



Conservation genomics of the imperiled Leon springs pupfish, *Cyprinodon bovinus*, across temporal and spatial scales

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Abstract

The Leon Springs pupfish, *Cyprinodon bovinus*, is federally listed as endangered and currently confined to spring-fed pools on the Diamond Y Spring Preserve in Pecos County, Texas, USA. A refuge population is maintained by U.S. Fish and Wildlife Service in Dexter, New Mexico, USA because of ongoing threats from hybridization with the introduced congener, the sheepshead minnow, *C. variegatus*. To inform continued conservation and management planning for this species, a comprehensive conservation genomics study using double digest restriction-site associated DNA sequencing was performed. No evidence of contemporary hybridization or recent introgression between *C. bovinus* and *C. variegatus* was found. The refuge population was significantly differentiated from wild samples collected at two locations, and the two wild populations (less than 1 km apart) also exhibited significant heterogeneity in allele frequencies. Estimates of within population diversity were smaller for wild locations than the refuge population. Correspondingly, the wild populations' contemporary effective population size estimates were smaller than the refuge's effective population size estimates (145–262 versus 679). Temporal analyses, involving genetic data collected from samples obtained in 2013, suggest an increased magnitude of drift acting on the wild populations relative to the reserve population. Based on the results of this study, it appears that steps taken in the early 2000s to eradicate genetically admixed individuals from the wild populations were successful. However, habitat fragmentation and small effective population sizes of the wild populations have led to accelerated drift and decreasing levels of within population genetic diversity. This may jeopardize the long-term persistence of *C. bovinus*.

Keywords Genetic drift · Temporal genetic analysis · Desert fishes · Next generation sequencing · Genetic management

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Introduction

Genetic monitoring programs have become important tools for conservation and management of many endangered freshwater fishes (Schwartz et al. 2007; Bernos et al. 2020). Many of these species have short generation times, small population sizes and fragmented distributions, making them at risk of rapidly losing genetic diversity due to genetic drift and inbreeding (Fagan 2002). Furthermore, the widespread introduction of non-native species poses threats in terms of competition and predation and, in the case of introduced congeners, hybridization (Perry et al. 2002). Hybridization can result in the introduction of maladaptive variation and in a worst-case scenario the genetic swamping and loss of native species (Allendorf et al. 2001; Todesco et al. 2016). To combat these problems, refuge populations are often

established and maintained in dedicated facilities, which can be used to augment or replace wild populations if necessary (Meretsky et al. 2006). However, refuge populations must be managed carefully to ensure that they maintain sufficient levels of standing genetic variation and remain genetically similar to wild populations (Love Stowell et al. 2017; Novak et al. 2020). Genomic assessments can provide insight into all of these processes by allowing researchers to assess the standing genetic variation (adaptive potential) of endangered species, detect inbreeding and hybridization, monitor captive breeding or reintroduction programs, and enable the identification of fine-scale population structure, all of which are essential for developing targeted conservation plans (Meffe and Vrijenhoek 1988).

One group of freshwater fishes of particular conservation concern are pupfishes in the genus *Cyprinodon* (Family Cyprinodontidae) in southwestern North America (swNA). The genus contains 31 currently recognized species in swNA (southwestern United States and northern Mexico; Echelle and Echelle 2020). Phylogenetic analyses suggest that a common ancestor to the group was widespread across a wetter swNA during the Pliocene (~3 MYA), but as the region became arid, populations became isolated in pockets of habitat that remained, driving speciation in the group (Echelle et al. 2005; Hoagstrom and Osborne 2021). Currently, many pupfish species in swNA have extremely limited ranges, with fragmented distributions and small population sizes (Echelle 2008). Because swNA is an arid region, human water usage has become a major threat to naturally limited pupfish habitat (Baugh and Deacon 1988; Lewis et al. 2013; Black et al. 2017). To address these issues, many of these habitats have been the focus of protection by state or federal agencies or non-government organizations and other conservation practices (Minckley and Deacon 1991). Furthermore, some species are now maintained in ex-situ refuge populations as a buffer against continued decline in the wild, including *C. diabolis* (Devils Hole pupfish), *C. elegans* (Comanche Springs pupfish) and the focal species of this study, *C. bovinus* (Leon Springs pupfish, Fig. 1; Baugh and Deacon 1988; Black et al. 2017).



Fig. 1 *Cyprinodon bovinus*, male, collected from Diamond Y pool within the Diamond Y Spring Preserve, Pecos Co., TX

Cyprinodon bovinus is currently restricted to a spring-fed system on the Diamond Y Spring Preserve (DY) in Pecos County, Texas. Like many other species of pupfish, *C. bovinus* is sexually dimorphic, with males typically reaching lengths of around 2.5 to 3.8 centimeters and females reaching slightly smaller sizes (Kennedy 1977). The species is also dichromatic, with large breeding male *C. bovinus* exhibiting a yellowish hue dorsally with flecks of blue and yellow on the body and a dark margin on the caudal fin (Fig. 1), while females have visible dark barring and are drabber. Breeding usually occurs during the spring and early summer months and involves large males guarding territory around the water's edge, with females selecting males and laying eggs in their territories to be fertilized (Al-Shaer et al. 2016; Bernos et al. 2020). *Cyprinodon bovinus* has a short generation time, reaching maturity between 4 and 6 months and living on average ~1 year (Kennedy 1977).

