



# Safeguarding *Iguana* diversity: enabling rapid and low-effort tracking of non-native iguanas through terrestrial eDNA innovations

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**Abstract** Reptiles have among the highest extinction risk across terrestrial vertebrates, with habitat fragmentation, habitat destruction, and invasive alien species being the primary causes of reptile species loss on a global scale. Invasive hybridization (i.e. hybridization between native and invasive alien species) is increasing globally, causing the extinction of native genotypes, and this phenomenon is particularly pervasive in Caribbean iguanas. The Lesser Antillean Iguana (*Iguana delicatissima*), a keystone species of Caribbean coastal ecosystems, has become critically endangered mainly due to ongoing hybridization with

the invasive Common Green Iguana (*I. iguana*). For impactful conservation intervention, the need for early detection of invasive animals and their progeny, or detection of surviving pure native animals, is urgent. We aimed to develop a novel environmental DNA (eDNA) toolkit using Kompetitive Allele Specific PCR (KASP) technology, a method of allele-specific amplification for cost-effective and efficient sampling of terrestrial substrates to aid in mapping the distribution of native *I. delicatissima*, invasive *I. iguana*, and signal potential invasive hybridization. We demonstrate proof-of-concept and successfully identified *I. delicatissima*, *I. iguana*, and their hybrids via blood samples using our primer sets, as well as successful detection of *I. delicatissima* in

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several ex-situ (Rotterdam Zoo) and in-situ (St. Eustatius) eDNA samples, collected with environmental swabs and tape-lifting. We found that sampling potential perching spots yielded the highest number of positive detections via environmental swabbing and tape-lifting. Our toolkit demonstrates the potential of terrestrial eDNA sampling for iguana conservation, enabling faster detection of putative invasive hybridization. Additionally, the method holds promise for other terrestrial cryptic species, contributing to broader collection of population-level information.

**Keywords** Green iguana · Hybridization · KASP · Lesser Antillean iguana · Non-invasive sampling · Vegetation sampling

## Introduction

Reptiles have among the highest extinction risk of all terrestrial vertebrates, with habitat fragmentation, destruction, and invasive alien species (IAS) being the primary causes of global species loss (Cushman 2006; Bland and Böhm 2016; Cordier et al. 2021). Of the approximate 70% decline in global vertebrate populations, indirect and direct translocation, introduction, and diffusion of IAS represent a major contributor to biodiversity decline (Bellard et al. 2016). Importantly, IAS not only endanger wildlife via predation, competition, and disease transmission (Ricciardi et al. 2017), they also threaten the genetic integrity of native taxa through hybridization (Meilink et al. 2015; van den Burg et al. 2023). In fact, invasive hybridization (hybridization between native and IAS) is underappreciated, but significant cause of species decline (Vuillaume et al. 2015), leading to the extinction of native genotypes, eliminating local adaptations or entire species and ultimately homogenizing biodiversity (Largiadèr 2008). The consequences of invasive hybridization can thus have far-reaching consequences, particularly when native individuals show lower fitness than their hybrid counterparts (Prentis et al. 2008). Therefore, for impactful conservation intervention, the need for early detection of hybridization or, depending on the stage of invasion, detection of (presumably rare) surviving pure native animals, is more urgent than ever.

One of the many indigenous and enigmatic insular species groups threatened by invasive

hybridization are West Indian Iguanas (Alberts et al. 2004). Herbivorous reptiles such as iguanas perform fundamental ecosystem services via herbivory, nutrient cycling, and seed dispersal of indigenous plants (Cooper and Vitt 2002; Traveset et al. 2016; Valido and Olesen 2019). The role of iguanas as keystone-species highlights the need for the protection of dry coastal and elevated coastal forests with their corresponding communities, as these threatened environments also harbor several other endemic species. Preservation of these communities for iguanas will in-turn contribute to the mitigation of the dramatic loss of biodiversity in the Caribbean (Knapp et al. 2014; Thibaudier et al. 2024).

The Caribbean Lesser Antilles are inhabited by high iguanid diversity, though these native populations can still hybridize with non-native iguanas (Stephen et al. 2013). In particular, the critically endangered Lesser Antillean Iguana (*Iguana delicatissima*) is at risk of hybridizing with non-native Common Green Iguanas (*Iguana iguana*), whilst its populations are also threatened by other anthropogenic factors (van den Burg et al. 2018a). *Iguana delicatissima* represents a unique component of Caribbean biodiversity, though only five populations remain that are free of the immediate threat of hybridization with non-native iguanas (van den Burg et al. 2018a; Pounder et al. 2020). St. Eustatius is home to one of the few remaining and very small *I. delicatissima* populations, with a recent estimate of ~1000 individuals (van den Burg et al. 2022). The compounded effect of invasive Green Iguanas and their ensuing hybridization on St. Eustatius is complicating population recovery (van den Burg et al. 2018b).

Displacement by, and hybridization with, *I. iguana* are the dominant factors in the decline of *I. delicatissima* across its range, with few island populations remaining that are not directly threatened by invasive *I. iguana* (Pounder et al. 2020). These negative processes have already led to the extinction of several *I. delicatissima* populations, e.g., several islands within the Guadeloupe Archipelago and in southern Martinique (Vuillaume et al. 2015). In fact, not only is identification and removal of invasive iguanas directly needed, the speed and spread of invasive hybridization is a compounding practical conservation hurdle. Hybridization initially poses a less visible threat, but quickly escalates once the invasive population grows.

Such rapid effects to *I. delicatissima* population sustainability and recovery prompts immediate choices for impactful detection and extraction.

Commonly used methods for individual detection and population monitoring are transect-based morphological surveys. These conventional surveys, however, often have low detection rates when applied to rare or cryptic species (Crawford et al. 2020; Matthias et al. 2021). Conventional morphologically-based surveys often require hand-capture and individual genotyping, causing risk to caught organisms, as well as much time and cost for accurate processing (Stewart and Taylor 2020). Importantly, morphologically-based surveys are also particularly unreliable for identifying hybrid individuals. Breuil (2013) and Vuillaume et al. (2015) have shown that even animals identified in the field as *I. delicatissima* were sometimes hybrid or introgressed individuals, and although recent research (van den Burg et al. 2024) has identified additional morphological characteristics that might be used to detect hybrids, detailed data needed for its application and a study on its broader utility remain lacking. Since morphology still does not provide identification certainty, there remains a need for alternative monitoring techniques for the detection of rare invasive or hybrid individuals. This is particularly true early during invasion before they spread and multiply, when eradication efforts are likely to have some chance at success (e.g., Debrot et al. 2022). Similarly, the detection of rare surviving native animals for removal to artificial breeding programs has high conservation value (e.g., Milinkovitch et al. 2013; Miller et al. 2017; Pounder et al. 2020).

