GENETIC SIGNATURES FOLLOW DENDRITIC PATTERNS IN THE EASTERN HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*)

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Abstract.—The Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) is a large paedomorphic salamander experiencing declines throughout much of its geographic range. Little is known regarding the effect of anthropogenic isolating mechanisms (stream alteration, habitat fragmentation, or dams) on levels of genetic diversity or structure. Conservation needs for this species include assessing levels of fine-scale genetic structure at the state-level and determining the number of discrete genetic groupings, genetic diversity, and effective population size (N_e) across Pennsylvania watersheds of the Allegheny, and Western Branch of the Susquehanna Rivers. These watersheds are located within the core of the Eastern Hellbender range and represent one of the few stable locations in the country. We examined the landscape genetics of 13 distinct stream reaches, represented by 284 Eastern Hellbenders, using both spatial and non-spatial Bayesian genetic approaches. Pennsylvania populations of Eastern Hellbenders are characterized by significant genetic structure that is partitioned among dendritic river drainages. Bayesian clustering analysis inferred four discrete genetic clusters (three within the Allegheny River drainage and one within the Susquehanna River drainage). Estimates of N_e for discrete genetic clusters were variable among clusters but higher within the northern Allegheny National Forest. Evidence for a population bottleneck was detected at one cluster; however, overall levels of genetic diversity were high among stream reaches. Based on our results, gene flow (possibly downstream drift) is high among watersheds based on genetic signatures from adults sampled. We suggest increased sampling of younger age classes, which may reflect more recent patterns of gene flow. These finding are important for future conservation management strategies within Pennsylvania watersheds and across the range of the species.

Key Words.—amphibian decline; effective population size; landscape genetics; microsatellites; salamander

INTRODUCTION

Conservation genetics has emerged as a valuable tool to address questions for species of conservation concern (Manel et al. 2003). Molecular approaches allow characterization of genetic patterns across spatial landscapes and are vital to our understanding of levels of population connectivity (gene flow). Although many terrestrial dispersing species have been extensively studied, there are relatively few studies conducted at fine spatial scales in amphibian species within river networks, where gene flow is confined to the complex dendritic-like Riverscape (Kanno et al. 2011). These Riverscape genetic approaches are useful for detecting genetic signatures of barriers corresponding to agents of fragmentation such as impoundments, hydroelectric dams, and tail waters (Hopken et al. 2013; Roberts et al. 2013). Spatial features unique to stream watershed topology, such as natural environmental barriers (geologic) or anthropogenic isolating mechanisms (dams), may restrict gene flow among populations leading to genetic structuring and differentiation

upstream and downstream of these barriers. Elucidating these mechanisms using conservation genetics is important to inform effective conservation management strategies for aquatic species, including giant salamanders (Crowhurst et al. 2011; Kanno et al. 2011).

Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are large, long-lived, fully aquatic salamanders first described from the Allegheny River system in northwestern Pennsylvania, USA (Nickerson and Mays 1973). Pennsylvania populations occur in either the Allegheny-Ohio River or Susquehanna River drainages, with the latter the only watershed within the range of the species that flows into the Atlantic Ocean (Mayasich et al. 2003). Populations in many portions of Pennsylvania were thought to be stable (Hillis and Bellis 1971; Mayasich et al. 2003); however, recent surveys (EJC unpublished data) indicate reductions in the number of captures similar to those reported throughout its range (Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011; Hiler et al. 2013), as well as low recruitment, and presence of the emerging infectious disease, *Batrachochytrium dendrobatidis* (*Bd*; Regester
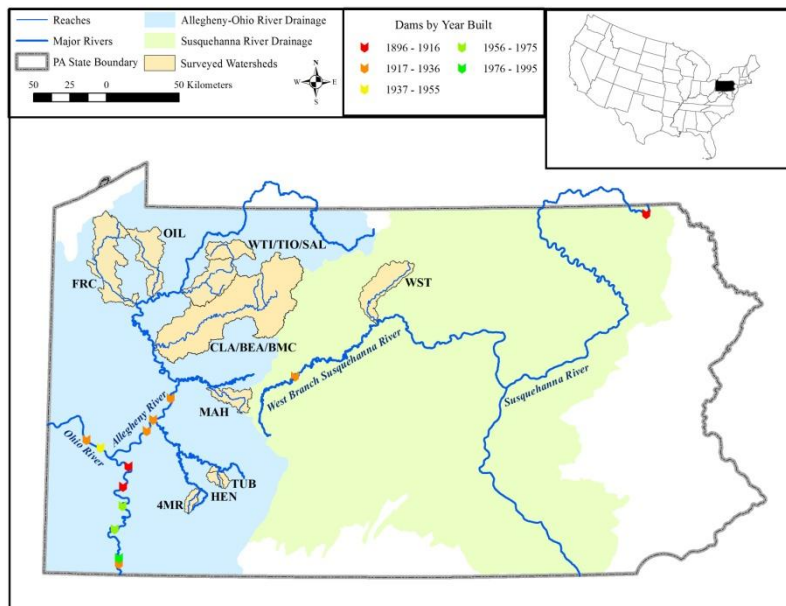
FIGURE 1. Map of major rivers, surveyed watersheds, and stream reach names for Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) within the Allegheny-Ohio River and Susquehanna River drainages in Pennsylvania, USA. Major Allegheny drainage dam locations depicted by chevrons according to year of dam construction.

et al. 2012; Quinn et al. 2013). Many states agencies are implementing conservation programs to address these pervasive declines, including artificial nest structures, captive propagation, head-starting, and translocations (Briggler and Ackerson 2012). To aid in the implementation of these various management actions, it is necessary to assess the underlying genetic structure across one of the few remaining core populations, those within Pennsylvania watersheds. Bayesian clustering methods offer a powerful statistical approach to infer the number of genetically distinct groups, or clusters, to which individuals within the Riverscape are probabilistically assigned. These approaches, when paired with traditional methods of genetic differentiation (*F*-statistics), allow managers to prioritize conservation action strategies within specific watersheds.

Another genetic metric commonly used by conservation managers for assessing overall population health is effective population size (N_e), which is defined as the size of an ideal population (N) that will result in the same amount of genetic drift as in the actual population being considered (Wright 1931). The dendritic nature of stream environments, coupled with the presence of isolating anthropogenic barriers, may hasten population declines, leading to river-specific population bottlenecks or reduction of N_e. Watersheds with small N_e may be vulnerable to genetic drift, loss of genetic diversity, and reductions in long-term viability of genetic populations (Frankham 1995; Hare et al. 2011).