Historically, *Cyprinodon bovinus* inhabited the clear, cool, spring-fed waters that occurred throughout the length of Leon Creek (Pecos Co., Texas), from the headwaters at Leon Springs (the type locality; Baird and Girard 1853) downstream towards the confluence with Comanche Creek (Echelle and Miller 1974; Hendrickson and Cohen 2024). However, due to issues with water usage, the species was extirpated from much of its range and thought to be extinct (Hubbs 1957) until it was rediscovered in the DY area (Minckley et al. 1991). The known historical distribution of *C. bovinus* once encompassed about 25 km of the Leon Creek basin (Echelle and Echelle 1997). However, by the 1970s, it occurred only in an 8–10 km segment that includes a 2-km upstream watercourse and a 4-km downstream watercourse in DY (Hubbs et al. 1978).

In 1980, the species was listed as endangered by the US Fish and Wildlife Service (USFWS; 45 FR 54678), and a recovery plan was adopted in 1985 (USFWS 1985), and includes the maintenance of a refuge population at the Southwestern Native Aquatic Resources and Recovery Center (SNARRC) in Dexter, New Mexico. *Cyprinodon bovinus* readily hybridizes with the widely introduced sheepshead minnow *C. variegatus* (Kennedy 1977; Echelle and Echelle 1997), and much of the conservation effort since 1976 has been concerned with culling *C. variegatus* and admixed individuals, followed by restocking with pure *C. bovinus* (reviewed in Black et al. 2016). Currently, the wild population seems to occur only in the upper watercourse (see Methods), and it is the result of a restoration effort in 1998–2001, which involved nearly complete elimination of all pupfish via chemical treatment, followed by replacement using ~2,000 fish from SNARRC (Echelle et al. 2004).

Black et al. (2017) examined the degree of contemporary hybridization between *Cyprinodon variegatus* and *C. bovinus* as well as morphological and genetic divergence

between the refuge and wild populations of *C. bovinus* in DY using samples collected in 2013. They found significant differences in morphology and genetic variation between the refuge and the wild, but no significant evidence of contemporary hybridization between *C. bovinus* and *C. variegatus*. The study provided critical data as a first pass genomic assessment of *C. bovinus* but was a snapshot in time of processes (drift and hybridization) that are dynamic and span multiple generations. Black et al. (2017) also collected all individuals in DY from a single site in the upper range of the DY spring fed pool system. Historically, strong groundwater flow maintained connection between spring fed pools in DY, but there have been local and state-wide droughts that have left some pools completely disconnected for a portion of each summer since 2018 (Smith and Bean 2023; Ryan Smith, personal communication), which, given the species short generation time, is potentially enough time for significant divergence to occur. Furthermore, levels of interactions between potentially hybridizing species are dynamic and *C. variegatus* is still widely distributed in the Trans-Pecos region of Texas (Hendrickson and Cohen 2024), making hybridization a continuing threat. Therefore, this study used fine-scale contemporary sampling (one additional site was added beyond that examined by Black et al. 2017) to look for evidence of current hybridization between *C. bovinus* and *C. variegatus* and assess patterns of genetic diversity within *C. bovinus*. Temporal and fine-scale geographic comparisons enable a comprehensive assessment of population structure in the wild and provide an opportunity to better understand processes that might cause the wild and refuge populations to diverge genetically. Therefore, the data presented in Black et al. (2017) were utilized to assess changes in diversity over time.

Methods

Field sampling

Fin clips were collected from *Cyprinodon bovinus* at DY in March of 2020 and from the USFWS refuge population (hereafter CREF) at the SNARRC in September 2021. Four discrete sampling locations in DY, where *C. bovinus* were known to be present, were identified in advance (Ryan Smith, personal communication). However, one site (Lower Monsanto Pool) had unexpectedly desiccated, leaving no habitat, and the species was undetected at a second site (the lower reach of Diamond Y Draw below Euphrasia Spring), despite exhaustive sampling. Thus, fish were sampled from only two locations in DY: Karges Springs (KGS) and the Diamond Y Pool (DY Pool), which are only separated by ~0.5 km (Fig. 2). Fin clips were also collected from *C.*

variegatus from four locations across the species' range in Texas. All tissues were immersed in 20% salt-saturated dimethyl sulfoxide (DMSO) buffer (Seutin et al. 1991) upon collection and stored at room temperature until time of extraction. To capture individuals, a seine net was used when sites were large enough and dip nets and minnow traps at all other times. A sterile razor blade was used to remove the upper 25% of the caudal fin from each captured fish and all tools were cleaned using deionized water and 10% bleach solutions between fish. After handling, individuals were held temporarily in a bucket filled with water from their habitat, which was oxygenated using a bubbler, and placed in the shade to ensure survival after release. Individuals of *C. variegatus* were collected as described. Tissues were collected, and fish were euthanized with an overdose of clove oil and preserved in 95% ethanol. Sampling for *C. bovinus* within Pecos County was conducted under Federal permit number TE814933. Samples of *C. variegatus* were collected throughout Texas with permission from Texas Parks and Wildlife Department (permit numbers SPR-0614–111 and SPR-1010-173). All animal use and care was conducted under TAMU IACUC protocols 2021–0001, 2023–0216.0.

