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METHODS AND RESOURCES ARTICLE

Development and validation of four environmental DNA assays for species of conservation concern in the South-Central United States

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Abstract

We describe the development and testing of qPCR assays to detect four species of amphibians and reptiles of conservation concern in the South Central United States through environmental DNA (eDNA) samples. The target species include the Ringed Salamander (*Ambystoma annulatum*), Three-toed Amphiuma (*Amphiuma tridactylum*), Crawfish Frog (*Rana areolata*), and Chicken Turtle (*Deirochelys reticularia*). A set of primers and probes amplifying a 64–72 bp target regions were designed for each species from DNA sequence data for either the mitochondrial Cytochrome Oxidase I or Cytochrome B gene. All assays were assessed for target specificity, with no evidence of amplification in closely related or sympatrically-distributed non-target species. In vitro tests indicate that all assays consistently detect focal species down to concentrations of 2×10^{-9} pg/µL. We evaluated the utility of qPCR assays on the eDNA samples collected during field surveys across Eastern Oklahoma, focusing on counties with vouchered historical records for the target species. Although detection rates were low for field applications of the assays, positive detection of *Ambystoma annulatum*, *Rana areolata*, and *Deirochelys reticularia*, but not *Amphiuma tridactylum*, were recorded. These assays can provide a practical tool for a non-invasive, genetic monitoring program that allows for both rapid detection and tracking of native aquatic and semi-aquatic amphibian and reptile species of conservation concern.

Keywords *Ambystoma annulatum* · *Amphiuma tridactylum* · Cytochrome B · Cytochrome Oxidase I · *Deirochelys reticularia* · eDNA · Oklahoma · *Rana areolata*

As organisms interact with aquatic or semi-aquatic environments, they leave behind genetic material (e.g., urine, feces, skin, reproductive output) termed environmental DNA (or eDNA). Techniques have recently been developed to detect eDNA, which has applications to monitoring and conserving wild populations (Bohmann et al. 2014). In aquatic environments, the non-invasive, rapid approach using eDNA involving simple water sampling allows researchers to screen for the presence of rare or enigmatic species that are otherwise hard to find with traditional survey methods (Ficetola et al. 2019). In many cases, this eDNA technique results in more frequent detection of target species than traditional methods and has even detected focal species at locations where they have not been found previously (Hobbs et al. 2017; Wineland et al. 2019).

Species-specific quantitative PCR (qPCR) assays or genetic barcoding techniques, to detect an organism's DNA in an eDNA sample, are developed from available DNA sequence data. These qPCR assays consist of a primer-probe set that is designed to differentiate DNA signatures of the focal species from those of all other species occurring in the same environment. Additionally, other factors are now known to impact the probability of target species detection

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via eDNA in aquatic environments, including DNA shedding rates (Adams et al. 2019), water pH, water temperature, UV light levels (Pilliod et al. 2014; Strickler et al. 2015), substrate type (Buxton et al. 2017; Stoeckle et al. 2017), and water flow rates (Pilliod et al. 2014; Stoeckle et al. 2017).

We developed eDNA qPCR assays for three species of aquatic amphibians and one species of aquatic reptile found in eastern Oklahoma, USA: *Ambystoma annulatum* (Amphibia: Caudata: Ambystomatidae; Ringed Salamander), *Amphiuma tridactylum* (Amphibia: Caudata: Amphiumidae; Three-Toed Amphiuma), *Rana areolata* (Amphibia: Anura: Ranidae; Crawfish Frog), and *Deirochelys reticularia miaria* (Reptilia: Testudines: Emydidae; Western Chicken Turtle). All species are listed by the Oklahoma Department of Wildlife Conservation (ODWC) as Species of Greatest Conservation Need (Oklahoma Department of Wildlife Conservation 2016). These species make excellent candidates for developing a monitoring program using eDNA, because they are rare in Oklahoma, emerge only rarely or seasonally, and are difficult to locate with, or sensitive to, traditional visual survey methods. *Ambystoma annulatum* is solitary as an adult and lives under leaf litter and rotting logs within forested habitats, but congregates for breeding in vernal pools in August–October, where larvae subsist in the pools for 7–9 months before metamorphosis (Briggler et al. 2004; Semlitsch et al. 2014). *Amphiuma tridactylum* is a fully aquatic salamander that lives in swamps and ditches, spending its days underground in self-dug burrows, coming out only at night to forage (Fontenot 1999). *Rana areolata* is a secretive species, found singly inside of crayfish burrows (both in and surrounding bodies of water), and congregates during its breeding season in March–April (Heemeyer et al. 2012; Williams et al. 2012). Finally, *D. reticularia* inhabits ephemeral ponds and wetlands and is likely to estivate on land during dry periods (mid-summer–late winter; McKnight et al. 2015).

