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Natural Population Dynamics of Rock Iguanas in the Bahama Archipelago

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By

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Natural population dynamics of rock iguanas in the Bahama Archipelago

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Understanding whether groups of individuals represent a single panmictic gene pool, or multiple genetically structured populations across a species range should aid in predicting whether specific conservation strategies would be more or less effective for species preservation. Further, contrasting the population structures of multiple coexisting taxa could foster an even deeper understanding of evolutionary divergence among demes and potentially even suggest local adaptation in the form of tight coevolutionary relationships. Finally, the analysis of population dynamics within small and isolated populations could improve our understanding of the relative importance that different evolutionary mechanisms have in predicting population persistence in the wild.

Using microsatellite markers I characterized the population genetic structure in the critically endangered *Cyclura cychlura cychlura* iguanas on Andros Island. I found significant differences between inferred and realized rates of gene flow. This finding demonstrates that evolutionarily independent populations can occur even with high rates of dispersal. In the second and third study I contrasted patterns of genetic variability in *Cyclura cychlura* iguanas, ticks in the genus *Amblyomma* parasitizing these iguanas, and *Rickettsia* spp., potential pathogens transmitted by these ticks. I determined that genetic

differences among *Rickettsia* samples and *Amblyomma* samples are highly concordant with genetic divergence among iguana populations. This finding suggests largely vertical dispersal of ticks and their super-parasite, a high specificity of this reptile-tick interaction, and historically low rates of dispersal in iguanas. This finding also indicates that island populations of iguanas may be locally adapted due to tight coevolutionary relationships. Finally, I investigated the mechanisms that eliminate harmful mutations in small isolated and natural populations of the critically endangered *Cyclura carinata* iguanas. Using molecular tools I found indirect evidence suggesting that small natural populations can maintain significant levels of genetic variation in spite of strong selection acting against harmful mutations. Under regimes of random mating, the buildup of harmful mutations in small populations may result in a large number of inviable young. However, harmful mutations may also be eliminated when exposed to natural selection through increased competition, as population density increases. However, quantification of the relative role of competition was not feasible in this study.

DEDICATION

I dedicate this work to my father Alfredo, my brother Gabriele, and to the loving memory of my mother Maria Chiara. Dad, without your unconditional support and advice none of what I have accomplished to date could have ever been possible. Brother, you have always been, and always will be a model for me.

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CHAPTER I

INTRODUCTION

The evolutionary consequences of habitat fragmentation and inbreeding in natural populations have been central to discussions among population biologists since Fisher, Wright and Haldane first modeled population genetics (Provine 1971). The geographic distribution of individuals within and among populations, their density, intraspecific interactions, and ecology can have profound ramifications for the population and evolutionary dynamics of different taxa (Levins 1969; Hanski 1989; Hanski and Gilpin 1991; Hanski and Gaggiotti 2004). Depending on their size and degree of connectivity, populations, here defined as groups of interbreeding individuals of the same species persisting on a fragmented landscape, may express increased morphological and genetic variance. Making meaningful predictions concerning the evolutionary trajectories of populations, hence, requires a deep understanding of the possible long-term repercussions of such structure and dynamics (Templeton et al. 1990; Hoffmeister et al. 2005).

One of the major concerns associated with isolation and reduced population size is the increased probability of local extinctions. Ecological and genetic factors are the major contributors to the enhanced risk of local extinctions and, although their relative contribution has been debated in the past (Gilpin and Soulé 1986; Lande 1988; Pimm et al. 1988; Lande 1998), it is not unlikely that their synergistic effects ultimately drive populations to extinction (Gilpin and Soulé 1986; Frankham 2005; Frankham et al. 2010).

Saccheri and colleagues (1998) studied how the probability of extinction increases with fragmentation and reduced population size (Saccheri et al. 1998). They demonstrated that isolation and inbreeding could result in reduced viability, survivorship, and adult life expectancy in the fritillary butterfly *Meliatea cinxia.* More recently, Clark and colleagues (2011) described the combined negative effects of anthropogenic factors, climate change, and habitat fragmentation on populations of the timber rattlesnake, *Crotalus horridus*, varying in their degree of isolation and local density of individuals. There are now numerous examples and a general consensus that genetic factors and the deleterious effects of inbreeding both in captive and small natural populations are relevant factors limiting population growth and persistence (Bijlsma et al. 2000; Brook et al. 2002; Keller and Waller 2002; O'Grady et al. 2006; Keller et al. 2007).

Inbreeding *sensu stricto* refers to the process by which related individuals produce offspring with identical by descent (IBD) alleles (Allendorf et al. 2012). The mating dynamic responsible for siring inbred individuals typically involves a departure from random assortment of gametes, with a consequent increase in homozygosity in the population (Wright 1922). The phenotypic costs associated with inbreeding, i.e., inbreeding depression, are generally explained by two competing, but not mutually exclusive hypotheses: dominance and overdominance (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009). According to the former, the fitness reduction in inbred individuals is caused by the expression of recessive or partially recessive deleterious alleles (Lynch and Walsh 1998). The second mechanism assumes that heterozygous individuals have an advantage over both homozygous genotypes, hereby determining a mean fitness reduction in populations with high level of homozygosity at

overdominant genes (Bijlsma et al. 2000; Crow 2008). Dominance has been invoked to explain the majority of inbreeding depression (Charlesworth and Willis 2009) although overdominance is sometimes favored (Sommer 2005).

Inbreeding *sensu lato*, refers to the mechanism by which alleles in the offspring happen to be identical by state (IBS; Powell et al. 2010). In this case genetic drift contributes to the random loss and/or fixation of potentially deleterious alleles in small populations (Paland and Schmid 2003). Thus, even under regimes of random mating, we expect the offspring of smaller populations to be on average more homozygous and with lower fitness when compared to the offspring of larger populations (Lohr and Haag 2015).

A recent review on the fitness consequences of inbreeding in natural populations highlighted some of the major gaps in the study of inbreeding and inbreeding depression in the wild (Neaves et al. 2015). Among others Neaves and colleagues documented how some vertebrate taxa, including reptiles, have been relatively poorly investigated. Moreover the authors were concerned that the majority of studies in natural populations focused on the analysis of only one or few generations of inbreeding, and have not analyzed possible differences in response to variation in population size and dynamics within a species. Additionally, species ecology and non-prominent natural barriers can sometime contribute to fine scale patterning of genetic structure, hampering our understanding of populations' evolutionary and demographic cohesion (Row et al. 2010; Rutz et al. 2012). Finally, estimates of inbreeding depression are generally obtained using individuals with known pedigree information (Crnokrak and Roff 1999; Pemberton 2004) or measuring fitness performance in controlled crosses between outbred and inbred lines

(Crnokrak and Barrett 2002). These approaches are not always feasible in the wild and in the analysis of non-model organisms, due to the biology of the species, the presence of migrants, absence of precise pedigree information, multiple paternity, sampling bias and complex biogeographic histories (Charlesworth and Charlesworth 1987; Coltman and Slate 2003; Chapman et al. 2009).

In such context, populations on islands within archipelagoes offer many advantages to the study of how fragmentation and inbreeding depression could affect evolutionary dynamics. Reduced number of individuals, relative isolation and the possible conservation benefits originating from such analyses, make island populations a desirable system to investigate patterns of gene flow and inbreeding depression in the wild. From an experimental perspective species dwelling on islands and archipelagoes can sometimes present another advantage. Analyses aiming to investigate the possible relation between inbreeding and extinction in the wild sometimes lack sufficient statistical power. This is due to the inherently stochastic nature of the extinction process in the wild (Bijlsma et al. 2000). Compared to their mainland counterparts, species with island (or island like) distributions are more likely to present natural population replicates that can be used to more reliably investigate how inbreeding affects demographic dynamics and more accurately estimate extinction probabilities (Bijlsma et al. 2000).

Tropical islands harbor the largest percentage of endemic species threatened with extinction (Baillie et al. 2004). It has been estimated that since the 17^{th} century ≈ 75% of animal extinctions involved island species (Frankham 1998). Simberloff (2000) recognized four characteristics to explain why the majority of conservation concerns over the loss of biodiversity are island related. On average, island biotas are impoverished

(fewer species when compared to the mainland), unsaturated (with unexploited, available niches), disharmonic (with some taxa better represented than others) and with a high proportion of endemic species (Simberloff 2000). Isolation from potential migrant sources exposes populations on remote islands, and island-like systems such as mountain peaks, to greater risks posed by environmental stochasticity and genetic hurdles (Clark et al. 2011; Miller et al. 2011). Moreover, due to their isolated, impoverished and unsaturated characteristics, some have theorized that island populations are more susceptible to invasive species (Sax and Gaines 2008). Some authors found a positive correlation between the number of invasive mammals and the probability of extinction in 220 oceanic islands (Blackburn et al. 2004). For example, the near extirpation of a Turks and Caicos Rock iguana population by introduced cats and dogs was documented over a three-year period (Iverson 1978).

The goal of this doctoral work is to investigate spatial patterns of genetic variation and the potential fitness costs of inbreeding depression in endangered and critically endangered Rock Iguana species (genus *Cyclura*) inhabiting different islands within the West Indies. I will be testing different hypotheses concerning population structure and dynamics and their potential effect in regulating the local patterns of persistence in the studied taxa. More specifically, in Chapter II of this dissertation I will analyze the population genetic structure of *Cyclura cychlura cychlura* iguanas on Andros Island, Bahamas. I will be testing the specific hypothesis that gene flow measures, derived from the analysis of molecular data, are concordant with patterns of gene flow inferred using ecological and behavioral data. My results imply that gene flow estimates based solely on ecological data can sometimes be upwardly skewed. Chapter III expands on the analysis

of population structure in the Northern Bahamian Rock Iguana, *Cyclura cychlura*. In this section I use a multi-taxon approach and make inferences over the taxonomic characterization and potential patterns of dispersal in the *C. cychlura – Amblyomma* spp. complex in The Bahamas. The simultaneous distribution analysis of reptiles and their tick parasites provides interesting clues regarding the possible coevolutionary relationships between the two interacting taxa, and can improve our understanding of the mechanisms giving rise to the current species distributions. My results show a haplotype distribution pattern in iguana hosts and their tick ectoparasites supporting a vertical mechanism of dispersal (i.e., tick dispersal occurs primarily through their reptile hosts). This, compounded with evidence of historically low rates of gene flow, suggests a high degree of specificity in these interacting taxa and the potential for concordant evolutionary history.

Chapter IV elaborates on the patterns of concordance between *Cyclura cychlura* hosts and their *Amblyomma* parasites distributions. Ticks are known to be the vectors of a variety of bacteria, including multiple species in the genus *Rickettsia*. I, therefore, expanded on previous distribution analyses to investigate the possible presence of *Rickettsia* spp. in *Amblyomma* ticks parasitizing different populations of *C. cychlura* iguanas. I found evidence indicating that different *Amblyomma* species are infected with at least two different *Rickettsia* species. Even more interestingly, the distribution of genetic variation in these bacteria is concordant with that of the ticks and their hosts. Furthermore, one of the identified species, *Rickettsia tamurae*, has been associated with human diseases. Because *C. cychlura* iguanas are targets of the illegal pet trade and

unregulated translocations, their unsanctioned movements could potentially leave naïve and novel host populations at risk, and potentially spread this pathogen.

Finally, in Chapter V I investigate the long-term evolutionary consequences of inbreeding and inbreeding depression in small isolated populations of *C. carinata* iguanas in the Turks and Caicos Islands. Based on behavioral and experimental observations I formulated the hypothesis that density dependent selective processes (*"soft selection"*) may be favoring recruitment of heterozygous individuals in small isolated populations of iguanas. If heterozygote excess reflects soft selection, a competitive advantage for outbred individuals, then small populations should be more robust than expected assuming that heterozygous excess results from expressing lethal equivalents, i.e, hard selection. On the contrary, if observed attrition rates result from hard selection, then these small populations are more likely to be ephemeral, and would require relatively frequent recolonization. Using polymorphic microsatellites I found indirect evidence suggesting that small natural populations can maintain significant levels of segregating load. Under regimes of random mating this load can be exposed to natural selection through increased competition, as populations approach carrying capacity. However, quantification of the relative roles of hard and soft selection affecting the population dynamics of these iguanas was not feasible. Hence, the genetic resilience of small isolated populations of this species remains unclear.

CHAPTER II

INFERRED VS REALIZED PATTERNS OF GENE FLOW: AN ANALYSIS OF POPULATIONS STRUCTURE IN THE ANDROS ISLAND ROCK IGUANA

Abstract

Ecological data, the primary source of information on patterns and rates of migration, can be integrated with genetic data to more accurately describe the realized connectivity between geographically isolated demes. In this paper I implement this approach and discuss its implications for managing populations of the endangered Andros Island Rock Iguana, *Cyclura cychlura cychlura*. This iguana is endemic to Andros Island, a highly fragmented landmass of large islands and smaller cays. Field observations suggest that geographically isolated demes were panmictic due to high, inferred rates of gene flow. I expand on these observations using 16 polymorphic microsatellites to investigate the genetic structure and rates of gene flow in 188 Andros Iguanas collected across 23 island sites. Bayesian clustering of specimens assigned individuals to three distinct genotypic clusters. An analysis of molecular variance (AMOVA) indicates that allele frequency differences are responsible for a significant portion of the genetic variance across the three defined clusters ($F_{st} = 0.117$, $p << 0.010$). These clusters are associated with larger islands and satellite cays isolated by broad water channels with stronger water currents. These findings imply that broad water channels present greater obstacles to gene flow than previously inferred from field observation

alone. Additionally, rates of gene flow were indirectly estimated using BAYESASS 3.0. The proportion of individuals originating from within each identified cluster varied from 94.5 to 98.7%, providing further support for local isolation. This assessment reveals a major disparity between inferred and realized gene flow. I discuss these results in a conservation perspective for species inhabiting highly fragmented landscapes.

Introduction

Natural or anthropogenic habitat fragmentation may hinder or prevent animal dispersal. Inherently linked to dispersal, gene flow is also largely determined by geographic features such as fragmentation (Wang et al. 2009). High rates of dispersal and gene flow favor genetic homogenization across broad geographic ranges. In spite of high dispersal, a lack of gene movement between geographic isolates enhances the likelihood of local adaptation, random loss of genetic variability, and reduction in population size, which increase the probability of inbreeding depression and local extinction (Saccheri et al. 1998; Kawecki and Ebert 2004; Frankham et al. 2010; Janečka et al. 2011; Mattila et al. 2012).

Though life history data and field observation provide important insights regarding patterns of species dispersal, it is imperative that potential differences in perceived and realized gene flow be recognized if we are to implement effective conservation measures. For instance, adopting translocation as a strategy to reinforce declining populations could be harmful if the source population is genetically distant from the recipient one due to historical lack of gene flow (Allendorf et al. 2012). Moreover, inferring gene flow and dispersal based on landscape features alone can erroneously represent how genetic variation is spatially structured. For example,

molecular and landscape analyses in different species of salamander (genus *Ambystoma*) revealed unexpected high connectivity between geographically isolated demes (Purrenhage et al. 2009; Wang et al. 2009). These salamanders have the ability to migrate between ponds via habitats characterized by relatively high dispersal cost, hence reducing genetic differences between isolated ponds or demes (Wang et al. 2009). Species ecology also affects our perception of gene flow. It is expected, for example, that flight of highly mobile organisms like birds could mitigate differentiation between adjacent populations (Edwards et al. 2005). Bertrand and colleagues (2014), however, demonstrated that, even at short distances (< 26 Km), groups of island passerine birds, *Zosterops borbonicus*, exhibited extremely reduced rates of gene flow. Indeed, perceived migration rates fell short of realized gene flow rates determined by population genetic analyses. This discrepancy could be exacerbated in highly fragmented landscapes, and in species of conservation concern could lead to misguided management. In this paper I use molecular tools to investigate the conformity between inferred and realized patterns of gene flow in the endangered Andros Island Rock Iguana, *Cyclura cychlura cychlura*.

