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RESEARCH ARTICLE

Under the radar: genetic assessment of Rio Grande Shiner (*Notropis jemezanus*) and Speckled Chub (*Macrhybopsis aestivalis*), two Rio Grande basin endemic cyprinids that have experienced recent range contractions

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Abstract

The Rio Grande drainage of the southwestern United States and Mexico has undergone intense anthropogenic alteration by water diversions, extraction and associated habitat changes. These alterations have disproportionately impacted the pelagic broadcast spawning guild of minnows (pelagophils). Several Rio Grande endemic pelagophils, including the co-occurring Rio Grande Shiner (*Notropis jemezanus*) and Speckled Chub (*Macrhybopsis aestivalis*), have experienced dramatic recent range-wide declines yet have slipped under the radar of conservation efforts. The status of *N. jemezanus* and *M. aestivalis* in the Rio Grande and Pecos River was evaluated and standing genetic variation was characterized. Genetic evidence indicates that populations of both species found in the Rio Grande and Pecos River are genetically distinct. Additionally, 159 outlier loci were identified in *M. aestivalis* suggesting possible local adaptation in the Rio Grande and Pecos River populations. Though range-wide genetic data are limited, *N. jemezanus* populations in both rivers harbor considerable genetic diversity. Mitochondrial data from both taxa are consistent with a history of secondary contact between formerly isolated populations with deeply divergent haplotypes found within the Rio Grande and Pecos River populations of *N. jemezanus* and within the Rio Grande population of *M. aestivalis*. Extensive survey efforts in the lower Rio Grande and its tributaries in Texas document significant range contraction and near extirpation of *N. jemezanus* from this part of the basin; highlighting the need for immediate action to protect the species.

Keywords Cyprinidae · Pelagophilic · Great Plains · Chihuahuan Desert

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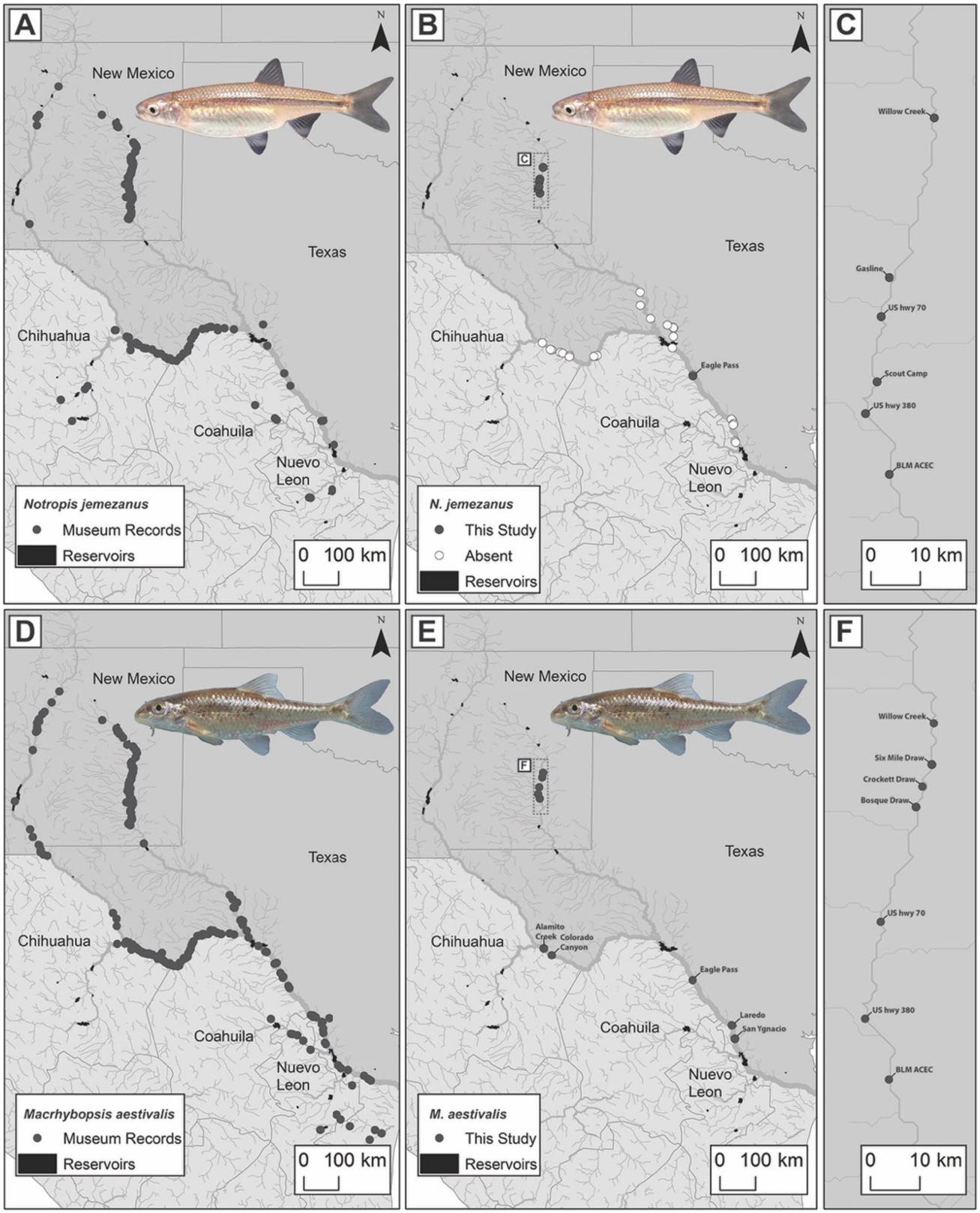
Introduction

The Rio Grande (RG) drainage of the southwestern United States (US) and Mexico (MX) has undergone intense anthropogenic alteration (Hoagstrom 2003, 2009). Changes associated with rapid agricultural development and municipal growth have drastically altered natural temperature and flow regimes and have reduced the quality and quantity of water reaching the Gulf of Mexico. Hydrologic changes resulting from water diversion, channelization, impoundments and introduction of non-native species have negatively impacted aquatic biodiversity, with recent, rapid population declines documented for endemic vertebrate and invertebrate taxa (e.g., Hubbs 1990; Karatayev et al. 2012). The recent demise of the RG freshwater fish fauna is especially alarming, with approximately half of the endemic


A
New Mexico
Texas
Chihuahua
Coahuila
Nuevo Leon
Notropis jemezanus
Museum Records
Reservoirs
0 100 km
B
New Mexico
Texas
Chihuahua
Coahuila
Nuevo Leon
Eagle Pass
N. jemezanus
This Study
Absent
Reservoirs
0 100 km
C
Willow Creek
Gasline
US hwy 70
Scout Camp
US hwy 380
BLM ACEC
0 10 km
D
New Mexico
Texas
Chihuahua
Coahuila
Nuevo Leon
Macrhybopsis aestivalis
Museum Records
Reservoirs
0 100 km
E
New Mexico
Texas
Chihuahua
Alamito Creek
Colorado Canyon
Eagle Pass
Laredo
San Ygnacio
Coahuila
Nuevo Leon
M. aestivalis
This Study
Reservoirs
0 100 km
F
Willow Creek
Six Mile Draw
Crockett Draw
Bosque Draw
US hwy 70
US hwy 380
BLM ACEC
0 10 km

◂**Fig. 1** **A** Distribution of museum vouchered material of *N. jemezanus* in US collections (based on 2,001 lots from 16 institutions). Dots on map may represent more than one record. **B** Distribution of sites visited in the lower Rio Grande in TX as part of this study. **C** Distribution of collection sites in the Pecos River NM. White and black dots indicate locations at which *N. jemezanus* was not collected or collected, respectively. **D** Distribution of museum vouchered material of *M. aestivalis* in US collections (based on 2,125 lots from 13 institutions). **E** Distribution of sites visited in the lower Rio Grande in Texas as part of this study **F** Distribution of collection sites in the Pecos River NM. Reservoirs are shown as these are potential barriers to these species

species now considered extinct or threatened with extinction (Jelks et al. 2008).

