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ORIGINAL ARTICLE

Development and validation of an environmental DNA protocol to detect an invasive Caribbean freshwater fish, the guppy (*Poecilia reticulata*)

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Abstract

We describe the development and validation of a qPCR assay to detect *Poecilia reticulata*, a highly invasive species of freshwater fish invasive to the Caribbean islands, through environmental DNA (eDNA) sampling. Originating from Trinidad, this species is invasive and detrimental to countless native tropical fish communities. A qPCR assay, consisting of a set of primers and a fluorescent probe, amplifying a 214 base pair target region of the mitochondrial Cytochrome B gene was designed for *P. reticulata* from existing DNA sequence data. The assay was assessed for target specificity, with no evidence of amplification in closely related or sympatrically distributed non-target species. In vitro tests indicate that the assay consistently detects *P. reticulata* down to concentrations of 2.0×10^{-5} ng/μl. The developed assay provides a new, practical tool for monitoring freshwater habitats throughout the Caribbean, allowing for early and rapid detection of invasive fish species of conservation concern.

KEYWORDS
cytochrome B, eDNA, Jamaica, quantitative PCR, trinidadian guppy, wildlife conservation

1 | INTRODUCTION

Environmental DNA (or eDNA) sampling is becoming a broadly employed technique for detecting the presence of an organism in its environment, often aquatic habitats, based on the genetic material it leaves behind (e.g., urine, feces, skin, reproductive output). This method of sampling has broad ecological applications for monitoring and conserving wild populations, and detecting invasive species (Bohmann al., 2014; Kamoroff & Goldberg, 2018; Thomas et al., 2019). Environmental DNA sampling is particularly useful for the detection of species that may be difficult to find through traditional survey methods (Ficetola et al., 2019; Hinlo et al., 2017). As a result, eDNA sampling often results in increased target species detection rates, even in habitats or localities where they have not previously been detected (Hobbs et al., 2017; Wineland et al., 2019). Additionally, eDNA field sampling requires little training and no species-specific collecting permits or specialized animal capture equipment (Ficetola et al., 2019; Ruppert et al., 2019). In aquatic environments particularly, eDNA provides a rapid non-invasive sampling approach via water collection and filtration. To detect a target species' eDNA from a filtered water sample, either a real-time or quantitative polymerase chain reaction (qPCR) assay, or genetic barcoding techniques, are developed using available DNA sequence data (Ficetola et al., 2019; Ruppert et al., 2019). Both methods must use specific targeted DNA assays designed to distinguish between closely related species that may be occurring in the same habitat (Ficetola et al., 2019).

We describe the development and validation of a novel eDNA qPCR assay to detect the invasive species of freshwater fish, *P. reticulata* (Actinopterygii: Cyprinodontiformes: Poeciliidae; Trinidadian Guppy). *Poecilia reticulata* is one of the most invasive fishes in the world (Deacon et al., 2011; Stockwell & Henkanaththegedara, 2011). It is found naturally in freshwater streams on Trinidad but now has a pantropical introduced distribution and has successfully established populations in warm aquatic habitats in temperate zones (Stockwell & Henkanaththegedara, 2011). These freshwater fish are sometimes actively introduced for mosquito control, but likely the main source of introductions are aquarium releases, as guppies are a very popular pet fish that breed easily in human care and excess individuals were presumably released into nature (Courtenay Jr. & Stauffer Jr., 1990). Consequently, in the neotropics, and especially in the Caribbean, *P. reticulata* establish easily, and often compete with other members of the family Poeciliidae and other non-related fish endemic to this biodiversity hotspot, such as *Limia* (Poeciliidae) in the Greater Antilles (Weaver et al., 2016), or *Gambusia* (Poeciliidae) in Jamaica (Rivas, 1963). For this project, we focused on the situation in Jamaica with invasive *P. reticulata* and endemic *Limia melanogaster*, as well as native *Gambusia*; however, parallel situations exist on Hispaniola and Cuba, where *P. reticulata* interact with other species of *Limia* and also *Poecilia*. For example, we collected *P. dominicensis* in the Dominican Republic together with guppies (pers. obs.). Additionally, in Mexico, guppies are one of several invasive livebearer species (Fuentes et al., 2021) and are often found with other species of *Poecilia* and *Xiphophorus*. The same is known for several other tropical countries (Deacon et al., 2011). The presence of guppies has several negative effects on native fishes through competition or behavioral interactions (Stockwell & Henkanaththegedara, 2011). In Mexico, for example, male guppies sexually harass females of *Skiffia bilineata* (Goodeidae) and are implicated in the decline of this and other species (Valero et al., 2008). Therefore, endemic and invasive guppies make excellent candidates for developing a monitoring program using eDNA, as their small body sizes make them difficult to detect, often requiring major sampling efforts by trained personnel using dip nets or seines to confirm presence in the wild.

