Biogeography and evolutionary history of the hellbender salamander (*Cryptobranchus alleganiensis*)

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In partial fulfillment of
The requirements for
The degree

Master of Science
In
Biology: Conservation Biology

By

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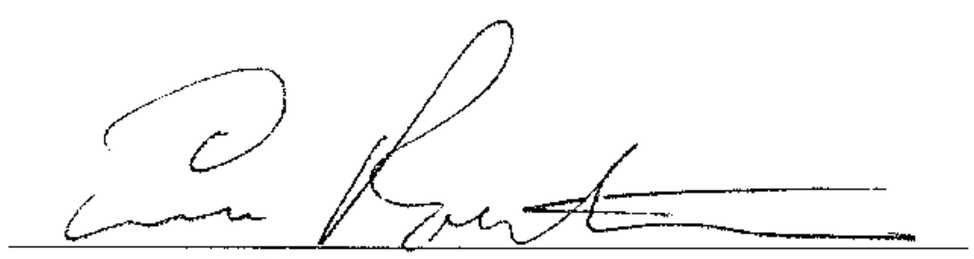
CERTIFICATION OF APPROVAL

I certify that I have read *Biogeography and evolutionary history of the hellbender salamander (Cryptobranchus alleganiensis)* by Stephen J Sabatino, and that in my opinion this work meets the criteria for approving a thesis submitted in partial fulfillment of the requirements for the degree: Master of Science in Biology: Conservation Biology at San Francisco State University.



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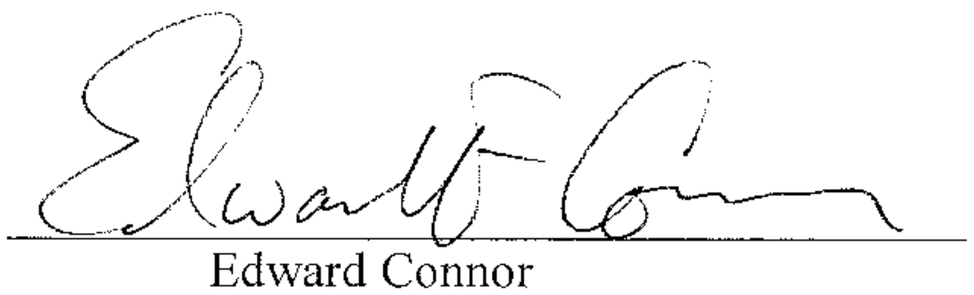

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Biogeography and evolutionary history of the hellbender salamander
(*Cryptobranchus alleganiensis*)

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I 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 (Cytb) and NADH dehydrogenase subunit 4 (ND4) mtDNA sequence, totaling 2160 nucleotides. Hellbender haplotypes differed by 0.1% to 5.9% maximum likelihood corrected sequence divergence (MLCSD). Phylogenetic analyses reveals hellbenders are separated into 8 reciprocally monophyletic populations or clades differentiated by 0.7% to 5.9% MLCSD, each of which constitutes a separate Management Unit (MU). High among population divergence and reciprocal monophyly suggest female-mediated gene flow is severely restricted or non-existent among each MU. Hellbenders are currently divided into two subspecies based on morphological characters, *C. a. alleganiensis* and *C. a. bishopi*. An important finding in my study is that phylogenetic analyses strongly indicate these subspecies are paraphyletic. Management priorities for the hellbender should be reconsidered in light of these new molecular data. Hellbender population structure appears to have been shaped by both pre- and post Pleistocene events. Results from Bayesian molecular clock 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.

I certify that the Abstract is a correct representation of the content of this thesis.

Chair, Thesis Committee

Date

PREFACE AND/OR ACKNOWLEDGEMENTS

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INTRODUCTION

Phylogeography and Conservation

Phylogeography aims to understand the geographic distribution of lineages within and among closely related species (Avise, 2000). Phylogeographic studies draw upon techniques from phylogenetics, population genetics and geography and can be used to elucidate the evolutionary history of a species. These fields can also provide information about species boundaries and the level of genetic diversity maintained within a population (Avise, 1995). Thus, phylogeography is of interest to both evolutionary and conservation biologists.

One persistent problem in conservation biology addressable through molecular phylogeography is the delineation of species boundaries. The term "species" is controversial and has resulted in a large number of "species concepts" (Futyama, 1997). The Ecological Society of America states: "§3.D.15 "species" includes any subspecies of fish or wildlife or plants, and any distinct population segment of any species of vertebrate fish or wildlife that interbreeds when mature." A subspecies definition is subjective, being loosely described as "populations partway through the evolutionary process of divergence towards full speciation (Frankham *et al.* 2003)." Common to these concepts is the idea that species are evolutionary lineages that are separated from other such

lineages. However, determination of what constitutes a separate lineage is also subjective. 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). The possession of unique alleles or sets of alleles for a number of different genetic loci or markers strongly indicates lineage separation, suggesting that the differences are genetic and involve multiple traits (Waples, 1995). Genetics is especially useful in the identification of cryptic species or subspecies where morphological differentiation is absent or misleading (Baric & Sturmbauer, 1999). Thus, molecular investigations into phylogeography can provide taxonomic information on closely related organisms where species or subspecies boundaries are unclear.

A second conservation issue is the importance of maintaining genetic diversity within populations of a threatened species. Genetic diversity is a determinant of phenotypic variation and evolutionary potential, which allows a species to adapt to environmental change (Ashley *et al.* 2003; Frankham, 1995). A decrease in population size or a bottleneck can result in an elevated rate of inbreeding, loss of alleles and fixation of mildly deleteriously alleles (Nei *et al.* 1975; Hedrick 2003). As a result, a species can experience lower competitiveness, disease resistance and overall survival due to inbreeding depression and increased genetic load (Keller & Waller, 2003; e.g. Rowe &

Beebee, 2003). Survival of a bottlenecked species or population, therefore, depends partly on whether it recovers from loss of genetic diversity (Mills & Smouse, 1994).

