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Certain detection of uncertain taxa: eDNA detection of a cryptic mountain sucker (*Pantosteus jordani*) in the Upper Missouri River, USA

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Abstract

A lineage of the Mountain Sucker species complex (*Pantosteus jordani*) exists as a genetically and morphologically distinct taxon restricted to the Missouri River basin. This species is thought to be declining throughout its range and is assumed to be extirpated from the southern portion of its distribution. We developed a quantitative PCR-based environmental DNA assay for *P. jordani* to help define and monitor its current range. The assay is both specific to *P. jordani* and sensitive to low amounts of DNA, with a detection limit of 10 DNA copies per reaction. In vitro experiments involved testing DNA from twenty tissue samples, collected from the Missouri River basin in Montana and Wyoming. The assay efficiently detected DNA of all *P. jordani* samples and did not amplify DNA of any closely related nontarget species. Additionally, 29 environmental DNA samples were taken in 19 waterbodies within *P. jordani* range and its presence or absence was determined prior to sampling at six of 29 sites. All sites where *P. jordani* was known absent produced negative results, and all sites where it was known present were confirmed with environmental DNA detections. The new assay was able to detect *P. jordani* at ten sites which were not previously known to contain individuals, demonstrating that this tool has the potential to rapidly expand the current understanding of this taxon's distribution.

KEYWORDS
Catostomidae, environmental DNA, quantitative PCR

1 | INTRODUCTION

The recent advent of environmental DNA (eDNA) approaches has enhanced the assessment and monitoring of rare native species' populations (McKelvey et al., 2016), identification of invasion fronts of non-native species (Rubenson & Olden, 2019), and the effectiveness of eradication efforts targeting invasive taxa (Kamoroff & Goldberg, 2018). Quantitative PCR-based (qPCR) eDNA assays typically target single species or groups of very closely related taxa (Goldberg et al., 2016). These assays can be highly sensitive and specific, capable of amplifying a single copy of DNA of the target species from an environmental sample containing vast quantities of DNA from nontarget

taxa (Wilcox et al., 2013). The effectiveness of an eDNA assay for detecting a species also relies on a taxonomy that corresponds to evolutionary history, because the sensitivity and specificity of a qPCR-based eDNA assay are correlated with the levels of divergence between the target species and all potentially sympatric taxa. In some cases, eDNA assays were recognized as being effective for detecting occupancy across most but not all of a species' range because high intraspecific diversity prevented development of a more comprehensive assay (Dysthe et al., 2017) or because the assay was intended for use in a limited area with a small pool of nontarget species (e.g., Franklin et al., 2018). When a named taxon, however, includes unrecognized biodiversity in the form of cryptic species, patterns of occupancy based on qPCR-based eDNA sampling may be spurious. One solution to this problem is to employ metabarcoding approaches using more general primers to amplify eDNA, but at present, these lack the sensitivity of qPCR-based eDNA assays (Harper et al., 2018). Alternatively, qPCR assays can be designed to be specific to monophyletic groups within a named taxon that contains multiple clades. These assays can be used individually within the spatial extent of a clade or can be mixed to simultaneously detect organisms from broader genetic groups (e.g., Dysthe et al., 2018).

Suckers in the genus *Pantosteus* are freshwater fishes in the family Catostomidae that are associated with fast-flowing, cool- to cold-water environments. Although initially regarded as distinct, they were synonymized with members of the genus *Catostomus* based on morphological characters (Smith, 1966). More recent genetic analyses led to the proposed resurrection of this genus (Bagley, Mayden, & Harris, 2018; Unmack et al., 2014), but there still remains uncertainty with regard to the number and distribution of its constituent species. For example, Mountain Sucker (*Pantosteus* (*Catostomus*) *platyrhynchus*) was thought to be present in headwater portions of the Colorado, Missouri, and Columbia River basins, the endorheic Bonneville and Lahontan Basins in the U.S. (Belica & Nibbelink, 2006; Page & Burr, 2011), and the Fraser and Saskatchewan River basins in Canada (COSEWIC, 2010). Subsequent assessments demonstrated that this name represented several geographically constrained and genetically and morphologically distinct groups that might be considered monophyletic taxa (Bangs, 2016; Smith, Stewart, & Carpenter, 2013; Unmack et al., 2014). Members of one such group are restricted to the Missouri River basin and were first described as *Pantosteus jordani* (Evermann, 1894). Populations likely to represent this species are regarded as stable in a number of occupied areas (COSEWIC, 2010), but are declining and effectively extirpated in the southern portion of their range (Belica & Nibbelink, 2006; Patton, Rahel, & Hubert, 1998; Schultz & Bertrand, 2012). These declines tend to be attributed to water development or habitat degradation, but indigenous suckers are also vulnerable to hybridization with non-native suckers that may lead to introgression or loss of reproductive effort (Mandeville et al., 2017).

