Development of a tetraplex digital PCR (dPCR) assay for the detection of invasive snake species in Florida, U.S.A.

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June 22, 2024

Abstract

Florida, U.S.A. is a hotspot of biological invasions with over 500 non-native species reported. Reptiles encompass the majority of non-native wildlife with over 50 species established, many of which are sympatric and are identified as invasive due to their impacts to the environment, economy, and human health and safety. Reports of new non-native reptiles occur and many established non-native reptiles continue to expand their ranges in Florida, increasing the need for multi-taxa detection and monitoring capabilities. Invasive constrictor snakes are a primary focus of management efforts due to life history traits that favor successful establishment and dispersal in Florida as well as their impacts to native wildlife and Everglades restoration efforts. While traditional survey methods that rely on visual detections fail to reliably detect invasive constrictors, environmental DNA (eDNA) has proven to be a promising method for detection of cryptic and rare species across the landscape. To address emerging needs for multi-species detection and monitoring in Florida we developed the first tetraplex dPCR assay designed for detection of four species of invasive constrictor snakes, including Burmese pythons (Python bivittatus), northern African pythons (P. sebae), boa constrictors (Boa constrictor), and rainbow boas (Epicrates cenchria). In this tetraplex assay, no cross-amplification across species was documented. This assay serves as a valuable tool for faster and more accurate monitoring efforts of these invasive species in south Florida.

Introduction

Florida is a hotspot of biological invasions, particularly for reptiles, due to a subtropical climate, peninsular geography, disturbed habitats, thriving exotic pet trade, and multiple major ports of entry to the U.S (Engeman et al. 2011). At least 54 species of non-native reptiles are established (i.e., breeding) in Florida, including 27% of all established non-native reptile species known to occur globally (Capinha et al. 2017). Since establishment, many of these invasive reptiles have increased in abundance and expanded their range, with numerous species becoming sympatric. Among the most concerning are invasive large constrictor snakes, including the Burmese python (*Python bivittatus*), northern African python (*P. sebae*), and boa constrictor (*Boa constrictor*) (Figure 1) due to their large size, high reproductive rate, long life span, and ability to disperse long distances (Guzy et al. 2023). In addition, evidence supports the probable establishment of a fourth species of non-native constrictor snake, the rainbow boa (*Epicrates cenchria*) (Figure 1), of which all size classes, including a gravid female, have been captured in the wild (MAM, pers. comm.; EDDMapS 2024). Due to known and potential impacts to native wildlife in Florida, these constrictors are considered priority species by environmental agencies (i.e., Florida Fish and Wildlife Conservation Commission and South Florida Water Management District) targeted for Early Detection and Rapid Response (EDRR) efforts, containment, or long-term management depending on the area invaded and the time since introduction (Florida Python Control Plan 2021).

Documented impacts are known primarily for Burmese pythons, the most studied of the invasive constrictor species in Florida and include severe reductions in populations of mammalian prey species (Dorcas et al. 2012;

McCleery et al. 2015), co-introduction of invasive parasites that have spread to infect native wildlife (Miller et al. 2018; Miller et al. 2020), and increased prevalence of zoonotic viruses (Hoyer et al. 2017). Impacts of other invasive constrictors may yield comparable results once fully examined. Of particular concern is how these invaders may affect Everglades restoration efforts, a multi-billion-dollar initiative to restore the quantity, quality, timing, and distribution of water for the benefit and protection of people, habitats, and wildlife (South Florida Ecosystem Restoration Task Force 2022). To address this issue, the Invasive Exotic Species Strategic Action Framework (South Florida Ecosystem Restoration Task Force 2020) was initiated to combat the impacts of invasive species, including constrictor snakes, on these efforts as the establishment of invasive species directly threaten restoration goals.

Substantial resources have been provided to support control efforts of invasive constrictors in Florida with 10.6 million dollars spent by federal and state agencies, and one non-governmental organization during 2004 – 2021 (2021 Florida Python Control Plan) for removal of Burmese pythons, the most abundant and widespread of these invasive snakes (EDDMapS 2024). Despite these unprecedented resources, python removal remains challenging due to their cryptic appearance and propensity for secretive behaviors and large periods of inactivity, which belies our ability to easily detect and remove this species (Guzy et al. 2023, Nafus et al. 2019). The detection probability of Burmese pythons using data from visual encounter surveys and radio telemetry, is estimated to be less than 5% (Nafus et al. 2019) and detection likely decreases with increasing habitat complexity. In addition to low detection, Burmese pythons inhabit vast remote interior locations within the Everglades with dense vegetation and seasonal flooding, often requiring specialized equipment for human access, which can further limit the utility of visual detection methods due to logistics or lack of resources to maintain sustained monitoring efforts. A delay in detection reduces the effectiveness of rapid response and removal efforts, decreasing the likelihood of successful eradication and increasing the probability an introduced species will become established and require long-term control. Therefore, development of methods to increase detection of invasive snakes are paramount for the success of subsequent eradication and control efforts (Hunter et al. 2015).