Genomic library preparation and sequencing

Genomic DNA was extracted from fin clips using Mag-Bind Blood and Tissue HDQ DNA extraction kits (Omega Bio-tek) and reduced representation libraries were assembled following a modified version of the double-digestion restriction-site associated DNA sequencing (ddRADseq; Peterson et al. 2012). Briefly, libraries were assembled using genomic DNA digested with the restriction enzymes *EcoRI* and *MspI* (New England Biolabs). For each sample, one of 48 barcoded adapters was ligated to the *EcoRI* site and a common adaptor ligated to the *MspI* site. Ligation reactions were pooled into one of four indexed libraries (Table S.2.1) and amplified using PCR for 14 cycles to incorporate flow cell adapters containing sequence primer binding sites, Illumina flow cell binding sites and an index sequence. Libraries were paired end sequenced on a single lane of an Illumina HiSeq 4000 (2 × 150 bp; Genewiz, Inc.).

Bioinformatics and filtering

For downstream analyses, three ddRAD datasets were produced and the code is available at https://github.com/marinegenomicslab/C_bovinus_2025. One dataset was created for hybrid analysis and contained all contemporary *Cyprinodon bovinus* and *C. variegatus* individuals. The second dataset included only contemporary *C. bovinus* individuals and was used to look at patterns of contemporary genetic structure and diversity. Finally, the third dataset combined SNP data

