Two novel qPCR assays to enhance black rail (*Laterallus jamaicensis*) eDNA surveys in the United States

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Abstract

Black rails (*Laterallus jamaicensis*) are a rare and protected bird species. They are also notoriously difficult to monitor. eDNA surveys have proven beneficial for the detection of this elusive species, but additional developments are needed to enhance eDNA survey utility (including reliability of outcomes) in light of black rail status and associated high-profile conservation actions. The use of multiple assays functions to increase eDNA detection confidence by increasing the probability of amplification in low concentration, highly degraded samples (i.e., reducing the risk of false negatives) and by providing a multiplicative test for ruling out contamination as the source of qPCR amplification (i.e., reducing the risk of false positives). The two eDNA assays developed, optimized, and validated in this study will prove valuable for the continued surveillance of the secretive black rail, offering data to measure its distribution and occurrence over time and as influenced by anticipated climate-change-induced disturbances and/or habitat restoration/creation initiatives. The assays are applicable to both Eastern black rails and California black rails, and do not exhibit qPCR amplification in non-target, co-distributed taxa (including other Rallidae species) in the United States.

Keywords Bird · eDNA · qPCR · Rallidae · Survey · Syringe

Introduction

The black rail (*Laterallus jamaicensis*; BLRA) is a rare and elusive, sparrow-sized marsh bird protected by the United States Fish and Wildlife Service (USFWS) and the Endangered Species Act (ESA; USFWS 2020). Over the last 20 years, BLRA has experienced substantial habitat loss, dramatic declines in population size, and significant contractions in distribution (Watts 2016; USFWS 2019). The distribution and site occupancy of BLRA is expected to continue shifting over time. One major factor influencing these shifts is climate change, which will impose risks to BLRA survival via anticipated increases in both habitat disturbance and habitat loss (USFWS 2019). Initiatives to restore or create BLRA habitat provide, on the other hand, countermeasures to this anticipated loss, and have been outlined as priority conservation actions (Atlantic Coast Joint Venture 2020). Consequently, there is a crucial need to monitor the presence of BLRA—and the success of conservation actions—through time, as well as across different habitat types (i.e., natural vs. created). Methods currently employed in BLRA surveillance (e.g., call-back surveys) provide, however, rather low detection rates and require substantial effort even under ideal survey conditions (e.g., Legare et al. 1999; Butler et al. 2015; Tolliver et al. 2019). As such, development of sensitive, highly efficient surveillance methods are needed to assist BLRA monitoring needs.

Survey methods targeting eDNA (Pawlowski et al. 2020; Rodriguez-Espeleta et al. 2021) have proven to be effective for many hard-to-detect taxa, including numerous imperiled species (e.g., Akre et al. 2019; Wineland et al. 2019; Lor et al. 2020). To-date eDNA surveys have been underutilized in birds (see Fig. 2 in Beng and Corlett 2020), but avian-focused methods are emerging (e.g., Anaya 2018; Ushio et al. 2018; Day et al. 2019; Rahlin et al. 2020; Schütz et al. 2020). In fact, one publication already details the development of a successful BLRA eDNA effort (Niece and McRae 2021). Here, the authors used gold standard eDNA approaches (Goldberg et al. 2016), employing extremely...
sensitive qPCR, and developing a single, BLRA-specific qPCR assay (i.e., primers and probe) targeting CoxI.

Surveys utilizing a single eDNA assay are common. Yet, the use of multiple eDNA assays may be especially beneficial when monitoring high-profile species, and where survey outcomes are often linked to costly management implications. Evidence suggests that the concurrent use of multiple eDNA assays (targeting different gene fragments) functions to increase confidence in eDNA survey results in two important ways. First, the incorporation of multiple assays improves the power of eDNA surveys by decreasing the risks of non-detection (i.e., not detecting an organism of interest despite the organism’s presence in the sampled environment) which can occur as a result of eDNA degradation/fragmentation, eDNA concentration, assay efficiencies, and/or PCR inhibition (e.g., Lance and Guan 2020; Wood et al. 2020). Second, multiple assays can be used as independent tests of detection, where qPCR amplification across different species-specific gene targets provides a multiplicative increase in detection certainty (Sepulveda et al. 2020). This helps to rule out contamination as the source of amplification, thereby reducing the risk of false positives (i.e., detecting the organism of interest even though the organism is absent in the sampled environment). Both risks (non-detection and false positives) contribute to eDNA uncertainty and potentially erode trust in eDNA survey outcomes (Darling et al. 2021; Jerde 2021). Thus, we aimed to develop additional BLRA eDNA assays that, in conjunction with the existing Neice and McRae (2021) assay, substantially increase the power of eDNA tools to serve BLRA conservation and management.

