# An integrative approach to assess non-native iguana presence on Saba and Montserrat: Are we losing all native *Iguana* populations in the Lesser Antilles?

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## Abstract

Invasive alien species are among the main drivers of the ongoing sixth mass extinction wave, especially affecting island populations. Although the Caribbean is well-known for its high species richness and endemism, also for reptiles, equally important is the regional contribution of non-native species to island biodiversity. The Lesser Antilles encompass high genetic diversity in *Iguana*, though most native populations either have gone extinct or are declining following competitive hybridization with invasive non-native green iguanas. Here, we assessed non-native presence in two poorly-studied native melanistic *Iguana iguana* populations using available genetic tools and explored utilizing size-dependent body measurements to discriminate between native and non-native iguanas. Genetic samples from Saba and Montserrat were genotyped across 17 microsatellite loci with STRUCTURE, and multivariate analyses indicating non-native iguana presence only on Saba. This was corroborated by mtDNA and nDNA sequences, highlighting a non-native origin in Central America and the ABC islands. We identified preliminary evidence suggestive of hybridization. Morphological variation among size-dependent characteristics showed that non-native iguanas have significantly larger subtympanic plates than native iguanas. Non-native individuals also differed in scalation and coloration patterns. Overall, our findings demonstrate the need for continuous monitoring of non-native iguanas within remaining native *Iguana* populations in the Lesser Antilles, as those not directly threatened by non-native green iguanas are restricted to only 8.7% of the historic range. Although genetic data allow for the identification of non-native or hybrid iguana presence, this field-to-lab workflow is time-consuming. Rapid *in-situ* identification of non-native individuals is crucial for conservation management. In addition to patterns of scalation and coloration, we have highlighted the utility of size-dependent variables for rapid diagnosis. We urge regional partners to build morphometric databases for native *Iguana* populations allowing the quick detection of future incursions of non-native green iguanas and the rapid implementation of effective countermeasures during the early phase of invasion.

## Introduction

Invasive alien species (IAS) are among the most prominent threats to global biodiversity (Bax *et al.,* 2003; Butchart et al., 2010), contributing to what has been named the sixth mass extinction event (McGeoch *et al.,* 2010; Bellard,

Cassey, & Blackburn, 2016). Although natural introductions (e.g., following hurricanes) of non-native species have shaped current patterns of species diversity and biogeography (Heinicke, Duellman, & Hedges, 2007; Fonte, Mayer, & Lötters, 2019; Kennedy *et al.,* 2020), anthropogenically-mediated introductions continue to increase in frequency and

are redefining biogeographic patterns (Capinha *et al.,* 2015). IAS can impact native species through a range of mechanisms, for example, direct predation, competition, replacement, and hybridization, and their various combinations (Reaser *et al.,* 2007).

Isolated insular populations and species are especially vulnerable to IAS (Tershy *et al.,* 2015), including those of the Greater Caribbean region (Gleditsch *et al.,* 2022). Within this region, human impact, especially in terms of high inter-island transport and poor biosecurity, drives the high occurrence of non-native species, especially reptiles (Cox *et al.,* 2022; Jesse *et al.,* 2022). In addition, hurricane events can also translocate invasive alien reptile species to other islands (Censky, Hodge, & Dudley, 1998), as can recovery aid campaigns triggered by natural disasters (van den Burg *et al.,* 2021a).

Within the Greater Caribbean region, insular iguana species are being impacted by recently-arrived non-native iguanas (Knapp *et al.,* 2021). While on Little Cayman the intergeneric hybridization of the native *Cyclura nubila caymanensis* with non-native *Iguana iguana* is alarming (Moss *et al.,* 2017), the main invasive iguana hotspot is in the Lesser Antilles (Fig. 1). There, especially *Iguana delicatissima* has been impacted by non-native iguanas, which are considered the major factor behind its range-wide decline and its recent assignment as Critically Endangered (van den Burg, Breuil, & Knapp, 2018a). Non-native iguanas are currently competitively hybridizing and outcompeting native *I. delicatissima* populations throughout the French West Indies (Vuillaume *et al.,* 2015; Angin, 2017), on Anguilla (Pounder *et al.,* 2020), on St. Eustatius (van den Burg *et al.,* 2018b), and most recently also in the Commonwealth of Dominica (van den Burg, Brisbane, & Knapp, 2020). Besides *I. delicatissima*, several native populations of the *I. iguana* complex also occur on Lesser Antillean islands (Stephen *et al.,* 2013; Iguana Taxonomy Working Group *et al.,* 2016, 2022). Although these populations have received less attention, research and conservation interest in these native gene pools has increased recently.

Iguanas on St. Lucia, St. Vincent, and the Grenadines were recently described as subspecies and later as the species *Iguana insularis*, with two subspecies (Breuil *et al.,* 2019, 2022). The IUCN-SSC Iguana Specialist Group's Taxonomy Working Group (ITWG) currently does not recognize the proposed full species status, retaining the subspecies status for *Iguana iguana insularis* and *I. i. sanctaluciae* as originally described by Breuil *et al.* (2019) until such time at which further evidence may be published (Iguana Taxonomy Working Group *et al.,* 2022). Among these southern Lesser Antillean islands, non-native iguanas are also established on or have hybridized with native iguanas, on St. Lucia, St. Vincent, and several Grenadine islands (Fig. 1; Morton, 2008; Breuil *et al.,* 2019, 2022). For Grenada, some evidence suggests non-native presence, but no samples have been analyzed so far to confirm their presence and potential hybridization (Breuil *et al.,* 2019).

