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METHODS AND RESOURCES

Development, verification, and implementation of an eDNA detection assay for emydids with a case study on diamondback terrapins, *Malaclemys terrapin*, and red eared slider, *Trachemys script elegans*

Andrew T. Fields[1] · Elizabeth P. Hunt[1] · Katherine Lanoue[1] · Aaron S. Baxter[2] · Riley S. Sebby[1] · W. Jay Tarkington[3] · David S. Portnoy[1]

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Abstract
Globally, many pond turtles (Family Emydidae) are of conservation concern due to contracting ranges, increasingly fragmented habitats, and declining populations. One of these turtles, the diamondback terrapin (*Malaclemys terrapin*), is of concern within the United States, but surveying potential estuarine habitat for the presence of the species requires high levels of time and effort. Here a species-specific, probe-based qPCR assay for identifying terrapin DNA from environmental DNA (eDNA) is described. To expand the utility of this tool, the assay was designed to allow for potential detection of at least 19 other pond turtles taxa and the utility was tested by designing a second species-specific probe for red-eared slider (*Trachemys scripta elegans*) with a different fluorophore, which can be used simultaneously with the terrapin probe to detect either species. Probes were found to be species-specific and effective at detecting low levels of DNA for the target species, the diamondback terrapin (10 fg/µL) and the red-eared slider (100 fg/µL) in laboratory conditions. The assay also showed species-specific detection in environmental samples taken from field locations where one species or the other were known to reside. Through citizen science approaches, this assay could be used for discovering novel terrapin habitats, monitoring invasive red-eared slider populations or surveying other emydids, with species-specific probes, at a fraction of the cost of traditional surveys.

Keywords Emydidae · qPCR · eDNA · Diamondback terrapin · Red-eared slider

Introduction

Turtles are long lived, late maturing animals with low reproductive output. As with many aquatic species, habitat loss and fragmentation, consumption by humans as well as competition from invasive species have threatened them with extinction. Over 50% of turtle and tortoise species world-wide are categorized as Vulnerable, Endangered or Critically Endangered by the IUCN (Stanford et al. 2020). Within North America, 11 out of 12 freshwater turtle species in Canada are at a risk of extinction (Desforges et al. 2022) and the US Endangered Species Act (ESA) lists 32 turtle species, seven of which are freshwater species found in the United States.

This is typified by the diamondback terrapin (*Malaclemys terrapin*; terrapin), which are the only brackish water species of pond turtle (Family Emydidae) in North America, inhabiting coastal areas from Cape Cod, MA to Corpus Christi, TX (Ernst et al. 1994). Terrapin habitat includes marshes, tidal creeks/rivers and embayments, but diamondback terrapins face many dangers including habitat loss and fragmentation, pollution, boat strikes, and bycatch mortality associated with crab traps (Seigel and Gibbons 1995); leading to them being listed as Vulnerable to extinction by the IUCN (Roosenburg et al. 2019). This species often occurs in small, localized populations and it can be easily extirpated from an area

✉ Andrew T. Fields
Andrew.Fields.biologist@gmail.com

✉ David S. Portnoy
David.Portnoy@tamucc.edu

[1] Marine Genomics Laboratory, Texas A&M Corpus Christi, 6300 Ocean Dr, Corpus Christi, TX, USA

[2] Coastal Bend Bays & Estuaries Program, 1305 N Shoreline Blvd #205, Corpus Christi, TX, USA

[3] Palacios Marine Agricultural Research Inc, 555 N Carancahua St, Ste 240, Corpus Christi, TX, USA

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with little, to no chance, for natural re-population because dispersal potential is low (Gibbons et al. 2001). Thus, the conservation initiatives for the species require continuous monitoring effort.

Due to habitat fragmentation, vast amounts of potential habitat, and the reclusive nature of the species, monitoring can be time consuming and costly. Traditional sampling methods require the use of small vessels and traps and, by necessity, sampling all potential terrapin habitat, which is time intensive and expensive, though not unprecedented (Guillen et al. 2015; Pearson and Wiebe 2018). Environmental DNA, or eDNA, is an alternative technique for detecting species presence that relies on taking environmental samples that contain trace amounts of DNA shed by the target organisms, isolating that DNA, and amplifying it, thereby demonstrating the presence of a species in a particular area. These trace amounts of DNA are present in the environment because organisms leave behind cells through the release of skin, fecal matter, blood, etc. that can be found in sediment, water and even air (Leempoel et al. 2020; Havermans et al. 2022; Lynggard et al., 2022). Sample collection can also be done with minimum training, allowing for a citizen science approach (e.g., Biggs et al. 2015) or at reduced field costs (Biggs et al. 2015; Huver et al. 2015; Sigsgaard et al. 2015). When properly analyzed, this technique can be used to indicate the presence of the focal species and is especially suitable for reclusive species, such as diamondback terrapins.

Environmental DNA assays have been developed for a number of turtle species including the bog turtle (*Glyptemys muhlenbergii*; Kirtane et al. 2019), the alligator snapping turtle (*Macrochelys temminckii*; Feist et al. 2018) and the wood turtle (*Glyptemys insculpta*; Lacoursiere-Roussel et al. 2016). An eDNA assay has also been developed to detect diamondback terrapin eDNA through a dye-based qPCR assay (Conner 2020), following other assays designed for pond turtle detection (Davy et al. 2015; de Souza et al. 2016). While the terrapin assay was successful in detecting captive terrapins held in an estuary, it failed to detect non-captive terrapins in field sampling, though it is unclear if this was due to the method or the absence of terrapin DNA. Dye-based qPCR assays are also subject to false positives due to unexpected amplification, such as the presence of primer dimer and non-specific target amplification (Conner 2020) and can only be used on a single species at a time. Probe-based qPCR assays are generally more specific, less likely to register false positives due to primer dimer and can also be used to target more than one species at a time (Conner 2020; Yang et al. 2002), although each set of species-specific probes require rigorous validation to guard against false positives and false negatives, as do all qPCR assays. Therefore, a probe-based qPCR assay was designed to detect diamondback terrapins, with built in flexibility to allow for design and incorporation of species-specific probes to detect other pond turtle species. A species-specific probe for red-eared sliders (*Trachemys scripta elegans*; sliders) was also designed and implemented, to display this method's versatility.