Methods and results

Tissue (DNA) samples

Twenty-seven tissue samples representing 14 Aves species were acquired from the genetic resource collections of five museums (Table 1). Eight of these tissue samples were BLRA. The others belonged to 13 non-target species (NTS; Table 1) with distributions overlapping that of BLRA. Different samples were used throughout different stages of assay development, as indicated in the text below. Genomic DNA was extracted from all tissues using Qiagen DNeasy Blood and Tissue Kits, following the manufacturer’s protocol.

| Target | Species common name | Species scientific name | Loaning institution: institution’s accession # for each supplied tissue | Specimen origin |
|--------|---------------------|------------------------|---------------------------------------------------------------------|----------------|
| Black rail | Laterallus jamaicensis | FLMNH: 71852, 81777 | CA, FL, LA, TX |
| Non-target, Rallidae | American coot | Fulica americana | MVZ: 189449 | CA |
| | Clapper rail | Rallus crepitans | LSU: B-3725, B-10168 | CA, LA, MS |
| | Common gallinule | Gallinula galeata | SDNHM: 52165 | CA |
| | King rail | R. elegans | LSU: B-20977, B-28944 | LA |
| | Purple gallinule | Porphyrio martinica | FLMNH: 71652, 96637, 96962 | FL |
| | Ridgway’s rail | R. obsoletus | SDNHM: 52750, 53162 | CA |
| | Sora | Porzana carolina | SDNHM: 54148 | CA |
| | Virginia rail | R. limicola | SDNHM: 54296 | CA |
| | Yellow rail | Coturnicops noveboracensis | FLMNH: 91692 | FL |
| Non-target, Non-Rallidae (Aves) | Belted kingfisher | Megaceryle alcyon | LSU: B-38699 | TX |
| | Hooded merganser | Lophodytes cucullatus | LSU: B-3998 | LA |
| | Marsh wren | Cistothorus palustris | LSU: B-47132 | TX |
| | Ringed kingfisher | Megaceryle torquatus | LSU: B-41615 | Panama |
Inclusion of the Neice and McRae (2021) CoxI assay

We included the Neice and McRae (2021) CoxI assay in all stages of this project, beginning at in vitro checks for specificity and continuing through eDNA sample analysis. We refer to it as Neice-CoxI.

Generation of mitogenome data

Sequence data representing BLRA mtDNA is sparse within public repositories (e.g., NCBI GenBank, Sayers et al. 2020). Thus, we sequenced the mitogenomes of four BLRA from across four states within the current distribution so as to improve candidate options for eDNA assay development. We also attempted to sequence the mitogenomes of three NTS, as these would prove useful for increasing BLRA specificity: Ridgway’s rail (Rallus obsoletus; RIRA), Virginia rail (Rallus limicola; VIRA), yellow rail (Coptruncops noveboracensis; YERA). In the end, one complete mitogenome was accessioned into Genbank for BLRA (OM677841), and partial mitogenomes (d-loop/control region absent) was accessioned into Genbank for BLRA (OM992118 and OM992117, respectively). For details regarding methods used in this effort, please see Suppl. File, Supporting Text and Table S1.

eDNA assay design and candidate assay specificity checks (in silico + in vitro validation)

The Primer3 design tool in Geneious was used to search for and design candidate BLRA-specific eDNA assays according to guidance outlined in Klymus et al. (2020a). Numerous gene regions were assessed for suitability using alignments containing the mitogenomes produced for this project, all publicly available BLRA mtDNA sequences, and publicly available mtDNA for NTS. Following close inspection, we narrowed interest to CoxI, ND2, ND4, and ND5.

In addition to visual inspection of our Geneious alignments—and in accordance with design guidance—candidate assays were tested in silico for BLRA specificity using both BLAST and Primer-BLAST (Ye et al. 2012). In BLAST, we used three different algorithms to search for sequences that were highly similar, somewhat similar, and more dissimilar. For Primer-BLAST, under Primer Pair Specificity Checking Parameters, we set the query to (nr), and searched both the Aves and Rallidae organismal databases (separately). In all steps, we searched and opted for assays that maximized the number of nucleotide mismatches between BLRA and co-distributed NTS, with particular focus on utility/specificity for BLRA in the United States (US).