Herein, we describe a qPCR-based eDNA assay for the monophyletic clade named *Pantosteus jordani* by Evermann (1894). The assay was developed in three phases—in silico, in vitro, and in vivo—and challenged against a representative panel of nontarget species that are potentially sympatric, as well as two congeneric species common in other basins. The assay was found to be highly sensitive and specific in laboratory tests, and patterns of detections of *P. jordani* in field tests were consistent with expectations (Figure 1).

2 | METHODS

For names, we follow Eschmeyer's Catalog of Fishes (accessed 08 July 2019, https://www.calacademy.org/scientists/projects/catalog-of-fishes). To develop an eDNA assay specific to *P. jordani*, we examined partial sequences of the cytochrome b (*cytb*) mitochondrial gene available from GenBank ($n = 2$), as well as sequences from 11 sympatric nontarget species (Table 1). To increase our representation of diversity within *P. jordani*, we produced *cytb* sequences from 11 additional fish collected in Montana and Wyoming and additionally produced new *cytb* sequences from the closely related taxon *P. platyrhynchus* (Table 1). *Cytb* data were generated using PCR products amplified with primers from Dowling, Tibbets, Minckley, and Smith (2002), including two internal sequencing primers. PCR took place in 40 μl reactions with 4 μl (~4–20 ng) DNA template, 4 μl of 10× PCR buffer, 4 μl $MgCl_2$ (2.5 mM), 1 μM of each primer, 200 μM of each dNTP, 1 unit Titanium® Taq DNA Polymerase (Takara Bio USA, Inc.), and the remaining volume filled with PCR-grade distilled water. Thermocycling conditions involved an initial denaturation at 95°C for 2 min, followed by 12 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 1.5 min. An additional 12 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and extension at 72°C for 1.5 min were then completed. Final extension took place at 72°C for 5 min before PCR products were cleaned with an ExoSAP-IT™ PCR Product Cleanup Reagent kit (Life Technologies). Sequencing reactions were conducted with an ABI 3730XL sequencer located at the Eurofins Genomics facility. We processed the raw sequence data with Sequencher v5.4.6 (Gene Codes Corporation) and trimmed consensus sequences to the 1,140 base-pair length of the *cytb* gene. Sequence data are available from GenBank (Accession nos. MN917148-MN917162), and additional data are available on reasonable request from the corresponding author. We aligned all sequences in MEGA 7 (Kumar, Stecher, & Tamura, 2016) and scanned the data visually to identify candidate primer sites that would ultimately amplify a 119-nucleotide region in our alignment that was unique to *P. jordani* (Table 2). Within this amplicon, we designed a FAM-labeled, minor-groove-binding, nonfluorescent quencher (MGB-NFQ) probe (Table 2). We maximized nucleotide mismatches between oligonucleotides and nontarget sequences to avoid instances of primer competition and cross-amplification of the probe (Wilcox et al., 2016). We used Primer Express 3.0.1 (Life Technologies) to adjust primer and probe lengths to optimize annealing temperatures and screened them for secondary structures using the IDT OligoAnalyzer web application (https://www.idtdna.com/calc/analyzer). Using the NCBI nucleotide BLAST tool, we further examined the specificity of the assay in silico to reduce the potential for detecting nontarget taxa. Each

