Genetic Analysis of Ozark Hellbenders Utilizing RAPD Markers

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Abstract: Ozark hellbenders *(Cryptobranchus alleganiensis bishopi)* are large aquatic salamanders found in flowing waters. The abundance of this species is thought to have declined over the long term. What had been the most abundant population, in Spring River, Arkansas, appears to have declined precipitously in the last decade. The possibility of supplementing the population through captive propagation has been suggested, raising concerns about genetic issues. Random amplified polymorphic DNA (RAPD) was evaluated for assessing genetic variability among and within populations of Ozark hellbenders from Spring River (Ark.), Eleven Point River (Ark.), and North Fork White River (Mo.). Six primers were tested, and all generated reproducible RAPD profiles. Forty RAPD bands were generated of which 20% exhibited within-population variation and an additional 5% exhibited between-population variation. Analysis using primer OpB12 produced 2 fixed markers that distinguished hellbenders from the Spring River and North Fork White River from those in Eleven Point River. The distinct RAPD profiles of different populations suggest a lack of gene flow and possible effects of inbreeding and random genetic drift. This is in agreement with previous studies that examined isozymes and mitochondrial DNA (mtDNA). Previous isozyme data, which is based on actual proteins and phenotypic variation indicate that Spring River and White River are identical populations and North Fork White River could be used for restoration efforts.

RAPD and mtDNA data combined, which are based on non-coding regions of the genome indicate that the 3 populations examined are uniquely different from each other and should not be mixed. No data on the quantitative genetic variation is available. In light of the population structure of these hellbenders, apparent strict reproductive isolation and the development of highly homozygous lines, and possible habitat degradation, intentional crossing of those populations could actually increase their fitness and viability. To provide genetic information useful for restoration efforts, further studies are needed to assess the population structure and geographical distribution of various genotypes.

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The hellbender, *Cryptobranchus alleganiensis*, is a large, aquatic salamander. They are found throughout much of the Ohio River basin with disjunct populations in the Ozarks of Missouri and Arkansas (Conant and Collins 1991).

The taxonomic status of this species is not clear. Some of the populations in the Ozarks have been designated as a subspecies referred to as the Ozark hellbender, *Cryptobranchus alleganiensis bishopi*, separate from the eastern hellbender, *Cryptobranchus alleganiensis alleganiensis*. Collins (1991) considered *C. bishopi* to be a species. Isozyme data shows the 2 subspecies to be similar (Merkle et al. 1977, Shaffer and Breden 1989). mtDNA analysis (Routman 1993) shows the 2 subspecies to be distinctive, and the DNA content and red blood cell size of the 2 subspecies is distinct (Merkle et al. 1977).

The Ozark hellbender is the only subspecies that has been found in Arkansas. Populations are present in the Spring River in Fulton County (Black and Dellinger 1938, Dowling 1957, Dundee and Dundee 1965, Ratliff 1965, Nickerson and Mays 1973, Peterson 1985) and the Eleven Point River in Randolph County (Trauth et al. 1993). There have also been unverified reports from the Black River, and a single specimen was taken in 1966 from the White River in Baxter County (Trauth, pers. commun.). Routman's (1993) analysis of mitochondrial DNA in the genus found differences between populations of *C. a. alleganiensis*, supporting its status as a distinct evolutionary unit, be it species or subspecies. The study even identified a difference in 1 restriction site between *C. a. bishopi* populations in the Spring River (Ark.) and the North Fork White River (Mo.). Routman's study did not include the Eleven Point River population. This hellbender has likely experienced declines since European settlement of Arkansas. The species was considered to be vulnerable in 1974 (Reagan 1974) and further evidence of declination was reported (Trauth et al. 1992*a*, 1992*b*, 1993). Trauth (pers. commun.) attributed this decline to over-collection, habitat alteration, pollution, and siltation due to riparian clearing for agriculture, industrial use, and human occupation. By 1990, the Spring River hellbender population was in serious decline (Trauth et al. 1992*a*, 1992*b,* 1993). A tag and release study was conducted on a 26-km stretch of the Spring River to confirm the population decline (Trauth, pers. commun.). The survey concluded that low numbers of capture,

