

Within-river gene flow in the hellbender (*Cryptobranchus alleganiensis*) and implications for restorative release

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Abstract Understanding how populations are genetically and demographically connected is beneficial for species management, since gene flow and dispersal contribute to genetic diversity and population persistence. For hellbenders (*Cryptobranchus alleganiensis*), an aquatic salamander species experiencing dramatic declines in population size, fine-scale (i.e. within river) patterns of genetic diversity and gene flow are not well understood. Previous findings indicate that hellbenders are habitat specialists that exhibit extreme site fidelity and low vagility, suggesting that gene flow is restricted among the several, discrete habitat patches within a river. Using 15 polymorphic microsatellite loci and 497 hellbender samples from four Missouri rivers, we assessed fine-scale patterns of genetic diversity in order to infer population connectivity and aid in population management. Results indicate moderate levels of genetic variation ($H_O = 0.66\text{--}0.78$) with little differentiation among habitat patches (avg. $F_{ST} = 0.002$) and no evidence of isolation by distance. Our data suggest that hellbender gene flow has

been extensive even among habitat patches separated by distances greater than >100 km. These results are useful for hellbender management, especially in terms of making informed decisions regarding restorative releases of cap- tively propagated individuals.

Keywords Hellbender · Conservation genetics · Gene flow · Dispersal · Restorative release

Introduction

Demographic stability and genetic diversity are two important factors to consider when making management decisions for species of conservation concern. In addition to being characterized by small numbers, endangered populations may exhibit diminished genetic variation as a result of inbreeding and genetic drift (Frankham 1995). The deterioration of genetic diversity is further exacerbated when habitat is marked by discontinuity and movement between isolated habitat patches is restricted (Wright 1943). Once isolated populations begin to differentiate, separate and individualized management plans may be required (reviewed in Fraser and Bernatchez 2001).

The mechanism that prevents isolation and drives demographic and genetic connectivity is dispersal. As long as it is followed by successful reproduction, dispersal provides a buffer against population differentiation by facilitating the movement of alleles between populations (i.e. gene flow; Slatkin 1985). Gene flow, by contributing to population connectivity, helps to maintain genetic diversity, and thus, can promote the adaptive potential of species (Reed and Frankham 2003). Conversely, gene flow, in the presence of local adaptations, can introduce mal- adaptive alleles into a population, potentially resulting in

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negative fitness consequences for immigrants and their offspring (Lenormand 2002).

The hellbender (*Cryptobranchus alleganiensis*), an elusive, long-lived and fully aquatic salamander species, was once abundant throughout large portions of the eastern United States. However, in the past few decades, hellbenders have undergone precipitous declines throughout much of their distribution (Gates et al. 1985a; Pflingsten 1989; Trauth et al. 1992; Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011b). In the state of Missouri, the only location where both hellbender subspecies (eastern hellbender, *C. a. alleganiensis*, and Ozark hellbender, *C. a. bishopi*) can be found, population sizes have decreased by more than 75 % (Wheeler et al. 2003). In an effort to augment hellbender numbers, the Missouri Department of Conservation and the Saint Louis Zoo have invested in a captive breeding program in conjunction with the U.S. Fish and Wildlife Service, U.S. Forest Service, and National Park Service (Briggler et al. 2012; Ettling et al. 2013). Before propagated individuals can be used to augment wild populations, a more detailed understanding of population connectivity and gene flow is needed as current knowledge regarding within river population genetic structure is lacking. An understanding of the population genetic structure in Missouri hellbenders will help to prevent, during restorative release, the disruption of any naturally established genetic pools and avoid the potential introduction of incompatible (i.e. poorly adapted) alleles (Storfer 1999).

From previous studies, we know that clear genetic structuring occurs at two hierarchical levels, with genetic differentiation identified between the eastern and Ozark subspecies, and among river basins within subspecies (Templeton et al. 1990; Sabatino and Routman 2009; Crowhurst et al. 2011; Unger et al. 2013). Based on broad-scale genetic observations, populations within rivers appear to be panmictic (Templeton et al. 1990; Routman 1993). However, these broader-scale conclusions were based on a small number of within-river samples analyzed with allozymes and/or mitochondrial DNA. These loci may not have the power to detect the finer-scale patterns of genetic structure that can be distinguished using microsatellite data, which are highly polymorphic and have greater power to resolve population differentiation (e.g. Estoup et al. 1998; Ross et al. 1999). Indeed, observations based on movements of adult and/or larger juvenile hellbenders conflict with an assertion of panmixia, and suggest that additional genetic structuring may be present. Telemetry and mark-recapture studies have found that larger hellbenders are rather sedentary, exhibiting extreme fidelity to a small home range (Nickerson and Mays 1973; Peterson and Wilkinson 1996). Home range sizes in Missouri are especially small, averaging less than 100 m² for both the Ozark

(Coatney 1982) and eastern subspecies (Peterson and Wilkinson 1996). In other regions, however, home range sizes have been found to be substantially larger (for example, 1,545 m² in Indiana, Burgmeier et al. 2011a). Additionally, hellbenders are habitat specialists that require cool, swift flowing streams with ample rock cover (Smith 1907; Fobes 1995; Bodinof et al. 2012b). In Missouri streams, these habitats are often isolated from one another (>1 km), with distances between habitat patches being typically greater than maximum distances observed in hellbender movements (Nickerson and Mays 1973; Gates et al. 1985b; Burgmeier et al. 2011a; Bodinof et al. 2012a). There are no data available regarding larval and or young juvenile dispersal.

Therefore, a fine-scale assessment of genetic structuring is needed to better understand how population decline, life history, and local environments impact connectivity between hellbender habitat patches within a single stream. To gain a more thorough knowledge of population connectivity, we used microsatellite loci to investigate fine-scale patterns in genetic structure within rivers. We address the following questions: (1) do Missouri hellbender populations exhibit fine-scale genetic structuring? (2) do levels of diversity differ between habitat patches within a single stream? We conclude with a discussion of implications for the management of this species based upon investigated population connectivity.

Due to the patchiness of hellbender habitat and observed low vagility of adults, we predicted that we would observe significant differentiation between spatially discrete habitat patches. In addition, if a stream is divided into discrete subpopulations that possess their own distinct gene pools, we hypothesized that diversity would appear to be high when these subpopulations are pooled together even if diversity within subpopulations is low. Therefore, we predicted genetic diversity would be high within streams but low within patches.

Methods

Sample collection and laboratory procedures

Hellbender tail clippings ($n = 497$) were collected over a 7 year period (2005–2011) by the Missouri Department of Conservation. Upon capture, hellbenders were PIT-tagged to facilitate future identification and to prevent repeat sampling. Each hellbender was categorized by life stage (adult or juvenile) based upon size (weight and total length; reviewed in Petranka 1998) and/or evidence of sexual maturity (e.g. swollen cloaca). Samples were collected from four Missouri rivers, with each subspecies represented by two rivers (Table 1). For the eastern hellbender,

Table 1 Sample size and extent and characteristics of hellbender habitat within the four rivers analyzed

River	No. of hellbenders sampled	Total habitat length ^a (km)	Average distance between habitat patches (km)	No. of habitat patches	No. of patches with ≥ 5 hellbenders
Ozark hellbender (<i>C. a. bishopi</i>)					
Current	92	169	6.0	25	6
North Fork of White	159	29	1.7	21	7
Eastern hellbender (<i>C. a. alleganiensis</i>)					
Big Piney	131	108	4.5	28	7
Niangua	115	49	2.4	18	6

^a Total habitat length = distance from the most upstream site to the most downstream site, including the intervening, non-suitable habitat