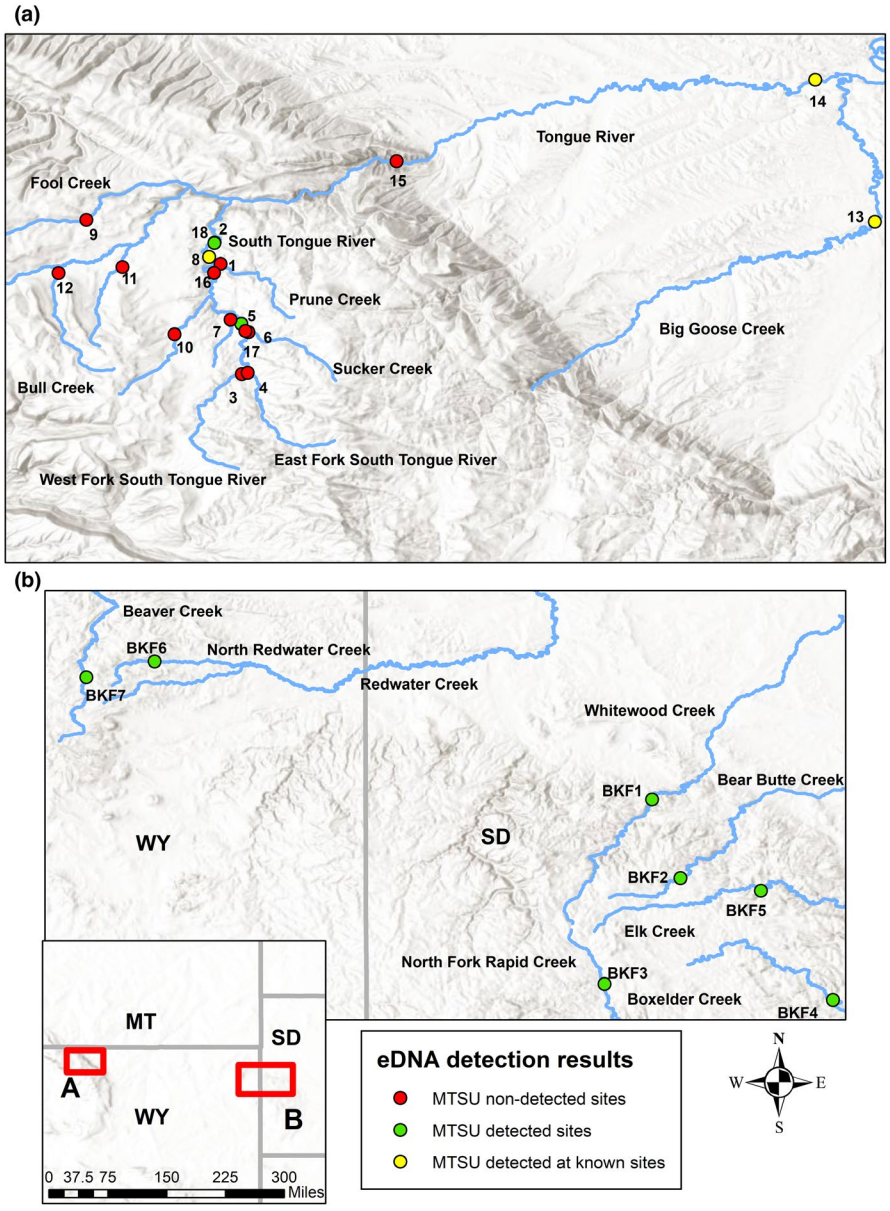

FIGURE 1 Map showing eDNA sample collection locations, sample streams, and eDNA detection results for sites sampled during efforts to validate the *Pantosteus jordani* (MTSU) eDNA assay in the Upper Missouri basin of Wyoming and South Dakota. The inset map shows the extent of panels A and B outlined in red, and the state boundaries in gray. The scale bar represents distances shown within the inset map. Several sites from the South Tongue basin in Wyoming appear as overlapping dots in panel A, but all sampling sites are included in the map

oligonucleotide was examined individually in this manner before the complete assay was assessed using Primer BLAST and the full NCBI nucleotide collection.