The Andros Rock Iguana is endemic to Andros Island in the Bahamas (Knapp and Buckner 2004) which is a composite of four major landmasses and hundreds of satellite cays separated by water channels, called bights, and smaller saline creeks (Fig. 2.1a). Further, the island is composed of multiple habitat types, including pine woodlands with open or closed broadleaf understory, dry evergreen scrublands and intertidal mangroves (Knapp and Owens 2005). Despite this naturally fragmented and mosaic landscape, dispersal and gene flow may be high in the Andros Rock Iguana. There is anecdotal and documented evidence of this species dispersing across water channels (Knapp 2005a).

Additionally, *C. c. cychlura* hatchlings have been documented to readily disperse from nesting areas across a variety of different habitat types (Knapp et al. 2010). This system, hence, provides a valuable opportunity to investigate the conformity between perceived and realized migration patterns and to estimate the relative importance of different types of barriers to gene flow.

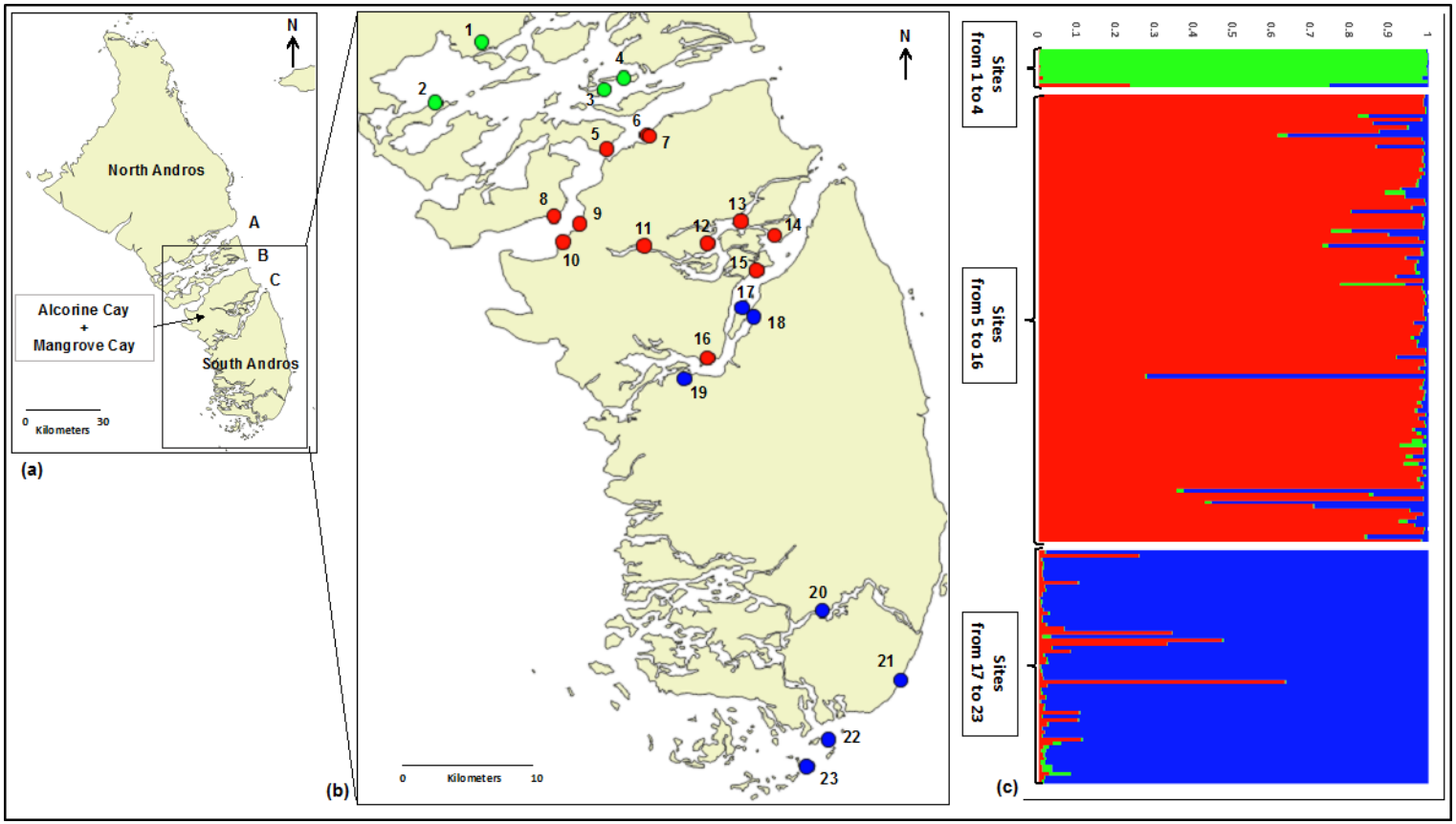

Figure 2.1 Andros Island Map

(a) North Andros, Alcorine Cay, Mangrove Cay and South Andros. Capital letters A, B and C indicate North, Middle and South bight respectively. The map lacks enough resolution to depict the separation of Mangrove and Alcorine Cays by Lisbon Creek; **(b)** The 23 sampling locations. Sites are colored accordingly to the Bayesian clustering output. **(c)** Bayesian clustering output produced using STRUCTURE v2.3.4 (Pritchard et al. 2000) and analyzing 188 individuals genotyped at 16 microsatellite loci.

Current conservation plans for the Andros Island Rock Iguana (Knapp and Pagni 2011) were informed largely by a long-term ecological study of the species (Knapp et al. 2006; Knapp and Owens 2008; Knapp et al. 2010). In the present study I expand on these investigations to infer metapopulation dynamics of these iguanas using neutral molecular markers. I used 16 polymorphic microsatellites to define the population genetic structure of *C. c. cychlura* on Andros Island. I then examined the population structure in the context of the species ecology and the associated landscape to infer patterns of gene flow. I hypothesized that the high dispersal rates inferred from anecdotal accounts and telemetry studies (Knapp 2005a; Knapp et al. 2010) limit genetic divergence among local demes of *C. c. cychlura* on Andros Island despite their patchy distribution across geographic isolates. If true, I anticipate limited evidence for genetic structure across the island.

Materials and Methods

Ethical Statement

I thank the Bahamans Environment, Science and Technology (BEST) commission for permission and permits to conduct the study. I thank the Bahamas National Trust for permission to work in the West Side National Park. The Bahamas Ministry of Agriculture issued the CITES export permits. This work was made possible through the help of Shedd Aquarium volunteer research assistants. The Shedd Aquarium research review committee approved methodologies for this study.

Study System and Samples Collection

Andros is the largest island in the Bahamian archipelago, encompassing an area of 5,959 km^2 and supporting a human population between 8000 and 9000 individuals concentrated along the eastern coast (Layman et al. 2011). This subtropical island is a composite of four main islands (North Andros, Mangrove Cay, Alcorine Cay and South Andros; Fig. 2.1a), along with hundreds of associated cays separated by wide (≥ 5 Km) saline tidal channels and smaller saline creeks. The substrate consists of oolitic and bioclastic limestone, and from east to west a thin costal ridge (to 30 m elevation) gives way to a flat and pine forested interior. Approximately halfway across the island, in a westerly direction, forest degrades into extensive scrubland, mudflats, and mangroves as the water table reaches the surface. Knapp and colleagues (2010) provide detailed descriptions of primary plant communities from the study area.

Less than 5,000 iguanas remain on the island and, when present, occur in low densities (0.5 – 2.5 adults/ha; Knapp et al. 2006). The sampling effort over the years covered the whole island. Iguanas on North Andros are rarely encountered due to habitat degradation from historic logging practices, poaching and predation from non native mammals (Knapp 2005a). Therefore, fieldwork was focused between the southern extent of North Andros Island (24˚ 22.103'N) and the southern most cays associated with the island (23˚ 38.569'N). Accessing study sites and locating individuals presented serious logistical challenges, as much of Andros Island is extremely remote and iguanas occur in low densities.

Tissue samples were collected from animals captured during fieldwork from 1999 to 2013 using fish-landing nets or nooses. A total blood volume of 1-2 ml was drawn

from each individual via the ventral coccygeal vein using a heparinized syringe. Blood samples were stored in SDS lysis buffer (0.1M Tris-HCl pH 8.0, 0.1M EDTA, 0.01M NaCl, 2% SDS) at ambient temperature prior to long term storage at -80 ºC (Longmire et al. 1997).

DNA Extraction and Genotyping

Approximately 20 µL of blood lysate were digested for 5 hours in a 65 ºC water bath with Proteinase-K (20 mg/mL) and a digestion buffer with 17 mM Tris-HCl, 1.7 mM $CaCl_2$ and 50% glycerol (Sambrook et al. 1989). Following digestion, genomic DNA was extracted using the ABI PRISM-6100 Nucleic Acid Prep Station and proprietary chemistry (Applied Biosystem, Foster City, California, USA). Successful DNA extraction was assessed through electrophoretic migration in a 1% agarose gel. A suite of 23 microsatellites developed for congeneric species were screened for positive amplification and variability (An et al. 2004; Rosas et al. 2008; Lau et al. 2009; Welch et al. 2011). Three primer PCR (Schuelke 2000) was performed in 10 µL volume with ≈ 10 ng DNA, 2 mM $MgCl_2$, 30 mM tricine (pH 8.4) KOH, 50 mM KCl, 100 µM of each dNTP, 200 nM of reverse primer and M-13 forward primer (CACGACGTTGTAA-AACGAC) labeled with a fluorescent dye (HEX, NED, FAM, VIC or PET), between 40 and 150 nM forward primer with the M-13 extension, and 0.4 U of Taq DNA polymerase. Touchdown PCR (Don et al. 1991) profiles were set with an initial denaturation period of 5 min at 94 ˚C followed by 10 touchdown cycles with 30 sec at 94 ˚C, 30 sec at annealing temperature and 30 sec at 72 ˚C. In touchdown protocols the initial annealing temperature is 10 ˚C above the final annealing temperature. In each successive PCR cycle the annealing temperature drops by 1 ˚C. The remaining 25 cycles

had thermal cycling profiles of 30 sec at 94 ˚C, 30 sec at 52 ˚C, and 30 sec at 72 ˚C. A final elongation phase of 7 min at 72 ˚C completed the PCR cycle profiles. Fragment analysis was performed on ABI 3730 capillary sequencer (ABI, Foster City, CA) at the Arizona State University DNA Laboratory using LIZ-500 as size standard (GeneScan – 500 LIZ Size Standard - Applied Biosystems). Genotypes were visually scored using PEAK-SCANNER v1.0 (Applied Biosystems).

Additional microsatellite markers were developed specifically for *C. c. cychlura.* Tandem repeat regions were identified using the subtracting hybridization method of Glenn and Schable (Zimmer and Roalson 2005). Digested DNA was enriched for eight oligonucleotide repeats $(AC)_{15}$, $(AG)_{15}$, $(AAC)_{10}$, and $(AGG)_{10}$. Enriched PCR products were cloned using the pGEM-T cloning kit (Promega – Madison, WI) with color screening. Ninety-three color positive (i.e., white) colonies were suspended in 50 µL TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, pH 8.0). A PCR was used for insert of suitable size for sequencing. Reactions were 10 µL in volume and contained 0.5 µL template DNA, 0.3 µM each of primers pUC-M13F and pUC-M13R (Integrated DNA Technologies), 1X GoTaq Flexi Buffer (Promega – Madison, WI), 2 mM $MgCl_2$, and 160 mM of dNTPs. PCR profile consisted of 3 min at 95 ˚C, followed by 35 cycles 95 ˚C for 30 sec, and 50 ˚C for 30 sec, 72 ˚C for 1.5 min, and lastly a final extension period at 72 ˚C for 7 min. Amplicons were electrophoresed in 1.5% agarose TBE gels and visualized by ethidium bromide and UV light. Clones that exhibited a single amplified band of 500-1000 base pairs were cleaned with 16 U of Exonuclease I and 3 U of Antarctic Phosphatase (New England Biolabs, Ipswich, MA) followed by ethanol precipitation. Cleaned PCR products were sequenced using the pUC-M13F primer, Big Dye

Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) in 10 μL reactions including 0.5 μL Big Dye, 0.3 μL primer, 0.87X sequencing buffer (Applied Biosystems), and 2 μL of cleaned PCR product. Sequence reactions were cleaned using columns packed with Sephadex and then electrophoresed at the Arizona State University DNA Lab. Microsatellites were identified using TANDEM REPEATS FINDER (Benson 1999) and primer designed using PRIMER3 (Rozen and Skaletsky 1999).

Data Analysis

I first tested for evidence of Linkage Disequilibrium (LD) between pairs of loci using the exact probability test implemented in GENEPOP ON THE WEB v4.2 (Raymond and Rousset 1995; Rousset 2008). The Markov-Chain parameters were set to compute 1000 dememorisation steps, 100 batches and 1000 iterations per batch. Observed and expected heterozygosity (H_o and H_e respectively) were calculated according to Nei (Nei 1987) and using ARLEQUIN v3.5 (Excoffier et al. 2010). I additionally tested for any significant departure from Hardy Weinberg Equilibrium (HWE) following Guo and Thompson (1992) using GENEPOP ON THE WEB v4.2 (Guo and Thompson 1992; Raymond and Rousset 1995; Rousset 2008). The Markov-Chain parameters were set to compute 5000 dememorisation steps, 100 batches and 5000 iterations per batch. The sequential Bonferroni correction was used to adjust significance thresholds (Holm 1979) when necessary.

Population Structure

To test the hypothesis of high rates of dispersal and gene flow, and thus panmixia among iguanas on Andros, I performed a Bayesian based individual assignment test using

STRUCTURE v2.3.4 (Pritchard et al. 2000). The program assigns individuals to inferred populations based on the posterior probability that a certain genotype is sampled from a modeled allelic distribution. An ancestral iguana population was assumed to have recently split due to rising sea levels associated with the current interglacial period (Malone et al. 2003). I assumed an admixture ancestry model with correlated allele frequencies and no prior information on sampling locations. A total of 10^6 Markov chain Monte Carlo (MCMC) iterations were calculated, and the first 100,000 replicates were discarded as burn-in. I assumed K putative populations ranking in number from one to 10 and performed 10 iterations of the MCMC sampling procedure for each K value. The most likely number of clusters was estimated using the Evanno method, based on the second order of difference in likelihood function of K (i.e., ΔK) and implemented in the web tool STRUCTURE – HARVESTER (Evanno et al. 2005; Earl and vonHoldt 2012).

