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Objective II.

Development of environmental DNA methodology for early detection of aquatic invasive species in Loxahatchee NWR.

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Summary of major findings

1) Species-specific primers for eDNA detection via traditional PCR agarose gel visualization and qPCR were developed for *Hemichormis letourneuxi* and *Channa marulius.*

2) Theoretical qPCR detection threshold levels for *H. letourneuxi* and *C. marulius* were approximately 0.0002 ng/uL (R^2 = 0.89) and 0.005 ng/uL (R^2 = 0.94) at a PCR cycling threshold of 28.5-29 and 22-23 amplification cycles, respectively

3) 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.

4) Environmental DNA persisted in controlled tank experiments for up to 24 days post removal of *H. letourneuxi* and *C. marulius* with minimal degradation, but between 24 and 31 days DNA concentration and 260/280 optical density readings decreased significantly.

5) The only significant (P = 0.0299) factor influencing DNA persistence in controlled tank experiments for the eight estimated abiotic parameters held over an eight day period was temperature. Degradation of DNA occurred between 25°C and 33°C

6) The use of qPCR for eDNA detection along with confirmation from direct sequencing of positive PCR reactions should provide a reliable method for the detection of *H. letourneuxi* and *C. marulius* when their densities are greater than threshold values and PCR inhibition is minimized.

INTRODUCTION

Environmental DNA (eDNA) refers to tissue fragments that a species leaves behind in the environment. Therefore to test for the presence/absence of an aquatic species, tissues suspended in the water column can be collected and a known volume of water filtered on fine micron screens to trap the tissue. The eDNA can then be extracted from the tissue on the filter and screened for the presence of a taxon (or taxa) of interest via the polymerase chain reaction (PCR) and molecular markers specific to the target taxon. Presence is typically confirmed by one or all of the following methods: agarose gel electrophoresis of PCR product, quantitative PCR (qPCR), or direct sequencing of the PCR product.

The basic technique outlined above, raises the possibility to monitor and detect representatives of target taxa in an environmental sample that are extremely rare and eliminate the extraneous noise generated by the multiplicity of non-target taxa. Thus eDNA as a monitoring method will have broad research and management applicability in freshwater, estuarine, and marine ecosystems for threatened and endangered species and for invasive species. However, recognizing this rare signal can be arduous because identification of the specimens requires both accuracy and sensitivity (Darling and Blum 2007; Dejean et al 2012).

The goal of this study was to explore the utility of eDNA detection methods for use with two (*Hemichromis letourneuxi and Channa marulius*) aquatic invasive species that are of concern to Loxahatchee NWR (NWR). We achieved this goal with the following five objectives: 1) development of species specific molecular markers for *H. letourneuxi* and *C. marulius*), 2) estimation of the theoretical detection threshold levels for qPCR using known control amounts of DNA, 3) comparison of eDNA detection methods from aquarium trials where known numbers of individuals in known volumes of waters were used, 4) estimation of detection probabilities for each eDNA detection method, 5) assessing the influence of abiotic factors on eDNA persistence,

and 6) test newly designed qPCR primers and probe using water samples collected from known areas where *H. letourneuxi* occur.

Considered a predatory cichlid fish, the African jewelfish, *H. letourneuxi*, was introduced in Florida during the early 1960s (Rivas 1965) and has expanded throughout Florida and has been observed in canals adjacent to Loxahatchee NWR. To date there are no reports of this species in Loxahatchee NWR. Regardless, *H. letourneuxi* has been thought to compete with native sunfishes (Schofield et al 2007) along with other non native species (Porter et al 2012) such as the spotted tilapia (*Tilapia mariae*), blue tilapia (*Oreochromis aureus*), the Nile tilapia (*O. nilotica*), Mayan cichlids (*Cichlasoma urophthalmus*) and the black acara (*Ci. bimaculatum*). The introduction of this predator represents a significant threat to aquatic species within the confines of Loxahatchee NWR.

Very little is known regarding the interaction of *C. marulius* (bullseye snakehead) with native aquatic species; however, given the closely related congener *C. argus* (the northern snakehead) it is presumed to be top predator, competing for habitat, spawning areas and prey with native species at the same trophic level. The species, which is native to Asia, was introduced to Florida during the early 2000s and has rapidly spread throughout northern Florida and potentially other southern states. Individuals of the species were first reported in the Loxahatchee NWR in 2012 (J. Galvez, USFWS, personal communication). Like *H. letourneuxi* the introduction of *C. marulius* poses a potential threat to all levels of community structure for aquatic fauna in Loxahatchee NWR.

Our investigation provides new genetic tools for aquatic invasive species detection in an effort to potentially reduce time and cost of traditional inventory and detection methods. This research also establishes a standard protocol for eDNA detection that can be use for detection and monitoring of other aquatic species across the United States.

MATERIAL AND METHODS

Collection and DNA extraction of tissue samples.

Tissue samples of *H. letourneuxi* (n= 10) and *C. marulius* (n = 19) were obtained by United States Fish and Wildlife (USFWS) biologists via boat electrofishing at Hillsboro canal (Broward county) FL and placed in individually labeled vials containing 1 mL 95% non denatured ethanol. All tissue samples were archived at the USFWS Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted from each tissue sample using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California). Final DNA templates were eluted in 150 µL of AE buffer (QIAGEN, Inc), which yielded DNA concentrations ranging from 50-150 ng/µL.