Environmental DNA (eDNA) sampling can aid in detection of invasive wildlife, improve early detection, inform occupancy modeling, help determine invasion fronts, and provide managers a method to monitor and assess eradication efforts (Hunter et al. 2015, Hunter et al. 2019, Orzechowski et al. 2019, Morisette et al. 2021, Keller et al. 2022; Carim et al. 2019). This method has proven to be a viable tool for increasing detection of cryptic invasive species, including Burmese pythons (Hunter et al. 2015, Orzechowski et al. 2019, Hunter et al. 2019). Using eDNA assays and occupancy modeling, Hunter et al. (2015) estimated the detection probability of pythons to be greater than 91%, demonstrating the utility of eDNA for python detection compared to the less than 5% detection probability of pythons using visual encounter surveys (Nefas et al. 2019). However, despite advantages for increasing detection, particularly of secretive or rare species, eDNA has yet to be widely deployed as a detection tool for invasive species management. A contributing factor may result from a lack of confidence in eDNA detections due to the potential for false positives and false negatives, which can confound management decisions. For example, a positive eDNA detection of an invasive species that cannot be verified by other means of detection may trigger a costly response that may not be warranted (Jerde 2019). The potential for false-negative detections may increase when environmental inhibitors, preventing detection of DNA, are present or when samples contain trace amounts of DNA that may be undetected by the eDNA assay utilized.

Recent advances in digital PCR (dPCR) assays show promise for addressing many of these issues due to increased specificity and accuracy while allowing for multiplex testing for detection of multiple target species within a sample (Gaňová, et al. 2021). Multiplex eDNA assays can be conducted rapidly and efficiently for detection of up to six target species simultaneously with reduced resources compared to traditional eDNA assay methodologies (Gaňová et al. 2021). Development of multiplex dPCR assays is an emerging need to meet demands for early detection and monitoring of multiple target species in systems with multiple invasion events, such as South Florida. Additionally, a streamlined, automated workflow has potential to reduce resources required for implementing multiplex dPCR as a detection tool for large scale application across the landscape, which may increase the accessibility of eDNA for use by natural resource managers for

implementing monitoring programs.

Towards this goal, we developed a robust multiplex assay for rapid, accurate, and cost-effective detection of environmental DNA of multiple target species within a sample that meet the needs of natural resource managers. Specifically, our multiplex dPCR assay is designed to detect invasive constrictor snakes (Burmese python, boa constrictor, northern African python, and rainbow boa) in Florida. We provide a detailed account of assay development and discuss potential advantages of this methodology for increasing effectiveness of invasive species monitoring programs in multi-invaded systems.

Materials and Methods

Animal Tissue Source and

DNA Extraction

Animal tissues were harvested from specimens (Northern African python, Burmese python, boa constrictor, and rainbow boa) previously collected during research projects conducted by the University of Florida (UF IACUC protocol number 202200000027; FWC EXOT-23-63a; FWC EXOT-23-83) or from specimens donated by the Florida Fish and Wildlife Conservation Commission acquired through invasive species removal efforts.

Muscle tissue (100 mg) was excised from each specimen and transferred to a 1.5 ml microcentrifuge tube. Total DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions with slight modifications. In the initial lysis step, tissue was not macerated, it was left intact and allowed to lyse over a 24-hour period at 56 °C. Final eluate had DNA concentration and purity measured using a NanoDropLite spectrophotometer (ThermoFisher) and was diluted to 25 ng/µl for running PCR assays.

PCR Assay Design

The gene and region selected for assay design was the cytochrome*c* oxidase subunit I (COI) barcoding region (5'-half) due to high levels of variability among species (and even among populations; Kundu et al. 2020, Liu et al. 2020) allowing for easy development of species-specific assays without cross-amplification. Template obtained from *B. constrictor* , *E. cenchria* , *P. sebae* , and*P. bivittatus* , was screened in the initial assay using the universal COI primers LCO1490 (forward) and HCO2198 (reverse) from Folmer et al. (1994). Reactions were run in volumes of 25 µl and were comprised of 5X GoTaq Flexi Buffer (Promega, Madison, Wisconsin, USA), 25 mM MgCl2, 200 µM dNTPs, 0.5 µM of forward and reverse primer, 2% PVP-40, 1 U GoTaq Flexi DNA polymerase (Promega, Madison, Wisconsin, USA), and 2 µl of DNA template with sterile water to increase the final reaction volume to 25 µl. Utility of these primers in snakes was previously unknown so insect DNA extract using the same protocol as that for snakes from the planthopper *Pelitropis rotulata* was used as a positive control and molecular grade water was used as a non-template control. Thermal cycling conditions were as follows; initial denaturation at 95 °C for 2 min. followed by 35 cycles of denaturation at 95 °C for 30 sec., annealing for 30 sec. and extension at 72 °C, followed by a final extension at 72 °C for 5 min (Table 1). Products obtained from PCR reactions were run on 1.5% agarose gel stained with GelRed (Biotium) and amplicons of the correct size, relative to the positive control, were sent for Sanger sequencing (Eurofins Genomics, Louisville, Kentucky, U.S.A.).

Sequence data was assembled using DNA Baser (Version 4.36) (Heracle BioSoft SRL, Pitesti, Romania) and aligned using Clustal*W* as part of the MEGA7 package (Kumar et al. 2016). Sections of 100 bps displaying variability among the four species included in this study were selected and uploaded to OligoArchitect™ Online (Sigma-Aldrich) using the "Dual-Labeled Probe" tab. Each resulting assay was subsequently purchased as a TaqMan® MGB (minor grove binder) probe with a 5' FAM label and 3' nonfluorescent quencher (NFQ) for optimization along with corresponding primers. Each assay was screened against its corresponding snake species with species-specific primers only by standard PCR using a gradient to establish optimal annealing temperatures for the assay. For the gradient PCR, reactions were performed using the same concentrations as the initial PCR assays listed above with a gradient of 50 °C to 60 °C. Each resultant assay was labeled according to abbreviated common names of the snake species for ease of labeling and presentation;

