A Genetic Analysis of the Invasive Green Iguana (*Iguana iguana*) in the Cayman Islands

By

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CHAPTER 1
INTRODUCTION

1.1 Summary

Green iguanas (*Iguana iguana*) are a highly invasive lizard species that have been introduced to multiple continental regions, as well as many Caribbean and Pacific islands. The introduction of this species has yielded apprehension because of the potential influences they might have on endemic flora and fauna, and even ecosystem function. This concern is heightened within island ecosystems due to their increased levels of endemism and potential sensitivity to invasives. It is due to these apprehensions that evaluating the modes of introduction, colonization, and the subsequent population dynamics of Green Iguanas in its introduced range are of the utmost importance if insight into the full impact of this species is to be understood and ultimately managed. Once collective knowledge of these dynamics is attained, and proper management practices are applied, the invasive species could potentially be eradicated in critical areas of introduction to mitigate their influences. In this study, our goal was to use a molecular genetic approach to evaluate three different invasive Green Iguana populations found in the Cayman Islands of the Caribbean, where this species has been successful at colonization and poses a threat to the endemic, critically-endangered iguana populations. The use of molecular methods has become increasingly popular in assessing the relatedness within and among populations. Therefore, given the prior success of these forensic approaches, it was deemed appropriate for reconstructing the invasion history of these iguanas. We predicted that our results would yield two patterns: (1) the small Sister Isles populations are related to the larger invasive colony on Grand Cayman and (2) these populations have much less genetic variation. While the Sister Isles would be composed of closely related individuals, they would exhibit lower levels of

diversity due to infrequent introductions from Grand Cayman, which harbors the largest successful invasive colony in the islands. If it were determined that the animals within these three populations were not closely related, or that the Sister Isles maintained moderate to high levels of variation, this would inform us that biocontrol is likely ineffective at managing this invasive species. Excessive levels of variation on the Sister Isles would be largely signified by the presence of an equal or greater number of alleles across multiple molecular markers when compared to the population on Grand Cayman. Higher levels of genetic variation among the Sister Isles Green Iguana populations could have directly resulted from either the consistent introduction of invasives that are successfully reproducing, or invasive colonies have been breeding for a sufficient time frame, allowing for the generation of adequate levels of variation among the recently sampled populations. If management and biocontrol have been successful, we should find a more genetically diverse population on Grand Cayman that is also clearly related and ancestral to the Sister Isle Green Iguana populations. Our results yielded a greater amount of genetic variation on Grand Cayman. Further, some genetic variants, private alleles, were found in the Grand Cayman Green Iguana population that were absent from both Little Cayman and Cayman Brac (i.e. the Sister Isles). These results were consistent with our predictions and showed that the Sister Isles Green Iguana populations were related to the larger Grand Cayman colony due to the alleles that were shared between them. The presence of private alleles within the Grand Cayman populations also supported the hypothesis that these individuals possessed greater levels of genetic diversity than the Sister Isles populations. Considering these results, it can be deduced that the Grand Cayman colony is likely the primary source of invasive individuals that have been introduced to the Sister Isles, but that management has been

ineffective in mitigating their introduction due to the present levels of variation among the Sister Isles iguanas.

1.2 Invasive Species and Establishment

Invasive species are a threat whose origins and subsequent dispersal can be traced back in antiquity to anthropogenic influences (Leuven et al. 2009, Pickering et al. 2011, Meyerson and Mooney 2007, Hulme 2009). Invasive organisms, which are also commonly referred to as non-native or alien, are all equally applied when defining a species that has been introduced to a region(s) outside of the original historical range and are represented by a wide array of organisms, including multiple microorganisms, plants and fungi, vertebrates, and invertebrates (IUCN 2000). While the dispersal of alien species by humans has been occurring for millennia, rates of introduction have only increased through the centuries, and within the past 25 years dispersal rates have reached their highest records (Hulme 2009). It is a direct result of these exponentially increasing introductions, which are largely expected to continue rising in incident, that concern regarding the health and integrity of native ecosystems has peaked (Lodge et al. 2006, Sutherland et al. 2008, Bauer 2012). The heightened awareness that detrimental effects could arise given the introduction of an alien species has led to the publication of numerous studies which have sought to define the relationship between introduced species and non-native habitats. While there are numerous accounts of non-native organisms being dispersed accidently, alien species have been introduced purposefully for multiple reasons, predominantly as biological controls of a more harmful invasive (McFadyen 1998, Thomas and Reid 2007, Messing and Wright 2006). When managed effectively, specifically when limiting risks of unintentional and uncontrolled dispersals, alien species can even fill critical niches in an

ecosystem that was originally occupied by a native species that has since been lost (Schlaepfer et al. 2011).

While there can be benefits for the intentional introduction of non-native species, their costs, if unregulated, can greatly outweigh their value. Invasive species, once established and dispersed, can negatively impact biodiversity, directly affect native species, and further threaten endangered populations (Kairo et al. 2003, Lee 2002, Alonso et al. 2001, Abdelkirm et al. 2005, Vitousek et al. 1997). In fact, introduced species represent the main cause of endemic species extinction throughout many ecosystems, especially those of islands (Reaser et al. 2007**,** Gurevitch et al. 2004, Clavero et al. 2009, Blackburn et al. 2004, Sax and Gaines 2008). Within the US, impacts of invasive species on the extinction and endangerment of natives is cited as the second leading factor, falling only behind direct human influence (Pimentel et al. 2005). While studies have assessed a discouraging relationship between the presence of an alien species and the ecosystem in which it has been introduced, the introduction of non-native individuals does not guarantee establishment and subsequent colonization success (Kolar and Lodge 2001, Williamson and Fitter 1996). While a relatively large number of non-native species have been introduced to novel ecosystems, a limited number have resulted in successful invasive establishment (Kolar and Lodge 2001). For an alien species to be considered truly invasive (i.e. maintain an actively breeding colony that furthers establishment), individuals must first be introduced, then establish themselves by adjusting to new selective pressures and produce fit offspring, and then finally spread to regions further within the non-native range (Figure 1, Kolar and Lodge 2001). Therefore, each of these steps may supply distinct opportunities to limit successful invasions once they are identified, allowing the management of invasives to become proactive before a colony has been established (Wilson et al. 2009).

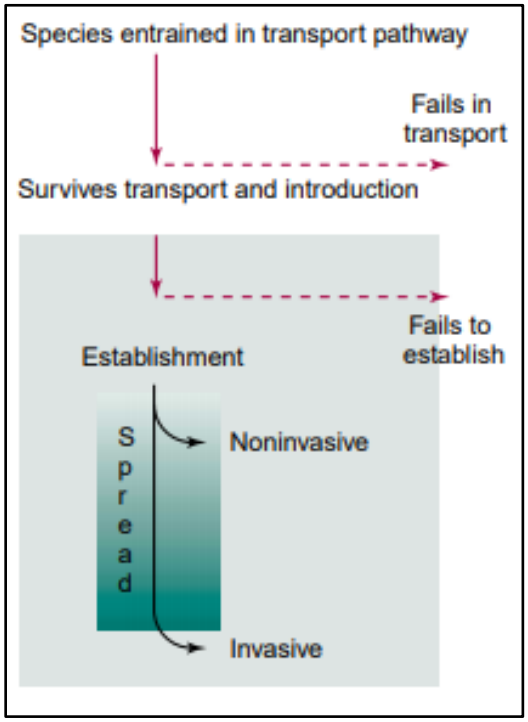

Figure 1: Diagram of the steps an introduced species must follow to become invasive in a non-native ecosystem. The dotted arrows represent the failure of the species to proceed through the transition, preventing it from becoming invasive. Solid arrows signify the species has overcome the dispersal barrier. Each of these points during the process of an invasion allow for management to act proactively. Image from Kolar and Lodge 2001.

