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The Endangered White Sands pupfish (Cyprinodon tularosa) genome reveals low diversity and heterogenous patterns of differentiation

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Abstract

The White Sands pupfish (Cyprinodon tularosa), endemic to New Mexico in Southwestern North America, is of conservation concern due in part to invasive species, chemical pollution, and groundwater withdrawal. Herein, we developed a high quality draft reference genome and use it to provide biological insights into the evolution and conservation of C. tularosa. Specifically, we localized microsatellite markers previously used to demarcate Evolutionary Significant Units, evaluated the possibility of introgression into the C. tularosa genome, and compared genomic diversity among related species. The de novo assembly of PacBio Sequel II error-corrected reads resulted in a 1.08Gb draft genome with a contig N50 of 1.4Mb and 25,260 annotated protein coding genes, including 95\% of the expected Actinopterigii conserved orthologs. Many of the previously described C. tularosa microsatellite markers fell within or near genes and exhibited a pattern of increased heterozygosity near genic areas compared to those in intergenic regions. Genetic distances between C. tularosa and the widespread invasive species C. variegatus, which diverged \textasciitilde 1.6-4.7 MYA, were 0.027 (nuclear) and 0.022 (mitochondrial). Nuclear alignments revealed putative tracts of introgression that merit further investigation. Genome-wide heterozygosity was markedly lower in C. tularosa compared to estimates from related species, likely because of smaller long-term effective population sizes constrained by their isolated and limited habitat. These population inferences, generated from our new genome assembly, provide insights into the long term and contemporary White Sands pupfish populations that are integral to future management efforts.

Introduction

The integration of genomics into conservation biology has enabled the incorporation of genome-wide diversity metrics within the context of the demographic history of threatened and endangered populations (Leroy et al. 2018). For example, whole-genome sequence analysis of Grey wolf (\textit{Canis lupus}) revealed patterns of low heterozygosity and high inbreeding depression, despite considerable amounts of detected historical introgression from domesticated dog (\textit{C. lupus familiaris}; Gómez-Sanchez et al. 2018). Such genome-wide assessments can provide key insights into historical population dynamics and are critical for guiding contemporary management actions. While overall genetic diversity is a key predictor of long-term population persistence, conservation geneticists have historically relied on tools that provide data from a limited number of anonymous, putatively neutral genetic markers (e.g. microsatellites) to quantify genetic diversity (Wan et al. 2004) and identify conservation units (Moritz, 1994). However, important differences in population and genome-wide patterns of variability can emerge when whole genome data are used compared to genotypes from a few microsatellite loci (Fischer et al. 2017). Specifically, genomics offers a more reliable
approach to achieve a high resolution image detailing patterns of diversity and population differentiation within and among endangered species (Lehmann et al. 2019; Shingate et al. 2020).

The pupfishes are a group of 10 genera of temperature and salinity-tolerant fishes (Cyprinodontidae) with a broad Nearctic and Neotropical distribution (Smith and Miller, 1986; Wildekamp, 1995). Many Nearctic pupfish species are restricted to small isolated desert springs (Miller, 1981), which makes them highly susceptible to groundwater withdrawal (Deacon, 1979), habitat loss (Black et al. 2016) and interspecific hybridization with introduced congeners such as those used as bait by recreational fishers (e.g., C. variegatus; Echelle and Echelle, 1997). Among the pupfish complex, there are currently 12 pupfish species listed as endangered or critically endangered and 14 others identified as threatened or vulnerable (IUCN 2020). Because of this, many pupfish species are commonly maintained in captive breeding facilities or artificial refugia to provide stock for reestablishing or augmenting declining wild populations, or as a hedge against the imminent extinction of natural populations (Koike et al. 2008; Martin et al. 2016; Black et al. 2017).

The present paper focuses on the White Sands pupfish (C. tularosa), which is endemic to the Tularosa Basin of Southern New Mexico. The species is listed as Endangered by IUCN, as threatened by the New Mexico Department of Game and Fish, and is under review as a possible endangered species by the U.S. Fish and Wildlife Service. Ancestral native populations of C. tularosa were first documented during the early 1900s at two ecologically distinct locations: Malpais Spring and Salt Creek. Using allozyme, mitochondrial, and microsatellite markers, Stockwell et al. (1998) found evidence for significant genetic differentiation between the two native populations and demarcated two Evolutionary Significant Units (ESUs). Furthermore, Collyer et al. (2005) demonstrated substantial adaptive body shape variation between individuals collected from the Malpais Spring and Salt Creek ESUs, putatively driven by salinity differences between these two habitats. Since recognition of the genetic and morphologic differences between these two native C. tularosa populations, nine additional microsatellite markers have been developed to aid in the classification and management of these ESUs (Iyengar et al. 2004). Additionally, two refuge populations (Mound Spring and Lost River) were founded by the Salt Creek ESU and established to safeguard against any future and unforeseen population extirpations (Stockwell et al. 1998).

