Conservation Genetics (2021) 22:1005–1021
https://doi.org/10.1007/s10592-021-01391-w

RESEARCH ARTICLE

Post-delisting genetic monitoring reveals population subdivision along river and reservoir localities of the endemic Concho water snake (*Nerodia harteri paucimaculata*)

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Received: 18 November 2020 / Accepted: 6 August 2021 / Published online: 10 August 2021

Abstract

The Concho water snake (*Nerodia harteri paucimaculata*), endemic to the Colorado and Concho Rivers in Texas, was removed from the U.S. Endangered Species Act in 2011. Monitoring of a species removed from threatened/endangered status is required to assess long-term management goals. As such, we conducted extensive surveys along with population genetic structure assessment at the time of delisting. No Concho water snakes were captured along the Concho River, part of the previously known range. Compared to older studies, fewer snakes were captured on the Colorado River. Several individual-based population genetic analyses showed concordant patterns of population substructure along the Colorado River. Specifically, there was significant genetic differentiation, with a signature of isolation by distance, among sampled riffle habitats and the O. H. Ivie Reservoir population. At the level of sampling locations, effective size estimates were low (10 to 20) and bottlenecks were detected. Habitat modification and low water flows during periods of drought may have contributed to existing patterns. Overall, genetic-based results demonstrate that the Concho water snake exists as small subpopulations along the Colorado River. The latter result coupled with the failed detection along the Concho River raises concern for this delisted species. Because much of our sampling was conducted during a drought, we recommend additional monitoring for which our genetic and demographic results will provide a much-needed baseline.

Keywords Concho water snake · Post-delisting monitoring · Population genetics · Conservation

Introduction

The Concho water snake (*Nerodia harteri paucimaculata*) was listed as threatened under the Endangered Species Act (hereafter ESA) in 1986 and removed in 2011. This natricine snake has one of the most restricted ranges of any snake in the U.S.A., occupying a maximum of 450 km of river habitat along the Colorado and Concho Rivers including 64 km of shoreline within two reservoirs in central Texas (Fig. 1) (Scott et al. 1989; Rose 1989). Despite its restricted range, Tinkle and Conant (1961) noted that Concho water snake populations occurred at high densities within suitable habitat (e.g., 114 individuals collected from a single location on the upper Colorado River in Coke County Texas in only 3 days). A subsequent study noted similarly abundant populations with 236 individuals captured in E.V. Spence Reservoir in 1990–1992 (Whiting et al. 1998).

Riffles and rocky shores characterize the microhabitat of *N. h. paucimaculata* where both adults and juveniles prey on small minnows (Cyprinidae) (Greene et al. 1994; Scott et al. 1989). Adults have also been observed in deeper pools (Whiting et al. 1997). In reservoirs, Concho water snakes occupy rocky shoreline with shallow slopes and low wave action, but also, have been found in rocky cliffs that end abruptly at the water (Whiting et al. 1997). Concho water snakes are rarely encountered more than a meter from the water because of their habitat affinity and potentially high rates of evaporative water loss due to their small size, 260

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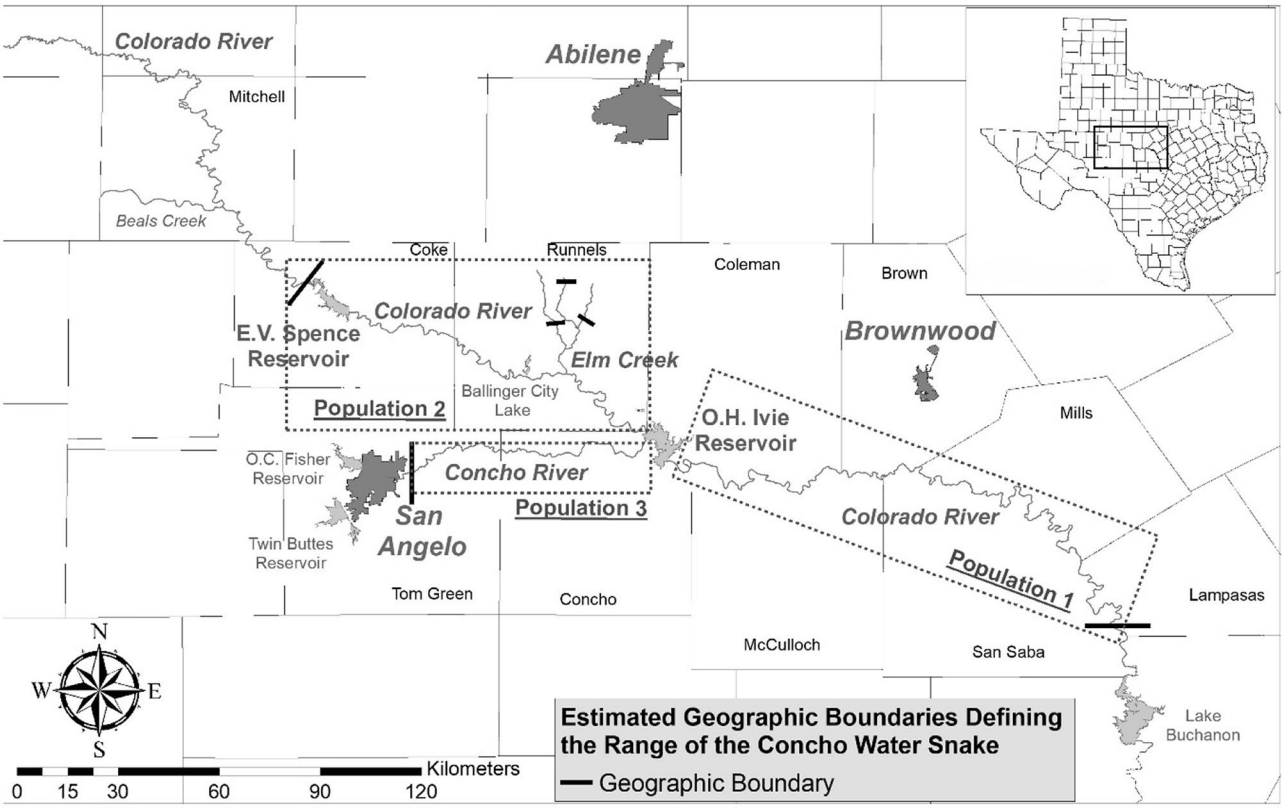

Fig. 1 Estimated range of the Concho water snake. The Concho water snake occurs only along the Colorado and Concho River and in Elm Creek, a tributary of the Colorado River. Reservoir populations have been documented in E.V. Spence and O.H. Ivie Reservoirs and in Ballenger City Lake. Grey boxes denote the three potential populations of concern separated by the OHIR that were delineated in the original post-delisting monitoring call (USFWS). Geographic boundary lines (thick black lines) demark the most upstream and downstream historical distribution of the Concho water snake at the time of de-listing. Texas State county names are listed within their respective demarcated county boundaries

to 477 mm in total length (Scott et al. 1989; Winne et al. 2001). They are thought to have a low dispersal capacity, moving longer distances (4 km) only in response to habitat loss (Whiting et al. 1997). Several authors have suggested that the persistence of Concho water snakes in novel lake and reservoir habitat is an indication that it may not be as specialized in its habitat as was once believed (Greene et al. 1994; Scott et al. 1989; Whiting et al. 1997; Whiting et al. 2008). In general, their restricted range, habitat specialization, high site fidelity and low dispersal capability suggested that Concho water snake populations would be vulnerable to habitat loss and degradation.

The Colorado and Concho Rivers are the primary source of water for several municipalities in semi-arid west Texas and provide an important irrigation source for crops and livestock. As such, several reservoirs have been built along these rivers. The E.V. Spence Reservoir (hereafter EVSR) was completed in 1969 on the upper Colorado River within the northern most extremes of the Concho water snake's range. Along the Concho River, O.C. Fisher Reservoir and Twin Buttes Reservoir constrict water flow from upstream confluences of the primary Concho River near San Angelo, Texas (Fig. 1). Concho water snakes were extirpated upstream of San Angelo although a small population was located at the Bell Street Bridge (San Angelo, Tom Green County, Texas) in 2008 (Scott et al. 1989; USFWS 2011).

The construction of O.H. Ivie Reservoir (hereafter OHIR) at the confluence of the Colorado and Concho Rivers, within the core range of the Concho water snake, prompted the USFWS to list it as Threatened in 1986. Construction of OHIR was completed in 1989, inundating approximately 25% of the core habitat of the snake and potentially creating three disconnected populations: (1) lower Colorado River (LCR) populations downstream of OHIR, (2) upper Colorado River (UCR) populations upstream of OHIR and (3) Concho River populations upstream of OHIR (Whiting et al. 2008) (Fig. 1). Extensive field work in the late 1980s and early 1990s suggested that the Concho water snake was not restricted to the riffle sections, but that it would also use habitats within lakes, especially areas with rocks along the

shoreline (Greene et al. 1994, 1999; Whiting et al. 1997, 2008). Based on 10 years of intermittent survey data, Whiting et al. (2008) found the Concho water snake was able to persist in Lake Ballinger, EVSR and OHIR reservoirs at low densities. In 2011, the Concho water snake was removed from the ESA in 2011 (Federal Register 76:66780–66804; July 8, 2011). The reasons being: (1) Concho water snakes can survive in lower flows than previously thought, (2) Populations were detected in all three reaches of the species' range, (3) Concho water snakes are reproducing the reservoir and (4) Concho water snakes have been detected during droughts and in degraded habitats (USWFS 2011).

