Conservation Genetics Resources
https://doi.org/10.1007/s12686-024-01375-1

METHODS AND RESOURCES

From jaguars to pathogens: simultaneous genetic detection of diverse taxa across biodiversity hotspots in the U.S. arid southwest

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Received: 31 May 2024 / Accepted: 20 November 2024
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Abstract
The management of biodiversity on arid landscapes can be challenging: Arid landscapes are often highly vulnerable to environmental and land-use changes. Moreover, these landscapes are home to rare and endemic species which are difficult to monitor. Environmental DNA sampling—the inference of species presence from genetic material in the environment—using high-throughput quantitative PCR (HT-qPCR) panels may be an efficient, cost-effective monitoring tool when multiple, taxonomically diverse taxa are of conservation concern. We validated and applied a HT-qPCR panel to detect ten native, invasive, and pathogenic taxa of relevance to conservation in the arid southwestern United States. We found that the sensitivity and specificity of our HT-qPCR platform was similar to that of single-taxon qPCR (ST-qPCR) (concordance on environmental samples 0.900–1.000, n = 315 samples) and that landscape patterns of detection were largely concordant with previous, conventional sampling. For example, our data reconstructed previously documented ecological patterns: Amphibian pathogens *Batrachochytrium dendrobatidis* (*Bd*) and *Ranavirus* were rare in samples without detection of invasive American bullfrog (*Rana catesbeianus*; 1.9% and 1.2% respectively), but common where bullfrogs were detected (29.1% and 21.8% of samples). Data generation with HT-qPCR required substantially less time and reagents than would be required using ST-qPCR alone.

Keywords Environmental DNA · eDNA · Biodiversity · HT-qPCR · Freshwater

Introduction

Arid landscapes present unique challenges for the persistence of life. Within these harsh environments, small aquatic habitats such as springs, seeps, and streams represent crucial refuges for biodiversity, hosting a diverse array of aquatic organisms (Davis et al. 2017; Fensham et al. 2023; Fernández-Martínez et al. 2024; Rossini et al. 2018). Despite their small size, these aquatic habitats may play a large role in the ecological connectivity of arid landscapes. Acting as essential corridors, they facilitate the movement and dispersal of species across vast expanses of inhospitable terrain (Sheldon et al. 2010). Not only are these habitats occupied by aquatic (and often highly endemic [Stevens and Meretsky 2008]) taxa, but they support outsized diversity of riparian-associated terrestrial species (Shepard 1993) and attract occasional use of many additional upland terrestrial species as thermal refugia and sources of drinking water (Tockner et al. 2021). The ecological significance of these aquatic habitats is increasingly threatened by anthropogenic activities and climate change (Zhang et al. 2023). As human populations continue to expand and natural habitats are increasingly fragmented and degraded, the resilience of arid ecosystems and the species they support are put at risk (Bogan et al. 2014). For example, in a survey of 34 Endangered Species Act listed or candidate species found on southwestern Unites States Department of Defense lands, Tizak and Martin (2002) found that 100% have been impacted by habitat loss and degradation and 49% by hydrological alterations. In the preceding century, a notable decline in biodiversity has been observed across arid ecosystems (Zhang et al. 2023). Consequently, there is an urgent need for effective monitoring and subsequent conservation strategies to safeguard the biodiversity of these unique environments.

Traditional methods of monitoring populations in arid landscapes (i.e. track-based surveys, camera-traps, visual observations, netting, and auditory surveys) are often constrained by logistical challenges and resource limitations

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Published online: 19 December 2024

(Southwell et al. 2023). Conducting surveys in these environments can be labor-intensive and time-consuming, particularly given the low population densities, cryptic behaviors, and vast spatial scales involved (Dickman et al. 2018). Because of this, aquatic habitats within arid landscapes may be a particularly efficient place for biological monitoring using environmental DNA (eDNA) sampling, which can potentially detect a wide range of aquatic and terrestrial taxa from shared water samples. Environmental DNA sampling has been most widely applied for the detection of aquatic animals and their pathogens (e.g., Huver et al. 2015; McKelvey et al. 2016; Rees et al. 2014), but recent studies have also demonstrated the potential to detect terrestrial visitors to these aquatic habitats (Farrell et al. 2022; Lyet et al. 2021; Schenekar et al. 2024; Wilcox et al. 2021). The ability to detect a diverse array of taxa spanning various taxonomic groups enables the determination of species composition and diversity within a given ecosystem. Moreover, it provides an avenue to infer species interactions such as competition, predation, or pathogen transmission dynamics (Congram et al. 2022; Hossack et al. 2023; Wilcox et al. 2018).

The complex nature of eDNA samples, which may contain DNA from a diverse array of species across a wide taxonomic breadth, presents unique analytical challenges. Environmental DNA analysis pipelines tend to fall into two categories: Targeted detection and high-throughput, non-targeted assessment. Targeted detection employs techniques such as quantitative real-time PCR (qPCR) which are ideal for detecting specific taxa with high sensitivity (Klymus et al. 2020; Wilcox et al. 2020). However, qPCR analysis is constrained by taxonomic resolution; only one to a few taxa may be analyzed at a time, which makes analysis of multiple target species expensive and time-consuming. The second category is high-throughput, non-targeted. High-throughput, non-targeted assessments based on sequencing include shotgun sequencing, capture enrichment, and amplicon sequencing. The most common of these approaches is sequencing amplicons generated with conserved primers, known as metabarcoding (Deiner et al. 2017). Although targeted amplicon sequencing can be highly sensitive (McCarthy et al. 2023), metabarcoding across taxa tends to be less sensitive and more susceptible to errors (both non-detection and false positive detection) than targeted approaches (Harper et al. 2018; Wood et al. 2019).

High-throughput qPCR (HT-qPCR) offers a promising intermediate method for targeted, multi-species detection. In HT-qPCR, the species-specific qPCR assays and samples are robotically loaded into a high-density chip containing thousands of individual reactions (i.e., species by sample site combinations). The resulting data are directly comparable to ST-qPCR but with significantly reduced reagent use, 90% less DNA per taxon, and less technician time, leading to an estimated 40% cost reduction compared to ST-qPCR (Elmore et al. 2024). Reaction volumes on the HT-qPCR platform are small (< 0.1 µl), typically necessitating a multiplex PCR pre-amplification to enrich target loci. However, this pre-amplification step carries potential risks, such as increased contamination and primer-primer interactions, which may reduce sensitivity (Wilcox et al. 2020). While optimizing the pre-amplification step can be time-intensive, once completed, HT-qPCR data can produce detection profiles that are nearly identical to those of ST-qPCR when applied to environmental samples (e.g., Bernardi et al. 2021; Van der Pouw Kraan et al. 2023; Elmore et al. 2024).Unlike metabarcoding, HT-qPCR is limited to a predefined list of species, making it most suitable for scenarios with a well-defined set of priority species where highly accurate presence/absence inference is required.

Here, we validated a HT-qPCR panel which screens for a wide range of taxa—aquatic endemics, wetland-dependent terrestrials, pathogens, and invasives. This HT-qPCR panel holds particular relevance to ongoing conservation efforts in the southwestern United States. Despite ongoing decision-making processes in this region, there remains a lack of knowledge regarding both endemic biodiversity and invasive and pathogenic threats. This HT-qPCR chip may be used to provide valuable insights into ecosystem dynamics, species interactions, and disease transmission dynamics crucial for creating effective conservation strategies in these unique and imperiled desert ecosystems.

Materials and methods

Focal taxa selection

This work was funded by the Department of Defense Strategic Environmental Research and Development Program (SERDP) funding scheme (RC18-1348). One of the tasks in this contract was to identify priority taxa of high conservation concern on and surrounding Department of Defense managed landscapes in the arid southwestern United States. In consultation with Department of Defense, USDA Forest Service, and the US Fish and Wildlife Service as well as state, academic, and non-profit wildlife professionals working in the region. We specifically focused on elusive, data-depauperate native species that are either known to occur or suspected to occur on Department of Defense lands, as well as invasive and pathogenic species that pose significant threats to native biodiversity. We identified four native taxa: northern Mexican gartersnake (*Thamnophis eques megalops*), Chiricahua leopard frog (*Rana chiricahuensis*), jaguar (*Panthera onca*), and ocelot (*Leopardus pardalis*), three invasive taxa: American bullfrog (*Rana catesbeianus*), virile crayfish (*Faxonius virilis*), and New Zealand mudsnail (*Potamopyrgus antipodarum*), and three pathogens:

Batrachochytrium dendrobatidis, amphibian *Ranavirus*, and *Ophidiomyces ophiodiicola* (causative agent of snake fungal disease; Table 1) for inclusion on the HT-qPCR chip.

eDNA assay validation

We assembled taxon-specific, hydrolysis (i.e., Taqman) qPCR assays for each of the focal taxa. We revalidated previously published assays for Chiricahua leopard frog (Goldberg et al. 2017), northern Mexican gartersnake (Goldberg et al. 2014), snake fungal disease (Allender et al. 2015), and amphibian chytrid fungus (Boyle et al. 2004). We made minor modifications to the probe and reverse primer annealing loci for the amphibian *Ranavirus* assay (globally distributed double-stranded DNA viruses that infect cold-blooded vertebrates, often causing mass mortality in amphibians; Brunner 2004) to increase generality across genetic strains described since the original manuscript publication (see Appendix 1 for details). The assays for American bullfrog, virile crayfish, and New Zealand mudsnail are described in Kronenberger et al. (2024) virile crayfish Assay B used in this study) using the same structure as assay validations in this study. The assay for jaguar was previously described in Wilcox et al. (2021). The original assay for ocelot used on the HT-qPCR chip had poor performance (see Results), so we developed a second taxon-specific assay for ocelot and analyzed 17 samples from priority habitats in the Huachuca Mountains (Arizona) on ST-qPCR.

Each of these assays was assessed for sensitivity and specificity following the framework described in Kronenberger et al. (2024). Briefly, assays were designed based on alignments of sequences from target and non-target confamilial taxa collected from the GenBank nucleotide (nr) database (Sayers et al. 2019). These alignments were first assessed in silico using the eDNAssay tool (Kronenberger et al. 2022), a machine-learning model that predicts the probability of qPCR assay amplification. For northern Mexican gartersnake there were multiple non-target confamilial taxa without sequence data, so we assessed cross-amplification risk of these unsampled non-targets using the probability density function of eDNAssay assignment probabilities for confamilial taxa with sequence data as in Wilcox et al. (2024). As suggested in Kronenberger et al. (2022), except where co-occurrence was very unlikely, non-target templates with an eDNAssay assignment probability > 0.3 (i.e., < 99.9% probably of specificity; see Wilcox et al. 2024) were assessed in vitro with ST-qPCR. For all assays, the probes each contain a 3′-minor groove binding (MGB) moiety, which increases the effect probe Tm (Kutyavin et al. 2000). All assays were optimized for two-step thermocycling with a 60 °C annealing and extension step. Primer concentrations were optimized by comparing all possible combinations of 100, 300, 600, and 900 nM of the forward and reverse primers (n = 16) while holding the probe concentration at 250 nM in a ST-qPCR. The limit of detection (LOD; lowest eDNA concentration with a 95% amplification rate as in Bustin et al. 2009) was determined based on serial dilutions of a synthetic DNA fragment quantified on a fluorometer (Qubit; Life Technologies). We also tested the performance of each assay in situ by testing assays on environmental or other non-invasive samples. Full details of assay validation and performance are described in Appendix 1 in an Assay Description Document format, as described in Kronenberger et al. (2024).

ST-qPCR analysis

ST-qPCR reactions were run on StepOnePlus™ and QuantStudio™ 3 Real-Time PCR Systems (ThermoFisher Scientific) in triplicate. Each well contained 7.5 µL of TaqMan® Environmental Master Mix 2.0 (2×; ThermoFisher Scientific), 4 µL of DNA, and 0.75 µL of assay mix (20×), and sterile water to 15 µL total. The thermal cycling profile was 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Environmental and non-invasive samples were only handled in dedicated laboratory facilities where no high-quality DNA or PCR products are handled. Each plate contained triplicate positive and negative controls for each assay in accordance with the National Genomics Center for Wildlife and Fish Conservation (NGC) eDNA Program Standard Operating Procedure (SOP; e.g., Carim et al. 2016; Vanderpool et al. 2024; Wilcox et al. 2020). Environmental samples on their first ST-qPCR analysis were analyzed in conjunction with an internal positive control (Exogenous Internal Positive Control Kit; ThermoFisher Scientific) to test for the presence of PCR inhibitors. Any samples with inhibition (> 1 Ct shift in the IPC relative to the positive control samples) were cleaned (see extraction protocol below) and re-analyzed.