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 segment of the mtDNA *cytochrome oxidase I* gene (*COI*). Specifically, 658 and 560 nucleotide (nt) segments of *COI* from *H. letourneuxi* and *C. marulius* were PCR amplified using *COI* primers known to amplify in these two species (Table 1). Reactions (20 µL reaction volume) contained 4 µL DNA (15-200ng), 2.0 µL of 5× *Taq* reaction buffer (GoTaq Flexi, Promega, Madison, WI), 2.5 µL $MgCl_2$ (25mM) 0.5 µL of each dNTP (1mM), 1 µL of each primer (10 µM each), and 0.20 µL *Taq* DNA polymerase (5 U/µL; 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 for *H. letourneuxi* or 56˚C for *C. marulius* (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, cleaned using the QIAquick Purification Kit (QIAGEN, Inc), and eluted with 30 µL 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 XTerminator 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 sequence data and that published in Genbank (four sequences of *H. letourneuxi* and two sequences of *C. striata*) we developed species-specific PCR primers for each taxon (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 using *Micropterus salmoides* (fish), *Amblema neislerii* (freshwater mussel) and *Cittarium pica* (marine gastropod) as template DNA (PCR reaction conditions given previously).

***qPCR* primers and probe design**

While traditional agarose gel electrophoresis of PCR products can be used to detected species presence, qPCR is often more sensitive than traditional detection methods. This technique relies on the development of two primers and a probe internal to each species specific PCR primer set. Internal *COI* primers and probe sequence for each species were designed from *COI* alignments for *H. letourneuxi* and *C. marulius* using Primer Express 3.0 (Applied Biosystems, Inc) and corroborated by the online software program Genscript (http://www.genscript.com). As above primer specificity was tested by comparing the selected primer sequences to all previously published sequence data using the Basic Local Alignment Search Tool

Theoretical lower limit of detection

We used a qPCR standard curve analysis to determine theoretical threshold levels of DNA for qPCR detection. Specifically, we started with a 20 ng/µL sample for both species and performed a 1:10 serial dilution to $1:1x10^{-6}$. For standard curve analyses, we randomly selected three pure DNA samples per species (i.e., samples whose DNA was used for sequencing and marker development). Taqman assays (Applied Biosystems, Inc.) consisted of 20 µL reaction volumes and contained 4 µL of DNA solution from each dilution, 2.0uL of 5× *Taq* reaction buffer (Applied Biosystems, Inc), 2.5 µL $MgCl_2$ (25mM), 0.5 µL of each dNTP (1 mM), 1 µL of each primer (10uM each), 0.4 µL species specific probe (10 µM), 0.5ul AmpErase (Uracil-N-glycosylase), and 0.20 µL *Taq* DNA polymerase (5 U/µL; Amplitaq Gold, Applied Biosystems, Inc). All qPCR Taqman assays were run using the following thermal profile: 60˚C (1min), initial denaturation at 95˚C for 10min., 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.).

Field trials: proof-of-concept

For each species, 30 individuals were collected from Hillsboro canal (Broward County, FL), transported to a quarantine facility located at the USFWS Warm Springs Regional Fisheries Center (aquarium trials were conducted in March 2012 for *H. letourneuxi*, and June 2012 for *C. marulius*), and held at the quarantine facility during one week aquarium trials. Aquarium trials consisted of four treatments (0, 1, 3, and 6 individuals per gallon) with three replicates per treatment. Each treatment consisted of a 94.6 L aquarium filled with 75.7 L of water, an aquarium heater (set to 26˚C, which approximated the temperature of the canal water), and an air stone. After an initial acclimation period of three days, a 1 L water sample was taken from each aquarium on days 3, 5, and 7 of the experiment. Each water sample was treated with 1 mL of 3 M sodium acetate (pH 5.2) and 33 mL 95% non-denature ethanol for DNA preservation (see

Appendix 1). 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 µm). 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 70ul 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/µL and optical density readings (260/230) lower than 1.5, standard ethanol precipitation of DNA was conducted (Sambrook et al. 1989) to increase DNA concentration. All template pellets were re-suspended with 25 µL AE buffer (QIAGEN, INC).

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 a gel to qPCR that detects and measures the number of gene copies during every cycle of the PCR. For each species, we performed PCR and qPCR from aquarium trials as outlined above and recorded either the presence or absence of the DNA template for each detection method. If presence was observed, then we sequenced each PCR product for species confirmation.

We performed generalized linear regression (probit link function) where the response variable was the observed presence or absence of eDNA for each water sample and continuous predictor variables were fish density (no. of fish/tank), DNA concentration of extracted water sample, and time of water collection (days). Note that we 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. In some cases ANOVA

analyses (Sokal and Rohlf 1995) were used instead of generalized linear regression due to the lack of variability among treatments (see Results). We tested for significance of eDNA detection among the two competing methods using the Kruskal-Wallis rank sum test (Sokal and Rohlf 1995). All statistical analyses were conducted using the program S-Plus v7.0 (Insightful Corp., New York, NY)

We estimated detection probabilities for each eDNA detection method using the following logit back-transformed equation:

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

where intercept and slope values were estimated for generalized linear regression (logit link function) using detection method as the dependent variable and density as the independent variable. Note that the above technique was done for each species and detection method except for *H. letourneuxi* qPCR due to the lack of variability in the datasets (see Results).