The application of environmental DNA (eDNA) is a well-suited and powerful, yet relatively underused tool in terrestrial biodiversity monitoring (Barnes and Turner 2015; Thomsen and Sigsgaard 2019; Valentin et al. 2021; Banerjee et al. 2022; Gregorič et al. 2022; Lyman et al. 2022; Wilcox et al. 2022; Aucone et al. 2023; Lynggaard et al. 2024). This is surprising considering it is non-invasive, non-destructive, time and cost efficient, and eliminates the considerable sampling effort and animal-handling expertise often needed with conventional methods (Stewart et al. 2017; Qu and Stewart. 2019; Didaskalou et al. 2022). Fortunately, sampling of the terrestrial environment for reptile eDNA is a growing field of research (Hunter et al. 2015; Kucherenko et al. 2018; Katz et al. 2021; Kyle et al. 2022; Nordstrom et al. 2022;

Bell et al. 2024). Our objective is to advance such methods with the use of Kompetitive Allele-Specific PCR (KASP) to map putative invasive hybridization. For this, we aim to optimize species-specific markers for the use of eDNA sampling within terrestrial environments, by first testing these markers on blood samples from native, invasive and hybrid individuals, and subsequently testing these approaches both ex-situ (zoo enclosure) and in-situ (St. Eustatius). Additionally, we investigate the dynamics of terrestrial eDNA, including different sampling approaches and substrates, aiming to enhance our understanding of its suitability as a detection tool.

## Methods

To address our research objectives, we developed species-specific KASP assays. KASP involves fluorescent allele-specific amplification through which species-specific Single Nucleotide Polymorphisms (SNP) variants (homozygous: parental species; heterozygous: hybrids) are determined with two allele-specific primers, requiring no sequencing (Semagn et al. 2013). Testing relied on blood sample assays, and we conducted eDNA sampling both ex-situ at the Rotterdam Zoo and in-situ on the island of Sint Eustatius. Employing surface swabbing and tape-lifting techniques, we collected eDNA samples to assess the efficacy of our assays and compared the levels of positive and negative detection outcomes to determine an optimal sampling strategy.

## Sample collections

To accurately identify and differentiate between pure *I. delicatissima* and hybrids with *I. iguana*, we obtained blood samples derived from previous studies and Rotterdam Zoo collections, all from various genetically confirmed individuals (van den Burg et al. 2018b, 2023) (Appendix I). We included two *I. iguana* specimens from Bonaire and Saba (samples: TBB65, SAB46), three *I. delicatissima* specimens from St. Eustatius (samples: EUX4b, Z18114, Z0208), and one confirmed *I. iguana* x *I. delicatissima* hybrid specimen from St. Eustatius (sample: H3). These samples had been stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  prior to extraction.

To test the efficacy of terrestrial eDNA collections for iguana detections, we first collected different ex-situ samples consisting of sterile swabs (QIAGEN, Germany) and tape-lifts (sterile PCR film, Sarstedt), from an *I. delicatissima* exhibit at the Rotterdam Zoo (Rotterdam, the Netherlands). The exhibit housed two adult *I. delicatissima* individuals, one male and one female. The exhibit itself consisted of an enclosed large terrarium located within the Aquarium building of the zoo, kept at a stable 27 °C with a relative humidity ranging between 65 and 80%. A misting system was used to maintain the desired humidity, with additional showers (two times a week) with a garden hose. A natural lighting cycle was provided via a transparent ceiling, supplemented with additional UV lights from 7:30 am till 6:30 pm. The interior of the exhibit contained artificial plants and several branches, as well as rock claddings to provide a safe retreat from the viewing window (Fig. 1).

All exhibit swabs (n=60) were taken from the available environmental surfaces following the sampling protocol of Valentin (2020). A total of 30 dry swabs and 30 wet swabs were taken from three different surfaces: branches, artificial plants, and artificial

rock claddings. The swabs were taken with an even pressure by rubbing the swab flat against the aforementioned surfaces in triplicate. The replicates were taken right next to the original sample to minimise the risk of lowering eDNA concentrations by sampling on the exact same spot. A parallel number of 10 wet samples were taken by spraying deionized water on the environmental surface prior to swabbing. Once swabbed, samples were stored in 1 ml Longmire's Solution (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 0.5% SDS, 0.2% sodium azide) in 2 ml Eppendorf tubes to stabilize the available eDNA, and subsequently stored at -20 °C (Longmire et al. 1987). Blank field swabs (both dry and wet) were taken during sampling to track potential contamination.

Sterile PCR plate seals were used as tape-lifts to sample potential eDNA from branches, artificial plants, and artificial rocks in triplicate. The replicates were taken as explained previously. Wet samples (N=5) and dry samples (N=5) were taken of three different surfaces (broad leaves, branches, and rock claddings), where the area for the wet samples was sprayed with deionized water prior to taking the sample. Samples were obtained by wrapping a



**Fig. 1** *Iguana delicatissima* exhibit at the Rotterdam Zoo (ex-situ sampling site). Image taken from main viewing window. Image taken by J. van Kuijk



sterile PCR plate seal tightly over the desired surface to maximize the contact area. Tape lifts were then gently peeled loose and placed in a sterile 50 ml collection tube containing 30 ml Longmire's solution with sterile forceps.

Opportunistic environmental samples were taken in-situ at two locations on St. Eustatius (Fig. 2). There, upon encountering an iguana (identified as *I. delicatissima* based on morphology) perching on a branch (n=1) or resting on concrete ground (n=1), surface-samples were taken following the protocol of Valentin (2020). Wet and dry swabs were taken in duplicate for both locations, together with a wet and dry negative control during sampling (n=8). Once swabbed, samples were stored in 1 ml Longmire's in 2 ml Eppendorf tubes to stabilize the available eDNA and placed in a refrigerator on St. Eustatius then stored at -20 °C in the Netherlands.

### Sample extraction

DNA from all samples, blood as well as ex-situ and in-situ eDNA samples, were extracted via the isopropanol DNA extraction protocol from the Promega Wizard® Genomic DNA purification kit. Sample material was digested in 300 µl of Nuclei Lysis Solution (Promega) and 3.0 µl Proteinase K. Proteins were precipitated with 100 µl Protein Precipitation Solution (Promega). The supernatant was transferred to a new microcentrifuge tube and 300 µl of 100% isopropanol (kept at -20 °C) was added to precipitate the DNA pellet. The DNA pellet was washed with 300 µl 70% ethanol and resuspended in 100 µl TE buffer (Fischer). To give us an estimate of how much general eDNA is collected during sampling, total eDNA concentrations were estimated using a Nanodrop (ND-1000 UV-Vis Thermo Scientific) and aliquoted as undiluted and diluted to 1:10, 1:20, 1:50, and 1:100 concentrations for testing in the KASP assay (see Primer Optimization).