Herein, we present and evaluate a new draft genome assembly for a White Sands pupfish collected from the Upper Lost River population (a part of the Salt Creek ESU) and compare this assembly to genomes of other related fishes. We then leverage the new C. tularosa assembly to determine the genomic position of microsatellites previously assumed to be “neutral” markers in ESU classification. Next, we evaluate the C. tularosa genome for evidence of introgression via historical interspecific hybridization by estimating pairwise genetic distances at nuclear (nuDNA) and mitochondrial (mtDNA) sequences. Finally, we evaluate genome-wide diversity (Heterozygosity, H) for C. tularosa and compare this diversity estimate to several other fish genomes represented among the order Cyprinodontiformes. The overarching goal of this work is to lay the groundwork and genomic infrastructure to enable future high-resolution assessments of the conservation status and evolutionary potential of this imperiled species.

**Methods and Methods**

**Sample Collection, Library Construction and Sequencing**

In September 2018, a deceased heterogametic male (wsp-4) was collected from the upper reach of Lost River (32deg 54' 2.88" N, 106deg 6' 54" W), stored in 95% ethanol and deposited in a -80°C freezer until processing. For Illumina library preparations, a Qiagen DNeasy Blood and Tissue kit was used to extract DNA from fin tissue. For the PacBio library preparations, high molecular weight DNA was extracted from skeletal muscle tissue by Polar Genomics (Ithaca, NY) to meet the integrity requirements for PacBio long read sequencing.

The TruSeq PCR-free protocol was used to prepare the Illumina library in the core genomics center at Purdue University (West Lafayette, IN) using an Illumina NovaSeq with 2x151bp chemistry. The SMRTBell Express Template Prep kit 2.0 was used on genomic DNA sheared to a mean fragment size of 30Kb and sequenced using two 8M single-molecule real-time cells (SMRTcells) of a PacBio Sequel II at the University
Quality Control and Genome Assemblies

Quality assessment of Illumina short-read sequences was conducted by visual examination of fastqc (v.0.11.7; Andrews, 2017) quality score distributions and the presence / absence of sequencing adapters. Following quality assessment, reads were filtered with trim galore (v.0.6.5; Krueger, 2015) by clipping identifiable Illumina adapters, removing low quality bases (Phred <20), and discarding any processed reads that were less than 30-nucleotides in length.

PacBio subread binary alignment mapping files from the two SMRTcells were converted over to fastq file format using the SMRTLink v8.0 command bam2fastq and concatenated. Reads <1Kb or >50Kb in length were removed using the program seqkit (v.0.12; Shen et al. 2016) prior to assembly. To generate a de novo assembly of both nuDNA and mtDNA genomes, the program wtdbg2 was used to create consensus contigs from a fuzzy de Bruijn graph (v.2.2; Ruan and Li, 2020). PacBio reads were sub-sampled at two different coverage levels (50x and 90x) to determine the optimal depth for assembly, as excessive reads can lead to subpar assemblies due to the accumulation of base-call errors. After each depth iteration, quast (v.3.2; Gurevich et al. 2013) was used to generate summary statistics (e.g., N50) to identify the optimal depth.

PacBio subreads from both SMRTcells were then mapped back to the optimal assembly with minimap2 (v.2.11; Li, 2018), followed by consensus calling with arrow (v.2.3; Chin et al. 2013). Following long-read polishing, the quality controlled Illumina short-reads were used for additional error correction using the polca script compiled with the assembler MaSuRCA (v.3.41; Zimin et al. 2017). Changes to consensus assembly quality began to plateau after several iterations of short-read polishing, so three total error-correction iterations were conducted (Figure S1). High coverage heterozygous haplotypes that were assembled as separate contigs were identified and removed using the program purge_haplotigs (v.1.0; Roach et al. 2018). Primary haplotype sequences were then evaluated for contaminants using blobtoolkit2 (v.2.1; Challis et al. 2020) and those that were identified to have been derived from non-target species were removed. The curated genome assembly was then scanned for Benchmarking Universal Single-Copy Orthologs (busco; v.4.0.6; Seppey et al. 2019) to quantitatively assesses genome completeness by evaluating structural integrity (e.g., full:partial genes) and comprehensiveness (proportion of target genes in the assembly). Completeness was evaluated by performing local alignments between the assembly and the Actinopterigii__obd10 database, which contained a set of 3,640 core genes.

The complete mtDNA sequence of C. tularosa was reconstructed using the cleaned Illumina paired-end reads. To help prevent the inadvertent incorporation of Nuclear Translocations (NUMTs) into the mtDNA assembly, we first used coalqc (v.0.1; Patil et al. 2020) and samtools (v.1.17; Li et al. 2009) to extract Illumina reads that aligned to the Devils Hole pupfish (C. diabolis ) mtDNA assembly (NC_030345.1; Lema et al. 2016). The resulting bam file was then converted back to fastq file format with bedtools (v.2.29.0; Quinlan and Hall, 2010) and used alongside the C. diabolis full mitogenome as a backbone for the C. tularosa mtDNA genome assembly with mitobim (v.1.8; Hahn et al. 2013).

