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RESEARCH ARTICLE

Phylogeography and conservation genetics of the hellbender salamander (*Cryptobranchus alleganiensis*)

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Abstract We investigated hellbender phylogeography through phylogenetic analyses of individuals sampled from 16 locations throughout their range in the eastern United States. Analyses were conducted on concatenated cytochrome-oxidase I (COI), cytochrome-*b* (Cyt*b*) and NADH dehydrogenase subunit 4 (ND4) mtDNA sequence, totaling 2160 nucleotides. Hellbender haplotypes differed by 0.1 to 5.8% maximum likelihood (ML) corrected sequence divergence. Phylogenetic analyses reveal that hellbenders are separated into 8 reciprocally monophyletic populations or clades differentiated by a minimum of 0.7 to 5.4% sequence divergence, each of which constitutes a separate Management Unit (MU). High among population divergence and reciprocal monophyly suggest that female-mediated gene flow is severely restricted or non-existent among each MU. Hellbenders are currently divided into two subspecies, *Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi* based on morphological characters. The phylogenetic analyses presented here strongly indicate that these subspecies are paraphyletic. Management priorities for the hellbender should be reconsidered in light of these new molecular data. Results from Bayesian rooting indicate the root of the hellbender mtDNA tree lies on the branch leading to hellbender haplotypes from the Current, Eleven Point and New Rivers. The rooted tree suggests that a common ancestor in the southern Ozarks and/or southern Appalachians gave rise to northern hellbender populations, consistent with a Pleistocene refuge hypothesis.

Keywords *Cryptobranchus alleganiensis* Conservation Phylogeography mtDNA Management unit

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Introduction

Phylogeography and conservation

One persistent problem in evolution and conservation biology, addressable through molecular phylogeography (Avise 2000), is the delineation of species boundaries. The terms "species" and "subspecies" are controversial, which has resulted in numerous definitions for each in the literature (Futuyma 1997). A common thread among several widely accepted definitions is the idea that species or subspecies are lineages that are evolving independently from other lineages. Genetic differentiation among groups of related organisms may be the best evidence of lineage separation as it is indicative of reproductive and/or ecological isolation and is the result of long-standing trends in demographic history (Avise 1995). Genetic data is especially useful for identification of cryptic species or subspecies where morphological differentiation is absent or misleading (Baric and Sturmbauer 1999).

Molecular phylogeography can also be used to measure the genetic dependence of conspecific populations, or population structure. The degree to which populations are independent of one another depends on the level of gene flow among the populations. For example, a species may exist as a single panmictic population or separate populations among which migrants are exchanged

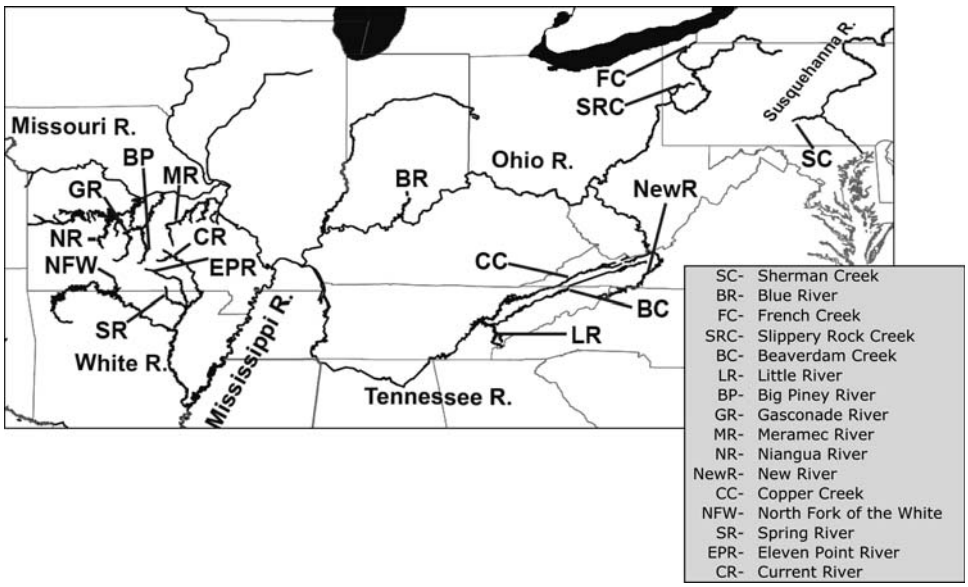

Fig. 1 Map of the eastern USA showing the locations where hellbenders were sampled for this study

intermittently, resulting in some level of gene flow between them. The population structure of a species must be considered when predicting how conservation measures targeted at one group of individuals will affect the rest of the species. Relatively independent populations are more likely to be fixed for unique alleles associated with local adaptations. The preservation of locally adapted populations and maintenance of their genetic integrity is important since they are likely to be significantly contributing to the overall adaptive potential of the species (Lacy 1987; Templeton 1987). The genetic integrity of locally adapted populations can be compromised via management practices implemented to increase population sizes and/or genetic diversity of endangered species such as breeding programs or the translocation of individuals among currently isolated populations (Tallmon et al. 2004). This risk stems from the fact that adaptive phenotypes of individuals within independent populations are often controlled by unlinked multi-gene complexes (Wright 1968; Matoli and Templeton 1999). If individuals from such genetically distinct populations are crossed, either through breeding programs or translocations, their hybrid offspring may receive portions of co-adapted gene complexes that are incompatible or unsuited to each other since they evolved in unique genetic backgrounds (i.e., outbreeding depression; Hedrick 2001). Outbreeding depression leads to lower individual fitness (Hedrick 2001; Lenormand 2002; Gharrett et al. 1999) and can therefore hamper conservation efforts. Molecular phylogeography can therefore be used to identify which populations should be preserved and assess risks associated with conservation strategies that involve cross-breeding of independent populations.