*Iguana melanoderma* was described by Breuil *et al.* (2020) for the Lesser Antillean iguana populations of Saba and Montserrat, while the ITWG still considers these as distinct subpopulations of *Iguana iguana iguana*, until further evidence calls for a revision may become available (Iguana Taxonomy Working Group *et al.,* 2022). Here, we acknowledge that the taxonomic position of these melanistic populations is under discussion and for now choose to follow the proposed ITWG taxonomy. Breuil *et al.* (2020) found no evidence for the presence of non-native iguanas on Saba and Montserrat based on morphological characters and limited microsatellite and mtDNA sequence data. However, more extensive yet preliminary data collected during 2021 suggested that a few non-native iguanas might have already been or since arrived on Saba (van den Burg, Madden, & Debrot, 2022). Reports of an apparent sudden increase in iguana numbers on Montserrat (pers. comm. Ernestine Corbett; pers. comm. Stephen Mendes) also give reason for concern about the possible presence and spread of non-native iguanas with higher reproductive potential.

Non-native presence and potential hybridization with native *Iguana* populations are mostly assessed using microsatellite and sequence data in conjunction with genetic databases for native populations (e.g., Vuillaume *et al.,* 2015; van den Burg *et al.,* 2018b, 2021b; Pounder *et al.,* 2020), most notably for hatchling and juvenile iguanas given their high morphological similarity between island populations. Although some comparisons on scale and coloration patterns can also be used (Breuil, 2013), the data underlying this reference dataset only cover a small part of the *Iguana iguana* complex and numerous characteristics are not diagnostically informative given the absence of quantitative data and lack of definitions for qualitative categories. These morphological characteristics are mostly useful to identify non-native *I. iguana* and *I. iguana* x *I. delicatissima* hybrids within native *I. delicatissima* populations (Vuillaume *et al.,* 2015; van den Burg *et al.,* 2018b, 2020; Pounder *et al.,* 2020). However, correct diagnostic characterization of hybrid status using these patterns is not guaranteed (Vuillaume *et al.,* 2015). Hence, non-native and hybrid identification within native *I. iguana* populations is more difficult, and additional characteristics should be explored to strengthen rapid *in-situ* field identification.

Here, based on preliminary data from Saba and Montserrat, we aimed to assess the presence of non-native iguanas on two of the last Lesser Antillean islands that are still home to native *Iguana* populations. For this, we implemented genetic reference tools representing most of the extended native range of *Iguana iguana* (spanning tropical South and Central America). We also explored the use of additional variables to identify non-native and hybrid iguanas by assessing size-corrected body measurements for native and non-native iguanas.

## Materials and methods

Fieldwork was conducted during August–September 2021 and July–December 2021 on Saba and Montserrat, respectively. We captured iguanas by means of lasso and pole, by hand, or using cage traps. We then collected photographic images of the entire body as well as all sides of the head, and collected a genetic sample, either blood (ventrally from
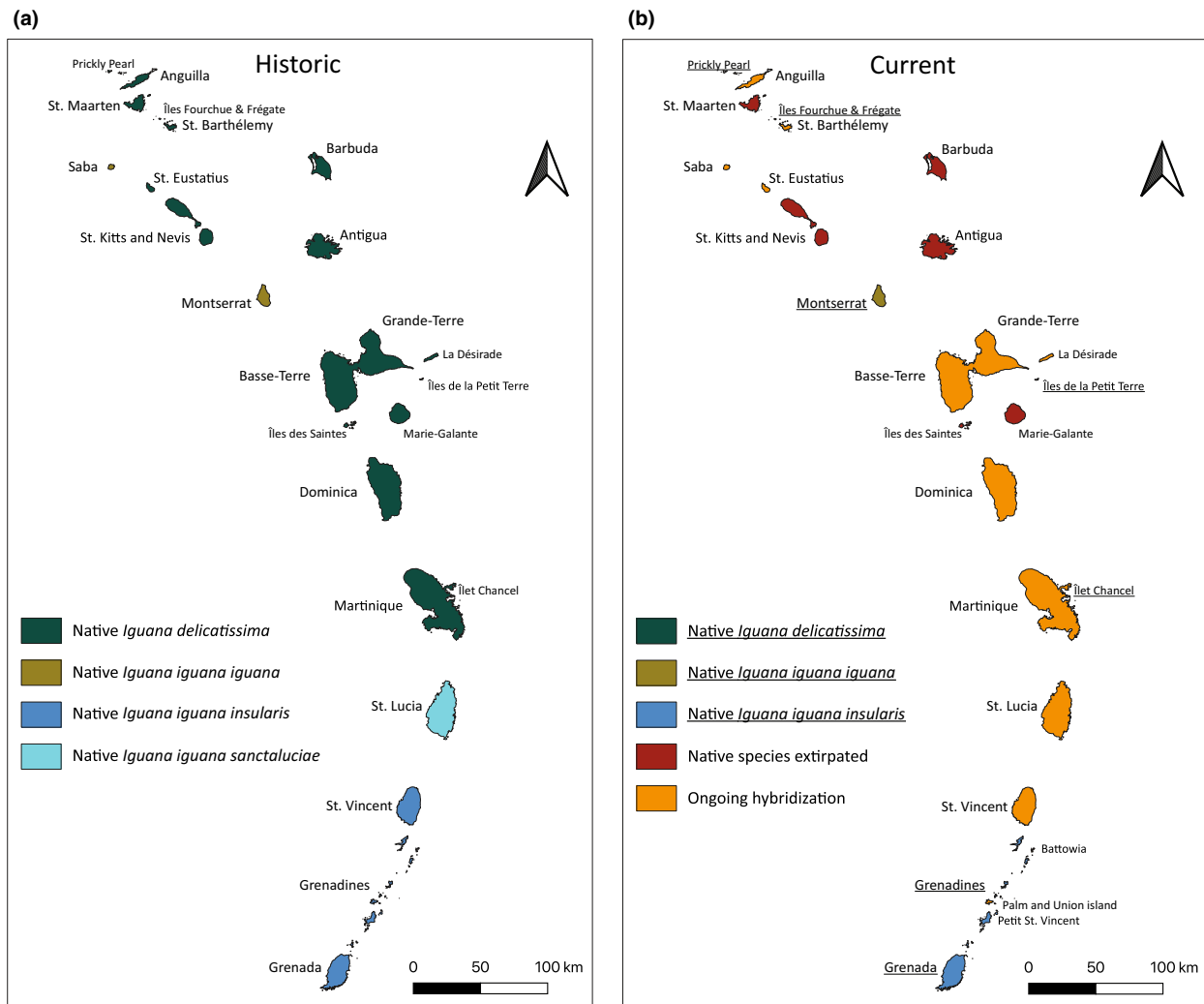
**Figure 1** Historic (a) and current (b) status of *Iguana* sp. populations within the Caribbean Lesser Antilles, including ongoing hybridization and extirpated populations. Taxonomy follows the Iguana Taxonomy Working Group Supplement 2022. Only the Grenadines islands with current hybrid populations are individually mentioned. Some areas on islands identified as having 'ongoing hybridization' still have isolated native populations