lack of young individuals, and absence of eggs indicated low levels of reproduction. The U.S. Fish and Wildlife Service reviewed the status of the Ozark hellbender in 1993, finding that protection under the Endangered Species Act was not warranted (LaClaire 1993). The Service recommended a survey of the Spring River population comparable to the work of Peterson (1985) to better define the magnitude of the apparent decline. Although the cause of the decline of the hellbender population on the Spring River remain undetermined, the Arkansas Game and Fish Commission decided to consider augmenting the hellbender population in the Spring River through stocking either by transplanting individuals from more abundant populations or by release of young from a captive-breeding program. However, concerns existed over the effect of stocking on the genetic composition of the population, particularly in consideration of its geographic isolation and lack of ability to migrate. Hellbenders appear to exist in reproductively isolated populations that have limited adaptive potential. Thus, evaluation of genetic composition of hellbender populations was needed.

Previous studies using allozymes (Merkle et al. 1977, Shaffer and Breden 1989) revealed little genetic variability throughout the range of hellbenders. Merkle et al. (1977) examined 2 populations of *C. a. bishopi* (Spring River and North Fork White River) and 10 populations of *C. a. alleganiensis* for isozyme variation. Mean heterozygosities were extremely low, 0.002 and 0.003 for North Fork White River and Spring River, respectively, and percent polymorphic loci was 4.2%. Typical values for vertebrates and fish are 0.06 for mean heterozygosity and 10%–20% for loci polymorphic (Merkle et al. 1977). However, it is not unusual to find individuals/populations that are monomorphic for all isozyme loci (Phillipp et al. 1983). Variant alleles at the single polymorphic locus for Spring River and North Fork White River were at almost identical frequencies. Nine of the 10 populations of *C. a. alleganiensis* were homozygous at all isozyme loci (Merkle et al. 1977) which is unusual. Shaffer and Braden (1989) examined 2 populations in Missouri, in the Gasconde and Big Piney rivers, which, based on location, should be *C. a. alleganiensis*. Mean heterozygosities, 0.018–0.021, and percent loci polymorphic (11%–14%) were higher than the values found by Merkle (1977) for *C. a. alleganiensis* and *C. a. bishopi*. Shaffer and Braden (1989) conducted an analysis that showed that paedomorphic urodeles, non-transforming amphibians that have larval reproduction, have lower genetic variability than transforming species and concluded that this fact as well as population structure explain low genetic variation in *C. a. alleganiensis*. Another point not previously discussed is that isozyme variation in *C. a. alleganiensis* is much higher west of the Mississippi River (Merkle et al. 1977). Nine of the 10 populations of *C. a. alleganiensis* of the east of the Mississippi are monomorphic and the tenth population has minimal isozyme variation. Two of 3 populations of *C. a. alleganiensis* west of the Mississippi exhibit moderate levels of isozyme variability and a fourth population has low levels of isozyme variation. Low variability due to population bottlenecks and temporal instability of aquatic habitats (Shaffer and Breden 1989) coupled with such low heterozygosity may be detrimental to the species' persistence, through reduced adaptive potential.

