MICROSATELLITE VARIATION AND PHYLOGEOGRAPHY OF THE HELLBENDER, *CRYPTOBRANCHUS ALLEGANIENSIS*

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by

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CERTIFICATION OF APPROVAL

I certify that I have read *Microsatellite variation and phylogeography of the hellbender, Cryptobranchus alleganiensis* by Maria Tonione, and that in my opinion this work meets the criteria for approving a thesis submitted in partial fulfillment of the requests for the degree: Masters of Science in Biology: Conservation Biology at San Francisco State University.

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MICROSATELLITE VARIATION AND PHYLOGEOGRAPHY OF THE HELLBENDER, *CRYPTOBRANCHUS ALLEGANIENSIS*

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We investigated genetic diversity of the hellbender (*Cryptobranchus alleganiensis*) throughout its range in the eastern U.S. using nuclear markers and compared our results to previously published mitochondrial analyses. Autosomal genetic variation was surveyed using a variety of single copy nuclear markers and was found to have very low levels of DNA sequence variation. Final analyses were done using four microsatellite loci. These microsatellite loci showed moderate among population sharing of alleles at these loci, in contrast to mitochondrial DNA. However, analysis using F-statistics and Bayesian clustering algorithms showed considerable population subdivision and clustered hellbender populations into the same major groups as the mtDNA. The microsatellites combined with the mtDNA suggest that gene flow is severely restricted or non-existent among 8 major groups, and potentially among populations (rivers) within groups. Management strategies are suggested in light of these new data.

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

Chair, Thesis Committee

Date

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Introduction

In attempting to conserve biodiversity it is imperative that genetic diversity be incorporated into conservation planning (Moritz 2002; DeSalle and Amato 2004; Schwartz et al. (2007). As species become increasingly fragmented into small isolated populations, heterozygosity is lost and gene diversity is redistributed into between-population genetic variance (Templeton et al. 1990). These population fragments have an increased risk of extinction through inbreeding depression and reduced capacity to respond to selection (Reed and Frankham 2003). Another important factor is that when populations have been isolated, they are likely to possess unique local adaptations acquired through selection. Without knowledge of the genetic relationships of populations, management resources could be inadvertently diverted away from genetically unique populations. In addition, by managing genetically distinct populations as connected populations, breeding programs or translocations could be detrimental by lowering population mean fitness via outbreeding depression (Gharrett et al. 1999; Hedrick 2001; Lenormand 2002). Using phylogeography to understand the distribution of genetic variation within and among geographically isolated populations will help to determine conservation priorities and management strategies.

Mitochondrial DNA (mtDNA) is frequently used as an initial marker in phylogeography because of the ease of data collection, lack of recombination, faster rates of evolution (as compared to nuclear DNA), and effective haploidly. The latter means

that reciprocal monophyly evolves more quickly for mtDNA than for nuclear genes (Moore 1995; Edwards and Beerli 2000; Hudson and Turelli 2003). However, mitochondrial gene trees are often criticized as not being representative of the actual species tree because of problems associated with incomplete lineage sorting, introgression, and sex-biased gene flow (Moore 1995). There can be discordant patterns seen in morphological, mitochondrial, and nuclear gene trees. Thus, analysis of multiple independent loci can be more informative than single gene trees.

World-wide, amphibians have been declining for decades (Houlahan et al. 2000; Stuart et al. 2004). The most commonly cited reasons for this decline have been habitat loss, overexploitation, and disease, though in many instances unidentified processes threaten declining species (Stuart et al. 2004). One of these declining amphibians, the hellbender, (*Cryptobranchus alleganiensis*) is among the largest of the salamanders (up to 74cm in length). This primitive salamander is completely aquatic throughout its 30+ year lifespan (Nickerson and Mays 1973) and inhabits clear rocky fast-flowing streams in the eastern U.S. (Fig. 1). Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are found throughout the Appalachian Mountains from southern New York to northern Georgia and in rivers draining northward from the Ozarks. A second subspecies, the Ozark hellbender (*Cryptobranchus a. bishopi*), inhabits streams that drain south out of the Ozark Plateau in Missouri and Arkansas and differs from the nominal subspecies in minor morphological characters (Grobman 1943). Recent studies have concluded that many populations of both subspecies are experiencing population declines

(Williams et al. 1981; Wheeler et al. 2003; Routman, personal observation of the Mermec River population; Ettling and Wanner 2008), and the U.S. Fish and Wildlife Service (USFWS) is considering listing both subspecies as endangered under the Endangered Species Act (Amy Salveter, USFWS, pers. comm.). Because hellbenders breathe primarily through the skin, they are dependent on cool, well-oxygenated, flowing water (Guimond and Hutchison 1973). This highly adapted physiology and specialized habitat requirements make hellbenders extremely vulnerable to the effects of habitat destruction including damming, increased sedimentation of rivers due to land development, and pollution. Other factors cited as reasons for their decline include over-harvesting and infection by the chytrid fungus (Williams et al. 1981; Briggler et al. 2007).

A previous allozyme study by Merkle et al. (1977) showed low within- and between-population genetic variation even between the two subspecies. They analyzed 24 loci from 12 populations sampled throughout the hellbender's range. Twenty-two of these loci were monomorphic in all populations. The two remaining loci were polymorphic, but only in one population each. More recently, Routman et al. (1994) and Sabatino and Routman (2008) used mitochondrial DNA to gain insights into the population structure of hellbenders. Routman et al. (1994) found high levels of among-population variation using restriction enzyme digests of the entire mtDNA genome. Sabatino and Routman (2008) conducted a DNA sequence analysis using 3 mitochondrial genes. They determined hellbender populations were highly divergent with 8 reciprocally monophyletic

Management Units (MU) (Fig. 3). According to both Routman et al. (1994) and Sabatino and Routman (2008), the two named hellbender subspecies are paraphyletic if the mtDNA phylogeny reflects the population phylogeny.

In this study, we will attempt to examine discrepancies between the morphology and past genetic studies of *C. alleganiensis* with multiple independent nuclear markers. The nuclear loci in combination with the mitochondrial data will help establish subspecies or ESU status and suggest which populations, if any, are suitable for interbreeding or which populations should be maintained as separate populations.

Materials and Methods

Sampling

Samples analyzed include both subspecies from 18 different rivers and streams throughout the current distribution. Most blood and/or tissue samples used in this study were from previously collected samples (Fig. 1 and Table 1; see Routman 1993; Routman et al. 1994). The Eleven Point River samples were collected by Dr. Jeff Briggler of the Missouri Department of Conservation.