Medium and large sand-bed rivers of the Great Plains and Chihuahuan Desert ecoregions of North America are inhabited by a reproductive guild of cyprinid fishes in which females release non-adhesive, semi-buoyant ova directly into the water column (Bottrell et al. 1964). Members of this group are referred to as pelagophilic. Following fertilization, embryos develop during a drifting phase lasting several days (Bottrell et al. 1964). These species share traits characteristic of opportunistic fishes (sensu Winemiller 2005) including small body size, high mortality in the first years of life and short generation time (Hoagstrom et al. 2015; Horwitz et al. 2018). Maintenance of upstream populations of pelagophils depends on retention of eggs and larvae and/or upstream dispersal of young-of-year or adults (Fausch and Bestgen 1997; Archdeacon et al. 2018; Platania et al. 2020). To ensure successful development and recruitment these species require some combination of: (i) long stretches of free-flowing river, (ii) natural river-channel morphology, and (iii) a natural flow regime to provide spawning cues to which each population is adapted, avoid excessive downstream displacement of drifting embryos, and stimulate instream productivity to support annual recruitment (Dudley and Platania 2007; Hoagstrom and Turner 2015; Osborne et al. 2005). Although pelagophils are adapted to highly variable flow regimes (Fausch and Bestgen 1997), numerous authors (*e.g.,* Dudley and Platania 2007, Hoagstrom et al. 2008a) have demonstrated that these cyprinids are susceptible to river fragmentation by reservoirs, water diversions and habitat changes that may limit retention of eggs in nursery habitat. Likewise, stream dewatering may preclude recruitment and decimate adult populations (Archdeacon 2016; Perkin et al. 2019). The short generation times of these species mean that a single stochastic event, such as a drought, may be sufficient to eliminate entire populations or species, especially in river reaches that are already environmentally degraded (Perkin et al. 2015a, b; Perkin et al. 2019). Consequently, many pelagophilic cyprinids have disappeared from large portions of their historic range (reviewed in Worthington et al. 2018), including the Rio Grande Silvery Minnow (*Hybognathus amarus*, Bestgen and Platania 1991), Arkansas River Shiner (*Notropis girardi*, Wilde 2002), Peppered Chub (*Macrhybopsis tetranema*, Luttrell et al. 1999; Pennock et al. 2017), Speckled Chub (*M. aestivalis,* Bestgen and Platania 1990) and Rio Grande Shiner (*N. jemezanus,* Hoagstrom and Brooks 2005).

Declines of two pelagophils endemic to the RG basin (Pecos Bluntnose Shiner *Notropis simus pecosensis* and *H. amarus*) have received considerable attention from scientists and managers, leading to state- and federal-lead conservation programs designed to stem further declines and provide protection under the Endangered Species Act (Worthington et al. 2018). These and two extinct endemic RG basin pelagophils (Phantom Shiner *Notropis orca* and Rio Grande Bluntnose Shiner *Notropis simus simus*) experienced declines and range contractions in association with water withdrawals, and habitat degradation (e.g., Bestgen and Platania 1990; Edwards and Contreras-Balderas 1991; Dudley and Platania 2007). However, two other RG endemic pelagophils—*N. jemezanus* and *M. aestivalis*— have experienced severe declines yet have largely slipped under the radar of conservation. Prior to 2004, *M. aestivalis* was recognized as polytypic species comprising seven subspecies (Moore 1950). Based on morphological analyses, Eisenhour (2004) elevated five subspecies to the species level, including *M. aestivalis* which was restricted to the Rio Grande and Río San Fernando MX. Recent genetic analysis has revealed additional cryptic variation within the *M. aestivalis* complex (Echelle et al. 2018). *Notropis jemezanus* and *M. aestivalis* were historically widespread throughout the main stem RG and its tributaries in the US and MX (Sublette et al. 1990; Miller et al. 2005; Fig. 1).

Range contractions of both *N. jemezanus* and *M. aestivalis* have resulted in fragmented distributions, though both are still found in the Pecos River (PR) New Mexico (NM) (between the Fort Sumner Diversion Dam and Brantley Reservoir; Sublette et al. 1990), and in the RG along the US/MX border (between the Río Conchos and Río Salado confluences; Hendrickson and Cohen 2015). In a section of the RG (Río Conchos to PR), *N. jemezanus* was once abundant (Hubbs 1990; Edwards et al. 2002a, b), but recent surveys have produced alarmingly low numbers (*i.e.* < 10; Heard et al. 2012). Elsewhere, reports indicate both species remain patchily distributed in the Río Conchos (Edwards et al. 2002a, b) and *M. aestivalis* persists in the Río San Fernando (de León et al. 2005). The status of each species in the ríos San Juan and Salado of MX is unknown.

Conservation efforts depend on protecting standing genetic diversity across the geographic distribution of species. Genetically diverse populations are more able to adapt to changing environmental conditions (e.g., Therkildsen et al. 2019). Likewise, understanding the genetic component of biodiversity can be used to identify areas of high conservation value (Moritz 1995). Highly polymorphic microsatellites can provide reliable estimates of effective population size (Waples and Do 2010), genetic diversity and population

structure (Jeffries et al. 2016). When sample size is small, data from single nucleotide polymorphisms (SNPs), produced using next generation sequencing technologies, can provide even more reliable detection of population structure because many more loci are assayed (Jeffries et al. 2016). Moreover, retaining multiple SNPs within the same contig (i.e., microhaplotype/SNP containing loci) can provide increased power for population genetic analyses (Baetscher et al. 2017). This approach retains low frequency variants and can improve detection of recently diverged populations (Hendricks et al. 2018). The trajectory of genetic change between populations can be driven by neutral and selective evolutionary forces and genomic data allow these to be disentangled. Detection of genomic regions showing evidence of local adaptation can be useful when delineating conservation units within a species (Funk et al. 2012). Here data from microsatellites, mitochondrial DNA and SNP-containing loci are used to provide the first evaluation of genome-wide genetic diversity across the US range of *N. jemezanus* and *M. aestivalis.* These data provide insights into the demographic history of both species and can be used to inform future conservation actions.

Methods

Sampling

Tissues (fin clips) for both species were obtained via fieldwork in Texas (TX) and NM (Table 1 and S1). Distribution maps for *N. jemezanus* (Fig. 1A) and *M. aestivalis* (Fig. 1D) were created in ArcMap v.10.5.1 using records from US collections (accessed via FishNet2; http://www.fishnet2.net/). Microsatellite and mtDNA analyses for *N. jemezanus* included individuals from one RG locality (n = 4, collected in 2017; Fig. 1B) and five PR sites (microsatellites n = 153 and mtDNA n = 121, collected in 2007 and 2013; Fig. 1C). Microsatellite and mtDNA analyses for *M. aestivalis* included individuals from four sites along the RG in TX (microsatellites n = 63 and mtDNA n = 57; collected in 2011, 2015, 2016 and 2017; Fig. 1E) and eight PR sites (microsatellites n = 169 and mtDNA n = 142; collected in 2007 and 2013; Fig. 1F).

RADseq data for *N. jemezanus* included individuals from one RG locality (n = 4; collected in 2017; Fig. 1B) and five PR sites (n = 55; collected in 2017; Fig. 1C). RADseq data for *M. aestivalis* included individuals from two RG localities (n = 35; collected in 2015 and 2017 Fig. 1E) and from five PR sites (n = 52; collected in 2017; Fig. 1F). Complete overlap of individuals in the microsatellite/mtDNA and RADseq datasets was not possible because of limitations in the amount of tissue and subsequent DNA availability.

Microsatellites and mtDNA

DNA for microsatellite and mtDNA analyses was extracted from fin clips using proteinase-K digestion and phenol/chloroform (Hillis et al. 1996). *Notropis jemezanus* and *M. aestivalis* were screened for genetic variation at ten and nine microsatellite loci respectively, using primers and conditions

Table 1 Genetic diversity statistics for *N. jemezanus* and *M. aestivalis* collected from the Rio Grande and Pecos River

	Microsatellites				SNP-containing loci			
	n	A_R	H_E	H_O	n	A_R	H_E	H_O
N. jemezanus								
Rio Grande 2017*	4	2.704 ± 0.918	0.700 ± 0.092	0.592 ± 0.020	4	1.477 ± 0.703	0.484 ± 0.006	0.441 ± 0.005
Pecos R. 2007	25	2.673 ± 1.010	0.644 ± 0.110	0.591 ± 0.010	–	–	–	–
Pecos R. 2013	128	2.803 ± 0.941	0.681 ± 0.093	0.620 ± 0.090	–	–	–	–
Pecos R. 2017		–	–	–	55	1.500 ± 0.601	0.501 ± 0.005	0.459 ± 0.004
M. aestivalis								
Rio Grande 2011*	10	5.206 ± 2.348	0.796 ± 0.056	0.815 ± 0.081		–	–	–
Rio Grande 2015*	33	5.475 ± 2.231	0.778 ± 0.075	0.733 ± 0.060	26	2.071 ± 0.771	0.337 ± 0.004	0.322 ± 0.004
Rio Grande 2016*	10	4.681 ± 2.262	0.693 ± 0.094	0.668 ± 0.100		–	–	–
Rio Grande 2017*	10	5.755 ± 2.253	0.724 ± 0.104	0.667 ± 0.096	9	2.058 ± 0.822	0.334 ± 0.004	0.324 ± 0.004
Pecos R. 2007	26	5.243 ± 2.500	0.733 ± 0.099	0.720 ± 0.086	–	–	–	–
Pecos R. 2013	143	5.582 ± 2.583	0.745 ± 0.090	0.728 ± 0.100	–	–	–	–
Pecos R. 2017	–	–	–	–	52	1.992 ± 0.755	0.318 ± 0.004	0.307 ± 0.004

