



Environmental DNA Monitoring of Invasive Central American Boas in St. Croix at Salt River Bay National Historical Park and Ecological Preserve (SARI)



Caitlin Beaver, U.S. Geological Survey, and Wilfre Fuentes Santos, USDA Wildlife Services, sampling for environmental DNA (eDNA) in the western forests of St. Croix, U.S. Virgin Islands.

USGS

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Caitlin E. Beaver ¹, Alexis B. Tays², Wilfre Fuentes Santos³, Madison E. A. Harman ²,
Kristen A. Ewen⁴, Evan L. Gwilliam⁴, Margaret E. Hunter ¹

¹ U.S. Geological Survey
Wetland and Aquatic Research Center
7920 NW 71st St.
Gainesville, FL 32653

² Cherokee Nation System Solutions
Contractor to the U.S. Geological Survey
7920 NW 71st St.
Gainesville, FL 32653

³ U.S. Department of Agriculture
Animal and Plant Health Inspection Service
9600 Two Notch Rd., Ste 10
Columbia, SC 29223

⁴ National Park Service
Christiansted NHS
2100 #100 Church St.
Christiansted, VI 00820

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Abstract

Invasive Central American boas (*Boa imperator*) have established a reproducing population on the western side of St. Croix, U.S. Virgin Islands but prevalence throughout the island is largely unknown. The large snakes threaten endemic and endangered species through competition and predation, jeopardizing island biodiversity. Environmental DNA (eDNA) methods were used to investigate occurrence and focal areas for management efforts in the Salt River Bay National Historical Park and Ecological Preserve (SARI). To validate a previously developed assay, we collected tissue samples and 13 × 60 mL water samples from a captive boa enclosure in St. Croix. We implemented this assay for both a pilot and main field sampling effort. The pilot in December 2023 resulted in analysis of 7 × 60 mL water samples per site from SARI (3 sites) and from the western forests where boas are established (1 site). The main sampling event in July 2024 collected 15 × 60 mL water samples per site within SARI (11 sites) and western forests (4 sites). Pilot sample replicates were filtered individually, while main samples were consolidated into groups, resulting in seven replicates for pilot sites and five replicates for main event sites, totaling 103 environmental samples. eDNA was isolated using a modified phenol-chloroform isolation method to remove PCR inhibitors, and target eDNA was amplified using droplet digital PCR technology. Water samples from the captive boa amplified target eDNA in 12 of 13 samples, indicating assay effectiveness ex-situ. Low concentrations of eDNA (below the 95% limit of detection) were amplified in 4 of 5 sites in the western forest and in 8 of 14 sites within the National Historic Park. Overall, boa eDNA concentrations were consistently low, as expected in water samples targeting a semi-arboreal snake species with a low rate of eDNA shedding. Further optimization of methods could enable recovery of greater eDNA concentrations in future studies. Additional eDNA method testing and ground-truthing may help to improve the assessment of invasive *Boa imperator* in St. Croix.

List of Acronyms

CI: Confidence interval

ddPCR: Droplet digital PCR

DNA: Deoxyribonucleic acid

eDNA: Environmental DNA

IPC: Internal positive control

IRK: OneStep PCR Inhibitor Removal Kit

LOB: Limit of blank

LOD: Limit of detection

LOQ: Limit of quantification

PCI: Phenol-chloroform-isoamyl alcohol

PCR: Polymerase chain reaction

Acknowledgments

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Introduction

The Caribbean region functions both as a source and a recipient of invasive species, a dynamic facilitated by its generally calm and stable environmental conditions (Powell et al. 2011; Treglia et al. 2013). The translocation of species across geographic boundaries is commonly driven by international shipping, the pet trade, and cross-border travel (Kraus 2009). Among the taxa frequently involved in such movements are constrictor snakes, which are often kept as pets and subsequently released into the wild. These species exhibit a high potential to become invasive, particularly in tropical and subtropical environments (Perring et al. 2015; Reed and Rodda 2009; Wiens and Hobbs 2015). The ecological and economic implications of such introductions are significant. For instance, the management of invasive giant constrictors in Florida has incurred expenditures over \$15 million in 2008, underscoring the substantial costs associated with the establishment of reproducing invasive populations (USFWS 2012).

Established populations of boas have been documented in Puerto Rico (Reynolds et al. 2013), Aruba (Bushar et al. 2015), and St. Croix (Angeli et al. 2019). In the Caribbean, these invasive boas have demonstrated significant ecological impact, driven by their generalist and opportunistic predatory behavior, high reproductive output, and broad habitat tolerance. These traits underscore their potential as a high-risk invasive species and highlight the importance of early intervention and eradication efforts (Bushar et al. 2015). Invasive boas have been observed preying upon a wide range of native species (Gilliland et al. 2023; Quick et al. 2005; Vázquez 2023). In Aruba, their presence has been associated with significant ecological changes and the decline of native species, with some evidence pointing to a potential risk of ecosystem collapse (Goessling et al. 2015).

Invasive boas were first reported on the island of St. Croix in 2012 (Angeli et al. 2019). The pet trade is considered the most probable source of introduction, as St. Croix is separated from nearby islands such as Puerto Rico by more than 80 km of deep ocean with strong currents, making natural, unaided dispersal highly unlikely (Angeli et al. 2019). Further, genetic analysis by Angeli et al. 2019 demonstrated that the St. Croix population matched boas in the Yucatán Peninsula of Mexico. This lineage is currently identified as the Central American boa, *Boa imperator* (Thorpe and Malhotra 2023), hereafter referred to as “boa” throughout this study. Since 2012, invasive boas have spread from the western forests of St. Croix, U.S. Virgin Islands and are estimated to be established across 28.2–62.4% of the island, as far east as the Salt River (Angeli et al. 2019; Gilliland et al. 2023). Conventional visual searching or trapping technologies have shown inadequate detection probability, which limits population distribution estimation capabilities (Reed et al. 2011). Current occurrence estimates and range delineations for boas throughout St. Croix rely on human searching and physical captures, but detection and removal of the species by these methods is inefficient due to their cryptic coloration and behavior. Alternative detection methods, such as detector dogs, were tested in St. Croix Salt River Bay National Historic Park and Ecological Preserve (SARI) but had limited success due to the challenging terrain and climate at the time of searching (Michaels 2024).

Large population size and persistent predation by boas could impact imperiled or at-risk native species on St. Croix. Native birds and reptiles have declined in other parts of the Caribbean where

boas have been introduced (Martínez-Morales and Cuarón 1999). Populations of native and endemic species, especially threatened and vulnerable species like zenaida doves (*Zenaida aurita*) or bridled quail-doves (*Geotrygon mystacea*), may suffer from sustained predation by these invasive constrictor snakes (Gilliland et al. 2023). St. Croix's biodiversity is distinctive since it is a landbank island with indigenous species and unique conservation difficulties (Pregill and Olson 1981). The need for early detection of invasive species to inform practical management is particularly urgent in the SARI region, an ecosystem of international and archeological significance (Capinha et al. 2017; Kraus 2015).

The SARI staff are actively restoring the east side of the park by planting native trees and removing invasive species. The park intends to use information from this inventory to help develop a boa removal program and increase the success of restoration. This information can also be used to protect sensitive native species like the ground-nesting least tern (*Sternula antillarum*), the St. Croix ground lizard (*Pholidoscelis polops*), and hawksbill sea turtle (*Eretmochelys imbricata*) nests. Increasing detection rates will be critical to observing and responding to new introductions or recently established populations of invasive boas on the island, as control and eradication of invasive species becomes more difficult and costly after the initial invasion stages (Darling and Mahon 2011; Kolar et al. 2010; Lodge et al. 2006). To complement ongoing survey efforts, we conducted environmental DNA (eDNA) surveillance as a sensitive, non-invasive detection method for boas.

Environmental DNA originates from cellular material shed by organisms (e.g., via skin, excrement, etc.) in water and soil and can be used for species identification (Dejean et al. 2011; Thomsen et al. 2012). Environmental DNA detection has been proven to assist with the early detection of novel invasive species and range delimitation or expansion for established species, as well as being time and cost-effective in several environments (Goldberg et al. 2011; Jerde et al. 2011; Mahon et al. 2013; Takahara et al. 2012). The use of eDNA monitoring coupled with the highly sensitive droplet digital PCR (ddPCR) platform has been employed to detect eDNA from a similar large, cryptic constrictor species, the invasive Burmese python, in southern Florida since 2013. These methods have identified a sustained python population in Loxahatchee National Wildlife Refuge since 2014 (Hunter et al. 2019a). Traditional visual searching or trapping tools proved inadequately low for this species (<1%), whereas eDNA proved more effective at detecting population level occurrence rates (90–100%; Hunter et al. 2015). This method has shown the potential to aid short- and long-term management decisions by estimating python occurrence, improving detection estimates, and identifying newly colonized areas.

