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RESEARCH ARTICLE

Microsatellite variation, population structure, and bottlenecks in the threatened copperbelly water snake

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Abstract Habitat loss and isolation is pervasive in the Midwest U.S. Wetlands are experiencing particularly dramatic declines, yet there is a paucity of information on the genetic impacts of these losses to obligate wetland vertebrates. We quantified the genetic variation of extant populations of a shallow wetland specialist and evaluated potential reductions in population size (i.e. bottlenecks) using seven polymorphic microsatellite DNA markers. We analyzed 228 copperbelly water snakes (*Nerodia erythrogaster neglecta*), representing populations from three states. Moderate genetic differentiation exists among all three regions ($F_{ST} = 0.12$, $P < 0.001$), with evidence for low levels of differentiation within the federally protected Ohio region ($F_{ST} = 0.025$, $P = 0.007$), and moderate to strong differentiation within the Indiana region ($F_{ST} = 0.23$, $P < 0.001$). Furthermore, Bayesian clustering (i.e. STRUCTURE) supports the separation of the Indiana sites, both from each other and from all other sampling sites. However, it does not support the separation of the Ohio sites from the Kentucky sites. Differentiation among sampling sites did not appear to be related to geographic distance, but rather depended on the quality of terrestrial corridors used for dispersal. Mode shifts in allele frequencies and excess heterozygosity tests were negative, while *M*-ratio tests were nearly all positive, indicating the likelihood of historical rather than contemporary population bottlenecks. However, potential subspecific intergradation in the Kentucky region may have artificially lowered the *M*-ratio, and we suggest caution when using the *M*-ratio approach if intergradation is suspected. Our results have conservation implications for wetland management and management of the copperbelly populations, and emphasizes the importance of protecting wetland complexes.

Keywords Population structure · *Nerodia erythrogaster neglecta* · Microsatellite · Bottleneck · *M*-ratio

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Introduction

Habitat fragmentation (i.e. loss and isolation) negatively impacts ecosystem functioning (Naeem et al. 1994) and is a dominant force in the reduction of biodiversity across virtually all taxa (Fischer and Lindenmayer 2007). Despite pervasive habitat fragmentation in many landscapes worldwide, we lack the most basic understanding of its impact on genetic diversity for most taxa (Debinski and Holt 2000). Wetland habitats have experienced particularly dramatic declines in the last 200 years in the U.S. In the Midwestern U.S. alone, wetland acreage has been reduced by at least 85% (Dahl and Allord 1994, Unpublished), resulting in pervasive habitat fragmentation. This places obligate wetland species at a greater genetic risk, since fragmentation of populations increases the chance of extinction by reducing genetic diversity, increasing inbreeding, and increasing susceptibility to stochastic demographic processes (Lacy 1987; Frankham 1995).

Furthermore, it is becoming increasingly apparent that terrestrial travel corridors are critical for many obligate

wetland vertebrates, especially reptiles (e.g. Roe and Georges 2007; Lesbarreres et al. 2006; Ficetola and De Bernardi 2004). The value of such travel corridors are often underestimated or ignored in wetland management practices (Semlitsch and Bodie 2003; Roe and Georges 2007), and thus potential corridors are often developed or converted to agricultural land. It is still unclear how corridor characteristics affect corridor quality, and thus dispersal and population connectivity in obligate wetland vertebrates.

The copperbelly water snake, *Nerodia erythrogaster neglecta*, a subspecies of the plainbelly water snake (Conant and Collins 1991), primarily inhabits shallow wetland systems consisting of sloughs, oxbows, river floodplains, and buttonbush (*Cephalanthus occidentalis*) swamps (Coppola 1999; Kingsbury and Laurent 2000; Hyslop 2001), much of which have been lost or heavily fragmented (Pruitt and Szymanski 1997). In addition, the copperbelly is known to rely extensively on terrestrial habitat to traverse between spatially and temporally unpredictable wetland resources (Roe et al. 2003), offering an ideal system to investigate the role of terrestrial habitat on wetland connectivity. Presently, the copperbelly exists mainly as isolated, often small, populations separated by as much as 300 km. Moreover, northern populations were listed as threatened by the United States Fish and Wildlife Service (USFWS), and endangered by the states of Indiana, Michigan, and Ohio (Pruitt and Szymanski 1997). Populations in southern Illinois, southern Indiana, and northern Kentucky are currently afforded only limited state protection.

Even though immediate conservation needs exist in many obligate wetland reptiles (Buhlmann and Gibbons 1997; Gibbons et al. 2000), few studies of obligate wetland snakes incorporate genetic analyses (e.g. Lawson et al. 1991; Prosser et al. 1999), and none specifically in the context of a fragmented landscape.

In an effort to address the lack of information available regarding the population structure and connectivity of the copperbelly, we quantified the genetic variation among extant populations using microsatellite DNA, which have been shown to be superior to other genetic markers in detecting fine-scale population subdivision in snakes (Lougheed et al. 2000). In addition, we also evaluated the magnitude of potential recent and historical reductions in population size using bottleneck detection techniques. Bottlenecks and low genetic diversity have been implicated in increased birth deformities, chromosomal abnormalities, and reduced juvenile survival in snakes (e.g. Madsen et al. 1995; Gautschi et al. 2002; Ujvari et al. 2002). Thus, it is important to identify populations most susceptible to inbreeding depression. Additionally, this information will not only be valuable for the management of the copperbelly, but will also increase our understanding of how an obligate wetland species responds to habitat fragmentation.