**Fig. 2** Overview of the in-situ sampled surfaces after a successful iguana encounter. **A** Male *Iguana delicatissima*. **B** Sampled surface consisted of the perching spot (branch) of the

iguana in **A**. **C** Female *I. delicatissima*. **D** Second sampled surface consisted of the resting spot (concrete) of the iguana in **C**. Images taken by M.P. van den Burg

## Primer development

We used published and unpublished genetic data from across the Iguana range to assess the presence of species-diagnostic SNPs for several markers and designed small (< 150 bp) nuclear eDNA primers for downstream analysis (Stephen et al. 2013; Malone et al. 2017; van den Burg et al. in prep.). We focused on a subset of four nuclear markers: polymerase alpha catalytic subunit (PAC), neurotrophin subunit 3 (NT3), MutL homolog (MLH3), and the oocyte maturation factor (C-MOS) (Appendix II). Sequences were taken from GenBank (Accession numbers in Appendix II). Species diagnostic SNPs were determined by aligning and checking the sequences in Geneious Prime (version 2022.2).

The program Kraken (LGC, Biosearch Technologies) was then used to design two forward primers carrying the standard FAM and HEX tails, with the targeted SNP at the 3' end, and a common reverse primer. The HEX tail is connected to the *I. delicatissima* SNP, whereas the FAM tail is connected to the *I. iguana* SNP. The SNP sequences from the desired markers with 15 bp left-flanking sequence and 14 bp right-flanking sequence of each SNP site were used in this design.

## Primer optimization and sample testing

A total of four KASP markers were tested on whole blood samples (Appendix I) in 384 well plates with 3 µl KASP primer mix and 3 µl DNA template volume. PCR reactions were performed in a HydroCycler (LGC, Biosearch Technologies) using the following program: initial denature at 94 °C for 15 min, 10 touchdown cycles of 94 °C for 20 s and 61–55 °C (dropping 0.6 °C per cycle) for 1 min; followed by 26 cycles of 55 °C. Plates were read using the PHERAstar plate reader (BMG labtech), and recycled in the thermocycler with 3 additional annealing cycles of 57 °C.

All ex-situ and in-situ samples were tested and optimized in 384 well plates with a similar reaction set up of 3 µl KASP primer mix and different DNA template volumes (3 µl, 6 µl, and 9 µl). As KASP amplification has never before been performed on eDNA samples, we used this opportunity to optimize template volume for an effective assay (i.e. to amplify enough nuclear DNA for our SNPs). We

were also unsure of the impact of potential inhibitory substances, and thus further tested our samples at five different dilutions; pure, 1:10, 1:20, 1:50, 1:100. PCR reactions were performed using the following program: initial denature at 94 °C for 15 min, ten touchdown cycles of 94 °C for 20 s and 61–55 °C (dropping 0.6 °C per cycle) for 1 min; followed by 26 cycles of 55 °C. Plates were read using the PHERAstar plate reader (BMG labtech), and recycled in the thermocycler with four additional annealing cycles of 57 °C.

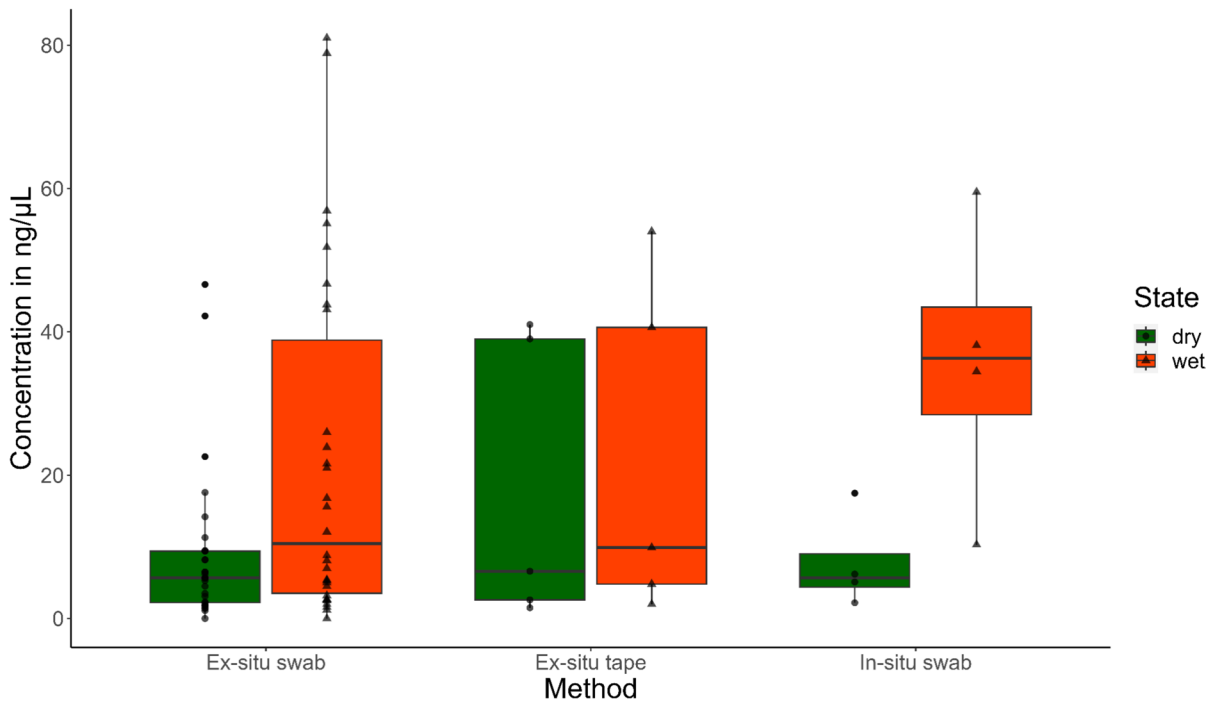
## Data analysis

Data was imported from the PHERAstar plate reader and visualized in RStudio (RStudio team, 2023) using ggplot2 (Wickham 2016) and Cowplot (Wilke 2020). The statistical analysis to determine differences between the sampling methods was done via *t*-tests due to this dataset demonstrating normality via Shapiro–Wilk's test. Comparisons between sample surfaces were made by testing for normality using Shapiro–Wilk's test. Further downstream analysis was achieved via the Kruskal–Wallis test, selected due to non-normal data distribution, with a post hoc Dunn's test from the FSA package (Ogle 2018) to determine differences between sample surfaces. The obtained *p*-values from the Kruskal–Wallis tests were adjusted to account for multiple testing via the benjamini–hochberg procedure.

## Results

### Collected DNA

All together, we were able to collect eDNA from both methods, both ex-situ from the zoo exhibit and in-situ from St. Eustatius. The average total eDNA concentration per sample method showed that ex-situ swabs yielded an eDNA concentration of 15.33 ng/µl ± 19.55 and the ex-situ tape-lift samples yielded 20.2 ng/µl ± 20.71, whereas the in-situ swabs yielded 21.67 ng/µl ± 20.37 (Fig. 3). It should be noted that Nanodrop concentration quantification does not discern whether it is measuring iguanid DNA or DNA from other organic sources. A one-way ANOVA did not reveal a significant difference between the total eDNA concentration and the different methods ( $F_{2,75} = 0.552, p > 0.05$ ).



