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Research article

Development and assessment of an environmental DNA (eDNA) assay for a cryptic *Siren* (Amphibia: Sirenidae)

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ABSTRACT

Environmental DNA (eDNA) assays have become a major aspect of surveys for aquatic organisms in the past decade. These methods are highly sensitive, making them well-suited for monitoring rare and cryptic species. Current efforts to study the Rio Grande Siren in southern Texas have been hampered due to the cryptic nature of these aquatic salamanders. Arid conditions further add to the difficulty in studying this species, as many water bodies they inhabit are ephemeral, sometimes constraining sampling efforts to a short window after heavy rain. Additionally, sirens are known to cease activity and reside underground when ponds begin to dry or as water temperatures increase. Conventional sampling efforts require extensive trap-hours to be effective, which is not always possible within the required sampling window. This study presents the development of a novel eDNA assay technique for this elusive species using conventional PCR and Sanger sequencing and compares eDNA sampling results with simultaneous trapping at multiple sites to assess the relative effectiveness of the procedure. Rio Grande Siren detection via eDNA sampling was significantly higher at all sites compared to trapping, confirming the utility of this assay for species detection. This methodology gives promise for future work assessing the distribution and status of the Rio Grande Siren and has potential for use on other southern Texas amphibians.

1. Introduction

The salamander genus *Siren* is currently known to encompass three species native to the southeastern United States: the Lesser Siren (*Siren intermedia*; found from Virginia south to central Florida, along the coastal plain west to southern Texas, and along the Mississippi drainage north to Michigan), Greater Siren (*S. lacertina*; found from Virginia south to southern Florida, and west to southeastern Alabama), and Reticulated Siren (*S. reticulata*; found in southern Alabama and the Florida panhandle; Petranka, 1998; Graham et al., 2018). Sirens are fully aquatic, neotenic salamanders with long and eel-like bodies, small, reduced forelimbs, and no hindlimbs (Petranka, 1998), and they are most often found in shallow freshwater habitats with minimal flow, ranging from resacas (also called oxbow lakes) and ponds to drainage ditches. Despite their relatively large size and abundances, sirens are generally understudied and poorly understood.

The taxonomic identity of sirens in southern Texas has challenged herpetologists for over 80 years. Populations from Aransas and Live Oak counties, west to Maverick County and south to the Rio Grande, in some part, have been assigned to *Siren intermedia, S. i. nettingi, S. i. texana, S. lacertina, S. i. nettingi* × *S. i. texana* hybrids, or an endemic species, *S. texana* (Frost and Lannoo, 2005; Tipton et al., 2012; Dixon, 2013). Given the confusion surrounding the taxonomic assignment of sirens from this region, Tipton et al. (2012) withheld a formal taxonomic assignment but noted differences between southern Texas *Siren* and *S. intermedia* found elsewhere in Texas. Recent analysis with cytochrome b suggests that sirens from eastern and southern Texas differed markedly from the *S. lacertina/S. intermedia* group from Arkansas, eastern Louisiana, and Florida (Graham et al., 2018). Currently, Texas Parks and Wildlife Department (TPWD) lists sirens in southern Texas as "South Texas Siren (large form), *Siren* sp. 1", but are more often referred to as Rio Grande Sirens, *Siren intermedia texana*, which is the taxonomy we

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follow here. Regardless of their name, these sirens are classified as state-threatened by TPWD (Davis and LaDuc, 2022), making their study imperative.

Detection of aquatic salamanders can be challenging. Cryptic species that evade detection may be incorrectly labeled as rare, and the exclusion of certain species due to lack of capture may produce inaccurate information regarding species richness (Evans et al., 2016). Common methods for amphibian detection include visual encounter surveys (Biagas et al., 2012), call surveys (Guzy et al., 2014), funnel trapping (Buech and Egeland, 2002), and dip netting (Denton and Richter, 2013), but success with these methods can vary greatly depending on species, life stage, and environmental conditions (Farmer et al., 2009). Detection of sirens is most often limited to trapping; however, dip netting and visual encounter surveys may be effective in shallow water with high siren densities (McDaniel, 1969). Altogether, trap success for sirens can be highly variable. Setting traps consistently even in an area known to be populated with sirens does not guarantee detection (Sorensen, 2003). Because of this, new alternative methods for detection are needed to monitor cryptic species such as sirens.

Environmental DNA (eDNA) sampling allows for the detection of aquatic species without needing to physically capture, observe, or hear the species in the field. eDNA sampling can be used to detect species that traditional survey methods (dip netting, funnel trapping, visual encounters, and call surveys) may miss (McKee et al., 2015; Spear et al., 2015). eDNA surveys are also less invasive, are less time and labor intensive, and have been found to be a more cost-effective method of detection than traditional sampling (Ficetola et al., 2008; Goldberg et al., 2011, 2016; Taberlet et al., 2012; Hoddman et al., 2016; Brozio et al., 2017).