Methods

Sampling localities and tissue collection

Most tissue samples were collected by walking wetland edges and hand capturing individuals, although some were collected from fresh roadkills. Tissue was obtained from small clippings of ventral scales, a non-invasive procedure that minimizes discomfort to the animal. Scale clippings were preserved in 95% EtOH and then stored at 4°C.

Three regional sampling areas were chosen for tissue collection to represent known disjunct populations (Fig. 1; Table 1). Three locations near the La Su Ann Wildlife Management Area located in northwest Ohio and southern Michigan were sampled; LSA, Pioneer, and Clear Fork (CF). These sites are within three km of each other and consist of meandering streams flanked by numerous oxbows, forested, shrub wetlands and open aquatic habitats of variable sizes and hydroperiods (Roe et al. 2003). These sites also represent the largest federally protected populations of the copperbelly. The second regional sampling site was conducted in a disjunct population along the Muscatatuck River, in the Muscatatuck National Wildlife Refuge

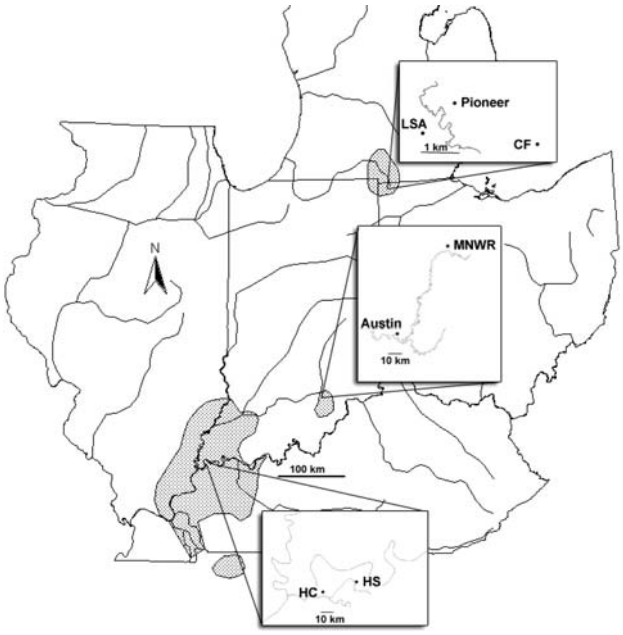

Fig. 1 Estimated distribution of extant populations of *Nerodia erythrogaster neglecta* given by shaded areas. Inserts refer to specific locations for tissue sampling (see also Table 1). Note differences in spatial scales indicated by scale bars

Table 1 Sample sizes and summary statistics of genetic diversity (SE) for the seven sampling locations

Region	Collection site	Sample size	H_E	H_O	A	Private Alleles	F_{IS}
Ohio/Michigan	La Su Ann WMA (LSA)	22	0.70 (0.03)	0.64 (0.04)	5.13 (0.53)	1	0.09
	Pioneer	11	0.70 (0.04)	0.69 (0.07)	5.71 (0.83)	1	0.02
	Clear Fork (CF)	22	0.69 (0.04)	0.70 (0.04)	5.47 (0.60)	1	−0.01
Indiana	Muscatatuck NWR (MNWR)	64	0.61 (0.02)	0.53 (0.03)	4.64 (0.20)	7	0.13
	Austin	21	0.57 (0.04)	0.53 (0.06)	3.99 (0.45)	1	0.08
Kentucky	Hardy Slough (HS)	58	0.76 (0.02)	0.64 (0.02)	7.15 (0.38)	21	**0.16**
	Highland Creek (HC)	30	0.74 (0.02)	0.68 (0.03)	6.42 (0.39)	6	0.09

H_E, mean expected heterozygosity; H_O, mean observed heterozygosity; A, mean allelic richness adjusted for sample size; F_{IS}, mean level of inbreeding observed. Bootstrapping was used to create 95% confidence intervals (CI) for F_{IS} estimates, values where CI did not include 0.0 are indicated in bold

(MNWR) in Jackson County, Indiana, and at a wetland 29 river km south of MNWR in Washington, County, outside of Austin, Indiana (Austin). The third sampling region was in the southern portion of the copperbelly range and included two locations, 16 river km apart, within the Sloughs State Fish and Wildlife Area in Henderson County, Kentucky; Hardy Slough (HS) and Highland Creek Management Unit (HC). These sites were chosen because they occur in the most contiguous portion of the copperbelly range, and previous censuses have indicated large population sizes (e.g. Laurent 2000).

Total genomic DNA was extracted from scale clippings using either DNeasy Tissue Kits (Qiagen) or ammonium acetate purification (Puregene^{TM} kit, Gentra Systems). Eight microsatellite primer sets developed for the northern water snake, *Nerodia sipedon sipedon* (Prosser et al. 1999), and ten primer sets developed for garter snakes, *Thamnophis* spp. (McCracken et al. 1999; Garner et al. 2004), were screened for consistent amplification and polymorphism in our sample.