Generally, we considered any linear amplification in at least one PCR replicate to indicate a positive detection on the ST-qPCR platform. Occasionally, samples with single-well amplifications that are adjacent to a positive control or other strongly positive wells were re-analyzed in accordance with NGC eDNA Program (SOP). In these cases, if subsequent analyses did not result in amplification, qPCR results were scored as negative.

HT-qPCR chip protocol

The HT-qPCR analysis protocol mirrored that described in Elmore et al. (2024). Briefly, each sample first underwent a pre-amplification step composed of 22.5 µL TaqMan Environmental Master Mix 2.0 (2×; ThermoFisher Scientific), 0.4 nM each species-specific assay primer, and internal control assay primers (see below), 12 µL template, 1 µL IPC

Table 1 Focal taxa for environmental DNA assay design and validation based on conversations with regional resource managers

Focal taxon		Oligos	Conc (nM)	LOD	Target gene & Amplicon length	Special considerations
Destructive pathogens						
Snake fungal disease	F	5′-TGTTTCTGTCTCGCT CGAAGAC-3′	900	2	Internal transcribed spacer 1 (*ITS1*) (67 BP)	None
Ophiomyces ophiodiicola	R	5′-AGGTCAAACCGGAAA GAATGG-3′	600			
Allender et al. (2015)	P	5′-FAM-CGATCGGCG CCCGTCGTC-MGB-NFQ-3′	250			
Ranavirus	F	5′-ACACCACCGCCCAAA AGTAC-3′	300	10	Major capsid protein (*MCP*) gene (76 BP)	Potential cross-amplification with some fish viruses
Iridoviridae	R	5′- ATCGAGCCGTTCATG ATGC/CATCGAACC GTTCATGATGC -3′	900			
Brunner et al. (2004)*	P	5′- FAM- CCATCAACC ACAACATTA -MGB -NFQ-3′	250			
Chytrid fungus	F	5′- CTTGATATAATACAG TGTGCCATATGT -3′	900	10	Internal transcribed spacer 1 (*ITS1*) and 5.8S (145 BP)	Potential reduced sensitivity for one, rare lineage
Batrachochytrium dendrobatidis	R	5′-AGCCAAGAGATCCGT TGTCAAA-3′	900			
Boyle et al. (2004)*	P	5′-FAM-CGAGTCGAA CAAA-MGB-NFQ-3′	250			
Invasive species						
New Zealand mudsnail	F	5′-TGTTTCAAGTGTGCT GGTTTAYA-3′	600	10	Cytochrome *b* (*cytb*) (92 BP)	None
Potamopyrgus antipodarum	R	5′-CAAATGGRGCTAGTT GATTCTTT-3′	900			
Goldberg et al. (2013)	P	5′-FAM-CCTCGACCAATA TGTAAAT-MGB-NFQ-3′	250			
Virile crayfish	F	5′-CCGAGTAGAGTTAGG YCAGCCA-3′	900	25	Cytochrome *c* oxidase I (*COI*) (142 BP)	Potential cross-amplification with congenerics
Faxonius virilis	R	5′-AAGGAATTAACCAGT TCCCGAAA-3′	900			
Kronenberger et al. (2024)	P	5′-FAM-TCGTCCCCAATC AA/TCATCCCCAATT AATCT-MGB-NFQ-3′	250			
American bullfrog	F	5′-TTTTCACTTCATCCT CCCGTTT-3′	900	50	Cytochrome *b* (*cytb*) (83 BP)	Potential cross-amplification with eastern congenerics
Rana catesbeianus	R	5′-GGGTTGGATGAGCCA GTTTG-3′	900			
Strickler et al. (2015)	P	5′-FAM-TTATCGCAGCAG CAAGT-MGB-NFQ-3′	250			
Rare native species						
Ocelot	F	5′-GGGATACCTAATCTC CTACAACATT-3′	600	10	NADH-ubiquinone oxidoreductase chain 5 (*ND5*) (219 BP)	Potential lack of generality for southern sub-species
Leopardus pardalis	R	5′-TGGGAGGCGGTGTAT TACGG-3′	900			
This study	P	5′-FAM-CTCCAACCTATT GGGGTAC-MGB-NFQ-3′	250			

Table 1 (continued)

Focal taxon		Oligos	Conc (nM)	LOD	Target gene & Amplicon length	Special considerations
Jaguar	F	5′-AACAATCGTCTAATC TCACTCCAACAG-3′	900	10	Mitochondrially encoded ATP synthase membrane subunit 6 (*ATP6*) (141 BP)	None
Panthera onca	R	5′-CCAACAGGTTTGTTG ATCCAATG-3′	900			
Wilcox et al. (2020)	P	5′-FAM-CTTGGGCTCTAA TACTC-MGB-NFQ-3′	250			
Chiricahua leopard frog	F	5′-GGTACCGCTCATATC ATGACTACTTG-3′	900	50	Control region, D-loop (83 BP)	Potential cross-amplification with northern leopard frog
Rana chiricahuensis	R	5′-TCCAGTTGGACTCAC TTAGGAATG-3′	900			
Goldberg et al. (2017)	P	5′-FAM-TAGGACCTTCGC TTGTTAT-MGB-NFQ-3′	250			
Northern Mexican garter-snake	F	5′-CGAAAAGGCCCTAAC CTGG-3′	900	2	NADH dehydrogenase 1 (*ND1*) (133 BP)	Potential cross-amplification with congenerics
Thamnophis eques megalops	R	5′-CTATGATTGGTGAYA GGGTGAACAGT-3′	900			
Goldberg et al. (2014)	P	5′-FAM-AATAGGCCT TCTACAGCCTA-MGB-NFQ-3′	250			

We selected ten focal taxa which are rare native (n = 4), invasive (n = 3), or pathogenic (n = 3) species of high conservation concern in the southwestern United States for design and validation of taxon-specific qPCR assays. For each assay we list the species name, oligonucleotide sequences (F: forward primer, R: reverse primer, P: hydrolysis probe; where multiple oligos are used, both are listed), optimized oligonucleotide concentration, limit of detection (LOD; copies/reaction), target gene/amplicon length (given respectively), and special considerations for application of the assay in some regions. An asterisk indicates two assays (ranavirus and chytrid fungus) which were previously described in the literature and modified to improve generality as part of this study. Details for assay validation are described in Appendix 1

template (approximately 10 copies/µL), and molecular-grade water to a total reaction volume of 45 µL. The thermocycling conditions were 95 °C 10 min, (95 °C 15 s, 60 °C 3 min) × 12 cycles, then held at 12 °C. To simultaneously monitor for contamination between samples during PCR setup and PCR inhibition, we alternatingly tiled an internal positive control (IPC) with 'A' and 'B' variants, as described in Elmore et al. (2024). Amplification of assay 'A' in a 'B' well, or vice versa, indicates a possible cross-well contamination event. If the assigned IPC variant fails to amplify, we considered the sample inhibited.

Following pre-amplification, each sample underwent an exonuclease cleaning by adding 4.5 µL ExoSAP-IT and incubating at 37 °C for 30 min and then 80 °C (deactivation step) for 15 min. Each exonuclease-cleaned, pre-amplified sample was arranged on a 384-well plate in quadruplicate for analyses following the SmartChip MyDesign Gene Expression protocol (60° extensions/annealing) for a 12 assay/384 sample format (10 taxon-specific assays and both IPC assays). The sample plate was loaded with Takara SmartChip Probe qPCR Master Mix and an assay plate was loaded with 5 × assays for each taxon and the two IPC assays, along with ROX normalization dye as described in the manufacturer's protocol. These plates were shipped frozen to the Iowa State University Veterinary Diagnostics Laboratory (Ames, Iowa, USA) for robotic loading into an aluminum HT-qPCR chip, amplification, and visualization. Setup for pre-amplification occurred in the same dedicated space as for ST-qPCR analysis. Exonuclease clean setup and plating for HT-qPCR analysis occurred in a different dedicated UV hood which was irradiated for one hour prior to each use. When analyzing environmental samples, we included eight no-template control wells in each HT-qPCR analysis. Each no-template control was analyzed in quadruplicate resulting in 320 replicates tested against ten taxon-specific assays on each chip and 1,600 control analyses total over the course of the study.

For each sample, we visually inspected the PCR results for amplification of each assay using WaferGen qPCR Software (version 2.8.33.0, Wafergen Biosystems, Inc.). We scored any replicate positive when there was amplification with a linear phase at any point before 50 PCR cycles. As in Elmore et al. (2024), we required amplification in at least two replicates for a given assay to call the sample "positive."

HT-qPCR chip validation

Primer-primer interactions during preamplification can negatively impact sensitivity on the HT-qPCR platform. We screened for potential primer-primer interactions in the pre-amplification reaction in silico using MFEprimer-3.0 (Wang et al. 2019). Where there were mixed bases in an oligo, we considered both possible sequence versions and considered all possible primer-primer interactions between all assays (n = 12). The lowest predicted dimer had a Δ-G = − 5.29 kcal/mol, which is much greater than the maximum value we've observed impacting pre-amplification efficiency in previous tests (data not shown). We further tested sensitivity by including eight replicate pre-amplifications of a known-composition mock community sample composed of a mix of all the target taxa (10 copies from each taxon per 4 µL template; 320 replicates across five chips; mock community samples built from synthetic genes; Integrated DNA Technologies).

HT-qPCR concordance testing

The primary test of platform sensitivity and specificity was an assessment of concordance with ST-qPCR on the same samples. Given several of our focal taxa are extremely rare, we selected eDNA samples with pre-existing ST-qPCR data to enrich for positive detections that might otherwise be rare or absent from unknown-status locations sampled as part of this project. Some of the samples were sourced from the Aquatic eDNAtlas Project (Young et al. 2018); a database containing thousands of samples previously analyzed using ST-qPCR with the same species-specific assays employed on the HT-qPCR chip. Overall, we selected 315 samples. Of these, 279 samples were new, unknown-status samples collected as part of this project and 36 samples were included from the eDNAtlas archives. All 315 samples were analyzed on the HT-qPCR platform, with samples divided across five chips. There was a contamination event for Chiricahua leopard frog on the third chip (see Results and Discussion). To confirm that this contamination issue was fully resolved, four samples were analyzed a second time on a subsequent chip.

Of the new, unknown-status samples collected as part of this project, 196 were analyzed on ST-qPCR for American bullfrog, primarily prior to HT-qPCR analysis. Other ST-qPCR data were produced because samples were specifically selected from the eDNAtlas to include relevant data or ST-qPCRwas conducted after HT-qPCR analysis to (1) investigate specific HT-qPCR detections that were unexpected and (2) increase the sample size for concordance assessment, with a focus on balancing the confusion matrix to maximize power to detect concordance issues. This sampling design may somewhat bias concordance estimates downward relative to a random sample (i.e., estimates may be conservative because low probability HT-qPCR amplifications were more likely to be re-assessed with ST-qPCR).

Based on these paired data, we calculated several concordance metrics to assess HT-qPCR performance relative to ST-qPCR: (1) Concordance (i.e., raw concordance), being the proportion of results in agreement between the two methods, (2) recall, being the proportion of ST-qPCR detections also detected on HT-qPCR, and (3) precision, being the proportion of ST-qPCR non-detections that were also non-detections on HT-qPCR. The implicit assumption in the use of the terms "recall" and "precision" is that ST-qPCR represents "truth" against which HT-qPCR performance is being evaluated. Although false negative and false positive errors do occur with ST-qPCR, this method represents the highest available sensitivity and specificity platform for eDNA analysis (e.g., Harper et al. 2018) and so is an appropriate benchmark against which to compare other laboratory methods.