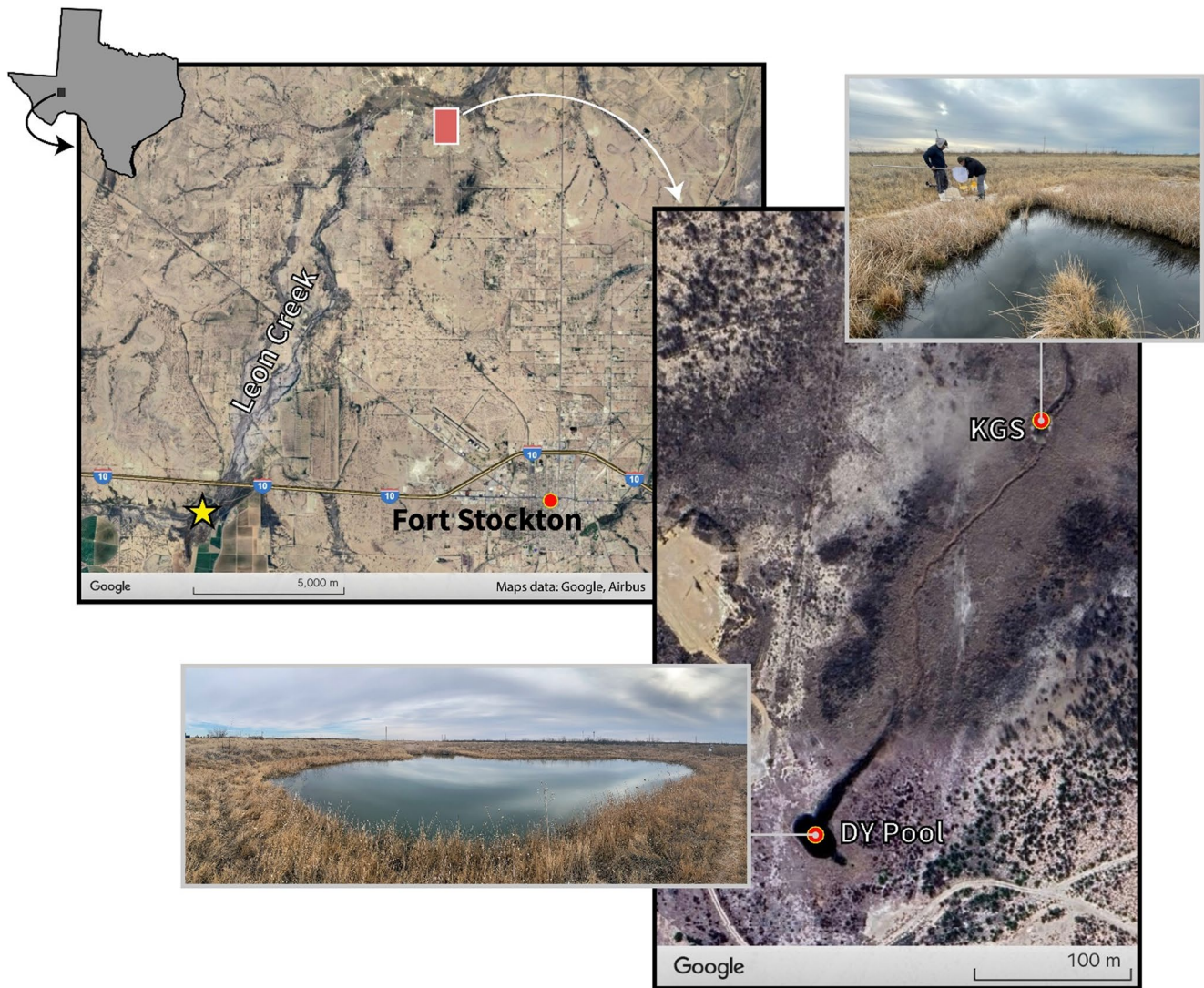


Fig. 2 Map of sampling locations for *Cyprinodon bovinus* within Diamond Y Spring Preserve (DY Pool, Diamond Y Pool; KGS, Karges Springs). Star denotes type locality. Satellite imagery available from Google Earth

from contemporary individuals of *C. bovinus* with SNP from historical samples of *C. bovinus* provide by Andrew Black (Black et al. 2016, 2017) to assess temporal changes in diversity using the historical data.

To create the datasets, raw Illumina reads for *Cyprinodon bovinus* and *C. variegatus*, and reads from Black et al. (2017), were demultiplexed using *process_radtags* v2.62 (Catchen et al. 2011) and processed using the DDOCENT v2.9.1 pipeline (Puritz et al. 2014). For each dataset, appropriate demultiplexed sequences were quality trimmed with *fastp* v0.23.4 and stacked into orthologous scaffolds using CDHIT v4.8.1 with a range of K1, K2 and c-values to determine which created an optimal de novo reference. Quality trimmed sequences were mapped onto the appropriate species-specific de novo reference with *BWA* v0.7.17 (Li 2013) and genomic variants were scored and compiled

into a VCF file using *Freebayes* v1.3.7 (Garrison and Marth 2012). Variants were then quality filtered using a combination of VCFtools v0.1.17 (Danecek et al. 2011) and various BASH and Perl scripts. Filtering followed guidance of O'Leary et al. (2018) to iteratively remove low quality or artefactual SNPs, potential paralogs and low-quality individuals resulting in datasets with a minimum genotype depth of 3 and an average genotype depth of 20, a maximum missing data per locus of 5% and a maximum missing data per individual of 20%. A minor allele frequency cut-off of 0.01 was implemented for temporal analysis. Using *rad_haplotype* v1.1.0, SNP variants found in the same contig were phased into microhaplotypes (hereafter loci) following (Willis et al. 2017), rather than thinning the data to one SNP per contig. Pair-wise relatedness (Wang 2014) was used to confirm duplicate pairs (technical replicates) and

identify potentially related individuals in *adegenet* v.2.5.1 (Jombart 2008). A Bayesian approach for outlier detection was implemented using BAYESCAN v2.0 (Foll and Gaggiotti 2008), with 30 pilot runs of 5,000 iterations, followed by a burn in of 50,000 iterations and 500,000 iterations sampled 5,000 times. Significance was assessed at a q -value of 0.05. In addition, *OutFLANK* (Whitlock and Lotterhos 2015) was used to screen for outliers (q -value < 0.05), with the inferred distribution of neutral F_{ST} having the highest and lowest 5% of values trimmed. While outlier loci provide evidence for localized adaptation, they may cause bias in estimating some population parameters. Because the focus of this study was to understand patterns of population structure and drift, significant outliers were removed from downstream analyses.

Hybrid analysis

Using the dataset, reads were mapped to a multispecies reference and 4,734 loci with alleles that were completely fixed in alternative states between the species (diagnostic loci) were used to identify a set of individuals that were pure *Cyprinodon bovinus* or pure *C. variegatus*. Individuals were then assigned back to one of five categories, i.e., the two parent species and three hybrid classes (F1, F1 x *C. bovinus*, and F1 x *C. variegatus*), using the Bayesian approach implemented in NEWHYBRIDS v2.0 (Anderson and Thompson 2002). A secondary approach for hybrid detection was implemented in *adegenet* in R. For this approach, 30 F1 hybrids, 30 F1 x *C. bovinus* backcrosses, and 30 F1 x *C. variegatus* backcrosses were simulated. Hybrid individuals and sampled individuals were then plotted using PCA in *adegenet*, with missing data imputed using empirically derived allele frequencies and the function `scaleGen()`, to see how sampled individuals grouped with simulated individuals. Finally, a subset of individuals from both species was selected haphazardly for sequencing 681 bp of the mitochondrially encoded (mtDNA) cytochrome oxidase subunit I gene (COI), using universal primers (Ward et al. 2005). Species identities based on COI were compared to species identities based on nuclear data to validate hybrid analyses and assess for potential mtDNA introgression.