PCR was performed in 15 µl reactions containing 1X reaction buffer, 200 µM dNTP, 2 mM $MgCl_2$, 0.8 mg BSA, 167 nM each forward and reverse primer, 0.6 U *Taq* DNA polymerase (Promega), and 50 ng of DNA template. Cycling was as follows: 94°C, 4 min; (94°C, 30 s; annealing temperature, 30 s; 65°C, 1 min) × 32; 72°C, 5 min; 4°C until end. Appropriate template DNA from either *Nerodia sipedon* or *Thamnophis sauritus* were used as positive controls. Negative controls did not contain template DNA. PCR products were diluted twenty times and subsequently sorted by size using 5% polyacylamide gels on a ABI 377 with GENESCAN software (PE Applied Biosystems). Allele sizes calculated and analyzed using GENOTYPER software (PE Applied Biosystems).

Genotyping errors were minimized in several ways. About ten percent of the samples were haphazardly chosen for reanalysis for quality assurance; genotyping errors were summarized for each locus and across all loci (DeWoody et al. 2006). Additionally, the possibility of scoring errors resulting from the production of 'stutter' bands (i.e. *Taq* polymerase slippage), preferential amplification of smaller alleles (i.e. large-allele dropout), and the presence of null alleles (i.e. non-amplifying alleles) were evaluated using Micro-Checker (Oosterhout et al. 2004).

Data analysis

Adherence of individual loci to Hardy-Weinberg equilibrium and levels of nonrandom association of alleles for each pair of loci were tested using exact tests with 3,200 iterations as implemented in GDA (Lewis and Zaykin 2001). Fixation indices (F_{IS}) were also calculated using GDA, with significance of deviations from zero assessed through bootstrapping 1,000 replicates across loci to generate 95% confidence intervals.

Genetic diversity

Genetic diversity was evaluated by calculating allelic richness (A), corrected for sample size (FSTAT 2.9.3.2, Goudet 1995) and as observed (H_O) and expected (H_E) heterozygosity (GDA, Lewis and Zaykin 2001). Differences in expected heterozygosities between sampling locations were assessed using a Friedman test, which uses loci as the blocking factors, accounting for interlocus variation (Zar 1996). To test the significance of differences in allelic richness between the three sampling regions, we used a nonparametric test implemented in FSTAT with 10,000 permutations. Friedman statistical comparison and the multidimensional scaling analysis were performed using SPSS 15.0 for Windows (SPSS Inc.).

Population structure

The presence of population subdivision was evaluated using multiple analytical techniques. Initially a Bayesian clustering approach was implemented (i.e. STRUCTURE,

Pritchard et al. 2000). This approach simultaneously estimates population allele frequencies and assigns individuals to populations probabilistically. The model accounts for the presence of Hardy-Weinberg or linkage disequilibrium by introducing population structure and attempting to find population groupings that are in Hardy-Weinberg equilibrium (Pritchard et al. 2000). The program was run with a burn-in time of 10,000 repetitions and 500,000 iterations (MCMC) using K (predicted number of populations) from 2–10. Estimated log probabilities of data $\Pr(X \mid K)$ for each value of K were evaluated by calculating ΔK, the rate of change in the log probability of data between successive K values (Evanno et al. 2005). The rate of change in the log probabilities has been shown to be a more reliable predictor of the true number of populations, relative to selection of K with the highest posterior probability (Evanno et al. 2005).

Population subdivision was also evaluated by testing for heterogeneity in allele frequencies using the exact probability test implemented in GENEPOP (Raymond and Rousset 1995), for each pair of sampling sites. In addition an F_{ST} analysis was conducted using θ, an F_{ST}-estimator (Weir and Cockerham 1984), hereafter referred to as F_{ST}. F-statistics were calculated using SPAGeDi version 1.2e (Hardy and Vekemans 2002). F_{ST} values were tested for significance using 10,000 permutations. An analysis of molecular variance (AMOVA) was used to determine the hierarchical distribution of genetic variance within populations, among populations within a region, and among regions using GENALEX (Peakall and Smouse 2006). Visualization of the pattern of genetic differentiation was facilitated by a multidimensional scaling plot based on Nei's unbiased genetic distance (Nei 1978). All input file preparations were made using CONVERT version 1.31 (Glaubitz 2004).

Detection of bottlenecks

We used three approaches to determine which populations may have undergone significant reductions in size. We used BOTTLENECK v1.2.02 (Piry et al. 1999), to test for allele frequency mode-shifts (i.e. distortion away from the typical L-shape distribution). Secondly, we tested for the presence of an excess of observed heterozygotes by using the Wilcoxon signed rank test to evaluate deviations from 50:50 deficiency/excess (Cornuet and Luikart 1996, Luikart and Cornuet 1998). Heterozygote excess was tested under all three mutation models, infinite alleles (IAM), two-phase (TPM), and the step-wise mutation model (SMM). For TPM we set $ps = 0.9$ (the frequency of single step mutations) and the variance of those mutations as 12. These are generic values typical for many microsatellite markers (e.g. Busch et al. 2007).

