Development of environmental DNA markers for three aquatic invasive species for Savannah National Wildlife Refuge

PI: Gregory R Moyer[1]

greg_moyer@fws.gov

Co-PI: Edgardo Díaz-Ferguson [1,2]

edgardo_diaz-ferguson@fws.gov

[1]U.S. Fish & Wildlife Service (USFWS), Warm Springs Fish Technology Center, 5308 Spring Street, Warm Springs, GA 31830.

[2]Department of Fisheries and Allied Aquacultures, Auburn University, 203 Swingle Hall, Auburn, Alabama 36849

December 2013

Summary of major findings

1) Species-specific primers for eDNA detection via traditional PCR agarose gel visualization and qPCR were developed for three aquatic invasive species of concern for Savannah National Wildlife Refuge using amplicons of the following mitochondrial DNA gene segments: COI (for Mayan cichlids), 16S (for Asian swamp eels) and D-Loop (for lion fish).

2) The lower detection value for all species was determined through qPCR standard curves base on seven DNA serial dilutions. For Mayan cichlids, the lower limit of detection was 0.0024ng/uL with a minimum number of qPCR cycles required (C_T= 32). For the Asian swamp eel and the lion fish lower limit of detection and C_T were 0.001 ng/uL and 33 cycles respectively.

3) Primers and probes were tested in water samples using lyophilized tissue of the target species and with the presence of common organic inhibitors for freshwater systems (simulated by using pond fertilizer and fish food both rich in common organic inhibitors). Different DNA extraction kits (Rapid Water DNA Isolation kit vs Power Water DNA Isolation kit) were tested for their ability to deal with the potential organic inhibition. Power Water DNA Isolation kit was more effective than Rapid Water DNA Isolation kit at eliminating potential PCR inhibitors.

4) Presence and absence of the target species was monitored at three sites in the Savannah National Wildlife Refuge using the new developed markers. A total of 27 water samples (1 L each) were screened for presence of the target species DNA. No eDNA from the target species was found from the three analyzed sites.

INTRODUCTION

Traditionally, detection and surveillance of aquatic invasive species (AIS) has been conducted either by direct observation or capture of the focal species. A relatively new detection approach, which does not require the observation of the target species, thus reducing the time and cost of the AIS monitoring, is the detection of environmental DNA (eDNA). The method consists of detecting genetic material from non-living components of the environment, such as water or soil, using species specific molecular markers. Our study developed markers for eDNA detection of three species of concern to the Savannah National Wildlife Refuge: the Mayan cichlid (*Cichlasoma urophthalmum*), the red lionfish (*Pterois volitans*) and the Asian swamp eel (*Monopterus albus*).

Native to the Atlantic slope of Guatemala and Mexico, the Mayan cichlid was first reported in the Everglades National Park, Florida in 1983 (Nico et al. 2007; Valdez-Moreno et al. 2009 Kline et al. 2012) and since then has been observed throughout numerous freshwater systems of Florida. Asian swamp eels were first introduced to the United States in Hawaii during 1900's and confirmed as inhabiting the continental United States in 1994 near Atlanta, Georgia (Fulton County, ponds of the Chattahoochee Nature Center and Chattahoochee River system; Collins et al. 2002, Long et al. 2011). In addition, two populations were discovered in South Florida in 1997 --one in the Homestead area (Miami-Dade and Broward counties) close to the Everglades National Park and the other near Tampa (Nico et al. 2011). Introductions of this species also have been reported recently in the state of New Jersey (Nico et al. 2011). The Asian swamp eel is considered a species complex (Collins et al. 2002; Matsumoto et al. 2010). For example; individuals from Homestead, Florida have mtDNA haplotypes consistent with those from Indochina. In contrast, Tampa and North Miami individuals appear related to populations from Southern China, and swamp eels from Georgia are related to Japan and Korea populations (Collins et al. 2002). These findings support the existence of three independent introductions of Asian swamp eels in the United States.

The red lion fish is the most successful fish invader in the Western Atlantic and the Caribbean (Green et al. 2012). Since its 1980's introduction in South Florida, the lion fish has expanded its range from the east coast of the United States to the Caribbean Sea, where it is reported in coastal ecosystems of seven countries and two US territories (Barbour et al. 2010). The red lion fish has not been detected in the Savannah National Wildlife Refuge; however, the species has been reported in the neighboring states of Florida, South Carolina as well as North Carolina.

The goals of this study were to provide information for the AIS Monitoring and Surveillance program for the Savannah National Wildlife Refuge and to generate the first data (species-specific molecular markers, establish the lower limit of detection and test the new markers in field samples from Savannah NWR) for eDNA detection of these invasive species.

In order to accomplish our research goals we conducted the following research objectives:

1. To develop qPCR primers and probes for the Mayan cichlid, the red lionfish and the Asian swamp eel.

2. To estimate the lower limit of qPCR detection from standard curve analysis for each species using known quantities of DNA.

3. To test developed markers on field samples collected from three sites adjacent to the refuge.

MATERIALS AND METHODS

We conducted a review of previous genetic work on focal species (Collins et al. 2002; Sparks and Smiths 2004; Freshwater et al. 2009) in order to select the gene target segment and initial primer set. Gene segments were selected based on the existence of taxon specific markers that amplified a base line sequence that was used as a sequence template for internal design of qPCR species specific primers and a probe. Thus, searches for base line reference sequences of the target gene segments in DNA databases such as GENBANK and FISHBOL were conducted (Collins et al. 2002; Sparks and Smiths 2004; Freshwater et al. 2009).

DNA extraction from tissues

Specimens and tissue samples from Mayan cichlids (n=18) were obtained by United States Fish and Wildlife (USFWS) biologists via boat electrofishing. All Mayan cichlid samples were collected from Canals C-111 (25.45N, -80.56W), L31N (25.54N, -80.56W) and C-31N (25.67N, -80.49W) bordering the Everglades National Park. For lion fish, nine samples were collected from two deep water reefs located near the Miami area by USFWS personnel (28.637N, -80.126W). Three additional lion fish samples were collected in Biscayne Bay, Florida (Biscayne National Park, near Homestead) by staff of the Biscayne Bay National Park. For Asian swamp eels, 14 samples were collected from C-113 Canal near Homestead, Florida (outside of the Everglades National Park) and two from the Manatee River located in Tampa, Florida (27.66 N, -82.35 W). An additional 10 samples of this species were collected from the Chattahoochee River, Georgia (34.00N, -78.38W).