the caudal vein) or tissue (1 cm clip from largest dorsal spine), from the captured animals. On both Saba (58) and Montserrat (80), all caught iguanas were used in genetic analyses (Fig. 2a,b). Morphology was used to pre-identify potential non-native iguanas using the following characteristics: body coloration, the presence/absence of a melanistic patch between the eye and subtympanic plate, and the presence/absence of enlarged nasal scales (Breuil *et al.,* 2020).

On Saba, sampling was done opportunistically during a population assessment (van den Burg *et al.,* 2022), where we also collected the following morphometric measurements: snout-vent length, tail length, upper frontleg length, lower frontleg length, upper hindleg length, lower hindleg length, length of 4th toe, head width, head length, snout length, eye length, mouth length, head depth, tympanum height, tympanum width, subtympanic plate height, subtympanic plate width, mid-body spine length, and the presence or absence

of an enlarged nasal scale ('horn') (for methodology see Supplementary material); lateral facial measures were taken from the right side. All variables were measured given the knowledge of their SVL dependence and absence of allometry across the *I. iguana* species complex; topics that will be addressed in a separate manuscript (van den Burg et al. in prep.). All measurements were taken by the first author. Although melanism was defined as one of the main characteristics of these populations (Breuil *et al.,* 2020), a subsequent study on the Saba population found that very few animals were completely melanistic (van den Burg *et al.,* 2022). Therefore, knowledge about native body coloration is still in need of further assessment. Consequently, for this assessment, we sufficed by recording only the presence of a melanistic patch between the eye and tympanum in adult iguanas as the key distinguishing color characteristic (Gerber, 1999; Breuil *et al.,* 2020).
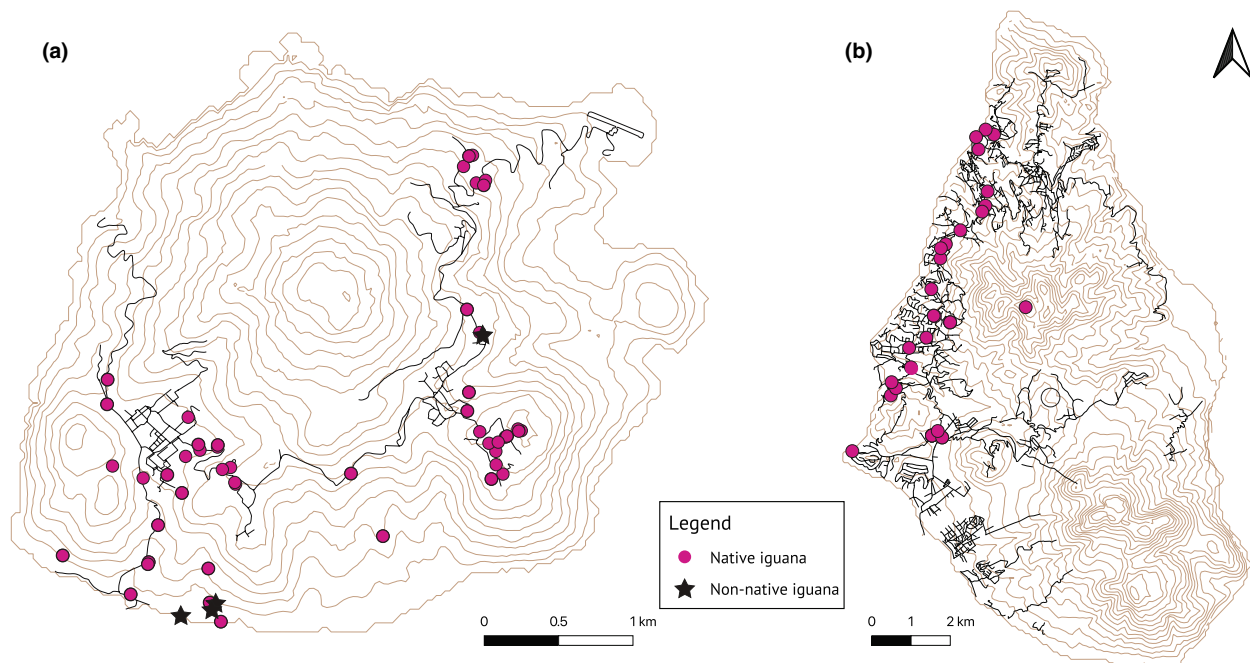
**Figure 2** Maps of Saba (a) and Montserrat (b) showing locations of collected genetic samples, and the distribution of non-native iguanas on Saba. Contour lines are at 50-meter intervals

Microsatellite laboratory procedures at Labofarm-GenIndexe (France) were used to amplify 17 microsatellite loci (see van den Burg *et al.*, 2021b), using identical methodologies as presented in Valette *et al.* (2013). Subsequently, PCR product analyses and allele scoring were performed following van den Burg *et al.* (2021b) and using sample IGD277 to standardize allele sizes.