The hellbender salamander

Hellbenders are large (up to 74 cm) aquatic salamanders found in clear fast-flowing streams in the eastern U.S. (Fig. 1). There are currently two recognized hellbender subspecies. The Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) inhabits streams that drain south out of the Ozark Plateau in the highlands of Missouri and Arkansas. All other populations of hellbenders, including those in rivers draining northward from the Ozarks, belong to the nominal subspecies, *C. a. alleganiensis*. The Ozark hellbender is mainly characterized by its smaller spiracle size relative to the eastern hellbender, its coloration pattern and a few other minor morphological characteristics (Grobman 1943).

Due to recent declines in Ozark hellbender populations and the degradation of their habitat, the U.S. Fish and Wildlife Service is considering designating *C. a. bishopi* a candidate for the Endangered Species List (*Federal Register*/ Vol. 71, No. 176/Tuesday, September 12, 2006/Proposed Rules). Many populations of *C. a. alleganiensis* are also smaller than in the recent past and face similar threats (Wheeler et al. 2003). Riverine amphibian species such as hellbenders are threatened by anthropogenically induced environmental change (Blaustein and Kiesecker 2002). Increased sedimentation and siltation of rivers due to land development, pollution and over-harvesting have all been implicated in hellbender declines (Nickerson et al. 2002). Amphibian species are often distributed as multiple independent populations among which gene flow is restricted by natural barriers (Shaffer et al. 2004). As riverine habitats become degraded, resident species become increasingly fragmented, which may exacerbate the deleterious genetic effects of small population size. Thus, "evolutionarily

enlightened" management (Ashley et al. 2003) strategies based on solid molecular data are needed to help shape conservation efforts for declining hellbender populations.

The most recent investigation into hellbender population genetics, using restriction enzyme digestion of the entire mtDNA genome, found high levels of genetic differentiation (between .2 and 4% sequence difference) among most populations within each putative subspecies (Routman et al. 1994). The two named hellbender subspecies *C. a. alleganiensis* and *C. a. bishopi* were also shown to be paraphyletic. However, the relationships among major clades of mtDNA haplotypes were not well resolved and the phylogeny was midpoint rooted. One major problem encountered by Routman et al. (1994), which is often found in molecular phylogeography, was the inability to reliably root the phylogenetic tree using the closest outgroup, in this case the giant Chinese salamander, *Andrias davidianus*. If the closest extant relative of the ingroup has been independently evolving for a long time, it will be disproportionately divergent with respect to divergences among ingroup populations. Over-divergent outgroups confound phylogenetic analysis due to extensive homoplasy and multiple evolutionary changes at many nucleotide sites (Felsenstein 2004). This problem may be widespread among phylogeographic and biogeographic studies, yet it is rarely recognized. Huelsenbeck et al. (2002) showed that reliable roots can be obtained by generating trees with Bayesian analysis while enforcing a molecular clock, especially when the data are from lineages that have diverged at similar rates (i.e. in a clock-like fashion). Bayesian rooting was successfully implemented by Steele et al. (2005) to root the mtDNA tree of the aquatic salamander genus *Dicamptodon* and thus may hold promise for hellbenders.

Here we explore the molecular phylogeography of the endangered hellbender salamander, *Cryptobranchus alleganiensis*, using mtDNA sequence data. Our goals are to better understand the recent and historical relationships among hellbender populations, to test subspecies boundaries within the species, and to identify which hellbender populations may be particularly important to the long-term viability of the species. These data can be used to shape conservation management of hellbender populations.

Methods

Hellbender sampling

Blood and/or tissue samples were collected from hellbenders caught in 15 different rivers and streams throughout the Appalachian and Ozark Mountains of the eastern US (Fig. 1, Table 1; see Routman 1993; Routman et al. 1994 for details). Tissue samples from Eleven Point River hellbenders were collected by Dr. Jeff Briggler of the Missouri Department of Conservation and stored in 95% ETOH. We extracted DNA from hellbender blood and tissue samples using the standard phenol/chloroform method outlined in Routman (1993). We further cleaned the DNA obtained from blood by adding 500 ng of it to 100 µl of 5% Chelex (BioRad), boiling the mixture for 2 min and isolating the supernatant. All DNA samples were stored at −20 C.

Table 1 Sampling locations, major drainages into which the rivers flow, and hellbender subspecies designation

Sampled river	Major drainage	Subspecies
Gasconade River (GR)	Missouri	*alleganiensis*
Big Piney River (BP)	Gasconade—Missouri	*alleganiensis*
Niangua River (NR)	Osage—Missouri	*alleganiensis*
Meramec River (MR)	Mississippi	
Little River (LR)	Tennessee	*alleganiensis*
Beaverdam Creek (BC)	Holston—Tennessee	*alleganiensis*
Copper Creek (CC)	Clinch—Tennessee	*alleganiensis*
Blue River (BR)	Ohio	*alleganiensis*
French Creek (FC)	Allegheny—Ohio	*alleganiensis*
Slippery Rock Creek (SRC)	Connoquenessing—Ohio	*alleganiensis*
New River (NR)	Kanawha—Ohio	*alleganiensis*
Sherman Creek (SC)	Susquehanna	*alleganiensis*
North Fork of the White River (NFW)	White	*bishopi*
Spring River (SR)	Black—White	*bishopi*
Current River (CR)	Black—White	*bishopi*
Eleven Point River (EPR)	Black—White	*bishopi*