**Fig. 3** Overview of the average total DNA concentration per sampling method in ng/μL, measured via Nanodrop

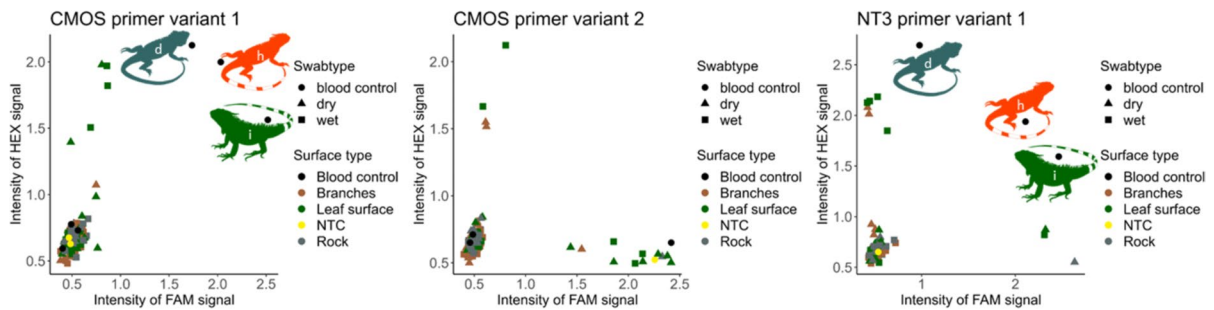
### KASP interpretation

For both ex-situ and in-situ tests, we used positive control samples from known *I. delicatissima*, *I. iguana* and hybrid individuals to ensure accuracy and reliability of our results. These control samples were accepted if they produced a signal that reached over a value of 1.0 on the KASP fluorescence graph, which was indicative of a strong, reliable match, with clusters based on allele-specific fluorescence signals. A result was considered positive for *I. delicatissima* when the fluorescence signal closely clustered to that of the control samples. This method allowed us to confidently differentiate between *I. delicatissima* and *I. iguana*, minimizing the risk of misclassification. Of the four nuclear genes tested, 10 SNPs in PAC, 17 SNPs in MLH3, 4 SNPs in NT3, and 2 SNPs in C-MOS, three SNPs were found to be functional in the KASP assays (1 SNP in CMOS-1, 1 SNP in CMOS-2, and 1 SNP in NT3).

### Ex-situ swabs

Ex-situ swabs from the Rotterdam Zoo exhibit were tested with KASP at a sample dilution of 1:20 with 2 different DNA template volumes (6 μL, and 9 μL), which resulted in 13 positive detections of *I. delicatissima* out of 62 swabs (10.5%). No significant correlation between sample total eDNA concentration and positive iguanid detections was found ( $R^2=0.01$ ,  $F=3.142$ ,  $df=178$ ,  $p>0.05$ ).

None of the samples tested positive on all three SNPs. The 6 μL template volume showed amplification at both CMOS-1 and NT3 primers (Fig. 4). The CMOS-2 assay was rejected from further analyses on ex-situ swabs due to non-functional positive controls and potential contamination on the 6 μL template volume plate. The 9 μL DNA template volume did not produce any reliable amplifications and was therefore likewise rejected in further analysis (Fig. 4). A *t*-test on the remaining individual amplifications of the 6 μL template DNA run did not reveal a significant difference in the detection of iguanid DNA between wet swabs and dry swabs ( $t$ -value=0.331,  $df=166$ ,  $p>0.05$ ).



**Fig. 4** Comparison of the different ex-situ surfaces of the 6  $\mu$ l template volume, on three different markers from the C-MOS gene and NT3 gene. Surfaces and Swab type did not have a significant influence on the strength of the HEX signal. (d)

*Iguana delicatissima*, (i) *Iguana iguana*, and (h) *delicatissima* x *iguana* hybrid. The NTC stands for the non template control to detect potential contaminations

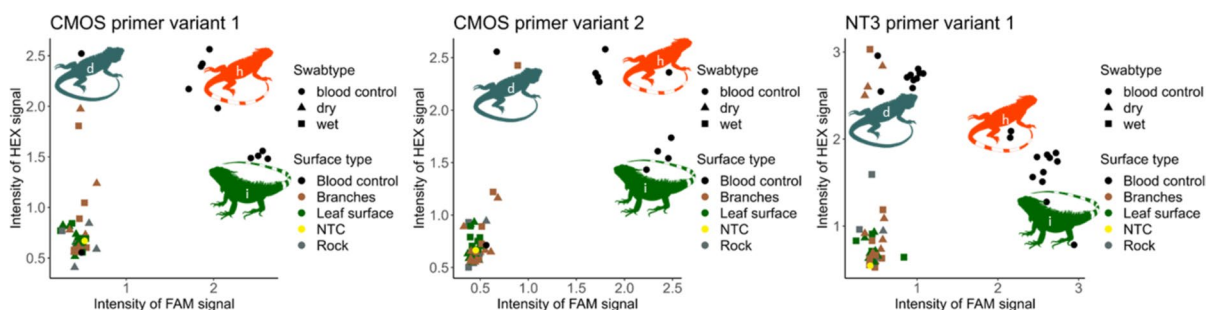
A Kruskal–Wallis multiple comparison was conducted to compare the effects of different surfaces on the HEX signal strength. The data included samples tested on three surfaces (Branches, Leaf surfaces, Rocks) and two markers (CMOS-1 and NT3) (Fig. 4). A threshold was set at a HEX signal strength of 1.0, removing all samples below 1.0 that did not show amplification. No significant difference between the three sampled surfaces was found ( $X^2=4.1511$ ,  $df=2$ ,  $p>0.05$ ).

#### Ex-situ tape-lift samples

Three different surfaces (Branches, Leaf surfaces, and Rocks) were tested for iguana eDNA via tape-lifting, with ten PCR seals as sampling medium, and compared to determine the best sampling surface (Fig. 5). A similar threshold was set at a HEX signal strength of 1.0, removing all samples below 1.0 that did not

show amplification. The 6  $\mu$ l DNA template volume resulted in a total of 14 positive detections (28%), with two samples that tested positive on all three SNPs (samples: TD1, TD2) while tested with undiluted samples. It is important to note that the ex-situ tape-lift CMOS-2 assay included functional positive controls and was therefore incorporated. No significant correlation between sample total eDNA concentration and positive iguanid detections was found ( $R^2=0.03$ ,  $F=2.749$ ,  $df=147$ ,  $p>0.05$ ).