Sample size (n), allelic richness (A_R) and gene diversity (H_E), heterozygosity (H_O) are shown for microsatellites and SNP-containing loci. Standard deviations are provided following the diversity metric

*Rio Grande 2011: Colorado Canyon, Big Bend; Rio Grande 2015: Laredo; Rio Grande 2016: Alamito Creek confluence; Rio Grande 2017: Eagle Pass

Table 2 Mitochondrial DNA diversity statistics for *N. jemezanus* and *M. aestivalis* from the Rio Grande and Pecos River

	n	N haps	$h \pm$ se	$\pi \pm$ se	pw	H_R	D	Fs
N. jemezanus								
Rio Grande 2017	4	4	1.0 ± 0.177	0.015 ± 0.010	12.177	1.000	− 0.311	0.561
Pecos R. 2007	16	8	0.825 ± 0.076	0.002 ± 0.002	2.042	2.113	− 0.908	− 2.782*
Pecos R. 2013	105	29	0.872 ± 0.02	0.003 ± 0.003	2.455	2.333	**− 2.243****	**− 20.432*****
M. aestivalis								
Rio Grande 2011	8	4	0.750 ± 0.139	0.013 ± 0.007	10.464	2.75	1.857	4.348
Rio Grande 2015	32	16	0.887 ± 0.044	0.014 ± 0.007	11.761	4.33	0.250	0.654
Rio Grande 2016	10	6	0.844 ± 0.103	0.009 ± 0.005	7.756	3.733	− 0.408	1.460
Rio Grande 2017	7	4	0.810 ± 0.130	0.020 ± 0.011	16.19	3.000	0.955	4.822
Pecos R. 2007	26	10	0.837 ± 0.049	0.003 ± 0.002	2.326	3.592	− 0.877	− 2.911
Pecos R. 2013	116	11	0.807 ± 0.018	0.003 ± 0.002	2.207	3.183	− 0.393	− 1.081
Pecos R. 2017	–	–	–	–	–	–	–	–

Included are sample size (n), number of haplotypes (N haps), haplotype diversity (*h*), nucleotide diversity (π), standard error (se), average number of pairwise difference (pw), haplotype richness (H_R), Tajima's D (D) and Fu's Fs (Fs). Significant *p*-values for Fu's Fs and Tajima's D are in bold and significance levels are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

detailed in Table S2. All samples were run on an automated ABI 3130 DNA sequencer and analyzed with GENEMAPPER vers. 4.0 (ABI).

For both species, genetic variation was characterized for 813 (*N. jemezanus*) and 823 (*M. aestivalis*) base pairs of the mitochondrially-encoded NADH-ubiquinone oxidoreductase chain 4 (ND4) gene using the PCR primers as described in Table S2. Sequencing was conducted using the ABI Big Dye Kit Version 1.1. DNA sequences were visualized and aligned using SEQUENCHER v.4.9 (Genecodes). To verify unique haplotypes, amplicons were sequenced in the forward and reverse directions. DNA sequences were submitted to Genbank with the following accession numbers: MT667318 – MT667350 for *N. jemezanus* and MT667287 – MT667317 for *M. aestivalis.*

Illumina sequencing

Double-digest RAD libraries were prepared as in Portnoy et al. (2015) and sequenced on one Illumina HiSeq 4000 lane with replicate samples sequenced across indices. Bioinformatics, including filtering and quality control, followed procedures outlined in Hollenbeck et al. (2019) and O'Leary et al. (2018). SNPs recovered from the same fragment were haplotyped (Willis et al. 2017), resulting in final data sets of 3,588 loci containing 12,741 SNPs for *N. jemezanus* and 3,806 loci containing 10,368 SNPs for *M. aestivalis*. A full description of laboratory and bioinformatic procedures are provided in the supplementary material.

Genetic variability

For both species, conformance to expectations of Hardy–Weinberg equilibrium (HWE) in temporal and spatial samples was assessed for microsatellites and SNP-containing loci using the R package PEGAS vers. 0.9 (Paradis 2010). GENEPOP'007 vers. 4.2 (Rousset 2008) was used to test for global patterns of linkage disequilibrium between microsatellite loci. Sequential Bonferroni correction (Rice 1989) was applied to account for multiple comparisons. Nei's unbiased gene diversity (H_E, Nei 1987), observed heterozygosity (H_O) and standard deviations (sd) were calculated using the program GENODIVE vers. 3.02 (Meirmans and Van Tienderen 2004). Rarefied averaged allelic richness across loci (A_R) was calculated using the R package HIERFSTAT (Goudet 2005). Genetic diversity statistics were calculated by geographic sampling site and across pooled sites within NM and within TX. Microsatellite diversity values were compared between the RG and PR collections of *M. aestivalis* with the OSx-statistic with significance evaluated using 9,999 permutations using FSTAT vers. 2.93 (Goudet 1995). This analysis was not conducted for *N. jemezanus* due to small sample size from the RG and was not conducted with SNP-containing loci because there was only a single PR temporal (2013) sample.

For mitochondrial DNA, haplotype diversity (*h*), nucleotide diversity (π) and average pairwise differences were calculated with ARLEQUIN vers. 3.5.1.3 (Excoffier and Lischer 2010) and haplotype richness was calculated using CONTRIB vers. 1.02 (Petit et al. 1998). Twenty-four alternative substitution models were compared using Bayesian Information Criterion (BIC) in MEGAX (Stecher et al. 2020). Pairwise divergence among haplotypes was calculated using the model with the lowest BIC score (Tamura-Nei). To visualize relationship between haplotypes, median joining networks (Bandelt et al. 1999) were constructed using POPART (http://popart.otago.ac.nz). Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) were calculated to examine whether population size has remained stable over

evolutionary timescales using ARLEQUIN. Significant departures from zero for either statistic indicates that neutrality or population stability can be rejected.

BEAST vers 2.5.1 (Bouckaert et al. 2014) was used to estimate divergence times among mitochondrial haplotypes for *N. jemezanus* and *M. aestivalis* with a per lineage clock rate of 1.0% per mya (Osborne et al. 2016). A strict clock was used as suggested for relatively young clades where rate variation among branches is expected to be low (Brown and Yang 2011). An outgroup was not included, following Drummond and Bouckaert (2015). bModeltest add-on for BEAST (Bouckaert and Drummond 2017) was used to infer the base substitution model during the Bayesian run. All other methods for divergence dating followed Osborne et al. (2016). Divergence time estimates among haplotypes were calculated to allow results to be compared with previous studies on RG drainage fishes (e.g., Osborne et al. 2016; Kim and Conway 2014).

Populations structure

For both species, each complete dataset of SNP-containing loci was screened for outlier loci (putatively under selection) using the Bayesian modeling approach implemented in BAYESCAN vers. 2.1 (Foll and Gaggiotti 2008; Supplementary Material). Neutral and outlier loci were then analyzed separately to evaluate the relative effects of genetic drift and localized selection on population structure. To assess for homogeneity of allele distributions among geographic samples we conducted a single level Analysis of Molecular Variance (AMOVA) for *N. jemezanus* and a hierarchical AMOVA for *M. aestivalis* using the program GENODIVE vers. 3.02 (Meirmans and Van Tienderen 2004), with significance assessed using 9999 permutations. To evaluate whether there was genetic differentiation between sampling sites of each taxa, pairwise F_{ST} was calculated following Weir and Cockerham (1984) for each pair of samples using the program GENODIVE. Significance was assessed using 9,999 permutations. For *N. jemezanus*, these analyses were only conducted using neutral SNP-containing loci and not microsatellites due to small sample size of the RG collection.

Population structure was also evaluated in each species using discriminant analysis of principle components (DAPC) as implemented in the R package ADEGENET using *k*-means clustering (Jombart and Ahmed 2011) where *k* is the number of groups. To identify the number of principle components we used the cross-validation procedure (Jombart et al. 2010) with a training set comprising 90% of the data and 50 replicates. Bayesian Information Criteria were used to compare different values of *k*. For *N. jemezanus*, this analysis was conducted using neutral SNP-containing loci. For *M. aestivalis*, microsatellites, neutral and outlier SNP-containing loci were analyzed using DAPC.

For *M. aestivalis*, a hierarchical AMOVA based on mtDNA data was used to assess whether a significant portion of variance was attributed to differences between rivers and/or among samples within rivers using ARLEQUIN vers. 3.5.1.3 (Excoffier and Lischer 2010). Significance was assessed using 9999 bootstrap replicates. *Post-hoc* estimates of pairwise Φ_{ST} were also calculated using ARLEQUIN and significance assessed as above. The above analyses were not conducted for *N. jemezanus* using mtDNA due to the small sample size from TX.