1.2.1 Green Iguanas: An Invasive Species

An example of a successful invasive species is the Green Iguana (*Iguana iguana*). These are highly invasive lizards that have colonized multiple regions outside of their historical range of Central and South America (Villanueva 2016, Krysko et al. 2007, Falcón et al. 2013). The introduction of this species to new areas has been largely facilitated by human-mediated modes of dispersal, and their invasiveness is due to multiple ecological and evolutionary factors (Villanueva 2016, Krysko et al. 2007, Falcón et al. 2013). Green Iguanas mature quickly with males able to reach sexual maturity around 20 months and females within about 31 months (Sementelli 2008, Meshaka et al. 2007). This species also produces large clutch sizes, and individuals can be long lived (Sementelli 2008, Meshaka et al. 2004). In addition, most of the

ecosystems where the Green Iguana has successfully invaded are tropical and do not support large, terrestrial endemic predators, allowing for increased offspring survivorship and atypically high population densities (Meshaka et al. 2009, Smith et al. 2007). Such population explosions within short timeframes can present multiple deleterious effects to human communities and native ecosystems. Throughout many areas where this species has been introduced, they are considered a pest, due largely to their destructive digging activities, which destroy residential and commercial landscape vegetation and infrastructure (Krysko et al. 2007, López-Torrez et al. 2012). In southern Florida, a region where this species has flourished, damage caused by burrowing females to canals, levees, and dikes, which are required for flood control and water management, have resulted in substantial hydraulic structure failure and an estimated cost of $2,480/hectare where these iguanas are present (Sementelli 2008). Large numbers of basking adults have also posed airstrike hazards on runways in Puerto Rico (Engeman et al. 2005). Increased numbers of invasive iguanas also impact endemic flora and fauna. The herbivory of such a large number of individuals could potentially eradicate multiple native plant species as well as assist in the establishment of multiple non-natives through fecal distributions (Krysko et al. 2007, Townsend et al. 2003, Falcón et al. 2013). The species has also been observed usurping the burrows of the Florida burrowing owl (*Athene cunicularia floridana*) and the gopher tortoise (*Gopherus polyphemus*), which is a species vulnerable to endangerment and considered a keystone species (Sementelli 2008). While there are documented cases of the Florida Burrowing Owl feeding on invasive juvenile iguanas, the disruption to the population dynamics of this species by burrow usurpers likely exceeds any benefits and costs the state of Florida $500 for each lost owl (McKie et al. 2005, Florida Administrative Code 39). There is also evidence supporting the depredation of egret eggs by invasive iguanas, which also has a cost of $500 per

incident and could contribute to population sensitivity in these areas if predation is not managed (Sementelli 2008, Arendt 1986, Florida Administrative Code 39).

While all endemic fauna is at risk of the detrimental impacts generated by the invasive iguana, native species with similar resource-dependency, particularly those that are closely related to the invasive, are of special concern (Keitt et al. 2017). Many of the islands where the Green Iguana has been introduced host endemic iguanas, and a majority of them, including those of the Blue Iguana (*Cyclura lewisi*) of Grand Cayman and the Sister Islands Rock Iguana (*Cyclura nubila caymanensis*) of Cayman Brac and Little Cayman (i.e. the Sister Isles), are critically endangered. The introduction of predatory mammals, particularly those of domesticated origin such as cats and dogs, habitat fragmentation, and increased vehicular traffic have all directly impacted these endemic iguana populations (Alberts, 2004). Hence, the introduction of the Green Iguana likely acts synergistically, amplifying population declines of these native species. While documented evidence that competition for resources, including food and nesting sites, is scarce, the overlapping territories and similar resource-dependency of the invasive Green Iguanas and endemic iguanas contribute to the likelihood of competition occurring if resources were limited. Additionally, concern regarding the transmission of novel diseases to the critically endangered populations is also under review. Furthermore, hybridization events between native and invasive iguanas have been confirmed to occur in the Caymans when putative hybrids between the Sister Isles Rock Iguana and the Green Iguana were captured and later authenticated through a genetic analysis in 2017 (Moss et al 2017). This threat of hybridization is magnified due to the pronounced effect it has already had on other iguana species in the Caribbean, such as *Iguana delicatissima* of the Lesser Antilles where it is deemed the greatest risk to the persistence of this species throughout its range (Moss et al. 2017, Vuillaume et al. 2015, Oppel et al. 2017).

High rates of hybridization threaten species with “genomic extinction,” whereby the natural population is replaced by the invasive population through piecemeal introgression (Fitzpatrick et al. 2009, Rhymer and Simberloff 1996, Mooney and Cleland 2001). Therefore, if all anthropogenic stressors that resulted in the initial population decline of these species were remedied, the endemic iguana populations could still experience extinction if hybridization events between the endemics and invasives is not eliminated.

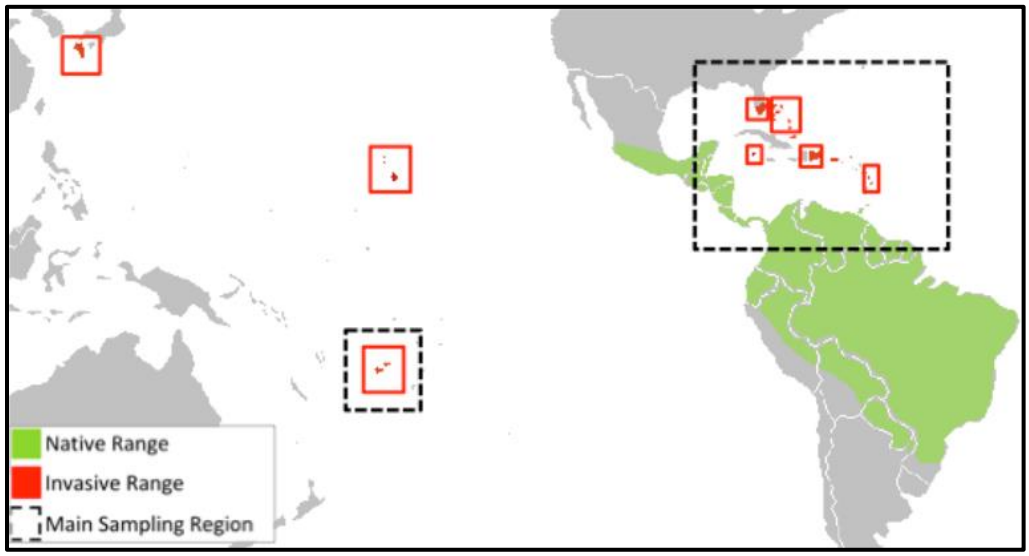

Figure 2: Historical vs Introduced Distribution of the Green Iguana. Image from Villanueva 2016.