Herein, we use multilocus microsatellite genotypes to investigate the Riverscape genetics of Eastern Hellbenders throughout Pennsylvania. For this study, we used fine-scale genetic approaches and Bayesian assignment tests to characterize the number of discrete genetic clusters and demographic parameters (genetic diversity, effective population size, and presence of potential population bottlenecks) of Eastern Hellbenders within the Allegheny-Ohio and Western Branch of the Susquehanna River drainages. Fine-scale population genetic analyses are critical for future conservation management of Eastern Hellbenders within Pennsylvania and across the species range.

MATERIALS AND METHODS

Field.—Survey locations for Eastern Hellbenders in Pennsylvania occurred primarily within tributaries of the Allegheny-Ohio River Drainage, including the Little Mahoning, French, Clarion, Tionesta, and Tubmill watersheds consisting of 13 distinct streams, Tionesta (TIO), West Branch of Tionesta (WTI), Salmon Creek (SAL), Oil Creek (OIL), French Creek (FRC), Clarion River (CLA), Big Mill Creek (MIL), Bear Creek (BEA), Little Mahoning Creek (MAH), Tubmill Creek (TUB), Hendricks Creek (HEN), Four Mile Run (4MR), and Kettle Creek (WST; Fig. 1). The Little Mahoning watershed comprises a mixture of agriculture lands, deciduous forests, residential development, and light industry before it empties into Mahoning Creek, and

then the Allegheny River. The Upper Allegheny watersheds (French, Clarion, and Tionesta) are all tributaries of the Allegheny River and are dominated by forests of the Allegheny National Forest with some agriculture land. Both Tubmill/Hendricks Creek and Four Mile Run watersheds flow into the Conemaugh River, before draining into the Allegheny River.

We conducted stream reach surveys 1–27 July 2010 and 20 June to 11 August 2011. Stream reaches within the Allegheny River drainage averaged about 400 m in length. Surveys consisted of 8–10 people walking upstream through shallow stream reaches lifting suitable rock habitat. Peter Petokas provided samples from the Western Branch of the Susquehanna River drainage. We captured hellbenders by hand or net. We obtained tissue samples from each individual by clipping off a small section of the tail and we placed tissue samples in 100% ethanol until DNA extraction. We recorded GPS locations at each individual capture location and also recorded a general survey site GPS location approximating the middle of individual capture locations for each stream reach. Therefore, our sampling hierarchy consists of individual capture locations, stream reaches, watersheds comprised of several stream reaches, and major river drainages (Fig. 1).

Laboratory.—We extracted high quality genomic DNA from all tissue samples using a standard phenol-chloroform extraction (Sambrook and Russell 2001). We used 12 highly polymorphic microsatellite markers grouped in four multiplex Polymerase chain reactions (PCR) and genotyped using GENEMAPPER following the methods described in Unger et al. (2010, 2012). Quality control methods included a positive and negative control for each 96-well plate and blind scoring 10% of previously typed individuals to resolve ambiguities. Prior to statistical analysis, we tested our microsatellite data for the presence of null alleles and scoring errors using MICRO-CHECKER (Oosterhout et al. 2004).

Genetic analyses.—We measured genetic diversity by estimating the observed heterozygosities (*Ho*), expected heterozygosities (*He*), and allelic richness (*A*) in GenAlex 6.41 (Peakall and Smouse 2006). We calculated rarified average allelic richness (*Ar*) using HP-rare (Kalinowski 2005) to correct for sample size, by sampling eight individuals (16 allele copies) at random. Because of the limited number of samples collected for some stream reaches, we calculated estimates for genetic diversity only for stream reaches with a minimum of eight individual samples. We set the minimal sample size number to eight individuals because it allowed inclusion of our Four Mile Run samples, representing the Loyalhanna River Watershed. Deviations from Hardy–Weinberg equilibrium expectations and linkage disequilibrium were calculated in Genepop version

4.0.10 (Rousset 2008). We calculated the presence and frequency of private alleles (alleles unique to a single stream reach) in HP-RARE (Kalinowski 2005).

We used two Bayesian methods for clustering individuals based on likelihood, GENELAND (Guillot et al. 2005), which incorporated spatial coordinates into genetic clustering, and STRUCTURE (Pritchard, J., and W. Wen. 2003. Documentation for STRUCTURE software:Version 2. http://pritch.bsd.uchicago.edu. [Accessed 15 January 2013]), which we used with no spatial priors and infers clusters based solely on genetic data. Both these programs infer the number of discrete genetic clusters, or *K*, and assign probable population membership (%) to individuals within each cluster using a Markov chain Monte Carlo (MCMC) algorithm, with each cluster characterized by a set of allele frequencies at each loci (Pritchard et al. 2000). We used the uncorrelated allele frequency model for both programs to avoid overestimation of *K* (Rieux et al. 2011). We selected the best supported number of discrete genetic clusters for STRUCTURE, or *K*, by using both the highest mean estimated log probability (Pritchard et al. 2000) and the ΔK method of Evanno et al. (2005). We determined that when the best supported *K* is reached, likelihoods for larger plateau of Ks and the variance among runs increases following Pritchard and Wen (2003). Results for both these methods in STRUCTURE were visualized in STRUCTURE HARVESTER (Earl and vonHoldt 2012). For both programs, we performed 10 runs at each value of *K* (for *K* = 1 to *K* = 15) based on initial runs of *K* = 20, indicating no additional signatures of structure. These methods for Bayesian assignment models are described in full elsewhere (Unger et al. 2013). Briefly, our parameters include 1,000,000 iterations (with thinning every 100th iteration, spatial coordinates uncertainty as 0, and a maximum rate of Poisson process fixed to 300 for GENELAND) after a burn in of 100,000 to ensure stabilization of our MCMC (Markov chain Monte Carlo). We set criteria for assigning individuals to specific clusters at > 50%. To validate STRUCTURE and GENELAND concordance, we implemented an additional individual assignment test in GENECLASS2 using 10,000 simulated individuals and rejection level of $P = 0.01$ (Piry et al. 2004).