Genetic effective population size

The single sample linkage disequilibrium method (Hill 1981) was used to estimate effective population size (N_e) from microsatellite DNA and SNP-containing loci for *N. jemezanus* and *M. aestivalis* using the program NeEstimator Vers. 2.0 (Do et al. 2014). We used $P_{crit} = 0.02$ or $P_{crit} = 0.03$ as suggested when the number of individuals sampled is > 25 or < 25 respectively (Waples and Do 2010). N_e was not calculated when sample size was less than 20 individuals. Upper- and lower-bound 95% confidence intervals for N_e were calculated using the jack-knife approach (Jones et al. 2016).

Results

Surveys for *N. jemezanus* were conducted at 44 sites within the RG drainage in TX and along the US/Mexico international border between 2013 and 2018. Based on museum vouchered material, *N. jemezanus* had been previously collected from 22 of the 44 sites. Multiple sites were surveyed more than once resulting in a total of 51 sampling events (Table S1). This included three sites within the PR basin (including Independence Creek), five sites within the Devils River basin (including Dolan Creek), and 35 sites located along the main stem of the RG or its tributaries in six TX counties, including Brewster (15), Webb (6), Presidio (5), Maverick (3), Terrell (4), Zapata (2) and Val Verde (1). A complete list of species collected during the 51 sampling events is available upon request from the authors. *N. jemezanus* (n = 4) was encountered at one site on the RG (site 18, Table S1). Although not the original target of surveys, *M. aestivalis* had been previously collected from 22 of 44 sites and was encountered at 16 sites in the new surveys including those along the main stem of the RG in Presidio (4), Brewster (4), Maverick (3), Webb (3), and Zapata (2) counties.

Notropis jemezanus- genetic variability

For *N. jemezanus*, there were no departures from the expectations of HWE following Bonferroni correction (α = 0.002) in the RG sample at any microsatellite loci, while two loci (*Lco3* and *Lco6*) departed from HWE in the 2013 PR sample. All loci were retained for downstream analyses. There was no indication of linkage disequilibrium among any pairs of loci in either the RG or PR sample. Allelic richness of the RG sample (A_R = 2.704) was very similar to the temporal samples from the PR (2007, A_R = 2.673; 2013, A_R = 2.803). Gene diversity for *N. jemezanus* collected from the RG was 0.700 and for the PR temporal samples H_E was 0.644 in 2007 and 0.681 in 2013 (Table 1).

A total of 298 SNP-containing loci that departed from HWE after adjusting for inflated Type I errors (α = 0.000007) in the PR sample were removed before calculating diversity metrics, leaving 3290 loci. One (644 loci) to eight alleles (mean = 2.773, sd = 1.414) were detected per SNP-containing locus in the RG sample. Across the PR samples there was one (117 loci) to 37 alleles per locus (mean = 6.838, sd = 4.519). Allelic richness (A_R = 1.477) and gene diversity (H_E = 0.484) in the RG were smaller than in individual PR sites and all metrics of genetic diversity were similar among PR sites (average H_E = 0.501; H_O = 0.459; A_R = 1.497 (Table S3).

Sequences of ND4 were obtained for 125 N. *jemezanus* (Table 2). Thirty-two haplotypes were identified with three exclusive to the RG, 28 exclusive to the PR and one shared between rivers (A1). Two haplotypes (A and A1) were found in 40% of individuals while the remainder were infrequent (< 8%). The majority of haplotypes were separated from one another by 1–3 substitutions with the exception of four haplotypes (Tx1, Tx2, Tx3 and W; Fig. 2). The number of pairwise differences between haplotypes in the RG was 12.177, while the average number of pairwise differences in the PR was 2.249 (Fig. 2a). Percent sequence divergence between *N. jemezanus* haplotypes ranged from 0.1 to 2.85%. Haplotype diversity was 1.0 for the RG, *i.e.* each individual had a unique haplotype, and 0.825 and 0.872 for the PR in 2007 and 2013 respectively. Nucleotide diversity was 0.015 for the RG and 0.002–0.003 for the PR samples. Tajima's D and Fu's Fs were positive and not significantly different from zero for the RG. Tajima's D was significantly negative for the PR in 2013 and Fu's Fs was significantly negative in the PR in both years. Negative values of Tajima's D and Fu's Fs may be indicative of population expansion. For *N. jemezanus* the cluster of haplotypes predominantly found in RG (W, Tx1-3) diverged 1.294 (0.794–1.810) mya from the cluster containing haplotypes mainly from the PR (Fig. 2b).

Notropis jemezanus- population structure

For *N. jemezanus* a single outlier locus was detected and removed prior to analysis of population structure. Single-level AMOVA based on SNP-containing loci revealed significant heterogeneity among all *N. jemezanus* samples (F_{ST} = 0.088, p = 0.001). Estimates of pairwise F_{ST} between PR samples and the RG sample were all significantly different from zero after correction for multiple comparisons with the exception of Willow-Eagle Pass comparison (Table S4). Estimates of pairwise F_{ST} between PR samples were not significantly different from zero. SNP-containing loci assigned individuals to two clusters based on the lowest BIC value; one cluster corresponded to all of the PR samples and the other to the RG with the first discriminant explaining 64.7% of the variance between groups (Fig. 3, Fig. S1). All individuals were accurately assigned to the RG (n = 4) or the PR (n = 55). Two outlier points were apparent in the scatterplot (Fig. S1). These samples did not have large amounts of missing data or increased homozygosity that might suggest coverage problems and/or the presence of another species. Likewise, they did not have high levels of heterozygosity indicative of incorrect SNP calling and/or paralogs. However, these samples had relatedness values consistent with half siblings. Both randomly collected samples were retained as suggested by Waples and Anderson (2017).

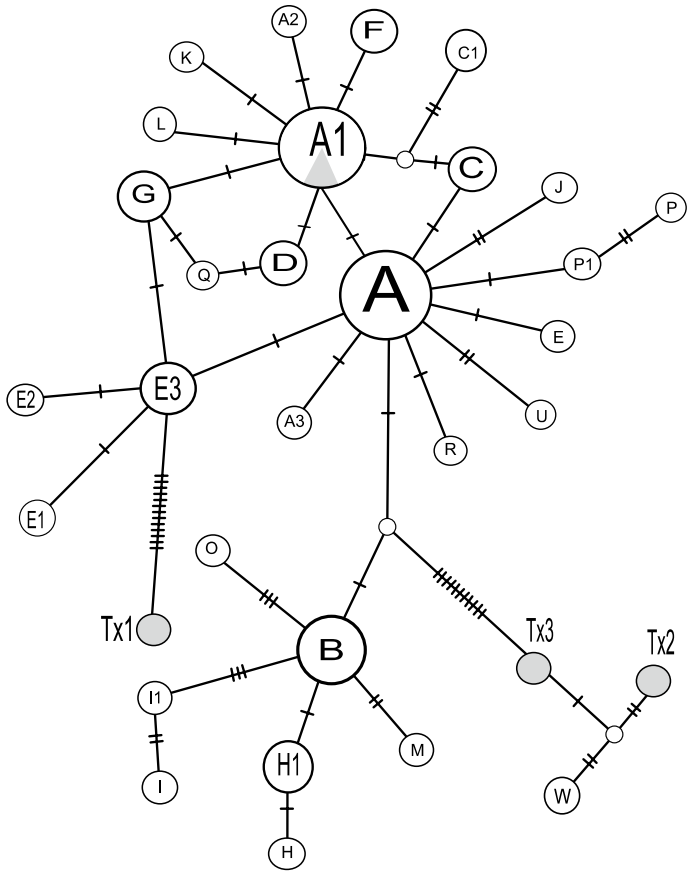
Notropis jemezanus- genetic effective population size

The small sample size for the RG population of *N. jemezanus* precluded meaningful estimation of N_e from either microsatellites or SNP-containing loci. Based on microsatellites, N_e estimates for the PR population of *N. jemezanus* in 2007 was 259 (95% CI 47–infinity) and in 2013, indistinguishable from infinity (95% CI 693–infinity, Table S9). N_e based on SNP-containing loci for the PR population in 2017 was also large (N_e = 8,443, 95% CI 668–infinity).