Nuclear and Mitochondrial Genome Annotation

After draft genome curation, transposable element families were identified in the C. tularosa mtDNA assembly with repeatmodeler (v.1.09; Smit and Hubley, 2008) and regions matching known proteins were removed from the library (±50 nucleotides) with ProtExcluder (v.1.2; Campbell et al. 2014). Using the custom C. tularosa transposable element library, along with the Zebrafish (Danio rerio ) RepBase library, gene annotation were then accomplished by performing: 1) local alignments of translated protein and transcriptome sequences to the assembly and 2) ab initio gene predictions with the maker pipeline (v.2.31; Cantarel et al. 2008). To help annotate the nuDNA assembly, protein and transcriptome sequences were used from related species: Sheepshead Minnow (C. variegatus ), Zebrafish (D. rerio ) and the Southern Platypfish (Xiphophorus maculatus ). Among all three species, 70,576 protein sequences were downloaded from swiss-prot (UniProt Consortium, 2019) and 54,466 cDNA sequences were obtained from Ensembl (Cunningham et al. 2019) prior to mapping these features to the assembly with blast (v.2.10; Gertz et al. 2006) and screening alignments with...
exonerate (v.2.2.0; Slater and Birney, 2005). Ab initio gene predictions were made using Augustus (v.3.3.2; Hoff and Stanke, 2019) with the D. rerio and X. maculatus training sets as well as two rounds of snap (v.0.15; Korf, 2004). The gene models were then merged and redundancy removed, prior to functional annotation with the UniProtKB database and blastp. The assembled mtDNA sequence was annotated with geseq (v.1.84; Tillich et al., 2017), visualized with ogdraw (Lohse et al. 2013) and protein sequence similarity was compared to the RefSeq C. tularosa assembly (NC_028292.1) with blatn (v.35; Kent, 2002). For a visual representation of the methods used to assemble the C. tularosa nuDNA and mtDNA genomes, see Figure S2.

Assembly metrics

For perspective and to provide a comparative framework, we then sought to compare our new C. tularosa genome assembly to other published reference-enabled Cyprinodontiformes. Two scaffold-level pupfish reference genomes were available at the time of our analyses, C. variegatus (GCF_000732505.1) and the Amargosa pupfish (C. nevadensis; GCA_000776015.1). Three other Cyprinodontiformes were also included, Guppy (Poecilia reticulata; GCF_000633615.1), D. rerio (GCF_002775205.1) and the Annual Killifish (Austrofundulus limnaeus; GCF_001266775.1). For all six genomes (including the new C. tularosa reference), assembly statistics and annotation completeness were assessed by busco and quast using a minimum sequence length of 5Kb. The raw sequence data used for each assembly (i.e., Illumina fastq files for each Biosample) were obtained and cleaned with trim galore, using the same methods for C. tularosa (see above). Draft genome assemblies and raw shotgun sequence data for all species were downloaded from NCBI using the RefSeq (or GenBank) FTP site and Sequence Read Archive (SRA) files were obtained using the sra-toolkit on 15 August 2020.

Microsatellite Identification

Earlier work to define C. tularosa ESUs was based on putatively neutral microsatellite data, along with allozyme and mtDNA D-loop control regions (Stockwell et al. 1998). To help gauge whether any of these ‘neutral’ microsatellite markers might be subject to genetic hitchhiking associated with selection on a functional gene (Maynard Smith and Haigh, 1974), we leveraged our genome assembly to determine the distances from each microsatellite locus to the nearest suspected functional gene(s). To this end, the full microsatellite sequences were obtained from Iyengar et al. (2004) and aligned to the C. tularosa genome with bwa (v.0.717; Li and Durbin, 2009). primersearch (v.6.31; Rice et al. 2000) was used to determine the location of each Stockwell et al. (1998) primer set in the C. tularosa assembly, requiring a zero mismatch rate for both forward and reverse primers. All mapped microsatellite sequences and amplimer lengths were then manually reviewed in Integrative Genome Viewer (IGV) to determine the proximity to neighboring genic regions. For each microsatellite marker, we report the distance (in Kb) to the nearest predicted protein coding gene (or if a microsatellite occurred within a gene, the feature that it was found in) and prior estimates of genetic diversity at each microsatellite marker. This includes estimates of observed (H_{obs}) and expected heterozygosity (H_{exp}) as well as the number of alleles (A) at each locus (Stockwell et al. 1998; Iyengar et al. 2004).

For each ESU, the relationship between these microsatellite loci (N=11), their observed homozygosity and the distance to the nearest predicted gene were then fit using a linear model (H_{obs} ~ Distance + ESU) using the lm function in base R. Because of the non-normal distribution of distances between a given microsatellite and the nearest annotated gene (see Results), we conducted two regressions, one using all of our data associated with the microsatellite loci and another using only data associated with microsatellites found < 50Kb from a gene. In both cases, significance was evaluated by permuting the model 1,000 times and plotting the results with ggplot2 (v.3.3.2; Wickham, 2016). To evaluate neutrality at the two microsatellite markers used in ESU demarcation (WSP-2 & WSP-11), genotypes were obtained from Stockwell et al. (1998). The 20 samples (N=10 / ESU) genotyped at these two loci were tested for departure from Hardy-Weinberg Expectations (HWE) as well as population differentiation. Default parameters were used for the HWE test (Guo and Thompson, 1992) and the log-likelihood exact G-test (Goudet et al. 1996), as implemented in genepop (v.4.6; Raymond, 1995).
Genomic diversity

Genome-wide heterozygosity ($H$) was estimated for six Cyprinodontiformes. First, to determine mappability at each site in each reference genome, genmap (v.1.3.0; Pockrand et al. 2020) was run using 100-mer and allowing up to two mismatches. Repeatmasker (v.4.07; Smit et al. 2015) was then used to identify repeats using the D. rerio RepBase library as a reference. Sites with mappability <1 and repeated regions were excluded from downstream analyses (Table S1). Scaffolds >100Kb in length were removed and $H$ was calculated using angsd (v.0.93; Korneliussen et al. 2014) using the unfolded site frequency spectrum (SFS), requiring a minimum mapping quality of 30 and base quality >20. The results were visualized with ggplot2.