A more recent study (Routman 1993) reported high levels of variation in mitochondrial DNA between populations, but the variation within populations was lower than in other vertebrates. Population structure based on mtDNA and isozyme analysis differs, but conclusions on overall species genetic variability are the same. These conflicting results may be explained by the nature of the genetic markers being used in the studies. Allozymes exhibit low levels of polymorphism because of the limited numbers of loci available, the low numbers of alleles at a specific locus and they are actual proteins, therefore, less variable. While the evolutionary rate of mitochondrial DNA is generally high, leading to high levels of polymorphisms. In this case, differences at the base pair level rather than amino acid changes (with differing electrical charge) are detected. Additionally, mtDNA reveals only maternal lineage. Nevertheless, Routman (1993) found distinct mitochondrial restriction patterns for proximate populations, again indicating high levels of reproductive isolation among populations. Two populations in the North Fork White River had identical and non-variable haplotypes. A second haplotype was fixed in the Spring River and a third haplotype was fixed in the Current River. Spring River, Eleven Point River, and Current River are all in close proximity and these rivers and North Fork White River are all part of the Black River Drainage. Based on the mtDNA, F_{ST} was 0.865, indicating that *C. a. alleganiensis* populations were structured essentially like isolated inbred lines (1.00 is the maximum F_{ST}). Reproductive isolation between populations, when coupled with population numbers, can result in long-term adverse impacts to the viability of an organism.

Randomly amplified polymorphic DNA markers are DNA sequences separated by gel electrophoresis after polymerase chain reaction (PCR) using short random oligonucleotide primers. Usually, only 1 primer is used in RAPD reactions. When the primer binds to the opposite strand of DNA at close sites, a PCR product may result. RAPD polymorphism is caused by length changes between the 2 closest primer-binding sites due to insertions or deletions and by base changes in the primer binding sites. Despite its shortcomings of lack of high reproducibility due to low temperature PCR, RAPD is a rapid and highly economical approach for genetic analysis. However, we (Liu et al. 1998) demonstrated that when high stringency is utilized, RAPD is quite repeatable. RAPD is a useful technique for species such as hellbenders for which there is little previously known genetic information available. It has been used to reveal genetic variations at the species levels or at the subspecies among populations in a number of aquatic organisms (Dinesh et al. 1993, Johnson et al. 1994, Foo et al. 1995, Caccone et al. 1997, Liu et al. 1998, 1999). In addition, the PCR-based RAPD approach required neither large samples nor sacrifice of animals.

The objectives of this research were to evaluate the potential of using random amplified polymorphic DNA (RAPD) for the analysis of genetic composition of hellbender populations and to confirm results from early studies using allozymes and mitochondrial DNA markers. Here we report our initial studies of RAPD genetic variation in Ozark hellbender populations, particularly the populations of the Spring River (Ark.), the Eleven Point River (Ark.), and North Fork White River (Mo.).

Methods

Sample locations are shown in Fig. 1. Tail clips from Spring River and Eleven Point River samples were taken from individuals held at the Spring River Hatchery. North Fork White River samples were collected in the field and the animals immediately returned to the river. A total of 12 individuals from the Spring River and 8 from the Eleven Point River were sampled, as population numbers are low in the natural environment. For comparison, samples were taken from 23 individuals from the North Fork White River. Samples were shipped on dry ice to the Molecular Genetics and Biotechnology Laboratory in the Department of Fisheries and Allied Aquacultures at Auburn (Ala.) University, and stored at –80 C until preparation of DNA. DNA was prepared using the C-TAB method (Saghai-Maroof et al. 1994). DNA was amplified in 50 µl of a reaction mixture containing 10 mM Tris-HCl, pH 9.0 (at 25 C), 50 mM KCl, 0. 1% Triton X-100, and 1.5 mM $MgCl_2$, 100 mM dNTPs, 4.5 mM primer, 25 ng DNA template, and 0.75 units of *Taq* DNA polymerase (Life Tech., Bethesda, Md.). After an initial heat denaturation of 5 minutes at 94 C, the reaction mixtures were subjected to amplification in a thermocycler (MJ Research, Boston, Mass.) for 40 cycles. The PCR profiles consisted of 1 minute at 94 C, 1 minute at 36 C, and 2 minutes at 72 C. Oligonucleotide primers were obtained from Operon Technologies, Inc. (Alameda, Calif., Table 1). Six primers were evaluated to compare Spring River and Eleven Point populations. After this analysis, the only primer with