Where available, we compared our eDNA sampling data with previous information on species distributions including the Nonindigenous Aquatic Species (NAS) database (https://nas.er.usgs.gov), peer-reviewed literature, agency reports, and direct consultation with installation and other agency partners. In some cases, specific location information for sampling or known populations represents a security or sensitive species risk and so that information will not be reported to the Aquatic eDNAtlas. In most cases, comparisons between eDNA and conventional sampling (i.e., visual, netting, and auditory survey data) were made without statistical analysis due to the lack of a standardized conventional sampling protocol for comparison. However, an exception exists with paired conventional and eDNA sampling at pond habitats on Ladder Ranch (New Mexico). On Ladder Ranch, Samaniego (2020) conducted eDNA sampling alongside visual, auditory, and dipnet surveys conducted in 2019, totaling twelve surveys per site, encompassing both day and night surveys across 13 habitats. One eDNA sample was collected per site each year 2019 and 2020. We conducted a comparison of naïve occupancy estimates derived from conventional sampling, ST-qPCR, and HT-qPCR to assess detection concordance for American bullfrog and Chiricahua leopard frog across various sites on Ladder ranch annually.

Field sampling and eDNA extraction

This study aimed to pilot eDNA sampling-based monitoring of arid landscapes surrounding military installations in the southwestern United States. Sampling sites were determined through aquatic habitat inventories and consultation with the local resource managers. Focused efforts were conducted in five priority landscapes: White Sands Missile Range, Fallon Naval Air Station, Nellis Air Force Base, Camp Navajo,

and Fort Huachuca. Additional samples were collected from Ladder Ranch (New Mexico) in conjunction with amphibian surveys in 2019 and 2020 by the University of New Mexico. At White Sands Missile Range, 87 samples were collected primarily from identified habitats based on a previous inventory. Fallon NAS yielded 60 samples from sites selected based on Dixie Valley toad survey information and other inventories. Nellis AFB provided 13 samples from escorted sampling of spring habitats and four environmental samples from adjacent lands. Camp Navajo contributed 25 samples collected over two visits guided by the local staff. Fort Huachuca provided 62 samples, selected using various databases and plans, with additional sampling from surrounding areas (Fig. 1).

All samples were collected using the protocol described in Carim et al. (2016). Briefly, an electronic peristaltic pump was used to draw 5 L of water through a 1.5-micron pore size glass microfiber filter in the field. If the filter clogged prior to reaching 5 L of water, up to three filters were used per sample and pooled for extraction. Samples were preserved in silica desiccant and then frozen within two weeks of collection and mailed to the NGC. Sampling supplies were prepared at NGC in Missoula, Montana (USA) in a dedicated laboratory facility and provided to partners individually bagged to avoid contamination. All environmental samples underwent DNA extraction using a QIAGEN DNeasy protocol as described in Franklin et al. (2019). Extractions were conducted in a dedicated, restricted-access laboratory space where only environmental samples are handled. Any sample with detection of PCR inhibitors was cleaned with an inhibitor removal column (One-Step Inhibitor Removal kit; Zymo; McKee et al. 2015).

Results and discussion

eDNA assay validation

Generally, the revalidated assays demonstrated broad applicability to the genetic diversity of the focal taxon while maintaining specificity against non-target taxa within the study region. However, in some cases, assay generality or

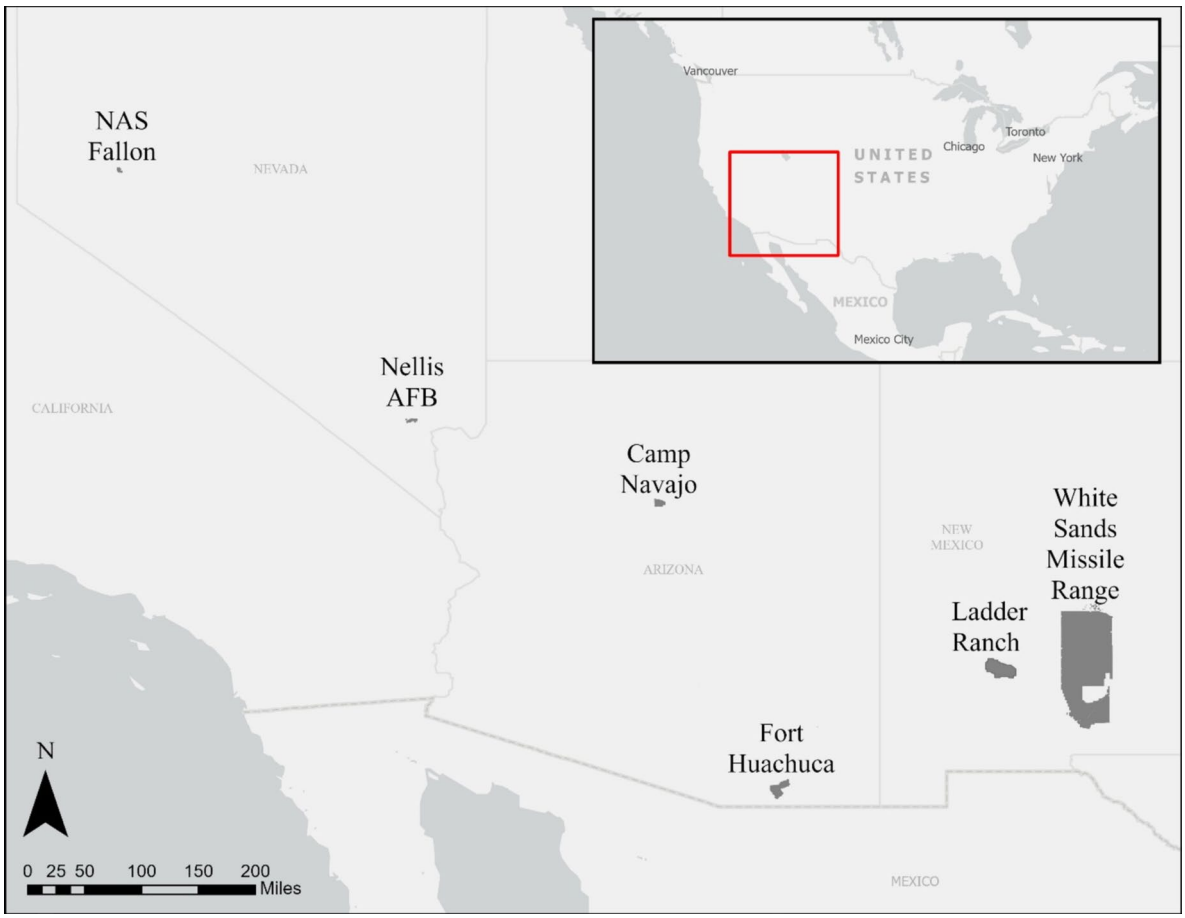


Fig. 1 The five priority landscapes within our study: White Sands Missile Range, Fallon Naval Air Station, Nellis Air Force Base, Camp Navajo, and Fort Huachuca—along with additional sampling site, Ladder Ranch

specificity have special considerations for application where certain closely related, non-target taxa or lineages of the target taxon may occur (Table 1). However, as applied in this study, the interpretation of these assays is simple. For example, although assays for Chiricahua leopard frog and northern Mexican gartersnake have the potential for cross-amplification with certain congenerics (e.g., Goldberg et al. 2014, 2017) which occur in portions of the southwestern United States, all detections in this study occurred at sites with only known focal species occurrence. Further, our Chiricahua leopard frog detections occurred outside of the distribution of northern leopard frog, which could result in cross-amplification (gbif.org). Although less information is available on congeneric distributions for Northern Mexican gartersnake, all detections were associated with the presence of a known individual. We encourage future researchers applying these assays to new regions to consider the scope of applicability for each specific assay (Appendix 1).

HT-qPCR chip validation

Although our first HT-qPCR chip had good performance on environmental samples, amplification rates for the IPC assays and positive control samples were low (13.3% and 9.4% respectively). We increased sample PCR reagent mix volumes from 7 to 9.4 µL for subsequent chips, which improved performance. For chips 2–5, the IPC was positive in 98.18% of samples, of which 95.23% had amplification in all four replicate wells. For chips 2–5, the positive control amplification rate was 99.22%. On the third chip, there was contamination for Chiricahua leopard frog (31.25% of no-template control wells) and so data for this assay/chip combination were excluded. For the remaining 352 no-template controls/assay combinations, there were single-well amplifications in 12 controls, but zero no-template controls met the > 1 replicate threshold used to indicate detection for an environmental sample. Overall, 99.15% of no-template control wells had no amplification. In addition, four samples were analyzed twice on two different HT-qPCR chips. This was done to confirm Chiricahua assay performance under normal circumstances after contamination was detected on the third chip. On this re-run, all four samples were negative for Chiricahua leopard frog (as expected and concordant with ST-qPCR), but all other assays had identical results to the first analysis.

HT-qPCR concordance testing

Overall, there were 323 HT-qPCR/ST-qPCR paired analyses. There were no ST-qPCR data generated for snake fungal disease because there were no potential detections on HT-qPCR (see *Snake Fungal Disease*). Estimating recall is impossible if there are no detections and estimates of concordance and precision will be biased high if all samples are truly negative. The number of HT-qPCR/ST-qPCR comparisons ranged 6–211 (mean = 40) per assay for the other eight assays. Concordance between platforms on environmental samples ranged from 0.900 to 1.000 (mean = 0.918; Table 2), obtaining similar levels of concordance to those reported in Wilcox et al. (2020; n = 5 assays, concordance range = 0.917–1.000, mean concordance = 0.958) and Elmore et al. (2024; n = 10 assays, concordance range = 0.833–0.965 mean concordance = 0.919).

In most cases where historical sampling data were available, HT-qPCR detections occurred only at habitats where the species was previously known to occur. There were no detections of jaguar, ocelot (ST-qPCR only; see Appendix 1), or snake fungal disease, which is consistent with the rarity or likely absence of these taxa on the landscape. Both jaguar and ocelot are only represented by occasional individuals in southern Arizona (Culver et al. 2016, US Fish and Wildlife Service 2016) and although eDNA sampling has been demonstrated for rare carnivores (e.g., Wilcox et al. 2021; Franklin et al. 2019), sampling of drinking water also may miss many taxa, particularly those that are rare and only visit water sources briefly (e.g., Schenekar et al. 2024). Snake fungal disease has not yet been widely documented in snake populations of western North America (Lorch et al. 2016) and the sensitivity of detection from environmental samples is still unclear and may be low (Baker et al. 2020).

All New Zealand mudsnail, virile crayfish, and northern Mexican gartersnake detections on the HT-qPCR chip, which were also analyzed on ST-qPCR, were concordant, and all detections at sites with historical sampling data were consistent with known species occurrence. The only New Zealand mudsnail detections on the HT-qPCR platform were samples from the eDNAtlas with known species presence in Idaho (Snake and Bear Rivers) and Montana (Bitterroot River) based on previous ST-qPCR data. Of the 26 virile crayfish detections, 20 could be related to known species occurrence (NAS Database, Montana Natural Heritage Program, Egly and Larson 2018, Jeff Sorensen *personal communication*). Other detections lacking conventional sampling data occurred on Camp Navajo (n = 5 samples across three habitats) and Ladder Ranch (n = 1 sample). Northern Mexican gartersnake was only detected in three samples collected downstream from a known individual in the Bill Williams River National Wildlife Refuge. Although northern Mexican gartersnakes are known to occur in the Huachuca region (US Fish and Wildlife Service 2021), we didn't expect detections at any of the sites where we sampled. Additionally, most sampling on the Huachuca landscape was conducted in the months of February and October (2019) when temperatures may have been low for snake activity to occur.