B. constrictor specific assay = BC, *E. cenchria* specific assay = RB, *P. sebae* specific assay = NAP and *P. bivittatus* specific assay = BP.

qPCR and dPCR optimization

All assays designed were subsequently screened against the original template used to generate sequences that resulted in the corresponding assays. Each assay specific to a snake species was screened in triplicate against template for the same snake species (representing positive controls) and also screened in triplicate against the other three snake species template (negative controls) to ensure no cross-amplification occurs. All assays were run on a QuantStudio 6 Flex qPCR system (Applied Biosystems by Thermo Fisher Scientific). Reactions were performed in volumes of 20 µl and comprised of 10 µl of TaqMan Universal Master Mix II with UNG, 10 µM for each oligonucleotide (forward primer, reverse primer, and probe), 10% polyvinylpyrrolidone (PVP-40), 1 µl of DNA template with sterile dH_2O added to reach final volume (20 µl). Thermal cycling conditions for qPCR assays were as follows; initial hold at 50 °C for 2 min, initial denaturation at 95 °C for 10 min. followed by 35 cycles of denaturation at 95 °C for 15 sec. and annealing/extension at 58 °C for 1 min.

Amplicons from the gradient PCR for each snake species were cloned using the pGEM-T Easy Vector kit (Promega) following the manufacturer's instructions. The cloned vectors were then transformed into NEB Turbo Competent *E. coli* (New England BioLabs) and plated on Lysogeny broth (LB) plates containing 100 mg/mL of Ampicillan. Plates were incubated overnight and transformed colonies were screened for the clones with correct inserts using M13F/M13R primers. Clones with an insert of the correct size were incubated at 37 °C overnight on a shaker with 250 rpm in 20 ml of LB broth containing 100 mg/ml of Ampicillan. Finally, plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen) per the manufacturer's instructions and sent for Sanger sequencing (Eurofins Genomics) to confirm identity of the inserts. Plasmid eluate was subsequently diluted to 10^7copies/µl followed by a serial dilution to 10^1copies/µl.

Serially diluted plasmids for NAP were subsequently run with corresponding assay on the QuantStudio Absolute Q Digital PCR System (dPCR) to establish optimal dilution concentration. In addition, eluate from the extraction protocol from raw tissue for NAP was diluted to 25 ng/µl, then subsequently serially diluted (10:1) three times and screened with the corresponding assay to determine optimal concentration for total DNA samples. Optimal concentration was determined only using NAP because the same target is being evaluated across taxa, so with standardized concentrations for both samples and plasmids, optimal concentrations can be determined and extrapolated to the other species.

Multiplex optimization

The multiplex assay was purchased directly from ThermoFisher. The 5'-ends of each species-specific probe were labeled accordingly; FAM-NAP, VIC-RB, ABY-BP and JUN-BC. All assays tagged on 3' end with MGB-NFQ quencher.

Plasmid standards and samples for each snake species with the optimal concentrations were screened using the multiplex assay. In addition, samples with optimal concentrations for each snake species were mixed in all possible combinations (C(n,r)=n!/r!(n-r!)) to reflect the possibility of an eDNA sample containing multiple targets.

All reactions were run in volumes of 9 µl and comprised of 1.8 µl Absolute Q Master Mix (5x), 0.45 µl dPCR assay (20x), 1 µl DNA template and the remaining volume made up with UltraPure water. Thermal cycling conditions were as follows; 10 min. initial denaturation at 95 °C followed by 25 cycles of denaturation for 15 sec. at 95 °C and annealing/extension for 1 min. at 58 °C. All reactions were run on the QuantStudio Absolute Q Digital PCR System (ThermoFisher Scientific).

Results

Assay design and qPCR optimization

The initial PCR reactions on all four snake species using the universal primer set resulted in positive amplification. For each species, a 621 bp product was obtained. Sequences for each species were deposited in GenBank; *B. constrictor* = PP556870, *E. cenchria* = PP556871, *P. sebae* = PP556869 and *P. bivittatus* = PP556868 (Table 1). Resulting primers and probes for each respective snake species are presented in Table 1 along with optimal annealing temperatures and times. All four assays were found to run optimally at 58 degC for 1 min based on the gradient PCR. When run by qPCR with the TaqMan probe included, all assays were successful in amplifying all three replicates of the target species without cross amplification of non-target species (Table 2). Therefore, the BC assay successfully amplified from all three replicates of the *B. constrictor* sample but failed to amplify the target for *E. cenchria* , *P. sebae*and *P. bivittatus* . The RB assay successfully amplified from all three replicates of the *E. cenchria* sample but failed to amplify the target for *B. constrictor* , *P. sebae* and *P. bivittatus* . The NAP assay successfully amplified from all replicates of the *P. sebae* sample but failed to amplify the target for *B. constrictor* , *E. cenchria* and *P. bivittatus* . Finally, the BP assay successfully amplified from all replicates of the *P. bivittatus* sample but failed to amplify the target for *B. constrictor* , *E. cenchria* and *P. sebae* .

dPCR assay validation and optimization for concentration levels and thresholds

Serially diluted plasmids for NAP successfully amplified at all concentrations using the FAM labeled probe. Digital PCR chips were fully saturated (100% positive wells) at concentrations of 10^7 copies/µl to 10^4 copies/µl. At the 10^3 copies/ µl concentration, approximately 69% of the wells displayed successful amplification, with 10% and 1% successful amplification observed for 10^2 and 10^1 copies/µl concentrations, respectively. The optimal concentration for the NAP plasmids was between 10^2 and 10^1 copies/µl, having 95% confidence intervals (CI) values 10% and below (Table 3).

