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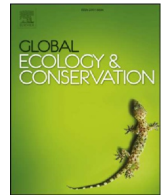
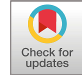


An eDNA diagnostic test to detect a rare, secretive marsh bird



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ABSTRACT

We describe a novel method to detect a rare, secretive marsh bird using environmental DNA (eDNA). The Black Rail (*Laterallus jamaicensis*) occurs in visually concealing habitats and is most commonly surveyed by auditory callback. This method does not detect unresponsive individuals, is constrained seasonally and temporally, and requires significant personnel effort. New minimally invasive detection methods are needed to determine distribution and habitat use of this threatened species. We developed a highly species-specific quantitative PCR assay. We conducted callback surveys targeting Black Rails at sites on the mid-Atlantic coastal plain to collect samples for validation of eDNA diagnostics. Our assay reliably produced a signal when sufficient copies of Black Rail template were present. We successfully amplified Black Rail eDNA from 47% of the environmental samples taken from locations with detections. We tested whether environmental factors (water depth, salinity, air temperature), or sampling and handling procedures (time between collection and DNA extraction, storage temperature before filtering, field detection method, time between detection and sample collection) affected eDNA detectability. Only water depth had a significant positive effect on amplification success, emphasizing the importance of small pools as reservoirs of eDNA for terrestrial vertebrates. Our technique can be used in combination with other conservation strategies such as measuring occupancy in conjunction with habitat restoration efforts and resurveying coastal marshes after extreme weather events. It is adaptable to other elusive species of concern.

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1. Introduction

Animals release DNA into the environment during everyday activities resulting in shedding skin cells or depositing intestinal epithelial cells along with waste (Valière and Taberlet, 2000). This 'environmental' DNA (hereafter, eDNA) can be extracted from a substrate sample to confirm the target organism's presence in the area based on a unique sequence signature. Environmental DNA can provide early detection of small numbers of individuals, such as can be found at the invasion frontiers of two alien species of Asian carp (*Hypophthalmichthys molitrix* and *H. nobilis*) in tributaries of the Great Lakes (Jerde et al., 2011). It has been used successfully to detect the presence of rare species such as the great crested newt (*Triturus cristatus*) in the United Kingdom (Biggs et al., 2015), and holds promise for other elusive species.

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The Black Rail is a sparrow-sized rail found in high marsh (saltmarsh, brackish or freshwater) or infrequently flooded upland wetlands generally dominated by shorter grasses (Flores and Eddleman, 1995). Comparatively little is known of its ecology and behavior because its concealing habitat and secretive nature make visual detection difficult. Black Rails are currently listed as globally Endangered (BirdLife International, 2019). An alarming loss of inland marsh habitat has driven Black Rails, among other wetland species, to fragmented, marginal habitat and to coastal marshes that are impacted by human development and sea level rise (Conway and Sulzman, 2007; Dahl and Stedman, 2013). Having a reliable and time-efficient detection method to assess the range and current occupancy of this species is of critical importance to their persistence.

The Black Rail was recently uplisted federally to Threatened under the Endangered Species Act (U.S. Fish and Wildlife Service, 2018, 2020). Intensive breeding season surveys over much of its historic eastern range in recent years have yielded few detections, and it is suspected to have been extirpated in some states (Watts, 2016). Black Rails are notoriously difficult to detect due to their scarcity, diminutive size, and crypsis. In addition, they are found in habitat that is difficult to traverse. They prefer marshes with relatively shallow water dominated by *Spartina* marsh grass species and have been found to occupy managed impoundments (Roach and Barrett, 2015). Rails are weak flyers and tend to opt for a speedy foot retreat. They only flush when necessary making them even less likely to be detected visually (Stuart, 1920; Davidson, 1992).

Currently, the main method for detecting secretive marsh birds is the standardized North American marsh bird monitoring protocol (Conway, 2011). Surveys are carried out by trained observers in suitable habitat at multiple points. Auditory callback of species-specific calls solicits vocal responses as evidence of occupancy. Surveys must be completed in prescribed time windows during the morning (thirty minutes before sunrise until two hours after) or evening (two hours before sunset until thirty minutes after). Ideally, surveys should be conducted at the same point location three times during the breeding season with at least 10 days between visits. Black Rails vocalize more readily during the breeding season from March to late June (Kerlinger and Wiedner, 1990; Flores and Eddleman, 1995; Spear et al., 1999).

Callback surveys have certain drawbacks. They are less effective during the non-breeding season. They rely on the bird making its presence known through a vocal response, and they are likely only to elicit a response from breeding males (Legare et al., 1999). Their effectiveness relies on the trained surveyor hearing and recognizing a responding individual, and they require the effort of visiting multiple sites repeatedly over an extended period of time. A lab-based molecular detection method could circumvent some of these limitations.

Few studies have so far attempted to detect eDNA from birds. Metabarcoding has been used to detect avian species from eDNA in water. Universal bird primers were designed that targeted the 12S subunit of RNA, and sequences were generated with MiSeq technology (Ushio et al., 2018). The technique was validated using water samples collected from the Yokohama zoo, and confirmed the presence of reads corresponding to the non-native species in the enclosures and not from local wildlife. The zoo sample results corresponded to the species present in the respective enclosures. The presence of a smaller number of reads from species from other enclosures was attributed to the zoo staff transporting DNA and sharing husbandry equipment. They also collected samples from a local pond from which they detected DNA sequences from expected local bird species.

While not the focus of the study, seabirds have been detected in eDNA samples taken off the coast of Denmark that were collected to ascertain the biodiversity of the coastal area and detect a rare vagrant fish, the European pilchard (*Sardina pilchardus*) (Thomsen et al., 2012). In addition to fish, metabarcoding detected red-throated loon (*Gavia stellata*) DNA which was later validated by a bird watch database confirming the species presence during the time of sampling. Seabird eDNA (from cormorants *Phalacrocorax* spp.) was also identified in near shore water samples of California kelp forests (Port et al., 2016). Metabarcoding samples from U.K. pondscapes not only found DNA from aquatic avian species, such as ducks and coots, but also terrestrial species such as jays, owls, and even mammals (Harper et al., 2020). Thus, birds leave detectable amounts of their DNA in the environment.