Different environments hold unique challenges for aquatic eDNA studies (Harrison et al., 2019). Filtration is the most common method of obtaining eDNA, with 78% of studies using this method, but factors such as pore size and filter material may have an impact on eDNA collection (Tsuji et al., 2019). Preservation and extraction of eDNA from filters also varies based on filter type and environment (Goldberg et al., 2016; Tsuji et al., 2019). Following eDNA collection and extraction, samples can be amplified with polymerase chain reaction (PCR) using species-specific primers, often targeting the cytochrome oxidase 1 (CO1) region due to its high specificity and availability of sequences in reference databases (Smith et al., 2008; Tsuji et al., 2019). Primers must be designed to be specific to the species without creating dimers, PCR conditions must be optimized for amplification of small amounts of DNA, sampling procedures must be tested to eliminate contamination, preservation and extraction procedures must be assessed, and field tests must be completed to ensure the entire process is functioning adequately; in short, an assay must not only be designed, but also validated (Goldberg et al., 2016; Thalinger et al., 2021). Depending on the specificity and efficiency of these primer sets, products may be visualized on a gel, quantified via quantitative PCR (qPCR), purified and sequenced, or run through nested or semi-nested PCR to improve detection capabilities.

Comparisons between eDNA surveys and traditional detection methods are necessary to determine the ideal method balancing detection with cost, time, and labor. This can be accomplished through simultaneous use of traditional and molecular detection methods and comparison of the success of each using statistical tools such as occupancy models (Dejean et al., 2012; Takahara et al., 2013; Smart et al., 2015; Smith 2017; Dorazio and Erickson, 2018; Wineland et al., 2019; Rose et al., 2019). Detection rates can be compared directly via percent of positive detection for each method, or by comparing catch per unit effort, from which statistical significance can be determined; methodology varies between studies, but the majority find eDNA sampling to have a higher detection rate than traditional sampling (Jerde et al., 2011; Dejean et al., 2012; Takahara et al., 2013; McKee et al., 2015; Smart et al., 2016; Smith, 2017). The use of eDNA sampling methods has been applied numerous times to survey amphibians, particularly those which are largely or completely aquatic (Goldberg et al., 2011; Pilliod et al., 2013, 2014; Rees et al., 2014; Spear et al., 2015; Eiler et al., 2018; Wineland et al., 2019). Here, we developed a novel eDNA assay for the detection of the Rio Grande Siren (*S. i. texana*) and compared eDNA sampling to traditional trapping methods over 12 months to assess the effectiveness of each method on species detection.

2. Material and methods

2.1. PCR protocol

Initial and semi-nested primer sets were designed targeting the cytochrome oxidase I (CO1) mitochondrial gene region using sequences available from GenBank (Table 1; GenBank accession numbers: KU871392, KU904482–KU904487, KU904489). Using the software Primer3 (Rozen and Skaletsky, 1999), we developed primer sets specific to Rio Grande Siren with similar melting temperatures and minimal dimer formation. As eDNA is often degraded, the primer sets were developed to amplify a relatively small region. While the target for the primer design was the Rio Grande Siren, amplification of other subspecies was not intentionally avoided due to the taxonomic uncertainty surrounding this group. Primers were tested for specificity using queries in GenBank; any matches in primer sequences were examined to ensure the exclusion of potentially sympatric species. All primers and Sanger sequencing services were ordered from Eurofins Genomics LLC (Louisville, KY, USA). Primer sets were optimized by adjusting the annealing temperature using the results of gradient PCR.

The forward and reverse primers of the initial set amplified a 182-bp fragment in the CO1 region (Table 1), and the semi-nested primer set amplified a 176-bp fragment. The same forward primer sequence was used for both initial and semi-nested sets. Due to the nature of nested PCR, no measures of sensitivity were possible. For PCR, 25 μL reactions were used consisting of 12.5 μL master mix (GoTaq G2 HotStart MasterMix, Promega, Madison, WI, USA), 5.5 μL H_2O, 1 μL 2 μM CO1B-forward primer (FW), 1 μL 2 μM CO1B-reverse primer (RV), and 5 μL sample and amplified for 35 cycles (initial denaturing: 95°C for 30 s; denaturing: 95°C 15 s; annealing: 57 °C for 30 s; extension: 72 °C for 15 s; cooling: 4°C for 10 min). For semi-nested PCR, the protocol was the same with the following changes to reagents: CO1B-FW and CO1B-RVnest were used at 5 μM, and 5 μL of product (purified using Monarch PCR & DNA Cleanup Kits [New England BioLabs, Ipswich, MA, USA]) from the first round of PCR was used.

In developing this assay, we tested numerous primer sets across the mitochondrial genome of sirens prior to this CO1B primer set. Additionally, we developed both SYBR and probe-based qPCR assays to improve detectability. While these primer sets and qPCR assays were effective in detecting siren eDNA from aquarium samples, amplification was highly inhibited when water from our field locations was added as a dilutant, making confident detection and quantification unfeasible. The water at sites known to contain sirens is opaque green to brown and is very turbid containing urban runoff, agricultural runoff, pesticides, herbicides, and humic acids, leading to the observed inhibition. Inhibitor removal cocktails, changes to extraction procedures, and modification of PCR protocol did not improve these assays. By using the semi-nested protocol described above, we were able to detect sirens despite the difficulties with inhibitors present in water samples.

PCR products from Rio Grande Siren tissue DNA (extracted using GenCatch Blood and Tissue Genomic DNA kits [Epoch Life Science, Sugar Land, TX, USA]) using the above PCR protocols were run on a 1% agarose EtBr gel electrophoresis, resulting in high fluorescent intensities when viewed under a UV transilluminator and resulting in a 100% match for Rio Grande Siren when sent for Sanger sequencing and searched using NCBI Blast (blast.ncbi.nlm.nih.gov/blast.cgi). The primer sets were also tested with DNA from Lesser Siren tissue samples from Harris County, Texas, as well as individuals from Arkansas, Mississippi, and Oklahoma, USA (Table 2). Primers were also tested against tissue samples from 13 southern Texas sympatric amphibians to ensure

Table 1
Sequences and melting temperatures for initial and semi-nested primers, and full amplified sequences for each primer set. Both primer sets used the CO1B-FW primer. Underlined sections of full and semi-nested sequences indicate primers.

