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Research Article

Development of molecular markers for eDNA detection of the invasive African jewelfish (*Hemichromis letourneuxi*): a new tool for monitoring aquatic invasive species in National Wildlife Refuges

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Abstract

The genetic material (DNA fragments of cellular or extracellular origin) that organisms leave behind in nonliving components of the environment such as water, soil, or sediments is defined as environmental DNA (eDNA). Recently, the use of eDNA has been recognized as an effective method for aquatic invasive species early detection and surveillance. We developed molecular markers for eDNA detection of the African jewelfish (*Hemichromis letourneuxi*) in and around Loxahatchee National Wildlife Refuge, Florida. The lower limit of detection using these markers was determined as well as the effect of fish density and time on detection using controlled experiments. Specificity and sensitivity of these markers was tested in aquarium trials and also in field samples. Results showed that developed markers (probe and primers) for Taqman assays were sensitive and specific for eDNA detection via traditional and quantitative PCR methods (qPCR). The observed theoretical minimal qPCR detection level, based on standard curve analysis for this species, was approximately 0.0002 ng/uL (R^2 = 0.90) at a PCR cycling threshold (C_T) of 28.5–29. There was a positive and significant relationship between fish density and eDNA detection with detection probabilities ranging from 0.32–1.00 depending on fish density. A negative and significant relationship between average C_T values and density further corroborated our findings that target eDNA increased with increasing fish density. Developed markers detected the presence of *H. letourneuxi* in the canal adjacent to but not in Loxahatchee National Wildlife Refuge. The single positive found in the canal adjacent to Loxahatchee National Wildlife Refuge showed a similar C_T value to the observed average for density of three fish per aquarium.

Key words: eDNA, qPCR, *Hemichromis*, detection probability, density, molecular markers, Loxahatchee National Wildlife Refuge

Introduction

The introduction of invasive species to natural ecosystems can reduce biodiversity at all levels and produce a significant alteration of the community structure (Lodge et al. 2006; Olden et al. 2004). In freshwater ecosystems, impacts of aquatic invasive species (AIS) are considered even greater since biological invasions are considered a leading cause of extinction in fish communities (Witte et al. 1992; Light and Marchetti 2007; Ricciardi and MacIsaac 2011).

Traditionally, detection and management of AIS has been conducted using conventional methods of population dynamics requiring direct observation, tagging, or capture of the target species (e.g., density and abundance assessments). The arrival of molecular methods has provided new tools for identification, surveillance and monitoring of AIS (Darling and Mahon 2011). In particular, the use of environmental DNA (eDNA) in freshwater ecosystems is being applied as an alternative method for early detection and surveillance of AIS (Minamoto et al. 2012). The method can detect presence/absence of a species through cells or tissues suspended in the water column containing the genetic material. Genetic material is then collected via water filtration through a micron screen and tested for presence of the target species, using specific genetic

markers via polymerase chain reaction (PCR), quantitative PCR (qPCR) or direct sequencing of the PCR product.

The eDNA methodology outlined above raises the possibility to monitor and detect individuals of a target species using an environmental sample from a site where the organism is extremely rare and even eliminate the extraneous noise generated by the multiplicity of non-target species. Thus eDNA, as a monitoring method, will have broad research and management applications in freshwater ecosystems including biodiversity assessment, understanding of trophic interactions and evaluation of changes in species distribution due to habitat fragmentation and climate change (Yoccoz 2012; Takahara et al 2013). However, recognizing the eDNA signal can be an arduous task because identification of the specimens requires both specificity and sensitivity of the designed markers (Darling and Blum 2007; Dejean et al. 2012).

The African jewelfish, *Hemichromis letourneuxi* (Sauvage 1880), is a cichlid native to tropical West Africa. The species was introduced in Florida during the early 1960s (Rivas 1965) and expanded its range in subsequent decades (Loftus and Kushland 1987). The species is currently considered among the most widespread and abundant invasive species throughout the Everglades National Park (Kline et al. 2013). There are also reports of *H. letourneuxi* in Big Cypress National Preserve and in coastal habitats of elevated salinity (Langston 2010). Although there are no reports of this species within the A. R. Marshall Loxahatchee National Wildlife Refuge (LOX NWR or Refuge), African jewelfish has been observed at low densities in canals adjacent to LOX NWR (Rehage and Kline personal communication). LOX NWR is an important conservation area and forms the remainder of the northern portion of the Everglades ecosystem. The Refuge is surrounded by an interconnected system of canals, large urban areas, intensive agriculture and a high density of roads making LOX NWR a susceptible area for aquatic invasions. Because of the proximity and potential introduction to the Refuge, *H. letourneuxi* represents a threat as a predator and competitor to the native aquatic diversity of LOX NWR.