We designed four species-specific primer and probe sets for qPCR assays targeting either the mitochondrial Cytochrome B (*Amphiuma tridactylum*, *R. areolata*, and *D. reticularia*) or Cytochrome Oxidase I (*Ambystoma annulatum*) genes (Table 1; Online Resources 1–4). DNA sequences for the focal species, closely related species, and additional species that occur sympatrically with the focal species were obtained from GenBank, and datasets were further supplemented with novel sequence data collected from vouchered tissue samples or blood samples (Online Resources 1–4). For novel sequencing efforts, DNA from vouchered tissue samples was extracted via a high salt extraction method (Esselstyn et al. 2008), and from *D. reticularia* blood samples via a DNeasy Blood & Tissue Kit (Qiagen), with extracts stored at -20 °C until used for qPCR screening. Whenever possible, we collected sequence data from individuals from multiple populations across Oklahoma to represent the genetic diversity of the focal species and outgroups. The species *Amphiuma tridactylum* and *D. reticularia* are poorly represented in museum tissue collections, therefore we also included individuals from neighboring states in our datasets.

All four assays were developed using Primer Express v3.0.1 (Applied Biosystems) following the protocol for Designing Primers and Probes for Allelic Discrimination Assays (Chap. 3, ABI Primer Express Software Version 3.0 Getting Started Guide), as described hereafter in brief. For each species, DNA sequences were aligned using Geneious version v9.0.5 (Biomatters), and single nucleotide polymorphism (SNP) sites were identified in which the nucleotide at the site was unique and conserved for the focal species. In doing so, we found that the Cytochrome B sequences

Table 1 Primer and probe information for the four developed eDNA assays

Assay (Genus/species)	Primer	Primer sequence (5′–3′)	Probe	Probe sequence (5′–3′)
Ambystoma annulatum eDNA COI	A_ annulatum_COI_F1	GAGTTGAAGCAGGTG CTGGAA	A_ annulatum_COI_probe	TGGACTGTGTACCCG
	A_ annulatum_COI_R1	ATGGGCTAAATTACC AGCAAGTG		
Amphiuma tridactylum eDNA CytB	A_tridactylum_CytB_F1	AGCCACTCTCACCCG ATTCTT	A_tridactylum_CytB_ probe	TTCCATTTTACTCTTCCG
	A_tridactylum_CytB_R1	TGGATGATACTTGTT CCGATAATGA		
Rana areolata eDNA CytB	R_areolata_CytB_F1	TCGGAACTAACCTTG TCCAATGA	R_areolata_CytB_probe	TACCGAGAATCCG
	R_areolata_CytB_R1	CGGGTTAGGGTGGCA TTGT		
Deirochelys reticularia eDNA CytB	D_reticularia_CytB_F1	CCTACCATGAGGCCA AATATCC	D_reticularia_CytB_probe	AGGCGCAACTGTTA
	D_reticularia_CytB_R1	ATATATGGAATGGCT GAGAGGAGATT		

of *D. reticularia* were substantially differentiated (pairwise sequence divergence up to 17%) as expected among the three distinct subspecies recognized in *D. reticularia* (*D. r. chrysea* [Florida Chicken Turtle], *D. r. miaria* [Western Chicken Turtle], *D. r. reticularia* [Eastern Chicken Turtle]; Buhlmann et al. 2008), and we were unable to identify the SNP sites that are identical for all of the *D. reticularia* sequences and distinctive from the other taxa included in the alignment. Therefore, we designed the primers and probe specific only to the *D. reticularia* samples from Oklahoma and surrounding states (Arkansas), so that the assay can be used for the populations of the subspecies *D. r. miaria*.

