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The Lesser Antillean Iguana (*Iguana delicatissima*) on St. Eustatius: Genetically Depauperate and Threatened by Ongoing Hybridization

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Abstract

The Lesser Antillean Iguana (*Iguana delicatissima*) is an endangered species threatened by habitat loss and hybridization with non-native Green Iguanas (*Iguana iguana*). *Iguana delicatissima* has been extirpated on several islands, and the Green Iguana has invaded most islands with extant populations. Information is essential to protect this species from extinction. We collected data on 293 iguanas including 17 juveniles from St. Eustasius, one of the few remaining *I. delicatissima* strongholds. Genetic data were leveraged to test for hybridization presence with the Green Iguana using both mitochondrial and nuclear genes, including 16 microsatellite loci. The microsatellites were also analyzed to estimate genetic diversity, population structure, and effective population size. Using molecular and morphological data, we identified 286 *I. delicatissima* individuals captured during our first fieldwork effort, and 7 non-native iguanas captured during a second effort, showing hybridization occurs within this population. Comparing homologous microsatellites used in studies on Dominica and Chancel, the *I. delicatissima* population on St. Eustatius has extremely low genetic diversity (H_O = 0.051; H_E = 0.057), suggesting this population is genetically depauperate. Furthermore, there is significant evidence for inbreeding (F_{IS} = 0.12) and weak spatial genetic structure (F_{ST} = 0.021, P = 0.002) within this population. Besides immediate threats including hybridization, this population's low genetic diversity, presence of physiological abnormalities and low recruitment could indicate presence of inbreeding depression that threatens its long-term survival. We conclude there is a continued region-wide threat to *I. delicatissima* and highlight the need for immediate conservation action to stop the continuing spread of Green Iguanas and to eliminate hybridization from St. Eustatius.

Keywords: bottleneck, conservation, endangered species, genetics, inbreeding, microsatellites

Endangered species with small population sizes are especially susceptible to anthropogenic disturbance and stochastic events. Fluctuations in the size of small populations are more likely to result in decreased genetic diversity, isolation of populations, and reduced fitness due to inbreeding depression. Another major threat for populations of endangered species is hybridization following the introduction of non-native species, with possible subsequent introgression. The role of hybridization with non-native species as a conservation risk can be controversial because it may occur naturally, but is typically viewed as actionable when introductions have anthropogenic origins (Dierking et al. 2014). In such cases, hybridization can be deemed the principle threat to endangered species, and has even lead to documented extinctions (Rhymer and Simberloff 1996; Todesco et al. 2016). Population genetic analyses are widely used to estimate genetic diversity, the effects of population bottlenecks, and to determine whether admixture has occurred. Such genetic analyses allow the presence and magnitude of demographic processes and hybridization to be estimated using multiple population parameters (Lowe and Allendorf 2010; Allendorf et al. 2013).

The Caribbean islands are a hotspot of biodiversity, thanks to their diversity of landscapes, their large combined coastline and their complex geological past that includes many vicariance events, volcanic activity, and sea level changes. Since European settlement of these islands, this biodiversity has come under increasing pressure. As the islands have historically served as a hub for intercontinental trade, there has been a strong increase in the human population, leading to large-scale habitat destruction and depletion of natural resources. For example, the island of St. Eustatius was once described as being "cultivated to the very summit" of the Quill volcano (Chambers and Chambers 1842). International trade also brought an influx of invasive species, which have further compromised the already fragile ecosystem.

The Lesser Antillean Iguana, *Iguana delicatissima*, is an endemic species of the Lesser Antilles that has disappeared from a large number of islands in the region since the start of European settlement (Vuillaume et al. 2015). The reasons for this disappearance are thought to include habitat loss, introduction of predators, human consumption, and introduction of the closely related Green Iguana, *I. iguana* (Breuil et al. 2010a; Knapp et al. 2014). *Iguana iguana* is thought to threaten the native *I. delicatissima* populations in 2 ways. First, as a competitor, *I. iguana* is more aggressive, has larger clutch sizes and reaches reproductive maturity more readily (Breuil et al. 2010a). Second, the 2 species can hybridize. Non-native *I. iguana* from South America was first introduced in the Lesser Antilles to the island of Les Saintes around 1860 (Breuil 2013). Since then, *I. iguana* from Les Saintes, South America and Central America have invaded additional islands with native populations of *I. delicatissima*, leading to subsequent hybridization (Knapp et al. 2014; Vuillaume et al. 2015). Iguanas have been found as stowaways on boats, but also to traverse between islands on drifting material following hurricanes (Censky et al. 1998; Day et al. 2000). Although *I. iguana* and *I. delicatissima* diverged 8.9 million years ago (Malone et al. 2017), Vuillaume et al. (2015) showed that hybridization and introgression occur (St. Barthélemy, Basse-Terre, Grand-Terre, and Les Saintes) by identifying backcrossed individuals with both parental species, highlighting the threat to the genetic integrity of invaded *I. delicatissima* populations. Furthermore, previous work showed that *I. iguana* and *I. delicatissima* have genetic (Stephen et al. 2013; Martin et al. 2015), and morphologically distinct characteristics (Breuil 2013) allowing hybrids to be identified using these characteristics.

St. Eustatius holds one of the last potentially pure *I. delicatissima* populations. Historical data on this population are absent, but the dense human population during the 19th century implies a scarcity of natural vegetation and a reduced population size of *I. delicatissima*. Recently, population assessments estimated the population to consist of between 300 and 425 individuals (Reichling 2000; Fogarty et al. 2004; Debrot et al. 2013), with the latest study suggesting this estimate to be at the lower end of this range. The survival of this population is threatened by its small size and low density, which could be caused by anthropogenic mortality and habitat degradation (Debrot et al. 2013). *Iguana iguana* was believed to be absent from St. Eustatius until February 2016 when an adult Green Iguana was found (Jesse et al. 2016), an additional 6 hybrid iguanas have since been recovered. The *I. iguana* individual was likely an illegally imported pet that was released (Jesse et al. 2016), although *I. iguana* individuals also arrive on boats from neighboring islands (the current article). The extent of hybridization within the *I. delicatissima* population on St. Eustatius is currently unknown, but this has important implications for future conservation management.

