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# Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi*) and Paiute cutthroat trout (*Oncorhynchus clarkii seleniris*) detection from environmental DNA samples: a dual-purpose assay

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Abstract.—Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi*) and Paiute cutthroat trout (*Oncorhynchus clarkii seleniris*) are rare taxa of conservation concern. Their generally low population densities and remote distribution make traditional sampling difficult, but they primarily exist in small streams, a habitat type where environmental DNA (eDNA) sampling has proven extremely effective. We developed a single eDNA assay that allows sensitive detection of both taxa, which occur separate from each other in different regions of the Lahontan Basin. Performance of the assay was evaluated using most lineages of Lahontan cutthroat trout and Paiute cutthroat trout as well as tissues of other cutthroat trout and rainbow trout that have been introduced within the Lahontan Basin. The assay amplified DNA only from the target taxa, and only from eDNA samples collected where those taxa were known to be present. This assay should enhance the ability of managers to more precisely and efficiently describe the distribution of these taxa.

Resumen.—La trucha degollada de Lahontan (*Oncorhynchus clarkii henshawi*) y la trucha degollada de Paiute (*Oncorhynchus clarkii seleniris*) son taxones raros de interés para la conservación. Sus densidades de poblacionales generalmente bajas, así como sus distribuciones remotas dificultan el muestreo tradicional. No obstante, se encuentran principalmente en arroyos pequeños, un tipo de hábitat donde el muestreo de ADN ambiental (eDNA) ha demostrado ser extremadamente efectivo. En este estudio llevamos a cabo un único análisis de eDNA que permite la detección sensible de cualquiera de los miembros del taxón, que se encuentran en diferentes regiones de la cuenca de Lahontan. El resultado del análisis se evaluó utilizando la mayoría de los linajes de la trucha degollada de Lahontan y la trucha degollada de Paiute, así como tejidos de otras truchas degolladas y la trucha arco iris que se han introducido en la cuenca de Lahontan. El análisis permitió únicamente la amplificación de ADN de los taxones objetivo, y sólo de las muestras de eDNA recolectadas donde se sabía que esos taxones estaban presentes. Este análisis mejorará la capacidad de los administradores para describir la distribución de estos taxones de manera más precisa y eficiente.

The explosion of interest in environmental DNA (eDNA) sampling is largely due to its exceptional specificity and sensitivity for detection of target species, especially when based on single-species, quantitative PCR-based (qPCR) assays (Goldberg et al. 2016, Mason et al. 2018). Although initial applications often involved detection of nonnative species (Ficetola et al. 2008), another major objective of eDNA sampling has been to characterize the presence and distribution of rare native species (e.g., McKelvey et al. 2016, Franklin et al. 2018). For many of these taxa, eDNA sampling has provided better estimates of habitat occupancy than alternative methods (e.g., Wilcox et al. 2016, Robinson et al. 2019) despite not requiring direct observation of the target species. This latter characteristic can be an advantage in instances where traditional sampling may lead to injury or mortality of the target species, where field identification of species is uncertain, or where sampling is costly, difficult, or inefficient (Evans et al. 2017).

Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi*) and Paiute cutthroat trout