In addition, we also calculated the M-ratio, the mean ratio of the number of alleles (k) to the range in allele size (r) using the software M_P_VAL (Garza and Williamson 2001). This method takes advantage of the size specificity of microsatellite allelic states. That is, during a bottleneck rare alleles are lost quickly thus reducing the number of observed allelic states (k) faster than the size range of those alleles (r), resulting in a reduced M-ratio ($M = k/r$). To determine the significance of a ratio we compared our observed value to a distribution of values obtained from a simulated population given our sample size and mutation model (e.g. TPM). Critical values (M_c) set at the lower 5% tail of the distribution were determined using the program CRITICAL_M, below which it can be assumed that an observed ratio is from a population that has experienced a significant reduction in size (Garza and Williamson 2001). To calculate M_c we estimated three TPM parameters: p_s, Δ_g (the size of non one-step changes) and $\theta = 4N_e\mu$. Critical values can be sensitive to these parameters (e.g. Busch et al. 2007). However, we did not have species-specific information on p_g, Δ_g and θ. Instead we varied Δ_g and θ, which are more influential than p_s (Garza and Williamson 2001). For Δ_g we used the values 2.8 (mean value determined from a literature review by Garza and Williamson (2001), 3.5 (default value), and 5.0. We varied θ from 0.01 to 50, encompassing a wide range of biologically plausible values. To ensure this range of θ values was relevant, we estimated θ using a common microsatellite mutation rate (μ) recommended by Garza and Williamson (2001): 5.0×10^{-4} mutants/generation/locus (Weber and Wong 1993). To estimate plausible values for pre-bottleneck N_e we used two long-term estimators (e.g. Busch et al. 2007). The two models are rearrangements of Eq. 3.15 from Hartl and Clark (1989) and Eq. 7 from Ohta and Kimura (1973), respectively:

$$N_e = \frac{H_E}{4\mu(1 - H_E)} \quad (1)$$

and

$$N_e = \frac{\left(\frac{1}{(1-H_E)}\right)^2 - 1}{8\mu} \quad (2)$$

The first equation assumes IAM and the second SMM, which represent the two extremes of the mutation process.

Given the number of hypothesis tests performed, Type I error rate (the probability of falsely rejecting the null hypothesis) is a concern. To reduce experiment-wise (EW) Type I error, we reduced critical values (α) using a correction procedure that balances risks of Type I and Type II errors called the False Discovery Rate (FDR) (Narum 2006). FDR entails controlling the expected proportion of falsely rejected hypotheses rather than controlling all

falsely rejected hypotheses (Narum 2006). We chose the B-Y method (Benjamini and Yekutieli 2001), which controls for the proportion of false discoveries and EW Type I error (Narum 2006).

Results

Genetic data analysis

Seven microsatellite loci were used to genotype 228 individuals representing seven sampling sites in three regional locations (Table 1). Six loci were from *Nerodia sipedon* (Prosser et al. 1999), while only one locus screened from *Thamnophis* (Garner et al. 2004) was informative (Table 2). Scoring errors were minimal (Table 2), and there was no evidence for stuttering errors or large allele dropout. Results from Micro-Checker did suggest the presence of null alleles for locus NSu6. However, removal of this locus from analyses did not qualitatively change the results, so it was retained.

Exact tests (3,200 iterations) for Hardy-Weinberg disequilibrium revealed that all sampling sites had at least one locus not in equilibrium. LSA, Pioneer, and HC each had a single locus not in equilibrium (Nsu 7, Nsu3, and Nsu9b, respectively). CF, Austin, and HS each had two (Nsu10, Nsu9b; Nsu3, Nsu6; Nsu6, Nsu10, respectively). MNWR had four loci not in equilibrium (Nsu2, Nsu6, Nsu9b, and Ts1Ca4). Alpha significance was corrected for 49 tests and set at 0.011. Tests for nonrandom association of alleles indicated that LSA had four significant pairwise comparisons, Pioneer and MNWR each had two, CF and HC each had one, with Austin and HS having none (Alpha significance was corrected for 147 tests and set at 0.009). Only HS had an F_{IS} significantly different from zero (Table 1).

Table 2 Summary of microsatellite loci used to genotype *N. erythrogaster neglecta*

Locus	Size range (bp)	Number of alleles	Scoring error rate
NSu2	152–174	8	1
NSu3	146–222	34	2
NSu6	100–166	23	0
NSu7	102–184	11	1
NSu9b	202–258	15	1
NSu10	122–144	12	1
Ts1Ca4	114–160	13	0

All NSu loci are from (Prosser et al. 1999), Ts1Ca4 taken from (Garner et al. 2004). Error rate refers to the number of mistypes per 25

Patterns of genetic diversity

Expected heterozygosities (H_E) ranged from 0.57 (Austin) to 0.76 (HS); however, there was no significant difference between sampling locations (Friedman Test, $P = 0.10$). Allelic richness ranged from 3.99 (Austin) to 7.15 (HS), and was significantly different between regions sampled; Kentucky had significantly more alleles than Indiana ($P = 0.004$), but not Ohio ($P = 0.20$), and Indiana and Ohio were not significantly different ($P = 0.28$).

Population subdivision

Evaluation of ΔK, the rate of change in the log probability of data between successive K values, indicated the most likely number of clusters is two or three. The two clusters represent a conglomerate of LSA, Pioneer, CC, HS, HC, and Austin, with the other Indiana site (MNWR) as the second cluster. However, there was also considerable support for three clusters. This clustering scenario separates the Ohio and Kentucky sampling sites (which includes Austin), with MNWR as the third.