Here, we study the population genetics of the *I. delicatissima* population on St. Eustatius. First, we aim to assess the degree to which hybridization has historically impacted this population and if hybridization is currently occurring. Second, we aim to quantify the population's genetic diversity and to identify whether this population suffers from inbreeding depression. Finally, we aim to characterize this populations' genetic structure and test the impact of potential dispersal barriers. Given the paucity of prior evidence for non-native iguanas on St. Eustatius, we anticipate a recent hybridization origin. Further, as the estimated population size and density are low, we expect the genetic diversity of the Lesser Antillean Iguana on St. Eustatius to be low. Lastly, we expect that the Lesser Antillean Iguana population on St. Eustatius consists of 2 subpopulations separated by cultivated fields, the airport and the town of Oranjestad due to anthropogenic factors such as airport fences and roads, which can prevent migration, as well as a lack of suitable habitat. These areas could thereby serve as an obstacle to gene flow. This study will assess these hypotheses using genetic data from mitochondrial and nuclear markers. Our genetic results will also be used to assess this population's status and current and future implications for conservation management.

Methods

Fieldwork

In 2015, an island-wide survey of St. Eustatius was conducted, and blood samples were collected from *I. delicatissima* (n = 255). To compare the genetics of *I. delicatissima* with *I. iguana*, we also sampled *I. iguana* (n = 30) individuals on St. Martin. We searched for iguanas both opportunistically and based on information from residents and evidence of iguana presence such as shed skin, feces, tail markings, hides, and nest holes. The latter were documented because there seem to be few nesting sites on the island (Debrot et al. 2013). Iguanas were captured by noose or hand. Blood was collected from the caudal vein using a syringe, following Gerber and Pasachnik (2014). All samples were stored in lysis buffer at a blood–buffer ratio of 1:2 (Longmire et al. 1992; Gerber and Pasachnik 2014). During fieldwork, all blood samples were stored at 4 °C, and afterwards at −80 °C for long-term storage. After blood collection, iguanas were uniquely marked for future identification and to avoid resampling. Juveniles and subadults were marked with a nontoxic marker, and

adult iguanas were bead tagged using colored beads following Binns and Burton (2015). These identification methods are widely used in iguana research (Rodda et al. 1988; Pasachnik et al. 2012).

In 2016, the year after the island-wide survey was completed, one adult female *I. iguana* individual was found on St. Eustatius (Jesse et al. 2016), and subsequent surveys identified 6 individuals with intermediate characteristics. We were able to obtain morphological information from all 7 of these individuals and genetic samples for 4 individuals, adding these to our dataset. The 3 individuals for whom no blood samples are available were not live caught, but found several days after they died or were killed by collision with a vehicle. During the preparation of this manuscript an additional 4 *I. iguana* individuals arrived on St. Eustatius on a cargo ship hidden in cinder blocks from St. Martin, and, unfortunately, only one individual was captured.

Additionally, species status was determined based on the morphological characteristics illustrated by Breuil (2013). Breuil (2013) shows that both *Iguana* species and their hybrids have unique morphological characteristics and can be used to identify species status, even for backcrossed individuals. Species status was determined in situ, and again ex situ using 3 facial images taken from both sides of the head and from the top of the head. This method allows individuals to be classified as *I. delicatissima*, *I. iguana* or as hybrids. The sex of subadults and adults was determined to identify sex bias in the population and the direction of inheritance for possible hybrid iguanas. Finally, physical measurements, presence of abnormalities, GPS coordinates, and presence of parasites were noted. The GPS coordinates were used for the spatial genetic analyses, but since it concerns an endangered species, they will not be made public to prevent them from being used by poachers (Lindenmayer and Scheele 2017). Physical measurements include snout–vent length (SVL; to 0.1 cm), tail length (TL; to 0.1 cm), and body mass (BM; to 5 g). Individuals that were larger than 25 cm were classified as adults following Judson et al. (2018).

DNA Isolation

Laboratory procedures were performed at the University of Amsterdam (UvA) and at Mississippi State University (MSU). At the UvA, DNA was extracted using a DNeasy Kit (QIAGEN, Germany) and at MSU using a Maxwell 16 Nucleic Acid Extraction System with the Maxwell 16 Tissue DNA Purification Kit (Promega). DNA isolation was successful for all samples except for one non-native iguana sample, possibly due to inappropriate sample storage, leaving only 3 of the 4 putative non-native iguana samples with successful DNA isolation.

Detection of Hybrids

Testing for the possible presence of Green Iguana DNA on St. Eustatius was done using DNA sequence analysis, restriction fragment length polymorphism (RFLP) analysis of nuclear and mitochondrial genes, and through genotyping 17 microsatellites found to be variable in *I. delicatissima*. Previous work on *Iguana* species identified fixed, diagnostic interspecific differences for one mitochondrial and several nuclear loci allowing genetic identification (Stephen et al. 2013; Martin et al. 2015). From these, we used the mitochondrial ND4 gene and the nuclear genes PAC (polymerase alpha catalytic subunit), and NT3 (neurotrophin-3). ND4 has around 75 interspecific sites depending on haplotypes being compared (Stephen et al. 2013; Martin et al. 2015); PAC has 2 diagnostic indels in *I. iguana* alleles that allow distinction from *I. delicatissima* alleles; for NT3 there are 4 interspecific nucleotide sites (Stephen et al. 2013).

To detect hybrids in the island-wide survey, we used the RFLP approach. RFLPs have been used to identify hybridization in other study systems (Paige and Capman 1993; Marcilla et al. 2002) and here, we show this technique can be applied for *I. delicatissima* and *I. iguana* hybrids as well. First, for both species all GenBank sequences for ND4 and PAC were obtained (Stephen et al. 2013; Martin et al. 2015), providing a range-wide haplotype dataset for both species. Then MEGA 6 (Tamura et al. 2013) was used to identify diagnostic nucleotides for the 2 species. Hence, we acknowledge that Green Iguanas with different geographical backgrounds occur in the region and thus a large number of different *I. iguana* haplotypes could be present (Breuil et al. 2010b, 2011; Stephen et al. 2013; Vuillaume et al. 2015). PCR–RFLPs for these diagnostic sites between all *I. iguana* and *I. delicatissima* haplotypes were then designed using Restriction Mapper V3 (http://www.restrictionmapper.org/). PAC and ND4 were found to be reciprocally monophyletic, and specific enzymes were identified that are uniformly present or absent in all haplotypes of each species, *Pst*I and *Eco*RII, respectively. The *I. delicatissima* PAC haplotype has one cut site for *Pst*I that was absent from the *I. iguana* haplotypes. In ND4, 2 *Eco*RII restriction sites were absent in *I. iguana* haplotypes but present in *I. delicatissima* haplotypes. Hence, restriction profiles for *I. delicatissima* ND4 and PAC amplicons should result in 3 and 2 fragments, respectively, whereas *I. iguana* amplicons for the same regions should be intact following attempted digests.