The ESA Section 4(g)(1) requires that the United States Fish and Wildlife Service (hereafter USFWS), in conjunction with state agencies, conduct at least 5 years of population monitoring following delisting of a species to ensure the biological status of the species does not change. Employment of both demographic and genetic-based methods would be ideal for such post-delisting monitoring. However, the life history traits and behaviors of some species (e.g., the Concho water snake) may preclude cost-effective or logistically tractable estimates of population demographics via field-based surveys (e.g., mark–recapture). For example, snakes are regarded as one of the most difficult vertebrate groups to study owing to their short activity periods, cryptic coloration, and secretive behavior (Durso et al. 2011; Willson et al. 2011). Previous detection probability estimates based on site occupancy modeling of water snakes ranged from 0.03 to 0.46 with considerable interspecific variability, but increase in detectability when densities are high (Durso et al. 2011). Stochastic environmental variation in temperature and precipitation can further reduce snake detectability (Durso et al. 2011). These factors are often compounded for snakes that inhabit inaccessible aquatic or fossorial habitat, and/or that occupy privately-owned lands with restricted access. Fortunately, genetic monitoring has proven a useful tool in assessing the effects of anthropogenic habitat modification, determining population stability, assessing the efficacy of reintroductions, and identifying subpopulations with declines in genetic diversity (Cain et al. 2011; Hieb et al. 2014; Schwartz et al. 2007; Sork et al. 2002). Among snakes, genetic monitoring is especially valuable in detecting changes in population connectivity and declines resulting from habitat alteration because field-based demographic estimates are cost-prohibitive or logistically difficult (Gautschi et al. 2002; Gibbs and Chiucchi 2012; Jansen et al. 2007; Marshall et al. 2009).

Our overarching goal in this study was to apply genetic monitoring as part of Phase I of the post-delisting monitoring plan for the Concho water snake. We had four primary objectives: (1) conduct survey work along the known range of the Concho water snake including sites previously positive for the snake, (2) develop new species-specific microsatellite markers that would be informative for fine-scale population genetics analysis, (3) characterize the local scale genetic diversity and population structure of the Concho water snake that exists at the time of post-delisting, and (4) estimate local effective population sizes along the species range. In addition, population genetics work on *N. h. paucimaculata* was conducted prior to its delisting (samples from 2004 to 2008; Rodriguez et al. 2012). Where possible, we make comparisons to our results, which were based on samples from 2013 to 2015, roughly five generations after the Rodriguez et al. (2012) sampling effort. Our study establishes a baseline for the conservation genetics of the Concho water snake at the time of de-listing, makes available 13 novel microsatellite markers, and provides critical data to aid future efforts in the long-term conservation of this endemic species.

Methods

Surveys and sampling

Surveys were conducted from 2013 to 2015 at 19 sites along the Colorado and Concho Rivers spanning the known range of the Concho water snake (Fig. 2). Sites were selected to include riffles previously reported to be occupied by Concho water snakes. Each site consisted of a minimum of 1 mile of river that encompassed the majority of the riffle habitat in that location. Snakes were captured with partially submerged minnow traps baited with little stinker catfish bait (to lure in small fishes) or captured by hand during active searches. Each site was surveyed twice in the spring and summer. Sites where Concho water snakes were detected during the spring/summer surveys were surveyed again in the fall for the presence of neonates. Catch per unit effort was calculated as the number of unique individual Concho water snakes caught by hand divided by the total number of search hours or number caught by trap per reach of the river divided by trap hours per reach for each year (see Supplemental Table S1). Tissue samples were collected by removing a small clip of caudle scale and stored in 70% EtOH. Each individual was marked with a PITT tag prior to release. The research protocols (i.e., capture and handling) in this study were approved by the Institutional Animal Care and Use Committee at Texas A&M University. We note our original intention was to incorporate mark–recapture data to complement the genetic results presented herein. However, small sample sizes and low recapture rates precluded a formal mark–recapture analysis (see "Results" section).

Hydrological data was collected from 7 of the 8 USGS stream flow gauges from 2010 to 2015. Steam flow data ($ft.^3/s$) was collected from site 08138000 in the Colorado River at Winchell, TX for only 2010 and 2011 as monitoring by the USGS at this site was discontinued after 2011.

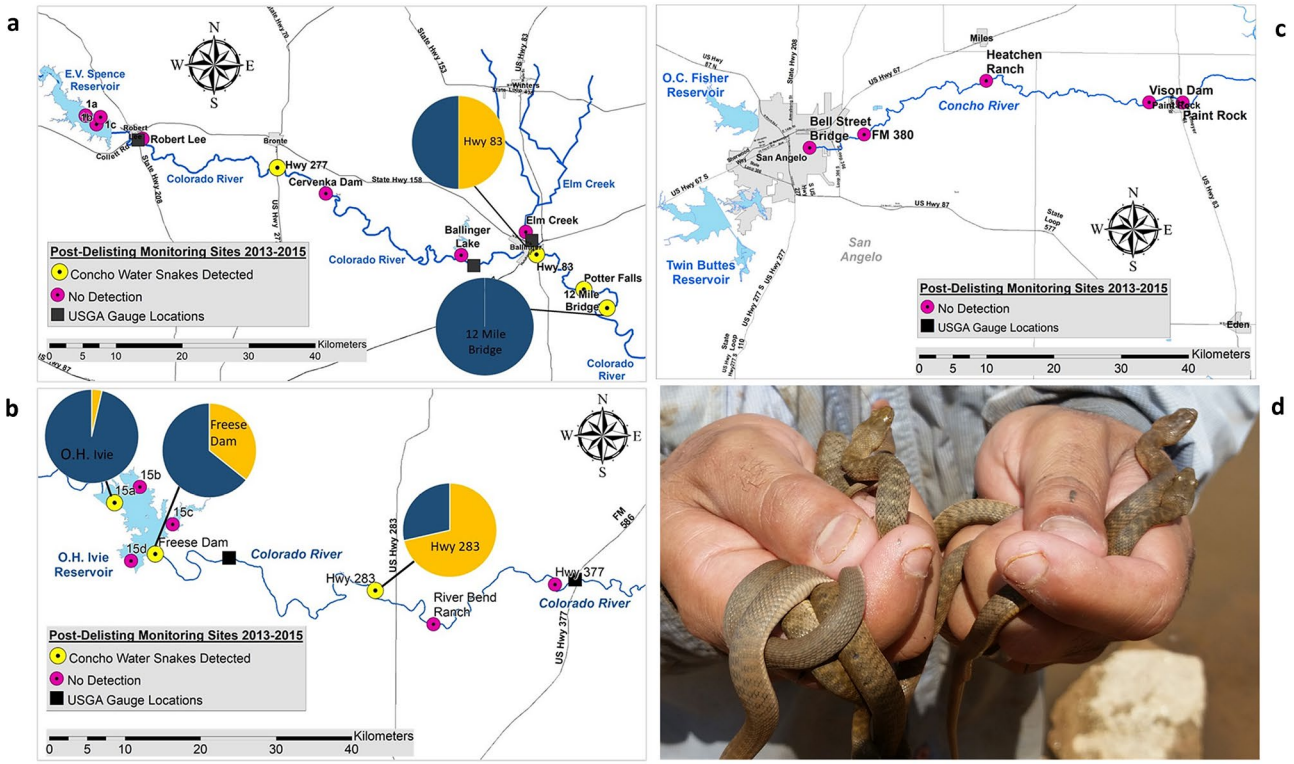

Fig. 2 Post-delisting monitoring sites, occupancy status of sites, and mtDNA haplotype frequency for *N. h. paucimaculata*: **a** upper Colorado River upstream of O.H. Ivie Reservoir, **b** O.H. Ivie Reservoir and lower Colorado River downstream of the confluence of the Conch and Colorado Rivers, and **c** the Concho River upstream of O.H. Ivie Reservoir. Purple dots show monitored sites where Concho water snakes were not detected in our surveys. Sites positive for Concho water snakes are shown as yellow dots and include four locations on the upper Colorado River (Hwy 277, Hwy 83, Potter Falls, and 12 Mile Bridge), one location within O.H. Ivie Reservoir (Concho Recreation Area), and two locations on the lower Colorado River (Freese Dam and Hwy 283). Sites where we obtained mtDNA sequence data are shown as pie charts depicting the proportion of each haplotype from each site: haplotype NpB in blue and NpA in yellow (sample sites give in Table S12). **d** Neonate Concho water snakes captured in the O.H. Ivie Reservoir

Microsatellite development and genotyping

A tissue sample collected from a single individual from the Hwy 277 survey site in 2013 was submitted to the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (Ithaca, NY) to develop a microsatellite library following protocols described in Nali et al. (2014) (see also van Paridon et al. 2016; Detwiler and Criscione 2011).

