



Development, verification, and implementation of an eDNA detection assay for diamondback terrapins in the Texas Coastal Bend

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Executive Summary

Diamondback terrapin distribution in Texas waters is poorly understood and the necessary field sampling to characterize the species distribution is expensive and time consuming. Therefore, an environmental DNA (eDNA) qPCR assay was developed to test for the presence of diamondback terrapins which uses water samples that could easily be collected by researchers and/or citizen scientists. To increase the utility of the assay, primers were designed to amplify a fragment of mtDNA in all Emydidae turtles. Probes designed for diamondback terrapin and red-eared slider were found to be species-specific, detected DNA in very small concentrations and worked on all individuals tested of both species. Tank experiments demonstrated the ability to detect both species in water samples taken as many as seven days after the turtles had been removed and showed both species could be detected in mixed water samples. Field collected water samples were tested, and terrapin and red-eared sliders were detected in locations where species presence could be verified visually. The results of the study demonstrate that the eDNA assay designed is reliable and can be used to detect diamondback terrapin (or red-eared slider) using water samples collected in suspected habitat.

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Introduction

Diamondback terrapins (terrapins) are North America's only brackish water species of pond turtle (Family Emydidae), inhabiting coastal areas from Cape Cod, MA to Corpus Christi, TX. Terrapin habitat include marshes, tidal creeks/river, and embayments, but many aspects of the species biology in Texas are still poorly understood, and as a result, management efforts are somewhat compromised. The largest obstacle facing state resource managers is a lack of distributional data for this species. There are still large, unsampled areas of seemingly appropriate habitats for this species along the Texas coast that may, or may not, contain terrapin populations.

Range wide population declines have been documented for terrapins and populations in Texas face threats such as habitat loss and fragmentation, pollution, boat strikes, and bycatch mortality associated with crab traps. Because this species often occurs in small, localized populations, it can be easily extirpated from an area, with little, to no chance, for natural re-population because dispersal potential is low (Seigel and Gibbons, 1995, Gibbons *et al.*, 2001). For these reasons, it is imperative to identify locations that contain terrapins so that state resource managers can mitigate habitat threats and assess resilience of local populations.

Traditional sampling methods require the use of small vessels and traps and, by necessity, sampling all potential terrapin habitat would be time intensive and expensive. Due to limited funding, only a few select locations have been adequately sampled for the presence of terrapins, leaving gaps in our understanding of their distribution in Texas. 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). When properly analyzed, this technique can be used to indicate the presence of the species of focus and the method is especially suitable for species that are difficult to catch, such as diamondback terrapins.

Methods

Sample Collection

All sample collection was conducted under TPWD Scientific Research Permit #SPR-0220-026 and TAMUCC IACUC Protocol 2020-09-009. Terrapin, and red-eared slider, were captured using modified crab traps, which allowed captured turtles to breathe when inside the trap.

Tissue samples were collected from wild caught terrapins using a 4 mm biopsy punch. Each sample was collected from the webbing between the toes on one of the animal's back feet. Tissue samples were immediately placed in a vial containing a DMSO buffer solution and stored for transport to the Marine Genomics Lab at TAMUCC. Terrapin tissue samples were collected from Nueces Bay (n = 13) and Goose Island State Park (n = 4; Fig. 1). Tissue samples were collected, stored, and transported in the same manner for red-eared slider at Lakeview Pond (n = 10) in Corpus Christi, TX (Fig. 1). DNA was extracted from these samples using Mag-Bind Tissue DNA kits (Omega Bio-Tek) following the tissue protocol.

Primer design

Mitochondrial genomes from eleven individuals across three genera were downloaded from GenBank (Table 1). Genomes were aligned using Clustal Omega (Sievers *et al.*, 2011) and alignments checked by eye in BioEdit v 7.2.5 (Hall, 1999). Regions of conserved DNA sequence between the genomes were identified and primers were designed using Primer3 (Untergasser *et al.* 2012). All designed primer sets were tested on using PCR. Reactions included 1 uL of DNA, 1x Buffer, 200 uM each dNTPs, 1.5 mM MgCl₂, 0.25 uM of each primer and 1 unit of GoTaq in a 25 uL reaction. PCR cycling consisted of an initial denaturation at 95°C for 2 mins, followed by 35 cycles of denaturation at 95°C for 60 seconds, annealing at 54-57°C (Table 2) for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. Success of amplification was evaluated using electrophoresis on a 1% agarose gel.