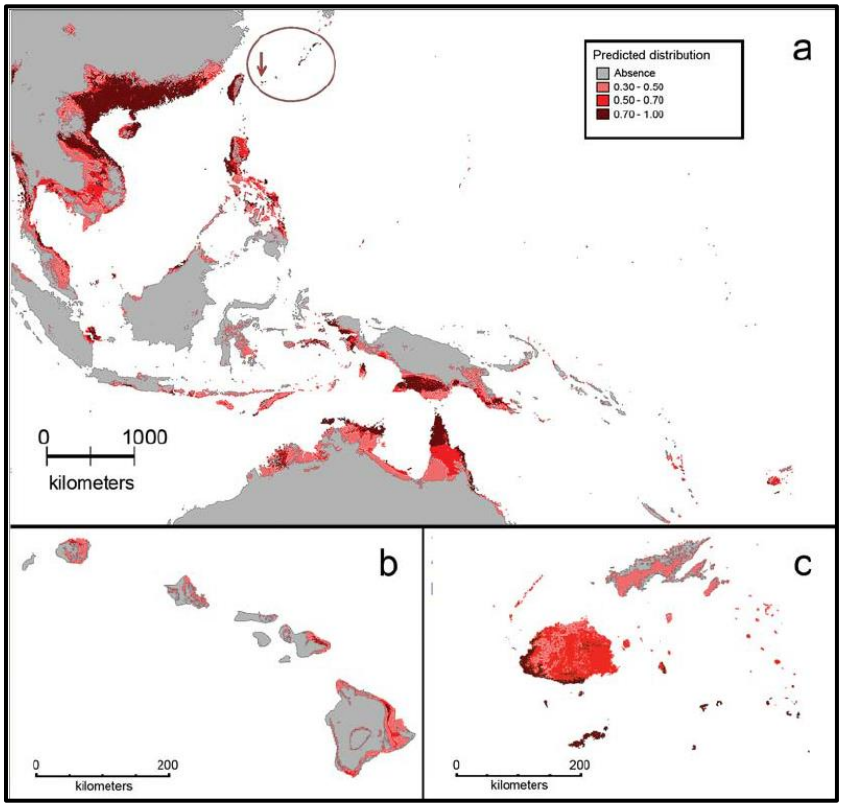

Figure 3: Predicted distribution for *Iguana iguana* in the Pacific (a), Hawai'I (b), and Fiji (c). This image reinforces the notion that Green Iguanas are highly successful invaders that, if not monitored and controlled, can extend their introduced ranges exponentially. Image from Falcón et al. (2013).

1.3 Green Iguanas in the Cayman Islands

The Cayman Islands are a British Overseas Territory within the western Caribbean Sea that includes three islands: Grand Cayman, Cayman Brac, and Little Cayman. Grand Cayman is the only location where the endangered Blue Iguana (*Cyclura lewisi*) can be found in the wild while Cayman Brac and Little Cayman support the last remaining wild populations of critically endangered Sister Islands Rock Iguana (*Cyclura nubila caymanensis*). As discussed, the introduction of the Green Iguana to these islands represents a major concern that they may further impact these endangered species and hamper ongoing attempts to restore stability. The invasive iguanas are thought to have been imported to Grand Cayman more than 30 years ago as

exotic pets (Serju 2019). It is speculated that multiple Green Iguanas escaped into the wild from captivity following the destruction of Hurricane Ivan in 2004 (Serju 2019). Instances of intentional releases are also speculated to have contributed to the appearance of numerous wild Green Iguanas on Grand Cayman. Initially, their presence raised no alarm, so their populations were not monitored or controlled. This lack of control mechanisms resulted in an exponential population explosion, where Green Iguana numbers were estimated to range between 1.1 and 1.6 million individuals in 2018 (Whittaker 2018). To mediate their impact and drastically reduce the population size, an island-wide one-year culling program was implemented in October 2018 where cullers were offered CI$5 a head for each reptile (Serju 2019, RCIPS 2020). The end of the program in 2019 marked the extermination of over a million individuals, and a new culling season was approved and continued in January 2020 with 594 iguanas already culled to date (Connolly 2019, Ragoonath 2020).

While the appearance of Green Iguanas on the Sister Isles was first reported in 2007, these islands have yet to witness a population explosion comparable to Grand Cayman's (Moss et al. 2017). This is likely due to heightened awareness of the invasive potential of the species which allowed for successful removals when individuals were captured early in their invasion pathway (Moss et al. 2017). However, Green Iguanas, while low in numbers, still persist within the Sister Isles. With the demonstrative effectiveness of culling efforts on Grand Cayman, similar programs have been implemented on both Cayman Brac and Little Cayman for several years. Unfortunately, despite annual culling success, it is a course of action that must be revisited every year to thwart invasive Green Iguana colonization on the Sister Islands. Until the modes of invasive introductions to Cayman Brac and Little Cayman are determined and the effects of invasions on the population dynamics of the species are understood, Green Iguanas will continue

to remain on the islands which will allow for the threat of genomic extinction to persist for the Sister Islands Rock Iguana.

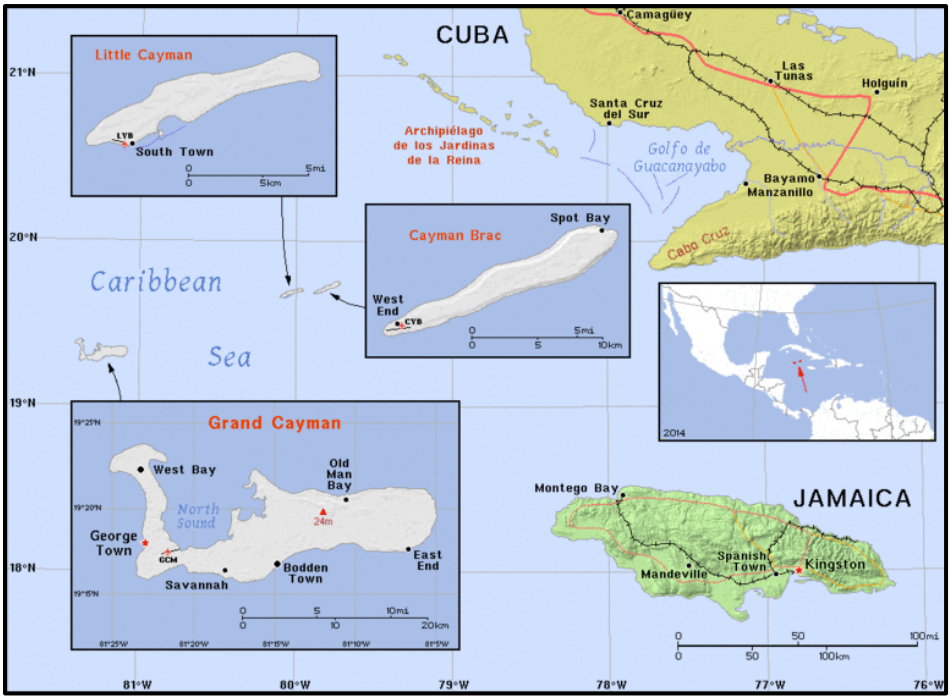

Figure 4: Atlas of the Cayman Islands. Image released into public domain by author Ian Macky (2017).

Figure 5: Location map of the Cayman Islands and their endemic critically endangered iguana species. The introduction of the Green Iguana to these islands has raised considerable concern that their presence will further harm the population statuses of these species.