The M13 method was used for screening (6 individuals) and subsequent genotyping (109 individuals). An 18-bp M13 tag (TGTAAAACGACGGCCAGT) was attached to the 5′ end of the forward primer (Schuelke 2000). A short tail sequence (GTTTCTT) was added to the 5′ end of the reverse primer to reduce polyadenylation (Brownstein et al. 1996). The DNA was extracted from tissue taken from a small, ventral scale clip using a solution of 5% Chelex and 0.2 mg/mL Protienase K in a 200 µL volume. The samples were incubated at 56 °C for 12 h and then boiled at 100 °C for 8 min. PCR amplification was performed in 10 µL reactions containing 3.1 µL ultrapure water, 5 µL 2 × Qiagen Type-IT Kit Master Mix, 0.16 µL of 10 µM fluorescent-labeled M13 primer (Applied Biosystems: FAM), 0.08 µL M13-tagged forward primer, 0.16 µL of 10 µM reverse primer and 1.5 µL of genomic DNA. The thermocycler profile was 94 °C for 5 min, 31 cycles of 94 °C for 30 s, 56 °C for 45 s, 65 °C for 45 s, followed by nine cycles of 94 °C for 30 s, 53 °C for 45 s 65 °C for 45 s and extension at 65 °C for 10 min. PCR-product was visualized on a 2% agarose gel run in 0.5 × TBE buffer at 95 °C for 45 min. Primers that yielded discrete bands in the expected product size range were sent to the DNA Analysis Facility on Science Hill at Yale University (Princeton, NJ, USA) and visualized on a 3730xl 96-capillary Genetic Analyzer with 500-LIZ size standard. Samples were genotyped on GeneMarker 2.6.4.

Initially, we tested primer pairs for 48 loci in a subset of 6 Concho water snakes. Loci were included in our final panel if there was allelic variation, if alleles could be scored without ambiguity, and alleles were reproducible in repeated samples. The final panel consisted of 13 novel loci (Table 1).

Table 1 The 13 microsatellite loci developed for *N. h. paucimaculata* in this study

Primer name	Primer sequence (5′ to 3′)	Repeat motif	Size range (bp)
NePa 1470	F: TCATAAGAATCTGTTGTCTAGTCACAC	AGAT	289–313
	R: AATTGTTCACTGCCCAGAATCATAG		
NePa 5932	F: ACAACGACCTCTTTATGTAATGCAG	AAGT	330–342
	R: ACAAATGTATATTGCTGGTCTTTGAC		
NePa 12681	F: TATCAACCATTAGCACCAGTCAATG	AAC	196–208
	R: CAAGTTGAAATCCATAGCACGTTTC		
NePa 21288	F: TATGTCCATGCATTTCTTCCTCAAG	AAC	167–182
	R: CTGCCTGTCTGATCAATACCAAAC		
NePa 2349	F: AGTGGTAGAATTACACATCAACACC	ACAT	276–3517
	R: TTCTGACTATGACAACAATGGATTC		
NePa 9687	F: TCCGTTGAAGTAAGAGTTTCCAAAG	ACAT	278–286
	R: CAACAGTTCAGGAAGGTTTCAATTG		
NePa 1277	F: ATTGCTAGTCCATGTATGATATGCC	AAAC	350–362
	R: AATCATTCCCAACATTAGGCTCTTC		
NePa 2048	F: ATCTTAGCCACTGAGCCACTG	ACAT	350–358
	R: TGTCATATCACACAAACAGCAATTG		
NePa 2287	F: TGATCTTAACCAGTTCCAAGTTAGC	ACT	200–215
	R: TGTCTTCCTACATTCTCCCAGTTG		
NePa 11248	F: ATGGATGCAATGAACATGAATTTGG	AAC	260–265
	R: TGAATGTCTTGGCTGAATATCACTC		
NePa 12577	F: CTTGCTAAGTGATGTATTTGAATGATAC	ACT	230–254
	R: TCTTGGCAGATAGAACTTGTCAATG		
NePa 22153	F: TACATTCGGAGTATAAGACACACCC	ACAG	169–185
	R: ACTGGAGGTTGTTGATGGAATAATAG		
NePa 9972	F: AAATTTGATATCCCTTCAGTAGCCG	ACAG	317–321
	R: GGCCACAACAGGTAGGTAGG		

In addition, we genotyped the five loci used by Rodriguez et al. (2012) on the Concho water snake. These five loci were developed in other snake species and included: Nsu3 and Nsu4 (Northern water snake, *Nerodia sipedon*, Prosser et al. 2002), 3Ts (Common garter snake, *Thamnophis sirtalis*, Garner et al. 2002), Ts3 (Common garter snake, McCraken et al. 1999), and Ebou l (Black rat snake, *Pantherophis obsoleta*, Blouin-Demers and Gibbs 2003). The 13 species-specific novel loci and the 5 cross-amplified loci were genotyped in the captured Concho water snakes during our survey. However, of the five cross-amplified loci, two (Ts3 and Ebou 1) were monomorphic across all 109 genotyped snakes. Hence, all of our population genetic analyses used 16 loci in total.

Genetic data analyses

Sibling identification

Because several snake captures from the same location and same day consisted of neonates (especially in the OHIR), we suspected that sibling groups were represented in the data set. Indeed, exploration of the data revealed multilocus genotypes that differed at just one or two loci. To test, for the presence of siblings we used COLONY v2.0.6.5 (Wang et al. 2012). We ran COLONY with all captured individuals included. The following settings in COLONY were used: male and female mating system was set to polygamy without inbreeding; the full-likelihood analysis method, with the length of run set to long, high likelihood precision and a weak sib-ship prior was used; sibship sizes for male and female were set to 1 and 1; and false allele rate was held constant at 0.01. Four allele dropout rates (0.1, 0.01, 0.05, and 0.001) were explored, but sibling groups were consistently identified regardless of dropout rate, thus we report only the results from the 0.001. Using the output files of the Fullsib and Halfsib dyad output files, we identified sibling groups (pairwise relationships) as full or half sib that were identified with a probability of 95% or higher.

The COLONY results indicated there were siblings in the data set (see "Results" section). The presence of full siblings within a dataset has been demonstrated to inflate estimates of population subdivision (Allendorf and Phelps 1981). To test for the influence of siblings on subsequent data analyses, we created a reduced data set. The reduced data set consisted of a single representative individual (randomly selected) from

each full sibling group. Subsequent individual-based clustering analyses (unless noted below) were performed on both the full data set ($N = 109$) and a reduced dataset ($N = 92$) to determine if the presence of siblings among the samples influenced within or among population genetic results.

Individual-based clustering and isolation by distance analyses

We conducted four individual-based clustering analyses that do not depend on a priori delimitations of populations as some locations had sparse sampling. First, we used principal coordinate analysis (PCoA) as implemented in GENALEX 6.502 (Peakall and Smouse 2012). Second, we used the model-based Bayesian clustering implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), which partitions individuals based on HWE and linkage equilibrium. The input parameters for STRUCTURE were set to correlated allele frequencies and the admixture model. STRUCTURE was run with 5,000,000 iterations with a burn-in of 500,000 iterations for K (i.e., the number of possible clusters) values 1 to 10 with 10 replications of each possible K value. Third, spatial (using geographic coordinates) and non-spatial Bayesian clustering assignment were implemented in the program BAPS 6.0 (Corander et al. 2008). Ten iterations of each K from 2 to 10 were repeated. Clustering assignment was performed at the level of the individual. Finally, we used the Bayesian clustering approach implemented in GENELAND 4.0.6 (Guillot et al. 2005) on the full dataset only ($N = 109$), which also incorporates geographic coordinates of samples into the analysis. GENELAND (Guillot et al. 2005) analysis was performed to test for the number of clusters using the correlated allele frequency model. Ten independent runs evaluating $K = 1$ to 10 were performed with 200,000 chains, thinning of 100, and burnin of 200. Maps with geographic sampling locations were reconstructed showing probability densities of population membership for the run with the highest log posterior probability score.

Patterns of isolation by distance were examined by testing the correlation between individual-based genetic distance and geographic distance for the full data set only ($N = 109$). The genetic distance matrix was created in GENEPOP 4.6 for pairwise comparisons between individuals (â; Rousset 2000) as implemented in R (Rousset 2008) (R core Development Team 2017). A geographic Euclidean distance matrix was generated in adegenet 2.1.1. (Jombart 2008). The Mantel test for isolation by distance for pairwise comparisons between individuals was implemented with 1000 permutations and visualized in adegenet 2.1.1 (Jombart 2008).

Based on the clustering results, there was clear substructuring across the sampled locations (see "Results" section). In four of these identified subpopulations, which corresponded to collection locations, we had sample sizes greater than 10 that enabled additional population genetic analyses. All subsequent analyses were performed for the following four subpopulations: 12 Mile Bridge, OHIR, Freese Dam, and Hwy 283. In addition, the analyses based on these four subpopulations were performed on both full and reduced data sets, where the latter used one randomly selected representative of a sibling group (see Table 2 for subpopulation specific sample sizes).