Name	Sequence 5'–3'	Melting Temp. (°C)
CO1B-FW	ACGCTATTCCGATTATCCAG	58.4
CO1B-RV	GACATCCGTGAAGTCATTC	58.0
CO1B-RVnest	CGTGAAGTCATTCTACATTAGTTG	59.4
Full Sequence	ACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCATCAATT GGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATCATTTGAGAAGCCTTCTCA GCTAAACGAGAAGTTAAGTCCACTGAACTAACTTCAACTAATGTAGAATGACTTCACGGATGTC	N/A
Semi-nested Sequence	ACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCATCAA TTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATCATTTGAGAAGCCTT CTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAACTTCAACTAATGTAGAATGACTTCACG	N/A

specificity of primers for *Siren* (Table 2). To validate the primers with water samples, pond water spiked with water from an aquarium containing two captive Rio Grande Sirens at a concentration of 5% was filtered and extracted following the eDNA collection and extraction protocols described below.

2.2. eDNA collection and extraction protocols

Water from field sites was collected using a pitcher attached to an extendable telescoping pole and care was taken to not stir up sediment while obtaining water, as eDNA from sediment may persist longer than aquatic eDNA (Goldberg et al., 2016). The filtration device consisted of a 47-mm filter cup (XX1104700, MilliporeSigma, Darmstadt, Germany) on a PVC arm that could be inserted into a hand-powered automotive fluid evacuator (MV7400, Mityvac, St. Louis, MO, USA; Fig. 1A). Other filtration methods were tested, and this was found to be the most effective for filtering water quickly in the field without requiring carrying heavy equipment, an important consideration for locations where immediate chilling or preservation of water samples for future filtration is not possible. Filters used were Whatman Grade 4 cellulose filters (1004047, GE Healthcare, Chicago, IL, USA) with an approximate pore size of 25 μm.

Prior to sampling, all equipment was washed with a 50% chlorine bleach solution followed by a 0.1 M sodium thiosulfate wash and distilled water rinse. For each sample, one field blank was collected by passing 1 L of distilled water through the filter, and then three field samples were taken, where up to 1 L of site water was passed through the filter (or until the filter became clogged). After filtration, filters were promptly placed in 2-mL microcentrifuge tubes pre-filled with 700 μL of DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA; Chomczynski et al., 1997). Fresh nitrile gloves were used for each sample (1 field blank + 3 eDNA filters).

The filter eDNA extraction protocol was adapted from the DNAzol manual (Bare, 2018). Filters were stored in the dark in DNAzol for up to 6 months at room temperature before extraction procedures. Samples stored in DNAzol at room temperature are expected to be viable for a year or more. The samples were then heated at 55 °C for 30 min, vortexed, and centrifuged for 1 min at 5000 *g*. Filters were then removed from heat and squeezed using clean forceps to collect all DNAzol in each microcentrifuge tube. Next, 500 μL of chloroform was added to each tube, vortexed, let stand 1 min, and centrifuged 2 min at 12,000 *g*. Supernatants were extracted into clean 1.5-mL microcentrifuge tubes. 500 μL of absolute EtOH was added to each tube, inverted until mixed, and centrifuged for 10 min at 16,000 *g* to pellet the DNA. Supernatants were discarded, and pellets were washed with 500 μL 95% EtOH, followed by a second wash in 500 μL 75% EtOH. Pellets were air-dried for 30 min before being dissolved in 22 μL dilute Tris-EDTA buffer solution (3 mM Tris-Cl, 0.3 mM EDTA, pH 8.0) at 55 °C.

All extracted samples were quantified for total DNA concentration using a Qubit 3.0 Fluorometer (Q33216, ThermoFisher Scientific, Waltham, MA, USA) with a Qubit dsDNA high sensitivity assay kit (Q32851, ThermoFisher Scientific, Waltham, MA, USA) and stored at -20 °C. All eDNA extractions took place in a separate clean lab from the PCR and tissue processing lab to help prevent contamination of samples (Goldberg et al., 2016). Benchtops and micropipettes were cleaned with 50% chlorine bleach solution before extractions, and only sterile filter pipette tips were used.