While eDNA offers high sensitivity compared to many conventional survey methods, it presents challenges when applied to detecting ambush predator snakes, like pythons and boas. Reptiles tend to shed relatively low amounts of DNA, making their detection through eDNA methods more difficult (Hunter et al. 2015, 2019a). Most vertebrates, such as fish and mammals, shed skin cells and other DNA-containing materials more consistently as they move through their environment. For example, fish can release eDNA through sloughing of mucus, shedding of skin cells, and water passing through their gills, which can release cellular material. In contrast, reptiles shed their skin infrequently, primarily in full-body shedding events. Their lower metabolic rates and feeding behaviors further

reduce eDNA availability; they often consume large meals at extended intervals and may not defecate daily. Additionally, as sit-and-wait, or ambush-foraging reptiles, species like giant constrictor snakes tend to remain stationary for long periods, further limiting opportunities for DNA to enter the environment. These behavioral and physiological traits make sit-and-wait reptiles particularly challenging targets for eDNA monitoring and highlight the need for tailored approaches when studying these species.

Additional challenges for eDNA detection include the warm environments where giant constrictors live, with water containing numerous microbes that rapidly degrade eDNA. The environments can also contain high levels of PCR inhibitors, such as tannins and humic acid from mangroves, pine trees and soils, which can reduce the ability to detect eDNA or cause false negative results. Access to water for sampling can be limited in St. Croix due to tropical savanna and semi-arid conditions in lower elevations. During the dry season, ephemeral pools and puddles can be scarce and may concentrate inhibitors as they dry down.

The boas assessed here are classified as semi-arboreal and often spend time in trees. While they are found near water and are capable of swimming, they may not frequent the water, unlike the semi-aquatic pythons that have been studied extensively using eDNA methods with water samples. For this study, water was selected as the sample type due to the optimized filtration and eDNA extraction methods (Hunter et al. 2019a, 2019b). Furthermore, water can be rapidly filtered to concentrate eDNA from a larger volume, while an alternative sample type of soil can require extensive sampling and often produces lower detection estimates (López-González et al. 2025). This study system of low DNA shedding snakes that may not frequent the water and occupy inhibitor-laden, warm, tropical environments may result in low concentration eDNA detections. Depending on the abundance of boas and the habitat utilized, eDNA concentrations in St. Croix may be lower than those found in Florida for semi-aquatic pythons.

The standard methods for eDNA analysis include the calculation of the limit of detection (LOD) and the limit of quantification (LOQ; Hunter et al. 2017; Klymus et al. 2020). The LOD is calculated as the lowest detected dilution step with >95% of PCR replicates (e.g., 5 out of 5) amplifying target eDNA. The LOQ is the lowest standard concentration with a percent Coefficient of Variance (%CV) <35% (Klymus et al. 2020). It is critical to calculate the LOD and LOQ metrics to understand the confidence estimates of the assay at the lower limits of its dynamic range of concentrations and for comparison of results across different studies with the same assay, or across different assays. Due to these guidelines for interpreting eDNA detections, concentrations are reported above LOQ, whereas concentrations between LOD and LOQ are reported as detections (with no concentration specified). Concentrations below LOD represent values that are detected less than 95% of the time (i.e., they are at a lower concentration than can be consistently detected with a 95% confidence interval) but can still be considered true detections (Klymus et al. 2020).

The use of proper controls and the ddPCR instrument, which allows for absolute quantification and the ability to detect a single copy of DNA within a droplet, allows for the inclusion of concentrations <LOD. Indeed, Klymus et al. (2020), stated, “detections of target eDNA at copy numbers below

those of the LOD may be expected due to the rarity of target species. Such detections may still be true positives and are too important to disregard (Ellison et al. 2006; Hunter et al. 2017; Kralik and Ricchi 2017). The observation of eDNA detections below the LOD concentration indicates that there is less than the desired confidence level of detecting the target sequence, when it is present at that low concentration.”

This study aimed to detect Central American boa eDNA at focal areas in SARI. We had three objectives: 1) assess the developed boa eDNA marker for specificity and sensitivity to the invasive St. Croix population; 2) design an eDNA sampling plan to assess Central American boa occurrence at SARI in St. Croix; and 3) conduct eDNA surveys in SARI, using water, to assess areas for detection and non-detection of Central American boa eDNA. We expected boa eDNA occurrence probability to be highest in the western forests and to decline progressively for sites located further east. Survey sites in more developed or residential areas were likely to contain different densities of boas compared to natural environments due to habitat availability and prey species abundance.

Methods

Tissue DNA Extraction and Sequencing

We utilized St. Croix boa specimens previously collected by the general public as part of a bounty program and deposited to the U.S. Virgin Islands Department of Planning and Natural Resources (DPNR) in January and February 2024. National Park Service staff then collected tissues from these specimens for our use as DNA source tissues. All Central American boa tissue samples were collected by the National Park Service under Invasive Species Collection permit DFW23015X issued by the DPNR office. We sampled both juvenile ($n = 12$, <1 m estimated length) and adult ($n = 14$, 1–4 m estimated length) snakes to assess whether the previously designed eDNA assay by Hunter et al. (2015) was specific and sensitive to the genetic diversity found in the St. Croix invasive population.

Metadata for these specimens was minimal (Table 1). Specimens were stored at -20°C and defrosted for 10–15 minutes before sampling. New gloves, a clean area, and a sterile scalpel were used to clip a small piece of tissue from areas of each specimen as detailed in Table 1. The tissue was clipped and put in tissue buffer (250 mM sodium tetra ethylenediaminetetraacetic acid [EDTA], glacial acetic acid to pH 7.5, 3.5 mM sodium chloride, 20% of total volume dimethyl sulfoxide [DMSO], and 70% of total volume ultrapure water). Tissue samples were stored at room temperature until extraction. A shed skin ($n = 1$) was also bagged and kept at room temperature until extraction. All samples were isolated following Qiagen's DNeasy Blood and Tissue Kit (Valencia, CA, USA) protocol and the quality of the DNA was assessed using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Filter pipet tips were used throughout the study.

Table 1. Metadata for boa tissue samples deposited with the Department of Planning and Natural Resources in St. Croix between January and February 2024. Tissue samples for genetic analysis were collected February 22, 2024.

Sample ID	Sample type	Body part	Location of capture (latitude °N, longitude °W)	Age class	Total length (ft)
BC01	Tissue	Near head	17.471840, -64.8712748	Juvenile	1
BC02	Tissue	Midbody, near tail	–	Juvenile	1
BC03	Tissue	Tail	–	Juvenile	1
BC04	Tissue	Tail	–	Adult	3
BC05	Tissue	Midbody	–	Juvenile	2
BC06	Tissue	Midbody	–	Adult	5
BC07	Tissue	Internal organ	–	Juvenile	1.5
BC08	Tissue	Midbody	–	Adult	3
BC09	Tissue	Midbody	–	Juvenile	1.5
BC10	Tissue	Tail	–	Juvenile	1.5
BC11	Tissue	Midbody	–	Adult	6

Table 1 (continued). Metadata for boa tissue samples deposited with the Department of Planning and Natural Resources in St. Croix between January and February 2024. Tissue samples for genetic analysis were collected February 22, 2024.