Genetic data

Whole genomic DNA was extracted from blood and tissue samples using a standard phenol/choloform method as outlined in Routman (1993). We further cleaned the DNA obtained from blood by adding 500ng of it to 100µl of 5% Chelex (BioRad), boiling the mixture for two minutes and then isolating the supernatant. All DNA samples were stored at -20°C.

A total of eight nuclear introns were surveyed for variation in a subsample of 3-9 individuals. One of these autosomal markers was obtained from the literature, while the other seven were developed *de novo*. Primers were developed from existing GenBank sequences, with the exception of the GATA locus. The primers for this locus were designed from a sequence of a clone obtained from the microsatellite library used for the

microsatellite analysis and amplified a unique sequence flanking region of the microsatellite GATA (Table 2).

Myosin Heavy Chain, Steel, and 5S primers were designed from conservative regions in GenBank genomic DNA sequences, while the Translation initiation factor 2 subunit 1 (eif2s1), acidic ribosomal phosphoprotein P0 (RPLP0), and Elongation factor-1 alpha subunit1 (Ef-1α) primers were designed from cDNA library clones from the liver of *Andrias davidianus* (Table 2). Because they were cDNA sequences that only contain the conserved exon regions of the genome, we needed to annotate them to try and capture intron information. To do this, the unique sequences were translated into proteins and then blasted to locate the most appropriate gene family for that sequence via Metazome (www.metazome.com). Only hits that scored as $\leq 1 \times 10^{-50}$ were considered conserved enough to be useful for primer design. The successful hits were then aligned to the *Xenopus* homolog and proper alignment was confirmed manually. Degenerate primers were designed from the exon flanking regions between *Xenopus* and *Andrias* and used to sequence *C. alleganiensis*. Genes that were likely to be present as single copies were selected to avoid the difficulties associated with amplifying pseudogenes. The resulting PCR products did not align with the original *A. davidianus* exon sequences but this was not surprising because the product should span the intron, which is not expressed in cDNA library. These unmatched products may represent the correct gene homologue, but lack significant exonic sequence to allow alignment to GenBank sequences, nevertheless, they are named according to the corresponding *Xenopus* homologous genes.

In this study, four microsatellite loci were analyzed: 3 dinucleotide loci (CRAL1, CRAL4 and CRAL9) and 1 tetranucleotide locus (GATA) (Johnson et al. 2009). Microsatellite loci were amplified using fluorescent polymerase chain reaction (PCR) as described by Johnson et al. (2009). The forward primer was 5'-labelled with a florescent tag and the fluorescent product was then genotyped on an ABI 3100 sequencing machine (Applied Biosystems) and visualized using GeneScan Analysis Software (version 3.1; Applied Biosystems) with Filter Set D.

Data analysis

To estimate the frequency of null alleles and scoring errors due to stuttering in our dataset we used MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). Several genetic statistics were calculated on the populations (number of alleles (Na), number of private alleles, and overall heterozygosity (H)) using the program GenAlEx version 6.1 (Peakall and Smouse 2006). We used ARLEQUIN version 3.11 (Excoffier et al. 2005) to estimate observed (H_O) and expected (H_E) heterozygosities at each site and locus for statistically significant deviations from Hardy-Weinberg equilibrium (HWE). Significance was evaluated using MCMC (Markov chain parameters: 100,000 dememorization; 1,000,000 steps per chain) and we applied a Bonferroni correction (adjusted P value < 0.0009, $\alpha = 0.05$). To test for linkage disequilibrium, we used GENEPOP version 4 (Raymond and Rousset 1995) with a Bonferroni correction (adjusted P value <0.0008, $\alpha = 0.05$).

We used STRUCTURE version 2.2 (Pritchard et al. 2000) to infer population structure and assign individuals to genetic clusters. This software uses a Bayesian algorithm that creates clusters maximizing both Hardy-Weinberg equilibrium and gametic phase equilibrium within clusters and disequilibrium between clusters. Ten iterations were run for each value of K= 1 (rivers constitute a single panmictic population) through 20 (two more than the number of rivers sampled) we ran the independent model, with a burn-in of 50,000 and MCMC values of 500,000. We used the method described by Evanno et al. (2005) to identify the number of hierarchical clusters in our dataset. The program *Distruct 1.1* (Rosenberg 2004) was used to display the data graphically.

We used Arlequin to analyze population structure using Wright's F-statistics (Wright 1968). This analysis requires the populations and groups of populations be known *a priori*. Therefore, we used the algorithm to test the hypothesis of populations relationships by clustering population samples into groups based on our mtDNA phylogeny, STRUCTURE clusters, or named subspecies and compared the values of the F statistics.

Results

Single copy nuclear markers

We were able to amplify and sequence eight single copy nuclear markers. They were screened for variation in both subspecies, at several geographic regions to maximize the chances of finding variation (Table 3). β-crystallin, eif2s1, RPLP0, and Ef-1α had no variation, while Myosin heavy chain, 5S, and GATA had low levels of polymorphisms, and the Steel locus did not amplify consistently. Because single copy nuclear loci were difficult to analyze and/or lacked variation in these preliminary surveys, we decided to focus on microsatellite variation.

Microsatellite loci

Microsatellite variation was determined for 147 individuals, from 18 rivers, at 4 loci (Table 4). All four loci were polymorphic: GATA had 19 alleles (136 – 212bp), CRAL4 had 17 alleles (161 – 201bp), CRAL9 had 13 alleles (119 – 147bp), and CRAL13 had 10 alleles (187 – 205bp). Our loci showed per population allele numbers ranging from an average of 5.50 alleles (WV, CR) to 1.75 alleles (EPR). Rivers had low numbers of private alleles (Fig. 2).

Genotype frequencies generally conformed to Hardy-Weinberg Equilibrium (HWE) in all populations across all loci, with one exception at Copper Creek (locus CRAL9). Observed and expected heterozygosities for each locus at all populations are

shown in Table 4. Null alleles were found in the Spring River population in one locus (CRAL9). To test if the null allele or the deviation from Hardy-Weinberg affected our results, we ran the complete dataset for all analyses as well as the dataset excluding the CRAL9 locus in the Spring River and the Copper Creek population. There were only minor changes in the results, so we felt we were able to include this locus in our analyses.

