Amended Project Completion Report

Project: Rio Grande sucker and chub eDNA marker development
Contractor(s): Turner Ranch Properties, L.P. – Ladder Ranch
Investigator(s): Dr. Carter Kruse and Dr. Kellie Carim
Date: June 25, 2017, amended November 3, 2017
Contract #: NMDGF 16-516-0000-00034
Work supported through funding from the Share with Wildlife program and State Wildlife Grant T-32-4 #14

Project Objective: This proposed effort will explore development of eDNA markers for detecting presence of Rio Grande chub (*Gila pandora*) and Rio Grande sucker (*Catostomus plebeius*) in New Mexico streams. If species specific markers are identified, initial testing would be conducted in natural stream systems to determine eDNA presence and field test sensitivity (based on distance and biomass) for Rio Grande chub.

Project Need: Rio Grande sucker and chub have experienced declines in distribution and are considered Species of Greatest Conservation Need in New Mexico. Both species have been petitioned for listing under the Endangered Species Act. Information regarding the historical and contemporary distribution, as well as life history and habitat needs, of these two non-game species is limited; future management, conservation and restoration will depend, in part, on effective sampling methodology to detect presence in a waterbody. Environmental DNA is an emerging technique for inventorying and monitoring aquatic species and has allowed biologists to sample large areas at lower cost and detect rare species more often than traditional sampling methods, such as electrofishing or netting. Development of a reliable eDNA marker has high potential to provide valuable information to agencies that manage and conserve rare or declining taxa, including the Rio Grande chub and sucker. Specifically, successful implementation of this project should assist NM Department of Game and Fish in responding to the listing petitions for these species and developing long term management and recovery strategies by providing the best tools available to improve our understanding of species presence and distribution.

Proposed Primary Tasks:

1. Development of Rio Grande chub eDNA marker. Principal Investigator(s) would survey relevant biologists and scientists across the range of the species (i.e., southern CO and NM) and develop a list of extant Rio Grande chub populations. Field crews would visit and collect nonlethal tissue samples (e.g., fin clip preserved in ethanol) with electrofishing from a portion of these populations in a manner designed to capture as much genetic variation across the species as possible. Total populations visited and tissue samples collected (approximately five to 10 individual samples per population) would depend on the spatial extent and separation among remaining populations. Tissue samples from any closely related species would also be collected to ensure that marker development can differentiate target species from any congener. No closely related species to Rio Grande chub have been identified for collection at this time, but any potential species that should be included would be determined in conversations with biologists prior to starting field work. Tissue samples from three to five individuals of fish species that commonly occur in the study area would also be collected for laboratory screening and validation of marker specificity. Tissue samples would be transferred to the National Genomic Center for Fish and Wildlife Conservation (Center) in Missoula, MT for analyses and development of the eDNA marker.

   Rio Grande chub eDNA reliability and sensitivity testing. A reach of stream on the Ladder Ranch (Turner Ranch Properties, LP; Sierra County, NM) that previously did, but does not currently, contain Rio Grande chub would be selected to conduct eDNA sensitivity tests. A known biomass of Rio Grande chub would be captured and transferred to the identified vacant habitat and held either in cages (e.g., live cars) or in a contained reach (e.g., block nets) for a minimum of 24 hrs to allow eDNA to build and disperse in the environment. Subsequently, eDNA samples would be taken according to established eDNA collection protocols at 100 m intervals downstream of the

holding site for at least 500 meters. Replicate samples would be taken at each collection site resulting in 12 total samples (two samples each at 0 m, 100 m, … out to 500 m) per test. We propose to test at least two different amounts (50 and 100 gm) of Rio Grande chub. We will add a third (250 gm) if time and funding allow.

Concurrent with eDNA marker development, field crews would collect Rio Grande cutthroat trout eDNA samples on the Ladder Ranch (Turner Ranch Properties, LP; Sierra County, NM) to determine the viability, reliability, and sensitivity of a Rio Grande chub eDNA test. Four drainages (Cuchillo Negro, Palomas, Seco, and Las Animas) flow west to east across the Ladder Ranch from Black Range of the Gila National Forest to the Rio Grande River. Field crews would conduct electrofishing surveys in Palomas, Seco, and Las Animas Creeks on the Ladder Ranch to confirm expected Rio Grande chub and sucker distributions based on recent survey results. Reaches where Rio Grande chubs are present will be identified and eDNA samples (n=5 per reach) would be collected from at least three reaches in two different streams known to contain Rio Grande chub following established eDNA collection protocols. An effort would be made to select reaches with differing densities of Rio Grande chub, if possible. Relative Rio Grande chub density per reach would be estimated with a 50-m, three-pass electrofishing depletion effort conducted after eDNA samples have been collected. Environmental DNA samples would be submitted to the Center for analyses

Accomplishments:

Primary Task 1. Development of Rio Grande Chub eDNA marker. Working with NMDGF and Colorado Division of Wildlife staff we developed a list of 47 potential collection sites for Rio Grande chub and/or Rio Grande sucker. From June 2 – July 24, 2016, six field staff visited 39 locations around New Mexico and Colorado and collected 30 viable (>five individuals) Rio Grande chub samples (Figure 1; Table 1) and 17 viable Rio Grande sucker samples (Figure 2; Table 1). The tissue samples were sent to the Center for analyses. Development of eDNA markers for Rio Grande chub and Rio Grande sucker is complete (see attached Supplemental Report A).

Primary Task 2. Rio Grande chub eDNA reliability and sensitivity testing. In July 2016, a trial was conducted to test the sensitivity of the eDNA marker for Rio Grande chub detection. Over three consecutive days 130 gr (n=8 fish), 265 gr (n=7 additional fish or 15 total), and then 577 gr (n=14 additional fish or 29 total) of Rio Grande chubs were held in cages (Figure 3) in a fishless reach of stream (Seco Creek Box) on the Ladder Ranch. 24 hours after the initial and two subsequent introductions of Rio Grande chub, eDNA samples were taken at 0 m, 100 m, 200 m, 300 m, 400 m, and 500 m downstream of the cages. The chub from each test were left in the stream and used in each subsequent test, thus the original eight individuals were held for 72 hours by the time the three sets of samples were collected. One additional eDNA sample was taken above the cages on the second day as a null or blank test. Rio Grande chub eDNA was detected in all samples except the blank sample above the holding cages (Table 2). Quantities of eDNA filtered generally declined with distance downstream from the cages (Figure 4). Flows in Seco Creek were estimated to be approximately 1.5-2.0 cfs during sensitivity testing.