Methods

Sample collection

Tissue samples were collected for many *Emydidae* taxa (n = 20), gathered throughout the Southern United States, to test the functionality of the primers across taxa, and from several local diamondback terrapins and red-eared sliders to test amplification within the focal species. Samples from many taxa were provided through museum loans (Table 1), while additional tissue samples of diamondback terrapin and red-eared slider were collected under TPWD Scientific Research Permit #SPR-0220-026 and TAMU-CC IACUC Protocol 2020-09-009. Terrapins and sliders were captured using modified crab traps, which allowed captured turtles to breathe when inside the trap. Tissue samples were taken from wild caught turtles using a 4 mm biopsy punch to collect tissue from the webbing between the toes on one of the animal's back feet. Tissues were immediately placed in a vial containing a 20% DMSO-0.25 M EDTA-saturated NaCl buffer (Seutin et al. 1991) and stored for transport to the Marine Genomics Lab at Texas A&M University—Corpus Christi. Terrapin tissues were collected from Nueces Bay, TX (n = 13) and Goose Island State Park, TX (n = 4) while slider tissues were collected from Lakeview Pond, TX (n = 10; Fig. 1). DNA was extracted from these samples using Mag-Bind Tissue DNA kits (Omega Bio-Tek, Norcross, GA) following the tissue protocol.

Primer design

Mitochondrial genomes from eleven individuals across three genera were downloaded from GenBank (Table S1), aligned using Clustal Omega (Sievers et al. 2011) and alignments checked by eye in BioEdit v 7.2.5 (Hall 1999). Regions of DNA sequence conserved among the genomes were identified and primers designed for a region starting in tRNA-Gln and ending in NADH dehydrogenase subunit 2 (ND2) using Primer3 (Untergasser et al. 2012). DNA hairpins, homodimers, annealing temperature and heterodimers were all considered using the IDT OligoAnalyzer Tool (IDT, Iowa. USA) and multiple occurrences of the primer region were tested for using NCBI BLAST (Altschul et al. 1990). Tissue from specimen mentioned above, were extracted with Mag-Bind Blood and Tissue DNA kits (Omega Bio-Tek, Norcross, GA), following the standard protocol, yielding DNA concentrations ranging from 0.1 to 104 ng/uL according to the

Table 1 Vouchered tissue samples used to test the primer set as well as the species specificity of the qPCR probes including the turtle catch location and the adjusted signal to noise (S/N) ratio of each species-specific probe (diamondback terrapin and red-eared slider)

Collection code	Catalog number	Species	Catch Location	Terrapin Adjusted S/N	Slider Adjusted S/N
UF	166880	*Chrysemys picta*	Maine, USA	0.20	0.21
UF	145038	*Clemmys guttata*	North Carolina, USA	0.22	0.23
UF	162285	*Deirochelys reticularia*	Florida, USA	0.21	0.21
OMNH:OCGR	12314	*Graptemys ouachitensis*	Oklahoma, USA	0.19	0.22
TCWC	104222	*Graptemys pseudogeographica*	Texas, USA	0.19	0.35
KUH	289649	*Graptemys pseudogeographica kohnii*	Louisiana, USA	0.21	0.20
UF	152776	*Graptemys versa*	Texas, USA	0.19	0.19
UF	151506	*Pseudemys concinna*	Florida, USA	0.18	0.21
UF	151371	*Pseudemys floridana*	Florida, USA	0.20	0.25
TT	RG01	*Pseudemys gorzugi*	Texas, USA	0.19	0.22
UF	162158	*Pseudemys nelsoni*	Florida, USA	0.19	0.20
UF	151505	*Pseudemys peninsularis*	Florida, USA	0.19	0.19
UF	173227	*Terrapene carolina*	Florida, USA	0.19	0.20
KUH	276516	*Terrapene ornata*	Kansas, USA	0.21	0.21
TCWC	104200	*Trachemys scripta*	Texas, USA	0.18	0.74
UF	151289	*Trachemys scripta elegans*	Florida, USA	0.19	1.00
UF	189570	*Trachemys scripta scripta*	South Carolina, USA	0.19	0.24
UF	157188	*Trachemys terrapen*	Eluthrea, The Bahamas	0.20	0.20
UF	157304	*Trachemys venusta*	Florida, USA	0.20	0.20
TT	NU02	*Malaclemys terrapin*	Texas, USA	1.00	0.22
NA	NA	No template control	NA	0.19	0.19

Adjusted S/N calculated as the ratio of the 40th qPCR cycle divided by the 1st over the S/N ratio from the target species

Tissue contributing facilities were Texas A&M University Biodiversity Research and Teaching Collection (BRTC), the University of Kansas Herpetology collection (KU), the Oklahoma Collection of Genomic Resources (OMNH), Texas Turtles (TT) and the Florida Museum of Natural History Genetic Resource Repository (UF)

AccuBlue high sensitivity dsDNA quantification (Biotium, Fremont, CA) analyzed on a plate reader. The samples were PCR amplified using 1uL of DNA, 1x GoTaq buffer, 200 µM each dNTPs, 1.5 mM $MgCl_2$, 0.25 µM of each primer and 1 unit of GoTaq in a 25 µL reaction. PCR cycling consisted of an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Success of amplification was evaluated using electrophoresis on a 1% agarose gel. PCR products were purified using Mag-Bind TotalPure NGS (Omega Bio-Tek, Norcross, GA) and Sanger sequenced on an ABI 3730 using just the forward primer.