2 | MATERIALS AND METHODS

2.1 | Sequence alignment and assay development for target species

We designed species-specific primer and probe sets for qPCR assays targeting the mitochondrial Cytochrome B (CytB) gene using sequences available on GenBank (Table 1). DNA sequences for CytB for the focal species (*P. reticulata*), one closely related species (*P. mexicana*), and eight additional species that occur sympatrically with the focal species were obtained from GenBank (*Gambusia affinis*, *G. holbrooki*, *G. melapleura*, *G. puncticulata*, *G. wrayi*, *Limia melanogaster*, *Xiphophorus hellerii*, and *X. maculatus*). Two potential assays were developed (Table 1) using NCBI's Primer Blast tool, as described hereafter in brief (Ye et al., 2012). All sequences were input into Primer Blast via accession numbers and the default parameters were used. In silico tests for *P. reticulata* specificity of both assays were then checked in Primer3 v0.4.0 against our focal species to determine suitability (presence of single-nucleotide polymorphism [SNP] sites unique and conserved for the focal species; Table 2). We aligned all sequences from all species in MEGA v10.0.4 (Kumar et al., 2018; Stecher et al., 2020) to design the two probes. A 20 base pair long sequence was selected manually between primer sites to optimize

TABLE 1 Primer–probe assays developed and tested for use as a biosurveillance monitoring method for the invasive species *P. reticulata* in the Caribbean

Assay	Primer/Probe combination	Name	Sequence (5′–3′)
ASSAY 1	Forward primer 1	CytB_Poecilia_retic_F1	CTCCACGAAACCGGATCCAA
	Reverse primer 1	CytB_Poecilia_retic_R1	CTGGCTTAATGTGTGGGGGT
	TaqMan MGB Probe 1 (6-FAM)	CytB_Poecilia_retic_probe1	ACCCTAGCCCTGTTTTCTCC
	Amplified DNA segment		CTCCACGAAACCGGATCCAATAACCCAA TCGGATTAAACTCCGACGCCGACAAA ATTTCATTCCACCCTTATTTCTCATATAAAG ACCTTCTAGGATTCATT TTAATACTTACTGCACTAATTACCCTAGCCCTGTTTTCTC CTAACCTATTAGGAGATCCTGA AAACTTCACTCCTGCAAATCCA CTCGTTACACCCCCACACATTAAGCCAG
ASSAY 2	Forward primer 2	CytB_Poecilia_retic_F2	ATCCTAATAGTTGTTCCCCTATTACAC
	Reverse primer 2	CytB_Poecilia_retic_R2	ATGCTGCGGTGGGAATCAA
	TaqMan MGB Probe 2 (6-FAM)	CytB_Poecilia_retic_probe2	CATACCTGTCGAACACCCA

Note: Assay 1, which amplified most consistently during in vitro testing and was subsequently employed in testing assay performance on control samples collected in mesocosm environments at the ARF, is shown with the associated amplified DNA segment.

TABLE 2 In silico test results for *P. reticulata* specificity of both assays

Species Name	Collection locality	GenBank accession no.	Forward primer mismatches	Reverse primer mismatches	Probe mismatches
Gambusia affinis	Missouri, USA	EF017514.1	2	5	5
Gambusia holbrooki	Not reported	KP013115.1	2	5	5
Gambusia melapleura	Not reported	U18216.1	–	–	–
Gambusia puncticulata	Not reported	U18221.1	–	–	2
Gambusia wrayi	Jamaica	EF017516.1	2	4	8
Limia melanogaster	Not reported	EF017534.1	4	5	4
Poecilia mexicana	Mexico	HQ677873.1	4	5	4
Poecilia reticulata	Trinidad and Tobago	EF017536.1	0	0	0
Xiphophorus hellerii	Not reported	AY056056.1	1	5	4
Xiphophorus maculatus	Not reported	EF017551.1	1	4	5

the number of mismatches at the 3' end. Our probe sequences were then tested in Primer3 alongside the associated primer pairs to ensure compatibility. Based on the methods described above, we selected two primer–probe assays for testing (Table 1); both assays were ordered from Sigma-Aldrich Products. The TaqMan MGB (minor groove binder) probes contained a 5' fluorescent reporter dye (FAM) and a 3' nonfluorescent quencher (NFQ).