Probes were designed for red-eared slider and diamondback terrapin to compliment products that successfully amplified using the given primer sets. Quantitative PCR (qPCR) was then run in triplicate, to optimize the probes and determine if they were species specific. Once qPCR was optimized and probes verified to be species specific, a DNA dilution series was used to determine the detection limit for each probe. Reactions included 2 uL of DNA, 1x Buffer, 200 uM dNTPs, 3.25 mM MgCl₂, 0.25 uM of each primer, 0.2 uM of each probe, 1 unit of GoTaq

and 0.5 uM ROX reference dye in a 10uL reaction. PCR cycling consisted of a denaturation at 95°C for 3 mins, followed by 45 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 68°C for 60 seconds with a fluorescence check after the 68°C step, run on a StepOnePlus thermocycler (Thermo Fisher Scientific). The full assay was then performed on diamondback terrapins and red-eared sliders collected from the Coastal Bend to verify that it worked on the local populations.

Turtle eDNA tank trials

For the dilution trials, a single diamond-backed terrapin and a single red-eared slider were placed in separate 50-gallon lidded troughs (Fig. 2) with constant circulation held at a constant room temperature with lights controlled with timers to mimic natural day night cycles. A separate trough without a lid or turtle was used to control for airborne transmission between tanks. Instant Ocean (Instant Ocean, Blacksburg, VA) was added to distilled (DI) water to create water of salinity 28 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 hours and then the turtles and lids were removed. Two and a half liters of water were sampled with replacement from each of the troughs when the turtles were removed, as well as one day, two days, four days and seven days after the turtles were removed. Water samples were immediately transported to the Marine Genomics Laboratory at Texas A&M University – Corpus Christi, Texas, and were either immediately filtered or frozen and maintained at -20°C until being filtered. Filtering involved passing 500 mL of water through a 0.45um nylon membrane filter, performed in triplicate. All filters were frozen in a -20°C laboratory grade freezer until extraction. Equipment was cleaned and UV sterilized after filtering a sample and nanopure water was passed through a filter before any samples were filtered in the apparatus for the day and after all of 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. A 50:50 mixture of red-eared slider and terrapin water from each time point was also filtered and extracted as a part of the study, to understand how the assay performed when more than one species was present.

A quarter of each filter was removed, cut into strips and DNA extracted using a modified Qiagen Blood and Tissue Kit extraction protocol, while the rest of the filter was returned to the freezer. The modified extraction procedure included incubating the filter in 500uL of 0.5% SDS lysis

buffer with 20uL of Proteinase K at room temperature for 2-3 hours on an orbital shaker platform. Filters were then incubated at 55°C for 10 mins 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 Qiagen spin column, AL buffer and ethanol were also increased to 500uL. All other steps were performed as detailed in the Qiagen protocol manual, including spinning the tissue lysis through the spin column, washing with buffers AW1 and AW2 as well as eluting with 75uL of AE buffer.

Extracted DNA was passed twice through a Zymo inhibitor removal column to reduce the amount of inhibitors in the samples. The optimized multiplex qPCR including both primer and probe sets was performed, in triplicate, upon each extraction. A dilution series of both terrapin and slider DNA ranging from approximately 10 fg/uL to 0.1 ng/uL and a qPCR negative template control (water) were also included in triplicate for each qPCR plate.

Turtle eDNA field trials

Prior to beginning field trials for terrapins, DNA extraction methods were tested using water collected from the Lakeview duck pond, a local pond site which contained one of our target species, red-eared slider (Fig. 1). Water was filtered and DNA was extracted as described in the laboratory trial but was found to contain large amounts of PCR inhibitors even after passing through the Zymo inhibitor removal columns. Samples were subsequently reextracted using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's protocol and then the DNA was passed through the Zymo inhibitor removal columns, and this was found to remove the inhibitors to the qPCR reaction.

Water was then collected in the Oso Bay wetlands, known to have a terrapin turtle population (Fig. 1), and was kept on ice until returned to the molecular lab where it was frozen at -20°C. Water from the field was filtered and DNA was extracted following using DNeasy PowerSoil Pro Kit and passed through the Zymo inhibitor removal column twice. Quantitative PCR was performed on each of the samples in triplicate as described for the laboratory samples.