The goals of this research are to develop genetic tools for the detection of *H. letourneuxi* and to explore the reliability of eDNA detection methods in an effort to increase the sensitivity and reduce time and cost of traditional inventory and detection methods for AIS within units of the National Wildlife Refuge System, especially LOX NWR. In order to achieved our research goals we had five specific objectives: 1) development of eDNA molecular markers for *H. letourneuxi*, 2) estimation of the theoretical detection threshold levels for qPCR using known amounts of DNA, 3) comparison of eDNA detection methods and estimation of detection probabilities for each detection method, 4) test the effect of fish density and time on eDNA detection using controlled experiments 5) field testing of the newly developed primers on water samples taken in LOX NWR (presumed absence of *H. letourneuxi*) and in the canal system adjacent to LOX NWR (confirmed presence of *H. letourneuxi*).

Methods

Collection and DNA extraction of tissue samples

Tissue samples of *H. letourneuxi* (n=10) were obtained by U.S. Fish and Wildlife Service biologists via boat electrofishing at the Hillsboro canal (Broward County, FL), placed in individually labeled vials containing 1 mL 95% ethanol and archived at the USFWS Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California). Final DNA templates were eluted in 150 uL of AE buffer (QIAGEN, Inc), which yielded DNA concentrations ranging from 50-150 ng/uL.

Molecular marker development

Molecular marker development is a critical first step in eDNA aquatic species monitoring and detection because the marker must be species-specific to ensure species detection. We targeted a partial coding 658 nucleotide (nt) segment of the mtDNA cytochrome oxidase I gene (COI) which was PCR amplified using COI primers known to amplify in cichlid fish (Table 1). Amplification reactions for this segment (20 µL reaction volume) contained 4uL DNA (15-200ng), 2.0uL of 5X Taq reaction buffer (GoTaq Flexi, Promega, Madison, WI), 2.5uL $MgCl_2$ (25mM) 0.5uL of each dNTP (1mM), 1uL of primers PROS1 and PROS2 (10uM each), and 0.20uL Taq DNA polymerase (5U/uL; GoTaq, Promega). Optimized thermal cycle conditions for COI were an initial 94˚C (5 min.) denaturation followed by 35 cycles of 95˚C (1 min.), 62˚C (1.30 min.), and 72˚C (1 min.). An additional 7 min. extension at 72˚C was added at the end of the reaction. PCR products were visualized on 1% agarose gels,

Table 1. PCR and Taqman qPCR primers/probes used to amplify different amplicons of the mtDNA COI gene for *H. letourneuxi.*

Name (direction)	Sequence (5`-3`)	Detection method used	Primer combination and amplicon size (nt)	Citation or NCBI accession number
PROS1 (forward)	TTCTCGACTAATCACAAAGACATYGG	PCR	PROS1/PROS2 (650nt)	Sparks and Smiths (2004)
PROS2 (reverse)	TCAAARAAGGTTGTGTTAGGTTYC	PCR		Sparks and Smiths (2004)
AJFF3 (forward)	ATCCCCCTCTAGCAGGCAACCTCG	PCR	AJFF3/PROS2 (240nt)	
AJFq3 (forward)	CCCTCTAGCAGGCAACCTC	qPCR	AJFq3/AJFR2q2 (64nt)	
AJFR2q2 (reverse)	GTGGAGGGAGAAGATGGCTA	qPCR		
PCOAJF6 (probe)	6FAM-CCACGCCGGACCTT CCGTAGAC-TAMRA	qPCR		Pr028373859

cleaned using the QIAquick Purification Kit (QIAGEN, Inc), and eluted with 30uL EB buffer (QIAGEN, Inc). Cycle sequencing was conducted following the Big Dye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) using forward and reverse primers outlined above under the following PCR thermal profile: 25 cycles of 96˚C for 10 s, 50˚C for 5 s and 60˚C for 4 min. Cycle sequencing PCR products were purified using the BigDye X Terminator Purification kit (Applied Biosystems, Inc.) and then run on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Inc.). All sequences were imported into BioEdit Sequence Alignment Editor (Hall 1999), ends trimmed, and the remaining sequence aligned by eye. From the obtained sequence data and those published in Genbank (JQ667546.1, JN026744.1, JN026743.1, GU817297) we developed species-specific PCR (AJFF3) and qPCR (AJFq3 and AJFR2q2) primers for *H. letourneuxi* (Table 1). Primers were designed using Primer Express 3.0 (Applied Biosystems, Inc). Primer specificity was tested by comparing the selected primer sequences to all previously published sequence data using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast; default settings). We also tested for cross species amplification through qPCR and PCR using templates from the following species: *Micropterus salmoides* (Largemouth bass - fish), *Amblema neislerii* (fat three-ridge - freshwater mussel), *Cichlasoma urophthalmum* (Mayan cichlid - fish) and *Cittarium pica* (West Indian Topshell - marine gastropod) as template DNA (using the same PCR and qPCR reaction conditions given previously).

qPCR primers and probe design

While traditional agarose gel electrophoresis of PCR products can be used to detect species presence, qPCR is often more sensitive than traditional detection methods. This technique relies on the development of two primers and an internal probe. The internal COI primers and probe sequence for each species were designed from COI alignments for *H. letourneuxi* using Primer Express 3.0 (Applied Biosystems, Inc) and corroborated by the online software program Genscript (http://www.genscript.com). Probe design included up to three mismatches at the probe region. Taqman probes can produce measurable signals with two or three base pair mismatches (Herrero et al. 2010; Wilcox et al. 2013).