When conservation of genetic diversity within a species is an issue, it is useful to categorize populations based on genetic uniqueness. A widely accepted measure of genetic independence of populations is the Evolutionary Significant Unit (ESU), which is strictly defined as a population that shares no mtDNA alleles with other populations (with reciprocal monophyly) and has significant divergence of allele frequencies at nuclear loci (Moritz 1994; Waples, 1995). Genetic diversity at neutral loci serves as a proxy for evolutionary potential. Therefore, it may be prudent to conserve populations with lesser degrees of genetic uniqueness than those specified by the ESU definition. Mortiz (1994) considers such populations Management Units (MU), or those with significant divergence of allele frequencies at nuclear or mitochondrial loci, "regardless of the phylogenetic distinctiveness of the alleles." The identification of ESUs and MUs within a threatened species enables managers to prioritize recovery efforts to conserve genetic diversity.

Threatened species with a fragmented habitat may be especially at risk of extinction for several reasons. In general, fragmented species are divided into completely independent populations among which there is little or no gene flow (Larson *et al.*, 1983). Small, independent populations are subject to accelerated rates of random loss of alleles due to genetic drift relative to larger populations (Hartl & Clark, 1997). Genetic drift can

act quickly to fix alleles in independent populations of a fragmented species (Templeton *et al.*, 2001). At the same time, restricted gene flow promotes the evolution of local adaptations in independent populations, which will elevate genetic diversity for the species as a whole (Templeton *et al.*, 1990). Since each independent population of a species with a fragmented habitat may be fixed for unique alleles associated with local adaptations or due to genetic drift, its extinction would constitute a decrease in genetic diversity and evolutionary potential for the species as a whole (Lacy, 1987). Theory predicts a lower extinction risk for a species distributed as a metapopulation than if it were a single population, although the probability of any single subpopulation going extinct is relatively high (Driscoll, 1998). This general principle applies to true metapopulations where there is low but recurrent migration among subpopulations. Independence among subpopulations within a species with a fragmented habitat precludes re-colonization as a means to replace an extinct population, and its loss will permanently decrease overall population size. For these reasons, it is crucial to determine the amount of gene flow occurring among populations of a threatened species when considering conservation measures (Templeton, *et al.* 1990).

Theoretical (Wright, 1968) and empirical (e.g. Matioli &Templeton, 1999) work has shown genetically independent populations will readily evolve locally adapted multi-gene complexes for some fitness traits. If individuals from such genetically distinct populations are crossed, their hybrid offspring may receive portions of co-adapted gene

where model parameters were fixed and tree topology was free to vary. The molecular clock hypothesis was tested for the data using a likelihood ratio test (Felsenstein, 2004). I calculated pairwise genetic distances in **PAUP** using the ML corrected substitution model parameter estimates. Hereafter the minimum ML corrected sequence divergence between haplotypes from different populations or groups of populations is referred to as the "MLCSD".

To root the hellbender mtDNA tree by enforcing a molecular clock, I first randomly pruned the mtDNA dataset to include one representative haplotype from each clade or population differentiated by 0.7% or greater MLCSD. Bayesian trees were generated using the software program **MrBayes** (Huelsenbeck and Ronquist, 2001) as follows: Because the mtDNA data analyzed here is all coding sequence, where the first, second and third positions of an amino acid are likely to be evolving at different rates, I treated each position in each gene as an independent model parameter. I ran two Monte Carlo Markov Chain heated chains, each of which was composed of four Metropolis-coupled chains and started with a random tree. Each Bayesian run was sampled every 1000 generations until it reached stationarity (see Huelsenbeck & Ronquist, 2001). The Bayesian posterior probability (BPP) that a node is the root of the tree is the percentage of sampled trees with that particular root when a molecular clock has been enforced (see Huelsenbeck *et al.* 2002). I calculated the BPP for each root position by importing all the

complexes that are incompatible or unsuited to each other since they evolved in unique genetic backgrounds (i.e., outbreeding depression; Hedrick, 2001). Empirical examples of outbreeding depression are becoming widespread (see Lenormand, 2002; e.g. Gharrett *et al.*, 1999) and in some cases have been explicitly linked to cyto-nuclear interactions (e.g. Christopher *et al.*, 2003). Two management practices commonly implemented to increase population size and/or genetic diversity are breeding programs and the translocation of individuals among currently isolated populations (Storfer, 1997; Tallmon *et al.*, 2004). If subspecies, ESUs or MUs are interbred via these practices it could be counterproductive due to outbreeding depression. Therefore, at the very least, the taxonomy and population genetic structure of a species must be well understood prior to the implementation of management practices that alter their natural breeding structure.

The Hellbender Salamander

The hellbender salamander, *Cryptobranchus alleganiensis*, inhabits clear rocky streams in the eastern United States. There are currently two recognized hellbender subspecies (Nickerson, 1972). 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 (Nickerson & Mays 1973).

Due to recent declines in both Eastern and Ozark hellbender populations, the U.S. Fish and Wildlife Service (USFWS) is considering listing them as endangered under the Endangered Species Act (ESA) (Wheeler *et al.* 2003). Riverine amphibian species such as hellbenders are threatened by anthropogenically induced environmental change (Blaustein & Kiesecker, 2002). Increased sedimentation and siltation of rivers due to land development, pollution and over-harvesting have all been implicated in hellbender declines (Nickerson, 2002). Amphibian species such as hellbenders are often distributed as multiple independent populations among which gene flow is restricted by natural barriers (Shaffer *et al.*, 2004). As riverine habitats are degraded, resident species are increasingly fragmented, which may exacerbate the deleterious genetic effects of small population size. Thus, "evolutionarily enlightened" management (Ashley, 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 divergence) 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 represent non-natural groups (i.e.

sampled Bayesian trees into **PAUP** and filtering them with constraint trees for each possible root placement on the 7 taxa tree (See Figure 3).