The results of the STRUCTURE analysis estimated the most probable value of K to be 11 clusters (Bayesian posterior probability = 0.954). However, when populations are distributed in hierarchical groups, in which migration among populations within a group is greater than migration among groups, Evanno et al. (2005) showed that the most likely value of K does not provide an accurate estimate of the number of higher level groups. To estimate the number of population groups, we employed the method outlined in Evanno et al. (2005) which uses the variance adjusted rate of change in the likelihood of K (ΔK) to estimate the number of clusters. Using this approach, the highest ΔK was found at K = 8 (Fig. 4).

The genetic clusters found by STRUCTURE show a high level of concordance with the mtDNA groupings found by Sabatino and Routman (2008). Figure 3 compares the STRUCTURE results for K = 8 with the phylogeny of the mtDNA from Sabatino and Routman (2008). Their mitochondrial tree showed 8 reciprocally monophyletic lineages that corresponded to river drainages or geographic region. These are: (1) Northern Ozarks, (2) Tennessee River, (3) Beaverdam Creek, (4) Little River, (5) Copper Creek,

(6) North Fork of the White, (7) Spring River and (8) New River. Most of these groups are also evident in the microsatellites, because the clusters (colors in Fig. 3) tend to found with high probability in only one of the 8 clusters above. The one exception is the New River sample (2 specimens from the New River and 6 specimens from the West Fork of the Greenbrier River), which was clustered with the Tennessee River lineage (7 specimens cluster with Copper Creek and 1 specimen (from the New River) clusters with the Little River and Beaverdam Creek). However, when the data were reanalyzed omitting the CRAL9 locus from the Spring River (which showed null alleles) and Copper Creek samples (which were out of Hardy-Weinberg) the New River drainage specimens belong to a separate cluster (data not shown).

Figure 5 compares the STRUCTURE output for K = 8, and 11. Although the additional clusters of individuals have greater ambiguity as to cluster membership, the basic geographic division into 8 groups is the same with the exception of only a few individuals within a river.

In order to determine which genetic structure is best supported by our data, we calculated Wright's hierarchical F-statistics among-group (F_{CT}), among-populations-within-groups (F_{SC}), and within-population (F_{ST}) (Excoffier et al. 1992). We partitioned the data into groups in three ways: using the mitochondrial groups found by Sabatino and Routman (2008), the STRUCTURE results from this study, and by named subspecies (Table 5). For all three hypotheses, the F_{ST} values were very similar, ranging from 0.42 – 0.44. The

among-group variation increases when populations are clustered as either the mtDNA groups (F_{CT} = 0.39) or the STRUCTURE groups (F_{CT} = 0.37) as compared to the subspecies grouping (F_{CT} = 0.11). However, the among-populations-within-groups variation (F_{SC}) shows the opposite pattern; the subspecies grouping shows an increased F_{SC} (0.36) as compared to the mtDNA and STRUCTURE groups (F_{SC} = 0.08 and 0.09 respectively). The F-statistics among populations within group variation is reduced for the hellbenders grouped as subspecies, which suggests that either the mtDNA groups or STRUCTURE groups represent genetically differentiated population groups (Table 5).

Discussion

The patterns of microsatellie variation in the hellbenders revealed nuclear clusters with similar geographic patterns as the mtDNA. When combined with the mtDNA data (Sabatino and Routman 2008) they suggest that most populations surveyed in this study are genetically distinct. Populations of *Crytobranchus alleganiensis* comprise at least 8 distinct units: 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. In addition, the STRUCTURE and F-statistics results are consistent with conclusion that the two subspecies are paraphyletic and that at a minimum each subspecies contains highly divergent populations that should not be treated as if they were identical. It is not unexpected that the sequenced nuclear introns had low sequence variation in contrast to high sequence variation in the mtDNA. This is caused by higher rates of substitution in the mitochondrial DNA.

In our analysis, there is only one group discordant with the mitochondrial findings: the New River samples cluster with the Copper Creek River populations. In the mitochondrial analysis, the hellbenders from the New River group with the highly divergent Current River/Eleven Point River samples, which comprises the sister clade to the rest of the samples. However, based on the microsatellites, the New River and Copper Creek River samples are consistently grouped together even at K=11 as indicated by the fact that on average the probabilities of cluster assignments are similar. Because the New

River drainage hellbenders belong to a unique cluster when the CRAL9 locus was omitted from the Copper Creek and Spring River samples, the association between the New River and the Tennessee River populations based on STRUCTURE analysis of all 4 microsatellites might be an artifact. On the other hand, the association could reflect incomplete lineage sorting from the common ancestor of the New and Tennessee River populations while the clustering of the New River mtDNA haplotypes with the Current River/Eleven Point haplotypes could reflect a lack of concordance between the gene tree and the population tree. Regardless of the process, the New River and Copper Creek hellbenders have very different mitochondrial haplotypes and so warrant status as separate groups.

The microsatellites have considerable allele sharing among populations as shown by low numbers of private alleles (Fig. 2). This lack of population specific autosomal markers could mean the populations are not as evolutionarily isolated as the mitochondrial phylogeny suggests or that there is some level of gene flow among populations. However, the complete reciprocal monophyly, high divergence, and geographically structured lineages within the mitochondria would mean that ongoing gene flow would have to be strongly male biased. According to Nickerson and Mays (1973), Peterson (1987), and Routman (unpublished data), hellbenders show low within-river movement and philopatry for both genders of adult hellbenders. Gene flow mediated by larvae (which may have a higher propensity to be washed downstream) are unlikely to exhibit male bias.

Sharing of alleles may occur without ongoing gene flow. One explanation for microsatellite allele sharing in the presence of mtDNA isolation among populations is that shared microsatellite alleles may have evolved independently (Culver et al. 2001). Another explanation might be the longer coalescence time for autosomal markers. Coalescence theory (Kingman 1982) predicts that the average time to the common ancestor for a sample of autosomal genes is approximately $4N_e$ generations, where N_e is the effective population size of the population. Because the mitochondrial genome is effectively haploid and maternally inherited, the N_e is one quarter that of nuclear genes (assuming a 1:1 sex ratio), which means lineages achieve reciprocal monophyly faster for mtDNA than neutral nuclear markers and sharing of alleles may represent the retention of ancestral polymorphisms for nuclear DNA but not for mtDNA (Moore 1995). This does not mean the mtDNA results are misleading, but that recently isolated populations might still be sharing neutral autosomal alleles long after mitochondrial genes have become reciprocally monophyletic.