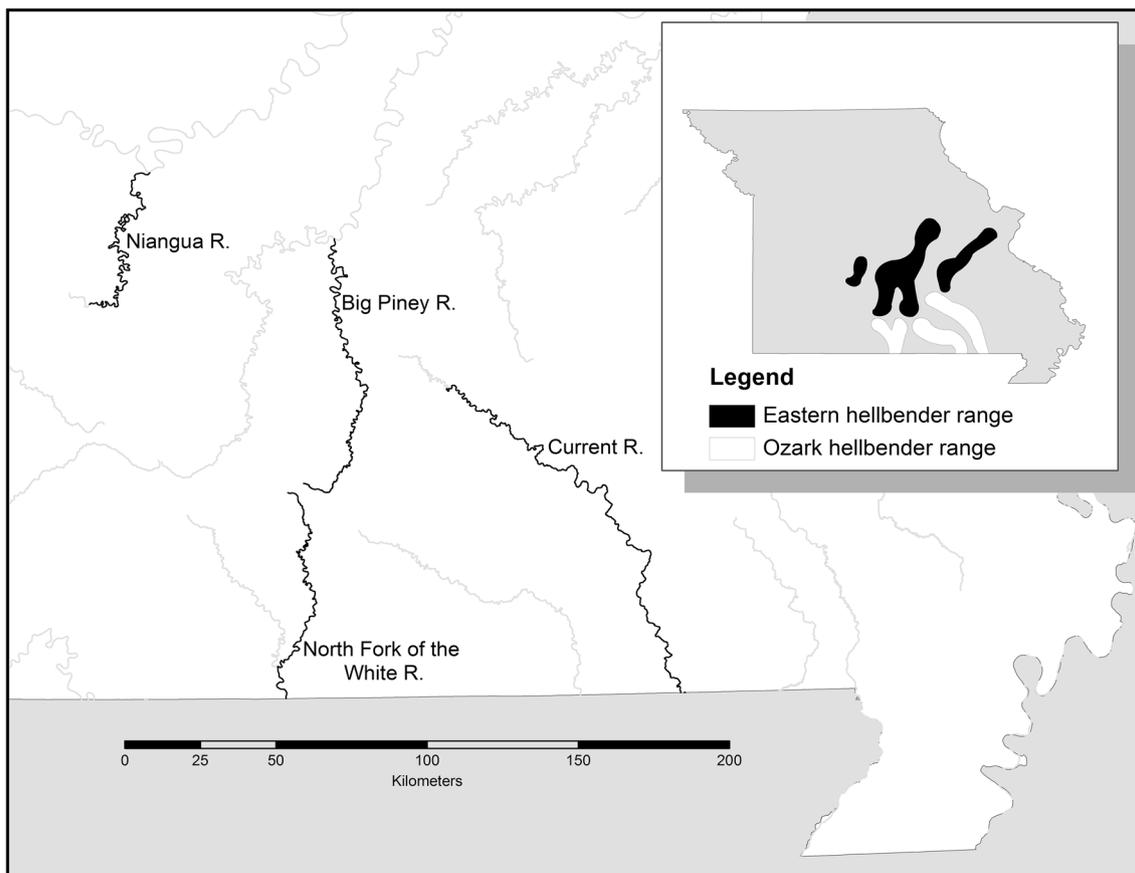


Fig. 1 Location of the four rivers where samples were collected. Map inset shows the distribution of eastern (*C. a. alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders in the state of Missouri (USA)

samples originated from the Niangua and Big Piney Rivers; Ozark hellbender samples were obtained from the North Fork of the White River and Current River (Fig. 1). The total length of habitat (measured as the stream distance between the most upstream site and the most downstream site), and the number of habitat patches along this length, varied among rivers (Table 1). Total habitat lengths ranged from 29 to 169 km, and lengths (measured as the distance between center points) between habitat patches within

rivers ranged from 1.7 to 6 km. Characteristics of habitat patches vary (for example, see Bodinof 2010).

Samples were preserved in absolute ethanol and stored frozen. Extractions were carried out using Qiagen DNeasy kits (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. We amplified 15 polymorphic microsatellite loci (Johnson et al. 2009; Unger et al. 2010), in four multiplexed polymerase chain reactions (PCR; Supplementary Table S1). Reactions were carried out in

8 μ l volumes according to the protocol outlined in the Qiagen multiplex kit (adjusted for a reduced volume), incorporating approximately 7.5 ng DNA and 0.65 μ l bovine serum albumin (BSA). Amplifications were performed on Eppendorf ep (Eppendorf, Hamburg, Germany) thermocyclers and were initiated at 95 °C for 15 min, followed by 35 cycles of: 94 °C for 30 s, multiplex specific T_A (Supplementary Table S1) for 90 s, 72 °C for 60 s. A final extension of 60 °C was carried out for 30 min. Positive and negative controls were included in all reactions to ensure allele scoring consistency and absence of reagent contamination, respectively.

Fragment analysis was performed at the University of Missouri DNA Core facility on an ABI 3730xl DNA analyzer (Applied Biosystems), where fragment peak sizes were standardized by the addition of LIZ 600 (Genescan). Resulting chromatograms were visualized and genotyped in GENEMARKER software, version 1.95 (Softgenetics). After all samples were genotyped, a genotyping error rate was calculated by reanalyzing approximately 8 % randomly chosen samples which were subjected to a second round of PCR using fresh aliquots of extracted DNA.

Statistical analyses

Crowhurst et al. (2011) identified genetic differentiation among all rivers included in this study. Since our goal was to investigate fine-scale (or within river) patterns of genetic diversity, we conducted the analyses for the four rivers separately unless otherwise noted.

To test whether the loci had sufficient power to differentiate individuals, a probability of identity, or $P_{(ID)}$ test (Paetkau et al. 1995) was performed in GENALEX v. 6.41 (Peakall and Smouse 2006). Significance for $P_{(ID)}$ was set at $p \leq 0.001$ following the suggested threshold proposed by Waits et al. (2001). Resulting values determine the minimum number of loci each sample must be successfully genotyped at to reliably identify individuals even among siblings.

Prior to pooling samples across years, we performed an analysis of molecular variance (AMOVA) in ARLEQUIN v. 3.5 (locus by locus, 1,000 permutations; Excoffier and Lischer 2010). No significant temporal genetic variation was observed (<0 %, p value = 1.00), and thus samples from all years were pooled for subsequent analyses.

We tested for deviations from genotype frequencies expected under Hardy–Weinberg equilibrium and for linkage disequilibrium in GENEPOP v. 4.0.10 (Rousset 2008). Because these tests involve multiple comparisons, a Bonferroni correction (Rice 1989) was applied to decrease the chance of Type I error. We tested for the presence of null alleles using MICROCHECKER v. 2.2.3 (Van Oosterhout et al. 2004). The program LOSITAN (Antao et al.

2008) was used to test for evidence that the loci conform to the general expectation of neutrality, where the presence of an F_{ST} outlier would indicate nonconformity, suggesting the influence of selection. Analyzing all four rivers at once with each river representing a single population, we had the program estimate a forced F_{ST} and an F_{ST} under neutral expectations using 100,000 simulations and both the Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM). A locus was considered to be under the influence of selection only if it was observed to be a potential candidate for selection in both the SMM and IAM models at the 95 % confidence level.

To address potential concerns involving sampling bias—specifically, whether or not samples represent highly related individuals—we used COANCESTRY (Wang 2011) to estimate both population mean relatedness (R) and relatedness between all possible pairs of hellbenders within river using Wang's (2007) triadic likelihood estimator (TrioML).

Within river genetic diversity

We used GENALEX (Peakall and Smouse 2006) to calculate heterozygosity and allelic richness. We further investigated allelic richness by adjusting for differences in sample size (i.e. rarified allelic richness) among rivers and among habitat patches within a river, and looking for differences in private alleles with HP-Rare (Kalinowski 2005).

Within river population genetic structure

Using AMOVA, we evaluated genetic partitioning at the following fine-scale hierarchical levels: among rivers supporting the same subspecies, among habitat patches within rivers, among individuals within habitat patches, and within individuals. The AMOVA was performed in ARLEQUIN (Excoffier and Lischer 2010) using the locus-by-locus calculation and 1,000 permutations.

To estimate genetic distances between habitat patches, we compared differences in allelic frequencies between patches using the permutation calculation for population pairwise F_{ST} and 10,000 permutations in ARLEQUIN (Excoffier and Lischer 2010). Because sampling was uneven among patches within a stream, we limited the pairwise F_{ST} calculation to only those patches that supplied ≥ 5 samples to ensure a more accurate comparison. The resulting F_{ST} matrix was utilized in a Mantel test, in conjunction with associated stream distances, to test for isolation by distance in GENALEX (Peakall and Smouse 2006).

An additional Mantel test was performed in the R package ECODIST (Goslee and Urban 2007). This test

plotted relatedness values, obtained in COANCESTRY, between pairs of hellbenders against the pair's associated stream distance (km) to illustrate how related individuals are distributed in space.