For the RFLP analysis, we amplified ND4 and PAC regions from 193 individuals using polymerase chain reaction (PCR); ND4: following denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 58°C for 30 s, 72°C for 90 s, ending with a final elongation at 72 °C for 7 min; PAC: 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s, ending with a final elongation at 72 °C for 7 min. Both regions were amplified for 160 putative *I. delicatissima* individuals from St. Eustatius, 3 putative hybrids from St. Eustatius and 30 *I. iguana* individuals from St. Martin. When PCR amplification was successful, 5 µL of PCR product was digested with restriction enzyme. All samples were incubated for 60 min, at 37 °C for PAC and 60 °C for ND4. Restriction digests and controls were analyzed using gel electrophoresis to identify haplotypes present in the 193 individuals.

Besides the described RFLP method, electropherograms from DNA sequences were used to verify the species status of the putative hybrid individuals. Because of 2 diagnostic indels in PAC, hybrid sequence data will no longer run synchronous from basepair (bp) 69 onwards (first indel, 1 bp), or backwards from basepair 427 (second indel, 5 bp). For NT3, the 4 interspecific nucleotide sites are heterozygous for F1 hybrids. NT3 was amplified following denaturation at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, ending with a final elongation at 72 °C for 7 min. The mitochondrial ND4 gene sequences are haploid, but allow determining the maternal species of hybrids; for hybrids with *I. iguana* mothers, the variation in these genes allows alleles to be appointed to the possible region of origin (Stephen et al. 2013). Sequence data were obtained for the 3 putative hybrids, 1 pure *I. delicatissima* from St. Eustatius, and 4 pure *I. iguana* from St. Martin.

Genetic Diversity at Microsatellite Loci

To study the genetic diversity and population structure of the *I. delicatissima* population from St. Eustatius, we used 17 microsatellite loci developed in earlier studies: Ccste02 (Rosas et al. 2008); CycCar177 (Welch et al. 2011); D105, D110, D135, and D136 (Lau

et al. 2009); IgdL11, 12, 14, 17, 19, 20, 21, 22, and 24 (Valette et al. 2013); 60HDZ13 and 148 (An et al. 2004). All IgdL loci have been developed specifically for *I. delicatissima* and *I. iguana*, and although the other 8 have been developed for *Cyclura* species, Judson et al. (2018) showed that these amplify and are variable in *I. delicatissima*. Samples from 254 iguanas were genotyped for 13 microsatellite loci (Valette et al. 2013; Judson et al. 2018) using PCR. Another 4 loci (D105, D135, D136, and 60HDZ13) were only genotyped for 160 individuals as all these individuals were completely monomorphic for these loci. PCR was performed following 1 of 2 protocols with different annealing temperatures. The protocol for markers D105 and D135 consisted of denaturing on 98 °C for 30 s followed by 10 cycles of 10 s at 98 °C, 10 s at 58 °C for annealing and 10 s elongation at 72 °C. Followed by 25 cycles for which only the annealing temperature differed (53 °C) and a final elongation of 60 s at 72 °C. The protocol for markers D136 and 60HDZ13 only differed for the first annealing temperature, which was 55 °C instead of 58 °C. Furthermore, a touchdown protocol was used for the following markers: D110, IgdL11, IgdL12, IgdL14, IgdL17, IgdL19, IgdL20, IgdL22, and IgdL24. This protocol consisted of denaturing on 94 °C for 5 min, followed by 10 cycles beginning with 30 s at 94 °C, 30 s annealing starting at 65 °C, 45 s elongation at 72 °C, during which the annealing temperature decreased with 1 °C per cycle to 55 °C, then another 25 identical cycles with only an annealing temperature of 55 °C and a final elongation of 7 min. Final touchdown annealing temperatures for markers IgdL21, Ccste02, and CycCar177 were different with 57 °C, 57 °C and 53 °C, respectively. These protocols were performed in 10 µL reactions, with a final DNA concentration of approximately 10 ng. Afterwards, PCR amplification was verified using gel electrophoresis.

As laboratory work was divided over 2 labs, 8 individuals were re-genotyped for 3 loci (all other loci were only genotyped in one of the labs) to prevent lab-based bias and align allele binning and calling. Results from 2 loci were entirely consistent between both labs, however results for Ccste02 were inconsistent and thus, we excluded this locus from further analyses. For this locus, UvA results were monomorphic, but MSU results indicated the presence of a small number of heterozygous individuals (<5) with a presence of 2 alleles at this locus.

After PCR product verification on gels, fragment analysis was performed at the UvA or at Arizona State University using Applied Biosystems (ABI) 3130 sequencers. Fragment data was analyzed using Peak Scanner software v. 1.0 (Applied Biosystems) and GeneMapper® v. 4.1 (Applied Biosystems). Individuals were scored as heterozygous when a second peak had a minimum height of 50% compared to the highest peak. Microsatellite data for all individuals can be found in the Supporting Information.

Microsatellites can be subject to genotyping errors due to stutter, large allele dropout and null alleles. Loci with these genotyping errors can often be identified through departures from Hardy–Weinberg equilibrium (HWE). We performed a quality check on each locus using MICRO-CHECKER (version 2.2.3, van Oosterhout et al. 2004). However, we discarded the program's correction for null alleles, since this may also obscure any true patterns of excess homozygosity stemming from low genetic diversity and inbreeding, which we expect to occur in the small *I. delicatissima* population. Overall, PCR and fragment analysis were robust, the highest failure for a single marker was 4 individuals. Further, MICRO-CHECKER results indicated no null alleles or scoring errors due to stutter.

In total, 254 individuals from the island-wide survey were genotyped for the described microsatellites. Individuals scored at fewer than 9 loci were excluded from further analyses. This resulted in a dataset consisting of 247 individuals that were retained for the population genetic analyses.

Data Analysis

For each locus, the following genetic parameters were calculated using GenAlEx 6.502 (Peakall and Smouse 2012): number of alleles, effective number of alleles, expected heterozygosity, observed heterozygosity, fixation index, and departure from Hardy–Weinberg equilibrium.