Sequences were quality trimmed using fastp v 0.23.4 (Chen et al. 2018), trimming both the beginning and the end of the sequences with a 5 bp sliding window until the average mean quality was 15 within the window. Trimming also removed bases until at least 50% of retained bases had a quality score above 15. Trimmed sequences were aligned using Clustal Omega (Sievers et al. 2011) to look for base pair differences between taxa for *in silico* probe design (GenBank Accession: PP230170 - PP230203). Species-specific probes to compliment 26–29 bp of the PCR products, with at least two base pair differences between the target taxa and others, were independently designed for red-eared slider and diamondback terrapin (Table 2). Complementary fluorophores were added to the terrapin and slider probes (FAM and HEX, respectively) so both could be used in the same quantitative PCR (qPCR) assay. When designing the probes, oligo parameters (e.g., homodimers, annealing temperature) were considered. The magnesium chloride concentration and annealing temperatures were optimized for the multiprobe qPCR assay using repeated runs with DNA from both terrapin and slider DNA. To test that probes species-specificity in vitro, the multiprobe qPCR assay was performed using 0.5 ng of DNA from each of the 20 taxa for which there were tissues, in triplicate. Amplicons were visualized using electrophoresis on a 1% agarose gel to verify that the samples PCR amplified when qPCR results did not indicate binding of probes. Reactions included 2 µL of template DNA, 1x GoTaq Buffer, 200 µM dNTPs, 3.25 mM $MgCl_2$, 0.25 µM of each primer, 0.2 µM of each probe, 1 unit of GoTaq and 0.5 µM ROX reference dye in a 10 µL reaction. The qPCR cycling consisted of a denaturation at 95 °C for 3 min, followed by

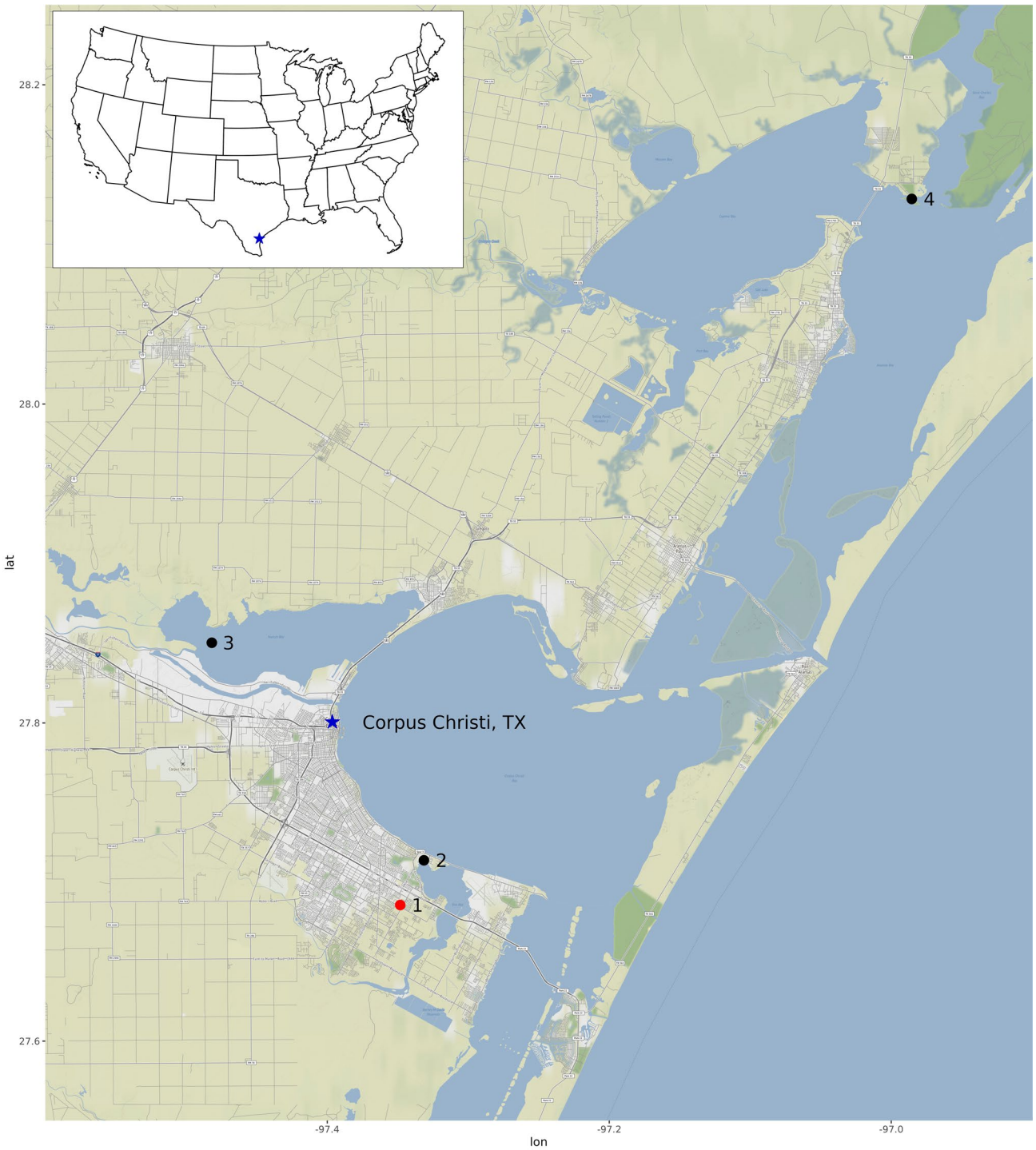

Fig. 1 Map of the sampling locations with black dots indicating the locations terrapins were sampled and the red dot showing where the red-eared sliders were sampled (1. Lakeview Pond; 2. Oso Bay Wetlands; 3. Nueces Bay; 4. Goose Island State Park). Only the two southern locations (Oso Bay Wetlands and Lakeview Pond) were used for eDNA field sampling

45 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 68 °C for 60 s with a fluorescence check after the 68 °C step, and were run on a StepOnePlus thermocycler (Thermo Fisher Scientific). The full multiprobe qPCR assay was performed on locally sampled diamondback terrapins and red-eared sliders collected from the Coastal Bend of Texas to ensure consistent results across the species.