We chose to evaluate the number of distinct genetic clusters (K) both between and within rivers using two approaches, each of which do not require a priori delineations of population structure and/or information regarding sampling locality per individual (i.e. habitat patch). Firstly, we used STRUCTURE v. 2.3.4 (Pritchard et al. 2000) in conjunction with STRUCTURE HARVESTER (Earl and vonHoldt 2012), which allowed us to estimate K following a Bayesian approach and the Evanno et al. (2005) method. According to the Evanno method, the most likely number of K clusters is identified by the second order rate of change in the log likelihood of the probability of K (ΔK). Using the admixture model and assuming correlated allele frequencies, STRUCTURE was run at two separate levels: among rivers and within each river. STRUCTURE parameters were set to a burn-in length of 10^5 , followed by 10^5 Monte Carlo Markov Chain repetitions, and 10 independent runs per K , where $K = 1-10$. Because STRUCTURE may not be able to detect population genetic substructure when F_{ST} values are small ($F_{ST} < 0.03$; Latch et al. 2006), we also evaluated K using the ADEGENET package (Jombart 2000), implemented in R v. 2.15.1 (R Core Team 2012). ADEGENET is a multivariate approach which applies discriminant analysis of principal components (DAPC) and Bayesian Information Criterion (BIC) to partition any existing genetic structure. Again, we evaluated the potential for one to 10 populations among rivers, and then in separate analyses, evaluated for one to 10 populations within each river. Here the optimal number of clusters is identified by the lowest BIC value, with attention paid to the amount of change occurring between BIC values (i.e. where the amount of change becomes negligible, and thus, indicates that additional clusters no longer add significantly to the model).

Genetic consequences of population decline

Documented declines in hellbender population size suggest that Missouri populations may have experienced a genetic bottleneck. Populations that have undergone a recent reduction in effective population size (N_e) will exhibit a reduction in allelic diversity, but because heterozygosity levels decrease at slower rate than the loss of alleles (Nei et al. 1975), heterozygosity levels appear excessive when compared to the number of alleles present (Cornuet and Luikart 1996; Luikart and Cornuet 1998). Data were analyzed for heterozygosity excess, which would be indicative of a relatively recent (within $\sim 2-4N_e$ generations) bottleneck event, using the program BOTTLENECK (Piry et al.

1999). We adjusted the parameters to 10,000 replications and utilized the infinite allele model (IAM, Kimura and Crow 1964), the strict stepwise mutation model (SMM, Kimura and Ohta 1978), and the two-phase model (TPM, Di Rienzo et al. 1994). TPM parameters were set according to the author's suggestion with 95 % adherence to SMM and the variance of multistep mutations as 12.

Results

Our $P_{(ID)}$ evaluation indicated that samples should be genotyped at a minimum of 9 loci to differentiate between siblings. Samples genotyped at less than 9 loci were excluded from analyses. The observed genotyping error rate across all loci was 1.7 % among the reanalyzed 8 % randomly chosen samples. When there was a disagreement in allele calls, the sample was reanalyzed until a consensus call was achieved. Three of the four rivers deviated from Hardy–Weinberg equilibrium after Bonferroni correction due to a significant deficit in heterozygosity at a single locus (Table 2): Big Piney River, Call 347 ($p < 0.0001$); Current River, Call 266* ($p < 0.0001$); North Fork of the White River, Call 4 ($p = 0.0018$). The Niangua River conformed to expectations under Hardy–Weinberg equilibrium when analyzed across all loci. However, Call 266* was out of equilibrium in this river ($p = 0.0014$) and exhibited heterozygosity deficit ($p < 0.0001$).

Several pairs of loci were in linkage disequilibrium after Bonferroni correction but the pairs varied among rivers: Big Piney River, Call 13 and Call 9 ($p = 0.0014$); Current River, Call 127 and Call 204 ($p < 0.001$); North Fork of the White River, Call 4 and Call 26 ($p = 0.0017$), Call 17 and Call 347 ($p = 0.0032$), and Call 127 and Call 341 ($p = 0.0032$). Because of the inconsistency across rivers, no loci were removed from analyses as a result of linkage disequilibrium.

Analysis in MICROCHECKER identified several loci that had a high probability of null alleles. Call 266* was removed from the Current and Niangua River analyses (null alleles at 13.76 and 7.36 %, respectively). Call 347 was removed from the Big Piney River analyses (null alleles at 28.44 %).

Four of the 15 genotyped microsatellite loci presented F_{ST} outliers at the 95 % confidence level in LOSITAN, thus indicating a potential influence of natural selection. These “non-neutral” loci identified were some of the least polymorphic, typically possessing less than 3 alleles in each river—most of which were unique to the river and/or subspecies. Inspection of the published sequences for these loci revealed no high similarity to any described genes in the National Center for Biotechnology Information (NCBI) GenBank database. Although it is unlikely that

Table 2 Hellbender genetic summary statistics for each river analyzed. A, A_E, H_O, and H_E represent number of alleles, number of effective alleles, and observed and expected heterozygosities

Locus	Current River <i>n</i> = 92				North Fork of the White River <i>n</i> = 159				Big Piney River <i>n</i> = 131				Niangua River <i>n</i> = 115			
	A	A _E	H _O	H _E	A	A _E	H _O	H _E	A	A _E	H _O	H _E	A	A _E	H _O	H _E
Cral 13 ^S	4 ^S	1.22 ^S	0.18 ^S	0.18 ^S	2 ^S	1.04 ^S	0.04 ^S	0.04 ^S	3 ^S	1.64 ^S	0.39 ^S	0.39 ^S	2 ^S	1.03 ^S	0.03 ^S	0.03 ^S
Call 26	13	9.81	0.89	0.90	11	4.99	0.83	0.80	10	6.38	0.79	0.84	12	8.39	0.88	0.88
Call 127	9	3.72	0.68	0.73	4	2.27	0.55	0.56	8	5.93	0.76	0.83	6	3.90	0.77	0.74
Call 351 ^S	14 ^S	10.03 ^S	0.92 ^S	0.90 ^S	10 ^S	4.98 ^S	0.82 ^S	0.80 ^S	12 ^S	6.38 ^S	0.79 ^S	0.84 ^S	10 ^S	6.07 ^S	0.85 ^S	0.84 ^S
Call 204	11	7.09	0.81	0.86	11	2.97	0.61	0.66	11	3.90	0.75	0.74	11	7.22	0.85	0.86
Call 282	9	6.11	0.78	0.84	7	3.18	0.70	0.69	6	2.38	0.55	0.58	8	4.18	0.78	0.76
Cral 17	19	11.07	0.91	0.91	7	3.20	0.67	0.69	9	3.27	0.64	0.69	7	5.29	0.77	0.81
Cral 10	10	2.96	0.64	0.66	4	2.04	0.50	0.51	3	1.37	0.29	0.27	3	2.11	0.51	0.53
Cral 4 ^S	3 ^S	1.03 ^S	0.03 ^S	0.03 ^S	3^{N,S}	1.53^{N,S}	0.26^{N,S}	0.35^{N,S}	3 ^S	1.77 ^S	0.45 ^S	0.44 ^S	3 ^S	2.12 ^S	0.52 ^S	0.53 ^S
Call 232	14	7.75	0.86	0.87	6	3.01	0.69	0.67	7	5.74	0.85	0.83	6	1.34	0.29	0.27
Call 205	9	4.62	0.73	0.78	12	4.54	0.74	0.78	7	3.17	0.70	0.69	7	4.43	0.78	0.77
Call 341	15	7.53	0.85	0.87	8	4.05	0.75	0.75	8	2.13	0.54	0.53	9	2.04	0.49	0.51
Call 266*	13^N	8.19^N	0.63^N	0.88^N	13	4.89	0.77	0.80	6	3.63	0.73	0.72	8^N	6.61^N	0.72^N	0.85^N
Cral 9 ^S	3 ^S	1.02 ^S	0.02 ^S	0.02 ^S	8 ^S	1.00 ^S	0.00 ^S	0.00 ^S	10 ^S	1.38 ^S	0.28 ^S	0.28 ^S	7 ^S	1.48 ^S	0.35 ^S	0.32 ^S
Call 347	7	2.54	0.60	0.61	1	5.20	0.78	0.81	4^N	5.90^N	0.34^N	0.83^N	2	4.46	0.81	0.78
Average ^A	10.20	5.65	0.64	0.67	7.13	3.26	0.58	0.59	7.13	3.66	0.59	0.63	6.73	4.05	0.63	0.63
Average ^R	11.60	6.32	0.78	0.80	8.27	3.67	0.69	0.70	7.50	3.79	0.66	0.67	7.60	4.34	0.69	0.69

Parameters in bold indicate significant deviation from HWE with a Bonferroni correction ($p \leq 0.0033$)

^S The locus was removed because it was a candidate for selection ($p \leq 0.05$)

^N The locus was removed due to null alleles observed at a frequency >7 %

^A Averaged across all 15 loci

^R Averaged across a reduced number of loci (only those remaining after HWE/null alleles, selection tests)

microsatellite loci themselves would be the target of selection, Cral 4, Cral 9, Cral 13, and Call 351 were removed from additional analyses as their inclusion may have introduced bias.