To assess if the airport and town of Oranjestad restrict migration between individuals living on either side of this potential barrier to gene flow, we used F_{ST} to test for population differentiation between the 2 sides (Wright 1949; Holsinger and Weir 2009). For this, we used an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) in the program GenoDive (Meirmans and van Tienderen 2004); significance was tested using 9999 permutations. Besides population subdivision, another factor that can explain spatial genetic differences is isolation by distance (IBD) (Meirmans 2012). Here, we used redundancy analyses (RDA) in the R package "vegan" (Oksanen et al. 2014) to investigate how IBD and gene flow barriers affect F_{ST} values. For this, we calculated within-individual allele frequencies, which we used as a response variable in the RDA. Explanatory variables for IBD and population subdivision were the GPS coordinate data and a categorical factor representing the 2 hypothesized subpopulations. To distinguish between population subdivision and IBD, both explanatory variables were used as a co-variable.

Despite being a small island, other gene-flow barriers and genetically distinct populations might be present on St. Eustatius. Significant genetic structure has been shown over a narrow geographic range in other iguana species (Colosimo et al. 2014). Therefore, we used Structure v2.3.4 (Pritchard et al. 2000) to analyze population structure with the number of possible populations (K) ranging from 1 to 10. Per value of K 10 repeats were run, each with a burn-in period of 100 000 followed by 1 000 000 MCMC repeats. The optimal number of clusters was estimated using Structure's ln(prob) statistic; we did not implement Delta K (Evanno et al. 2005) as it cannot be calculated for $K = 1$ which might be the true value of K. All structure analyses were run both with and without the admixture model and with and without correlated allele frequencies. However, these settings did not have a large influence on the results.

Presence of inbreeding depression can be assessed by analyzing relationships between fitness-correlated traits and multi-locus heterozygosity (MLH), by using heterozygosity-fitness correlations (HFC). Though there are multiple estimators for MLH, these are all highly correlated (Chapman et al. 2009). Here, we used the standardized heterozygosity (SH, Coltman et al. 1999) as this MLH measure is widely used. The R package "Rhh" (Alho et al. 2010) was used to calculate SH values. Appropriate fitness traits were carefully selected for calculating HFCs, because HFCs are only biologically relevant for traits that influence individual fitness (Chapman et al. 2009). Fitness in lizard species is influenced by body size that, in turn, is commonly quantified using BM and SVL (Le Galliard et al. 2004). In addition, these variables are known to influence female clutch size and male social dominance (Perry and Garland 2002) in iguanas (Alberts et al. 2002). HFCs were run in R using only the adult individuals without missing microsatellite data. To prevent sex-based bias in both BM and SVL variables, z-scores were calculated per sex per variable after which the z-scores from both sexes were pooled (Berk 2013).

The effective population size (N_e) of the adult iguana population was estimated using the corrected linkage disequilibrium (LD) method of Waples and Do (2008), as implemented in NeEstimator V2.01 (Do et al. 2014). This single-sample method uses LD

differences between microsatellites to estimate N_e and assumes neutrality of microsatellites and random mating. Default settings were used and 95% confidence intervals (CI) were calculated using the parametric method of Waples and Do (2008) for alleles with a frequency threshold larger than 0.05.

Results

Census of St. Eustatius' Iguana Population

During the island-wide survey, which covered 82% of the islands' surface, we counted 324 unique individuals and captured 286 of those. Of the captured iguanas, 196 were bead tagged for future identification and monitoring, and we collected blood samples from 255 iguanas. Overall, we found a male:female sex ratio of 0.52:1, and a remarkably low number of juveniles (n = 17; 5.2% of 324). Four nest holes were counted of which one was previously unknown.

Species Identification and Morphological Characteristics

All 286 iguanas that were captured during the island-wide survey in 2015 were morphologically identified as *I. delicatissima* and no evidence of *I. iguana* or hybridity was found (Supporting Materials). All 6 putative hybrids, captured in 2016 and early 2017, had morphological characteristics consistent with *Iguana* hybrids (Figure 1b): 9 gular spines, intermediate subtympanic region, few and small nuchal tubercles and faded black rings. The morphology of the *I. iguana* individual caught on St. Eustatius (Figure 1a) matched with Central American individuals: presence of nasal horns, high number of prominent nuchal tubercles, very large subtympanic plate, and 10 gular spines.

Among the captured individuals, 7 had physical abnormalities—all individuals that were morphologically and genetically pure *I. delicatissima*. These abnormalities consisted of an additional appendage attached to each forelimb (n = 1) (Figure 2), a normal looking but boneless tail (n = 1), and abnormal eye coloration (n = 5) (Figure 3). Abnormal eye colorations were either red or black, with the later the iris appears damaged (Figure 3a); black eyes may be associated with blindness, as these individuals did not respond to nearby movement.

Hybrid Presence on St Eustatius

RFLP results (provided in Supporting Materials) for ND4 and PAC after digestion revealed that 160 analyzed samples from St. Eustatius had *I. delicatissima* haplotypes for both the mitochondrial and the nuclear gene. In addition, no alleles suggestive of hybridization were recovered from the nuclear microsatellite genotypes of the island-wide survey. All 3 putative hybrids from St. Eustatius had an *I. iguana* mitochondrial haplotype with both an *I. iguana* and *I. delicatissima* nuclear PAC haplotype. In addition, all 30 analyzed samples from St. Martin showed *I. iguana* haplotypes for both the nuclear and mitochondrial gene.

Nuclear PAC and NT3 sequences, visualized using electropherograms (Figure 4 and Supplementary Figure S1, respectively), revealed that all putative hybrids, captured in 2016 and 2017, were heterozygous for diagnostic alleles. For PAC, the *I. delicatissima* individual was homozygous for this species' monotypic allele; the 4 St. Martin individuals were all heterozygous for intraspecific *I. iguana* PAC alleles (Figure 4) with alleles previously found to be specific for Curacao, Ecuador/Peru, and Central America. For NT3, *I. delicatissima* was homozygous for this species' monotypic allele; the St. Martin *I. iguana* carried alleles from Central America and South America, and one unpublished allele. For the mitochondrial ND4 gene, the 3 putative hybrids had 2 different *I. iguana* haplotypes; 1 had an unpublished haplotype and 2 had a haplotype previously found in El Salvador and Honduras. The *I. delicatissima* haplotype differed by one nucleotide from this populations' other haplotype (Stephen et al. 2013; Martin et al. 2015). Haplotypes from the St. Martin *I. iguana* individuals showed high similarity to Curacao (n = 3) and Caribbean/South America haplotypes (n = 1) (Stephen et al. 2013). All sequences were deposited on GenBank (Supporting Materials).