All drainages eventually flow into the Mississippi River except Sherman Creek (Susquehanna) which terminates in the Chesapeake Bay (see Fig. 1)

Table 2 Primer sequences for mitochondrial genes sequenced for hellbenders

Primer name	Primer sequence (5′–3′)	Reference
cyt*b*		
tRNA-Glucine	TTGTATTCAACTATAAAAAC	Steele et al. (2005)
SAL-R1	ACTTAACCTCCTGTTGGTCA	
Cyt*b*-CA-F1	TTCATTTATTGACCTACCAACC	
Cyt*b*-CA-R1	GATAATTGACACTAAGGCTCAG	
COI		
LCO 1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
COI-CA-F1	TTAAGCTTATTAATCCGAGCAG	
COI-CA-R1	TGGCTGATGTGAAATAAGCTCG	
ND4		
ND4-Nex-H	TGGGGGCTACGGCATAATAC	Hardy et al. (2002)
ND4-Nex-L	CCAATGGATGAACTATTATCCT	Hardy et al. (2002)
ND4-Sal-F1	AAAATACCACTCTATGGTGC	
ND4-Sal-R1	GTTCATAACTTTCACTTGGA	

Polymerase chain reaction and sequencing

Initially we used the Polymerase Chain Reaction (PCR) to amplify hellbender cytochrome-*b* (cyt*b*) with a forward primer tRNA-Glucine designed for *Dicamptodon* (see Table 2 for all primer sequences) and a reverse primer we designed from the consensus sequences of *Ambystoma laterale* (Genbank accession number: AY728227), *Desmognathus fuscus* (AY728218) and *Andrias davidianus* (NC_004926). The DNA template used for this particular PCR was extracted from hellbender ova, which contain a high amount of mtDNA relative to somatic tissue. The result was a 900 bp fragment of cyt*b*, which we sequenced and used to design the hellbender specific primers (Cyt*b*-CA-F1, Cyt*b*-CA-R1) that amplify a 750 bp fragment. We amplified cytochrome oxidase I (COI) using universal primers LCO 1490 and HCO 2198 (Folmer et al. 1994), which yielded a 500 bp fragment. Because a larger fragment of COI is desirable, we aligned the resultant sequence with that of *Andrias davidianus* (NC_004926) and used it to design the primers COI-CA-F1 (based on *Andrias* sequence alone) and COI-CA-R1 (based on *Andrias/Cryptobranchus* alignment), which amplify a 750 bp fragment. We amplified NADH dehydrogenase subunit 4 (ND4) with ND4-Nex-F and ND4-Nex-R from Hardy et al. (2002). These primers did not work for a number of hellbender samples, so we designed the hellbender specific primers ND4-Sal-F1 and ND4-Sal-R1 from *Andrias davidianus* (NC_004926) and hellbender sequence, which yielded a similar sized fragment. Ampli-taq chemistry (Applied Biosystems (ABI)) was used for PCR under the following conditions: 94 C (120 s) initial denature, {94 C (30 s), 58 C (30 s), 72 C (60 s)} 30×, and a final extension at 72 C (120 s). We sequenced DNA with ABI Big Dye chemistry on an ABI 377 sequencer according to manufacturer's recommendations.

We assessed within-population genetic diversity by conducting cyt*b*, COI and ND4 allele counts. For every novel cyt*b* or COI haplotype recovered, at least one individual that possessed it was also sequenced at ND4; in this way, we hoped to recover much of the genetic diversity in the hellbender populations studied and ensure there was sufficient statistical power for phylogenetic analyses.

Phylogenetic analyses

We conducted maximum likelihood (ML) phylogenetic analyses on the concatenated cyt*b*, COI and ND4 sequences. Redundant haplotypes were removed from the dataset prior to all phylogenetic analyses. We ran a maximum likelihood analysis (MLA), with and without *Andrias davidianus* sequence included as an outgroup, using the program *PAUP 4.0b10* (Swofford 2001). To choose a substitution model for MLA and Bayesian analysis (see below), we used the software program *Modeltest 3.7* (Posada and Crandall 1998). We conducted the following iterative MLA rather than a full one. We began by calculating the parameter values for the substitution model based on fixed tree topology generated via the bio-neighbor-joining algorithm in *PAUP* (Distance model = ML parameter estimates generated by *ModelTest*). The parameter estimates were then fixed and a heuristic ML search was run to find the most likely tree topology. The resultant tree was in turn used to re-estimate model parameter values. We continued the iterative process of estimating model parameters based on a fixed tree topology, and vice versa, until all model parameters, including the tree topology, stabilized. Independent estimation of model parameters

and tree topology has been shown to accurately converge on the ML tree found when conducting a full MLA while dramatically increasing computational efficiency (Sullivan et al. 2005). We conducted bootstrap analysis using 500 ML bootstrap pseudoreplicates of the dataset, fixing model parameters at maximum likelihood values and estimating tree topology.

To root the hellbender mtDNA tree by enforcing a molecular clock, we first pruned the mtDNA dataset to include one representative haplotype from each of the 8 major monophyletic clades. The molecular clock hypothesis was tested for the data using a likelihood ratio test (Felsenstein 2004). We determined which evolutionary model to use to test the molecular clock hypothesis using *Modeltest* and conducted the phylogenetic analysis in *PAUP*.