Macrhybopsis aestivalis- genetic variability

For *M. aestivalis,* there were five significant departures from HWE expectations out of 54 comparisons following Bonferroni correction (α = 0.0009). These occurred at *Nme232* (PR 2007, RG 2015), *Loc1* (RG 2011 and 2016) and at *Ppro118* (RG 2015). In all cases, there was an excess of homozygotes. All loci were retained for further analyses. There was no evidence of linkage disequilibrium between any pairs of loci. For the RG, A_R ranged from 4.681 in 2016 to 5.755 in 2017 (Table 1). For the PR, A_R was 5.243 in 2007 and 5.582 in 2013. Gene diversity was 0.796 in the 2011 collection and 0.778 in the 2015 collection from the RG as compared to samples collected in 2016 (0.693) and 2017 (0.724). These

a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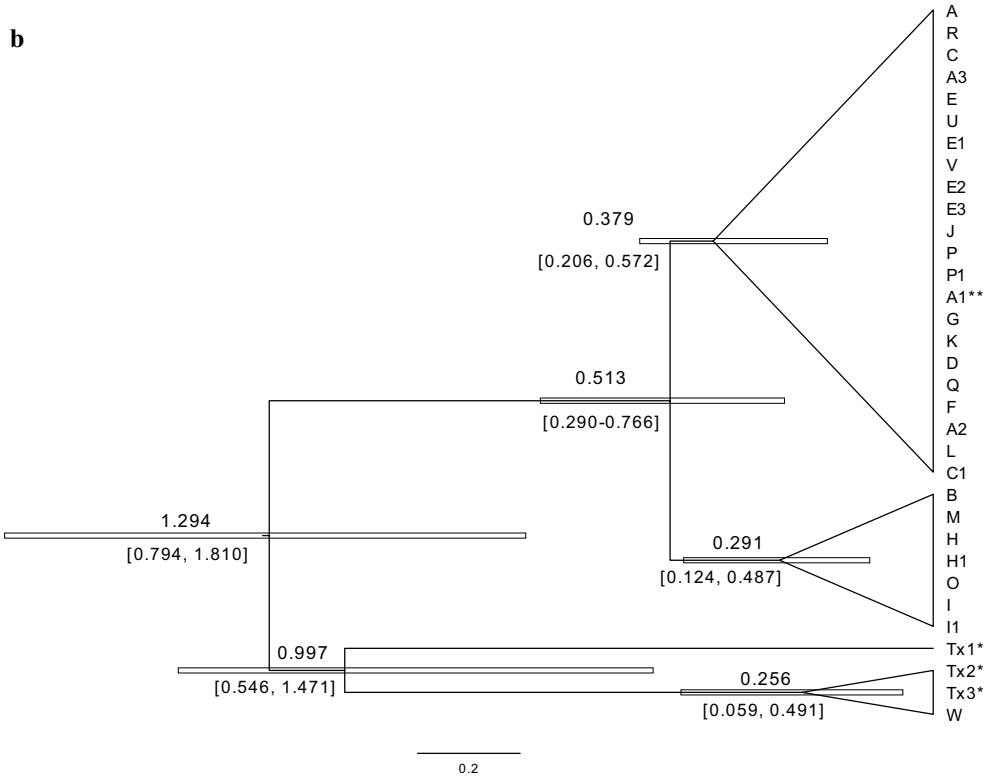
0.379
[0.206, 0.572]
0.513
[0.290-0.766]
1.294
[0.794, 1.810]
0.291
[0.124, 0.487]
0.997
[0.546, 1.471]
0.256
[0.059, 0.491]
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Tx3*
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◂**Fig. 2** **a** ND4 haplotype network for *N. jemezanus*. Nucleotide changes are indicated by bars on lines connecting haplotypes. Circle size reflects haplotype frequencies. Grey shading indicates samples collected from the Rio Grande in Texas, white indicates haplotypes detected in the Pecos River New Mexico. **b** Chronogram shows mean estimated time (mya) to most recent common ancestor. 95% HPD (highest posterior density) intervals are shown by blue bars and values in parentheses, letters refer to distinct ND4 haplotypes. Unshaded clades correspond to predominantly Pecos River haplotypes. * exclusive Rio Grande haplotypes, ** shared Rio Grande/Pecos River haplotypes

metrics were not significantly different between the RG and PR (H_E- $p = 0.296$; H_O- $p = 0.488$, A_R- $p = 0.803$).

Within the PR sample (combined across sites) and/or RG sites, 143 SNP-containing loci departed from HWE after adjusting for inflated Type I errors ($\alpha = 0.000004$). The remaining 3663 loci that conformed to HWE were used for calculation of genetic diversity and N_e. Between one (347 loci) and 19 alleles (mean = 2.915) were detected per SNP-containing locus in the RG. Across the PR samples, one (507 loci) to nine alleles (mean = 2.713) were detected. In the RG collection A_R varied from 2.058–2.071 and in the PR sample, A_R was 1.992.

Sequence data from ND4 were obtained for 199 M. *aestivalis.* Thirty-one haplotypes were identified, and these were divided into two haplogroups largely associated with either the RG or PR (Fig. 4). Eighteen haplotypes were unique to the RG, ten were unique to the PR and three were shared between rivers. In the RG, one haplotype was prevalent (TxB) and found in 18% of individuals and other haplotypes were present at lower frequencies (< 8%). In the PR, five haplotypes were common (A1, A2, B, D and F) accounting for 89% of individuals. The average number of pairwise differences between haplotypes in the RG was 11.543, while the average number of pairwise differences in the PR was 2.267 (Fig. 4a). Haplotype diversity was 0.750 (2011) to 0.877 (2015) for the RG, and 0.837 (2007) and 0.807 (2013) for the PR. Nucleotide diversity was 0.013–0.020 for the RG and 0.003 for the PR in 2007 and 2013. Percent sequence divergence between haplotypes ranged from 0.1 to 5.6%. Neither Tajima's D or Fu's Fs were significant in any case. For *M. aestivalis,* haplotype clusters unique to the RG diverged 1.1 (0.646–1.510)–1.6 (1.01–2.170) mya from the cluster containing haplotypes shared between drainages (Fig. 4b).

Macrhybopsis aestivalis- population structure

AMOVA based on microsatellites indicated that a significant proportion of variance was attributable to differences between the RG and PR ($F_{CT} = 0.124$, $p = 0.0001$). There were also significant differences among sites within rivers ($F_{SC} = 0.020$, $p < 0.0001$). Values of pairwise F_{ST} were significant for three comparisons of temporal/spatial RG samples following Bonferroni correction (Table S5). Estimates of pairwise F_{ST} were small and not significantly different from zero among PR temporal/spatial samples following Bonferroni correction. DAPC based on *k*-means clustering of microsatellite data revealed two clusters corresponding to the RG and PR. The first axis explained 84% of the between group variance (Fig. 5). Using the groupings identified from *k*-means, posterior assignments of individuals to either the RG or PR was achieved for 98% and 99% of individuals, respectively.

For *M. aestivalis*, 159 candidate outlier loci were detected from the complete dataset. The majority of these (155) had an alpha value greater than zero, indicating possible directional selection, while the remainder (4) had an alpha value less than zero, indicating possible balancing selection. AMOVA based on neutral SNP-containing loci indicated that a significant proportion of variance was attributed to differences between the RG and PR ($F_{CT} = 0.164$, $p < 0.0001$) but not between sites within rivers ($F_{SC} = -0.001$, $p = 0.700$). Pairwise F_{ST} from neutral SNP-containing loci between RG spatial/temporal samples and between PR spatial/temporal samples were not significantly different from zero (Table S7). When only outlier loci were analyzed, the majority of variance was attributed to differences among the RG and PR ($F_{CT} = 0.601$, $p < 0.0001$) and F_{SC} was small (0.001) and not significantly difference from zero ($p = 0.135$). Pairwise F_{ST} values between RG and PR sites ranged from 0.537 to 0.611 and were highly significant (Table S8). Pairwise F_{ST} between the two RG sites was 0.008 ($p = 0.043$) and significant before but not after Bonferroni correction.

Neutral and outlier SNP-containing loci assigned individuals to two clusters based on the lowest BIC values; one cluster corresponded to all PR samples and the other, RG samples (Fig. S2). For neutral loci, the first linear discriminant explained 87.3% of between group variance (Fig. 6A). For outlier loci, the first linear discriminant explained 95.7% of the between group variance (Fig. 6B). Posterior assignment of individuals accurately placed all individuals in either the RG or PR when either neutral or outlier loci were considered.

AMOVA based on mtDNA haplotype frequencies found a significant proportion of variance attributed to differences between the RG and PR ($\phi_{CT} = 0.473$, $p = 0.003$) and between samples within these groups ($\phi_{SC} = 0.130$, $p < 0.0001$). At the site level, there were 20 significant pairwise comparisons between RG and PR samples after correction for multiple comparisons (Table S6).