An additional five blind eDNA samples were collected from sites on Palomas and Las Animas creeks (Ladder Ranch) to test efficacy of the two eDNA markers under field conditions. An eDNA sample was collected (Figure 5) and then the stream was electrofished to determine if, and how many, Rio Grande chubs and Rio Grande suckers were present in the stream above the eDNA sample site. The length of stream electrofished above the eDNA sample location was variable as the electrofishing was only intended to confirm presence or absence of either species, and provide a rough approximation of how many, and how close, fish were to the eDNA sampling location. Results are shown in Table 3. Rio Grande chub eDNA was correctly detected in all five blind samples, even for one sample for which Rio Grande chubs were more than 150 m upstream from the eDNA sample site. Rio Grande sucker eDNA was correctly detected in the three of five sites where Rio Grande suckers were confirmed present with electrofishing. Observationally, there did not seem to be a clear relationship between amount of eDNA filtered and the number or proximity of the fish in the stream.

Table 2. Rio Grande chub eDNA sensitivity results. Sample (A) represents the blank collected above the cages.

Stream	Site #	Date	Chub eDNA detected	Distance downstream from cage (m)	DNA quantity (copies/L)	Log (DNA quantity)
Seco	130-0	7/19/2016	Y	0	303	2.4814
Seco	130-1	7/19/2016	Y	100	41	1.6128
Seco	130-2	7/19/2016	Y	200	35	1.5441
Seco	130-3	7/19/2016	Y	300	167	2.2227
Seco	130-4	7/19/2016	Y	400	25	1.3979
Seco	130-5	7/19/2016	Y	500	13	1.1139
Seco	256-0	7/20/2016	Y	0	1118	3.0484
Seco	256-1	7/20/2016	Y	100	1266	3.1024
Seco	256-2	7/20/2016	Y	200	720	2.8573
Seco	256-3	7/20/2016	Y	300	273	2.4362
Seco	256-4	7/20/2016	Y	400	703	2.8470
Seco	256-5	7/20/2016	Y	500	210	2.3222
Seco	500-0	7/21/2016	Y	0	12411	4.0938
Seco	500-1	7/21/2016	Y	100	1279	3.1069
Seco	500-2	7/21/2016	Y	200	987	2.9943
Seco	500-3	7/21/2016	Y	300	1294	3.1119
Seco	500-4	7/21/2016	Y	400	344	2.5366
Seco	500-5	7/21/2016	Y	500	693	2.8407
Seco	256-A	7/20/2016	N	-5	0	0.0000

Table 3. Results of blind field tests for Rio Grande chub and Rio Grande sucker eDNA.

Stream	Site #	Date Collected	eDNA detected	DNA (copies/L)	Log (DNA Q)	Field notes
Rio Grande chub (*Gila pandora*)						
Palomas	01	7/18/2016	Y	3943	3.5959	Blind eDNA sample, then shocked 50 m above sample location. First Rio Grande chub at 40 m above sampling site (N=6 total in reach). Longfin dace and Rio Grande sucker present.
Lower Palomas	01	7/18/2016	Y	13625	4.1344	Blind eDNA sample, then shocked 60 m above sample location. Captured Rio Grande chub (N=9), most between 35 and 60 m above sample location. Rio Grande sucker present.
Lower Palomas	02	7/20/2016	Y	9507	3.9781	Blind eDNA sample, then shocked 150 m above sample location. No Rio Grande chub in shocked reach, but they are present upstream. Longfin dace and Rio Grande sucker present.
Las Animas	01	7/19/2016	Y	5161	3.7129	Blind eDNA sample, then shocked 175 m above sample location. Rio Grande chub (N=16 total) captured at 80 m (N=3) , 110 m (N=2), 141 m (N=2), 164 m (N=3), and 175 m (N=6) above sample location. Longfin dace, green sunfish, and largemouth bass present.
Las Animas	02	7/19/2016	Y	6760	3.8300	Blind eDNA sample, then shocked 50 m above sample location. Rio Grande chub (N=19 total) first captured 15 m above sampling location, and all 19 within 50 m. Longfin dace present.
Rio Grande sucker (*Catostomas plebeius*)						
Palomas	01	7/18/2016	Y	7082	3.8500	Blind eDNA sample, then shocked 50 m above sample location. Rio Grande suckers (N=66) throughout reach. Longfin dace and Rio Grande chub present.
Lower Palomas	01	7/18/2016	Y	15441	4.1887	Blind eDNA sample, then shocked 60 m above sample location. Captured Rio Grande suckers (N=10), most between 35 and 60 m above sample location. Rio Grande chub present.
Lower Palomas	02	7/20/2016	Y	11082	4.0446	Blind eDNA sample, then shocked 150 m above sample location. Captured Rio Grande suckers (N=3) at 65 m. Longfin dace present.
Las Animas	01	7/19/2016	N	0	0	Blind eDNA sample, then shocked 175 m above sample location. No Rio Grande sucker captured. Rio Grande chub, longfin dace, green sunfish, and largemouth bass present.
Las Animas	02	7/19/2016	N	0	0	Blind eDNA sample, then shocked 50 m above sample location. No Rio Grande sucker captured. Rio Grande chub and longfin dace present.

Figure 1. Rio Grande sucker collected at Alamosa Warm Springs

Figure 2. Rio Grande chub collected in lower Palomas Creek.

Figure 3. Rio Grande chub holding cages in Seco Creek box.