Overall genetic variability across putative genetic isolates was also quantified. For each population identified by the Bayesian clustering the percentage of polymorphic loci and private alleles (P_a) were calculated using GENALEX v6.5 plugin for EXCEL (Peakall and Smouse 2012). ARLEQUIN v3.5 (Excoffier et al. 2010) was used to calculate H_o and H_e. Due to the differences in sample sizes across different clusters, and given that allelic richness should be a function of sample size, I used an allelic richness index (A_r) that rarefies the number of alleles according to the number of genes examined in the smallest population (Goudet 2005). A_r was calculated using HIERFSTAT (Goudet 2005), a package designed for use in the R statistical software (https://www.r-project.org).

AMOVA and Gene Flow

To test the significance of any genetic isolates and to infer the rate of gene flow among hypothesized clusters, hierarchical Analyses of Molecular Variance (AMOVAs) were performed using ARLEQUIN v3.5 (Weir and Cockerham 1984; Excoffier et al. 1992; Excoffier et al. 2010). The proportion of genetic variation attributable to STRUCTURE grouping (F_{st}) was quantified and tested for statistical significance. I also performed a pairwise comparison (pairwise-F_{st}) in order to estimate migration rates between pairs of clusters. Inferences of gene flow based on F statistics following $4N_em=1/F_{st}-1$ (Wright 1931; Wright 1984) are relatively insensitive to rare alleles and in general are based on rather simplistic assumptions (Whitlock and McCauley 1998; Wilson and Rannala 2003). I hence used the Bayesian approach developed by Wilson and Rannala in BAYESASS 3.0 to infer migration rates (Wilson and Rannala 2003). The program uses genotypic data and MCMCs to more accurately infer recent patterns of gene flow. This approach does not explicitly calculate the number of migrants, unless the analyzed populations have equal number of individuals, but it returns the proportion of immigrants within each population, allowing for indirect estimates of gene flow (Wilson and Rannala 2003). I performed five independent analyses. In each run the program computed 10^7 MCMCs and discarded 10^6 chains as burn-in. Chains were sampled every 2,000 generations. To ensure sufficient mixing of the MCMCs and to improve the coverage of the probability space I adjusted the acceptance rate for estimated allele frequencies and inbreeding coefficients. I hence increased the mixing parameters for both allele frequencies (ΔA) and inbreeding coefficient (ΔF) to 0.30 as suggested by the authors (Wilson and Rannala 2003). Each independent run started with a different

random seed. Mixing and convergence of MCMCs were visually assessed using TRACER v1.6 (Rambaut and Drummond 2013) Among the five independent runs, I chose the one with lowest Bayesian deviance in the logProb calculated using the r-function provided by Faubet et al. (2007) and as suggested in Meirmans (2014). The function is also available in the Appendix (A.1).

Results

One hundred ninety two individuals were sampled from 23 sites. Identifiers for each sampled location and number of individuals sampled per site are listed in Tab. 2.1. Due to the extremely low density of iguanas in the northern region, no captures were possible on North Andros. Sample sizes reflect exhaustive efforts to sample animals. Low sample numbers indicate extremely low density, not scarce sampling effort.

Table 2.1 *Cyclura cychlura cychlura* sampling sites

Site ID	Name	N	H_o	H_e	HWE p-val
1	NMB1	4	0.525(0.26)	0.435(0.17)	ns
2	NMB2	1	–	–	–
3	NMB3	4	0.568(0.32)	0.575(0.12)	ns
4	NMB4	1	–	–	–
5	NMB5	2	0.700(0.421)	0.700(.13)	ns
6	NMB6	4	0.461(0.431)	0.557(0.13)	ns
7	MA1	1	–	–	–
8	NMB7	4	0.571 (0.28)	0.625 (0.17)	ns
9	MA2	1	–	–	–
10	MA3	37	0.575 (0.27)	0.551 (0.26)	ns
11	MA4	5	0.723 (0.26)	0.664 (0.17)	ns
12	MA5	36	0.523 (0.21)	0.569 (0.18)	***
13	MA6	4	0.613 (0.26)	0.654 (0.14)	ns
14	MA7	6	0.547 (0.22)	0.615 (0.17)	ns
15	MA8	7	0.615 (0.27)	0.576 (0.21)	ns
16	MA9	10	0.564 (0.23)	0.577 (0.19)	ns
17	SA1	36	0.594 (0.23)	0.581 (0.18)	ns
18	SA2	4	0.569 (0.11)	0.639 (0.11)	ns
19	SA3	4	0.533 (0.30)	0.617 (0.21)	ns
20	SA4	4	0.553 (0.26)	0.609 (0.18)	ns
21	SA5	2	0.692 (0.25)	0.666 (0.18)	ns
22	SA6	7	0.564 (0.27)	0.505 (0.19)	ns
23	SA7	4	0.440 (0.26)	0.550 (0.19)	ns

Site ID, Name (NMB = North Middle Bight; MA = Mangrove Alcorine; SA = South Andros), number of individuals (N), observed heterozygosity and its standard error (H_o(s.e.)) and expected heterozygosity and its standard error (H_e(s.e.)), and significant departure from HWE for each sampling location (ns = non significant; *** = significance at p = 0.01)

Of 23 microsatellite markers developed in congeneric *Cyclura* species and analyzed, 18 were successfully amplified, and 13 were polymorphic in *C. c. cychlura* (Tab. 2.2). In addition, we successfully designed three species-specific polymorphic markers. Of 93 sequenced inserts, 15 contained short tandem repeats and were used for designing species-specific microsatellite primers. PCR primers and protocols were successfully designed for amplification of nine microsatellite loci. Three of these loci are polymorphic and were genotyped in all sampled animals (Tab. 2.3).

Table 2.2 Molecular marker information for loci characterized in congeners of *Cyclura cychlura cychlura.*

Name	Reference	Size Range	N_a	H_o	H_e
F478	(An et al. 2004)	208-226	9	0.651 (0.06)	0.490 (0.03)
F519	(An et al. 2004)	331-355	9	0.577 (0.06)	0.494 (0.04)
C6	(Lau et al. 2009)	125-145	8	0.646 (0.05)	0.512 (0.03)
C124	(Lau et al. 2009)	210-248	9	0.677 (0.06)	0.571 (0.04)
D136	(Lau et al. 2009)	162-210	13	0.690 (0.06)	0.552 (0.04)
Z13	(An et al. 2004)	269-283	4	0.094 (0.03)	0.177 (0.03)
Z65	(An et al. 2004)	160-181	5	0.544 (0.06)	0.477 (0.05)
Z99	(An et al. 2004)	122-135	5	0.219 (0.05)	0.226 (0.04)
Z106	(An et al. 2004)	179-192	6	0.530 (0.06)	0.499 (0.04)
Z154	(An et al. 2004)	214-231	4	0.267 (0.06)	0.333 (0.05)
Z494	(An et al. 2004)	197-214	5	0.466 (0.06)	0.414 (0.05)
CCSTE02	(Rosas et al. 2008)	288-299	6	0.671 (0.06)	0.515 (0.04)
CIDK177	(Welch et al. 2011)	258-285	12	0.587 (0.07)	0.502 (0.05)

Name, reference, size range, number of alleles (N_a) observed heterozygosity and its standard error (H_o(s.e.)) and expected heterozygosity and its standard error (H_e(s.e.)). Summary statistics are based on the total number of sampled iguanas.

Table 2.3 Molecular marker information for newly characterized loci.

Name/Acc.	Sequence (5'-3')	Mot.	T	Range	N_a	H_o	H_e
CycCyc9	F: TGCAGTTTGTCCTTTGTTGC	(GT)	52	206-221	3	0.197 (0.05)	0.164 (0.0)
KF646798	R: CTTGAGCCACCCATTTCTTG						
CycCyc16	F: TGGCAACCCTGTAAATCCTC	(CA)	52	167-187	5	0.553 (0.07)	0.444 (0.04)
KF646799	R: TGAGACTGGAAGATTGCCTTG						
CycCyc31	F: TGGCCCAAGCATTAATAAAA	(CTA)	52	170-197	5	0.311 (0.08)	0.253 (0.05)
KF646800	R: CATGGGAGGGAGGGAATAAT						

Name, GenBank Accession number, forward (F) and reverse (R) primer sequence, repeat motif (Mot.), annealing temperature in ºC (T), number of alleles (N_a) observed heterozygosity and its standard error (H_o(s.e.)) and expected heterozygosity and its standard error (H_e(s.e.)). Summary statistics are based on the total number of sampled iguanas.

A total of 16 polymorphic markers were hence used. Four individuals did not yield usable DNA and the total number of genotyped individuals used for the study was 188 out of the 192 collected. Of the overall possible genotypes (188 * 16), 91% were successfully scored. Among the 16 microsatellites analyzed, D136 was the most variable locus (N_a = 13; H_o = 0.690) while Z13 and CycCyc9 were the least variable (N_a = 4; H_o = 0.094 and N_a = 3; H_o = 0.197 respectively (Tab. 2.2 and 2.3).

I did not find any evidence for LD between microsatellite markers. Of the 2760 pairwise comparisons between loci I found significant gametic disequilibrium in only 12 instances (≈ 0.5%) a value that does not deviate significantly from random expectation at $\alpha = 0.05$. A single sampling site exhibits significant deviation from HWE (Site 12, Tab. 2.1). This site is actually a peninsula with three distinct patches of appropriate iguana habitat. Considering the short geographic distance between each of these patches and the fact that they are actually connected by land, collections made in these three patches were treated as a single site during analysis. One explanation for the departure from HWE seen at Site 12 would be the presence of fine scale genetic structure within the peninsula. An ad hoc analysis of individuals sampled in each of the three habitat patches (10 iguanas

from the north patch, 25 iguanas from the middle patch and one iguana from the south patch) was conducted to test this hypothesis. Individuals from the north patch showed a slight but non-significant excess of homozygotes (F_{is} = 0.087, p = 0.193). Individuals from the middle patch showed a slight but non-significant excess of heterozygotes (F_{is} = -0.014, p = 0.626). An overall analysis of deviation from HWE using GENEPOP ON THE WEB v4.2 revealed no significance (p = 0.053 north patch; p = 0.074, middle patch). This lack of significance may reflect diminished sample sizes. However, the fact that directionality of deviation from HWE differs between the two samples suggests these deviations may simply reflect sampling variance. The deviation from HWE and deficit of homozygosity detected at Site 12 may also reflect some fine scale genetic structuring. Other explanations considered include the presence of null alleles, non-random mating and heterozygote advantage. However, the presence of genotyping artifacts and null alleles was not detected during analysis with MICRO-CHECKER (Van Oosterhout et al. 2004). The small sample sizes for each patch prevent a more detailed investigation into the non-random mating and the heterozygote advantage hypotheses.

Population Structure and Variability Analyses

Individual assignment tests suggest three clusters based on the largest values of ΔK method implemented in STRUCTURE – HARVESTER (Evanno et al. 2005; Earl and vonHoldt 2012; Fig. 2.2). Individuals from Site 1 to Site 4 were grouped in a single cluster (green, Fig. 2.1b-c; North Cluster hereafter). Specimens collected from Site 5, Site 6, Site 8 and all samples from Alcorine Cay and Mangrove Cay constitute the second cluster (red, Fig. 2.1b-c; Central Cluster hereafter). The remnant individuals captured south of South Bight, including Site 17, compose the third cluster (blue, Fig. 2.1b-c;

South Cluster hereafter). All clusters were characterized by the presence of private alleles (Tab. 2.4). The South cluster was characterized by the highest value of Expected Heterozygosity (H_e = 0.616, s.e. = 0.177), though the Central cluster was the only group polymorphic at all 16 loci and with the highest score of Allelic Richness (A_r = 3.842; Tab. 2.4).

Table 2.4 Summary statistics for the clusters identified in STRUCTURE

Cluster	N	L	H_o	H_e	A_r	P_a
North	10	13	0.446(0.20)	0.599(0.15)	3.042	7
Center	117	16	0.540(0.22)	0.580(0.22)	3.842	16
South	61	15	0.558(0.18)	0.616(0.12)	3.663	6

Cluster, number of individuals (N), number of polymorphic loci (L), observed heterozygosity and its standard error (H_o(s.e.)) and expected heterozygosity and its standard error (H_e(s.e.)), allelic richness (A_r), and private alleles (P_a).

Table 2.5 Pairwise F_{st} between genetic clusters on Andros

	North	Central	South
North	–	p << 0.01***	p << 0.01***
Central	0.273	–	p << 0.01***
South	0.234	0.072	–

Cells in the lower diagonal show pairwise F_{st} values. Cells in the upper diagonal show the significance level of each comparison.

Table 2.6 Indirect estimates of gene flow between genetic clusters on Andros

	North	Central	South
North	0.945(-0.03)	0.025(-0.02)	0.030(-0.03)
Central	0.003(-0.003)	0.987(-0.008)	0.010(-0.007)
South	0.005(-0.005)	0.015(-0.01)	0.980(-0.01)

Values in the form m_{ij} (with i = rows and j = columns) represent the proportion of individuals in the i_{th} population that originated from the j_{th} population per generation. Values in parentheses are standard deviations of the probability distribution.

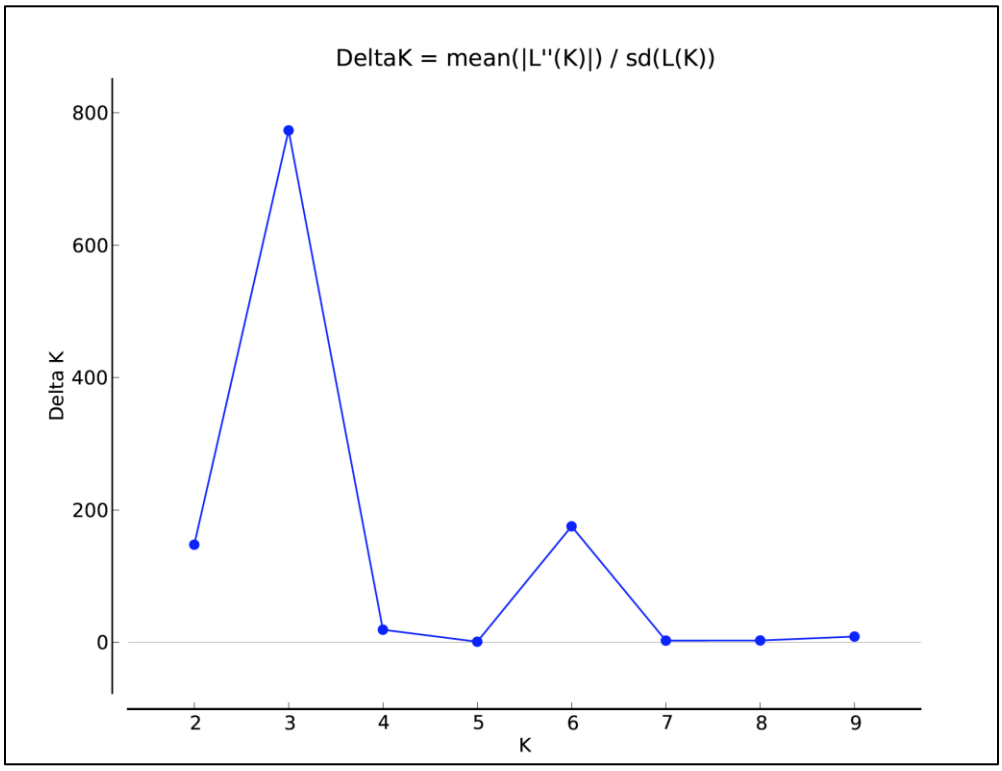

Figure 2.2 STRUCTURE HARVETSER's output.