We calculated pairwise F_{st} estimates (Weir and Cockerham 1984) between all stream reaches and inferred genetic clusters in FSTAT version 2.9.3 (Goudet, J. 2002. Fstat 2.9.3.2 URL:http//www2.unil.ch/popgen/softwares/fstat.htm. [Accessed 15 January 2013]). To characterize isolation by distance (IBD), we performed a Mantel test in ALLELES IN SPACE (Miller 2005), which test for significance correlation between pairwise genetic and geographic distance matrices. Presence of an IBD pattern would indicate geographically proximate individuals to be more similar genetically than geographically distant ones, whereas

TABLE 1. Major river drainages, watershed names, stream reaches, stream reach abbreviations, and sample sizes of Pennsylvania Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) sampled in this study in Pennsylvania, USA.

Major River Drainage	Watershed	Stream Reach	Abbreviation	Sample Size
Allegheny-Ohio	Middle Allegheny/Tionesta	Tionesta	TIO	76
		West Branch of Tionesta	WTI	4
		Salmon Creek	SAL	2
		Oil Creek	OIL	11
	French Creek	French Creek	FRC	38
	Clarion River	Clarion River	CLA	2
		Big Mill Creek	MIL	1
		Bear Creek	BEA	1
	Mahoning Creek	Little Mahoning Creek	MAH	92
	Tubmill Creek	Tubmill Creek	TUB	27
		Hendricks Creek	HEN	13
	Loyalhanna River	Four Mile Run	4MR	8
Susquehanna	West Branch of Susquehanna	Kettle Creek	WST	9

absence of IBD could indicate a lack of structure, genetic drift, landscape processes not correlated with measured distances between sites, or panmixia across sample locations. We used individual capture locations within stream reaches (GPS coordinates) for testing IBD. To further examine the role of F_{st} across the dendritic riverscape, we measured the within stream distance (km) between stream reach sites (general survey location GPS) in ArcGIS 10.1 (ESRI) and we regressed F_{st} against within stream distance.

We used the program NeEstimator v2 to estimate effective population size (N_e) and confidence intervals for N_e estimates. This program uses linkage disequilibrium and heterozygote-excess data from microsatellite loci requiring only a single temporal sample to infer N_e (Do et al. 2014). We performed estimates of N_e for major stream reaches and for discrete genetic clusters inferred by Bayesian analyses. The number of migrants per generation (N*m*) was calculated in Genepop version 4.0.10 (Rousset 2008). N*m* values greater than one indicate gene flow may counteract genetic drift, resulting in overall low levels of genetic differentiation (Nei 1987). To evaluate evidence for recent population bottlenecks within each discrete genetic cluster, we used the program BOTTLENECK version 1.2 (Piry et al. 1999), which detects deficiency or excess in heterozygotes based on a specific mutation model. We used a two-phase mutation model (TPM) and step-wise (SMM) mutation model with Wilcoxon sign-rank test with variance for TPM set to 5% and proportion of step-wise (SMM) in TPM set at 95% with 10,000 iterations across all loci.

RESULTS

Field and Laboratory.—We collected 284 adult individuals across 13 distinct stream reaches (Table 1). We collected most salamanders from tributaries within the Allegheny River drainage, with fewer collected from the Susquehanna River Drainage. We successfully extracted DNA from all tissue samples and we used the 12 microsatellite loci to genotype all individuals. MICRO-CHECKER detected the presence of null alleles for three loci within MAH stream reach, albeit at low frequency (5.7–6.4%).

Genetic analyses.—The observed and expected heterozygosities for stream reaches with a minimum of eight individuals ranged from 0.648 to 0.832 and from 0.605 to 0.848, respectively (Table 2). Most stream reaches exhibited high levels of allelic richness (Table 2). We detected private alleles for TUB and WST stream reaches (Table 2). No loci were in linkage disequilibrium after Bonferroni correction for multiple comparisons across stream reaches, amd no loci were significantly out of Hardy-Weinberg equilibrium (Table 3).

The program STRUCTURE consistently grouped hellbenders into four discrete genetic clusters across Pennsylvania; three clusters within the Allegheny River drainage and one cluster within the Susquehanna River drainage. These groupings largely reflected major watershed topology, with specific cluster/stream reach assignment as follows: 1) a Tubmill and Hendricks Creeks cluster, TUBM/HEND (TUB and HEN cluster), 2) a Little Mahoning Creek cluster, MAHN (MAH), 3) an Upper Allegheny River cluster, or UPALL (TIO,

TABLE 2. Genetic diversity estimates including mean observed and expected heterozygosities (Ho and He, respectively), allelic richness (A), allelic richness rarified to eight individuals (A_r), number of private alleles and frequency across twelve loci in each of the eight stream reach sampled with a minimal n = 8 for Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Pennsylvania, USA.

Stream reach	Ho	He	A	A_r	Private alleles	Private allele frequency
Tionesta	0.811	0.840	10.15	7.03	0	0
Oil Creek	0.818	0.802	7.67	6.85	0	0
French Creek	0.815	0.848	10.34	7.19	0	0
Little Mahoning Creek	0.832	0.843	9.18	6.81	0	0
Tubmill Creek	0.747	0.777	8.80	5.98	1	0.17
Hendricks Creek	0.724	0.729	6.00	5.23	0	0
Four Mile Run	0.771	0.750	5.75	5.75	0	0
Kettle Creek	0.648	0.605	3.83	3.73	1	0.08

FRC, OIL, 4MR, WTI, BEA, SAL, and CLA), and 4) a Susquehanna cluster, or SUSQ (WST; Fig. 2). Surprisingly, STRUCTURE did not differentiate 4MR from the Upper Allegheny stream reaches (FRC, TIO, WTI, SAL, BMC, BEA, CLA) as genetically distinct even though stream reach 4MR is situated along the southern extent of the Allegheny River drainage. GENELAND consistently inferred $K = 4$ in 10 out of 10 runs with spatial data incorporated in modeling (Fig. 3). The highest mean estimated log probability for STRUCTURE corresponded to $K = 4$ (LnP (K) = -10,215.3) reaching a plateau after which the standard deviation increased, whereas the highest ΔK value corresponded to $K = 2$ ($\Delta K = 578.3$) with a secondary peak at $K = 4$ ($\Delta K = 276.3$; Fig 4). Investigations of STRUCTURE runs at $K = 2$ indicated one cluster comprised entirely of MAH stream reaches and another cluster consisting of all remaining stream reaches. STRUCTURE at $K = 2$ was unable to differentiate individuals from the majority of Allegheny River stream reaches from the Susquehanna drainage. Moreover, the

TABLE 3. Summary of Hardy-Weinberg equilibrium test for heterozygote excess for 12 microsatellite loci (Unger et al. 2010) for Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Pennsylvania, USA.