The molecular clock rooting analysis was conducted using the program *MrBayes 3.1.2* (Huelsenbeck and Ronquist 2001). Since it is possible to specify separate evolutionary models for different parts of a dataset in *MrBayes 3.1.2* and the 1st, 2nd and 3rd nucleotide positions within the protein-coding genes that make up the composite haplotypes are likely to be evolving at different rates, we used a separate model for each position. We determined which model to use for each position using the software program *Modeltest*. Bayesian trees were generated using the program *MrBayes* (Huelsenbeck and Ronquist 2001) as follows: we ran two replicate searches, each of which was composed of four Metropolis-coupled chains and started with a random tree. Each chain ran for 1.0×10^7 generations and was sampled every 1000 generations. The chains were run until the average standard deviation of split frequencies among them reached .001, which yielded 10,000 trees from each run. The last 5,000 trees generated in each run (for a combined total of 10,000) were used to determine the root. The Bayesian posterior probability (BPP) that a node is the root of the tree was calculated as the percentage of the 10,000 trees with that particular root. We calculated the BPP for each root position by importing all 10,000 Bayesian trees into *PAUP* and filtering them with constraint trees for each possible root placement on the 8 haplotype tree (See Fig. 3).

Results

Sequencing

A total of 26 cyt*b* ($N = 88$), 19 COI ($N = 91$) and 17 ND4 ($N = 61$) haplotypes were recovered from 16 hellbender populations (see Table 3). Most individuals were sequenced at both cyt*b* and COI, but this was not always the case due to variation in PCR and sequencing success. Concatenation of the three mtDNA gene sequences for individuals with complete data resulted in 32 unique mtDNA haplotypes that were 2160 bp in length (807 bp—cyt*b*, 705 bp—COI and 648 bp—ND4) after sequence editing. To ensure that mtDNA genes and not nuclear pseudogenes were sequenced, each sequence was translated, checked for stop codons and compared to the amino acid sequence of *Andrias davidianus* to ensure the reading frame was intact.

Considerable among-population genetic independence was observed, even at individual loci. Ten of 16 hellbender populations surveyed in this study share no cyt*b*-COI-ND4 haplotypes with other populations. The 6 hellbender populations with shared haplotypes comprise the following 3 pairs that share haplotypes within pairs but not among them: (Big Piney River, Gasconade River; Blue River, Slippery Rock Creek; French Creek, Sherman Creek). The Big Piney River is a tributary of the Gasconade River, and these hellbenders could be considered a single population. The other four rivers contain haplotypes that are all closely related (see below). In contrast, haplotypes within each population appear closely related, differing by a maximum of 7 bases out of over 2000 bases sequenced.

Phylogenetic analyses

Modeltest selected the TrN+G model to be the most appropriate substitution model for the mtDNA haplotype dataset with no outgroup using the Akaike Information Criterion (AIC). Maximum likelihood analysis resulted in the following model parameter estimates: $\Pi_A = 0.356$, $\Pi_C = 0.162$, $\Pi_G = 0.180$, $\Pi_T = 0.301$; AC = 1.000, AG = 20.979, AT = 1.000, CG = 1.000, CT = 26.965, GT = 1.000 and Γ-distribution shape parameter $\alpha = 0.161$. The midpoint rooted ML phylogeny (Fig. 2) has a likelihood score of $-\ln L = 4591.762$. The outgroup-rooted ML tree including the *Andrias* haplotype (tree not shown) has the same overall topology as the mid-point rooted tree except it places the Current River haplotype as a sister taxon to all others. However, because 20% sequence divergence exists between the *Andrias* haplotype and those of Current River hellbenders, Andrias is clearly a poor outgroup and the exact root placement is questionable. As a result, we used the Bayesian analysis to determine the root of the hellbender phylogeny.

The dataset used for Bayesian rooting included one haplotype from the following hellbender populations: Big Piney/Gasconade River, New River, Spring River, Current River, Little River, Slippery Rock Creek, Copper Creek and the North Fork of the White River. The results of *Modeltest* showed the GTR+G model to be most appropriate for the entire pruned dataset used to test the molecular clock hypothesis. The likelihood scores for the tree estimated with and without enforcing a molecular clock were 4238.54 and

Table 3 Haplotype distribution among sampling localities for three separate mitochondrial genes and 3-locus composite haplotypes