Theoretical lower limit of detection and Taqman assays conditions

We used a qPCR standard curve analysis to determine the theoretical minimum amount of DNA as well as the cycle threshold (C_T) value (C_T is defined as the number of cycles required for the fluorescent signal to cross the threshold i.e., exceeding the background level and obtaining an amplification of the target DNA sequence). For standard curve quantification analyses, we randomly selected three pure DNA samples of the target species, standardizing each sample to an initial DNA concentration of 20 ng/uL and performed a 1/10 serial dilution to 0.0002 ng/uL (i.e., five serial dilutions with three replicates per dilution value) (Figure 1). Taqman assays (Applied Biosystems, Inc.) consisted of 20 uL reaction volumes and contained 4uL of DNA solution from each dilution, 2.0 uL of 5× Taq reaction buffer (Applied Biosystems, Inc), 2.5 uL $MgCl_2$ (25mM), 0.5 uL of each dNTP (1mM), 1uL of primers AJFq3 and AJFR2q2 (10 uM each), 0.25 uL species probe (10 uM), 0.5 ul AmpErase (Uracil-N-glycosylase), and 0.20uL Taq DNA polymerase (5 U/uL; Amplitaq Gold, Applied Biosystems, Inc). All qPCR Taqman assays

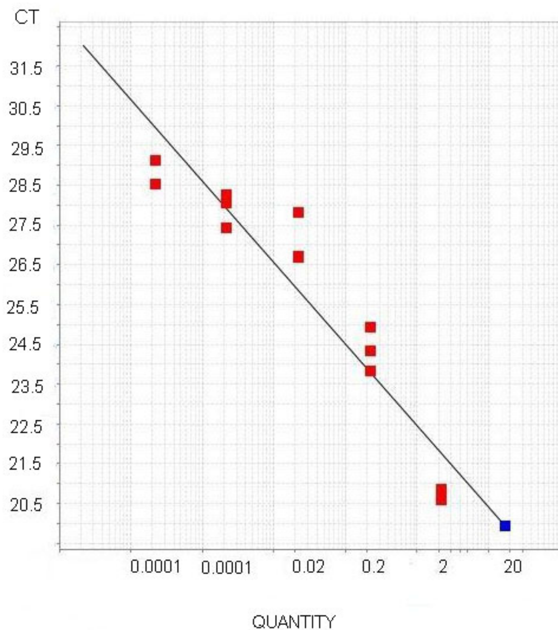

Figure 1. Graph of PCR cycle threshold (CT) vs. DNA quantity for standard curve analyses. Red squares represent qPCR results for 1/10 serial dilutions using an initial DNA template of 20ng/uL (blue square). Each group of red squares represents a serial dilution from the initial template. First serial dilution value 2ng/uL (first group of red squares from the right), second 0.2ng/uL, third 0.02ng/uL, fourth 0.002ng/uL and fifth 0.0002ng/uL.

were run using the following thermal profile: 60˚C (1 min), initial denaturation at 95˚C for 10 min., followed by 40 cycles of 95˚C (15 s) and 60˚C (1 min.). Detection of DNA from each dilution and random sample was performed using a 7500 Fast Real Time PCR machine (Applied Biosystems, Inc.). Quality controls in Taqman assays consisted in repetition of qPCR results two times and inclusion of negative and positive controls on each qPCR plate.

Aquarium trials

Thirty individuals of the target species were collected from the Hillsboro canal (Broward County, FL) and transported to a quarantine facility located at the USFWS Warm Springs Regional Fisheries Center. Aquarium trials, conducted for one week in March 2012, consisted of four treatments (0, 1, 3, and 6 individuals/tank) with three replicates per treatment. Water used for the experiment was city water that was dechlorinated naturally over a 48 hour period and tested for the absence of chlorine via a litmus paper test kit (Franklin Machine Products, Lumberton, New Jersey). Each treatment consisted of a covered 94.6 L aquarium filled with 75.7 L of water, an aquarium heater (set to 26˚C, which approximated the temperature of LOX NWR and adjacent canal water at collection time), and an air stone. After an initial acclimation period of three days, a 1 L water sample (autoclaved 1 L plastic bottle) was taken from each aquarium on days 3, 5, and 7 of the experiment (to avoid contamination, only one aquarium was open at a time and new surgical gloves were worn for each sampling event). Each water sample was treated with 1 mL of 3M sodium acetate (pH 5.2) and 33 mL 95% non-denature ethanol for DNA preservation. All samples were stored at 4˚C until DNA extraction was performed. At the end of each trial, all fish were weighed, euthanized with MS-222, and stored at -20˚C.