A consensus sequence for each focal species was generated in Geneious and imported into Primer Express for assay design. For each focal species-specific SNP site in the consensus sequence, we searched for possible primer-probe sets using the TaqMan MGB Allelic Discrimination option with default parameter settings (Online Resource 5) and selected the primer-probe set with the lowest penalty score and amplicon length for the assay (Online Resources 1–4). Each primer-probe set targeted regions of 64–72 base pairs in length. TaqMan MGB (minor groove binder) probes with a 5′ fluorescent reporter dye and a 3′ nonfluorescent quencher (NFQ) were ordered from ThermoFisher Scientific, and primers were ordered from either ThermoFisher Scientific or Eurofins Genomics.

We conducted qPCR tests for each primer-probe set with DNA extracts from individuals that were not included in the original sequence assay development dataset whenever possible (Tables 2, 3, 4 and 5). For each test, DNA from several ingroup individuals from different populations was tested against several closely related or sympatric outgroup individuals to verify the specificity of each assay (Tables 2, 3, 4 and 5). DNA extracts were quantified using a Quantus Fluorometer (Promega) and diluted to 20 ng/µL to standardize each extract. The extracts were then further diluted using serial dilutions to 1/1000 and 1/10,000 concentrations (down to 2×10^{-9} pg/µL) to simulate the low DNA concentrations found in environmental DNA. We tested ingroup and outgroup samples against the assay individually to verify assay specificity.

The qPCR reactions were set up in duplicate on an Applied Biosystems MicroAmp Fast 96-well Reaction plate and run on a QuantStudio 3 (Applied Biosystems) using the Presence/Absence experiments option of the QuantStudio Design and Analysis Software v1.4. The 10 µl PCR reaction cocktail for each reaction was composed of 0.75 µl of molecular grade sterile water, 5.0 µl of TaqMan Fast Advanced Master Mix, 0.5 µl each of the 10 µM forward and reverse primers, 0.25 µl of 10 µM TaqMan MBG probe and 3 µl of eDNA extract. For the negative control, 3 µl of molecular grade water was used in place of eDNA extract in the reaction. After each well was filled, the plate was covered with an Applied Biosystems MicroAmp Optical Adhesive Film. The qPCR thermal cycling conditions were as follows: 1 cycle of 95 °C for 20 s, 60 cycles of 95 °C for 1 s and 60 °C for 20 s, and 1 cycle of 60 °C for 30 s. A target DNA presence test was considered positive if the intensity of the fluorescence (amplification) was above the call threshold algorithmically determined by the QuantStudio Design and Analysis Software. In vitro tests showed all four assays consistently amplify target species DNA with 100% specificity down to the lowest DNA concentrations tested (2×10^{-9} pg/µL) with no cross-amplification observed for congener or sympatric species DNA (Tables 2, 3, 4 and 5).

Table 2 Summary of the results of in vitro species specificity tests for the *Ambystoma annulatum* assay using vouchered genomic DNA extracts, deposited at the Sam Noble Oklahoma Museum of Natural History (OMNH), for the target species and closely related or sympatrically distributed congeners

Individual species/community pools	Museum voucher numbers	Amplification
Ambystoma annulatum	OMNH 40366	+
Ambystoma annulatum	OMNH 42636	+
Ambystoma annulatum	OMNH 44725	+
Ambystoma annulatum	OMNH 44727	+
Eurycea longicauda	OMNH 45978	–
Eurycea lucifuga	OMNH 45994	–
Eurycea tynerensis	OMNH 46000	–
Ambystoma maculatum	OMNH 46211	–
Ambystoma maculatum	OMNH 44740	–
Ambystoma maculatum	OMNH 44761	–
Ambystoma opacum	OMNH 47603	–
Ambystoma opacum	OMNH 44744	–
Ambystoma texanum	OMNH 44746	–
Ambystoma texanum	OMNH 44747	–

Table 3 Summary of the results of in vitro species specificity tests for the *Amphiuma tridactylum* assay using vouchered genomic DNA extracts, deposited at the Louisiana State University Museum of Zoology (LSUMZ) and the Florida Museum of Natural History (FLMNH), for the target species and closely related congeners