Environmental DNA has great potential as a tool for identifying habitat and distributions of birds of conservation concern. A recent study sampled drinking water sources from both captive and wild populations of the Gouldian finch (*Erythrura gouldiae*), an endangered species from Australia. In the desert, finch flocks congregate daily at watering holes and the authors collected samples from these water sources to examine the efficacy of using eDNA to detect specifically this species in the wild (Day et al., 2019). They detected Gouldian finch DNA from watering holes where mixed wild finch flocks had been observed. Detectable eDNA persisted in captive finches' water dishes up to 144 h after the dishes had been removed from the enclosure and exposed to sunlight. Another innovative method to collect eDNA non-invasively from birds was to source saliva left on food remains. In Costa Rica, scarlet macaw (*Ara macao*) DNA recovered from partially eaten almond fruits was of sufficient quality to conduct PCR-based sex diagnosis and amplify several microsatellite loci (Monge et al., 2020).

In developing species-specific genetic tools for diploid species, mitochondrial genes have the advantage that individuals carry only one haplotype inherited through the maternal line. Mitochondrial DNA (mtDNA) is highly variable among species, with higher rates of mutation accumulation than nuclear genomes due to haploid inheritance and small effective population size (Neiman and Taylor, 2009). Metabarcoding studies employ mitochondrial sequences that can be amplified with universal primers, are short, and unique to species. Cytochrome c oxidase subunit I (COI), the "bar-coding" gene, works well in distinguishing among species because of its low within-species and high between-species sequence variability (Hebert et al., 2003). Over 260 bird species can be distinguished using one 648-base pair region of the COI gene (Hebert et al., 2004).

Our objectives were to develop and validate a molecular diagnostic test using eDNA to detect Black Rails. To our knowledge, this is the first study to investigate whether DNA from a secretive marsh bird can be detected from environmental samples. Our specific aims were (1) to develop a highly sensitive diagnostic PCR-based test specific to Black Rail DNA, (2) to demonstrate the efficacy of the test using environmental samples collected from locations where occupancy was confirmed, and (3) to identify

Table 1
Primer and probe sequences (5′ - 3′) used in this study that amplify portions of the COI gene in rails. Our final eDNA assay used the BLRA COI2 primers in combination with the BLRA Affinity Plus probe. The '+' signs in the probe sequence indicate the bases to which the Affinity Plus ® (IDT) molecule was added. The 3′ end IABkFQ refers to the Iowa Black ® (IDT) quencher.

Primer or probe name	Sequence
Rail COI primers	Product size: 610 bp
RailCOI1F	ACCTAATCTTTGGGGCCTGA
RailCOI1R	GGGTCGAAGAATGTGGTGTT
Laterallus COI primers	Product size: 591 bp
LaterallusCOIF	AGCCGGCATAATTGGTACTG
RailCOI1R	GGGTCGAAGAATGTGGTGTT
BLRA COI2 primers	Product size: 219 bp
BLRA COI2F	CTTCCTCCCTCTTTCCTGCT
BLRA COI2R	GGATAGTGCGGGTGGTTTTA
Affinity Plus probe (IDT)	
BLRA AP probe	6-FAM-CTA+C+TA+GCTT+C+A+TCA-IABkFQ

factors affecting detectability to be able to make recommendations about sample collection and handling for future eDNA studies.

2. Materials and methods

2.1. Species-specific assay development

The Rallidae family is an ancient bird lineage (Taylor and Van Perlo, 1998) with high sequence divergence from other living bird species. For developing an eDNA diagnostic test, this has the advantage of reduced likelihood of having false positives from other avian DNA. However, universal bird primers for the COI gene published by Hebert et al. (2004) would not amplify rail mtDNA sequences. Therefore, the development of our assay primer design was completed in two stages: first, we designed primers to sequence a large segment of the COI gene for a selection of related rail species that co-occur in North America, then we designed unique primers for the Black Rail.

First, rail-specific primers within the COI gene were designed based on published sequences for rails in GenBank (*Laterallus* and Rail COI primers, Table 1). We used these to sequence part of the COI gene using blood samples from eastern Black Rails contributed by collaborator E. Johnson (Louisiana Audubon). We also sequenced this region from samples of confamilial species from North Carolina: two king rails (*Rallus elegans*) (Brackett et al. 2013), and two clapper rails (*Rallus crepitans)* contributed by a local hunter (G. Huntsman).

We extracted genomic DNA using a DNeasy Blood and Tissue Kit (Qiagen). We amplified 2 μL of DNA template in 25 μL reactions with 11.5 μL nuclease free water, 2.5 μL Apex 10x Mg-free PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer (Laterallus COI or Rail COI), and 1.25 Units Taq Polymerase (Apex, Genesee Scientific). Thermocycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a 72 °C hold for 5 min. We then sequenced the 591 bp product for the eastern Black Rail (*Laterallus* primers, Table 1), and the 610 bp product for the *Rallus* species (Rail COI primers, Table 1). Sanger sequencing was performed using a standard Big Dye protocol on an ABI 3130 Genetic Analyzer.

Sequences were trimmed and aligned to published COI sequences downloaded from GenBank from all other rail species sympatric over at least part of the eastern Black Rail's U.S. range: yellow rail (*Coturnicops noveboracensis*), Virginia rail (*Rallus limicola*), common gallinule (*Gallinula galeata*), sora (*Porzana carolina*), and American coot (*Fulica americana*) (Table A1). Targeting regions of sequence dissimilarity with the other rails, we designed species-specific primers for the Black Rail using the online program Primer3 (Koressaar and Remm, 2007). The selected primer pair amplified a 219-bp segment of the Black Rail COI gene (BLRA COI2; Table 1).

We tested the primers for cross-species amplification with genomic DNA from blood or tissue samples of sympatric rail species (king rail, clapper rail, yellow rail, common gallinule, Virginia rail, sora) using traditional PCR (BioRad T100 thermocycler). The 10 μL reactions included 5.6 μL nuclease-free water (Fisher Scientific), 1X Apex Mg-free PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each BLRA COI2 primer, 0.5 Units Taq Polymerase (Apex, Genesee Scientific), and 2 μL genomic DNA template. Thermal cycling conditions were set at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a 72 °C hold for 5 min. PCR products were visualized by electrophoresis on a 2% agarose gel. The primers were also tested for their detection limits using the same PCR reaction conditions and a dilution series of Black Rail PCR genomic DNA.