Final eluate from the total DNA extractions of NAP also had successful amplification for all concentrations. At concentrations of total DNA of 25 ng/µl and 2.5 ng/µl, chips were fully saturated (100% of wells reading as positive) while concentrations of 0.25 ng/µl and 0.025 ng/µl total DNA yielded approximately 31% and 3% positive wells. The calculated copy number for concentrations of 0.25 ng/µl and 0.025 ng/µl were 901.56 (21.7 95% CI) and 74.16 (5.52 95% CI), respectively (Table 3).

Multiplex validation and optimization

The multiplex assay successfully amplified for all reported dyes/assays for the corresponding snake species with no cross-amplification across snake species detected (Figure 2). For the BC plasmid (10^2 copies/µl) and sample (0.25 ng/µl) with a florescence threshold of 128.615, there were 21.7% and 29.7% positive wells detected for the JUN-BC probe, with zero positive wells for the ABY-BP, FAM-NAP and VIC-RB probes in both the plasmid and sample tests. For the BP plasmid (10^2 copies/µl) and sample (0.25 ng/µl) with a florescence threshold of 655.377, there were 9.6% and 31.41% positive wells detected for the ABY-BP probe, with zero positive wells for the JUN-BC, FAM-NAP and VIC-RB probes in both the plasmid and sample tests. For the NAP plasmid (10^2 copies/µl) and sample (0.25 ng/µl) with a florescence threshold of 61.813, there were 18.1% and 33.26% positive wells detected for the FAM-NAP probe, with zero positive wells for the JUN-BC, ABY-BP and VIC-RB probes in both the plasmid and sample tests. For the RB plasmid (10^2copies/µl) and sample (0.025 ng/µl) with a florescence threshold of 453.251, there were 37.6% and 11.29% positive wells detected for the VIC-RB probe, with zero positive wells for the JUN-BC, ABY-BP and FAM-NAP probes in both the plasmid and sample tests (Table 4).

All samples with mixed template screened with the multiplex assay displayed positive/negative patterns that directly corresponded to which species were present in the sample (Figure 3). Generally, the difference in efficiency and copy number estimate did not significantly change among two-species, three-species, and four-species mixtures. At low concentrations, the addition of additional template did not influence the assay. The 0.025 ng/µl concentration for RB, while yielding approximately 11 copies/µl by itself, consistently yielded significantly higher estimates in mixtures with other species. In addition, mixtures with RB template did display a reduction in copy number estimate as the amount of template increased, dropping from approximately 140 copies/µl in two-species combinations to approximately 70 copies/µl when mixed with all

three other species.

Discussion

The development of a multiplex dPCR assay that allows for detection of four invasive species of snake in Florida is a critical advancement in the ability to analyze eDNA and will significantly strengthen early detection and monitoring efforts in the Florida Everglades. Previous studies have utilized dPCR for analyzing eDNA samples in Florida to monitor for pythons (Hunter et al. 2015, Orzechowski et al. 2019, Hunter et al. 2019). However, methods used were limited to detection of only one species (in that instance *P. bivittatus*). With the current Absolute Q dPCR system, used in this study, a total of four reporter dyes are available, allowing for the development of more robust multiplex assays and with the system being automated, the workflow for setting up, running and analyzing samples is significantly reduced. To the authors knowledge, this represents the first tetraplex dPCR assay for snakes. Other systems that have reported utility of this technology include salmonid pathogens (von Ammon et al. 2022), breast and ovarian cancer screening (Oscorbin et al. 2019), lung cancer screening (Oscorbin et al. 2022), and GMOs (genetically modified organisms) detection in food crops (Dobnik et al. 2016). The utilization of this technology in areas such as human medicine, especially with regard to cancer, and large-scale agricultural monitoring highlight the power of this technology and validate the need to adapt this tool to invasive species monitoring where critical ecosystems are at stake. While the tetraplex dPCR assay for invasive snakes represents a new, cutting-edge tool to improve monitoring efforts, the utility and value of tetraplex assays based on standard and quantitative PCR (non-dPCR) has been previously demonstrated and include food allergens (Köppel et al. 2010), eDNA of food animals (Safdar et al. 2014, Hossain et al. 2017, Kaltenbrunner et al. 2018), drug resistant fungus (Arastehfar et al. 2018), paternity cases (Seidl et al. 1996) and influenza viral types (Henritzi et al. 2019). The development of a reliable and time efficient tetraplex dPCR is a necessary tool for wildlife monitoring as the rate of habitat encroachment by humans in Florida is exponentially increasing with Florida having the highest net migration rate from July 2021 to July 2022 (U.S. Census Bureau, 2021).

Cross-amplification was not observed for any of the species analyzed in this study despite the close phylogenetic relationship among constrictor species examined. As invasive constrictor snakes in the families Pythonidae and Boidae share more genetic similarity compared to other families (Colubridae, Elapidae, and Viperidae) of native snakes found within Florida (Burbrink et al. 2020), we do not anticipate issues of cross-amplification with native snakes in our assay should DNA of native snakes be collected inadvertently in environmental samples. Previous dPCR assays, while designed to avoid cross-amplification, were not evaluated against closely related taxa.

Numerous factors are responsible for increased sensitivity and reliability of dPCR results over other protocols (standard PCR and quantitative PCR). The physical partitioning of a sample over approximately 20,000 wells functionally allow for replicating a sample 20,000 times where detection of single target (gene representing a target species) is possible. Additionally, the same partitioning of a sample also significantly reduces impact of environmental inhibitors (substances that can inhibit PCR amplification, and hence detection of eDNA) due to significant reduction/elimination of inhibitor concentration per reaction well. This degree of sensitivity allows for detection of trace amounts of DNA in the environment (either terrestrial or aquatic), which can provide an effective means for early detection monitoring efforts of target invasive species. This may be particularly useful for detection of species that exist at low population densities or when only one individual of a non-native species is reported.

