

Genetic status assessment of Chihuahua chub in the Mimbres basin.

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Final Report submitted for period ending December 30th 2019

Submitted to:

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Introduction

The Chihuahua chub (*Gila nigrescens*) is native to the Mimbres River, New Mexico, as well as the Guzman and Laguna Bustillos basins in Chihuahua, Mexico (Smith & Miller 1986). The Chihuahua chub was first collected from the Mimbres River in 1851 (Baird & Girard 1854) and later (1975) another population was discovered in Moreno Spring (Rogers 1975) within the Mimbres River drainage. The Chihuahua chub has declined in abundance and has disappeared from much of its former range (Miller and Chernoff 1979; Propst and Stefferud 1994) and as such is listed as a threatened species under the Endangered Species Act (U.S. Fish and Wildlife Service 1983). It is also listed at the state level as an endangered species (New Mexico Department of Game and Fish 1976), as a ‘critically imperiled species’ and a ‘Species of Greatest Conservation Need’ under the State Wildlife Action Plan for New Mexico (NMDGF 2016). The Chihuahua chub has a very restricted range in New Mexico and is only regularly found in Moreno Spring and in a roughly 15 km stretch of the Mimbres River downstream from the confluence of Allie Canyon (Propst 1999). A ‘new’ population was discovered in the upper Mimbres River near Cooney Place by a biologist from the US Forest Service (DL Propst Pers. Comm.) in 2008 and additional individuals have been encountered further upstream near Monument Canyon more recently (2016). A previous genetic assessment suggested that the population at Cooney Place was likely a remnant population rather than a population recently established by migration from an effort to establish a population in McKnight Creek or by upstream movement of individuals from below Allie Canyon (Osborne et al. 2012). The genetic affinity of the individuals collected near Monument Canyon is unknown. However, PIT-tagged individuals were collected at Monument Canyon, providing evidence that Chihuahua chub are moving from sites lower in the Mimbres River, where PIT-tagged fish were released, to upstream sites. Genetic data has also show that there is substantial genetic divergence between populations in Mexico and those in the United States such that they comprise distinct Evolutionarily Significant Units (ESUs) (Osborne et al. 2012). In addition to these wild populations, a captive population is maintained at Southwestern Native Aquatic Resource and Recovery Center (Southwestern Native ARRC).

In addition to the threats posed to the Chihuahua chub through degradation of its habitat, small and isolated populations are at risk from factors resulting from loss of genetic diversity. Maintenance of natural levels of genetic variation in imperiled species, like the Chihuahua chub, is a critically important

conservation goal. Genetic diversity allows species to respond to changing environmental conditions, such as exposure to new diseases and pathogens. All Mimbres basin populations of Chihuahua chub are small and are subject to periodic extirpations due to factors including ash-flows following wildfires such as occurred after the Silver fire in 2013. This fire impacted 139,000 acres including the headwaters of the Mimbres, Allie and Powderhorn Canyons on the Mimbres River.

The aim of this project was to assess current levels of genetic variation and spatial structure in New Mexico populations of Chihuahua chub in the Mimbres River and in the population held at Southwestern Native ARRC, which was sampled in 2013 and 2016. Genetic diversity was compared to previous data (Osborne et al. 2012) to assess the trajectory of genetic change in Chihuahua chub populations. We also calculated the contemporary genetic effective size to further characterize the genetic status of the population.

Methods

Sampling and Molecular Methods

Chihuahua chub populations in the Mimbres River (at properties owned by the Nature Conservancy [TNC] and New Mexico Department of Game and Fish [NMDGF]) and at Moreno Spring in southwestern New Mexico were sampled by personnel from the New Mexico Department of Game and Fish in 2016 and 2018 (Figure 1). Moreno Spring is located on private land along the Mimbres River. In addition to the wild populations of Chihuahua chub, we genotyped material sampled from the captive population held at Southwestern Native ARRC, which were stocked in the Mimbres River in both 2013 and 2016 at the property owned by the New Mexico Department of Game and Fish. We included previous data collected in 2009 and 2010 (Osborne et al. 2012) from the Mimbres River (TNC and NMDGF properties and Moreno Spring) and the hatchery population held at Southwestern Native ARRC to provide context for the current results. All samples for genetic analysis were collected non-destructively by taking a small piece of caudal fin. Samples were deposited at the Museum of Southwestern Biology. DNA was extracted from fin clips using proteinase-K digestion and standard phenol/ chloroform isolations methods (Hillis et al. 1996). Chihuahua chub populations were screened for variation at nine microsatellite loci as described in Osborne et al. (2012). We did not assess Chihuahua chub for variation in mitochondrial DNA as previous efforts did not identify any genetic variation in two genes (ND2 and ND4) across the populations in New Mexico (Osborne et al. 2012).