Results

Sequencing

A total of 24 Cytb (N=72), 22 COI (N=74) and 26 ND4 (N=63) haplotypes were recovered from 16 hellbender populations (see Table 1). Predominantly the same individuals were sequenced at both Cytb and COI, but it was not always the case due to variation in PCR and sequencing success. Concatenation of the three mtDNA gene sequences resulted in 31 unique mtDNA haplotypes that were 2160bp in length (807bp-Cytb, 705bp-COI and 648bp-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 without conducting any phylogenetic analyses. Ten of 16 hellbender populations surveyed in this study are monophyletic and share no Cytb-COI-ND4 haplotypes with other populations. The 6 paraphyletic hellbender populations comprise the following 3 groups that share haplotypes within, but not among, them: (Big Piney River, Gasconade River), (Blue

they are paraphyletic). However, the phylogeny of mtDNA alleles was not well resolved and was midpoint rooted. One major problem encountered by Routman *et al.* (1994), which is often encountered in molecular biogeography and phylogeography, was inability to reliably root the phylogenetic tree using the closest outgroup, *Andrias davidianus*. If the closest extant relative to 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, 1988). This problem may be widespread among phylogeographic and biogegraphic studies, yet it is rarely recognized. Huelsenbeck *et al.* (2002) showed reliable roots can be obtained by generating trees with Bayesian analysis while enforcing a strict molecular clock, especially when the data is from lineages that all have diverged at similar rates (i.e. in a clock-like fashion). Molecular clock rooting was successfully implemented by Steele *et al.* (2005) to root the *Dicamptodon* mtDNA phylogenetic tree and thus may hold promise for hellbenders.

I investigated the phylogeography and population structure of hellbenders using mtDNA sequence data. My goal was to better understand the recent and historical relationships among hellbender populations, to delineate subspecies boundaries and provide additional information on within- and among-population genetic diversity. These data should be used to shape conservation efforts to manage hellbender populations

Methods

Hellbender Sampling

Dr. Eric Routman and colleagues collected blood and/or tissue samples from hellbenders caught in 15 different rivers and streams throughout the Appalachian and Ozark Mountains of the Eastern US (Figure 1 and Table 1; see Routman *et al.* 1993 and 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. I extracted DNA from hellbender blood and tissue samples using the following phenol/chloroform method: I placed 10 to 30 µl of blood (depending on blood cell density) or 2mm^2 of tissue in 500µl of STE lysis buffer [0.5% SDS, 5mM EDTA, 100mM Tris, pH7.0] with 25ng of Proteinase K and incubated it at 55°C for 1 hour. After centrifuging the mixture, I removed the supernatant and washed it once with 1:1 phenol/chloroform and three times with 1:1 chloroform/isoamyl alcohol using Phase Lock Gel (Eppendorf) according to the manufacturer's directions. Following the final chloroform wash, I removed the supernatant and added to 500µl of 95% ETOH, vortexed it briefly, and then centrifuged it to pellet the DNA. After removing the ETOH, I washed the DNA pellet twice with 75% ETOH in the same manner. After all the ETOH had evaporated the cleaned DNA was dissolved in water. I further cleaned the DNA obtained from blood by adding 500ng of it to 100 µl of 5% Chelex (BioRad), boiling the mixture for 2 minutes and isolating the supernatant. All DNA samples were stored at -20°C.

River, Slippery Rock Creek) (French Creek, Sherman Creek). In contrast, hellbenders within each population appear closely related. Only the Spring and New River populations harbored mtDNA haplotypes that differed by more than two mutations (6 and 7 mutations, respectivcly). All other populations contained haplotypes differing by a maximum of two mutations.

Phylogenetic Analyses

Modeltest found the GTR+G 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: Π_A=.289, Π_C=.202, Π_G=.139, Π_T=.370; AC=1.940, AG=42.742, AT=.732, CG=2.06, CT=22.085, GT=1.00 and Γ-distribution shape parameter α=.161. The ML phylogeny had a likelihood score of $-\ln L$= 4537.88. When a molecular clock was enforced and a MLA conducted, the resulting tree had a $-\ln L$=-4557.95. These two tree scores were not significantly different (Chi-square δ=40.14; p=.082, df =29), therefore, the molecular clock assumption could be used to estimate divergence times of lineages (Felsenstein, 2004) and to root the tree (Huelsenbeck *et al.*, 2002) with greater confidence. Since the molecular clock assumption could not be rejected, I generated a ML tree where a molecular clock was enforced as my final tree. **Modeltest** found the Trn+I+G substitution model for the outgroup-rooted tree using the AIC. The MLA generated the following model parameter estimates: Π_A=.293, Π_C=.210, Π_G=.134,

Polymerase Chain Reaction & Sequencing

Initially I used the Polymerase Chain Reaction (PCR) to amplify hellbender cytochrome-b (Cytb) with a forward primer designed for *Dicamptodon* (tRNA-Glucine: 5'-TTG TAT TCA ACT ATA AAA AC- 3'; Steele *et al.* 2005) and reverse primer Cytb-SAL-R1: 5'-ACT TAA CCT CCT GTT GGT CA- 3', which I designed from the consensus sequences of *Ambystoma laterali* (Genbank accession number (GAN): AY728227), *Desmognathus fuscus* (GAN: AY728218), and *Andrias davidianus* (GAN: NC_004926). The DNA template used for this particular PCR was extracted from hellbender ova, which contains a high amount mtDNA relative to somatic tissue. The result was a 900bp fragment of Cytb, which I sequenced and used to design the following hellbender specific primers that amplify a 750bp fragment (Cytb-CA-F1: 5'-TTC ATT TAT TGA CCT ACC AAC C- 3', Cytb-CA-R1: 5'- GAT AAT TGA CAC TAA GGC TCA G- 3'). I amplified cytochrome oxidase I (COI) using universal primers (1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G -3', 2198 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA-3'; Folmer *et al.*, 1994), which yielded a 500bp fragment. Since a larger fragment of COI is desirable, I aligned the resultant sequence with that of *Andrias davidianus* and used it to design the following primers, which amplify a 750bp fragment (COI-CA-F1: 5'-TTA AGC TTA TTA ATC CGA GCA G-3', COI-CA-R1: 5'-TGG CTG ATG TGA AAT AAG CTC G-3'). I amplified NADH dehydrogenase subunit 4 (ND4) with ND4-Nex-R: 5'- TGG GGG CTA CGG CAT AAT AC- 3' and

ND4-Nex-R: 5'- CCA ATG GAT GAA CTA TTA TCC- 3' from Hardy *et al.* (2002). These primers did not work for a number of hellbender samples, so I designed the following primers from *Andrias davidianus* and hellbender sequence (ND4-Sal-F1: 5'-AAA ATA CCA CTC TAT GGT GC-3'-; ND4-Sal-R1: 5'-GTT CAT AAC TTT CAC TTG GA-3'), which yielded a similarly sized fragment.