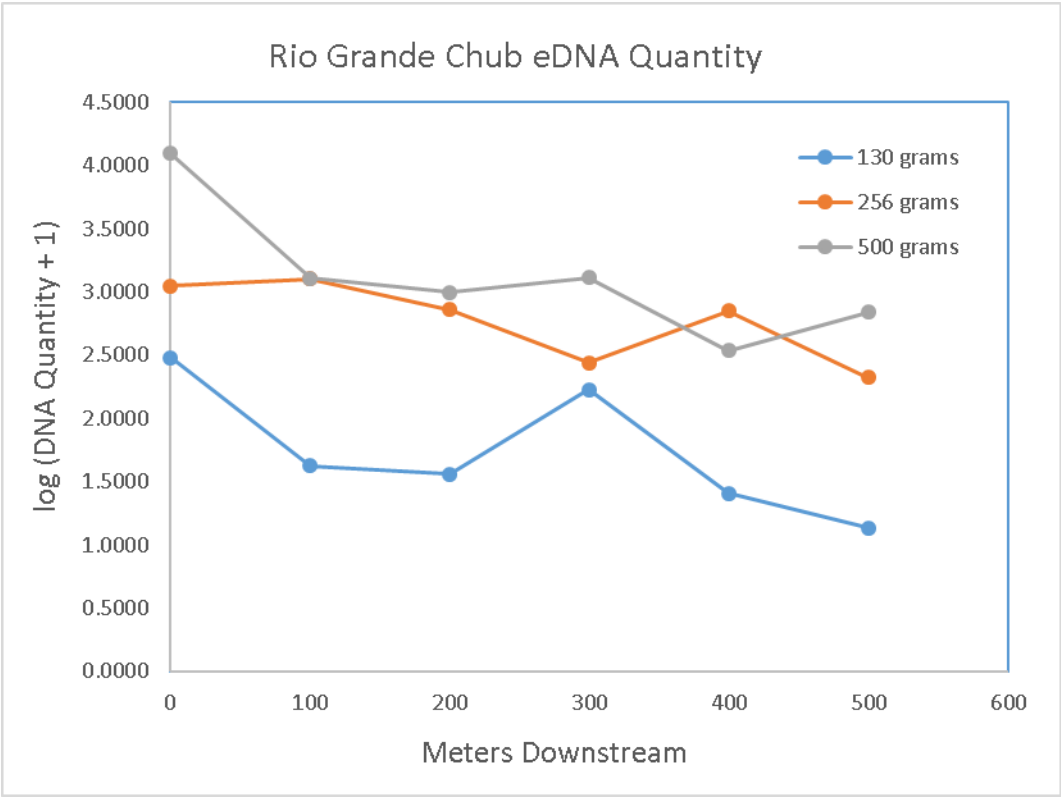

Figure 4. eDNA quantities.

Figure 5. Sampling apparatus for collection of eDNA samples.

Supplemental Report A: Rio Grande chub (*Gila pandora*) and Rio Grande sucker (*Catostomus plebeius*) eDNA assay development

Dr. Kellie Carim and Dr. Carter Kruse

Methods

Development of eDNA assays for Rio Grande chub and Rio Grande sucker
To develop separate eDNA assays for detecting Rio Grande chub and Rio Grande sucker, we first compiled GenBank sequence data of the cytochrome b oxidase (cytb) mitochondrial gene for Rio Grande chub and 13 non-target species of the same genus (Table 1). We also compiled GenBank sequence data for Rio Grande sucker, however, the genetic sequences were so diverse within this species it was impossible to design an eDNA assay to detect all variants without detecting non-target species. There was up to 10% sequence divergence between individuals labeled as Rio Grande sucker. Usually 2.5% divergence is considered enough to distinguish between species. As a result, we generated cytb DNA sequence data from tissues of 34 Rio Grande suckers from 18 locations throughout their distribution in the United States (Table 2; see below for collection and DNA extraction details). We generated DNA sequences for two individuals from each location, except from the Ojo Caliente River, NM, where we only obtained one specimen, and Skates Canyon, NM, where we successfully sequenced just one individual. To generate cytb sequence data, we amplified a 1116 bp fragment of the cytb mitochondrial gene using primers LA-a, HD-a, LD-RBS, and HA-a from Dowling et al. (2002, 2016) with modified cycling conditions. The first segment (using primers LA-a and HD-a) of all samples and the second segment (using primers LD-RBS and HA-a) of 21 specimens used thermocycler conditions as follows: 94 °C for 2 min; 12 cycles of 94 °C for 30 s, 55°C for 40 s, and 72 °C for 90 s; 12 cycles of 94 °C for 30 s, 52°C for 40 s, and 72 °C for 90 s; 12 cycles of 94 °C for 30 s, 48°C for 40 s, and 72 °C for 90 s; and a final extension stage of 72 °C for 5 min. The second segment of the remaining 14 samples used thermocycler conditions as follows: 94 °C for 2 min; 12 cycles of 94 °C for 30 s, 52°C for 40 s, and 72 °C for 90 s; 12 cycles of 94 °C for 30 s, 50°C for 40 s, and 72 °C for 90 s; 12 cycles of 94 °C for 30 s, 48°C for 40 s, and 72 °C for 90 s; and a final extension stage of 72 °C for 5 min. We cleaned the PCR product using ExoSAP-IT™ PCR Product Cleanup Reagent (Life Technologies) and sent it to Eurofins Genomics where they generated sequences on an ABI 3730XL sequencing machine.

We screened the sequence data *in silico* with *R* v 3.3.2 (R Core Team 2016) using the *DECIPHER* package (Wright et al. 2014) to generate candidate primers that amplify a fragment of cytb in each species. We aligned the candidate primers with the genetic sequence data in MEGA 7.0 (Kumar et al. 2015) and manually adjusted primer positions and length to maximize base-pair differences with non-target species and optimize annealing temperatures. The resulting primers amplify a 120-base and 146-base fragment of the cytb mitochondrial gene in Rio Grande chub and Rio Grande sucker, respectively (Table 3). We then aligned additional sequence data of potentially co-occurring, non-target fishes (Table 1) previously detected throughout the Rio Grande Basin during surveys conducted with traditional sampling methods (Propst et al. 1987; Platania 1991; Platania 1993; Rinne & Platania 1995; Rees et al. 2005). We designed FAM-labeled, minor-groove-binding, non-fluorescent quencher (MGBNFQ) probes manually from the sequence alignments, maximizing base-pair differences with non-target species (Table 3). Each primer-probe set was evaluated for potential secondary structure formation using IDT OligoAnalyzer (https://www.idtdna.com/calc/analyzer), and annealing temperatures were assessed in Primer Express 3.0.1 (Life Technologies; Table 3). In addition, a nucleotide BLAST search was performed on each primer and probe to confirm the specificity of each assay *in silico*.