Delta K value estimated using the Evanno method (Evanno et al. 2005) on 188 individuals collected on Andros and genotyped at 16 microsatellite markers.

AMOVA and Gene Flow

Analysis of molecular variance (AMOVA) among the genetic clusters resulted in an F_{st} value of 0.117 ($p << 0.01$). Such an F_{st} value can sometimes be associated with a migration rate of ≤ 1 individual per generation (Mills and Allendorf 1996; Whitlock and McCauley 1998; Wang 2004). All pairwise comparisons among clusters revealed significant differences (Tab. 2.5). Results for the Bayesian estimation for non-symmetrical rates of gene flow are reported in Tab. 2.6. The proportion of individuals originating from within each identified cluster varied from 94.5 to 98.7%, with the highest value found in the Central cluster. Each independent run of BAYESASS

converged towards similar values of logProb despite different starting seeds. Moreover, visualization of the MCMC trace output confirmed mixing and movement in the parameter space (Fig. 2.3) and the posterior probability values of migration obtained from the run with the lowest estimate of Bayesian deviance (Faubet et al. 2007; Meirmans 2014) suggests strong isolation for all the inferred clusters.

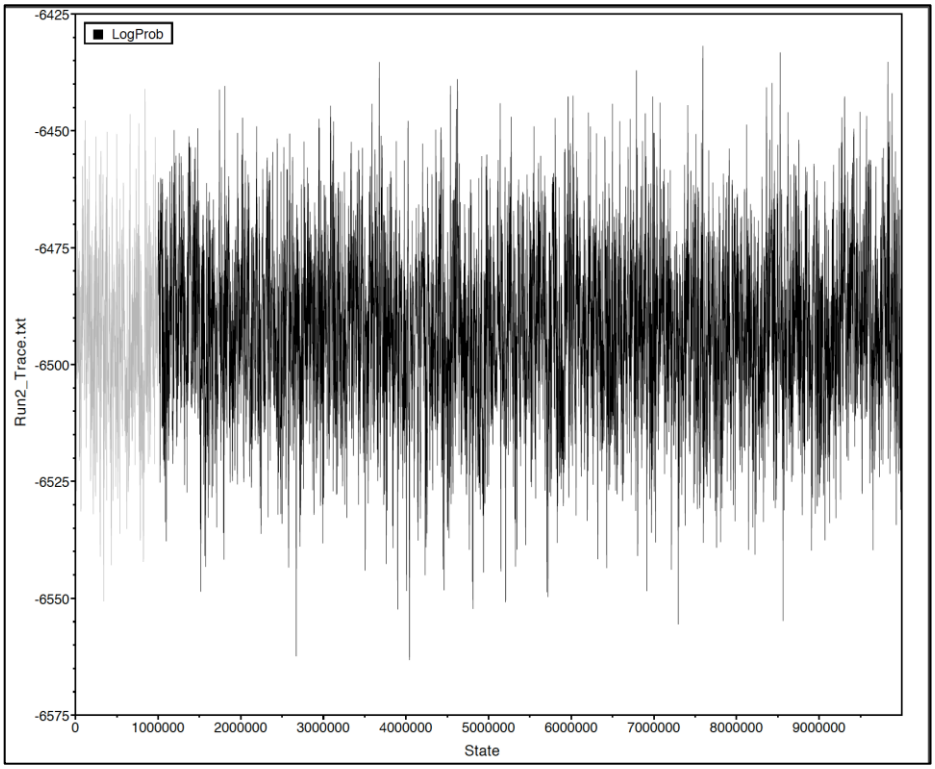

Figure 2.3 MCMC trace output from BAYESASS 3.0

MCMC trace output from BAYESASS 3.0. Grey lines corresponds to MCMC iterations discarded as burn-in. Black lines corresponds to the MCMC iterations used to investigate the posterior probability space. The fluctuations from the central value (ca. -6500) are small, indicating that the chain may have reached the right distribution. Also, the chain is mixing well: it is exploring the distribution by traversing to areas where its density is very low.

Discussion

I investigated the genetic structure of the endangered Andros Iguana to test the hypothesis that rates of gene flow across the island are high. The results of this study indicate that *Cyclura cychlura cychlura* iguanas do not belong to a single, large panmictic population as previously inferred. That is, gene flow is much lower than estimates based on field observations would suggest. Managing isolated local populations as a single entity, without considering metapopulation dynamics and patterns of genetic diversity can be dangerous (Greig 1979; Hanski 1989; Hedrick et al. 1996; Hanski and Gaggiotti 2004; Schwartz et al. 2007). Although the importance of barriers is highly dependent on the species ecology (Slatkin 1987), fine scale genetic structure analyses demonstrated that perceived rates of gene flow may not reflect realized ones, despite the apparently high dispersing potential of a species (Dever 2007; Rutz et al. 2012; Bertrand et al. 2014). Iguanas in the genus *Cyclura* tend to experience genetic structuring, presumably because these large-bodied, terrestrial lizards have difficulties dispersing across certain types of physical barriers and establishing viable populations, or because they are tightly associated with local selective pressures such as the availability of forage. Examples include *C. carinata* in the Turks and Caicos Islands (Welch et al. 2004; Bryan et al. 2007), and *C. cychlura figginsi* and *C. cychlura inornata* in the Exuma Islands chain, Bahamas (Malone et al. 2000). By contrast surveys and field observations of the Andros Rock Iguana suggested high dispersal capability (Knapp 2005a; Knapp et al. 2010). In particular, iguana hatchlings have been observed dispersing across a variety of different terrestrial habitats on Andros such as pine woodland, scrubland and mangroves and even water channels (Knapp 2005a; Knapp et al. 2010). The results presented in this study

reject the initial hypothesis of a panmictic iguana population on Andros and are indicative of historically limited patterns of gene flow. I foresee two possible explanations that could account for high dispersal yet interrupted gene flow. First, high overland dispersal rate may attenuate over narrow waterways and not extend over broader channels with stronger water currents. This scenario is in line with evidence indicating geography as an important component in lizard isolation and differentiation (Wang et al. 2013). Second, dispersal could be relatively high but successful migrant recruitment to the breeding populations could be low due to selection acting against migrants. This could occur if there is a direct cost to dispersal or if migrants are poorly adapted to local conditions (Nosil et al. 2005).

Fine-scale genetic structuring in reptiles, and particularly iguanine lizards, is not uncommon (Welch et al. 2004; Bryan et al. 2007; Row et al. 2010; Ng and Glor 2011). Limited gene flow has been detected even within the only iguana adapted to a marine environment, *Amblyrhynchus cristatus* (Steinfartz et al. 2009; Lanterbecq et al. 2010). The structure analysis presented here suggests that samples from Andros are best partitioned into three genetically distinct clusters. It could be argued that some portion of the inferred structure reflects recent changes in population dynamics (Moore et al. 2008). However, these results are most likely influenced by historical landscape fragmentation and not recent anthropogenic perturbations because all samples were collected in uninhabited and remote areas of Andros. Still, some portion of the inferred genetic structure may reflect biased estimates of allele frequencies due to sampling error. Small population sizes should result in greater rates of genetic drift, enhancing allele frequency differences among subpopulations, and greater fragmentation means there should be

fewer opportunities for gene flow to offset change due to genetic drift. Further, reduced population size and density are responsible for small sample sizes for many locations. However, increased variation among sampling sites within groups should make it less likely for differences among groups to appear statistically significant. Moreover, the results of the individual assignment tests demonstrate that these potential biases obstructing current perspectives on historic population genetic structure cannot explain all of the genetic divergence found among sampling sites. In particular, almost all individuals assigned to a specific genetic cluster were collected from clearly defined geographic regions divided by intervening large water channels (Fig. 2.1b-c). The geographic boundaries of the genetic clusters identified by STRUCTURE correspond well with the two bights separating Mangrove Cay and Alcorine Cay from North and South Andros. Individuals sampled from Sites 5, 6 and 8 represent the only notable exception. Despite being located on cays within Middle Bight they were grouped with the middle cluster (Fig. 2.1b-c). This pattern is consistent with the empirical observation of individuals floating across water channels. Water currents passing through the bights run east to west (tidal dependent), and tidal flow is further influenced by the easterly trade winds. I suspect that iguanas entering the water at the north edge of Mangrove Cay could disperse passively over water in a westerly direction. Some cays in Middle Bight (e.g., Sites 5, 6 or 8) are situated in the path of potential dispersers from Mangrove Cay as Middle Bight veers south (Fig. 2.1b-c). Cays farther north may be more difficult to reach given the tidal currents and influence of easterly trade winds and thus remain genetically isolated from the southern populations. However, the presented results suggest that

observations of iguanas crossing water barriers (Knapp 2005a) rarely result in successful migration.

Overwater dispersal has been documented to play an important role for colonization, particularly in lizards (Glor et al. 2005). The presented results suggest that the terrestrial ecology of *Cyclura* iguanas makes them less adapted to swim or withstand strong water currents that flow across the bights of Andros. Still, iguanas are particular good at rafting, and are known to be highly salt tolerant (Censky et al. 1998). It is hence conceivable that dispersal occurs more frequently across narrower or slower moving channels and is highly influenced by the directionality of the water current.

Consistent with evidence suggesting historically low inferred rates of gene flow, private alleles were restricted to each of the three geographic clusters further supporting that Middle and South Bights serve as major barriers to successful iguana dispersal. Differences in genetic makeup that generate genetic structure build up over time in the form of variance in allele frequencies across subpopulations and the emergence of private alleles due to random genetic drift or mutational events (Haasl and Payseur 2011). The inferred estimates of gene flow also contradict genetic homogeneity across Andros. The significant AMOVA value ($F_{st} = 0.117$, $p << 0.01$) for genetic clusters identified by STRUCTURE indicates that more than 11% of the total genetic variance on Andros reflects significant differences in allele frequencies among the three clusters. This value is in accordance with other estimates of genetic isolation documented in other iguana taxa (*Amblyrhynchus cristatus* $F_{st} = 0.002–0.011$, Lanterbecq et al. 2010; *Cyclura carinata* $F_{st} = 0.18–0.43$, Alberts 2004; Welch et al. in review). Pairwise estimates of genetic differentiation (Tab. 2.5) indicate that the Center and South clusters are more closely

related to each other than either is to the North cluster (Central-South F_{st} =0.072, p << 0.01; Center-North F_{st} = 0.273, p << 0.01) suggesting a genetic uniqueness of the North cluster. Moreover, the Bayesian inference of recent migration rates substantiates the existence of at least three distinct iguana populations on Andros (Tab. 2.6). The proportion of individuals originated locally within each identified cluster varied from 94.5 to 98.7% indicating that there is little gene flow between any of these populations. The small percentage of migrant genotypes found within each cluster could represent ancient polymorphisms retained among the genetic and geographic isolates of today.

Conclusion

I document that *C. c. cychlura*, despite its dispersal potential, shows significant genetic structuring and that natural landscape features (i.e. water channels) influence successful migration and thus genetic structure across the island. That is not to say that anthropogenic activities are not important in further compromising the extant metapopulation dynamics on the island, and the extremely low density of individuals on North Andros (see Knapp 2005 for details) is a clear example of what could happen when human development goes unregulated. These data also suggest that, in accordance with ecological observations, the identified cluster on Alcorine and Mangrove Cays harbors significant genetic diversity relative to other populations. Genetic diversity is the fundamental requirement for adaptive evolution in response to environmental changes and should be correlated with the resilience of populations to novel environmental selective pressures (Reed and Frankham 2003; Liao and Reed 2009). In addition, reduction in genetic diversity and heterozygosity is tightly linked to inbreeding depression and can enhance the probability of extinction in stressful environments

(Armbruster and Reed 2005; Liao and Reed 2009; Fox and Reed 2011). Although the genetic data presented here are not directly indicative of any enhanced adaptive potential for iguanas in the Central cluster, it would be wise to focus the limited conservation resources on the subpopulation showing higher degree of variability at neutral-nuclear markers and higher density of individuals. In 2009, the West Side National Park was expanded, in part based on ecological and population studies of *C. c. cychlura* (Knapp and Pagni 2011). The expanded boundaries include Alcorine Cay and segments of Mangrove Cay. Molecular data confirm the strategic importance of the new boundaries. The extension of the national park now includes a much larger portion of the island, enhancing the future prospects for the iguanas in the central region of Andros Island. However, the prospects for the two iguana populations associated with North and South Andros are far less certain. The data suggest that these populations, in particular the one north of North Bight, are genetically unique. Given their uniqueness and that they reside largely outside park boundaries additional efforts should be taken to ensure that these populations also receive protection.

CHAPTER III

MULTI SPECIES INTERACTIONS: ANALYZING CONCORDANT PATTERNS OF POPULATION STRUCTURE IN A CARIBBEAN HOST-PARASITE SYSTEM (*CYCLURA-AMBLYOMMA*)

Abstract

Host-parasite interactions have often been used to support hypotheses explaining current biogeographic patterns, resolve species dispersal routes, and investigate population structure. This approach is particularly useful when habitat fragmentation and geological history make biogeographic assessments difficult. In this study I compare the distribution of genetic variability in *Cyclura cychlura* iguanas and their tick ectoparasites (genus *Amblyomma*) in The Bahamas. My goal is to investigate how organismal dispersal affects current biodiversity patterns on these islands, and whether the distribution of the two interacting taxa could suggest co-adaptive patterns. Specifically I tested the hypothesis that *Amblyomma* ticks in The Bahamas disperse "vertically", i.e., in parallel with their hosts' dispersal. Alternatively these parasites could disperse "horizontally", i.e., via multiple hosts besides iguanas. I sampled *Amblyomma* ticks and *C. cychlura* iguanas on Andros and the Exuma Islands, on the edges of the once emergent Great Bahama Bank. Mitochondrial DNA was extracted from sampled individuals and a series of haplotype networks were constructed to compare the variability and phylogeographic distributions of the analyzed taxa. Additionally, I explored the population structure of *C.*

cychlura figginsi within the Exuma Islands. Although non-definitive, I found a phylogeographic pattern supportive of vertical dispersal and suggesting vicariance as a main driver of current biodiversity patterns. In both *Amblyomma* and *C. cychlura* I identified three concordant hotspots of diversity characterized by divergent mitochondrial sequences. These results indicate a high degree of specificity in this reptile-tick system. Moreover, when compounding mtDNA information with microsatellite structure results from this and other studies it is possible to infer historically low rates of dispersal in iguanas, possibly even when the Great Bahama Bank was emergent and Andros and the Exuma Islands Chain were connected by land. Finally, my results also suggest that a taxonomic revision of iguanas in The Bahamas is advisable.