A subset of five samples per island was then processed at the University of Amsterdam where DNA isolation and PCR methodology were performed to amplify the NADH dehydrogenase subunit 4 (ND4) and the MutL homolog 3 (MLH3) using PCR, following van den Burg *et al.* (2018b) and Malone, Reynoso, & Buckley (2017), respectively. These samples were selected based on microsatellite and morphology results being suggestive of non-native origin. Successful amplification was confirmed using gel electrophoresis, whereafter resulting PCR fragments were both reverse and forward-sequenced. Chromatograms were assessed, curated, and aligned using Geneious Prime (2021.1.1). Sequences were uploaded to GenBank (OQ556117-OQ556120), and the microsatellite data were added to the IguanaBase database (van den Burg *et al.*, 2021c).

## Data analyses

Genetic analyses using microsatellites were only performed on samples with <20% missing data. These samples were combinedly analyzed with those from IguanaBase (van den Burg *et al.*, 2021c) to assess the extent of clustering with samples previously collected on Saba and Montserrat, as well as to identify the potential presence of non-native iguanas and their geographic origin. First, we assessed non-native iguana presence

through the implementation of the predict.dapc (DAPC) function from the *adegenet* package (Jombart, 2008) within the R environment (R Core Team, 2022). This function assigns individuals to populations using a provided reference dataset, in this case IguanaBase. We performed multiple analyses on the reference data that differed in PCA axes inclusion in order to test assignment robustness. Thereafter, we performed an additional assignment analysis following van den Burg *et al.* (2021c) by running STRUCTURE (Pritchard, Stephens, & Donnelly, 2000) within GENODIVE (Meirmans, 2020) using identical settings for K (ranging from 1 to 8) and prepared subsequent visualizations using bar plots through Structure Harvester (Evanno, Regnaut, & Goudet, 2005; Earl & von Holdt, 2012) and distruct (Rosenberg, 2004). Given the large number of samples collected in 2021 compared to population sample sizes in IguanaBase, we ran STRUCTURE using half of the Saba and Montserrat samples, specifically including samples that were (partially) assigned as non-native following the DAPC analysis. The allele ranges for all loci were then visually compared with those published in IguanaBase (van den Burg *et al.*, 2021c), especially for those loci so far regarded as fixed in the Saba and Montserrat populations; L3, L8, L13, L16, L17, L24. Lastly, relative allelic richness was calculated for Saba and Montserrat separately using the *GenPopReport* package (Adamack & Gruber, 2014). Basemaps were created in QGIS 3.8.0 Zanzibar (QGIS.org, 2022) and finalized in Adobe Illustrator 25.3.1.

Mitochondrial (ND4) and nuclear (MLH3) sequence data were compared with data from the native *Iguana iguana* range available on GenBank and from a large unpublished dataset (van den Burg et al. unpublished data). For both

markers, the dataset included native samples from Saba and Montserrat, collected prior to 2010. Pairwise differences were used to identify available sequences with the highest nucleotide similarity and their geographic origin.

Morphological analyses were done on native iguanas by regressing individual length variables against SVL to assess size-dependence, whereafter we compared residuals of adults (>20 cm SVL) between sexes to assess potential sex-linked differences using *t*-tests. Analyses of these variables across the *Iguana iguana* complex showed their SVL dependence, with an absence of allometry (van den Burg et al. in prep). Variables were initially checked for normality and equality of variance. We repeated SVL regressions for a dataset including non-native iguanas and used the resulting residuals to assess differences between sex and species status. These were tested using a two-way ANOVA or Kruskal-Wallis test depending on the results from the homogeneity of variance and normality tests. Data handling and analyses were performed in RStudio Version 1.2.5033 (RStudio Team, 2019). Morphometric data are available through *figshare* (van den Burg *et al.,* 2023).

## Results

### Genetic results

#### Microsatellite data

Samples with <20% missing data included 57 individuals from Saba and 76 from Montserrat. DAPC posterior scores clustered all except four samples within the genetic morphospace from previously collected Saba and Montserrat samples, and minimally with *I. i. iguana* (Fig. 3). Specimen SAB58 was assigned to *I. i rhinolopha*, while population assignment of the other three Saba specimens differed depending on the number of included PCA axes. Affinities for the latter three specimens were as follows: SAB04: *I. aff. iguana* Clade I; SAB57: *I. i iguana*; SAB08: mixed partial assignments to *I. i rhinolopha*, *I. aff. iguana* Clade I, *I. i iguana*, as well as Saba and Montserrat. All except three specimens from Montserrat were consistently assigned to the IguanaBase group of Saba and Montserrat; Mont33, Mont39, and Mont40 were also assigned to *I. i. iguana*.

Results from STRUCTURE analyses for K = 6 (Figs. 4a, b), based on taxonomic and geographic clusters, indicated that all 2021-captured individuals had assignment scores of >0.90 for the cluster with melanistic *I. iguana* from Saba and Montserrat, except for four samples from Saba and three from Montserrat (marked with asterisk in Fig. 4b). For Saba, specimen SAB58 was assigned to *I. i. rhinolopha* (0.92), while the other three specimens indicated mixed origins for *I. i. rhinolopha* (SAB08, 0.42; SAB57, 0.29), *I. i. rhinolopha,* and *I. aff. iguana* Clade I (SAB04, 0.13, 0.43). For Montserrat, the three specimens (Mont33, 0.35; Mont39, 0.22; Mont40, 0.10) had partial assignments to *I. i. iguana* and to several reference samples from Montserrat collected before 2021.