Results

Primer design

Four different regions of mtDNA were found to have conserved regions close enough to each other for primer design. Of these, two of the regions amplified in all of the samples, so probes were designed for them (Table 2; Table S1). The probe for the mtDNA amplicon starting in the tRNA-Gln and ending in the ND2 was found to be species specific. The detection limit of the terrapin probe (10 fg/uL) was found to be lower than the red slider probe (100 fg/uL). The efficiency of the terrapin qPCR was between 87.9 and 89.7 percent while the efficiency for the red-eared slider was 82.0 to 87.9 percent. The assay was found to amplify all of the diamondback terrapin and red-eared slider samples sampled in the Coastal Bend.

Turtle eDNA tank trials

Blank filters with pure water passed through them at the beginning and end of the filtering for the day did not amplify with the qPCR assay (Table 3), though after passing through the inhibitor removal column, the fluorescence-based DNA quantification method used to detect DNA did record elevated fluorescence over the baseline. The fluorescence-based quantification of these negative filter controls quantified prior to inhibitor removal did not show any DNA present. Zymo has acknowledged that the resin in the inhibitor removal plates does filter through during inhibitor cleanup, though they say it is inert in a PCR assay. Quantifiable levels of DNA were found in all the extractions from the water taken from the laboratory experiment, including the container which had housed no turtle (Table 4). All of the time points from the red-eared slider tank were found to have only red-eared slider DNA. The amount of 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 trial (Table 3). All of the terrapin time points were found to have terrapin DNA. As with the slider, 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 enough PCR inhibitors for the assay to work. The number of positive qPCRs increased between day 0 and day 1 and then decreased through the rest of the trial (Table 3). While DNA was detected in

the bin which did not house a turtle, only day 1 had a single positive qPCR assay which detected small amounts of terrapin DNA (7.91 fg/uL) and no slider DNA. This detection is likely due to a sampling error during day 0 when some drops of the water which housed the terrapin were accidentally transferred into the empty bin.

DNA was found to be present in the mixed samples at much lower concentrations relative to the unmixed water by itself (means of 1.92 and 7.10 ng/uL, respectively). The qPCR results found a higher amount of terrapin DNA in the day 0 mixed samples, but less in the following time points. The detectability of terrapin DNA also changed with more qPCRs detecting DNA in day 0 samples and fewer qPCRs detecting the DNA in the later time periods (days 1-7; Table 5). The slider DNA was found at lower concentrations in the mixed samples as well as fewer qPCRs detecting slider DNA. This was all despite technical difficulties which resulted in the mixed water going through multiple freeze thaw cycles.

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 wetland, where a known population of terrapins is located. Slider DNA was also detected in the duck pond at Lakeview Park where red-eared sliders have been observed. No other DNA was detected in the field qPCR assays included the blanks (Table 6).

Discussion

Overall, this assay was found to be effective at detecting terrapins and red-eared sliders. Turtle DNA was detected throughout the entire laboratory experiments and in the field indicating that these assays can be successfully employed in turtle monitoring as a complementary method to field observations.

Inhibitors were a large factor in the experiment, but when accounted for, assays worked well to identify the species in question. This assay was also designed so that it could be easily expanded to any pond turtle species including 20 found in Texas which the PCR assay was found to successfully amplify.

Laboratory trials did not find DNA degradation to be a large factor in detection, particularly in the red-eared slider assay. If there was an attempt to assess the quantity of turtles from the qPCR assay, then DNA degradation and the number of assays which identified the presence of the species would both be metrics to better understand. It would also be essential to carry out the eDNA degradation experiment in a natural environment to further assess environmental effects as well as turtle quantity upon eDNA quantity and degradation rate.

There was high variability in the concentrations of the turtle DNA between water samples taken at the same day in the same location, likely due to the filter storage. Because only a portion of the filter was used for DNA analysis and the filters were folded for storage, there was 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. These detections were found in both the laboratory study and the field sampling.