DNA extraction from water samples

Using a vacuum pump, we filtered each 1 L water sample through a sterile cellulose nitrate filter (0.45 um). After filtration, filters were dried at 56˚C for 10 min. and DNA extracted using the protocol outlined by the Rapid Water DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). Extracted DNA was suspended in 70 uL of buffer provided by the kit and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA). For DNA yields lower than 15 ng/uL and optical density readings (260/280) lower than 1.5, standard ethanol precipitation of DNA was conducted to increase DNA quality and concentration.

Detection of eDNA from aquarium trials and estimation of detection probabilities

There are various methods used to detect eDNA from water samples. We compared the utility of standard visualization via staining a PCR product in an agarose gel to qPCR that detects and measures the number of gene copies during every amplification cycle of the PCR. For each species, we performed PCR and qPCR, on each DNA extraction from the aquarium trials as outlined above and recorded either the presence or absence of the DNA template for each detection method. Positive PCR reactions consisted of the presence of band/PCR product in an agarose gel while positive qPCR reactions were determined

by obtaining an amplification curve of the probe (positive/presence of the target).

A typical amplification curve of a positive sample must show four distinct sections (a. baseline, b. exponential, c. linear, b. plateau) (Figure 2). In addition, the initial C_T should be in the range of our previous C_T values determined by the standard curve quantification analysis. Positive reactions should also have ΔRn (defined as the magnitude of the change in the fluorescent signal generated by the given set of qPCR conditions i.e., DNA concentration) above the threshold ΔRn that was previously set based on the minimum C_T and amount of DNA required to produce amplification of the target and provided by the standard curve quantification analysis.

Any positive reaction was sequenced for confirmation using procedures described in the molecular marker development section. All PCR and qPCR results were replicated two times. For PCR and qPCR reactions, we ran a series of positive and negative controls. For a negative control, double distilled water was substituted for template DNA, and for a positive control we replaced the template DNA with DNA (20 ng/uL) extracted from *H. letourneuxi* tissue (as outlined above). Note that we were not concerned with PCR inhibition in our aquarium trial experiment because the water was dechlorinated city water.

We performed generalized linear regression where the response variable was either the observed presence or absence of eDNA for each water sample or the C_T value and continuous predictor variables were fish density (no. of fish/tank), DNA concentration of extracted water sample, and time of water collection (days). We also explored using a random intercept (data not shown) to account for dependence among each of the three replicate samples, but results were similar indicating dependence was not a significant problem. We estimated detection probabilities for two eDNA detection methods (PCR and qPCR) using the following logit back-transformed equation:

$$\text{Prob. (PCR detect)} = 1/(1+\exp(-(\text{intercept} + \text{slope*density})))$$

where intercept and slope values were estimated for generalized linear regression (logit link function) using eDNA detection (binary coded) as the dependent variable and density as the independent variable. All statistical analyses were conducted using the program S-Plus v7.0 (Insightful Corp., New York, NY).

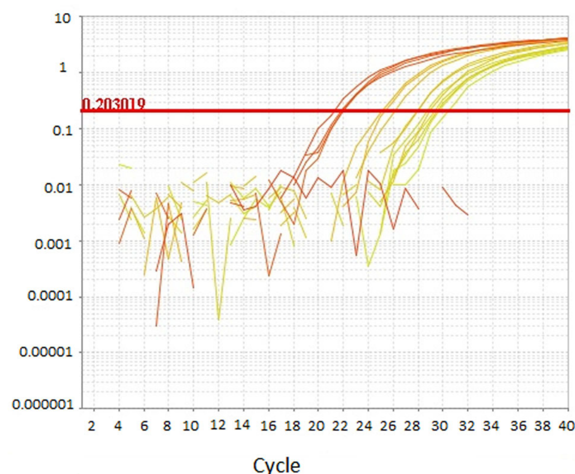

Figure 2. Typical amplification curve for *Hemichromis letourneuxi* samples at different target concentration. The plot also shows the ΔRn (magnitude of the change in fluorescent at each point of the reaction) threshold and the number of cycles required for target amplification.

eDNA detection from samples collected at LOX NWR and Hillsboro canal, Florida

A total of 18 water samples from two sites (LOX NWR and Hillsboro canal) were collected using a 3*3 grid sampling design (three transects at each site at three different depths) (see Table 5 for transect location and depth strata). Distance between each sampling point within transects and depth strata are described in Table 5. Samples were collected using a sterile collection bottle (1 L plastic bottle) and at each sampling event new surgical gloves were applied. In order to preserve the genetic material, 1 mL of sodium acetate (3M) and 33mL of ethanol 95% were added to each sample. Water samples from field sites were filtered, DNA extracted, and tested for target eDNA presence via qPCR (using both negative and positive controls). Any positive qPCR reaction was sequenced using procedures outlined previously. Due to the potential presence of PCR inhibitors in water samples, we ran a further positive control in which we spiked a water sample from Hillsboro canal and another from LOX NWR with 5-10 mg/uL of lypholized tissue from *H. letourneuxi*. These samples were filtered, DNA extracted and DNA used as positive confirmation that our qPCR reactions were working correctly in the presence of potential inhibitors.