2.3. eDNA detection at field sites

eDNA samples were collected at a total of 10 sites across southern Texas, USA to test the efficacy of the newly designed assay (Fig. 2A,B). Five lentic sites were chosen near Brownsville, Cameron County based on known Rio Grande Siren occurrence (Fig. 2B). These sites included: Resaca de la Palma State Park (RP; 25.98760°N, 97.56434°W), the former TPWD Olmito Fish Hatchery (FH; 25.98602°N, 97.53138°W), UTRGV Lozano Banco Resaca (LB; 25.89503°N, 97.48720°W), Sabal Palm Sanctuary (SP; 25.85004°N, 97.41927°W), and TNC Southmost Preserve, Black Willow Resaca (SM; 25.85428°N, 97.39193°W). An additional five lotic sites were selected along the Rio Grande drainage (Fig. 2A). These sites included: Rio Grande, near Eagle Nest Canyon (EN; 29.80829°N, 101.54893°W), Rio Grande, below Amistad Dam (AD; 29.42455°N, 101.04118°W), Fort Clark Springs, Headwater Pond (FC; 29.30944°N, 100.42125°W), Rio Grande, at Eagle Pass (EP; 28.70416°N, 100.51046°W), and Rio Grande, below Falcon Dam (FD; 26.54608°N, 99.17093°W). Of the five sites along the Rio Grande drainage, records of Rio Grande Sirens exist only near one site (EP), where seven individuals were collected and vouchered from Upson, Maverick County in 1880 (Goin, 1957). These sites were chosen because they were unlikely, but not out of the range of possibility, to contain Rio Grande Siren as they occur along the western edge of the species' range and were visited one time each for water collection. For a positive eDNA detection, a band of the correct size needed to be observed for at least two of the three replicates when visualized on the agarose gel. All positive bands from semi-nested PCR were sequenced for further confirmation, requiring a ≥95% match to published Rio Grande Siren sequences to be considered positive, as well as a top alignment match of Rio Grande Siren. Lack of sequence confirmation resulted in a negative detection, regardless of band presence. If any amplification in field blanks indicated potential contamination, all replicates from those samples were excluded. If any amplification was detected in the no-template controls, all samples were re-run to ensure exclusion of false positives.

2.4. eDNA sampling vs. trapping comparison

A comparison of eDNA sampling to the traditional method of trapping was conducted at the five lentic sites with known populations of Rio Grande Sirens in Cameron County, Texas (see *eDNA detection at field sites* above; Fig. 2B). All locations were visited monthly from March 2019 to

Fig. 1. eDNA and *Siren* Collection
A) eDNA collection equipment, consisting of a 47-mm filter cup on a PVC arm that is inserted into a hand-powered automotive fluid evacuator; B) trapped Rio Grande Siren (*Siren intermedia texana*) in a vinyl-coated metal minnow funnel trap.

February 2020. We attempted to sample in the middle of each month, but this was dependent on equipment availability, weather, and the presence of water at these sites. When no water was available, sites were not sampled.

At each site each month, we collected a single eDNA sample (1 field blank + 3 eDNA samples) and set eighteen traps overnight to sample for sirens. Occasionally, the amount of water present at a site was not adequate to place the full number of traps, and the numbers of traps were reduced accordingly. The traps used were vinyl-dipped, two-piece metal minnow traps, cylindrical in shape with funnel ends, that had enlarged openings to accommodate larger individuals (Fig. 1B). Traps were baited with fresh chicken liver in small, perforated plastic containers within the traps, and were set with small foam flotation devices to avoid accidental drownings. Traps were set in shallow water along the edge of the water body within vegetation to maximize the chances of siren capture. Traps were set overnight with the average time set of 21.96 h (standard deviation: ± 1.79 h). Trapped sirens were counted, tissued, swabbed, and released at the site of capture. All trapping was conducted under an approved UTRGV IACUC protocol (AUP #18-28) and under an approved TPWD Scientific Collecting Permit (SPR-1018-294), both issued to DRD.

2.5. Statistical analyses

A Fisher's exact test was used in R v3.4.4 software (R-project, 2013) to determine whether the difference in detection between trapping and eDNA sampling was statistically significant. A P value < 0.05 was considered as statistically significant.

3. Results

3.1. Detection and sequencing of eDNA samples

Both the initial and semi-nested primer sets successfully amplified DNA from sirens collected from Cameron and Kinney counties, confirming the effectiveness of the eDNA assay for Rio Grande Sirens. The primer sets also amplified DNA from Lesser Sirens from Harris County, Texas, and Arkansas, Mississippi, and Oklahoma (Table 2). Additionally, our primer sets did not amplify DNA from tissue samples of other southern Texas amphibian species (Table 2). PCR products from pond water spiked with captive Rio Grande Siren aquarium water (5%) and run on 1% agarose EtBr gel electrophoresis resulted in high fluorescent intensities when viewed under a UV transilluminator. When eDNA samples were run through the semi-nested PCR and sequenced, sequences were identified as Rio Grande Siren DNA with high confidence (≥95% similarity) based on an NCBI BLAST search (Fig. 3). For most samples, a single round of PCR was not adequate to produce enough copies of the target region for Sanger sequencing; this was likely due to inhibition, which was overcome by using the semi-nested PCR procedure to produce adequate copies for DNA for sequencing.

3.2. eDNA detection of sirens across southern Texas

Our assay successfully detected Rio Grande Siren eDNA in field samples collected at all five lentic sites in Cameron County, Texas (Fig. 2A) where sirens are previously known to exist based off vouchered specimens, citizen science observations, and literature reports. For the lotic sites further up the Rio Grande drainage, we detected Rio Grande Siren eDNA at two sites (Fig. 2B). These sites include the Rio Grande, at Eagle Pass (EP), which is near a locality where sirens have not been detected since 1880, and the Rio Grande, below Amistad Dam (AD), which represents the furthest area up the Rio Grande drainage that sirens have been detected.