Table 2 Comparison of ST-qPCR qPCR and HT-qPCR detection results on environmental samples for each assay on the HT-qPCR chip

	Concordance (n)	Recall (n)	Precision (n)
American bullfrog	0.933 (211)	1.000 (33)	0.921 (178)
Chiricahua leopard frog*	0.833 (6)	1.000 (1)	0.800 (5)
Northern Mexican gartersnake	0.917 (12)	0.750 (4)	1.000 (8)
New Zealand mudsnail	0.947 (19)	0.889 (9)	1.000 (10)
Ranavirus	0.909 (22)	1.000 (10)	0.833 (12)
Virile crayfish	1.000 (20)	1.000 (10)	1.000 (10)
Chytrid fungus	0.905 (21)	0.778 (9)	1.000 (12)
Jaguar	0.900 (10)	1.000 (5)	0.800 (5)
Snake fungal disease	–	–	–
Ocelot	–	–	–
Mean	0.918 (40)	0.927 (10)	0.919 (30)
Median	0.913 (20)	1.000 (9)	0.960 (10)

Columns indicate number of comparisons, concordance (total proportion matching amplification/non-amplification across platforms), recall (proportion ST-qPCR amplifications also amplifying on HT-qPCR), and precision (proportion ST-qPCR non-amplifications also not amplifying on HT-qPCR). Sample sizes for each comparison are in parentheses after each. No comparisons were available for snake fungal disease (*Ophidiomyces ophiodiicola*) and ocelot. Due to the poor performance of the original assay, the ocelot data from the HT-qPCR platform were disregarded. Subsequently, a revised ocelot assay was exclusively analyzed using ST-qPCR on samples from the Huachuca Mountains. Four samples underwent analysis twice using two different HT-qPCR chips. This was necessary due to a contamination event on the third chip for Chiricahua leopard frog. Our objective was to verify the assay's performance under standard conditions on the HT-qPCR platform. Upon reanalyzing the samples on the subsequent chip, all four yielded negative results for Chiricahua leopard frog reflected in the precision estimate (indicated by asterisk)

HT-qPCR discordance

There were some cases of discordance between HT-qPCR and known species occurrence for Chiricahua leopard frog and American bullfrog, but most of these cases were traced to a single contamination event. A laboratory error occurred with the third HT-qPCR chip in this project, leading to contamination of Chiricahua leopard frog and American bullfrog DNA across multiple samples on the chip. To confirm that subsequent chips produced reliable data for this assay, we re-ran four samples that had amplification for Chiricahua leopard frog initially but were expected to be negative based on the sampling location. All four samples were negative for Chiricahua leopard frog when re-analyzed. After excluding the third chip (n = 32 suspect amplifications) there were two detections for Chiricahua leopard frog on the HT-qPCR platform, which were both known-occupancy habitats (location data redacted to protect sensitive populations; Audrey Owens; *personal communication*). One of these two samples was negative on ST-qPCR. This sample showed high levels of PCR inhibitors, even after an inhibitor clean-up. Previous tests of the HT-qPCR platform suggest that the protocol is more resistant to inhibitors than ST-qPCR (Elmore et al. 2024), which might explain why this detection was missed on the ST-qPCR platform.

For American bullfrog, there were 14 HT-qPCR detections not concordant with ST-qPCR or historic species distributions. Of the 14 discordant samples, only one exhibited inhibition, indicating a minimal probability that inhibition played a causal role in the observed discordance. However, the third HT-qPCR chip in this project had a laboratory error that appears to also have resulted in contamination for American bullfrog affecting 10 out of the 14 discordant HT-qPCR detections. Three samples from Fort Huachuca and one sample from Nellis AFB were positive on HT-qPCR, but all samples on these two military lands were negative on ST-qPCR, so these likely represent errors possibly resulting from contamination during the HT-qPCR process, e.g., from handling highly-concentrated pre-amplification products. Other detections of American bullfrog from the focal study areas were concordant with previous data on the species including USGS NAS records on lands near Fort Huachuca (Parker Canyon Reservoir, Canelo Hills, and the San Pedro River) and Fallon NAS (Forrest et al. 2023, ManTech International Corporation 2019).

Conventional sampling concordance

The Ladder Ranch system provided an opportunity to compare eDNA sampling results on the HT-qPCR and single-species platforms with conventional sampling for amphibians. High throughput-qPCR and conventional surveys were 85.2% concordant for American bullfrog (n = 26) and 76.2% concordant for Chiricahua leopard frog (n = 26), but all HT-qPCR data for this comparison were associated with the third chip with known contamination issues. ST-qPCR

and conventional surveys were 84.6% concordant for American bullfrog (n = 26) and 100% concordant for Chiricahua leopard frog (n = 10). In consultation with local partners, we identified some additional cases where eDNA sampling on both platforms failed to detect a species that is known or suspected in the area (American bullfrog, Chiricahua leopard frog, and *Bd*). We did not attempt to quantify levels of concordance outside of Ladder Ranch in part because this lack of detection could be related to fine-scale differences in sampling locations between methods (e.g., Franklin et al. 2018). There are additional considerations in complex wetland systems. First, as water bodies dry out in hot or dry seasons, biodiversity is often concentrated, potentially reducing the number of sites that need to be sampled. However, this also limits the number of available habitats, and there is a risk that researchers may hike to a site only to find it dry, rendering the site unsampleable. This underscores the challenge of timing eDNA sampling to coincide with the presence of water in these dynamic environments. Second, optimizing the sampling design to better capture spatially heterogenous eDNA signals, which is particularly important for eDNA detection of amphibians (e.g., Bedwell and Goldberg 2020). In subsequent field protocol testing at other wetlands, we found that although the Carim et al. (2016) protocol used here misses some American bullfrog populations in lentic systems, detection can achieve 100% concordance with known species occurrence by combining smaller, spatially replicated sub-samples (Hernandez et al. 2024).

It is difficult to assess concordance of eDNA detection of *Ranavirus* and amphibian chytrid fungus (*Bd*) because (1) historic datasets are limited, (2) pathogen occupancy and abundance may be dynamic over short time-scales (e.g., Voyles et al. 2014), and (3) pathogens may be detectible at low levels in the environment without infection outbreaks in the amphibian population (Kamoroff and Goldberg 2017). However, our detections were consistent with previous eDNA studies which found both pathogens to be relatively widespread on the landscape (e.g., Hossack et al. 2023; Vilaça et al. 2020). Co-occurrence patterns were also consistent with previous studies showing that *Ranavirus* and *Bd* are more common where American bullfrog occur. Among 260 samples negative for American bullfrog on the HT-qPCR platform, only 1.9% were positive for *Bd* and 1.2% for *Ranavirus*. In contrast, where bullfrogs were detected, detection rates of *Bd* and *Ranavirus* were ~ 10 × higher (29.1% for *Bd* and 21.8% for *Ranavirus*). Hossack et al. (2023) sampling in Arizona, New Mexico, and Sonora (Mexico) similarly found that *Bd* and *Ranavirus* were associated with American bullfrog: proportion of sites occupied was estimated to be 31–47% with versus 23% without American bullfrog for *Bd* and 10–33% with versus 3% without bullfrog for *Ranavirus*. A high throughput chip similar to that described here may provide a useful tool to cost-effectively further explore interaction dynamics between native and invasive amphibians and their pathogens as in Hossack et al. (2023).

Conclusion

The adoption of high-throughput quantitative PCR (HT-qPCR) chips represents a significant advancement in eDNA analysis, offering an approach to simultaneously screen a wide range of taxa, including aquatic endemics, wetland-dependent terrestrials, invasive species, and pathogens. This technological innovation is particularly crucial in the context of arid ecosystems, where urgent conservation action is needed due to the observed decline in biodiversity driven by anthropogenic disturbances and climate change. Notably, invasive American Bullfrogs have been linked to the decline of native amphibians, primarily through predation and pathogen transmission. In regions where bullfrogs were detected, the rates of *Bd* and *Ranavirus* detection were approximately tenfold higher compared to samples devoid of bullfrogs, corroborating findings by Hossack et al. (2023). Our study underscores the importance of identifying drivers of pathogen presence for the implementation of effective conservation strategies, emphasizing the need for continued monitoring and management efforts to mitigate the threats posed by invasive species. Moving forward, further refinement of HT-qPCR protocols and expansion of chips to include additional target species will enhance the utility of this approach for use in arid ecosystems to understand pathogen dynamics and other types of species interactions.

Appendix 1: Assay description documents

We revalidated previously published assays for Chiricahua leopard frog (Goldberg et al. 2017), northern Mexican gartersnake (Goldberg et al. 2014), snake fungal disease (Allender et al. 2015), and amphibian chytrid fungus (Boyle et al. 2004). The assay for amphibian *Ranavirus* was modified from Brunner (2004), shifting the probe and reverse primer locations to increase assay generality across genetic strains described since the original assay validation. New assays were developed for ocelot. Each of these assays is described below using a standardized framework as described in Kronenberger et al. (2024).

Chiricahua leopard frog (*Rana* [*Lithobates*] *chiricahuensis*)

Assay region: Control region, D-loop.

Forward primer: 5′-GGTACCGCTCATATCATGACT ACTTG-3′

Reverse primer: 5′-TCCAGTTGGACTCACTTAGGA ATG-3′

Probe: 5′-FAM-TAGGACCTTCGCTTGTTAT- MGB-NFQ -3′

Optimized primer concentrations: 900 nM Forward, 900 nM Reverse.

LOD: 50 copies/reaction.

A note on the target species

The Chiricahua leopard frog is endemic to Mexico and to Arizona and New Mexico, USA. It has undergone considerable population declines and is listed as Threatened under the Endangered Species Act (USFWS 2002). There are no known nonnative occurrences. The Chiricahua leopard frog is often placed in the genus *Lithobates* (sensu Frost et al. 2006), but this genus has been found to cause paraphyly in other Ranidae genera, prompting the reallocation of its members into *Rana* (Yuan et al. 2016). Many sources—including AmphibiaWeb (amphibiaweb.org; a premier online amphibian taxonomy database)—now consider *Rana* the preferred genus. We therefore use this updated, phylogenetically-based taxonomy to refer to non-target species.

Original assay description

The Chiricahua leopard frog assay was originally designed and validated by Goldberg et al. (2017) for use within the species' native range in Arizona and New Mexico. Goldberg et al. (2017) confirmed assay generality in silico by creating consensus sequences from multiple Chiricahua leopard frogs prior to assay design (number and origin unspecified) and in vitro by testing genomic DNA from at least 10 individuals. The authors confirmed assay specificity in silico via Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast; Ye et al. 2012) and in vitro by testing DNA from at least five individuals each of 13 non-target species, including Great Plains toad (*Anaxyrus cognatus*), red-spotted toad (*Anaxyrus punctatus*), Woodhouse's toad (*Anaxyrus woodhousii*), Sonoran Desert toad (*Incilius alvarius*), barking frog (*Craugaster augusti*), canyon treefrog (*Hyla arenicolor*), Arizona treefrog (*Hyla wrightorum*), western narrow-mouthed toad (*Gastrophryne olivacea*), American bullfrog (*Rana catesbeiana*), Tarahumara frog (*Rana tarahumarae*), lowland leopard frog (*Rana yavapaiensis*), Couch's spadefoot (*Scaphiopus couchi*), and Mexican spadefoot (*Spea multiplicata*). The authors used QuantiTech Multiplex PCR Mix (QIAGEN) and a 60 °C annealing temperature.

Assay performance and extent of validation

The Chiricahua leopard frog is placed in family Ranidae, which has a global distribution and currently contains 432 accepted species among 24 genera. There are currently 30 accepted ranid species within the continental United States, all within the genus *Rana*. Within the study area applicable to this taxon (Arizona and New Mexico) we identified seven non-target confamilials (Table 3). Sequence data were available for six of the seven non-target confamilials (no sequence data available for Tarahumara frog; *R. tarahumarae*). Maximum eDNAssay assignment probabilities were ≥ 0.3 for four species. Primer-BLAST (Ye et al. 2012) flagged an additional 13 non-target species, all of which were anurans. However, none of the flagged species occur within the Chiricahua leopard frog's native range.

All potentially co-occurring confamilials were tested for specificity in vitro either by Goldberg et al. (2017) or in revalidation, with the exception of relict leopard frog (*R. onca*) which is currently restricted to Lake Mead National Recreation Area (eDNAssay assignment probability = 0.304). All in vitro tests indicated specificity except for northern leopard frog (*R. pipens*). Thus, the assay should be applied with caution where this taxon may also occur.

Assay generality was tested in silico based on alignment with existing sequence data from GenBank (n = 12; Goldberg et al. 2017). The eDNAssay assignment probability for all 12 sequences was 0.964. Goldberg et al. (2017) also confirmed assay generality in vitro by testing genomic DNA from ten individuals of the target species collected throughout its native range.