We tested the specificity of the assay in vitro using a QuantStudio 3 Real-time PCR Instrument (Life Technologies) in 15-µl reactions containing 7.5 µl Environmental Master Mix 2.0 (Life Technologies), 900 nM each forward and reverse primer, 250 nM of probe, 4 µl DNA template (~0.4 ng), and PCR-grade water for the remaining volume. Thermocycler conditions were 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Pipettes, tube racks, and consumables were irradiated with UV light in a hood for 1 hr prior to set-up. We screened DNA extracted from 20 *P. jordani* tissues collected from Montana and Wyoming, and from 48 additional nontarget species (Table 3). DNA used for in vitro screening was obtained from archival samples, or from small fin clips collected from fish that were immediately released at the point of capture. Tissues from fin clips were collected by personnel affiliated with the Wyoming Game and Fish Department and Montana Fish, Wildlife and Parks who were authorized to make such collections. Fin clips were stored in ≥95% ethanol until DNA was extracted using the DNeasy Tissue and Blood Kit (Qiagen, Inc) according to the manufacturer's protocol. Prior to extraction, we rinsed the tissues with a 10% sodium hypochlorite solution to remove eDNA from co-occurring species that may have been on the tissue surface, then thoroughly rinsed each tissue with deionized water to minimize destruction of target DNA.

We optimized primer concentrations by testing a single *P. jordani* DNA sample with concentrations of each primer at 100, 300, 600, and 900 nM, resulting in 16 unique assay concentrations (Wilcox et al., 2016). We selected the assay concentration that displayed a high relative end-point fluorescence and the lowest C_t value for use in subsequent analyses (Table 2). Using the optimal concentrations of 600 nM of forward and 900 nM reverse primer and the same qPCR conditions as above, we tested assay sensitivity and

TABLE 1 Species, sample size (*n*), GenBank accession numbers, and minimum number of primer mismatches for DNA sequences used for in silico Mountain sucker assay development

					Mismatches		
Family	**Species**	**Common name**	***N***	**Accession(s)**	**Forward**	**Reverse**	**Probe**
Targets							
Catostomidae	*Pantosteus jordani*	Mountain sucker (in part)	13	KJ441276-7; MN917148-58	0	0	0
Nontargets							
Cyprinidae	*Cyprinella lutrensis*	Red shiner	4	GQ275188–9; GQ275190; GQ275194	9	5	3
Catostomidae	*Carpiodes carpio*	River carpsucker	7	AF454867; JF799431; JN053177; JN053185; JN053187-8; JN053260	6	5	4
	Catostomus catostomus	Longnose sucker	5	AF454871; EU676808; JX258854-6	8	3	5
	Catostomus commersonii	White sucker	5	JF799435-7; JX488781; KU697932	5	4	4
	Pantosteus platyrhynchus	Mountain sucker (in part)	4	MN917159-62	6	2	4
	Cycleptus elongates	Blue sucker	2	AF454868; JF799439	10	6	4
	Ictiobus bubalus	Smallmouth buffalo	5	FJ226281; FJ226285; FJ226287-89	7	7	2
	Ictiobus cyprinellus	Bigmouth buffalo	5	FJ226257-60	7	7	2
	Moxostoma macrolepidotum	Shorthead redhorse	3	AF454890; JF99473-4	6	4	2
Salmonidae	*Coregonus clupeaformis*	Lake whitefish	2	JX960775-6	10	8	4
	Salvelinus namaycush	Lake trout	4	JX960858; KT630743; KU761868-9	13	4	3

Note: The common names of members of the genus *Pantosteus* are further described with "in part" to indicate that the current understanding of these lineages' divergence from each other has not yet been incorporated in to separate common names for each taxon.

TABLE 2 Optimized environmental DNA assay for detecting Mountain sucker using qPCR

Assay component	**Sequence (5'–3')**	**Tm (°C)**	**Optimal concentration (nM)**
Forward primer	TGTTGTGCTTCTCCTGTTAGTGATG	59.4	600
Reverse primer	ACATAAGGGACTGCGGACAAA	58.2	900
Probe	FAM-TTGTTGGGTACGTACTTC-MGBNFQ	69	250

efficiency by analyzing a seven-level dilution sequence created from a commercially synthesized gBlock gene fragment (Integrated DNA Technologies), rehydrated and quantified on a Qubit 2.0 fluorometer, then serially diluted in sterile TE buffer to 31 250, 6 250, 1 250, 250, 50, 10, and 2 copies per 4 μl. We analyzed the dilution sequence across six replicates of each concentration on a single 96-well qPCR plate.