It is important to note that although there is considerable allele sharing, STRUCTURE was able to define clusters (or groups of clusters) that are almost identical to the mitochondrial lineages without *a priori* information about the geographic location for the samples. This result suggests the 8 groups found in this study comprise distinct evolutionary units.

Conservation implications

The congruence of two independent types of markers (mitochondrial and autosomal) suggests that the mitochondrial phylogeny may reflect the underlying phylogeny of the populations of this species. This means that named subspecies are not natural groups and should not be used to make management decisions for hellbenders. Treating each subspecies as a management unit will, at best, combine very divergent populations and, at worst, combine populations that are not even each other's closest relatives.

Instead, most populations in the study are genetically distinct and should be managed as at least 8 distinct Evolutionary Significant Units (Moritz 1994). The finding of 8 groups of hellbender microsatellites that match well with the mtDNA groups demonstrates that these groups have had time to evolve differences in these (presumably) selectively neutral markers. Because we are using neutral markers, we are measuring changes in allele frequency based on a relatively slow nonadaptive process: genetic drift. It takes less time for selection to create differences among isolated populations than by genetic drift alone; therefore, these groups are likely to have evolved local adaptations to their environments. This means that the 8 groups should be managed independently to prevent mixing of populations and the disruption of coadapted gene complexes and possible outbreeding depression.

Conversely, the opposite strategy should be employed in the event of complete population extinctions. If faced with the scenario where captive breeding is required to conserve the entire species or a large part of the species, it might be best to intentionally mix individuals from different groups. By doing so, you will be creating a varied gene pool on which selection may operate. Some individuals might have a reduced fitness as a result of outbreeding depression, but as a whole, the increase in variation might be what allows the species to survive. Templeton et al. (1990) used this approach successfully to reintroduce collared lizards to glades in the Missouri Ozarks. In this system, individuals from genetically distinct lineages were with low population sizes to increase the genetic variation and allow selection to occur on the founder populations. A similar approach may prove useful for the hellbender.

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Table 1. Sampled localities

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	*alleganiensis*
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)	Algeheny-Ohio	*alleganiensis*
Slippery Rock Creek (SRC)	Connoquenessing-Ohio	*alleganiensis*
New River (NewR)	Kanawha-Ohio	*alleganiensis*
West Fork of the Greenbrier River (WFGR)	New-Kanawha-Ohio	*alleganiensis*
Wabash River (WR)	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*

Sampling locations (abbreviations); major drainages into which the rivers flow; hellbender subspecies designation.

Table 2. Autosomal marker primer design

Locus	Primer	Sequence (5' – 3')	Reference	GenBank Accession no.
β-crystallin	CRYBA1Ls	CGCCTGATGTCTTTCCGCC	Dolman and Philips(2004)	AY508951 – AY508952
	CRYBA2Ls	CCAATGAAGTTCTCTTTCTCAA		
Myosin heavy chain	MYH2-F10	TGCACTCACAGAACACCAG	Schinske, J. pers. comm.	AY508930
	MYH2-R	TGGTGTCCTGCTCCTTCTT		
Steel	STl-CA-F1	ACGCCAGGTATGCCGAGG	Sabatino, S. pers. Comm.	
	STl-CA-R2	GTGATGTTAGGAGTCAGTGC		
5S	5S-F1	GGGAGACTGCCTGGGAATAC	This study	AB066111 – AB066113
	5S-R1	CGCATTCAGGGTGGTATGG		
eif2s1	Euktrans_x4	GGTCTGAGNTGTTCCACAGA	This study	EH168568
	Euktrans_x5	CNTANCGAGGAGGAGCAATC		
RPLP0	Ribpro_x1	CAGGGAAGACAGGGCTACNT	This study	EG018546
	Ribpro_x2	CCCCCACAATGAAGCATTT		
Ef-1α	Elofact_x6	CCNATGTGNGTGGAGAGCTT	This study	ES272822
	Elofact_x7	CATGTCACGGACAGCAAAAC		
GATA	GATAF1	AGGGATCCCCTGACAGAAGT	Johnson et al. 2009	
	GATAR	GCTTTGACTGGGCCATTCTA		

Primer sequences (5' – 3') with references and GenBank Accession numbers for the original locus.

Table 3. Characterization of nuclear loci for *C. alleganiensis*

Locus	Product size	SNPs found	Indels	Population successfully amplified
β-crystallin	186	0	0	BP, CC, NFW*, SR*
Myosin heavy chain	254	1	0	BP, CR*, FC
Steel	388	10	2 (2, 5)	BP, CC, CR*, EPR*, GR, LR, NewR, NFW*, SC, SR*
5S	278	9	0	BC, BP, CC, CR*, EPR*, FC, LR, NewR, NFW, SR*, SRC
eif2s1	422	0	0	BR, EPR*, NFW*, SRC
RPLP0	248	0	0	FC, NFW*, SR*, SRC
Ef-1α	296	0	0	NFW*, SR*, SRC
GATA	659	6	1 (2)	BP, CC, CR*, NewR, SRC, SR*

Approximate size of allele in base pairs; the number of polymorphic sites; the number of sites with insertions/deletions (indels) and size of indels in parentheses; population in which the locus was amplified: Beaverdam Creek (BC), Big Piney (BP), Copper Creek (CC), Current River (CR), Eleven Point River (EPR), French Creek (FC), Gasconade River (GR), Little River (LR), New River (NR), North Fork of the White (NFW), Sherman Creek (SC), Spring River (SR), Slippery Rock Creek (SRC). Localities for *C. a. bishopi* are denoted by an asterisk (*).