Individual species/community pools	Museum voucher numbers	Amplification
Amphiuma tridactylum	LSUMZ 2426	+
Amphiuma tridactylum	LSUMZ 2793	+
Amphiuma tridactylum	LSUMZ 2885	+
Amphiuma tridactylum	LSUMZ 876	+
Amphiuma means	FLMNH 173152	–
Amphiuma means	FLMNH 180421	–
Amphiuma pholeter	FLMNH 165477	–
Amphiuma pholeter	FLMNH 178204	–

Table 4 Summary of the results of in vitro species specificity tests for the *Rana areolata* assay using vouchered genomic DNA extracts, deposited at the Sam Noble Oklahoma Museum of Natural History (OMNH), for the target species and closely related or sympatrically distributed congeners

Individual species/community pools	Museum voucher numbers	Amplification
Rana areolata	OMNH 39698	+
Rana areolata	OMNH 39878	+
Rana areolata	OMNH 42015	+
Rana areolata	OMNH 42016	+
Rana areolata	OMNH 43585	+
Rana catesbeiana	OMNH 44491	–
Rana catesbeiana	OMNH 45421	–
Rana catesbeiana	OMNH 44460	–
Rana catesbeiana	OMNH 44463	–
Rana clamitans	OMNH 45560	–
Rana clamitans	OMNH 44533	–
Rana clamitans	OMNH 44538	–
Rana palustris	OMNH 46578	–
Rana palustris	OMNH 46588	–
Rana sphenocephalus	OMNH 44576	–
Rana sphenocephalus	OMNH 44580	–
Rana sphenocephalus	OMNH 44571	–
Acris blanchardi	OMNH 44149	–
Acris blanchardi	OMNH 44300	–
Acris blanchardi	OMNH 44273	–

Table 5 Summary of the results of in vitro species specificity tests for the *Deirochelys reticularia* assay using genomic DNA extracts of the target species (non-cataloged samples[a]) and closely related or sympatrically distributed congeners (vouchered at the Sam Noble Oklahoma Museum of Natural History [OMNH])

Individual species/community pools	Museum voucher numbers	Amplification
Deirochelys reticularia	DERE 111[a]	+
Deirochelys reticularia	DERE 112[a]	+
Deirochelys reticularia	OMNH 44,172	+
Chelydra serpentina	OMNH 44305	–
Kinosternon subrubrum	OMNH 44355	–
Pseudemys concinna	OMNH 44307	–
Sternotherus odoratus	OMNH 44364	–
Terrapene carolina	OMNH 44323	–
Terrapene carolina	OMNH 44320	–
Trachemys scripta	OMNH 44345	–
Trachemys scripta	OMNH 44325	–

[a]Non-destructive blood samples provided by D. Ligon (DERE = *Deirochelys reticularia*).

To conduct an empirical test of assay performance in the field, we collected 565 water samples from 79 wetlands across 11 counties in eastern Oklahoma (Table 6). Although no visual confirmation of any of the focal species were made during sample collection efforts, we targeted sites in counties with vouchered historical records of the focal species or those in close proximity to locations with historical records (Table 6; Online Resource 6). Samples of 500–600 mL of water were collected 7–10 cm below the water surface (Kamoroff and Goldberg 2018) and stored in sterile 36 oz., one-time use Whirl-Pak sampling bags (Wineland et al. 2019). A single negative control was created for each waterbody by filling a water sample bag with molecular grade water, sealing it, and dipping the sealed bag into the water for ca. 30 s. All samples were stored in a cold, dark cooler and filtered within 12 h of collection in the field (via a portable VWR vacuum pump) or the lab (via the vacuum pump installed in fume hood) depending on proximity and timing.

Water was homogenized in the sample bag, then poured into sterile, one-time use 500 mL polyethersulphone (PES) membrane filters, with a 75 mm filter diameter and a 0.45 µm pore size (various vendors: ThermoScientific Nalgene, Fisher Scientific, VWR, Foxx Life Sciences Autofil). Field and negative samples were vacuum filtered until the membranes became clogged or until 500 mL was filtered (whichever came first), cut out of the filter membrane using a sterile, one-time use 11-blade scalpel to avoid cross-contamination of samples, and placed into a 10 mL cryovial with 95% ethanol for −20ºC freezer storage until the time of extraction (< 6 mo) (Online Resource 6). Prior to sample filtration, all work surfaces were sterilized with ELIMINase (Decon Labs) or 10% bleach.