Finally, we applied the assay in vivo by screening eDNA samples collected from 19 waterbodies in the western U.S. within the range of *P. jordani* (Figure 1, Table 4). The eDNA samples were collected by filtering 5 L of water through a 1.5 μm pore sized glass filter as per Carim, McKelvey, and Young (2016). DNA was extracted from the filters with the DNeasy Tissue and Blood Kit (Qiagen, Inc) following a modified protocol (Carim et al., 2016) which included extraction controls. Using the optimized qPCR conditions, the extracts were then analyzed along with a TaqMan Exogenous Internal Positive Control (1.5 μl of 10X IPC assay and 0.15 μl of 50X IPC DNA per reaction; Life Technologies), to screen for qPCR inhibition by environmental contaminants. An environmental sample was considered inhibited if there was a > 1 cycle-threshold (Ct) shift in the IPC relative to the curve from the no-template control. All in vivo analyses were performed across three replicates and included a no-template control substituting distilled water for eDNA. To confirm the specificity of the assay in vivo, we pooled the qPCR products from each triplicate of a subset of samples (products from replicate wells pooled, but products from different samples kept separate) which demonstrated amplification curves, cleaned the products using an ExoSAP-IT kit (Life Technologies), and sequenced the amplicons using the eDNA

TABLE 3 Species used for in vitro testing of the *Pantosteus jordani* assay. Origin refers to the region/waterbody for *P. jordani* and to the state for all other samples

Family	Species	Common name	N	Origin
Acipenseridae	*Scaphirhynchus platorynchus*	Shovelnose sturgeon	1	MT
Catostomidae	*Carpiodes carpio*	River carpsucker	1	MT
	Catostomus catostomus	Longnose sucker	1	MT
			3	WY
	Catostomus commersonii	White sucker	1	MT
			3	WY
	Pantosteus discobolus	Bluehead sucker	1	WY
	Pantosteus platyrhynchus	Mountain sucker (in part)	5	WY
	Pantosteus jordani	Mountain sucker (in part)	2	Eastern MT
			6	Goose Creek, WY
			6	North Redwater Creek, WY
			6	South Tongue River, WY
	Ictiobus bubalus	Smallmouth buffalo	1	MT
	Moxostoma macrolepidotum	Shorthead redhorse	1	MT
Centrarchidae	*Lepomis cyanellus*	Green sunfish	1	CO
	Micropterus dolomieu	Smallmouth bass	1	MT
	Micropterus salmoides	Largemouth bass	1	MT
	Pomoxis nigromaculatus	Black crappie	1	MT
Cottidae	*Cottus cognatus*	Rocky Mountain slimy sculpin	1	MT
	Cottus sp.	Rocky Mountain sculpin	1	MT
Cyprinidae	*Cyprinella lutrensis*	Red shiner	1	NM
	Cyprinus carpio	Common carp	1	WY
	Hybognathus argyritis	Plains minnow	1	MT
	Macrhybopsis gelida	Sturgeon chub	1	MT
	Macrhybopsis meeki	Sicklefin chub	1	MT
	Notropis atherinoides	Emerald shiner	1	MT
	Notropis stramineus	Sand shiner	1	MT
	Pimephales promelas	Fathead minnow	1	ID
			1	MT
	Platygobio gracilis	Flathead chub	1	MT
	Rhinichthys cataractae	Longnose dace	1	ID
			1	WY
Dreissenidae	*Dreissena polymorpha*	Zebra mussel	1	Unknown
Esocidae	*Esox lucius*	Northern pike	1	AK
			1	MT
	Esox masquinongy	Muskellunge	1	MN
Fundulidae	*Fundulus zebrinus*	Plains killifish	1	CO
Hiodontidae	*Hiodon alosoides*	Goldeye	1	MT
Ictaluridae	*Ictalurus natalis*	Yellow bullhead	1	MT
	Ictalurus punctatus	Channel catfish	1	MT
	Noturus flavus	Stonecat	1	MT
Lotidae	*Lota lota*	Burbot	1	MT

(Continues)

TABLE 3 (Continued)

Family	Species	Common name	*N*	Origin
Percidae	*Etheostoma exilie*	Iowa darter	1	CO
	Perca flavescens	Yellow perch	1	WA
			1	MT
	Sander candensis	Sauger	1	MT
	Sander vitreus	Walleye	1	WA
			1	Unknown
Salmonidae	*Coregonus clupeaformis*	Lake whitefish	1	MT
	Oncorhynchus clarkii bouvieri	Yellowstone cutthroat trout	1	WY
	Oncorhynchus mykiss gairdneri	Rainbow trout	2	MT
	Oncorhynchus nerka	Kokanee salmon	1	MT
	Prosopium williamsoni	Mountain whitefish	1	MT
	Salmo trutta	Brown trout	1	OR
	Salvelinus fontinalis	Brook trout	1	ID
	Salvelinus namaycush	Lake trout	1	MT
	Thymallus arcticus	Arctic grayling	1	MT
Sciaenidae	*Aplodinotus grunniens*	Freshwater drum	1	MT
Umbridae	*Umbra limi*	Central mudminnow	1	MT