For PCR I used Ampli-taq chemistry (Applied Biosystems (ABI)) under the following conditions: 94°C (120s) initial denature, {94°C (30s), 58°C (30s), 72°C (60s)} 30x, and a final extension at 72°C (120s). I sequenced DNA with ABI Big Dye chemistry on an ABI 377 sequencer according to manufacture's recommendations. I used PCR primers for all sequencing reactions although the annealing temperature was raised to 60°C.

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

Phylogenetic Analyses

I conducted maximum parsimony and maximum likelihood (ML) phylogenetic analyses on the concatenated Cytb, COI and ND4 sequences. The total haplotype (sets of

Π_T=.363; AG=42.742, CT=17.175; the proportion of invariable sites =.631 and Γ-distribution shape parameter α=2.125.

The dataset used for molecular clock rooting included one haplotype from the following hellbender populations: New River, Spring River, Current River, Little River, Meramec River, Copper Creek and North Fork of the White. **Modeltest** found the GTR+I+G model to be most appropriate for the pruned dataset using the AIC. The Bayesian analysis reached stationarity after generating $1x10^5$ trees, 500 of which were used to calculate Bayesian posterior probabilities. Ninety-six percent 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 (Figure 3). All other nodes were found to be the root less than 1.0% of the time. This result is consistent with mid-point rooting of the tree. The outgroup-rooted ML tree using all available haplotypes has the same overall topology as the mid-point rooted tree except it places the Current (plus the Eleven Point River) haplotypes as sister to all others (see Figure 4). However, since 20% MLCSD exists between *Andrias* and Current River hellbenders, it is clearly a poor outgroup and the exact root placement is questionable. It is worth noting that the outgroup, molecular clock and midpoint rooted trees agree that the Current River, Eleven Point River, and New River populations are sister taxa to the rest of the hellbender populations. The molecular clock and midpoint

sequence data for one individual) length for these three genes is 2160 nucleotides. Redundant haplotypes were removed from the dataset prior to all phylogenetic analyses. I ran a maximum likelihood analysis (MLA) with *Andrias davidianus* included to root the tree and a MLA and parsimony analysis without an outgroup using the program **PAUP** 4.0 (Swofford, 1999). For the parsimony analysis, I conducted a heuristic search using **PAUP** default settings except the addition of taxa was set to random with 10 replicates. One thousand parsimony bootstrap replicates of the data were used to evaluate branch support. To choose a substitution model for all MLA and Bayesian analysis (see below), I utilized the software program **Modeltest** (Posada & Crandall, 1998). I conducted the following iterative MLA rather than a full one. MLA 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. I 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. Estimation of model parameters and tree topology independent of one another 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). I conducted 100 ML bootstrap replicates of the dataset

sequence divergence from those in the Current River (Table 2). Paraphyletic relationships can arise for different reasons including a lack of statistical power or past hybridizations (Funk & Omland, 1999). Strong bootstap 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, 1998). Discordance between cytoplasmic and nuclear genomes has been used to implicate hybridization as the cause of phylogeographic incongruities (Redenbach & 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 & Bernatchez, 1998). To better understand and fully establish the relationship between the two subspecies, data from nuclear loci need to be obtained.

The placement of southern Ozark populations as sister to the rest of the populations on the rooted mtDNA tree is consistent with the hypothesis that they 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). Ozark and Tennessee River populations, on the other hand, are located south of the latitude to which glaciers reached during the last ice age and therefore may have persisted. Moreover, Southern Ozark hellbender populations and

rooting trees are in agreement and therefore these approaches are used to root the ML molecular clock tree presented here.

Parsimony and ML phylogenetic analysis yielded trees with the same overall topology (See Figure 2). Phylogenetic analyses reveals hellbenders are separated into 8 reciprocally monophyletic populations or clades differentiated by 0.7% to 5.9% MLCSD. The 8 groups, named by population (i.e. sampling location) or geographic region, are: 1. Northern Ozarks (MO) 2. Eleven Point/Current River (MO) 3. North Fork of the White (MO) 4. Spring River (AR) 5. New River (VA) 6. Copper Creek (VA) 7. Tennessee River (TN) and 8. Ohio/Susquehanna River (IN, PA). Moderate (above 70%) to strong (above 90%) ML bootstrap support was found for the placement of all clades separated by more than 0.7% MLCSD except of that in Copper Creek (bootstrap support = 68%). In general, resolution at the tips of clades is weak. Three exceptions are strong bootstrap support for monophyly of hellbender populations within the Meramec, Current and Eleven Point Rivers.

Discussion

Phylogeography

The geology of eastern North America has undergone dramatic changes throughout the time hellbenders are likely to have inhabited their current range. Amazingly, a 160 million year old fossil of a cryptobranchid recently found in Asia is

morphologically similar to present-day hellbenders (Gao & 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 that hellbenders or their ancestors evolved in Asia and reached North America via a land bridge prior to 56mya (Gao & Shubin, 2003). Indeed, the earliest cryptobranchid fossil found in North America so far is from the Paleocene around 65mya (Naylor, 1981). Since the Paleocene, the Ozark highlands have flattened and have risen again (Nickerson and Mays, 1973). In addition, numerous rivers and lakes, including those from glacial runoff, have formed and dissipated throughout Eastern North America (Peliou, 1996). Such geologic changes would have created and destroyed river habitats and migratory routes available to organisms such as the hellbender. It is therefore plausible that aspects of current hellbender population genetic structure can only be explained by pre-Pleistocene relationships established when the geology of the region was vastly different from its current state.