Table 2 Primers and probes designed for this experiment including used to detect red-eared slider and diamondback terrapin in both the number of taxa successfully amplified for each primer set. field and the laboratory experiments

Primer set	Primer	Sequence (5'–3')	Tm	Annealing temp (°C)	Amplicon size
tRNA-Gln to ND2	F	AGGACTCGAACCTACACCAGA	57	57	294
tRNA-Gln to ND2	R	GCTTCAATTGCTCGTGGGTG	57	57	294
Probe set	Species	Sequence (5'–3')	Tm	Fluorophore	
tRNA-Gln to ND2	Terrapin	CCCTTCCTCTACTAATAAATCCGC ATGC	59	FAM	
tRNA-Gln to ND2	Slider	AACCGGTCTAGAAATCAGCACCCT AGC	62	HEX	

Fig. 2 Laboratory set up used to test the transfer of DNA from live turtles to the water and the degradation of eDNA, including a bin without any turtles physically present in the water (Empty). The photo was altered by adding labels to the containers

Turtle eDNA tank trials

Degradation trials were performed to understand how long the multiprobe assay would continue to be useful in detecting the species after the turtles were removed from the environment. A single diamondback terrapin and a single red-eared slider were placed in separate 50-gallon lidded troughs (Fig. 2) with independent, constant water circulation, and held at a constant room temperature with full spectrum aquarium lighting controlled by timers to mimic natural day-night cycles. A separate trough without a lid or turtle was used as a control for transmission (e.g., airborne or splashes) of DNA between tanks. Instant Ocean (Instant Ocean, Blacksburg, VA) was added to deionized (DI) water to create water of salinity of 28 ppt in the terrapin trough and no salts were added to the DI water in the red-eared slider or empty trough. Turtles were placed in the trough for 24 h and then the turtles and lids removed. Two and a half liters of water were sampled, subsurface with replacement, from each of the troughs immediately after the turtles were removed, as well as one day, two days, four days and seven days later. Water samples were immediately transported across the street to the Marine Genomics Laboratory at Texas A&M University—Corpus Christi, Texas and filtered. They were stored at 4 °C while awaiting filtering. Filtering involved passing 500 mL of water through a single 0.45 µm nylon membrane filter, performed in triplicate; therefore, three filters were used for each tank at each timepoint. The extra liter of water was stored at − 20 in case it was needed. All filters were frozen in a − 20 °C laboratory grade freezer until extraction. Equipment was cleaned and UV sterilized after each filtering and nanopure water was passed through a sterile filter before any samples were filtered in the apparatus for the day and after all the samples had been processed for the day to create "blank filters." Blank filters were extracted and run alongside treatment filters to verify that there was no contamination.

Subsequently, a quarter of each filter was excised, cut into strips and DNA extracted using a modified Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany) extraction protocol; the rest of the filter was returned to the freezer. The modified extraction procedure included incubating the filter in 500 µL of 0.5% SDS lysis buffer with 20 µL of Proteinase K at room temperature for 2–3 h on an orbital shaker platform. Filters were then incubated at 55 °C for 10 min without shaking to increase the activity of the Proteinase K and finish lysing any cells present. To provide the appropriate chemical concentrations for DNA binding to the silica in the spin column, AL buffer and ethanol were also increased to 500 µL. All other steps were performed as detailed in the protocol manual, including spinning the tissue lysis through the spin column, washing with buffers AW1 and AW2 and eluting with 75 µL of AE buffer.

Extracted DNA was passed twice through a OneStep PCR Inhibitor Removal Kit (Zymo Research Corp, Irving,

CA) inhibitor removal column to reduce the amount of inhibitors in the samples and total DNA was quantified using an AccuBlue High sensitivity dsDNA kit and plate reader. The optimized multiprobe qPCR, including the primers and both probes, was used to test each extraction, including the filter blanks, in triplicate. A dilution series of terrapin DNA and a dilution series of slider DNA, each ranging from approximately 10 fg/uL to 0.1 ng/µL, and a qPCR negative template control (water) were also included in triplicate for each qPCR plate. A result was considered positive for eDNA when the amplification of the sample was above the threshold automatically determined by the StepOne Software v2.2.2 (Applied Biosystems, Foster City, CA, USA). One sample from the Day 0 terrapin and slider tanks was amplified, sequenced and trimmed as described above and compared to the NCBI database using the BLAST algorithm (Altschul et al. 1990) to ensure species-specific products were present.

Turtle eDNA field trials

Prior to beginning field trials for terrapins, DNA extraction methods were tested using subsurface water collected from the Lakeview Pond, a local pond site which contained one of our target species, red-eared slider (Fig. 1). A water sample was filtered and DNA was extracted as described in the laboratory trial, but while sliders were visually present when samples were collected and DNA was present in the extraction according to the AccuBlue High sensitivity dsDNA quantification, no slider DNA was found in the multiprobe qPCR assay. A literature review (e.g., Jane et al. 2015, McKee et al. 2015, Lance and Guan 2020) suggested that PCR inhibitors, such as tannic or humic acids from plant material, are common in the environment and may require additional cleaning to remove. Samples were subsequently reextracted using the Dneasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and then the DNA was passed through the Zymo inhibitor removal columns. The new extractions tested with the multiprobe qPCR assay resulted in detectable levels of slider DNA in the environmental samples leading to the conclusion that this extraction adequately removed inhibitors.