Sample ID	Sample type	Body part	Location of capture (latitude °N, longitude °W)	Age class	Total length (ft)
BC12	Tissue	Tail	–	Adult	5
BC13	Tissue	Midbody	17.7271640, -64.8712749	Adult	4
BC14	Tissue	Midbody	17.73735, -64.838097	Adult	6
BC15	Tissue	Tail	–	Juvenile	1.5
BC16	Tissue	Midbody	–	Adult	3.5
BC17	Tissue	Midbody	–	Juvenile	2
BC18	Tissue	Midbody	–	Adult	>1
BC19	Tissue	Tail	–	Adult	12
BC20	Tissue	Tail	–	Juvenile	1.5
BC21	Tissue	Midbody	–	Adult	8
BC22	Tissue	Midbody	–	Juvenile	1.5
BC23	Tissue	Midbody	–	Adult	8
BC24	Tissue	Midbody	–	Juvenile	1
BC25	Tissue	Midbody	–	Adult	4
BC26	Tissue	Midbody	–	Adult	5
BC27	Shed skin	–	–	Undocumented	–

PCR for Tissue DNA Sequencing

Primers previously developed for the mitochondrial cytochrome-b region of snakes, L14910 F and H16064 R, were used to sequence the DNA extracted from St. Croix boa tissue (Burbrink et al. 2000; de Queiroz et al. 2002). Conventional PCR reactions were run with a total volume of 25 µL reagent mix per reaction including 1 µL of 10 ng DNA, 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl; Sigma-Aldrich, Inc., St. Louis, MO, USA), 200 µM dNTP, 3.5 mM MgCl₂, 0.25 µM of each primer, 0.0625 mg/mL Bovine Serum Albumin, and 0.02 units of Sigma Jump Start Taq DNA polymerase. The PCR cycling conditions were 5 min at 95°C; then 35 cycles of 30 sec at 95°C, 30 sec at 48°C, and 1 min at 72°C; then 5 min at 72°C and a holding at 4°C. The results were visualized on a 1% agarose gel. The PCR products were purified and bi-directionally sequenced by a third-party sequencing company, Eurofins. Sequences were reviewed for QA/QC, directions were assembled, and sequences aligned using Geneious v. 11.1.5. The newly generated DNA sequences from St. Croix boa, as well as cytochrome-b sequences from closely related non-target species, were aligned to evaluate eDNA assay efficiency in-situ. The non-target species included the Dumeril's boa (*Acrantophis dumerili*), carpet python (*Morelia spilota*), and Burmese python (*Python bivittatus*). The diverse target species included the red-tailed boa constrictor (*Boa constrictor*), Central American boa (*B. imperator*), and long-tailed boa (*B. c. longicauda*). All sequences are provided in Beaver et al. (2025).

Assay Optimization

A previously developed eDNA assay for boas was optimized on the Bio-Rad droplet digital PCR QX200 system (ddPCR) to provide absolute quantification and detection capabilities nearing single molecules of target eDNA. We optimized conditions on target and non-target species DNA in 25 μ L reactions with 4 μ L template. The optimal annealing temperature was tested using a gradient thermocycler with 4-step intervals set at 57°C, 57.9°C, 59.8°C, and 62°C. The optimal temperature was determined by only amplifying target species DNA and finding the greatest separation in amplitude between the positive and negative droplets, which was found at 57°C. The PCR cycling conditions for all testing and subsequent reactions were 95°C for 10 min, 40 cycles of 94°C for 30 sec and 57°C (annealing temperature) for 60 sec; then 98°C for 10 min and a 4°C hold indefinitely. Then, optimal primer and probe concentrations were tested in various concentration combinations with primers ranging from 350 to 950 nM in 150 nM increments and probes ranging from 100 to 250 nM in 50 nM increments. The optimal concentration combination of 800 nM (primer) and 250 nM (probe) was chosen based on the greatest separation in amplitudes of positive and negative droplet clouds while yielding the highest concentration of target eDNA.

The limit of detection (LOD) and limit of quantification (LOQ) were determined using a synthetically derived sequence (gBlocks; Integrated DNA Technologies, Coralville, IA, USA) of the target amplicon for the eDNA assay previously identified in Hunter et al. (2015; Klymus et al. 2020). To test the discrete LOD, a boa gBlocks was diluted in an 8-step standard series that ranged from 3,613.24 copies/ μ L to 1.33 copies/ μ L in dilution increments of 1 in 5 and 1 in 2. A second plate to verify the LOD was run with additional dilution steps that ranged from 2.90 copies/ μ L to <0.01 copies/ μ L in 1:5 dilution steps. The discrete LOD is calculated as the lowest dilution step with >95% of ddPCR replicates amplifying target eDNA, indicating reliable detection. A modeled LOD was calculated using the R function “LODmodel.glm” (Hunter et al. 2017). The discrete LOQ was calculated as the lowest standard concentration with a percent Coefficient of Variance (%CV; standard deviation/mean $\times$ 100) <35% according to Klymus et al. (2020) as applied to droplet digital PCR eDNA concentrations by Taylor et al. (2017).

Study Design

The sampling design aimed to gather information on the detection of boa eDNA in water samples and estimate boa occurrence within SARI, while working within budgetary and logistical constraints. Environmental DNA water samples were collected at sites where boas were expected to be present around the western forests near Creque Dam and Mahogany Road, and from sites where the prevalence of boas was unknown within protected lands of SARI (Figure 1; Michaels 2024). An initial pilot study was planned in St. Croix to sample various habitats on the island to determine assay efficiency and the detection rate of boa eDNA from environmental water samples. These pilot data were used to refine the total water volume sampled or PCR replicate numbers to increase probability of detecting boa eDNA and highlight areas of interest for further sampling.

For the main sampling event, sites within SARI were chosen based off their proximity to natural locations where boas were previously spotted, areas that were flagged by dog detection teams, or high priority areas for park management. Site selection also aimed to represent a variety of habitat

types: wetlands, mangrove, forest, and disturbed areas. The timing of sampling was planned to coincide with periods of moderate rain that create standing water, but not heavy, frequent rain that could dilute eDNA from the waterway.

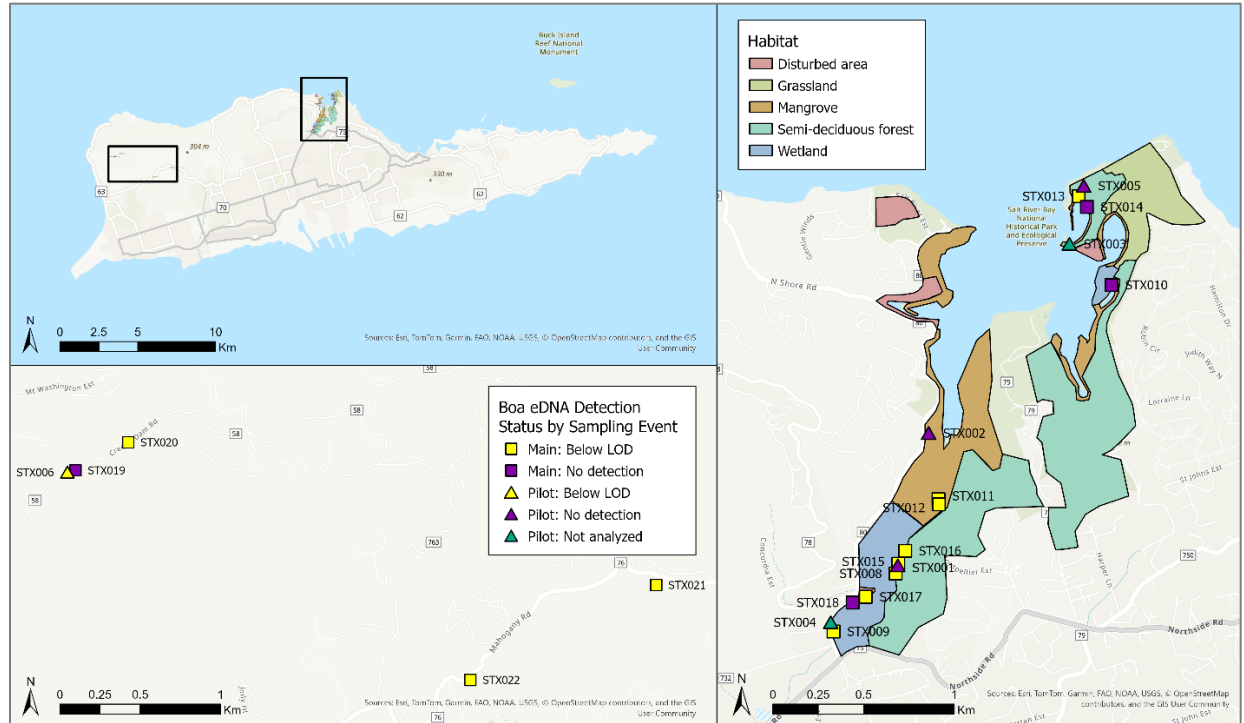


Figure 1. Maps depicting environmental DNA (eDNA) sampling in St. Croix, U.S. Virgin Islands (top left), with insets of sample collection sites in the western forests (bottom left) and Salt River Bay National Historical Park and Ecological Preserve (SARI; right). The pilot event (December 2023) sites are indicated by triangles and the main event (July 2024) sites by squares. The symbol fill color corresponds to the detection status of *Boa imperator* eDNA, where yellow indicates eDNA amplification below the limit of detection (LOD), purple indicates no detection, and blue indicates samples were collected but not analyzed. Symbols are enlarged for ease of visualization. Habitat types within SARI are shown with color-coded polygons, including disturbed area (pink), grassland (green), mangrove (tan), semi-deciduous forest (blue-green), and wetland (blue).