With the capability of multiplex testing, researchers and natural resource managers can reliably survey for multiple target species of interest simultaneously from an individual sample. This can reduce resources required for bio-surveillance programs as well as increase the efficiency of monitoring programs in areas where numerous invasive species are present, which can increase the ability to initiate an effective rapid response effort. Additionally, multiplex dPCR testing can be developed for any combination of target species, fitting the real time needs for detection of invasive species across landscapes with multiple invasions. For instance, the range of Burmese pythons encompasses the distribution of northern African pythons, boa constrictors, and rainbow boas in Miami-Dade County, FL (Figure 4) and having this tetraplex dPCR

assay will allow for a more time and cost-effective means to evaluate overlapping ranges and how (and if) these overlaps change over time. The capacity to test for multiple target species can also facilitate rapid and accurate confirmation of a species during EDRR efforts when reported sightings of non-natives are ambiguous concerning species identification. Accurate species identification can aid managers in decisions regarding the appropriate response, or depending on the situation, whether to respond (i.e., a large constrictor is reported but the species identification is confirmed to be a Burmese python occurring in an area known to be heavily infested with pythons), which can inform prioritization of resources.

In addition to early detection efforts, multiplex dPCR testing may be an effective way to assess the outcome of removal efforts. For example, when post-removal surveys for an invasive species are desired to demonstrate a decrease in occupancy or repeated zero detections (maximum control; Godfrey et al. 2023), use of dPCR can provide a reliable surveillance method for target species of interest that is accurate, rapid, and cost effective relative to traditional visual survey methods. This can be especially useful when species of interest for detection are cryptic or rare (i.e., snakes, crocodylians), and otherwise hard to detect using other methodologies. When management outcomes of interest are not based on repeated zero detections of a target species, but on the response of a native species impacted by an invader, multiplex testing may provide an additional advantage. For example, multiplex dPCR assays could be developed to assess the impact of python removal efforts on python occupancy across space and time, while simultaneously monitoring occupancy rates of mammals (i.e., opossums, racoons, and marsh rabbits) known to be impacted by pythons through predation (Dorcas et al. 2012; McCleery et al. 2015). An increase in occupancy of impacted native mammals over space and time may be used as an ecological proxy indicative of ecosystem recovery.

While utilizing multiplex dPCR assays for early detection and monitoring has advantages, these benefits are only advantageous if they are accessible to natural resource managers. As the number of invasive species continues to rise, the need for monitoring programs targeting multiple species increases. Use of dPCR assays can increase the efficacy of eDNA monitoring programs through simultaneous monitoring of multiple target species. The dPCR assay developed herein for detection of four invasive constrictor species increases the capacity for rapid and accurate detection of insipient populations and range expansions as well as long-term monitoring for assessment of control efforts. Data gained from monitoring programs can aid natural resource managers in informed decision-making concerning an appropriate response as well as allow managers to prioritize resources relative to the situation. For example, a manger may prioritize resources to respond to detection of target invasive species near biologically sensitive areas. Additionally, with the ability to detect multiple target species in a sample and a reduced workflow for assays, multiplex dPCR may be a more cost-effective means for implementing eDNA monitoring programs.

This study documents successful development of a novel multiplex assay designed to detect eDNA of four species of invasive constrictors rapidly and accurately. Future work will assess multiplex testing under semi-natural conditions with known presence of target species to validate results obtained in this study in the field, which will facilitate development of a monitoring program for invasive constrictors in Florida. As Florida is a hotspot of biological invasions, additional multiplex assays targeting other established invasive taxa (i.e., lizards, fish, and crocodylians) negatively impacting native ecosystems, as well as non-native species with a high risk of adverse effects if they become established, are needed and should be a focal point of future studies to facilitate multi-species eDNA monitoring programs.

Acknowledgements

We thank current and former members of the Croc Docs research team for their help collect the specimens used in this study. We thank the South Florida Water Management District and the UF/IFAS Invasion Science Research Institute for providing funding for this project. Funding information: South Florida Water Management District Agreement 4600004856.

References

Arastehfar, A., Fang, W., Badali, H., Vaezi, A., Jiang, W., Liao, W., Pan, W. and Hagen, F. 2018. Low-cost tetraplex PCR for the global spreading multi-drug resistant fungus, *Candida auris* and its phylogenetic

relatives. *Frontiers in Microbiology* , 9, p.366803.

Burbrink, F.T., Grazziotin, F.G., Pyron, R.A., Cundall, D., Donnellan, S., Irish, F., Keogh, J.S., Kraus, F., Murphy, R.W., Noonan, B. and Raxworthy, C.J., 2020. Interrogating genomic-scale data for Squamata (lizards, snakes, and amphisbaenians) shows no support for key traditional morphological relationships. *Systematic Biology* , 69(3), pp.502-520.

Capinha, C., Seebens, H., Cassey, P., García-Diaz, P., Lenzner, B., Mang, T., Moser, D., Pyšek, P., Rödder, D., Scalera, R. and Winter, M., 2017. Diversity, biogeography and the global flows of alien amphibians and reptiles. *Diversity and Distributions* , 23(11), pp.1313-1322.