Population genetic analyses

For the contemporary *Cyprinodon bovinus* dataset, a single-level analysis of molecular variance (AMOVA) was carried out in ARLEQUIN (Excoffier and Lischer 2010), using a locus-by-locus framework to account for uneven levels of missing data across loci (Weir and Cockerham 1984).

Significance was determined at an α -level of 0.05 by permuting individuals among locations 10,000 times and 95% confidence intervals determined using 20,000 bootstrap replicates. Post-hoc pairwise F_{ST} was then estimated in ARLEQUIN with significance determined as above and 95% confidence intervals determined using 20,000 bootstrap replicates. To assess the number of genetic groups present in the data, discriminant analysis of principal components (DAPC; Jombart et al. 2010) was implemented in *adegenet* using K -means, testing K from 2 to 6 (twice the number of sampled units). Missing data was imputed as above and the number of clusters was selected by comparing Bayesian information criterion values (BIC). The optimal number of PCs to retain was then determined using cross validation and membership probabilities for each individual to the inferred clusters were calculated. Effective population size (N_E) was estimated for each genetic group using the linkage disequilibrium approach with an allele frequency cut-off of 0.01 and 95% confidence intervals determined using 1,000 jackknife replicates, as implemented in NEESTIMATOR v2.1 (Do et al. 2014). To account for potential physical linkage between pairs of loci, all contemporary N_E estimates were adjusted following Waples et al. (2016) based on the haploid number of chromosomes for *Cyprinodon* (24; Uyeno and Miller 1971; Echelle and Connor 1989). Mean expected heterozygosity (H_E ; Nei 1973) and rarefied allelic richness (A_R ; El Mousadik and Petit 1996) were estimated using *hierfstat* (Goudet 2005). Friedman's tests were conducted on both metrics to assess for homogeneity among locations using R (R Core Team 2025), and post-hoc paired Wilcoxon signed-rank tests conducted to assess pairwise differences using the R package *coin* v.1.3.1 (Hothorn et al. 2008).

For the contemporary/historical dataset from the refuge and DY pool populations, F_{ST} was estimated using ARLEQUIN between samples over time: contemporary refuge (CREF) vs. historical refuge (HREF) and contemporary wild (CWILD) vs. historical wild (HWILD), and across samples taken at approximately the same time: CREF vs. CWILD and HREF vs. HWILD, with significance and 95% confidence intervals determined as above. Clustering was also visualized using PCA as implemented in *adegenet* with missing data imputed as above. Lastly, estimates of temporal N_{Et} (Waples 1989) and confidence intervals were made for the refuge (across seven generations) and wild (across seven generations) using Pollack's F (Pollak 1983) and a minimum allele frequency of 0.01 in NEESTIMATOR. To assess for potential changes in within group diversity over time in the wild and at the refuge, H_E and A_R were estimated and compared using paired Wilcoxon signed-rank test as above.

Table 1 AMOVA results (averaged over 4,567 neutral loci) for three contemporary *Cyprinodon bovinus* populations; showing sum of squares (SS), variance components (VC) and percentage of variance (%)

	SS	VC	%
Among locations	5,506.10	42.69	5.29
Within locations	10,6647.92	764.82	94.71
Total	112,154.02	807.51	

Table 2 Pairwise F_{ST} estimates between contemporary *Cyprinodon bovinus* populations below the diagonal with p -values above the diagonal. Locations are Karges springs (KGS), diamond Y pool (DY pool), and the refuge population (CREF)

	KGS	DY Pool	CREF
KGS	-	0.0004	<0.0001
DY Pool	0.0065 (0.0059–0.0084)	-	<0.0001
CREF	0.0711 (0.0670–0.0734)	0.0637 (0.0603–0.0664)	-

Results

Hybrid analysis

After filtering, the final hybrid dataset contained 112 individuals genotyped at 4,734 loci, with an average of 6.7 alleles (microhaplotypes) per ~300 bp locus. No hybrids were detected with NEWHYBRIDS and no empirical samples grouped with simulated hybrids or backcrosses using PCA (Figure S1). Therefore, no individuals were removed from the contemporary *Cyprinodon bovinus* dataset. Individual species identities inferred from COI and nuclear data corresponded, providing no evidence of mtDNA introgression.