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Captive Boa Samples

To test assay efficacy in a controlled environment, water samples were collected from two different sources used by a captive boa, about 0.6 m in length, kept by Virgin Islands DPNR Division of Fish and Wildlife. Previously sterilized water bottles were used to collect water. The water samples were immediately preserved with Longmire's Buffer (AquaPhoenix Scientific, Hanover, PA, USA) in a 1:3 ratio to water volume collected. Samples were put on ice after collection and transported or frozen at National Park Service headquarters until shipment could occur.

For the pilot effort, the body of the snake was dipped in a bucket of water and allowed to move freely (~15 min). We collected 7 × 60 mL water samples from the water bucket after the snake was

removed. The main sampling event utilized samples from the water bowl within the captive boa's habitat. We collected 6×60 mL from a water bowl that was in the habitat for at least seven days. The water bowl was then replaced with fresh water, and a 60 mL sample was collected before the water was put in the habitat. Finally, 1,350 mL of water was collected after four days of being in the habitat.

Field Work

Environmental water samples were collected using the same general protocols as the captive boa samples. The initial pilot was conducted December 18–19, 2023, by U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS). For the pilot sampling, we collected 7×60 mL water samples per site within SARI (5 sites) and the western forests (1 site; Figure 1, Table 2). The western site was visited after sampling was completed within the park to avoid cross contamination. Due to the low concentrations of eDNA in preliminary data from the pilot effort, we doubled the volume of water collected and filtered from each site to increase the probability of detection if boa eDNA was present at a site. The main sampling event occurred July 16–18, 2024, by U.S. Geological Survey (USGS) and USDA-APHIS personnel. For the main sampling event, we collected 15×60 mL water samples per site within SARI (11 sites) and the western forests (4 sites; Figure 1, Table 2). Available standing water was limited during both sampling events, which resulted in sampling design changes within SARI.

Table 2. Abiotic and collection information from each geographic location sampled. Locales are Salt River Bay National Historical Park and Ecological Preserve (SARI) and the western forests (West) of St. Croix, U.S. Virgin Islands. Abiotic measurements from the geographic locations where samples were collected are reported for water salinity, temperature, depth, pH, and conductivity.

Effort	Locale	Habitat	Date	Site ID	Latitude	Longitude	Water volume collected (mL)	Salinity (ppt)	Temp (°C)	Depth (in)	pH	Conductivity (µS/cm)
Pilot	SARI	Wetland	12/18/2023	STX001	17.76237	-64.76182	420	1.1	26.2	2	6.95	2255
Pilot	SARI	Mangrove	12/18/2023	STX002	17.76891	-64.76029	420	0.4	25.2	1	7.31	400 ^A
Pilot	SARI	Forest	12/18/2023	STX003	17.77827	-64.75332	420	33.7	27.2	2	8.33	53,525
Pilot	SARI	Wetland	12/19/2023	STX004	17.75955	-64.76514	420	0.6	23.5	2	7.85	1163
Pilot	SARI	Forest	12/19/2023	STX005	17.78114	-64.75261	420	33.2	26.5	3	7.76	2195
Pilot	West	Forest	12/19/2023	STX006	17.7449	-64.87871	420	0.5	24.6	2	7.47	1044
Main	SARI	Wetland	7/16/2024	STX008	17.76192	-64.76192	900	3.2	27.1	3	6.85	6126
Main	SARI	Wetland	7/16/2024	STX009	17.75905	-64.765	900	1.3	28.3	3	6.83	2701
Main	SARI	Wetland	7/16/2024	STX010	17.77619	-64.75123	900	OVER	33.7	2	7.92	121,699
Main	SARI	Mangrove	7/17/2024	STX011	17.76559	-64.7598	900	9.8	33.6	2	8.08	19,649
Main	SARI	Mangrove	7/17/2024	STX012	17.76534	-64.75978	900	27.0	29.0	4	7.75	45,496
Main	SARI	Forest	7/17/2024	STX013	17.78058	-64.75286	840	36.2	32.3	>60	7.60	62,788
Main	SARI	Forest	7/17/2024	STX014	17.78005	-64.75245	900	38.3	32.9	>60	8.84	66,915
Main	SARI	Wetland	7/17/2024	STX015	17.7624	-64.76179	900	2.3	27.8	12	6.76	4590
Main	SARI	Forest	7/17/2024	STX016	17.76304	-64.76145	900	1.8	27.1	12	6.97	3621
Main	SARI	Disturbed	7/17/2024	STX017	17.76077	-64.7634	810	1.9	29.2	2	6.95	4011
Main	SARI	Wetland	7/17/2024	STX018	17.76048	-64.76405	790	1.3	27.6	2	6.93	2737
Main	West	Forest	7/18/2024	STX019	17.74496	-64.87825	900	0.6	27.4	16	7.31	1266
Main	West	Forest	7/18/2024	STX020	17.74652	-64.8753	900	0.5	26.3	24	7.96	1140
Main	West	Forest	7/18/2024	STX021	17.73855	-64.84578	900	0.7	27.1	4	7.73	1363
Main	West	Forest	7/18/2024	STX022	17.73325	-64.85617	900	0.6	28.0	1	7.77	1320

^A Reading of conductivity may not be accurate.

Water was collected in sterile 60 mL water bottles, preserved, and kept cold or frozen until filtration. To preserve eDNA, 15 mL of Longmire's buffer was added to each 45 mL of water immediately upon collection before being stored on ice or icepacks in insulated coolers and backpacks for transportation back to the National Park Service headquarters. Two samples collected in the pilot study on December 19, 2023 were instead preserved with 15 mL 98% EtOH. New gloves were used at each site and efforts were taken to decontaminate equipment between sites with 7% sodium hypochlorite solution. Environmental covariates were collected at each site, including collection date and time, GPS coordinates, water temperature, habitat type, and salinity (Table 2). Upon return to headquarters, samples were frozen at -20°C for storage until shipment. All samples were shipped to the laboratory for processing in insulated coolers with icepacks. Upon reaching the laboratory, samples were frozen at -20°C for short-term storage, then thawed overnight at 4°C the day prior to filtration. However, the shipment of pilot samples to the laboratory was delayed enroute by customs, which could have resulted in eDNA degradation during time spent at higher temperatures.

Water Filtration

The 60 mL water samples collected during the pilot effort were filtered individually, resulting in seven sample replicates per site, while the main event water samples were consolidated and filtered in groups of three, resulting in five sample replicates per site. We followed the methodology outlined by Renshaw et al. (2015), collecting eDNA from water on 1.2 μm , 47 mm polyethersulfone (PES) filters (Sterlitech, Kent, WA, USA) using vacuum filtration (Hunter et al. 2019b). During sample filtration, six filtering controls (sterile water) were run every ~ 12 samples. Funnels were rinsed with 1X phosphate-buffered saline (pH 7.4) to flush eDNA bound to the sides of the plastic. Filters were placed in individual microcentrifuge tubes containing 900 μL of modified CTAB buffer (EDTA 0.5 M [0.04 mL/mL], Tris 1 M [0.1 mL/mL], NaCl 5 M [0.28 mL/mL], PCR H_2O [to 1 mL], CTAB [0.02 g/mL], PVP [0.04 g/mL], β -mercaptoethanol [5 $\mu\text{L/mL}$]) and incubated at 4°C for 3 to 7 days until isolation was started. To assess contamination during the isolation processes, four isolation controls were implemented intermittently during processing by placing a clean filter into the modified CTAB buffer and proceeding with the protocol.