Carim, K.J., Caleb Dysthe, J., McLellan, H., Young, M.K., McKelvey, K.S. and Schwartz, M.K., 2019. Using environmental DNA sampling to monitor the invasion of non-native *Esox lucius* (northern pike) in the Columbia River basin, USA. *Environmental DNA* , 1(3), pp.215-226.

Dobnik, D., Štebih, D., Blejec, A., Morisset, D. and Žel, J. 2016. Multiplex quantification of four DNA targets in one reaction with Bio-Rad droplet digital PCR system for GMO detection. *Scientific Reports* , 6(1), p.35451.

Dorcas, M.E., Willson, J.D., Reed, R.N., Snow, R.W., Rochford, M.R., Miller, M.A., Meshaka Jr, W.E., Andreadis, P.T., Mazzotti, F.J., Romagosa, C.M. and Hart, K.M., 2012. Severe mammal declines coincide with proliferation of invasive Burmese pythons in Everglades National Park. *Proceedings of the National Academy of Sciences* , 109(7), pp.2418-2422.

Early Detection & Distribution Mapping System (EDDMapS)., 2024. The University of Georgia Center for Invasive Species and Ecosystem Health. Available online at http://www.eddmaps.org/; last accessed March 17, 2024.

Engeman, R., Jacobson, E., Avery, M.L. and Meshaka Jr, W.E., 2011. The aggressive invasion of exotic reptiles in Florida with a focus on prominent species: A review. *Current zoology* , 57(5), pp.599-612.

Esri Inc. (2023). ArcGIS Pro (Version 3.2). Esri Inc. https://www.esri.com/en-us/arcgis/products/arcgis-pro/overview

Florida Python Control Plan. 2021. //flpythoninvasion.org/media/xpzkw2pl/fpcp_compliant.pdf

Last accessed March 17, 2024.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R., 1994. DNA primers for

amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology 3*, pp.294-299.

Gaňová, M., Zhang, H., Zhu, H., Korabečná, M. and Neužil, P., 2021. Multiplexed digital polymerase chain reaction as a powerful diagnostic tool. *Biosensors and Bioelectronics* , 181, p.113155.

Godfrey S.T., S.A. Balaguera-Reina, E.F. Metzger III, M.R. Rochford, M.A. Squires, E.V. Gati, A. Godahewa, F.J. Mazzotti. 2023. What is the Potential for Extirpating Spectacled Caiman from Comprehensive Everglades Restoration Plan Projects in South Florida? Management of biological invasions. 14(3): 403 – 419. 10. 3391/mbi.2023.14.3.02

Guzy J.C., Falk B.G., Smith B.J., Willson J.D., Reed R.N., Aumen N.G., Avery M.L., Bartoszek I.A., Campbell E., Cherkiss M.S., Claunch N.M., Currylow A.F., Dean T., Dixon J., Engeman R., Funck S., Gibble R., Hengstebeck K.C., Humphrey J.S., Hunter M.E., Josimovich J.M., Ketterlin J., Kirkland M., Mazzotti F.J., McCleery R., Miller M.A., McCollister M., Parker M.R., Pittman S.E., Rochford M., Romagosa C., Roybal A., Snow R.W., Spencer M.M., Waddle J.H., Yackel Adams A.A., Hart K.M. (2023) Burmese pythons in Florida: A synthesis of biology, impacts, and management tools. *NeoBiota,* 80,1–119. https://doi.org/10.3897/neobiota.80.90439

Henritzi, D., Hoffmann, B., Wacheck, S., Pesch, S., Herrler, G., Beer, M. and Harder, T.C. 2019. A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza and Other Respiratory Viruses* , 13(1), pp.71–82.

Hossain, M.M., Ali, M.E., Sultana, S., Asing, S.Q.B., Kader, M.A. and Rahman, M.A. 2017. Quantitative tetraplex real-time polymerase chain reaction assay with TaqMan probes discriminates cattle, buffalo, and porcine materials in food chain. *Journal of Agricultural and Food Chemistry* , 65(19), pp.3975–3985.

Hoyer, I.J., Blosser, E.M., Acevedo, C., Thompson, A.C., Reeves, L.E. and Burkett-Cadena, N.D., 2017. Mammal decline, linked to invasive Burmese python, shifts host use of vector mosquito towards reservoir hosts of a zoonotic disease. *Biology letters* , 13(10), p.20170353.

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T., Reed, R.N. and Hart, K.M., 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PloS one*, *10*(4), p.e0121655.

Hunter, M.E., Meigs-Friend, G., Ferrante, J.A., Smith, B.J. and Hart, K.M., 2019. Efficacy of eDNA as an early detection indicator for Burmese pythons in the ARM Loxahatchee National Wildlife Refuge in the greater Everglades ecosystem. *Ecological Indicators* , *102* , pp.617-622.

Jerde, C.L., 2021. Can we manage fisheries with the inherent uncertainty from eDNA?. *Journal of Fish Biology* , 98(2), pp.341-353

Kaltenbrunner, M., Hochegger, R. and Cichna-Markl, M., 2018. Tetraplex real-time PCR assay for the simultaneous identification and quantification of roe deer, red deer, fallow deer and sika deer for deer meat authentication. *Food Chemistry* , 269(10), pp.486–494.

Keller, A.G., Grason, E.W., McDonald, P.S., Ramón-Laca, A. and Kelly, R.P., 2022. Tracking an invasion front with environmental DNA.*Ecological Applications* , 32(4), p.e2561.

Koppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A. and Waiblinger, H.U., 2010. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *European Food and Research and Technology*, 230(3), pp.367–374.