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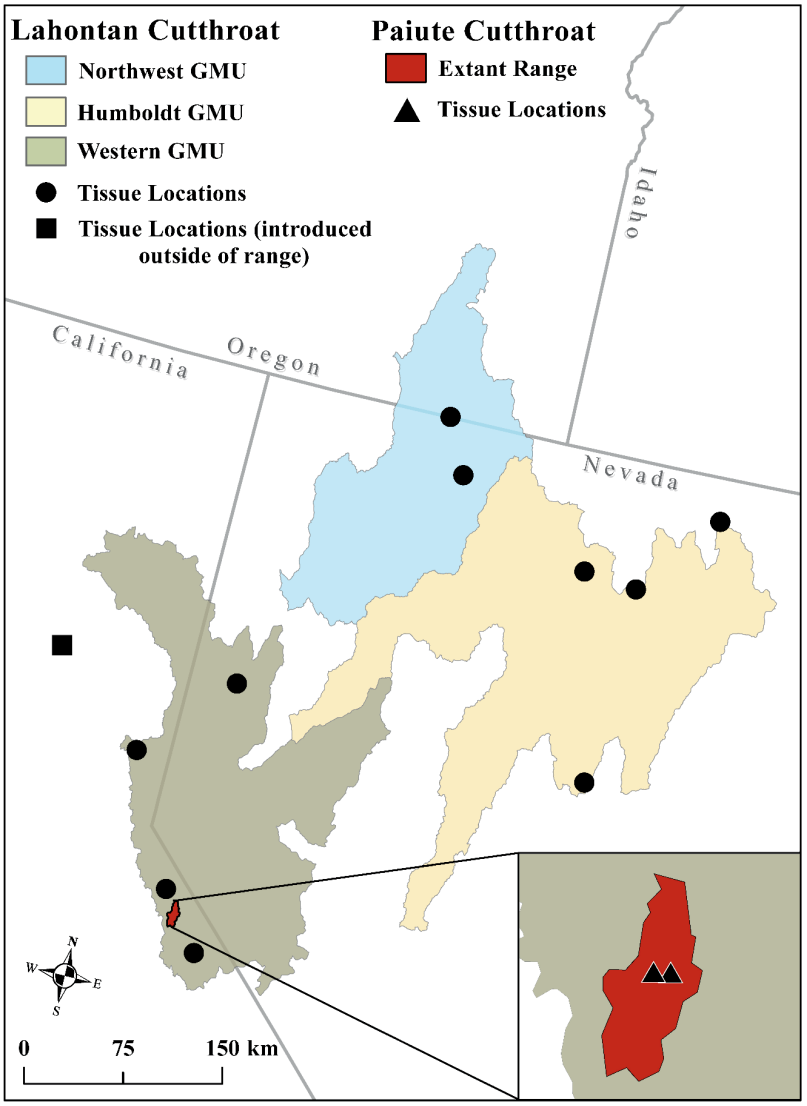


Fig. 1. Map of Lahontan and Paiute cutthroat trout ranges and sites where tissue-derived DNA was obtained for assay development. The Lahontan range is divided into 3 geographic management unit (GMU) areas: blue represents the Northwest GMU, yellow represents the Humboldt GMU, and green represents the Western GMU. Dark red represents the extant Paiute cutthroat trout range. Circles denote Lahontan cutthroat trout sites where tissue-derived DNA was obtained, and a square denotes a Lahontan cutthroat trout site where tissue-derived DNA was obtained from a population of fish introduced outside of the species' native range. Triangles denote Paiute cutthroat trout sites where tissue-derived DNA was obtained.

(*O. c. seleniris*) are salmonid fishes endemic to the Pleistocene Lahontan Basin in the southwestern United States (Fig. 1). Both taxa are listed as threatened under the U.S. Endangered Species Act (USFWS 2009, 2013) and are recognized as distinct but sister taxa (Saglam et al. 2017, Peacock et al. 2018). Lahontan cutthroat trout were once relatively widely distributed among over 400 streams and 11 lakes, but invasions by nonnative fishes, water diversion, and habitat disruption have reduced their distribution by over 90% (USFWS 2009). Aside from a recent reintroduction, Paiute cutthroat trout now exist exclusively outside their historic range in streams isolated from invasions by nonnative

fishes (USFWS 2013). Assessing whether these taxa are present in particular waters, as well as their distribution within these waters, is crucial to evaluating their conservation status and the effectiveness of management actions (e.g., reintroductions). This is particularly true for Lahontan cutthroat trout in those portions of their range where sampling has been sporadic in space and time and where current occupancy for some streams has been inferred based on correlative models or professional opinion (Leasure et al. 2019, Neville et al. 2020). Detection of these species using traditional methods has proven difficult due to their low densities (<100 fish/km) across the majority of their fluvial habitat (USFWS 2009, 2013). Furthermore, they are primarily found in small, remote headwater streams (<3 m wetted width; USFWS 2009, 2013). Because eDNA sampling is highly effective and efficient for detecting low-density species across broad geographic ranges with greater detection probabilities (McKelvey et al. 2016, Jerde 2021), it may be a critical addition to the toolbox of biologists charged with managing these species. Also, eDNA sampling has met the criteria for legal evidence in many courts when applied thoughtfully (Court of Appeals for the Seventh Circuit 2011, Sepulveda et al. 2020). Therefore, proper application of eDNA assays such as those presented here has the potential to direct the management of federally threatened species such as Lahontan and Paiute cutthroat trout.