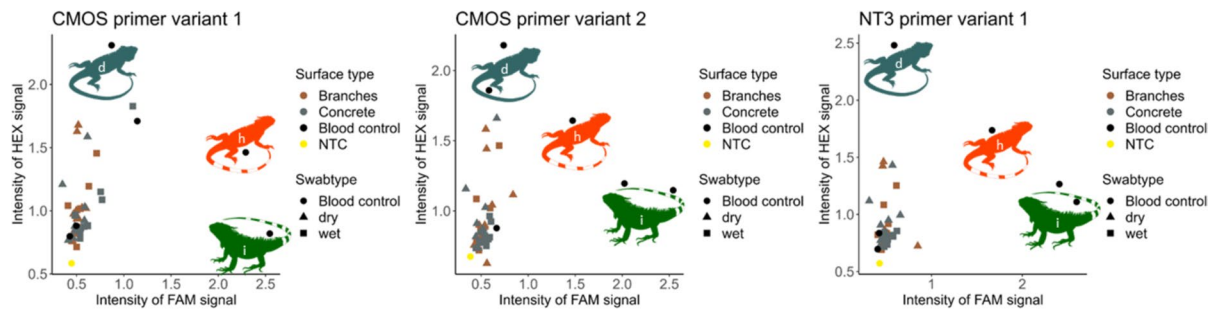
A Kruskal–Wallis multiple comparison was used to compare the three sampled surfaces ( $X^2=18.087$ ,  $df=2$ ,  $p<0.001$ ). A post hoc Dunn's Test revealed a significant increase of on branches compared to the other surfaces after multiple testing correction (benjamini-hochberg,  $p<0.05$ ). A  $t$ -test revealed no significant difference in the detection of iguanid eDNA between wet swab and dry swab sampling ( $t$ -value=0.852,  $p$ -value>0.05,  $df=31$ ).



**Fig. 5** Comparison of the different ex-situ surfaces sampled, combined with a comparison of sample types (wet/dry tape lifts), tested on three different markers from the C-MOS gene

and NT3 gene. (d) *Iguana delicatissima*, (i) *Iguana iguana*, and (h) *delicatissima* x *iguana* hybrid. The NTC stands for the non template control to detect potential contaminations





**Fig. 6** Comparison of the different in-situ different surfaces, combined with a comparison of sample types (wet/dry swabs), tested on three different markers from the C-MOS gene and NT3 gene. (d) *Iguana delicatissima*, (i) *Iguana iguana*, and (h)

*delicatissima x iguana* hybrid. Swabs were taken from the surface on which the iguana was encountered. The NTC stands for the non template control to detect potential contaminations

### In-situ samples

A total of eight different in-situ swabs were sampled on the island of St. Eustatius. The 9  $\mu$ l DNA template volume showed high amplification with the pure samples across all SNPs (Fig. 6) and resulted in a total of 31 positive detections (77.5%), with 6 undiluted samples tested positive across all three SNPs (samples: 1Wa, 1Wb, 1 Da, 1Db, 2 Da and 2Db). It is important to note that the in-situ CMOS-2 assay included functional positive controls and was therefore incorporated. A significant correlation between sample total eDNA concentration and positive detections was found ( $R^2=0.18$ ,  $F=13.02$ ,  $p<0.05$ ,  $df=147$ ). The outcomes of the two sampled surfaces (Branches, Concrete) were compared with a Kruskal–Wallis multiple comparison to determine a difference in outcomes. A threshold was set at a HEX signal strength of 1.0 to remove all samples below that did not show amplification. No significant difference between the sampled surfaces was found ( $X^2_{8,1}=0.0369$ ,  $p>0.05$ ). Swab types were also compared with a  $t$ -test to determine differences in swab types. No significant difference in the detection of iguanid eDNA between

wet and dry swab sample collection was found ( $t=-1.688$ ,  $df=147$ ,  $p>0.05$ ).

### Overview

In summary (see Table 1), we found that 16,1% of the ex-situ swabs resulted in positive detections for two SNPs (the third SNP marker failed), tested with a templated volume of 6  $\mu$ l and a dilution of 1:20. Within the ex-situ tape-lift samples, we found that 28% resulted in positive detections, when tested with a 6  $\mu$ l template volume and in a 1:20 dilution. Finally, we found that 77.5% of the in-situ samples resulted in positive detections for all three SNPs, while testing undiluted samples with a template volume of 9  $\mu$ l. Furthermore, a significant correlation was found between the total eDNA concentration of the in-situ samples and the number of positive detections.

### Discussion

Here, we develop a new, non-invasive approach for the detection of invasive species and putative invasive

**Table 1** Summary of the results from the different sample techniques used in this study. Included are the optimal DNA template volume, optimal sample dilution, and sample success rate for each sampling technique

Summary table	In-situ swabs	Ex-situ swabs	Ex-situ tape-lift
Optimal template volume	9 $\mu$ l DNA template	6 $\mu$ l DNA template	6 $\mu$ l DNA template
Optimal dilution	Undiluted samples	1:20	1:20
Markers	Cmos1, Cmos2 & NT3	Cmos1, NT3	Cmos1, Cmos2 & NT3
Percentage positive detections	77.5%	16.1%	28.0%

hybridization in iguanas using terrestrial eDNA. Specifically, we designed species-specific, nuclear SNPs, and examined different eDNA sampling strategies as well as investigated different substrate locations for terrestrial eDNA. Our results show that we are able to successfully detect the presence of *I. delicatissima* in ex-situ and in-situ samples collected from both swabs, and tape-lifting. This study thus provides the first proof-of-concept evidence that eDNA, a novel cost-effective and efficient sampling method, can be utilized to survey terrestrial habitats across different substrates for iguana presence, as well as possible invasive hybridization. Notably, this is also the second study (see Schabaker et al. 2020) to combine fast and low-cost KASP analysis to eDNA collections, helping to process and expedite bi-allelic nuclear data, a necessary first-step in detecting potential hybrid individuals.

The ability to detect rare non-native animals and their hybrids, or rare surviving native animals, is especially critical at different spectra of the invasion process and for opposite conservation reasons. For example, eradication of invasive animals at the onset of invasion, or the identification and rescue of last native survivors before they too are usurped when invasion proceeds to full extinction of the native populations, is vital for safeguarding biodiversity (e.g., Milinkovitch et al. 2013; Miller et al. 2017; Debrot et al. 2022; Pounder et al. 2020). The application of eDNA is thus likely to be of great value for early invasion confirmation, particularly for presumed presence or absence of the target species (Rout et al. 2014; Russell et al. 2017), be it invasive species or rare, threatened native taxa (Stewart et al. 2017). Additionally, eDNA enables the simultaneous monitoring of multiple species from a single sample, offering a comprehensive view of ecosystem health. Of note, using eDNA methods also eliminates the need for CITES permits, significantly speeding-up workflows.

While other terrestrial eDNA studies have used universal mitochondrial markers that have a high success rate due to high copy number compared to nuclear DNA (Valentin et al. 2020; Andres et al. 2021; Lyet et al. 2021), we used custom-made nDNA SNP markers derived from previous studies (Malone et al. 2017; van den Burg et al. 2018b). This offered us more detailed insights into an individual's genotype, allowing for more in-depth genetic analysis and conservation strategies. However, during our

experiments, the markers PAC and MLH3 did not function as hoped. This issue may be attributed to the high density of SNPs closely clustered around the SNP of interest, leading to excessive individual variation and primer mismatches (Wilcox et al. 2013). Additionally, the presence of highly similar homoeologous or paralogous sequences could have contributed to these mismatches (Makhoul et al. 2020). As a result, both markers were excluded from the experiments. Furthermore, the C-MOS 2 marker showed inconsistent performance; it did not function in the ex-situ analysis in which we would expect high residual eDNA of interest, but worked as intended in the in-situ analysis. This discrepancy might be due to technical errors or contamination during preparation and processing of the ex-situ plate despite repeated tests. Though this seems unlikely, it certainly warrants further investigation.