Note: The common names of members of the genus *Pantosteus* are further described with "in part" to indicate that the current understanding of these lineages' divergence from each other has not yet been incorporated in to separate common names for each taxon.

assay primers. As before, sequencing reactions were conducted with an ABI 3730XL sequencer located at the Eurofins Genomics facility. We processed the raw sequence data with Sequencher v5.4.6 (Gene Codes Corporation) and trimmed consensus sequences to the length of the eDNA amplicon for comparison.

2.1 | RESULTS and DISCUSSION

The assay detected DNA from all *P. jordani* tissue samples and did not detect DNA from the nontarget species or within the no-template controls. The standard curve demonstrated a reaction efficiency of 103.154% (R^2 = 0.989, y-intercept = 40.482, slope = −3.249) and a limit of quantification (the lowest number of copies that can be precisely quantified, (EP17-A C.L.S.I. CLSI; Wayne, 2004) of 10 copies per reaction; DNA was detected in all six replicates at this concentration and in two of six replicates with an average of 2 copies per reaction. There were two catostomid species (*Ictiobus cyprinellus* and *Cycleptus elongatus*) sympatric with *P. jordani* that we were unable to test against the assay during in vitro validation, due to lack of available DNA. However, the assay has a total of 16 and 20 nucleotide mismatches with *cytb* sequences published from these species, respectively; we consider it extremely unlikely that the presence of eDNA from these species would lead to misleading results, since we observed complete specificity of the assay against nontarget templates with fewer mismatches (Table 1) and results from similar assay designs support the robustness of this assumption (Franklin et al., 2018; Mason et al., 2018). Additionally, primer BLAST results did not suggest any affinity of the primers for DNA from these taxa.

During in vivo validation, *P. jordani* DNA was not detected in any environmental samples taken where the species was expected to be absent, or in any of the extraction controls. We detected *P. jordani* at three sites that were known to be supporting *P. jordani* populations, confirming the marker's effectiveness (Table 4). Initial site occupancy status was based on previous electrofishing data conducted by Wyoming Game and Fish Department. Notably, *P. jordani* was also detected by eDNA methods in ten samples from sites which had previously held unknown occupancy status. Inhibition was not detected in any eDNA reactions. Amplicon sequences obtained from positive eDNA samples matched the known *cytb* sequence of *P. jordani* in the assay region (S3).

One of the greatest limitations to effective conservation of nongame or less charismatic species is a lack of data, particularly on their distributions. Such is the current situation for *P. jordani* (Belica & Nibbelink, 2006). While consideration of the scientific and technological limitations (see Thomsen & Willerslev, 2015 for a review of eDNA tools) remains important, surveys based on eDNA sampling can help to remedy this problem because of their lower costs and greater efficiency (Wilcox et al., 2016). Of particular relevance may be the application of eDNA sampling to find remnant populations at the southernmost extent of its range. In fact, although this project was not intended as a distributional study, the eDNA assay detected several populations in the Black Hills of South Dakota (Table 4, BKF1-7) whose status was unknown. The assay could also easily be applied to other regions where uncertainty persists, or to areas where the taxon has been believed to be absent entirely. Although historically reported from Nebraska in the Niobrara River, the species is currently regarded

TABLE 4 Collection information and detection results for in vivo testing of the *Pantosteus jordani* assay