2.2 | In vitro assay testing via quantitative PCR

We conducted an in vitro qPCR test for both primer–probe sets (Assays 1 and 2) with tissue samples obtained from captive-raised animals housed in tanks (213 cm long, 56 cm wide, 23 cm deep) at the Aquatic Research Facility (ARF) at the University of Oklahoma (OU) for *P. reticulata*, two congeners (*P. latipinna* and *P. mexicana*), and three other species encountered commonly in Jamaica (*Gambusia* sp., *Limia melanogaster*, and *Xiphophorus hellerii*; Table 3). DNA was extracted from the tissues via the high salt extraction method of Esselstyn et al. (2008). DNA extracts were quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and diluted to 50 ng/μl to standardize each extract. The extracts were then further diluted using serial dilutions to 1/1000 (0.05 ng/μl) and 1/10000 (0.005 ng/μl) concentrations to simulate the low DNA concentrations found in natural eDNA water samples. The diluted DNA extracts from individuals of the six fish species were screened using qPCR technology to determine assay specificity (Table 3). DNA extracts from two individuals of the five non-target species (*Gambusia* sp., *L. melanogaster*, *P. latipinna*, *P. mexicana*, and *X. hellerii*) were screened in triplicate for each dilution (six wells per species per dilution), and DNA extracts from three individuals of the target species (*P. reticulata*) were screened in triplicate for each dilution (nine wells for each dilution). To test each assay's ability to detect *P. reticulata* in the presence of a broader community of fishes, we also combined extracted DNA into two simulated fish communities. Community 1 included 5 μl of DNA from all five non-target species, with no DNA added for *P. reticulata*; Community 2 included 5 μl of DNA from all five non-target species plus *P. reticulata* (Table 3). Each community was screened in triplicate for each dilution (six wells in total).

The qPCR reactions were set up 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 (Applied Biosystems). 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 DNA 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 MicroAmp Optical Adhesive Film (Applied Biosystems). We utilized filter pipette tips for each step of the DNA extraction protocol and the qPCR plate setup. 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) in two or more wells was above the call threshold algorithmically determined by the QuantStudio Design and Analysis Software. The goal of this test was to determine whether the developed assays detected accurately the presence/absence of the target species DNA; therefore, we did not include standards in the initial in vitro assay test. Based on the results of these in vitro tests, we selected a single primer–probe set (Assay 1) to screen field-collected eDNA samples (Table 1). All assay testing and qPCR screening occurred at the Genomic Core Facility (GCF) located within the Sam Noble Oklahoma Museum of Natural History (SNOMNH).

2.3 | Assessing the performance of assay 1

To assess the performance of Assay 1, we created serial dilutions from DNA extraction aliquots of two vouchered *P. reticulata* tissue samples, starting from a standardized concentration of 20 ng/μl: 20.0, 2.0, 0.2, 0.02, 2×10^{-3}, 2×10^{-4}, 2×10^{-5}, 2×10^{-6}, 2×10^{-7}, and 2×10^{-8} ng/μl. Each dilution was run in 14 replicates (seven replicates per vouchered tissue extract), along with 12 no template controls (NTC). The results of this test were used to obtain parameter estimates for performance validation of Assay 1, including values for

TABLE 3 Summary of the results of in vitro species specificity tests for the two developed *P. reticulata* assays using vouchered genomic DNA extracts of the target species and closely related congeners