Introduction

Understanding and explaining current biodiversity patterns is a major goal of biogeography (Lomolino et al. 2010). The complex geological history of regions such as West Indies, and the often scant and fragmentary taxonomic information available for species found in these regions, make assessing such patterns a difficult task (Woods and Sergile 2001). Two main mechanisms are often invoked to explain why species are found where they are: vicariance and dispersal, with the relative contribution of the two often debated, especially when different time and spatial scales are analyzed (Croizat et al. 1974; Savage 1983; Zink et al. 2000). To overcome some of the ambiguities associated with the aforementioned paradigms, researchers have often investigated patterns of concordance in biogeographic distribution of multiple interacting species (Croizat et al. 1974; Savage 1983; Lomolino et al. 2010). Most species have a unique set of ecological requirements and independent evolutionary histories. This suggests that concordant

patterns of distribution in different taxa are more likely to arise by a common biogeographic history (Savage 1983). For example, some authors have suggested that concordance in phylogenetic signal of multiple species is most likely explained by segregating events scattering widespread ancestors (Croizat et al. 1974; Rosen 1975; Zink et al. 2000). Other authors have argued that congruence in such phylogenetic tracks may simply reflect similarity in dispersal routes (Hedges et al. 1992; Hedges 1996a; Hedges 1996b). Although this controversy is far from settled (Woods and Sergile 2001), host-parasite interactions have been widely used as a tool to understand and explain contemporary relationships of geographically structured populations, especially in those host species with low genetic variability and confounding phylogenetic signals (Whiteman and Parker 2005; Nieberding and Olivieri 2007; Whiteman et al. 2007; Nieberding et al. 2008). The advantage of using parasites originates from the tight association that often evolves with their host species. The rationale for this multi-taxon approach, hence, relies on the assumption of vertical transmission of parasites along with their hosts, or, in other words, the parallel dispersal of the two interacting taxa (Whiteman and Parker 2005; Whiteman et al. 2007). Wirth and colleagues demonstrated the efficacy of this method in their analysis of the distribution of human ethnic groups inferred using genetic sequences of *Helicobacter pylori*, a bacterium known to parasitize the human gut (Wirth et al. 2004).

Investigating host-parasite interactions on islands and archipelagoes can help elucidate historical patterns of dispersal for species whose biogeographic history is not well established, or even open the field to alternative hypotheses explaining current patterns of species distributions (McCoy et al. 2005). Moreover, the analysis of specific

host-parasite interactions in fragmentary habitats is key to the understanding of reciprocal evolutionary processes acting on both parasites and their hosts, and to elucidate the potential fitness cost that parasites may exert on the host's genetic diversity (Coltman et al. 1999; Carius et al. 2001; Rijks et al. 2008; Lion and Gandon 2015).

Cyclura cychlura iguanas in the Bahamas are characterized by a disjunct distribution: *C. c. cychlura* occurs on the large and fragmented island of Andros while *C. c. figginsi* and *C. c. inornata* are distributed along the Exuma Island Chain (Malone et al. 2003; Fig. 3.1). These two currently separated island groups were once connected, when the now partially submerged Great Bahama Bank, a carbonate platform that has been accumulating since the Cretaceous period, was above sea level during the Wisconsinan glaciation, ≈ 18,000 years ago (Short and Blair Jr. 1986; Schwartz 2005). The geological history of this area, thus, suggests that the currently separated populations of *C. cychlura* were once interconnected and possibly characterized by high rates of gene flow (Malone et al. 2000; Malone et al. 2003; Knapp and Pagni 2011).

Iguanas in the genus *Cyclura* throughout The Bahamas, The Turks and Caicos Islands, and the other islands in the West Indies are parasitized by ticks in the genus *Amblyomma* (Durden and Knapp 2005; Lemm and Alberts 2012). Recent biogeographic analyses of iguana-ectoparasite associations in The Bahamas revealed that *A. albopictum* ticks are found in populations of *C. c. cychlura* on Andros Island and *C. c. figginsi* in the central Exuma Islands, while *A. torrei* is known only from populations of *C. c. figginsi* in the southern Exuma Islands (Durden and Knapp 2005; Durden et al. 2015). Interestingly, no ticks have been reported from *C. c. inornata* populations in the northern Exuma Islands where *C. c. inornata* iguanas are found (Durden et al. 2015). This distribution

pattern could be explained by current limitations in the dispersal ability of the iguana hosts and their ectoparasites; alternatively, historically low rates of dispersal in *C. cychlura* iguanas, even before the vicariant event submerging most of the Great Bahaman Bank, could account for the current patterns of biodiversity.

In this study I investigate the genetic diversity and distribution in endemic Bahamian Rock Iguanas and their tick ectoparasites. My goal was to explore how organismal dispersal is affecting current biodiversity patterns in The Bahamas, and whether the distribution of the two interacting taxa could suggest patterns of coadaptation. The specific hypothesis tested here is that the dispersal of *Amblyomma* ticks in The Bahamas is "vertical". In this study I use the terms “vertical transmission” with a different meaning than the one usually found in host-parasite and symbiotic literature, i.e., the transmission of parasites to their hosts through the host’s germ line (see for example Nobre et al. 2010, and Nobre and Aanen 2010). Since ticks are ectoparasites, they are unlikely to be transmitted transovarially. Therefore, I refer to “vertical dispersal” in this system, and specifically in ticks, as a dispersal mechanism limited by the dispersal of their iguana hosts. Alternatively, tick distributions could be limited by ecological factors such as the presence of other appropriate hosts to complete different stages of their life-cycle, or they could disperse "horizontally" across multiple islands via other hosts such as birds (Johnson et al. 2002; Štefka et al. 2011). If the vertical hypothesis is supported, I expect the relatedness among island populations of ticks to be concordant with the relatedness of iguana populations across those same islands. To test this hypothesis I compared *C. cychlura-Amblyomma* phylogenetic networks obtained by

sequencing mtDNA genes in these taxa. I discuss these results accounting for the possibility of historically low rates of iguana dispersal across the Great Bahaman Bank.

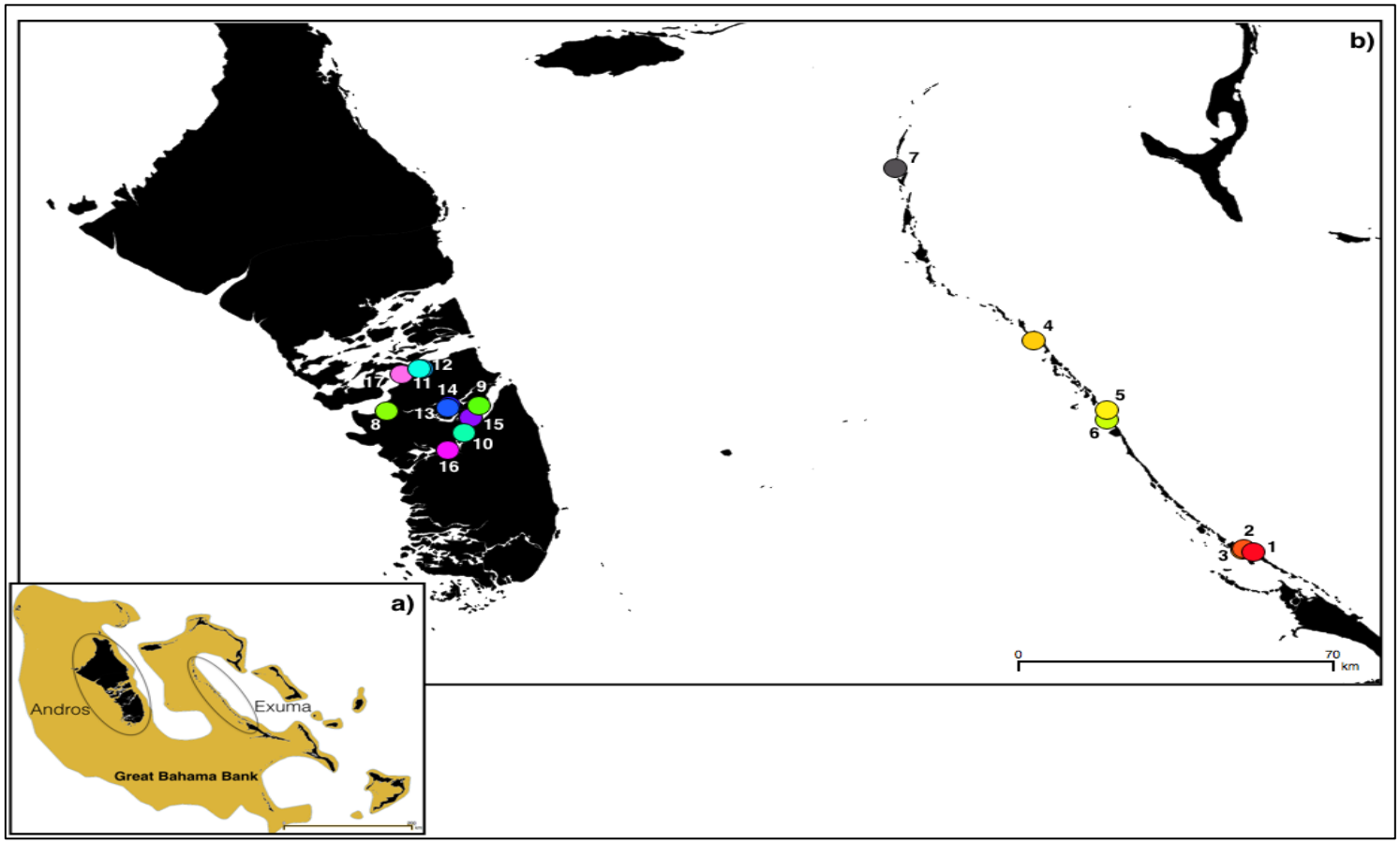

Figure 3.1 Map of the Bahamas

a) Map of the Bahamas. The brown/yellow area of the Great Bahama Bank represents its inferred above-water surface during the Wisconsinan glaciation (≈ 18Ky ago); **b)** Colors used for sampling locations are the same used in network analyses (see Materials and Methods and Figs. 3.2 and 3.3). For details on sampling locations and number of sampled individuals (including GenBank sequences) see Tab. 3.2.

Materials and Methods

Study System

Three subspecies of *Cyclura cychlura* iguanas are currently recognized in the Bahamas: *C. c. cychlura* on Andros, *C. c. inornata* and *C. c. figginsi* on the Exumas (Fig. 3.1). Details on sub-species distribution, natural history and conservation status have been extensively described elsewhere (Knapp and Malone 2003; Malone et al. 2003; Knapp 2005b; Knapp and Pagni 2011; Colosimo et al. 2014; Aplasca et al. 2016).

Amblyomma ticks are associated with many Caribbean reptiles. A few species, however, have been described as primarily parasitic of *Cyclura* Rock Iguanas in the West Indies: *A. cruciferum* in Haiti and Puerto Rico, *A. torrei* in the Cayman islands, Cuba, Puerto Rico and the Exuma islands, *A. antillorum* in the British Virgin islands, Dominica and East Caicos island, and *A. albopictum* known from Cuba, the Dominican Republic, Haiti and The Bahamas (Woods and Sergile 2001; Voltzit and Keirans 2003; Durden and Knapp 2005). Distributions of these tick ectoparasites have accumulated only recently (Morand and Krasnov 2010). For example, prior to Durden and Knapp (2005), *A. torrei* had been known only from iguanas on Cuba (*Cyclura nubila*) and on Mona Island (*Cyclura stejnegeri*). Another species, *A. albopictum*, has only recently been recovered from *Cyclura cychlura* iguanas on the Exuma Islands, Bahamas, where it had not previously been reported (Durden and Knapp 2005; Durden et al. 2015). Moreover, the majority of the taxonomic work done so far relies almost entirely on morphological recognition and little if any molecular data have been used to describe the current diversity of ticks parasitizing iguanas in The Bahamas (Durden and Knapp 2005; Durden et al. 2015).

Data Collection

Since 1997, all islands where *Cyclura cychlura* occur naturally have been visited as part of a population ecology research program for the species (e.g., Aplasca et al., 2016; Colosimo et al., 2014; Durden and Knapp, 2005; Durden et al., 2015; Knapp and Alvarez-Clare, 2016). While collecting morphometric data on iguanas, ticks were removed using forceps and stored in the field in 70% Et-OH. Ticks were identified to species level using morphological characters (Durden and Knapp 2005; Durden et al. 2015). A list of sampling sites from where tick samples have been collected is provided in Tab. 3.1 and Fig. 3.1. For the purposes of this study I analyzed iguana samples collected from 5 different locations: 5 individuals from White Bay Cay (collection site ID 1), 5 individuals from Noddy Cay (collection site ID 2), 5 individuals from North Adderly Cay (collection site ID 3), 5 individuals from Gaulin Cay (collection site ID 6), and 5 individuals from Alcorine (collection site ID 8; Fig. 3.1, Tab. 3.2). I focused only on these locations because previous work on these iguanas revealed low genetic variability at the mtDNA level across their range (see Malone et al 2003 for details).

Table 3.1 Tick sampling details

Site ID	N	Morpho ID	Molec. ID	Host
1	4	*A. torrei*	*R. sanguineus*	*C.c.figginsi*
2	2	*A. torrei*	*R. sanguineus*	*C.c.figginsi*
3	2	*A. torrei*	*R. sanguineus*	*C.c.figginsi*
4	2	*A. torrei*	*R. sanguineus*	*C.c.figginsi*
5	5	*A. albopictum*	*A. sabanareae*	*C.c.figginsi*
6	3	*A. albopictum*	*A. sabanareae*	*C.c.figginsi*
7	NA	NA	NA	NA
8	2	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
9	1	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
10	14	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
11	1	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
12	1	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
13	2	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
14	1	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
15	5	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
16	2	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*
17	1	*A. albopictum*	*A. sabanereae*	*C.c.cychlura*

Sampling sites, number of analyzed individuals per site (N) morphological and molecular identification and iguana host species for tick samples.

Table 3.2 Iguana sampling details

Site ID	N	Species
1	5	*C. c. figginsi*
2	5	*C. c. figginsi*
3	5	*C. c. figginsi*
6	5	*C. c. figginsi*
8	5	*C. c. cychlura*
AF217780*	1	*C. c. cychlura*
AF217781*	1	*C. c. cychlura*
AF217776*	1	*C. c. figginsi*
AF217775*	1	*C. c. figginsi*
AF217774*	1	*C. c. inornata*

Sampling site ID, number of analyzed individuals per site (N) and taxonomic identification of iguana samples. Entries characterized by an asterisk refer to iguana haplotypes downloaded from GenBank.

DNA extraction and sequencing

Genomic DNA extraction procedure and mtDNA marker amplification protocols in iguanas have been described elsewhere (Malone et al. 2003; Colosimo et al. 2014). Briefly, 20 µL of blood lysate were mixed with Proteinase-K (20 mg/mL) and a digestion buffer (17 mM Tris-HCl, 1.7 mM $CaCl_2$ and 50% Glycerol, Sambrook et al. 1989) prior to genomic DNA extraction using the ABI-6100 Nucleic Acid Prep Station and proprietary chemistry (ABI, Foster City, CA). I targeted the same mtDNA region previously used in other systematic work in *Cyclura* (Arevalo et al. 1994; Malone et al. 2000; Malone et al. 2003). The region encompasses ≈ 900 bp including Histidine and Serine. It is flanked by NADH-dehydrogenase subunit 4 (ND4) and Leucine, and has been proven variable and useful in other phylogenetic studies with *Cyclura* iguanas and other reptiles (Arevalo et al. 1994; Malone et al. 2000; Malone et al. 2003). For simplicity I will refer to this region as *ND4* gene throughout the rest of the study.