Genetic Diversity and HWE

Population genetic analysis of 16 microsatellite loci showed a low genetic diversity within the St. Eustatius population (Table 1), with 9 loci being completely monomorphic. Across all loci, the average number of alleles (Na) was 2.0 and the average effective number of alleles (Ae) was 1.149. The average observed (H_O) and expected heterozygosity (H_E) across loci were 0.051 and 0.057, respectively. Disregarding rare alleles with frequency ≤0.05, only 2 out of 16 loci (12.5%) were polymorphic. Overall, we found a significant excess of homozygotes, F_{IS} = 0.12 (P = 0.0004).

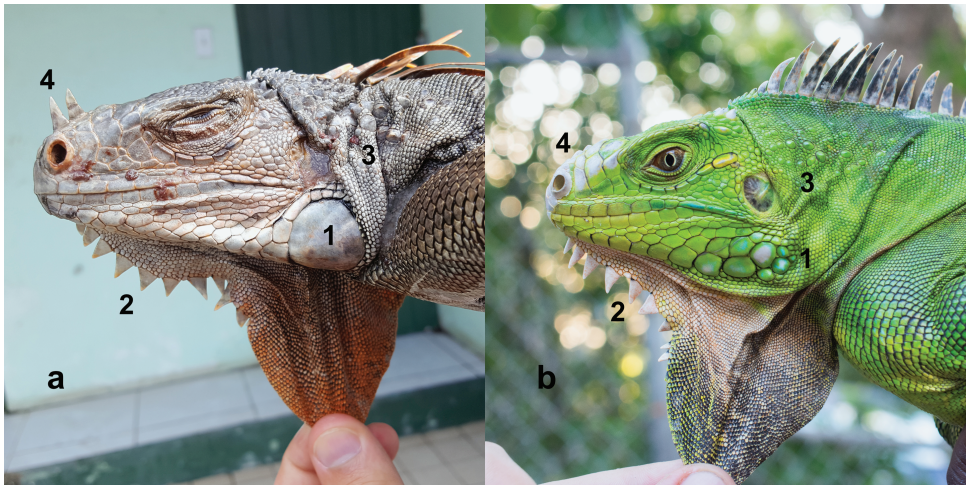

Figure 1. Non-native iguanas captured on St. Eustatius. (**a**) Individual with *Iguana iguana* characteristics; (**b**) individual with hybrid characteristics (following Breuil 2013). (1) Subtympanic plate, (2) gular spikes, (3) nuchal tubercles, (4) nasal horns. *Iguana iguana* photo taken by T. P. van Wagensveld, all other photos by M. P. van den Burg.

Figure 2. Captured iguana with additional claws attached to both forelimbs, only one is shown.

The low diversity affected multi-locus heterozygosity calculations, as the genetic diversity among loci was quantified by only 3 standardized heterozygosity values. As HFCs based on so few values are not informative and violate basic assumptions, we could not assess if this population suffers from inbreeding depression.

Population Structure

The populations on either side of the airport/town area (North = 106; South = 141) were slightly differentiated from each other; multilocus F_{ST} was 0.021 (P = 0.002). The RDA showed that this differentiation was better explained by population subdivision than by IBD. When the presence of population subdivision was tested, using IBD as a co-variate, 2.2% of the total genetic variance was explained (P = 0.003); when the presence of IBD was tested, using the 2 subpopulations as a covariate, only 0.8% of the variance was explained and this was not significant (P = .494). In contrast, STRUCTURE analyses with K values ranging from 1 to 10 indicated no clear population clusters (optimal K = 1), suggesting that no gene-flow barriers are present on St. Eustatius (Supplementary Figure S2). It is also important to note that if any *I. iguana* or hybrids would have been present in this sample, they would have been recognized as such by STRUCTURE, see Vuillaume et al. (2015).

Effective Population Size

Using data from the 191 adult iguanas for which all polymorphic markers were scored, NeEstimator estimated the effective population size of the iguana population on St. Eustatius to be comprised of 86.7 individuals with a 95% CI ranging from 1.4 to infinite.

Discussion

Hybridization

As hybridization with the non-native and invasive Green Iguana threatens populations of the native Lesser Antillean Iguana, we assessed the genetic identity of the iguana population on St. Eustatius. Morphological data, as well as RFLP (n = 160) and microsatellite (n = 247) results from our 2015 fieldwork effort show the absence of non-native iguana and hybridization, which, if present, should have been identified by these analyses (Vuillaume et al. 2015). Despite this absence of non-native iguanas in our initial population survey in 2015, during follow-up fieldwork since 2016 7 non-native individuals were captured. Morphological data showed these individuals had hybrid (n = 6) or *I. iguana* characteristics (n = 1). Additionally, genetic data showed all 3 analyzed hybrid individuals had *I. iguana* mitochondrial haplotypes and interspecific heterozygosity for the nuclear PAC and NT3 genes. Combined, our fieldwork covered 82% of the islands' surface, allowing us to capture 286 unique *I. delicatissima* during our initial fieldwork effort, and 7 non-native iguanas during a second effort. These numbers suggest that the first introduction of *I. iguana* and subsequent hybridization did not occur before 2016, or had occurred only at extremely low frequencies on St. Eustatius before that date. This shows that the Lesser Antillean Iguana population on St. Eustatius is threatened by hybridization, and highlights the need for immediate conservation management to eliminate this threat.

The size measured for the captured hybrids conflicts with the lack of hybrids observed in our initial island-wide survey. The captured hybrids measured between 19.3 and 33 cm SVL, suggesting these animals are 2–3 years of age (Wikelski and Romero 2003); the genetic data indicate 2 independent reproduction events. On the other hand, the low number of hybrids in our dataset following an island-wide collection effort suggests that hybridization is of recent origin. Clutch sizes for *I. delicatissima* (4–30; Knapp et al. 2014) are much lower than those for *I. iguana* (9–71; Bock 2014), but no literature exists on hybrid clutch size. Assuming hybrids have an intermediate clutch size, the resulting higher population growth rates of *I. iguana* and hybrids will lead to displacement and elimination of *I. delicatissima* (genetic swamping; Todesco et al. 2016).