While sterilization protocols are essential for this type of research, it was found that clean room procedures were not necessary to avoid contamination, though sampling does require care and therefore implementation for citizen science would require minimal training for the water collection, but a high degree of training for the filtering and qPCR portion of the experiment. Due to technical difficulties beyond our control, the mixed samples from the laboratory trial went through three freeze-thaw cycles before they could be extracted. This led to a lower quantity of DNA being present in our final assay, but both species were still detectable throughout most of the time course. While ideally water samples would be filtered as soon as they were taken and those filters frozen to prevent DNA degradation, if this project was expanded to 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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Figures

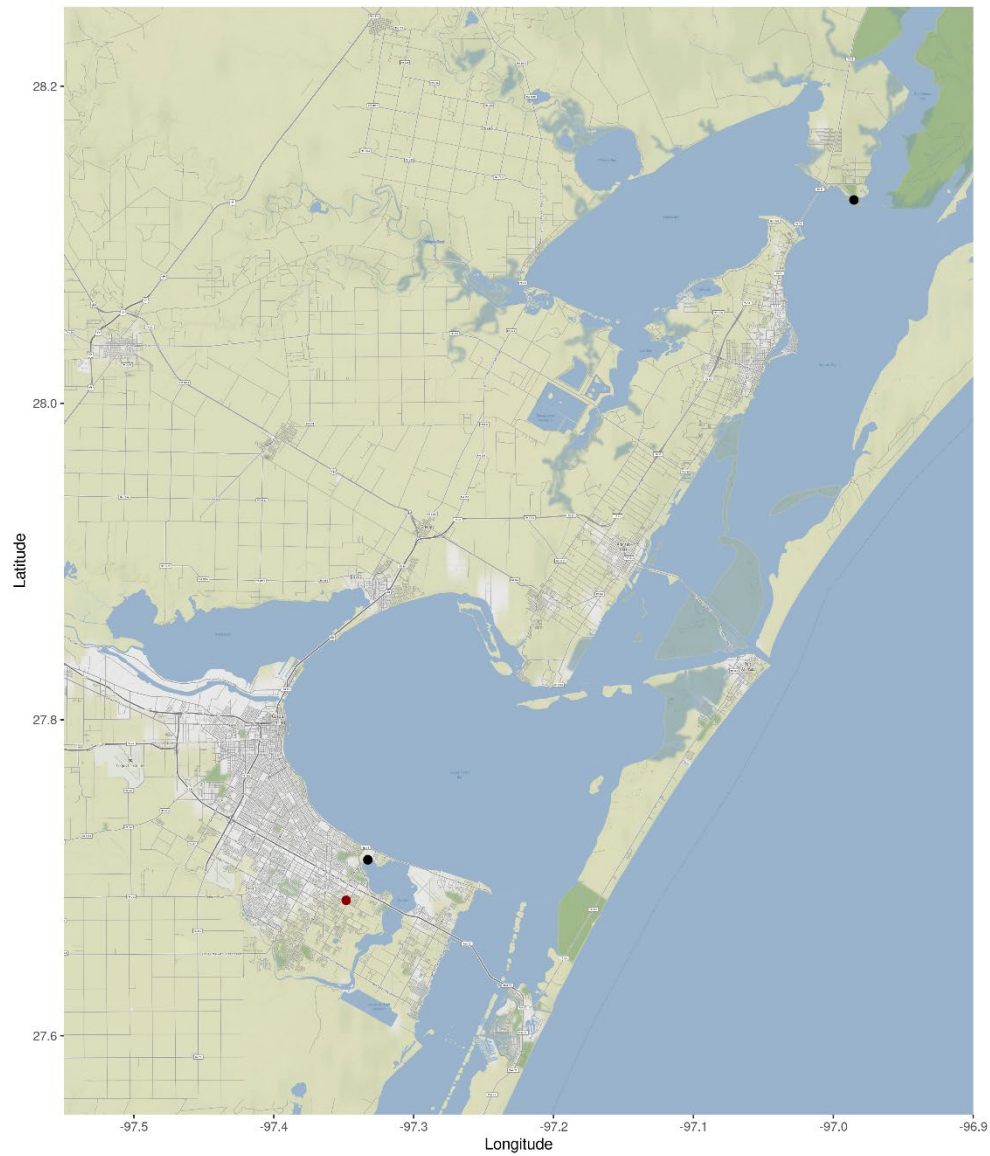


Figure 1. A map of the sampling locations with black dots indicating the locations terrapins were sampled and the red dot showing where the red-eared sliders were sampled. Only the two southern locations were used for eDNA field sampling.



Figure 2. Laboratory tanks used to test the transfer of DNA from live turtle specimen to the water and the degradation of eDNA including a bin without any turtles physically present in the water (Empty).

Tables

Table 1. Pond turtle mitochondrial DNA sequences downloaded from GenBank for primer design.