Results

Marker development

From 10 aligned sequences of *H. letourneuxi*, specific COI primers were developed (Table 1). Primers AJFF3 in combination with PROS2 amplified a 240 nt COI segment in *H. letourneuxi*. We used this 240 nt segment of *H. letourneuxi* to develop internal primers AJFq3 and AJFR2q2 for Taqman qPCR assays along with probe PCOAJF6 (Table 1) that yielded a PCR fragment 64 nt in length. Note that the 5' and 3' end of the probe was labeled with a fluorescent dye (6-FAM) and a TAMRA quencher, respectively (Table 1). When sequences from the *H. letourneuxi* primer pair were subjected to the Basic Local Alignment Search, the only reported query to return both primer sequences was for *H. letourneuxi.* No base pair mis-matches were found in the probe region of the 10 lab sequences. However two sequences showed one base pair mismatch (A/C) in the reverse primer region.

New developed primers and probe produced typical amplification curves when qPCR assays were ran using target DNA extracted from *H. letourneuxi* tissue. We observed no cross species amplification through qPCR using other genomic DNA from fish, freshwater mussel, and gastropods.

Theoretical lower limit of detection

Based on a serial dilution of five known amounts of DNA from the target; the lower limit of eDNA detection for *H. letourneuxi* was 0.0002 ng/µL. Target DNA amount and C_T were correlated (R^2 = 0.90) and a C_T of 28.5-29 was required to detect the minimum amount of DNA from serial dilution (Figure 1).

Detection of eDNA from aquarium trials and estimation of detection probabilities

For *H. letourneuxi,* average biomass per tank was 5.49 g (SD = 1.63; 1 fish), 15.82 g (SD = 1.88; 3 fishes) and 30.71 g (SD = 3.58; 6 fishes). All positive detections by PCR and qPCR were confirmed to be *H. letourneuxi* via sequencing except two *H. letourneuxi* PCR samples in the density 1 trials (Table 2). For these samples, a positive (but faint) band was detected on the agarose gel; however, qPCR failed to detect the presence of *H. letourneuxi*, and sequencing of the PCR product was unsuccessful. We designated the

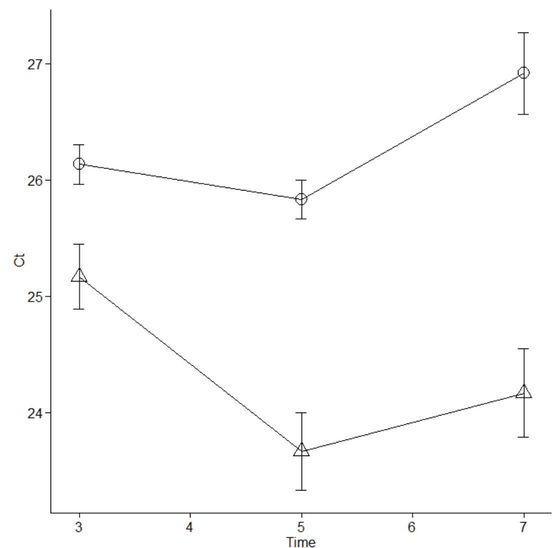

Figure 3. Average cycle threshold (CT) by fish density and by time (days). Open circles represent Ct values at densities of 3 fish/75.7 L and open triangles represent Ct values at densities of 6 fish/75.7 L. The bars in the figure show the standard errors to Ct scores for each density and time.

two observations as false positives. Qualitative inspection of *H. letourneuxi* presence and absence data for aquaria trials indicated that qPCR was a more sensitive and reliable method for eDNA detection than standard PCR visualization on an agarose gel (Table 2). At densities of one fish/tank all methods failed to detect the presence of eDNA in the water column (Table 2). Generalized linear regression results for the traditional PCR detection method indicated that there was no significant relationship between time and detection or DNA concentration (i.e., average DNA concentration of replicate water filtered samples); however, the relationship between density (no. fish/tank) and PCR detection was positive and significant (Table 3). The average C_T values for qPCR reactions at densities of three and six fish were 26.29 (SD = 0.74) and 24.33 (SD = 1), respectively. There was a negative and significant relationship between C_T and density (Figure 3; Table 4). However, the relationship between C_T and time was non-significant (Table 4).