Using a ViiA 7 Real-Time PCR System (Applied Biosystems; hereafter ViiA7) and diluted DNA from 13 NTS (Table 1), candidate assays and Neice-CoxI underwent in vitro checks for BLRA specificity. Each NTS was evaluated for qPCR amplification using triplicate reactions, Taqman Environmental Master Mix (EMM; Applied Biosystems), an annealing temperature of 60 °C, and 40 to 45 qPCR cycles. Our candidate CoxI assay proved to be nonspecific, amplifying in Hooded Merganser (Lophodytes cucullatus). Candidate assays targeting ND5 proved to be either extremely inefficient (large cycle threshold (C_T) values; this was observed for a probe modified with locked nucleic acid, or LNA, bases) or amplified in a number of NTS. Thus, candidate CoxI and ND5 assays were removed from further testing and optimization.

eDNA assay optimization and evaluation

Assays targeting ND2 and ND4 (plus Neice-CoxI; Table 2) underwent optimization for primer and probe concentrations with primers at 200, 300, and 400 nM, and probes at 25% and 50% of the primer concentration, using a 20 µl (total volume) EMM chemistry and 0.8 ng BLRA DNA. The ViiA7 cycling protocol followed EMM recommendations (10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s). Each assay concentration was analyzed in triplicate. Differences were negligible across and within tested concentrations (e.g., observed cycle threshold values, variation across replicates of a concentration). Thus, in all subsequent qPCRs, and for all three assays, we used a concentration of 200 nM primer and 50 nM probe.

We evaluated the efficiency and sensitivity of our assays using a gBLOCK synthetic DNA standard. The gBLOCK contained our target eDNA sequences and was serially diluted, producing a series of decreasing concentrations. For each assay: A standard curve was generated using six gBLOCK concentrations (31,250, 6250, 1250, 250, 50, and 10 copies per reaction) at three qPCR replicates each; the limit of detection (LOD) was determined using 24 replicate qPCRs for each of four gBLOCK concentrations (16, 8, 4, and 2 copies per reaction); the limit of quantification (LOQ) was determined using eight replicate qPCRs for each of eight gBLOCK concentrations (128, 64, 32, 16, 8, 4, 2, and 1 copies per reaction). We defined and calculated each assay’s LOD and LOQ following Klymus et al. (2020a, b).

Here and throughout eDNA analysis, each qPCR was prepared in a UV–sterilized hood within a room separate from PCR machinery and free of amplified DNA. Total qPCR volumes equaled 17 µl, and consisted of 10 µl EMM, 0.4 µl of each 10 µM primer (forward and reverse), 0.2 µl of 5 µM probe, 5 µl PCR grade H2O, and 1 µl gBLOCK (at each of the pre-defined, diluted concentrations). The cycling protocol began with 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Amplification and subsequent analyses occurred on ViiA7, with critical thresholds...
of fluorescence automatically determined by Viia7 software (i.e., not manually set/adjusted).

**Assay efficiencies and sensitivities**

Table 3 provides outcomes of the efficiency and sensitivity tests for all assays. Our novel ND2 and ND4 assays performed equally well, with LODs observed at 16 copies per reaction. The LOD was indeterminable for Neice-CoxI using the gBLOCK concentrations employed in this study, but evidence from our evaluation suggests the detection limit is > 250 copies per reaction (with no amplification observed in dilutions ≤ 250 copies per reaction); this result aligns with published sensitivity calculations for the assay, where Neice and McRae (2021) observed an LOD and LOQ of 109 and 901 copies per reaction, respectively.

**Field samples and in situ validation of optimized eDNA assays**

Ninety eDNA samples (plus field negative controls) were collected from 10 unique sites across four US locations within the current BLRA distribution (Table 4). Of these four locations, two had known recent BLRA occupancy (detected via vocalization). Due to habitat characteristics (including both the availability and depth of water), different sites were more amenable to different eDNA capture methods. As such, samples were collected in the form of water (in both small and large volumes; see next two paragraphs for details) and in the form of 1 ml surface soil (Table 4). All water was filtered on-site, with eDNA filters subsequently folded and preserved in silica beads. All soil samples were preserved in 5 ml Longmire buffer. Regardless of method, and to prevent cross-contamination, we ensured bleach-sterilized equipment and/or single-use consumables were used between locations and sites.