Nuclear and mitochondrial genomic divergence

For related pupfish species with an available reference genome (C. tularosa, C. variegatus and C. nevadensis), and for context X. maculatus (GCF_002775205.1), homologous nuDNA regions were identified by first breaking each genome into 30Kb sections using the splitter function implemented in emboss (v.6.60; Rice et al. 2000). Pairwise local alignments were then performed against each congenic repeat masked genome using blastn with the following non-default parameters (-perc_identity 3, -qcov_hsp_perc 3, -max_target_seqs 1, -evalue 50, -max_hsp 1, -culling_limit 1). Following local pairwise alignments, genetic distances were estimated between each aligned sequence (>2Kb in length) using the Kimura-2 parameter (K2P; Kimura, 1980) generated with the R package ape (v.5.4.1; Paradis et al. 2004). Results were visualized with ggplot2 to evaluate patterns of pairwise genomic differentiation.

To compare the matrilineal relationship among pupfish species, all Cyprinodontidae mtDNA genomes available as of 15 August 2020 were downloaded from NCBI for the following species: C. variegatus (KT288182.1), C. diabolis (KX061747.1), C. nevadensis amargosa (KU883631.1), the Desert pupfish (C. macularius; KM985373.1) and the Red River pupfish (C. rubrofluviatilis; NC_009125). In addition to the new C. tularosa mtDNA assembly reported herein, the previously archived C. tularosa RefSeq mtDNA assembly was also included (NC_028292.1). The X. maculatus mtDNA assembly (NC_011379.1) was used as an outgroup. Therefore, eight full mitogenome sequences were used for phylogenomic analysis; two for C. tularosa, five from other Cyprinodontidae and an outgroup. Multiple sequence alignment of full length mitogenome sequences was then conducted using clustalw (v.2.1; Thompson et al. 1994) and a Neighbor Joining (NJ) distance matrix was generated using a Jukes-Cantor substitution model using 100 bootstrap replicates within megax (Kumar et al. 2018). Molecular time estimates were obtained from Timetree (Kumar et al. 2017) and fixed rates were used as time constraints at each available node using the reltime-ML function (Tamura et al. 2018). Estimated mtDNA divergence times were then annotated to the NJ tree, exported in Nexus format and rendered using phlo.io (Robinson et al. 2016). In addition to creating a timetree, mean pairwise genetic distances (K2P) between species mtDNA sequences was estimated with megax. Our intention was not to perform a systematic review of Cyprinodontidae phylogeny, but to provide evolutionary context for our pairwise comparisons.

Results

Quality Control and Genome Assemblies

Two PacBio Sequel II SMRT cells yielded 165Gb worth of sequence data among 16 million reads with a mean N50 polymerase read length of 22Kb. After extracting reads between 5-50Kb, 100Gb remained among 14.3 million reads for use with genome assembly (~100x coverage). The Illumina sequencing platform yielded 43Gb of raw sequence data among 286 million 151-bp paired-end reads. After quality filtering, there were 284.9 million quality controlled reads remaining (99.6% of total) with an estimated genome coverage of ~40x.

Using the optimal 90x coverage level, the assembler wtdbg2 yielded 1.09Gb among 2,606 contigs, with a N50 of 1,357Mb and a GC content of 39%. The assembly was then polished using PacBio subreads from the two SMRT cells and error corrected using three iterations of short-read polishing. The program purge_haplotigs discarded 332 contigs from the assembly as haplotigs (total length 3.5Mb) and 12 as artifactual (total length 596Kb), resulting in 2,009 contigs. blobtools2 was then used to identify non-target DNA sequenced in the
pupfish libraries. Overall, five contigs (68Kb) were not assigned as Actinopterigii sequences (classified as ‘no-hits’, Arthropoda, or Chytridiomycota) and were subsequently filtered out of the assembly. The curated fasta file containing 2,004 contigs was then used for downstream analyses.

The complete mtDNA sequence of *C. tularosa* was reconstructed using congeneric (*C. diabolis*) aligned Illumina short-reads. The assembly of the *C. tularosa* mitogenome produced a 16,508 nt contiguous sequence similar in length to other published *Cyprinodon* mtDNA assemblies (16,499-16501 nt). The *C. tularosa* mtDNA assembly had a 100% identity match with contig125 within the primary assembly and was subsequently removed from the mtDNA sequence file prior to annotation.

**Nuclear and Mitochondrial Genome Annotation**

maker identified the genomic coordinates of 25,260 protein coding genes in the curated *C. tularosa* assembly, which is generally congruent with the 23,019 coding genes annotated in the current genome release of *C. variegatus* (GCF_000732505.1). Functional annotation of the new *C. tularosa* mtDNA assembly illustrated that these annotations were also consistent with other published *Cyprinodon* mitogenomes, with the correct ordering of 13 polypeptides, 22 tRNA genes, 2 rRNA genes, and one control region. A sequence similarity search between the two *C. tularosa* mitogenomes revealed pls scores of 97-100% among protein coding genes.