All samples were placed in individually labeled vials containing 1 mL 95% non-denatured ethanol. All tissue samples were archived at the USFSW Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted for each tissue sample 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 50-150ng/uL.

Initial PCR reactions for the three target species were conducted for 35 cycles at a specific annealing temperature (Ta) using universal primers of the following genes: COI, 16S and D-Loop. For Mayan cichlids, COI segment amplification was conducted at a Ta of 55°C (Ward et al. 2005). For Asian swamp eels, PCR reactions were conducted using 16S as target gene segment with a Ta of 52°C (Kocher et al. 1989; Collins et al. 2002; Cai et al. 2012). For lion fish, a D-Loop segment was selected as target gene and PCR reactions were conducted at a Ta of 50.5 °C (Freshwater et al. 2009). All PCR reactions were optimized for 20uL (final reaction volume) using the following reagent amount and concentrations: 4uL of DNA extracted from tissue at concentration between 50-150ng/uL, 1uL of Taq reaction buffer (0.5X; Go-Taq Flexi, PROMEGA, Madison, WI), 1 uL of dNTP's (1mM), 2.5uL of $MgCl_2$ (25mM), 1uL of each primer set (10uM each) and 0.1uL of Taq (5U/uL; Go-Taq Flexi, PROMEGA, Madison, WI).

PCR products of all three species were cleaned using the QIAquick Purification kit (QIAGEN, Inc.). In order to sequence these products, cycle sequencing reactions were conducted following the Big Dye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) 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 GENEIOUS 4.8.5 (Drummond 2010), ends trimmed, and aligned by eye. All sequences were compared for base pair composition and similarity with other sequences of these taxa previously deposited in GENBANK or FISHBOL.

Internal molecular marker development for target taxa

For each taxon, all sequences were edited (i.e., all sequences were check for total number of base pairs, base pair composition, and quality) and aligned using GENEIOUS 4.8.5 (Drummond et al. 2010). Edited sequences from each taxa were then imported the software *Primer express* 3.0 where qPCR specific primers and a probe were designed for each species.

In order to avoid cross species amplification, the Basic Alignment Search Tool (BLAST) in GENEBANK (http://www.ncbi.nlm.nih.gov/tools/primer-blast) was implemented on each primer set and amplicon to search for similar reference sequences that the primers might amplify. We also tested the newly designed molecular markers for cross amplification in Mayan cichlids, Asian swamp eel, lion fish and African jewelfish.

All qPCR reactions were conducted for 35 cycles. For all three species, qPCR reactions were optimized for 20uL using the following final concentration of Taqman core reagents: 4uL of DNA solution from each dilution, 2.0uL of 5× Taq reaction buffer (Applied Biosystems, Inc), 2.5uL $MgCl_2$ (25mM), 0.5uL of each dNTP (1mM), 1uL of each primer (10uM each), 0.5uL AmpErase (Uracil-N-glycosylase), and 0.20uL Taq DNA polymerase (5U/uL; Amplitaq Gold, Applied Biosystems, Inc). Probe concentration varied among species Taqman assays; thus 0.4uL of probe PCOICU (10uM) was used for Mayan cichlid reactions, 0.3uL of probe PDLLF (10uM) for lion fish and 0.25uL of probe P16SSE (10uM) for Asian swamp eel. 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.).

Lower limit of qPCR detection

The sensitivity of the designed qPCR primers and probes was tested by conducting presence/absence Taqman qPCR assays (reaction conditions were described in the previous section). We also conducted standard curve analysis to estimate the lower limit of eDNA detection for each species and its C_T value (number of qPCR cycles required for amplification of specific amount of DNA). For this purpose we used a serial dilution of DNA from known concentrations of tissue and DNA (starting from a stock of 20ng/uL to 0.0001ng/uL). Reproducibility of these results was ensured by duplicating the standard curve experiments.

Effectiveness of developed primers and probes in field samples

To test the presence of the target AIS species in Savannah National Wildlife Refuge samples, 2L water samples (1L bottles,-2 L per sample) were collected from three sites using a Van Dorn vertical sampler. Site one was located at Little Bank River (32.17N, -81.11W), while site two and three were located at two different locations on the Savannah River (32.23N, -81.15W and 32.29N, -81.15). Each site consisted of 3 x 3 transect where water was sampled from surface, middle and bottom of the water column. Each sample (collection water bottle) contained the following information: site, sample number, transect number and depth. Genetic material collected in the water samples was preserved using 1ml of sodium acetate and 33mL of 95% ethanol per 1L bottle. DNA extraction from water samples was conducted using the Power Water DNA Isolation kit (MOBIO Laboratories Inc) following manufacture's guidelines. Samples were tested for presence and absence of the target species using the developed molecular markers. Additional qPCR runs were conducted for the detection of African jewelfish and the bullseyes snakehead (Moyer and Díaz-Ferguson in Herod et al. 2013). All qPCR sample plates were tested with positive controls using different DNA concentration of the target species in order to determine the concentration of possible positives samples.

Negative controls were also implemented in sample plates using water as a template instead of DNA.

Effects of PCR inhibitors on detection using two extraction kits

Efficiency to remove qPCR inhibitors from water samples using two commercial DNA extraction kits (Power Water DNA Isolation kit and the Rapid Water DNA Isolation kit) was tested through the following experiment. Four water samples (1L each) collected from hatchery ponds were spiked with a known amount of lyophilized tissue containing DNA from two of the target species (1-5mg). Two additional bottles containing distilled water were spiked with target species tissue. PCR inhibitors such as food fish (50mg) and pond fertilizer (50mg) (organic compounds) were also placed in these water bottles. Six additional water samples (four water samples from ponds of the hatchery and two filled with distilled water) were also spike with lyophilized tissue of the target species; however, no inhibitors were added.

For each experimental treatment (i.e., water bottles with inhibitors or water bottles without inhibitors) three samples were extracted using the Rapid Water DNA Isolation kit while DNA from other three bottles was extracted using the Power Water DNA Isolation kit (Appendix 1-Table 1). DNA extracted from each kit was quantified and check for OD ratios. DNA extracted from each kit was tested for presence and absence through qPCR Taqman assays as outlined above.