## Methods

Although the Paiute cutthroat trout is recognized as a distinct taxon, it exhibits relatively little mitochondrial divergence from other members of the Lahontan cutthroat trout lineage (Loxterman and Keeley 2012). This, and the fact that the 2 taxa occupy nonoverlapping ranges, led us to design a single eDNA assay for both. Initially, we compiled genetic data of the cytochrome c oxidase subunit 3 (CO3) mitochondrial gene from sympatric target and nontarget species (Table 1). Most genetic data were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), but in addition we generated sequences from Lahontan ($n$ = 10), Paiute ($n$ = 6), and Yellowstone (*O. c. bouvieri*; $n$ = 10) cutthroat trout tissues to increase our genetic representation of these lineages. To generate these sequences, we amplified a 798-bp fragment using forward primer 5′-GTTTAATGGCAC ACCAAGCACA-3′ and reverse primer 5′-AGAAAGATTATGAGCCTCATCAGTA AATAG-3′ in 40-µL reactions containing 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 each dNTP, 25 µg BSA, 1 Unit Titanium® Taq DNA Polymerase (Takara Bio USA, Inc.), and PCR-grade distilled water for the remaining volume. The thermocycling conditions contained an initial denaturation at 95 °C for 12 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1.5 min; there was a final extension stage at 72 °C for 5 min. We cleaned PCR products using ExoSAP-IT™ PCR Product Cleanup Reagent (Life Technologies) and generated sequences on an ABI 3730XL sequencing machine at Eurofins Genomics. We processed the raw sequencing data in Sequencher v 5.4.6 (Gene Codes Corporation; listed as NGCWFC in Table 1).

We aligned sequence data in MEGA 7.0 (Kumar et al. 2016) and manually trimmed sequences to the same length (727 bp). We generated candidate eDNA primers using the DECIPHER package (Wright 2016) in R v. 3.3.2 (R Core Team 2016) and added them to our alignment. Primer lengths and positions were adjusted to maximize base-pair mismatches with nontarget sequences and to concentrate mismatches on or near the 3′ ends. We then visually designed a FAM-labeled, minor-groove-binding, nonfluorescent quencher (MGB-NFQ) probe unique to Lahontan and Paiute cutthroat trout (Table 2). The annealing temperatures of the primers and probe were evaluated using Primer Express 3.0.1, and we assessed potential dimerization and hairpin formation using the IDT OligoAnalyzer web application (https://www.idtdna.com/calc/analyzer). To evaluate the specificity of the assay in silico, we compared the primers and probe to the entire NCBI nucleotide collection using Primer-BLAST (Ye et al. 2012) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

To ensure the specificity of the assay in vitro, we performed qPCR analyses with tissue-derived DNA extracted from 60 Lahontan cutthroat trout, 6 Paiute cutthroat trout, and 28 nontarget species (Fig. 1, Table 3). The

TABLE 1. Species and GenBank accession number for cytochrome c oxidase subunit 3 (CO3) DNA sequences used for in silico development of the Lahontan and Paiute cutthroat trout eDNA assay. Species are limited to those sympatric with the target species. Also included is the maximum number of mismatches between the eDNA marker and the sequences screened.