1.4 Previous Hybridization Project Revealed Allelic Disparity among Sister Isles Green Iguanas

In a previous project conducted in 2018, 6 hybrid hatchlings and 14 Green Iguana hatchlings captured on Little Cayman in 2016 and 2017 were evaluated to determine if they were dammed by the same Green Iguana. While evaluating the sibship and parentage of these hatchlings, the genotypes of other Green Iguana individuals from Little Cayman and Cayman Brac were also analyzed as controls. Determining if a single female Green Iguana dammed both clutches signified whether the number of breeding invasives on the island of Little Cayman had been kept under control. Unfortunately, the results were largely inconclusive due to a lack of genetic variation found in the limited number of molecular markers used. However, incompatibilities at 4 of 16 microsatellite loci warn of the possibility that more than one Green Iguana female was breeding on the island. If confirmed, this finding would imply successful colonization of the invasive on Little Cayman. The analysis also revealed a similar lack of molecular variation across all Green Iguana individuals from both islands, but, when mitochondrial DNA (mtDNA) of these individuals were sequenced, there were notable differences between Cayman Brac and Little Cayman Green Iguanas. Of the Cayman Brac individuals (n = 41), 95% were identical and aligned to one mtDNA sequence (Haplotype 1) while 96% of the Little Cayman Green Iguanas (n = 25) were identical for an alternative mtDNA sequence (Haplotype 2). Since mitochondria are maternally-inherited, mtDNA sequence patterns, or haplotypes, can yield insight into the relatedness of individuals within and among populations (Allard et al. 1994, Avise et al. 1987, Hufbauer et al. 2004). Therefore, considering that a few Green Iguanas from both islands aligned to the corresponding majority haplotype of the other island, it can be inferred that individuals of similar genetic stock colonized the Sister Isles. Additionally, since two distinct haplotypes were derived from all individuals, it is likely that only

a few distinct maternal lines were most successful at reproduction on Little Cayman and Cayman Brac, generating the excessive mtDNA sequence bias. These inferences would account for the lack of molecular variation across the Sister Isles as well as the reasoning behind such distinct maternal haplotype designations.

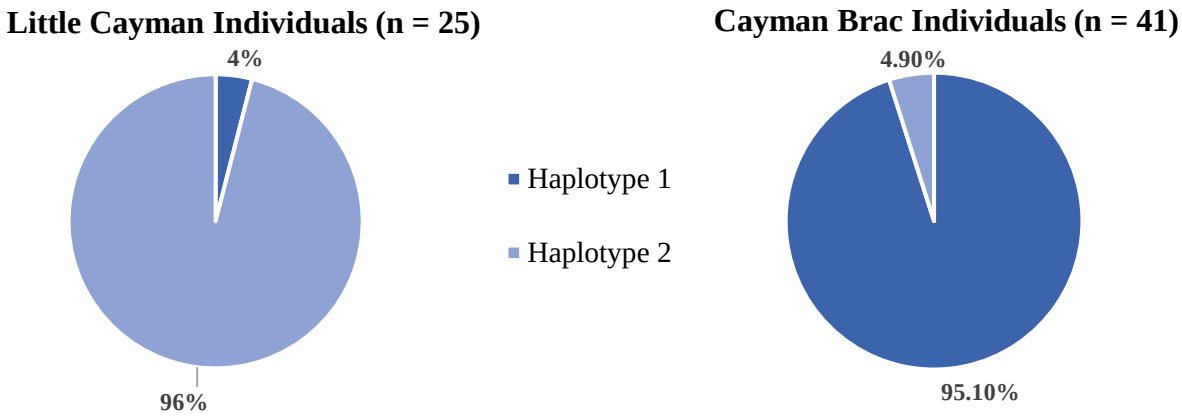

Figure 6: Mitochondrial Haplotype Distributions of Little Cayman vs Cayman Brac samples from previous study (Green Clutch, n = 14; Hybrid Clutch, n = 6; Fite's Farm female, n = 1; Reference Cayman Brac Greens, n = 41; Reference Little Cayman Green, n = 4).

1.4.1 Relevance to Project

This study sought to further evaluate the lack of molecular variation and differences in mtDNA haplotype frequencies obtained from the invasive Green Iguana populations found on the islands of Little Cayman and Cayman Brac. To reiterate, the previous analysis revealed a similar lack of nuclear molecular variation across all individuals from both Little Cayman and Cayman Brac, yet there were notable differences in mtDNA haplotype frequencies (Moss et al. 2017). This implied a disparity between the spread of nuclear and mitochondrial markers during the colonization of these islands. Given this finding, we hypothesized that individuals colonizing both islands were of similar genetic stock originating from the island of Grand Cayman, where

this species has been firmly established, but only a few females have successfully reproduced on Little Cayman and Cayman Brac. This would simultaneously account for the similarity in nuclear molecular variation and distinct mtDNA haplotype frequencies across the Sister Isles. Therefore, to further test the hypothesis that genetically similar individuals populated the Sister Isles, but that only a distinct few maternal lines were highly successful, the genetic variation of invasive Green Iguanas found on the island of Grand Cayman was characterized. If far more molecular variation is uncovered on this island when compared to Little Cayman and Cayman Brac as well as the presence of a distinct number of shared alleles between all three populations, it would be clear that a limited number of individuals is reaching the Sister Isles and successfully reproducing. Further, if true, this pattern of limited genetic diversity on the Sister Isles when compared to that on Grand Cayman would also indicate that the Green Iguana populations on Cayman Brac and Little Cayman are likely experiencing founder events and subsequent population bottlenecks; a founder effect occurs when a small number of individuals populate a new region, resulting in a population with reduced genetic diversity (Nei et al. 1975, Abdelkrim et al. 2005, Dlugosch and Parker 2007, Kolbe et al. 2004). While a founder event would indicate that limited individuals are currently breeding on the Sister Isles, this would still suggest that current biosecurity parameters are ineffective in preventing the distribution of invasive iguanas if such an event occurred recently. Therefore, while culling has proven to be an effective eradication method for removing a large number of individuals within a limited timeframe, delegating extensive amounts of funds and workforce are less effective if their modes of distribution are not controlled. If opportunities for the introduction of the Green Iguana to the Sister Islands are available, no amount of culling will completely eradicate them from these ecosystems. However, if we determine that genetic variability is similar across all islands, this

will inform us that either increased numbers of Green Iguanas are in fact reaching the Sister Islands and proliferating or that the invasive populations have already achieved colonization status. Therefore, to limit the current effects of the invasives on the islands, culling would be the most effective method in reducing the current population status of Green Iguanas to a manageable number in the short-term. Once the invasive populations have been culled to a size that their effects on the ecosystems, and particularly their influence on the endangered Sister Islands Rock Iguana, can be ruled as negligible, evaluation and implementation of effective biosecurity controls to limit new opportunities for invasive individuals to be introduced will be appropriate.

CHAPTER 2
OBJECTIVES & HYPOTHESIS

2.1 Objectives

The objective of this study was to evaluate genetic variability within the Grand Cayman Green Iguana population. This assessment was deemed necessary to better understand the spread of this invasive species throughout the Cayman Islands. By determining whether the genetic variation found in the Sister Isles Green Iguana populations in a previous study (Moss et al. 2017) could have come from Grand Cayman, we hope to better inform management of the invasive. Further, if Grand Cayman is the source of Green Iguanas in the Sister Isles then quantifying the proportion of the genetic variation present in each of the populations have direct implications regarding the potential effectiveness of specific biosecurity measures. If the Sister Isles are relatively depauperate in genetic variation, then simpler biocontrol methods may be suitable. Whereas more extreme measures might be necessary if the majority of genetic variation on Grand Cayman is finding its way to the Sister Isles. This would imply a steady influx of

Green Iguanas from the source population to the Sister Isles. To accomplish this objective, samples from all three invasive Green Iguana populations (i.e. those found on Grand Cayman, Cayman Brac, and Little Cayman) were assessed for genetic variation using a set of nuclear molecular markers known as microsatellites to explore these potential patterns of molecular variation across the Cayman Islands.

2.2 Hypothesis

We hypothesized that the invasive populations on Little Cayman and Cayman Brac originated from Grand Cayman. We further hypothesized that the Sister Islands population of Green Iguanas are experiencing genetic bottlenecks due to limited dispersal events from Grand Cayman. Predictions consistent with these hypotheses are that most if not all variation present in the Sister Isles is also present on Grand Cayman, and that genetic variation in the Grand Cayman population across the microsatellite markers evaluated exceeds that found in the Sister Isles populations.