2.2. qPCR with probe chemistry yields a diagnostic test for Black Rail eDNA

Quantitative PCR (qPCR) is vastly more sensitive than conventional PCR to low template amount. Early tests of our BLRA COI2 primers in qPCR successfully amplified product from very small amounts of Black Rail genomic DNA template. However, they also amplified DNA from the other rail species we tested. To increase specificity and resolve problems with cross-species amplification, we added a fluorescent probe to be used in conjunction with the BLRA COI2 primers. Our custom hydrolysis probe was designed within the 219-bp product (BLRA AP probe, Table 1) and increased specificity due to requiring complementarity with both primers and the probe. The Affinity Plus probe's Iowa Black dark quencher (Integrated DNA Technologies, proprietary) has locked nucleotide monomers placed strategically at sites on the probe sequence that help stabilize and increase the melting temperature of the probe/template complex (Owczarzy et al., 2011). Signal is produced when a reaction takes place at each cycle that separates the quencher from the reporter causing it to fluoresce, thereby eliminating background signal from non-specific amplification. Separate primer and probe aliquots allowed us to vary their concentrations to optimize the assay.

To quantify eDNA sensitivity with the qPCR assay, a purified product standard was prepared by running a traditional PCR in 25 µL reactions with 11.5 µL nuclease free water, 2.5 µL Apex 10x Mg-free PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each BLRA COI2 primer, 1.25 Units Taq Polymerase (Apex, Genesee Scientific), and 2 µL template (DNA extracted from a single Black Rail blood sample). Approximately 8 µL of the PCR product was visualized on a 2% agarose gel. The remainder was cleaned and purified using a MoBio Ultraclean Gelspin DNA Purification Kit per the manufacturer's instructions. We quantified this with a Qubit 3 fluorometer (Invitrogen) using Qubit high sensitivity dsDNA assay reagents (ThermoFisher). We calculated the number of copies = (DNA amount in ng * 6.022 × 10^{23})/(length in base pairs * 650 g/mole * 10^9 ng/g). The end concentration was 1.29 ng/µL (or 5.46 × 10^9 copies per µL). A standard curve from 2.58 × 10^{-4} ng target DNA (i.e. ~109,145 copies) to 2.58 × 10^{-11} ng target DNA (~1 copy) in 10-fold dilutions was made with which to quantify unknown samples and determine a detection limits for the assay.

To generate quantification standards, fresh purified product was prepared from Black Rail DNA. We ran five replicates of a standard standard dilution series ranging from 109,145 target copies to 1 copy, in 10-fold dilutions. On the same qPCR plate, we tested our panel of non-target species for cross-species amplification: two individuals per species, in duplicate, of king rail, clapper rail, Virginia rail, sora, yellow rail, and common gallinule. Real-time PCR (Bio-Rad CFX Connect Real-Time System) was performed with reactions containing 3.31 µL nuclease free water (Fisher Scientific), 0.75 µL of each 10 µM BLRA COI2 primer (final concentration = 0.625 µM), 0.19 µL of 10 µM Affinity Plus probe (final concentration = 0.156 µM), 5 µL 2X TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 2 µL template (total volume = 12 µL). This resulted in a 0.83X concentration of the environmental master mix. Based on the Applied Biosystems TaqMan protocol, thermal cycling conditions were set at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

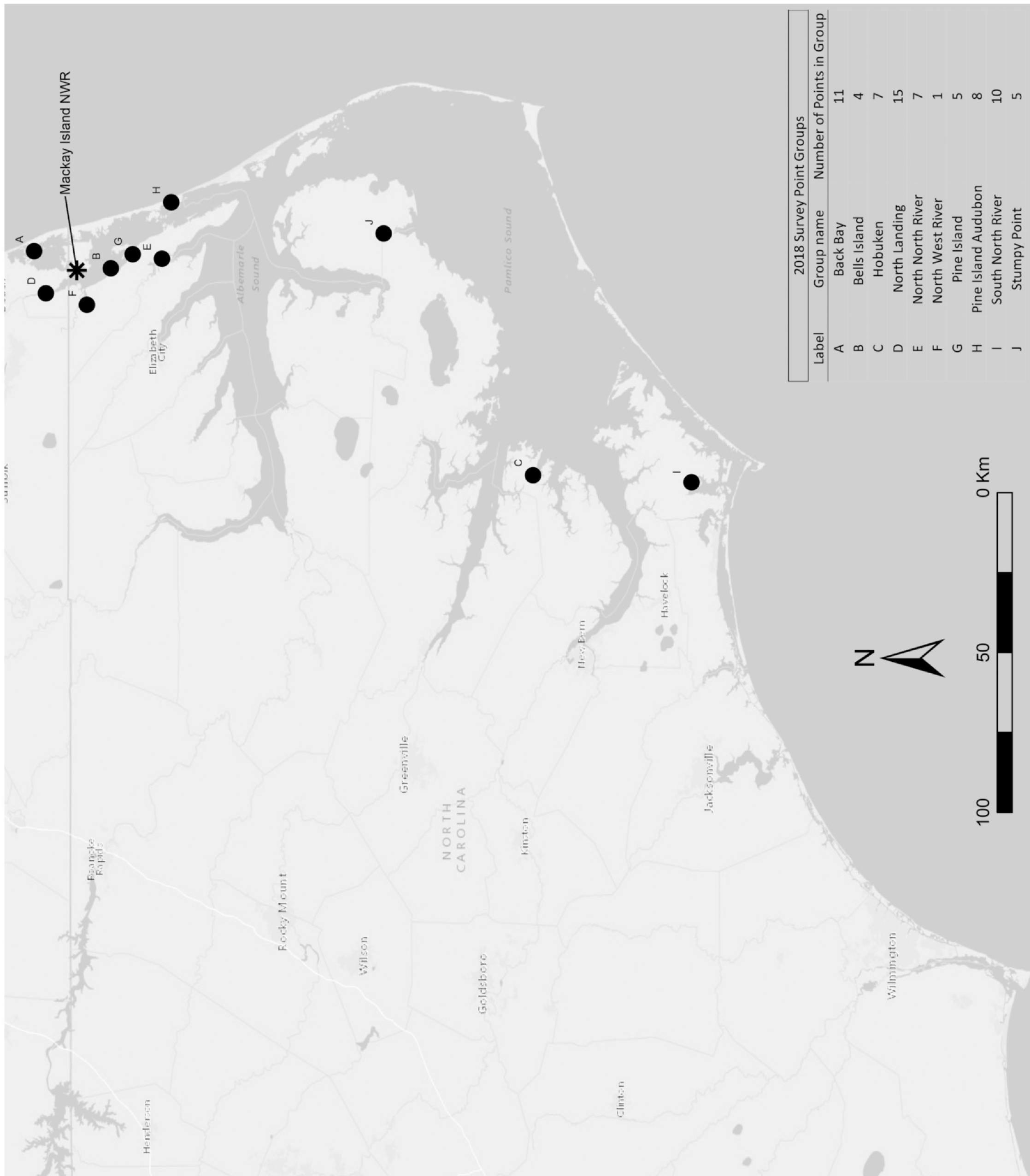
Following publication standards for qPCR experiments, we set the limit of quantification (LOQ) at 95% amplification calculated from the five replicates of a dilution series of purified product (Bustin et al., 2009). We set the limit of detection (LOD) as the point where only one out of five replicates produced a signal (Dunn et al., 2017; Harper et al., 2020). We validated our assay using the field-collected eDNA samples. For an eDNA sample to be considered positive for Black Rail DNA, it had to produce a qPCR signal at a cycle number (C_q) below or at the LOD. Only samples amplifying with a C_q at or below the LOQ were considered quantifiable. This five-replicate dilution series enabled us to reduce the total number of standards needed on each qPCR plate. We ran duplicates of two standard dilutions above the LOQ and two dilutions below the LOQ (including the LOD) on each subsequent plate to ensure eDNA results were comparable.