These challenges might contribute to the observed low iguana eDNA detection rates in our study. A minority (c. 16%) of swabs taken at the iguana exhibit at the Rotterdam Zoo resulted in positive detection. One explanation for this low eDNA detection rate could be the insufficient presence of nuclear eDNA available on the sampled surface (Adams et al. 2019a, b; Sigsgaard et al. 2020) due to any number of possible biotic and abiotic forces dictating eDNA shedding, transport or degradation (Stewart 2019). An alternative explanation could be the shortage of iguana eDNA on artificial surfaces, or simply the concentration of eDNA in the samples we collected due to methodological shortcomings. Contrary to expectations, this study did not find a significant difference between swab types (wet or dry) and sample surfaces, whereas Valentin (2018) argued that water is the dominant mechanism of eDNA transport even for terrestrial systems. Sample collection via tape-lifting yielded a higher proportion of positive detections (28%) and as far as we know, has not been tested previously. Our findings indicate that branches, which serve as natural perching spots for iguanas, resulted in a significantly higher number of positive detections compared to rocks and leaves, suggesting that species-specific behaviour is important to consider for eDNA sampling. The in-situ samples from St. Eustatius directly taken after spotting an iguana had a considerably higher success rate for positive iguana eDNA detection (77.5%). It should be noted, however, that sampling directly after encounters ensured

minimal environmental degradation of the sample DNA.

In this study we found three successful nDNA SNPs that can be applied to genotype and distinguish putative hybrid iguanas via DNA samples derived from blood and eDNA. When comparing sampling methods, we observed no significant difference in swab types used for *Iguana* eDNA detection. In general, we found a tendency for perching spots such as broad branches to exhibit a higher rate of amplification when compared to other surfaces. A possible explanation for this phenomenon is that iguanid lizards mark their territory via waxy lipids released from their femoral pores (Alberts 1990). The femoral pores are holocrine secretory glands located on the thighs and are found in all members of the Iguanidae (Mayerl et al. 2015). Since iguana's mark perching spots as part of their territory, it is reasonable to assume that DNA is also deposited on these sites. Therefore, sampling perching spots or similar objects in the habitat that are likely to be marked with waxy lipids by iguanas could therefore increase the overall detection of iguanid lizards. Additionally, future studies could assess variation between sex and eDNA deposition as *Iguana* sexes are known to differ in femoral pore sizes (Bakhuys 1982).

We demonstrate the viability of detecting *Iguana* eDNA in terrestrial environments, from surfaces and vegetation using various sampling techniques. It should be noted that our markers and approach worked “in silico”, as well as with blood samples from both species and hybrids. However, we were only able to test environmental samples from *I. delicatissima* due to logistical constraints. We would recommend an additional study to test the approach on environmental samples from *I. iguana* and their hybrids. Sampling strategies should focus on the specific microhabitat use and life cycle of the target species (Adams et al. 2019a), and monitoring threatened or endangered species should prioritize the minimizing of potential sampling hazards to the focal organism or habitat. For example, we currently are unable to ascertain how long this *Iguana* eDNA lasts on environmental surfaces. If prolonged detection is possible, we foresee these terrestrial eDNA approaches to be useful in sampling environmental surfaces to prospect for potential invasive or hybrid

presence (i.e. sight unseen). This eDNA method could also be valuable in establishing the probability of hybrid individuals that do not demonstrate obvious morphological signs of genetic admixture. This would enable more targeted response measures after eDNA analysis. Additionally, rigorous validation and optimization steps of assays are required to enhance our understanding of any confounding factors that might influence the performance and efficiency of eDNA detection in cryptic taxa.

## Conclusion

This research is relevant to a broad spectrum of terrestrial biodiversity managers and researchers as it provides a practical solution for conservation practitioners, particularly those working to protect the Lesser Antillean Iguana and potentially other cryptic terrestrial species facing similar threats. The implications of our findings extend beyond the Caribbean, offering a methodology that could be adapted for use in other regions and for other species at risk of hybridization or extinction due to invasive species. We believe that our study will be of significant interest given the global relevance of biodiversity conservation and the increasing threat of invasive species. The novel application of terrestrial eDNA sampling techniques for species conservation represents a significant advancement in the field, offering a new tool that can enhance the speed and accuracy of conservation interventions.