eDNA persistence

We performed several experiments to assess the influence of abiotic factors on eDNA persistence. First, upon completion of our aquarium trials, we removed all fish from aquaria and allowed any eDNA to persist in the water for up to 31 days post fish removal. Persistence of the DNA molecule was evaluated by removing 1 L of water at 7, 14, 24 and 31 days and testing for the incidence of eDNA using the DNA preservation, extraction, and qPCR approaches outlined above. Significance of factors (time, and density) on eDNA detection was assessed via ANOVA as implemented by S-PLUS. Second, at the end of the *H. letourneuxi* aquarium trials, we obtained 500 mL water samples from one tank (density of three fish, replicate II) and subjected these samples to four temperatures (8°C, 15°C, 25°C and 33°C). On days 0, 4, and 8 we estimated the following eight water quality parameters: pH, ammonia nitrogen, nitrate nitrogen, alkalinity, carbon dioxide, chlorine, hardness, and dissolved oxygen. Water quality parameters were

estimated using the Fresh Water Aquaculture Test Kit (LaMotte, Chestertown, Maryland) following the manufacturer's instructions. From these same samples, we extracted DNA, estimated DNA concentration (and 260/280 optical densities), and visually assessed DNA quality via agarose gel electrophoresis (methods outlined above). An ANOVA was performed to assess the significance of abiotic factors on DNA concentration and 260/280 optical density readings.

Detection of eDNA from Hillsboro Canal and NWR water samples

A total of 18 water samples were collected from Loxahatchee NWR (no known occurrence of *H. letourneuxi*) and Hillsboro Canal (contains *H. letourneuxi*). Sampliing consisted of a 3 x 3 grid transected across the water column. Samples were collected using a sterile collection bottle (1L). In order to preserve the genetic material 1ml of sodium acetate (3M) and 33ml of ethanol 95% were added to each sample (Appendix). Water filtration, DNA extraction, eDNA detection, and eDNA confirmation followed the methods outlined above.

RESULTS

Molecular marker development

From aligned sequences for each species, specific *COI* primers for *H. letourneuxi* and *C. marulis* were developed (Table 1). Primers AJFF3 and PROS2 amplified a 240 nt *COI* segment in *H. letourneuxi*. We used this 240 nt segment of *H. letourneuxi* to develop primers AJFq3 and AJFR2Q2 along with probe PCOAJF6 (Table 1). For *C. marulius,* specific primers CMnewF1 and FishR1 amplified a 439 nt segment of *COI* from which primers FCM2 and Rcomp2C and probe P2CMCO1 were developed (Table 1). Note that the 5' and 3' end of each probe was labeled with florescent dyes 6-FAM and Tamra, 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.* We found identical results for *C.*

marulius. We observed no cross species amplification using fish, freshwater mussel, and gastropod DNA for PCR amplification.

Theoretical lower limit of detection

Using serial dilutions of known amounts of DNA, we found that the lower limit of eDNA detection for *H. letourneuxi* was approximately 0.0002 ng/µL (R^2 = 0.89) at a PCR cycling threshold of 28.5-29 amplification cycles (Fig. 1a). In contrast, the lower limit for *C. marulius* was approximately 0.005 ng/µL (R^2 = 0.94) at a cycling threshold of 22-23 amplification cycles (Fig 1b).

Detection of eDNA from aquarium trials and estimation of detection probabilities

For *H. letourneuxi,* average biomass per tank (75.7 L water) at different densities was 5.49 g (1 fish), 15.82 g (3 fishes) and 30.71 g (6 fishes). Average biomass for *C. marulius* was 44.46, 97.88, and 197.94 g for 1, 3, and 6 fish per 75.7 L water, respectively. While there was no significant (both $P > 0.70$) difference among detection methods for each species, qualitative inspection of *H. letourneuxi* presence and absence data for aquarium trials indicated that qPCR was a more sensitive method for eDNA detection than standard PCR visualization on an agarose gel (Tables 2 and 3). At densities of one fish/tank for *H. letourneuxi*, all methods failed to detect the presence of eDNA in the water column (Table 2), but not for *C. marulius* (Table 3). For both species, generalized linear regression of traditional PCR detection method data indicated that there was no significant relationship between time and detection or DNA concentration (i.e., average DNA concentration of replicate water filtered samples) and detection; however, the relationship between density (no. fish/tank) and PCR detection was positive and significant (Tables 4 and 5). 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 a detection (Tables 2 and 3). ANOVA analyses of these data indicated a

positive and significant (sums of squares = 6.857; df = 1 $P < 0.0001$) relationship between density (no. fish/tank) and qPCR detection method.

For *H. letourneuxi,* we estimated detection probabilities for the traditional PCR eDNA detection method using the following logit back-transformed equation:

prob.(PCR detect) = 1/(1+exp(-(-intercept + slope*density))),

given values estimated in Table 4. Detection probabilities were 0.32, 0.54, and 0.82 for 1, 3, and 6 fish/tank, respectively. In contrast, *H. letourneuxi* eDNA detection probabilities for qPCR and sequencing methods were 0.00, 1.00, 1.00 for 1, 3, and 6 fish/tank (Table 2). For *C. marulius,* using the equation above and values given in Table 5, detection probabilities for the tradition PCR detection method were 0.42, 0.53, and 0.63 for 1, 3, and 6 fish per tank, respectively. Using the same equation but changing the intercept and slope values for the qPCR *C. marulius* data yielded detection probabilities of 0.46, 0.59, and 0.76 for 1, 3, and 6 fish/tank, respectively. Finally, all positive detections were confirmed to be either *H. letourneuxi* or *C. marulius* via sequencing of the detected PCR product except two *H. letourneuxi* samples (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.

eDNA persistence

Environmental DNA persisted in the water column for up to 24 days post removal of *H. letourneuxi* and *C. marulius* with minimal degradation, but between 24 and 31 days DNA concentration and optical density (260/280) readings decreased (Table 6). We found a negative and significant relationship between DNA concentration and time, as well as, optical density readings and time (Table 7). The only significant ($P = 0.0299$) factor influencing DNA persistence for the eight estimated abiotic parameters held over an eight day period was temperature (Tables 8 and 9). Degradation of DNA occurred between 25°C and 33°C (Table 8; Fig. 2).