Data Analyses- Genetic Variability and Spatial Structure

GENEPOP Version 3.1d (Raymond & Rousset 1995) was used to conduct the modified exact test to determine whether the observed genotype frequencies conformed to Hardy-Weinberg expectations and to conduct the global test for linkage disequilibrium among loci. Sequential Bonferroni correction (Rice 1989) was applied to account for multiple comparisons. Measures of diversity, particularly allelic richness, are dependent on sample size, so we used a resampling procedure to calculate all diversity measures, which include: Nei's unbiased gene diversity (H_{exp}) (Nei 1987), heterozygosity (H_{obs}) and allelic diversity (N_{AC}). For each temporal and spatial sample 1000 random subsamples were drawn without replacement to a minimum sample size of $n=22$ (the smallest sample size). The average inbreeding co-efficient (F_{IS}) was calculated using the R package *HIERFSTAT* (Goudet and Jombert 2015). To evaluate whether there was genetic differentiation between sampling sites, we separated the samples collected from the upper Mimbres from those collected at the TNC and NMDGF properties and then calculated pairwise F_{ST} following Weir and Cockerham (1984) for each pair of samples using the program *GENODIVE* (Meirmans and Van Tienderen 2004). F_{ST} values evaluate differences in allele frequencies between samples and can range from 0 (there is no variance in allele frequencies between locations) to one (populations do not share any alleles). Significance was assessed using 999 permutations.

Data Analyses - Genetic Effective Size

The single sample linkage disequilibrium method (Hill 1981) was used to estimate the effective number of breeders (N_{eD}) from microsatellite DNA data for Chihuahua chub using the program *NeEstimator* Vers. 2.0 (Do et al. 2014). This program implements a correction factor to account for bias that may occur when the sample size is less than the real (unknown) effective number of breeders (England et al. 2006). *NeEstimator* calculates estimates after excluding all alleles with frequencies of less than a specified critical value. Here we used $P_{crit}=0.02$ as is suggested for use where the number of individuals sampled is greater than 25 (Waples and Do 2010). This value of P_{crit} generally provides a good balance between precision and bias (Waples and Do 2010). Upper- and lower-bound 95% confidence intervals for N_{eD} were calculated using the jackknife approach as implemented in *NeEstimator*.

Results and Discussion

Genetic Variability, Spatial Structure, and Genetic Effective Size

We assessed genetic variation at nine microsatellite loci in 128 Chihuahua chubs collected from Mimbres River populations as well as the refugial population held at Southwestern Native ARRC. The number of alleles by locus ranged from six at *Cyp2* to a high of 15 at *Lco8*. There were two departures from Hardy-Weinberg expectations. These occurred at the loci *Cyp45* (Mimbres River collection) and at *Lco8* (Southwestern Native ARRC from 2013). In both cases, there was an excess of heterozygotes. There was evidence of linkage disequilibrium following Bonferroni adjustment between *Cyp26* and *Lco1*, *Ca8* and *Cyp45*, and *Lco8* and *Ca6* and between *Cyp5* and *Lco1* and *Ca8*. Linkage disequilibrium between loci can be caused by mixing two different populations when equilibrium has not yet been achieved. Genetic drift resulting from small population size can also disrupt equilibrium.

For both the Mimbres River samples (collected in 2018) and the samples from the captive population at Southwestern Native ARRC collected in 2013 and 2016, observed heterozygosity exceeded expected heterozygosity (Table 1). This result is reflected in the observed negative values for the average inbreeding co-efficient. Allelic diversity was lower in these 2018 and Southwestern Native ARRC samples compared to previous (2010 and 2009) collections from both the Mimbres (including Cooney Place) and Moreno Springs populations. Gene diversity was virtually identical between all collections and observed heterozygosity was highest in the 2018 Mimbres River sample. There are a number of potential explanations for an excess of heterozygotes in a population including: (1) occurrence of a population bottleneck (i.e., reduction in population size) and (2) gene flow between populations with different allele frequencies. When a population has been reduced in size, rare alleles are initially lost through genetic drift. Hence, after a population bottleneck, there will be a deficit of rare alleles compared to the number expected in a population that is at equilibrium. However, rare alleles contribute little to expected heterozygosity, so there will be an excess of observed heterozygosity compared to a population at equilibrium that has an equivalent number of alleles (Cornuet and Luikart 1996). Allele frequencies also differ between the hatchery population and the population in the Mimbres River likely due to genetic drift (which is stronger when the population size is small). Significant values of pairwise F_{ST} between the Mimbres and the Southwestern Native ARRC samples are

consistent with this explanation.