Locus	HWE *P*-value
Call 171	0.317
Call 127	0.313
Call 351	1.000
Call 204	1.000
Call 205	1.000
Call 232	0.087
Call 347	0.971
Call 282	0.143
Call 341	0.240
Call 261	0.992
Call 26	0.990
Call 266	0.442

program STRUCTURE may be unable to correctly assign individuals to the most biologically meaningful number of clusters (K) at low levels of differentiation (F_{st} values < 0.03) as observed in our study (Table 4; Latch et al. 2006). Therefore, $K = 4$ provided the most detailed and biologically meaningful genetically distinct clusters for Eastern Hellbenders in Pennsylvania.

Overall, there was agreement between GENELAND and STRUCTURE in the number of individuals assigned to each of the four discrete genetic clusters. However assignment probabilities were noticeably lower in GENELAND (TUBM/HEND = 88.9%, 49.2% MAHN = 70.4%, 50.13%, SUSQ = 99.9%, 48.9%, UPALL = 64.35%, 47.7% for STRUCTURE and GENELAND, respectively). The number of individuals assigned to clusters that did not include their stream reach of origin was low: TUBM/HEND (1), MAHN (4), SUSQ (0), and UPALL (14), indicating high concordance between programs. Results from GENECLASS2 individual assignment test were consistent with GENELAND and STRUCTURE groupings and correctly assigned individuals to their four genetic clusters (78.9%).

We observed detectable levels of stream reach differentiation with F_{st} estimates consistent with the number and geographic distribution of clusters detected by Bayesian analyses. Stream reach WST (Susquehanna River drainage) was the most differentiated from remaining Pennsylvania stream reaches within the Allegheny River drainage (F_{st} 0.118–0.199; Table 4). F_{st} values for discrete genetic clusters were consistent with stream reach F_{st} values, with SUSQ cluster the most differentiated from remaining clusters within the Allegheny River Drainage (0.113–0.165; Table 5). Within the lower section of the Allegheny River Drainage, the two clusters MAHN and TUBM/HEND were the most differentiated from each other with an F_{st} value of 0.049 (Table 5). These two clusters are separated by a minimum of six potentially isolating barriers (two large dams within tributaries and four locks within the main-stem Allegheny River) with a within stream distance of 152.4 km for MAH and TUB stream reaches (Table 4). Mantel tests detected a significant,
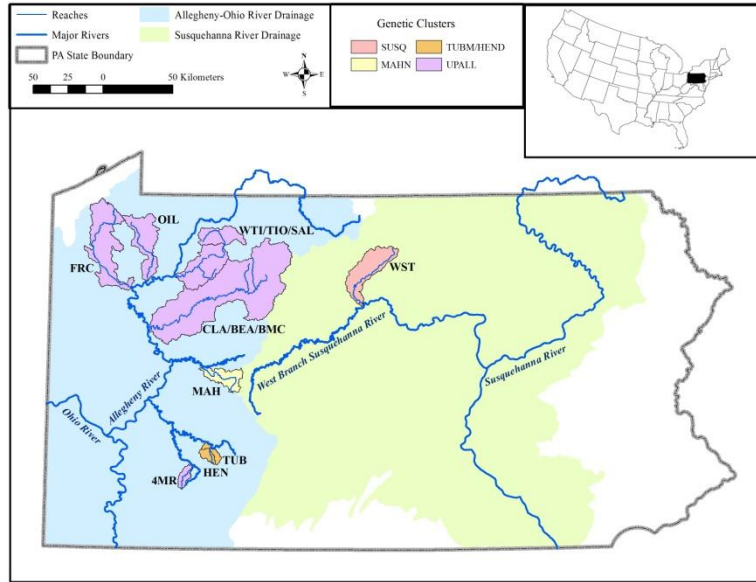
FIGURE 2. Major rivers, surveyed watersheds, and stream reach names for Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) within the Allegheny-Ohio River and Susquehanna River drainages of Pennsylvania, USA. Discrete genetic clusters inferred by Bayesian analyses of Pennsylvania Eastern Hellbenders includes SUSQ (Susquehanna cluster), MAH (Little Mahoning Creek cluster), TUBM/HEND (Tubmill and Hendricks Creek cluster), and UPALL (Upper Allegheny River cluster).

weak ($r = 0.10$, $P < 0.001$) pattern of isolation by distance (IBD). The regression of F_{st} and within stream distance was not significant ($F_{1,19} = 1.773$, $P = 0.199$).

In bottleneck analyses, we found evidence for recent reductions in population size only in one discrete genetic cluster, (MAHN; $P < 0.001$) using Wilcoxon test under
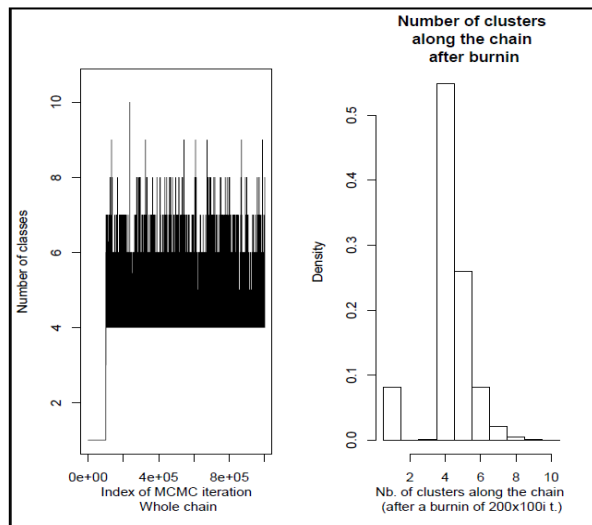

FIGURE 3. Plot of the number of genetic clusters simulated from the posterior distribution along the MCMC chain obtained with GENELAND for Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Pennsylvania, USA, with the highest density corresponding to four genetic clusters.

the TPM model. Estimates for effective population size (N_e) using NeEstimator v2 included values from 14.5 to about 1,315.1 with several estimates including infinity (∞; Table 6). N_e estimates were larger for discrete genetic clusters within the northern Allegheny River system (UPALL cluster; based on the lower 95% confidence interval of 1,315.1) and lowest for the SUSQ (14.5) and TUBM/HEND (16.4) clusters. N_e estimates for major stream reaches followed similar patterns as estimates for genetic clusters (Table 7). The number of migrants (Nm) after correction for sample size was 3.91.