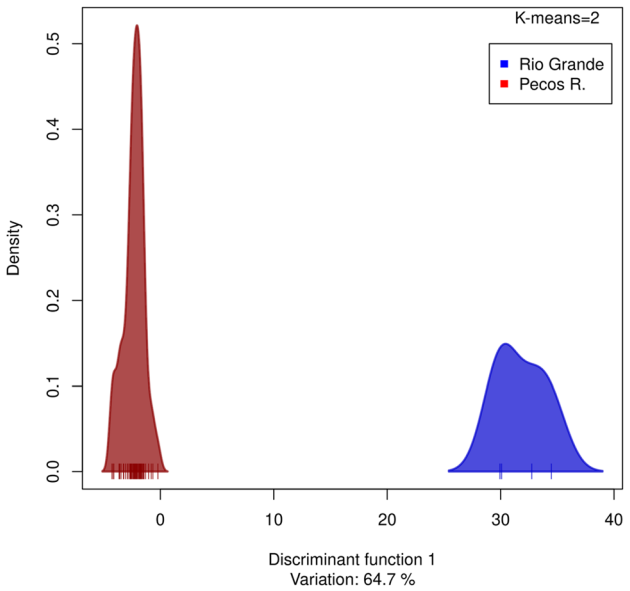

Fig. 3 Discriminant analysis of principal components based on SNP-containing loci between Rio Grande and Pecos River samples for *N. jemezanus* for *k*-means = 2. The x-axis represents the first Linear Discriminant (LD)

Macrhybopsis aestivalis- genetic effective population size

Four finite estimates of N_e were obtained from microsatellite data from *M. aestivalis.* For the RG, N_e was 176 in 2015 (95% CI 58–infinity) and 206 in 2016–2017 (95% CI 46–infinity). Point estimates were larger for the PR population ranging from 2019 in 2007 (95% CI 25–infinity) to 698 (95% CI 292–infinity) in 2013. N_e based on SNP-containing loci was 5,250 (95% CI 201–infinity) in 2015. For the PR population, N_e was indistinguishable from infinity in 2017.

Discussion

This study documents significant range contraction and near extirpation of *N. jemezanus* from the lower RG in TX and provides a genomic assessment for this species and *M. aestivalis* to aid conservation efforts. Highly divergent mtDNA haplotypes were identified within both species, likely indicative of fragmentation within ancestral populations and subsequent secondary contact in refugia during the Pleistocene, followed by more recent anthropogenic fragmentation between remnant populations. The unique genetic variation present in the RG (along the TX/MX border) and PR (NM) for both species suggests that both regions should be a focus of conservation and monitoring. Collectively, results highlight the critical need to evaluate the status of these taxa in the lowermost RG, including Mexican tributaries, and for *M. aestivalis* in the Río San Fernando.

Contracting range of *N. jemezanus*

Despite being once widespread throughout the main stem of the RG, *N. jemezanus* and *M. aestivalis* have long been extirpated from the RG in NM (Bestgen and Platania 1990; Eisenhour 2004), but were still considered "common" in the RG in TX (Hubbs 1990; Page and Burr 2011). For the majority of surveyed sites on the main stem RG (TX), *N. jemezanus* had been collected within the last 30 years, suggesting that their absence reported here, reflects a recent decline. While *M. aestivalis* remained relatively widespread, only four *N. jemezanus* were collected from one locality, in a survey of 44 sites distributed throughout the main stem RG (TX) (Fig. 1B). Prior to this, *N. jemezanus* was lost from confluent portions of the Río Conchos, Chihuahua (Edwards et al. 2002a, b, 2003); strongly suggesting that the range of *N. jemezanus* has contracted. Although the 44 survey sites were distributed across a broad area (including sites in seven TX counties and through remote areas of the Big Bend and Lower Canyons), it is extremely difficult to sample extensively in such a broad geography and *N. jemezanus* could persist in low numbers in these areas. This highlights the importance of further intensive survey work in the Big Bend and lower canyons region of the Rio Grande along the US/Mexico border to establish if *N. jemezanus* is truly on the brink of extirpation from the entire main stem RG.

The looming loss of *N. jemezanus* from the RG in TX contrasts with its persistence in the PR between Fort Sumner Irrigation District Dam and Brantley Reservoir in NM. There are no diversions or storage dams within this 330-km section of river. Although PR flows are regulated, these impacts are buffered by periodic floods and inputs of alluvial sediment provided by unregulated tributaries (Hoagstrom and Brooks 2005). Likewise, inputs from tributaries and groundwater seepage maintain base flows in some sections (Mower et al. 1964; Mourant and Shomaker 1970). Hoagstrom et al. (2015) identified a 151 km 'relict ecosystem' that maintains high concentrations of pelagophils because of its wide river channel and persistent base flows (Hoagstrom et al. 2008a). This section of channel also retains drifting propagules in situ (Dudley and Platania 2007), while river connectivity allows some propagules displaced downstream to eventually return upstream (Chase et al. 2015). Although streamflow of this river segment can become intermittent during dry years (Hoagstrom et al. 2008b), absence of instream barriers means that some fish can periodically avoid drying sections by occupying wetted refugia either above or below the drought-prone reach, and recolonizing the dried sections once flows return. Understanding habitat/environmental differences between the RG and PR is critical

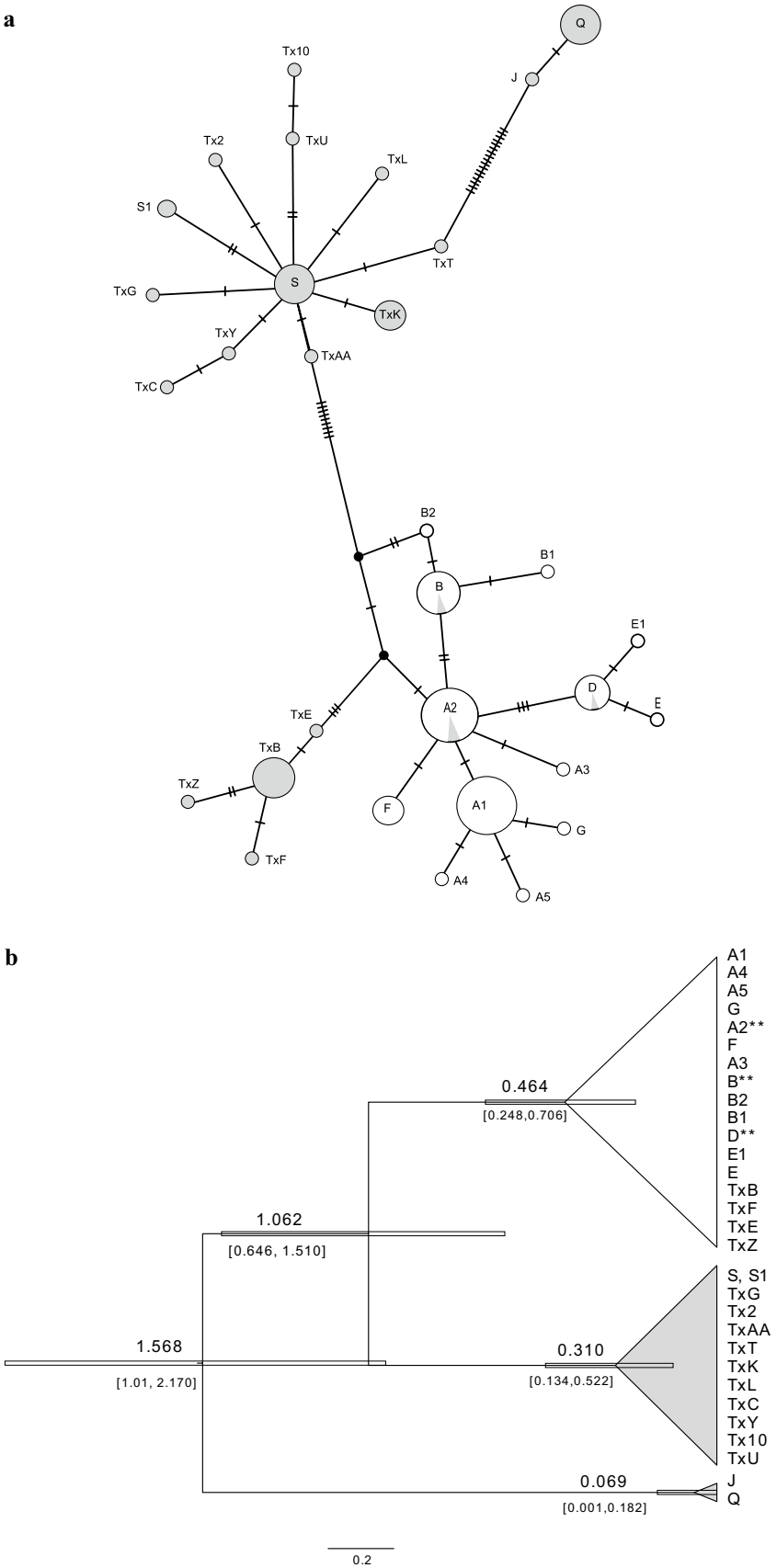

Fig. 4 **a** ND4 haplotype network for *M. aestivalis.* Circle size reflects haplotype frequency. Grey fill indicates haplotypes detected in the Rio Grande, white indicates haplotypes detected in the Pecos River. **b** Chronogram showing mean estimated time (mya) to most recent common ancestor. 95% HPD (highest posterior density) intervals are shown by blue bars, letters refer to distinct haplotypes. Grey shaded clades-haplotypes exclusive to the Rio Grande. Unshaded clades correspond to predominantly Pecos River haplotypes. Grey shading exclusive Rio Grande haplotypes, ** shared Rio Grande/ Pecos River haplotypes