RESULTS

Mayan cichlids molecular marker development

From ten aligned COI sequences of Mayan cichlids (402bp) amplified with primers FISH F1 and FISH R1 (Ward et al. 1995), we designed internal specific primers (FCOICUq and RCOICUq) along with a probe (PCOICU) to produce an amplicon of 64bp (Table 1). The developed primers and probe were tested using known quantities of DNA through qPCR Taqman assays. The Taqman assays for this species showed a successful qPCR amplification curve (Fig 1).

Swamp eel molecular marker development

Ten aligned 16S sequences of the Asian swamp eel (431bp) amplified with 16SAR and 16SBR primers (Kocher et al. 1989; Palumbi 1991) were used to design internal specific primers (F16SSEq and R16SSEq) along with an internal probe (P16SSE) to produce an amplicon of 87bp (Table 1). Amplification was negative for other fish species while positive for all swamp eel clades (Homestead, Tampa, and Georgia). Nonetheless, Georgia samples had a higher C_T value suggesting lower specificity for the initial designed primer (F16SSEq) (Appendix 1-Figure 1). Based on this result we designed an additional forward primer (FqSEGA) that was able to detect all clades with a similar C_T (Table 1).

Lion fish molecular marker development

Ten D-Loop sequences of the lion fish (420bp) amplified with primers LionB-L and LionB-H (Freshwater et al. 2009) were used to design internal specific primers (FDLLFq and RDLLFq) an internal probe (PDLLF) to produce an amplicon of 90bp (Table 1). The developed primers and probe were tested using known quantities of DNA through qPCR Taqman assays. The Taqman assays for this species showed a successful qPCR amplification curve (Fig 3).

Lower limit of qPCR detection in target species

The lower limit of detection for each species was obtained running a standard curve analysis through qPCR. Standard curves for all three species showed regressions with R^2 values above 0.90 with a range between 0.90 and 0.995 (Figures 4-6). Mayan cichlid samples showed a lower limit of detection of 0.0024ng/uL with a C_T of 32 cycles, the Asian swamp eel had a lower limit of detection of 0.001ng/uL with a C_T of 33 (Fig. 5), and the lion fish showed a lower limit of detection of 0.001ng/uL with a C_T of 33 cycles (Fig. 6).

Effects of PCR inhibitors on lower limit detection

Results of PCR inhibition tests concluded that DNA extracted using Power Water DNA Isolation kit reliably detected eDNA via qPCR for each treatment (i.e., 100% detection) even for samples containing high concentration of PCR inhibitors. In contrast, the Rapid Water DNA Isolation kit failed to detect eDNA via qPCR in samples containing inhibitors (Table 3).

Effectiveness of developed primers and probes in field samples

Presence and absence of the target species was monitored along three sites of the Savannah National Wildlife Refuge. A total of 27 water samples (2L each) were processed for eDNA extraction. For each target species, qPCR failed to detect the presence of that taxon's eDNA from each sampled location. Primers and probes previously developed for the African jewelfish and the Bullseyes snakehead (Moyer and Díaz-Ferguson in Herod et al. 2013) were also tested using eDNA extracted from Savannah samples. No positives were detected using these additional markers. Note that all positive qPCR controls using DNA of the target species showed successful amplification curves indicating that our qPCR reactions were working properly.

DISCUSSION

Marker development and lower limit of detection

Genetic markers for qPCR detection were successfully developed for Mayan cichlid, Asian swamp eel and red lion fish. A literature search suggested that these genetic markers are the first eDNA molecular markers developed for these taxa. Markers developed for lion fish are the first developed for eDNA qPCR detection of a marine invasive species since eDNA efforts in the marine environment are mainly focused on marine mammal detection and biodiversity of temperate communities (Foote et al. 2012; Thomsen et al. 2012). For the Asian swamp eel an additional forward primer was developed due to mismatches observed in the Chattahoochee River, Georgia samples when those were ran using an initial primer set based on Tampa and Homestead sequences (Appendix 1-Figure 1). This new swamp eel forward primer (FqSEGA) simultaneously tested with Fq16SSE will allow managers and scientists to discriminate between putative introduced populations of this species (Appendix 1-Figure 1-2).

Specificity of all markers was tested by running qPCR reactions for other fish species using the new developed markers (cross species amplification test) and also by comparing target sequences obtained in the laboratory with all possible sequences deposited in the GENBANK using BLAST (Table 2). Sensitivity of the new developed markers was demonstrated by running standard curves for each species (Figure 1-6) in order to determine the lower level of qPCR detection for each target species. Lower limits of detection ranged from 0.001 to 0.003 ng/uL. Similar lower limits of detection have been determined for the African jewelfish (Moyer and Díaz-Ferguson 2013 in Herod et al. 2013) and the brook trout (Blankenship et al. 2011). Lower levels of detection, as well as, C_T threshold values for the studied species were between 33 to 35 cycles (Figures 4-6). These values are important for future studies of biomass since the amount of DNA in the environment could be a good estimator of a species' biomass contribution in the ecosystem (i.e., the lower the C_T value, the higher the concentration

of target DNA in the system and the higher the biomass of the species releasing this DNA; Takahara et al. 2012). In addition, understanding the spatial variations of the amount of eDNA detected in a particular water body could be used as an estimator of the distribution and abundance of the target species or source of eDNA (Lodge et al. 2012; Takahara et al. 2013).

Inhibition control experiment

Humic substances are common components of freshwater ecosystems (Hessen 1998). Organic components and humic substances are present in freshwater samples in the form of humic acid, fulvic acid, phenols, and carbon structured molecules such as proteins. These substances are considered strong inhibitors of molecular reactions such as PCR, qPCR, and DNA sequencing (Matheson et al. 2010). Since these components are relatively resistant to chemical and biological degradation, scientists have found several ways to reduce their presence in soils, sediments, and freshwater samples (i.e., use of BSA, DMSO, dilution of the DNA template, increasing the Taq polymerase concentration, inhibitor size exclusion by chromatography and/or using an specialize DNA extraction kit with additional steps for inhibitors removal). In order to make sure that our selected protocol and DNA extraction kit successfully removed inhibitors, we conducted a test comparing the effectiveness of two MOBIO DNA extraction kits (Power Water DNA Isolation kit vs Rapid Water DNA Isolation kit) in removing potential PCR inhibitors. Results indicate that the additional step for inhibitor removal used in the Power Water DNA Isolation kit is effective. All target DNA extracted with the Power Water Isolation kit was successfully detected through qPCR even in samples containing high concentration of inhibitors (Table 3).