3.3. eDNA sampling vs. trapping comparison

A total of 66 Rio Grande Sirens were captured over 12 months of sampling at the five Cameron County sites (Table 3). Sirens were successfully trapped at four sites; Sabal Palm Sanctuary (SP) proved to be the most productive site for siren capture, with a total of 34 sirens captured, and no sirens were ever captured at the former TPWD Olmito Fish Hatchery (FH). The most productive month for siren capture was October 2019, with 15 sirens captured, and no sirens were captured in June or August at any site. Of the 56 trapping events, there were 25 (44.64%) positive events, as any number of sirens per site per month was considered a positive detection. A lack of water on four occasions prohibited sampling. eDNA was detected at all sites. A total of 55 (98.21%) eDNA samples had positive detection of sirens with at least two samples matching the sequences for siren, meeting the threshold for a positive eDNA detection. Detection methods were compared via a Fisher's exact test and indicated a significantly higher detection rates for eDNA than for trapping ($P < 0.0001$).

4. Discussion

The described eDNA assay with initial and semi-nested primers was able to successfully amplify and provide matching sequences to the GenBank database for Rio Grande Sirens from numerous localities across southern Texas. Additionally, the primer sets and PCR protocol successfully amplified tissue from other Lesser Sirens, indicating the potential application of the assay across the species' range. The specificity of the primers to Rio Grande Sirens was confirmed by testing them against other sympatric amphibian species from southern Texas, thus ruling out the potential for false positives. The ability to store the samples at room temperature for over six months will benefit long term

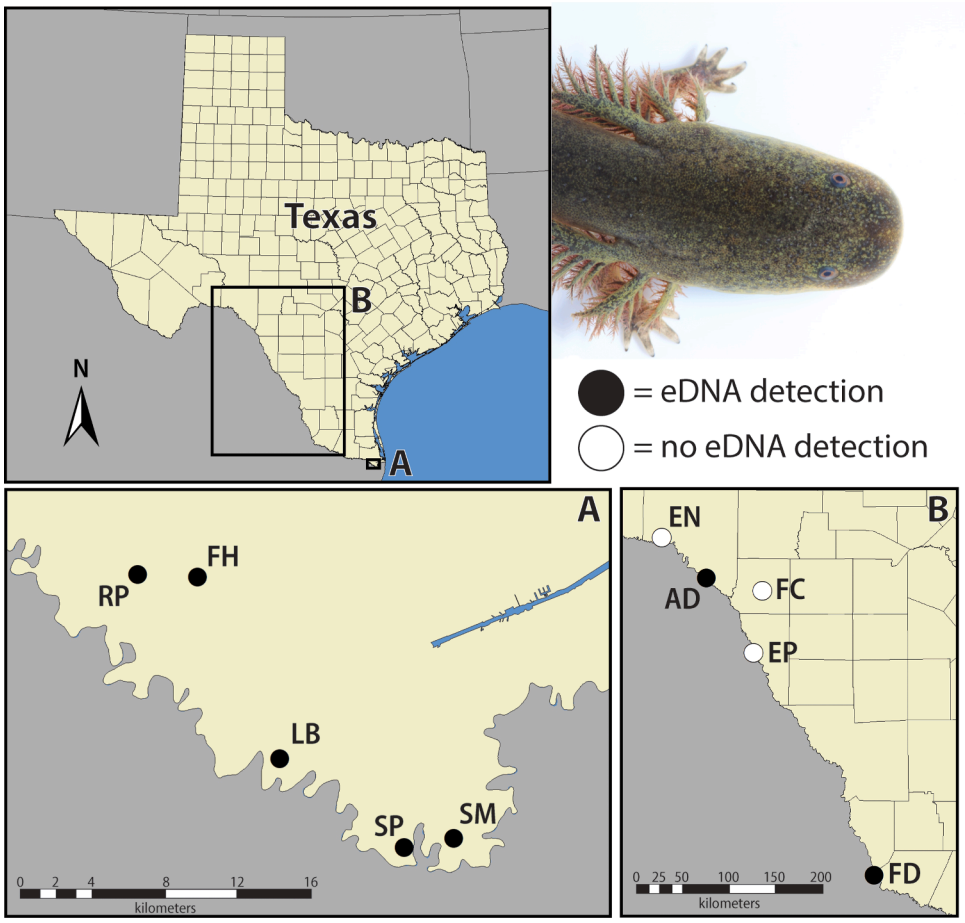

Fig. 2. Sampling Sites
Map of Rio Grande Siren (*Siren intermedia texana*) sampling sites across southern Texas, USA: A) five sites located in Cameron County, Texas where eDNA was sampled and where the eDNA sampling vs. trapping comparison was conducted; B) five sites located along the Rio Grande drainage where additional eDNA sampling was conducted. Black circles indicate positive siren eDNA detection and white circles indicate no siren eDNA detection. Complete locality information on sampling sites is described within the text.

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Amplified Region     ACGCTATTCCGATTATCCAGATGCATATACGCTATGAAATTCCATCTCAT
Siren i. texana      ACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCAT
Siren intermedia     ACGCTATTCTGATTACCCAGATGCATATACGCTATGAAATTCCATCTCAT
Siren reticulata     ACGATACTCAGACTACCCAGATGCATACACCCTCTGAAATTCTGTTTCAT
Siren lacertina      ACGTTACTCTGATTACCCAGACGCCTATACTTTATGAAATTCTATTTCAT
                     *** ** ** ** ** ***** ** ** **  * ********  * ****

Amplified Region     CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
Siren i. texana      CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
Siren intermedia     CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
Siren reticulata     CAATCGGATCACTAATTCATTAGTAGCAGTTATTATAATAATATTTATT
Siren lacertina      CAATCGGATCATTAATCTCACTAGTAGCAGTTATCATAATAATATTTATT
                     **** *****  **** *** ************ **************