During early stages of sampling, we collected large volume water samples using a battery-powered vacuum pump and filter apparatus, following methods similar to Carim et al. (2016) with the filter apparatus placed directly into marsh habitat (typically large bodies of standing water). The actual volume of water successfully filtered varied (Table 4) and was largely dependent on (1) the sampling effort (where goals for total volumes differed), as influenced by (2) the amount of water each filter could tolerate before clogging (likely a result of both habitat and filter traits). Our earliest sampling effort utilized 47 mm 0.2 micron cellulose nitrate (CN) filters. Here, we aimed to collect 5 l of water, using

| Assay                              | Sequence (5′ to 3′)                                      |
|-----------------------------------|--------------------------------------------------------|
| **Novel BLRA ND2 assay (178 bp)** | CAGCTCCTACTACTAAACAGCC                                  |
| 278F                              | FAM-TGATTCCCA-ZEN-GAAACCTCCAAAGGT-3IABkFQ              |
| 340P                              | GTTGGGTTAAGTGAGTGTGAGGT                               |
| 455R                              |                                                        |
| **Novel BLRA ND4 assay (131 bp)** | GCCTCAACTGACACACAGC TG                                 |
| 76F                               | HEX-CGCCACCTT-ZEN-AAGCCTCAGTGT-3IABkFQ                |
| 102P                              | GATAGTGAGTAGTGAGGGATGAG                               |
| 206R                              |                                                        |
| Neice and McRae (2021) published BLRA CoxI assay (219 bp) | COI2F CTTCCCTCTCTTTCCTGCT                              |
|                                  | AP probe HEX-ctaCTaGcttCAT-ABCkFQ                       |
|                                  | COI2R GGATGTGCWGTTGTTTATA                               |

For the Neice and McRae (2021) CoxI assay, LNA technology (IDT Affinity Plus) is denoted within the probe by uppercase nucleotides.

### Table 3

Efficiency and sensitivity of two novel and one published (Neice and McRae 2021) black rail eDNA assays, as based on serial dilutions of gBLOCK DNA

|                  | Slope  | Y-intercept | R²     | Efficiency (%) | Limit of detection (LOD), mean C_T | Limit of quantification (LOQ) |
|------------------|--------|-------------|--------|----------------|-----------------------------------|-----------------------------|
| ND2              | − 3.176| 39.23       | 0.995  | 106.45         | 16 copies, 36.91                   | 64 copies                   |
| ND4              | − 3.217| 40.20       | 0.989  | 108.81         | 16 copies, 37.29                   | 64 copies                   |
| Neice-CoxI       | − 4.069| 51.12       | 0.983  | 76.10          | ND, but > 250 copies               | ND, but > 250 copies        |

The y-intercept represents the expected critical threshold (C_T) value for a qPCR containing 1 copy of the target fragment, and directly relates to assay performance (not necessarily eDNA outcomes). ND, not determinable within serial dilution series utilized.

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Table 4 Ninety black rail eDNA samples were collected at four locations and 10 unique sites