Mean relatedness values (*R*), based upon the TrioML estimator in COANCESTRY, indicated that sampling was not biased towards highly related individuals. Relatedness was low in all rivers and only a few instances of parent-offspring, full-sib, and half-sib relationships were identified. The Current River had the fewest number of highly related individuals ($R \geq 0.50$; Current River, $n = 4$; North Fork of the White River, $n = 100$; Big Piney River, $n = 63$; Niangua River, $n = 44$; $n =$ the number of within-river pairwise relatedness comparisons where R was found to be ≥ 0.50 , total number of comparisons were in the thousands for each river). Furthermore, relatedness at the within river level (mean $R = 0.058-0.039$) was comparable to relatedness within habitat patches, where mean $R \leq 0.056$.

Genetic comparisons between rivers

Because a different set of loci remained in the analyses for each river, comparisons of genetic diversity among rivers

were based on the entire set of loci genotyped (Table 2). Observed allelic richness averaged across these 15 loci was similar for North Fork of the White, Big Piney, and Niangua Rivers with an average of 7.13, 7.13, and 6.73 alleles per locus, respectively. The Current River had higher observed allelic richness with an average of 10.2 alleles per locus. The Current River also had the highest observed heterozygosity (averaged across all 15 loci) at 0.64. Observed heterozygosity (averaged across all 15 loci) for the other three rivers was again similar (0.58–0.63). The observed heterozygosity was slightly lower than the expected heterozygosity for all rivers except for the Niangua River where the observed heterozygosity matched expected heterozygosity.

Within river genetic diversity and population structure

Using the pruned set of loci (ten for all rivers except the North Fork of the White River which had 11), and making comparisons among habitat patches within a river, analysis in HP-Rare indicated that no habitat patch was more genetically diverse than another. All habitat patches shared similar levels of allelic richness with no stream segment

Table 3 AMOVA assigning hierarchical amounts of genetic variation in hellbender (*C. a. bishopi* and *C. a. alleganiensis*) populations

Source of variation	Fixation indices	Percent variation	<i>p</i> value
Ozark hellbenders			
Between rivers (ϕ_{CT})	0.255	25.463	<0.001
Among habitat patches within rivers (ϕ_{SC})	0.003	0.220	0.559
Within habitat patches (ϕ_{IS})	0.039	2.892	0.001
Within individuals (ϕ_{IT})	0.286	71.425	<0.001
Eastern hellbenders			
Between rivers (ϕ_{CT})	0.161	16.079	<0.001
Among habitat patches within rivers (ϕ_{SC})	-0.002	-0.167	0.727
Within habitat patches (ϕ_{IS})	0.027	2.291	0.004
Within individuals (ϕ_{IT})	0.182	81.797	<0.001

(i.e. upstream, midstream, downstream) containing more private alleles.

AMOVA revealed no significant genetic differentiation among habitat patches in a single river for either subspecies (*C. a. alleganiensis*, $p = 0.73$; *C. a. bishopi*, $p = 0.56$; Table 3). Instead significant amounts of variation were observed among individuals within habitat patches (*C. a. alleganiensis*: 2.29 %, $p < 0.01$; *C. a. bishopi*: 2.89 %, $p = 0.001$), with the greatest amount of variation harbored within individuals (*C. a. alleganiensis*: 81.8 %, $p < 0.001$; *C. a. bishopi*: 71.43 %, $p < 0.001$), as expected.

No significant differences in genetic distance (F_{ST} ; ARLEQUIN) were found between within-river pairs of habitat patches in the Current, North Fork of the White, and Niangua Rivers, and only one habitat patch within the Big Piney River was significantly different from two of the 7 other habitat patches after Bonferroni correction (p values ≤ 0.0036). The most distant habitat patches within a river were not proportionally more differentiated than habitat patches in close proximity (Table 4).

No significant correlation between genetic distance (F_{ST}) and geographic distance was observed in any river using the Mantel tests performed in GENALEX ($r = 0.12-0.27$, $p > 0.05$), indicating no statistical support for isolation by distance. Similarly, no significant correlation was observed between relatedness and geographic distance in the ECODIST Mantel test ($r = -0.03-0.01$, $p > 0.05$; Fig. 2). A variety of relatedness values were found at each distance interval—including at the smallest distance interval (0 km)—indicating that some highly related individuals as well as unrelated individuals occupy the same habitat patch. There was no perceptible pattern in kinship across the stream length for any of the four rivers (Fig. 2).

When evaluating population genetic structuring at the among-rivers level, the most likely number of *K* clusters was evident in both STRUCTURE (Table 5) and ADEGENET (Table 6). In agreement with previous research (Crowhurst et al. 2011), four distinct populations were identified—each of which represented one of the four rivers analyzed with 100 % of individuals assigning to the river of origin. However, at the within-river level, there

Table 4 Genetic differentiation (F_{ST}) between hellbender habitat patches within the (a) current (above the diagonal) and Niangua (below the diagonal) rivers, and within the (b) North Fork of the White (above the diagonal) and Big Piney (below the diagonal) rivers

CU							
NI	Habitat 1	Habitat 2	Habitat 3	Habitat 4	Habitat 5	Habitat 6	
a							
Habitat 1	–	0.002	0.003	0.001	0.008	–0.004	
Habitat 2	–0.011	–	0.030	0.009	0.007	0.006	
Habitat 3	–0.029	–0.017	–	0.016	–0.005	–0.009	
Habitat 4	–0.013	0.000	–0.005	–	–0.009	–0.019	
Habitat 5	–0.013	–0.005	–0.020	0.004	–	–0.016	
Habitat 6	–0.001	0.007	–0.007	0.001	–0.012	–	
NF							
BP	Habitat 1	Habitat 2	Habitat 3	Habitat 4	Habitat 5	Habitat 6	Habitat 7
b							
Habitat 1	–	–0.006	–0.025	0.007	–0.021	–0.007	0.002
Habitat 2	0.059	–	–0.031	–0.019	–0.003	–0.003	–0.000
Habitat 3	0.007	0.030	–	0.010	–0.016	–0.011	–0.001
Habitat 4	0.004	0.045	–0.008	–	–0.003	0.015	0.019
Habitat 5	–0.013	0.033	–0.019	–0.013	–	–0.012	–0.003
Habitat 6	0.007	0.047	0.007	0.007	–0.011	–	0.004
Habitat 7	–0.006	0.038	0.003	–0.003	–0.003	0.016	–