River	*N*	Cyt*b* haplotype	*N*	COI haplotype	*N*	ND4 haplotype	3-locus haplotype name	3-locus composite haplotype
Big Piney River	6	4	13	2	3	3	BP_GR-1	4_2_3
	2	6	1	3	2	4	BP_GR-2	5_2_3
	4	5					BP-1	6_3_4
Gasconade River	3	5	4	2	3	3	BP_GR-2	5_2_3
	4	4	2	9	2	12	BP_GR-1	4_2_3
	2	6					GR-1	6_9_12
Niangua River	4	6	5	17	3	3	NR-1	21_17_3
	1	21					NR-2	6_17_3
Meramec River	8	16	7	12	6	3	MR-1	17_12_3
	2	17	1	13			MR-2	16_13_3
Beaverdam Creek	4	1	6	1	2	1	BC-1	2_1_2
	1	3			1	2	BC-2	1_1_1
	1	2					BC-3	3_1_1
Little River	5	13	5	1	2	1	LR-1	14_10_1
	1	15	1	11	1	13	LR-2	13_1_1
	1	14	1	10			LR-3	13_1_13
Copper Creek	9	8	8	5	3	7	CC-1	8_5_7
Slippery Rock Creek	1	25	3	4	1	10	SRC-1	24_4_6
	3	7	1	7	4	6	SRC_BR-1	7_4_6
	1	24	1	19			SRC-2	7_19_6
							SRC-3	25_7_10
New River	2	20	1	14	1	14	NewR-1	20_14_14
			1	15	1	15	NewR-2	20_15_15
Blue River	4	7	4	4	1	5	SRC_BR-1	7_4_6
					1	6	BR-1	7_4_5
French Creek	4	11	5	4	4	10	FC_SC-1	11_7_10
	1	12	3	7	3	6	FC-2	12_4_6
			1	8	2	11	FC_4	11_8_10
							FC-3	11_4_10
Sherman Creek	2	11	1	7	1	10	FC_SC-1	11_7_10
North Fork of the White River	4	19	6	16	2	16	NFW-1	19_16_16
	2	18					NFW-2	18_16_16
Spring River	1	22	1	18	6	17	SR-1	22_18_17
	1	23					SR-2	23_18_17
Current River	2	9	6	6	2	9	CR-1	9_6_9
					1	8		
Eleven Point River	1	10	3	3	3	9	EPR-1	10_3_9
	1	26						

N = number of individuals sequenced for corresponding gene. Sample size for the three locus haplotypes is the minimum sample size for the three individual loci comprising the haplotype

4236.61, respectively. The likelihood scores for the two trees are not significantly different ($X^2 = 3.86$, $P = 0.70$, $df = 6$) and the molecular clock hypothesis can not be rejected. Molecular clock rooting has been shown to be most accurate when sequences are evolving in a clock-like fashion and is therefore appropriate in this case.

The application of *Modeltest* indicated the GTR+G, Trn and the GTR+G models to be most appropriate for 1st, 2nd and 3rd codon positions, respectively, for the dataset used in the Bayesian molecular clock rooting analysis. Since it is not possible to specify the Trn model in *MrBayes* and Bayesian analysis is robust to slight over-parameterizations of models

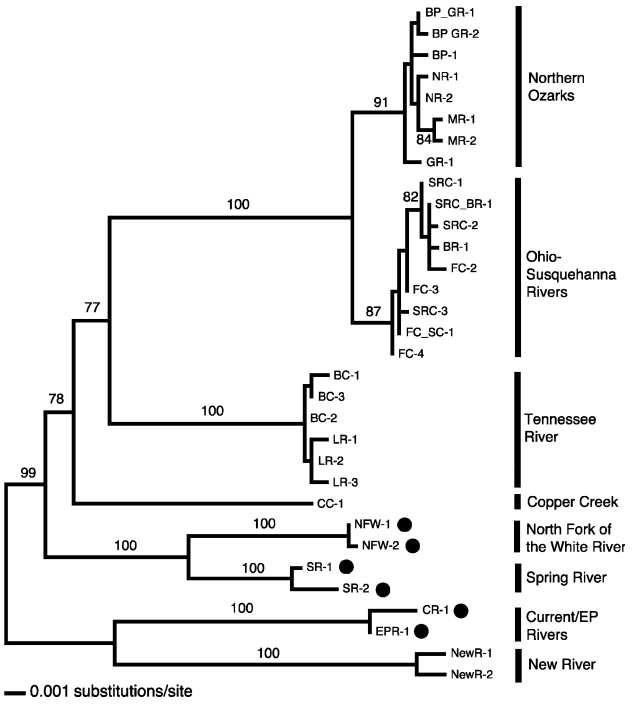

Fig. 2 Midpoint-rooted maximum likelihood (ML) phylogeny of hellbender mtDNA 3-locus haplotypes. The numbers on branches represent ML bootstrap values (500 replicates). Haplotypes are labeled with letters indicating the river in which they were found and a number to separate different haplotypes in the same river. Haplotypes found in more than one river are given both river names. More inclusive clades are named by major drainage or geographic region. Haplotypes followed by a black circle were those found in hellbenders currently designated *C. a. bishopi*; all other haplotypes are considered to be *C. a. alleganiensis*

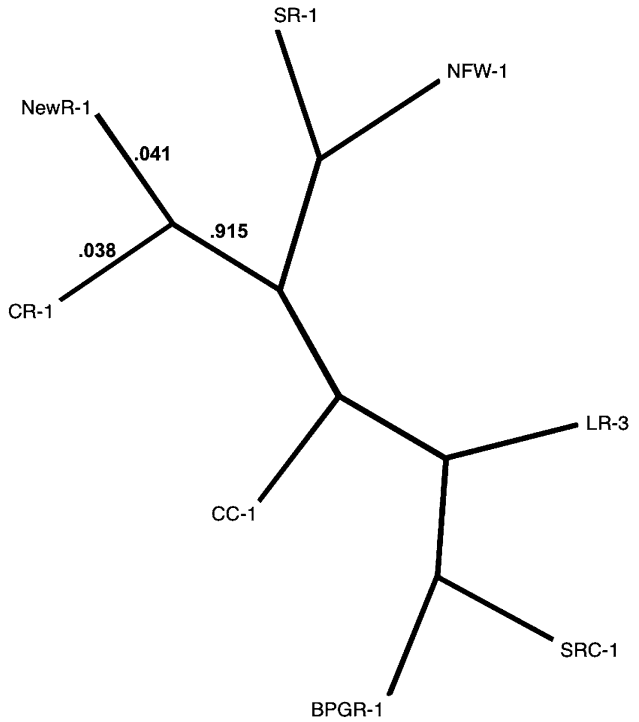

Fig. 3 One of the constraint trees used to filter the Bayesian trees generated under a molecular clock. The branch leading to the New River and Current River clade was found to be the root in 91.5% of the trees sampled from the posterior distribution