I used genomic DNA extracted from ticks to conduct analyses on tick specific molecular markers. To maximize DNA yield and prevent the hard chitin scutum from hampering nucleic acids extraction I based the tick extraction protocol on Halos et al (2004). Briefly, specimens were first mechanically sheared using a scalpel in a lysis solution (NaCl 0.1M, Tris-HCl 0.21M, pH8 EDTA 0.05M, SDS 0.5%). The tissue was then homogenized in a proteinase-K solution (20 mg/mL) using a bead mill (MM301-Retsch, Newton, PA). Genomic DNA was then extracted using the Maxwell–16™ Research instrument and proprietary chemistry (Promega, Madison, WI). Successful DNA extraction of tick genomic DNA was tested via Polymerase Chain Reaction (PCR) targeting the mtDNA- Cytochrome Oxidase 1 (*COI*) region of *Amblyomma* ticks. I

focused on this mtDNA gene because previous work on Ixodidae showed this marker to be a reliable tool for species differentiation (Cruickshank 2002; Cruickshank and Hahn 2014). However, markers specifically designed for *A. albopictum* and *A. torrei* were not available. I therefore used mtDNA *Amblyomma* sequences downloaded from GenBank and the online tool PRIMER3 (Rozen and Skaletsky 1999) to design new primers that could target and amplify ≈ 650 base pairs of the *COI* gene in *A. torrei* and *A. albopictum* (Tab. 3.3). The PCR profile for amplification in tick samples was set with an initial denaturation period of 5 min at 94 ˚C. I then used 5 ramp-up cycles with 30 sec at 94 ˚C, 30 sec at the annealing temperature and 30 sec at 72 ˚C. In ramp-up protocols, the annealing temperature raises 1 ˚C each cycle. I used this approach to maximize chances of DNA amplification in analyzed specimens. I set the initial annealing temperature at 45 ˚C. The remaining 30 cycles of the PCR were 30 sec at 94 ˚C, 30 sec at annealing temperature of 48 ˚C, and 30 sec at 72 ˚C, with a final elongation phase of 7 min at 72 ˚C.

Table 3.3 *Amblyomma COI* primer details

Name	Sequence (5'-3')	T ˚C
AmbCOI-F	GGT CAA CAA ATC ATA AAG ATA TTG G	48
AmbCOI-R	TAA ACT TCA GGG TGA CCA AAA AAT CA	

Name, forward (F) and reverse (R) sequences, annealing temperature (T ºC) for primers developed to amplify *COI* gene in *Amblyomma* ticks.

Amplicons were electrophoresed in 1% agarose TBE gels and visualized by ethidium bromide and UV light. Successful PCR products were shipped to Arizona State University for purification and sequencing. This facility uses Big Dye V3.1 chemistry and an Applied Biosystems 3730*xl* DNA Analyzer Instrument (ABI, Foster City, CA) for sequencing. Shipped samples were sequenced with forward and reverse primers. DNA

sequences were visualized and edited using SEQUENCHER v4.7 (© Gene Code Corporation). When possible, reverse and forward sequences were combined together to build a consensus sequence.

Molecular identification, sequence variability and haplotype network

Each mtDNA sequence obtained was first compared with the National Center for Biotechnology and Information (NCBI) nucleotide database for molecular identification using the Basic Local Alignment Search Tool – BLAST (Altschul et al. 1990), and optimizing the search for highly similar sequences (i.e., megablast). I used DNAsp (Librado and Rozas 2009) to calculate DNA sequence variability indexes including nucleotide diversity (π) and the average number of nucleotide differences (k). For this analysis I grouped individuals based on recognized taxonomy. To compare patterns of dispersal and to infer potential barriers to parasite movements across islands, I constructed and compared two phylogenetic networks. In each taxon, individual haplotypes were first aligned and trimmed to the same length using MEGA 5.2.2 (Tamura et al. 2011). Aligned sequences were exported and converted to nexus files using FABOX (Villesen 2007). Phylogenetic networks were plotted using the Minimum Spanning Network (MSN) algorithm implemented in POPART (Leigh and Bryant 2015). Given a set of pairwise distances representing the degree of dissimilarity among haplotypes, the MSN depicts the haplotypes as nodes connected by edges so to minimize the distance among haplotypes, and without creating a cycle with the already existing links (Bandelt et al. 1999). To investigate the recent population dynamics of *Amblyomma* ticks, I used two metrics to test for neutral patterns of molecular evolution. Specifically, I focused on Fu and Li's D* and F* (Fu and Li 1993; Fu 1997). These metrics should be

more reliable at estimating departures from random expectations when using non recombining regions such as mtDNA (Ramírez-Soriano et al. 2008). For these calculations I used the software DNAsp v5 (Librado and Rozas 2009). For sampling sites with five or more individual sequences I used a tool implemented in POPART (Leigh and Bryant 2015) to estimate Φ statistics, measures of genetic differentiation analogous to Wright's F-indexes but applied to DNA sequence data (Holsinger and Weir 2009). Significant differences in variance partitioning among samples were tested with 1000 permutations.

Results

Samples analyzed

I obtained 712 base pairs of the *ND4* gene for 25 iguana individuals, five from sites ID1, ID2, ID3, ID6 and ID8 respectively (Fig. 3.1, Tab. 3.2). I also added to my dataset 5 *ND4 C. cychlura* sequences downloaded from GenBank (Malone et al. 2003; AF217774, *C. c. inornata*; AF217775, AF217776, *C. c. figginsi*; AF217780, AF217781, *C. c. cychlura;* Tab. 3.3).

Out of the 85 tick individuals processed for DNA extraction 48 (56%) yielded DNA of sufficient quality and quantity to PCR amplify. This low yield is probably due to the suboptimal storage condition for some of these samples. At the time of processing in the laboratory many samples had enough Et-OH to preserve the specimen for morphological recognition, but probably not enough to preserve nucleic acids. For successfully processed specimens I obtained 590 base pairs of the mtDNA *COI* gene (details on number of sampled individuals and provenience are provided on Tab. 3.1).

Molecular identification, sequence variability and haplotype network

Sequence analysis of iguana mtDNA revealed 6 unique haplotypes. No new haplotypes were discovered in *C. c. figginsi* samples, while two new haplotypes were identified in *C. c. cychlura* on Andros Island. Interestingly, I found that the number of mutations separating *C. c. figginsi* sequences sampled at sites ID1, ID2 and ID3 from *C. c. cychlura* haplotypes on Andros equaled that separating *C. c. figginsi* sequences (sites ID1, ID2 and ID3) from other *C. c. figginsi* sampled at site ID6 (Fig. 3.2). DNA diversity statistics based on the currently recognized taxonomy are reported in Tab. 3.5.

At the time of these analyses the NCBI database did not have any mtDNA sequence directly derived from *A. albopictum* or *A. torrei* tick species. Therefore, the program returned *Rhipicephalus sanguineus* as the closest match for sequenced samples morphologically identified as *A. torrei* and collected in the southern and northern ends of the Exuma Islands (collection sites 1, 2, 3 and 4; 590 bp; Query Cover: 99%; E-value: 0.0; Ident.: 87%; accession number KM494916). No haplotypic variation was found within this group (Tab. 3.2 and Fig. 3.3). *Rhipicephalus sanguineus,* also known as the brown dog tick, has a cosmopolitan distribution and is mostly known to parasitize mammals, although there also have been cases in which this parasite has been associated with birds and reptiles (Gray et al. 2013).

The BLAST search for ticks collected from the remaining sites (central Exuma Islands and Andros Island) and morphologically identified as *A. albopictum* returned *Amblyomma sabanareae* as the closest match (590 bp; Query Cover: 96%; E-value: 3e-170; Ident.: 86%; BLAST results were similar for all specimens). *Amblyomma sabanerae* is known to parasitize primarily reptiles, and its distribution is largely restricted to

Suriname and Central America, with some records from the Nearctic Region (Voltzit and Keirans 2003). Throughout the rest of this study I will refer to these ticks using the taxonomy based on morphological characteristics adopted by Durden et al. (2015). The network in Fig. 3.3 shows the presence of three main separated haplogroups within analyzed ectoparasites (Tab. 3.2 and Fig. 3.3). DNA diversity statistics are reported in Tab. 3.6.

The number of haplotypes and the number of *A. albopictum* individuals (N = 38) were sufficient for testing for departures from neutral evolutionary rates using sequence data. Both of Fu and Li's parameters D* and F* were negative and significant when considering all the *A. albopictum* sequences together (-2.596, $p < 0.05$, and -2.691, $p < 0.05$ respectively). Given the relative geographic isolation of the Central Exuma (Sites 5 and 6, N = 8) and Andros haplogroups (Sites 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17, N = 30), I performed a hierarchical AMOVA between these two groups. I found a significant degree of genetic differentiation ($\Phi_{ct} = 0.906$, $p < 0.01$). I then performed group specific tests of population dynamics using the available haplotype diversity. Ticks collected on Andros Island showed significant departure from random expectations (N = 30, D* = -4.126, $p < 0.02$; F* = -4.194, $p < 0.02$), while the tick samples coming from the central Exuma Islands did not (N = 8, D* = -4.126, $p > 0.1$; F* = -4.194, $p > 0.1$).

Table 3.4 *Cyclura cychlura* sequence diversity statistics

Taxon	N	N-sites	H	S	π	Hd
C. cychlura	30	712	6	9	0.004	0.676
C. c. cychlura	7	712	3	2	0.001	0.714
C. c. figginsi	22	712	2	4	0.002	0.416
C. c. inornata	1	712	1	na	na	na

Diversity statistics for *C. cychlura* mtDNA. Statistics are calculated at the species and intra-specific level according to the currently recognized taxonomy. Statistics include the number of samples (N), the number of base pairs analyzed (N-sites), number of haplotypes (H), number of segregating sites (S), nucleotide diversity (π), and haplotype diversity (Hd). Sequences downloaded from GenBank were included when making these calculations.

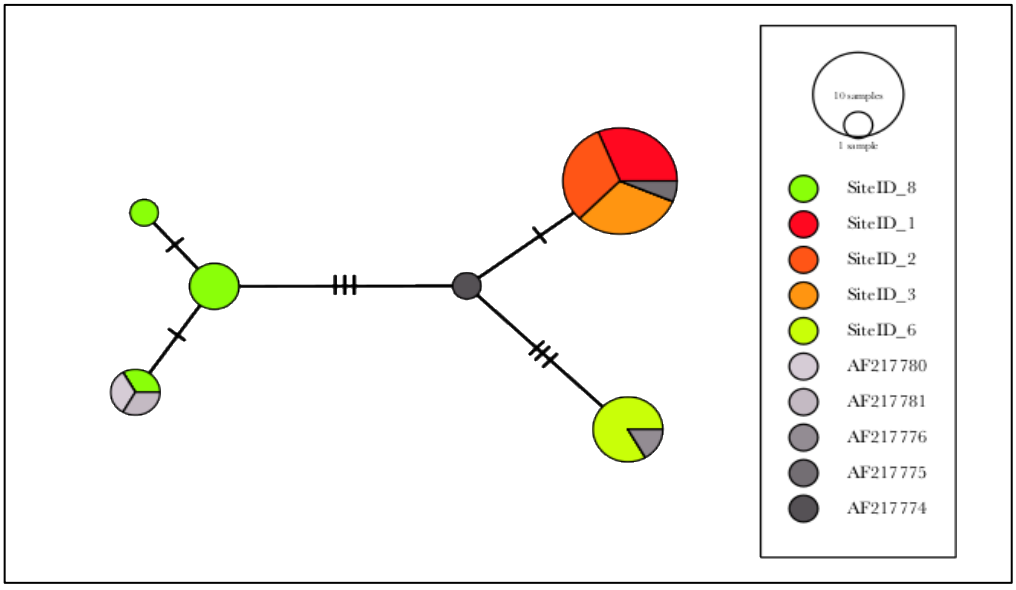

Figure 3.2 *Cyclura cychlura ND4* minimum spanning network.

Minimum spanning network based on 712 base pairs for the *ND4* gene in *C. cychlura*. Marks across lines represent the number of mutations separating the haplotypes. Color-coding is the same as that used in Fig. 3.1. GenBank Haplotypes depicted in gray-scale. Note that all previously recorded haplotypes were recovered in this study.

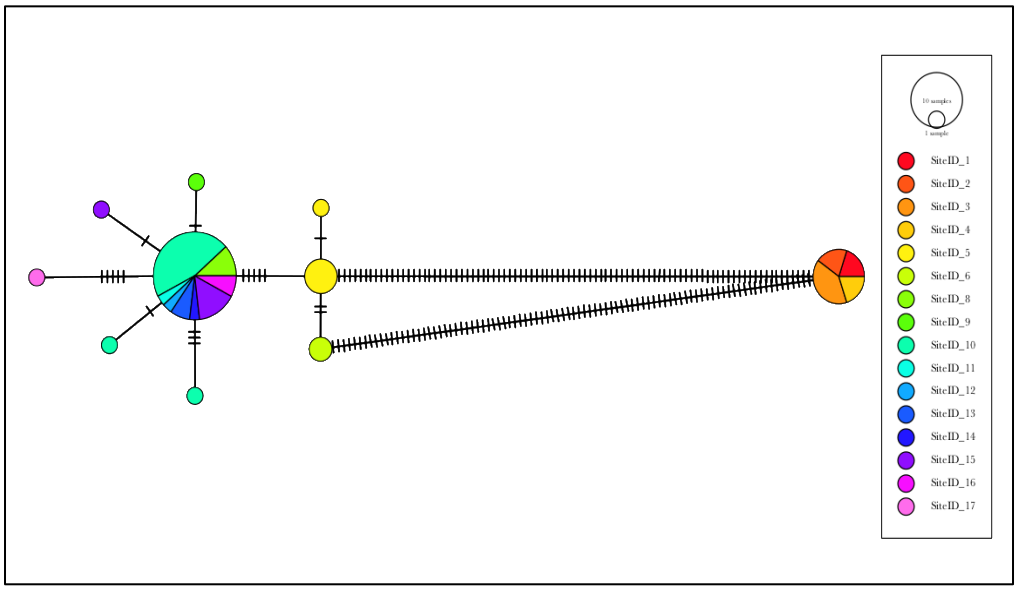

Figure 3.3 Tick *COI* minimum spanning network.

Minimum spanning network on 590 base pairs for the *COI* gene in ticks. Marks across lines represent the number of mutations separating the haplotypes. Color-coding is the same as that used in Fig. 3.1.

Table 3.5 Tick sequence diversity statistics

Taxon	N	N-sites	H	S	π	Hd
A. albopictum	38	590	9	17	0.003	0.528
A. torrei	10	590	1	na	na	na

Mitochondrial DNA diversity statistics for ticks parasitizing iguanas. The taxonomic identification reported in the table is reported for both the morphological ID. Number of samples (N), base pair length of the analyzed fragment (N-sites), number of haplotypes (H), number of segregating sites (S), nucleotide diversity (π), haplotype diversity (Hd).