Species/Community	Number of Individuals (N)	DNA Concentration (ng/μl)	Contains Target Species (Yes/No)	Assay 1 Amplification (+/−)	Number Wells+/N	Ct Mean	Assay 2 Amplification (+/−)	Number Wells+/N	Ct Mean
Gambusia sp.	2	0.05	No	−	0/6		−	0/6	
		0.005	No	−	0/6		−	0/6	
Limia melanogaster	2	0.05	No	−	0/6		−	0/6	
		0.005	No	−	0/6		−	0/6	
Poecilia latipinna	2	0.05	No	−	0/6		+	**1/6**	**10.529**
		0.005	No	−	0/6		+	**1/6**	**38.187**
Poecilia mexicana	2	0.05	No	−	0/6		−	0/6	
		0.005	No	−	0/6		−	0/6	
Poecilia reticulata	3	0.05	Yes	+	9/9	28.226	+	9/9	30.121
		0.005	Yes	+	9/9	32.136	+	9/9	35.277
Xiphophorus hellerii	2	0.05	No	−	0/6		+	**1/6**	**10.939**
		0.005	No	−	0/6		−	0/6	
Community 1 (*Gambusia* sp., *L. melanogaster*, *P. latipinna*, *P. mexicana*, and *X. hellerii*)	1 per species	0.05	No	−	0/3		−	0/3	
		0.005	No	−	0/3		−	0/3	
Community 2 (*Gambusia* sp., *L. melanogaster*, *P. latipinna*, *P. mexicana*, *P. reticulata*, and *X. hellerii*)	1 per species	0.05	Yes	+	3/3	29.934	+	3/3	32.102
		0.005	Yes	+	3/3	34.130	+	3/3	36.543

Note: Bold indicates false positive detection events for a sample in the absence of DNA of the target species.

the r^2, slope, efficiency, the Limit of Quantification (LoQ), and the Limit of Detection (LoD; Klymus et al., 2020).

To test Assay 1 performance in environmental settings more similar to the wild, we collected water samples from live fish mesocosms (213 cm long, 56 cm wide, 23 cm deep) maintained at the ARF on the OU campus to create positive and negative controls. Three samples were collected with sterile equipment and gloves from tanks with guppies (positive controls) and an additional three samples from a separate water tank containing non-target fish species (negative controls). All control samples consisted of 500–650 ml of water collected in 1 L Whirlpak sterile plastic bags. Control samples, fish tissue DNA extractions, eDNA filter extractions, and preparation of the qPCR plates occurred in the same laboratory at the GCF within the SNOMNH.

Prior to sample filtration and extraction, all work surfaces were cleaned with ELIMINase (Decon Labs) or 10% bleach, and nitrile gloves were changed between handling each sample. Before vacuum filtration, water samples were homogenized by gently agitating each Whirlpak sample bag prior to pouring the water into sterile, one-time use 500 ml polyethersulphone (PES) membrane filters, with a 75 mm filter diameter and a 0.45μm pore size (manufactured by either Thermo Fisher Scientific [Item No. FB1256651] or VWR [Item No. 10040–468]). Once a total of 500 ml of water was processed, filters were cut out of the filter cup using a sterile, one-time use 11-blade scalpel to avoid cross-contamination of samples and placed into a sterile 10 ml cryovial with 95% ethanol for −20°C freezer storage until the time of extraction. To extract eDNA from the filters, we used the Qiagen DNeasy Blood and Tissue Kit and QIAshredder Kit, as well as the Zymo OneStep PCR Inhibitor Removal Kit, following the protocol outlined in Siler et al. (2021).

For the screening of all mesocosm control samples, qPCR reactions utilizing Assay 1 were set up in sextuplicate, run on the QuantStudio 3, and analyzed in QuantStudio Design and Analysis Software as described in the in vitro assay test methods (section 2.2). Individual control samples were considered positive, indicating the focal species was detected in the sample, if two of the sextuplicate reactions were positive in the initial screening. If only one of the sextuplicates was positive in the initial screening, the qPCR assay was repeated for the sample and the target species was considered present only if two or more of the sextuplicate samples were positive in the repeat screening. Filter pipette tips were used for each step of the eDNA extraction protocol and qPCR plate setup. Finally, negative controls were filtered and extracted alongside control samples collected at the ARF to monitor for potential contamination.