Testing the development primer in field samples collected from the Savannah National Wildlife Refuge.

Primers and probes were tested during the experimental phase of this project for sensitivity using DNA from the target species at different DNA concentration and for

specificity using DNA from other fish species (cross amplification test). However, we wanted to expose the primer and probe sets to water samples taken from the field because it tests the primer sets for the potential to generate false positives (i.e., it tests for the potential for other species that could possibly be amplified using the primers and probes). No positives samples were detected; thus confirming species specificity of these markers for Savannah National Wildlife Refuge. Positive and negative controls were run with all samples to avoid false negatives due to failure in the Taqman assay. Obtained results suggest that the developed molecular tool could be used for species detection and incorporated into management, comprehensive conservation, AIS early detection and rapid response plans of Region 4. Absence of positives found in this study represented a complementary result to current inventory and monitoring efforts of the Service related to the detection of AIS in Savannah National Wildlife Refuge. Nonetheless, our finding about the complete absence of these target species along the refuge should be interpreted with caution. The information presented here is only a snapshot of a particular area at a specific time scale based on 27 samples from three sites. Additional water sampling (i.e., more sites or more water samples per site) may be required to detect eDNA especially for species at low densities (Darling and Mahon 2011).

Future experiments using controlled field data and by species will be important in order to provide more information about the effectiveness of these new tools (i.e., sampling water from known locations where densities of the target taxon can be measured using a robust spatial-temporal design and establishing the minimum number of samples that should be collected base on life history and habitat features). These future experimental trials will be especially important in order to assess biomass, abundance and distribution of these species in natural systems using eDNA. In addition, development of occupancy models of larger artificial systems such as ponds and rivers for Asian swamp eels and Mayan cichlilds; and coastal ecosystems for lion fish (i.e.,

sampling coral reefs where lion fishes have been reported) will give us a robust evidence of the utility and reliability of these new eDNA markers.

REFERENCES

Barbour AB, Montgomery ML, Adamson AA, Díaz-Ferguson E, Silliman BR (2010) Mangrove use by the invasive lionfish *Pterois volitans*. Marine Ecology Progress Series 401:291-294.

Blankenship S, Teply M, Dominguez L, Schumer G (2011) Sampling and analysis to assess brook trout (*Salvelinus fontinalis*) population trends in high Lake (Oregon) using environmental DNA monitoring. Technical report, Cramer Fish Sciences. 18p.

Cai X, Shumin Yu, Tserangdonko M, Zhang X, Yue B (2012) Phylogenetic lineages of *Monopterus albus* (Synbranchiformes: Synbranchidae) in China inferred from mitochondrial control region. Journal of Zoological Sistematics and Evolutionary Research 51 (1): 38-44.

Collins T, Trexler J, Nico L, Rawlings T (2002) Genetic diversity in a morphologically conservative invasive taxon: multiple introductions of swamps eels to the southeastern United States. Conservation Biology 16: 1024-1035.

Darling J, Mahon A (2011) From molecules to management: Adopting DNA based methods for monitoring biological invasions in aquatic environments. Environmental Research 111:978-988.

Drummond A (2010) Geneious version 4.8.5 available from http://www.geneious.com

Foote A, Thomsen P, Sveegaard S, Wahlberg M, Kielgast J, Kyhn L, Salling A, Galatius A, Orlando L, Thomas M, Gilbert T (2012) Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLoS one 7(8):e4178.

Freshwater D, Hines A, Parham S, Wilbur A, Sabaoun M, Woodhead J, Whitfield P, Paris C. (2009) Mitochondrial control region sequence analyses indicate dispersal from the US East Coast as the source of the invasive Indo-Pacific lionfish *Pterois volitans* in the Bahamas. Marine Biology 156(6): 1213-1221.

Green S, Akins J, Malkovic A, Cote I (2012) Invasive lionfish drive Atlantic coral reef declines. PLoS ONE 7(3): e32596

Herod J, Sinclair T, Brown A, Jackson A, Galvez J, Díaz-Ferguson E, Moyer G, Pelizza S (2013) Development of biometric and environmental DNA standardized protocols for early detection and population assessment of aquatic invasive species for Arthur R. Marshal Loxahatchee NWR. USFWS Project Report. 76p

Hessen D, Tranvik L (1998) Humic acid substances: ecology and biochemistry. Berlin, Springer. ISBN 3-540-63910.

Kline J, Loftus W, Kotun K, Trexler J, Rehage J, Lorenz J, Robinson M (2012) Recent fish introductions into Everglades National Park: An unforeseen consequence of water management? Wetlands DOI: 10.1007/s13157-012-0362-0.

Kocher T, Thomas W, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences USA 86:6196-6200.

Lodge D, Cameron T, Jerde C, Barnes M, Chadderton L, Egan S, Feder J, Mahon A, Pfrender M (2012) Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. Molecular Ecology 21:2555-2558.

Long J, LaFleur C (2011) Estimation of daily age and timing of hatching of exotic swamp eels *Monopterus albus* (Zuiew 1793) in a backwater marsh of the Chatahoochee River, Georgia USA. Journal of Applied Ichthyology 27:1019-1022.

Matheson C, Gurney C, Esau N, Lehto R (2010) Assessing PCR inhibition from humic substances. The open enzyme inhibition journal 3: 38-45.

Matsumoto S, Nagai S, Imanishi S, Hasuike H, Mosonori (1998) An expanding distribution of a swamp-eel *Monopterus albus* (Teleostei: Synbranchidae) in Nara Prefecture. Japanese Journal of Ecology 48:107-116 (in Japanese with English abstract).