(Huelsenbeck et al. 2002), we used GTR for 2nd position nucleotides. A total of 10,000 trees were used to calculate the posterior probability of each possible root position in the 8-haplotype tree. About 92% of the sampled Bayesian trees generated while enforcing a molecular clock rooted the mtDNA tree on the long branch between the New River/Current River hellbender haplotypes and the rest of the tree (Fig. 3). The root was found to be on the branch leading to the Current River hellbender haplotype or New River one in 3.8 and 4.1% of the trees, respectively. All other nodes were found to be the root less than 1.0% of the time. This result is consistent with the mid-point rooted tree.

The rooted ML phylogeny (Fig. 2) reveals hellbender mtDNA haplotypes are separated into 8 reciprocally monophyletic clades differentiated by 0.7 to 5.4% sequence divergence. The 8 groups, named by drainage (see Fig. 1, Table 1) or geographic region, are: (1) Northern Ozarks, (2) Eleven Point/Current River, (3) North Fork of the White, (4) Spring River, (5) New River, (6) Copper Creek, (7) Tennessee River, and (8) Ohio/Susquehanna River. Moderate (70–80%) to strong (above 90%) ML bootstrap support was found for the placement of all clades separated by more than 0.7% sequence divergence. In general, resolution at the tips of clades is poor, except for the Meramec River clades, whose monophyly is supported by a bootstrap frequency of 87%, and a subset of the Ohio-Susquehanna Rivers clade (82% bootstrap support).

Discussion

Phylogeography

Most hellbender populations surveyed in this study are genetically distinct, evidenced by mutually exclusive haplotypes for most populations surveyed and reciprocal monophyly among 8 major groups of hellbender haplotypes. These data suggest female gene flow among hellbender populations is restricted, which is consistent with results from mark-recapture studies showing low within-river movement and remarkable philopatry (for both genders) in this species (Nickerson and Mays 1973; Peterson 1987; Routman et al. 1994). Hellbenders have very specific habitat requirements and are only found in rocky streams with clean, clear, cold, well-oxygenated water. The rarity of such habitat, especially at low elevations, may severely limit hellbender migration between rivers and render the range of hellbenders highly fragmented. In

addition, hellbenders have external fertilization and as a consequence colonization of a new population requires at least a breeding pair, rather than a single inseminated female. This life history trait reduces the chances for successful colonization via serendipitous events such as floods which move hellbenders to new locations. Successful migration and colonization may only occur when geologic or climatic changes result in the formation of migratory paths suitable to hellbenders. Geologic and climatic history is therefore likely to have played a significant role in shaping the distribution of mtDNA variation observed today (Routman et al. 1994; Kozak et al. 2006).

The geology of eastern North America has undergone dramatic changes throughout the time hellbenders are likely to have inhabited their current range. Remarkably, a 160-million-year old cryptobranchid fossil found in Asia is morphologically very similar to hellbenders found in eastern North America today (Gao and Shubin 2003). Based in part on the fact that younger cryptobranchid fossils have been found in Asia, Europe, and North America (Stebbins and Cohen 1995), a reasonable hypothesis is hellbenders or their ancestors evolved in Asia and reached North America via a land bridge prior to 56 mya (Gao and Shubin 2003). Indeed, the earliest cryptobranchid fossil found in North America so far dates back to the Paleocene around 65 mya (Naylor 1981). Since the Paleocene, the Ozark highlands have flattened and have risen again (Nickerson and Mays 1973). In addition, Pleistocene glaciations formed, reformed and dissipated numerous rivers and lakes throughout eastern North America (Pielou 1991). Such geologic changes would have created and destroyed river habitats and migratory routes available to organisms such as the hellbender. It is therefore likely that aspects of current hellbender population genetic structure can only be understood by considering past geologic and climatic events. The deep genetic divergences among some populations of hellbenders observed in our ML tree suggest ancient events separated these populations.

Our ML tree and Bayesian rooted tree (see Figs. 2, 3) strongly support a sister taxon relationship between the New River and Current/Eleven Point Rivers (hereafter abbreviated C/EP Rivers) hellbender populations. This is a surprising result given the New River drains into the Ohio River in West Virginia and the C/EP Rivers drain into the Mississippi River via the White River in southern Arkansas (Fig. 1). Hellbenders inhabit parts of the Ohio and Tennessee Rivers, which lie between and connect the C/EP Rivers and New River. However, hellbenders from these populations are not closely related to those in either the C/EP Rivers or New River, which would be the case if they were remnant populations from a migratory event between the southern Ozarks and New River. The New River hellbender haplotypes differ from those of C/EP Rivers hellbenders by a minimum of 4.3% sequence divergence, which is comparable to the difference between the most distantly related hellbender populations surveyed in this study (5.4%). This suggests an old relationship. The Teays River existed prior to the Pleistocene glaciations and was the major river system in the region where the Ohio River now runs, connecting the Appalachians and the Old Mississippi River. The New River is one of the oldest rivers in the United States and widely accepted to be a remnant of the ancient Teays River (Berendzen et al. 2003). The relationship between New River and C/EP Rivers hellbenders may reflect a pre-Pleistocene migratory event along the Teays River. This hypothesis is supported by a similar phylogeographic pattern found in the northern hogsucker (*Hypentelium nigricans*), where individuals from the New River were most closely related to those in the northern and southern Ozarks (Berendzen et al. 2003).

