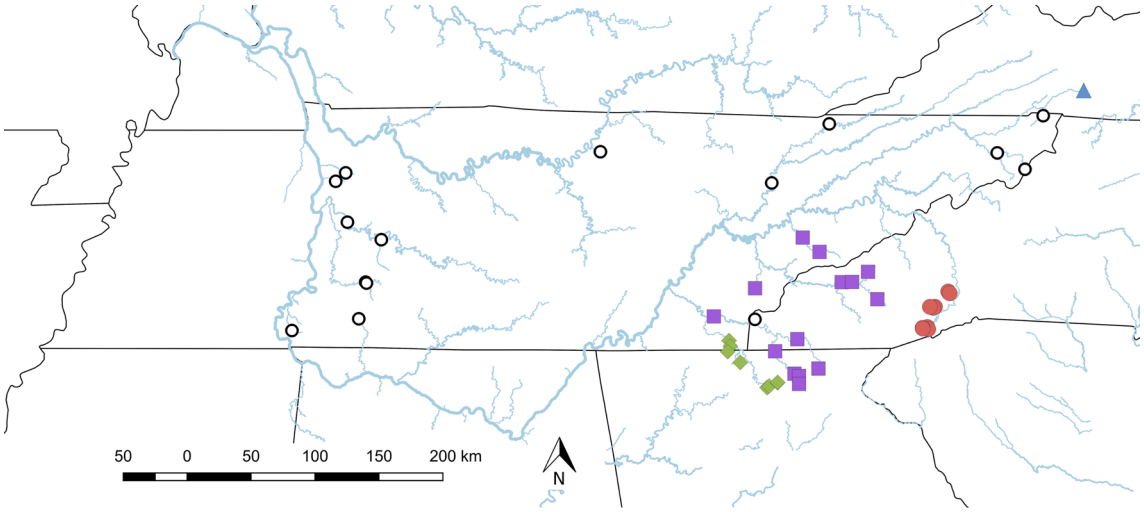

Fig. 5 Evidence of strong concordance between genetic cluster and watershed for Ocoee/Toccoa (diamonds), Hiwassee/Little Tennessee/Little (squares), French Broad (circles), Holston (triangles). Open circles indicate populations that were not clearly assigned to a genetic cluster

Little Tennessee/Little watersheds by excluding all other populations from the analysis; under this analysis, Little River clustered strongly from the other populations, and this clustering was also evident for STRUCTURE analysis with $K=5$. Remarkably, the Ocoee/Toccoa watershed forms a separate cluster, even though the Ocoee/Toccoa is a tributary of the Hiwassee, and hellbenders are found in the Hiwassee within 9 km of their confluence. It is possible that the genetic differentiation between these populations was established in the distant past, or is a product of recent anthropogenic changes to both watersheds. The Hiwassee has four hydroelectric dams (all within North Carolina), while the Ocoee/Toccoa has three hydroelectric dams in Tennessee and one in Georgia. Moreover the Ocoee River within Tennessee was massively impacted by the Copper Basin mining operations that effectively defaunated the mainstem river in the late 1800's and early 1900's as a result of acid mine drainage. It is tempting to imagine that the complete isolation produced by these changes may have contributed to the observed divergence between Hiwassee and Ocoee/Toccoa populations. However, there are numerous populations within each watershed that are also effectively isolated from each other by impoundments, and these clearly cluster by watershed, suggesting that any additional isolation caused by impoundments and mainstem impairment has been too recent to drive the observed patterns of differentiation. Therefore, it seems most likely that the divergence of Hiwassee and Ocoee/Toccoa hellbenders predates recent anthropogenic impacts and is quite puzzling given the strong clustering of Hiwassee populations with much more distant Little Tennessee and Little River populations. Possibly there have been historic stream capture or isolation events that have produced somewhat anomalous patterns of differentiation in some geographic locations such as Ocoee and Little River. Sabatino and Routman (2009) observed similar patterns for mitochondrial haplotypes in the southern Ozarks where hellbenders from rivers all draining into the White River and separated by only tens of miles exhibited up to 5.3% sequence divergence, which was much greater than the sequence divergence (0.7%) observed between North Ozark, Ohio and Susquehanna drainages which are separated by many hundreds of kilometers. This suggests very different patterns of geological history driving invasion and isolation events across the geographic range of hellbenders.