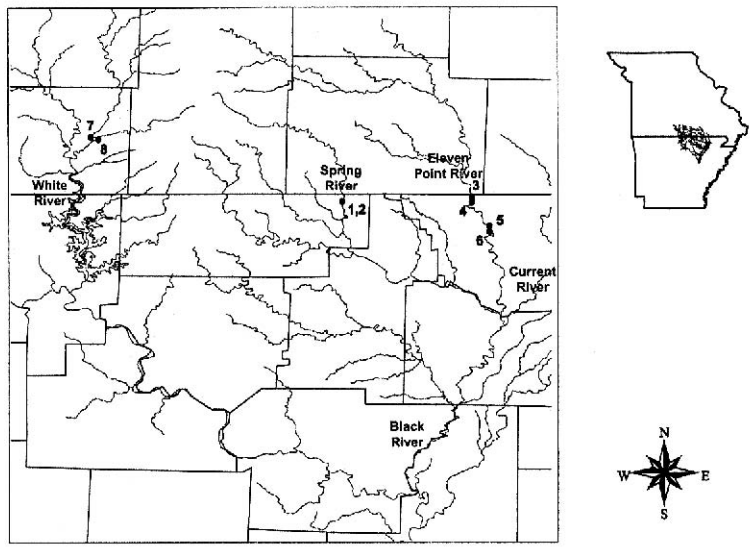

Figure 1. Collection locations for Ozark hellbender genetics samples. Location 1 and 2, Spring River, Arkansas; location 3, 4, 5 and 6, Eleven Point River, Arkansas; location 7 and 8, North Fork White River, Missouri.

fixed difference, opB12 was used to compare North Fork White River population with the other 2 populations. After completion of RAPD PCR, amplified products were analyzed by agarose or acrylamide gel electrophoresis. Most gels were visualized by ethidium bromide staining. To enhance sensitivity, some gels were visualized by silver staining according to protocol described by Qiu et al. (1995). Briefly, acrylamide gels were impregnated with 1% (w/v) $AgNO_3$ for 15 minutes and developed in a silver developing solution containing 6% (w/v) Na_2CO_3 and 0.4% (w/v) $Na_2S_2O_3$ until the bands were visible. The staining reaction was stopped by submersion of the gel in 10% (v/v) acetic acid for 10 minutes. The gel was rinsed with distilled water and photographed. For variable bands, alleles or sequence difference frequencies between populations were compared between populations with a modified *t*-test (Hallerman et al. 1986). RAPD primers are inherited in a dominant fashion so it is not possible to identify heterozygotes. Allele frequencies/number of heterozygotes were estimated assuming the populations were at Hardy-Weinberg equilibrium.

Results and Discussion

As the initial study of the applicability of RAPD markers for population differentiation, 6 primers were tested (Table 1). All 6 primers produced reproducible RAPD profiles and therefore are technically good primers for RAPD analysis of hellbenders. Of the 6 primers, opA8, opA20, and opB12 generated polymorphic bands reflecting their potential as primers to produce informative RAPD profiles, but opA1, opC2 and opC13 produced non-variable RAPD profiles. Primer opB12 produced distinct RAPD profiles with fixed differences between the population from Spring River and the population from the Eleven Point River (Fig. 2). A prominent band with a size of approximately 450 base pairs (SR450) was present from all tested individuals of the hellbenders from Spring River, but absent from all individuals of those from the Eleven Point River (Fig. 2). Likewise, a second band with a size of approximately 500 bp (EPR500) was specifically amplified only from individuals of the hellbenders from the Eleven Point River, but not from those of Spring River. These population-

Table 1. RAPD primers (Operon Technologies, Alameda, Calif.) and their application in population studies of hellbenders, *Cryptobranchus alleganiensis bishopi* from Spring River and Eleven Point River in Arkansas and North Fork White River in Missouri