Many hellbender populations surveyed in this study are genetically distinct, evidenced by reciprocal monophyly for 10 of the 16 populations surveyed and 0.7% to 5.9% MLCSD among 8 major groups of hellbender populations. These data suggest that gene flow among hellbender populations is restricted, which is consistent with results from mark-recapture studies showing low within-river movement and remarkable philopatry in this species (Nickerson and Mays 1973; Peterson, 1987; Routman, 1994).

those in the vicinity of the Tennessee River would have been unaffected by glacial runoff (Routman, 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.

Although my phylogenetic tree can only tell us that the Southern Ozarks and New River hellbender populations are sister to all others, two findings in this study are consistent with the scenario that hellbenders from refugial populations colonized the Tennessee River, which subsequently invaded either the Ohio River or Northern Ozarks. First, there is low bootstrap support for the placement of Copper Creek hellbenders as sister to the other Tennessee River populations and those in the northern Ozarks and Ohio/Susquehanna Rivers. This may mean that Copper Creek populations were intermediate between other Tennessee River populations and the Northern Ozarks and Ohio/Susquehanna River populations. Secondly, the southern Ozark hellbenders are genetically more similar to hellbenders from the Tennessee River drainages than they are to the hellbenders from the northern Ozarks and Ohio/Susquehanna river populations (Table 2). It remains unclear whether the ancestors of Tennessee River hellbenders colonized both the Ohio River and the Northern Ozarks rivers, or whether colonization was sequential (e.g. Tennessee River → Northern Ozarks → Ohio River)

There is little phylogeographic concordance between hellbenders and other species with similar ranges (Routman, *et al.*, 1994). Hardy *et al.* (2002) studied the

One implication of extremely low gene flow is the expectation that geologic history has played a significant role in shaping the distribution of mtDNA variation observed today (Routman, 1994).

My ML molecular clock tree (see Figure 2) strongly supports a sister relationship between the New River and Current River hellbender populations. This is a surprising result given the New River drains into the Ohio River and the Current River drains into the Mississippi River. The major river connections between the Current and New River presently contain hellbender populations, yet they are more distantly related to the Current or New River hellbenders than the latter are to each other (see Table 2). The relationship between New and Current River hellbenders may reflect a pre-Pleistocene population with more extensive gene flow. It is worth noting that the New River hellbender haplotypes differ from those of Current River hellbenders by 4.2% MLCSD, which is comparable to the difference between the most distantly related hellbender populations (5.9%). This suggests an old relationship. Routman *et al.* 1994 found New River hellbenders to be most closely related to those in the Tennessee River using restriction enzyme data, but no statistical analysis was done on this relationship.

Hellbender populations within the Northern Ozarks, Ohio/Susquehanna River and Tennessee River are genetically similar. The MLCSD among populations within these regions is less than 0.1%. This result is not surprising given the populations are geographically associated and share river drainages. However, there is some evidence

that population genetic structure exists within these groups. The Meramec River enters directly into the Mississippi River while the Big Piney, Gasconade and Niangua Rivers are tributaries of the Missouri River. Monophyly of Meramec River hellbenders suggests the Mississippi River is a barrier to gene flow in the northern Ozarks. Hellbenders from the Little River and Beaverdam Creek, which are tributaries of the Tennessee River, form monophyletic clades but they are not statistically significant. In the Ohio and Susquehanna Rivers, Sherman Creek hellbenders share alleles with those in French Creek while Slippery Rock Creek individuals share alleles with Blue River individuals, but all four of these populations have haplotypes that are closely related. It would be interesting to study these closely related hellbender populations using hypervariable markers such as the control region of the mitochondria or microsatellites to investigate whether fine scale genetic structure exists.

The Sherman Creek hellbender population is the only one sampled from a waterway that does not flow into the Mississippi River (it drains into the Susquehanna River). Therefore, 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, 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

phylogeography of the slender madtom (*Noturus exilis*), which was chosen precisely because it has similar ecological requirements to the hellbender, yet they found few similarities in their geographic distribution when genetic relationships were considered. A recent review of Ozark biogeography included in Fetzner & 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 phylogeographies.

Conservation Genetics

The mtDNA data presented here show a total of 8 monophyletic populations separated by 0.7 % to 5.9% MLCSD. Each of these groups meets the requirements of being an MU and therefore should be considered as such. Each MU may constitute an ESU depending on whether the pattern holds for nuclear loci. Crossing or translocating members of these populations for conservation purposes should be done cautiously. An important finding supported by these data is the currently named subspecies are paraphyletic. Therefore, these recognized putative subspecies should not be given management priority over other genetically distinct lineages. However, it is important to emphasize that the conclusion of paraphyly needs to be supported with data from nuclear genes.

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 (MLCSD = 0.7%) genetic divergence between the 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. This shallow divergence indicates one of these populations colonized the other recently compared to the events that separated other hellbender populations (Routman, 1994). The relationship between the Northern Ozark and Ohio River hellbender populations sharply contrasts that of the Southern Ozarks, which are extremely divergent 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 2% and 5.4% MLCSD, 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), hellbenders from the Spring River are much more closely related to those in the North Fork of the White River. Clearly long-standing barriers to gene flow exist in parts of the White River drainage 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, undoubtedly due to dissimilar geologic histories.

The relationship of Copper Creek hellbenders to all others is unresolved. Low bootstrap support for the Copper Creek hellbender haplotype stems from differences among each individual mtDNA loci in the concatenated dataset (ML trees not shown). However, both the molecular clock and outgroup rooted ML trees place the Copper Creek hellbender haplotype between the Tennessee River/Ohio/Susquehanna River clades and the North Fork of the White/Spring River clade. Routman *et al.* (1994) found a sister relationship between Copper Creek hellbenders and those in Tennessee River populations, which seems reasonable from a geographic perspective and is not in conflict with my findings. However, given statistical significance for support of this relationship is lacking in both studies, it needs to be explored further.