Table 4. Expected and observed heterozygosities

	n	GATA	CRAL4	CRAL9	CRAL13
BC	6	1.00/0.79	0.83/0.85	0.50/0.59	0.83/0.71
BP	24	0.63/0.73	0.22/0.37	0.26/0.37	0.83/0.61
BR	6	0.83/0.86	1.00/0.79	0.33/0.71	0.50/0.56
CC	11	0.73/0.74	0.73/0.52	**0.64/0.80**	0.89/0.78
CR	6	1.00/0.97	0.17/0.17	0.17/0.17	0.17/0.17
EPR	4	1.00/0.68	0.25/0.25	-	-
FC	12	0.83/0.86	0.66/0.74	0.25/0.23	0.83/0.63
GR	9	0.67/0.80	0.22/0.21	0.11/0.29	0.78/0.70
LR	7	0.57/0.84	1.00/0.86	0.86/0.70	1.00/0.73
MR	14	0.67/0.59	-	-	0.75/0.55
NR	2	1.00/1.00	1.00/1.00	0.50/0.83	1.00/0.67
NFW	13	0.69/0.75	0.46/0.37	-	0.15/1.00
NewR	10	0.60/0.80	0.70/0.57	0.10/0.10	0.80/0.50
SC	2	0.50/0.83	-	0.50/0.50	0.00/0.67
SR	11	0.90/0.90	-	0.09/0.26	-
SRC	5	1.00/0.84	0.80/0.64	0.60/0.60	0.60/0.64
Wa	1	1.00/1.00	1.00/1.00	1.00/1.00	1.00/1.00
WFGR	6	1.00/0.92	0.67/0.88	0.50/0.68	0.67/0.68

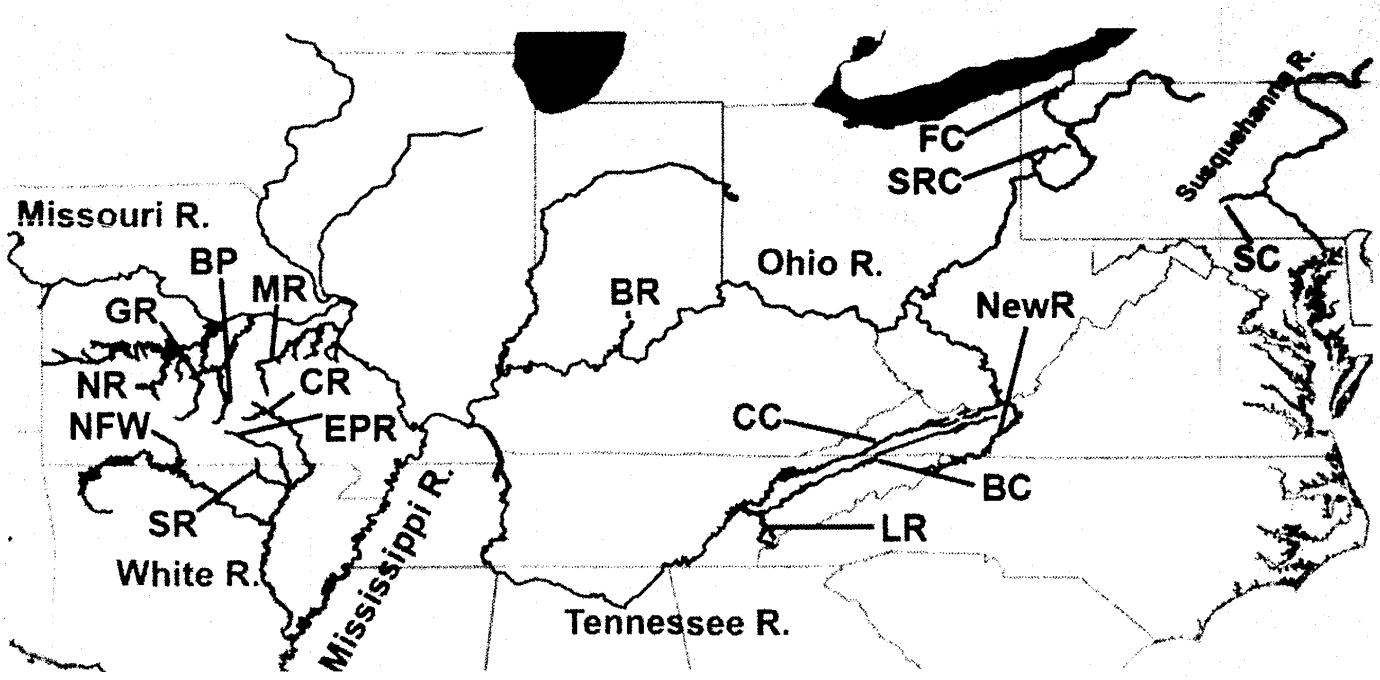
Heterozygosities estimated (Ho/He) at four microsatellite loci in populations of *Cryptobranchus alleganiensis*. For each river, n equals the number of individuals genotyped and dashes (-) indicate the locus was monomorphic in that population. Results in bold indicate populations that do not conform to HW expectations.

Table 5. Wright's F-statistics

	Structure tested	F_{ST}	F_{SC}	F_{CT}
mtDNA groups	(BP, GR, MR, NR) (BR, FC, SC, SRC) (BC, LR) (CC) (EPR, CR) (NFW) (SR) (NewR)	0.44	0.08	0.39
STRUCTURE groups	(BP, GR, MR, NR) (BR, FC, SC, SRC) (BC, LR) (CC, NewR) (EPR, CR) (NFW) (SR)	0.42	0.09	0.37
subspecies	(BP, GR, MR, NR, BR, FC, SC, SRC, BC, LR, CC, EPR) (NFW, SR, NewR, CR)	0.43	0.36	0.11

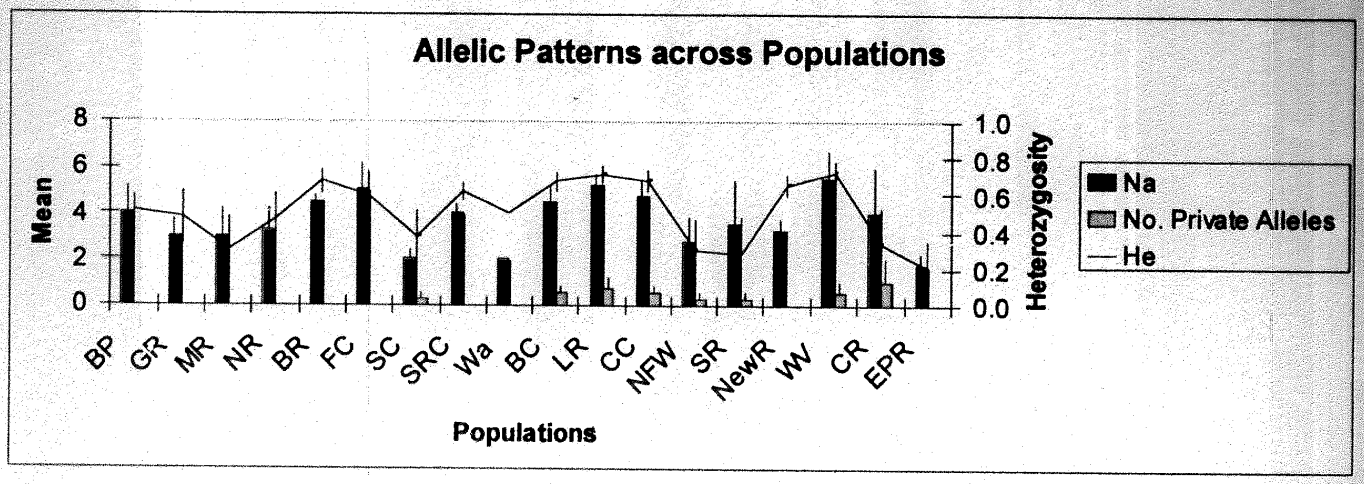
AMOVA statistics for the three hypotheses. F_{ST} represents within-populations variation, F_{SC} represents among-populations-within-groups variation, and F_{CT} represents among-group variation.