There was an overall significant F_{ST} value (0.12, Fisher's method $P < 0.001$; $\alpha = 0.014$). Pair-wise comparisons indicated modest divergence of the Ohio sampling sites ($P = 0.010$). There was no divergence between LSA and Pioneer, but both diverged to some degree from CF. There was also no divergence between Kentucky sampling sites ($P = 0.045$), but strong divergence between Indiana sites and all other pairings (Table 3).

The AMOVA revealed that 79% of the molecular variance is explained by within population variation. The partitioning of genetic variation between the three regions ($P < 0.001$, 8%) and among populations within a region ($P < 0.001$, 13%) were also significant. AMOVA was also used to partition genetic variation within and among major watersheds. The Ohio sites are within the St. Joseph River watershed, which flows into Lake Michigan. The Indiana sites are within the White River watershed, which flows into the Wabash River, near where the Wabash River flows into the Ohio River (Fig. 1). Kentucky sites are near the confluence of those two rivers (Fig. 1). This AMOVA revealed that within population variation explains 77% of the total molecular variance, with among watersheds accounting for 6% ($P < 0.001$) and among populations within a watershed accounting for the remaining 17% ($P < 0.001$).

Multidimensional scaling analysis indicates the Kentucky and Ohio sampling sites are genetically more similar to each other than either is to the Indiana sites. Furthermore, the two Indiana sites are as different from each other as they are from any of the other sampling sites, despite their geographic proximity (Table 3, Fig. 2).

Table 3 Pairwise F_{ST} for all seven sampling locations

	Pioneer	CF	MNWR	Austin	HS	HC
LSA	**0.01 (0.07)**	**0.02 (0.02)**	0.17 (<0.001)	0.17 (<0.001)	0.07 (<0.001)	0.06 (<0.001)
Pioneer		0.04 (0.009)	0.17 (<0.001)	0.19 (<0.001)	0.08 (<0.001)	0.06 (<0.001)
CF			0.19 (<0.001)	0.16 (<0.001)	0.08 (<0.001)	0.08 (<0.001)
MNWR				0.23 (<0.001)	0.14 (<0.001)	0.13 (<0.001)
Austin					0.13 (<0.001)	0.14 (<0.001)
HS						**0.007 (0.045)**

P-values (in parentheses) were obtained through 10,000 permutations of individuals across sampling sites. Bold values indicate nonsignificance under the FDR correction ($\alpha = 0.014$)

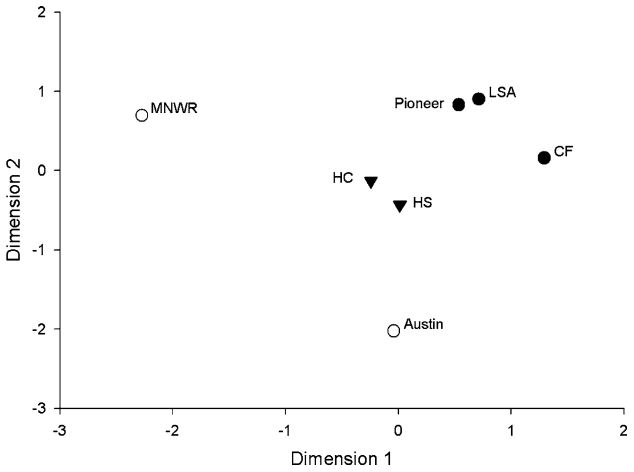

Fig. 2 Multidimensional scaling analysis based on Nei's unbiased genetic distance (Nei 1978). Dots in Ohio, circles in Indiana, and triangles in Kentucky (see also Fig. 1)

Bottleneck analyses

A key assumption of bottleneck analyses is the absence of population structure (Busch et al. 2007). To define populations we used a combination of results from STRUCTURE, F_{ST}, allele frequency distribution, and Nei's (1978) genetic distance analyses. STRUCTURE delineated significant population structure at the regional scale. In addition, F_{ST}, allele frequency distribution, and Nei's genetic distance further revealed population structure within regions (e.g. Table 3 and Fig. 2). In Ohio, there is no evidence of any significant differences between Pioneer and LSA. However, there is evidence that both of those sampling sites differ from nearby CF in allele frequency distributions and genetic distance (e.g. Fig. 2). The two Indiana sampling sites are quite different from each other under all analyses, but there are no differences between the Kentucky sampling sites. Thus, size reductions were evaluated within the following five populations: the Ohio sites joined into two populations, Pioneer and LSA combined (hereafter referred to as LSA), Clear Fork (CF), MNWR, Austin, and the Kentucky sites combined (hereafter referred to just as Kentucky).

Excess heterozygosity was detected in LSA, CF, MNWR only under the IAM mutation model (Table 4). The mode-shift test did not detect any evidence of a bottleneck (Table 4). In the case of chronic bottlenecks that last multiple generations, allelic variation may decrease to an average of just 2–3 alleles/locus (Garza and Williamson 2001). The mean number of alleles across all populations is 7.2, ranging from 4.6 in Austin to 10.6 in Kentucky, indicating that allelic variability remains high.