Novel haplotypes for these populations were found across 15 loci (Fig. S1). Compared to previously genotyped
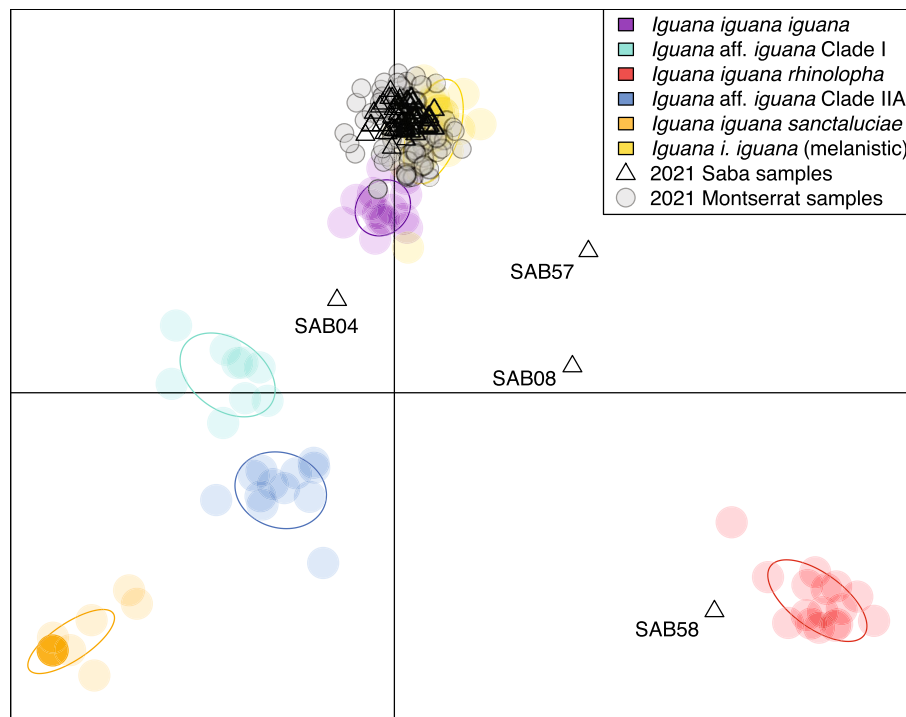


**Figure 3** Discriminant analysis of principal components (PC) scatterplot representing 17 microsatellite loci from reference IguanaBase individuals (colored circles) and assigned 2021-sampled individuals (57 iguanas from Saba and 76 from Montserrat; black triangles) in the PC morphospace
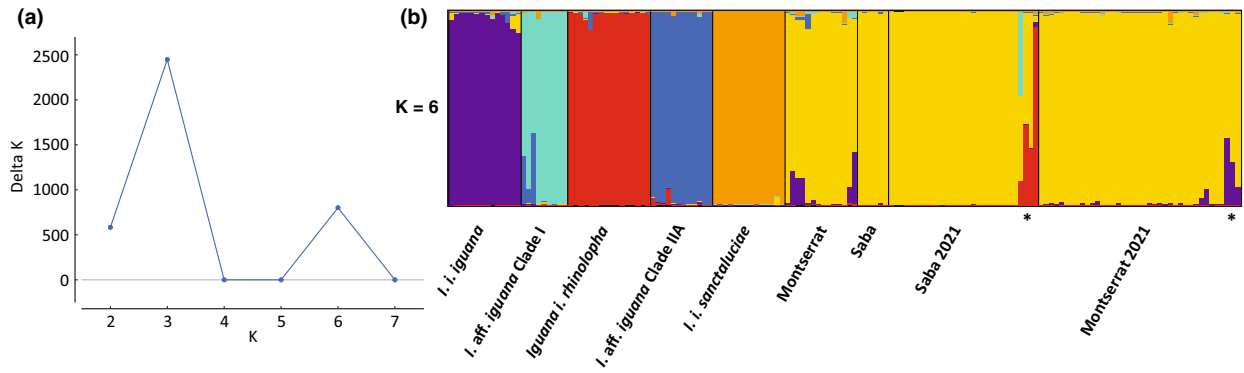
**Figure 4** Genetic variation among 17 microsatellites for samples collected on Saba and Montserrat, and referenced to the *Iguana iguana* complex following IguanaBase (van den Burg *et al.*, 2021c). (a) Delta K and (b) STRUCTURE plots for K = 6, differentiating among five taxonomic units, and pre- and post-2021-sampled individuals on Saba and Montserrat. Asterisks for Saba and Montserrat indicate samples highlighted in the text: SAB04, 08, 57, 58, and Mont33, 39, 40.

samples, 23 new haplotypes were identified from Saba, and five from Montserrat of the latter of which two overlapped. For 13 loci these new haplotypes fell outside the known size ranges for the Saba and Montserrat populations.

On Saba, mean allelic richness was 2.57 for all 57 genotyped samples, 1.67 when excluding the four identified non-native iguanas, and 1.82 when combining the 53 native samples with six Saba samples from IguanaBase. For the 76 2021-sampled iguanas from Montserrat, mean allelic richness was 2.33, and 2.50 including 14 Montserrat IguanaBase samples.

## Sequence data

Generated ND4 (789 bp) and MLH3 (778 bp) sequences all matched 100% to published and unpublished available sequences from the native *I. iguana* range. For ND4, all five Montserrat specimens and Saba specimen SAB02 had the Caribbean CAR2 haplotype (GenBank accession HM352505, Stephen *et al.*, 2013). From Saba, SAB08, SAB57, and SAB58 had the mtDNA Central-American haplotype CA4 (GenBank accession HM352508, Stephen *et al.*, 2013), while SAB04 had a haplotype only known from the ABC islands (SA16; van den Burg et al. unpublished data).

For MLH3, both islands had one sample with unreadable chromatogram data. The remaining four Montserrat specimens, as well as SAB02, were homozygous for a haplotype only found within *I. i. iguana* including a native pre-2021 sample from Saba (H18; van den Burg et al. unpublished data). While SAB08 and SAB57 were heterozygous for H18 and, respectively, a haplotype known from El Salvador (H16; van den Burg et al. unpublished data) and one known only from Honduras (H15; van den Burg et al. unpublished data). Finally, SAB58 was heterozygous for H15 and H16.

## Morphology results

Considering all life stages of native iguanas, each measured length variable was found to be size-dependent, see Table 1.