Low Genetic Diversity

The Lesser Antillean Iguana population on St. Eustatius showed low levels of genetic diversity, with low heterozygosity (H_O = 0.05) and allelic diversity (Ae = 1.15) found for 16 microsatellite loci known to be variable in *I. delicatissima* (Valette et al. 2013; Judson et al. 2018). Nine loci were monomorphic and only 2 had more than one common allele. This low diversity is not a property of the loci per se, but rather of the St. Eustatius population; in other populations of *I. delicatissima*, these same loci showed much higher heterozygosity (Valette et al. 2013; Judson et al. 2018). Comparing all 9 loci (IgdL11, 12, 14, 17, 19, 20, 21, 22, and 24) homologous between Valette et al. (2013) and this study, heterozygosity was much lower on St. Eustatius (21 km^2; H_O = 0.022) than on the much smaller island of Îlet Chancel (0.8 km^2; H_O = 0.44). Similarly, when comparing the 6 loci (CycCar177, D105, D110, D135, D136, 60HDZ13, and 60HDZ148) homologous with Judson et al. (2018), the heterozygosity on St. Eustatius (H_O = 0.004) was much lower than on Dominica (751 km^2; H_O = 0.43). Variability of microsatellite loci in *Cyclura*, the other genus of iguana native to the Caribbean, is consistent with that observed on Îlet Chancel and Dominica (Colosimo et al. 2014; Aplasca et al. 2016; Welch et al. 2017). This demonstrates that the

Figure 3. Cropped images showing the eyes of 2 different iguanas captured on St. Eustatius. (**a**) An abnormal *Iguana delicatissima* eye; (**b**) a normal eye.

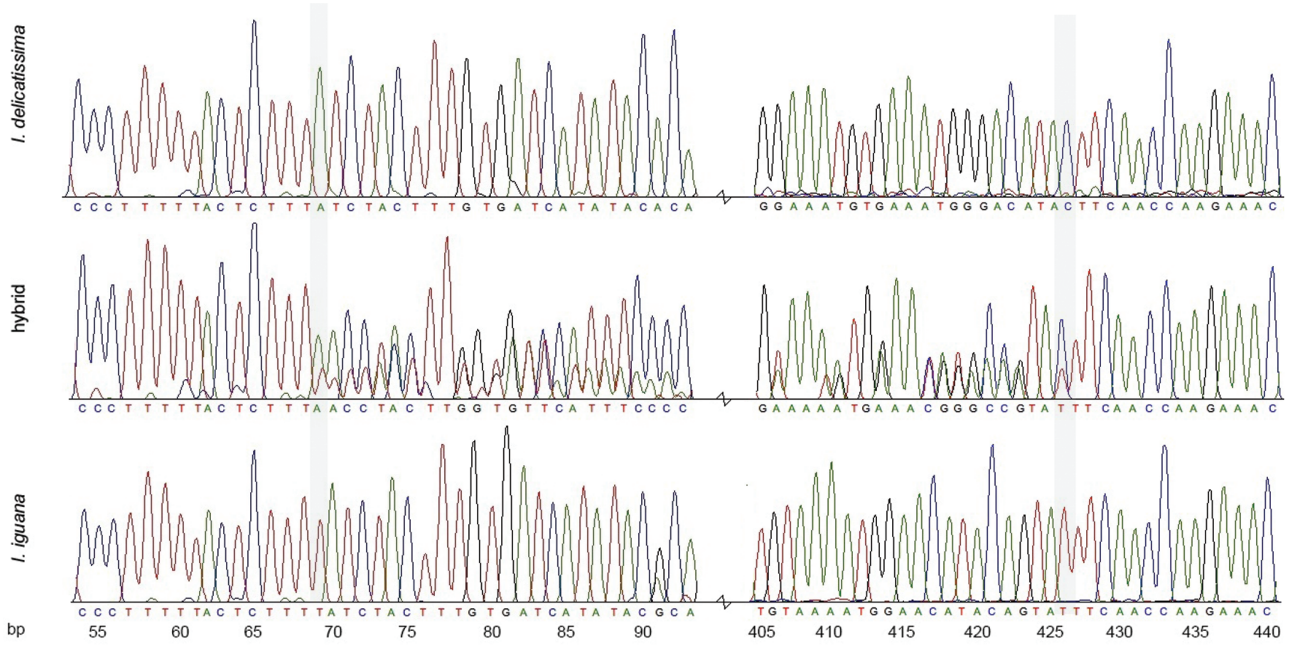

Figure 4. PAC electropherograms from *Iguana delicatissima,* hybrid and *Iguana iguana* individuals. Interspecific *Iguana* indels exist for PAC alleles: hybrid electropherograms no longer run synchronous after these indels, indicated using gray boxes. Data show one sequence per species/hybrid for visual clarity, for each category every individual showed the visualized pattern.

population on St. Eustatius is extremely depauperate in terms of its genetic variability.

There are several historical processes that may have jointly caused the low diversity. First, the population might have originated from a small founder population, either through natural dispersal or facilitated by Amerindians, as suggested for this species (Martin et al. 2015) and for other species (Censky et al.1998; Bryan et al. 2007; Hedges and Heinicke 2007). Secondly, postcolonization bottlenecks could have decreased the population's genetic diversity, especially when its effective population size remained small for long periods of time (Amos 1996; Landergott et al. 2001; Schultz et al. 2009). Bottlenecks might have occurred in the 18th and 19th century as natural habitat would have been scarce due to extensive agriculture (Chambers and Chambers 1842). Ironically, the lack of genetic diversity prevented us from making an accurate estimate of the effective population size. This also occurred in a study on Australian Lungfish (Hughes et al. 2015), despite their total heterozygosity being higher than presented here. Despite the lack of a statistical estimate of N_e, it is clear from the low diversity and the small census size that N_e must be small. During our island-wide survey, we counted 324 individuals, which is only slightly higher than the last estimate of 300 individuals (Debrot et al. 2013). Currently, due to continuing conservation efforts 434 animals have now been tagged with a PIT tag, bead tag or both (RAVON, unpublished data).

Inbreeding in small populations is almost certain to occur and increases the chance of homozygosity of deleterious alleles and inbreeding depression (Amos 1996; Charlesworth and Willis 2009; Allendorf et al. 2013). This especially holds for populations that recover slowly from bottleneck events and reduced effective population size (Weber et al. 2004). Inbreeding and inbreeding depression can

lead to an increase in abnormalities, disorders and diseases (Madsen et al. 1996; Olsson et al. 1996). During our fieldwork, 3 types of abnormalities were identified; eye abnormalities, extra nonfunctional appendages and a primary but boneless tail. Of these, at least the latter 2 are likely to have a genetic origin. The population's low genetic diversity and the presence of abnormalities provide evidence consistent with inbreeding depression. Unfortunately, the effects of the genetic load present in this population cannot be assessed directly with the molecular and morphological data currently available. Although we found supporting evidence for inbreeding and nonrandom mating within this population (F_{IS} = 0.12), a heterozygosity–fitness correlation analysis was inconclusive because the genetic diversity is so low that MLH values approach zero. We propose continuing ecological monitoring and research to obtain more information on the diversity and severity of abnormalities in this population.