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 relationship between New River (*C. a. alleganiensis.*) hellbenders and those in the Current and Eleven Point Rivers (*C. a. bishopi.*) in the unrooted ML tree. In the outgroup-rooted ML tree these taxa are not sister, but the New River populations are sister to 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. Secondly, the North Fork of the White and Spring River hellbenders (both are *C. a. bishopi*) are sister to *C. a. alleganiensis* populations and exhibit greater

The greatest number of Cytb-COI-ND4 haplotypes found in any population was 3 (see Table 1). Additionally, only the Spring River and New River populations harbor haplotypes that differ by more than two mutations. The number of haplotypes recovered is expected to be proportional to sampling effort (Hartl & 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. Between the two studies, more than 6 individuals were characterized from all populations except those in the New River, Eleven Point River, Blue River and Slippery Rock Creek, which need to be further studied. All populations that were sufficiently sampled show low within-population genetic diversity, which suggests they have been bottlenecked. Measures should be taken to preserve the remnant within-population mtDNA genetic diversity that exists in each bottlenecked population.

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Table 2. Minimum percentage of sequence divergence among hellbender populations. The values are maximum likelihood corrected sequence divergences.

	SC	BR	FC	SRC	NewR	BP	GR	MR	NR	BC	LR	CC	NFW	SR	CR	EPR
Sherman Creek	0															
Blue River	0.2	0														
French Creek	0.0	0.2	0													
Slippery Rock Creek	0.1	0.0	0.1	0												
New River	5.9	5.9	5.9	5.8	0											
Big Piney River	0.8	1.0	0.8	0.9	5.9	0										
Gasconade River	0.8	1.0	0.8	0.9	5.6	0.2	0									
Meramec River	0.9	1.1	0.9	1.0	5.8	0.3	0.3	0								
Niangua River	0.7	0.9	0.7	0.9	5.7	0.1	0.1	0.1	0							
Beaverdam Creek	3.1	3.2	3.1	3.2	5.3	3.5	3.4	3.5	3.4	0						
Little River	3.2	3.4	3.2	3.3	5.5	3.6	3.5	3.6	3.6	0.2	0					
Copper Creek	3.5	3.5	3.5	3.4	4.7	3.9	3.8	3.8	3.7	3.2	3.3	0				
North Fork White River	4.4	4.5	4.4	4.5	5.3	4.4	4.2	4.4	4.3	3.8	3.9	4.0	0			
Spring River	4.2	4.3	4.2	4.3	4.9	4.3	4.1	4.3	4.3	3.7	3.8	3.4	2.0	0		
Current River	5.5	5.8	5.5	5.7	4.2	5.5	5.5	5.7	5.6	5.0	5.2	4.9	5.4	5.1	0	
Eleven Point River	5.1	5.5	5.1	5.4	3.8	5.1	5.1	5.3	5.2	4.6	4.7	4.4	5.0	4.7	0.3	0

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Figure 1. Map of the Eastern US showing the locations where hellbenders were sampled for this study. See Table 1 for abbreviations.