Environmental DNA samples are notorious for containing compounds that inhibit polymerase activity, and sample dilution has been found to be an effective solution (Biggs et al., 2015; Dunn et al., 2017). We diluted our reactions to reduce the effects of inhibitors on qPCR efficacy. Once optimized, the adjusted master mix contained 4.21 µL nuclease-free water (Fisher Scientific), 0.64 µL of each 10 µM BLRA COI2 primer, 0.16 µL of 10 µM Affinity Plus probe, 4.35 µL 2X TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and was added to 2 µL template. This resulted in 0.725X environmental master mix, while maintaining a consistent primer:probe ratio of 4:1, and a total reaction volume of 12 µL. Following the Applied Biosystems TaqMan protocol, thermal cycling conditions were set at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. This dilution was adopted for processing the eDNA samples.

2.3. Field collection of eDNA samples

To validate the diagnostic eDNA assay, we collected samples from sites of Black Rail detections to serve as positive controls. During the breeding season, we conducted callback surveys according to the standardized marsh bird survey protocol (Conway, 2011), in appropriate emergent vegetation wetlands in the North Carolina coastal plain. We selected sites based on accessibility and habitat suitability: wetland habitats at least half a hectare in size, dominated by native marsh grasses (particularly *Spartina* spp.), and irregularly flooded to a depth at or below 15 cm. These included oligohaline estuarine and freshwater landlocked marshes on public and private lands.

Between April 17th and July 8th, 2018, we conducted 186 surveys at 66 individual survey points. We made three visits to each site with at least nine days between visits (Fig. 1). A few individual survey points were visited only twice: points were added when new suitable habitat was discovered, and occasionally points became inaccessible due to tidal activity. Between March 22nd and July 14th, 2019, we conducted 434 callback surveys for Black Rails at 155 sites, with three visits to each site at least 17 days between visits (Fig. 2). In 2019, 14 points at 3 sites near Wilmington, NC were surveyed once and abandoned due to



2018 Survey Point Groups

Label	Group name	Number of Points in Group
A	Back Bay	11
B	Bells Island	4
C	Hobuken	7
D	North Landing	15
E	North North River	7
F	North West River	1
G	Pine Island	5
H	Pine Island Audubon	8
I	South North River	10
J	Stumpy Point	5

Fig. 1. Locations of Black Rail surveys conducted during the 2018 breeding season. The letter next to each point corresponds to the group label in the table listing the number of individual survey points at each site. The environmental negative controls were collected from Mackay Island NWR (*).