Delimited subpopulation structure analyses

Pairwise tests of genetic differentiation and estimation of F_{ST} among the four subpopulations (12 Mile Bridge, OHIR, Freese Dam and Hwy 283) were conducted in FSTAT 2.39 at 1000 randomizations of genotypes among locations. Pairwise tests for allelic differentiation (*Jost's D*) were calculated in GENALEX 6. 502 (Peakall and Smouse 2012) at 999 randomizations of genotypes among locations.

Within subpopulation genetic diversity and equilibrium tests

Gene diversity (H_s), the number of alleles per locus (A_N), and allelic richness (A_R) (rarefied to the lowest sample size) were calculated in FSTAT 2.39 (Goudet 1995). Estimates of F_{IS} (Weir and Cockerham 1984), which quantifies the proportional change in heterozygosity due to deviations in Hardy–Weinberg equilibrium, were conducted in in FSTAT 2.39 (Goudet 2001). To test if F_{IS} values deviated from 0, 1000 randomizations of alleles among individuals within locations were carried out in FSTAT. Tests for linkage disequilibrium (LD) were performed using GENEPOP with 5000 dememorizations, batches, and iterations per batch using the genotypic disequilibrium option (Rousset 2008). LD analyses were conducted within each of the four subpopulations. Statistical tests for differences in allelic richness,

Table 2 Sample sizes of Concho water snakes genotyped from the 2013 to 2015 post-delisting monitoring period

Occupied site name	Individuals caught per sampling year			Total sample size	Sample size with siblings removed
	2013	2014	2015		
Hwy 277	1			1	1
Hwy 83	4	1	3	8	7
Potter Falls			1	1	1
12 Mile Bridge			12	12	10
O.H. Ivie Reservoir			56	56	43
Freese Dam	1	2	15	18	18
Hwy 283		4	9	13	12
All sites				**109**	**92**

gene diversity and F_{IS} values among the four subpopulations (12 Mile Bridge, OHIR, Freese Dam, and Hwy 283) were conducted using the non-parametric Kruskal–Wallis test implemented in R using the dplyr and coin libraries.

Within subpopulation effective population size and detection of bottlenecks

The effective population sizes (N_e) for the four subpopulations (12 Mile Bridge, OHIR, Freese Dam and Hwy 283) was estimated using the bias-corrected linkage disequilibrium estimator (LD–N_e) of Waples (2006) as implemented in the software NEestimator V2.1 (Do et al. 2014). The primary assumption behind using the linkage disequilibrium method (Waples 2006) is that genetic drift is the only cause of linkage disequilibrium in the sample. Thus, the method assumes a closed population (i.e., no migration; Waples and England 2011). If in the population sample there are a few migrants that originated from highly diverged populations, LD–N_e estimates of N_e may sometimes be decreased due to the linkage disequilibrium generated by admixed individuals (Waples and England 2011).

The software BOTTLENECK 1.2.02 (Piry et al. 1999) was used to test for the presence of recent bottlenecks in the four subpopulations (12 Mile Bridge, OHIR, Freese Dam and Hwy 283). The Wilcoxon signed rank test was used to test for an excess of gene diversity (i.e., heterozygosity under HWE) relative to the gene diversity expected based on the observed allelic diversity under mutation-drift equilibrium. This test of a bottleneck is based on the concept that when a population undergoes a large reduction in N_e, the decrease in number of alleles will occur at a faster rate than the gene diversity of the population. The presence of bottlenecks was tested at 10,000 coalescent simulations with two mutation models, the infinite alleles model (IAM), and two-phase mutation model (TPM). The TPM model was implemented with four different multistep change proportions (30, 10, 5, and 1%) and the default variance of 30. We only ran the bottleneck tests after reducing sibling groups to one representative per family due to results we found with the LD–N_e estimates (see "Results" section).

Mitochondrial sequencing and analysis

In a previous study on the Concho water snake, Rodriguez et al. (2012) sequenced 920-bp of cytochrome b (MT-CYTB) in 34 snakes from the Colorado River and 30 snakes from the Concho River. They identified only two haplotypes differing by a single substitution. To compare the change in the frequency of these two haplotypes since their study, we designed species-specific primers from the GenBank deposited sequences (JQ743512.1 and JQ743513.1) that flanked the polymorphic site and amplified a shorter 136-bp fragment: NePaCytb-650-F (5′-CGA CCC AGA AAA CTT CTC AAA-3′) and NePaCytb-915-R (5′-TGG CTG ATC AGG TGA TTA TGA-3′). The PCR amplifications were performed in 20 µL volumes using 6 µL of DNA template, 10 µL of Amplitaq Gold 360 Master Mix (Applied Biosystems), 0.40 µL of 20 µM forward primer and 0.4 µL of 20 µM reverse primer, and 3.20 µL of ultrapure DNase/RNase-free distilled water (Invitrogen). Thermocycling conditions began with an initial denaturation step of 95 °C for 10 min, then 35 cycles of 95 °C for 30 s, 56 °C for 1 min, and 72 °C for 45 s and an extension of 72 °C for 7 min. To clean the PCR product, 2 µL of Exo-SAP-IT (USB Corp.) was added to 5 µL of PCR product and incubated at 37 °C for 35 min and at 80 °C for 15 min. Cleaned amplicons were submitted to the DNA Analysis Facility at Yale University (New Haven, CT, USA) for sequencing. Primer sequences and low-quality reads were trimmed in Sequence Scanner v2.0 (Applied Biosystems). The resulting sequences were aligned with MUSCLE using the default settings in MegaX 10.0.5 (Kumar et al. 2018). A Fisher exact test was used to determine statistical differences in haplotype frequency between the Rodriguez et al. (2012) populations and those sampled in this study.

Results

Surveys and sampling

A total of 112 individual Concho water snakes were captured at seven of the 19 surveyed sites and we genotyped 109 (Fig. 2; Table 2). Tissue samples from three neonate individuals captured in the OHIR during the fall of 2015 were inadvertently not collected. Importantly, we did not capture or observe any Concho water snakes at the five locations sampled over 3 years on the Concho River itself (Fig. 2). In the positive locations along the Colorado River, multiple individuals were captured at each riffle site, except for Hwy 277 and Potter Falls, where a single individual was captured from the riffles (Fig. 2). Of the 112 individuals captured: 61 were neonates, 28 were juveniles, and 23 were adults [classification of age group was based on snout-vent-length (SVL): adult males > 380 mm SVL, adult females > 420 mm SVL, juvenile females < 420 mm SVL, neonates < 250 mm SVL; Greene et al 1999]. We had seven recapture events, all from 2015, three from OHIR, one from Freese Dam, and three from Hwy 283 (two of which involved the same individual). All recaptures were for individuals captured twice in 2015 except for one female from Hwy 283, which was originally marked in 2014 and recaptured twice in 2015. With too few recaptures, we were not able to conduct formal mark–recapture analyses to assess the demographic population size.

The total number of trap hours completed during the post-delisting monitoring was 120,728 across the three years, augmented by 1645.5 active search hours. In the UCR, where snakes were captured each year from the 2013 to 2015 surveys, the CPUE ranged from 1 Concho water snake per 156 active search hours in 2013 to 1 Concho water snake per 17.92 active search hours in 2015 when there was more rainfall (Table S1). In general, a significant amount of time was required to capture a single individual. Mean annual stream flow in the UCR near Hwy 277 was 0.015 ft.^{3}/s and 0.031 ft.^{3}/s in 2013 and 2014. Mean annual stream flow increased to 0.73 ft.^{3}/s in 2015 (Tables S2, S3, S4). The 2015 spring and summer sampling season experienced the largest influx of rain that west-central Texas had experienced during the study period, with mean annual stream flows increasing at all USGS monitoring sites on the Colorado and Concho Rivers (Tables S2, S3, S4). Despite the heavy rains, the sites on the UCR upstream of Hwy 277 and south of Bronte, TX did not have water in the riverbed.

Sibling identification

Nine groups of full siblings were identified with a high probability (≥ 95%), ranging from pairs to a family group of eight individuals (Table S5). All sibling groups consisted of neonate snakes captured during the fall sampling period in early September. Most of the siblings were encountered at the OHIR location near the Concho recreation area (Fig. 2d). No half siblings were identified among the neonates. No full- or half-siblings were identified within either the juvenile or adult age classes at a probability of 95% or higher.