Detection of eDNA from Hillsboro Canal and NWR water samples

We successfully extracted DNA from 16 out of 18 water samples. The concentration of DNA ranged from 14.5 to 141 ng/uL. Among the analyzed samples, *H. letourneuxi* eDNA was detected from only one water sample (sample 13). The water sample was taken from Hillsboro Canal in an area known to contain *H. letourneuxi.* The eDNA fragment detected by qPCR was confirmed using traditional sequencing. The obtained sequence for Hillsboro Canal sample 13 was 99% similar to *H. letourneuxi* sequences found in Genbank.

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 and 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. 2011). Rather recently, genetic techniques have been introduced for species detection and monitoring in freshwater ecosystems (Ficetola et al. 2008; Mahon et al. 2010; Takahara et al. 2012). In particular, eDNA detection for aquatic invasive species has taken center stage due to the high profile eDNA evidence implicating invasive bighead and silver carps may be in close proximity to or possibly above barriers that were intended to prevent their dispersal into Lake Michigan (Jerde et al 2011). The methodological advantage of using eDNA detection techniques is its presumed sensitivity for species detection (Jerde et al 2011; Thomsen et al 2011) and cost effectiveness (Goldberg et al 2011). For example, the use of such a technique to detect and monitor for aquatic invasive species invasion 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 aquatic species using eDNA methods appears to be a promising tool that can be incorporated into management,

comprehensive conservation, and detection/rapid response plans for aquatic invasive species. Our study sought to develop eDNA techniques and provide proof-of-concept for use of this tool in monitoring for the presence of *H. letourneuxi* and *C. marulius* within the Loxahatchee NWR.

Molecular marker development

Molecular marker development is a critical first step in eDNA aquatic species monitoring and detection because the marker(s) 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), and 2) testing for cross-species amplification (or lack thereof) in other taxa. We had high primer specificity to deposited Genbank sequences of the target taxon and observed no cross-species amplification for species specific primer pairs. These observations imply that each primer pair should reliably amplify target species eDNA in water samples assuming that the DNA concentration extracted from water samples is above threshold levels. Although our primer pairs for each species appeared specific to the taxon in question, the potential for cross species amplification with other taxa still remains. To reduce these potential risks requires an understanding of the genetic diversity for *H. letourneuxi* and *C. marulius* throughout their native ranges and is beyond the scope of this study. This is a topic of concern when basing management decisions on eDNA results. To avoid potential cross species amplification issues, we advocate sequencing of all eDNA samples tested as positive (via PCR or qPCR detection) for either *H. letourneuxi* and *C. marulius* within the Loxahatchee NWR. Furthermore, the risk that primers developed in this study could amplify closely related congeners while unknown, should minimal because *H. letourneuxi* and *C. marulius* are non-native and have no closely related native taxa proximate to Loxahatchee NWR. Thus any positive eDNA sample, if not the correct species, should at least identify other closely related (and invasive) *Hemichromis* or *Channa* species (or closely related

congeners) in the Loxahatchee NWR. On the other hand, if these markers are truly species specific, then any other *Hemichromis* or *Channa* species that has invaded Loxahatchee NWR will go undetected using this technique. Our results suggest that the newly developed primer sets for *H. letourneuxi* and *C. marulius* should reliably amplify eDNA for these two taxa in water samples (assuming that the concentration of DNA from tissue is above threshold levels), but we urge caution when using these primers on other systems.

Theoretical lower limit of detection

Our observed theoretical lower limit of detection for qPCR and results from aquarium trials highlighted the sensitivity and accuracy of qPCR as method of eDNA detection. While Jerde et al. (2011) reported lowest detection of pure DNA extracts from Asian carp species at levels ranging from 3.30×10^{-8} to 7.25×10^{-11} ng/µL using traditional detection techniques, it is unclear how these numbers were derived. Standard curve analyses indicated that our detection threshold for *H. letourneuxi*, which was approximately 0.0002 ng/µL, was an order of magnitude less than that of *C. marulius* (ca. 0.005 ng/µL). These results indicated that differing Taqman assay primers and probes have the potential to influence theoretical eDNA detection limits using qPCR and highlighted the importance of performing standard curve analysis for each taxon and for a variety of primers and probes so as to achieve the lowest possible limit of eDNA detection.