DISCUSSION

The role of dams as isolating mechanisms in streams has been documented in many fully aquatic species (Meeuwig et al. 2010). However, elucidating dam-induced genetic effects on aquatic organisms have been mixed and in many cases appear to be species specific, influenced by life-history traits, directional dispersal ability, or dam age (Neville et al. 2006; Abernathy et al. 2013). The level of recent gene flow across our study system was high based on the low occurrence of private alleles and Nm values (3.91 migrants per generation). Moreover, estimates of F_{st} were relatively low across most stream reaches within the Allegheny River drainage and followed predicted patterns based on stream topology (geographically proximate stream reaches characterized by low levels of genetic differentiation). Based on these observations, Eastern
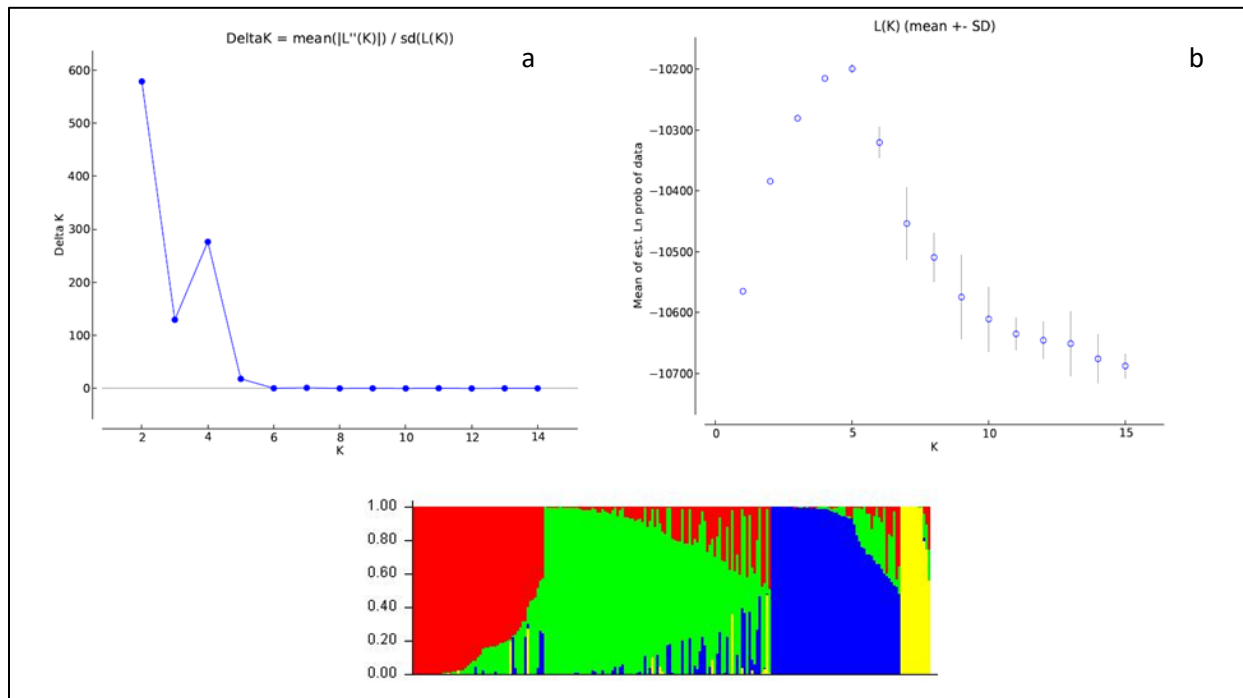
FIGURE 4. Plot of estimate of *K* across runs (upper left), mean likelihood values across *K* (upper right, and plot of Q values for *K* = 4 according to STRUCTURE (bottom center). We performed 10 runs at each ideal number of genetically distinct clusters, *K*. Results shown only for *K* =1 to 14, 15, respectively for a), b). Q values correspond to TUB/HEND (*red*), UPALL (*green*), MAH (*blue*) and SUSQ (*yellow*) genetic clusters of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*).

Hellbenders appear to be highly vagile because of either downstream dispersal (stream drift) or gene flow occurring prior to construction of dams and reservoirs across the Allegheny River and its tributaries from 1920–1951, when most dams were built (Cowell and Stoudt 2007). Given the relatively long-lived nature of Eastern Hellbenders (about 30 y; Nickerson and Mays 1973), there has likely been insufficient time to develop higher levels of genetic differentiation in response to the reduced gene flow and genetic drift that results from the dam barriers (Landguth et al. 2010). This is consistent with other studies in long-lived species, including mussels (Galbraith et al. 2015) and tuataras (Moore et al. 2008), whereby longevity and large historical population sizes likely mask genetic signatures of recent disturbance events. However, we may have detected early signs of genetic differentiation in two discrete genetic clusters located at the southern extend of the Allegheny River Drainage, MAHN and TUBM/HEND. The sites are separated by only about 153 stream km between stream reach distances, yet have high levels of differentiation compared to other clusters ($F_{s t}$ = 0.049). Both MAHN and TUBM/HEND clusters and stream reach 4MR are found upstream of dams and presumably unsuitable lake habitat that may limit the ability of individuals to colonize upstream or disperse downstream. This study underscores how stream topology can influence genetic structure at a large spatial scale within the Allegheny River drainage while presence of barriers to dispersal (dams) or other factors (unsuitable reservoir or stream habitat) may be inflating levels of genetic differentiation within the lower Allegheny River drainage stream reaches.