Waterbody	Site	Latitude	Longitude	Date	Expected occupancy	DNA detected	Mean Ct	Ct Std Deviation
Prune Creek	1	44.76777	−107.46520	9/26/2018	?	N	Undetermined	–
South Tongue River	2	44.78426	−107.46984	9/26/2018	?	Y	34.872	0.415
West Fork South Tongue River	3	44.68316	−107.44893	9/26/2018	?	N	Undetermined	–
East Fork South Tongue River	4	44.68423	−107.44438	9/26/2018	?	N	Undetermined	–
South Tongue River	5	44.72174	−107.44937	8/26/2019	?	Y	36.225	0.296
Sucker Creek	6	44.71543	−107.44385	9/26/2018	?	N	Undetermined	–
Copper Creek	7	44.72489	−107.45760	9/26/2018	?	N	Undetermined	–
South Tongue River	8	44.77308	−107.47409	8/26/2019	Y	Y	36.976	–
Fool Creek	9	44.80168	−107.56836	9/25/2017	N	N	Undetermined	–
Owen Creek	10	44.71384	−107.50068	9/25/2017	?	N	Undetermined	–
Big Willow Creek	11	44.76540	−107.54072	9/25/2017	N	N	Undetermined	–
Bull Creek	12	44.76068	−107.58973	9/25/2017	N	N	Undetermined	–
Big Goose Creek	13	44.80021	−106.96244	10/5/2017	Y	Y	36.929	0.555
Tongue River	14	44.90929	−107.00832	10/5/2017	Y	Y	37.492	1.421
Tongue River	15	44.84669	−107.32999	10/5/2017	?	N	Undetermined	–
South Tongue River	16	44.76089	−107.47034	10/6/2017	?	N	Undetermined	–
Sucker Creek	17	44.71621	−107.44617	10/6/2017	?	N	Undetermined	–
South Tongue River	18	44.78384	−107.47006	8/26/2019	?	Y	38.564	0.657
South Tongue River	19	44.76743	−107.46854	8/26/2019	?	N	Undetermined	–
South Tongue River	20	44.76572	−107.47446	9/6/2019	?	N	Undetermined	–
South Tongue River	21	44.72174	−107.44937	9/26/2018	?	N	Undetermined	–
South Tongue River	22	44.76216	−107.47516	9/6/2019	?	N	Undetermined	–
Whitewood Creek	BKF1	44.40933	−103.69608	11/8/2018	?	Y	34.597	0.609
Bear Butte Creek	BKF2	44.31072	−103.66056	11/8/2018	?	Y	36.626	0.292
North Fork Rapid Creek	BKF3	44.17803	−103.75596	11/8/2018	?	Y	34.574	0.15
Box Elder Creek	BKF4	44.15788	−103.46911	11/8/2018	?	Y	35.132	0.895
Elk Creek	BKF5	44.29484	−103.55962	11/8/2018	?	Y	38.546	–
North Redwater Creek	BKF6	44.58262	−104.32055	11/9/2018	?	Y	33.027	0.191
Beaver Creek	BKF7	44.56286	−104.40610	11/9/2018	?	Y	37.054	1.375
Extraction Ctrl1	–	–	–	4/13/2018	–	N	Undetermined	–
Extraction Ctrl2	–	–	–	3/14/2019	–	N	Undetermined	–
Extraction Ctrl3	–	–	–	4/14/2019	–	N	Undetermined	–
Extraction Ctrl4	–	–	–	9/10/2019	–	N	Undetermined	–
Extraction Ctrl5	–	–	–	10/21/2019	–	N	Undetermined	–

Note: Expected occupancy is based off of previous electrofishing data collected by Wyoming Game and Fish Department. Sites with uncertain expected occupancy status at the time of sampling are indicated by "?" in the appropriate column.

as extirpated (Belica & Nibbelink, 2006). The lack of additional observations in the Niobrara River led to speculation that this historical record was in error, but discovery of late Pleistocene fossils from Kansas (Smith et al., 2013) lent credence to the notion that *P. jordani* may have occurred this far south in the last century. Given declines in this species in portions of its range in the last few decades (Patton et al., 1998; Schultz & Bertrand, 2012), and the likelihood that populations are persisting in isolated fragments (Bertrand, VanDeHey, Pilger, Felts, & Turner, 2016), eDNA sampling may provide a strategic alternative to more costly traditional sampling and could be used to relatively rapidly establish a modern benchmark for the distribution of this phylogenetically recognizable group, regardless of its taxonomic status. Despite sampling a small portion of the distribution of *P. jordani*, this study detected

the taxon at ten new sites from eight streams where its presence was unknown, emphasizing the impact that this sensitive and efficient approach can provide.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The raw data from the qPCR experiments are available from the Dryad repository at https://doi.org/10.5061/dryad.t1g1jwt0h.

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