We had trouble fitting a generalized linear model to the *H. letourneuxi* qPCR data presumably because densities of 3 and 6 always resulted in detections, whereas densities of 1 never resulted in detection (Table 2). For standard PCR, estimated detection probabilities were 0.00, 0.54, and 0.82 for 1, 3, and 6 fish/tank, respectively. In contrast,

Table 2. Results of eDNA detection for *H. letourneuxi* using PCR and qPCR methods. PCR and qPCR positive results were confirmed by DNA sequencing. Average [DNA] is the average DNA concentration of water filtered samples for each treatment.

				Replicate (PCR)			Replicate (qPCR)			Replicate (sequence confirmation)		
Day	Treatment	No. fish/ treatment	Ave. [DNA] ng/uL	I	II	III	I	II	III	I	II	III
3	I	0	21.6	-	-	-	-	-	-	-	-	-
	II	1	16.2	-	+	-	-	-	-	-	-	-
	III	3	49.5	+	+	-	+	+	+	+	+	+
	IV	6	25.9	+	+	-	+	+	+	+	+	+
5	I	0	19.6	-	-	-	-	-	-	-	-	-
	II	1	7.4	-	+	-	-	-	-	-	-	-
	III	3	22.9	+	+	-	+	+	+	+	+	+
	IV	6	6.7	+	+	+	+	+	+	+	+	+
7	I	0	12.25	-	-	-	-	-	-	-	-	-
	II	1	11	-	-	-	-	-	-	-	-	-
	III	3	19.41	+	+	-	+	+	+	+	+	+
	IV	6	24.2	+	+	+	+	+	+	+	+	+

Table 3. Generalized linear regression results of potential factors influencing eDNA PCR detection when visualized on an agarose gel for *H. letourneuxi* aquarium trials. Time was measured in days, density was number of fish per treatment, and DNA refers to average DNA concentration. Data are given in Table 2.

Value	df	Estimate	Std. Error	t-value	p-value
(Intercept)		-2.13	1.04	-2.04	
time	32	0.15	0.17	0.88	0.39
density	32	0.45	0.13	3.44	0.001
DNA	32	0.01	0.01	0.86	0.40
(Intercept)	34	-1.19	0.38	-3.14	
density	34	0.46	0.13	3.54	0.001

Table 4. Generalized linear regression results for qPCR cycle threshold (C_T) values as a function of fish density and time. Time was measured in days, and density was number of fish per treatment.

	df	Estimate	Std. Error	*t*-value	*p*-value
(Intercept)	33	28.39	0.65	43.50	<0.001
Density	33	-0.65	0.10	-6.61	<0.001
time	33	-0.03	0.09	-0.30	0.77
(Intercept)	34	28.23	0.46	61.09	<0.001
density	34	-0.65	0.10	-6.70	<0.001

Table 5. Results of eDNA detection from Loxahatchee National Wildlife Refuge and adjacent Hillsboro canal based on qPCR analysis. The abbreviation DNA conc. indicates the concentration of extracted DNA from water filtered samples. Positive eDNA detection is indicated by + and confirmed by DNA sequencing. Total transect length for both sites was approximately 30 meters and distances between sample points within the transect 10 meters.

Location	Transect location and strata (depth)	Depth (ft)	DNA conc. (ng/uL)	qPCR detection
LOX NWR	Marsh (surface)	0	14.5	-
	Marsh (middle)	2.5	32.7	-
	Marsh (bottom)	5.0	71.9	-
	Center (surface)	0	31.8	-
	Center (middle)	7	31.1	-
	Center (bottom)	14	70.8	-
	Levee (surface)	0	63.6	-
	Levee (middle)	2.5	93.9	-
	Levee (bottom)	5.0	28.9	-
HILLSBORO	South Levee side (surface)	0	106.1	-
	South Levee side (middle)	2.5	141	-
	South Levee side (bottom)	5.0	99	-
	Center (surface)	0	71.9	+
	Center (middle)	7	37.7	-
	Center (bottom)	14	0	-
	North Levee Side (surface)	0	74.5	-
	North Levee Side (middle)	2.5	41.9	-
	North Levee Side (bottom)	5.0	0	-

observed eDNA detection probabilities for qPCR were 0.00, 1.00, 1.00 for 1, 3, and 6 fish/tank (Table 2).

eDNA detection from water samples collected at LOX NWR and Hillsboro Canal, Florida

We successfully extracted DNA from 16 of 18 water samples. The DNA concentration from field water samples ranged from 14.5 to 141 ng/µL (Table 5). One sample out of nine collected at the Hillsboro canal site was positive via qPCR. This positive sample was confirmed via sequencing. The obtained C_T value for the positive sample was 26. The obtained sequence for the Hillsboro canal sample showed 95-97% similarity to other *H. letourneuxi* sequences (Genbank accession numbers JQ667546.1, JN026744.1 and JN026743.1). No positives were obtained from samples collected in LOX NWR. We obtained a positive qPCR reaction in each positive qPCR inhibition control indicating PCR inhibition was negligible.