Three subsurface water samples of 500 mL were then collected in the Oso Bay Wetlands, an area known to have resident terrapins (Fig. 1). Samples were kept on ice until returned to the laboratory where they were frozen at − 20 °C until filtered and extracted. After thawing, each 500 mL sample was passed through a separate 0.45 µm nylon membrane filter with blank filters before and after filtering the field samples. DNA extracted using the Dneasy PowerSoil Pro Kit and passed through the Zymo inhibitor removal column twice as with red-eared slider samples. Quantitative PCR was used to assess the presence of terrapin DNA in each of the 500 mL sample in triplicate as described for the laboratory samples. A result was considered positive for eDNA when the amplification of the sample was above the threshold automatically determined by the StepOne Software v2.2.2.

qPCR limit of detection and efficiency

Determination of the limit of detection (LOD) followed Forootan et al. (2017) and amplification efficiency followed Kubista et al. (2006). The qPCR standard curves, using the assay with both probes present, were generated nine times for each species with DNA concentrations from 10^{-2} to 10^{-6} ng/uL. LOD was then calculated as the percentage of time that a standard at a specific concentration was above the detection threshold automatically determined by the StepOne Software v2.2.2. A concentration was considered successful if it amplified greater than 75% of the time, as this assay was designed for presence/absence detection involving multiple replicate environmental samples. The efficiency of a qPCR was determined using the slope of the standard curve between log of DNA mass and the cycle threshold value (Ct), in the equation (Kubista et al. 2006):

$$E = -1 + 10^{\left(-\frac{1}{slope}\right)}$$

Given that each probe has a different color fluorophore and is species-specific, the efficiency was determined in the multiprobe assay. Signal to noise (S/N) ratios were calculated by comparing the fluorescence levels within a sample at cycle 1 and cycle 40 for a given probe for both target and non-target taxa (n = 19). The adjusted signal to noise ratio was then calculated by dividing the S/N of each non target taxon by S/N of the target species, and then an averaged adjusted S/N was calculated for each probe (Wilcox et al. 2013).

Results

Primer design

The PCR primers for the multiprobe assay amplified all 20 taxa tested (Table 1) and the probes were found to provide a qPCR signal only for the specific species they were designed to target. It has been suggested that species-specific primers and probes are required to have sufficient S/N for species-specific detection (Wilcox et al. 2013); but the signal to noise ratio for the terrapin-specific probe with universal primers had an averaged adjusted signal to noise ratio (0.20) very similar to what was found in Wilcox et al. 2013 (0.19) as well as to the adjusted S/N ratio of controls with no template in this experiment (0.19; Table 1). For the

red-eared slider probe, the average adjusted S/N ratio was slightly higher than the terrapin result (0.25) driven by a couple taxa including the pond slider (*Trachemys scripta*; 0.72) and the false map turtle (*Graptemys pseudogeographica*, 0.33; Table 1). All terrapin samples from the coastal bend area of Texas were detected with the terrapin probe in the multiprobe assay with a S/N ratio averaging 6.4. All the red-eared slider samples from the coastal bend area of Texas were detected by the slider probe in the multiprobe assay with a S/N ratio averaging 6.5.

qPCR limit of detection and efficiency

Species-specific fluorescence above the automated threshold was recorded in every instance for DNA concentrations from 10^{-2} to 10^{-5} ng/uL. The 10^{-6} ng/uL concentration had a signal above the threshold in the terrapin 77% of the time and in the slider 22% of the time; therefore, the detection limit for the terrapin probe was found to be 10 fg/µL and the detection limit for the slider probe was found to be 100 fg/µL. The amplification efficiency of the terrapin probe in the multiprobe assay averaged 88.2% with the coefficient of determination (R^2) of 0.986. The amplification efficiency of the slider probe averaged 84.3% with the coefficient of determination (R^2) between 0.993 (Table S2).

Turtle eDNA tank trials

Fluorescence-based quantification of the total DNA quantity in the filter blanks using a dye interpolation (e.g., AccuBlue) did not show any DNA present prior to inhibitor removal, however, after passing blanks through the inhibitor removal column, fluorescence above the baseline (0 ng/uL) was recorded. This occurred because the resin in the inhibitor removal plates passes through the filter during cleanup, but it is inert in a qPCR assay (Zymo per. comm.). Supporting this, no blank filters showed any terrapin or red-eared slider DNA in the multiprobe qPCR assay (Table 3). Total DNA concentration, using the dye interpolation (e.g., AccuBlue), was higher than the blank filter baseline in all the extractions from the water taken from the laboratory experiment, including the container which had housed no turtle (Table 4). Using the multiprobe assay, all of the time points from the red-eared slider tank were found to have only red-eared slider DNA and no terrapin DNA. The BLAST analysis of the sequencing confirmed the presence of slider DNA. The amount of red-eared slider DNA present increased between day 0 and day 1, and then proceeded to decrease through the rest of the trial, and the number of qPCR replicates which yielded positive results decreased throughout the time (Table 3). Using the multiprobe assay, all of the terrapin time points were found to have terrapin DNA, but not slider DNA. The BLAST analysis of the sequencing confirmed the presence of terrapin DNA. The amount of terrapin DNA increased between day 0 and day 1 and then proceeded to decrease the rest of the trial; however, the ability to detect the DNA in the terrapin tank on day 1 was contingent upon extracting the sample with the DNeasy PowerSoil Pro Kit to remove PCR inhibitors. The number of positive qPCRs increased between day 0 and day 1 and then decreased through the rest of the trial (Table 3). While small amounts of terrapin DNA (7.91 fg/uL) were detected in the bin which did not house a turtle, it happened only on day 1 in a single replicate, out of three, and is likely due to an error

Table 4 The mean concentration of total DNA (in ng/uL) according to a fluorescence-based measurement (AccuBlue dsDNA quantification) in the DNA extractions after removing inhibitors with the Zymo inhibitor removal column in the empty control bin as well as bins containing terrapins and sliders