Population genetic analyses

After filtering, the contemporary *Cyprinodon bovinus* data contained 74 individuals (from KGS, DY Pool, and CREF) genotyped at 4,597 loci, with an average of 2.4 alleles (microhaplotypes) and a range of 2–9 alleles per ~300 bp locus. Thirty loci were found to be putatively under selection using BAYESCAN (no outliers were detected with OUTFLANK) and removed to avoid confounding demographic processes with selective processes, leaving 4,567 neutral loci. Two pairs of duplicate individuals had relatedness values greater than

Table 4 Post-hoc Wilcoxon test for H_E with test statistic (W) and p -values for *Cyprinodon bovinus* between wild populations and the refuge population. Locations are Karges springs (KGS), diamond Y pool (DY pool), and the refuge population (CREF)

	W	p -value
KGS x DY Pool	-2.01	0.045
KGS x CREF	-11.57	<0.001
DY Pool x CREF	-11.40	<0.001

0.99, leading to the removal of one individual from each of the pairs. Two pairs of related individuals were found in the DY Pool ($r=0.46$ and 0.52). The final dataset contained 72 individuals genotyped across 4,567 loci (DY Pool, $N=21$; KGS, $N=20$; CREF, $N=31$) with an average allele per locus count of 2.35 (range 2–9). The component of genetic variation attributable to differences among locations was highly significant ($\%V=5.29$, $P<0.0001$; Table 1). The post-hoc estimate of pairwise F_{ST} was significant between all locations but were an order of magnitude greater in comparisons involving CREF relative to the comparison involving the two wild locations (Table 2). The minimum BIC value was obtained for $K=2$ (Figure S2), concordant with a refuge group and a wild group (Figure S3), and 100% of individuals assigned back to their group of origin.

Estimated contemporary N_E for KGS, DY Pool, and CREF were 262, 145, and 698 respectively (Table 3). Estimates of H_E were 0.32, 0.32 and 0.35, and estimates of A_R were 2.05, 2.08, and 2.21, for KGS, DY Pool, and CREF respectively (Table 3). Friedman's tests for differences in within population diversity among populations were significant for H_E ($H_E: X^2_{(2)}=101.77$, $P=2.2 \times 10^{-16}$) but not A_R . All post-hoc Wilcoxon tests for H_E were significant with CREF having significantly higher diversity than the wild populations (Table 4).

The final contemporary/historical dataset contained 117 individuals (HWILD, $N=24$; HREF, $N=21$; CWILD, $N=41$; CREF, $N=31$) genotyped at 3,281 shared loci, with an average of 2.09 alleles per locus. Sixty-five loci were found to be putatively under selection and removed to avoid confounding demographic processes with selective processes, leaving 3,216 neutral loci. Pairwise estimates of F_{ST} were nearly an order of magnitude greater for spatial comparisons (HWILD vs. HREF; CWILD vs. CREF) than temporal comparisons (HREF vs. CREF; HWILD vs. CWILD).

Table 3 Contemporary effective population size (N_E) estimates estimated using the linkage disequilibrium approach, with lower and upper 95% confidence intervals obtained using a jackknife method, point estimates, and mean observed heterozygosity (H_O), mean expected heterozygosity (H_E), and mean allelic richness (A_R) for all three contemporary *Cyprinodon bovinus* populations including sample numbers (n); locations are Karges springs (KGS), diamond Y pool (DY pool), and the refuge population (CREF)

	n	N_E			H_O	H_E	A_R
		Lower	Point	Upper			
KGS	20	170	262	546	0.329	0.320	2.049
DY Pool	21	73	145	1,604	0.334	0.323	2.081
REF	31	425	679	1,649	0.353	0.353	2.208