To examine the specificity of the assays *in vitro*, we tested DNA extracted from 87 Rio Grande chub, 85 Rio Grande sucker, and 39 non-target species (Table 2). Fin clips from Rio Grande chub, creek chub (*Semotilus atromaculatus*), Rio Grande sucker, and white sucker (*C. commersonii*) were collected by Carter Kruse and Eric Leinonen under New Mexico Scientific Collecting Permit #3261-2016. To collect the fin clips, fish were captured by electrofishing and a small amount of tissue was excised from the caudal fin and placed in a vial of 95% ethanol. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Inc.) following manufacturer's procedures, except final elution was into Buffer TE instead of AE. For all other samples, we used DNA archived at the National Genomics Center for Wildlife and Fish

Conservation, USDA Forest Service, Rocky Mountain Research Station, Missoula, MT, that was previously extracted (using the same methods) for other projects.

Next, we screened each assay against DNA from these tissue samples on StepOne Plus Real-time PCR Instrument (Life Technologies) or QuantStudio 3 Real-Time PCR System (Life Technologies). Each sample was tested in a 15-µl reaction consisting of 7.5 µl Environmental Master Mix 2.0 (Life Technologies), 900 nM of each primer, 250 nM probe, 4 µl DNA template (~0.4 ng), and the remaining volume was deionized water. The thermocycler profile included initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. All qPCR consumables and pipettes were irradiated with UV for 1 h before set-up and all experiments were set up inside a UV sterile hood. For each experiment, we included a no-template control with distilled water substituted in place of DNA template.

We then optimized primer concentrations following methods in Wilcox et al. (2015) and determined the sensitivity of the assay with a standard curve analysis. Briefly, we used the same qPCR recipe above except varied the concentrations of each primer (100, 300, 600, 900 nM) for a total of 16 unique combinations, and analyzed each combination in triplicate reactions. To create a standard curve, we purified qPCR product of each target species using GeneJET PCR Purification Kit (ThermoFisher Scientific), quantified it on a Qubit 2.0 fluorometer (ThermoFisher Scientific), and diluted it into sterile TE. A seven-level serial dilution (31 250, 6 250, 1 250, 250, 50, 10, and two copies per reaction) was created and each level was analyzed in six replicate reactions.

Finally, we tested the reliability of each assay *in vivo* by analyzing environmental samples collected from field sites where the species are known to occur and where the species are not expected to occur. In addition, we analyzed five eDNA samples taken from hatchery tanks containing known fishes (Table 4). Environmental DNA samples were collected following methods of Carim et al. (2016a) using a peristaltic pump to filter 5 L of water at field sites and 1 L of water at hatchery sites. Environmental DNA was extracted in a dedicated room using the DNeasy Blood & Tissue Kit (Qiagen, Inc.) following a modified protocol described in Carim et al. (2016b). Extracts were stored at -20 °C until qPCR analysis. We analyzed these samples in triplicate, 15-µl reactions using the qPCR recipe and thermocycler profile above, except optimized primer concentrations (Table 3) were used, and a TaqMan Exogenous Internal Positive Control (Life Technologies), including 1.5 µl of 10X IPC assay and 0.30 µl of 50X IPC DNA, was used in place of deionized water. We also included a no-template control with deionized water used in place of DNA template on each analysis.

Results

Rio Grande chub assay

The Rio Grande chub assay successfully detected DNA in all 87 Rio Grande chub tissue samples from 31 locations throughout Colorado, New Mexico, and Texas (Table 2). The assay also amplified DNA of three, non-target *Gila* species: Chihuahua chub (*G. nigrescens*), roundtail chub (*G. robusta*), and Utah chub (*G. atraria*; Table 2.) Interestingly, the assay did not amplify DNA of roundtail chub samples collected in Arizona (n=6), but did amplify a subset of roundtail chub samples collected in Colorado (n=4 of 6). Additionally, the assay was not screened against DNA from Salinas chub (*G.* modesta), however the nucleotide BLAST search of the Rio Grande chub assay indicated that this species was an exact match to all three components of the assay, meaning that amplification of Salinas chub DNA is certain. However, Salinas chub is not present within the geographic range of Rio Grande chub, making the false positive detection of Rio Grande chub unlikely in our study area. While these four species have not been documented to co-occur with Rio Grande chub, cautious interpretation of results must be exercised when sampling in areas where these fishes may be sympatric. The assay did not amplify DNA from the remaining 36 non-target species or in any of the no-template controls, and the BLAST search did not identify any other species with genetic sequences similar to more than two components of the assay.

Optimization tests identified 600 nM as the optimal primer concentrations for both the forward and reverse primers (Table 3). The standard curve experiment had an efficiency = 97.74% (r^2 = 0.99, y-intercept = 40.35, slope = -3.38) and a limit of detection (defined as the lowest concentration with >95%

amplification success; Bustin et al. 2009) at 10 mtDNA copies per reaction. The Rio Grande chub assay detected DNA in four of six replicates at two copies per reaction. Rio Grande chub DNA was detected in all environmental samples taken where the species is expected to occur, and did not detect DNA in samples collected where Rio Grande chubs were expected to be absent (Table 4).