F_{ST} values significant after Bonferroni correction are indicated in bold

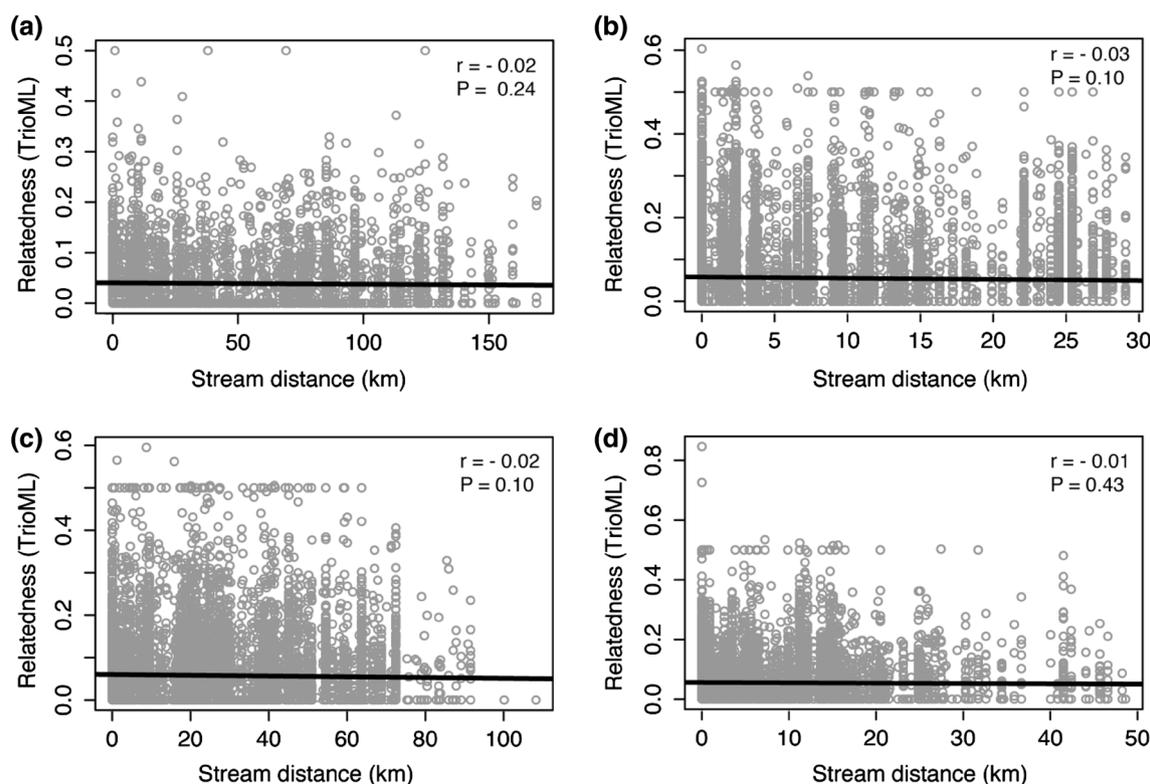


Fig. 2 Mantel graphs indicating the correlation between relatedness (TrioML) and spatial proximity in hellbenders for the **a** Current River, **b** North Fork of the White River, **c** Big Piney River, and **d** Niangua

River. *Trend line* for each river is indicated with a **bold black line**. Note the low levels of highly related (TrioML ≥ 0.50) individuals, as well as the distance between these individuals

Table 5 STRUCTURE HARVESTER results, indicating the average log likelihood for the probability of each K cluster (Avg LnP (K)) and the second order rate of change in the probability (ΔK) at $K = 1-10$ distinct genetic clusters or sub-populations

K	All rivers		Big Piney River		Niangua River		Current River		North Fork of the White River	
	Avg LnP (K)	ΔK	Avg LnP (K)	ΔK	Avg LnP (K)	ΔK	Avg LnP (K)	ΔK	Avg LnP (K)	ΔK
1	-30370.20	-	-3758.92	-	-3488.99	-	-3641.73	-	-5147.95	-
2	-25180.50	8.204	-3852.85	0.414	-3548.95	2.561	-3654.13	1.925	-5165.91	4.346
3	-22210.23	2.770	-3923.98	0.236	-3524.26	1.822	-3687.16	0.972	-5220.37	0.547
4	-20663.43	423.600	-4016.93	0.194	-3576.88	0.927	-3668.27	1.541	-5255.05	0.073
5	-20736.81	0.697	-4130.75	0.460	-3566.65	0.141	-3702.49	1.502	-5294.70	0.900
6	-20858.05	0.312	-4174.16	0.367	-3546.02	0.768	-3852.59	0.962	-5282.04	0.767
7	-21048.84	0.492	-4290.54	0.654	-3588.28	1.175	-3845.29	0.450	-5330.49	0.660
8	-21100.17	0.400	-4274.16	0.223	-3535.12	3.210	-3924.47	0.557	-5307.26	0.376
9	-21250.61	0.730	-4176.46	0.881	-3648.95	0.700	-3857.94	0.141	-5355.39	0.529
10	-21724.15	-	-4405.62	-	-3606.23	-	-3828.31	-	-5317.00	-

Large support for population structuring is seen at the among-river level (i.e. all rivers), but lack of support is evident for additional sub-structuring within rivers. Analyses were run at two levels, independently: among rivers and within a river

ΔK values in bold indicate the most likely number of clusters (K)

was no support for population sub-structuring in either STRUCTURE (Table 5) or ADEGENET (Table 6), indicating that each river is likely one population or genetic cluster with no habitat patch representing a unique subpopulation.

Genetic consequences of population decline

None of the rivers exhibits the signature of a recent genetic bottleneck, except under the IAM model where all rivers show significant heterozygosity excess at p values ≤ 0.05

Table 6 Bayesian Information Criterion (BIC) values from ADE-GENET revealing clear population genetic structure among all four hellbender rivers ($K = 4$, BIC value indicated in bold), but no support for population genetic structure within rivers

BIC values					
K	All rivers	Big Piney River	Niangua River	Current River	North Fork of the White River
1	895.67	166.51	146.99	134.57	221.84
2	787.55	164.04	145.06	134.50	216.99
3	721.24	162.38	144.77	135.49	215.90
4	680.48	162.49	145.17	137.02	215.72
5	676.79	163.34	146.36	138.45	216.82
6	676.25	164.94	147.67	140.59	218.21
7	676.01	166.40	149.70	142.82	219.22
8	678.00	168.88	151.07	145.15	222.09
9	676.93	169.88	153.10	147.50	223.83
10	678.03	172.60	155.05	–	225.82

Analyses were run at two levels, independently: among rivers and within a river

with the Wilcoxon one-tailed test. Because the IAM is prone to erroneous detection of bottlenecks (Luikart and Cornuet 1998), and no support for a genetic bottleneck was detected with the SMM or TPM models, we do not consider this result to be informative.

Discussion

Gene flow and inferred dispersal

Among the several habitat patches that we analyzed, we found only one instance of genetic differentiation and/or isolation. The lack of within river structuring suggests extensive within river gene flow—a conclusion that aligns well with the results of Templeton et al. (1990) and Routman (1993), but contradicts predictions based on hellbender movement patterns extrapolated from behavioral and direct observational data. Movement can be categorized into dispersal and migration, where dispersal indicates movement from the natal habitat patch to future reproductive patches and migration is any movement from one habitat patch to another patch following natal dispersal and first reproductive event (i.e. movement between breeding sites). It is possible that a genetic measurement of movement in this study does not accurately capture population demographics; discrepancies between observational measures of dispersal and those measures inferred from estimates of gene flow are not uncommon because direct and indirect methods are actually measuring differing components of population connectivity (demographic vs. genetic connectivity) at differing time scales

(“instantaneous” vs. ecological timescales; Slatkin 1987; Lowe and Allendorf 2010). Because these populations have experienced relatively recent declines in population size, connectivity may be overestimated and recent shifts in the rate of gene flow may not have yet been manifested in the observed genetic structure due to associated lag times (Nei and Chakravarti 1977; Landguth et al. 2010).

Given the disparity between movement estimates from genetic and observational data, we conclude that hellbender movement is more complex than previously thought. Studies investigating hellbender movement patterns have utilized larger (i.e. older) individuals that appear to exhibit extreme site fidelity, garnering the hellbender recognition as a “sedentary” species. However, dispersal often occurs during the juvenile stage in amphibian species (Semlitsch 2008), and it is possible that significant amounts of movement may be occurring in either larval or juvenile hellbenders, which is very difficult to detect. If dispersal occurs during one of these younger life stages, studies of only sub-adult or adult hellbenders would inevitably underestimate dispersal.

We can only speculate about the mechanisms facilitating dispersal and/or movement because gene flow estimates did not shed light on that behavior. Habitat patch connectivity may be promoted via environmental characteristics, and by the interaction between those characteristics and hellbender movement behavior. As obligate stream species, hellbenders occupy highly dynamic, aquatic environments that are susceptible to both change and degradation. Within a hellbender lifetime, several perturbations (e.g. floods) have likely occurred within the river and as a result, these disturbances may have altered not only the quality of habitat patches (e.g. loss of available shelter and nesting sites and decreased prey density due to increased sediment load, discussed in Keitzer 2007; Quinn et al. 2013), but also the distribution of individuals via displacement (Humphries 2005). Changes in stream depth, velocity, and discharge may affect hellbender settlement and movement, and may facilitate permanent dispersal from the natal habitat patch to future reproductive patches and/or infrequent migrations after initial settlement.