Individual-based clustering and isolation by distance analyses

Using the full data set ($N = 109$), the PCoA demonstrated strong geographic clustering of individuals from OHIR in the multivariate spaces along axes 1 vs. 2 and axes 1 vs. 3 (Fig. 3). Axis 1 (17.7% of the variance) separated individuals from the OHIR population from the other reaches of the

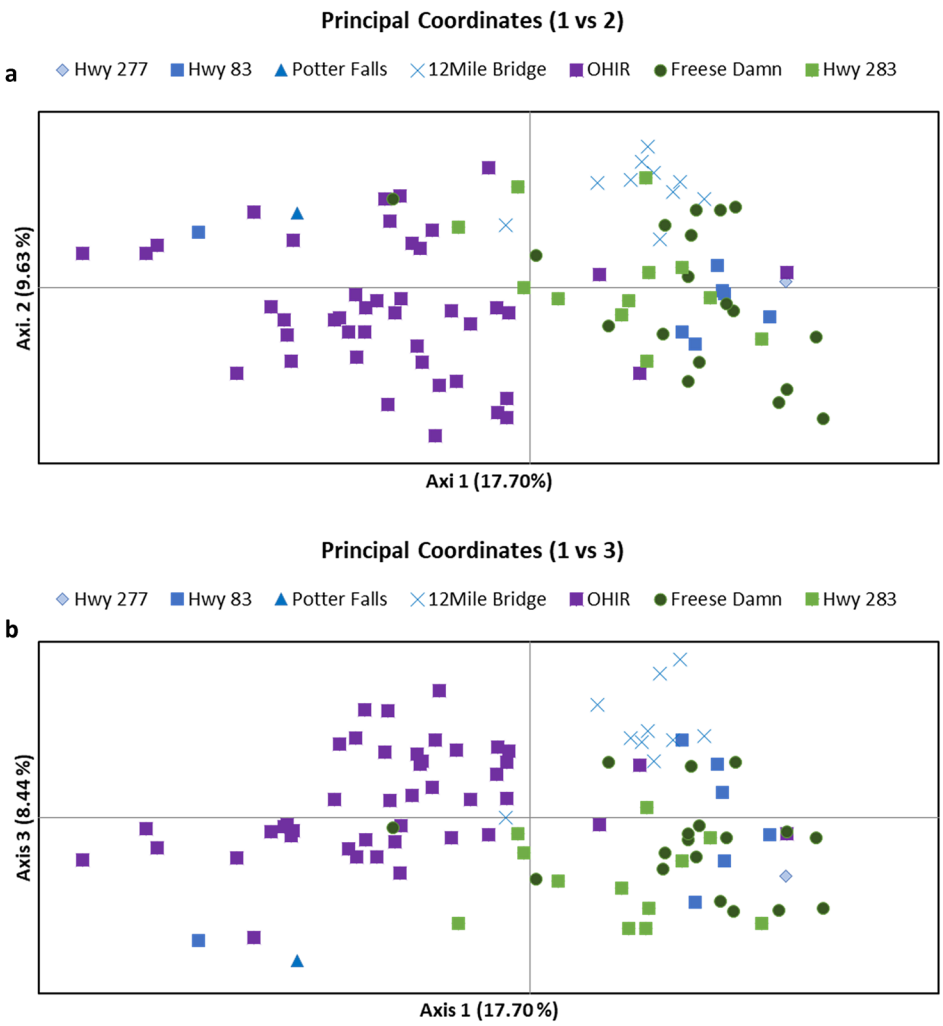

Fig. 3 Results of the PCoA for the complete data set ($N = 109$) using 16 loci. **a** Comparison of axis 1 vs. 2 largely delineates the OHIR (purple) from the other reaches of the Colorado River along axis 1. **b** Separation of the OHIR on axis 1 continues to be visible on PCoA of axis 1 vs. 3, but additional separation of individuals between the UCR (blue) and the LCR (green) is visible along axis 3. Finer scale separation between collection sites, particularly for 12 Mile Bridge (blue dashes), is also apparent along this axis

Colorado River. Axes 2 and 3 showed mild delineation of sites between the UCR (in blue) and LCR (in green), in particular for individuals collected from 12 Mile Bridge. The clusters, however, are not completely distinct even within the reservoir population. A scree plot of the Eigen values for axes 1–10 are given in Fig. S1.

The Bayesian clustering analyses revealed fine levels of substructure for sites along the Colorado River. In the STRUCTURE analyses, the OHIR population is visibly divergent at $K=2$ from both the UCR and LCR regions. At $K=4$, population substructure associated with the riffle collection sites is apparent. The ln $P(D)$ continued to increase beyond $K=4$ and an asymptote was reached at about $K=7$ (Fig. S2). But, because of the high variance in runs at $K=5$, we restrict our interpretation at $K=4$ (Fig. S2). Similarly, the BAPS analysis indicated that the most likely number of genetic clusters was 4 in both the spatially explicit (i.e., using geographic coordinates of samples in the analysis) and the non-spatially explicit models (Fig. 4b). In GENELAND, the highest posterior probability was obtained for $K=5$ in all 10 independent runs (Fig. S3). Maps with geographic sampling locations were reconstructed showing probability densities of population membership for $K=5$ clusters in GENELAND for the run with the highest log posterior probability score (−2560.47) (Figs. S3, S4). The cluster assignments from the BAPS, STRUCTURE, and GENELAND analyses were largely concordant in that they largely delineated individuals collected at the same riffle location. These results indicated local levels of population structure strongly associated with the riffle sites where the snakes were captured (Fig. 4). Interestingly, however, the samples from the two most upstream collection sites (Hwy 277 and

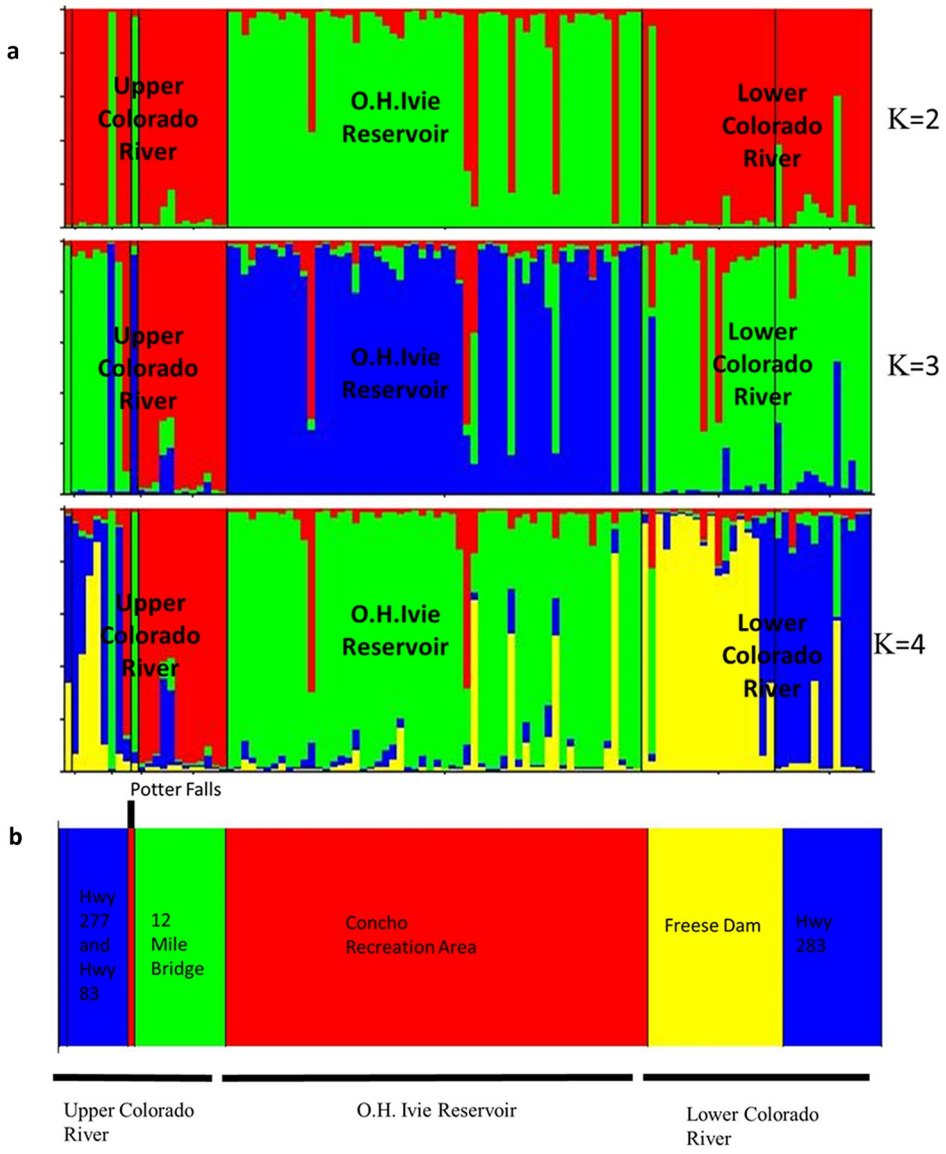

Fig. 4 Results from the Bayesian clustering analyses for the complete data set ($N=109$) of *N. h. paucimaculata* individuals using 16 microsatellite loci. Individuals are arranged from most upstream to most downstream collection site. Each color represents a genetic cluster. **a** Results from STRUCTURE. The y-axis is the Q-value, i.e., the proportion of an individual's genome belonging a cluster. **b** Results from BAPS. The program identified 4 as the most likely number of clusters with a probability of 0.97

Hwy 83, Fig. 4b) fell into a cluster that contained samples in the LCR (Hwy 283 in the BAPS analysis and a mixture of Freese Dam an Hwy 283 in the STRUCTURE analysis; compare $K=4$ in Fig. 4a, b). GENELAND, however, treated individuals from Hwy 277 and Hwy 83 as a discrete cluster in all runs, possibly due to weighting the spatial information incorporated into the analysis itself (Fig. S4). In general, the PCoA and the STRUCTURE results were similar in that the same individuals were classified as "admixed" and/or were assigned to a genetic cluster different than their location of origin (see overlapping symbols in Fig. 3). Qualitatively, the clustering results from the reduced data set ($N=92$) were the same for the PCoA, BAPS and STRUCTURE analyses (Figs. S5, S6).