**Assembly metrics**

For the White Sands pupfish draft genome, assembly statistics on 1,995 contigs (those greater than 5Kb in length) showed a total length of 1.086Mb, a max contig size of 8.12Mb and a N50 of 1.36Mb (Table 1). Thus, the new *C. tularosa* assembly is more contiguous than the scaffold-level *C. variegatus* assembly (contigs=4,093; max contig size=4.51Mb; N50=31.4Mb) but less contiguous than the chromosome-level *P. reticulata* assembly (contigs=8,438; max contig size=46.3Mb; N50=31.4Mb) or the *X. maculatus* assembly (contigs=97; max contig size=35.3Mb; N50=31.5Mb; Table 1). Using the Actinopterigii database of 3,640 orthologs, busco identified 3,452 complete single-copy genes (C 95%) in the *C. tularosa* assembly with little inherent fragmentation (F 1%). Genome completeness, based upon these values, was moderately lower than the *X. maculatus* assembly (C 96.6%; F 0.3%), equivalent to the chromosome-level *P. reticulata* assembly (C 95%; F 1%), similar to *C. variegatus* (C 94%; F 1%) and *A. limnaeus* (C 92%; F 2%), but markedly improved over the *C. nevadansis* assembly (C 70%; F 13%; Figure 1).

**Microsatellite Identification**

Each of the 16 microsatellites previously used to characterize *C. tularosa* populations were successfully located in the *C. tularosa* genome assembly. Three were found within an intron of a gene, five were located within 10Kb of a gene(s), five were located between 10-100Kb of one or more genes and three were mapped to a location >100Kb from any gene (Table 2). Using 11/16 loci which had previously reported heterozygosity estimates, our comprehensive regression suggested no significant relationship between microsatellite heterozygosity and distance to the nearest gene or ESU (Figure 2A; Intercept: Estimate=0.265, SE=0.082, p < 0.001; Distance: Estimate=0.000, SE=0.000, p-value=0.780; ESU: Estimate=0.020, SE=0.110, p-value=0.850; R²=0.055; F=0.050; DF=2,19). Upon limiting our regression to microsatellite loci that were <50Kb, we found that genic distance was negatively related to H⁰ (Figure 2B; Intercept: Estimate=0.414, SE=0.092, p-value < 0.001; Distance: Estimate=-0.008, SE=0.003, p-value=0.022; ESU: Estimate=0.003, SE=0.103, p-value=0.953; R²=0.302; F=3.349; DF=2,15). Both microsatellite markers used to inform ESU demarcation (Stockwell et al. 1998) conformed to Hardy-Weinberg Expectations within their respective ESU (p-values=0.195-1.00). An exact G-test at these loci illustrated significant (p-values=0) levels of differentiation (WSP-2, FST =0.350; WSP-11, FST =0.624) and were located nearby (<2Kb) predicted genes in the *C. tularosa* genome (Table 2).

**Genomic diversity**

Genome-wide heterozygosity (H) was then estimated for all six reference enabled Cyprinodontiformes (Table S1). Diversity values among all six species illustrated that H estimates ranged from 0.01225 (*C. variegatus*) to 0.00003049 (*X. maculatus*), which we note was derived from a highly inbred line. Estimated H for *C.*
*tularosa* (0.00053) was found at the low end of these estimates, near *C. nevadensis* (0.00116) and the inbred *X. maculatus* line. According to the IUCN, both vulnerable (*C. nevadensis*) and endangered (*C. tularosa*) pupfish species were found at the low end of our diversity estimates (Figure 3).

**Nuclear and mitochondrial genomic divergence**

Pairwise local alignments of [??]30Kb sequences were performed between reference enabled species to: 1) compare levels of nuDNA genome sequence divergence between related species; 2) compare levels of nuDNA and mtDNA divergence; and 3) assay the *C. tularosa* genomic landscape for signatures of a) potential introgression with *C. variegatus* and/or b) localized windows within the genome that may be involved with selective sweeps. We found that nuDNA sequence divergence generally mirrored the mtDNA genome tree (Figs. 4 and 5). Genetic distances were approximately 2x greater for mtDNA sequences compared to nuDNA data, with mean (+-SD) pairwise nuDNA K2P values of 0.0309+-0.010 (*C. tularosa* vs *C. nevadensis*) and 0.0304+-0.009 (*C. variegatus* vs *C. nevadensis*). Across 20,000 alignments averaging 5,528 nt in length, mean nuDNA K2P values were lower between *C. tularosa* and *C. variegatus* (0.0269) with highly variable distances (K2P range =0.0004-0.265; Figure 4A). Following multiple sequence alignment of full mitogenomes, mean Kimura 2 values (K2P; Kimura, 1980) and divergence times (MYA) were estimated for all species pairs. As expected, the greatest genetic distances were observed between the outgroup *X. maculatus* and other pupfish species (K2P range=0.2655-0.2712). Pairwise mean K2P values using the new *C. tularosa* mtDNA assembly ranged from 0.0009 (RefSeq,*C. tularosa*) to 0.0691 (*C. diabolus*; Figure 4B). Mean genetic distance between the White Sands pupfish and the Sheepshead minnow (*C. variegatus* vs *C. tularosa*; K2P=0.0222; timetree estimates an estimated divergence time of 1.6-4.7 MYA) were ~3x lower than other congener comparisons (K2P range=0.053-0.069; Figure 4B). The clades recovered in our phylogenic assessment (Figure 5) were largely congruent with previous assessments of *Cyprinodontidae* evolutionary history (e.g., Echelle *et al.* 2005).