Isolation

Environmental DNA methods were optimized to maximize eDNA yield and reduce environmental inhibitors, following Hunter et al. (2019b). After incubation, proteinase K (90 μL , 20 mg/mL) was added and samples were incubated at 55°C for 1 hour. Briefly, eDNA was isolated according to the phenol-chloroform-isoamyl alcohol (PCI) method described in Renshaw et al. (2015) with slight modifications. Centrifugation after the addition of PCI and chloroform-isoamyl alcohol was done at 15,000 RPM for 5 minutes. The aqueous layer was moved to a new 2 mL tube, then 1.2 mL PEG (30% polyethylene glycol/2.5 M NaCl) and 0.48 μL of glycogen (20 mg/mL) were added to each sample and inverted several times for thorough mixing (Kirtane et al. 2024). The addition of the PEG modification was in response to heavy inhibitor loads found in southern Florida water samples (Hunter et al. 2019a, 2019b). Samples were vortexed lightly for 3 minutes, then precipitated overnight at 4°C . The next day, the DNA was pelleted by centrifugation at 15,000 RPM for 10 minutes before all liquid was removed via decantation and discarded. The eDNA pellet was washed with 1 mL of cold 70% ethanol, and centrifuged at 15,000 RPM for 5 minutes, then the liquid was

discarded This process was repeated for a second wash, after which eDNA pellets were allowed to air-dry until all residual ethanol had evaporated. Extracted eDNA was suspended in 100 μ L of 1X Low EDTA TE Buffer and quantified using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The OneStep PCR Inhibitor Removal Kit (IRK; Zymo Research, Irvine, CA, USA) was utilized to eliminate PCR inhibitors from the extracted eDNA. Samples underwent a maximum of five rounds of inhibitor removal, based on the observed pigment and extent of PCR inhibition, in accordance with Hunter et al. (2019b).

Environmental DNA Amplification

All environmental and control samples were processed on the QX200 droplet digital PCR system in 25 μ L total volume reactions containing 4 μ L of template, 12.5 μ L of ddPCR mix (Bio-Rad, Hercules, CA, USA), a final concentration of 250 nM probe (VIC), and 800 nM of each primer. An internal positive control (IPC) was run concurrently with the target reaction to identify any PCR inhibition and help delineate the potential of false negatives. The IPC consisted of *Moa* primers with probe (FAM) previously published for an extinct species (*Eurapteryx curtus*; Ramón-Laca et al. 2021) and a synthetic gBlocks template. PCR cycling conditions for each reaction were run at 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 57°C for 60 seconds, then 98°C for 10 minutes and a 4°C hold indefinitely.

Droplet counts from the reader were run through a code to determine concentration of target eDNA detected using the volume of droplets with a Poisson distribution. The maximum concentration of target eDNA for any negative field or laboratory control reaction became the limit of blank (LOB). All reactions with a target concentration equal to or less than the LOB were changed to zero positive droplets, then the code was rerun. Additionally, any ddPCR wells that produced less than 12,000 acceptable droplets were removed from the analysis according to manufacturer recommendations describing statistically reliable data (Bio-Rad). We interpreted detections of *boa* eDNA from environmental samples in two ways: 1) *positive detections* were declared if the concentration of *boa* eDNA amplified was greater than the LOD, or 2) detections $<LOD$ were declared if the concentration of *boa* eDNA amplified was greater than the LOB, but less than the LOD. The concentration of *boa* eDNA detected in each sample is only reported if it is greater than the LOQ. Any concentrations reported in copies/ μ L units are for the sample elution and have incorporated the template and reaction volumes for each reaction ($\times 25/4$).

Statistical Analyses

Naïve occurrence, availability, and detection estimates for target eDNA were calculated at the level of sample site, sample replicate, and ddPCR replicate, respectively, to inform data interpretation. Naïve occurrence estimates were calculated by dividing the number of sites with amplified target eDNA by the total number of sites and samples assessed. The naïve availability of eDNA was calculated by dividing the number of isolated samples with target eDNA by the total number of samples analyzed. The naïve detection of eDNA was calculated by dividing the number of ddPCR wells that amplified target eDNA by the total number of ddPCR wells analyzed. Naïve estimates were used to account for the smaller sample sizes in some locations.

Results

Tissue DNA Extraction Sequencing/PCR

The tissue samples were sequenced at the mitochondrial cytochrome-b region and resulted in 27 sequences, all an identical haplotype 863 base pairs long with >89.0% high quality nucleotide calls. The haplotype is the same as one previously published in GenBank (accession number MK330861.1) by Angeli et al. (2019), where a single haplotype was found in St Croix boa tissue samples that matched a Central American lineage.

Assay Optimization

We determined the eDNA assay to be quite sensitive to low concentrations of boa eDNA during laboratory optimization (Hunter et al. 2015). The initial LOD test resulted in 100% detection rates at all dilution steps, down to a concentration of 1.33 copies/ μ L. A second LOD plate was run with low concentrations. This second plate had 100% detections at the 2.9 copies/ μ L dilution, but reduced to 90% detections at both 2.40 and 0.70 copies/ μ L before dropping to 40% at 0.07 copies/ μ L. As a result, the discrete LOD was conservatively determined to be 2.9 copies/ μ L. The modeled LOD was calculated to be 0.34 copies/ μ L (95% CI = 0.30–0.38). The %CV was calculated and LOQ was 36.3 copies/ μ L.

Captive Boa Samples

Most water samples collected from the bucket or captive boa's water bowl had reproducible, but low concentrations of boa eDNA ($n = 11$ of 13). One sample collected in December 2023 had a positive detection above the LOD (Table 3). Only one captive boa sample collected in July 2024 had no detection of boa eDNA.

Field Work

We collected environmental water from a total of 21 geographic sites, including 16 in SARI and 5 in western forests, in December 2023 and July 2024. The sampling efforts targeted four different habitats (Figure 1): wetland ($n = 6$), mangrove ($n = 3$), forest ($n = 4$ inside and $n = 5$ outside the park), and disturbed areas ($n = 1$). A total of 279 water samples were collected across SARI ($n_{\text{pilot}} = 35$ and $n_{\text{main}} = 165$), the western forests ($n_{\text{pilot}} = 7$ and $n_{\text{main}} = 60$), and negative controls ($n_{\text{pilot}} = 6$ and $n_{\text{main}} = 6$).

Water Filtration and Isolation

The pilot sampling effort resulted in a final volume of 420 mL water collected and filtered from each site (STX001–006). The main sampling effort resulted in final volumes ranging from 790–900 mL water collected and filtered for each site (STX008–022). Water samples from two pilot SARI sites (STX003, STX004) were not analyzed, as they were taken as biological replicates for habitats previously sampled (STX005, STX001, respectively). After pilot site removal and main site replicate pooling (see Methods), a total of 103 field samples were analyzed; sample sizes totaled 76 from SARI ($n_{\text{pilot}} = 21$ and $n_{\text{main}} = 55$), 27 from western forests ($n_{\text{pilot}} = 7$ and $n_{\text{main}} = 20$), and 12 negative field controls. An additional 10 lab controls were analyzed, including six filtering negatives and four isolation negatives. All 13 captive boa samples were analyzed ($n_{\text{pilot}} = 7$ and $n_{\text{main}} = 6$).

PCR inhibitor loads varied by site, with some sites having samples with no inhibition and others with high inhibitor loads similar to the Florida Everglades, leading to the number of utilized Inhibitor Removal Columns ranging from zero to five (Appendix A, Table 4; Hunter et al. 2019b). Five sites had samples with evidence of PCR inhibition that required Inhibitor Removal column treatment, but three of those sites still had samples with boa eDNA detections <LOD. Controls and captive boa samples did not undergo inhibitor removal.

Table 3. Naïve estimates of availability and detection of Central American boa environmental DNA (eDNA). Naïve estimates were calculated by comparing the proportion of isolated filters that contained eDNA out of the total number of isolated filters analyzed (naïve availability of eDNA in water samples) and the proportion of digital droplet PCR (ddPCR) wells that amplified target eDNA out of the total number of ddPCR wells analyzed (naïve detection of eDNA in ddPCR analysis). Sites STX006 and STX019–022 are located in the western forests of St. Croix, U.S. Virgin Islands. All other site identifiers (ID) with STX are sites located in Salt River Bay National Historical Park and Ecological Preserve (SARI). Sites with “Captive” in the identifier are water samples collected from a captive boa habitat.