The two other well-supported clusters comprised all French Broad populations and the mainstem South Fork Holston populations respectively. Populations in the Watauga tended to show mixed ancestry drawn from both the Holston and Hiwassee/Little Tennessee/Little clusters, as did Holston1; this seems plausible given their intermediate geographic location between those watersheds. We had the most difficulty in assigning middle/western Tennessee River populations to any cluster, with no consistent geographic pattern that might explain the mixed ancestry. In many of these rivers sample size was low, which is primarily attributable to dramatic declines in these populations over recent

Table 4 AMOVA using the results of the STRUCTURE analyses to group sample sites into genetic populations

Source of variation	*df*	% of var.	*F* statistic	*P*
Within individual	683	0.300	R_{IT}=0.700	–
Among individual within population	678	0.039	R_{IS}=0.115	0.001
Among populations	4	0.660	R_{ST}=0.660	0.001

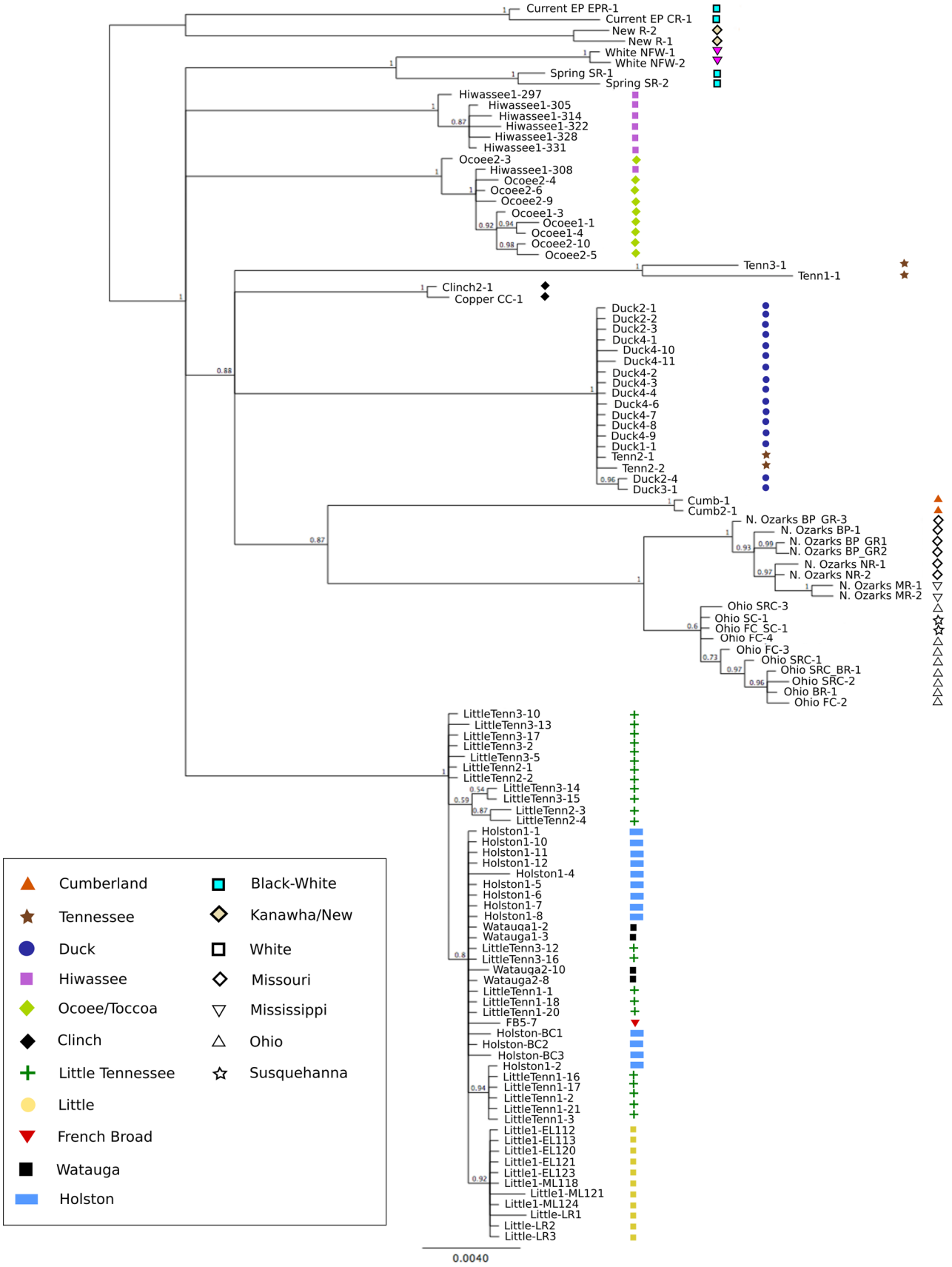

Fig. 6 Mitochondrial phylogeny of hellbenders based on concatenated *COI-CytB* sequences

Table 5 Haplotype (H_D) and nucleotide (N_D) diversity for concatenated Tennessee Valley hellbender *COI-Cytb* sequences

Population no.	Location, State	N	H_N	H_D	N_D
1	Cumb1, TN	2	1	0.000	0.00000
3	Tenn2, TN	2	2	1.000	0.00062
6	Duck2, TN	4	2	0.500	0.00031
8	Duck4, TN	10	3	0.378	0.00025
9	Hiwassee1, TN	7	6	0.952	0.00799
17	Ocoee1, TN	3	3	1.000	0.00144
18	Ocoee2, TN	6	6	1.000	0.00169
27	LittleTenn1, TN	8	2	0.536	0.00033
28	LittleTenn2, NC	4	3	0.833	0.00144
29	LittleTenn3, NC	9	5	0.861	0.00075
32	Little, TN	8	2	0.250	0.00031
43	Watauga1, TN	2	1	0.000	0.00000
44	Watauga2, TN	2	2	1.000	0.00062
45	Holston1, TN	10	3	0.378	0.00049

N is the number of sequences, and H_N is the number of observed haplotypes