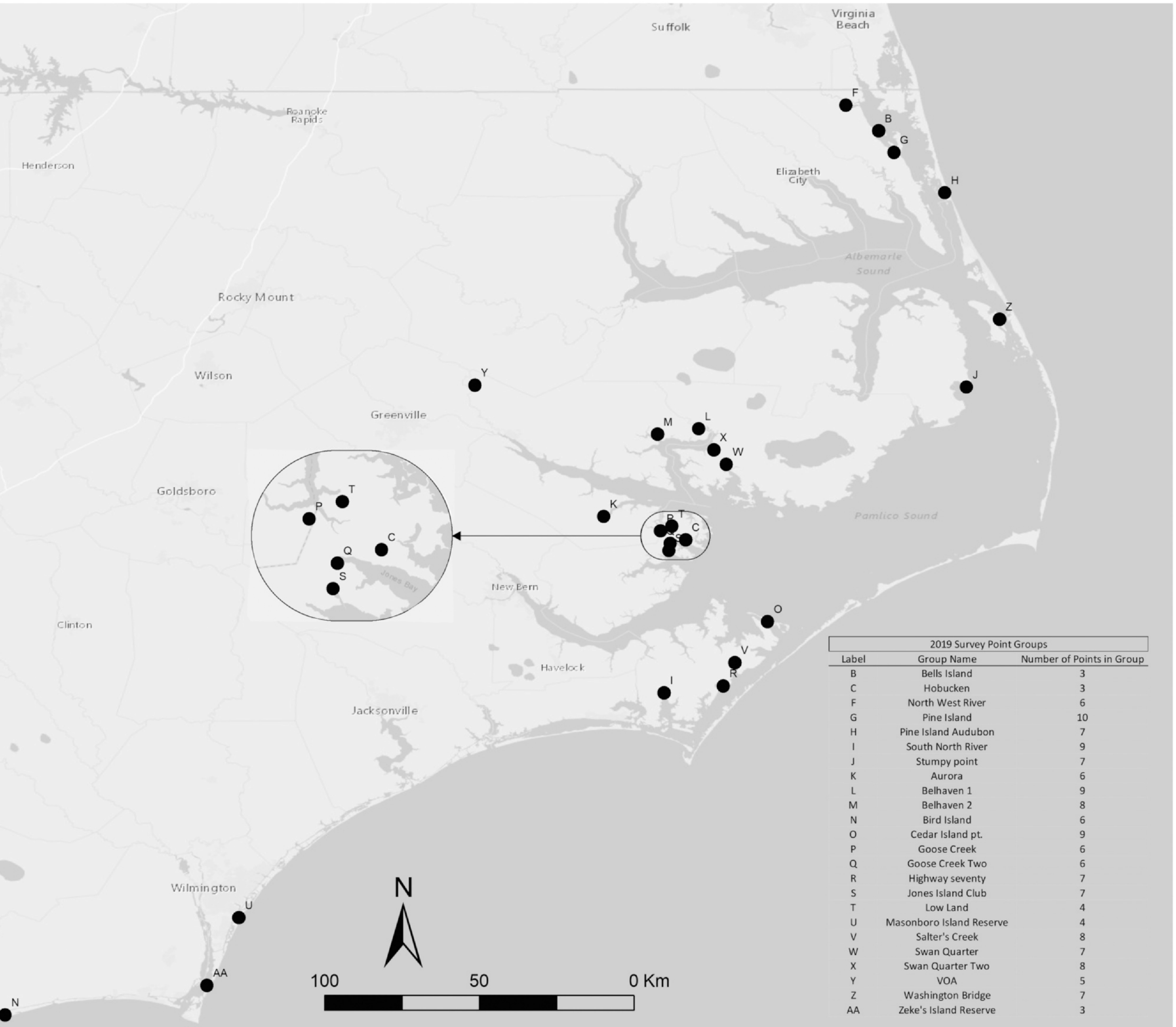


Fig. 2. Locations of Black Rail surveys conducted during the 2019 spring/summer breeding season. The letter next to each point corresponds to the group label in the legend listing the number of individual survey points at each site.

Table 2
Summary of North Carolina and Virginia callback surveys and eDNA sample collection. In the "Both years" column, statistics are totals for both years, except for the "Total survey points", "Survey points with detections", and "Locations sampled" (shaded grey) that represent the overlap in sites surveyed or sampled in both years.

Survey summary	2018	2019	Both years
Total surveys	186	434	620
Total number of survey points	66	155	26
Survey points with only 2 visits	10	23	N/A
Points abandoned after first survey	1	14	N/A
Surveys with detections	5	7	12
Survey points with detections	3	6	2
Individual black rails detected	6	7	13
eDNA samples collected	9 (6 water, 3 soil)	10 (3 water, 7 soil)	19
Locations sampled	2	5	1

logistical constraints. These sites had sparse monotypic vegetation structure and the high, tidally-influenced water level suggested marginal habitat for Black Rail. Of 115 points surveyed in 2019, 26 had also been surveyed in 2018 (Table 2). We collected 19 eDNA samples in the course of these surveys.

We also received samples (12 water and 27 soil) from collaborators working on Black Rails: 34 from South Carolina and 5 from Florida. Motion sensor camera traps were used at the Bear Island Wildlife Management Area site in South Carolina to collect visual data on Black Rails during both the 2018 and 2019 seasons (Hand et al., 2019). C. Hand and SBM collected environmental samples where Black Rails were detected using trail cameras (Fig. 3). Auditory surveys for Black Rails by USFWS

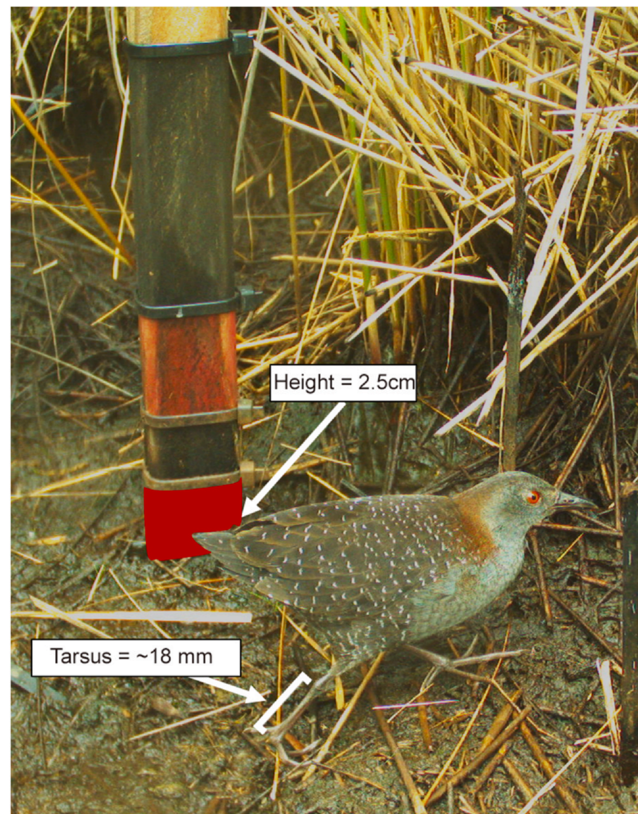


Fig. 3. Adult Black Rail captured on film by a trail camera in South Carolina (Christy Hand/South Carolina Department of Natural Resources). The wooden stake is a water depth marker and indicator of scale.

Table 3
Summary of the sample type, detection type, sample state, and water storage condition of eDNA samples collected during each year of the study. Under Detection type, 'No detection' refers to sites where Black Rails had been detected previously during that breeding season, but were not detected at the time of sampling.

		2018	2019
Detection type	Auditory	19 (8 water, 11 soil)	17 (7 water, 10 soil)
	Visual	0	2 (soil)
	Camera	12 (5 water, 7 soil)	16 (5 water, 11 soil)
	No detection	0	11 (4 water, 6 soil)
State of origin	North Carolina	9 (6 water, 3 soil)	28 (11 water, 17 soil)
	South Carolina	17 (7 water, 10 soil)	17 (5 water, 12 soil)
	Florida	5 (soil)	0
Water sample storage temperature	Frozen (−20 °C)	10	13
	Refrigerated (4 °C)	3	3
No. locations sampled		7	7
Total samples		31 (13 water, 18 soil)	45 (16 water, 29 soil)

biologists in Spring 2018 at three different refuges in Florida: St. Marks NWR, St. John NWR, and St. Vincent NWR yielded 5 samples from sites of positive detections (summarized in Table 3; for a full listing of all eDNA samples, see Supplementary Table A2).