Table 3 Testing summary for all potentially sympatric confamilial species including number of sequences tested in silico, maximum eDNAssay assignment probability, number of samples tested in vitro, and results of in vitro testing for the Chiricahua leopard frog assay originally described by Goldberg et al. (2017)

Species	In silico tests	Max AP	In vitro tests	In vitro results
Rio Grande leopard frog *(Rana berlandieri)*	1	0.472	1	No Amp
Plains leopard frog *(Rana blairi)*	1	0.476	1	No Amp
American bullfrog *(Rana catesbeiana)*	1	0.256	≥ 5	No Amp
Relict leopard frog *(Rana onca)*	1	0.304	–	–
Northern leopard frog *(Rana pipiens)*	1	0.545	5	Amp
Tarahumara frog *(Rana tarahumarae)*	–	–	≥ 5	No Amp
Lowland leopard frog *(Rana yavapaiensis)*	1	0.297	≥ 5	No Amp

Goldberg et al. (2017) tested the assay in situ using eDNA samples collected at Fort Huachuca, Arizona, in conjunction with aural and visual amphibian surveys. Chiricahua leopard frog eDNA was detected at 10 of 12 sites where the species was known to be present, with both non-detections occurring at sites with very low population densities. The estimated per-sample eDNA detection probability based on single-species occupancy modeling was 0.62 (95% CI 0.43–0.81; Tables 3, 4, 5 in the original report). The assay was further assessed in situ on field samples as part of this study, particularly on known occupancy status habitats on Ladder Ranch (New Mexico).

Northern Mexican gartersnake (*Thamnophis eques megalops*)

Assay region: NADH dehydrogenase 1 (*ND1*).

Forward primer: 5′-CGAAAAGGCCCTAACCTGG-3′

Reverse primer: 5′-CTATGATTGGTGAYAGGGTGA ACAGT-3′

Probe: 5v-FAM-AATAGGCCTTCTACAGCC TA- MGB- NFQ-3v.

Optimized primer concentrations: 900 nM Forward, 900 nM Reverse.

LOD: 2 copies/reaction.

A note on the target species

The northern Mexican gartersnake is endemic to Mexico and to Arizona and New Mexico, USA in the western portion of USFWS Region 2. It has undergone considerable population declines and is listed as threatened under the Endangered Species Act (USFWS 2014). There are no known nonnative occurrences.

Original assay description

The northern Mexican gartersnake assay was originally designed and validated by Goldberg et al. (2014) for use within the species' native range in Arizona and New Mexico. Goldberg et al. (2014) did not specify their strategy for confirming assay generality in silico but did confirm generality in vitro by testing genomic DNA from 10 individuals. The authors confirmed assay specificity in silico using sequences from an unspecified set of closely related species and in vitro by testing DNA from the black-necked gartersnake (*Thamnophis cyrtopsis*; n = 2) and checkered gartersnake (*Thamnophis marcianus*; n = 1). The authors used QuantiTech Multiplex PCR Mix (QIAGEN) and a 60 °C annealing temperature.

Assay performance and extent of validation

The northern Mexican gartersnake is placed in the largest snake family, Colubridae, which has a global distribution and currently contains 1,902 accepted species among 257 genera. There are currently 137 accepted Colubridae species within the continental United States. Of these, 51 potentially occur within the northern Mexican gartersnake's native distribution. Of these, 21 taxa had relevant sequence data available (Table 4). For these, maximum eDNAssay assignment probabilities were ≥ 0.3 for six species, all of which are congenerics. Primer-BLAST (Ye et al. 2012) did not identify any additional non-target taxa of potential concern.

Because we could not obtain relevant sequences or DNA for 30 of the 51 confamilial species that potentially cooccur with northern Mexican gartersnake within the continental United States we estimated the probability of cross-amplification with unsampled taxa using the framework described in Wilcox et al. (2024). The probability density function describing the assay's assignment probability distribution indicates that untested confamilials have a 17.3% (95% CI: 13.2–23.2) probability of exceeding the 0.3 assignment probability threshold and a 0.3% (95% CI: 0.1–1.9) probability of exceeding the 0.5 assignment probability threshold. However, when congenerics were excluded (all relevant congenerics tested in vitro; see below), the probability of exceeding the 0.3 assignment probability threshold was only 1.5% (95% CI 0.5–5.3) and the probability of exceeding the 0.5 assignment probability threshold was 0.0% (95% CI 0.0–0.0). Probability of exceeding the threshold is estimated per taxon. The probability of at least one of n taxa exceeding the threshold is $1-(1-p)^n$ (Kronenberger et al. 2022).

We tested all seven congeneric taxa (eDNAssay assignment probability 0.184–0.544) in vitro using synthetic gene fragments based on the GenBank record with the highest eDNAssay assignment probability for each taxon. Of these, three species amplified (*T. elegans, T. radix, T. sirtalis*). *Thamnophis elegans* and *T. sirtalis* have a widespread distribution that overlaps with the target species, but *T. radix* is not found in Arizona. Results from this assay should be interpreted with caution where these taxa might occur.

Goldberg et al. (2014) confirmed assay generality in silico by aligning assay oligonucleotides with northern Mexican gartersnake sequences (provided by Dustin Wood), but did not specify the number sequences assessed nor the location where sequenced individuals were captured. We also confirmed coverage of the assay for a sequenced individual collected at Tonto Creek in central Arizona (also provided by Dustin Wood). The assignment probability for the given sequence was 0.982. We therefore find strong evidence that the assay can detect northern Mexican gartersnakes throughout their native range. Goldberg et al. (2014) confirmed

Table 4 Testing summary for all potentially sympatric confamilial species including number of sequences tested in silico, maximum eDNAssay assignment probability, number of samples tested in vitro, and results of in vitro testing for the northern Mexican gartersnake assay originally described by Goldberg et al. (2014)

Species	In silico tests	Max AP	In vitro tests	In vitro results
Arizona elegans	1	0.172	–	–
Bogertophis subocularis	1	0.183	–	–
Coluber constrictor	2	0.180	–	–
Diadophis punctatus	–	–	–	–
Drymobius margaritiferus	–	–	–	–
Gyalopion canum	–	–	–	–
Gyalopion quadrangulare	–	–	–	–
Heterodon kennerlyi	–	–	–	–
Heterodon nasicus	–	–	–	–
Hypsiglena chlorophaea	5	0.156	–	–
Hypsiglena jani	5	0.161	–	–
Lampropeltis californiae	–	–	–	–
Lampropeltis gentilis	–	–	–	–
Lampropeltis knoblochi	–	–	–	–
Lampropeltis pyromelana	1	0.194	–	–
Lampropeltis splendida	–	–	–	–
Masticophis bilineatus	–	–	–	–
Masticophis flagellum	2	0.232	–	–
Masticophis taeniatus	–	–	–	–
Nerodia fasciata	5	0.123	–	–
Opheodrys vernalis	–	–	–	–
Oxybelis microphthalmus	–	–	–	–
Pantherophis emoryi	–	–	–	–
Phyllorhynchus browni	–	–	–	–
Phyllorhynchus decurtatus	–	–	–	–
Pituophis catenifer	2	0.128	–	–
Rhinocheilus lecontei	1	0.169	–	–
Salvadora deserticola	–	–	–	–
Salvadora grahamiae	–	–	–	–
Salvadora hexalepis	–	–	–	–
Senticolis triaspis	1	0.202	–	–
Sonora annulata	–	–	–	–
Sonora cincta	–	–	–	–
Sonora episcopa	–	–	–	–
Sonora occipitalis	5	0.160	–	–
Sonora palarostris	1	0.149	–	–
Sonora semiannulata	–	–	–	–
Tantilla hobartsmithi	–	–	–	–
Tantilla nigriceps	–	–	–	–
Tantilla wilcoxi	–	–	–	–
Tantilla yaquia	–	–	–	–
Thamnophis cyrtopsis	4	0.434	3	No amp
Thamnophis elegans	1	0.544	1	Amp
Thamnophis marcianus	2	0.408	1	No amp
Thamnophis proximus	1	0.184	1	No amp
Thamnophis radix	1	0.384	1	Amp
Thamnophis rufipunctatus	5	0.396	1	No amp
Thamnophis sirtalis	4	0.357	1	Amp
Trimorphodon lambda	–	–	–	–
Trimorphodon vilkinsonii	–	–	–	–
Tropidoclonion lineatum	1	0.237	–	–

assay generality in vitro by testing genomic DNA from 10 individuals of the target species, but did not specify the sampling locations.

Goldberg et al. (2014) tested the assay in situ using eDNA samples collected at and around Fort Huachuca and the Santa Cruz River, Arizona, in conjunction with visual surveys and trapping conducted by the Arizona Game and Fish Department. Northern Mexican gartersnake eDNA was detected at two of five sites where the species was known to be present. Estimates of detection probability ranged from 0 to 0.75 and may have been positively correlated with water conductivity at the sampling site. The authors posited that generally low detection probabilities may have been due to low DNA shedding rates of semi-aquatic snakes relative to other aquatic taxa.

Snake fungal disease (*Ophiomyces ophiodiicola*)

Assay region: Internal transcribed spacer 1 (*ITS1*).

Forward primer: 5v-TGTTTCTGTCTCGCTCGA AGAC-3′

Reverse primer: 5′-AGGTCAAACCGGAAAGAA TGG-3′

Probe: 5′-FAM-CGATCGGCGCCCGTCGTC- MGB NFQ-3′

Optimized primer concentrations: 900 nM Forward, 600 nM Reverse.

LOD: 2 copies/reaction.

A note on the target species

The causative species of snake fungal disease (*Ophiomyces ophiodiicola*) is an emerging pathogen affecting a wide variety of snake species. It is often lethal and has been implicated in the decline of at least one population (Clark et al. 2011). The fungus has thus far been documented in wild snakes throughout the eastern United States (USFWS Regions 3, 4, and 5), but may also be present in the western United States at a lower prevalence or disease severity (Lorch et al. 2016).

The species implicated in snake fungal disease is the sole member of its genus in family Onygenaceae. This family is currently comprised of 189 accepted species among 26 genera. Of these, 24 species have documented occurrences within the continental United States, according to the Global Biodiversity Information Facility (gbif.org; Table 5).

Original assay description

The snake fungal disease assay was originally designed and validated by Allender et al. (2015) for clinical diagnosis of snake disease via swabs of nasolabial pits and lesions. Allender et al. (2015) did not discuss their method for confirming assay generality in silico, but did confirm target amplification in vitro using DNA isolated from an infected massasauga rattlesnake (*Sistrurus catenatus*). They also did not discuss any in silico testing of assay specificity, but did confirm specificity in vitro by testing DNA from 14 non-target species that are common in soil and clinical isolates: *Alternaria* sp., *Aspergillus flavus*, *Aspergillus terreus*, *Blastomyces dermatitidis*, *Chrysosporium vallenarense*, *Cladosporium cladosporioides*, *Colletotrichum acutatum*, *Fusarium lateritium*, *Geomyces* sp., *Mucor racemosus*, *Penicillium verrucosum*, *Pseudogymnoascus destructans*, *Pseudogymnoascus pannorum*, and *Simplicillium cylindrosporum*. The number and geographic source of tested non-targets was unspecified. The authors used TaqMan Platinum PCR SuperMix-UDG (Invitrogen) and a 60 °C annealing temperature.