| Family | Species | Common name | Accession(s) | Mismatches | | |
|---|---|---|---|---|---|---|
| | | | | Forward | Reverse | Probe |
| Salmonidae | *Oncorhynchus clarkii henshawi* | Lahontan cutthroat trout | AY88676; NGCWFC ($n$ = 10) | 0 | 0 | 0 |
| | *Oncorhynchus clarkii seleniris* | Paiute cutthroat trout | NGCWFC ($n$ = 6) | 0 | 0 | 0 |
| Centrarchidae | *Lepomis macrochirus* | Bluegill | MF621714 | 5 | 6 | 7 |
| | *Micropterus dolomieu* | Smallmouth bass | MF621711 | 6 | 4 | 6 |
| | *Micropterus salmoides* | Largemouth bass | DQ536425 | 6 | 3 | 8 |
| | *Pomoxis nigromaculatus* | Black crappie | MF621715 | 5 | 4 | 6 |
| Cyprinidae | *Cyprinus carpio* | Common carp | MG670426 | 6 | 5 | 8 |
| | *Rhinichthys osculus* | Speckled dace | KT424924 | 1 | 4 | 7 |
| Ictaluridae | *Ameiurus nebulosus* | Brown bullhead | MF621734 | 4 | 6 | 6 |
| | *Ictalurus punctatus* | Channel catfish | MF621718 | 2 | 5 | 6 |
| Leuciscidae | *Notemigonus crysoleucas* | Golden shiner | MG570438 | 3 | 3 | 10 |
| | *Siphateles bicolor mohavensis* | Mohave tui chub | MF972070 | 5 | 3 | 8 |
| Poeciliidae | *Gambusia affinis* | Western mosquitofish | AP004422 | 4 | 1 | 7 |
| Salmonidae | *Oncorhynchus clarkii bouvieri* | Yellowstone cutthroat trout | NGCWFC ($n$ = 10) | 1 | 2 | 2 |
| | *Oncorhynchus mykiss* | Rainbow trout | KP013084 | 3 | 2 | 4 |
| | *Oncorhynchus nerka* | Sockeye salmon | KU872726 | 2 | 2 | 3 |
| | *Prosopium williamsoni* | Mountain whitefish | JQ390061 | 1 | 4 | 7 |
| | *Salmo trutta* | Brown trout | MF621762 | 4 | 2 | 4 |
| | *Salvelinus fontinalis* | Brook trout | KU872727 | 3 | 1 | 4 |
| | *Salvelinus namaycush* | Lake trout | MF621744 | 2 | 2 | 6 |

Table 2. Optimized environmental DNA assay for detecting Lahontan and Paiute cutthroat trout using qPCR.

| Assay component | Sequence (5′ to 3′) | Temp. (°C) | Optimal concentration (nM) |
|---|---|---|---|
| Forward primer | GCATCTGGTGTTACCGTCACG | 60.0 | 300 |
| Reverse primer | ACGCCGTCAGCAATTGTAAAC | 58.8 | 600 |
| Probe | FAM-CTCTTACTCTAACCATTCTACTGG-MGBNFQ | 69.0 | 250 |

tissue and DNA samples used were obtained from collections originating from previous studies at the National Genomics Center for Wildlife and Fish Conservation (NGC; Missoula, MT) and the University of Nevada, Reno. All tissues and DNA used in this study were from archived samples collected for other projects under appropriate state and federal permits or by the respective state agency. Previously unextracted tissue samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc.) following the manufacturer's protocol at the NGC. Prior to extraction, the surface of the tissue was cleaned with a 10% bleach solution followed by a rinse with distilled water to remove residual DNA from nontarget taxa. We analyzed the DNA extracts with a QuantStudio 3 Real-time PCR Instrument (Life Technologies) in 15-µL reactions comprising 7.5 µL Environmental Master Mix 2.0 (Life Technologies), 900 nM of each primer, 250 nM of probe, 4 µL DNA template (~0.4 ng), and PCR-grade water for the remaining volume. The thermocycling conditions included an initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. These thermocycling conditions were used for all qPCR analyses.