**Discussion**

The conservation of endangered species is increasingly informed by genomic data. To that end, we have generated a high quality draft genome assembly for the endangered White Sands pupfish (*C. Tularosa*). Our 1.08Gb assembly is largely complete (~25k protein coding genes including 95% of conserved *Actinopterygii* genes) with very little fragmentation (1%). Furthermore, 95% of the quality controlled Illumina paired-end reads aligned to our *C. tularosa*assembly (data not shown), demonstrating the utility that this new draft genome will have in future conspecific, as well as many heterospecific, re-sequencing and/or transcriptomic studies. Finally, our ~16Kb *C. tularosa* assembled mitogenome was consistent with other pupfish mtDNA annotations (13 polypeptides, 22 tRNA genes, 2 rRNA genes and one control region). Below, we briefly discuss the results and implications of our work.

**Intraspecific evaluation**

Heilveil and Stockwell (2017) demonstrated that the Lost River refuge population showed reduced genetic diversity and the current work validates and extends their insight to the genomic level. Heterozygosity estimates revealed low levels of genomic diversity in the single *C. tularosa* specimen (*wsp-4*) collected from the upper reach of Lost River and used for sequencing and assembly. Compared to the other *Cyprinodon* examined, *C. tularosa* showed a genome-wide heterozygosity that was one (or two) orders of magnitude less than other species diversity estimates (Figure 3). If this *C. tularosa* sample is representative of the heterozygosity of the refuge population, this may be of conservation concern as low genetic diversity can constrain adaptive potential in light of future environmental change (e.g., Finger *et al.* 2013). Lost River was founded by 30 individuals translocated from Salt Creek circa 1970, with approximately "bi-annual translocations of 40 fish (from Salt Creek) beginning in 2008 (Carman, 2010). It is currently unknown what, if any, affect these translocations have on the genetic diversity of the Lost River refuge population but the low level of heterozygosity of our study suggests that there may have been a substantial reduction in genomic diversity due to the founder effect and/or drift.
From a genetic management perspective, refuge populations should strive to replicate their source populations and maintain original levels of genetic diversity. If the other *C. tularosa* populations are as homogenous at the whole genome level, facilitated local gene flow (i.e., genetic rescue) may be one approach to help increase heterozygosity (Petit and Excoffier, 2009). However, to do so effectively, the source population needs considerably more genetic variation than the recipient population (Rails *et al.* 2020). More extensive population genomic surveys of the Lost River and Salt Creek populations are required for certainty, but this refuge population may be similar to an insurance policy with insufficient coverage for future losses. Furthermore, it is unclear if the potential cost of homogenizing locally adapted / differentiated gene-pools, through the act of small reciprocal inoculations between ESUs (Arnold, 2016), would outweigh the need to preserve and protect the two separate *C. tularosa* ESUs. These are conservation issues that will require the careful planning and examination of individual- and population-level diversity metrics in future whole genome re-sequencing studies.

**Microsatellite Identification**

Our intention was to identify the genomic coordinates of microsatellites previously used to define and monitor ESU in the White Sands pupfish. We successfully identified the location of all 16 microsatellite markers within the *C. tularosa* draft genome. Many of these loci were either within 10Kb of predicted genes (5/16) or inside an intron of a gene (3/16), suggesting they may not have evolved in a strictly neutral fashion. Indeed, we found a negative correlation between heterozygosity and genic proximity, suggesting that selective processes differ between genic and intergenic regions within the *C. tularosa* genome. However, for the two loci where genotypic data were publicly available (WSP-02 & WSP-11; Stockwell *et al.* 1998), we found no deviation from Hardy-Weinburg Expectations. This is only a weak test of natural selection and our assessments of genomic provenance are not formal tests of microsatellite neutrality, but the data provided herein add considerable context to earlier microsatellite studies that helped define the two ESUs. High resolution neutrality tests may provide future genomic insights into local adaptation associated with the two *C. tularosa* ESUs.

**Nuclear and mitochondrial genomic divergence**

Our mtDNA based phylogeny provided 100% bootstrap support at each node (Figure 5) and was largely in agreement with the evolutionary history of *Cyprinodontidae* (e.g., Echelle *et al.* 2005; Săgîlam *et al.* 2016). In addition to the phylogenomic tree, we also used our mtDNA assembly to quantify genetic distance with a previously assembled *C. tularosa* mitogenome that was sourced from the Salt Creek ESU on December 1983 (http://fishnet2.net/; MSB:Fish:96664). The genetic distance estimate between these two *C. tularosa*mitogenomes was essentially zero (K2P=0.0009; Figure 4B), which is what one would expect to observe between individuals collected from the same ESU and sampled less than 50 years apart. As translocation of 40 individuals have occurred on a semi-annual basis since 2008 (Carman, 2010), and there was little difference between mitochondrial genomes, we expect little genomic divergence between these two populations (Salt Creek and Upper Lost River). However, Heilveil and Stockwell (2017) demonstrated moderate population structure between the Upper and Lower Lost River sub-population ($F_{ST}$ = 0.024) which suggests that future research is required to evaluate levels of genomic differentiation between these two waterways within the Lost River refuge population.