The results of the Mantel test showed a weak, but significant pattern of isolation by distance ($r=0.28$, $p=0.001$, Fig. 5). The overall pattern of isolation by distance was reduced by individuals from the UCR at the Hwy 277 and Hwy 83 locations, which had higher genetic similarity to individuals collected from Hwy 283 (the most downstream sampling location in the LCR), despite their greater geographic distance (see far right data points in Fig. 5). This pattern is congruent with the individual-based clustering patterns seen in the STRUCTURE and BAPS analyses.

Given the individual-based clustering analyses were consistent in indicating substructuring that was largely concordant with sampling locations, we carried out additional analyses at the level of cluster-identified sampling locations where we had total sample sizes of $N>10$. There were four subpopulations that met this criterion: 12 Mile Bridge, O.H. Ivie Reservoir, Freese Dam, and Hwy 283 (Table 2; Fig. 4).

Delimited subpopulation structure analyses

There was significant pairwise structure identified among the four locations with similar F_{ST} values among pairwise comparisons (Table 3). Likewise, pairwise measures of allelic differentiation (*Jost's D*) were similar and significant among all four sampling locations (Table 3). When siblings group were reduced to one representative of each family, the F_{ST} values and *Jost's D* values remained significant and similar to their full data set counterparts (Tables S6, S7).

Table 3 Pairwise F_{ST} (below diagonal) and Jost's D (above diagonal) for the Concho water snake (*N. h. paucimaculata*) from the four cluster-identified subpopulations with sample sizes above 10 and using all genotyped snakes

	12 Mile Bridge	OHIR	Freese Dam	Hwy 283
12 Mile Bridge	–	0.197	0.164	0.187
OHIR	0.186	–	0.178	0.166
Freese Dam	0.171	0.165	–	0.111
Hwy 283	0.182	0.148	0.108	–

All pairwise tests of differentiation were significant at $p=0.001$

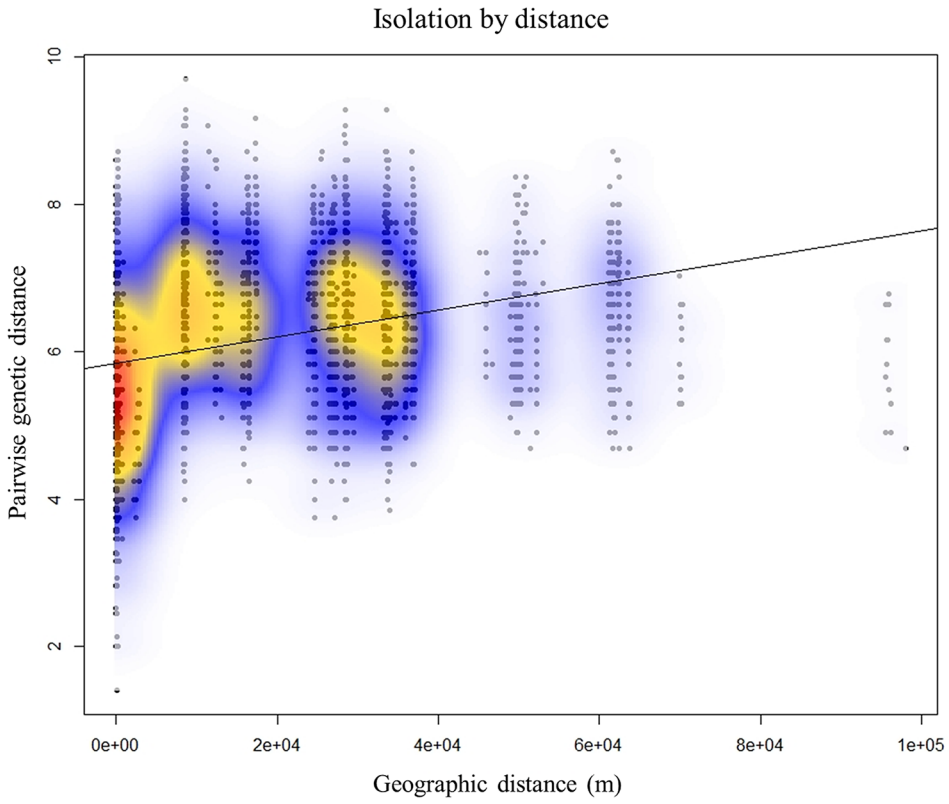

Fig. 5 Correlation between geographic distance and individual-based pairwise genetic distance (Rousset 2008) along the Colorado River (Mantel test, $r=0.286$, $p=0.001$). Heat map is overlaid to show local density of data, discontinuity indicates variation in local density, which corresponds to riffle habitats along the Colorado River. Analysis is based on 16 microsatellite loci from the full data set ($N=109$)

Within subpopulation genetic diversity and equilibrium tests

Microsatellite and within-subpopulation genetic statistics for the four cluster-identified subpopulations with sample sizes greater than 10 and using all genotyped samples from these locations are given in Table 4. A per locus breakdown of these summary statistics for each of these four subpopulations and using all genotyped samples is given in the supplemental information (Tables S8, S9, S10, S11). There were no significant differences in allelic richness (A_R; $p = 0.612$) or gene diversity (H_s; $p = 0.539$) among the four populations. There was a significant difference in average F_{IS} ($p = 0.037$) among the four locations where Freese Dam had a significant value of 0.167, but the other three locations were not significantly different than 0. There was significant overall linkage disequilibrium detected at three of the four sites: 12 Mile Bridge, OHIR, and Hwy 283 (exact binomial test on whether more pairs tested significant than expected by chance at $\alpha = 0.05$; Table 4).

Only one (Freese Dam) of these four subpopulations did not have siblings. For the other three subpopulations, the reduced data sets (where sibling groups were reduced to one random representative per family) had F_{IS} values that remained non-significant, but all showed drastic reductions in LD (Table 5). In particular, 12 Mile Bridge and Hwy 283 no longer showed overall LD and OHIR went from 36% of pairs of loci testing significant to 21% (compare Tables 4, 5).

Within subpopulation effective population size and detection of bottlenecks

When using the full data sets of the four subpopulations, the LD–N_e method returned very small effective size estimates ranging from 8.6 to 13.1 (Table 4). After reducing sibling groups to one individual per family, N_e estimates in 12 Mile Bridge, OHIR, and HWY 283 slightly increased with a range from 11.7 to 19.1 (compare Tables 4, 5). Hence, sibling groups, which increased LD (compare Tables 4, 5), were slightly driving down the N_e estimates. With sibling groups reduced to one representative per family, recent bottlenecks

Table 4 Microsatellite and within-subpopulation genetic statistics for the Concho water snake (*N. h. paucimaculata*) from the four cluster-identified subpopulations with sample sizes above 10 and using all genotyped snakes

Subpopulation	N	A_N	A_R	H_O	H_S	F_{IS}[a]	LD[b]	N_e[c]
12 Mile Bridge	12	2.81	2.81	0.411	0.418	0.017	**16/105**	8.6 (5.9–12.4)
O.H. Ivie Reservoir	56	3.625	3.26	0.491	0.48	−0.023	**38/105**	13.1 (10.2–16.8)
Freese Dam	18	3.56	3.33	0.381	0.457	**0.167**	2/120	11.9 (0.4–20.3)
Hwy 283	13	3.25	3.25	0.538	0.494	−0.09	**15/120**	12.2 (8.9–16.8)

N number of individuals genotyped, A_N the number of alleles per locus, A_R allelic richness, H_O observed heterozygosity, H_S gene diversity

[a]Fixation index: bold indicates significant deviation from 0 ($p < 0.05$)

[b]Genotypic disequilibrium: the number of loci pairs that tested significant over total possible tested pairs. Bold indicates more pairs tested significant than expected by chance at $\alpha = 0.05$ (binomial exact test)

[c]Effective population size based on the LD–N_e method (95% confidence intervals). Lowest allele frequency cutoff was 0.02 for 12 Mile Bridge and, 0.03 for all other sites based on the guidelines of Waples and Do (2010)

Table 5 Microsatellite and within-subpopulation genetic statistics for the Concho water snake (*N. h. paucimaculata*) from three of the cluster-identified subpopulations with sample sizes above 10 and where sibling groups were reduced to one random representative per family

Subpopulation	N	A_N	A_R	H_O	H_S	F_{IS}[a]	LD[b]	N_e[c]
12 Mile Bridge	10	2.81	2.81	0.406	0.41	0.011	4/105	11.7 (3.2–134.5)
O.H. Ivie Reservoir	43	3.43	2.99	0.481	0.487	0.028	**19/91**	15.7 (11.7–21.2)
Hwy 283	12	3.25	3.16	0.54	0.505	−0.072	4/105	19.1 (8.1–166.4)

Freese Dam was not included as it did not have any siblings so there was no change from Table 3

N number of individuals genotyped, A_N the number of alleles per locus, A_R allelic richness, H_O observed heterozygosity, H_S gene diversity

[a]Fixation index: bold indicates significant deviation from 0 ($p < 0.05$)

[b]Genotypic disequilibrium: the number of loci pairs that tested significant over total possible tested pairs. Bold indicates more pairs tested significant than expected by chance at $\alpha = 0.05$ (binomial exact test)

[c]Effective population size based on the LD–N_e method (95% confidence intervals). Lowest allele frequency cutoff was 0.02 for 12 Mile Bridge and, 0.03 for all other sites based on the guidelines of Waples and Do (2010)

were detected in all four populations under the IAM model. Also, as the TPM approached a pure stepwise model, significance diminished in the OHIR (Table 6).