Evidence suggests that larval stream salamanders are susceptible to downstream drift and current-mediated advection (Johnson 1975; Stoneburner 1978; Bruce 1985, 1986; Petranka and Sih 1986; Petranka et al. 1987; Thiesmeier and Schuhmacher 1990). While evidence would suggest that hellbender dispersal is also commonly aided by the direction of stream flow (Gates et al. 1985b; Bodinof et al. 2012a), movement solely in the downstream direction should result in higher genetic diversity at downstream sites and lower diversity at upstream sites. Yet, all habitat patches in this study—regardless of position—exhibited similar levels of variation. Differentiation,

however, may be buffered if passive dispersal is subsequently accompanied by a more active movement with individuals choosing when and where to enter or exit the current (Johnson 1975; Stoneburner 1978; Thiesmeier and Schuhmacher 1990), and demonstrating purposeful upstream orientations (Bruce 1986; Ferguson 1998). For hellbenders, research demonstrates that juvenile and adult hellbenders orient downstream but are also capable of moving against the current towards upstream habitats (Gates et al. 1985b; Topping and Peterson 1985; Peterson 1987; Bodinof et al. 2012a). Such upstream movements may help to counteract any larval downstream drift.

Population structure and genetic diversity

Of the four rivers analyzed, the Current River exhibited the greatest amount of genetic diversity. When compared to the North Fork of the White, Big Piney, and Niangua Rivers, the Current River contains the longest stretch of hellbender habitat (Table 1), is the most spring-fed (Vineyard and Feder 1974), has the highest elevation (stream) gradient (http://mo.water.usgs.gov/fact_sheets/fs-092-01-wilson/report.pdf), and the highest stream discharge rate (<http://mdc.mo.gov/landwater-care/stream-and-watershed-management/missouri-watersheds>). These habitat characteristics may influence genetic diversity and gene flow especially if undetected local adaptation occurs at some of the Current River spring locations and/or elevation gradients.

Where there is local adaptation, cryptic population differentiation may also exist among ecologically important traits (i.e. phenotype, morphology, life history) which are subject to differing selective pressures in contrasting environments. In fact, the lack of genetic differentiation found within our rivers using neutral molecular markers (F_{ST}) does not necessarily mean that there is also a lack of adaptive divergence (or quantitative trait divergence, Q_{ST}) among habitat patches. After reviewing several $Q_{ST} - F_{ST}$ studies involving a number of different taxa, Merilä and Crnokrak (2001) concluded that Q_{ST} is typically larger than F_{ST} . In a later, more robust meta-analysis, Leinonen et al. (2008) reaffirmed this conclusion, further stating that weak population structure (F_{ST}) may mask levels of Q_{ST} . For amphibians, strong adaptive divergence has indeed been demonstrated among intraspecific populations inhabiting dissimilar environments (Palo et al. 2003; Cano et al. 2004; Hangartner et al. 2011)—even in the presence of moderate gene flow, where isolation by distance is absent (Gomez-Mestre and Tejedo 2004) or population genetic structure is weak (Richter-Boix et al. 2013). Because of the likely correlation with fitness, divergence in ecologically important traits (i.e. adaptive divergence) is an important management and conservation matter (Lynch 1996, Palo et al.

2003; Leinonen et al. 2008). Consideration of such factors is critical in translocation and/or reintroduction efforts (including the release of propagated individuals), as maladaptive traits/alleles could prove disastrous for individual fitness and population viability (reviewed in Storfer 1999).

If we compare our levels of genetic diversity to those found in other hellbender populations, we see that the average number of alleles across all four rivers (Missouri River and White River drainages, avg. $N_A = 7.79$) is slightly lower than the average number of alleles observed across hellbender rivers within the Ohio and Tennessee River drainages (avg. across avg. N_A for each population within drainages = 8.84 and 9.84, respectively; Unger et al. 2013). In their range wide study of the eastern hellbender, which included genetic analyses at multiple spatial scales, Unger et al. (2013) observed similar levels of within-river allelic richness for the Big Piney and Niangua Rivers as those observed in our study. However, the average number of alleles per locus for these rivers (Big Piney and Niangua) was smaller than that observed in most of the other Unger et al. (2013) rivers, which exhibit levels of genetic diversity more similar to levels of genetic diversity observed in our Current River analysis.

Because Missouri hellbender populations have undergone dramatic declines in population size, the presented levels of genetic diversity and the lack of evidence for a recent bottleneck are quite interesting. Although demographic instability often results in a reduction of genetic variation, evidence suggests that this is not true for all populations as species longevity may help slow the process of genetic erosion. Results from our study are congruent with the findings of Kuo and Janzen (2004), Lippe et al. (2006), and Pittman et al. (2011) who suggest that in declining populations of long-lived species, the retention of genetic variability is attributable to the high proportion of mature individuals remaining in the population, as they represent multiple generations and various decades of reproduction. The majority (93 %) of hellbenders sampled in this study were presumed to be adults given their size and capture history. Although adults can range anywhere in age from 5 to 30 years (Taber and Wilkinson 1975; Peterson and Wilkinson 1983), adults in this study are estimated to be >10 years old (J. Briggler, unpubl. data). During our 7 year survey period, only 7 % ($n = 35$) of sampled individuals were considered to be juveniles. Of those 7 %, 74 % came from a single river (North Fork of the White River).

Larval samples are absent in this study, making it difficult to draw conclusions about current rates of gene flow. Genetic patterns based solely upon adult genotypes may be reflective of patterns from several decades past, and are not likely to help us understand how standing genetic variability is being reallocated in present-day progeny.

Nevertheless, we assert that in the populations studied, newer generations of hellbenders would exhibit robust variation as the observed levels of heterozygosity, allelic richness and low levels of relatedness—even at the habitat patch level—suggest that matings should be occurring between genetically diverse, unrelated individuals. Thus, we believe nonrandom mating, and inbreeding depression, are not yet major problems in these populations.

Management/conservation implications

Our results indicate that Missouri hellbender populations possess levels of genetic diversity greater than we anticipated given the observed declines in population size and isolation from each other and other populations. Unfortunately, we are unable to compare current levels of diversity to historic levels. Yet, we know that in populations of long lived species with overlapping generations, a disproportionate amount of the observed genetic diversity may be harbored in the oldest cohorts. If that is true for the populations analyzed here, much of the observed genetic diversity could be lost as these individuals cease reproduction and eventually die. This is especially problematic given the lack of evidence for juvenile recruitment. Our investigations into relatedness reveal only a small proportion of highly related individuals (i.e. parent-offspring, full-sibs, half-sibs; Fig. 2). Thus, it is unclear whether the lack of relatedness is due to a lull in reproduction or if some factor is restricting the successful recruitment of juveniles into the population. A number of factors, such as habitat and water degradation, disease, and predation by non-native fish may limit juvenile recruitment (Briggler et al. 2008; Gall and Mathis 2010; USFWS 2011).

To maintain the genetic integrity of hellbenders, and the high levels of diversity observed in this study, multiple individuals representing the full spectrum of genetic variation should be used for propagation (Frankham 1995). If offspring result from a limited set of mating pairs, the genetic potential of the species will be restricted—not only in terms of genetic diversity, but also in their capacity to adapt to future conditions. The captive breeding program could benefit from increased knowledge regarding the genetic makeup of the captive breeding stock, especially with regards to the representation of allelic diversity and the degree of relatedness among breeders.

Furthermore, propagated individuals should be returned to the river of origin (i.e. the broodstock's river of origin) following the recommendations of Bodinof et al. (2012b) and Briggler et al. (2012). Given the genetic homogeneity demonstrated within rivers, releases do not need to be restricted to a particular habitat patch as long as the strict recommendation for river origin is met. However, in accordance with our discussion regarding adaptive

divergence (Q_{ST}), released individuals should be monitored to determine whether or not they survive and reproduce. If adequate resources (i.e. shelter, nesting sites, prey base, water velocity, etc.) are available to support additional individuals, attention should be placed on augmenting the number of hellbenders present in less-populated habitat patches so as to encourage future mating opportunities.

With a carefully chosen, genetically diverse broodstock, and effective translocations of captive-bred offspring (i.e. restorative releases), population sizes can be bolstered for this endangered species while maintaining genetic diversity. However, the future trajectory of these populations will depend on being able to identify, address, and resolve the environmental factors that have contributed to population declines. Until then, the bolstering of population sizes will likely be temporary and hellbender persistence will rely on continual human intervention and repetitive restorative release events.