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## Declarations

**Conflict of interest** The authors have not disclosed any competing interests.

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## References

- Adams CIM, Hoekstra LA, Muell MR, Janzen FJ (2019a) A brief review of non-avian reptile environmental dna (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA Under Field Conditions. *Diversity* 11:50. <https://doi.org/10.3390/D11040050>
- Adams CIM, Knapp M, Gemmell NJ et al (2019b) Beyond biodiversity: can environmental DNA (eDNA) Cut It as a population genetics tool? *Genes* 10:192. <https://doi.org/10.3390/GENES10030192>
- Alberts AC (1990) Chemical properties of femoral gland secretions in the desert iguana, *Dipsosaurus dorsalis*. *J Chem Ecol* 16:13–25. <https://doi.org/10.1007/BF01021264/METRICS>
- Alberts AC, Carter RL, Hayes WK (2004) Iguanas: Biology and Conservation. 374.
- Andres KJ, Sethi SA, Lodge DM, Andrés J (2021) Nuclear eDNA estimates population allele frequencies and abundance in experimental mesocosms and field samples. *Mol Ecol* 30:685–697. <https://doi.org/10.1111/mec.15765>
- Aucone E, Kirchgeorg S, Valentini A et al (2023) Drone-assisted collection of environmental DNA from tree branches for biodiversity monitoring. *Sci Robot*. <https://doi.org/10.1126/scirobotics.add5762>
- Bakhuys WL (1982) Size and sexual differentiation in the lizard Iguana iguana on a semi-arid island. *J Herpetol* 16(3):322–325
- Banerjee P, Stewart KA, Dey G, Antognazza CM, Sharma RK, Maity JP, Saha S, Doi H, de Vere N, Chan MWY (2022) Environmental DNA analysis as an emerging non-destructive method for plant biodiversity monitoring: a review. *AoB Plants* 14:4
- Barnes MA, Turner CR (2015) The ecology of environmental DNA and implications for conservation genetics. *Conserv Genet* 17:1–17. <https://doi.org/10.1007/S10592-015-0775-4>
- Bell KL, Campos M, Hoffmann BD, Encinas-Viso F, Hunter GC, Webber BL (2024) Environmental DNA methods for biosecurity and invasion biology in terrestrial ecosystems: progress, pitfalls, and prospects. *Sci Total Environ* 926:171810. <https://doi.org/10.1016/j.scitotenv.2024.171810>
- Bellard C, Cassey P, Blackburn TM (2016) Alien species as a driver of recent extinctions. *Biol Lett* 12(2):20150623. <https://doi.org/10.1098/RSLB.2015.0623>
- Bland LM, Böhm M (2016) Overcoming data deficiency in reptiles. *Biol Conserv*. <https://doi.org/10.1016/j.biocon.2016.05.018>
- Breuil M (2013) Caractérisation morphologique de l'iguane commun Iguana iguana (Linnaeus, 1758), de l'iguane des Petites Antilles Iguana delicatissima Laurenti, 1768 et de leurs hybrides. *Bull Soc Herp Fr* 147:309–346
- Van den Burg MP, Breuil M, Knapp C (2018a) *Iguana delicatissima*. The IUCN red list of threatened species 2018: eT10800A122936983. Available from: <https://doi.org/10.2305/IUCN.UK.2018-1.RLTS.T10800A122936983.en>. Accessed on 31 January 2023.
- Cooper WE, Vitt LJ (2002) Distribution, extent, and evolution of plant consumption by lizards. *J Zool* 257:487–517. <https://doi.org/10.1017/S0952836902001085>
- Cordier JM, Aguilar R, Lescano JN et al (2021) A global assessment of amphibian and reptile responses to land-use changes. *Biol Conserv* 253:108863. <https://doi.org/10.1016/J.BIOCON.2020.108863>
- Crawford JA, Dreslik MJ, Baker SJ et al (2020) Factors affecting the detection of an imperiled and cryptic species. *Diversity* 12:177. <https://doi.org/10.3390/D12050177>
- Cushman SA (2006) Effects of habitat loss and fragmentation on amphibians: a review and prospectus. *Biol Conserv* 128:231–240. <https://doi.org/10.1016/J.BIOCON.2005.09.031>
- Debrot AO, Boman BE, Madden H (2022) Case study of a rapid response removal campaign for the invasive alien green iguana. *Iguana Iguana Manag of Biol Invasions* 13(2):449–465. <https://doi.org/10.3391/mbi.2022.13.2.11>
- Didaskalou EA, Trimpos KB, Stewart KA (2022) Environmental DNA. *Curr Biol*. <https://doi.org/10.1016/j.cub.2022.09.052>
- Gregorič M, Kutnjak D, Bačnik K et al (2022) Spider webs as eDNA samplers: biodiversity assessment across the tree of life. *Mol Ecol Resour* 22:2534–2545. <https://doi.org/10.1111/1755-0998.13629>
- Hunter ME, Oyler-McCance SJ, Dorazio RM et al (2015) Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive burmese pythons. *PLoS ONE* 10(4):e0121655. <https://doi.org/10.1371/journal.pone.0121655>
- Katz AD, Harper LR, Sternhagen EC et al (2021) Environmental DNA is effective in detecting the federally threatened Louisiana Pinesnake (*Pituophis ruthveni*). *Environ DNA* 3:409–425. <https://doi.org/10.1002/EDN3.126>



- Knapp C, Breuil C, Rodriguez C et al (2014) Lesser Antillean Iguana: *Iguana delicatissima*. Conservation Action Plan, 2014–2016.
- Kucherenko A, Herman JE, Iii EME, Urakawa H (2018) Terrestrial snake environmental DNA accumulation and degradation dynamics and its environmental application. *Herpetologica* 74:38–49. <https://doi.org/10.1655/HERPETOLOGICA-D-16-00088>
- Kyle KE, Allen MC, Dragon J et al (2022) Combining surface and soil environmental DNA with artificial cover objects to improve terrestrial reptile survey detection. *Conserv Biol* 36(6):e13939
- Largiadèr CR (2008) Hybridization and introgression between native and alien species. *Biol Invasions* 193:275–292. [https://doi.org/10.1007/978-3-540-36920-2\\_16](https://doi.org/10.1007/978-3-540-36920-2_16)
- Longmire JL, Albright KL, Lewis AK et al (1987) A rapid and simple method for the isolation of high molecular weight cellular and chromosome-specific DNA in solution without the use of organic solvents. *Nucleic Acids Res* 15:859–859. <https://doi.org/10.1093/NAR/15.2.859>
- Lyet A, Pellissier L, Valentini A et al (2021) (2021) eDNA sampled from stream networks correlates with camera trap detection rates of terrestrial mammals. *Sci Rep* 11:1–14. <https://doi.org/10.1038/s41598-021-90598-5>
- Lyman JA, Sanchez DE, Hershauer SN, Sobek CJ, Chambers CL, Zahratka J, Walker FM (2022) Mammalian eDNA on herbaceous vegetation? Validating a qPCR assay for detection of an endangered rodent. *Environ DNA* 4:1187–1197. <https://doi.org/10.1002/edn3.331>
- Lynggaard C, Frøslev TG, Johnson MS, Olsen MT, Bohmann K (2024) Airborne environmental DNA captures terrestrial vertebrate diversity in nature. *Mol Ecol Resources* 24:e13840. <https://doi.org/10.1111/1755-0998.13840>
- Makhoul M, Rambla C, Voss-Fels KP et al (2020) Overcoming polyploidy pitfalls: a user guide for effective SNP conversion into KASP markers in wheat. *Theor Appl Genet* 133:2413–2430. <https://doi.org/10.1007/S00122-020-03608-X>
- Malone CL, Reynoso VH, Buckley L (2017) Never judge an iguana by its spines: systematics of the Yucatan spiny tailed iguana, *Ctenosaura defensor* (Cope, 1866). *Mol Phylogenet Evol* 115:27–39. <https://doi.org/10.1016/J.YMPEV.2017.07.010>
- Matthias L, Allison MJ, Maslovat CY et al (2021) Improving ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under artificial cover objects. *Ecol Indic* 131:108187. <https://doi.org/10.1016/J.ECOLIND.2021.108187>
- Mayerl C, Van Damme R, Baeckens S (2015) Evolution and role of the follicular epidermal gland system in non-ophidian squamates. *Amphib-Reptil* 36:185–206. <https://doi.org/10.1163/15685381-00002995>
- Meilink WRM, Arntzen JW, van Delft JJCW, Wielstra B (2015) Genetic pollution of a threatened native crested newt species through hybridization with an invasive congener in the Netherlands. *Biol Conserv* 184:145–153. <https://doi.org/10.1016/J.BIOCON.2015.01.022>
- Milinkovitch MC, Kanitz R, Tiedemann R, Tapia W, Llerena F, Caccone A, Gibbs JP, Powell JR (2013) Recovery of a nearly extinct Galapagos tortoise despite minimal genetic variation. *Evol Appl* 1752–4571:377–383
- Miller JM, Quinzin MC, Poulakakis N, Gibbs JP, Beheregaray LB, Garrick RC et al (2017) Identification of genetically important individuals of the rediscovered Floreana Galápagos Giant Tortoise (*Chelonoidis elephantopus*) provides founders for species restoration program. *Sci Rep* 7:11471
- Nordstrom B, Mitchell N, Byrne M, Jarman S (2022) A review of applications of environmental DNA for reptile conservation and management. *Ecol and Evol* 12(6):e8995
- Ogle, 2018 DH Ogle (2018) Introductory fisheries analyses with r <https://doi.org/10.1201/9781315371986>
- Pounder K, Mukhida F, Brown R et al (2020) Testing for hybridisation of the critically Endangered *Iguana delicatissima* on Anguilla to inform conservation efforts. *Conserv Genet* 21:405–420. <https://doi.org/10.1007/s10592-020-01258-6>
- Prentis PJ, Wilson JR, Dormontt EE et al (2008) Adaptive evolution in invasive species. *Trends Plant Sci* 13:288–294. <https://doi.org/10.1016/J.TPLANTS.2008.03.004>
- Qu C, Stewart K (2019) Evaluating monitoring options for conservation: comparing traditional and environmental DNA tools for a critically endangered mammal. *Sci Nat* 106:9. <https://doi.org/10.1007/s00114-019-1605-1>
- Ricciardi A, Blackburn TM, Carlton JT et al (2017) Invasion science: a horizon scan of emerging challenges and opportunities. *Trends Ecol Evol* 32:464–474. <https://doi.org/10.1016/J.TREE.2017.03.007>
- Rout TM, Kirkwood R, Sutherland DR, Murphy S, McCarthy MA (2014) When to declare successful eradication of an invasive predator? *Anim Conserv* 17(2):125–132. <https://doi.org/10.1111/acv.12065>
- Russell JC, Binnie HR, Oh J, Anderson DP, Samaniego-Herrera A (2017) Optimizing confirmation of invasive species eradication with rapid eradication assessment. *J Appl Ecol* 54(1):160–169
- Schabaker JC, Amish SJ, Ellis BK, Gardner B, Miller DL, Rutledge EA, Sepulveda AJ, Luikart G (2020) Increased eDNA detection sensitivity using a novel high-volume water sampling method. *Environmental DNA* 2(2):244–251. <https://doi.org/10.1002/edn3.63>
- Semagn K, Babu R, Hearne S, Olsen M (2013) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. *Mol Breeding* 33:1–14. <https://doi.org/10.1007/s11032-013-9917-x>
- Sigsgaard EE, Jensen MR, Winkelmann IE et al (2020) Population-level inferences from environmental DNA—Current status and future perspectives. *Evol Appl* 13:245–262. <https://doi.org/10.1111/EVA.12882>
- Stephen CL, Reynoso VH CWS, Hasbun CR, Breinholt JW (2013) Geographical structure and cryptic lineages within common green iguanas. *Iguana Iguana J Biogeogr* 40(1):50–62
- Stewart KA (2019) Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. *Biodivers Conserv* 28:983–1001