Time Period	Negative filter	Empty	Terrapin	Slider
Day 0	0.47	2.31	1.95	1.7
Day 1	0.53	11.87	16.18	3.79
Day 2	0.47	14.53	19.31	4.33
Day 4	0.47	13.9	8.43	6.53
Day 7	0.6	12.23	2.47	6.35

Table 3 The mean terrapin and red-eared slider DNA concentration (in ng/uL) for each filter treatment over the time course of the experiment including the numberfraction of qPCR assays which detected the species in parentheses

Time period	Negative Filter	Empty	Terrapin	Slider
Day 0	0 (0/6)	0 (0/9)	2.26E-04 (7/9)	5.33E-03 (9/9)
Day 1	0 (0/12)	7.91E-06 (1/9)	8.13E-04 (9/9)*	7.97E-03 (9/9)
Day 2	0 (0/12)	0 (0/9)	1.53E-04 (9/9)	3.06E-03 (9/9)
Day 4	0 (0/12)	0 (0/9)	6.29E-05 (6/9)	9.47E-04 (8/9)
Day 7	0 (0/12)	0 (0/9)	1.93E-05 (3/9)	6.48E-04 (8/9)

The sample with the star (*) was only detected after extracting with the Dneasy PowerSoil Pro Kit

For the Day 0 filtration, only one filter apparatus was used so only two negative controls filters were available and qPCR was performed in triplicate

The other days had two filtration setups in separate laminar flow hoods, doubling the number of negative controls

when some drops of the water which housed the terrapin were accidently transferred into the empty bin.

Turtle eDNA field trials

Terrapin DNA was detected in all three qPCR replicates of one of the three water samples taken from the Oso Bay Wetlands (Table 5), where a known population of terrapins is located. Slider DNA was detected in the duck pond at Lakeview Park where red-eared sliders were collected and observed. No terrapin DNA was detected in Lakeview Park and no slider was detected in Oso Bay and all field blanks and qPCR no template controls failed to detected any of either target species (Table 5).

Discussion

Assay evaluation

Overall, the probes were found to be species-specific and the multiprobe qPCR assay was found to be effective at determining the presence of diamondback terrapins and red-eared sliders in a sample. Turtle DNA was detected throughout the entire laboratory experiment and in the field indicating that these assays can be successfully deployed in turtle monitoring as a complementary method to field observation. In the laboratory, the eDNA concentration oddly appeared to increase after the removal of the turtles. DNA has been found to bind to soil particles (Lorenz et al. 1981) and large particles settle in aqueous systems. After removing the turtle, the water moved more freely in the tank, possibly suspending more of these particles and allowing our water sampling to recover them. The terrapin used for this study was also covered in schmutz (biofouling) and even shed a scute while in the tank, while the slider did not have visible growth on its shell. The "dirty" nature of this terrapin and DNA absorption or PCR inhibition by particles deposited off of the shell (Lorenz et al. 1981; Lance and Guan 2020) are likely why the day 1 sampling of the terrapin tank required additional steps to remove enough inhibitors for qPCR to function properly.

This study does not provide an exhaustive analysis of either focal species and, therefore, it is possible that the probes designed within would not work across the entire range of both species. Given the anthropogenic migration of diamondback terrapins within the US (Converse et al. 2017), it is likely that this assay will work in all US terrapins, but testing of local populations is prudent. The results from the red-eared slider probe in the multispecies test were not as robust as the terrapin probe. The subspecies of the *Trachemys scripta* samples are not known and based upon these results, would suggest that they could be red-eared slider because the yellow-bellied slider (*Trachemys scripta scripta*), also a subspecies of pond slider, had less than a quarter of the S/N signal of the red-eared slider. Though not as evolutionarily close to red-eared slider as the yellow-bellied slider, the false map turtle did have the highest adjusted S/N ratio of the non-target species, but was still less than half the S/N ratio of the red-eared slider and can be controlled for with keeping one as a standard to make certain that the qPCR threshold is high enough to exclude this species. Testing of the red-slider probe on local populations of slider as well as other possible sympatric turtle species would also be prudent as Sanger sequencing is not a useful means of differentiating species with this assay, though a metagenomic approach could be applied.

Table 5 The mean diamondback terrapin (A) and red-eared slider (B) DNA concentration (in ng/uL) for each filtered field site including the numberfraction of qPCR assays which detected the species in parentheses

Time period	Terrapin	Slider
Negative filter	0 (0/4)	0 (0/4)
Oso bay	6.70E-05 (3/9)	0 (0/9)
Lakeview	0 (0/1)	6.00E-03 (1/1)

As Lakeview was a trial grounds for understanding the best extraction technique, only one trial was performed with the Power Soil Kit and it was not qPCR amplified in triplicate

Implications for emydid turtles

Diamondback terrapins live in small, isolated populations in estuaries throughout the western North Atlantic, including the Gulf of Mexico (Ernst et al. 1994). Due to habitat fragmentation, anthropogenic movement of terrapins (Converse et al. 2017) and extirpation from portions of their distribution, the location and size of many of these populations is unknown (Seigel and Gibbons 1995). This qPCR tool provides a resource for detecting locations of terrapin populations. Due to the simple nature of water sampling for eDNA, this protocol could be expanded to include citizen scientists who would engage in water collection, supplying those samples to a well-trained laboratory for water analysis. Given GPS capabilities of most mobile devices, accurate locations could also be recorded by citizen scientists and associated with each of multiple samples collected throughout an estuary, providing a means for triangulating the locations of diamondback terrapins. This type of citizen science approach has proven highly successful for researchers wishing to canvas large areas but within a reasonable time frame and for modest cost (Florisson et al. 2018; Agersnap et al. 2022).