**Table 1** Morphometric analysis results for iguanas on Saba. $R^2$-values of snout-vent length regressions provided for native iguanas, as well as results of two-sample *t*-tests for size-corrected residual comparisons between the sexes. Significant *t*-test results are indicated by an asterisk for $P < 0.05$. Non-native and native iguana comparisons are addressed in footnotes

| | $R^2$ | *t*-value | df | P |
|---|---|---|---|---|
| Tail length | 0.98 | | | |
| Upper frontleg length | 0.96 | −1.8988 | 39 | 0.065 |
| Lower frontleg length | 0.98 | −3.756 | 39 | 0.0005638* |
| Upper hindleg length | 0.98 | −1.4096 | 39 | 0.1666 |
| Lower hindleg length | 0.98 | −3.0723 | 39 | 0.003862* |
| Length of 4th toe[a] | 0.91 | −2.7554 | 38 | 0.008951* |
| Head width[a] | 0.96 | −2.5122 | 39 | 0.01625* |
| Head length[a] | 0.95 | −3.6412 | 39 | 0.0007873* |
| Snout length[a] | 0.97 | −2.3068 | 39 | 0.02647* |
| Eye length | 0.94 | −1.0546 | 39 | 0.2981 |
| Mouth length[a] | 0.97 | −2.1523 | 39 | 0.03762* |
| Head depth[a] | 0.94 | −3.9221 | 39 | 0.0003454* |
| Tympanum height[a] | 0.88 | −2.2588 | 35 | 0.03014* |
| Tympanum width[a] | 0.72 | −1.7774 | 39 | 0.08332 |
| Subtympanic plate height[b] | 0.92 | −1.9112 | 39 | 0.06334 |
| Subtympanic plate width[a] | 0.78 | −3.1321 | 39 | 0.003286* |
| Mid-body spine length[a] | 0.82 | −5.9037 | 39 | 7.047 e-07* |

[a] $P < 0.05$ for size-corrected comparison between sexes for non-native and native split dataset.

[b] $P < 0.05$ for size-corrected comparison between status for non-native and native split dataset.

On Saba, differences in size-corrected residuals between native female and male adult iguanas were significant for 11 of 16 tested variables (Table 1). Tail length could not be tested given the high percentage of animals with a broken tail. Considering differences in sex and native/non-native status, ten length variables differed significantly between sex but not native/non-native status, while subtympanic plate height differed significantly for status but not sex (Table 1, Figs 5 and 6).
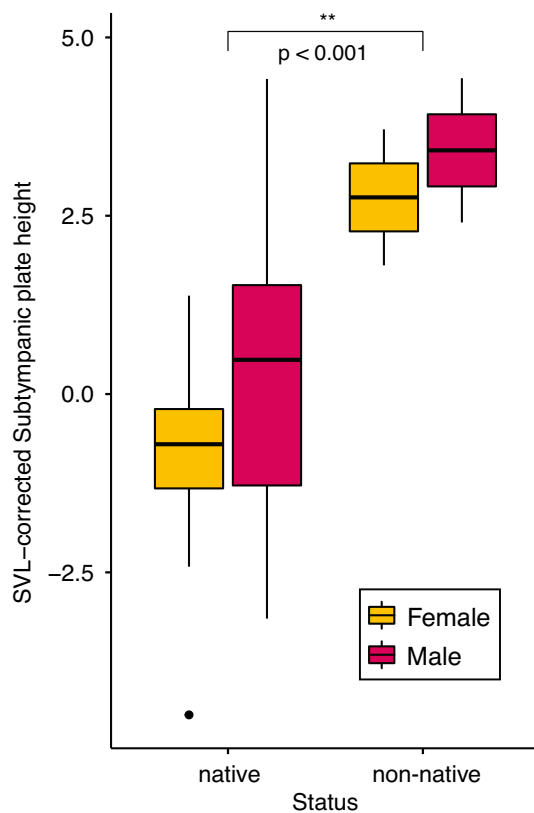
**Figure 5** Differences in the height of the subtympanic plate between non-native and native iguanas on Saba. Data are SVL-corrected residuals for genetically-assessed native adult iguanas, >20 cm SVL.

Nasal scalation assessments indicated that three iguanas from Saba (SAB08, SAB57, and SAB58) had an enlarged nasal scale (Fig. 6b,c), while such was absent in all iguanas from Montserrat. The melanistic patch between the eye and tympanum was absent in two iguanas (SAB57 + 58) but faintly present in the two other iguanas (SAB04 + 08) from Saba (Fig. 6c). On Montserrat, all iguanas had melanistic scales within this patch; however, for Mont36 and Mont42, this feature was very limited.

## Discussion

The Lesser Antilles are plagued by the continuing presence and spread of non-native *Iguana iguana*, which is a major threat to the native insular *Iguana* populations. Here we report on the assessment of non-native iguana presence on Saba and Montserrat, two of the last remaining Lesser Antillean islands previously believed to lack such invasive populations. Our integrated analysis of multiple genetic and morphological data flags a recent incursion of non-native iguanas on Saba, while we found no evidence of incursions for Montserrat. In addition to scale and coloration patterns, we highlight how morphometrics can aid the *in-situ* identification of non-native and hybrid iguanas within native populations, especially in absence of rapid genetic analytic techniques.

Passenger and cargo transport to Saba are mainly channeled through St. Maarten, although private vessels often have other origins. A recently reinstalled ferry service also first passes St. Eustatius, which is home to a native *I. delicatissima* population. Additionally, a biweekly service delivers cargo from Martin County (Florida, USA), where non-native *I. iguana* is present (Meshaka, Butterfield, & Hauge, 2004; iNaturalist, 2022). On St. Maarten, *I. delicatissima* has already been extirpated, and currently, a large non-native *I. iguana* population is present. Based on microsatellite, ND4, and MLH3 data, our results identify that non-native iguanas on Saba have their ultimate genetic origin in Central America, as well as in the ABC islands. However, given the high inter-island connectivity with St. Maarten and since iguanas from the same genetic backgrounds have been identified there (van den Burg *et al.*, 2018b), their more likely



**Figure 6** Lateral view of three iguanas captured on Saba in 2021. (a) Native female (SAB09, 390 mm SVL) showing the absence of an enlarged nasal scale and the presence of a melanistic patch between the eye and tympanum. (b) Non-native female (SAB58, 375 mm SVL) showing the presence of enlarged nasal scale and the absence of a melanistic patch between the eye and tympanum. (c) Non-native male (SAB08, 345 mm SVL) showing the presence of enlarged nasal scales and the faint presence of a melanistic patch between the eye and tympanum.

immediate origin is the large non-native iguana population in St. Maarten. This is made even more likely considering that two other non-native reptile species originating from St. Maarten have recently been documented from Saba (van den Burg, Hylkema, & Debrot, 2021d).