Genetic effective size was lower in the Mimbres River population (2018) compared to previous estimates made in 2010 (Osborne et al. 2012). Genetic effective size was also lower in the Southwestern Native ARRC sample that was used to stock the Mimbres in 2013 compared to samples from Southwestern Native ARRC collected in either 2010 or 2016. Together these results are consistent with a population bottleneck having occurred in the Mimbres River population, presumably associated with the Silver fire and subsequent stocking from the refugial population at Southwestern Native ARRC.

The F_{ST} values provided no evidence of different allele frequencies between the sample collected in the upper Mimbres and those from the Nature Conservancy and Department of Game and Fish properties (Table 2). However, only 8 samples were collected from the upper Mimbres. The detection of PIT-tagged fish in the upper Mimbres confirms that movement is occurring between these sites. The 2013 Silver fire and associated impacts are thought to have eliminated Chihuahua chub from the Mimbres, so the majority of individuals now present are either stocked fish or their progeny. This highlights the importance of maintaining a genetically diverse captive population as well as protecting the Mimbres basin populations of Chihuahua chub. There were numerous significant F_{ST} values between other collections that reflect allele frequency differences (likely a result of genetic drift) between these samples. Conservation efforts for Chihuahua chub should continue to focus on protecting and building the Mimbres River population, as well as exploring whether Chihuahua chub populations could be established elsewhere within the drainage. The exceedingly small range of Chihuahua chub makes it extremely vulnerable to stochastic events.

Acknowledgements

Bryan Ferguson and Virginia Seamster (New Mexico Department of Game and Fish), Andy Dean and Wade Wilson (US FWS), Dustin Myers and Jerry Monzingo (US Forest Service), are gratefully acknowledged. Funding for this project was provided by New Mexico Department of Game and Fish Share with Wildlife Program and a State Wildlife Grant (T-60-R-1). UNM IACUC: Protocol 19-200808-MC.

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Figure 1. Collection localities for Chihuahua chub on the Mimbres River are shown by black dots.