We specifically tested whether the airport and the town of Oranjestad represent a barrier to migration and gene flow as these urban areas could limit iguana dispersal. We found a small but significant degree of genetic differentiation (F_{ST} = 0.021, P = 0.002) between these groups of individuals that was not consistent with isolation by distance. This result suggests that there is indeed somewhat reduced migration. However, this pattern says nothing about the timescale of the barrier to gene flow; such a small F_{ST} would be consistent with either a relatively high rate of gene flow that has been stable over time, or a recent more complete barrier to gene flow. The area where the current airport runway lies was previously cultivated by plantations and lacked natural vegetation (Hellebrand W, personal communication). Therefore, migration between both sides might have been limited for more than 2 centuries. Currently, the newly renovated fences of the airport (as of December, 2015) may further restrict dispersal as stone slabs have been placed to prevent holes from being dug underneath it. This forces large iguanas to pass through the fence itself, causing them to become stuck, a known source of mortality (the current article; see also Figure 5).

Table 1. Population genetic parameters from 16 microsatellite markers for *Iguana delicatissima* individuals from St. Eustatius

Marker	Na	Ae	H_O	H_E	HWE	F
CycCar177	1	1	0	0	—	—
D105	1	1	0	0	—	—
D110	8	3.102	0.583	0.678	***	0.14
D135	1	1	0	0	—	—
D136	2	1.006	0.006	0.006	NS	−0.003
IgdL11	1	1	0	0	—	—
IgdL12	1	1	0	0	—	—
IgdL14	2	1.008	0.008	0.008	NS	−0.004
IgdL17	1	1	0	0	—	—
IgdL19	4	1.029	0.029	0.028	NS	−0.01
IgdL20	2	1.004	0.004	0.004	NS	−0.002
IgdL21	2	1.2	0.159	0.167	NS	0.046
IgdL22	1	1	0	0	—	—
IgdL24	1	1	0	0	—	—
60HDZ13	1	1	0	0	—	—
60HDZ148	3	1.029	0.02	0.028	***	0.277
Average	2	1.149	0.051	0.057	—	0.063

Parameters are the number of alleles (Na), effective number of alleles (Ae), observed heterozygosity (H_O), expected heterozygozity (H_E), fixation index (F) and Hardy–Weinberg equilibrium. With HWE, NS indicates a nonsignificant departure from HWE, * indicates a significant departure (P < 0.05) from HWE and *** indicates a stronger departure (P < 0.001) from HWE.

Status of the Iguana populations on the Lesser Antilles

The changed status of the *I. delicatissima* population of St. Eustatius from "pure" to "invaded" and additional recent events necessitates an update for the status of *I. delicatissima* populations across its historic range. Currently *I. delicatissima* is extirpated on Antigua, Barbuda, St. Kitts, Nevis, St. Martin (both the Dutch and French sides), Marie-Galante, Les Saintes, and Grande-Terre (Breuil et al. 2010a; Vuillaume et al. 2015). Further, *I. iguana* is currently present on several islands where it is a major threat and contributor to the decline of the local *I. delicatissima* population: Anguilla, St. Eustatius, St. Barthélemy, Basse-Terre, Martinique, and La Ramier (Vuillaume et al. 2015; Curot-Lodéon 2016; the current article). A recent discovery of one *I. iguana* adult on La Désirade is extremely worrisome and implementation of future surveys need to identify if hybridization has already occurred (Association Ti-Tè 2017). Hence, the remaining islands that only harbor *I. delicatissima* individuals are limited to Dominica, and 6 small satellite islands belonging to other islands: Prickly Pear East (Anguilla), Îlet Fourchue and Fregate (St. Barthélemy), La Désirade and Petite Terre (Guadeloupe), and Îlet Chancel (Martinique) (Figure 6). This means that only 2 populations of this species occur on islands larger than 1.5 km^2 (Dominica, 751 km^2; La Désirade, 21 km^2), indicating the extreme vulnerability of this species.

Figure 5. Adult iguana that became stuck in a harmonica-wire fence after which it probably died of dehydration.

Furthermore, the vulnerability of several of these remaining populations could have severely increased due to the 2017 hurricane season, during which 2 major hurricanes (Maria and Jose) hit the Lesser Antilles. The impact of these events on the Lesser Antillean Iguana populations of Dominica, St. Barthélemy, and Anguilla still needs to be assessed.

The origin(s) of the *I. iguana* population on St. Martin is unclear, but can include Florida, the Greater Antilles or Basse-Terre (Breuil et al. 2010b, 2011). Our genetic data indicates intraspecific hybridization on St. Martin between individuals from 3 *I. iguana* clades (Stephen et al. 2013); Curaçao, Central America, and Caribbean/NE South America. Thus, providing new evidence of intraspecific hybridization within *I. iguana* and additional iguana translocations throughout the region.

Conservation Strategy

Our results highlight the need for a species recovery plan for the *I. delicatissima* population on St. Eustatius. The short-term survival of this population is under immediate threat by hybridization with the non-native *I. iguana* and hybrids. In addition, the low genetic diversity present in this population may limit its ability to adapt to a changing environment. Further reasons for concern are the apparent low recruitment and the presence of morphological abnormalities within this population that may be indicators of inbreeding depression. Given the presence of hybridization within this population, the main conservation efforts should be aimed at eliminating this threat. Such an effort will require routine surveys and a systematic study with continuous follow-up efforts, requiring persistent effort and a large financial investment.

In addition to conducting regular surveys for non-native iguanas on St. Eustatius, other methods are recommended. Female iguanas can be targeted during nesting season when they migrate in search of appropriate nesting sites. Further, known nesting sites could be monitored and fenced off during hatching season allowing conservationists the opportunity to identify and capture hybrid individuals when these hatch. The measure, suggested by Bock et al. (2016) to target invasive iguanas, to spread substances with the ability to deactivate the microbial intestinal flora of iguanas would not be recommended here because *I. delicatissima* would also likely be effected by these measures. Behavioral or ecological differences of hybrid iguanas have not been characterized, but would be of great interest to conservationist on islands where *I. delicatissima* occur. Finally, given that several *I. iguana* individuals have recently arrived on St. Eustatius as stowaways on boat traffic (the current article), it is clear that regional and local biosecurity improvements are of the highest priority.