Detection of eDNA from aquarium trials and estimation of detection probabilities

Results from our aquarium trial experiments showed support that eDNA detection was positively correlated with target taxon density, which support the findings of Dejean et al (2011) and Takahara et al (2012). For *H. letourneuxi* there was a significant and positive relationship between density and eDNA detection. These findings also translated to an increase in detection probability (i.e., as fish density increased so did the detection probability). Detection of eDNA using traditional PCR visualization was never 100%; however, using odds ratios, we estimated

that for *H. letourneuxi*, every 1 unit (fish) increase in fish density, the species was 1.58 times [exp(0.4564)] more likely to be detected. For *C. marulius,* every 1 unit (fish) increase translated to the species being 1.26 times [exp(0.2321)] more likely to be detected. In contrast, qPCR methods for *H. letourneuxi* produced reliable (100% detection) results for all but the lowest densities suggesting that the use of qPCR should be a reliable method for the detection of *H. letourneuxi* eDNA at densities greater than threshold values. For *C. marulius* there was also a significant and positive relationship between density and eDNA detection with every 1 unit (fish) increase translating to the species being 1.26 times [exp(0.2321)] more likely to be detected using the traditional PCR approach versus 1.32 times [exp(0.2652)] more likely using qPCR methods.

We successfully detected *C. marulius* eDNA at the lowest fish density aquarium trial, which was in contrast to that of *H. letourneuxi.* These findings seem counterintuitive given that the lower limit of qPCR detection for *H. letourneuxi* was less than that of *C. marulius;* however, the average weight of *C. marulius* was greater than six times that of *H. letourneuxi* and might explain the discrepancy in detection at the lowest fish density between the two taxa. Also of disparity between taxa were detection probabilities for qPCR that were always less than one for *C. marulius*. The stark contrast to *H. letourneuxi* aquarium trials emphasizes some of the potential hurdles associated with eDNA detection from water samples. On day two of the *C. marulius* trials, we began to notice a fungal outbreak in all but the lowest density tanks. Actions of fungi in the environment are known to degrade DNA (Takahara et al 2012). Estimates of eDNA detection probabilities for *C. marulius* experiments; therefore, may be conservative having been potentially influenced by fungal degradation. Regardless, we still observed a positive and significant relationship between eDNA detection and density suggesting, like *H.*

letourneuxi, that the use of qPCR (or sequencing) should be a reliable method for the detection of *C. marulius* eDNA (when the density is greater than threshold values).

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). DNA persistence is defined as the continuance of DNA after the removal of its source (Dejean et al. 2011). Persistence of DNA in our study was observed to be between 24 and 31 days at an average temperature of 26˚C and similar to that of Dejean et al (2011) who reported that DNA persisted until 25 and 21 days for American bullfrog and Siberian sturgeon respectively. Other abiotic factors (e.g., pH, conductivity) may also influence DNA persistence (Thomsen et al 2011). Our controlled experiments; however, showed that the only factor influencing eDNA persistence and detectability was water temperature (given the range of water quality parameters estimated in this study). The quality of DNA decreased drastically in experiments at temperatures of 33˚C.

A fundamental question still remaining about eDNA detection is how many water samples are needed to detect the presence of eDNA when the density of the fish in the system is unknown? Our study showed that for densities greater than three fish (15.82 g) in approximately 76 L of water, one sample of water was sufficient to detect *H. letourneuxi* eDNA. For densities less than this, some value greater than three water samples will be needed to detect *letourneuxi* eDNA. For example, if we were to sample water from a 500 m^3 pond presumed to have *H. letourneuxi*, then, given the findings of our study and assuming no abiotic factors are limiting our detection, any number less than approximately 20,000 fish in a pond of

this size would require greater than one water sample for the detection of *H. letourneuxi* eDNA. At densities lower than approximately 6500 fish in a 500 m^3 pond (approximately equivalent to three fish or 15.82 g in 76 L of water) , greater than three water samples would be necessary to achieve any detection of *H. letourneuxi* eDNA. How many more water samples will be contingent on the density of *H. letourneuxi* in the system? Given that most aquatic invasions are at densities below these threshold values, a critical facet of eDNA detection will be understanding the relationship between detection probability, density, and number of water samples, which is an area of active research in our lab.

In conclusion, our pilot study employing molecular techniques for aquatic invasive species detection shows great potential application for monitoring of *H. letourneuxi* and *C. marulius* in Loxahatchee NWR. While more costly, we advocate qPCR techniques over that of the traditional eDNA detection (i.e., via visualization of PCR products on an agarose gel) because of the observed increased sensitivity of qPCR. Regardless of the detection method, all positive detections should be confirmed via sequencing of the PCR product to alleviate concerns about false positives. Finally, while abiotic factors other than temperature did not influence our results, PCR inhibition due to unknown abiotic factors in uncontrolled environments has the potential to greatly influence eDNA detection. To detect PCR inhibition, we recommend at least one water sample for every sampling location be spiked with DNA from the target taxon as a control for subsequent PCR, qPCR, and sequencing analyses (for a qPCR positive control we advocate using the lowest detectable DNA concentration found in standard curve analysis). These recommendation along with more controlled field data (i.e., sampling water from know locations where densities of the target taxon can be measured) and occupancy models should provide a rather cost effective and efficient detection method for *H. letourneuxi* and *C. marulius* in Loxahatchee NWR.

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Table 1. PCR, sequencing and Taqman qPCR primers/probes used to amplify a segment of the mtDNA COI gene for *H. letourneuxi* and *C. marulius*.