CHAPTER 3
MATERIALS & METHODS

3.1 Project Design

3.1.1 Sample Collection and DNA Extraction

The Green Iguana samples that were included in this project were all collected from culling efforts. If the animal was collected alive, blood samples, snout-vent and vent-tail measurements, and sex, if it could be confidently determined, were taken and recorded before dispatch. Weight and estimated age were recorded after dispatch. Tissue samples, including sections of toes and/or tails, were collected from individuals that were already deceased when the

Cayman Islands Department of Environment arrived to collect samples. Green Iguanas were sampled from all 3 islands: Little Cayman (n = 23), Cayman Brac (n = 58), Grand Cayman (n = 24) captured between the years 2015-2019. Blood was collected in amounts between 0.5-2.0 ml, depending on animal size and body temperature. Blood was drawn from the caudal vein of the tail with a syringe. Blood was preserved in vials containing 0.5% SDS blood buffer that were labeled with the island of origin, date, location of capture, and a unique identification code. When blood could not be drawn or animals were dead upon arrival, tissue samples were collected. Either 1-2 toes or 1-2 inches of the midsection of the tail were clipped from a deceased animal and placed in vials labeled accordingly. All tissue samples were stored in 90% alcohol. All samples were stored in a freezer or fridge when available during travel. Upon arrival into the laboratory at Mississippi State University, all samples were kept in a freezer until DNA isolation.

The type of samples available (i.e. blood or tissue samples) for each population were as follows: all 23 Little Cayman samples were blood, 4 out of the 58 total Cayman Brac samples were tissue, and all 24 Grand Cayman samples were tissues. DNA was extracted from blood samples via blood extraction protocols utilizing the Maxwell® 16 Tissue DNA Purification kits. Before running any tissue samples through the extraction protocol, all samples had to be prepped to ensure optimal extractions. Toe and/or tail samples were removed from their vials and pieces approximately ½ cm were cut using sanitized knife and forceps. Sample pieces were then placed into individual vials and 200-300 uL of TE buffer and 3-4 nickel-plated buckshot were added; buffer quantity and amount of buckshot depended on sample size. Vials, which included tissue samples, TE buffer, and beads, were then placed into a bead mill for cycles of 3 minutes for homogenization. Homogenized samples were then centrifuged for 20-30 seconds. Milled toe and TE buffer were then placed in Maxwell 16 ® for DNA extraction cycle. Due to the increased

exposure and less stable storage conditions of all tissue samples in alcohol, DNA extraction from tissue samples had to be conducted multiple times for a sufficient yield for molecular analysis. The discrepancies between blood and tissue extraction protocols was considered when optimizing protocols for working with each of the microsatellites used.

Figure 7: Collection of a blood sample from a juvenile Green Iguana captured on Cayman Brac in July 2018. Photo taken by Sophie O' Hehir.

3.1.2 Molecular Methods

This study used molecular methods to assess population genetic structures and largely focused on the distribution of genetic variation within and among populations. These microsatellite-based methods have been commonly used in ecological and evolutionary studies to reconstruct the evolutionary history of invasions (Cristescu 2015, LaRue et al. 2011). Unfortunately, while Green Iguanas are one of the most notable and widespread iguana species, an extremely small number of species-specific markers have been designed and developed. Therefore, part of my task was to reference papers that had conducted similar population genetic

surveys for closely related species. Because these markers were successful in their use for other projects, we anticipated that some degree of variation would exist at these same loci in the Green Iguana. Microsatellites represent tandem repeats of one to six bases and are highly polymorphic (Cheng and Crittenden 1994). These polymorphisms are also subject to simple Mendelian inheritance patterns, meaning patterns of inheritance and inferences regarding population structure can be asserted when assessing these markers (Kaya and Yildiz 2008, MacAvoy et al. 2007).

3.2 Microsatellite Markers and Polymerase Chain Reactions

To assess genetic variability between the three islands, a total of 26 microsatellite markers were used. Of these 26, 12 microsatellite markers optimized for *Cyclura nubila caymanensis* (Moss et al. 2017, An et al. 2004, Welch et al. 2011, Malone et al. 2003, Rosas et al. 2008, Lau et al. 2009), 7 microsatellite markers optimized for *Cyclura cychlura cychlura* (Colosimo et al. 2014), and 7 microsatellite markers optimized for *Iguana delicatissima* (Valette et al. 2013) were used in genotyping all 105 Green Iguana individuals (Grand Cayman n = 24, Cayman Brac n = 58, Little Cayman n = 23). PCR was conducted in 10 uL reactions with approximately 7.1 uL ddH2O, 1.2 uL of Master Mix (i.e. 100 uL dNTP + 1000 uL ddH2O), 0.06 uL Forward primer, 0.3 uL Reverse primer, 0.3 uL M13 Tag, 0.1 uL *Taq* DNA polymerase, and 1.0 uL of DNA. Each locus was amplified with one or two replicates according to an optimized touchdown PCR protocol (ranging from 50°-57°). The thermal cycling profiles followed touchdown cycle guidelines (Welch et al. 2011). Gel electrophoresis was conducted upon thermal cycling completion to ensure PCR product was present.

Table 1: Microsatellite Loci (n=26), Referenced Paper, Source Species (*I. delicatissima* [n=7], *C. n. caymanensis* [n=12], *C. c. cychlura* [n=7]), M13 Tag, and Touchdown PCR Temperature

Locus	Reference	Source Species	M13 Tag	T (°C)	Locus	Reference	Source Species	M13 Tag	T (°C)
1) Igdl11	Valette et al. 2013	I. delicatissima	Ned	55	14) Z154	An et al. 2004	C. n. caymanensis	Pet	55
2) Igdl12	Valette et al. 2013	I. delicatissima	Fam	56	15) Z419	An et al. 2004	C. n. caymanensis	Fam	54
3) Igdl14	Valette et al. 2013	I. delicatissima	Vic	54	16) Z494	Colosimo et al. 2014	C. c. cychlura	Ned	54
4) Igdl17	Valette et al. 2013	I. delicatissima	Pet	53	17) Z780	An et al. 2004	C. n. caymanensis	Vic	55
5) Igdl19	Valette et al. 2013	I. delicatissima	Ned	56	18) CIDK177	Welch et al. 2011	C. n. caymanensis	Ned	54
6) Igdl20	Valette et al. 2013	I. delicatissima	Fam	55	19) F436	Malone et al. 2003	C. n. caymanensis	Ned	56
7) Igdl24	Valette et al. 2013	I. delicatissima	Pet	56	20) F519	Colosimo et al. 2014	C. c. cychlura	Pet	56
8) Z13	An et al. 2004	C. n. caymanensis	Vic	54	21) CCSTE02	Rosas et al. 2008	C. n. caymanensis	Pet	56
9) Z99	Colosimo et al. 2014	C. c. cychlura	Ned	55	22) C6	Colosimo et al. 2014	C. c. cychlura	Fam	50
10) Z106	Colosimo et al. 2014	C. c. cychlura	Vic	56	23) C124	Colosimo et al. 2014	C. c. cychlura	Fam	55
11) Z132	An et al. 2004	C. n. caymanensis	Ned	50	24) D9	Lau et al. 2009	C. n. caymanensis	Hex	57
12) Z148	An et al. 2004	C. n. caymanensis	Vic	53	25) D110	Lau et al. 2009	C. n. caymanensis	Fam	54
13) Z151	An et al. 2004	C. n. caymanensis	Ned	53	26) D136	Colosimo et al. 2014	C. c. cychlura	Fam	56