Genetic structure within the Exumas

The phylogenetic network built using *ND4* sequences in iguanas indicates the presence of genetic structure within *C. c. figginsi*. This result is corroborated by the presence of two clearly distinct tick taxa on southern and central Exuma Islands

respectively. Malone and colleagues (2003) briefly described the distribution of genetic variability in *C. c. figginsi*, suggesting restricted gene flow between the central and southern islands in the Exuma chain. To best describe and quantify this hypothesized isolation and to corroborate inferences obtained from mtDNA I decided to ran a population structure analysis on iguana samples collected on sites ID1, ID2, ID3 and ID6, where the majority of differentiation seems to reside. I extracted genomic DNA from 47 iguanas collected from site ID1, 47 iguanas from site ID2, 38 from site ID3 and 43 from site ID6 (see Materials and Methods for DNA extraction protocol). Individuals were genotyped at 12 polymorphic microsatellites (Tab. 3.8). I used GENALEX v6.5 to calculate summary statistics on these samples (Peakall and Smouse 2012), and I used GENEPOP v4.2 (Rousset 2008) to investigate patterns of Linkage Disequilibrium (LD). Of the 66 possible multiple marker comparisons, only loci Z50 and C124 showed a significant departure from independent assortment after Bonferroni correction (Z50–C124 p-val = 0.000474). I investigated patterns of population structure using the Bayesian framework implemented in the software STRUCTURE v2.3.4 (Pritchard et al. 2000). I used $1*10^5$ MCMC as burn-in and $9*10^5$ MCMC to calculate the posterior probability of each individual belonging to a unique genetic cluster and I assumed that K, the number of possible population clusters, was between 1 and 5. I repeated the analysis 20 independent times. I took advantage of the parallel computing capabilities of the R package *ParallelStructure v.1.0* (Besnier and Glover 2013) to run this analysis on a MacOS 10.11.6 (code is available in the Appendix B.1). I used the web-tool STRUCTURE-HARVESTER to estimate the most likely number of genetic clusters following Evanno et al. (2005) as modified by Earl and VonHoldt (2012).

Figure 3.4 Molecular marker information for loci characterized in congeners of *Cyclura cychlura figginsi*

Name	Reference	Size Range	N_a	H_o	H_e
Z13	(An et al. 2004)	269-276	3	0.130	0.138
Z50	(An et al. 2004)	295-303	4	0.165	0.426
Z106	(An et al. 2004)	173-188	6	0.233	0.299
F519	(An et al. 2004)	340-350	6	0.364	0.377
D111	(Lau et al. 2009)	142-146	2	0.306	0.284
D136	(Lau et al. 2009)	161-201	11	0.430	0.467
C124	(Lau et al. 2009)	210-252	9	0.581	0.652
CIDK101	(Welch et al. 2011)	250-263	4	0.354	0.415
CIDK109	(Welch et al. 2011)	360-384	7	0.422	0.420
CCSTE02	(Rosas et al. 2008)	287-291	4	0.204	0.339
CIDK135	(Welch et al. 2011)	417-425	4	0.557	0.571
CCYC31	(Colosimo et al. 2014)	179-181	3	0.179	0.130

Name, reference, size range, number of alleles (N_a) observed heterozygosity (H_o) and expected heterozygosity (H_e). Summary statistics are based on the total number of analyzed iguanas.

The Evanno algorithm (Evanno et al. 2005) identified K = 2 as the most likely number of clearly distinct genetic clusters (K = 2, Fig. 3.5). In Fig 3.5 I also report the output for values of K = 4. I used the two clusters identified in STRUCTURE to perform an analysis of molecular variance (AMOVA) using ARLEQUIN v3.5 (Excoffier et al. 2010). I used ≈ 1000 permutations to test for significant differences in allele frequency among the central and southern clusters. The program reported an F_{st} value of 0.373 (p-val << 0.01). Moreover, in both clusters I found significant evidence of departure from random mating expectations (F_{is}-central Exuma = 0.121, p-val = 0.012; F_{is}-southern Exuma = 0.212, p-val << 0.01).

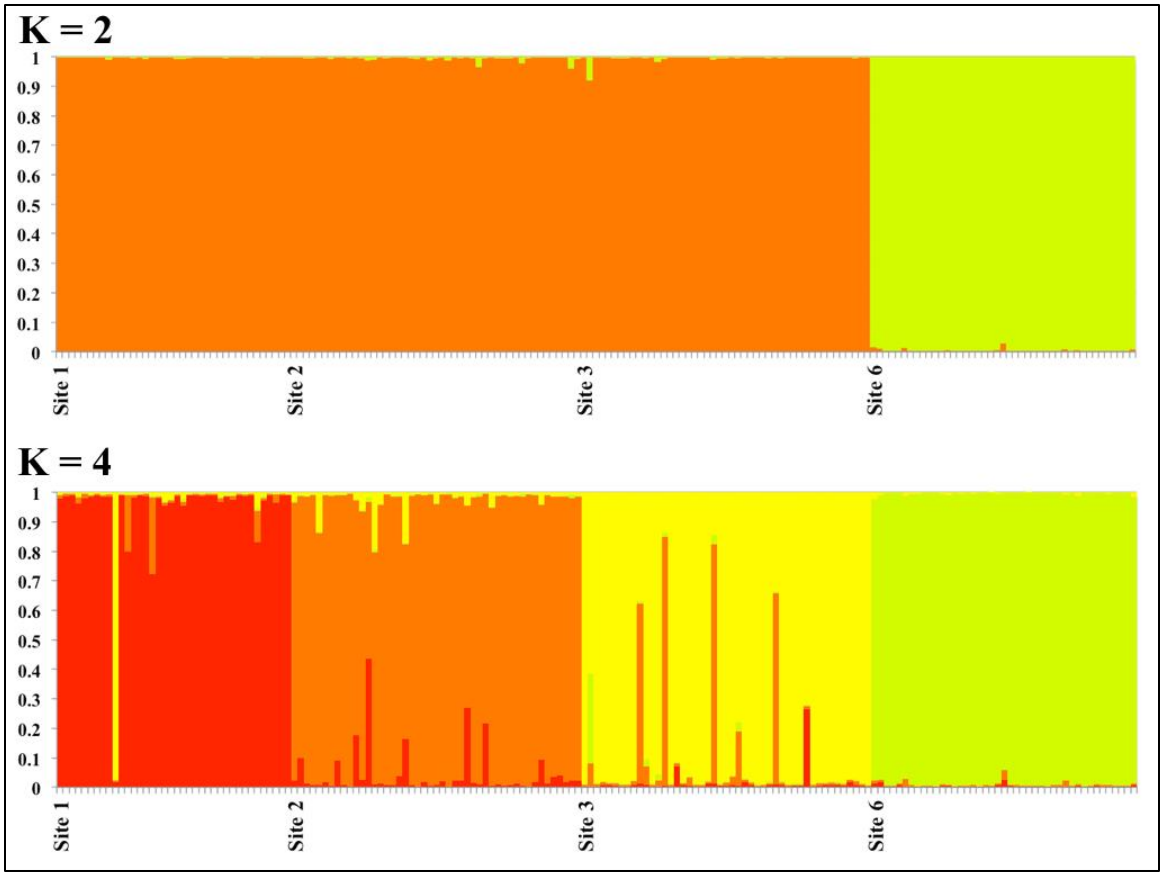

Figure 3.5 *Cyclura cychlura figginsi* structure results

Bayesian clustering output based on the analysis of 12 microsatellites in 175 *C. c. figginsi* iguanas sampled across four cays in the Exumas (see site IDs in Fig. 3.1 for details). I report the output for K = 2 and K = 4.

Discussion

Much can be learned from the comparative study of interacting species' phylogeographies. This approach can lead to a better understanding of the processes contributing to the current species' distributions (Lion and Gandon 2015), and to the acquisition of further insights on species ecology (Olszewski et al. 2009). The parallel analysis of multiple taxa can also foster our understanding of the variables influencing co–evolutionary interactions (Sorci et al. 1997; Lion and Gandon 2015), and the possible

fitness costs associated with different types of parasites (Salvaudon et al. 2005; Salvaudon et al. 2007). Finally, a greater knowledge on the type and nature of multi-species interaction could be used to inform on the potential conservation risks associated with moving endangered hosts around for translocation purposes (McCoy et al. 2005). In this study I examined patterns of variability and distribution in the Bahamas rock iguana *C. cyclura* and I compared these results to those obtained for its tick ectoparasites in the genus *Amblyomma*. One of the goals of this study was to elucidate mechanisms that regulate ectoparasite dispersal. My results support a vertical mechanism of dispersal across Bahamian islands, indicating a high level of specificity in this host parasite system. Moreover, these data, compounded with previous research already published, favor a scenario of historically low rates of admixture in *C. cychlura* populations on the Great Bahama Bank.

C. cychlura diversity and distribution

Results obtained using the *ND4* mtDNA marker in *C. cychlura* iguanas are in agreement with what had been described for this taxon (Malone et al. 2003). This analysis discovered two additional haplotypes in samples of *C. cychlura cychlura* collected on Andros Island, suggesting that the mtDNA variability in this taxon may be larger than previously thought (Fig. 3.2). I also described patterns of population relatedness that are inconsistent with currently recognized taxonomy. First, I found that the individuals of *C. c. figginsi* collected in the southern Exumas are genetically distinct from *C. c. figginsi* in the central Exumas. In fact, these *C. c. figginsi* populations are no more similar to each other than either is to *C. c. cychlura* on Andros (Fig. 3.2). Clearly this result is unexpected given the current taxonomy. However, previous work on *C.*

cychlura revealed unresolved phylogenetic relationships among populations in the Exumas (Malone et al. 2003). Specifically, these authors found that mtDNA sequences of *C. c. inornata* iguanas were nested with *C. c. figginsi* samples in an unresolved polytomy (Malone et al. 2003). When analyzing variation at 12 microsatellite markers greater details in these patterns of population structure become evident. Within the *C. c. figginsi* taxon I found two clearly identifiable genetic clusters (Fig. 3.5). The overall proportion of variance explained by genetic differences in these two clusters (F_{st} = 0.373) is one of the largest reported so far among populations within a single species of *Cyclura* iguanas (*C. c. cychlura* F_{st} = 0.117, Colosimo et al 2014; *C. carinata* F_{ct} = 0.202, Welch et al. in review; *C. c. inornata* F_{st} = 0.298, Aplasca et al. 2016; *C. cornuta* F_{st} = 0.154, Carreras De León 2015) suggesting a high degree of isolation between the central and southern Exuma Islands.

Tick diversity and distribution

Molecular data confirm what was anticipated based upon morphological analyses on ticks parasitizing iguanas in the Bahamas (Durden et al. 2015). I found that different populations of *C. cychlura* are parasitized by multiple species of ticks, and that the geographic isolation of these ectoparasites seems even more pronounced than that in their iguana hosts. The molecular data collected from ticks show the presence of at least two different taxa: *Amblyomma albopictum* (see "Results - Molecular identification, sequence variability and haplotype network" section for details), restricted to the central area of the Exuma (Sites ID5 and ID6) and across Andros, and *Amblyomma torrei* (see "Results - Molecular identification, sequence variability and haplotype network" section for details) found only in the southernmost cays of the Exuma Islands (Sites ID1, ID2 and ID3).

Iguanas on Site ID4 are also parasitized by *A. torrei*, a finding consistent with the fact that this population of iguanas was introduced on this island during a translocation project that used Leaf Cay as a source population, a small island located northeast of Norman's Pond Cay in the geographic vicinity of sites ID1, ID2 and ID3 (Knapp 2002). Previous work on ticks parasitizing Bahamian iguanas originally described the two taxa of ticks as *A. dissimile* and *A. torrei*, respectively, using only morphological characteristics (Durden and Knapp 2005). This taxonomic identification was then updated and the two tick taxa were re-classified as *A. albopictum* and *A. torrei* (Durden et al. 2015).

The genetic diversity of the analyzed ticks shows the presence of three well-defined geographic groupings consistent with that in their host's (Fig. 3.1, 3.2). Ticks parasitizing iguanas in the southern Exumas are clearly distinct from ticks elsewhere in the central Exuma and on Andros Island (16.27% sequence divergence). I identified a single haplotype from the 10 samples collected in this area. This finding is consistent with a recent bottleneck, perhaps, or this population of ticks has only recently established itself in the southern Exumas.

Ticks collected from the central Exuma and Andros Islands are all classified as *A. albopictum*. Sequence analysis revealed nine distinct haplotypes across 38 tick samples. Three of these haplotypes are restricted to the central Exumas (Sites ID5 and ID6) while the remaining six are only found among Andros Island populations. These data suggest that ≈ 91% of the total genetic variance found among analyzed samples can be attributed to the disjunct geographic distribution of these haplotypes ($\Phi_{ct} = 0.906$, $p < 0.01$).

The identification of geographically restricted haplotypes and the absence of shared haplotypes across The Bahamas suggest isolation for a prolonged period of time. Such isolation supports a recent vicariant event as a major driver of the current biodiversity pattern. Three lines of evidence from the tick data are in agreement with this scenario. First, the tick haplotype distribution on Andros Island is concordant with patterns of genetic structure described for their iguana by Colosimo et al. (2014). The two most distinct *COI* sequences identified, were collected on sites 10 and 17, and distinguished five and three SNPs, respectively, from the most abundant haplotype. These sites are separated from the majority of *C. c. cychlura* populations by two water channels that appear to be significant barriers to iguanas migration (Colosimo et al. 2014). Second, within the Exuma island chain, central and southern iguana populations are parasitized by different species of ticks, consistent with reptiles serving as the primary dispersal vector for these ectoparasites. Finally, ticks are not known to parasitize *C. c. inornata* populations in the northernmost islands in the Exumas. Combined, these findings suggest that ticks colonized these iguana populations after the vicariance event associated with rising sea levels, or that local tick population extinctions have occurred since vicariance. The limited distributions of ticks also support the original hypothesis that the iguanas largely disperse ticks vertically.

Host-Parasite structure, co-evolutionary potential and taxonomic considerations

The comparative analysis of population structure in a host-parasite system can provide valuable information about the potential for co-evolutionary interactions (McCoy et al. 2005). On one hand we can learn more about the interaction's spatial scale, while

disentangling how selection and gene flow contribute to the current patterns of variability (McCoy et al. 2003; McCoy et al. 2005).

Previous research analyzing species interactions have produced discordant results concerning the similarity in patterns of population structure, especially in host-parasite systems (Parker and Spoerke 1998; Althoff and Thompson 1999; Delmotte et al. 1999; Mutikainen and Koskela 2002; Burban and Petit 2003). Only with a high degree of specificity should species interactions result in correlated population structures between taxa (McCoy et al. 2005). Hence, these results support a vertical mechanism of dispersal in ticks parasitizing iguanas in The Bahamas due to the implied high degree of host specificity of *Amblyomma* ticks. *Amblyomma albopictum* is largely restricted to the Caribbean region (Cuba, Hispaniola, Swan Islands, Cayman Islands and the Bahamas). Some record exist for this species in Central America and Brazil, although reports from these latter locations remain questionable (Voltzit and Keirans 2003). The species is typically associated with iguanas (*Cyclura* spp., *Iguana* spp.) but it has also been sampled on other reptiles (*Leiocephalus* spp., *Alsophis cantheringerus* and *Epicrates angulifer*; Woods and Sergile 2001; Durden et al. 2015). *Amblyomma torrei* has a more restricted distribution (Cuba, Cayman Islands, Puerto Rico and The Bahamas). This species is also typically found associated with *Cyclura* spp. iguanas (Woods and Sergile 2001; Voltzit and Keirans 2003; Durden et al. 2015). The data presented here corroborate this specificity. No haplotypes in either the hosts or their parasites had a widespread distribution, and in all cases I was able to find concordance in patterns of molecular variability and distribution, indicating comparable levels of genetic structures.