After successful elimination of the hybridization threat, other actions should be undertaken to address conservation issues identified by this study; low recruitment and population size. Head-starting is a well-known method and could be considered to boost the small population size as has been done for several other species of iguana (Alberts et al. 2004; Grant and Hudson 2014). In addition, the low number of juveniles that we observed in this population is of concern. Previous studies indicate that recruitment can be limited by inbreeding depression acting on early life stages, but also by low availability of nest sites (Madsen et al. 1996; Olsson et al.

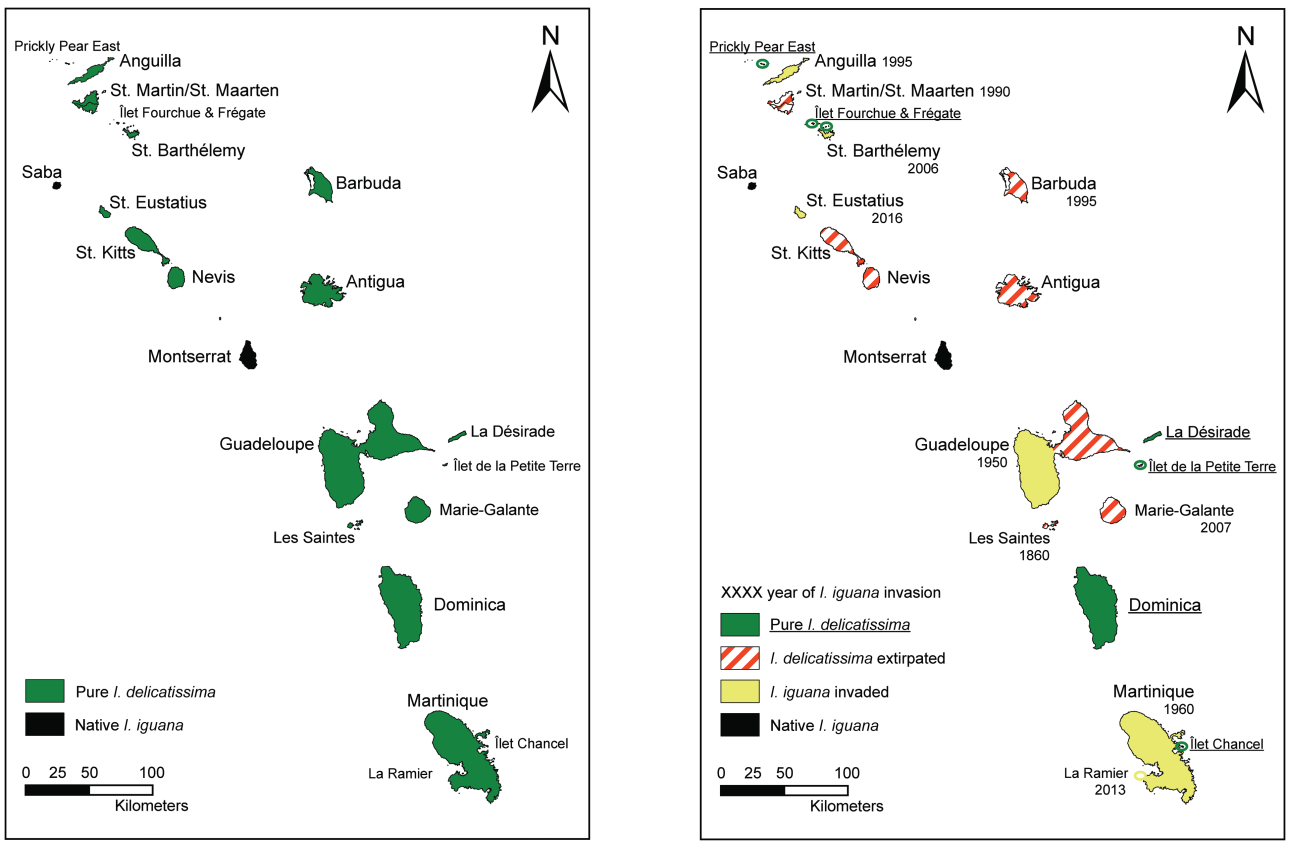

Figure 6. Historic and current distribution of *Iguana* species in the Lesser Antilles.

1996; Slate and Pemberton 2002). Indeed, currently only 11 sites are known on St. Eustatius (Debrot et al. 2013; van Wagensveld 2016; the current article). Several of the sites identified by Debrot et al. (2013), especially the main nesting area, appeared no longer in use and overgrown with high grass and some shrub during our fieldwork efforts. Therefore, a comprehensive survey of nest site usage and presence should be conducted to estimate hatching success, overall recruitment and whether inbreeding depression influences recruitment in this population.

However, when using these techniques, any present problems concerning inbreeding depression due to lack of genetic diversity would not be mitigated. Therefore, we propose to translocate iguanas from genetically different (but pure *I. delicatissima*) populations to St. Eustatius. These individuals could then be used in a captive breeding and head-starting program to increase the genetic diversity, potentially facilitating genetic rescue (Whiteley et al. 2015). Although arguments against genetic rescue include the possibility of outbreeding depression, evidence for an increase in individual fitness after translocation has been found by numerous studies (Whiteley et al. 2015). Also, Martin et al. (2015) indicate that most of the *I. delicatissima* island populations share the same mitochondrial haplotype, suggesting a lack of evolutionary divergence that would decrease the chances of outbreeding depression between individuals of these islands. In addition, genomic analyses might aid in identifying the most suitable source population from which animals should be translocated, thereby further reducing the chance of outbreeding depression (Whiteley et al. 2015).

Conclusion

We show that the *I. delicatissima* population on St. Eustatius is threatened by inbreeding, low genetic diversity, and above all by ongoing arrival of non-native iguanas and subsequent hybridization. Without an immediate systematic study and fulltime monitoring, hybridization and aggressive displacement will lead to its extinction. Although expensive and time consuming, a lack of initiative at this crucial stage will lead to an uncontrollable and unchangeable situation with large numbers of non-native iguanas and extirpation of *I. delicatissima* on St. Eustatius. Since only a few pure populations of the Lesser Antillean Iguana remain, the protection of the St. Eustatius population is important for the survival of this species.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

All relevant data are available in the text or in the Supporting Information.

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