Moyer G and Díaz-Ferguson E (2013) Development of environmental DNA methodology for early detection of aquatic invasive species in Loxahatchee National Wildlife Refuge. In Herod J et al (2013) Development of biometric and environmental DNA standardized protocols for early detection and population assessment of aquatic invasive species for Arthur R. Marshal Loxahatchee NWR. USFWS Project Report. 76p

Nico L, Beamish W, Musikasinthorn P (2007) Discovery of the invasive Mayan Cichlid Fish "*Cichlasoma*" *urophthalmus* (Gunther 1962) in Thailand, with comments in other introductions and other potential impacts. Aquatic Invasions 2(3): 197-214.

Nico L, Sharp P, Collins T (2011) Imported Asian swamp eels (Synbranchidae: *Monopterus*) in North American live food markets: Potential vectors of non-native parasites. Aquatic Invasions Vol 6 (1):69-76.

Palumbi S, Martin A, Romano S, McMillian W, Stine L, Grabowski G (1991) The simple fool guide to PCR, v.2.0. Honolulu: Department Zoology, Kewalo Marine Laboratory, University of Hawaii.

Sparks L, Smith W (2004) Phylogeny and biogeography of cichlid fishes (Teleostei: Perciformes). Cladistic 20: 501-517.

Thomsen P, Kielgast J, Lonsmann L, Moller P, Rassmusen M, Willerslev E (2012) Detection of diverse marine fish fauna using environmental DNA from seawater samples. PLoS ONE 7(8): e41732.

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of fish biomass using environmental DNA. PLoS ONE 7(4): e35868.

Takahara T, Minamoto T, Doi H (2013) Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLoS ONE 8(2): e56584.

Valdez-Moreno M, Ivanova N, Elias-Gutierrez M, Contreras-Balderas S, Hebert P (2009) Probing diversity in freshwater fishes from Mexico and Guatemala. Journal of Fish Biology 74:377-402.

Ward R, Zemlak T, Innes B, Last P, Hebert P (2005) DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B 360:1847-1857.

TABLES AND FIGURES

Table 1. PCR, sequencing and Taqman qPCR primers/probes used to amplify mtDNA amplicons of the target taxa. Probes can be differentiated from primers by the presence of a fluorescent label Fam at the 5' extreme of the sequence and a quencher –TAMRA at the 3' extreme.

Taxon	Name	Sequence (5`-3`)	Citation
Cichlasoma urophthalmum	FCOICUq	5' -ACTGCTCCCCCCCTCATTC- 3'	
	RCOICUq	5' –GTTCCAGCACCGGCTTCA- 3'	
	PCOICU	5'-Fam-TGCTCCTCCTCGCTTCCTCAGGTG- 3'TAMRA	
	FISHR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	Ward et al. 1995
Monopterus albus			
	FqSEGA	5'-AACTGTCTCCTCATTAAAGT-3'	
	F16SSEq	5'-CGAGGGCTTAACTGTCTCCTCAT- 3'	
	R16SSEq	5'-GGGTCTTCTCGTCTTATGGTGTTATC-3'	
	P16SSE	5'-Fam-AATAAAATTGATCTCCCCGTGCAGAAGCG-3'TAMRA	
	16SBr	5'-CCGGTCTGAACTCAGATCACGT-3'	Kocher et al. 1989
Pterois volitans			
	FDLLFq	5'-TCATCGACGCTTGCATAAGTT -3'	
	RDLLFq	5'-AAGGAACCAGATGCCCGATG -3'	
	PDLLF	5'-Fam-AGCGGGAGCAACCCCCATGCCGAGCGTT -3'TAMRA	
	LionB-L	5'-CATATCAATATGATCTCAGTAC-3'	Freshwater et al. 2009

Table 2. qPCR amplicon region and size in base pairs for the studied target taxa

SPECIES	SEQUENCE	AMPLICON SIZE	% OF SIMILARITY WITH GENEBANK SEQUENCES
Cichlasoma urophthalmum	ACTGCTCCCCCCCTCATTCCTGCTCCTCCTCGCTTCCTCAGGTGTTGAAGCCGG TGCTGGAAC	64bp	100% *Cichlasoma urophthalmum*
Monopterus albus (Tampa & Homestead Miami clades)	CGAGGGCTTAACTGTCTCCTCATTAAAGTCAATAAAATTGATCTCCCCGTGCA GAAGCGGGGATAACACCATAAGACGAGAAGACCC	87bp	100% *Monopterus albus*
Monopterus albus (Georgia clade)	AACTGTCTCCTCATTAAAGTCAATAAAATTGATCTCCCCGTGCAGAAGCGGGG ATAACACCATAAGACGAGAAGACCC	78bp	100% *Monopterus albus*
Pterois volitans	TCATCGACGCTTGCATAAGTTAATGGTGGAAAACATAAGCGGGAGCAACCCC CATGCCGAGCGTTCTTTCCATCGGGCATCTGGTTC	90bp	100% *Pterois volitans*

Table 3. PCR inhibition experiments summary of results. Testing the efficiency of the Power Water DNA Isolation kit vs the Power Water DNA Isolation kit in removing inhibitors from samples. Treatment 1 (no inhibitors were added to water samples). Treatment 2 (inhibitors added to water samples).

Treatment	*Kit*	*DNA [ng/ul]*	*OD ratio*	qPCR detection
1 pond water	Rapid Water	40.1	1.90	+
1 pond water		32.3	1.83	+
1 dist water		27.5	1.75	+
1 pond water	Power Water	28.7	1.93	+
1 pond water		30.5	2.01	+
1 dist water		40.0	2.00	+
2 pond water	Rapid Water	16.3	1.80	-
2 pond water		28.2	1.74	-
2 dist water		20.0	1.68	-
2 pond water	Power Water	11.5	1.75	+
2 pond water		15.4	1.80	+
2 dist water		11.9	1.50	+

Note: treatment 1 (1L Clean water + lyophilized tissue target species, no inhibitors), treatment 2 (Pond water + lyophilized tissue, inhibitors added to water)