Using both nuDNA and mtDNA sequences, pairwise comparisons between related species demonstrated that genomic distance (Figure 4B) increased with taxonomic divergence (Figure 5) with a range in pupfish divergence times of 0-10 MYA. For the most part, differentiation values reported herein were 4-5x higher in mtDNA divergence (relative to nuDNA) with genetic distances roughly equivalent to those reported in Willoughby *et al.* (2020) at both nuDNA (*C. variegatus* vs *C. nevadensis* =0.04) and mtDNA (*C. variegatus* vs *C. nevadensis* =0.06) regions. We note that K2P variability was substantially lower in nuDNA alignments (K2P=0.01-0.05) relative to mtDNA (K2P=0.006-0.137) alignments (Willoughby *et al.* 2020). This disparity in variance is likely a consequence of sequence mappability; the entire mtDNA assembly is well-known and annotated so sequence mappability is relatively high and the K2P variance likely represents the biological variation inherent to hypervariable regions (e.g., the D-loop) compared to constrained regions (e.g., tRNA sequences and ND4). In contrast, nuDNA genomes are still incomplete even when considering high-quality
chromosome-level assemblies. Furthermore, our strategy was designed to reduce spurious matches, but this means we almost certainly homogenized mappable regions and necessarily underestimated genetic distances in the nuDNA data. Thus, absolute K2P estimates of genomic divergence are provisional for nuDNA data relative to mtDNA sequences.

Both mtDNA and nuDNA divergence rates showed a positive correlation ($R^2=0.99$) among all six pairwise comparisons (Figure 4C). For 5/6 pairwise comparisons, results demonstrated a 1.9-2.2x increase in pairwise mtDNA sequence divergence compared to nuDNA divergence, in agreement with the well-recognized higher levels of mitochondrial mutation rates first reported by Brown et al. (1979). However, we found similar estimates of genetic distances between the C. tularosa and C. variegatus mtDNA (K2P distance = 0.022) and nuDNA (K2P distance = 0.0269) sequences, suggesting that our mtDNA estimates may not have fully captured the true patterns of sequence divergence (Figure 4A). Alternatively, this raises the possibility of contemporary introgression from C. variegatus into a C. tularosa population(s), which could have partially homogenized the C. tularosa gene pool and led to this reduction in nuDNA (and perhaps mtDNA) divergence. Furthermore, evaluating the distribution of all pairwise sequence alignments between C. tularosa and C. variegatus illustrated a highly variant mosaic of homologous blocks between C. tularosa and C. variegatus (K2P range =0.0004-0.264). Here, we suggest that contigs with near zero K2P values may represent C. variegatus introgressed regions (Figure 4A). However, extensive sampling and re-sequence data will be better suited to confirm (or reject) these potential tracts of introgression within the C. tularosa genome. Nevertheless, identification of the genomic patterns of introgressions, and how this influenced evolution of C. tularosa, is important because it may alter how we manage this endangered desert fish. For example, an extreme but often utilized approach in managed desert fish populations is to cull any ‘genetically compromised’ fish and reintroduce or translocate ‘representative’ samples back into the original population (e.g., Echelle et al. 1997). Thus, we think the data and analyses presented herein provide a significant resource to the broader community of conservation biologists, especially those working with the pupfishes.

Conclusions

We present the first draft of an annotated genome for C. tularosa and find that it is among the best assemblies available for this group of fishes. To add context to our assembly and to more broadly illustrate its potential as a community resource, we performed a number of exploratory analyses related to the diversity and divergence of C. tularosa. We found that heterozygosity in our assembly is strikingly low relative to related species, including other pupfish, and that population genomic surveys are warranted to determine if the lack of heterozygosity extends to individuals from other populations and/or encompass large runs of homozygosity (e.g., due to recent inbreeding). Our retrospective analyses of microsatellite heterozygosity indicates that population genetic diversity at this suite of “neutral” markers is not distributed randomly, but is partitioned relative to genomic distance from annotated functional genes. We also used our assembly to evaluate genome-wide divergence from related species to help identify outlying contigs that could represent regions homogenized due to introgression (a major conservation concern) or divergent regions that could represent targets of selection (e.g., adaptation to different salinity or parasite regimes). Overall, we think these new resources and analyses will benefit future ecological and evolutionary studies of the Cyprinodontidae and ultimately, hope they contribute to pupfish conservation.

Acknowledgements

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References


**Competing Interests**

All authors declare that they have no competing interests.

**Data Accessibility**


Scripts used to perform assembly and comparative genomic analyses: https://doi.org/10.5061/dryad.p8cz8w9nt

**Author Contributions**
A.N.B, J.R.W, B.L.P. and JAD contributed to the sample collection and design of the study. A.N.B and A.B.O analyzed the data. A.N.B and J.A.D. wrote the paper with input from all authors.