After confirming the specificity of the assay, we optimized primer concentrations and evaluated the assay's sensitivity. To optimize primer concentrations, we used the same qPCR recipe above but tested 4 concentrations of each primer—100, 300, 600, and 900 nM—for a total of 16 concentration combinations. Each combination was run in triplicate reactions, and the concentration resulting in the earliest mean cycle threshold (Ct) value while maintaining an exponential amplification curve with a high end-point-normalized fluorescence was selected for further testing. We assessed the sensitivity of the optimized assay by analyzing a 7-level sequential dilution containing 31,250, 6250, 1250, 250, 50, 10, and 2 copies of target DNA per reaction for each target species. The DNA for each species was derived from qPCR product that we cleaned with the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and quantified on a Qubit Fluorometer 2.0 (Thermo Fisher Scientific) before diluting the product in sterile TE buffer.

We then validated the assay in vivo by analyzing eDNA samples collected from areas where Lahontan and Paiute cutthroat trout were known to be present or absent based on previous recent electrofishing surveys (Table 4). Four samples from Independence Creek in California and one sample from Wildcat Creek in Nevada were expected to contain DNA from Lahontan cutthroat trout. Two samples collected from one site in Silver King Creek in California were expected to be positive for Paiute cutthroat trout. The remaining samples were not expected to contain DNA from Lahontan or Paiute cutthroat trout but were known to contain DNA from 1 of 2 closely related species (Loxtermann and Keeley 2012): Yellowstone cutthroat trout or rainbow trout (*O. mykiss*). To collect the eDNA samples, 5 L of water was pumped through a glass microfiber filter (pore size = 1.5 µm) using the technique developed by Carim et al. (2016) for collecting eDNA samples from streams. These samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc.) following a modified protocol including negative extraction controls (Franklin et al. 2019). The eDNA extracts were then analyzed with a QuantStudio 3 Real-time PCR Instrument (Life Technologies) in triplicate 15-µL reactions comprising 7.5 µL Environmental Master Mix 2.0 (Life Technologies), 300 nM forward primer, 600 nM reverse primer, 250 nM of probe, 4 µL eDNA extract (~0.4 ng), TaqMan Exogenous Internal Positive Control (IPC; Life Technologies), which included 1.5 µL of 10× IPC assay and 0.30 µL of 50× IPC DNA, and PCR-grade water for the remaining volume. We used the IPC to screen for

TABLE 3. Species and sample sizes (*n*) used for in vitro testing of the Lahontan and Paiute cutthroat trout assay. Origin refers to a waterbody for Lahontan and Paiute cutthroat trout samples and to a state or province for all nontarget samples. These origin locations are spatially displayed in Fig. 1.