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References

- Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: a workbench to detect molecular adaptation based on a Fst-outlier method. *BMC Bioinform* 9(1):323
- Bodinof C (2010) Translocation and conservation of hellbenders (*Cryptobranchus alleganiensis*) in Missouri. Thesis, University of Missouri, Columbia
- Bodinof CM, Briggler JT, Junge RE, Beringer J, Wanner MD, Schuette CD, Ettlting J, Gitzen RA, Millspaugh JJ (2012a) Postrelease movements of captive-reared Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). *Herpetologica* 68(2):160–173. doi:10.1655/herpetologica-d-11-00033.1
- Bodinof CM, Briggler JT, Junge RE, Beringer J, Wanner MD, Schuette CD, Ettlting J, Millspaugh JJ (2012b) Habitat attributes associated with short-term settlement of Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) salamanders following translocation to the wild. *Freshw Biol* 57(1):178–192. doi:10.1111/j.1365-2427.2011.02697.x
- Briggler JT, Larson KA, Irwin KJ (2008) Presence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on hellbenders (*Cryptobranchus alleganiensis*) in the Ozark Highlands. *Herpetol Rev* 39:443–444
- Briggler JT, Crabill TL, Irwin KJ, Davidson C, Civiello JA, Wanner MD, Schuette CD, Armstrong SL, Grant V, Davidson T, Ettlting JA (2012) Propagation, augmentation, and reintroduction plan for the Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*). Ozark Hellbender Propagation Committee, Jefferson City
- Bruce RC (1985) Larval periods, population structure and the effects of stream drift in larvae of the salamanders *Desmognathus quadramaculatus* and *Leurognathus marmoratus* in a Southern Appalachian stream. *Copeia* 1985(4):847–854. doi:10.2307/1445232

- Bruce RC (1986) Upstream and downstream movements of *Eurycea bislineata* and other salamanders in a Southern Appalachian stream. *Herpetologica* 42(2):149–155. doi:10.2307/3892380
- Burgmeier NG, Sutton TM, Williams RN (2011a) Spatial ecology of the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *Herpetologica* 67(2):135–145. doi:10.1655/herpetologica-d-10-00027.1
- Burgmeier NG, Unger SD, Sutton TM, Williams RN (2011b) Population status of the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *J Herpetol* 45(2):195–201. doi:10.1670/10-094.1
- Cano JM, Laurila A, Palo J, Merila J (2004) Population differentiation in G matrix structure due to natural selection in *Rana Temporaria*. *Evolution* 58(9):2013–2020
- Coatney CE (1982) Home range and nocturnal activity of the Ozark hellbender. Thesis, Southwest Missouri State University, Springfield
- Core Team R (2012) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
- Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144(4):2001–2014
- Crowhurst R, Faries K, Collantes J, Briggler J, Koppelman J, Eggert L (2011) Genetic relationships of hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*). *Conserv Genet* 12(3):637–646. doi:10.1007/s10592-010-0170-0
- Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple-sequence repeat loci in human populations. *Proc Natl Acad Sci USA* 91(8):3166–3170
- Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Res* 4(2):359–361
- Estoup A, Rousset F, Michalakis Y, Cornuet JM, Adriaumanga M, Guyomard R (1998) Comparative analysis of microsatellite and allozyme markers: a case study investigating micogeographic differentiation in brown trout (*Salmo trutta*). *Mol Ecol* 7(3):339–353
- Ettling JA, Wanner MD, Schuette CD, Armstrong SL, Pedigo AS, Briggler JT (2013) Captive reproduction and husbandry of adult Ozark hellbenders *Cryptobranchus alleganiensis bishopi*. *Herpetol Rev* 44(4):604–610
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* 14:2611–2620
- Excoffier L, Lischer HEL (2010) Arlequin suite ver. 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol Res* 10(3):564–567. doi:10.1111/j.1755-0998.2010.02847.x
- Ferguson H (1998) Demography, dispersal, and colonisation of larvae of Pacific giant salamanders (*Dicamptodon tenebrosus*) at the northern extent of their range. Thesis, University of British Columbia, Vancouver
- Fobes TM (1995) Habitat analysis of the Ozark hellbender, *Cryptobranchus alleganiensis bishopi*, in Missouri. Thesis, Southwest Missouri State University, Springfield
- Foster RL, McMillan AM, Roblee KJ (2009) Population status of hellbender salamanders (*Cryptobranchus alleganiensis*) in the Allegheny River drainage of New York State. *J Herpetol* 43(4):579–588. doi:10.1670/08-156.1
- Frankham R (1995) Conservation genetics. *Annu Rev Genet* 29:305–327
- Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Mol Ecol* 10(12):2741–2752. doi:10.1046/j.0962-1083.2001.01411.x
- Gall BG, Mathis A (2010) Innate predator recognition and the problem of introduced trout. *Ethology* 116:47–58
- Gates JE, Hocutt CH, Stauffer JR Jr, Taylor GJ (1985a) The distribution and status of *Cryptobranchus alleganiensis* in Maryland. *Herpetol Rev* 16:17–18
- Gates JE, Stouffer RH Jr, Stauffer JR Jr, Hocutt CH (1985b) Dispersal patterns of translocated *Cryptobranchus alleganiensis* in a Maryland stream. *J Herpetol* 19(3):436–438. doi:10.2307/1564279
- Gomez-Mestre I, Tejedo M (2004) Contrasting patterns of qualitative and neutral genetic variation in locally adapted populations of the natterjack toad *Bufo calamita*. *Evolution* 58(10):2343–2352
- Goslee SC, Urban DL (2007) The ecodist package for dissimilarity-based analysis of ecological data. *J Stat Softw* 22(7):1–19
- Hangartner S, Laurila A, Räsänen K (2011) Adaptive divergence in moor frog (*Rana arvalis*) populations along an acidification gradient: Influences from Q_{ST} to F_{ST} correlations. *Evolution* 66:867–881
- Humphries WJ (2005) *Cryptobranchus alleganiensis* displacement by a flood. *Herpetol Rev* 36(4):428
- Johnson JE, Goldberg AS (1975) Movement of larval two lined salamanders (*Eurycea bislineata*) in the Mill River, Massachusetts. *Copeia* 3:588–589. doi:10.2307/1443669
- Johnson J, Faries K, Rabenold J, Crowhurst R, Briggler J, Koppelman J, Eggert L (2009) Polymorphic microsatellite loci for studies of the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*). *Conserv Genet* 10(6):1795–1797. doi:10.1007/s10592-009-9818-z
- Jombart T (2000) Adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24:1403–1405
- Kalinowski ST (2005) Hp-rare 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol Ecol Notes* 5(1):187–189. doi:10.1111/j.1471-8286.2004.00845.x
- Keitzer SC (2007) Habitat preferences of the eastern hellbender in West Virginia. Thesis, Marshall University, Huntington
- Kimura M, Crow JF (1964) The number of alleles that can be maintained in a finite population. *Genetics* 49(4):725–738
- Kimura M, Ohta T (1978) Stepwise mutation model and distribution of allelic frequencies in a finite population. *Proc Natl Acad Sci USA* 75(6):2868–2872
- Kuo C-H, Janzen FJ (2004) Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). *Conserv Genet* 5(4):425–437. doi:10.1023/b:coge.0000041020.54140.45
- Landguth EL, Cushman SA, Schwartz MK, McKelvey KS, Murphy M, Luikart G (2010) Quantifying the lag time to detect barriers in landscape genetics. *Mol Ecol* 19(19):4179–4191. doi:10.1111/j.1365-294X.2010.04808.x
- Latch E, Dharmarajan G, Glaubitz J, Rhodes O (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conserv Genet* 7(2):295–302
- Leinonen T, O'Hara RB, Cano JM, Merila J (2008) Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. *J Evol Biol* 21(1):1–17
- Lenormand T (2002) Gene flow and the limits to natural selection. *Trends Ecol Evol* 17(4):183–189
- Lippe C, Dumont P, Bernatchez L (2006) High genetic diversity and no inbreeding in the endangered copper redhorse, *Moxostoma hubbsi* (Catostomidae, Pisces): the positive sides of a long generation time. *Mol Ecol* 15(7):1769–1780. doi:10.1111/j.1365-294X.2006.02902.x