Site ID	Naïve availability of eDNA in water samples (eDNA present/total filters)	Naïve detection of eDNA in ddPCR analysis (eDNA present/total reactions)
STX001	0.00 (0/7)	0.00 (0/35)
STX002	0.00 (0/7)	0.00 (0/35)
STX005	0.00 (0/7)	0.00 (0/35)
STX006	0.29 (2/7)	0.09 (3/35)
STX008	0.40 (2/5)	0.08 (2/25)
STX009	0.80 (4/5)	0.16 (4/25)
STX010	0.00 (0/5)	0.00 (0/25)
STX011	0.20 (1/5)	0.04 (1/25)
STX012	0.60 (3/5)	0.12 (3/25)
STX013	0.40 (2/5)	0.08 (2/25)
STX014	0.00 (0/5)	0.00 (0/25)
STX015	0.40 (2/5)	0.08 (2/25)
STX016	0.40 (2/5)	0.12 (3/25)
STX017	0.40 (2/5)	0.08 (2/25)
STX018	0.00 (0/5)	0.00 (0/25)
STX019	0.00 (0/5)	0.00 (0/25)
STX020	0.20 (1/5)	0.04 (1/25)
STX021	0.40 (2/5)	0.08 (2/25)
STX022	0.20 (1/5)	0.04 (1/25)
Captive19Dec2023	1.00 (7/7)	0.74 (26/35)
Captive15July2024	0.80 (4/5)	0.48 (12/25)
Captive19July2024	1.00 (1/1)	1.00 (5/5)

Environmental DNA Amplification

The LOB was calculated to be 0.38 copies/ μ L, which indicates minimal contamination of target eDNA in the field collection or laboratory processing. The ddPCRs resulted in 12,255–23,896 accepted droplet counts. Detection results differed slightly between the pilot and main sampling event, but no environmental samples yielded boa eDNA concentrations greater than the discrete LOD (LOD = 2.9 copies/ μ L). Sample concentrations are conventionally not reported when they are below the LOD because the concentration is not statistically detected >95% of the time. However, to help visualize the average amount of target eDNA obtained from each sample, all samples that amplified target eDNA compared to the LOD and LOQ are plotted in Appendix A, Figure 2.

Over both sampling events, 8 of 14 sites in SARI and 4 of 5 sites in the western forests of St. Croix resulted in boa eDNA detections <LOD. The analyzed water samples collected within SARI in December 2023 resulted in no detection of boa eDNA (STX001, 002, 005). That same sampling event found a boa eDNA detection <LOD in the western forests of St. Croix (STX006). Conversely, samples taken in July 2024 from the sites in SARI had higher water volumes and resulted in detections <LOD at 8 of the 11 sites. Detections <LOD of boa eDNA were also found at 3 of 4 western forest sites in the July sampling event. Four sites sampled in July 2024 resulted in no detection of boa eDNA, three of which were within SARI ranging from the northeastern (STX010, 014) to the southwestern (STX018) parts of the park and one in the western forests of St. Croix (STX019). All habitats had similar proportions (14–20%) of samples with detections <LOD compared to total samples collected.

Statistical Analyses

Naïve estimates for target eDNA were calculated at the site, sample, and PCR replicate level. Naïve occurrence estimates (proportion of sites with amplified target eDNA out of total sample sites) for sites within SARI were 0.00 (0/3) for the pilot and 0.72 (8/11) for the main sampling event. Naïve occurrence estimates for sites within western forests were 0.75 (3/4) for the pilot and 1.00 (1/1) for the main sampling event. The naïve availability of eDNA (percentage of isolated samples with target eDNA out of total samples analyzed) for analyzed water samples from SARI averaged 23.7% (18/76) overall, with 0.0% (0/21) for the pilot and 32.7% (18/55) for the main sampling event. Naïve availability of eDNA for western forest water samples averaged 22.2% (6/27) overall, with 28.6% (2/7) for the pilot and 20.0% (4/20) for the main sampling event. The availability from the captive snake habitat water samples was 92.3% (12/13; Table 3).

The naïve detection of eDNA (percentage of ddPCR wells with target eDNA amplified out of total ddPCR wells analyzed) in ddPCR sample replicates from SARI averaged 5.0% (19/380), with 0.0% (0/105) for the pilot and 6.9% (19/275) for the main sampling event. Naïve detection of eDNA from western forest ddPCR replicates averaged 5.2% overall (7/135), with 8.6% (3/35) for the pilot and 4.0% (4/100) for the main sampling event. The naïve detection of eDNA from the captive snake habitat was 66.2% (43/65; Table 3).

Conclusions

The boa eDNA assay developed by Hunter et al. (2015) was optimized and validated on 27 Central American boa tissue samples collected from St. Croix, U.S. Virgin Islands. The assay successfully amplified eDNA in environmental water samples collected from St. Croix, although the eDNA concentrations were low. Environmental DNA concentrations were found below the LOD in both the western forests (where boas are known to occur) and the Salt River Bay National Historical Park and Ecological Preserve, indicating a range expansion.

Our results indicated non-detections and detections below the LOD concentration in environmental water samples. This was expected given that boas are low-shedding, semi-arboreal reptiles and likely do not interact with water as much as other studied semi-aquatic snake species (Hunter et al. 2015, 2019a). It is important to note that results with concentrations <LOD are still meaningful and considered qualitative results since LOD is used as a comparative tool of statistical power across studies and assays (see details in the introduction). Therefore, concentrations below the LOD (<2.9 copies/uL) can still be considered detections of low concentrations of the target eDNA when appropriate QA/QC measures are applied. The QA/QC measures that we implemented to protect against contamination included the LOB, the use of ddPCR, conservative droplet thresholds and quality control, and ensuring reproducibility of data (Ellison et al. 2006; Hunter et al. 2017; Klymus et al. 2020).

Tissue DNA Extraction Sequencing/PCR

The single haplotype in the mitochondrial cytochrome-b region from 27 tissue samples indicated low genetic diversity of boas in St. Croix and matches the previous haplotype for boas on the island (Angeli et al. 2019). The results support the theory that the colonization of boas on the island likely came from a single genetic source (Angeli et al. 2019), or that the stock from the pet trade was reduced to a severe bottleneck and is highly uniform, as has been identified previously in pythons (Hunter et al. 2018). However, mitochondrial DNA is maternally inherited and often conserved within the genome, meaning that the genetic diversity inherited by the paternal snake and nuclear DNA regions that have a higher mutation rate are not represented in this analysis. A more thorough study of diverse specimens from St. Croix using multi-locus nuclear markers would be necessary to investigate the invasion history of the species on St. Croix.

Assay Optimization

PCR inhibition can cause false negative results when the chemical compounds bind to target eDNA and suppress PCR amplification. The suppression of eDNA amplification can be compounded when targeting a low shedding species where detection of eDNA is already expected to be difficult, as found with Burmese pythons in southern Florida (Hunter et al. 2019a, 2019b). The present study utilized several laboratory protocol modifications including the use of ddPCR technology, phenol-chloroform and chloroform-isoamyl-alcohol isolation methods, and inhibitor removal kits to mitigate the potential of PCR inhibitors (Hunter et al. 2019a, 2019b). The low number of sites that had evidence of PCR inhibition and no eDNA detections suggests that the rate of false negative results is relatively low in this study.

Study Design and Implications

There was a lack of boa eDNA detections in sites within the park during the pilot sampling effort (December 2023) as compared to the detections below LOD in nearby sites during the main sampling effort (July 2024). This could be a result of the sampling season, unequal sampling, issues with delayed shipments of pilot water samples to the lab, increased total water volume collected and filtered per laboratory sample in the main sampling, or differences in true boa occurrence. Alternative methods of filtering on site or hand carrying samples can be implemented in the future for faster preservation timelines. In relation to the main event in July, boas are likely to be more active in the summer and dry months when they are reproductively active in native areas (O'Shea 2007). Additionally, a larger volume of water was collected during the July sampling event compared to December, potentially increasing the likelihood of capturing target eDNA (Moyer et al. 2014). Sites with available water were more difficult to find in July 2024 due to a lack of rain, whereas the water tables were higher during sampling in December 2023; however, frequent rains can flush and dilute eDNA. Although sampling in the park during the summer and with enough water availability is important, it is difficult to plan and execute field work in this environment without local capacity and consistent sampling routines.