Tables:

Table 1. Quast assembly statistics for the six *Cyprinodontidae* assemblies, reported using contigs [?]5,000 nucleotides in length. The *C. tularosa* assembly is new, the other five assemblies were downloaded from NCBI.

<table>
<thead>
<tr>
<th>Accession</th>
<th>C. tularosa</th>
<th>C. variegatus</th>
<th>C. nevadensis</th>
<th>A. limneaus</th>
<th>P. reticulata</th>
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<td>Scaffold</td>
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<td>3,687</td>
<td>853</td>
<td>97</td>
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Table 2. Genic proximity to previously published microsatellite markers within the newly annotated *C. tularosa* genome. Each row corresponds to one microsatellite marker, the observed (*H*<sub>obs</sub>), expected Heterozygosity (*H*<sub>exp</sub>), and number of Alleles (*A*) at that locus within the Malpais Spring (N=10) or Salt Creek (N=10) population and the genic distance (Kb) downstream or upstream to the closest gene. Markers that fell within a genic area are classified according to the associated feature (i.e., *intron*). Protein coding genes of unknown function are labeled as “UKP”.

+ Microsatellite markers used in *C. tularosa*ESU classification calculated from genotypes in Stockwell et al.1998

++ *H*<sub>obs</sub>, *H*<sub>exp</sub> and*A* (only available for both populations) obtained from Iyengar et al. 2004
Figures Legends:

**Figure 1.** BUSCO results for analysis of genome completeness among six *Cyprinodontiformes*, using the *Actinopterigii* database containing 3,640 core orthologs. A star and bold font are used to designate the new *C. tularosa* assembly.

**Figure 2.** Genetic diversity ($H_{OBS}$) at each microsatellite marker (N=11) and the distance (Kb) to the closest predicted *C. tularosa* gene. The shape of each microsatellite locus depicts the source ESU (Malpais Spring or Salt Creek). The relationship between $H_{OBS}$, ESU and the distance to closest gene were tested using a linear model ($lm= H_{OBS}^*Distance+ESU$). A) For all available microsatellite markers, the regression analysis showed a non-significant (p-value=0.780) relationship ($R^2=0.005$) between heterozygosity and genic distance. B) Using only markers located 50Kb from neighboring genes revealed a significant (p-value=0.022) negative correlation ($R^2=0.30$) between $H_{OBS}$ and genic distance. $H_{OBS}$ estimates were obtained from Iyengar *et al.* (2004) and Stockwell *et al.* (1998).

**Figure 3.** Genomic estimates of heterozygosity ($H$) for the six *Cyprinodontiformes* examined. $H$ was estimated for each species using the unfolded site frequency spectrum, as implemented in angsd (Korneliussen *et al.* 2014). A red star is used to designate the new *C. tularosa* assembly. With the exception of the highly inbred *X. maculatus* line, heterozygosity in *C. tularosa* is greatly reduced compared to related species.

**Figure 4.** Nuclear and mitochondrial mean pairwise genetic distances among *Cyprinodontiformes*: a) Pairwise Kimura 2 distances (K2P) between aligned nuDNA reference sequences. Dashed red line signifies the mean nuDNA K2P value across >30kb windows for each comparison. Points colored red signify distances that were >0.99 or <0.01 percentiles; b) Upper left triangle matrix, mean genetic distances between aligned nuDNA sequences. Lower right triangle matrix, mean K2P genetic distances between aligned mtDNA sequences. Black boxes signify pairwise comparisons which had both nuDNA and mtDNA distances; c) scatterplot illustrating the correlation between mean K2P nuDNA and mtDNA divergence for the six pairwise comparisons. The dashed red line depicts a 1:1 ratio of nuDNA to mtDNA divergence, and the solid grey line is the K2P correlation among taxa. A star is used to designate the new *C. tularosa* assembly.

**Figure 5.** Neighbor Joining tree constructed using the Jukes-Cantor model, following multiple sequence alignment of full mtDNA sequences. Significance of 100% bootstrap support are signified by black circles and divergence times (MYA) are labeled under the tree. Estimated divergence times were generated using the *reltime-ML* function (Tamura *et al.* 2018) from constrained time estimates (min/max) obtained from TimeTree (Kumar *et al.* 2017). A red star is used to designate the new *C. tularosa* assembly.
A phylogenetic tree showing the distribution of Actinopterygii Core Orthologs (%).

The tree includes branches for Cyprinodontidae and Poeciliidae, with species such as Xiphophorus, Poecilia, and Austrofundulus. Each branch is color-coded to indicate the level of conservation:
- Blue: Complete (C) and duplicated (D)
- Red: Missing (M)
- Complete (C) and single-copy (S)
- Yellow: Fragmented (F)

Species and their corresponding ortholog counts are labeled on the branches:
- C:3547 [S:3519, D:28], F:13, M:80, n:3640
- C:3480 [S:3449, D:31], F:32, M:128, n:3640
- C:3478 [S:3452, D:26], F:35, M:127, n:3640
- C:3458 [S:3406, D:52], F:39, M:143, n:3640
- C:3371 [S:3335, D:36], F:74, M:195, n:3640
- C:2575 [S:2554, D:21], F:322, M:743, n:3640