Amplified Region     ATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAAC
Siren i. texana      ATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAAC
Siren intermedia     ATTTGAGAAGCCTTCTCAGCTAAGCGAGAAGTCAAGTCCACCGAACTAAC
Siren reticulata     ATTTGAGAAGCCTTCTCAGCCAAACGAGAAGTGAAATCTACTGAACTTAC
Siren lacertina      ATTTGAGAAGCCTTTTCAGCCAAGCGGGAAGTAAACTCTACAGAACTTAC
                     ************** ***** ** ** ***** ** ** ** ***** **

Amplified Region     TTCAACTAATGTAGAATGACTTCACGGATGTC
Siren i. texana      TTCAACTAATGTAGAATGACTTCACGGATGTC
Siren intermedia     TTCAACTAATGTAGAGTGACTTCATGGATGCC
Siren reticulata     ATCAACTAAAGTAGAATGACTTCACGGATGCC
Siren lacertina      ATCAACCAATGTAGAATGATTACATGGATGCC
                      ***** ** ***** *** * ** ***** *
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Fig. 3. Amplified Regions
Alignment of the amplified region with published sequences from Rio Grande Siren (*Siren intermedia texana*; GenBank accession number: KU904486), Lesser Siren (*S. intermedia*; MH888024), Reticulated Siren (*S. reticulata*; MH888031), and Greater Siren (*S. lacertina*; NC036927). Original primers are underlined, with mismatches identified in red text. One base pair in the forward primer was intentionally changed from T to C in order to prevent dimer formation. Percent overlap with the amplified region is 99.45% for *S. i. texana*, 95.63% for *S. intermedia*, 86.89% for *S. reticulata*, and 84.07% for *S. lacertina*. Asterisks indicate matching base pairs.

Table 2
List of specimens and tissue samples tested against designed siren primers to ensure species specificity. TNHC = Biodiversity Collections, The University of Texas at Austin; MMNS = Mississippi Museum of Natural Science; OMNH = Sam Noble Oklahoma Museum of Natural History, University of Oklahoma; DRD = Drew R. Davis Field Series.

Family	Species	State	County	Catalog Number
Sirenidae	*Siren intermedia*	TX	Cameron	TNHC 116623
Sirenidae	*Siren intermedia*	TX	Harris	TNHC 116622
Sirenidae	*Siren intermedia*	TX	Kinney	TNHC 112457
Sirenidae	*Siren intermedia*	AR	Craighead	DRD 5457
Sirenidae	*Siren intermedia*	MS	Perry	MMNS 5978
Sirenidae	*Siren intermedia*	OK	McCurtain	OMNH 48185
Ambystomatidae	*Ambystoma mavortium*	TX	Cameron	TNHC 114655
Salamandridae	*Notophthalmus meridionalis*	TX	Cameron	DRD 5165
Bufonidae	*Anaxyrus speciosus*	TX	Kenedy	TNHC 112166
Bufonidae	*Incilius nebulifer*	TX	Cameron	TNHC 112149
Bufonidae	*Rhinella horribilis*	TX	Willacy	TNHC 114653
Hylidae	*Smilisca baudinii*	TX	Cameron	TNHC 114656
Leptodactylidae	*Leptodactylus fragilis*	TX	Zapata	TNHC 114657
Microhylidae	*Gastrophryne olivacea*	TX	Hidalgo	TNHC 112082
Microhylidae	*Hypopachus variolosus*	TX	Hidalgo	TNHC 112004
Ranidae	*Rana berlandieri*	TX	Cameron	TNHC 112113
Ranidae	*Rana catesbeiana*	TX	Refugio	TNHC 114658
Rhinophrynidae	*Rhinophrynus dorsalis*	TX	Starr	TNHC 114654
Scaphiopodidae	*Scaphiopus couchii*	TX	Cameron	TNHC 112175

field studies and reduce storage costs. This benefit was also noted by Renshaw et al. (2015). Our results build upon others who have successfully developed eDNA assays to survey for cryptic amphibians, and show the utility of these methods (Rees et al., 2014; Fukumoto et al. 2015; McKee et al., 2015; Spear et al., 2015; Smith 2017; Vörös et al., 2017; Wineland et al., 2019).

When we tested this eDNA assay at sites where Rio Grande Sirens were not expected, three sites were negative, and two sites were positive for siren eDNA. Given the understudied nature of sirens, it is entirely possible that Rio Grande Sirens exist further up the Rio Grande drainage than previously recognized. This has been recently supported by new records of sirens collected at this western geographic extent within the Rio Grande drainage (Davis et al., 2019). These Rio Grande drainage sites are on the edge of the described range of sirens in Texas, and further surveys in suitable habitat in these areas may reveal previously unreported populations of sirens. It is important to note that these were lentic sites; it is possible that water flow could transport eDNA downstream (Stoekle et al., 2017), but no populations of sirens are known upstream of these positive eDNA detections (Frost and Lannoo, 2005; Tipton et al., 2012; Dixon, 2013).