Mitochondrial variation

Using a 136 bp fragment of the MT-CYTB gene for 61 Concho waters snakes from 5 of the collection sites (Fig. 2; Table S12), we were able to identify the same two haplotypes defined by a single SNP (NpA and NpB) as found by Rodriguez et al. (2012). Rodriguez et al. (2012) found a frequency of 0.9 ($n=30$) for NpB across samples from the Concho River. We cannot compare to this frequency since we did not find Concho water snakes along this river. From samples across the Colorado River, Rodriguez et al. (2012) reported a frequency of 0.824 ($n=34$) for NpA. Across the same stretch of the Colorado River, we found a dramatic statistically significant shift in haplotype frequency where NpB was more common (frequency = 0.77, $n=61$; $p<0.001$ Fishers Exact Test).

Discussion

No Concho water snakes were found among the monitoring sites along the Concho River. Concho water snakes were found along the Colorado River though recaptures were too rare for formal mark–recapture analysis. Four individual-based clustering methods showed concordant evidence of population substructure associated with riffle sampling locations and between OHIR and other sampling sites located on the Colorado River. Sibling groups were captured during fall surveys in 2015. The presence of siblings in the data set did not qualitatively alter clustering results nor estimates of population subdivision. However, sibling groups did increase linkage disequilibrium within analyzed subpopulations. Even after reducing sibling groups to one representative per family, estimates of population effective sizes were low and recent bottlenecks were also detected in all locations where sample sizes were large enough to conduct these analyses.

2013–2015 Surveys

Although not directly comparable because survey hours were not reported in many prior studies, we note that historical studies (between years 1987 and 1996) examining the natural history of the Concho snake were able to obtain samples sizes upwards of 300 individuals in a given year (Whiting et al. 2008). In contrast, we only captured a total of 112 Concho water snakes between 2013 and 2015. Greene (1993) reported 1461 snake captures (including recaptures) in 11,501 trap days (which included both trapping and active searches from April to October in 1991 and 1992), yielding at 12.7% trap success. Our survey included 5030 trap days and 119 captures (112 unique individuals, 7 recapture events) for a 2.3% trap success rate. However, these comparisons are also not directly comparable, as our survey times were selected to coincide the highest seasonal trap success days (April–-June 30th and September). We did not conduct surveys in July and August when trap success was reported to be lowest.

Of the total 112 we captured, 99 were captured in 2015, which was the year with the heaviest rainfall. The 2013 and 2014 surveys were conducted during extreme drought conditions (Tables S2, S3, S4). River habitat upstream of the Hwy 277 Colorado River crossing remained dry throughout the survey period (compare Fig. S7a–c). Previous studies have demonstrated a significant decrease in water snake catchability and detection during periods of drought (Green et al. 1994; Whiting et al. 1997; Scott et al. 1989). However, because there are no population size estimates prior to the delisting, it is not possible to determine if population sizes have decreased or if sampling was negatively impacted by extreme weather conditions. Water snakes are highly water-dependent and specialized for life in aquatic systems and thus, are susceptible to prolonged periods of drought. Indeed, drought may have influenced the number of snakes captured for this study. Additional future sampling seasons will be required to determine if the small sample sizes reflect snake activity in relation to weather conditions or if the Concho water snake populations do indeed have small and possibly reduced, census population sizes.

Table 6 Results of bottleneck tests in the four cluster-identified subpopulations with sample sizes above 10 and where sibling groups were reduced to one random representative per family

Population	N	IAM	TPM			
			30	10	5	1
12 Mile Bridge	10	**> 0.001**	**0.001**	**0.005**	**0.006**	**0.007**
OHIR	43	**0.009**	**0.04**	**0.04**	0.067	0.11
Freese Dam (LCR)	18	**> 0.001**	**> 0.001**	**> 0.001**	**> 0.001**	**0.001**
Hwy 283 (LCR)	12	**> 0.001**	**> 0.001**	**> 0.001**	**> 0.001**	**0.001**

Bold values indicate statistically significant excess of observed gene diversity ($\alpha < 0.05$)

N number of individuals, *IAM* infinite alleles model, *TPM* two-phased mutation model assessed at four multistep change proportions

The situation along the Concho River itself is also perplexing. The waterways along this stretch always had water, but the habitat is more akin to trenched canals rather than intermittent riffles, runs, and pools characteristic of the Colorado River. The hydrology of the river has been altered by multiple small private impoundments (Fig. S8), but the timing of the construction of these smaller dams is unclear. Regardless, the lack of detection of Concho water snakes along the Concho River in our surveys, despite extensive search hours (Table S1), should raise a warning flag about the current demographic status of *N. h. paucimaculata* along this stretch of its previously known range.

Individual-based analyses identify substructure

The four individual-based clustering methods were largely concordant in identifying substructure along the Colorado River. Specifically, the Bayesian clustering analyses identified substructure such that genetic clusters could largely be delimited among individuals collected locally at riffle habitat sites. These clustering patterns remained when sibling groups were reduced to one representative per family. Hence, at the scale of among-populations as identified with individual-based clustering analyses, the presence of siblings had no impact on the results. The substructuring along the Colorado River can be explained, in part, by an isolation-by-distance pattern as we detected a significant signature (Fig. 5). Though, the samples from Hwy 277 and Hwy 83 weakened the overall isolation by distance pattern (discussed next).

There was one unusual result from the individual-based analyses in that the individuals from the uppermost sites on the Colorado River (Hwy 277 and Hwy 83) consistently clustered with individuals from the lower Colorado River (except in the GENELAND analysis, which we believe is being weighted more by the geographic information rather than genetic). There are approximately 108 river kilometers between the Hwy 277 river crossing and the riffles downstream of Hwy 283 with OHIR between, thus long-distance migration between the two locations seems unlikely. Anecdotally, this pattern may reflect past successful translocation activities. The Colorado River Municipal Water District, which manages the regions' reservoirs, signed an agreement to carry out translocations between the upper and lower Colorado River as part of the proposed species delisting (USFWS 2011). However, information regarding the timing of the translocations, the locations, and the number of individual snakes involved was not documented. Nonetheless, the patterns of population structure detected in our study are consistent with these efforts. Whether our observed genetic patterns indicate translocated individuals and/or their offspring warrants further investigation.

Focusing in on the four cluster-identified subpopulations (12 Mile Bridge, OHIR, Freese Dam, and Hwy 283) due to their larger sample sizes, there was significant genetic differentiation between all paired combinations of subpopulations. In addition, F_{ST} values and Jost's D values are comparable and indicate moderate levels of differentiation (both measures ranging from approximately 0.11 to 0.19; Table 3). Again, reducing siblings within these subpopulations did not have any qualitative impact on among-population structure (Tables S6, S7).

Regardless of the unusual pattern noted above, it is apparent that local samples (i.e., riffle habitats) contain cohesive genetic signatures. In part, this may be driven by an isolation by distance mechanism. During drought periods, connectivity along the river can become discontinuous (personal observations, Figs. S6, S7) and may disrupt migration and thus, reinforce or make stronger the isolation by distance.

Within subpopulation genetic patterns

Microsatellite diversity was moderate and not significantly different among the four subpopulations regardless of the inclusion of siblings (H_s range 0.418–0.494, Table 4 and 0.41–0.505, Table 5). While not directly comparable, Rodriguez et al. (2012) also found low genetic diversity ($H_e = 0.43$) within the Colorado River from samples collected between 2004 and 2008. Given the larger sample sizes and additional loci included in our study, the consistent finding of low to moderate levels of genetic diversity across the sampling periods of the two sample collections is likely to be an accurate characterization of the species.

Levels of genetic diversity detected in this study are similar or lower than other snake species that have undergone recent bottlenecks or isolation due to habitat loss or degradation such as the Copperbelly water snake (*N. erythrogaster neglecta*) (Marshall Jr. et al. 2009), Western Massasauga (*Sistrurus catenatus*) (Gibbs and Chiucchi 2012; McCluskey and Bender 2015), Smooth snakes (*Coronella austriaca*) (Pernetta et al. 2011) and eastern Fox snakes (*Pantherophis gloydi*) (Row et al. 2010).