Figure 1



Map showing sampled localities for this study. Major rivers are spelled out. Rivers are abbreviated as follows: Gasconade River (**GR**), Big Piney River (**BP**), Niangua River (**NR**), Meramec River (**MR**), Little River (**LR**), Beaverdam Creek (**BC**), Copper Creek (**CC**), Blue River (**BR**), French Creek (**FC**), Slippery Rock Creek (**SRC**), New River (**NR**), West Fork of the Greenbrier River(**WFGR**), Sherman Creek (**SC**), North Fork of the White River (**NFW**), Spring River (**SR**), Current River(**CR**), Eleven Point River (**EPR**). The last four populations represent the subspecies *C. a. bishopi*. All other populations represent *C. a. alleganiensis*.

Figure 2



Allelic diversity patterns across rivers. Na.; Number of alleles. No.Private Alleles; alleles unique to that river, He; expected heterozygosity.

Figure 3

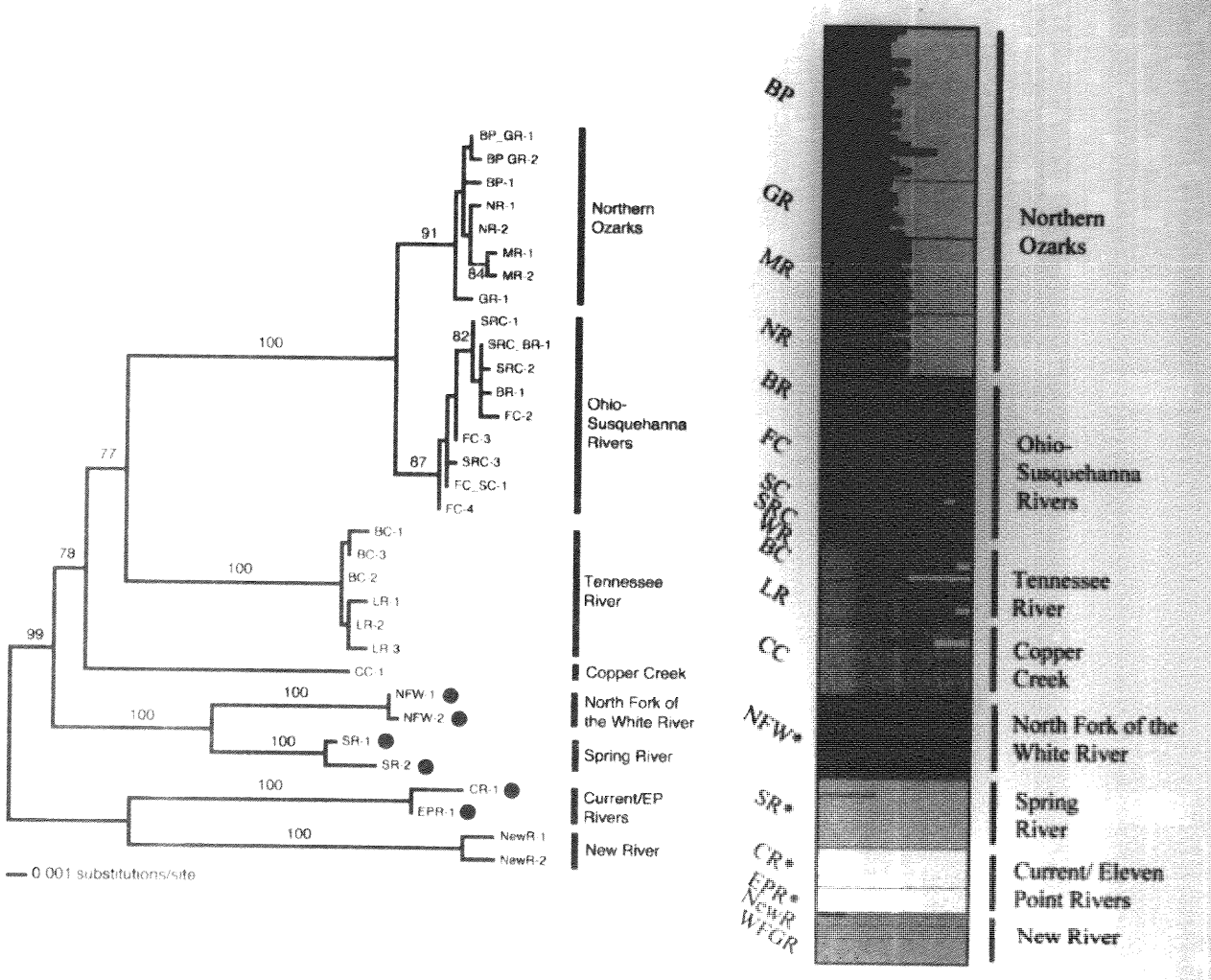
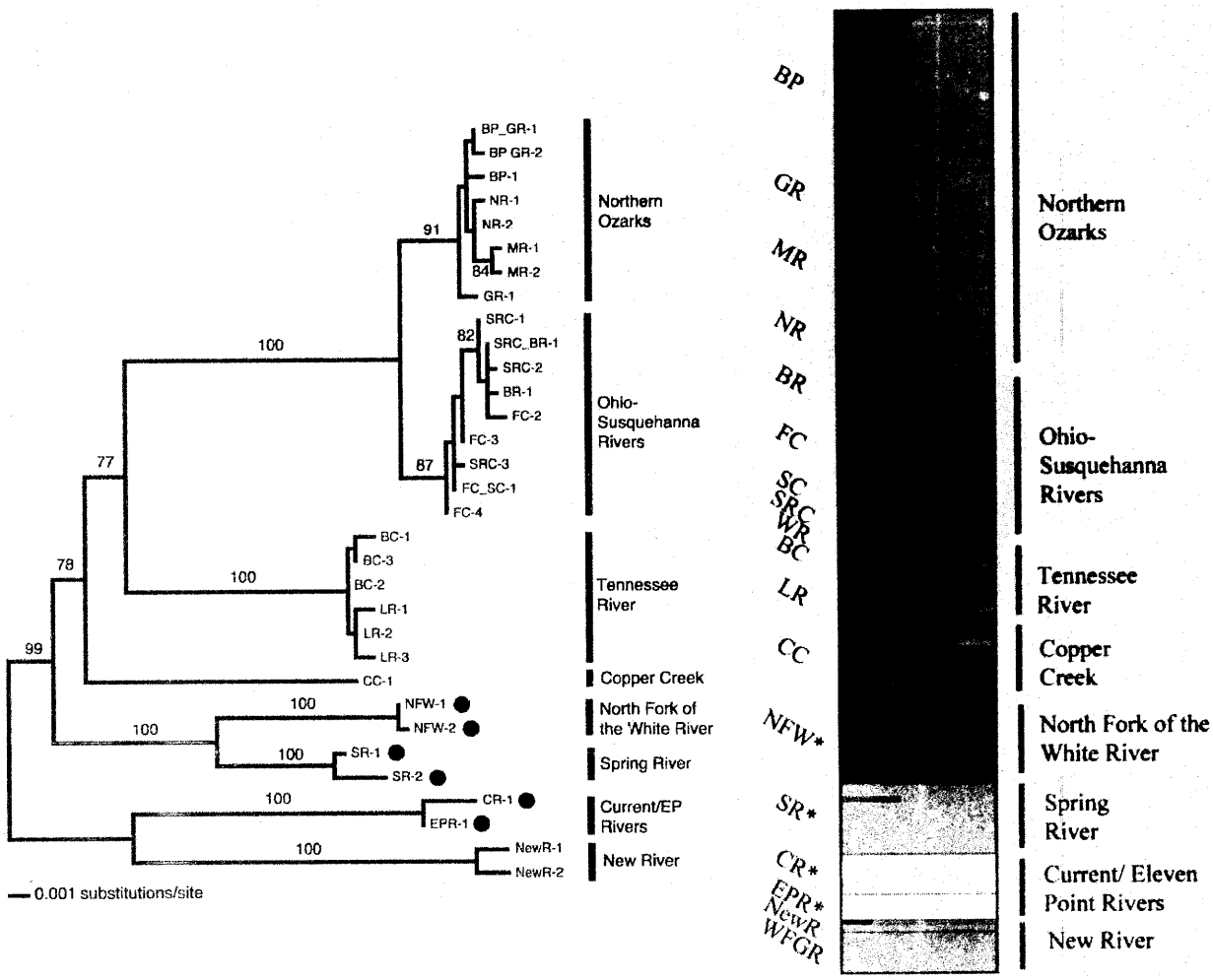
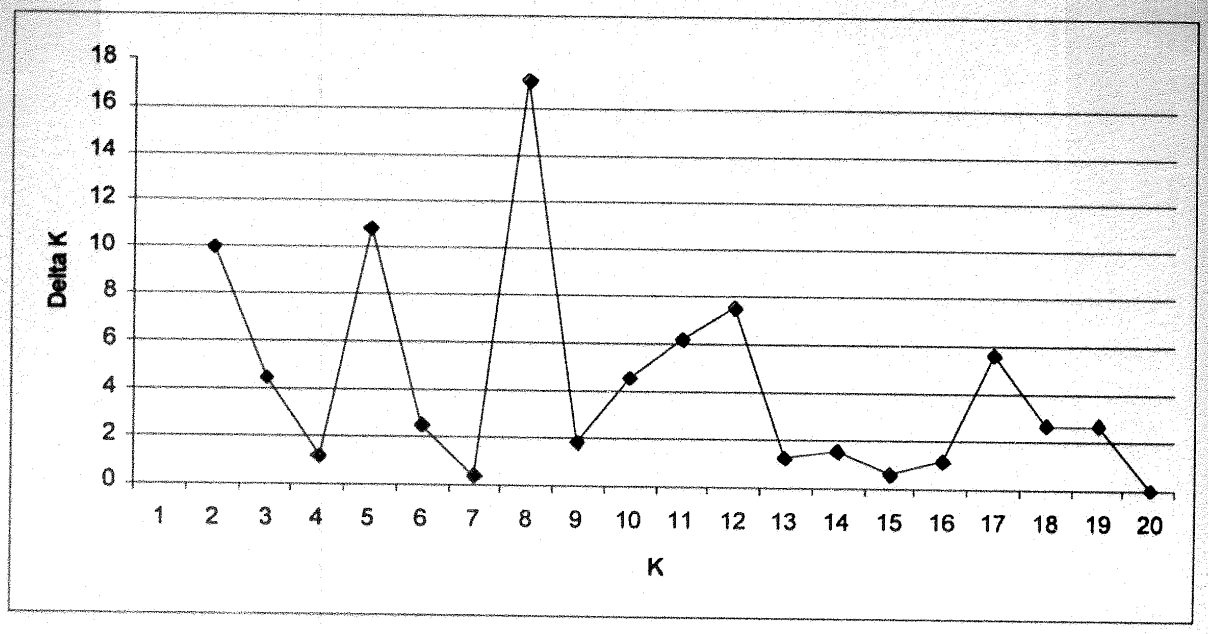