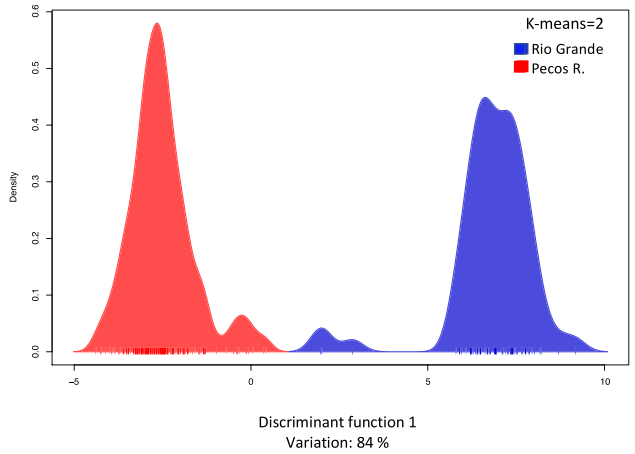

Fig. 5 Discriminant analysis of principal components based on microsatellites between Rio Grande and Pecos River samples for *M. aestivalis* for *k*-means = 2. The x-axis represents the first Linear Discriminant (LD)

in order to identify factors behind the decimation of the *N. jemezanus* population in the RG in TX. More importantly, this knowledge could be used to direct conservation actions for *N. jemezanus* in the RG that could simultaneously protect *M. aestivalis* and other aquatic species (*e.g.*, freshwater mussels; Inoue et al. 2015). In light of recent surveys documenting large range reductions and loss of suitable habitat for *N. jemezanus* and *M. aestivalis*, Texas Parks and Wildlife acted this year to add both species to the state threatened and endangered species list.

Historical demography

The RG and PR populations of both species had relatively high levels of mtDNA diversity, with *h* exceeding 0.8. The most common haplotypes for both species were shared between PR and RG populations, implying that these are likely ancestral (Watterson and Guess 1977). For the PR population of *N. jemezanus* the average number of pairwise differences between haplotypes was very small, with many rare haplotypes radiating in a star-like manner from ancestral haplotypes; consistent with relatively recent population expansion in the PR. This was further supported by significantly negative Fu's Fs for the PR population of *N. jemezanus* in both years of collection. Tajima's D was also significantly negative for the 2013 sample. The RG population featured four haplotypes scattered across the network; perhaps signaling a decline from a previously large population. Too few samples were collected to address this idea but it is consistent with population survey data indicating a population collapse.

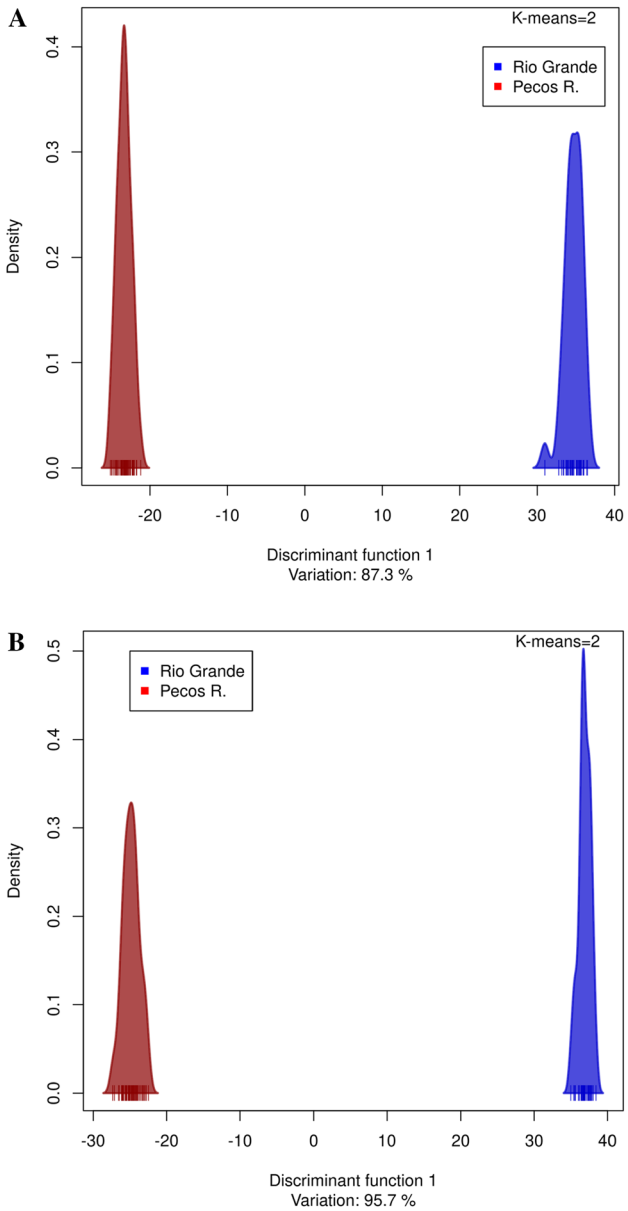

Fig. 6 Discriminant analysis of principal components from SNP-containing loci between Rio Grande and Pecos River samples of *M. aestivalis* for **A** neutral and **B** outlier loci for *k*-means = 2. The x-axis represents the first Linear Discriminant (LD)

In the PR population of *M. aestivalis,* five haplotypes were found at relatively high frequencies, while in the RG, most haplotypes were rare (although sample size was smaller). High levels of genetic diversity within a population (with all haplotypes closely related) can be a signature of population stability, which was supported by non-significant values of Fu's Fs and Tajima's D in both the RG and PR populations of *M. aestivalis.* Maintenance of high levels of variability also suggests that these populations have maintained large long-term N_e. The basal position of most

RG haplotypes for both species in the chronograms suggests that these species may have arisen in the lower RG and then expanded and colonized upstream reaches, as also proposed for Red Shiner *Cyprinella lutrensis* (Osborne et al. 2016). For *N. jemezanus*, *M. aestivalis* and *C. lutrensis*, divergence times between PR and RG haplotype clusters date to the Middle-Late Pleistocene. Texas hornshell mussel also shares this history (Inoue et al. 2015).

Both the RG and PR populations of *N. jemezanus*, and RG population of *M. aestivalis* had highly divergent haplotypes scattered across the network, perhaps indicative of mixing between previously isolated populations associated with climatic fluctuations. Osborne et al. (2016) proposed that canyon formation could account for divergence among *C. lutrensis* lineages because that species generally associates with unconfined, sediment-laden river channels; which may also apply to *N. jemezanus* and *M. aestivalis.* Canyons were likely least suitable for these species when being excavated, which was the long-term trend through the Middle-Late Pleistocene (Galloway et al. 2011; Repasch et al. 2017). However, periods of deglaciation may have temporarily inundated canyons with sediment, potentially increasing their suitability. If so, changing conditions among successive glacial and interglacial periods may have promoted periodic cycles of range expansion and fragmentation, allowing periods of divergence alternating with opportunities for subsequent mixing, as reported for *C. lutrensis* (Osborne et al. 2016), Longnose Dace *Rhinichthys cataractae* (Kim and Conway 2014) and Roundnose Minnows *Dionda* spp. (Schönhuth et al. 2012). The presence of divergent haplotypes within the RG population of *N. jemezanus* is encouraging, but potential restriction of this population to a relatively small area (around Eagle Pass, TX) indicates this population faces extreme risk of extirpation.

Contemporary fragmentation

Genetic data indicate that the RG populations of *N. jemezanus* and *M. aestivalis* are now genetically distinct from PR populations. Retention of shared haplotypes between populations is likely the result of fragmentation and subsequent genetic drift in the past rather than contemporary gene flow. Significant genetic divergence was detected between populations for both species, with the magnitude of F_{ST}/Φ_{ST} indicative of no biologically meaningful contemporary gene flow. The PR between Brantley Reservoir and Girvin, TX—a 500 km reach—has the longest history of degradation within the basin due to damming and dewatering (Hoagstrom 2003). Cumulative effects of intense and long-term degradation have caused persistent salinization and periodic toxic algal blooms, devastating the fish assemblage, including disappearance of *N. jemezanus* and *M. aestivalis* (Hoagstrom 2009; Cheek and Taylor 2016). Loss of connectivity between remnant populations in the PR upstream of Brantley Reservoir and in the confluent RG downstream has presumably allowed differences in allele/haplotype frequencies to accumulate via genetic drift.