The assay developed herein opens new avenues for siren detection and monitoring. As Rio Grande Sirens are a cryptic species that are difficult to detect through conventional means, this eDNA assay offers an exciting avenue to assist in studies of this threatened salamander. While the specific target of this study was the Rio Grande Siren, the successful amplification of Lesser Siren DNA from other regions introduces the prospect of widespread siren monitoring via eDNA sampling. The use of Sanger sequencing followed by a BLAST search in GenBank for each positive sample ensures the correct identification of the targeted species. Collectively, the development of this assay can be considered successful and applicable for the desired purposes and can function as another tool

Table 3
Results of paired trapping and eDNA surveys for Rio Grande Sirens (*Siren intermedia texana*) from March 2019–February 2020. Sampling month, site, trap detection, and eDNA detection are provided. Site Codes correspond with those shown in Fig. 3 and described in the text. Positive detection (Y) and no detection (N) are provided and the number in parentheses indicates the numbers of sirens trapped or the number of eDNA technical replicates that amplified siren eDNA. Occasions when sampling was not possible due to sites being dry are indicated by a dash.

Sampling Month	Site Code	Trap Detection?	eDNA Detection?
March 2019	LB	N (0)	Y (3)
	SM	N (0)	Y (2)
	FH	N (0)	Y (3)
	SP	Y (5)	Y (2)
	RP	Y (4)	Y (2)
April 2019	LB	Y (2)	Y (2)
	SM	N (0)	Y (3)
	FH	N (0)	Y (3)
	SP	Y (11)	Y (2)
	RP	Y (1)	Y (3)
May 2019	LB	Y (2)	Y (3)
	SM	Y (1)	Y (2)
	FH	N (0)	Y (3)
	SP	N (0)	Y (3)
	RP	N (0)	Y (3)
June 2019	LB	N (0)	Y (3)
	SM	N (0)	Y (3)
	FH	N (0)	Y (3)
	SP	N (0)	Y (3)
	RP	N (0)	Y (3)
July 2019	LB	N (0)	Y (3)
	SM	Y (1)	Y (3)
	FH	N (0)	Y (3)
	SP	–	–
	RP	N (0)	Y (2)
August 2019	LB	N (0)	Y (3)
	SM	–	–
	FH	N (0)	Y (3)
	SP	–	–
	RP	N (0)	Y (3)
September 2019	LB	Y (1)	N (1)
	SM	–	–
	FH	N (0)	Y (3)
	SP	Y (4)	Y (3)
	RP	Y (1)	Y (3)
October 2019	LB	Y (1)	Y (2)
	SM	Y (7)	Y (2)
	FH	N (0)	Y (2)
	SP	Y (6)	Y (3)
	RP	Y (1)	Y (2)
November 2019	LB	N (0)	Y (3)
	SM	Y (3)	Y (2)
	FH	N (0)	Y (3)
	SP	Y (1)	Y (2)
	RP	Y (2)	Y (2)
December 2019	LB	Y (2)	Y (3)
	SM	Y (1)	Y (2)
	FH	N (0)	Y (3)
	SP	Y (4)	Y (2)
	RP	N (0)	Y (2)
January 2020	LB	Y (1)	Y (2)
	SM	N (0)	Y (3)
	FH	N (0)	Y (3)
	SP	N (0)	Y (2)
	RP	N (0)	Y (2)
February 2020	LB	N (0)	Y (2)
	SM	Y (1)	Y (3)
	FH	N (0)	Y (2)
	SP	Y (2)	Y (2)
	RP	N (0)	Y (2)

in the toolbox of amphibian research and conservation (Heyer et al., 1994).

One noteworthy aspect of the assay was the successful amplification of environmental DNA from filters with a 25-μm pore size. Previous research suggests that filtering large amounts of water through large-pore filters is more effective at retrieving eDNA than small amounts of water through small-pore filters; however, the use of large-pore filters (>10 μm) for eDNA capture in highly turbid waters has not been tested (Turner et al. 2014; Minamoto et al. 2016). Filter pore sizes for eDNA studies typically range from 0.45–3 μm, but even the largest of these is not able to consistently filter 1 L of water from siren habitat (KMR, unpubl. data). Robson et al. (2016) tested polycarbonate filters up to 20 μm for use in turbid, tropical water but determined that 4 L would be necessary for detection based on work by Turner et al. (2014). Siren habitat in southern Texas does not necessarily allow the filtering of this much water before becoming clogged. Given small fragments of DNA, a small pore filter would prevent more fragments from passing through, thus catching more DNA as was found by Liang and Keeley (2013) and Eichmiller et al. (2016), but a large amount of water would give a greater sample volume, which has been found to increase the probability of detection (Lopes et al., 2017). We have illustrated that large pore filters can be effective in environmental DNA retrieval in highly turbid environments. eDNA detection has the added benefit of potentially being more cost-effective than traditional surveys, particularly for elusive or cryptic species such as sirens (Biggs et al., 2015; Davy et al., 2015; Sigsgaard et al., 2015). The exact cost is variable among studies; once an assay has been developed, the cost may be less thantraditional surveys, particularly in labs already equipped with the necessary equipment (Goldberg et al., 2011; Biggs et al., 2015; Smart et al., 2016). For elusive species, the cost of reagents for running eDNA samples may be preferable to the cost of employing individuals to set and check traps and/or conduct field surveys over long periods of time (Huver et al., 2015; Sigsgaard et al., 2015; Davy et al., 2015; Smart et al., 2015, 2016).

Semi-nested PCR proved to be an effective method in improving the detection of Rio Grande Siren eDNA via Sanger sequencing that we could not accomplish in our preliminary attempts with a single round of PCR or qPCR. By running two rounds of PCR, the specificity of amplification may be increased as two sets of primers are used (Erume et al., 2001; Jackson et al., 2017). Most noticeably, nested PCR may offset issues of low copy numbers that are often problematic in eDNA studies (Wilcox et al., 2013). Finally, the concentration of inhibitors would have been greatly reduced in the second round of PCR (Erume et al., 2001).