Samples of eDNA were collected as close as possible to where Black Rails were detected. We used cues of footprints and small tunnels in the vegetation, targeting areas of higher probability for rail traffic. A subset was collected at the same time and location as paired soil and water samples: 6 pairs (12 samples) from South Carolina and 11 pairs (22 samples) from North Carolina. We also collected eDNA samples from locations where a Black Rail had been detected earlier in the season, regardless of whether there was a detection at the time of sampling.

Soil samples were collected by filling clean 50 mL falcon tubes away from where the observer had walked to avoid possible contamination. Areas of exposed moist soil at gaps in the vegetation were targeted. Soil samples were stored at −20 °C until DNA extraction.

We collected 2 L water samples in a sterilizable PC square media bottle (TriForest) or a single-use Whirl-Pak (Nasco). Pools of water in Black Rail habitat are typically only a few centimeters deep, so an aluminum loop with a 0.5 m handle holding a single-use plastic disposable cup (Hefty) was used as a ladle. This was mounted to a pole to extend the reach of the observer when collecting water samples. The loop was sanitized with bleach and dried between uses. Other samples were collected directly into the storage vessel.

Water samples collected in the same manner from marshes at Mackay Island National Wildlife Refuge, Currituck County, North Carolina served as negative environmental controls. We have conducted intensive monitoring of breeding king rails at the refuge since 2011 (Clauser and McRae, 2017; Kolts and McRae, 2017; Schroeder and McRae, 2020) without detecting Black Rails.

2.4. Water filtering protocol

Water samples were either refrigerated at 4 °C and then vacuum-filtered within 24 h, or frozen at − 20 °C as soon after collection as possible and always within 8 h, to be vacuum filtered at a later date (mean = 162 days after collection, range = 6–640). Two water samples from North Carolina accidentally thawed prematurely and were not processed further making the final eDNA positive control sample count 74.

Table 4
Quantitative PCR results for the final eDNA assay. We ran 6 replicates of each eDNA sample ($N = 74$). Thresholds reported for this assay were the limit of quantification (LOQ = 901 copies of target DNA) and the limit of detection (LOD = 109 copies of target DNA). The cumulative percent is the percent of samples with that number or more positive replicates.

No. replicates with signal	Limit of detection (LOD)		Limit of quantification (LOQ)	
	No. samples	Cumulative % with signal	No. samples	Cumulative % with signal
6	8	11%	3	4%
5	1	12%	1	5%
4	4	18%	1	7%
3	5	24%	3	11%
2	4	30%	0	11%
1	13	47%	0	11%
No signal	39	53%	66	89%

To concentrate eDNA, we filtered water samples through a 47 mm diameter cellulose nitrate filter with a pore size of 0.45 μm (Whatman), using a vacuum pump (KNF, Trenton, NJ) and filtering apparatus (Nalgene). The filtering apparatus was disassembled and soaked in a bleach solution for a minimum of 10 min, thoroughly rinsed with deionized water, and dried between uses.

2.5. DNA extraction

Environmental samples contain compounds that can be inhibitory to PCR. We tested several extractions methods for their ability to remove these including an ammonium acetate and ethanol precipitation ('Salting-out method', protocol based on Bruford et al. (1992)), DNeasy Blood and Tissue Kit (Qiagen), PowerWater Kit (Qiagen), and PowerSoil Kit (Qiagen). For each extraction, we used either half a cellulose nitrate filter disk with deposited precipitate or 0.4–0.7 g of soil and followed the manufacturer's instructions for each kit. We cut filters into small pieces with a clean razor blade to facilitate lysis of cells on the exposed surfaces. We tested extracts for the presence of inhibitors by spiking the sample with target sequence and running a standardized PCR reaction. The PowerSoil Kit performed substantially better in removing inhibitors and was used to extract all subsequent eDNA samples.

2.6. Testing field samples

We tested 6 replicates of each eDNA sample: each sample was run in triplicate on two separate plates to reveal any plate-batch effects. Each plate also included a standard dilution series (four dilutions from 10,900 copies to 10 copies, in duplicate), as well as a nuclease-free water no template controls (NTC) (Fisher Scientific). Given the small size and mobility of the bird, and the large size of the marshes being surveyed, the likelihood that sufficient eDNA would be deposited at a given point location was low. Therefore, a single lab detection out of six replicates was considered a positive result, with detection in more than one replicate improving confidence.

2.7. Statistical analyses for testing environmental and sampling variables

For each eDNA sample, the number of replicates scored as positive (C_q below the LOD) was divided by the total number of replicates to produce an amplification success rate. This rate was related to variables pertaining to the sample's origin, collection and handling methods. Statistical analyses were performed in R (R Core Team, 2019).

To determine whether sample type, collection and storage methods, and environmental conditions affected amplification success rate of eDNA samples taken from sites of Black Rail detection, we conducted Generalized Linear Mixed Models with a binomial distribution (see Table 5 in Results). Mixed models allowed the inclusion of random effects of location and sample group in cases where locations had been sampled multiple times and where water and soil samples were taken from the same place at the same time. We treated samples taken from the same location at the same time as non-independent. All the samples with camera trap detections came from a series of camera traps set at different locations within Bear Island Wildlife Management Area. These provided an opportunity to investigate detection probability in relation to the passage of time between field detection and sample collection.

Table 5
Generalized linear mixed models for Black Rail eDNA detectability. Models were considered significant if $p < 0.05$ and the bootstrap confidence intervals did not include 0 (significant p-values bolded).

Fixed Effects	Random Effects	Category	*Pr* (>|z|)	95% Bootstrap Confidence Interval	
				0.0250	0.9750
Days between collection and DNA extraction[a]	Sample location / sample group	N/A	0.0511	-0.0598	0.0032
Water sample storage temperature[b]	Sample group[c]	4 °C	0.8700	-3.2030	2.4171
Sample state of origin	Sample group[c]	NC	0.0355	-0.2815	15.7573
		SC	0.0379	-0.5022	15.3692
Detection type	Sample group[c]	Camera	0.5980	-1.3293	2.2017
		No detection	0.6910	-3.1494	1.2914
Days between collection and last camera detection[d]	Camera sample group	N/A	0.5662	-0.4927	0.1289
Water sample salinity[b]	Sample group[c]	N/A	0.7880	-0.2956	0.1984
Air temperature	Sample group[c]	N/A	0.0638	-0.1447	0.0008
Estimated water depth at sample location	Sample group[c]	N/A	**0.0254**	0.0004	0.3707

[a] Model originally returned a scaling error which was fixed by dividing the number of days between collection and DNA extraction by 10.
[b] A subset of the data containing only water samples was used for this model.
[c] Due to small sample size some models returned a singular fit and model complexity needed to be reduced by dropping the sample location as a random effect.
[d] A subset of the data containing only samples with camera detections was used for this model.