Discussion

The use of genetic techniques to identify and monitor aquatic and terrestrial organisms has been shown to be an effective tool for many fields of biology (Taberlet et al. 2012) including forensic science (Ogden 2008, 2009), ecology (Valentini et al. 2009; Barbour et al. 2010), taxonomic identification (Moyer and Díaz-Ferguson 2012), and conservation biology (Godley 2009; Thomsen et al. 2012). Recently, this methodology has been introduced as a new tool for species detection and monitoring in freshwater ecosystems (Ficetola et al. 2008; Jerde et al. 2010). In particular, eDNA detection for aquatic invasive species has taken center stage due to the high profile eDNA evidence implicating invasive bighead and silver carp in Lake Michigan (Jerde et al. 2010). The methodological advantage of using eDNA detection techniques is its presumed sensitivity for species detection (Jerde et al. 2010; Thomsen et al. 2012) and cost effectiveness (Goldberg et al. 2011). For example, the use of such a technique to detect and monitor for AIS may be of great advantage to traditional detection methods since invasive species are usually reported at lower densities during early stages of their introduction (Harvey et al. 2009). Detection of AIS using eDNA methods appears promising as a tool that can be incorporated into management, comprehensive conservation and species early detection/rapid response plans (Díaz-Ferguson and Moyer, in press). Our study sought to develop eDNA techniques and provide proof-of-concept for use of this tool in future monitoring for the presence of AIS in units of the National Wildlife Refuge System, and specifically *H. letourneuxi* within the LOX NWR.

Molecular marker development

Molecular marker development is a critical first step in eDNA aquatic species monitoring and detection because the marker must be species-specific to ensure consistent species detection. We tested for species specificity by 1) comparing the selected markers to all previously published sequence data in Genbank (repository for sequence data), 2) testing for cross-species amplification (or lack thereof) in other taxa, and 3) confirming PCR and qPCR positives by sequencing. We had high primer specificity to deposited Genbank sequences of the target taxon and observed no cross-species amplification for species specific primer pairs even for other cichlid species (i.e., *Cichlasoma urophthalmum*) also invasive in the study area. These observations imply that each primer pair should reliably amplify the target species' eDNA in water samples, assuming that the DNA concentration extracted from water samples is above threshold levels. Although our primer pairs for this species appeared specific to the taxon in question, the potential for cross species amplification with other taxa still remains. To further reduce the potential risk of cross-species amplification would require a better understanding for the genetic diversity of *H. letourneuxi* throughout their native range; however, such an endeavor was beyond the scope of this study. This is a topic of concern when basing management decisions on eDNA results. Thus, to avoid potential cross-species amplification, we advocate sequencing of all eDNA samples tested as positive (via PCR or qPCR detection) for *H. letourneuxi* within the LOX NWR and replicate results. Furthermore, the risk that primers developed in this study could amplify closely related congeners, while unknown, should not be a major factor since *H. letourneuxi* is non-native and has no closely related native taxa proximate to LOX NWR. Thus any positive eDNA sample, if not the correct species, should at least identify other closely related (and invasive) *Hemichromis* spp. or closely related congeners in the LOX NWR. Thus, the newly developed

primer sets for *H. letourneuxi* appear to reliably amplify eDNA of the target species from water samples (assuming that the concentration of DNA from tissue is above threshold levels established by the standard curve); however, any detection of *H. letourneuxi* using these primers should be confirmed via sequencing of the amplicon.

Theoretical lower limit of detection

Our observed theoretical lower limit of detection for qPCR (0.0002 ng/uL) and results obtained from aquarium trials highlighted the sensitivity and accuracy of qPCR as method of eDNA detection. A similar theoretical lower limit of detection for qPCR was reported for the brook trout, the bull trout (Blankenship et al. 2011; Wilcox et al. 2013) and the Idaho giant salamander (Pilliod et al. 2014). Jerde et al. (2011) reported lower bounds for the detection of Asian carp species using traditional detection techniques that were much less than that reported above (i.e., 3.30×10^{-8} to 7.25×10^{-11} ng/µL); however, it is unclear how these numbers were derived.