Captive Boa Samples

Water samples collected in a controlled environment with a captive snake revealed low concentrations (primarily <LOD) of boa eDNA. This data further supports our hypothesis that constrictor snakes typically shed low amounts of eDNA, which will likely result in lower concentrations detected in the environmental samples due to degradation caused by factors like heat, microbes, time from deposition, ultraviolet radiation, and water movement. As expected, target eDNA in the captive snake water samples was more consistently detected compared to detections in environmental samples, as evident by the higher naïve availability. Additionally, one of the 13 captive boa samples resulted in a positive detection >LOD, but the concentration is not reported due to it being below the LOQ. This detection may indicate the collection of biologic material (e.g., clump of skin or fecal material).

Field Work

The Central American boa is a semi-arboreal species that is less likely to spend time in water, which may have contributed to the limited detection and <LOD concentrations. Environmental samples of soil or swabbing tree bark may yield increased eDNA concentrations or detections for boas relative to water samples (Kucherenko et al. 2018). The optimization and validation of a soil or swabbing method would also reduce the need for available water sources to sample, which was an issue when sampling in July 2024 in SARI. Because suitable water sources were scarce in forested areas of the park, these habitats could not be sampled, resulting in a notable data gap in the study. Collecting water samples during the wet season, when water sources are more abundant and likely to be used by snakes, or incorporating alternative methods such as swab, or soil, sampling could enable a more comprehensive assessment of boa occurrence across the park, particularly in habitats where boas are known to be prevalent. However, both soil and swab-based eDNA techniques are still in early stages of development and would require substantial validation to inform study design and to accurately interpret results, especially in cases of non-detection.

Previous efforts to estimate the prevalence of boas in SARI using detection dogs had limited success (Michaels 2024). The dogs exhibited alerting behavior for boas a number of times in the western forests but did not alert for any boa within SARI. One advantage of the detection dogs compared to eDNA sample collection was that dogs could ascend to higher elevations whereas eDNA water sampling was limited to ground water runoff at lower elevations within SARI. However, the climate and dense vegetation restricted access to some of the areas for dogs as well (Michaels 2024).

Statistical Analyses

The low number of sample sites may inherently provide bias in the statistical analyses used in this study. It also prevented us from statistically analyzing the potential effects of habitat on boa presence. Due to the low number of sites, naïve estimate calculations are provided at the level of study population and sampling event; however, they cannot account for imperfect detection and are likely to underestimate the true values of occurrence and detection. The samples in SARI and western forests found low detection of eDNA in ddPCR reactions, potentially due to PCR inhibition from various water chemistry factors. However, we report naïve occurrence, availability, and detection estimates to provide insight on the prevalence of boas in SARI, and guide managers on priority areas for ground truthing the results. Factors in low detection rates include the well-documented stochasticity of eDNA detections (Hunter et al. 2017) and the semi-arboreal lifestyle of this reptile, which limits the amount of DNA shed into the aquatic environment sampled.

Future Directions

The low concentrations of eDNA found in this study indicate that additional analysis of samples and ground-truthing of the results could help to improve future assessments. Additional testing and optimization of collection methods could help recover greater eDNA concentrations to improve the eDNA detections and range assessments of boas across various environments. While often less effective at detecting cryptic species, ground-truthing via additional survey efforts and encouraging citizen science reports of sightings could assist with confirming and monitoring range expansion across the island. Visual observations can also help to validate the low concentration eDNA detections achieved in these challenging conditions.

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Appendix A: Environmental DNA Results

Table 4 and Figure 2 describe the results of water sample analysis.

Table 4. Water samples collected and analyzed for the study. Site is the geographic identifier for the sample replicates collected at that location. Pilot samples are labeled as A–G where seven sample replicates were collected from each site. In the main effort, 15 sample replicates were collected per site, then filtered together in groups of three for a total of five analyzed replicates, labeled A–E. Captive boa samples were collected during both sampling events. The detection status is reported for each sample, where “no detect” indicates no amplified target environmental DNA (eDNA), “<LOD” indicates that target eDNA was amplified but not in quantities greater than the assay’s limit of detection (LOD) >95% of the time, and “positive detection” indicates target eDNA was amplified in quantities greater than the LOD. Each sample was run in five PCR technical replicates (total PCR replicates) and the number of reactions that amplified target eDNA is reported (amplified). The total number of Inhibitor Removal (IR) columns used to produce the final data for each sample are reported.

Sample type	Site	Sample	Detection status	Amplified/total PCR replicates	Total IR columns
Pilot	STX001	A	No detect	0/5	0
	STX001	B	No detect	0/5	0
	STX001	C	No detect	0/5	0
	STX001	D	No detect	0/5	0
	STX001	E	No detect	0/5	0
	STX001	F	No detect	0/5	0
	STX001	G	No detect	0/5	0
	STX002	A	No detect	0/5	0
	STX002	B	No detect	0/5	0
	STX002	C	No detect	0/5	0
	STX002	D	No detect	0/5	0
	STX002	E	No detect	0/5	0
	STX002	F	No detect	0/5	0
	STX002	G	No detect	0/5	0
	STX005	A	No detect	0/5	0
	STX005	B	No detect	0/5	0
	STX005	C	No detect	0/5	0
	STX005	D	No detect	0/5	0
	STX005	E	No detect	0/5	0
	STX005	F	No detect	0/5	0
	STX005	G	No detect	0/5	0
	STX006	A	No detect	0/5	0

Table 4 (continued). Water samples collected and analyzed for the study. Site is the geographic identifier for the sample replicates collected at that location. Pilot samples are labeled as A–G, where seven sample replicates were collected from each site. In the main effort, 15 sample replicates were collected per site, then filtered together in groups of three for a total of five analyzed replicates, labeled A–E. Captive boa samples were collected during both sampling events. The detection status is reported for each sample where “no detect” indicates no amplified target environmental DNA (eDNA), “<LOD” indicates that target eDNA was amplified but not in quantities greater than the assay’s limit of detection (LOD) >95% of the time, and “positive detection” indicates target eDNA was amplified in quantities greater than the LOD. Each sample was run in five PCR technical replicates (total PCR replicates) and the number of reactions that amplified target eDNA is reported (amplified). The total number of Inhibitor Removal (IR) columns used to produce the final data for each sample are reported.

Sample type	Site	Sample	Detection status	Amplified/total PCR replicates	Total IR columns
Pilot (continued)	STX006	B	No detect	0/5	0
	STX006	C	No detect	0/5	0
	STX006	D	<LOD	1/5	0
	STX006	E	No detect	0/5	0
	STX006	F	<LOD	2/5	0
	STX006	G	No detect	0/5	0
Main effort	STX008	A	No detect	0/5	0
	STX008	B	No detect	0/5	0
	STX008	C	No detect	0/5	0
	STX008	D	<LOD	1/5	0
	STX008	E	<LOD	1/5	0
	STX009	A	No detect	0/5	1
	STX009	B	<LOD	1/5	1
	STX009	C	<LOD	1/5	1
	STX009	D	<LOD	1/5	1
	STX009	E	<LOD	1/5	5
	STX010	A	No detect	0/5	1
	STX010	B	No detect	0/5	1
	STX010	C	No detect	0/5	1
	STX010	D	No detect	0/5	1
	STX010	E	No detect	0/5	1
	STX011	A	No detect	0/5	0
	STX011	B	No detect	0/5	1
	STX011	C	No detect	0/5	0
	STX011	D	No detect	0/5	0
	STX011	E	<LOD	1/5	0

Table 4 (continued). Water samples collected and analyzed for the study. Site is the geographic identifier for the sample replicates collected at that location. Pilot samples are labeled as A–G, where seven sample replicates were collected from each site. In the main effort, 15 sample replicates were collected per site, then filtered together in groups of three for a total of five analyzed replicates, labeled A–E. Captive boa samples were collected during both sampling events. The detection status is reported for each sample where “no detect” indicates no amplified target environmental DNA (eDNA), “<LOD” indicates that target eDNA was amplified but not in quantities greater than the assay’s limit of detection (LOD) >95% of the time, and “positive detection” indicates target eDNA was amplified in quantities greater than the LOD. Each sample was run in five PCR technical replicates (total PCR replicates) and the number of reactions that amplified target eDNA is reported (amplified). The total number of Inhibitor Removal (IR) columns used to produce the final data for each sample are reported.