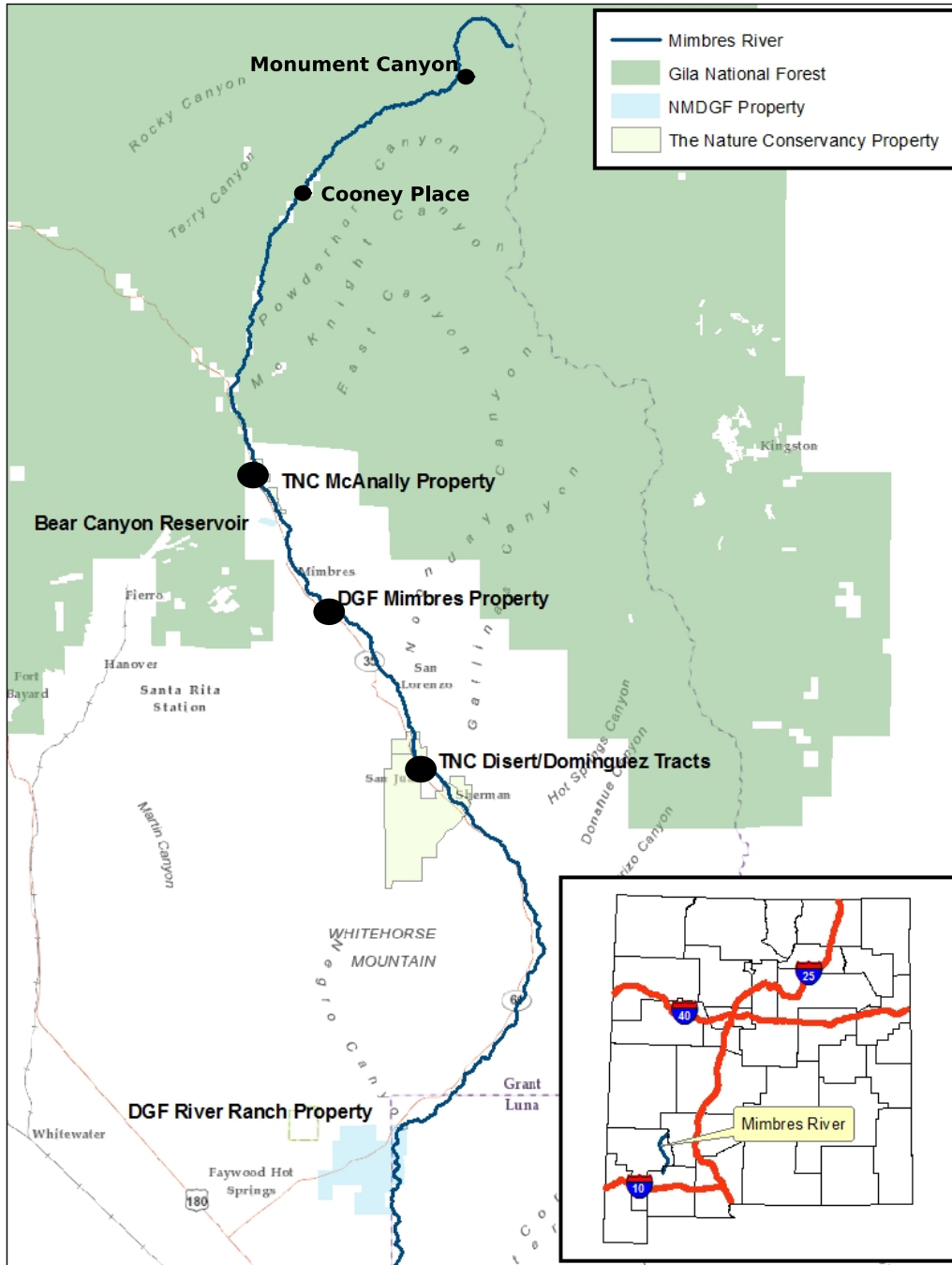


Table 1. Genetic diversity statistics for collections of Chihuahua chub from the Mimbres River population and the captive stocks held at Southwestern Native Aquatic Resource and Recovery Center. Sample size (n), allelic diversity (i.e., average number of alleles across loci; N_{AC}), Nei's gene diversity (H_{exp}), observed heterozygosity (H_{obs}) and inbreeding co-efficient (F_{IS}) are provided. Linkage disequilibrium estimates of effective number of breeders (N_{eD}) are also given. Lower and upper 95% confidence intervals are provided in parenthesis.

Population	n	N_{AC}	H_{exp}	H_{obs}	F_{IS}	N_{eD}
2018 Mimbres	45	4.8	0.550	0.618	-0.088	76 (34-920)
2016 SNARRC	33	3.5	0.500	0.529	-0.022	21 (10-68)
2013 SNARRC	50	3.7	0.506	0.555	-0.063	14 (6-39)
2010 SNARRC	60	3.7	0.500	0.476	0.088	24 (15-42)
2010 Moreno	22	5.2	0.561	0.552	0.054	33 (10- ∞)
2010 Mimbres	54	5.4	0.546	0.537	0.023	188 (70- ∞)
2009 Cooney	34	5.2	0.583	0.517	0.122	75 (32- ∞)

Table 2. Values of F_{ST} for all pairs of populations (P-values are above diagonal). Significant values are in bold. Significant values are indicative of different allele frequencies between samples. Mimbres 2018 samples were collected from The Nature Conservancy and New Mexico Department of Game and Fishes properties on the Mimbres River.

	Upper Mimbres 2016	Mimbres 2018	SNARRC 2016	SNARRC 2013	SNARRC 2010*	Moreno 2010*	Mimbres 2010*	Cooney 2009*
Upper Mimbres 2016	--	0.252	0.355	0.389	0.047	0.008	0.001	0.001
Mimbres 2018	0.006	--	0.001	0.001	0.001	0.001	0.001	0.001
SNARRC 2016	0.004	0.022	--	0.026	0.001	0.001	0.001	0.001
SNARRC 2013	0.001	0.03	0.015	--	0.001	0.001	0.001	0.001
SNARRC 2010*	0.029	0.047	0.054	0.06	--	0.001	0.001	0.001
Moreno 2010*	0.046	0.081	0.066	0.06	0.100	--	0.001	0.001
Mimbres 2010*	0.103	0.114	0.129	0.134	0.153	0.104	--	0.001
Cooney 2009*	0.103	0.112	0.142	0.14	0.126	0.115	0.047	--

* Data from Osborne et al. (2012)