While this multiprobe assay does provide a verified method for detecting diamondback terrapins, given that a number of pond turtle species are listed as in danger of

extinction (Stanford et al. 2020), this assay was also created so that other probes could be designed to aid in detecting other emydid turtles. Using readily available genetic data from public sources or DNA sequencing a few samples from a local population, probes for a number of species could be developed and verified within this same section of mtDNA. Given that a number of emydid species are found in closed freshwater systems, the likelihood of detecting their presence may be higher relative to diamondback terrapins, which live in a more open and labile environment and could allow for flushing of eDNA (Kelly et al. 2018). The red-eared slider, by contrast, has been categorized as one of the 'World's Worst Invasive Alien Species' (Lowe et al. 2000) and can be found on all of the continents except Antarctica (GISD 2015). This qPCR-based assay could also be used as a tool for monitoring invasions at the same time as surveying the range of native species through a single multiprobe assay.

eDNA lessons

This multiprobe qPCR assay performed well in a laboratory setting with ideal conditions and was able to detect terrapins and sliders in a known field setting, however, implementation of this assay in field studies would require careful planning and consideration to provide informative results. Inhibitors were a large factor in these experiments leading to new extraction protocols, but when accounted for the assays worked well to identify the species in question, however, field sites are likely to vary greatly in the amount and types of inhibitors present. The laboratory trials did not find DNA degradation to be a large factor in detection, particularly for red-eared slider, but a multitude of environmental factors are important in eDNA persistence in the field (Barnes et al. 2014) which cannot be not replicated in the laboratory. Furthermore, aspects of animal behavior can result in different eDNA shedding rates (Klymus et al. 2015) and local hydrology can determine where eDNA is located (Deiner and Altermatt 2014; Kelly et al. 2018; Inoue et al. 2022), suggesting that these are important considerations for planning field sampling activities. This assay was designed for emydid turtles, which have a limited home range, and therefore it may be possible to use it for longer-term monitoring of relative abundance, but additional field data would need to be collected such as turtle's initial numbers, sizes relative to DNA quantity for each population and seasonal habits which change eDNA distribution and concentration (Spear et al. 2021).

There was high variability in the concentrations of the turtle DNA between water samples taken on the same day in the same location. While variability has been found to increase with low DNA concentrations in qPCR (Doi et al. 2015) and DNA concentration can be variable within a water body due to many environmental factors (i.e., flow rate, inhibitors, shedding rates) and proximity to the organism (Strickler et al. 2015; Rourke et al. 2022), it is likely a portion of the variability in this experiment was due to the filter storage and sampling. Because only a portion of the filter was used for DNA analysis and the filters were folded for storage, there was likely some transfer of material between sides of the filter which likely contributed to variability in eDNA quantification. Even given this, the use of the technique for detecting the presence of a given species is still applicable since any positive detection would be motivation for a more in-depth analysis of the water body. A negative detection could indicate that the species is not present or in too low a concentration to detect (Rourke et al. 2022).

While sterilization protocols are essential for this type of research, it was found that positive pressure, ancient DNA clean room procedures (e.g., Knapp et al. 2012) were not necessary to avoid contamination. While dedicated equipment was used for these experiments, including sterilized pipettes, laminar flow hoods and bleach sanitized working areas, many of these measures can be taken in normal laboratory settings with adequate biosafety cabinets without renovating labs specifically for eDNA work. Sampling does require care and, therefore, implementation for citizen science would require some training for the water collection (e.g. Agersnap et al. 2022), but a high degree of training would be required for the filtering and qPCR portion of the experiment. While ideally water samples would be filtered as soon as they were taken and those filters frozen to prevent DNA degradation (e.g., Agersnap et al. 2022), freezing samples in other studies still allowed for the detection of target species (e.g., Jerde et al. 2011; Takahara et al. 2012). Therefore, for projects that include a citizen science portion, water samples could be frozen and shipped to a location where they could be processed with minimal loss in eDNA quantity.

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Declarations

Conflict of interest The authors declare no competing interests.

References

Agersnap S, Sigsgaard EE, Jensen MR, Avila MDP, Carl H, Møller PR, Krøs SL, Knudsen SW, Wisz MS, Thomsen PF (2022) A national scale bioblitz using citizen science and eDNA metabarcoding for monitoring coastal marine fish. Front Mar Sci 9:824100

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM (2014) Environmental conditions influence eDNA persistence in aquatic systems. Environ Sci Technol 48:1819–1827

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P, Williams P (2015) Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biol Conserv 183:19–28

Chen S, Zhou Y, Chen Y, Gu J (2018) Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinform 34(17):i884–i890

Connor NM (2020) Adapting new technologies for evaluating the ecology and distribution of the diamondback terrapin (*Malaclemys terrapin*) in Alabama. Dissertation, The University of Alabama at Birmingham

Converse PE, Kuchta SR, Hauswaldt JS, Roosenburg WM (2017) Turtle soup, prohibition, and the population genetic structure of Diamondback Terrapins (*Malaclemys terrapin*). PLoS ONE 12(8):e0181898

Davy CM, Kidd AG, Wilson CC (2015) Development and validation of environmental DNA -(eDNA) markers for detection of freshwater turtles. PLoS ONE 10(7):e0130965

de Souza LS, Godwin JC, Renshaw MA, Larson E (2016) Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. PLoS ONE 11(10):e0165273

Deiner K, Altermatt F (2014) Transport distance of invertebrate environmental DNA in a natural river. PLoS ONE 9(2):e88786

Desforges JE, Clarke J, Harmsen EJ, Jardine AM, Robichaud JA, Serré S, Chakrabarty P, Bennett JR, Hanna DE, Smol JP, Rytwinski T (2022) The alarming state of freshwater biodiversity in Canada. Can J Fish Aquat Sci 79(2):352–365

Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T (2015) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PLoS ONE 10(3):e0122763

Ernst CH, Lovich JE, Barbour RW (1994) Turtles of the United States and Canada. Smithsonian Institution Press, Washington