The limited studies examining broad-level genetic patterns in Eastern Hellbenders (Sabatino and Routman 2009; Unger et al. 2013) have revealed the Susquehanna River drainage to have different genetic signatures not only from other drainages within Pennsylvania but across the range, likely explained by its location near the eastern extent of the species range and drainage pattern of emptying into the Atlantic Ocean. This is consistent with our observation of the Western Branch of the Susquehanna River stream reach (WST) showing the highest level of differentiation to both Allegheny River drainage stream reaches and discrete genetic clusters according to F_{st} values (about 0.120–0.190; 0.113–0.165) even given our low sample size. Additional fine-scale genetic studies on both subspecies of Eastern and Ozark Hellbenders (*Cryptobranchus alleganiensis bishopi*) within Missouri found similar levels of within state genetic clustering by identifying three distinct genetic clusters (Crowhurst et al. 2011), indicating the level of gene flow, divergence, and isolation vary according to scale and phylogeographic history.

TABLE 4. Pairwise genetic differentiation estimates (F_{st}; upper diagonal) for Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) from seven stream reaches within the Allegheny River drainage of Pennsylvania, USA, sorted from upstream to downstream. Superscript * denotes significance. Lower diagonal is total waterway distance between average stream reach locations in kilometers. Last row includes F_{st} estimate comparisons for Allegheny River drainage stream reaches to the western branch of the Susquehanna stream reaches. Stream reaches with low sample size (less than eight) were not included in F_{st} analyses.

	TIO	OIL	FRC	MAH	TUB	HEN	4MR
TIO		0.006	0.006	0.018*	0.036*	0.039	0.016*
OIL	69.39		0.002	0.024*	0.051*	0.054	0.016
FRC	73.99	76.72		0.019*	0.036*	0.049*	0.024
MAH	237.2	172.3	220.1		0.052*	0.055*	0.038*
TUB	311.4	261.8	288.1	152.5		0.025	0.073*
HEN	313.3	263.6	290.0	154.4	1.870		0.077
4MR	290.2	266.2	322.5	210.9	211.0	212.9	
WST	0.199	0.131	0.118	0.129	0.166	0.193	0.139

We detected evidence for weak, yet significant IBD pattern indicating a potentially complex relationship between genetic and geographic distance in a species characterized by dendritic stream topology. Two of the stream reaches for this study (TUB and 4MR) appear to obfuscate the presence of a weak but significant IBD pattern detected in our study. These two stream reaches flow into the Conemaugh River before empting into Allegheny River and show high levels of differentiation (about 0.07) belonging to entirely discrete genetic clusters (TUB to TUBM/HEND and 4MR to UPALL), despite their relatively close geographic proximity (about 211 river km). The Conemaugh River, heavily affected by acid mine drainage (Sams and Beer 2000), along with the presence of the Conemaugh Dam and reservoir, located downstream of TUB may, in time act as an isolating barrier preventing gene flow from reaching upstream and eliminating the potential for genetic rescue. Alternatively, 4MR may have been assigned to the UPALL cluster due to low sample size (n = 8). Therefore, future studies should increase collection of individuals within streams both below and above dam barriers as well as incorporate sampling of younger age classes, which may reflect more recent levels of gene flow (Waples et al. 2014).

An interesting result of this study is the relatively higher N_e estimates for UPALL discrete genetic cluster, located within the predominantly forested northern Allegheny drainage cluster (UPALL). Younger age

classes (juveniles) have been recently detected at these clusters (Regester et al. 2012). Estimates for N_e within the MAHN cluster were markedly lower despite high sample (census) size. Therefore, the MAHN cluster may be at greater risk for genetic drift and lack of genetic rescue from other Allegheny individuals due to presence of potentially isolating anthropogenic factors (dams, reservoirs, etc.; Meeuwig et al. 2010; Hansen et al. 2014). Further sampling within the Allegheny drainage cluster (UPALL) may explain the discrepancy between assignment probabilities and reliability of higher N_e estimates. Estimates of N_e necessary to offset genetic drift vary, with a N_e of > 50 needed for short term conservation of heterozygosity while a N_e of > 500 is often thought to be needed for maintenance of long term evolutionary potential and retention of genetic diversity (Nelson and Soule 1987, Frankham 1995). Reduced N_e in amphibians suggest that genetic factors may play a role in amphibian declines (Funk et al. 1999) with the probability of population extinction increasing at smaller effective population sizes (Newman and Pilson 1997). Therefore, we recommend continued monitoring of Eastern Hellbender populations in Pennsylvania and increased surveys for younger age classes to increase the accuracy of N_e estimates that may reflect current trends of limited dispersal across watersheds.

We detected surprisingly high levels of genetic diversity across stream reaches compared to other

TABLE 5. F_{st} values for discrete genetic clusters of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Pennsylvania, USA. Abbreviations are MAHN = Little Mahoning Creek cluster, TUBM/HEND = Tubmill and Hendricks Creek cluster, SUSQ = Susquehanna cluster, and UPALL = Upper Allegheny River cluster.

	MAHN	TUBM/HEND	SUSQ
TUBM/HEND	0.049		
SUSQ	0.130	0.165	
UPALL	0.017	0.035	0.113

TABLE 6. Sample size (n) and effective population size (N_e) estimates for discrete genetic clusters of Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Pennsylvania, USA, and 95% confidence intervals (CI) for N_e calculated using NEestimatorv2 (Do et al. 2014) across 12 microsatellite loci. Estimates with ∞ denote infinite N_e and upper confidence intervals.

Discrete Genetic Cluster	n	N_e	95% CI for N_e
UPALL	142	∞	1,315.1–∞
MAHN	92	70.9	63.4–85.7
TUBM/HEND	40	16.4	13.9–19.4
SUSQ	9	14.5	4.7–∞

TABLE 7. Sample size (n) and effective population size (N_e) estimates for Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) from major stream reaches in Pennsylvania, USA, and 95% confidence intervals (CI) for N_e calculated using NEestimatorv2 (Do et al. 2014) across 12 microsatellite loci. Estimates with ∞ denote infinite N_e and upper confidence intervals.