3 | RESULTS

3.1 | Laboratory results

In vitro tests showed Assay 1 consistently amplified target species DNA in all replicates down to the lowest DNA concentration tested (0.005 ng/μl) with no cross-amplification observed (Table 3). Additionally, Assay 1 detected the presence of *P. reticulata* DNA when combined with DNA from all five non-target species (*Gambusia* sp., *L. melanogaster*, *P. latipinna*, *P. mexicana*, and *X. hellerii*) and did not incorrectly amplify in the simulated community (Community 1) without the target species DNA (Table 3). Assay 2 also consistently amplified target species DNA in all replicates down to the lowest DNA concentration tested (0.005 ng/μl); however, false positive detection events were observed in one reaction of *X. hellerii* and two reactions of *P. latipinna* (Table 3) DNA. Therefore, Assay 1 was selected for subsequent mesocosm-based empirical tests given its higher species specificity for *P. reticulata*. For Assay 1, the r^2 was 0.991, the slope was −3.428, and the efficiency was 95.794%. The LoQ for Assay 1 was 2.0×10^{-5} ng/μl, and the LoD was 2.0×10^{-6} ng/μl.

Tests of Assay 1 in the OU ARF mesocosms detected the presence of *P. reticulata* in all six replicate wells for each of the three positive control samples. One of the three negative control samples collected at the ARF amplified on the first round of screening (3/6 wells), so the sample was run again and also amplified on the second round of screening (2/6 wells), but none of the qPCR negative controls amplified.

4 | DISCUSSION

The objective of this study was to develop a novel monitoring program in the Caribbean for a highly invasive freshwater species of fish. We confirmed using in vitro and mesocosm control testing that eDNA primer–probe Assay 1 detects *P. reticulata* consistently at low DNA concentrations similar to the dilute nature of environmental DNA (Table 3). In line with our results, other studies have detected target fish species before the species' presence was detected using standard surveillance methods (Jerde et al., 2011; Sigsgaard et al., 2015), further emphasizing the ability of eDNA to increase detection of target fish species (Jerde et al., 2011; Sigsgaard et al., 2015; Wilcox et al., 2016).

False positive detection events, as observed for one of our ARF-collected negative control samples, are another common problem in eDNA sampling and may occur for various reasons including contamination during water collection, eDNA extraction, or qPCR/PCR amplification (Ficetola et al., 2015). False eDNA detection of an invasive species at a given site could lead to unnecessary expenditure of conservation resources in an attempt to eradicate a species that may be absent from the environment (Ficetola et al., 2016). However, strict sterile laboratory practices were employed, and no amplification events were recorded in any qPCR negative controls. Contamination of the one ARF-collected negative control may have occurred at the time of sample collection if *P. reticulata* were transferred between tanks within the same room, or at an earlier date (Maruyama et al., 2014). Although we are confident that the developed Assay 1 will consistently detect *P. reticulata* eDNA, future studies employing the assay should collect sufficient field samples from any focal waterbody (e.g., 10–15; Erickson et al., 2019) and employ multiple positive

and negative controls for each stage of the process (in-field sample collection, eDNA extraction, qPCR amplification).

To manage the spread of aquatic invasive species, early and accurate detection is critical to management and conservation efforts (Darling & Majon, 2011; Egan et al., 2015; Robson et al., 2016) and traditional sampling methods may be inefficient and labor intensive when individuals or species are difficult to detect due to small body sizes or cryptic behavior (Hinlo et al., 2017; Jerde et al., 2011; Ruppert et al., 2019; Sigsgaard et al., 2015). Our results contribute an effective and non-destructive tool for detecting *P. reticulata*, one of the most invasive fish species worldwide (Deacon et al., 2011). They occur on all continents except Antarctica and are known to compete with other small fishes (Stockwell & Henkanaththegadara, 2011), sometimes interfering with them directly (Valero et al., 2008). Utilization of this assay and eDNA methodology can alert wildlife managers and conservation biologists to the presence or absence of *P. reticulata*, allowing them to make informed management and conservation decisions concerning this species.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

I.S. and C.D.S. contributed to study conception and design. I.S. secured funding for assay development, testing, and field survey work. I.S., K.-A.B., and S.B. conducted field surveys in Jamaica; I.S. and J.L.W. oversaw collection of positive and negative control samples in Oklahoma. Assay design was undertaken by E.D.H. DNA extractions, *in vitro* assay tests, and assessment of assay performance was overseen by S.N.S. Data analysis and summary of qPCR screening results was performed by S.N.S. and C.D.S., with contributions from J.L.W. The first draft of the manuscript was written by S.N.S., with contributions and reviews by C.D.S., I.S., J.L.W., E.D.H., K.-A.B., and S.B. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The primer and probe genetic sequences are provided in Table 1 and are not currently archived in any other online repositories.

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