- Lowe WH, Allendorf FW (2010) What can genetics tell us about population connectivity? *Mol Ecol* 19(15):3038–3051. doi:10.1111/j.1365-294X.2010.04688.x
- Luikart G, Cornuet JM (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conserv Biol* 12(1):228–237
- Lynch M (1996) A quantitative genetic perspective on conservation issues. In: Avise J, Hamrick J (eds) *Conservation genetics: case histories from nature*. Chapman & Hall, New York, pp 471–501
- Merilä J, Crnokrak P (2001) Comparison of genetic differentiation at marker loci and quantitative traits. *J Evol Biol* 14(6):892–903
- Nei M, Chakravarti A (1977) Drift variances of F_{ST} and G_{ST} statistics obtained from a finite number of isolated populations. *Theor Popul Biol* 11:307–325
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* 29(1):1–10. doi:10.2307/2407137
- Nickerson MA, Mays CE (1973) A study of the Ozark hellbender *Cryptobranchus alleganiensis bishopi*. *Ecology* 54(5):1164–1165. doi:10.2307/1935586
- Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Mol Ecol* 4(3):347–354. doi:10.1111/j.1365-294X.1995.tb00227.x
- Palo JU, O'Hara RB, Laugen T, Laurila A, Primmers CR, Merilä J (2003) Latitudinal divergence of common frog (*Rana temporaria*) life history traits by natural selection: evidence from a comparison of molecular and quantitative genetic data. *Mol Ecol* 12(7):1963–1978
- Peakall ROD, Smouse PE (2006) Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6(1):288–295. doi:10.1111/j.1471-8286.2005.01155.x
- Peterson CL (1987) Movement and catchability of the hellbender, *Cryptobranchus alleganiensis*. *J Herpetol* 21(3):197–204
- Peterson CL, Wilkinson RF (1996) Home range size of the hellbender (*Cryptobranchus alleganiensis*) in Missouri. *Herpetol Rev* 27(3):126–127
- Peterson CL, Wilkinson RF Jr, Milton ST, Metter DE (1983) Age and growth of the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*). *Copeia* 1:225–231
- Petranka JW (1998) *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.
- Petranka JW, Sih A (1986) Environmental instability, competition, and density-dependent growth and survivorship of a stream-dwelling salamander. *Ecology* 67(3):729–736. doi:10.2307/1937696
- Petranka JW, Sih A, Kats LB, Holomuzki JR (1987) Stream drift, size-specific predation, and the evolution of ovum size in an amphibian. *Oecologia* 71(4):624–630. doi:10.2307/4218213
- Pfingsten RA (1989) The status and distribution of the hellbender, *Cryptobranchus alleganiensis*, Ohio. *Ohio J Sci* 89(2):3
- Piry S, Luikart G, Cornuet J-M (1999) BOTTLENECK: a computer program for detecting recent reduction in the effective population size using allele frequency data. *J Hered* 90:502–503
- Pittman S, King T, Faurby S, Dorcas M (2011) Demographic and genetic status of an isolated population of bog turtles (*Glyptemys mühlenbergii*): implications for managing small populations of long-lived animals. *Conserv Genet* 12(6):1589–1601. doi:10.1007/s10592-011-0257-2
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155:945–959
- Quinn SA, Gibbs JP, Hall MH, Petokas PJ (2013) Multiscale factors influencing distribution of the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*) in the northern segment of its range. *J Herpetol* 47(1):78–84. doi:10.1670/11-127
- Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conserv Biol* 17(1):230–237. doi:10.1046/j.1523-1739.2003.01236.x
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution* 43(1):223–225
- Richter-Boix A, Quintela M, Kierczak M, Franch M, Laurila A (2013) Fine-grained adaptive divergence in an amphibian: genetic basis of phenotypic divergence and the role of nonrandom gene flow in restricting effective migration among wetlands. *Mol Ecol* 22:1322–1340
- Ross KG, Shoemaker DD, Krieger MJB, DeHeer CJ, Keller L (1999) Assessing genetic structure with multiple classes of molecular markers: a case study involving the introduced fire ant *Solenopsis invicta*. *Mol Biol Evol* 16(4):525–543
- Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol Ecol Res* 8(1):103–106. doi:10.1111/j.1471-8286.2007.01931.x
- Routman E (1993) Mitochondrial DNA variation in *Cryptobranchus alleganiensis*, a salamander with extremely low allozyme diversity. *Copeia* 2:407–416
- Sabatino S, Routman E (2009) Phylogeography and conservation genetics of the hellbender salamander (*Cryptobranchus alleganiensis*). *Conserv Genet* 10(5):1235–1246. doi:10.1007/s10592-008-9655-5
- Semlitsch RD (2008) Differentiating migration and dispersal processes for pond-breeding amphibians. *J Wildl Manage* 72(1):260–267. doi:10.2193/2007-082
- Slatkin M (1985) Gene flow in natural populations. *Annu Rev Ecol Syst* 16:393–430
- Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science* 236(4803):787–792. doi:10.1126/science.3576198
- Smith BG (1907) The life history and habits of *Cryptobranchus alleganiensis*. *Biol Bull* 13:5–39
- Stoneburner DL (1978) Salamander drift. *Freshw Biol* 8(3):291–293. doi:10.1111/j.1365-2427.1978.tb01450.x
- Storfer A (1999) Gene flow and endangered species translocations: a topic revisited. *Biol Conserv* 87:173–180
- Taber CA, Wilkinson RF Jr, Milton ST (1975) Age and growth of hellbenders in the Niangua River, Missouri. *Copeia* 4:633–639
- Templeton AR, Shaw K, Routman E, Davis SK (1990) The genetic consequences of habitat fragmentation. *Ann Missouri Bot Gard* 77(1):13–27
- Thiesmeier B, Schuhmacher H (1990) Causes of larval drift of the fire salamander, *Salamandra salamandra terrestris*, and its effects on population dynamics. *Oecologia* 82(2):259–263. doi:10.2307/4219231
- Topping MS, Peterson CL (1985) Movement in the hellbender, *Cryptobranchus alleganiensis*. *Trans Missouri Acad Sci* 19:121
- Trauth SE, Wilhide JD, Daniel P (1992) Status of the Ozark hellbender, *Cryptobranchus bishopi* (Urodela: Cryptobranchidae), in the Spring River, Fulton County, Arkansas. *Arkansas Acad Sci* 46:83–85
- Unger S, Fike J, Sutton T, Rhodes O, Williams R (2010) Isolation and development of 12 polymorphic tetranucleotide microsatellite markers for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). *Conserv Genet Res* 2(1):89–91. doi:10.1007/s12686-009-9170-0
- Unger SD, Rhodes OE, Sutton TM, Williams RN (2013) Population genetics of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) across multiple spatial scales. *PLoS ONE* 8(10):e74180. doi:10.1371/journal.pone.0074180
- USFWS (U.S. Fish and Wildlife Service) (2011) Endangered and threatened wildlife and plants; endangered status for the Ozark hellbender salamander. *Fed Reg* 76(194):61956–61978

- Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* 4(3):535–538. doi:[10.1111/j.1471-8286.2004.00684.x](https://doi.org/10.1111/j.1471-8286.2004.00684.x)
- Vineyard JD, Feder GL (1974) Springs of Missouri. Missouri Geological Survey and Water Resources, Rolla
- Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: Cautions and guidelines. *Mol Ecol* 10(1):249–256. doi:[10.1046/j.1365-294X.2001.01185.x](https://doi.org/10.1046/j.1365-294X.2001.01185.x)
- Wang J (2007) Triadic IBD coefficients and applications to estimating pairwise relatedness. *Genet Res* 89(03):135–153. doi:[10.1017/S0016672307008798](https://doi.org/10.1017/S0016672307008798)
- Wang J (2011) Coancestry: a program for simulating, estimating and analysing relatedness and inbreeding coefficients. *Mol Ecol Res* 11(1):141–145. doi:[10.1111/j.1755-0998.2010.02885.x](https://doi.org/10.1111/j.1755-0998.2010.02885.x)
- Wheeler BA, Prosen E, Mathis A, Wilkinson RF (2003) Population declines of a long-lived salamander: A 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biol Conserv* 109(1):151–156. doi:[10.1016/s0006-3207\(02\)00136-2](https://doi.org/10.1016/s0006-3207(02)00136-2)
- Wright S (1943) Isolation by distance. *Genetics* 28(2):114–138