Taxon	Name (direction)	Sequence (5`-3`)	Citation
H. letourneuxi	PROS1 (forward)	TTCTCGACTAATCACAAAGACATYGG	Sparks and Smiths (2004)
	PROS2 (reverse)	TCAAARAAGGTTGTGTTAGGTTYC	Sparks and Smiths (2004)
	AJFF3 (forward)	ATCCCCCTCTAGCAGGCAACCTCG	
	AJFq3 (forward)	CCCTCTAGCAGGCAACCTC	
	AJFR2q2 (reverse)	GTGGAGGGAGAAGATGGCTA	
	PCOAJF6 (probe)	6FAM-CCACGCCGGACCTTCCG TAGAC-TAMRA	
C. marulius	FishF1 (forward)	ACTTCAGGGTGACCGAAGAATCAGAA	Benziger et al. 2011
	FishR1 (reverse)	GATAAAGGATAGGATCTCCTCCAC	Benziger et al. 2011
	CMnewF1 (forward)	ATTGGCGCCCCTGACATAGCATT	
	FCM2 (forward)	ATTCTAATCACCGCCGTACTTCTT	
	Rcomp2C (reverse)	TCGGTCTGTGAGTAGCATTGTAA	
	P2CMCO1 (probe)	6FAM-CCTCTCACTCCCAGTACTAGCCGCCG-Tamra	

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

Day	Treatment	No. fish/treatment	Ave. [DNA] ng/uL	Replicate (PCR)			Replicate (qPCR)			Replicate (sequence confirmation)		
				I	II	III	I	II	III	I	II	III
3	I	0	0.56	-	-	-	-	-	-	-	-	-
	II	1	17.56	+	+	+	+	+	+	+	+	+
	III	3	36.7	-	+	-	+	+	+	+	+	+
	IV	6	33.4	+	-	-	+	-	-	+	-	-
5	I	0	1	-	-	-	-	-	-	-	-	-
	II	1	18.3	-	+	-	-	+	-	-	+	-
	III	3	70.16	-	+	-	-	+	+	-	+	+
	IV	6	47.25	+	+	+	+	+	+	+	+	+
7	I	0	0.66	-	-	-	-	-	-	-	-	-
	II	1	26.9	+	+	+	+	+	+	+	+	+
	III	3	36.1	+	+	-	+	+	-	+	+	-
	IV	6	133.9	+	+	+	+	+	+	+	+	+

Table 4. 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	Std.	Std. Error	*t*-value	*p*-value
(Intercept)		-2.12915	1.0419815	-2.04337	
time	32	0.145289	0.1657775	0.876411	0.387339
density	32	0.448196	0.1301641	3.443315	0.001623
DNA	32	0.012162	0.0142032	0.856259	0.398221
(Intercept)	34	-1.1904	0.3789917	-3.14096	
density	34	0.4564	0.1289573	3.539153	0.001186

Table 5. Generalized linear regression results of potential factors influencing eDNA PCR (visualized on an agarose gel) or qPCR detection for *C. marulius* 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 3.

Method	Factor	df	Value	Std. Error	*t*-value	*p*-value
PCR	(Intercept)	32	-1.5619	0.815608	-1.91501	
	time	32	0.197209	0.146304	1.347943	0.187142
	density	32	0.269219	0.132082	2.038272	0.049858
	DNA	32	-0.00214	0.007341	-0.29193	0.772221
	(Intercept)		-0.57341	0.324048	-1.76953	
	density	34	0.232094	0.099999	2.320956	0.02642
qPCR	(Intercept)		-0.81271	0.785807	-1.03424	
	time	32	0.07637	0.14395	0.530528	0.599409
	density	32	0.263352	0.139139	1.89272	0.067471
	DNA	32	0.000948	0.008023	0.118185	0.922443
	(Intercept)		-0.41456	0.320989	-1.29151	
	density	34	0.265235	0.10784	2.459517	0.02705

Table 6. Results from eDNA persistence trials. The concentration of extracted DNA from water filtered samples was [DNA] and the abbreviation OD represents optical density as measured by a spectrophotometer.

Taxon	Time	Density	[DNA]	260/280 OD
H. letourneuxi	7	1	15	1.9
	14	1	12	1.85
	24	1	10	1.6
	31	1	0	1.1
	7	3	17.5	1.7
	14	3	13.5	1.53
	24	3	10.2	1.5
	31	3	0	1.2
C. marulius	7	1	21.06	1.95
	14	1	8.06	1.93
	24	1	11.6	1.74
	31	1	0	0
	7	3	20.5	1.93
	14	3	17.4	1.89
	24	3	21.8	1.93
	31	3	0	0

Table 7. ANOVA results of eDNA persistence trials for the influence of time (days) and density (no. fish/treatment) on DNA concentration ([DNA]) and optical density (OD).

Measurement	Taxon	Factor	df	Sum of Sq.	Mean Sq.	*F*-value	*p*-value
[DNA]	*H. letourneuxi*	time	1	256.6158	256.6158	33.4883	0.002169
		density	1	2.205	2.205	0.28775	0.614662
		Residuals	5	38.3142	7.6628		
	C. marulius	time	1	311.6854	311.6854	6.955412	0.046125
		density	1	45.0301	45.0301	1.004867	0.362151
		Residuals	5	224.0597	44.8119		
260/280 OD	*H. letourneuxi*	time	1	0.4275148	0.427515	20.54664	0.006209
		density	1	0.0338	0.0338	1.62445	0.258491
		Residuals	5	0.1040352	0.020807		
	C. marulius	time	1	3.311	3.311	7.867693	0.037773
		density	1	0.002113	0.002113	0.00502	0.946263
		Residuals	5	2.104175	0.420835		