Sample type	Site	Sample	Detection status	Amplified/total PCR replicates	Total IR columns
Main effort (continued)	STX012	A	<LOD	1/5	2
	STX012	B	<LOD	1/5	5
	STX012	C	No detect	0/5	5
	STX012	D	<LOD	1/5	5
	STX012	E	No detect	0/5	2
	STX013	A	<LOD	1/5	1
	STX013	B	No detect	0/5	5
	STX013	C	No detect	0/5	3
	STX013	D	No detect	0/5	5
	STX013	E	<LOD	1/5	3
	STX014	A	No detect	0/5	3
	STX014	B	No detect	0/5	1
	STX014	C	No detect	0/5	1
	STX014	D	No detect	0/5	1
	STX014	E	No detect	0/5	1
	STX015	A	No detect	0/5	0
	STX015	B	No detect	0/5	0
	STX015	C	<LOD	1/5	0
	STX015	D	<LOD	1/5	0
	STX015	E	No detect	0/5	0
	STX016	A	No detect	0/5	0
	STX016	B	No detect	0/5	0
	STX016	C	<LOD	2/5	0
	STX016	D	<LOD	1/5	0
	STX016	E	No detect	0/5	0
	STX017	A	No detect	0/5	0
	STX017	B	<LOD	1/5	0

Table 4 (continued). Water samples collected and analyzed for the study. Site is the geographic identifier for the sample replicates collected at that location. Pilot samples are labeled as A–G, where seven sample replicates were collected from each site. In the main effort, 15 sample replicates were collected per site, then filtered in groups of three for a total of five analyzed replicates, labeled A–E. Captive boa samples were collected during both sampling events. The detection status is reported for each sample where “no detect” indicates no amplified target environmental DNA (eDNA), “<LOD” indicates that target eDNA was amplified but not in quantities greater than the assay’s limit of detection (LOD) >95% of the time, and “positive detection” indicates target eDNA was amplified in quantities greater than the LOD. Each sample was run in five PCR technical replicates (total PCR replicates) and the number of reactions that amplified target eDNA is reported (amplified). The total number of Inhibitor Removal (IR) columns used to produce the final data for each sample are reported.

Sample type	Site	Sample	Detection status	Amplified/total PCR replicates	Total IR columns
Main effort (continued)	STX017	C	<LOD	1/5	0
	STX017	D	No detect	0/5	0
	STX017	E	No detect	0/5	0
	STX018	A	No detect	0/5	0
	STX018	B	No detect	0/5	0
	STX018	C	No detect	0/5	0
	STX018	D	No detect	0/5	0
	STX018	E	No detect	0/5	0
	STX019	A	No detect	0/5	0
	STX019	B	No detect	0/5	0
	STX019	C	No detect	0/5	0
	STX019	D	No detect	0/5	0
	STX019	E	No detect	0/5	0
	STX020	A	No detect	0/5	0
	STX020	B	<LOD	1/5	0
	STX020	C	No detect	0/5	0
	STX020	D	No detect	0/5	0
	STX020	E	No detect	0/5	0
	STX021	A	No detect	0/5	0
	STX021	B	No detect	0/5	0
	STX021	C	<LOD	1/5	0
	STX021	D	No detect	0/5	0
	STX021	E	<LOD	1/5	0
	STX022	A	No detect	0/5	0
	STX022	B	No detect	0/5	0
	STX022	C	No detect	0/5	0
	STX022	D	No detect	0/5	0

Table 4 (continued). Water samples collected and analyzed for the study. Site is the geographic identifier for the sample replicates collected at that location. Pilot samples are labeled as A–G, where seven sample replicates were collected from each site. In the main effort, 15 sample replicates were collected per site, then filtered in groups of three for a total of five analyzed replicates, labeled A–E. Captive boa samples were collected during both sampling events. The detection status is reported for each sample where “no detect” indicates no amplified target environmental DNA (eDNA), “<LOD” indicates that target eDNA was amplified but not in quantities greater than the assay’s limit of detection (LOD) >95% of the time, and “positive detection” indicates target eDNA was amplified in quantities greater than the LOD. Each sample was run in five PCR technical replicates (total PCR replicates) and the number of reactions that amplified target eDNA is reported (amplified). The total number of Inhibitor Removal (IR) columns used to produce the final data for each sample are reported.

Sample type	Site	Sample	Detection status	Amplified/total PCR replicates	Total IR columns
Main effort (continued)	STX022	E	<LOD	1/5	0
	Captive boa samples	–	–	–	–
	Captive19Dec2023	A	<LOD	4/5	0
	Captive19Dec2023	B	<LOD	1/5	0
	Captive19Dec2023	C	<LOD	5/5	0
	Captive19Dec2023	D	Positive	5/5	0
	Captive19Dec2023	E	<LOD	5/5	0
	Captive19Dec2023	F	<LOD	2/5	0
	Captive19Dec2023	G	<LOD	4/5	0
	Captive15July2024	A	<LOD	4/5	0
	Captive15July2024	B	<LOD	4/5	0
	Captive15July2024	C	<LOD	3/5	0
	Captive15July2024	D	<LOD	1/5	0
	Captive15July2024	E	No detect	0/5	0
	Captive19July2024	A	<LOD	5/5	0

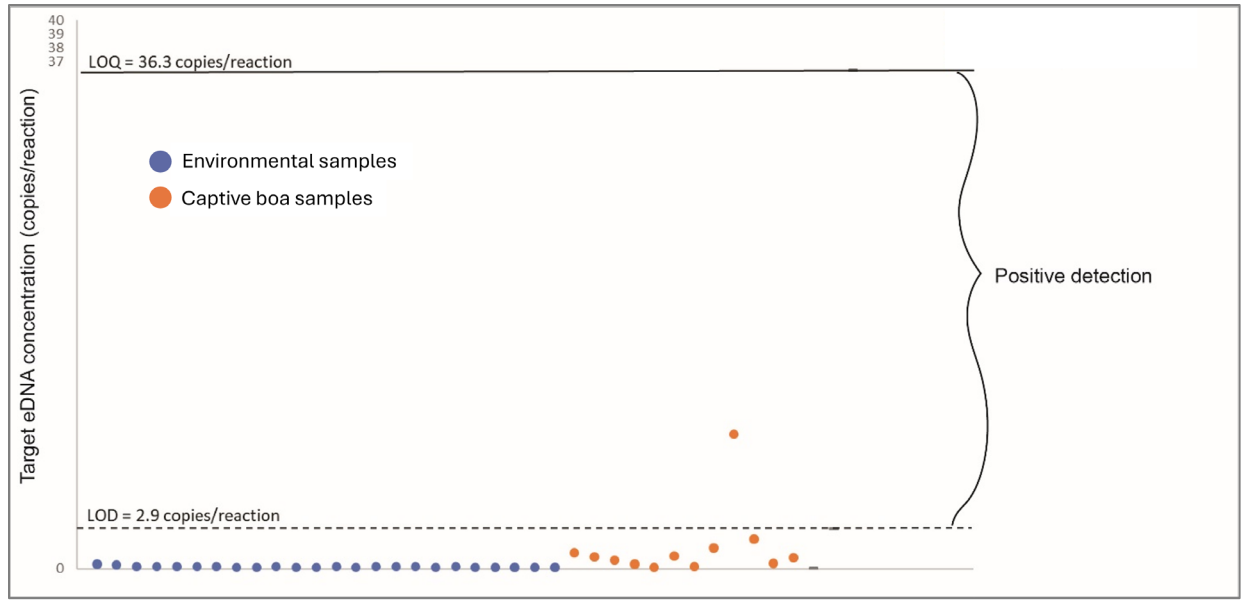


Figure 2. A plot depicting all sample concentrations compared to the limit of detection (LOD) and limit of quantification (LOQ) concentrations. The average target environmental DNA (eDNA) concentration of samples with amplified *Boa imperator* eDNA is displayed with points, where color differentiates water samples collected from the environment (blue) and those taken from water in the captive snake habitat (orange). Any eDNA concentration less than the LOD is interpreted as below the LOD (<LOD), and any eDNA concentration greater than the LOD, but less than the LOQ, is interpreted as a positive detection.

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1201 Oakridge Drive, Suite 150
Fort Collins, CO 80525