Among the Lesser Antillean islands, individual non-native *Iguana iguana* have been traced back to several native range origins, for example, mainland South America (Brazil), Central America (e.g., Honduras and El Salvador), the ABC islands, as well as Saba and St. Lucia (Vuillaume *et al.,* 2015; van den Burg *et al.,* 2018b; Breuil *et al.,* 2019; Pounder *et al.,* 2020). In addition, our work and that by others show that individuals from Lesser Antillean native *Iguana* populations must have also dispersed to other (neighboring) islands (Vuillaume *et al.,* 2015; van den Burg *et al.,* 2018b) in the past, be it either by natural or human-mediated means. The demonstrated occurrence of Saba/Montserrat haplotypes on St. Maarten (van den Burg *et al.,* 2018b) particularly shows the need to utilize multiple genetic markers when trying to identify non-native iguanas on Saba, as it cannot be excluded that introgressed Saban iguanas could re-invade their original native population. Considering mtDNA data across non-native populations present in the Greater (De Jesús Villanueva *et al.,* 2021) and Lesser Antilles (the current study and references above), non-native populations on Anguilla and St. Maarten stand out given their high genetic diversity with three out of the four major mtDNA clades of the *I. iguana* complex represented.

Although 26 novel alleles were identified across 15 loci for Saba and Montserrat, these can represent either undiscovered native variation or the presence of a non-native gene pool. As relatively few samples were previously analyzed from Saba and Montserrat it is not surprising that we identified novel alleles. Considering all loci examined, L20 stands out as novel alleles for this locus were identified on both islands, though we believe that their significance strongly differs. Namely, the 182 allele in this study identified for the L20 locus on Montserrat coincides closely with the previously documented diversity (see IguanaBase; van den Burg *et al.,* 2021c), which suggests that this constitutes a rare haplotype. By contrast, the novel L20 alleles from Saba suggest the presence of non-native iguanas given the large gap with known native genetic diversity (but see Estoup, Jarne, & Cornuet, 2002). Given the large pre- and post-2021 combined sample sizes from Saba (n = 63) and Montserrat (n = 90), the identification of novel alleles among fixed loci can aid in assessing the presence of non-native iguanas on the two islands. A total of four polymorphic loci (L3, L8, L16, L17) were fixed across both island populations with the exception of the four aberrant samples from Saba (Fig. 3 and Fig. S1) and provides additional evidence that non-native iguanas are present on Saba but absent on Montserrat. This was corroborated by our nDNA and mtDNA sequence data.

Eventhough a complete overview is still lacking, scalation and coloration patterns have already been found to be highly variable throughout the *I. iguana* complex. We assessed the presence of enlarged nasal scales and a melanistic facial patch in both the Saba and Montserrat population, given previous morphological assessments of these populations (Breuil *et al.,* 2020; van den Burg *et al.,* 2022). Among both populations, only three Saba animals had enlarged nasal scales (horn) and were additionally assigned as non-native by microsatellite and sequence data. In addition, these three iguanas, as well as a fourth, which were all genetically identified as non-native, lacked a clear melanistic black patch (though two had some faint partially black scales). On Montserrat, several adult iguanas genetically assigned as native had a patch with roughly 50% melanistic scales, while two individuals only had 10–20% melanistic scales. In other words, the melanistic patch of native animals from Montserrat was less pronounced than in native animals from Saba. As we further found no genetic evidence of non-native presence on Montserrat, we suggest that a less-pronounced melanistic patch is a native characteristic of the Montserrat population. This is corroborated by Breuil *et al.* (2020), who indicated that the Montserrat population was less melanistic.

In order to help distinguish non-native from native iguanas in invaded populations, we also explored the use of novel composite phenotypic tools in addition to scale and color pattern characters (Breuil, 2013) and molecular data (Stephen *et al.,* 2013; Martin *et al.,* 2015; Vuillaume *et al.,* 2015; van den Burg *et al.,* 2018b, 2021b; Miller *et al.,* 2019; Pounder *et al.,* 2020; Breuil *et al.,* 2022; Mitchell, Welch, & van den Burg, 2022). Size-dependent comparisons across 16 variables indicated that on Saba, genetically-assigned non-native iguanas have larger subtympanic plate scales than native iguanas (Figs 5 and 6). However, the diagnostic power and usage of these characteristics in distinguishing between non-native and native iguanas will depend not only on sample size but on the geographic and taxonomic origin and admixture of non-native individuals. In short, we suggest that other SVL-dependent variable(s) might be more useful to distinguish non-native animals with different genetic origins and admixture from native Saba iguanas. Therefore, additional analyses on intraspecific variation among the measured variables in *I. iguana* should be useful (and are ongoing; van den Burg et al. unpublished data). This approach is likely most applicable to small insular populations as opposed to widely-connected mainland populations given that the former tend to show less variation and are especially vulnerable to non-native iguana incursions. However, *ex-situ*, within the (illegal) pet trade (Noseworthy, 2017; van den Burg & Weissgold, 2020), these variables will remain of low value until a range-wide and high-sampled dataset is present. Therefore, we suggest that size-dependent variables will be of greatest value in distinguishing between more distantly related species and that they should especially be evaluated for use in conservation management for *I. delicatissima*.