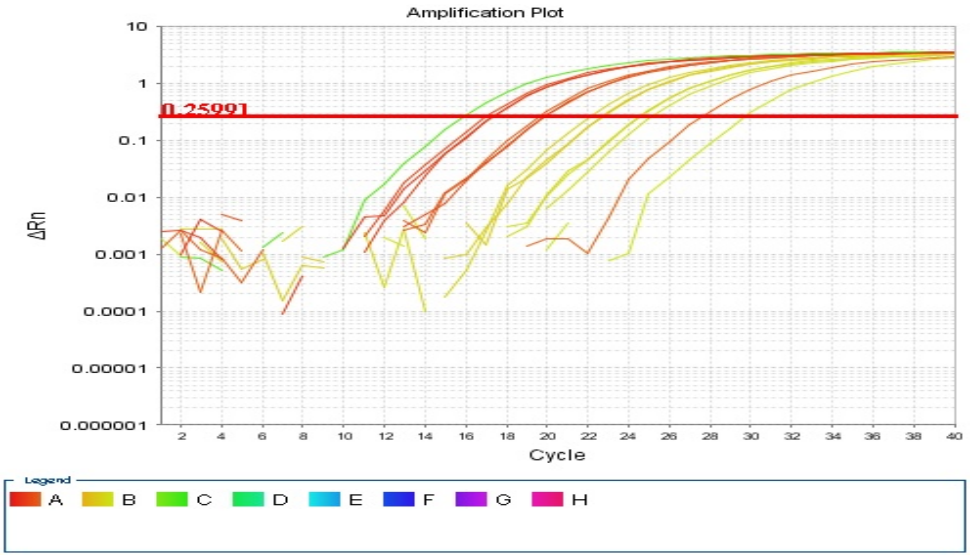

Figure 1. Amplification plot for *Cichlasoma urophthalmum* using known concentrations of DNA extracted from tissue samples.

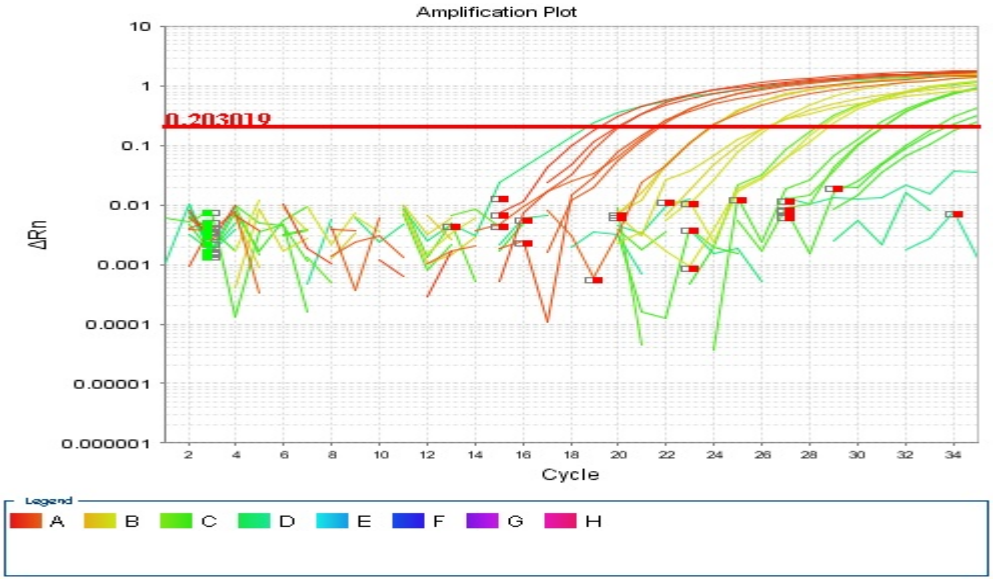

Figure 2. Amplification plot for *Monopterus albus* using known concentrations of DNA extracted from tissue samples.

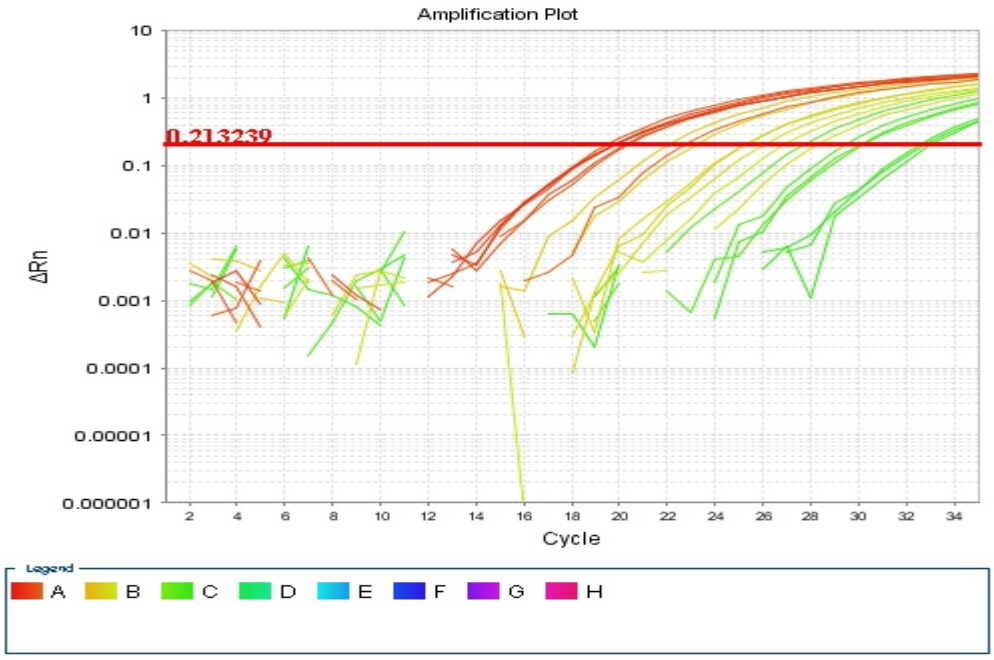

Figure 3. Amplification curve for *Pterois volitans* using known concentrations of DNA extracted from tissue samples.