The presence of siblings within a population sample tends to drive down F_{IS} and increase LD (Balloux 2004). In our data sets, the reduction of sibling groups to one representative of each family had little effect on F_{IS} where there were slight increases at OHIR and Hwy 283 and a slight decrease at 12 Mile Bridge. But, none of these significantly differed from 0 (Tables 4, 5). In contrast, upon reduction of sibling groups, 12 Mile Bridge and Hwy 283 no longer had overall signatures of LD. The percentage of loci pairs that had significant LD were also reduced in OHIR. Hence, the presence of sibling groups did have an influence on within subpopulation genetic patterns, which is important for the LD–N_e method of effective size estimation (discussed below).

Even after the reduction of sibling groups, the OHIR subpopulation still had substantial LD (Table 5). We originally hypothesized that the OHIR could have had an influx of snakes from the Concho River resulting in an admixed population. Ongoing admixture with random mating among individuals originating from Concho River populations and Colorado River populations could create LD in this location without an elevation in F_{IS} (random mating restores F_{IS} to 0 in one generation, but LD resulting from admixed populations takes longer to decay). Because our surveys did not yield any Concho water snakes along the Concho River, we cannot definitively determine what is going on in the OHIR. We attempted to get at this hypothesis by comparing to the results of Rodriguez et al. (2012). They captured 34 snakes spanning the upper and lower Colorado River and 30 snakes from the Concho River. Between the Concho and Colorado River samples, they found F_{ST} values of 0.27 and 0.68 based on five microsatellite loci and the two mtDNA haplotypes, respectively. If there was admixture, we might have expected to see a mid to high frequency of mtDNA haplotype NpB in the OHIR (using as a baseline the results of Rodriguez et al. 2012). While haplotype NpB was more common in the OHIR, we also found it was common in other locations across the Colorado River (Fig. 2; Table S12). The latter pattern significantly contrasts with the result of Rodriguez et al. (2012), who found a higher frequency of haplotype NpA in the Colorado. In addition, it is clear in our data that haplotypes vary in frequency across locations of the Colorado River (Fig. 2; Table S12). As different sample compositions from each of the locations in our collections compared to those of Rodriguez et al. (2012) could account for the differences in the studies, we find that the mtDNA data alone is inconclusive on whether admixture is occurring in the OHIR.

Effective population size and bottlenecks

N_e estimates at the level of riffles was low (ranged from 8.6 to 13.1; Table 4). Although the presence of sibling groups increased LD (and hence, reduced LD-based N_e estimates) within subpopulations, N_e estimates remained in the low teens (11.7–19.1, Table 5) even after the reduction of sibling groups. We recognize that sample sizes are greatly reduced by reducing the analyses to the level of sample locations. However, the clear substructure precludes combining the samples as excess LD would be generated via the Wahlund effect. Overall, there is consistency in that our estimates at each location suggest local effective breeding populations are small; i.e., on the order of 10–20 individuals per sampling location. In addition, Scott et al. (1989) estimated 1–3 adult females contributed to the yearly recruitment of Concho water snakes at each riffle site, which is consistent with the low effective size estimates from this study.

Bottlenecks were detected at 12 Mile Bridge (UCR), Freese Dam (LCR) and Hwy 283 (LCR); tests were highly significant regardless of model used (Table 6). The UCR is very likely to have been affected by drought and thereby, possibly causing drastic demographic fluctuations. Data collected by the USGS monitoring gauges estimate that between 2010 and 2014, there were between 160 and 229 days each year where stream flow was absent from the Colorado River between Robert Lee and Ballinger, TX. Hence, detection of a bottleneck at 12 Mile Bridge (UCR) is consistent with the low precipitation in this area. There was also reasonable support for a bottleneck in the OHIR sample (Table 6). A recent colonization (i.e., founder effect) of the OHIR (given the dam was constructed in 1986) might explain this pattern, but also, it has been noted that Concho water snakes occur at lower densities within the reservoir, possibly making them more susceptible to fluctuations in population sizes (Whiting et al. 2008). It is less clear why bottlenecks may have occurred at both sites along the LCR. The drought has been less severe along this section of the river, but may still be sufficient to affect the snakes, as stretches of dry riverbed were also observed in 2015 in the LCR even after significant rainfall (Fig. S7d).

Conservation implications and future monitoring efforts

Our results of this initial post-delisting survey are not meant to be a single, conclusive analysis regarding the status of the Concho water snake as a delisted species. Rather, they are meant to lay the groundwork for future genetic monitoring surveys and to establish an initial framework for research-based population management of this species. Our results do indicate that populations of Concho water snakes exhibit genetic differentiation that coincides with riffle systems and within the OHIR. Moreover, we found low effective population size estimates and significant detection of recent bottlenecks at the level of sampling locations. Taken together, genetic-based results demonstrate that the Concho water snake exists as small subpopulations along the Colorado River. The latter result coupled with the failed detection along the Concho River raises concern for this delisted species.

Previous conservation and management efforts in other systems have emphasized the importance of adaptive monitoring (Lindenmayer and Likens 2010; Lindenmayer et al. 2011). An adaptive monitoring program is one wherein the development of additional questions, experimental design and data collection are intrinsically modified by the results of previous efforts. Successful monitoring programs are required to adapt to new questions, and account for variation in conditions and information to improve future monitoring efforts. The first 2 years of the post-delisting

monitoring overlapped with a 5-year drought during which time no water was in the Colorado River upstream of the Hwy 277 crossing and downstream of E.V. Spence Reservoir. These conditions persisted in 2015 after the drought conditions ended downstream of Hwy 83 south of Ballinger, TX. Water snakes are susceptible to prolonged periods of drought, which are exacerbated by the presence of reservoirs (Rose and Todd 2017). Scott et al. (1989) and Whiting et al. (2008) noted significant decreases in Concho water snake detectability during prolonged periods of drought as was also observed in this study.

Research conducted by Winne et al. (2006) and Willson et al. (2006) suggest that migration and aestivation are common behavioral responses of aquatic snakes in response to drought. For example, aestivation during drought is known to be employed by the black swamp snake, *Seminatrix pygea*, another small-bodied highly aquatic snake. Yet, aestivation is associated with high survival costs, especially to females, during prolonged droughts (Winne et al. 2001). Migration is employed by several species of *Nerodia* that occupy seasonally variable wetland habitats (Willson et al. 2006). Previous work on the Concho water snake has documented migration driven by habitat availability within reservoirs (Whiting et al 1997). However, prolonged periods of drought in river systems surrounded by arid, unsuitable habitat has been documented to cause significant decreases in year to year survival, lower somatic growth rate, loss of large individuals from the populations, and local extinction (Rose and Todd 2014, 2017). We currently have no data on how the Concho water snake populations respond behaviorally or physiologically to prolonged drought, however severe droughts are predicted to continue and to become more severe in Texas due to climate change (Banner et al. 2010). We suggest that future investigations in this system address this aspect of the Concho water snake's biology to better manage populations in the future.

Because there are no population size estimates prior to the delisting, it is not possible to determine if population sizes have decreased or if sampling was negatively impacted by extreme weather conditions. Unfortunately, we had too few recaptures in our study to get a handle on current demographic sizes, but our genetic data suggest small local effective population sizes and recent bottlenecks. We also recommend that future studies keep track of sampling efforts as well as continue to use genetic monitoring methods. Continued monitoring will be needed to determine if the Concho water snake may require re-listing as threatened under the ESA to prevent these populations from becoming susceptible to extinction. Our study, as well as our original report to the Texas Parks and Wildlife (Janecka et al. 2016) provide explicit data on sampling locations and sampling effort and will help serve as a baseline for future studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10592-021-01391-w.

Acknowledgements We thank USWFS, CRMWD, TPWD, and the many private landowners involved in this project. Funding for this research was provided by TPWD Section Six Grant (to CDC and MJJ). Kevin Young, Abbie Ince, Andrew Sakla, Meghan Beatty, Chris Gonzales, Diego Araujo, and Hayden Kusy were instrumental in completing the fieldwork for the monitoring surveys. We thank Toby Hibbitts, Lee Fitzgerald and Jessica Stephenson for editing and valuable comments during the revision process.

Author contributions Conceptualization: MJ, CC; Methodology: MJ, CC; Data collection: MJ, AM; Formal analysis and investigation: MJ, CC; Writing-original draft preparation: MJ; Writing-review and editing: MJ, CC, JJ, AH; Funding acquisition: MJ and CC; Resources: JJ, CC; Supervision: JJ, CC.

Funding This research was funded by TPWD Section Six Traditional Grant (CDC and MJJ).

Data availability Sequence data will be deposited in GenBank and accession number will be provided once accepted. Microsatellite data will be deposited on DRYAD.

Declarations

Conflict of interest The authors have no relevant financial of non-financial interests to disclose.

Ethical approval All of the methods involving the capture and recapture of live animals were approved by the IACUC Committee at Texas A&M University. Collection permits were issued by Texas Parks and Wildlife Department.

Consent to Participate No human subjects were involved in this research.

Consent to Publish All authors have consented and approved the submitted manuscript.

Plant reproducibility No plants were involved in this research.

Clinical trials registration No clinical trials were involved in this research.

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