Estimates of N_e ranged from 563.8 to 1166.7 and from 882.5 to 2527.8 individuals (Eqs. 1 and 2, respectively). In addition, a mark-recapture analysis of the HS sampling site estimated the population size to be between 1,095 ± 492 (SE) and 2,079 ± 378 (SE) individuals for Jolly-Seber and Schnabel-Darroch equations, respectively (Laurent 2000). Thus, our biologically plausible pre-bottleneck N_e values were set to 5, 500, 1,000, 3,000, 6,000, and 25,000 (Table 4). The value five represents the most liberal test for significant reductions in population size by effectively reducing the pre-bottleneck θ, thereby increasing M_c values. However, we also used N_e values considerably larger than our estimates of the current N_e.

While using the default values of $p_s = 0.9$ and $\Delta_g = 3.5$, most *M*-ratio values, regardless of the assumed pre-bottleneck effective population size, were consistently below the critical value thresholds (Table 4). Only the two Ohio populations had scenarios with non-significant ratios. Changing μ ten fold either direction of the default value did not qualitatively alter any interpretations of M_c. Reducing Δ_g increases the bottleneck signature (Garza and Williamson 2001), thus increasing the possibility of a significant *M* ratio. We reduced Δ_g from 3.5 to 2.8 to determine if this would impact the simulation results for LSA and CF, the two populations with weak or no bottleneck signatures (Table 4). This Δ_g reduction in LSA reduced the percentage of simulated populations with lower ratios than observed from 7.18% to 0.99% for $\theta = 50$. All other simulations remained significant as

Table 4 Summary of the parameters and results for the *M*-ratio and BOTTLENECK analyses used to detect significant reductions in effective population sizes

Population	*M*-ratio					Bottleneck		
	N_e	θ[a]	*M*-ratio	M_c	Simulation results[b] (%)	Mode shift	Mutation model[c]	Heterozygote excess
LSA ($n = 33$)	5	0.01	0.61	0.83	0.00			
	500	1	0.61	0.76	0.12		IAM	$P = 0.012$*
	1,000	2	0.61	0.73	0.12	NS	TPM	NS
	3,000	6	0.61	0.71	0.57		SMM	NS
	6,000	12	0.61	0.66	0.94			
	25,000	50	0.61	0.60	7.18			
CF ($n = 22$)	5	0.01	0.70	0.83	0.20			
	500	1	0.70	0.75	1.46		IAM	$P = 0.008$*
	1,000	2	0.70	0.72	3.31	NS	TPM	NS
	3,000	6	0.70	0.66	13.71		SMM	NS
	6,000	12	0.70	0.63	27.45			
	25,000	50	0.70	0.54	90.91			
MNWR ($n = 64$)	5	0.01	0.55	0.83	0.00			
	500	1	0.55	0.76	0.01		IAM	$P = 0.004$*
	1,000	2	0.55	0.73	0.02	NS	TPM	NS
	3,000	6	0.55	0.70	0.02		SMM	NS
	6,000	12	0.55	0.70	0.00			
	25,000	50	0.55	0.67	0.00			
Austin ($n = 21$)	5	0.01	0.52	0.83	0.00			
	500	1	0.52	0.75	0.00		IAM	NS
	1,000	2	0.52	0.72	0.02	NS	TPM	NS
	3,000	6	0.52	0.66	0.02		SMM	NS
	6,000	12	0.52	0.63	0.14			
	25,000	50	0.52	0.54	2.63			
Kentucky ($n = 88$)	5	0.01	0.50	0.83	0.00			
	500	1	0.50	0.76	0.00		IAM	$P = 0.03$
	1,000	2	0.50	0.73	0.00	NS	TPM	NS
	3,000	6	0.50	0.71	0.00		SMM	NS
	6,000	12	0.50	0.71	0.00			
	25,000	50	0.50	0.69	0.01			

[a] $\theta = 4N_e\mu$

[b] The percent frequency smaller *M*-ratio values were obtained from 10,000 simulated populations

[c] TPM parameters set at $p_s = 0.9$, variance = 12

NS = Nonsignificance ($\alpha = 0.05$)

* Significance under FDR ($\alpha = 0.022$)

expected. Similar results were obtained for CF, where percentages decreased, but only with $\theta = 6$ was there a change in significance (i.e. 13.71% to 2.5%). Increasing Δ_g decreases the bottleneck signature (Garza and Williamson 2001). However, increasing Δ_g from 3.5 to 5.0 did not qualitatively alter interpretations of any ratio significance, thus only $\Delta_g = 3.5$ is reported (Table 4). Altering θ did have an impact of the strength of the bottleneck signal. LSA was non-significant under a θ value of 50, and CF with θ values of six or greater (Table 4).

Discussion

Population differentiation and genetic diversity

We detected fine-scale population structure in the copperbelly, which is not uncommon in snakes in human-dominated landscapes (e.g. Prior et al. 1997; Gibbs et al. 1997; Prosser et al. 1999). Furthermore, patterns of gene flow in snakes may often depend on the barriers found between populations (Prior et al. 1997; Bushar et al. 1998;

Manier and Arnold 2006), and not just on the geographic distance that separates them. For example, Prior et al. (1997) found that gene flow between black rat snake (*Elaphe obsoleta*) hibernacula was inhibited by urban development. Bushar et al. (1998) found that the distribution of suitable basking sites influenced patterns of gene flow in timber rattlesnakes (*Crotalus horridus*).