In developing this assay, conventional PCR was used rather than quantitative PCR (qPCR) or droplet digital PCR (ddPCR). Using either of these methods may have further expanded the applicability of our assay with the potential for quantification, improved detection sensitivity, and measures of assay sensitivity (Turner et al., 2014; Piggott, 2016; Williams et al., 2017). Further, ddPCR has been found to be less prone to inhibition, more sensitive, and more precise than qPCR (Doi et al., 2015; Hunter et al., 2016; Mauvisseau et al., 2019; Wood et al., 2019; Brys et al., 2020). However, the use of conventional PCR in eDNA assays is often more affordable than qPCR or ddPCR assays (Dejean et al., 2011; Piaggio et al., 2014; Davy et al., 2015; Davison et al., 2016; Simpfendorfer et al., 2016). As every sample was fully sequenced for use in species identification, the likelihood of false positive results from PCR artifacts is low (Davy et al., 2015; Schultz and Lance, 2015; Xia et al., 2018). Future improvement to the assay could allow for augmention with the addition of a probe to perform qPCR or ddPCR.

Though all sites sampled in Cameron County were known to contain Rio Grande Sirens, the consistent detectability of sirens via eDNA sampling was surprising. For the single negative eDNA sample, it is possible that the addition of another sample set (three eDNA samples from a different location in the water body) could be enough to detect sirens; however, as only one sample was negative at one site during one month, eDNA sampling remained highly efficient at detecting sirens, with ca. 98% of samples being positive for siren eDNA. Due to this high detection rate, seasonal trends in detection probability were not able to be determined. All negative controls yielded negative results, indicating that the sampling method was valid and was not subject to false positives, either from field or laboratory contamination. Though our results did not suggest false positives, the addition of more field and laboratory blanks in future studies could improve confidence in results given the possibility of contamination (Hutchins et al., 2021). Additionally, it is possible that the negative eDNA sample set is a false negative due to primer competition (Wilcox et al., 2013; Rees et al., 2014; Goldberg et al., 2016), as sirens were captured at the site during concurrent trapping. Though the eDNA sampling for this comparative study was limited (one eDNA sample per site per month), it is possible that the addition of more samples from more water bodies over a longer period could change the detection rate.

Though Rio Grande Sirens were not captured at the former TPWD Olmito Fish Hatchery (FH) during this study, eDNA sampling data indicates their continued presence at this site. In 1958, 81 sirens were captured and preserved from this location by A. G. Flury (specimens housed at the Biodiversity Collections, The University of Texas at Austin). Lafortune (2015) captured one siren at this location, and sirens have been anecdotally observed in the resaca from 2015–2018. It is possible that sirens were not captured due to the nature of the habitat at site: very little edge vegetation highly turbid waters, and no emergent or submerged vegetation.

When the two survey methods are compared, it is clear that eDNA sampling is far more effective at detecting Rio Grande Sirens. This follows similar findings in other studies regarding the utility of eDNA sampling compared to conventional surveys for amphibian detection (Fukumoto et al., 2015; McKee et al., 2015; Smith, 2017; Vörös et al., 2017; Eiler et al., 2018; Wineland et al., 2019) but is the first of its kind to document the efficiency of eDNA sampling for sirens. This opens possibilities for surveying and understanding siren distributions more effectively. As conventional trapping was found to be less consistent, eDNA sampling could be used to map siren distributions more effectively and efficiently without needing to capture sirens directly.

Additionally, the effort required for detecting sirens using eDNA sampling was lower than that for trapping. For trapping, setting traps required visiting the site twice. Preparing and placing the traps can take up to 1 h, and setting traps in wetlands can be physically disruptive to the habitat. Physically capturing and handling animals requires special collections permits, particularly for protected species; often, these permits are not required, or are easier to acquire, for the collection of eDNA samples. Collecting a single sample set for eDNA would take <0.5 h in the field without requiring wading into the water body, followed by lab work, including ca. 3 h for filter extraction and 6 h for nested amplification and sequencing preparation. As many samples can be processed simultaneously in the lab, the overall time invested in processing eDNA samples is less than with trapping, as well as requiring less travel and less intense field work. For species detection, eDNA sampling is clearly advantageous in terms of saving time and effort and successfully detects sirens.

Despite the promise of eDNA sampling, it is important to remember that a species' complete biology and ecology cannot currently be determined from eDNA samples alone, and since so little is known about sirens, further in-depth studies are necessary to better understand the needs of these species. As such, eDNA surveys could be used to screen for presence of sirens prior to using traditional methods for population assessments, thus potentially reducing the overall trap effort. Moreover, eDNA sampling is an additive tool for siren research, not one that should replace all conventional studies. The methods described in this study are successful for detecting Rio Grande Siren eDNA and may also work for additional subspecies and have the potential to improve siren detection without the need to visually observe this elusive species. When compared to trapping, eDNA sampling is significantly more effective at detecting Rio Grande Sirens, and as such, could be preferentially used in studies with the goal of siren detection.

CRediT authorship contribution statement

Krista M. Ruppert: Visualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Drew R. Davis:** Visualization, Formal analysis, Writing – review & editing. **Md Saydur Rahman:** Visualization, Writing – review & editing. **Richard J. Kline:** Visualization, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no financial interests that could have to influence the research findings reported in this paper.

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