Rio Grande sucker assay

The nucleotide BLAST search of the Rio Grande sucker assay indicated the one sequence of the bluehead sucker (*Catostomus discobolus*; accession KJ441236.1) was identical in the probe and reverse primer regions and differed by only one basepair in the forward primer region. We compared this sequence with all bluehead sucker sequence data available on GenBank (accessions: JX488782.1-JX488783.1; KJ441243.1-KJ441247.1; KJ441256.1-KJ441257.1) and found that it was divergent from all other bluehead sucker sequences. The unique bluehead sucker was over 5% diverged from all other bluehead sucker sequences on Genebank, but only 0.2 – 2.2% diverged from the 34 Rio Grande sucker cytb sequences generated in this study. (For reference, divergence within all 34 Rio Grande sucker sequences generated in this study ranged from 0-2.4%.) These data suggest that Genebank sequence KJ441236.1 is mislabeled as bluehead sucker. All other bluehead sucker sequences on Genbank have a minimum of two mismatches with the forward primer, two mismatches with the reverse primer, and two mismatches with the probe. Additionally, the mismatches for both primers are located at or within one basepair of the 3' end. Given the number and location of these basepair mismatches, amplification of bluehead sucker DNA with this assay is unlikely.

The Rio Grande sucker assay successfully detected DNA in all 85 Rio Grande sucker DNA extracts from 18 locations throughout its United States distribution. This assay did not detect DNA of any of the 39 non-target species tested. Optimization tests identified 100 nM and 600 nM as the optimal primer concentrations for the forward and reverse primers, respectively (Table 3). The standard curve experiment resulted in an efficiency = 99.91% (r^2 = 0.996, y-intercept = 37.20, slope = - 3.32) and the assay had a limit of detection at 10 mtDNA copies per reaction. The Rio Grande sucker assay detected DNA in five of six replicates at two copies per reaction. Finally, this assay detected Rio Grande sucker DNA in all environmental samples collected where the species is expected to occur, and did not detect DNA in samples collected where Rio Grande suckers were expected to be absent (Table 4).

References

Carim KJ, McKelvey KS, Young MK, Wilcox TM, Schwartz MK. Protocol for collecting eDNA samples from streams. Gen. Tech. Rep. RMRS-GTR-355. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 2016a; 18p. http://www.fs.fed.us/rm/pubs/rmrs_gtr355.pdf.

Carim KJ, Dysthe JCS, Young MK, McKelvey KS, Schwartz MK. An environmental DNA assay for detecting Arctic grayling in the upper Missouri River basin, North America. Conserv Genet Resour. 2016b Apr 01. doi: 10.1007/s12686-016-0531-1.

Dowling TE, Markle DF, Tranah GJ, Carson EW, Wagman DW, May BP. Introgressive hybridization and the evolution of lake-adapted catostomid fishes. PLoS ONE. 2016; 11(3): e0149884.

Dowling TE, Tibbets CA, Minckley WL, Smith GR. Evolutionary relationships of the plagopterins (Teleostei: Cyprinidae) from cytochrome *b* sequences. Copeia. 2002; 2002(3): 665–678.

Kumar et al. 2015. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Mol Bio and Evol. (submitted)

Platania SP. Fishes of the Rio Chama and upper Rio Grande, New Mexico, with preliminary comments on their longitudinal distribution. The Southwestern Naturalist. 1991;36(2): 186–193.

Patania SP. The fishes of the Rio Grande between Velarde and Elephant Butte Reservoir and their habitat associations. Report to the New Mexico Department of Game and Fish (contract 516.6-74-23) and U.S. Bureau of Reclamation (cooperative agreement O-FC-40-08870. 1993: 188 p.

Propst DL, Burton GL, Pridgeon BH. Fishes of the Rio Grande between Elephant Butte and Caballo Reservoirs, New Mexico. The Southwestern Naturalist. 1987;32(3): 408-411.

Propst DL. Threatened and endangered fishes of New Mexico. Technical Report No. 1. New Mexico Department of Game and Fish, Santa Fe, NM. 1999: 84 pp.

Rinne JN, Platania SP. Fish fauna. In: Finch DM, Tainter JA (Tech Eds.). Ecology, diversity, and sustainability of the Middle Rio Grande Basin. General Technical Report RM-GTR-268. Fort Collins: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. 1995: 165–175.

Rees DE, Carr RJ, Miller WJ. Rio Grande chub (*Gila pandora*): A technical conservation assessment. [Online]. USDA Forest Service, Rocky Mountain Region. 2005. Available: http://www.fs.fes.us/r2/projects/scp/assessments/riograndechub.pdf [accessed 6/15/2017].

R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2016. https://www.R-project.org/.

Schmidt TR, Bielawski JP, Gold JR. Molecular phylogenetics and evolution of the cytochrome *b* gene in the cyprinid genus *Lythrurus* (Actinopterygii: Cypriniformes). Copeia. 1998;1: 14–22.

Strange RM, Mayden RL. Phylogenetic relationships and a revised taxonomy for North American cyprinids currently assigned to *Phoxinus* (Actinopterygii: Cyprinidae). Copeia. 2009;3: 494–501.

Sublette JE, Hatch MD, Sublette M. The fishes of New Mexico. University of New Mexico Press, Albuquerque, New Mexico. 1990.

Wilcox TM, Carim KJ, McKelvey KS, Young MK, Schwartz MK. The dual challenges of generality and specificity with developing environmental DNA markers for species and subspecies of *Oncorhynchus*. PLoS ONE. 2015;10(11): e0142008.

Wright ES, Yilmaz LS, Ram S, Gasser JM, Harrington GW, Noguera DR. Exploiting extension bias in polymerase chain reaction to improve primer specificity in ensembles of nearly identical DNA templates. Environ Microbiol. 2014;16: 1354–1365.

Table 1. Species, sample size (*n*), and GenBank accession number for DNA sequences used for *in silico* eDNA marker development for Rio Grande chub (RGC) and Rio Grande sucker (RGS).