| Family | Species | Common name | *n* | Origin |
|---|---|---|---|---|
| Salmonidae | *Oncorhynchus clarkii henshawi* | Lahontan cutthroat trout | 5 | By-Day Creek, CA |
| | | | 5 | East Fork Carson River, CA |
| | | | 5 | Independence Lake, CA |
| | | | 5 | Milk Ranch Creek, CA |
| | | | 5 | Crowley Creek, NV |
| | | | 5 | Frazier Creek, NV |
| | | | 5 | Maggie Creek, NV |
| | | | 5 | Pyramid Lake, NV |
| | | | 5 | West Fork Mary's River, NV |
| | | | 10 | Line Canyon Creek, OR |
| | | | 5 | Willow Creek, NV |
| | *Oncorhynchus clarkii seleniris* | Paiute cutthroat trout | 2 | Corral Creek, CA |
| | | | 4 | Coyote Valley Creek, CA |
| Catostomidae | *Catostomus commersonii* | White sucker | 1 | CA |
| | *Catostomus lahontan* | Lahontan mountain sucker | 1 | NV |
| | *Pantosteus platyrhynchus* | Mountain sucker | 1 | WY |
| | *Lepomis cyanellus* | Green sunfish | 1 | WA |
| | *Micropterus dolomieu* | Smallmouth bass | 1 | OR |
| | *Micropterus salmoides* | Largemouth bass | 1 | MT |
| | *Pomoxis annularis* | White crappie | 1 | MT |
| | *Pomoxis nigromaculatus* | Black crappie | 1 | MT |
| Cottidae | *Cottus beldingii* | Paiute sculpin | 1 | CA |
| Cyprinidae | *Cyprinus carpio* | Common carp | 1 | OR |
| | *Rhinichthys cataractae* | Longnose dace | 1 | ID |
| | *Rhinichthys osculus* | Speckled dace | 1 | AZ |
| | *Richardsonius balteatus* | Redside shiner | 1 | ID |
| | *Richardsonius egregius* | Lahontan redside shiner | 1 | NV |
| Esocidae | *Esox lucius* | Northern pike | 1 | ID |
| Ictaluridae | *Ictalurus punctatus* | Channel catfish | 1 | MT |
| Salmonidae | *Oncorhynchus clarkii bouvieri* | Yellowstone cutthroat trout | 6 | ID, NV, WY |
| | *Oncorhynchus clarkii clarkii*[a] | Coastal cutthroat trout | 1 | British Columbia, Canada |
| | *Oncorhynchus clarkii lewisi*[a] | Westslope cutthroat trout | 1 | MT |
| | *Oncorhynchus clarkii pleuriticus*[a] | Colorado River cutthroat trout | 1 | WY |
| | *Oncorhynchus clarkii utah*[a] | Bonneville cutthroat trout | 1 | UT |
| | *Oncorhynchus mykiss* | Rainbow trout | 1 | WA |
| | *Oncorhynchus mykiss gairdneri* | Redband trout | 6 | CA, OR |
| | *Oncorhynchus nerka* | Sockeye salmon | 1 | OR |
| | *Prosopium williamsoni* | Mountain whitefish | 1 | MT |
| | *Salmo trutta* | Brown trout | 1 | OR |
| | *Salvelinus fontinalis* | Brook trout | 1 | WA |
| | *Salvelinus namaycush* | Lake trout | 1 | ID |

[a]Conspecific taxa representing different subspecies

Table 4. Collection information and detection results for eDNA samples used for in vivo validation of the Lahontan and Paiute cutthroat trout eDNA assay. Expected occupancy is based on previous surveys. DNA detected is based on environmental DNA sampling. N = no, Y = yes. Superscripted *L* indicates that Lahontan cutthroat trout DNA was expected, and superscripted *P* indicates that Paiute cutthroat trout DNA was expected.

| State | Site | Latitude | Longitude | Expected occupancy | DNA detected |
|---|---|---|---|---|---|
| AZ | Little Colorado River | 34.15181 | −109.30879 | N | N[a] |
| CA | Independence Creek | 39.43040 | −120.33096 | Y[L] | Y |
| CA | Independence Creek | 39.42849 | −120.33340 | Y[L] | Y |
| CA | Independence Creek | 39.42594 | −120.33576 | Y[L] | Y |
| CA | Independence Creek | 39.42434 | −120.33876 | Y[L] | Y |
| CA | Silver King Creek | 38.49895 | −119.60510 | Y[P] | Y |
| CA | Silver King Creek | 38.49895 | −119.60510 | Y[P] | Y |
| MT | Spring Gulch | 46.93244 | −113.95928 | N | N[a] |
| NV | Wildcat Creek | 41.63961 | −115.24567 | Y[L] | Y |
| WY | Bridge Creek | 44.52945 | −110.43810 | N | N[b] |
| WY | Clear Creek | 44.47393 | −110.27967 | N | N[b] |
| WY | Little Thumb Creek | 44.43687 | −110.58102 | N | N[b] |

[a]Rainbow trout DNA was detected in these samples using the rainbow trout eDNA assay described in Wilcox et al. (2015).
[b]Abundant Yellowstone cutthroat trout were observed upstream of these sample sites, and high levels of DNA were detected using the Yellowstone cutthroat trout eDNA assay described in Wilcox et al. (2015).

qPCR inhibitors that could reduce sensitivity of the assay. An environmental sample was considered inhibited if there was a >1 Ct shift in the IPC relative to the curve from the no-template control. All qPCR experiments included a qPCR-positive control containing 0.4 ng tissue-derived Lahontan cutthroat trout DNA and a negative control consisting of PCR-grade water in place of template. In addition, all experiments were set up in a hood in which all consumables, pipettes, and tube racks were previously exposed to UV light for at least 1 h.