Table 2: Primer Name and DNA Sequence

Primer Name	DNA Sequence	Primer Name	DNA Sequence
1) Igdl11	F: CACGACGTTGTAAAACGACGCTT CAGTGCATAGTTTCCTGTT R: TCATATATGCACTTCCCTCTCC	14) Z154	F: CACGACGTTGTAAAACGACATGTGCGGTC TCTCAGTTCTG R: AGTCTTGCTTACTTTCATCCTATTG
2) Igdl12	F: CACGACGTTGTAAAACGAGCCCA CCAATTAATGGAA R: TCTCTGTTGCAATCCAGCAA	15) Z419	F: CACGACGTTGTAAAACGACTCATTCT R: GACCACACACTCCCTTTTTTG
3) Igdl14	F: CACGACGTTAAACGACCCTACAG ATCATATCTTGTGCATTC R: TGGGAGAGATTCATCGGAAC	16) Z494	F: CACGACGTTGTAAAACGACCACAAG R: GGAGTGATTCCCTCGCCTC
4) Igdl17	F: CACGACGTTGTAAAACGACAACC ATAATGTCCATCCACACA R: TGGAAGTTCAGGTGAATCCAT	17) Z780	F: CACGACGTTGTAAAACGACGTTCA R: CCTCCTCTGTAGCAGAATGTATGT
5) Igdl19	F: CACGACGTTGTAAAACGACCCTG GTACCACTCAAGCTC R: GCTGCTGCAGAAGTCATAGC	18) CIDK177	F: CACGACGTTGTAAAACGACTGTGACA AATCCCTTCCCTAA R: GGAACAAAGGAGAGGGTTCC
6) Igdl20	F: CACGACGTTGTAAAACGACCCTG TGCTAGAACTTGCCATT R: GATGAAAAGTGCCTTCCTAGACA	19) F436	F: CACGACGTTGTAAAACGACAGCTGAA R: CAGGAGAGGGTAATGGAGACT
7) Igdl24	F: CCTGTGGCAGCCAATTCTAT R: GGGCAGGGAGGAATAGAGTAA	20) F519	F: CACGACGTTGTAAAACGACCACTGCA R: TGGCAACACTGACATCCTAA
8) Z13	F: CACGACGTTGTAAAACGACGGGG CTGGTGGGATTTAG R: CGGTTGGAACATTTGATTTTG	21) CCSTE02	F: CACGACGTTGTAAAACGACCAGTGTG R: CCCTTTCCTTTCTGCTTGTATTTTG
9) Z99	F: CACGACGTTGTAAAACGACATCAT CCCCTTTTCCACAGAC R: CAGTGACCCTCCACGTTCTC	22) C6	F: CAATGGTTACTCTGAAAGGAA R: ATAGCCCTGGAACTGAGAAC
10) Z106	F: CACGACGTTGTAAAACGACTTACA TA R: GGTCAACAGAGCCAGGGG	23) C124	F: CTCTCTCTCTTTTCCATCTCT R: AGAAGCCAATAACACACCTAA
11) Z132	F: CACGACGTTGTAAAACGACTCCCC R: GTTCCTAACCCCCTCCCC	24) D9	F: GTGCTCAAACCACTACATCAC R: GCCTATCTGCCTTTTTCAA
12) Z148	F: CACGACGTTGTAAAACGACCC CAC R: GTTCTGGCATTGTTGTTTGTG	25) D110	F: CCCCTAACCTCTGAGAGTTT R: GTCTTGTACCGAACAGTGTTG
13) Z151	F: CACGACGTTGTAAAACGACCCTT GCCTCATAAAACCCA R: GTTCAGACCGTGTAGTGTGGATA	26) D136	F: AGGCATGAAATAATGACCTG R: AACAAAGTGAACCCATCTTG

3.3 Microsatellite Fragment Analysis, Peakscanner, and GenAlEx

Once PCR product had been attained for all molecular markers and samples (105 Green Iguana samples x 26 markers = 2,730 PCR products in total), 3 uL of each product was shipped, along with size standard GS500LIZ, to the Arizona State University DNA Core Laboratory where fragment analysis was performed on ABI capillary sequencers. Alleles were scored visually using PeakScanner™ v 1.0 software.

The program GenAlEx is a Microsoft Excel add-on that is primarily used to estimate basic population genetic parameters and calculate deviation from Hardy-Weinberg equilibrium (Mtileni et al. 2016, Peakall and Smouse 2006, Larson et al. 2014). GenAlEx was used to infer levels of genetic variation after allele scoring was completed for all microsatellites. Specific parameters that were evaluated using this program was observed (H_O) and expected heterozygosity (H_e), number of alleles (A_n), effective number of alleles (A_e), and the number of private alleles. An AMOVA (Analysis of molecular variance) was used to evaluate how variation is partitioned within the total sample set at three different levels: among populations, within populations, and within individuals. These parameters were selected to investigate because they are standards in the field of population genetics and Green Iguanas in particular (Villanueva 2016).

Heterozygosity, or gene diversity, was used in this study as an estimate of genetic variation among and between the different populations (i.e. the level of heterozygosity among loci). If heterozygosity is low, or close to 0, we can infer that the population of breeding individuals is small, a finding that would be consistent with a population bottleneck and infrequent introduction events (Houlden et al. 1996). If heterozygosity is high, it can be inferred that the population likely has a large number of breeding individuals and that multiple

introduction events have occurred (Villanueva 2016). This implication can be further substantiated if observed heterozygosity is higher than the expected heterozygosity. Expected heterozygosity is an estimation of gene diversity within a population by accounting for sample size only and can decrease if individuals are related or inbred (Harris and DeGiorgio 2017). Observed heterozygosity considers the observed frequencies of alleles compared to the total number of copies in a population. If observed heterozygosity is lower than expected heterozygosity, this would be consistent with inbreeding or biased sampling; distinguishing between these two is beyond the scope of this study. The number of alleles also represents the presence of allelic variation when polymorphisms are present (i.e. the proportion of heterozygotes in the population). The number of polymorphic loci within the population can also indicate genetic diversity (Leberg 1992). The effective number of alleles is equivalent to the number of equally frequent alleles that would yield the same expected heterozygosity as at a given locus (Peakall and Smouse 2009).