					Nucleotide mismatches (RGC/RGS)		
Common name	**Family**	**Species name**	***n***	**GenBank accession**	**Forward Primer**	**Reverse Primer**	**Probe**
Rio Grande chub	Cyprinidae	*Gila pandora**	5	EU082467.1; EU747196.1; EU747198.1; JX443039.1; KF514227.1	0/6	0/6	0/4
Rio Grande sucker	Catostomidae	*Catostomus plebeius***	34	RMRS	11/0	5/0	5/0
			7	EU668771.1-EU668773.1; JX488803.1; KJ441235.1; KJ441237.1; KU697935.1	11/0	5/0	6/0
			4	EU668774.1-EU668776.1; KJ441238.1	11/1	6/0	5/0
			1	JX488804.1	11/2	6/0	5/0
			11	EU668762.1-EU668770.1; KJ441239.1-KJ441240.1	9/4	3/2	5/3
			21	EU668752.1-EU668761.1; EU668784.1; EU668786.1; EU668873.1-EU668880.1	9/3	3/2	4/1
Shovelnose sturgeon	Acipenseridae	*Scaphirhynchus platorhynchus*	4	U56984.1-U56986.1; U56988.1	9/7	5/5	7/7
American eel	Anguillidae	*Anguilla rostrata*	3	AB021767.1; AF006716.1-AF006717.1	12/7	3/2	3/5
Blue sucker	Catostomidae	*Cycleptus elongatus*	4	AF454868.1; EF062370.1; EF062372.1; JF799439.1	5/7	3/2	5/4
Bluehead sucker		*Catostomus discobolus***	9	JX488782.1-JX488783.1; KJ441243.1-KJ441247.1; KJ441256.1-KJ441257.1	10/2	2/2	4/2
Desert sucker		*Catostomus clarkii***	6	JX488779.1; KJ441258.1-KJ441261.1; KU697936.1	10/3	4/2	4/2
Flannelmouth sucker		*Catostomus latipinnis***	3	JX488788.1-JX488789.1; KU697930.1	7/5	3/3	5/2
Gray redhorse		*Moxostoma congestum*	4	JF799515.1-JF799518.1	9/5	6/6	6/3
Mountain sucker		*Catostomus platyrhynchus***	10	JX488801.1-JX488802.1; KJ441248.1-KJ441255.1	9/2	4/2	4/2
River carpsucker		*Carpiodes carpio*	4	AF454867.1; JF799431.1; JN053259.1-JN053260.1	10/5	6/4	5/3
Smallmouth buffalo		*Ictiobus bubalus*	4	FJ226361.1-FJ226364.1	7/6	4/2	4/5
Sonora sucker		*Catostomus insignis***	3	JX488786.1-JX488787.1; KJ441283.1	5/5	4/3	5/2
Tahoe sucker		*Catostomus tahoensis***	5	AF454874.1; JX488801.1-JX488808.1; KJ441282.1; KU697914.1	8/5	5/3	6/1
Utah sucker		*Catostomus ardens***	3	JX488771.1-JX488772.1; KJ441280.1	7/4	43	3/1
White sucker		*Catostomus commersoni***	6	HQ446762.1; JF799435.1-JF799437.1; JX488781.1; KU697932.1	7/4	5/3	4/4
Yaqui sucker		*Catostomus bernardini***	4	EU668838.1; EU668849.1; EU668864.1; EU668881.1	6/5	3/4	3/3
Black crappie	Centrarchidae	*Pomoxis nigromaculatus*	3	AY115991.1-AY115992.1; JF742840.1	5/7	3/5	4/4
Bluegill		*Lepomis macrochirus*	4	AY225667.1; AY828966.1-AY828968.1	5/7	6/7	5/5
Green sunfish		*Lepomis cyanellus*	4	AY115974.1; AY828958.1-AY828959.1; JF742828.1	8/8	5/5	6/5
Largemouth bass		*Micropterus salmoides*	4	KX588089.1-KX588092.1	6/7	5/4	4/5
Longear sunfish		*Lepomis megalotis*	4	AY828974.1-AY828977.1	5/7	4/8	3/5
Smallmouth bass		*Micropterus dolomieu*	4	HM070849.1; HM070897.1; HM070903.1-HM070904.1	5/7	4/5	4/5
Warmouth		*Chaenobryttus gulosus*	4	AY115971.1-AY115972.1; AY828963.1; JF742830.1	6/7	2/5	5/2
White crappie		*Pomoxis annularis*	3	AY115989.1-AQ115990.1; JF742839.1	5/6	3/5	3/3
Mexican tetra	Characidae	*Astyanax mexicanus*	4	FJ439338.1; FJ439344.1-FJ439346.1	5/7	6/9	5/4
Gizzard shad	Clupeidae	*Dorosoma cepedianum*	3	EU552584.1-EU552586.1	11/6	4/8	7/4
Threadfin shad		*Dorosoma petenense*	4	EU552581.1-EU552583.1; KF013218.1	11/6	2/8	5/4