Assay performance and extent of validation

We confirmed assay specificity in silico using available sequences from 37 confamilials (Table 5). Of the 37 non-targets tested in silico, maximum eDNAssay assignment probabilities were ≥ 0.3 for two species (*Mucor racemosus* and *Paranannizziopsis longispora*). Allender et al. (2015) found the assay to be specific against *M. racemosus* when tested in vitro. Based on the eDNAssay model, *P. longispora* is unlikely to cross-amplify (assignment probability = 0.344), but this taxon is also known to infect snakes and be associated with skin lesions (Lorch et al. 2016). All other confamilials with sequence data had an eDNAssay assignment probability < 0.255. It should be noted that many of the mismatches included in our counts are not mismatches per se, but indels in the assay region. The *ITS1* region is non-coding and therefore especially prone to developing mutations and indels over time. We designated these as mismatches because Wright et al. (2014) suggest that single insertions and deletions have a similarly negative impact on amplification efficiency as do single mismatches. Wright et al. (2014) also tested the effect of double deletions and found an even greater cumulative impact on amplification efficiency than both single and double basepair mismatches. Therefore, our inclusion of indels in mismatch counts for the eDNAssay model likely offers a highly conservative estimate of the degree of specificity. Primer-BLAST (Ye et al. 2012) did not flag any additional taxa of concern. Allender et al. (2015) also confirmed assay specificity in vitro by testing

Table 5 Testing summary for all potentially sympatric confamilial species including number of sequences tested in silico, maximum eDNAssay assignment probability, number of samples tested in vitro, and results of in vitro testing for the snake fungal disease assay originally described by Allender et al. (2015)

Species	In silico tests	Max AP	In vitro tests	In vitro results
Alternaria sp.	3	0.135	1	No amp
Amauroascus albicans	5	0.120	–	–
Amauroascus aureus	3	0.110	–	–
Amauroascus kuehnii	5	0.087	–	–
Amauroascus niger	4	0.171	–	–
Amauroascus verrucosus	2	0.254	–	–
Amauroascus volatilis-patellis	3	0.111	–	–
Aphanoascus keratinophilus	5	0.199	–	–
Aspergillus flavus	10	0.091	1	No amp
Aspergillus terreus	2	0.124	1	No amp
Auxarthron californiense	2	0.184	–	–
Auxarthron compactum	2	0.231	–	–
Auxarthron conjugatum	4	0.180	–	–
Auxarthron pseudauxarthron	4	0.083	–	–
Auxarthron umbrinum	5	0.126	–	–
Auxarthron zuffianum	5	0.094	–	–
Blastomyces dermatitidis	1	0.134	1	No amp
Chrysosporium carmichaelii	5	0.159	–	–
Chrysosporium georgiae	5	0.089	–	–
Chrysosporium lucknowense	5	0.156	–	–
Chrysosporium queenslandicum	2	0.198	–	–
Chrysosporium vallenarense	1	0.166	1	No amp
Cladosporium cladosporioides	3	0.182	1	No amp
Coccidioides immitis	5	0.108	–	–
Coccidioides posadasii	5	0.096	–	–
Colletotrichum acutatum	3	0.126	1	No amp
Fusarium lateritium	2	0.127	1	No amp
Geomyces sp.	2	0.116	1	No amp
Mucor racemosus	4	0.326	1	No amp
Neogymnomyces demonbreunii	6	0.074	–	–
Onygena corvina	1	0.101	–	–
Paranannizziopsis longispora	1	0.344	–	–
Penicillium verrucosum	5	0.135	1	No amp
Pseudogymnoascus destructans	5	0.128	1	No amp
Pseudogymnoascus pannorum	5	0.125	1	No amp
Renispora flavissima	4	0.146	–	–
Simplicillium cylindrosporum	9	0.152	1	No amp

DNA from 14 non-target species that are common in soil and clinical isolates.

We confirmed assay coverage for all sequences available on GenBank that spanned the full assay region (n = 15). Sequenced pathogens were collected from infected snakes in the United States, Australia, and England (n = 8, 1, and 1, respectively; Sigler et al. 2020), the United States territory of Guam (n = 1; Sigler et al. 2020), and Taiwan (n = 4; Sun et al. 2022). All sequences are an exact match with assay oligonucleotides.

This assay was previously validated by Allender et al. (2015) against 47 free-ranging eastern massasaugas (*Sistrurus catenatus*). Results using PCR and qPCR led to three and seven positive samples respectively. Baker et al. (2020) implemented the Allender et al. (2015) assay while attempting to detect *Ophiomyces ophiodiicola* in Clinton County (Illinois, USA) from soil samples. Despite positive controls amplifying in the study, none of the 100 environmental samples tested led to a detection. We tested the assay in situ using 15 aliquots of soil extracts sent to us by Mark Davis (Illinois State) known to be positive for *Ophiomyces*. These samples were collected in follow-up work associated with the Baker et al. (2020) study and tested using Allender

et al. (2015) assay. We managed to detect *Ophiomyces* in all samples using the Allender et al. (2015) assay.

Chytrid fungus (*Batrachochytrium dendrobatidis* (*Bd*-Global PanzooticLineage (GPL)))

Assay region: Internal transcribed spacer 1 (*ITS1*) and 5.8S.

Forward primer: 5′- CTTGATATAATACAGTGTGCC ATATGT -3′

Reverse primer: 5′- AGCCAAGAGATCCGTTGTCAAA -3′

Probe: 5′- FAM-CGAGTCGAACAAA-MGB-NFQ-3′

LOD: 10 copies/reaction.

Amplification efficiency: 91.317%

Optimized primer concentrations: 900 nM Forward, 900 nM Reverse.

A note on the target species

Batrachochytrium dendrobatidis (*Bd*) is a globally distributed aquatic fungus known to infect the epidermal tissue of many amphibian species and it has been implicated as a causative agent of large amphibian die-offs (Kamoroff and Goldberg 2017; Lips et al. 2006; Schloegel et al. 2006). Individuals with severe infections develop potentially fatal chytridiomycosis, but mortality rates are highly variable and *Bd* is sometimes highly prevalent in apparently stable populations even as other populations of the same species experience *Bd* infection and die-offs (Briggs et al. 2005). Intensity of chytridiomycosis is thought to be mediated by environmental conditions and affected by variation within *Bd* and the infected amphibian (Lips 2016).

Notable genetic diversity exists within *Bd*, and several *Bd* lineages appear to have spatially restricted distributions (Byrne et al. 2019). However, a single lineage is globally distributed (*Bd*-global panzootic lineage (GPL)), and this lineage has been most commonly associated with amphibian die-offs (Farrer et al. 2011). Therefore, we focused our effort on validating an assay which would sensitively detect *Bd*GPL.

Original assay description

In the initial study, Boyle et al. (2004) tested the assay for generality across culture isolates from four strains of *Bd,* and further tested for specificity against isolates from five closely related fungal species. To confirm that the assay could detect *Bd* from infected individuals, the authors also experimentally inoculated 30 great barred frogs (*Mixophyes fasciolatus*), took toe clips, and compared detection results from the qPCR assay to the results from histological examination, and found that the assay was often able to detect *Bd* infections in tissues earlier than histological examination could. In the original paper, generality and specificity tests used TaqMan Master Mix (Applied Biosystems) and 60 °C annealing. A subsequent study expanded on and upheld the findings of Boyle et al. (2004) with regard to the assay's sensitivity and specificity (Hyatt et al. 2007). The assay was validated by Walker et al. (2007) for application to environmental samples.

Assay performance and extent of validation

There are only two described members of the family Batrachochytriaceae. Since the original validation of this assay, a congeneric amphibian pathogen was described (*Batrachochytrium salamandrivorans* (*Bsal)*) and detected in Europe (Martel et al. 2013), but has not been detected in North America (Waddle et al. 2020; Hill et al. 2021). We assessed the specificity of the assay in silico against *Bsal* as well as 13 other chytrid fungi (Table 6). Of these, two had eDNAssay assignment probabilities > 0.3, but both were determined to be specific in vitro (Hyatt et al. 2007). Primer-BLAST (Ye et al. 2012) flagged six sequences of uncultured funguses isolated from water samples or leaves from Lithuania, Australia, or India (GenBank Accessions: MT237073, MT236834, KY649451, KY649450, KF522313, MW602952). There was no cross-amplification of non-target funguses tested in vitro in Hyatt et al. (2007). We did not test *Bsal* specificity in vitro, but eDNAssay assignment probability was low (0.211), indicating a very low probability of cross-amplification (Wilcox et al. 2024, Kronenberger et al. *in review*).

Boyle et al. (2004) designed the assay to encompass genetic diversity recovered from their four experimental strains, as well as sequence variation observed in wild strains which infected frogs in Australia, Panama, Ecuador, and the United States. These strains are similar to each other and are considered members of the *Bd* Global Pandemic Lineage (GPL). The authors further confirmed the assay's generality by comparing it to *Bd*-GPL sequences uploaded to NCBI's GenBank database. We re-examined the same database in September of 2021 and found that, since initial publication, there have been two *Bd* sequences produced which have mismatches with the forward primer (Accessions KX115400; *Bd*-China, JQ582886; *Bd*-Brazil), with one of these sequences also having a mismatch in the probe region (KX115400; eDNAssay assignment probability = 0.416). The isolate which produced KX115400 was collected from Hainan Province, China in 1982. A phylogenetic analysis of its relationship to other *Bd* strains suggests that it could be a newly discovered strain, endemic to the region (Wang et al. 2018).

Table 6 Testing summary for all potentially sympatric confamilial species including number of sequences tested in silico, maximum eDNAssay assignment probability, number of samples tested in vitro, and results of in vitro testing for the *Bd* assay originally described by Boyle et al. (2004)

Species	In silico tests	Max AP	In vitro tests	In vitro results
Batrachochytrium salamandrivorans	2	0.211	–	–
Allomyces arbuscula	2	0.217	1	No amp
Allomyces macrogynus	2	0.281	1	No amp
Catenaria sp.	1	0.266	1	No amp
Catenophlyctis sp.	1	0.198	1	No amp
Chytriomyces hyalinus	2	0.317	1	No amp
Cladochytrium sp.	1	0.231	1	No amp
Gonapodya sp.	1	0.238	1	No amp
Powellomyces sp.	1	0.220	1	No amp
Gorgonomyces haynaldii	1	0.336	1	No amp
Rhizophlyctis rosea	2	0.300	1	No amp
Rhizophlyctis sp.	2	0.284	1	No amp
Rhizophlyctis-Rhizophydium-like	–	–	1	No amp
Rhizophydium sp.	1	0.243	11	No amp
Spizellomyces sp.	1	0.281	3	No amp

The isolate which produced the JQ582886 haplotype was collected from an American bullfrog (*Lithobates catesbeianus*) purchased at a market in Michigan, USA. It shares its haplotype with a divergent strain of *Bd* thought to be native to Brazil, where bullfrog farming is a widespread practice (Schloegel et al. 2012) and live frogs are often exported. This haplotype has a single mismatch with the assay, located five nucleotides from the 5′ end of the forward primer. It's unlikely that this would eliminate eDNA detections entirely (eDNAssay assignment probability = 0.894), but sensitivity may be reduced for this strain. The presence of this strain in wild North American amphibian populations is unknown (Table 6).

Ranavirus (Iridoviridae)

Assay region: Major capsid protein (*MCP*) gene.

Forward primer: 5′- ACACCACCGCCCAAAAGTAC -3′

Reverse primer 1: 5′- ATCGAGCCGTTCATGATGC -3′

Reverse primer 2: 5′- CATCGAACCGTTCATGATGC -3′

Probe: 5′- FAM- CCATCAACCACAACATTA -MGB-NFQ -3′

LOD: 10 copies/reaction.

Amplification efficiency: 97.4452%

Optimized primer concentrations: 300 nM Forward, 900 nM Reverse.

Developed by: Dan Mason, based on the assay applied in Picco et al. (2007)

A note on the target species

Ranaviruses are a globally distributed group of double stranded DNA viruses which infect poikilothermic vertebrates (i.e., fish, amphibians, reptiles) and are associated with large die-off events in amphibians (Bollinger 1999; Docherty 2003; Jancovich 1997), although subclinical infections have also been observed. Viruses may be transmissible through contact with infected individuals or carcasses, including non-symptomatic individuals, and perhaps through environmental exposure (Williams et al. 2005).

Iridoviridae contains five genera, including *Ranavirus*. The six members of this genus demonstrate notable genetic and host species diversity, with genetic divergences of up to 30% (Ting et al. 2004) and frog, salamander and fish hosts. The difficulties of reconstructing viral phylogenies and the potentially emerging nature of these viruses obscures some of the finer details of relationships within the genus, but phylogenetic support for the group is strong (Williams et al. 2005).

Original assay description

This assay is derived from a qPCR assay described in Brunner (2004) and applied in Picco et al. (2007), designed in the major capsid protein (*MCP*) gene. In the initial study, Picco et al. (2007) do not report methods used to design and validate their assay. However, Hall et al. (2016) report that the assay is specific in silico and specifically that it does not amplify the *MCP* gene of viruses in the genera *Lymphocystivirus,* or *Megalocytivirus.* They also reported that the assay had mismatches with two members of the *Ranavirus* genus, but will amplify all known ranaviruses in North America (Hall et al. 2016, Appendix 1), and that in silico assessment of the assay identified similarity with *Nematostella vectensis* (Hall et al. 2016).