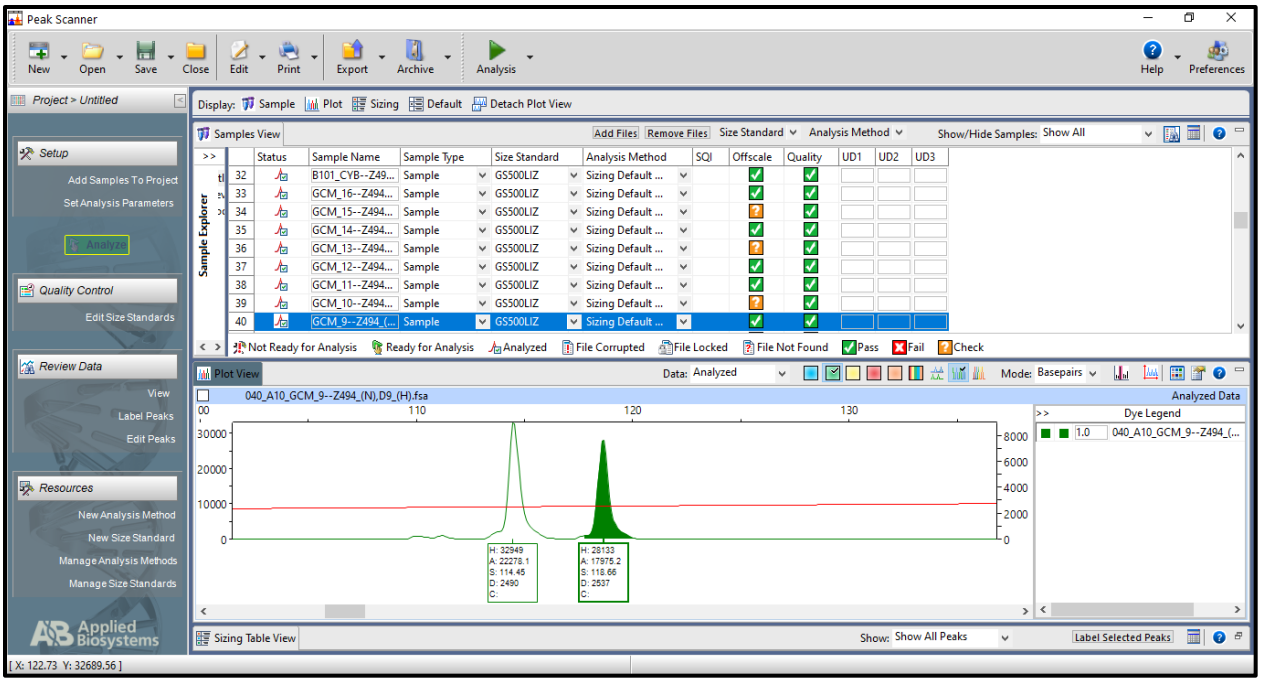

Figure 8: Screenshot of PeakScanner™ software and allele scoring.

CHAPTER 4

RESULTS

Only one molecular marker of X, Igdl-24, was dropped from the study due to a lack of successful PCR amplification across all populations. The number of alleles for the rest of the loci ranged from 4 - 21. Throughout the study, the Grand Cayman individuals were the most problematic due to reduced amplification success. It is suspected that these issues resulted from sample degradation as the Grand Cayman individuals were tissue stored in ethanol rather than the preferred blood in lysis buffer. Also as discussed in the methods section, DNA extraction from tissue samples required additional steps that could have also attributed to poorer DNA quality. The method of tissue storage could have also contributed to variable amplification success among samples considering they were stored in 90% alcohol, which does not stabilize DNA. Several estimates of population genetic variability were made using the genetic data

collected. These included observed (Ho) and expected heterozygosity (He), number of alleles (Na), effective number of alleles (Ne), and the number of private alleles. The percent of polymorphic loci across all populations was also determined. An AMOVA (Analysis of molecular variance) was also conducted to partition variation among and within populations and within individuals.

Observed heterozygosity was highest in the Little Cayman population (Ho = 0.670) while expected heterozygosity was highest in the Grand Cayman population (He = 0.574) (Table 3). Observed heterozygosity rates for the Sister Isles were both higher than their expected heterozygosity rates (Table 3). Grand Cayman's observed heterozygosity was lower than the expected (Table 3). The number of private alleles within all populations was around 2 (Table 3). AMOVA showed that the greatest proportion of variation (77%) was attributable to differences within individuals, 17% among populations, and the remaining 6% among individuals (Table 4, Figure 9).

Table 3: Genetic diversity statistics for Green Iguana populations in the Cayman Islands. Population names are followed by the mean values of the following: number of alleles (Na), observed (H_O), expected heterozygosity (H_e), effective number of alleles (Ne), and the number of private alleles. Standard error (SE) is presented in parenthesis for each value.

Population	**Na**	**SE**	**Ho**	**SE**	**He**	**SE**	**Ne**	**SE**	**% Polymorphic Loci**	**No. of Private Alleles**	**SE**
Little Cayman	5.08	0.535	0.670	0.062	0.523	0.044	2.544	0.227	96.00%	2.040	0.372
Cayman Brac	5.64	0.538	0.600	0.073	0.533	0.039	2.481	0.186	100.00%	1.800	0.412
Grand Cayman	5.84	0.663	0.507	0.061	0.574	0.040	2.911	0.288	100.00%	1.840	0.345

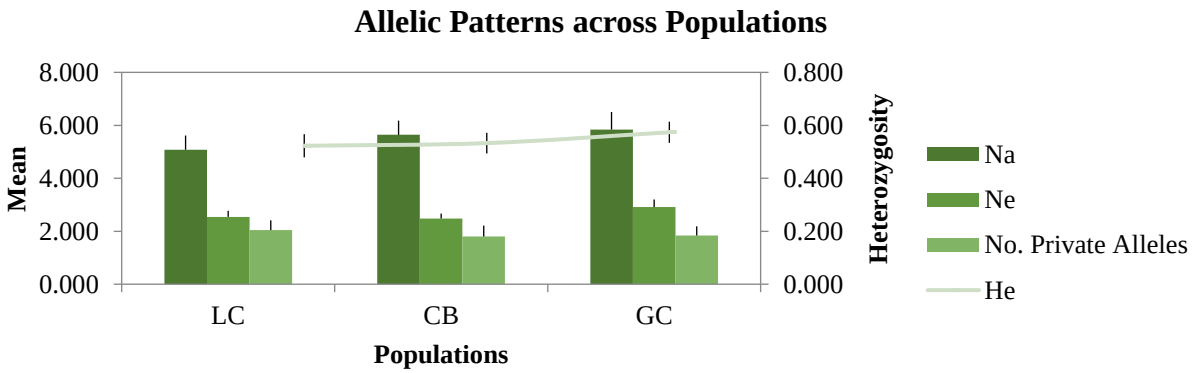

Figure 9: Bar Graph representing the patterns of number of alleles (Na), effective number of alleles (Ne), number of private alleles, and expected heterozygosity across the three populations.

Table 4: AMOVA assessment of variation.

Source	**Est. Var.**	**% Var.**	**Df**	**SS**	**MS**
Among Pops	1.44	16%	2	195.70	97.85
Within Pops	0.53	6%	102	799.03	7.83
Within Indiv	6.77	77%	105	710.5	6.77
Total	8.74	100%	209	1705.23	

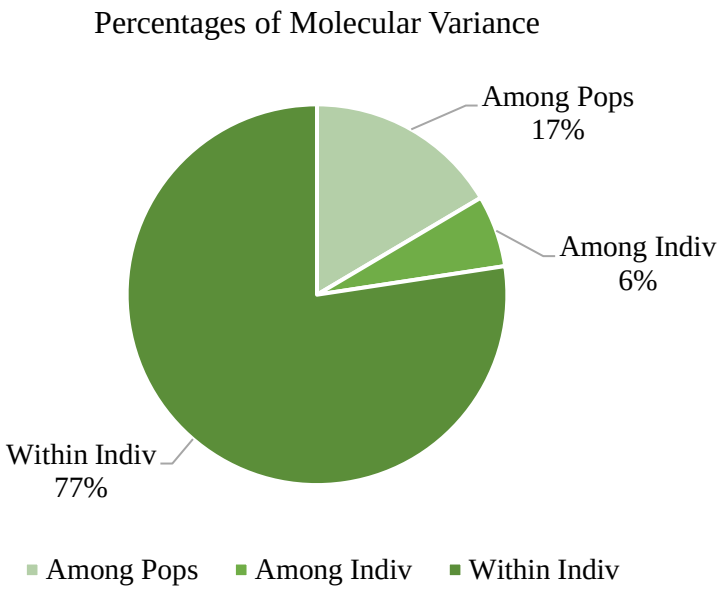

Figure 10: Variation percentages of AMOVA for the Green Iguana in the Cayman Islands.