Primer name	Primer sequence	*N* bands	Genetic variation	Presence of fixed markers
opA1	5' –CAGGCCCTTC–3'	3	no	no
opA8	5' –GTGACGTAGG–3'	7	yes	no
opA20	5' –GTTGCGATCC–3'	8	yes	no
opB12	5' –TGTCATCCCC–3'	10	yes	yes
opC2	5' –GTGAGGCGTC–3'	6	no	no
opC13	5' –AAGCCTCGTC–3'	6	no	no

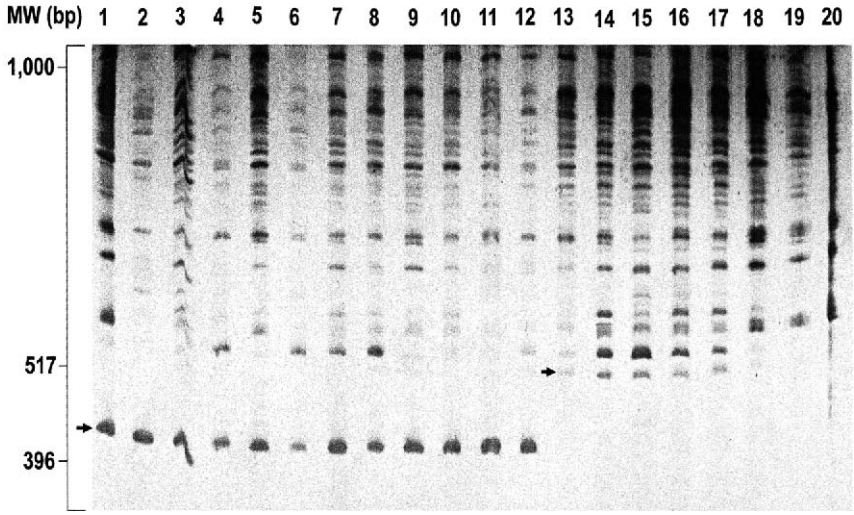

Figure 2. RAPD profiles of 20 hellbenders sampled from Spring River and Eleven Point River as revealed by primer opB12. Lines 1–12 are hellbenders from Spring River and lines 13–20 are hellbenders from Eleven Point River. Fixed RAPD markers SR450 for Spring River hellbender and EPR500 for Eleven Point River are indicated by arrows. DNA fragment sizes are in base-pairs.

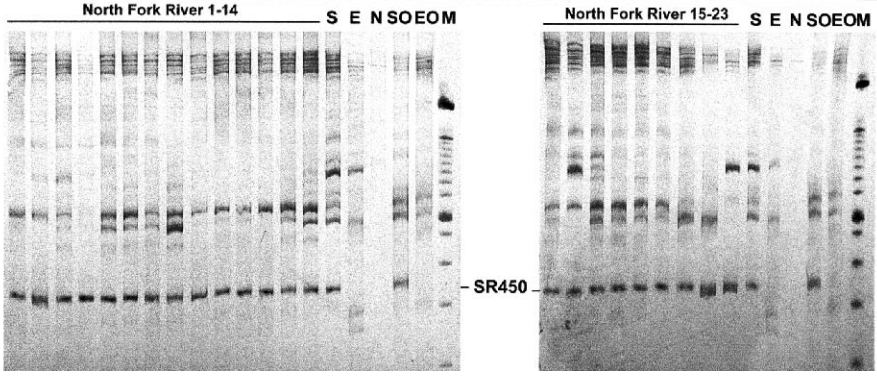

Figure 3. RAPD analysis of Hellbenders using primer OpB-12. Left plate, first 14 lanes, North Fork White River samples l–14; S, Spring River; E, Eleven Point River; N, negative control; SO and EO, Old Spring River and Eleven Point River PCR reactions; 100 base pair marker. Right plate, first 9 lanes, North Fork White River samples 15–23; S, Spring River; E, Eleven Point River; N, negative control; SO and EO, Old Spring River and Eleven Point River PCR reactions; 100 base pair marker.

specific bands may represent fixed RAPD markers of the populations. This fixation could be a result of inbreeding or random genetic drift due to small population sizes and lack of gene flow. Additionally, Shaffer and Breden (1989) would probably attribute part of the genetic uniformity to this non-transforming nature of *C a. bishopi*. If these bands prove to be fixed markers in future studies, they should be highly useful for the identification of populations.