Assay modifications

To make assay modifications, we examined sequences of the *MCP* gene region, downloaded from GenBank ($n = 33$). We aligned *MCP* sequences in MEGA 7 (Kumar et al. 2016) and aligned the forward primer from Picco et al. (2007) without modification, but manually made changes to the reverse primer and the probe to improve assay generality. To accommodate a pair of single nucleotide polymorphisms detected in four viral isolates in the original probe binding region, we shifted the probe 14 nucleotides in the 3′ direction. This shift caused the probe to overlap with the original reverse primer binding site, so we shifted the reverse primer eight nucleotides in its 5′ direction. The new reverse primer binding site also contained a polymorphic site, but we accommodated this by designing two reverse primers which bind to the same site; one primer with each base present at the polymorphic site. Despite this modification, there remain two strains of amphibian *Ranavirus* which have a mismatch within the probe binding site, eight strains which have a mismatch within the forward primer binding region (three isolated from amphibians, five from fishes), and one strain of frog *Ranavirus* which has an additional mismatch in the reverse region that is not accommodated (see below).

Assay performance and extent of validation

Iridoviridae is composed of two subfamilies: Alphairidovirinae (genera *Ranavirus, Lymphocystivirus*, and *Megalocytivirus*) and Betairidovirinae (genera *Iridovirus, Chloriridovirus,* and *Decapodiridovirus*). Testing in silico suggests that the assay is largely specific against non-targets across Iridoviridae (Table 7; n = 11 non-target iridovirid taxa all have eDNAssay assignment probabilities < 0.2). However, the assay may detect non-target viruses in two non-native (to North America) genera which are pathogens of fishes, *Lymphocystivirus* and *Megalocytivirus*. These two genera have the potential to infect an unknown but possibly significant portion of the North American fish fauna, but their status in wild fish populations is largely undocumented. Neither genus is known to infect amphibians, thus detection of the genera by the assay is not ideal. However, we were unable to modify the assay to detect a greater number of known amphibian ranavirus templates without causing this non-specificity. The assay will amplify some lineages of fish irridioviruses, but not all of them (Table 8). This may require some contextualization when fish-specific viruses may also be present at a site. The original assay from Picco et al. (2007) is a perfect match to several iridoviruses which infect fish, and for this reason, Hall et al. (2016) chose to apply it only to sample fishless ponds (Table 7).

Viruses in *Ranavirus* have been identified by a variety of methods; the type of clinical infection, the geographic region which the first isolate was detected from, or the host species the first isolate was detected from. However, the accuracy with which these characteristics represent relatedness amongst virus taxa is suspect. Genomic evidence suggests that viruses in this group demonstrate recombination at many genes (Epstein and Storfer 2016; Price et al. 2015), which complicates attempts to reconstruct phylogenies. Nonetheless, many sequences from the genus have > 90% sequence similarity in the *MCP* gene (Chinchar et al. 2017), and the region of the *MCP* which this assay was designed to match shows a high degree of nucleotide conservation. Gaps in scientific understanding of the taxonomy and geographic distribution of *Ranavirus* remain, but the existing information suggests that this assay has the taxonomic generality required for eDNA detection of the genus. Based on an assessment of 99 *Ranavirus* sequences in GenBank, the assay appears to have good generality across amphibian ranaviruses (minimum eDNAssay assignment probability > 0.7; Table 8).

Table 7 eDNAassay assignment probabilities for 11 non-target iridovirid taxa with data inGenBank

Taxon	N	Max. AP
Chloriridovirus sp.	4	0.148
Megalocytivirus sp.	1	0.102
Iridovirus sp.	1	0.100
Megalocytivirus sp	1	0.077
Lymphocystivirus sp.	3	0.050
Decapodiridovirus sp.	1	0.021

The original assay was applied by Picco et al. (2007) for detection of a ranavirus from experimentally infected mole salamanders and was later successfully used by Hall et al. (2016) for detection of ranavirus outbreaks using eDNA samples. This modified assay was tested in situ by analyzing two samples taken from a ranavirus culture, two swabs from sunfish skin, and 17 eDNA samples taken from waterbodies which had been observed to contain ranavirus infected amphibians in the recent past. The assay detected ranavirus at high concentrations from the cultures, and from the two fish swabs. However, based on this previous work, the efficacy of larger pore size filters (1.5 µm in this study) for ranavirus detection was unclear. We compared efficacy of these larger pore size filters with 0.22 µm pore size filters by collecting paired samples from ten putative positive waterbodies. *Ranavirus* was only detected at one site, but was positive in both filter pore sizes. *Ranavirus* was detected at three of four sites (17, 105, 108) in swab samples collected from animals shortly after the eDNA samples were collected (Joseph Mihaljevic, personal communication).

Table 8 Potential target sequences from GenBank ($n = 99$) with sample size and minimum and maximum eDNAssay assignment probabilities

Taxon	N	Min. AP	Max. AP
Ambystoma tigrinum virus	1	0.854	0.854
Common midwife toad virus	1	0.854	0.854
Epizootic haematopoietic necrosis virus	2	0.854	0.854
Frog virus 3	14	0.854	0.854
Unclassified ranavirus (amphibians)	31	0.716	0.854
Unclassified ranavirus (fish)	31	0.104	0.854
Unclassified ranavirus (reptiles)	9	0.685	0.854
Santee-Cooper ranavirus	4	0.333	0.333
Singapore grouper iridovirus	6	0.097	0.104

Ocelot (Leopardus pardalis)

Note: The original ocelot assay targeted an ND4 locus, but showed poor performance on environmental samples. This ND5 assay was designed and validated for single-taxon analysis on the qPCR platform, but not incorporated into the HT-qPCR chip.

Assay region: NADH-ubiquinone oxidoreductase chain 5 (*ND5*).

Forward primer: 5′- GGGATACCTAATCTCCTACAA CATT-3′

Reverse primer: 5′- TGGGAGGCGGTGTATTACGG-3′

Probe: 5′-FAM- CTCCAACCTATTGGGGTA C- MGB-NFQ3′

Optimized primer concentrations: 600 nM Forward, 900 nM Reverse.

LOD: 10 copies/reaction.

A note on taxonomy

Historically, there are up to nine recognized subspecies of ocelot (Wozencraft 2005). These groups were revisited by the Cat Classification Task Force of the IUCN/SSC Cat Specialist Group Kitchener et al. (2017). They found strong evidence for two groups which are potential species or subspecies: *Leopardus pardalis pardalis* (Northern portion of range; type locality Mexico) and *Leopardus pardalis mitis* (type locality Brazil), based on morphological and genetic studies by Ruiz-Garcia et al. (2012), Nascimento (2010), and Eizirik et al. (1998).

Species distribution

The core of the ocelot range is in northern South America, Central America, and Mexico in North America. Within the United States, there are two small populations in Texas and animals are occasionally detected in Arizona.

Closely related species

The other felids (confamilials) known to occur within the continental United States are jaguar (*Panthera onca*), domesticated cat (*Felis catus*), bobcat (*Lynx rufus*), Canada lynx (*Lynx canadensis*; range not overlapping), puma (*Puma concolor*), and potentially jaguarundi (*Puma yagouaroundi*). Margay (*Leopardus wiedii*), Andean mountain cat (*Leopardus jacobita*), Geoffroy's cat (*Leopardus geoffroyi*), oncilla (*Leopardus tigrinus*), and Pampas cat (*Leopardus colocola*) are not known to occur within the United States, but are closely-related felids found in South America and Mexico. Tiger (*Panthera tigris*), leopard (*Panthera pardus*), and African lion (*Panthera leo*) are not native to the United States, but escaped captive animals are possible. Species with an assignment probability ≥ 0.3 were included in in vitro testing to confirm assay specificity (Table 9).

Assay validation approach

For an eDNA assay to be valid it must have high generality (detecting all haplotypes of the target species) and specificity (detecting only the target species) within a defined geographic scope (sensu Wilcox et al. 2015). The gold standard for assay validation is in vitro testing, but obtaining the necessary DNA can be highly time- and resource-intensive. In vitro testing needs can be minimized in silico via eDNAssay (Kronenberger et al. 2022)—a machine learning classifier that has been trained on empirical qPCR results and is highly accurate at predicting assay specificity (Kronenberger et al. 2022). eDNAssay assigns each template a probability of belonging to the "amplify" class with a default threshold of 0.5. This threshold may be lowered to hedge against false negative errors when a template is predicted to *not* amplify but does amplify.

We used a combination of in silico and in vitro testing to validate the ocelot ND5 assay. Generality was demonstrated in silico via eDNAssay and in vitro by testing genomic DNA. Specificity was demonstrated in silico using eDNAssay to predict the amplification of one representative sequence from all non-target species with valid sequences on GenBank (ncbi.nlm.nih.gov/genbank). Species with maximum assignment probabilities < 0.3 were assumed to not amplify and were not necessarily tested in vitro. Species maximum assignment probabilities between 0.3 and 0.7 were tested in vitro using genomic DNA or synthetic gene fragments representing at least one sequence.

Testing summary

Target in silico *testing* The assay matches three of five ocelot mitochondrial genome sequences available in GenBank. The two sequences that failed to match were divergent and had low eDNAssay assignment probability of < 0.4. Accession

Table 9 Testing summary for the non-target confamilial species of potential concern

Species	Co-occurring	In silico tests	Max AP	In vitro tests	In vitro results
Leopardus pardalis		3	0.968	6	Amp
Acinonyx jubatus		4	0.269	1	No amp
Felis catus		128	0.18	4	No amp
Leopardus colocola		1	0.283	–	–
Leopardus geoffroyi		2	0.166	–	–
Leopardus guigna		3	0.122	–	–
Leopardus jacobita		3	0.228	–	–
Leopardus tigrinus		6	0.299	–	–
Leopardus wiedii		2	0.269	–	–
Leptailurus serval		2	0.179	1	No amp
Lynx canadensis		4	0.18	1	No amp
Lynx rufus		5	0.278	4	No amp
Panthera leo		5	0.182	1	No amp
Panthera onca		18	0.303	4	No amp
Panthera pardus		6	0.128	1	No amp
Panthera spelaea		6	0.167	–	–
Panthera tigris		49	0.153	1	No amp
Panthera uncia		5	0.141	–	–
Puma concolor		26	0.258	4	No amp
Puma yagouaroundi		2	0.15	1	No amp

For each non-target species, we specify: (1) filled boxes for co-occurring indicates that the non-target species may naturally co-occur or be introduced to the native range of ocelot (filled = present), (2) number of sequences tested in silico, (3) maximum eDNAssay assignment probability, (4) number of samples tested in vitro, and (5) the outcome of any in vitro testing

MW257208.1 (Hassanin et al. 2021) is labeled "*Leopardus pardalis mitisi*" and collection locality is noted as French Guiana. Due to the findings of the Cat Classification Task Force of the IUCN/SSC Cat Specialist Group (2017), we do not find this divergence to be problematic since we are targeting *L.pardalis* in the northern portion of its range. Additionally, accession KR132582.1 (Paijmans et al. 2016) may not be *Leopardus pardalis* at all. A GenBank BLAST of the COI sequence from this partial mitochondrial genome indicates top hits for *L. tigrinus*, *L. colocola*, and *L. wiedii* (94.2–99.6% identity) before the next most similar *L. pardalis* (93.9% identity). *L. pardalis* has low genetic structure in the northern portion of its range, particularly in the United States (Janecka et al. 2014). Accession OR822032.1 (Lescroart et al. 2023) is labeled "*Leopardus pardalis pardalis"* the sub-species known to occupy Mexico and the southern United States. With a maximum assignment probability of 0.968, we find strong evidence that the assay can detect *L. pardalis* throughout the northern extent of their range.

Target in vitro *testing* Five cotton swabs with saliva from two ocelots kept at Panther Ridge Conservation Center (Florida) were extracted with a QIAGEN DNeasy Blood and Tissue Kit and four microliters of elution were tested in triplicate for each swab. Both individuals were previously captive animals in the United States without available breeding records. Two of the five samples from Panther Ridge Conservation Center amplified. Additionally, an ocelot sample from Texas A&M University was tested and did not amplify.