Major Stream Reach	n	N_e	95% CI for N_e
Tionesta	76	526	297–∞
Oil Creek	11	76	45.7–∞
French Creek	38	178	107.8–451.8
Little Mahoning Creek	91	73.3	63.4–85.7
Tubmill Creek	27	17.4	14.3–21.7
Hendricks Creek	13	2.6	2.2–3.2
Four Mile Run	8	—	0–∞

studies (Crowhurst et al. 2011, Feist et al. 2014), which is suggestive of a healthy population that potentially can be used for various conservation measures, such as captive rearing and translocation. However, we caution interpretation of these results given the life history of Eastern Hellbenders (long lived) and biased sampling of mature adults in this study, which may mask signatures of demographic declines or genetic drift in discrete genetic clusters (Kimble et al. 2014). While we detected no difference in genetic diversity or allelic richness for any sample locations upstream of dams in this study, the unique dynamics of stream environments and presence of barriers to upstream clusters may in time limit connectivity across Allegheny River tributaries (Jager et al. 2001; Roberts et al. 2013).

These findings have implications for conservation within Pennsylvania and across the species range, where potential threats to current distributions include habitat destruction, emerging infectious diseases, drainages associated with natural gas well drilling, and erosion/sediment pollution (Evans and Ray 2010; Entrekin et al. 2011; Drohan et al. 2012; Regester et al. 2012). Moreover, in many geographic areas experiencing dramatic declines (Wheeler et al. 2003, Burgmeier et al. 2011), captive management may be the only strategy remaining for these populations, highlighting the need of conservation genetics to inform future management of Eastern Hellbender populations. Although one discrete genetic cluster (MAHN) does show evidence for a population bottleneck and reduced N_e, all genetically discrete clusters are characterized by high levels of genetic variation despite recent demographic declines and presence of potential isolating barriers. Our study of Eastern Hellbenders in Pennsylvania suggest that hydrological or ecological barriers have not yet resulted in significant losses of genetic diversity within the state and that either historical or recent downstream gene flow is sufficient to counteract genetic drift. However, our most differentiated stream reaches (MAH, TUB, HEN, and 4MR) are found within the lower section of the Allegheny River drainage where dams, reservoirs, or unsuitable habitat may act as isolating mechanisms. We suggest further sampling of younger age classes to refine estimates of N_e and genetic diversity patterns, which may reflect more current demographic trends within watersheds. Moreover, future research within the state should use these high levels of genetic variation for future captive management and continue to monitor Allegheny and Susquehanna River Drainages to decipher the enigmatic riverscape patterns of Eastern Hellbenders in Pennsylvania.

LITERATURE CITED

Abernathy, E., E. McCombs, L. Siefferman, and M. Gangloff. 2013. Effects of small dams on freshwater mussel population genetics in two southeastern USA streams. Walkerana 16:21–28.

Briggler, J., and J. Ackerson. 2012. Construction and use of artificial shelters to supplement habitat for Hellbenders (*Cryptobranchus alleganiensis*). Herpetological Review 43:412.

Burgmeier, N.G., S.D. Unger, T.M. Sutton, and R.N. Williams. 2011. Population status of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. Journal of Herpetology 45:195–201.

Cowell, C.M, and R.T. Stoudt. 2007. Dam-induced modifications to upper Allegheny River streamflow patterns and their biodiversity implications. Journal of the American Water Resources Association 38:187–196.

Crowhurst, R., K.M. Faries, J. Collantes, J.T. Briggler, J.B. Koppelman, and L.S. Eggert. 2011. Genetic relationships of Hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*). Conservation Genetics 12:637–646.

Do, C., R.S. Waples, D. Peel, G.M. Macbeth, B.J. Tillett, and J.R. Ovenden. 2014. NeEstimator v2: re-

implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. Molecular Ecology Resources 14:209–214.

Drohan, P., M. Brittingham, J. Bishop, and K. Yoder. 2012. Early trends in land cover change and forest fragmentation due to shale-gas development in Pennsylvania: a potential outcome for the north central Appalachians. Environmental Management 49:1061–1075.

Earl, A., and B. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetic Resources 4:359–361.

Entrekin, S., M. White, B. Johnson, and E. Hagenbuch. 2011. Rapid expansion of natural gas development poses a threat to surface waters. Frontiers in Ecology and the Environment 9:503–511.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611–2620.

Evans, R., and S. Ray. 2010. Distribution and environmental influences on freshwater gastropods from lotic systems and springs in Pennsylvania, USA, with conservation recommendations. American Malacological Bulletin 28:135–150.

Feist, S., J.T. Briggler, J.B. Koppelman, and L. Eggert. 2014. Within-river gene flow in the Hellbender (*Cryptobranchus alleganiensis*) and implications for restorative release. Conservation Genetics 15:953–966.

Foster, R.L., A.M. McMillan, and K.J. Roblee. 2009. Population status of Hellbender Salamanders (*Cryptobranchus alleganiensis*) in the Allegheny River Drainage of New York State. Journal of Herpetology 43:579–588.

Funk, C., D.A. Tallmon, and F.W. Allendorf. 1999. Small effective population size in the Long-toed Salamander. Molecular Ecology 8:1633–1640.

Frankham, R. 1995. Effective population size/adult population size ratios in wildlife: a review. Genetical Research 66:99–107.

Galbraith, H.S., D.T. Zanatta, and C. C. Wilson. 2015. Comparative analysis of riverscape genetic structure in rare, threatened and common freshwater mussels. Conservation Genetics 16:845–857.

Guillot, G., F. Mortier, and A. Estoup. 2005. GENELAND: a computer package for landscape genetics. Molecular Ecology Notes 5:712–715.

Hansen, M.M., M.T. Limborg, A. Ferchaud, and J. Pujolar. 2014. The effects of Medieval dams on genetic divergence and demographic history in brown trout populations. BMC Evolutionary Biology 14:122.

Hare, M., L. Nunney, M. Schwartz, D. Ruzzante, M. Burford, R. Waples, K. Ruegg, and F. Palastra. 2011.

Understanding and estimating effective population size for practical application in marine species management. Conservation Biology 25:438–449.

Hiler, W., B.A. Wheeler, and S.E. Trauth. 2013. The decline of the Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) in the Spring River, Arkansas, USA. Herpetological Conservation and Biology 8:114–121.

Hillis, R., and E. Bellis. 1971. Some aspects of the ecology of the hellbender *Cryptobranchus alleganiensis alleganiensis* in a Pennsylvania stream. Journal of Herpetology 5:121–126.

Hopken, M., M. R. Douglas, and M.E. Douglas. 2013. Stream hierarchy defines riverscape genetics of a North American desert fish. Molecular Ecology 22:956–971.