RAPD analysis was conducted using samples collected from North Fork White River (Fig. 3) to determine the genotype and presence of fixed markers using primer opB12. The North Fork White River population was identical to Spring River with regard to the 2 fixed markers. All the individuals from North Fork White River harbored SR450 band, but not the EPR500 band. This indicated that the fixed markers could only be used to differentiate the Spring River population and the Eleven Point River population.

OpA20, opA8, and opB12 primers revealed some genetic variation by producing variable RAPD profiles though not fixed markers (Tables 1, 2). These genetic variations would be important for quantitatively defining the populations using multiple RAPD markers by analysis of band sharing and heterogeneity. Band 3 and 7 of opB12 were polymorphic for Eleven Point and 7 and 8 were polymorphic for North Fork White River. Eleven Point River population was also polymorphic for bands 2, 5, and 7 of primer opA8, but Spring River was non-variable. Spring River was polymorphic for band 2 and 8 and Eleven Point River for band 8 of opA20. In total, 20% of the RAPD bands exhibited variability. This is more RAPD variability than that of channel catfish, *Ictalurus punctatus*, and blue catfish, *I. furcatus* (Liu et al. 1998), 2 species that conversely have much greater isozyme variability than Ozark hellbenders. In general, Spring River exhibited less RAPD genetic variability than Eleven Point River with 5% and 15% of loci polymorphic, respectively. Spring River was less variable than North Fork White River based on only the opB12 primer. If Hardy-Weinberg equilibrium is assumed and the number of heterozygote individuals was

Table 2. RAPD band(s) frequency for polymorphic bands of Ozark Hellbender *Cryptobranchus alleganiensis bishopi.* Primers opA1 (3 bands), opC2 (6 bands), and opC13 (6 bands) were monomorphic. OpA20, opA8, opB12 generated 8, 7, and 10 total bands respectively.

	Primer									
	opA20		opA8			opB12				
Populations (*N*)Band	2[a]	8	2[a]	5	7	3	7	8[a]	9[a]	10[a]
Spring River (12)	0.92	0.42	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Eleven Point River (8)	1.00	0.13	0.50	0.75	0.87	0.87	0.87	1.00	1.00	1.00
North Fork White River (23)	–	–	–	–	–	1.00	0.91	0.65	0.00	1.00

a. Band frequencies are different among populations at $P < 0.05$, modified *t*-test (Hallerman et al. 1986).

extrapolated (this cannot be done directly as RAPD markers are inherited as a dominant fashion), mean heterozygosity for RAPD markers was 0.05 and 0.07, respectively, for Spring and Eleven Point River populations. Additionally, although data is very limited, individuals that were variable for 1 RAPD band for an individual primer had a greater probability of being variable at a second band for that same primer, compared to other individuals, and this trend was independent of the genotypes seen for other primers.