| Sample Type | Total Volume (mL) | Total # of filters | + for BLRA eDNA | + with ND2 | + with ND4 | + with Neice-CoxI |
|-------------|------------------|--------------------|----------------|------------|------------|------------------|
| Imperial National Wildlife Refuge (Yuma, AZ), n = 16 |
| Unique Site A |
| 1 | Oct 2019 | Pump1 | 4750 | 3 | CTAB | No |
| 2 | Oct 2019 | Pump1 | 3500 | 3 | CTAB | Yes | 1/6, C<sub>1</sub> = 45 | 0 | 0 |
| 3 | Oct 2019 | Pump1 | 3000 | 3 | CTAB | No |
| 4 | Oct 2019 | Pump1 | 2700 | 3 | CTAB | No |
| 5 | Oct 2019 | Pump1 | 250 | 1 | CTAB | No |
| 6 | Oct 2019 | Pump1 | 5000 | 1 | CTAB | No |
| 7 | Oct 2019 | Pump1 | 5000 | 1 | CTAB | No |
| 8 | Oct 2019 | Pump1 | 5000 | 3 | CTAB | No |
| 9 | Oct 2019 | Pump1 | 5000 | 1 | CTAB | No |
| 10 | Oct 2019 | Pump1 | 1500 | 2 | CTAB | No |
| 11 | Oct 2019 | Pump1 | 5000 | 1 | CTAB | No |
| 12 | Oct 2019 | Pump1 | 4800 | 2 | CTAB | No |
| 13 | Oct 2019 | Pump1 | 5000 | 1 | CTAB | Yes | 0 | 1/6, C<sub>1</sub> = 43 | 0 | 0 |
| 14 | Oct 2019 | Pump1 | 5000 | 2 | CTAB | Yes | 2/6, C<sub>1</sub> = 41 | 0 | 0 |
| 15 | Oct 2019 | Pump1 | 5000 | 2 | CTAB | No |
| 16 | Oct 2019 | Pump1 | 5000 | 3 | CTAB | No |
| Sonny Bono Salton Sea National Wildlife Refuge (Calipatria, CA), n = 2 |
| Unique Site C |
| 17 | Oct 2019 | Pump1 | 2000 | 3 | CTAB | Yes | 1/6, C<sub>1</sub> = 45 | 1/6, C<sub>1</sub> = 44 | 0 |
| 18 | Oct 2019 | Pump1 | 150 | 3 | CTAB | Yes | 3/6, C<sub>1</sub> = 43 | 5/6, C<sub>1</sub> = 41 | 0 |
| Private Property* (Cameron Parish, LA), n = 29 |
| Unique Site D |
| 19 – 47 | Sept 2021 | Soil | 1 each | X | MPS | Yes | 1/6, C<sub>1</sub> = 39 | 0 | 0 |
| Galveston Island State Park* (Galveston, TX), n = 43 |
| Unique Site E |
| 48 | Aug 2021 | Pump2 | 1930 | 1 | GMD | No |
| 49 | Aug 2021 | Pump2 | 2060 | 1 | GMD | No |
| Unique Site F |
| 50 | Aug 2021 | Pump2 | 2420 | 1 | GMD | No |
| 51 – 52 | Jan 2022 | Soil | 1 each | X | MPS | No |
| Unique Site G |
| 53 | Aug 2021 | Pump2 | 1000 | 1 | GMD | No |
| 54 | Aug 2021 | Pump2 | 1710 | 1 | GMD | No |
| 55 | Aug 2021 | Pump2 | 1730 | 1 | GMD | No |
| 56 – 60 | Jan 2022 | Soil | 1 each | X | MPS | No |
| Unique Site H |
| 61 | Aug 2021 | Pump2 | 930 | 1 | GMD | No |
| 62 – 66 | Jan 2022 | Soil | 1 each | X | MPS | No |
| 67 | Jan 2022 | Syringe | 50 | 2 | GMD | Yes | 2/6, C<sub>1</sub> = 40 | 0 | 0 |
| 68 | Jan 2022 | Syringe | 55 | 2 | GMD | No |
| 69 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| 70 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| Unique Site I |
| 71 – 78 | Jan 2022 | Soil | 1 each | X | MPS | No |
| 79 | Jan 2022 | Syringe | 50 | 1 | GMD | No |
| 80 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| 81 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| Unique Site J |
| 82 – 86 | Jan 2022 | Soil | 1 each | X | MPS | No |
| 87 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| 88 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| 89 | Jan 2022 | Syringe | 120 | 1 | GMD | No |
| 90 | Jan 2022 | Syringe | 110 | 1 | GMD | Yes | 1/6, C<sub>1</sub> = 45 | 0 | 0 |
up to three filters. In a subsequent large volume effort, we utilized 47 mm 5.0 micron polyethersulphone (PES) filters, and aimed to filter 2 l of water using a single filter.

During later stages of sampling, and to optimize field methods using guidance from a BLRA expert, we attempted to (1) collect eDNA samples using 100 m transects (as based on home range sizes), and (2) collect soil and water samples side-by-side from suspected BLRA microhabitat (identified primarily as based on elevation gradients and vegetation, including marsh grasses, Spartina spp., salt grass, Distichlis spicata, and sea oxeye, Borrichia frutescens). Here, for water, we collected small volume samples (up to 120 ml, again dependent on filter clogging; Table 4) from puddles underlying suspected BLRA vegetative cover, or from the edge of small, shallow pools. We used (for each sample) two pulls of a 60 ml syringe fitted with a Pall Easy Pressure Syringe Filter Holder (PN 4320) housing a 25 mm mixed cellulose ester (MCE) filter with a 0.45 micron pore size (Millipore HAWP02500). Similar syringe methods have proved successful in other, recent eDNA studies (e.g., Barata et al. 2021). We anticipated that this method would be well-suited for targeted BLRA eDNA sampling as these small birds avoid entering large pools of water.

eDNA was extracted from field samples using three modified methods (Table 4): cetyltrimethyl ammonium bromide (CTAB); Doyle and Doyle 1987; Guan et al. 2019), Qiagen DNeasy Blood and Tissue Kit (Goldberg et al. 2011), and Qiagen DNeasy PowerSoil (Suppl. File, Supporting Text 2). At least one negative control was created for each set of extractions.