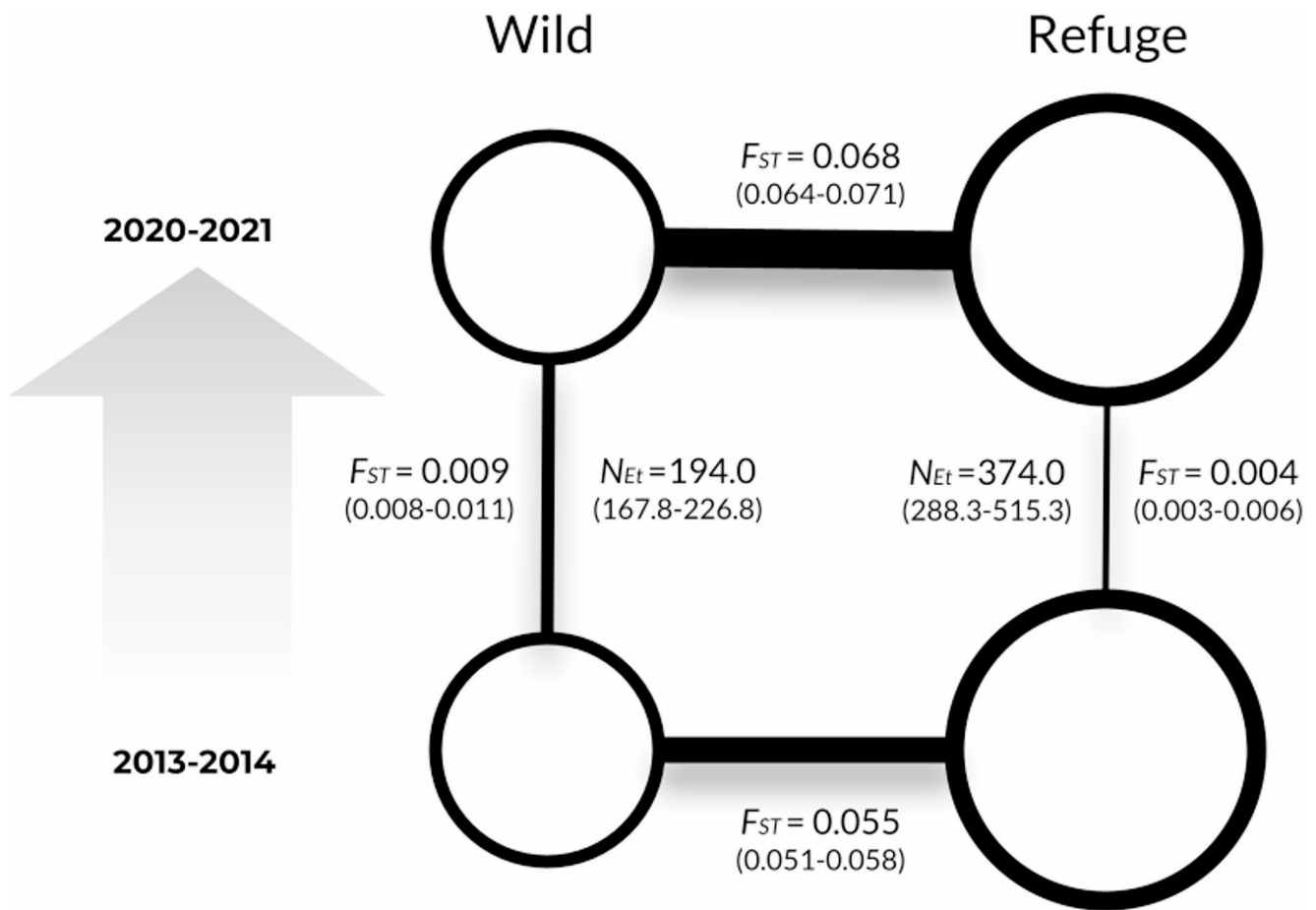


Fig. 3 Results of comparisons among historical and contemporary *Cyprinodon bovinus* samples collected in the wild and at the refuge, population differentiation estimated using fixation indices (F_{ST}) and

temporal effective size (N_{Et}). Line thickness denotes relative magnitude of F_{ST} . Confidence intervals for all estimated parameters are in parenthesis

The pairwise F_{ST} estimate between HREF and CREF was smaller than the estimate between HWILD and CWILD, and the former was not significant. The confidence intervals for all estimates did not include zero. Furthermore, the temporal samples from the refuge showed greater overlap in the PCA than the temporal samples from the wild, with the more recent wild sample occupying less space than the historic sample (Figure S4). Temporal estimates of N_{Et} were nearly two times larger for the refuge than the wild (194 vs. 374) and confidence intervals did not overlap (Fig. 3). Wilcoxon tests for H_E and A_R (Table S1) were significant ($p < 0.001$) indicating decreases in diversity over time in the wild population, but non-significant for the refuge.

Discussion

No contemporary hybrids or evidence of introgression (i.e., mismatch between nuclear and COI identity) were detected in this study, despite the presence of admixed populations of

Cyprinodon variegatus and *C. pecosensis* (Pecos Pupfish) at localities that are within 90 km of DY (Echelle and Echelle 1997). These results agree with the findings in Black et al. (2017) and suggest that extensive culling in DY was effective. No *C. variegatus* were encountered while sampling in DY, indicating that introduced *C. variegatus* in the surrounding areas have not yet returned to DY. Hybridization is common threat to species in the genus *Cyprinodon* (e.g., *C. elegans* and *C. variegatus*, Echelle and Echelle 1997; *C. pecosensis* and *C. variegatus*, and *C. bovinus* and *C. variegatus*, Echelle et al. 1987), and therefore, continued monitoring of Diamond Y for the presence of non-native pupfishes and admixed individuals will be necessary.

Population differentiation was found in the contemporary analysis among the wild locations and refuge populations, concordant with Black et al. (2017), but also between the wild populations sampled. Finding divergence among isolated groups of *Cyprinodon* in the wild is common and estimated levels of divergence between the wild samples here was low relative to values reported in other studies

(e.g., Duvernell and Turner 1998; Martin 2010; Black et al. 2024). Unlike those studies, divergence in *C. bovinus* has occurred despite the short geographic distance (~0.5 km) and periodic hydrologic connection between the locations. Likewise, individuals occupying those locations all descend from a single captive gene pool less than 25 years ago (see Black et al. 2016, 2017 for a review). Given the short generation time of this species (<1 year, Kennedy 1977) and the relatively small values of N_E reported here, theoretically little time would be required for genetic drift to create significant differentiation between populations (Wright 1931), if the effective number of migrants were persistently low. Prolonged periods of drought have affected the Trans-Pecos regions over the last two decades while groundwater usage has increased, leading to decreases in freshwater habitat quantity and quality, and habitat fragmentation across the region (Birdsong et al. 2021). Historically, groundwater flow to Diamond Y Spring maintained more consistent connections between DY Head Pool and the downstream KGS, potentially allowing continual gene flow between the locations to counteract drift. Since 2018 this flow has decreased, with the two habitats completely isolated, in terms of surface water, for approximately eight months per year. Furthermore, levels of flow when present are lower than they were historically, with depths of three inches or less in parts of the connecting channel, potentially limiting available habitat and/or movement between the two areas. (R. Smith, personal observation).