Finally, we evaluated the ability of the assay to distinguish target DNA in the presence of high concentrations of Yellowstone cutthroat trout DNA. The Yellowstone cutthroat trout is a closely related taxon that has been introduced into the range of, and has been mistaken for, Lahontan cutthroat trout (Pritchard et al. 2015). We diluted extracted Lahontan cutthroat trout DNA into Yellowstone cutthroat trout DNA (starting concentration = 0.1 ng/μL for both species) at ratios of 1:10, 1:100, and 1:1000 and compared these to Lahontan cutthroat trout DNA diluted at the same ratios in TE buffer. Each of these dilutions was analyzed in triplicate using the optimized assay concentrations and the same qPCR procedure described above.

## Results and Discussion

The developed assay was both sensitive and specific to the 2 target species. The standard curve analyses demonstrated a reaction efficiency of 95.58% ($R^2$ = 0.989, *y*-intercept = 39.64, slope = −3.43) and 101.21% ($R^2$ = 0.989, *y*-intercept = 38.90, slope = −3.29) when using Lahontan cutthroat trout DNA and Paiute cutthroat trout DNA, respectively. The limit of detection for both taxa, as measured by the lowest concentration with >95% amplification success (Bustin et al. 2009), was 10 copies per reaction. However, DNA was detected in 4 of 6 replicates at 2 copies per reaction, which is nearly equivalent to the expected number of replicates (5.16) that would contain any copies of DNA, so the realized sensitivity may be greater.

Specificity of the assay was confirmed in vitro; we detected DNA in all samples extracted from Lahontan and Paiute cutthroat trout and did not detect DNA from any non-target species samples, including Yellowstone cutthroat trout and other closely related taxa, i.e., Bonneville cutthroat trout (*O. r. utah*), coastal cutthroat trout (*O. r. clarkii*), Colorado River cutthroat trout (*O. r. pleuriticus*), rainbow trout, and westslope cutthroat trout (*O. r. lewisi*; Table 3). We developed this assay using individuals from populations across the range of these taxa, including representatives from 4 of the 5 extant evolutionary units representing the Lahontan cutthroat trout species complex (which includes Paiute cutthroat trout; Peacock et al. 2018); we therefore believe that this assay will perform across the entire range of both target species.