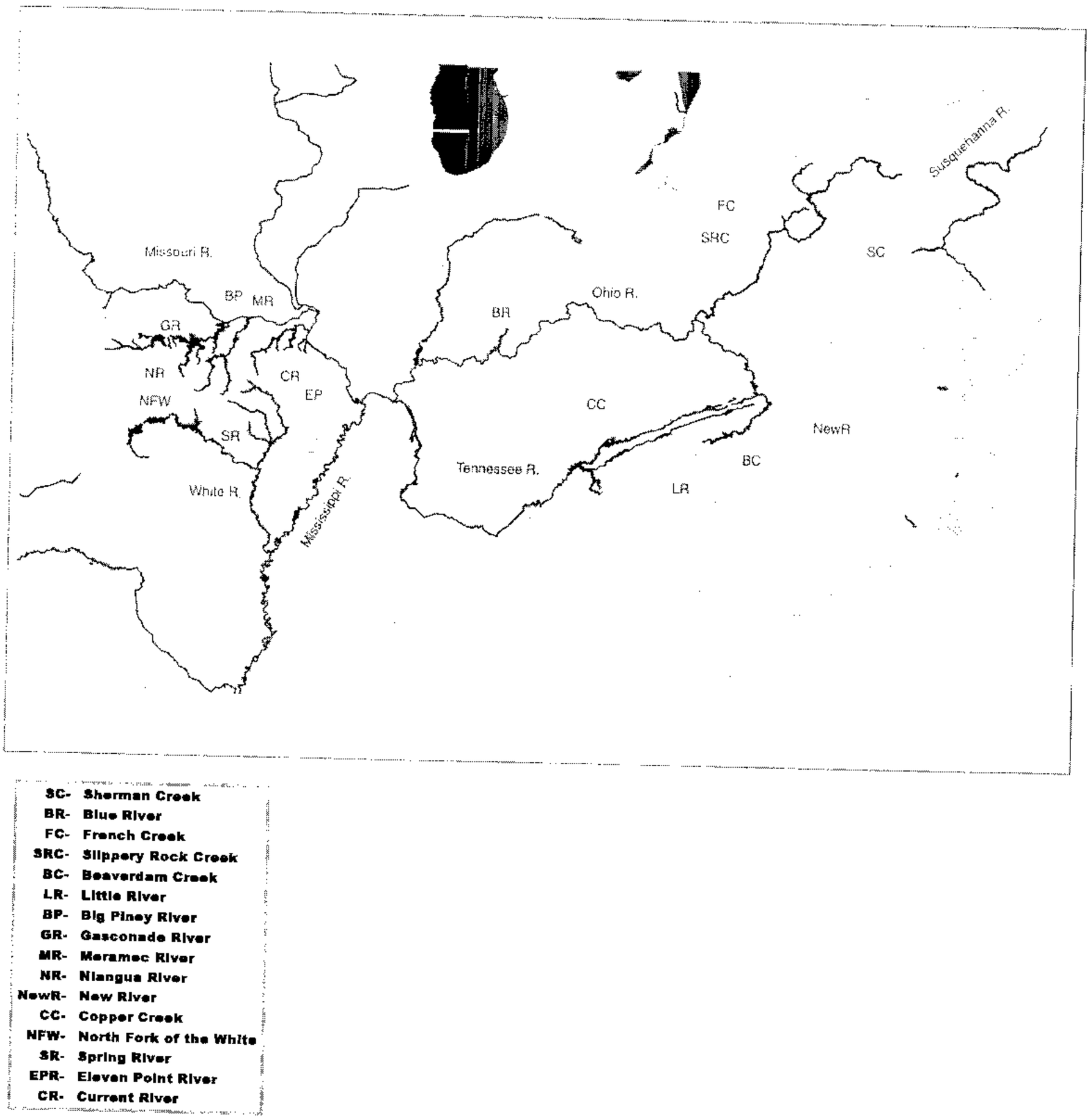

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Figure 2. Midpoint-rooted molecular clock maximum likelihood (ML) tree of hellbender mtDNA (Cytb, COI and ND4) haplotypes. The numbers atop branches represent ML bootstrap values (100 replicates) while those below are parsimony bootstrap values (1000 replicates). Hellbender clades are named by population or geographic region. *Cryptobranchus. a. bishopi* haplotypes are marked by a black circle.

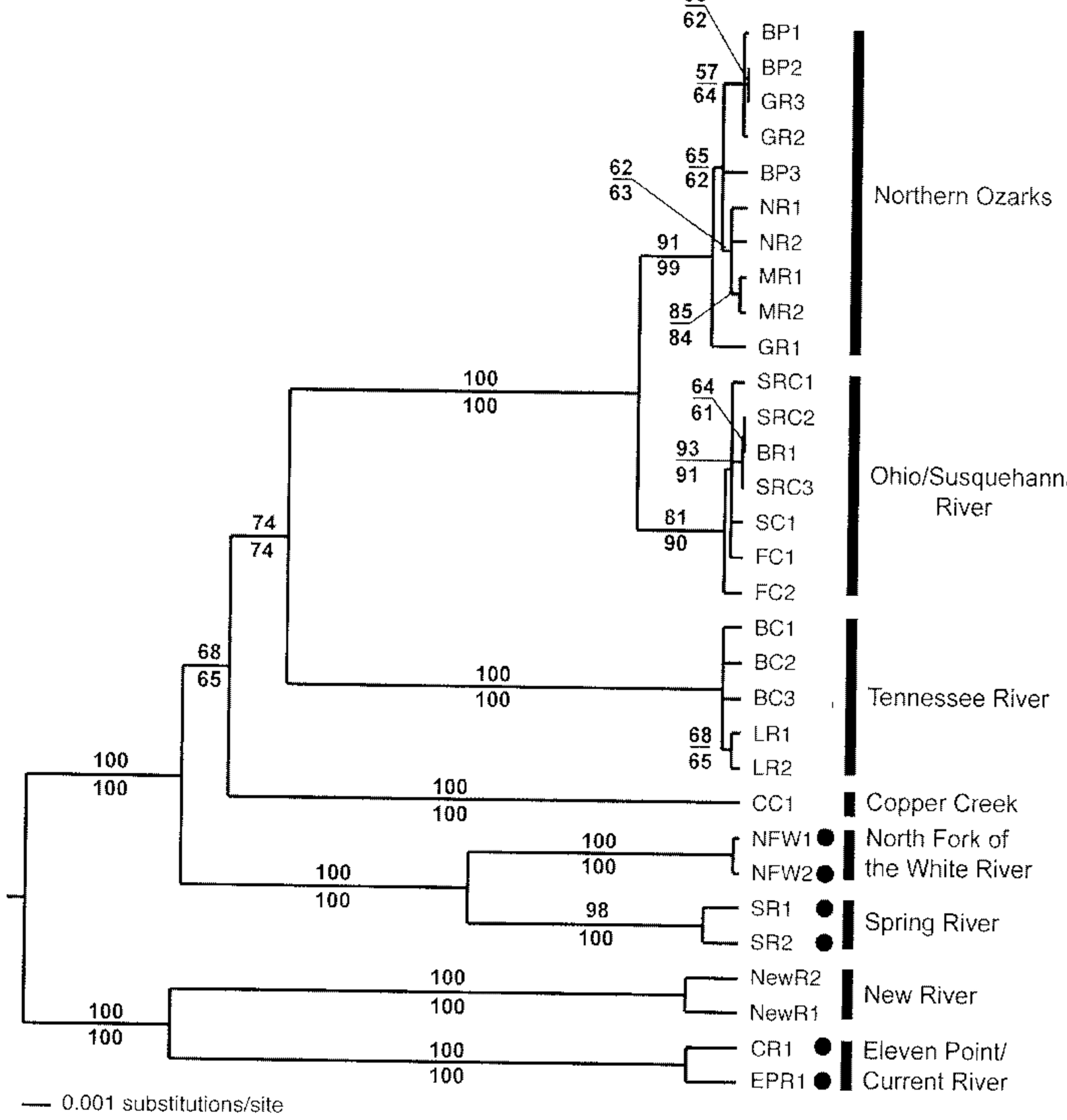

Table 1. The table below outlines all locations (see Figure 1) from which hellbenders were sampled, each of which is considered a separate population. Sampling locations are grouped geographically and by drainage. Included for each population are the number of individuals sequenced at each locus (N), the number of different haplotypes found (H), and its major river drainages (in order from upstream to downstream) and subspecies name. The two populations in which haplotypes that differ by more than 2 mutations were found are marked (*); these are the Spring and New River, which differed by 7 and 6 mutations, respectively.