Detection of putative outlier loci in *M. aestivalis* indicate that selective forces may have played a role in divergence of RG and PR populations, as estimates of pairwise F_{ST} obtained from outlier loci were almost five times greater in magnitude when compared to neutral loci. However, processes other than local selection can produce outlier loci including gene surfing (Excoffier and Ray 2008) and population bottlenecks, disparate levels of genetic differentiation among sampled populations and loci, and non-independence among loci (Foll and Gaggiotti 2008). Here, SNPs within a contig were collapsed into haplotypes to minimize the latter issue but further study of *M. aestivalis* within the RG relative to biotic and abiotic factors is warranted.

Environmental factors shown to be important selective forces in fishes include temperature, oxygen and salinity (e.g., Berg et al. 2015). Although the central core of the study area is the Chihuahuan Desert, the northernmost range is within the southwestern Tablelands and southernmost within the South Texas Plains, which differ substantially in climate (e.g., 180–200 versus 280–300 frost-free days; Griffith et al. 2006, 2007), and may correspond with interpopulation divergence in this study. In addition, pelagophils like *N. jemezanus* and *M. aestivalis* may also develop differing spawning ecology and life-history traits in response to differing geomorphic and hydrologic conditions (Hoagstrom and Turner 2015). Divergence estimates from mtDNA suggest that gene flow was likely limited between the RG and PR prior to anthropogenic impacts, therefore, it is possible that local selective pressures are historically responsible for restricting gene flow. Following anthropogenic modifications to the river, gene flow may have ceased entirely. More thorough sampling and analyses would be required to evaluate this scenario. Understanding potential adaptive differences between populations is crucial for effective *in-situ* conservation and should be better understood before translocations between populations are attempted.

At the local scale, all marker types failed to differentiate between PR sampling localities for both species. This finding, along with the life-history of these species and the absence of barriers within this stretch of the PR, suggests connectivity along this river segment. Connectivity among local populations of pelagophils is critical to their persistence, as dispersal can offset the downstream displacement of eggs and larvae. Dispersal can also facilitate recolonization of rewetted habitat patches following stream drying events. Results presented here are similar to findings and observations for other pelagophils for which mark-recapture and/or genetic data indicate substantial dispersal ability

(e.g., Platania et al. 2020; Osborne et al. 2010, 2012). Studies of otolith microchemistry in *N. s. pecosensis* (Chase et al. 2015) also demonstrate long-distance upstream dispersal, even by small juveniles.

By contrast, Amistad Dam and Reservoir, completed in 1969, separate populations in the RG upstream (Alamito and Colorado Canyon sites) and downstream (Eagle Pass, Laredo, and San Ygnacio sites). This must interrupt gene flow between these sites and could result in genetic divergence between populations of *M. aestivalis* and possibly *N. jemezanus*, if the later still persist in upstream locations. More comprehensive sampling is needed in the RG to identify spatial and temporal patterns of diversity within this river for *M. aestivalis* and to search for remnant populations of *N. jemezanus*.

Estimates of N_e from microsatellites and SNP-containing loci were large for the PR population of *N. jemezanus* in 2013 (microsatellites) and 2017 (SNP-containing loci). By contrast, the 2007 N_e estimate was small suggesting a period of reduced effective population size and, taken with the other results, fluctuations in abundance. For example, the 2000 – 2013 drought caused extreme low flows on the PR (Harley and Maxwell 2017), which negatively impacted some native fishes (e.g., Osborne et al. 2010; Hoagstrom 2014).

N_e estimates based on microsatellites for *M. aestivalis* collected from the RG were small ($N_e = 176–206$) for both temporal samples, while, N_e based on the SNP-containing loci for the RG (2015) and PR (2017) were considerably larger. For the 2015 RG sample, N_e obtained from microsatellites was very small compared to the estimate from SNP containing loci. This disparity may be a result of poor precision of the microsatellite estimate due to small sample size from a large population. Waples and Do (2010) found that when N_e is large, precision of N_e estimates will be poor unless large amounts of data are collected by either increasing sample size, number of loci or number of alleles. The number of loci and alleles was increased substantially by using SNP-containing loci, as such this estimate more likely reflects the effective size of the RG population. Other pelagophils native to the PR, including *N. simus pecosensis*, generally have large effective population sizes and estimates are positively associated with changes in density (Osborne et al. 2010). Maintenance of large effective population sizes in PR pelagophilic species of cyprinids highlights both the genetic and demographic benefit of having a contiguous river that maintains some wetted sections even during drought, that can serve as a refugium.

Taxonomic Implications for *M. aestivalis*

Two separate taxa within the *M. aestivalis* complex were historically recognized in the RG basin: (i) a reputed clear-water form (*M. aestivalis*) from the Río San Fernando and the RG downstream of the Río Salado confluence including ríos Salado and San Juan (RG tributaries) and (ii) a turbid-water form (referred to as *Macrhybopsis sterletus*, currently considered a junior synonym of *M. aestivalis*; Gilbert 1998) from the RG above the PR confluence, including the PR and Río Conchos (Moore 1950; Wallace 1978). However, detailed morphological study of museum specimens from throughout the basin suggested a longitudinal cline rather than two distinct taxa, with the RG between the Río Salado and PR confluences representing a transition zone between upstream and downstream forms (Eisenhour 2004). Unfortunately, none of our samples were taken within the historic range of the clear-water phenotype, thus it is unknown whether those populations previously recognized as the clear-water taxon are more highly divergent than those sampled. Nevertheless, samples from Eagle Pass, Laredo, and San Ygnacio occur within Eisenhour's transition zone and do not exhibit species-level divergence from those in the PR. Evidence presented here of shared mtDNA haplotypes between the PR and RG preliminarily supports Eisenhour's (2004) decision to recognize a single, morphologically variable species for the entire RG drainage, while emphasizing a need for genetic assessment of southern populations, if any persist.

The plight of pelagophils and conservation priorities

Pelagic spawning cyprinids have declined and suffered wholesale successive losses throughout the North American Great Plains and Chihuahuan Desert (Worthington et al., 2018). Historically, the RG drainage supported *Notropis simus simus*, which disappeared between 1940–1964, while *N. jemezanus* and *M. aestivalis* disappeared from the NM stretch of the RG between 1950–1960 (Bestgen and Platania 1990). *Notropis orca* although not common in the RG, NM, was extirpated after 1949 and while abundant in the lower RG, TX and MX (Robinson 1959) was extirpated by 1975 (Bestgen and Platania 1990). The sole remaining pelagophil in the upper RG, *Hybognathus amarus*, persists only because of ongoing conservation actions. Elsewhere, *N. girardi* was last collected in 1983 from the Arkansas and Ninnescah rivers, *Hybognathus placitus* persisted until 2006 and *Macrhybopsis tetranema* persisted in these streams until 2012 (Perkin et al. 2015b; Pennock et al. 2017). Collectively these examples document incremental losses across river basins of ecologically similar species and clearly demonstrate the vulnerability of all remaining pelagophils in Great Plains and Chihuahuan Desert rivers, highlighting the need for preemptive conservation measures including evaluating the status of all extant populations.

The history of losses of members of this guild across basins indicates that proactive conservation measures need to be implemented now to protect *N. jemezanus* and *M. aestivalis.* Basic research is urgently needed to fill knowledge gaps for both species, including circumscription of current geographic distribution (including MX), the identification of habitat preferences, spawning cues and requirements of early life history stages. Further genetic work will be needed to identify the number of populations that remain for each species, their effective sizes and levels of connectivity. Monitoring of both species within the RG would serve as a mechanism to detect changes to the status of populations. Effective monitoring, combined with the development of an emergency response plan (to be implemented if the status of either species changes) are likely realistic conservation measures that could protect *M. aestivalis* within the RG but more extreme conservation tools, including reintroduction to priority areas (e.g., Big Bend National Park) may be required to kick-start conservation of *N. jemezanus* in the RG unless a refugial population is discovered in poorly sampled reaches.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval Samples were collected under UNM IACUC protocol MSC10-100492-MCC and TAMU-IACUC protocol # 2017-0047.

Consent for publication All authors consent to publication.

Availability of data and material DNA sequences from this study have been deposited in GenBank, microsatellite and mtDNA data and SNP data are available on Dryad: https://doi.org/10.5061/dryad.4b8gthtb7. The commands used for bioinformatics analysis are available at: https://github.com/marinegenomicslab/Osborne-et-al.-2020-Under-the-radar.git.

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