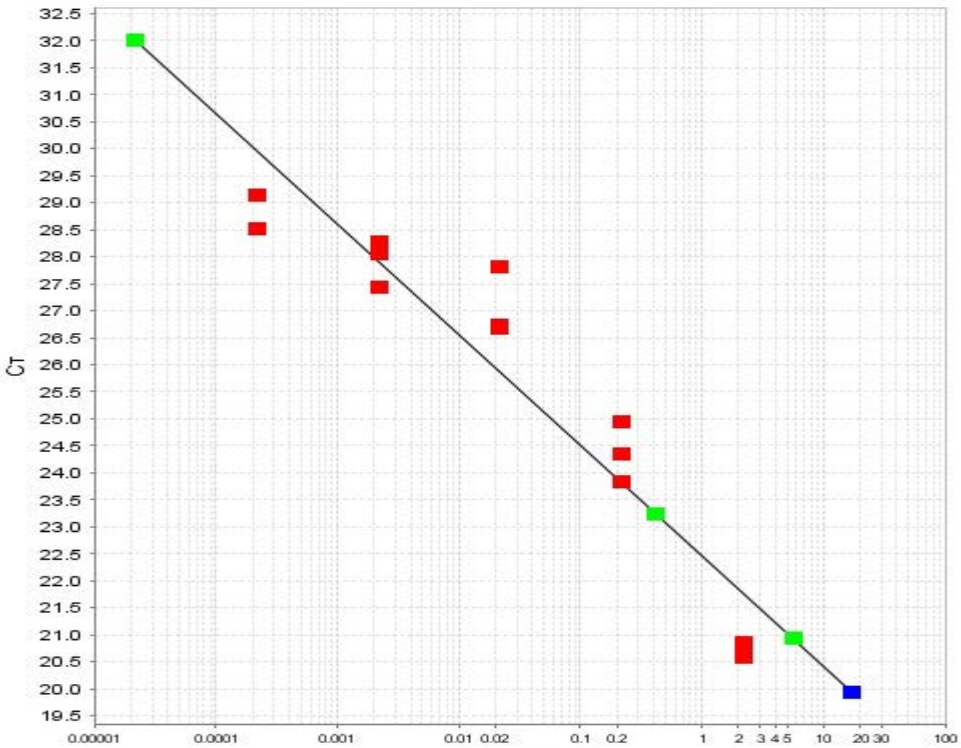
Table 8. Estimation of abiotic factors, DNA concentration ([DNA]), optical densities (260/280) and PCR eDNA detection (Detection). All abiotic parameters were recorded as ppm. Time and temperature (Temp) were recorded as days and ˚C, respectively.

Time	Temp	pH	Ammonia	Nitrogen	Alkalinity	CO_2	Chlorine	Hardness	O_2	[DNA]	260/280	Detection
0	8	7.5	1.5	0.05	20	3	12	20	5.8	22.9	2	+
0	15	6.5	0.4	0.05	23	2.5	15	18	5.6	21.6	2.05	+
0	25	6	0.8	0.05	15	2	14	12	5.9	24.9	2.06	+
0	33	5	0.6	0.05	30	2.5	16	8	5.4	27.8	2.07	+
4	8	6.5	1	0.05	20	3	12	8	5.81	50.4	1.9	+
4	15	5	1	0.05	18	2	16	8	5.8	50.9	1.92	+
4	25	6.5	1	0.05	26	2.5	16	8	5.4	38.6	1.89	+
4	33	5	0.6	0.05	30	2.5	17	8	5.4	2.6	1.23	-
8	8	6.5	3	0.05	20	3	10	8	5.4	47.3	1.86	+
8	15	6	2	0.05	19	3	15	8	5.6	47.9	1.88	+
8	25	6	1.5	0.05	24	2.5	14	10	5.3	35.7	1.84	+
8	33	8	2	0.05	25	2.5	13	10	2.4	2.3	1.13	-

Table 9. ANOVA results of eDNA persistence trials for the influence of time (days), temperature (Temp; ˚C), and abiotic factors on DNA concentration.

Factor	df	Sum of Sq.	Mean Sq.	F Value	Pr(F)
time	1	477.405	477.405	97.8153	0.064151
temp	1	2208.456	2208.456	452.4894	0.029906
ph	1	380.405	380.405	77.9409	0.071804
ammonia	1	94.997	94.997	19.464	0.141902
alkalinity	1	254.572	254.572	52.1592	0.087592
CO2	1	16.682	16.682	3.418	0.315652
Chlorine	1	13.129	13.129	2.69	0.348568
Hardness	1	213.939	213.939	43.8338	0.095434
O2	1	256.614	256.614	52.5775	0.087247
OD	1	93.202	93.202	19.0961	0.143217
Residuals	1	4.881	4.881		

A)



B)