Jager, H.I., J.A. Chandler, K.B. Lepla, and W.V. Winkle. 2001. A theoretical study of river fragmentation by dams and its effects on White Sturgeon populations. Environmental Biology of Fishes 60:347–361.

Kalinowski, S.T. 2005. HP-Rare 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187–189.

Kanno, Y., J.C. Vokoun, and B.H. Letcher. 2011. Fine-scale population structure and riverscape genetics of Brook Trout (*Salvelinus fontinalis*) distributed continuously along headwater channel networks. Molecular Ecology 20:3711–3729.

Kimble, S.J., O.E. Rhodes, and R.N. Williams. 2014. Unexpectedly low rangewide population genetic structure of the imperiled Eastern Box Turtle *Terrapene c. carolina*. PlosOne 9:e92274. doi:10.1371/journal.pone.0092274.

Landguth, E., S. Cushman, M. Schwartz, K. McKelvey, M. Murphy, and G. Luikart. 2010. Quantifying the lag time to detect barriers in landscape genetics. Molecular Ecology 19:4179–4191.

Latch, E.K., G. Dharmarajan, J.C. Glaubitz, and O.E. Rhodes. 2006. Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. Conservation Genetics 7:295–302.

Manel, S., M.K. Schwartz, G. Luikart, and P. Taberlet. 2003. Landscape genetics: combining landscape ecology and population genetics. Trends in Ecology and Evolution 18:189–197.

Mayasich, J., D. Grandmaison, and C. Phillips. 2003. Eastern Hellbender status assessment report. Natural Resources Research Institute, Technical Report 9:1–41.

Meeuwig, M., C. Guy, S. Kalinowski, and W. Fredenberg. 2010. Landscape influences on genetic differentiation among Bull Trout populations in a stream-lake network. Molecular Ecology 19:3620–3633.

Miller, M. 2005. Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. Journal of Heredity 96:722–724.

Moore, J.A., H.C. Miller, C.H. Daugherty, and N.J. Nelson. 2008. Fine-scale genetic structure of a long-lived reptile reflects recent habitat modification. Molecular Ecology 17:4630–4641.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, New York, USA.

Nelson, K., and M. Soule. 1987. Genetical conservation of exploited fishes. Pp. 345–368 *In* Population Genetics and Fisheries Management. Ryman, N. and F. Utter (Eds.). University of Washington Press, Seattle, USA.

Neville, H., J. Dunham, and M. Peacock. 2006. Landscape attributes and life history variability shape genetic structure of trout populations in a stream network. Landscape Ecology 21:901–916.

Newman, D., and D. Pilson. 1997. Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evolution 5:354–362.

Nickerson, M.A., and C.E. Mays. 1973. The Hellbenders: North America "Giant Salamanders." Milwaukee Public Museum Publication, Biology and Geology, Number 1, Milwaukee, Wisconsin, USA.

Oosterhout, C., W. Hutchinson, D. Wills, and P. Shipley. 2004. MICRO–CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Resources 4:535–538.

Peakall, R., and P.E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population software for teaching and research. Molecular Ecology Notes 6:288–295.

Piry, S., G. Luikart, and J. Cornuet. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90:502–503.

Piry, S., A. Alapetite, J. Cornuet, D. Paetkau, L. Baudouin, and A. Estoup. 2004. GeneClass2: a software for genetic assignment and first-generation migrant detection. Journal of Heredity 95:536–539.

Pritchard, J., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Quinn, S.A., J.P. Gibbs, M.H. Hall, and P.J. Petokas. 2013. Multiscale factors influencing distribution of the Eastern Hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*) in the northern segment of its range. Journal of Herpetology 47:78–84.

Regester, K., H. Simpson, E. Chapman, and P. Petokas. 2012. Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* among Eastern Hellbender populations (*Cryptobranchus a.*

alleganiensis) within the Allegheny-Ohio and Susquehanna River Drainages, Pennsylvania, USA. Herpetological Review 43:18–21.

Rieux, A., F. Halkett, L. deLapeyre deBellaire, M.F. Zapater, F. Rousset, V. Ravigne, and J. Carlier. 2011. Inferences on pathogenic fungus population structure from microsatellite data: new insights from spatial genetics approaches. Molecular Ecology 20:1661–1674.

Roberts, J.H., P.L. Angermeier, and E.M. Hallerman. 2013. Distance, dams and drift: what structures populations of an endangered, benthic stream fish? Freshwater Biology 58:2050–2064.

Rousset F. 2008. Genepop007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 8:103–106.

Sabatino, S.J., and E.J. Routman 2009. Phylogeography and conservation genetics of the Hellbender Salamander (*Cryptobranchus alleganienesis*). Conservation Genetics 10:1235–1246.

Sambrook, J., and D. Russell. 2001. Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Springs Harbor Press, Cold Springs Harbor, New York, USA.

Sams, J., and Beer, K. 2000. Effects of coal-mine drainage on stream water quality in the Allegheny and Monongahela River Basins-sulfate transport and trends. Water-Resources Investigations Report 99–4208. U.S. Geological Survey, Lemoyne, Pennsylvania, USA. 17 p.

Unger. S., J. Fike, T. Sutton, O. Rhodes, and R. Williams. 2010. Isolation and development of 12 polymorphic tetranucleotide microsatellite markers for the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*). Conservation Genetic Resources 2:89–91.

Unger, S., N. Burgmeier, and R. Williams. 2012. Genetic markers reveal high PIT tag retention rates in giant salamanders (*Cryptobranchus alleganiensis*). Amphibia-Reptilia 33:313–317.

Unger, S., O. Rhodes, T. Sutton, and R. Williams. 2013. Population genetics of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) across multiple spatial scales. PlosOne 8:e74180. doi:10.1371/journal.pone.0074180.

Waples, R.S., T. Antao, and G. Luikart. 2014. Effects of overlapping generations on linkage disequilibrium estimates of effective population size. Genetics 197:769–780.

Weir, B., and C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

Wheeler, B.A., E. Prosen, A. Mathis, and R.F. Wilkinson. 2003. Population declines of a long-lived salamander: a 20+ year study of Hellbenders,

Cryptobranchus alleganiensis. Biological Conservation 109:151–156.

Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:97–159.

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