Bonytail chub	Cyprinidae	*Gila elegans**	2	KF514187.1-KF514188.1	3/6	4/6	2/5
Chihuahua chub		*Gila nigrescens**	2	JX443049.1; KF514226.1	1/6	2/6	1/5
Conchos chub		*Gila pulchra**	2	KF514243.1; KF514253.1	0/4	1/6	2/4
Desert chub		*Gila eremica**	2	KF514192.1-KF514193.1	1/6	1/5	3/4
Gila chub		*Gila intermedia**	2	JX443036.1; KF514194.1	2/5	2/5	3/5
Headwater chub		*Gila nigra**	2	JX443028.1; KF514210.1	2/5	2/5	3/5
Humpback chub		*Gila cypha**	2	KF514181.1-KF514182.1	1/6	2/6	2/6
Mexican roundtail chub		*Gila minacae**	2	KF514199.1; KF514202.1	5/8	1/6	3/6
Nazas chub		*Gila conspersa**	2	KF514178.1-KF514179.1	0/5	3/6	4/4
Roundtail chub		*Gila robusta**	4	JX443033.1-JX443034.1; KF514254.1-KF514255.1	1/4	1/5	2/5
Shorttail chub		*Gila brevicauda**	2	KF514151.1; KF514153.1	1/6	1/6	2/4
Sonora chub		*Gila ditaenia**	2	JX443023.1; KF514186.1	2/5	2/6	3/4
Yaqui chub		*Gila purpurea**	2	JX443020.1-JX443021.1	2/4	1/5	3/4
Bullhead minnow		*Pimephales vigilax*	4	GQ184531.1-GQ184534.1	7/5	3/4	4/5
Central stoneroller		*Campostoma anomalum*	4	DQ486824.1; DQ486826.1-DQ486828.1	5/6	4/5	2/3
Colorado pikeminnow		*Ptychocheilus lucius*	2	JX443071.1-JX443072.1	1/6	1/7	3/4
Common carp		*Cyprinus carpio*	4	DQ868872.1-DQ878875.1	10/7	5/5	7/8
Fathead minnow		*Pimephales promelas*	4	GQ184519.1-GQ184521.1; GQ275159.1	8/5	4/3	2/5
Flathead chub		*Platygobio gracilis*	2	EU811100.1; JX442992.1	7/6	4/6	3/4
Golden shiner		*Notemigonus crysoleucas*	1	U01318.1	9/5	2/5	2/6
Goldfish		*Carassius auratus*	4	AB368694.1-AB368697.1	8/6	2/4	4/6
Longfin dace		*Agosia chrysogaster*	2	DQ324093.1; JX443014.1	7/5	3/4	2/4
Longnose dace		*Rhinichthys cataractae*	4	KF640154.1-KF640157.1	8/7	3/6	3/5
Mississippi silvery minnow		*Hybognathus nuchalis*	3	EU082469.1; EU811095.1-EU811096.1	9/6	5/5	2/5
Pecos bluntnose shiner		*Notropis simus pecosensis*	1	EU811099.1	9/6	4/6	3/6
Red shiner		*Cyprinella lutrensis*	4	GQ275188.1-GQ275190.1; GQ275194.1	6/5	3/6	3/5
Rio Grande shiner		*Notropis jemezanus*	2	AF352277.1; KT834520.1	10/5	7/5	2/7
Rio Grande silvery minnow		*Hybognathus amarus*	2	EU811097.1-EU811098.1	9/6	4/4	1/4
Roundnose minnow		*Dionda episcopa*	4	JN812387.1-JN812390.1	10/7	4/7	2/5
Speckled chub		*Macrhybopsis aestivalis*	1	JQ712319.1	8/5	4/7	3/3
Speckled dace		*Rhinichthys osculus*	4	DQ990313.1-DQ990316.1	11/7	3/6	2/3
Spikedace		*Meda fulgida*	4	AF452093.1-AF452094.1; JX443054.1-JX443055.1	8/6	5/6	3/4
Tench		*Tinca tinca*	4	HM167954.1-HM167957.1	6/6	3/6	3/6
Rainwater killifish	Cyprinodontidae	*Lucania parva*	2	GQ119769.1; KJ696823.1	8/5	4/7	4/6
Northern pike	Esocidae	*Esox lucius*	4	HM177469.1-HM177470.1; KT203379.1; KU659805.1	9/5	2/8	4/7
Plains killfish	Fundulidae	*Fundulus zebrinus*	4	GQ119765.1-GQ119766.1; KX359046.1-KX359047.1	7/4	4/4	3/2
Black bullhead	Ictaluridae	*Ameiurus melas*	2	AY184263.1; AY184273.1	9/7	5/6	6/7
Blue catfish		*Ictalurus furcatus*	3	AF484159.1; EF491729.1; KM264126.1	9/6	5/6	6/6
Channel catfish		*Ictalurus punctatus*	4	AB045119.1; AB069646.1; AY458886.1; EU490914.1	3/5	1/5	4/5
Flathead catfish		*Pylodictis olivaris*	3	AF484161.1; AY458887.1; DQ790748.1	6/6	4/6	7/6
Yellow bullhead		*Ameiurus natalis*	4	AF484158.1; AY184255.1; AY184265.1; AY458888.1	10/6	4/6	4/8
Longnose gar	Lepisosteidae	*Lepisosteus osseus*	3	JF912057.1-JF912059.1	9/5	5/9	6/6
White bass	Percichthyidae	*Morone chrysops*	3	AF240745.1; AY374295.1; AY770838.1	9/8	5/4	6/3
Walleye	Percidae	*Sander vitreus*	3	KC819819.1-KC819821.1	9/3	6/7	5/3
Yellow perch		*Perca flavescens*	4	EU348835.1-EU348838.1	6/6	3/6	4/4

Sailfin molly	Poeciliidae	*Poecilia latipinna*	4	FJ446153.1; KF276609.1; KJ696833.1; KP699889.1	8/6	5/6	4/4
Western mosquitofish		*Gambusia affinis*	2	EF017514.1; KP059011.1	5/5	2/4	4/2
Brook trout	Salmonidae	*Salvelinus fontinalis*	4	HQ167699.1; JX960851.1-JX960852.1; KU872718.1	11/6	7/6	6/6
Brown trout		*Salmo trutta*	4	KF985735.1-KF985738.1	8/5	7/6	5/7
Cutthroat trout		*Oncorhynchus clarki*	2	FJ435584.1-FJ435585.1	9/6	8/7	4/7
Rainbow trout		*Oncorhynchus mykiss*	4	AY032629.1-AY032632.1	9/6	8/7	5/7

*Sequences used for generating primers for Rio Grande chub using DECIPHER (Wright et al. 2014) in *R* v 3.3.2 (R Core Team 2016).

**Sequences used for generating primers for Rio Grande sucker using DECIPHER (Wright et al. 2014) in *R* v 3.3.2 (R Core Team 2016).

Table 2. Species used for *in vitro* testing of the Rio Grande chub (RGC) and Rio Grande sucker (RGS) quantitative PCR assays. Origin refers to the waterbody for each target species and to the state for all other samples.