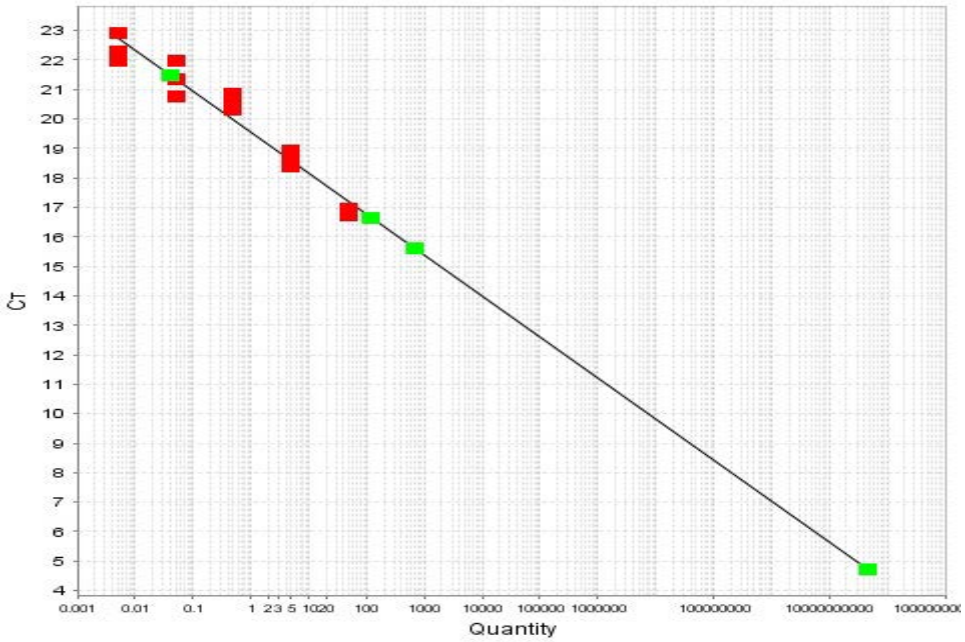


Figure 1. Graph of DNA quantity vs. PCR cycle threshold (CT) for standard curve analyses. A) results for *H. letourneuxi*; B) results for *C. marulius*. Red squares represent qPCR results for 1:10 serial dilutions of 20ng/uL template DNA (each dilution ran in triplicate). Blue or green squares represent random samples of DNA from tissue extracted samples.

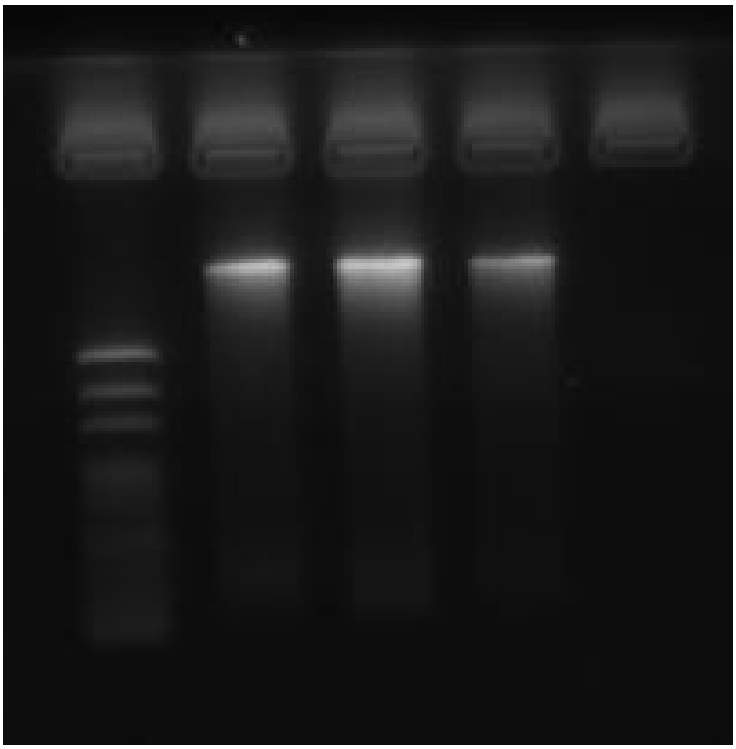


Figure 2. A representative 1% agarose gel image of stained genomic DNA from the DNA persistence study. From left to right the gel reads as follows: 1) 100 nt ladder; 2) genomic DNA whose tissue was held at 8˚C for four days; 3) genomic DNA whose tissue was held at 15˚C for four days, 4) genomic DNA whose tissue was held at 25˚C for four days; and 5) genomic DNA whose tissue was held at 33˚C for four days.

Appendix 1. eDNA preservation method for water samples

We conducted a controlled experiment to test the efficiency of ethanol and sodium acetate for preservation of tissue in water samples. Twelve water samples (1 L each) were collected from an aquaculture pond located at the USFWS Regional Fisheries Center, Warm Springs, GA. Each sample was treated with 10 mg of lyophilized tissue of our target species and six samples were treated with 3 M sodium acetate, pH 5.2 (1 mL) and 95% non denatured ethanol (33 mL). The remaining water samples went untreated as a control. Samples were stored either at room temperature or 4˚C for a period of 18 days before the DNA extraction. We estimated yield and quality of DNA extracted from water samples at day-9 and day-18 following the protocol establish in the Materials and Methods section.

Results from this experiment showed that DNA can be obtained from both preserved and unpreserved water samples; however, PCR detection of DNA in water samples was positive for all samples preserved with sodium acetate and ethanol.

Treatment	Time (Days)	Temp ˚C.	[DNA] (ng/µL)	PCR conf.	260/280 OD
NaOAc + EtOH - distilled water control	9	25	36	+	1.70
NaOAc + EtOH - pond water	9	4	95	+	1.83
NaOAc + EtOH - pond water	18	25	14.2	+	1.80
NaOAc + EtOH - pond water	18	4	91	+	2.00
No preservative - distilled water control	9	25	30	+	1.50
No preservative - pond water	9	4	86	-	1.30
No preservative - pond water	18	25	0.0	-	0.00
No preservative - pond water	18	4	16.5	-	1.20