Feist SM, Jones RL, Copley JL, Pearson LS, Berry GA, Qualls CP (2018) Development and validation of an environmental DNA method for detection of the alligator snapping turtle (*Macrochelys temminckii*). Chelonian Conserv Biol 17(2):271–279. https://doi.org/10.1371/journal.pone.0059520

Florisson JH, Tweedley JR, Walker TH, Chaplin JA (2018) Reef vision: a citizen science program for monitoring the fish faunas of artificial reefs. Fish Res 206:296–308

Forootan A, Sjöback R, Björkman J, Sjögreen B, Linz L, Kubista M (2017) Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol Detect Quantif 12:1–6

Gibbons JW, Lovich JE, Tucker AD, FitzSimmons NN, Greene JL (2001) Demographic and ecological factors affecting conservation and management of the diamondback terrapin (*Malaclemys terrapin*) in South Carolina. Chelonian Conserv Biol 4:66–74

Global Invasive Species Database (2015) Species profile: Trachemys scripta elegans. http://www.iucngisd.org/gisd/speciesname/Trachemys+scripta+elegans. Accessed 13 April 2023

Guillen G, Gordon ML, Oakley J, Mokrech M, Alleman B, George R, Bush D (2015) Population survey of the Texas diamondback terrapin. *Malaclemys terrapin littoralis*, in San Antonio Bay, Matagorda Bay, and Sabine Lake. EIH Report 15 – 001

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Havermans C, Dischereit A, Pantiukhin D, Friedrich M, Murray A (2022) Environmental DNA in an ocean of change: status, challenges and prospects. Arquivos De Ciencias do Mar 55:298–337

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol Appl 25:991–1002

Inoue N, Sato M, Furuichi N, Imaizumi T, Ushio M (2022) The relationship between eDNA density distribution and current fields around an artificial reef in the waters of Tateyama Bay, Japan. Metabarcoding Metagenom 6:e87415

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol Ecol Resour 15:216–227

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) Sight-unseen detection of rare aquatic species using environmental DNA. Conserv Lett 4:150–157

Kelly RP, Gallego R, Jacobs-Palmer E (2018) The effect of tides on nearshore environmental DNA. PeerJ 6:e4521

Kirtane AA, Wilder ML, Green HC (2019) Development and validation of rapid environmental DNA (eDNA) detection methods for bog turtle (*Glyptemys muhlenbergii*). PLoS ONE 14(11):e0222883

Klymus KE, Richter CA, Chapman DC, Paukert C (2015) Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. Biol Conserv 183:77–84

Knapp M, Clarke AC, Horsburgh KA, Matisoo-Smith EA (2012) Setting the stage–Building and working in an ancient DNA laboratory. Anat Anz 194(1):3–6

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Asp Med 27(2–3):95–125

Lacoursière-Roussel A, Dubois Y, Normandeau E, Bernatchez L (2016) Improving herpetological surveys in eastern North America using the environmental DNA method. Genome 59(11):991–1007

Lance RF, Guan X (2020) Variation in inhibitor effects on qPCR assays and implications for eDNA surveys. Can J Fish Aquat Sci 77(1):23–33

Leempoel K, Hebert T, Hadly EA (2020) A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. Proc R Soc B: Biol Sci 287:20192353

Lorenz MG, Aardema BW, Krumbein WE (1981) Interaction of marine sediment with DNA and DNA availability to nucleases. Mar Biol 64:225–230

Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the world's worst invasive alien species: a selection from the global invasive species database. The Invasive Species Specialist Group (ISSG), New Zealand

Lynggaard C, Bertelsen MF, Jensen CV, Johnson MS, Frøslev TG, Olsen MT, Bohmann K (2022) Airborne environmental DNA for terrestrial vertebrate community monitoring. Cur Biol 32:701–707

McKee AM, Calhoun DL, Barichivich WJ, Spear SF, Goldberg CS, Glenn TC (2015) Assessment of environmental DNA for detecting presence of imperiled aquatic amphibian species in isolated wetlands. J Fish Wildl Manag 6(2):498–510

Pearson SH, Wiebe JJ (2018) Considering diamond-backed terrapin (*Malaclemys terrapin*) nesting habitat and reproductive productivity in the restoration of Gulf of Mexico coastal ecosystems. Ocean Coast Manag 155:8–14

Roosenburg WM, Baker PJ, Burke R, Dorcas ME, Wood RC (2019) *Malaclemys terrapin*. The IUCN red list of threatened species. E.T12695A507698

Rourke ML, Fowler AM, Hughes JM, Broadhurst MK, DiBattista JD, Fielder S, Walburn JW, Furlan EM (2022) Environmental DNA (eDNA) as a tool for assessing fish biomass: a review of approaches and future considerations for resource surveys. Environ DNA 4:9–33

Seigel RA, Gibbons JW (1995) Workshop on the ecology, status, and management of the diamondback terrapin (*Malaclemys terrapin*). Chelonian Conserv Biol 1:240–243

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. Can J Zoo 69:82–90

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using clustal omega. Mol Syst Biol 7:539

Sigsgaard EE, Carl H, Møller PR, Thomsen PF (2015) Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Bio Conserv 183:46–52

Spear MJ, Embke HS, Krysan PJ, Vander Zanden MJ (2021) Application of eDNA as a tool for assessing fish population abundance. Environ DNA 3(1):83–91

Stanford CB, Iverson JB, Rhodin AG, van Dijk PP, Mittermeier RA, Kuchling G, Berry KH, Bertolero A, Bjorndal KA, Blanck TE, Buhlmann KA (2020) Turtles and tortoises are in trouble. Cur Biol 30(12):R721–R735

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol Cons 183:85–92

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. PLoS ONE 7(4):e35868

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40:e115

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. PloS One 8(3):e59520

Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE (2002) Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J Clin Microbiol 40(9):3449–3454