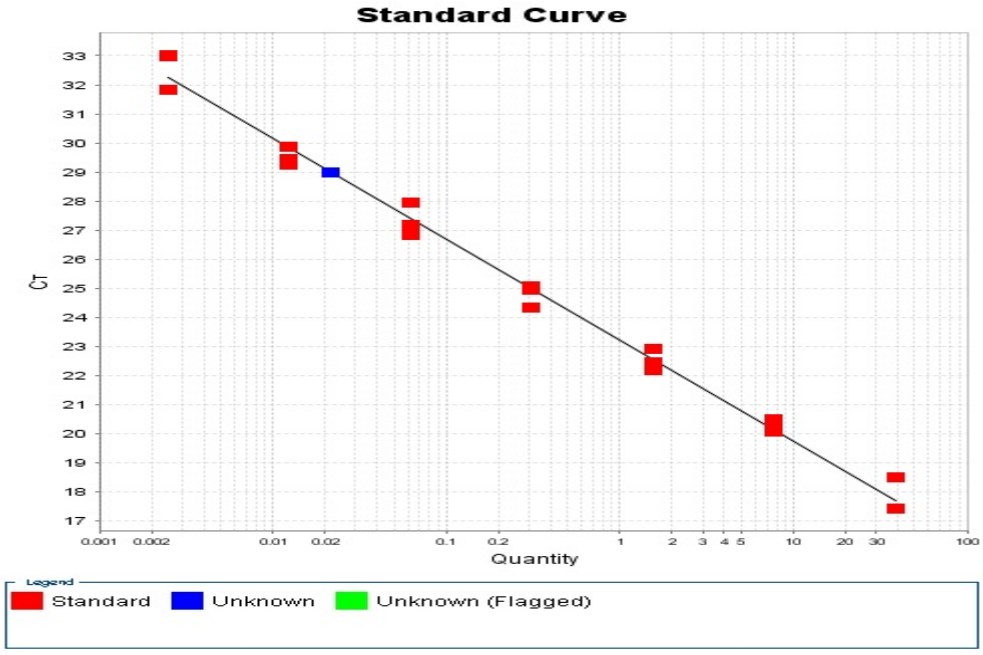

Figure 4. *Cichlasoma urophthalmum* standard curve using known concentrations of DNA extracted from tissue samples (R^2 = 0.992; lower limit C_T= 32; minimum amount detected 0.0024ng/ul).

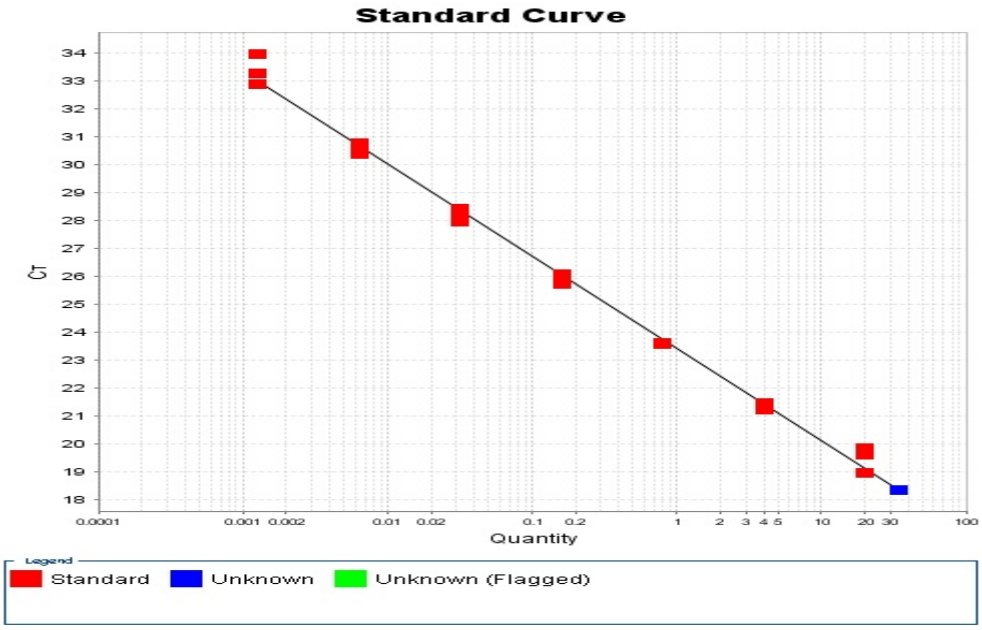

Figure 5. *Monopterus albus* standard curve using known concentrations of DNA extracted from tissue samples (R^2=0.995 ; lower limit C_T= 33 ; minimum amount detected 0.001ng/ul) .

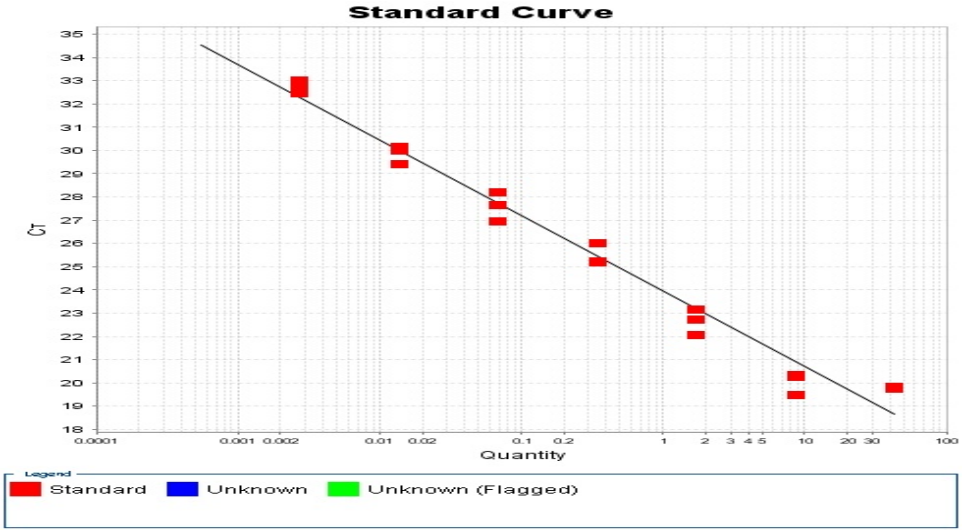

Figure 6. *Pterois volitans* standard curve using known concentrations of DNA extracted from tissue samples (R^2=0.90 ; lower limit C_T= 33 ; minimum amount detected 0.001ng/ul) .