A common concern in conservation planning is understanding the benefits of supplementation relative to the risk of reduced fitness when mixing refuge and wild populations (Bell et al. 2019). This study detected differences between the wild and refuge both in terms of standing contemporary genetic diversity (H_E) and allelic composition, with the latter difference increasing over time. In most cases increasing divergence over time between wild and refuge populations are attributed to captive breeding programs that may be constrained in terms of the number of reproducing individuals available, the lack of natural selection in the captive environment, and domestication effects (Frankham et al. 2014). For *Cyprinodon bovinus*, however, several lines of evidence suggest that increasing divergence is attributable to accelerated drift in the wild. The estimate of temporal N_{E_T} , which is the harmonic mean of N_E between the two sampling points (Waples 2005), was two times smaller for the wild than the refuge, indicating consistently smaller N_E and/or larger annual variation in N_E in the wild as compared to the refuge. Similarly, small estimates of contemporary N_E were found in wild populations relative to the refuge. Furthermore, the historical and contemporary wild samples were significantly differentiated, and levels of within population genetic variation have decreased significantly over time. On the other

hand, measures of differentiation between the historical and contemporary refuge samples were of a smaller magnitude and levels of within population genetic variation have remained stable, suggesting that the refuge population is being maintained in a way that reduces the effects of genetic drift and helps to maintain genetic diversity.

While the overall results of this study suggest that contemporary hybridization is currently not a threat for *Cyprinodon bovinus*, low levels of standing variation in the wild may be cause for concern, and genetic rescue via assisted migration (Whiteley et al. 2015) may soon be necessary. Temporal analyses conducted here suggest that if the two persisting wild populations remain largely isolated, they will likely continue to diverge while losing genetic diversity. Furthermore, estimates of N_E were in the low hundreds, indicating that wild populations are currently at risk of losing evolutionary potential ($N_E < 1000$) and could soon be at risk from inbreeding depression ($N_E < 100$), increasing the probability of extirpation/extinction (Frankham et al. 2014).

For many freshwater fishes of swNA, distributions are naturally fragmented (Lee et al. 1980), but ephemeral connections between occupied habitats likely allow for short-lived periods of connectivity, and species persistence may be supported by meta-population-like dynamics (Levins 1970). Drought, along with patterns of land and water usage in swNA, has increased the likelihood of the formations of isolated populations in discrete locations and decreased the potential for periods of connectivity, particularly during spring and summer mating (Kennedy 1977; Fagan et al. 2002), thereby decreasing the size of the gene pools that these populations can access (Jaeger et al. 2014). Assisted migration can provide benefits in terms of minimizing inbreeding depression, increasing fitness, and introducing adaptive and potentially adaptive variation (Frankham 2015) and supplementation programs have been successful at helping to maintain variation for a variety of North American freshwater fishes (e.g., *Plagopterus argentissimus*, Chen et al. 2011; *Hybognathus amarus*, Osborne et al. 2012, Archdeacon et al. 2022; *Xyrauchen texanus*, Dowling et al. 2013; Osborne 2024). However, caution will be needed before setting up such a program with *C. bovinus* as there is no direct relationship between levels of divergence and reductions of fitness caused by outbreeding (Edmands et al. 2007). Furthermore, this study cannot address the effects of localized adaptive variation in the wild, or potential domestication effects in the captive populations which can occur in a short number of generations (Finger et al. 2013). Therefore, it will be necessary to consider the possibility of deleterious alleles or maladaptive genotypes being introduced from a separate genetic unit (Dunham et al. 1999; Frankham et al. 2014) and balance that with the positive effects that outbreeding can provide (Edmands et al. 2007). Future studies should employ

whole genome analysis to deal with issues related to adaptive variation and compatibility, and continued monitoring of genetic diversity and hybridization will be required to be part of any future genetic management plan.

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Data availability Raw HiSeq reads are available in the NCBI SRA (BioProject PRJNA1226595). COI sequences were deposited in GenBank accession numbers PV351395–PV351454. Analysis scripts and data are available on GitHub (https://github.com/marinegenomicslab/C/_bovinus/_2025) and DataDryad (<https://doi.org/10.5061/dryad.h44j0zq0k>), respectively. Metadata associated with raw reads are available in the NCBI SRA (BioProject PRJNA1226595).

Declarations

Competing interests The authors declare no competing interests.

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