While the initial presence of non-native species can be expected to be highest around the incursion point, an initial presence at locations more distant from the incursion point can occur when biosecurity regulations are bypassed (Brisbane *et al.,* 2021). Saba knows two principal incursion points to the island. These are the Fort Bay harbor and the

Juancho E. Yrausquin airport, situated on the southwest and northeast sides of the island, respectively. Non-native iguanas were mainly located at a site 0.4 km east from the harbor, although one animal was found north of the settlement of Windwardside (Fig. 2). How this latter individual reached the center of the island is impossible to determine. We speculate that it might concern an intentionally and illegally released pet (Jesse *et al.,* 2016) arriving either via the airport or the habor or that it might have arrived as an intra-island stowaway that originally came in through the harbor. Occurrence of three of the four non-native iguanas documented for this island near the harbor strongly suggests their arrival via transport by boat or ship. In the case of the neighboring island of St. Eustatius, the harbor also appears to be the principal way of the incursion of non-native iguanas (Debrot, Boman, & Madden, 2022), and other non-native reptiles (Thibaudier *et al.,* 2023).

Introgressive displacement has been identified as the main threat to *I. delicatissima* (Vuillaume *et al.,* 2015). Hybrid and non-native iguanas are believed to be more territorially aggressive and have higher reproductive fitness thanks to larger clutch sizes compared with *I. delicatissima* (van Wagensveld & van den Burg, 2018). Although data on the clutch size from the Montserrat and Saba populations are practically unavailable (Blankenship, 1990; van den Burg *et al.,* 2022), insular *Iguana* populations have generally been found to have smaller clutch sizes than continental populations (e.g., Fitch & Henderson, 1977; van Marken Lichtenbelt & Albers, 1993; Bock *et al.,* 2018), even though this is believed to be partially climate dependent (e.g., Novosolov, Raia, & Meiri, 2013). The available data suggest that the Montserrat and Saba populations produce clutch sizes within the lower range recorded for *I. iguana*, potentially allowing reproductive and genetic swamping when non-native or hybrid iguanas would reproduce within these island populations.

Whether hybridization has occurred on Saba remains unresolved given our limited data, although preliminary data could suggest it is taking place. The presence of non-native iguanas on St. Maarten with a (partial) *I. i. iguana* origin limits our ability to assess whether the *I. i. iguana* nDNA from the three admixed iguanas of Saba represents native or non-native DNA. Although SAB58 could potentially be the maternal parent of SAB08 and 57 given mtDNA, nDNA (MLH3), and body size, our microsatellite allele data rejects this hypothesis. All data combined suggest that none of the non-native iguanas had an identical pair of parents. The relatively low number of non-native iguanas observed on Saba implies that on-island hybridization might still be absent or rare. However, the presence of two iguanas with faint melanism with the facial patch might suggest that these animals are hybrids. Additional fieldwork and genetic assessment are needed to determine whether hybridization is still truly absent.

Our results in any case show that there is a pressing need for rapid action to completely remove non-native iguanas from Saba, and that prospects for this are relatively good in light of apparently lacking or only limited hybridization.

However, only immediate action can result in the removal of non-native iguanas before they start interbreeding (Debrot *et al.,* 2022). Failure to act decisively at an early stage of the invasion process elsewhere has led to a strong growth of invasive iguana populations, and serious existential threats to or even extinction of the original native iguana populations, for example, on Grand Cayman (Rivera-Milán & Haakonsson, 2020) and in the French West Indies (Angin, 2017). Given this critical situation, the Saba subpopulation has recently been assessed as Critically Endangered following the IUCN Red List guidelines (van den Burg & Debrot, 2022).

The situation on Montserrat warrants further investigation, both into native population baseline numbers and to further corroborate the absence of non-native iguanas. The reports of a sudden, unexplained increase in iguana numbers there might be explained by other factors than invading non-natives, for example, observation bias or new agricultural expansions, techniques or crops. Unfortunately, no comparative data on past population numbers and distribution on Montserrat exist. The situation and potential factors explaining reported population increases should be followed up further with a view to pre-empt or mitigate a potential shift of public opinion against their largest native reptile. Especially a possible attempt to regulate iguana numbers on Montserrat should be preceded by further genetic testing, a thorough population estimate and better knowledge of the distribution across the island. Stringent biosecurity measures should be implemented on Montserrat's entry points to prevent non-native iguanas to enter given their widespread occurrence in the region (Knapp *et al.,* 2021). This would be especially important if it holds true that the island might still be the only Lesser Antillean main island without non-native iguanas. More extensive size-dependent morphometric data collection from the native population will additionally be essential to further help discern differences with non-native iguanas, which can help to rapidly identify and cull non-native and or hybrid iguanas.

We conclude by pointing out that our findings illustrate the need for continuous monitoring of non-native iguanas within the remaining native *Iguana* populations in the Lesser Antilles. Although genetic data from across the native *Iguana* range currently allows the identification of non-native or hybrid presence, this field-to-lab workflow is often excessively time-consuming. At present, genetic assessment requires the shipment of samples and involves delays due to the required permitting processes (e.g., CITES permits). Improved *in-situ* identification screening to rapidly distinguish non-native from native individuals is urgently needed. Here, in addition to scale and coloration patterns, we show that size-dependent morphological variables can also be utilized for rapid screening purposes. We urge our regional partners to build morphometric databases for native *Iguana* populations as soon as possible that can help to rapidly distinguish non-native iguanas for culling. Even if biosecurity is eventually improved, incursions will from time to time continue to take place and the need to be able to rapidly distinguish non-native iguanas for culling will remain important.

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## Authors' contributions

*MPB*, *MG,* and *AOD* conceived the ideas, designed the methodology, and acquired the funding; *MPB, LB, TSW,* and *KVR* collected the data; *MPB* analyzed the data; *MPB* led to the writing of the manuscript. All authors contributed critically to the drafts and gave the final approval for publication.

## Conflict of interest

The authors have no relevant financial or nonfinancial interests to disclose.

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# Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Allele variation across 17 microsatellites among six taxonomic and conservation units and the 2021-sampled individuals from Saba and Montserrat. Allele sizes denoted by the color gray have only been observed in hybrid specimens across different populations.

**Data S1** Supporting Information