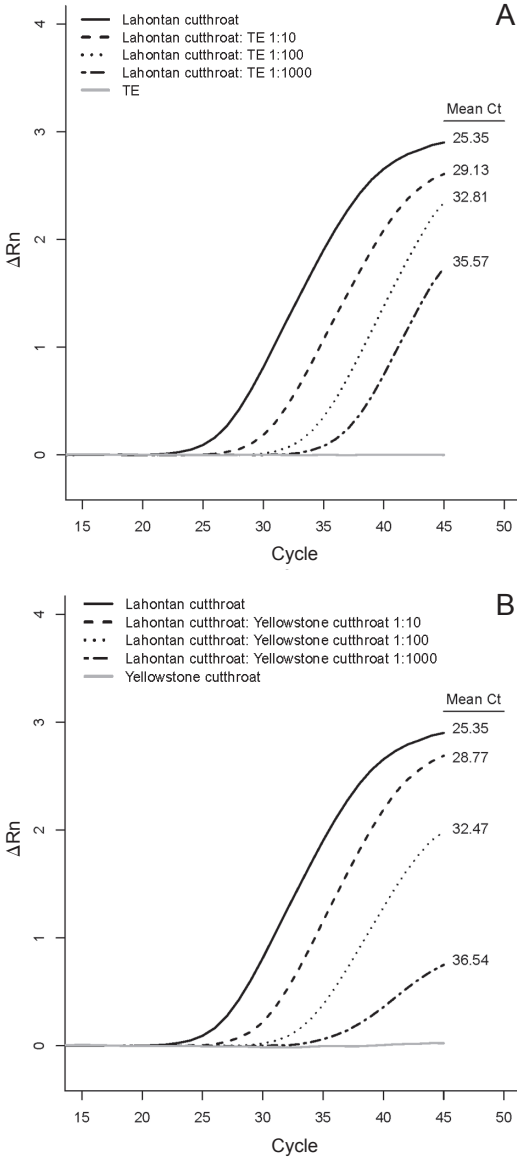


Fig. 2. Assessment of primer competition for the Lahontan/Paiute cutthroat trout assay when Lahontan cutthroat trout DNA is in the presence of a high concentration of DNA from closely related Yellowstone cutthroat trout. ***A***, Amplification curves of the assay when Lahontan cutthroat trout is diluted into sterile TE at ratios of 1:1, 1:10, 1:100, and 1:1000. ***B***, Amplification curves of the assay when Lahontan cutthroat trout is diluted into Yellowstone cutthroat trout DNA solution at ratios of 1:1, 1:10, 1:100, and 1:1000.

Analyses with the assay successfully detected Lahontan and Paiute cutthroat trout DNA in all environmental samples collected at locations where these taxa had been recently observed, but not in any of the samples collected where they were not expected to occur (Table 4). Although Lahontan cutthroat trout and Paiute cutthroat trout tend to be found at low densities, these are the conditions under which eDNA sampling has excelled. For example, Wilcox et al. (2016) estimated a detection efficiency of 84% for single eDNA samples from streams containing populations of stream-dwelling trout averaging 10 fish/km. This sensitivity will be especially useful for delineating the headwater extent of many populations of Lahontan cutthroat trout that might otherwise be approximated based on modeling elevation and geographic position (Warren et al. 2014).

The eDNA assay focused on a 160-bp fragment of the CO3 gene in Lahontan and Paiute cutthroat trout. Primer-BLAST indicated that an occasionally sympatric nontarget species (Yellowstone cutthroat trout) could amplify with the primers, though likely with reduced sensitivity due to a mismatch on the 3′ end of each primer. Amplification of the primers will not lead to false positive detections and would only be confounding in cases where DNA from large numbers of Yellowstone cutthroat trout would swamp DNA from less common target species (primer competition), resulting in failure to detect Lahontan or Paiute cutthroat trout (Wilcox et al. 2013). We subsequently tested primer competition and found that there was no effect on target DNA detection when the nontarget Yellowstone cutthroat DNA was 10 or 100 times more abundant than target DNA; however, there was a 1-Ct delay in Lahontan cutthroat trout DNA when Yellowstone cutthroat DNA was 1000 times more abundant (Fig. 2). There was also some diminished endpoint fluorescence at this dilution, but target detection was still distinguishable. The high specificity of this assay coupled with its resistance to primer competition even when Yellowstone cutthroat trout are more than 100× as common as the target species will be advantageous given that rainbow trout and Yellowstone cutthroat trout have been widely introduced throughout the range of Lahontan cutthroat trout (Pritchard et al. 2015). The ability of this assay to detect the target species in environments containing large populations of introduced salmonids, coupled with parallel sample analyses using assays developed for these

introduced salmonids (Wilcox et al. 2015, 2020), could identify areas of sympatry and likely hybridization (Pritchard et al. 2015); these assays are already being used to evaluate the results of chemical treatments to remove nonnative rainbow trout in preparation for Lahontan and Paiute cutthroat trout restoration (USFWS 2013; J. Stoller, Nevada Department of Wildlife, and W. Somer, California Department of Fish and Wildlife, personal communication).

Ongoing declines of Lahontan cutthroat trout, high temporal fluctuations in abundance, and uncertainty about the persistence of some populations (USFWS 2009, Peacock et al. 2018, Neville et al. 2020) demonstrate the need for continued monitoring and assessment of this taxon. Such actions will also be critical to evaluate attempts to restore Paiute cutthroat trout to their historical range (USFWS 2013). In those regards, this eDNA assay coupled with rigorous eDNA sampling protocols (Wilcox et al. 2018) can assist biologists attempting to precisely and efficiently describe the distribution of each of these taxa as part of inventory and monitoring efforts.

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