Models were assessed using the Wald test (*Pr* (>|*z*|)) and bootstrap confidence intervals were set at 95%. We tested for an effect on amplification detectability: the number of days between collection and DNA extraction, the storage temperature of the environmental sample before filtering (water samples only), sample location, field detection method (auditory, visual, camera trap, none), number of days since last camera detection before sample collection (for trail camera detections only), salinity (water samples only), air temperature, and estimated water depth at the sampling point. To examine the effect of sample type, we conducted a Wilcoxon rank test on paired water and soil samples collected at the same place and time.

3. Results

3.1. Species-specific eDNA test development

Two major considerations in developing a species-specific eDNA test are detectability of low copy number templates and high specificity of the assay. Considering low copy number first, we produced a dilution series of purified PCR product from the BLRA COI2 primers and Black Rail DNA isolated from blood to use as template. In traditional PCR, we found a loss of detectability at ~10,900 copies, whereas qPCR showed detectable fluorescence for as few as ~109 copies. When we tested our assay with the added BLRA AP probe and plotted the standard curve for the five-replicate dilution series, we found a limit of quantification (LOQ) of 901 template copies and a limit of detection (LOD) of 109 copies.

Next, we considered specificity of the assay. In conventional PCR, the BLRA COI2 primer pair worked only with Black Rail DNA as template; none of the other rail species produced a detectable signal as visualized on agarose. However, when we switched to quantitative PCR, cross-species amplification occurred. Using genomic DNA extracted from the blood of several non-target species (clapper rail, yellow rail, common gallinule, Virginia rail, sora), all but the clapper rail showed detectable amplification above or within the Black Rail standard curve. Our dilution series of purified product in these assays revealed an estimated detection limit of 1.18×10^{-6} ng or ~5000 copies of target Black Rail DNA.

Diluting the master mix improved reaction outcomes. Almost no NTCs or non-target samples produced any signal, but the reaction success of the standards was relatively unchanged. Only one out of six test plates produced a signal from a single NTC replicate, and the C_q for this signal was above the LOD threshold. We found either no amplification with non-target species template, or amplification below the LOD, therefore considered negative. Moreover, two environmental negative controls assayed in triplicate on two different plates did not produce a signal.

3.2. Field survey detections and eDNA sample collection

Of 620 callback surveys we conducted at 195 different survey points (66 in 2018, 155 in 2019, 26 points were surveyed both years), only 12 (2%) resulted in an auditory detection. Only 13 calling Black Rails were heard at 7 (4%) of the sites over two years. A total of 19 eDNA samples were collected from these sites at the time of detection (Table 2). Sixteen additional samples were collected without a callback survey at locations in North Carolina where Black Rails were detected earlier in the breeding season. Of these, 5 had previous detections at the time of sampling and 11 did not.

Of 34 samples received from South Carolina, 28 were from camera trap sites where a Black Rail had been detected visually from 10 days to 12 h before sampling. In a few cases more than one bird was seen multiple times in the 5 days prior to sampling (C. Hand, pers. comm.). The 5 eDNA soil samples contributed by the USFWS in Florida were all from federal refuge lands, collected in conjunction with auditory detections (Table 3).

3.3. Black Rail diagnostic test: validation using eDNA from sites of positive detections

Of 74 eDNA samples tested, 8 (11%) amplified consistently above the LOD and the same samples had at least three out of six replicates with a C_q at or below the LOQ (Table 4). In addition, 22 (30%) samples had at least two replicates with a C_q at or below the LOD, and 35 (47%) of them had at least one replicate with a C_q at or below the LOD. Thus, 47% met our criteria for a positive eDNA detection. The eDNA samples tested included 18 collected during callback surveys with positive Black Rail auditory detections; 9 (50%) of these had at least one positive replicate based on our criteria.

3.4. Environmental factors and effects of sample handling on Black Rail eDNA detectability

We investigated whether sampling methods and environmental parameters affected eDNA detection probability. With bootstrap confidence intervals, if 0 (the average of the distribution of means after resampling the full data set) is included in the 95% distribution of means from the resampling of data with variable of interest, then the variable is considered non-significant because the average is no different from the null. Two of the variables tested, the sample's state of origin (NC, SC, or FL) and the sample type (soil or water) had p-values < 0.05 but significance was not corroborated with the bootstrap confidence intervals (Table 5).

The only variable with both $p < 0.05$ and confidence intervals not including 0 was the estimated water depth, defined as the depth of any water in the vegetation surrounding the sample collection point within a 50 m radius, regardless of whether the water was sampled or not. The positive estimate for the model indicates that water depth has a positive correlation with detection rate. To gain a better understanding of this result we created three categories of water depth, 0–5 cm, 5–10 cm,

Table 6
Mean rate of detections using Black Rail eDNA assay in relation to environmental and sampling variables. The mean positive rate is based on six replicates per sample.

Variable	Category	Mean positive rate	No. of samples in category	No. of samples with detections
No. days between collection and extraction	<180 days	7%	12	4
	180–365 day	27%	46	22
	>365 days	27%	16	9
Water sample storage temperature	4 °C	21%	8	6
	-20 °C	54%	19	8
State of origin	North Carolina	21%	35	13
	South Carolina	25%	34	18
	Florida	30%	5	4
Detection type	Auditory	25%	38	18
	Camera trap	25%	25	13
	No detection	15%	11	4
No. days between camera detection and sample collection	≤3 days	19%	18	8
	>3 days	33%	10	7
Water sample salinity	$\leq 10^{0}/_{00}$	27%	14	7
	$>10^{0}/_{00}$	35%	13	7
Air temperature	≤26 °C	29%	33	16
	27–29 °C	23%	21	11
	>29 °C	16%	20	8
Estimated water depth	0–5 cm	20%	59	24
	5–10 cm	37%	10	6
	10–15 cm	37%	5	3
Paired water and soil samples	Soil	18%	17	7
	Water	29%	17	8

and > 10 cm. We then calculated the average detection rate (using the LOD as the positive threshold) for each category and found that the 5–10 cm and >10 cm categories had higher average detection rates ($mean_{5-10\,cm}$ = 37%, $mean_{>10\,cm}$ = 37%) than the 0–5 cm category ($mean_{0-5\,cm}$ = 20%) (Table 6). They also had a higher percentage of samples with at least one positive replicate in relation to the LOD.