Detection of eDNA from aquarium trials and estimation of detection probabilities

Results from our aquarium trial experiments supported the findings of Dejean et al. (2011) and Mahon et al. (2013) that eDNA detection is positively correlated with target taxon density. Our negative correlation between C_T values and density corroborated this relationship because lower C_T values are known to be inversely proportional to the amount of target eDNA in a particular sample (Wilcox et al. 2013). For *H. letourneuxi*, detection probabilities of eDNA using traditional PCR visualization were never 100%, but using odds ratios, we estimated that for every 1 unit (unit = fish) increase in fish density, the species is 1.56 times [exp(0.4564)] more likely to be detected. In contrast, eDNA detection of *H. letourneuxi* using qPCR produced reliable (100% detection probabilities) results for all but the lowest densities suggesting that the use of qPCR (with posterior sequencing confirmation) should be a reliable method for the detection of *H. letourneuxi* eDNA at densities greater than threshold values - a finding similar to Wilcox et al (2013) who showed that qPCR Taqman assays are substantially more target specific than traditional PCR for eDNA detection. The increase in target specificity may also reduce the presence of false positives as indicated by our false positive rate for traditional PCR (~ 6%) vs. qPCR (0%) reactions. Despite our findings from controlled aquarium trials, eDNA detection probabilities should be treated with caution because in more uncontrolled environments, the probability of detection could also be influenced by environmental factors such as radiation, temperature, endogenous nucleases, fungi, density of microbial community, protracted DNA persistence after death, contaminants and poor protocol conditions (Goldberg et al. 2011; Takahara et al. 2012; Dejean et al. 2012).

eDNA detection from water samples collected at LOX NWR and Hillsboro Canal south Florida

Our developed qPCR primers and probe were successfully tested on field samples from LOX NWR and Hillsboro canal site. These results represent the first eDNA analysis conducted on water samples directly extracted from a site where the target species is present (Hillsboro canal) and also from a site where it is considered absent (LOX NWR). While our results support the observation that *H. letourneuxi* is present in Hillsboro canal and absent in LOX NWR, our finding of absence from LOX NWR should be treated with caution due to the potential of false negatives arising from possible PCR inhibition and imperfect eDNA detection (Schmidt et al. 2013). The fact that we only obtained one positive sample in nine from Hillsboro canal stands in stark contrast to the 100% detection probabilities obtained during the density trials suggesting that PCR inhibition or imperfect eDNA detection may be influencing our field results. Our qPCR positive controls failed to detect any influence of PCR inhibition, thus field sampling eDNA detection probabilities appear lower than expected from our aquarium trials. The result becomes clear if we extrapolate our detection probabilities to a larger system, say an earthen pond (189000 L). In order to achieve a 100% probability of detecting eDNA from 1 L of water in the earthen pond, 7500 fish must be present in the pond. The actual density of *H. letourneuxi* in the Hillsboro canal remains unknown (however, see below); thus, our imperfect detection (approximately 11%) could be the result of lower densities for *H. letourneuxi* in the field compared to the aquaria trials, else other biotic or abiotic factors (e.g., flow) could be influencing our field detection probabilities. Our findings highlight a conundrum for biologists interested in using eDNA as a tool for AIS

monitoring or detection of threatened and endangered taxa. The probability of eDNA detection will vary depending on (among other variables) the volume of water sampled and the presumed (but usually unknown) density of the organism in question. The development of site-occupancy models that provide a means to account for imperfect detection of various sampling methods (Pilliod et al. 2013; Pollock et al. 2002, Royle and Dorazio 2008) including eDNA methods (Schmidt et al. 2013) should aid in the endeavor. Specifically, occupancy models can be used to study the effects of various abiotic and biotic factors that influence detection probabilities (both in the field and in the lab) and to determine the number of visits or volume of water needed to be confident that a species is absent from a site (Schmidt et al. 2013). The latter will be particularly important for monitoring of AIS along a leading edge of invasion.

Other important and applicable data obtained by the qPCR detection assays from natural systems was the relationship between the qPCR C_T value and the concentration of the target in the DNA sample. A C_T value can be used to measure of the relative amount of target sequence in the qPCR reaction (Wilcox et al. 2013). Thus in the case of eDNA, this value could be an indicator of species density or abundance (Takahara et al. 2012). Our positive sample observed in Hillsoboro canal exhibited the ideal amplification curve and amplified at 26 cycles. This information along with results from our standard curve analysis indicated that the DNA concentration in the positive environmental sample from the canal site was approximately 0.02–0.1 ng/µL. A similar C_T value was obtained during the density trials experiments for densities of three fish per tank suggesting that perhaps relatively high densities of *H. letourneuxi* might occupy this area. Unfortunately information about *H. letourneuxi* density, CPUE or abundance in the canals is currently unknown to corroborate the relationship between density and the obtained C_T value.

Based on results from this study, we advocate the use of qPCR techniques over traditional PCR eDNA detection (i.e., via visualization of PCR products on an agarose gel) because of the observed increased sensitivity of qPCR evidenced with higher detection probabilities for this method in the aquarium trials, and negligible false positive rate. Regardless of the detection method, positive detections should be confirmed via sequencing of the PCR product in order to avoid false positives. These recommendations, along with more controlled field data (i.e., sampling water from known locations where densities of the target taxon can be measured, minimum number of samples per site depending on life history and habitat features) and the development of occupancy models using larger artificial systems such as ponds or rivers should provide a rather cost effective and efficient detection method for *H. letourneuxi* in LOX NWR.

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