River/Tributary	Cytb		COI		ND4		Major River Drainages	Subspecies
	N	H	N	H	N	H		
Sherman Creek (SC)	2	1	1	1	1	1	Susquehanna-Chesapeake Bay	*alleganiensis*
Blue River (BR)	4	1	4	1	2	2	Ohio-Mississippi	*alleganiensis*
French Creek (FC)	5	1	9	3	10	2	Allegheny-Ohio-Mississippi	*alleganiensis*
Slippery Rock Creek (SRC)	5	3	5	3	5	2	Beaver-Ohio-Mississippi	*alleganiensis*
New River (NewR) *	2	1	2	2	2	2	Kanawha-Ohio-Mississippi	*alleganiensis*
Big Piney River (BP)	12	3	14	2	5	2	Gasconade-Missouri-Mississippi	*alleganiensis*
Gasconade River (GR)	8	3	7	2	5	2	Missouri-Mississippi	*alleganiensis*
Meramec River (MR)	10	2	9	2	6	1	Mississippi	*alleganiensis*
Niangua River (NR)	5	2	6	1	3	1	Osage-Missouri-Mississippi	*alleganiensis*
Beaverdam Creek (BC)	6	3	6	1	3	2	Holoston-Tennessee-Mississippi	*alleganiensis*
Little River (LR)	6	3	5	3	4	2	Tennessee-Mississippi	*alleganiensis*
Copper Creek (CC)	9	1	8	1	3	1	Clinch-Tennessee-Mississippi	*alleganiensis*
North Fork of the White (NFW)	5	2	6	1	2	1	White-Mississippi	*bishopi*
Spring River (SR) *	2	2	7	1	6	1	Black-White-Mississippi	*bishopi*
Eleven Point River (EPR)	1	1	3	1	3	1	Black-White-Mississippi	*bishopi*
Current River (CR)	2	1	6	1	3	2	Black-White-Mississippi	*bishopi*
Totals	84	24	98	22	63	25		

Figure 3. One of the constraint trees used to filter the Bayesian trees generated under a strict molecular clock. The branch leading to the New, Current and Eleven Point River clade was found 96% of the time.

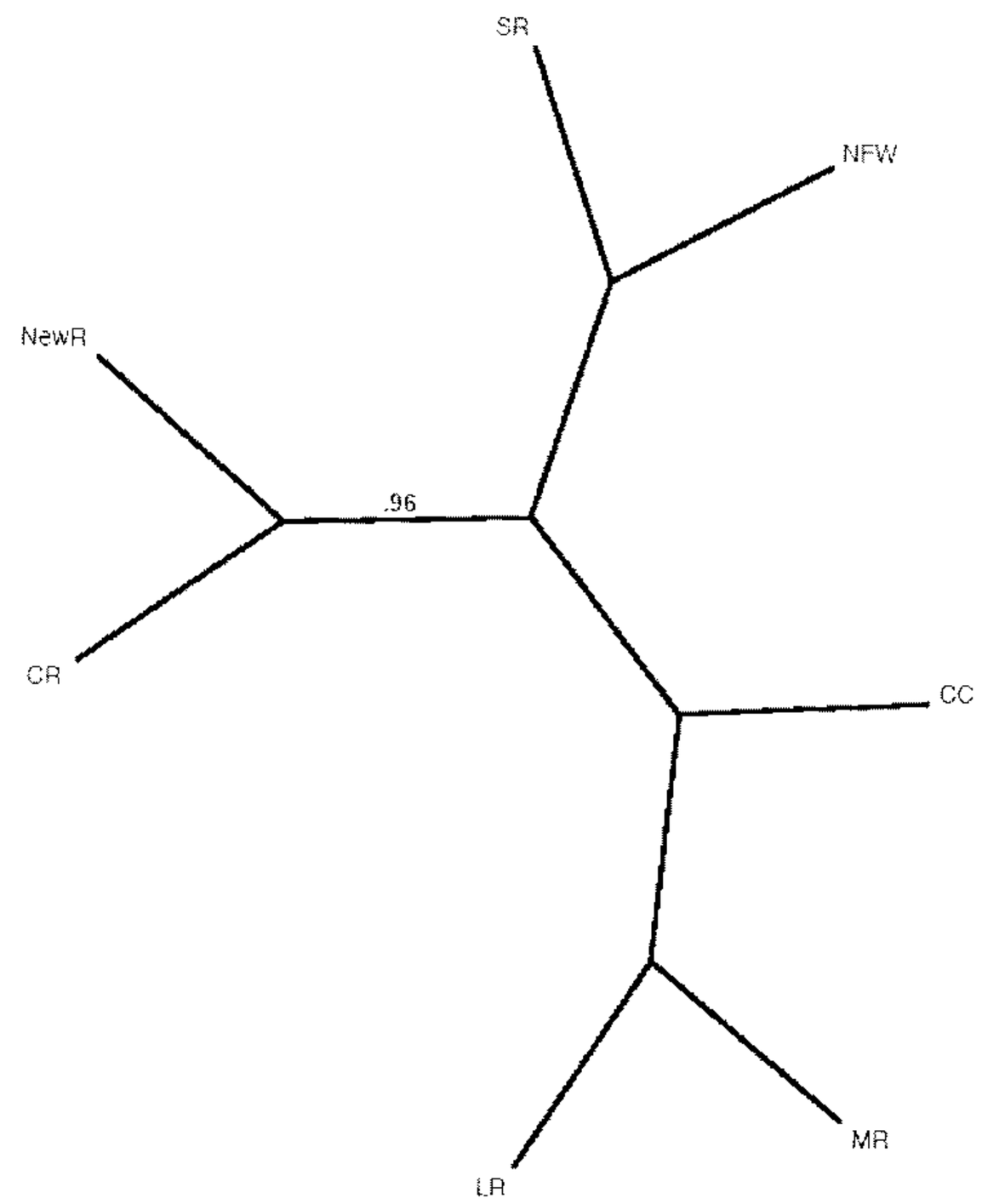

Figure 4. Outgroup-rooted maximum likelihood cladogram. The numbers on atop branches are bootstrap values (100 replicates). Hellbender clades are named by population or geographic region. *C. a. bishopi* haplotypes are marked by a black dot.