Non-target in silico *testing* We compared the assay sequence with a representative sequence from all felids found in the United States, as well as several felids found in Mexico and three African/Asian taxa which may be found in captivity in the United States (Table 9). We confirmed assay specificity by using eDNAssay—a machine learning classifier that has been trained on empirical qPCR results and is highly accurate at predicting assay specificity (Kronenberger et al. 2022). We used sequences from all closely related species found within the target's native range (Table 9). Of the 19 non-targets tested in silico, maximum eDNAssay assignment probabilities were ≥ 0.3 for 1 species (6%).

Although we used eDNAssay with sequences from closely related species in the United States to ensure specificity, it is possible that rare, undescribed, or introduced taxa may pose unexpected issues. To identify a broader suite of species that may cause spurious detections, we used Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast; Ye et al. 2012) with default settings to query all sequences contained in the NCBI nucleotide database. Ten non-target species were flagged, Pampas cat (*Leopardus colocola*), oncilla (*Leopardus tigrinus*), Andean Mountain cat (*Leopardus jacobita*), margay (*Leopardus wiedii*), bobcat (*Lynx rufus*), Pallas's cat

(*Otocolobus manul*), leopard cat (*Prionailurus bengalensis*), fishing cat (*Prionailurus viverrinus*), Iriomote cat (*Prionailurus iriomotensis*), flat-headed cat (*Prionailurus planiceps*). These species were tested using eDNAssay in which only one, Pallas's cat (*Otocolobus manul*), received a maximum assignment probability ≥ 0.3.

Non-target in vitro *testing* We confirmed assay specificity in vitro using genomic DNA or synthetic gene fragments from: cheetah (*Acinonyx jubatus;* n = 1), domestic cat (*Felis catus;* n = 4), serval (*Leptailurus serval;* n = 1), Canada lynx (*Lynx canadensis;* n = 1), bobcat (*Lynx rufus;* n = 4), lion (*Panthera leo;* n = 1), jaguar (*Panthera onca;* n = 4), leopard (*Panthera pardus;* n = 1), tiger (*Panthera tigris;* n = 1), mountain lion (*Puma concolor;* n = 4), and jaguarundi (*Puma yagouaroundi;* n = 1). There were no amplifications from any of the above non-target species.

Assay in situ *testing* Non-invasive samples (swabs) from two ocelots kept at Panther Ridge Conservation Center (Florida) were used for in vitro testing (described above). Additionally, seventeen samples from the Huachuca Mountains were run using this assay, of which none amplified (Table 9).

Assay specificity

The assay is specific against all non-targets species that may co-occur within *Leopardus pardalis* native range.

Conclusion

Following assay validation, we believe the ocelot assay can be confidently applied throughout the species' native range in Texas and Arizona. The assay does not appear to be general given in silico testing, although some uncertainty exists regarding the number and source of target individuals assessed. Furthermore, assay specificity against all closely related species co-occurring within the target area has been either directly confirmed in vitro or strongly suggested in silico via sequence divergence between non-targets and assay oligonucleotides.

The ocelot assay is likely to detect *Leopardus pardalis* at the northern extent of their range in Mexico and the southern United States. However, there is a degree of ambiguity concerning detections with this assay in the southern portion of their range. This is due to the divergence of *L. pardalis* into two potential subspecies: *Leopardus pardalis pardalis* (Northern portion of range; type locality Mexico) and *Leopardus pardalis mitis* (Southern portion of range; type locality Brazil). We predict that the assay will lose detection sensitivity at the southern portion of the species range where this potential split occurs. Our prediction is substantiated by the above in silico and in vitro testing.

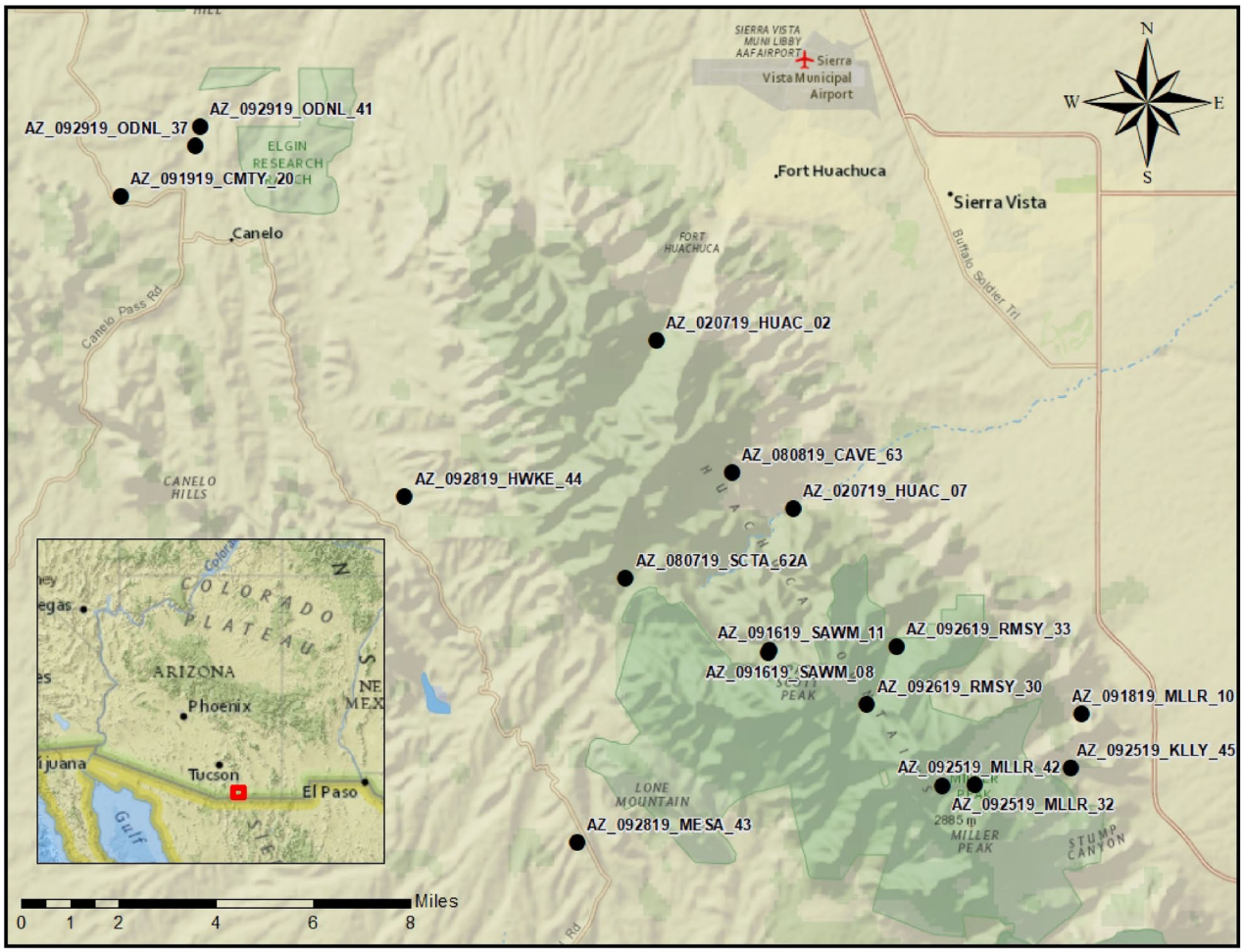
Accession MW257208.1 (Hassanin et al. 2021), labeled "*Leopardus pardalis mitisi,*" received an assignment probability of 0.359, making it unlikely to amplify, whereas accession OR822032.1 (Lescroart et al. 2023), labeled "*Leopardus pardalis pardalis,*" with an assignment probability of 0.968, is highly likely to amplify. In vitro testing reinforced this prediction by amplifying only two out of the six samples tested. Tested samples came from extracted saliva from two ocelots kept at Panther Ridge Conservation Center. Notably, two samples from one ocelot amplified, while three samples from the other ocelot did not. Although the ocelots' origins remain unknown since they were rescued from the pet trade, inference from the in silico testing suggests that the ocelot that amplified likely belongs to the northern subspecies *Leopardus pardalis pardalis.*

While there is always the possibility of unknown polymorphisms affecting assay performance, the rigorous validation this assay has undergone should legitimize its use within the target species' native range.

Ocelot follow-up testing

An initial ocelot assay was created targeting the mitochondrial gene NADH-ubiquinone oxidoreductase chain 4 (*ND4*). Upon its implementation via high-throughput quantitative polymerase chain reaction (HT-qPCR), we detected a pervasive contamination signal across samples, including negative controls. Additionally, the assay exhibited spurious amplification in environmental samples when applied on the ST-qPCR platform. Despite thorough investigations, the root cause of this issue remained elusive, highlighting the assay's suboptimal performance. As a result, we developed a new ocelot assay. The new assay (described above) was created targeting the mitochondrial gene NADH-ubiquinone oxidoreductase chain 5 (*ND5*) and then used to re-analyze a regionally relevant subset of environmental samples to resolve ocelot presence/absence data.

We selected 17 samples from the Huachuca Mountains, the sole habitat with purported ocelot presence within our study area. These samples were run on the ST-qPCRplatform. ST-qPCR reactions were run on StepOnePlus™ and QuantStudio™ 3 Real-Time PCR Systems (Thermo Fisher Scientific) in triplicate. Each well contained 7.5 µL of TaqMan® Environmental Master Mix 2.0 (2×; Thermo Fisher Scientific), 4 µL of target DNA, and 0.75 µL of assay mix, diluted to 15 µL with sterile water. The thermal cycling profile was 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The environmental samples were only handled in dedicated laboratory facilities where no high-quality DNA or PCR products are handled. Each reaction was run in triplicate and the plate contained triplicate positive and negative controls for each assay in accordance with the National Genomics Center for Wildlife and Fish Conservation eDNA Program Standard Operating Procedure. None of the 17 environmental samples amplified for ocelot when tested using the new assay.



Acknowledgements This work was in part supported by a co-led project with the authors as well as Rick Lance, Michael Young, and Kevin McKelvey (SERDP RC18-1348) who all contributed to initial study conceptualization. Field sampling was conducted by the authors as well as Rick Lance, Jennifer Hernandez, Rodrigo Sedeno, Alison Ochoa, Katherine Zarn, Joanna Elmore, and Daniel Vanderpool (USDA Forest Service), Hannah Telle (Camp Navajo), Jeff Sorensen (Arizona Game and Fish), Lyndsay Smithe (US Fish and Wildlife Service), and Wiebke Boeing and Lauren Samaniego (New Mexico State University). In addition, access and logistical coordination for Department of Defense and private lands by USDA Forest Service crews as part of this project were facilitated by Debbie Brewer (Fort Huachuca), Trish Cutler (White Sands Missile Range), Anna Johnson (Nellis AFB), Zackary Bowers (Fallon NAS), Hannah Telle (Camp Navajo), and Peter Leiterman (The Nature Conservancy). Samples from the Bill Williams were provided by Tiffany Love-Chezem and Chase Ehlo (US Fish and Wildlife Service). Both John Kronenberger and Joanna Elmore contributed insight and lab work in support of assay design and HT-qPCR protocol refinement. In addition, this manuscript was improved by input from project partners including those listed above, as well as Audrey Owens (Arizona Game and Fish) and Gita Bodner (The Nature Conservancy). This manuscript was improved by comments provided by Joanna Elmore.

Author contributions Michael Sleeting, Taylor Wilcox, and Michael Schwartz contributed to study conception and design for regional field sampling and HT-qPCR chip validation. Daniel Mason and Thomas Franklin conceived and executed validation of eDNA assays for Ranavirus and amphibian chytrid fungus. Material preparation, data collection, and analyses were performed by Michael Sleeting, Taylor Wilcox, Daniel Mason, and Thomas Franklin. The first draft of the manuscript

was written by Michael Sleeting and Taylor Wilcox and all authors contributed to additional writing and editing in subsequent drafts. All authors read and approved of the final manuscript.

Funding This work was funded by the Department of Defense Strategic Environmental Research and Development Program (SERDP RC18-1348) and the USDA Forest Service Rocky Mountain Research Station.

Data availability All assay validation is detailed in Appendix 1 and the underlying sequence data are available from GenBank. All eDNA sampling results approved for public release by partners are scheduled for the 2025 Aquatic eDNAtlas update (Young et al. 2018).

Declarations

Conflict of interest The authors declare no competing interests.

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