CHAPTER 5

DISCUSSION

In this study, the utilization of molecular markers to assess the genetic variation of Green Iguanas on the island of Grand Cayman yielded further insight to their patterns of dispersal and invasion. We hypothesized that the population of iguanas on Grand Cayman is the primary source of the invasive individuals being introduced to the Sister Isles and that few introductions

occurred, accounting for the low levels of genetic variation found in the Cayman Brac and Little Cayman populations. The mean number of alleles and the mean effective number of alleles were both highest in the Grand Cayman population (Table 3), which indicates greater genetic diversity is present on this island. While observed heterozygosity was lowest in the Grand Cayman population, this was likely due to the sampling used in this study. The sample size is small, and animals were taken from across the island. Only 24 Green Iguana samples were available from Grand Cayman. Hence, our estimates of allele frequencies might be modestly biased, and if there is some genetic structure in the Grand Cayman population, this would also increase the relative chances of sampling homozygotes. Due to our data suggesting that different alleles maintain varying frequencies within separate regions on Grand Cayman, it is likely that the Green Iguana colony on this island is not functioning as a panmictic population. A panmictic population is represented by random mating, which would mean the allelic diversity of each reproductive iguana would have an equal chance of being accurately represented within the population. This would be further reflected in allelic scoring data where alleles would be distributed equally across the entire island. However, it is evident that the Grand Cayman iguanas are not representative of a panmictic population and that mating is not random but is likely restricted to key parts of the island. Therefore, while a sample size of 24 individuals would be representative of a truly panmictic population, a larger sample size would have likely provided a broader scope of the allelic variation across Grand Cayman due to this population partaking in biased mating. Considering this, it is also possible that the higher observed heterozygosity rates of Little Cayman and Cayman Brac were due to the populations functioning as panmictic populations. The higher rates of observed heterozygosity compared to the expected rates among the Sister Isles populations provides further evidence that these populations are indeed small. Higher rates

of observed heterozygosity could be due to the chance differences in allele frequencies between the males and females sampled. The presence of some homozygotic alleles among these populations could also signify inbreeding depression. Due to these invasive populations being smaller in size and likely restricted to certain regions on the Sister Isles, it is possible that some individuals are mating with other closely related iguanas due to these combined pressures. While it is possible that inbreeding is occurring among these populations, the continued influx of new Green Iguana individuals from the source colony would allow for major effects of inbreeding depression on allelic variation to be diminished. Therefore, our data does not suggest that the Sister Isles invasive iguana populations are experiencing expansive genetic bottlenecks, which further signifies that a sufficient number of individuals have been continuously introduced to the islands.

The source of the Sister Isles invasive iguana populations has long been speculated to be Grand Cayman. This study provides evidence in support of this hypothesis because all islands share alleles for molecular markers evaluated. While each island possessed alleles private to their population, there were multiple alleles shared among them (Figure 8). Specifically, these shared alleles made up a subset that seemed to have derived from Grand Cayman. The percentage of molecular variance among populations being less than within individuals molecular variance also implies that, while variation is present among the populations, the three Green Iguana populations within the Cayman Islands are still relatively closely related. Therefore, it is likely that invasive Green Iguanas are being introduced from Grand Cayman. However, these data are also consistent with a small number of Green Iguanas on each island coming from other sources. The sources of these other individuals would be largely constituted by exotic pets that were purposefully released into the wild due to a number of reasons, including inability to provide

proper care. Once these pets were released, their survival on the islands would allow these iguanas to potentially interact and breed with other established individuals, contributing to the private alleles found within each population.

CHAPTER 6
CONCLUSION & FUTURE DIRECTIONS

The Green Iguana maintains an impressive ability to successfully establish itself in a wide range of tropical habitats. Due to the invasive potential of this species and concern that they might negatively impact native ecosystems and other endemic species, establishing sources of introduction and evaluating population dynamics of invading colonies is critical to eliminate continued dispersal. The Cayman Islands all currently host invasive Green Iguanas. However, their numbers vary drastically from island to island. Therefore, this study sought to infer a pattern of dispersal and genetic variability among these invasive populations. A previous analysis revealed little nuclear molecular variation within populations on either Cayman Brac or Little Cayman, yet notable differences in mtDNA haplotype frequencies yielded potential inferences of introduction (Moss et al. 2017). We hypothesized that individuals were being introduced from the established colony on Grand Cayman but that few iguanas were surviving to propagate, producing distinct maternal lineages on the Sister Isles that were closely related to each other and to the source population. To assess this hypothesis, the allelic variation of Green Iguana individuals from Grand Cayman were evaluated as a first step in determining pathways of introduction and dispersal. Our results provide support for the hypothesis that Grand Cayman is the main source of invasive individuals arriving in Sister Isles. However, nuclear genetic variation in these populations, unlike mtDNA variation, is at odds with the hypothesis that these populations are experiencing bottlenecks. This study also concluded that multiple individuals

have likely been introduced from Grand Cayman due to the presence of shared alleles among the populations. Due to this, eliminating the introduction of Green Iguanas from Grand Cayman is of the utmost importance if their dispersal is to be halted.

In addition to managing the introduction of Green Iguanas to the Sister Isles from Grand Cayman, continuing aggressive culling efforts on Cayman Brac and Little Cayman is suggested to ensure that the invasive populations do not successfully establish themselves on these islands. While isolating this species from the Sister Isles by establishing biosecurity controls in Grand Cayman would diminish a substantial introduction pathway, the presence of private alleles on Cayman Brac and Little Cayman that were not shared with the source population signified that other modes of introduction have been utilized by the invasive, likely in the form of released pets. Therefore, biosecurity controls to limit Green Iguana introduction from Grand Cayman will not completely inhibit this species from establishing on the Sister Isles if other forms of introduction are not equally managed. However, if dispersal management is paired with intensive, prolonged culling on Cayman Brac and Little Cayman for the following years, eliminating a successful colony on either island is possible. In addition, continued analysis through genetics methods is also suggested to evaluate the efficacy of implemented management practices. Assessing recently sampled Green Iguanas from the Sister Isles, particularly juveniles and hatchlings, would provide the most recent genetic material available to determine whether the allelic variation within the current population has shifted or remained the same. If individuals sampled present new alleles, this would suggest that ongoing introductions of invasive individuals has occurred outside of the primary pathway from Grand Cayman and that biosecurity has been effective in limiting introductions from the original source. In contrast, if similar allele frequencies are observed from this sampling event and continue to be represented

by shared alleles with Grand Cayman, it can be determined that control methods are ineffective and that iguanas are still managing to find ways to the Sister Isles from the primary source.

Additional sampling of Green Iguanas on Grand Cayman would also benefit a continued analysis. A broader sampling of invasive individuals from this island could provide further evidence that Grand Cayman is indeed the primary source of iguanas colonizing the Sister Isles. Similar to that of the proposed analysis on the Sister Isles, assessing this population for novel alleles could signify that other modes of Green Iguana introduction are still occurring even on Grand Cayman. If greater frequencies of new alleles are found among any of the Green Iguana populations in the Cayman Islands, tighter restrictions on exotic pet acquisition and registering of Green Iguanas as pets by private citizens to ensure escapes or releases are limited may need to be investigated. It is also suggested that an analysis of the mtDNA of Green Iguanas found on Grand Cayman be conducted. By evaluating the haplotypes found within this population, further inferences can be drawn regarding the relationships between Grand Cayman and those individuals on the Sister Isles. If Grand Cayman yields the same mtDNA sequence patterns that were found on Cayman Brac and Little Cayman (i.e. Haplotypes 1 and 2), this would provide further support for the results of this study which found that Grand Cayman is indeed the source population for the Sister Isles invasive iguana populations. Therefore, this proposed future direction provides an opportunity to continue assessing the efficacy of current biosecurity methods in preventing additional introductions and reducing the chances of detrimental impacts of the Green Iguana to the Cayman Islands as well as further solidifying the results of this study.

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