decades (Miller and Miller 2005). Incomplete lineage sorting may also contribute to the appearance of shared genetic variation, especially in long lived and/or recently isolated species (Hudson and Coyne 2002).

In general, the patterns of clustering we have identified are broadly consistent with the hypothesis that genetic structure in the Tennessee Valley is strongly driven by limited opportunities for gene flow in turbid, warm water mainstem rivers. The topography and hydrology in the eastern part of the Tennessee Valley has produced mainstem rivers such as the Hiwassee and French Broad that were historically suitable habitat, so it is not surprising that populations within these watersheds show little genetic differentiation since extensive gene flow is likely to have occurred until recent anthropogenic activities produced fragmentation and isolation within the watersheds. In contrast in most cases the watersheds that are separated by mainstem Tennessee River form distinct clusters consistent with historic isolation limiting gene flow.

Phylogeographic structure

Levels of mitochondrial genetic variation varied considerably, although this was confounded by low sample sizes in many rivers. However in populations with multiple samples, nucleotide diversity was very low (< 0.001) in most populations compared with the levels of diversity observed in other salamander species (Phillips 1994; Rissler and Apodaca 2007). Possible explanations for the low levels of genetic diversity include a range expansion by relatively few founder individuals, or a genetic bottleneck event (Sabatino and Routman 2009), or hitchhiking effects associated with strong selection (Smith and Haigh 1974). The only exceptions were Hiwassee1, Ocoee1 and LittleTenn28, which also had the highest haplotype diversities. Coupled with the microsatellite data, Hiwassee River appears to be the epicentre of genetic diversity in the Tennessee Valley.