Hellbender populations from within each of the northern Ozarks, Ohio/Susquehanna Rivers and Tennessee River regions are genetically similar. The sequence divergence among populations within each of these regions is <0.3%. This is an expected result given the populations are geographically associated and share river drainages. However, our data do suggest that some population genetic structure exists within these regions. In the northern Ozarks, the Meramec River enters directly into the Mississippi River while the Big Piney, Gasconade and Niangua Rivers are tributaries of the Missouri River (Table 1, Fig. 1). Monophyly of Meramec River hellbenders suggests the Mississippi River and/or the Missouri River are barriers to gene flow. Hellbender haplotypes from the Little River, which is a tributary of the Tennessee River, form a monophyletic clade, although with weak branch support. In the Ohio/Susquehanna Rivers region, Sherman Creek hellbenders share a haplotype with those in French Creek while Slippery Rock Creek individuals share a haplotype with Blue River individuals, but haplotypes found in all four of these populations are closely related. It would be interesting to study these relatively closely related hellbender populations using hypervariable markers such as the control region of the mitochondria or microsatellites to investigate whether significant fine scale genetic structure exists.

The Sherman Creek hellbender population is the only one sampled from a waterway that does not ultimately flow into the Mississippi River (it drains into the Susquehanna River, see Table 1, Fig. 1). Therefore, at present Sherman Creek hellbenders are completely isolated from all other sampled populations. The only Sherman Creek hellbender haplotype found in this study is shared by individuals within the Ohio River populations (French Creek), indicating the migration of hellbenders

into the Susquehanna or vice versa was relatively recent. Colonization may have occurred via stream capture or a recent connection between the two drainages (Routman et al. 1994). Since the Susquehanna River is presently isolated and the rooted mtDNA tree shows Sherman Creek hellbenders as one of the youngest lineages, it is reasonable to assume it was colonized by one of the Ohio River populations.

Surprisingly, there is relatively little (sequence divergence $\approx$ 0.7%) genetic divergence between the haplotypes from hellbenders found in rivers flowing north out of the northern Ozarks into the Missouri and Mississippi Rivers and those found in the Ohio and Susquehanna River tributaries (excluding the New River). This shallow divergence indicates a recent gene flow connection between these regions, compared to other hellbender populations (see also Routman et al. 1994). The relatively close relationship between the northern Ozark and Ohio/Susquehanna River hellbender populations sharply contrasts with relationships among rivers in the southern Ozarks, which contain populations that are extremely divergent from one another despite sharing the White River drainage and being separated by tens of river miles, as opposed to hundreds. For example, hellbenders in the North Fork of the White River differ from those in the Spring River and Current River by 1.7 and 5.3% sequence divergence, respectively. Astonishingly, despite the fact that the Spring, Current and Eleven Point Rivers drain into the same tributary of the White River (the Black River) at almost the same point, hellbenders from the Spring River are much more closely related to those in the North Fork of the White River. The relationship between the Spring River hellbenders and those in the North Fork of the White may have resulted from a headwater capture. It is clear, however, that long-standing barriers to gene flow exist in parts of the southern Ozarks while those dividing the northern Ozarks and the Ohio River and those within them are much younger or weaker. These remarkable contrasting results exemplify how the evolutionary history of hellbender populations varies by location, probably due to dissimilar geologic histories.

The relationship of Copper Creek hellbenders as a sister taxon to the (Northern Ozark, Ohio/Susquehanna River, Tennessee River) clade is moderately supported by maximum likelihood analysis. However, all our analyses (including parsimony and neighbor-joining distance analyses, data not shown) agree on this relationship. Routman et al. (1994) found a sister taxon relationship between Copper Creek hellbenders and those in Tennessee River populations, which seems reasonable from a geographic perspective and is not in conflict with our findings. However, given that statistical support of this relationship is not strong in either study, additional research is needed.

The placement of the (Current River/Eleven Point River, New River) clade as sister taxon to the rest of the hellbender haplotypes is consistent with the hypothesis that these rivers, or other southern rivers to which they were connected, served as Pleistocene glacial refugia. Pleistocene glaciations created the Ohio River (Ray 1974). Therefore, during the Pleistocene, glaciers must have extirpated most aquatic organisms inhabiting rivers at the same latitude or higher than the Ohio River currently sits (Pielou 1991). Southern Ozark, Tennessee River and New River populations, on the other hand, are located south of the latitude to which glaciers reached during the last ice age and therefore pre-Pleistocene relationships among those populations may have persisted through the Pleistocene glaciations. Moreover, southern Ozark hellbender populations and those in the vicinity of the Tennessee River would have been unaffected by glacial runoff and are the most likely source of post-Pleistocene recolonization (Routman et al. 1994). Thus the phylogeographic patterns observed in this study are consistent with reasonable expectations of how Pleistocene glaciations would have affected the evolutionary history of hellbenders.