We isolated total genomic eDNA from one-half of each vouchered filter membrane using a modified Qiagen DNeasy Blood and Tissue Kit protocol involving Qiagen QIAshredder and Zymo OneStep PCR inhibitor Removal Kit protocols (Online Resource 7). For each batch of DNA extractions, field negative controls and a lab negative control sample created by placing the same type of filter into sterile molecular grade water, were also extracted following the same protocols. The resulting DNA isolates were used in subsequent qPCR assay tests (Table 6).

The qPCR reactions were set up in triplicate, run on the QuantStudio 3, and analyzed in QuantStudio Design and Analysis Software as described in the method of the in vitro assay test above. Individual eDNA samples were considered positive, indicating the focal species was present, if two or three of the triplicates were positive in the initial screening. If only one of the triplicates was positive in the initial screening, the qPCR assay was repeated for the sample and the target species was considered present only if one or more of the triplicates was positive again in the repeat screening (Table 6).

Field applications of the assays detected the presence of *Ambystoma annulatum* at three distinct waterbodies across two counties (Adair, Cherokee), *R. areolata* at two

Table 6 Summary qPCR results of eDNA field surveys employing four designed assays. The total number of waterbodies and samples by county are shown (excluding field and lab negative controls), followed by the number of positive samples detected by county for each target species. N/A represents county samples that were not screened for target species in cases where those taxa have never been documented in the counties historically

County	No. samples /No. waterbodies	*Ambystoma annulatum*	*Amphiuma tridactylum*	*Rana areolata*	*Deirochelys reticularia*
		No. + samples (No. waterbodies with +); percent + samples (percent + waterbodies)			
Adair	24/3	16 (2) 66.7% (66.7%)	N/A N/A	0 0%	N/A N/A
Atoka	54/7	0 0%	N/A N/A	0 0%	1 (1) 1.1% (14.3%)
Bryan	18/4	0 0%	N/A N/A	0 0%	N/A N/A
Cherokee	92/12	1 (1) 1.1% (8.3%)	N/A N/A	1 (1) 1.1% (8.3%)	N/A N/A
Latimer	23/3	0 0%	N/A N/A	0 0%	N/A N/A
Le Flore	64/8	0 0%	N/A N/A	0 0%	N/A N/A
McCurtain	82/12	0 0%	0 0%	0 0%	0 0%
Muskogee	64/8	0 0%	N/A N/A	0 0%	N/A N/A
Pushmataha	22/6	0 0%	N/A N/A	0 0%	0 0%
Sequoyah	102/13	0 0%	N/A N/A	1 (1) 0.9% (7.7%)	N/A N/A
Wagoner	20/3	0 0%	N/A N/A	0 0%	N/A N/A
Total	565/79	17 (3) 3.0% (3.8%)	0 (0) 0%	2 (2) 3.6% (2.5%)	1 (1) 0.6% (4.0%)

waterbodies across two counties (Cherokee, Sequoyah), and *D. reticularia* at a single waterbody in Atoka County (Table 6). Field samples from McCurtain County in southeast Oklahoma screened for *Amphiuma tridactylum* returned a single positive sample; however, repeated screening of the eDNA extraction failed to amplify and all samples were treated as negative (Table 6). Overall, the low detection rates observed in the wild for three of the four focal species and failure to detect *Amphiuma tridactylum* during our field surveys is not surprising, given how rarely these species are observed and the seasonal nature of their activity cycles (Gibbons 1970; Briggler et al. 2004; Fontenot 1999; Heemeyer et al. 2012; Williams et al. 2012; Semlitsch et al. 2014). Additionally, abiotic factors at the waterbodies surveyed may impact detection success rates. Such factors should be investigated in future research employing these eDNA assays. The positive detection of three of the focal species adds to baseline occurrence data that will aid in spatial analyses and conservation planning. The results of this work support the application of the four designed eDNA assays as a viable biodiversity monitoring method that can aid in more rapid detection of four rare and threatened amphibian and reptile species in North America.

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