Our results indicate the presence of population structure in the copperbelly at a regional scale (Table 3, Fig. 2), with similar levels of heterozygosity, but differences in allelic richness among populations. Population structure, however, appears to not be simply due to differences in geographic distances between sampling sites. There is some suggestion of population structure among the three federally protected Ohio sampling sites, despite being at most 2 km apart. In contrast, the two Kentucky sampling sites were nearly identical genetically, even though they were 13 km apart. In addition, there is strong divergence between the two Indiana sampling sites, despite being along the same river, although 25 km apart.

The copperbelly is a highly terrestrial obligate wetland species (e.g. Roe et al. 2003) and may depend greatly on quality terrestrial habitat for dispersal. For instance, in Ohio, the terrestrial habitat between the Pioneer and LSA sampling sites is largely devoid of human perturbation, except for a gravel road, and consists of a meandering stream, flanked by wetlands of varying hydroperiods, upland forest and fallow fields. The quality of terrestrial habitat between those two sites and that of CF is considerably reduced, much of which is in annual agricultural rotation, which may be sufficient to deter dispersal despite close proximity.

The two Kentucky sampling sites are considerably further apart than any of the Ohio sampling sites, yet there is no evidence of any genetic divergence between them. These two sites are embedded in a 4,000 ha state-owned wildlife management area that is largely dedicated to providing habitat for migratory waterfowl and shorebirds. Extensive surveys between the two sites within the numerous cypress sloughs, oxbows, and shallow ephemeral wetlands suggest that copperbelly water snakes inhabit much of the intervening habitat (BA Kingsbury and M. Morton, personal communications). Consequently, these sites appear to represent a single large interbreeding population.

The strong divergence between the two Indiana sampling sites could result from several factors. They are the most distant of any two sampling sites within a region in our study; the Muscatatuck River that lies between them completely lacks a riparian buffer zone, and there is a near complete absence of wetland habitat. Furthermore, the riverbank is steep and thus maintains deep water levels, habitat that is marginal for copperbelly water snakes in other parts of their range (Laurent and Kingsbury 2003). Additionally, there is evidence that both populations have undergone significant size reductions sometime in the past (see below). Both a lack of dispersal and potentially small population sizes would facilitate genetic drift and subsequent genetic divergence.

An additional factor that could influence the magnitude of genetic divergence between sampling sites is post-Pleistocene range expansion (King and Lawson 2001). Two of our sampling regions (i.e. Ohio and Indiana) experienced glaciation. Populations found in glaciated regions may have reduced periods of isolation, thus may show lower levels of divergence than populations found in unglaciated regions (sensu King and Lawson 2001). *Nerodia* sampled in an unglaciated region at about the same spatial scale (10s–100s km) revealed levels of divergence (F_{ST}) as high as 0.29 (Lawson et al. 1991), whereas our greatest observed level of divergence was 0.23. Interestingly, the divergence between our most distant sampling sites (i.e. Ohio and Kentucky, ca. 500 km) was only between 0.06 and 0.08. Furthermore, the divergence of the Indiana sampling sites from the other two regions could be the result of a founding event or events. The Indiana sites are in the eastern reaches of the East Fork of the White River drainage, within the alluvial and outwash deposits found in the Illinoian glaciated region. However, for about 100 km downstream the river passes through the unglaciated region of Indiana, which does not support extensive wetlands (Phillip Owens, personal communication) and thus could be a barrier to dispersal.

Detection of significant reductions in population size

All sampled populations maintain rare alleles in frequencies expected for stable populations in mutation-drift equilibrium (Table 4). Likewise, there was modest support for heterozygosity excess in three populations (LSA, CF, and MNWR), but only under IAM (Table 4). Under IAM, microsatellite loci have been shown to exhibit heterozygosity excess in stable populations (Luikart and Cornuet 1998). In contrast, the *M*-ratio approach detected persistent bottleneck signatures in three populations, MNWR, Austin, and Kentucky, under all pre-bottleneck values of θ (Table 4). Both Ohio populations also show signatures of bottlenecks, but only under the most liberal of conditions (Table 4), suggesting a weak, if any, signal.

Despite these findings, we cautiously interpret our *M*-ratio results. First, we have no specific information on the mutation rate (μ) of our microsatellite suite, or of the average size of non-single step mutations (Δ_g), both of which can affect *M*-ratios (e.g. Garza and Williamson 2001; Busch et al. 2007). However, when we varied mutation rate (via changing θ) and Δ_g they largely did not alter our interpretations of our observed results. Second,

the assumption of closed populations might not be met. If a bottlenecked population receives alleles through migration from neighboring demes, and not through mutation events, then allelic states not otherwise occupied could be filled, raising the *M*-ratio. Essentially, neighboring demes would "rescue" the bottlenecked population and obscure any genetic signature of that population's decline (Busch et al. 2007). In the Ohio region there are at least two other areas that harbor small populations of the copperbelly water snake. It is possible that migrants from those other areas obscured genetic signatures of bottlenecks, although unlikely given their extremely small population sizes (Y. Lee, personal communication). Finally, intergradation between subspecies could also have significant impacts on *M*-ratios in an opposing direction to that of interdemic migration. Much of southern portion of the copperbelly water snake's distribution overlaps that of the northern extent of the yellowbelly water snake (*Nerodia erythrogaster flavigaster*), with numerous intergrades reported (Brandon and Blanford 1995). In fact, individuals were captured at both Kentucky sampling sites that share phenotypic attributes of both subspecies (e.g. yellow versus red venter, or some combination thereof). At all other sampling sites all individuals had consistent deep red to scarlet venters with mild to strong dorsal coloration encroachment on the venter, identical to that of their original description (Conant 1949). If populations of the yellowbelly contain allelic states out of the normal size range for the copperbelly water snake then intergradation could produce "artificial" gaps, thereby lowering the *M*-ratio, generating a bottleneck signature despite population size stability.