There is little broad-scale phylogeographic concordance between hellbenders and other species with similar ranges (Routman et al. 1994). Hardy et al. (2002) studied the phylogeography of the slender madtom (*Noturus exilis*) because it has similar ecological requirements to the hellbender, yet they found few similarities to hellbenders in their phylogeographic relationships. A review of Ozark biogeography included in Fetzner and Crandall's (2003) work on the Ozark crayfish (*Orconectes luteus*) shows there is little concordance among most Ozark species groups. Seemingly subtle differences in life history and the "shifting roles of dispersal and vicariance" (Zink et al. 2000) in shaping the distribution of populations within species may contribute to the wide diversity in the results of phylogeographic analyses of this region.

Conservation genetics

Phylogenetic analyses of the mtDNA sequence data presented here strongly support paraphyly for the two currently named subspecies for two reasons. First, the data show a strong (100% bootstrap support) sister taxon relationship between New River (*C. a. alleganiensis*) hellbenders and those in the Current and Eleven Point Rivers (*C. a. bishopi*) in the ML tree (In the outgroup-rooted ML tree these taxa are not sister taxa, but the New River haplotypes are sister taxa to those of all other hellbender populations (including *C. a. bishopi* populations), whereas the Current and Eleven Point River hellbender haplotypes are more distantly related. In either case, both subspecies are paraphyletic). Second, the North Fork of the

Table 4 Minimum % of sequence divergence (maximum likelihood distance) among populations for 3-locus haplotypes

	SC	BR	FC	SRC	New	BP	GR	MR	NR	BC	LR	CC	NFW	SR	CR	EPR
Sherman Creek	–															
Blue River	0.2	–														
French Creek	0.0	0.1	–													
Slippery Rock Ck.	0.1	0.0	0.1	–												
New River	5.7	5.5	5.4	5.4	–											
Big Piney River	0.7	1.0	0.7	0.8	5.2	–										
Gasconade River	0.7	1.0	0.7	0.8	5.2	0.0	–									
Meramec River	0.8	1.1	0.8	0.9	5.2	0.2	0.2	–								
Niangua River	0.7	1.1	0.7	0.8	5.3	0.1	0.1	0.1	–							
Beaverdam Creek	3.2	3.2	3.0	3.1	4.7	3.3	3.3	3.3	3.4	–						
Little River	3.2	3.3	3.1	3.2	5.2	3.4	3.4	3.4	3.5	0.1	–					
Copper Creek	3.5	3.5	3.4	3.4	4.5	3.7	3.7	3.6	3.7	3.1	3.2	–				
North Fork White	4.6	4.4	4.3	4.4	5.0	4.2	4.2	4.2	4.3	3.6	3.7	3.9	–			
Spring River	4.3	3.9	3.8	3.9	4.5	3.9	3.9	3.9	3.9	3.3	3.5	3.2	1.7	–		
Current River	5.7	5.8	5.5	5.6	4.3	5.5	5.5	5.5	5.6	5.0	5.1	4.9	5.3	4.8	–	
Eleven Point River	5.3	5.6	5.3	5.4	3.8	5.3	5.3	5.2	5.4	4.4	4.6	4.7	4.8	4.4	0.3	–

White and Spring River hellbenders (both are *C. a. bishopi*) are sister taxa to *C. a. alleganiensis* populations and exhibit greater sequence divergence from those in the Current River (Table 4). Paraphyletic relationships can arise for different reasons including a lack of statistical power, past hybridizations (Funk and Omland 2003) or differential sorting of ancestral polymorphisms. Strong bootstrap support for the data presented here suggests sufficient phylogenetic power. One possibility is the historical hybridization of eastern and Ozark hellbenders. Inter- or intra-specific hybridization can result in genetic exchange among two distinct organismal groups (i.e. introgression; Futuyma 1997). Discordance between cytoplasmic and nuclear genomes has been used to implicate hybridization as the cause of phylogeographic incongruities (Redenbach and Taylor 2002). In some cases it appears mitochondrial genomes of one species have introgressed into other species while the nuclear genome of the recipient remains unchanged (e.g. Wilson and Bernatchez 1998). Random sorting of polymorphism in a widespread ancestral population also cannot be ruled out. Nevertheless, our data support the idea that each named hellbender subspecies should not be given management priority over other genetically distinct lineages. However, it is extremely important to emphasize that the conclusion of paraphyly needs to be supported with data from nuclear genes.

The mtDNA data presented here show a total of 8 monophyletic clades separated by 0.7 to 5.4% sequence divergence. A widely accepted measure used in conservation to categorize populations according to their genetic independence is the Management Unit (MU) (Waples 1995). Populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, "regardless of the phylogenetic distinctiveness of the alleles" are considered an MU. Therefore populations from each of the 8 monophyletic clades should be considered a separate MU. Crossing or translocating members of these populations for conservation purposes should be avoided.

The greatest number of cyt*b*-COI-ND4 haplotypes found in any single population was 4 (see Table 3). Additionally, these haplotypes are all closely related. The number of haplotypes recovered is expected to be proportional to sampling effort (Hartl and Clark 1997), and in some cases sample sizes in this study are low. However, Routman et al. (1994) characterized mtDNA restriction site variation for the same populations used in this study and found similarly low within-population genetic diversity with larger sample sizes for several of the populations. Between the two studies, between 6 and 65 individuals were characterized from each population except those in the New River and the Eleven Point River, which need to be sampled more extensively. All populations show relatively low within-population genetic diversity, which suggests they have been bottlenecked. Low mitochondrial genetic diversity within each population is suggests that they may have experienced a population bottleneck, and measures should be taken to preserve the remnant within-population genetic diversity that exists in each population.

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