BP_GR-1
BP GR-2
BP-1
NR-1
NR-2
MR-1
MR-2
GR-1
SRC-1
SRC_BR-1
SRC-2
BR-1
FC-2
FC-3
SRC-3
FC_SC-1
FC-4
BC-1
BC-3
BC-2
LR-1
LR-2
LR-3
CC-1
NFW-1
NFW-2
SR-1
SR-2
CR-1
EPR-1
NewR-1
NewR-2
91
84
82
87
100
77
78
100
99
100
100
100
100
100
0.001 substitutions/site
Northern Ozarks
Ohio-Susquehanna Rivers
Tennessee River
Copper Creek
North Fork of the White River
Spring River
Current/EP Rivers
New River
BP
GR
MR
NR
BR
FC
SC
SRC
BC
LR
CC
NFW
SR
CR
EPR
NewR
WhR
Northern Ozarks
Ohio-Susquehanna Rivers
Tennessee River
Copper Creek
North Fork of the White River
Spring River
Current/ Eleven Point Rivers
New River

Figure 3


BP_GR-1
BP GR-2
BP-1
NR-1
NR-2
MR-1
MR-2
GR-1
SRC-1
SRC_BR-1
SRC-2
BR-1
FC-2
FC-3
SRC-3
FC_SC-1
FC-4
BC-1
BC-3
BC-2
LR-1
LR-2
LR-3
CC-1
NFW-1
NFW-2
SR-1
SR-2
CR-1
EPR-1
NewR-1
NewR-2
91
84
82
87
100
77
78
100
99
100
100
100
100
Northern Ozarks
Ohio-Susquehanna Rivers
Tennessee River
Copper Creek
North Fork of the White River
Spring River
Current/EP Rivers
New River
0.001 substitutions/site
BP
GR
MR
NR
BR
FC
SC
SRC
WR
BC
LR
CC
NFW*
SR*
CR*
EPR*
NewR
WFGR
Northern Ozarks
Ohio-Susquehanna Rivers
Tennessee River
Copper Creek
North Fork of the White River
Spring River
Current/ Eleven Point Rivers
New River

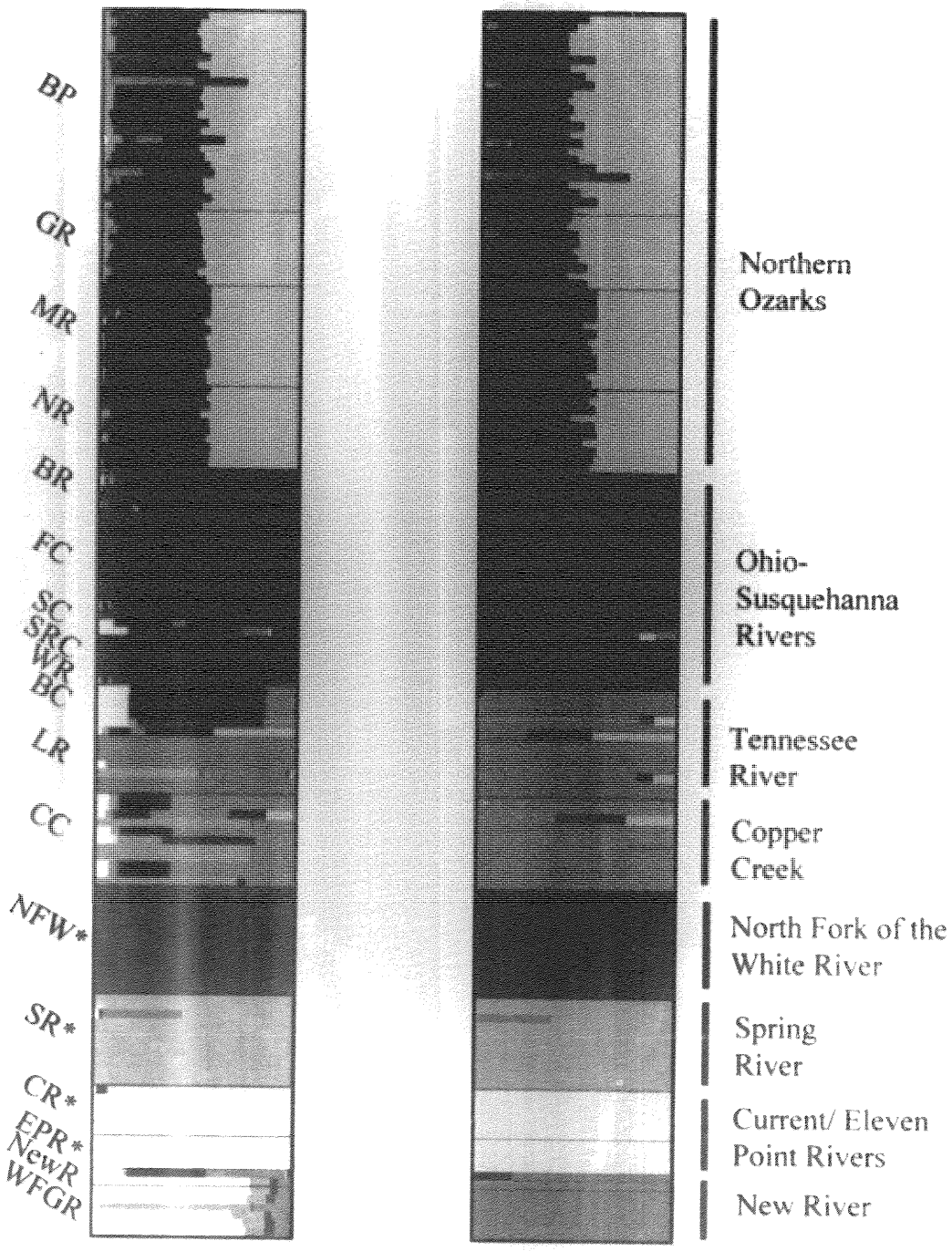
A. mitochondrial DNA phylogeny from Sabatino and Routman (2008). B. Results of the Bayesian assignment test based on four microsatellite loci. Each color represents one of the seven genetic clusters as determined by STRUCTURE. Colors in the STRUCTURE output represent different genetic clusters. Bars on the chart represent each individual, with the proportion or each color equaling the probability of that individual's membership in that cluster. Individuals are arranged by rivers sampled, with river abbreviations to the left of the STRUCTURE output and *a priori* mtDNA groups are shown to the right. Localities for *C. a. bishopi* resides are denoted with an asterisk. Width of population bars is proportional to the number of individuals sampled for each population.

Figure 4



Plot of ΔK for the microsatellite data. The peak at 8 suggests that populations are hierarchically grouped into 8 groups.

Figure 5.



A comparison of the clusters estimated with STRUCTURE for K=8 vs. K=11. Interpretation of the charts is as in Fig. 2.