Common name	Family	Species name	RGC testing sample size	RGS testing sample size	Origin	RGC detection (Y/N)	RGS detection (Y/N)
Rio Grande chub	Cyprinidae	*Gila pandora*	3	0	Crestone Creek, CO	Y	-
			3	0	Embudo Creek, CO	Y	-
			3	0	Hay Press Lake, CO	Y	-
			3	0	Hot Creek, CO	Y	-
			3	0	Hot Springs Creek, CO	Y	-
			3	0	Rio Grande del Rancho, CO	Y	-
			3	0	Saguache Creek, CO	Y	-
			3	0	San Antonio River, CO	Y	-
			3	1	San Luis Creek, CO	Y	N
			3	0	Almosa Creek, NM	Y	-
			2	0	Chamita River, NM	Y	-
			2	0	Cieneguilla Creek, NM	Y	-
			3	0	East Fork Jemez River, NM	Y	-
			3	0	El Rito Creek, NM	Y	-
			3	0	Jemez River, NM	Y	-
			3	0	Lower Las Animas Creek, NM	Y	-
			3	0	Lower Palomas Creek, NM	Y	-
			3	0	North Seco Creek, NM	Y	-
			3	0	Ojo Caliente River, NM	Y	-
			3	0	Pecos River	Y	-
			3	0	Pinos Negros Creek, NM	Y	-
			1	0	Rio Cebolla, NM	Y	-
			2	0	Rio de Las Vacas, NM	Y	-
			2	0	Rio Guadalupe, NM	Y	-
			3	0	Rio Nabor, NM	Y	-
			3	0	Rio Penasco, NM	Y	-
			3	0	Rio Tusa, NM	Y	-
			3	0	Rio Vallecitos, NM	Y	-
			3	0	Tio Grande Creek, NM	Y	-
			3	1	Upper Palomas Creek, NM	Y	N
			3	1	Rio San Antonio, TX	Y	N

Rio Grande sucker	Catostomidae	*Catostomus plebeius*	0	4	Crestone Creek, CO	-	Y
			0	5	Embudo Creek, CO	-	Y
			0	5	Hot Creek, CO	-	Y
			0	5	Almosa Creek, NM	-	Y
			1	5	Conones Creek, NM	N	Y
			0	5	East Fork Jemez River, NM	-	Y
			0	5	Jemez River, NM	-	Y
			0	5	Lower Palomas Creek, NM	-	Y
			0	1	Ojo Caliente River, NM	-	Y
			1	5	Pinos Negros Creek, NM	N	Y
			0	5	Rio Cebolla, NM	-	Y
			0	5	Rio de Las Vacas, NM	-	Y
			0	5	Rio Guadalupe, NM	-	Y
			1	5	Rio Tusa, NM	N	Y
			0	5	San Antonio Creek, NM	-	Y
			0	5	Skates Canyon, NM	-	Y
			0	5	Upper Palomas Creek, NM	-	Y
			0	5	Allie Canyon, TX	-	Y
Bluehead sucker		*Catostomus discobolus*	1	1	WY	N	N
Desert sucker		*Catostomus clarki*	1	1	NM	N	N
Longnose sucker		*Catostomus catostomus*	1	5	MT	N	N
Mountain sucker		*Catostomus platyrhynchus*	1	6	MT, WY	N	N
Razorback sucker		*Xyrauchen texanus*	1	2	AZ, UT	N	N
Sonora sucker		*Catostomus insignis*	1	2	NM	N	N
Utah sucker		*Catostomus ardens*	1	7	WY	N	N
White sucker		*Catostomus commersonii*	3	7	CA, MT, NM	N	N
Largemouth bass	Centrarchidae	*Micropterus salmoides*	1	1	MT	N	N
Smallmouth bass		*Micropterus dolomieu*	1	1	MT	N	N
Chihuahua chub	Cyprinidae	*Gila nigrescens*	2	2	NM	Y	N
Colorado pikeminnow		*Ptychocheilus lucius*	2	2	UT	N	N
Common carp		*Cyprinus carpio*	1	1	WY	N	N
Creek chub		*Semotilus atromaculatus*	2	2	NM	N	N
Fathead minnow		*Pimephales promelas*	1	1	NM	N	N
Loach minnow		*Rhinichthys cobitis*	1	1	AZ	N	N
Longfin dace		*Agosia chrysogaster*	1	1	NM	N	N
Longnose dace		*Rhinichthys*	1	1	ID	N	N

		cataractae					
Northern leatherside chub		*Lepidomeda copei*	2	2	WY	N	N
Red shiner		*Cyprinella lutrensis*	2	1	NM	N	N
Roundtail chub		*Gila robusta*	6	6	AZ	N	N
			4	4	CO	Y	N
Sacramento pikeminnow		*Ptychocheilus grandis*	1	1	CA	N	N
Speckled dace		*Rhinichthys osculus*	1	1	AZ	N	N
Spikedace		*Meda fulgida*	1	1	AZ	N	N
Utah chub		*Gila atraria*	2	2	ID	Y	N
Zebra mussel	Dreissenidae	*Dreissena polymorpha*	1	1	MN?	N	N
Muskellunge	Esocidae	*Exox masquinongy*	1	1	MN	N	N
Channel catfish	Ictaluridae	*Ictalurus punctatus*	1	1	MT	N	N
Burbot	Lotidae	*Lota lota*	1	1	MT	N	N
Walleye	Percidae	*Sander vitreus*	1	1	WA	N	N
Yellow perch		*Perca flavescens*	1	1	WA	N	N
Apache trout	Salmonidae	*Oncorhynchus apache*	1	1	AZ	N	N
Brook trout		*Salvelinus fontinalis*	1	1	ID	N	N
Brown trout		*Salmo trutta*	1	1	NM	N	N
Gila trout		*Oncorhynchus gilae*	1	1	NM	N	N
Redband trout		*Oncorhynchus mykiss gairdnerii*	1	1	OR	N	N
Yellowstone cutthroat trout		*Oncorhynchus clarkii bouvieri*	1	1	WY	N	N
Central mudminnow	Umbridae	*Umbra limi*	1	1	MT	N	N

Table 3. Quantitative PCR assay for detecting Rio Grande chub (RGC) and Rio Grande sucker (RGS) DNA in environmental samples. Tm = annealing temperature.

Assay component	Sequence (5'-3')	Tm (°C)	Optimal concentration (nM)
RGC forward primer	ATCTTTAGCATTATTTTCTCCCAACCTA	59	600
RGC reverse primer	ATGGCATAGGCAAATAAAAAATATCAC	58.8	600
RGC probe	FAM-CCACATATTCAGCCGGAGT-MGBNFQ	60	250
RGS forward primer	GCCACGGTGATTACTAACCTTTTG	59.8	100
RGS reverse primer	GCGGCGACTACAAATGGTAAT	57.8	600
RGS probe	FAM-TTGCCTACATAAGGGACTG-MGBNFQ	69	250