- Stewart KA, Taylor SA (2020) Leveraging eDNA to expand the study of hybrid zones. *Mol Ecol* 29(15):2768–2776. <https://doi.org/10.1111/mec.15514>
- Stewart K, Ma H, Zheng J, Zhao J (2017) Using environmental DNA to assess population-wide spatiotemporal reserve use. *Conserv Biol* 31:1173–1182. <https://doi.org/10.1111/COBI.12910>
- Thibaudier J, Debrot AO, van den Burg MP (2024) Inquilines highlight overlooked keystone species role for *Iguana delicatissima* Laurenti, 1768 as ecosystem engineer. *Herpetology Notes* 17:571–577
- Thomsen PF, Sigsgaard EE (2019) Environmental DNA metabarcoding of wild flowers reveals diverse communities of terrestrial arthropods. *Ecol Evol* 9:1665–1679. <https://doi.org/10.1002/ECE3.4809>
- Traveset A, Nogales M, Vargas P et al (2016) Galápagos land iguana (*Conolophus subcristatus*) as a seed disperser. *Integr Zool* 11:207–213. <https://doi.org/10.1111/1749-4877.12187>
- Valentin RE, Fonseca DM, Nielsen AL et al (2018) Early detection of invasive exotic insect infestations using eDNA from crop surfaces. *Front Ecol Environ* 16:265–270. <https://doi.org/10.1002/FEE.1811>
- Valentin RE, Fonseca DM, Gable S et al (2020) Moving eDNA surveys onto land: strategies for active eDNA aggregation to detect invasive forest insects. *Mol Ecol Resour* 20:746–755. <https://doi.org/10.1111/1755-0998.13151>
- Valentin RE, Kyle KE, Allen MC et al (2021) The state, transport, and fate of aboveground terrestrial arthropod eDNA. *Environ DNA* 3:1081–1092. <https://doi.org/10.1002/EDN3.229>
- Valido A, Olesen JM (2019) Frugivory and seed dispersal by lizards: a global review. *Front Ecol Evol* 7:49. <https://doi.org/10.3389/FEVO.2019.00049/BIBTEX>
- Van Den Burg MP, Meirmans PG, Van Wagensveld TP et al (2018b) The lesser antillean iguana (*Iguana delicatissima*) on St. Eustatius: genetically depauperate and threatened by ongoing hybridization. *J Hered* 109:426–437. <https://doi.org/10.1093/JHERED/ESY008>
- Van Den Burg MP, Goetz M, Brannon L et al (2023) An integrative approach to assess non-native iguana presence on Saba and Montserrat: are we losing all native *Iguana* populations in the lesser Antilles? *Anim Conserv*. <https://doi.org/10.1111/ACV.12869>
- van den Burg MP, Madden H, van Wagensveld TP, Boman E (2022) Hurricane-associated population decrease in a critically endangered long-lived reptile. *Biotropica* 54:708–720. <https://doi.org/10.1111/btp.13087>
- Van den Burg MP, Kappelhof J, Mitchell A, Debrot AO (2024) Exploring the potential of morphometrics to inform the conservation of *Iguana delicatissima*. *Conservation* 4(4):617–626. <https://doi.org/10.3390/conservation4040038>
- Vuillaume B, Valette V, Lepais O et al (2015) Genetic Evidence of Hybridization between the Endangered Native Species *Iguana delicatissima* and the Invasive *Iguana iguana* (Reptilia, Iguanidae) in the Lesser Antilles: management Implications. *PLoS ONE* 10:e0127575. <https://doi.org/10.1371/JOURNAL.PONE.0127575>
- Wickham H (2016) ggplot2. <https://doi.org/10.1007/978-3-319-24277-4>
- Wilcox TM, Jensen MR (2022) Drawing a line in the sand: environmental DNA population genomics. *Mol Ecol Resour* 22(7):2455–2457
- Wilcox TM, McKelvey KS, Young MK et al (2013) Robust detection of rare species using environmental dna: the importance of primer specificity. *PLoS ONE* 8:e59520. <https://doi.org/10.1371/JOURNAL.PONE.0059520>
- Wilke C (2020) cowplot: streamlined plot theme and plot annotations for “ggplot2”

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