Kumar, S., Stecher, G. and Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution* , 33(7), pp.1870-1874.

Kundu, S., Lalremsanga, H.T., Rahman, M.M., Ahsan, M.F., Biakzuala, L. Kumar, V., Chandra, K. and Siddiki, A.Z., 2020. DNA barcoding elucidates the population genetic diversity of venomous cobra species (Reptilia: Elapidae) in Indo-Bangladesh region. *Mitochondrial DNA Part B* , 5(3), pp.2525–2530.

Liu, B., Zhang, K., Zhu, K., Shafi, M., Gong, L., Jiang, L., Liu, L., Muhammad, F. and Lu, Z., 2020. Population genetics of *Konosirus punctatus* in Chinese coastal waters inferred from two mtDNA genes (COI and Cytb). *Frontiers in Marine Science* , 7, p.534.

McCleery, R.A., Sovie, A., Reed, R.N., Cunningham, M.W., Hunter, M.E. and Hart, K.M., 2015. Marsh rabbit mortalities tie pythons to the precipitous decline of mammals in the Everglades. *Proceedings of the Royal Society B: Biological Sciences* , 282(1805), p.20150120.

Miller, M.A., Kinsella, J.M., Snow, R.W., Hayes, M.M., Falk, B.G., Reed, R.N., Mazzotti, F.J., Guyer, C. and Romagosa, C.M., 2018. Parasite spillover: indirect effects of invasive Burmese pythons. *Ecology and Evolution* , 8(2), pp.830-840.

Miller, M.A., Kinsella, J.M., Snow, R.W., Falk, B.G., Reed, R.N., Goetz, S.M., Mazzotti, F.J., Guyer, C. and Romagosa, C.M., 2020. Highly competent native snake hosts extend the range of an introduced parasite beyond its invasive Burmese python host. *Ecosphere* , 11(6), p.e03153.

Morisette, Jeffrey, Stanley Burgiel, Kelsey Brantley, Wesley M. Daniel, John Darling, Jeanette Davis, Thomas Franklin et al. "Strategic considerations for invasive species managers in the utilization of environmental DNA (eDNA): steps for incorporating this powerful surveillance tool." *Management of biological invasions: international journal of applied research on biological invasions* 12, no. 3 (2021): 747.

Nafus, M.G., Mazzotti, F.J. and Reed, R.N., 2020. Estimating detection probability for Burmese pythons with few detections and zero recaptures.*Journal of Herpetology* , 54(1), pp.24-30.

Orzechowski, S.C., Frederick, P.C., Dorazio, R.M. and Hunter, M.E., 2019. Environmental DNA

sampling reveals high occupancy rates of invasive Burmese pythons at wading bird

breeding aggregations in the central Everglades. *Plos one* , 14(4), p.e0213943.

Oscorbin, I., Kechin, A., Boyarskikh, U. and Filipenko, M. 2019. Multiplex ddPCR assay for

screening copy number variations in BRCA1 gene. *Breast Cancer Research and*

Treatment , 178, pp.545-555.

Oscorbin, I.P., Smertina, M.A., Pronyaeva, K.A., Voskoboev, M.E., Boyarskikh, U.A., Kechin,

A.A., Demidova, I.A. and Filipenko, M.L. 2022. Multiplex droplet digital PCR assay for

detection of MET and HER2 genes amplification in non-small cell lung cancer. *Cancers* ,

14(6), p.1458.

Safdar, M., Junejo, Y., Arman, K. and Abasiyanik, M.F. 2014. A highly sensitive and specific tetraplex PCR assay for soybean, poultry, horse and pork species identification in sausages: development and validation.*Meat Science* , 98(2), pp.296–300.

Seidl, C., Jager, O. and Seifried, E. 1996. A tetraplex PCR system for the analysis of paternity cases. In *16th Congress of the International Society for Forensic Haemogenetics* ,*Santiago de Compostela* , pp.142–144.

Sepulveda, A.J., Hutchins, P.R., Forstchen, M., Mckeefry, M.N. and Swigris, A.M., 2020. The elephant in the lab (and field): Contamination in aquatic environmental DNA studies. *Frontiers in Ecology and Evolution* , 8, p.609973.

South Florida Ecosystem Restoration Task Force. 2022. Biennial Report. evergladesrestoration.gov. Last accessed March 24, 2024.

South Florida Ecosystem Restoration Task Force. 2020. Invasive Exotic Species Strategic Action Framework. https://www.evergladesrestoration.gov/report-indexquick-links. Last accessed March 24, 2024.

Von Ammon, U., Averink, T., Kumanan, K., Brosnahan, C.L., Pochon, X., Hutson, K.S and

Symonds, J.E. 2022. An efficient tetraplex surveillance tool for salmonid pathogens.

Frontiers in Microbiology , 13, p. 885585.

Figure Legend

Figure 1. Representative specimens of the species used to design the tetraplex digital PCR assay in this study; A) Burmese python (*Python bivittatus*), B) North African rock python (*Python sebae*), C) rainbow boa (*Epicrates cenchria*) and D) boa constrictor (*Boa constrictor*).

Figure 2. Chip images for the multiplex assay when screened against all four snake species (x-axis) with reporters/probes (y-axis) showing positive amplification for their corresponding species; red pixels = positive JUN florescence, yellow pixels = positive ABY florescence, green pixels = positive VIC florescence, blue pixels = positive FAM florescence, black pixels = no florescence/negative wells.

Figure 3. Absolute quantification of target DNA for each snake species when screened with tetraplex assay in mixed samples representing all possible combinations of multiple species present in a single sample.