Williams et al. (1981) considered the family *Cryptobranchidae* to be at a virtual evolutionary standstill, having a relict distribution typical of amphibian groups that are not currently undergoing dispersal or evolution. Previous genetic studies support this hypothesis by documenting low genetic variability throughout the range of hellbenders (Merkle et al. 1977, Shaffer and Breden 1989, Routman 1993). Electrophoretic data (Merkle et al.1977, Shaffer and Braden 1989) showed that the Susquehanna River population of *C. a. alleganiensis* was significantly different from the Mississippi basin populations of the same subspecies and Missouri populations differ from those east of the Mississippi River. In general, *C. alleganiensis* west of the Mississippi River have much greater isozyme variation than those east of the Mississippi River. Williams et al. (1981) contend that the unusually large degree of genetic uniformity throughout the hellbender's broad range is consistent with the species being a habitat specialist. Nevo (1978) suggested that specialization appears to be linked with low genetic variability. Williams et al. (1981) conclude that the hellbender's adaptation to a relatively constant environment has led to a number of niche-narrowing structural, behavioral, and physiological specializations. The sheltering from ecological variation afforded by the combination of these specializations with the stable environment has putatively resulted in failure of the species to maintain genetic variability. This level of specialization makes the hellbender very sensitive to habitat alteration.

The isozyme data (Merkle et al.1977, Shaffer and Braden 1989) did not show differences between Spring River and North Fork White River populations. Although data was minimal, no phenotypic differences were documented between the 2 populations. This hints that the mixing of these 2 populations may not have significant phenotypic or ecological impact, although again, minimal information was available on the overall phenotype and the information is difficult to measure in unreplicated natural populations.

The mtDNA data (Routman 1993) and the RAPD analysis indicated that there were fixed differences among the North Fork White, Spring, Eleven Point, and Current rivers, indicating that on the nucleotide level these populations were distinctive and reproductively isolated despite in some cases their close proximity to one another. Based on Routman (1993) and our data, Spring River, the population most in peril, was the least variable. Routman (1993) analysis clearly indicated that the maternal lineage of North Fork White, (2 sub samples), Spring, and Current rivers were different. Our RAPD analysis further showed paternal and maternal lineage of Eleven Point was different from Spring but that paternal lineage of Spring and North Fork White rivers could be similar. It should be understood that all these differences

were measured at the nucleotide level, and there is no evidence that they translate into any phenotypic variability, although that possibility or linkage to such exists.

Based on the data, one option would be to take the conservative genetic approach and protect these distinctive genetic units. In this case, restoration effects in the Spring River would need to be accomplished by captive breeding and stocking of hellbenders only from the Spring River. An additional assumption for this option would be that the individual populations are genetically most adapted and most fit for their individual environment because of the long history of reproductive isolation and natural selection for this specific habitat.

Option two would be to mix North Fork White River and Spring River populations. The rationale would be that the isozyme data was more meaningful than the DNA data, and inter-population enzyme differences were not observed.

The third option would be to intentionally cross any or all of the other 3 populations with the Spring River population. If indeed the decrease in population number and the decrease in the reproduction in the Spring River population are real, it could be partially explained by inbreeding depression. Inbreeding reduces growth rate, survival, vigor and reproduction and increases deformities, biochemical disorders and bilateral asymmetry. In this regard, the isozyme, mtDNA analysis and RAPD analysis all indicate extreme levels of homozygosity, and the mtDNA analysis and RAPD analysis indicates high population differentiation and development of lines indicative of inbreeding and random genetic drift. In this scenario, the intentional crossing could invigorate the Spring River population, counteract effects of inbreeding and random genetic drift, enhance reproductive ability and fitness, and infuse genetic variability. If the larger size of Spring River hellbenders is due to genetic rather than environmental effects such as increased food availability or decreased population numbers, then the mixing of the populations could reduce growth rate potential. However, the large size and increased growth rate could be undesirable if it promotes predation or is correlated with late or reduced sexual maturation as can be observed in fish (Dunham 1996). Additional justification would be that habitat alteration and degradation may have created an environment for which the genotype that the natural selection shaped is no longer highly adapted for the altered environment. Infusion of new genetic variation may be important for adaptation to the altered environments for which the hellbenders may be overspecialized and ill adapted.

All existing information, therefore, points toward protection and perhaps artificial stocking of hellbenders for increasing its population size. Further population genetic analysis should be of importance in directing such restoration efforts.

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