To evaluate the possibility that intergradation of the copperbelly and the yellowbelly lowered the *M*-ratio in Kentucky, we genotyped 21 yellowbelly water snakes from a location outside of the range of the copperbelly in northern Alabama (Perry Co., unpublished data). We used the same microsatellite loci with the above described protocols. We compared allelic size distributions of the Ohio and Indiana copperbelly populations to the Alabama yellowbelly population. The Alabama population contains 15 private alleles, five of which are out of the size distribution of any copperbelly population, however, all five are found in the Kentucky copperbelly population. This result suggests that intergradation may have lowered the *M*-ratio in the Kentucky copperbelly population. We cannot rule out the possibility of a bottleneck occurring, however, because intergradation does not account for all the unoccupied allelic states found within the normal allelic size distribution of the copperbelly. Nonetheless, we suggest caution when using the *M*-ratio approach to detect bottlenecks when intergradation is suspected.

Despite the influence of intergradation in Kentucky, the other bottleneck signatures (i.e. LSA, MNWR, and Austin) are likely genuine. The lack of mode shifts in allele frequencies or strong evidence for excess heterozygosity, yet significant *M*-ratios, suggests a more historical basis for those bottlenecks (Spear et al. 2006). Mode shifts and heterozygosity excess are transitory phenomena and will be erased in approximately 0.2–4N_e generations (Luikart and Cornuet 1998), whereas low *M*-ratios can persist for hundreds if not thousands of generations (Garza and Williamson 2001).

Conservation implications

A central goal for management of a species of conservation interest is the identification of demographically independent populations (i.e. management units (MUs)) (Palsboll et al. 2007). Although intuitively appealing, determining what level of genetic divergence corresponds to demographic independence remains challenging (Palsboll et al. 2007; Waples and Gaggiotti 2006). There is no contemporary gene flow between our three regions, given the extent of their geographic isolation from each other. Thus, our results argue for independent management of these three regions. Specifically, we advocate the continued federal and state protection of the Ohio region, but with the explicit understanding that there is fine-scale population structure likely resulting from fragmentation of terrestrial dispersal corridors. Additionally, the two sampling sites in Indiana are quite distinct, even though those two populations are near the same river. The almost complete lack of a riparian buffer zone and wetland habitat appears to significantly deter dispersal between the two. However, there are some recent efforts to purchase land between these populations and create shallow floodplain wetlands (Susan Knowles, personal communication). These efforts may increase gene flow and thereby homogenize these two sites. Nonetheless, we suggest protection of both locations. The Kentucky samples were taken from what is likely the largest remaining population of the copperbelly water snake, reflected in their higher levels of genetic diversity (Table 1). Higher levels of genetic diversity may reflect the influence of intergradation with the yellowbelly water snake. Although beyond the scope of this study, it would be interesting for future research to delineate the full extent of the intergradation and its impact on the population genetics of both the copperbelly and yellowbelly water snakes. Regardless, Kentucky remains a significant reservoir of genetic diversity, and should be afforded protection accordingly.

The seven sampling sites chosen in this study represent many of the remaining copperbelly populations, and it is encouraging to note that they all support relatively high levels of neutral genetic variation, despite some populations experiencing significant reduction in size (Table 1). Neutral genetic variation may not reflect functional genetic variation (e.g. DeWoody and DeWoody 2005; Madsen

et al. 2000); however, positive correlations with fitness related traits have been seen (e.g. Da Silva et al. 2006). Nonetheless, these populations appear to be large and robust. It is no coincidence that these areas also contain some of the largest remaining collections of shallow wetlands left in their range. It may be that, for the most part, copperbelly water snakes only inhabit areas consisting of multiple wetlands of varying sizes and hydroperiods. Extensive surveys in both the northern portion of their range (BA Kingsbury, personal observation) and southern portion (e.g. Laurent 2000) indicate they seldom occupy isolated wetlands, even if they appear to contain ideal habitat.

In conclusion, single-sample methods of bottleneck detection are powerful tools for inferring the demographic history of populations. However, these techniques have their limitations (e.g. Busch et al. 2007), and their results should be interpreted cautiously when the mutation process is uncertain and when populations can be considered open. Finally, conservation management in the copperbelly should focus on the protection of local populations and on dispersal corridors between them. Specifically, management plans need to explicitly consider the importance of riparian buffer zones and quality terrestrial habitat in maintaining natural heterogeneity of wetland complexes and in viable dispersal corridors between them (Roe and Georges 2007).

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