To detect BLRA, each eDNA sample underwent sextuplicate qPCR across all three assays (18 total replicates). We followed the qPCR chemistry volumes and ViiA7 cycling profile outlined for the efficiency and sensitivity tests (above), with two adjustments: instead of using 1 µl gBLOCK, we used 2 µl of eDNA extract (for a total volume of 18 µl), and we increased the number of qPCR cycles to 45. All eDNA samples underwent qPCR alongside their relevant field and extraction negatives, with one positive control (BLRA DNA at 0.03 ng/µl) and one negative control (H2O qPCR blank) included per assay. As with eDNA samples, all negative and positive controls underwent sextuplicate qPCR (per assay). A sample was considered positive for BLRA eDNA if at least one replicate amplified above the threshold level of fluorescence within 45 qPCR cycles (CT ≤ 45).

Table 4 (continued) Locations marked with * had known black rail presence. Sample Type: Pump1 = on-site filtering of water using large volumes and a vacuum pump with 0.2 µm cellulose nitrate (CN) filters; Pump2 = on-site filtering of water using large volumes and a vacuum pump with 5.0 µm polyethersulphone (PES) filters; Syringe = on-site filter of water using small volumes and syringes fitted with 0.45 µm mixed cellulose ester (MCE) filters, Soil = soil samples. Extraction Type: CTAB = cetyl trimethyl ammonium bromide; GMD = Goldberg Modified DNeasy; MPS = Modified Power Soil. Details for positive detections (+) are provided, including the number of positives observed (out of six qPCR replicates) for each sample per assay and as the average critical threshold value (CT) observed across associated positive replicates (rounded to nearest whole number). To ease visualization, samples with no positive detections are blacked out.

**eDNA detections**

We detected BLRA eDNA at all four locations sampled, at 5 of the 10 unique sites sampled, and in 8 of the 90 total samples collected across all locations and sites (Tables 4). This equates to detections of 100%, 50%, and 8.89% across locations, unique sites, and samples (respectively). BLRA eDNA was detected in 1 of 29 (3.45%) soil samples and in 7 of 61 (11.48%) on-site filtered water samples. Within the on-site filtered water samples, a wide range of volumes produced positive BLRA eDNA detections (where volumes were dictated by sampling method, i.e., pump vs. syringe, and associated pre-determined criteria for achieved volumes; i.e., not experimentally manipulated): 50 ml, 110 ml, 150 ml, 2 l, 3.5 l, and 5 l (Table 4).

Only two of the eight (25%) positive eDNA samples had detections across multiple assays. These detections were achieved using the two novel qPCR assays developed in this study, with ND2 outperforming ND4 in terms of the number of detections (ND2 produced detections in seven samples, whereas ND4 produced in three; Table 4). No detections were observed using Neice-CoxI. The number of positive qPCR replicates observed for each positive eDNA sample, and at a single assay, ranged from 1 to 5 (out of 6 possible replicates; Table 4), with a single replicate being the common outcome (60% of positive samples). Across all positive samples and all assays, average CT values ranged from 39.38 to 45 cycles (Table 4). All field, extraction, and qPCR negatives performed as expected (no qPCR amplification).

**Confirming eDNA results**

To test for qPCR inhibition, 10% of our non-amplifying eDNA samples (4 water, 6 soil) underwent an additional triplicate reaction using our FAM-labelled ND2 assay and an exogenous internal positive control (Applied Biosystems’ ExoIPC DNA and VIC-labelled Master Mix). No inhibition was observed (as defined by McKee et al. 2015; shift in ExoIPC CT values).
To ensure the correct target (BLRA) amplified, we Sanger sequenced 50% of positive eDNA samples using (FasTAP cleaned) qPCR amplicons as template and BigDye™ Terminator v3.1 Cycle Sequencing Kit with associated manufacturer protocols. Resulting sequences were visualized and aligned in Geneious, then checked for target accuracy using both a BLAST search and alignment with our mitogenome sequences. All detections were confirmed as BLRA.