All of the other variables (number of days between collection and DNA extraction, the storage temperature of water samples before filtering, field detection method (auditory, visual, camera trap, none), number of days since last camera detection before sample collection, water sample salinity, and air temperature) were non-significant with p-values >0.05 and confidence intervals that included 0 (Table 5). Paired water and soil samples had a non-zero difference between means ($mean_{water}$ = 29%, $mean_{soil}$ = 18%, $N = 17$) (Table 6), but this was not statistically significant ($p = 0.259$).

4. Discussion

We developed a diagnostic assay that can detect small quantities of Black Rail DNA from environmental samples and does not amplify DNA of sympatric North American rail species. The assay was validated using eDNA samples collected from sites of positive detection of Black Rails in the wild and did not amplify negative control samples. Our qPCR assay features a custom-designed Affinity Plus probe with fluorescent quencher reporter, and reliably detected as little as 109 copies of target DNA. This detection limit was within the same magnitude of sensitivity as described for another species-specific bird eDNA study (300 copy number limit of detection in Day et al. (2019)). Our assay outperformed traditional PCR using the same primers which had a detection limit of ~10,900 copies. Its specificity was demonstrated by the lack of a signal in qPCR reactions with genomic DNA from confamilial species as template.

Our auditory surveys detected a vanishingly small number of Black Rails. In spite of targeting locations where Black Rails have been detected in the recent past, our auditory surveys produced a 2% detection rate for both years and 4% occupancy for 2019 (12 out of 115 points). Unfortunately, these results exemplify the trends found at other sites on the east coast (Roach and Barrett, 2015; Wilson et al., 2015; Wilson et al., 2016; Watts, 2016).

Some eDNA samples were obtained in the course of auditory surveys. Several of the samples collected at sites of recent detections (11%) amplified Black Rail eDNA reliably. However, depending on the stringency of test criteria, 47% (one of six replicates) or 30% (for >1 of six replicates) of the eDNA samples collected at sites where presence or previous occupancy was confirmed by a field method produced a positive result with our diagnostic test. Lack of amplification from the others could have been due to (1) the eDNA sample not containing Black Rail DNA/the target sequence, (2) the eDNA containing Black Rail DNA but having degraded, (3) too few copies of the target sequence being present to be detected, or (4) qPCR inhibition. Considering the small size of the bird, the amount of DNA they shed into the environment is likely to be small. The size of the home range, compounded by variation in individual movements, dilutes the chances of capturing eDNA and contributes to sampling stochasticity.

Collecting water and soil samples at the same time from the same point provided the opportunity to test the effect of sample type on detectability. The eDNA concentrated from water samples was not significantly more likely to amplify than the soil samples. Samples with an estimated water level at or above 5 cm had a higher average percent positive detection rate. Deeper water is more mobile and could spread DNA farther from its source. This could dilute it over a wider area, leading to a greater likelihood of sampling the DNA. Environmental DNA is labile in water and can be transported over distance with sufficient current; in fast-moving water, aquatic invertebrate eDNA was detected as far as 10 km from the source (Deiner and Altermatt, 2014). Deeper water may also protect DNA from degradation by buffering temperature or sunlight, both of which have been shown to influence eDNA decay. Alien Asian carp eDNA was 8–1800 times more concentrated in sediment compared to water and lasted up to 132 days after the species was removed (Turner et al., 2015). A study looking at the effects of temperature, UV-B, and acidity on eDNA under controlled lab conditions, found that temperature was most strongly correlated with eDNA degradation and that the addition of UV-B light compounded this effect (Strickler et al., 2015).

Black Rails are unusual among waterbirds in that they inhabit moist soil wetlands with few pools of shallow water (Watts, 2016). Based on our findings, these pools are potential reservoirs of rail eDNA, but their presence, size and depth vary unpredictably. Nevertheless, this suggests some strategies for better detection using eDNA such as sampling standing water after rains. By contrast, for waterbirds in impounded areas, sampling impoundments soon after drawdown may concentrate eDNA to increase detection rates.

Assays for eDNA rely on the integrity of sample collection and handling in the field. Sampling eDNA for birds and other land vertebrates that range over large areas is inherently more challenging than sampling for aquatic species that reside in and shed DNA continuously into landlocked waterbodies (Takahara et al., 2013), and species that live their lives in smaller, more predictable ranges (Ficetola et al., 2008; Feng et al., 2020). Thus, we recommend informed field sampling where sites are inspected for physical evidence of occupancy. Cues such as footprints next to pools or in runways, presence of avian excrement or food remains can help guide strategic sampling.

A few previous studies have found an effect of sample storage on eDNA detectability (but see Hinlo et al. (2017)). We found no significant difference in detectability with water samples stored at 4 °C and filtered within 24 h and samples that were frozen and then later thawed directly before filtering. As in studies of eDNA collected in freshwater aquatic systems (Biggs et al., 2015), most environmental factors we tested did not affect amplification success. It is promising that eDNA detectability appears to have few environmental confounds.

In implementing the Eastern Black Rail Conservation Plan (Atlantic Coast Joint Venture, 2020), it will be critical to assess the distribution and occupancy patterns of Black Rails. In order to use eDNA testing effectively toward this goal, a better understanding of eDNA persistence in marsh environments will be needed. Testing should include the non-breeding period when the amount of DNA shed per unit area may be less due to greater movement and lower densities, but conditions for persistence may be improved with less sunlight and lower ambient temperatures (Strickler et al., 2015). Formal testing of these environmental factors on detectability would enable us to ameliorate the reliability of the eDNA assay.

5. Conclusions

Development of a reliable molecular diagnostic method to detect these imperiled marsh birds, represents an important addition to the conservation toolbox, complementing other methods to monitor occupancy and persistence. Environmental DNA offers an alternative to auditory callback, using minimally invasive field sampling techniques unconstrained by time of day or season. Overcoming challenges with the efficient processing and concentration of eDNA from marsh samples will increase consistency and reliability. For this tool to be transferable, future studies should focus on improving the consistency of amplification through optimizing sample collection, handling and extraction, and examine persistence of eDNA in marsh environments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2021.e01529.

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