Taxa	Common name	GenBank Accession
<i>Malaclemys terrapin terrapin</i>	Diamondback terrapin	KX774423.1
<i>Chrysemys picta</i>	Painted turtle	NC_002073.3
<i>Chrysemys picta</i>	Painted turtle	AF069423.1
<i>Chrysemys picta bellii</i>	Western painted turtle	KF874616.1
<i>Trachemys scripta</i>	Pond slider	MW122291.1
<i>Trachemys scripta</i>	Pond slider	NC_011573.1
<i>Trachemys scripta</i>	Pond slider	FJ392294.1
<i>Trachemys scripta elegans</i>	Red-eared slider	KM216748.1
<i>Trachemys scripta elegans</i>	Red-eared slider	MW019443.1
<i>Trachemys scripta scripta</i>	Yellow bellied slider	KM216749.1
<i>Trachemys scripta troostii</i>	Cumberland slider	MW122292.1

Table 2. Primer and probes designed for this experiment including the number of taxa successfully amplified for each primer set.

Primer Set	Primer	Sequence (5'-3')	Tm	Annealing Temp (°C)	Amplicon size	Taxa amplified
tRNA-Gln to ND2	F	AGGACTCGAACCTACACCAGA	57.1	57	294	20
tRNA-Gln to ND2	R	GCTTCAATTGCTCGTGGGTG	57.1	57	294	20
COI	F	TCAATAGGGGCTGTATTCGC	54.6	54	162	19
COI	R	TCCAGCAAGGCCTAGGAAAT	56.2	54	162	19
ATP6	F	GCCTCCGAAACCAACCAAC	56.9	56	199	17
ATP6	R	AATAGGGAGAGTGTTCGGT	56	56	199	17
CYTB	F	ACCCGAGACGTACAATACGG	56.4	56	221	20
CYTB	R	GCACCTCAGAAGGATATTTGGC	56	56	221	20

Table 3. The mean terrapin and red-eared slider DNA concentrations (in ng/uL) for each filter treatment over the time course of the experiment including the number of qPCR assays which detected the species in parentheses. The sample with the star (*) was only detected after extracting with the DNeasy PowerSoil Pro Kit.

Time period	Negative Filter	Empty tank	Terrapin tank	Slider tank
Day 0	0 (0)	0 (0)	2.26E-04 (7)	5.33E-03 (9)
Day 1	0 (0)	7.91E-06 (1)	1.33E-03 (9)*	7.97E-03 (9)
Day 2	0 (0)	0 (0)	1.53E-04 (9)	3.06E-03 (9)
Day 4	0 (0)	0 (0)	6.29E-05 (6)	9.47E-04 (8)
Day 7	0 (0)	0 (0)	1.93E-05 (3)	6.48E-04 (8)

Table 4. The average concentration of DNA (in ng/uL) according to a fluorescence-based measurement in the DNA extractions after removing inhibitors with the Zymo inhibitor removal column.

Time Period	Empty Tank	Terrapin tank	Slider tank
Day 0	2.31	1.95	1.70
Day 1	11.87	16.18	3.79
Day 2	14.53	19.31	4.33
Day 4	13.90	8.43	6.53
Day 7	12.23	2.47	6.35

Table 5. The average DNA concentration (in ng/uL) of the mixed and unmixed samples by species and sampling day accounting for different volumes filtered. The number of qPCR assays which had a positive result are in parentheses. The sample with the star (*) was only detected after extracting with the DNeasy PowerSoil Pro Kit.

Sampling	Terrapin		Slider	
	Unmixed	Mixed	Unmixed	Mixed
Day 0	2.26E-04 (7)	1.53E-03 (9)	5.33E-03 (9)	1.86E-03 (3)
Day 1	1.33E-03 (9)*	2.94E-04 (4)	7.97E-03 (9)	1.75E-03 (4)
Day 2	1.53E-04 (9)	5.29E-05 (6)	3.06E-03 (9)	7.75E-04 (5)
Day 4	6.29E-05 (6)	4.41E-05 (1)	9.47E-04 (8)	0 (0)
Day 7	1.93E-05 (3)	0 (0)	6.48E-04 (8)	0 (0)

Table 6. The mean terrapin and red-eared slider DNA concentration (in ng/uL) for each filtered field site including the number of qPCR assays which detected the species in parentheses.

Time period	Terrapin	Slider
Negative Filter	0 (0)	0 (0)
Oso Bay	6.70E-05 (3)	0 (0)
Lakeview	0 (0)	6.00E-03 (1)