**Discussion**

We designed and tested two qPCR assays for the species-specific detection of BLRA (in the US), and successfully demonstrated their use in BLRA eDNA surveys. Using our novel ND2 and ND4 assays, we detected BLRA eDNA in 100% of locations sampled, in 50% of sites within locations, and in 8.89% of all samples from across the entire eDNA sampling effort. Of the sampling methods used, filtered water samples provided greater detection success than soil samples. While this is contrary to the observations in Neice and McRae (2021), success with water vs. soil eDNA samples is likely site- and season-dependent, with factors such as weather, site attributes, and local BLRA behaviors (as well as surveyor knowledge of these behaviors) influencing outcomes.

Positive BLRA eDNA detections were achieved in both small and large volume water samples. Large volumes did not produce greater detection success than small volumes. In fact, in the eastern portion of the US-based BLRA distribution (where the species is ESA-protected), targeted small-volume-sampling of microhabitat using a syringe method increased detection success (see Galveston results in Table 4). Detection across > 1 assay was a rarity, with only two positive samples amplifying in both ND2 and ND4. Most detections produced only one qPCR amplification, out of a possible six replicates per assay. Although replicate success was low within positive eDNA samples, observed CT values indicate that our assays were performing at the limits of detection and in the presence of extremely low concentrations of BLRA eDNA—highlighting their sensitivity.

Despite similar performance in laboratory testing between our ND2 and ND4 assays, ND2 provided the greatest number of field-based BLRA eDNA detections. Neice-CoxI produced no positive BLRA detections. Sensitivity tests indicate that Neice-CoxI is less efficient than the two assays developed in this study. Yet, Neice and McRae (2021) had considerable success in their field validation, reporting up to 47% detection success across qPCR outcomes (using 50 qPCR cycles and sextuplicate reactions). Our success, even utilizing more sensitive and efficient assays, was much lower at 8.89%. Besides the obvious difference in the number of employed qPCR cycles, we hypothesize that discrepancies in detection success across studies may be attributable to field methodology. For example, Neice and McRae (2021) utilized BLRA experts to strategically collect their eDNA samples, using trail camera data and cues such as foot prints to pinpoint ideal eDNA sampling locations. Our hypothesis is supported by observations in this current study, where positive detections were secured in microhabitats suggested to us by BLRA experts (without the aid of additional cues to pinpoint ideal sampling locations). The hypothesis also emphasizes the importance of collaboration across field and lab disciplines, including utilization of species experts (or their advice) during eDNA sampling.

Our eDNA survey results are comparable to—and even surpass that of—BLRA detection rates using more traditional methods. For example, Tolliver et al. (2019) reported 5.55% detection success with call-playback surveys, where 239 BLRA audio detections were recorded over a 2-year period encompassing 3,425 surveys. Butler et al. (2015) reported 7.41% detection success, with 40 BLRA detections across a total of 540 callback surveys. We note, though, the focus of this project was assay development, with minimal effort placed on optimizing the field aspects of BLRA eDNA survey efforts. Field methodology can contribute substantially to eDNA survey success, and often requires target- and habitat-specific optimization. As such, BLRA eDNA surveys are likely to achieve even greater sensitivity and detection power with additional general and locale-specific sampling optimization.

Based on our results and observations, we suggest that future BLRA eDNA survey efforts (1) consult with/ utilize local species experts when conducting sampling, (2) maximize sampling effort (i.e., quantity of samples), and (3) incorporate multiple BLRA eDNA assays. We also reiterate the importance of eDNA quality assurance and quality control measures, ensuring careful adherence to sterile techniques while in the field and in the lab (including the incorporation of negative controls). By following these recommendations, surveyors can maximize BLRA eDNA detection success and improve utility of eDNA surveys for BLRA conservation and management purposes.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12686-022-01279-y.

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Park were authorized through Texas Parks and Wildlife Scientific Study Permits #81-21 and #109-21.

Author contributions RFL was responsible for study conception and provided overall guidance, insight, and supervision. SMF was the lead performer for all laboratory and analytical tasks, including design and optimization of assays. Field work was performed by all authors, except XG. XG and MPM assisted with laboratory tasks. The first draft of the manuscript was written by SMF. All authors reviewed and provided comments leading to the final, all-author-approved manuscript.

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Declarations

Competing interest The authors have no relevant interests to disclose.

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