Appendix 1

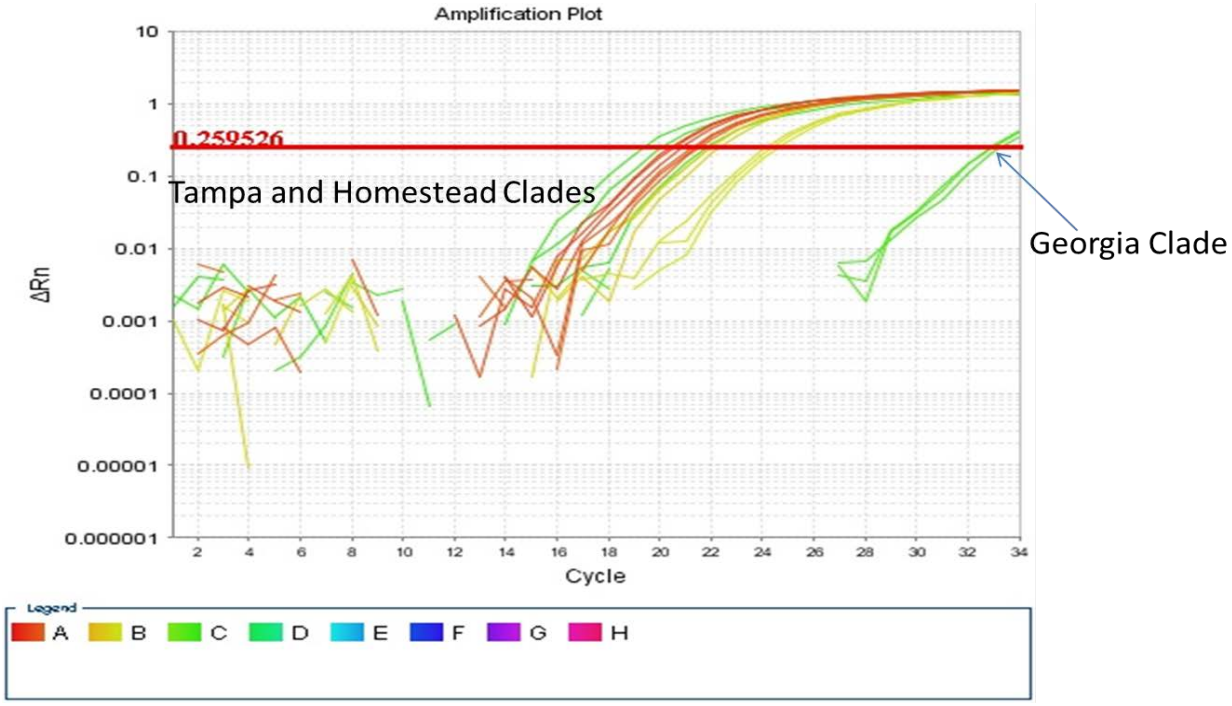

Figure 1. Amplification curve of the three clades of swam eels present in the United States using the F16SSEq forward primer. Georgia Clade samples showed higher C_T values or no amplification in comparison to Tampa and Homestead samples while using this primer. Higher C_T values observed for GA samples, using the same amount of DNA template and qPCR conditions indicate less specificity of F16SSEq marker to Georgia samples.

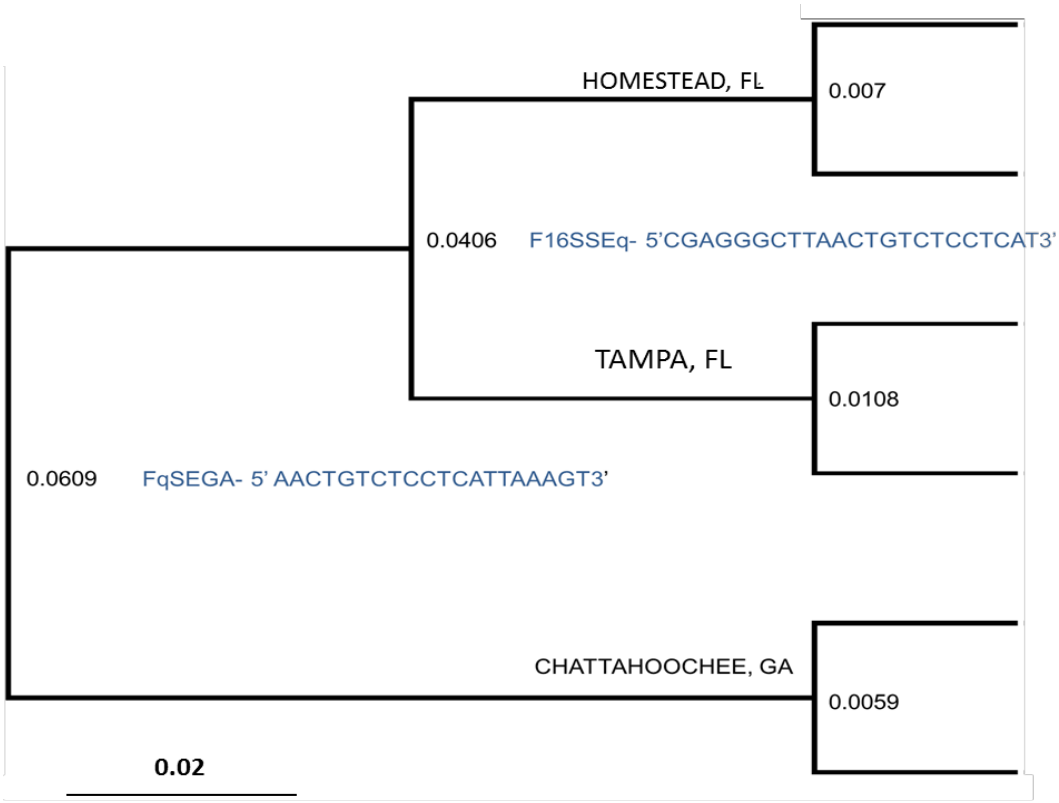

Figure 2. UPGMA dendrogram generated by the software GENEIOUS 4.5. comparing different laboratory sequences obtained from swamp eels collected in Tampa (FL), Homestead (FL) and Chattahoochee River, Georgia. Separation between branches is based on pairwise distances (values in the branches) between sequence data obtained from the three different locations. Position of the primer sequence in the dendrogram illustrate that FqSEGA sequence is sensitive for all clades while primer sequence F16SSEq is more specific for Tampa and Homestead samples.