Figure 4. Established populations of Burmese pythons (green), northern African pythons (blue), boa constrictors (purple), and rainbow boas (yellow) are shown for Miami-Dade County (blue outline) along with areas of range overlap among these species. Polygons representing each constrictor species were created using data available from EDDMapS (2024) using ArcGIS Pro, Version 3.2 and represent minimum bounding geometry.

Table 1. Primers and probes used for generating COI sequences and running qPCR and dPCR assays for optimization and developing the multiplex assay with corresponding critical PCR parameters.

Species	Oligo Name	Strand	Sequence (5' 3')	Annealing	Extension
Universal	LCO1490	Sense	GGTCAACAAATCATAAAGATATTGG	40 °C	1 min. 30
	HCO2198	Anti-sense	TAAACTTCAGGGTGACCAAAAAATCA	40 °C	1 min. 30
Boa constrictor (BC)	BC_COIF	Sense	CCTGCCTAAGCATCCTTA	58 °C	1 min.
	BC_COIR	Anti-sense	CTAGGACGTTGAAGATCTG	58 °C	1 min.
	BC_probe	Sense	CGAATGGAACTAACACAGCCCG	58 °C	1 min.
Epicrates cenchria (RB)	RB_COIF	Sense	TCACCACATGCATCAATA	58 °C	1 min.
	RB_COIR	Anti-sense	GCAGTGATTATAACAGATCAG	58 °C	1 min.
	RB_probe	Anti-sense	AACCAGCCTCAATACCAATATTTAACA	58 °C	1 min.
Python sebae (NAP)	NAP_COIF	Sense	TCCCACGAATAAATAACATAAG	58 °C	1 min.
	NAP_COIR	Anti-sense	CCAGCTTCTACGTATGAA	58 °C	1 min.
	NAP_probe	Sense	CGCTACTCCTCCTCCTGTCTT	58 °C	1 min.
Python bivittatus (BP)	BP_COIF	Sense	CCACTATCAGGCAATATG	58 °C	1 min.
	BP_COIR	Anti-sense	CAGCTAAGTGTAGTGAGA	58 °C	1 min.
	BP_probe	Sense	CCACTCAGGCCCATCAGTAGATC	58 °C	1 min.

Table 2. qPCR results for evaluating potential of cross-amplification across all four snake species for each species-specific assay; cycle threshold values (Ct) = average of triplicates, No Ct = no amplification.

	Assay	Assay	Assay	Assay
	Boa constrictor (BC)	*Epicrates cenchria* (RB)	*Python sebae* (NAP)	*Python bivittatus* (BP)
Species	Avg. Ct±SE	Avg. Ct±SE	Avg. Ct±SE	Avg. Ct±SE
Boa constrictor	19.29±0.04	No Ct	No Ct	No Ct
Epicrates cenchria	No Ct	18.50±0.44	No Ct	No Ct
Python sebae	No Ct	No Ct	18.58±0.09	No Ct
Python bivittatus	No Ct	No Ct	No Ct	16.30±0.11

Table 3. dPCR results for plasmid serial dilutions and sample dilutions for *Python sebae* (NAP) using FAM labeled probes.

Concentration	FAM-NAP	FAM-NAP	FAM-NAP	FAM-NAP
Plasmid	% (+) Wells	Total Wells	Conc. cp/µl	95% CI
10^7 copies/µl	100.00	20473	22978.85	4117.12
10^6 copies/µl	99.96	20450	17890.08	1449.88
10^5 copies/µl	99.98	20469	19769.38	2143.00
10^4 copies/µl	99.96	20446	18162.27	1535.00
10^3 copies/µl	68.80	20345	2696.42	46.83
10^2 copies/µl	9.62	20345	234.11	10.15
10^1 copies/µl	0.91	20427	21.06	2.83
Sample				
25 ng/µl	99.94	20455	17039.43	1212.64
2.5 ng/µl	99.99	19806	21297.67	2978.09
0.25 ng/µl	32.26	20317	901.56	21.70
0.025 ng/µl	3.15	20395	74.16	5.52

Table 4. dPCR results for the multiplex assay to validate function and verifying absence of cross amplification for the four snake species analyzed in this study based on optimal sample concentrations for plasmids and DNA extract. BC = Boa constrictor, BP = Burmese Python, NAP = North African python, RB = Rainbow Boa, CI = Confidence Intervals.

	Plasmid (10^2 copies/µl)	Plasmid (10^2 copies/µl)	Plasmid (10^2 copies/µl)	Plasmid (10^2 copies/µl
	JUN-BC	ABY-BP	FAM-NAP	VIC-RB
BC				
Positives Wells (%)	21.7	0.0	0.0	0.0
Total Wells	20364	20364	20364	20364
Conc. cp/µl	566.11	0.0	0.0	0.0
95% CI	16.49	0.0	0.0	0.0
BP				
Positives Wells (%)	0.0	9.6	0.0	0.0
Total Wells	20411	20411	20411	20411
Conc. cp/µl	0.0	233.69	0.0	0.0
95% CI	0.0	10.12	0.0	0.0
NAP				
Positives Wells (%)	0.0	0.0	18.1	0.0
Total Wells	20417	20417	20417	20417
Conc. cp/µl	0.0	0.0	463.38	0.0
95% CI	0.0	0.0	14.71	0.0
RB				
Positives Wells (%)	0.0	0.0	0.0	37.6
Total Wells	20324	20324	20324	20324
Conc. cp/µl	0.0	0.0	0.0	1091.35
95% CI	0.0	0.0	0.0	24.42