There was considerable concordance between our population genetic and phylogeographic analyses, with both data sets showing strong support for the major watersheds. The mitochondrial phylogeny supported the division of Hiwassee and Ocoee watersheds into separate clades, and significantly it also supported a Duck river clade in middle/west Tennessee that we were unable to resolve with microsatellites. Tonione et al. (2011) found that there was strong concordance between microsatellite and mitochondrial markers, with the exception of the New River drainage, which formed a strong monophyletic mitochondrial clade, but microsatellite markers tended to cluster with the Tennessee or Ohio watersheds depending on the value of K. Tonione et al. (2011) discussed possible explanations including the possibility that shared microsatellite markers may have evolved independently. However they argued that the longer coalescence time for autosomal microsatellite markers might result in reciprocal monophyly developing more quickly in maternally inherited haploid mitochondrial markers. Another possibility is that small sample sizes for the Duck watershed coupled with strong signals for other genetic populations may have caused STRUCTURE to have difficulty in resolving the group. Finally, male biased dispersal could allow the mitochondrial DNA markers to diverge while the autosomal markers still retain gene flow, although there is currently no evidence for male biased dispersal (Tonione et al. 2011). We believe the strong support for a monophyletic Duck river mitochondrial clade supports a separate and distinct evolutionary lineage. The Duck river is one of the most biologically diverse rivers in the United States with 146 species of fish, 53 species of freshwater mussels, and 22 freshwater snail species (Ahlstedt et al. 2004), with several endemic fish species.

Management implications

In contrast to the dramatic declines of eastern hellbenders across the rest of their range, many of the Tennessee Valley populations included in this study continue to persist at relatively high densities and with consistent recruitment, suggesting that the Tennessee Valley will play a disproportionately important role in preserving hellbenders from extinction. Moreover, the populations in the Tennessee Valley are spread over a wide geographic range and experience considerable diversity of physical conditions such as hydrology, geology, topography and climate. These factors should facilitate the three R's conservation framework of Shaffer and Stein (2000), incorporating wide geographic

representation, resilience to disturbance events, and redundancy in the case of catastrophic extinction events. Yet hellbender populations in the Tennessee Valley often occupy relatively short sections of river and are highly isolated (Freake and DePerno 2017). Thus they are individually at risk from demographic and environmental stochasticity leading to loss of fitness from genetic drift and inbreeding depression; and they are limited in their ability to share traits which may facilitate adaptation to human-induced environmental changes (Hendry et al. 2010; Lankau et al. 2011). In other words, these apparently healthy populations cannot be assumed to be safe in the foreseeable future, but may require interventions such as translocations and/or head-starting captive bred individuals to maintain appropriate demographic characteristics and genetic diversity. The combined mtDNA and microsatellite data indicate that major watersheds (Duck, Hiwassee, Ocoee, French Broad, Holston) should be considered separate management units. Our study suggests that when managing relatively healthy populations of hellbenders, movement of individuals within watersheds would be appropriate, but care should be taken when considering movements across watersheds to avoid introducing maladaptive alleles and promoting outbreeding depression. Unfortunately our approach of using neutral markers is useful for identifying patterns of isolation and differentiation, but provides no insight into site-specific differences in adaptive traits (Hedrick 2001). In contrast, declining populations may require a different strategy; population declines have been most severe in the Duck and Cumberland watersheds (Miller and Miller 2005; Freake and DePerno 2017) and so these populations are particularly in need of urgent intervention to prevent extinction. It will likely be very hard to set up captive breeding programs using adults drawn from these populations given the difficulty in even locating individuals in the wild, and so it may be necessary to rely on hellbenders from other quite distant populations drawn from divergent mitochondrial lineages. Using hellbenders from source populations with relatively high genetic diversity (e.g. Hiwassee) may actually increase the probability of successful translocations, and the increase in local genetic variation could enhance the resilience of the target populations to the emerging challenges of disease (Martel et al. 2014) and climate change (Caruso et al. 2014), outweighing the potential risk of outbreeding in some individuals (Tonione et al. 2011).

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