The overall low variability detected across analyzed taxa and their distribution is consistent with a recent bottleneck event, possibly triggered by a rise in sea level at the end of the Wisconsinan glaciation. Alternatively, a similar pattern of low sequence variability could have been caused by an intense selective sweep fixing one or few haplotypes over a large geographic area (Maynard Smith and Haigh 1974; Rato et al. 2010). With the available data I cannot completely rule out this latter hypothesis. The geographic distribution of host and parasites suggests that different populations of iguanas may be exposed to different biotic pressures. Parasite load can vary markedly across populations of iguanas in The Bahamas and, although the possible fitness costs associated with the presence of ticks is not known (Durden and Knapp 2005; Durden et al. 2015), lab reared reptiles with heavy ectoparasite loads eventually die (Barnard and Durden 1999). Moreover, abiotic conditions could also favor differential selective pressures across iguana populations. Despite most Bahamian islands being classified as "equatorial with dry winter" in the Köppen-Geiger climate scale (Kottek et al. 2006), these islands are also characterized by a north-south precipitation and temperature gradient (Carew and Mylroie 2004) and the presence of multiple plant assemblages on different islands (Correll and Correll 1996). However, low genetic variability in *Cyclura cychlura* iguanas and other congeneric species is also observed at the nuclear level (Malone et al. 2000; Knapp and Malone 2003; Welch et al. 2004; Colosimo et al. 2014; Aplasca et al. 2016), making selection acting at the mtDNA level a less likely scenario to explain current patterns of diversity and distribution.

From a conservation perspective, these data suggest that a taxonomic revision of iguanas in the Bahamas, especially along the Exuma Island chain, may be advisable. Not

only do I document the presence of two geographically isolated mtDNA haplogroups within the *C. c. figginsi* lineage, but estimates of population differentiation based on microsatellite data clearly suggest that these two groups of iguanas have been isolated for a significant amount of time and currently cannot be treated as a single panmictic group (F_{st} = 0.373, p-val << 0.01). Furthermore, the identification of geographically isolated groups of ticks should caution conservationists in considering translocations as a viable strategy to restock natural populations of these iguanas or to reintroduce them in their former range. Co-introductions of hosts and their parasites could, in fact, expose naïve species of reptiles to new selective pressures possibly affecting their fitness (El-Rashidy and Boxshall 2009; Lymbery et al. 2014).

CHAPTER IV

AMBLYOMMA SPP. TICKS PARASITIZING NATURAL POPULATIONS OF NORTHERN BAHAMIAN ROCK IGUANA (*CYCLURA CYCHLURA*) ARE CARRIERS OF *RICKETTSIA TAMURAE* AND *R. BELLII*

Abstract

This is the first report of bacteria (*Rickettsia* spp.) isolated from two species of hard ticks (genus *Amblyomma*) parasitizing iguanas in the genus *Cyclura* from multiple island populations in The Bahamas. Of DNA isolated from 48 ticks collected on iguanas, 12 were positive for *Rickettsia*. I was able to consistently amplify a 463-base pair amplicon from the citrate synthase gene (*gltA*), using *Rickettsia* genus-specific primers. I identified three geographically isolated rickettsial strains. The first was isolated from the tick species *Amblyomma torrei*, parasitizing *Cyclura cychlura figginsi* inhabiting the southern Exuma Islands, The Bahamas. This genotype had high sequence similarity to the intracellular bacterium *Rickettsia tamurae* (Ident.: 99%; GenBank accession # KX550952). The second and third strains, distinguished by a single nucleotide polymorphism, were isolated from the tick species *Amblyomma albopictum* parasitizing *C. cychlura cychlura* on Andros Island, The Bahamas. These latter sequences were consistent with *R. bellii* (Ident.: 99% GenBank accession #s KX550953, KX550954). Although sampled iguanas appeared to have normal physical condition, the possible fitness costs associated with parasite load and the presence of bacteria within them is

unknown. The characterization of rickettsial strains within *Amblyomma* ticks parasitizing *Cyclura* iguanas in The Bahamas is significant given that *Rickettsia tamurae* is known to be a human pathogen. Moreover, their presence suggests that other intracellular bacteria may be present. Because these iguanas are targets of the illegal pet trade, their unsanctioned movements could potentially leave naïve and novel host populations at risk.

Introduction

Amblyomma is a large genus of hard ticks (Acari: Ixodidae) comprising more than 120 species distributed across five continents (Voltzit and Keirans 2003). Forty-five of these *Amblyomma* species are endemic to the Neotropic ecozone and are know to be associated preferentially with reptiles and amphibians (Voltzit and Keirans 2003). Among these, *A. albopictum, A. torrei* and a few others are ectoparasites known to be specialists of iguanas (Guglielmone et al. 2003; Durden et al. 2015). One iguana host includes the Northern Bahamian Rock Iguana, *Cyclura cychlura*. This endemic species is restricted to the Great Bahama Bank (Knapp et al. 2011), and current taxonomy recognizes three subspecies with disjunct distributions: *Cyclura cychlura cychlura* on Andros Island, *C. c. figginsi* from the central and southernmost Exuma Islands, and *C. c. inornata*, restricted to few islands in the northern Exuma Islands (Knapp and Malone 2003; Knapp et al. 2011). With the exception of *C. c. inornata,* all of these iguana populations are parasitized by *Amblyomma* spp. (Durden and Knapp 2005; Durden et al. 2015). Tick species parasitizing iguanas in The Bahamas seem to have a disjunct pattern of distribution similar to that found in their hosts (Durden and Knapp, 2005; Durden et al., 2015; Colosimo et al., in prep). Specifically, *A. torrei* has been recorded only from iguana populations (*C. c. figginsi*) in the southern Exuma Islands, while *A. albopictum*

has been recorded from iguana populations in the central Exumas (*C. c. figginsi*) and from Andros Island (*C. c. cychlura*).

Ticks are blood-feeding arthropods known to carry an incredible variety of organisms, including potential pathogens for their hosts. Vectors of zoonotic diseases prevalently parasitize different tick genera within the Ixodidae family. For example, the causative agent for Lyme disease (*Borrelia burgdorferi*) is mostly found in species from the genus *Ixodes* (Parola and Raoult 2001). Several *Rickettsia* spp. have been detected from *Amblyomma* ticks (Parola and Raoult 2001; Parola et al. 2005), and many are the cases of *Rickettsia* spp. identified from *Amblyomma* ticks parasitizing reptiles (Reeves et al. 2006; Miranda and Mattar 2014; Sumrandee et al. 2014; Kho et al. 2015). Hence, I hypothesized that ticks collected from *Cyclura cychlura* rock iguanas may also harbor *Rickettsia*. To date no published results confirm the presence of *Rickettsia* in ticks parasitizing iguanas in The Bahamas. Interest in gathering information on the possible presence of bacterial strains in this system stems from the conservation concerns associated with these iguanas. Translocation of iguanas has been used as a conservation mitigation strategy (Malone et al. 2003; Knapp 2004), while unsanctioned translocations also occur for dubious reasons (Aplasca et al. 2016). Thus, there is potential for introducing host-associated parasites to a naïve ecosystem. Moreover, in light of the interest associated with the illegal pet trade of iguanas (Durden and Knapp 2005; Smith 2014), information on the presence of rickettsiae could help prevent the spread of exotic disease agents outside the Caribbean.

Materials and Methods

Data Collection

Since 1997, all islands where *Cyclura cychlura* occur naturally have been visited as part of a comprehensive research program for the species (e.g., Aplasca et al., 2016; Colosimo et al., 2014; Durden & Knapp, 2005; Durden et al., 2015; Knapp & Alvarez-Clare, 2016; see Fig. 1 for sampling locations). While collecting morphometric data on iguanas, ticks were removed using forceps and stored in the field in 70% Et-OH. Ticks were identified to species level using morphological characters (Durden and Knapp 2005; Durden et al. 2015).

DNA extraction, sequencing and molecular identification

To maximize DNA yield and prevent the hard chitin scutum from hampering extraction, I followed the extraction protocol of Halos et al (2004). Briefly, specimens were first mechanically sheared using a scalpel in a lysis solution (NaCl 0.1M, Tris-HCl 0.21M, pH8 EDTA 0.05M, SDS 0.5%). The tissue was then homogenized in a proteinase-K solution (20 mg/ml) using a bead mill. Genomic DNA was extracted using the Maxwell–16™ Research instrument and proprietary chemistry (Promega, Madison, WI). Successful DNA extraction on tick genomic DNA was tested via Polymerase Chain Reaction (PCR) targeting the ≈ 700 base-pairs mtDNA-*COI* region of *Amblyomma* ticks. Species specific primers for PCR amplification of *A. albopictum* and *A. torrei* mtDNA were unavailable. I therefore used mtDNA *Amblyomma* sequences available through GenBank and the online tool PRIMER3 (Rozen and Skaletsky 1999) to design new primers that could target and amplify ≈ 650 base pairs of the *Cytochrome Oxidase 1* (*COI*) in *A. torrei* and *A. albopictum* (AmbCOI-F: 5’ GGT CAA CAA ATC ATA AAG

ATA TTG G 3'; AmbCOI-R: 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'). The PCR profile for amplification in tick samples was set with an initial denaturation of 5 min at 94 ˚C followed by 5 ramp-up cycles of 30 sec at 94 ˚C, 30 sec at the annealing temperature and 30 sec at 72 ˚C. In the ramp-up protocol, the annealing temperature was increased by 1 ˚C with each subsequent cycle. I set the initial annealing temperature at 45 ˚C. The remaining 30 cycles of the PCR were conducted with 30 sec at 94 ˚C, 30 sec at the annealing temperature of 48 ˚C, and 30 sec at 72 ˚C, with a final elongation phase of 7 min at 72 ˚C. Successful tick DNA extractions were tested specifically for the possible presence of *Rickettsia* spp.. I used primers targeting specific regions in the *ompA* gene (primer names: Rr190.70p, Rr190.602n), the *gltA* gene (primer names: RpCS.877p, RpCS.1258n and Cs-5, Cs-1273), and the *17-kDa* gene (primer names: 17kD-1, 17kD-2 and 17k-5, 17k-3), all three loci are commonly used for the detection of *Rickettsiae* in animal hosts, including reptiles (Regnery et al. 1991; Labruna et al. 2004a; Labruna et al. 2004b). Established primer sequences and PCR conditions followed those of Labruna et al. (2004a, 2004b) and Regnery et al. (1991). Briefly, one initial cycle at 94 ˚C for 5 min, 40 cycles with 30 sec at 94 ˚C for denaturation, 30 sec at 45 ˚C for annealing, 30 sec at 72 ˚C for elongation, and a final elongation period of 7 min at 72 ˚C. Successful PCR products were shipped to Arizona State University for purification and sequencing. This facility uses Big Dye V3.1 chemistry and an Applied Biosystems 3730*xl* DNA Analyzer Instrument (ABI, Foster City, CA) for sequencing. Shipped samples were sequenced with forward and reverse primers. DNA sequences were visualized and edited using SEQUENCHER v4.7 (© Gene Code Corporation). When possible forward and reverse amplicons were used to build consensus sequences.

Each mtDNA sequence obtained was first compared with the National Center for Biotechnology and Information (NCBI) nucleotide database for molecular identification using the Basic Local Alignment Search Tool – BLAST (Altschul et al. 1990), and optimizing the search for highly similar sequences (i.e., megablast). To better analyze the distribution pattern of *Rickettsia* spp. I plotted a phylogenetic network using the minimum spanning network algorithm implemented in POPART (Leigh and Bryant 2015).

Results and Discussion

I successfully isolated DNA from 49 ticks that were collected from islands representing the full range in which *Amblyomma* are known to parasitize *Cyclura cychlura* (Fig. 1). One of these individuals was discarded from downstream analyses due to contamination with human DNA. All remaining ticks were assayed for the presence of rickettsiae. The *ompA* gene failed to amplify product in any of these samples. I obtained mixed results when attempting to amplify the *17-kDa* gene, but I was able to consistently amplify two fragments of the *gltA* gene for a total of 463 base pairs in 12 samples. From these 12 samples, I obtained DNA sequences from three distinct strains (Fig. 4.1). These sequences have been deposited in the NCBI database (accession #'s KX550952-KX550954). Based on these data I inferred ≈ 25% prevalence of rickettsial infection, a result comparable, if not higher, to other reported values of rickettsial strains in *Amblyomma* ticks (Fritzen et al. 2011; Fitak et al. 2014).

The NCBI database comparison identified two different species of *Rickettsia* (Fig. 4.1) including *R. tamurae* at sampling sites 1, 2 and 3 in the southern Exumas (*C.c. figginsi* host; number of processed samples = 5; number of haplotypes = 1; 463 bp; Query

Cover: 100%; E-value: 0; Ident.: 99%), and *R. belli* from sampling sites across Andros (*C. c. cychlura* host; number of processed samples = 7; number of haplotypes = 2; 463 bp; Query Cover: 99%; E-value: 0; Ident.: 99%; BLAST results were similar for the two haplotypes).

Interestingly, the geographic structure of genetic variability in *Rickettsia* spp. is concordant with the distribution of the two tick species. Specifically, *R. tamurae* is only identified from three sampling locations in the southern Exumas, where its host *A. torrei* is present. *Rickettsia bellii* was only found associated with *A. albopictum* from Andros Island (Fig. 4.1; see also Figs. 3.1, and 3.3). I failed to amplify *R. bellii* sequence from *A. albopictum* collected on *C. c. figginsi* in the Central Exumas. At this time I am not able to speculate on the possibility that rickettsial infection may be identifiable directly from genomic DNA extracted from iguana's blood. Failure in retrieving these bacteria directly from blood of a reptile host is not uncommon (Stenos et al. 2003; De Sousa et al. 2012), though it has been demonstrated more feasible from other tissues (De Sousa et al. 2012).

Although sampled iguanas appeared to have normal physical condition, the possible fitness costs associated with parasite load and the presence of bacteria within them is unknown. The characterization of rickettsial strains within *Amblyomma* ticks parasitizing *Cyclura* iguanas in The Bahamas should be considered when planning translocations, and is significant because *R. tamurae* is known to be a human pathogen. Indeed, while *R. bellii* has never been associated with any Spotted Fever Group or any Typhus Group (Ogata et al. 2006), *R. tamurae* has been described as the agent of spotted fever infection in Japan (Fournier et al. 2006; Imaoka et al. 2011; Sentausa et al. 2014). Moreover, its presence suggests that other intracellular bacteria may be found. Because

these iguanas are targets of the illegal pet trade (Smith 2014), their unsanctioned movements could potentially put naïve and novel host populations at risk, and may implicate reptile ectoparasites as a potential reservoir of zoonotic disease. Thus, further investigations are warranted.

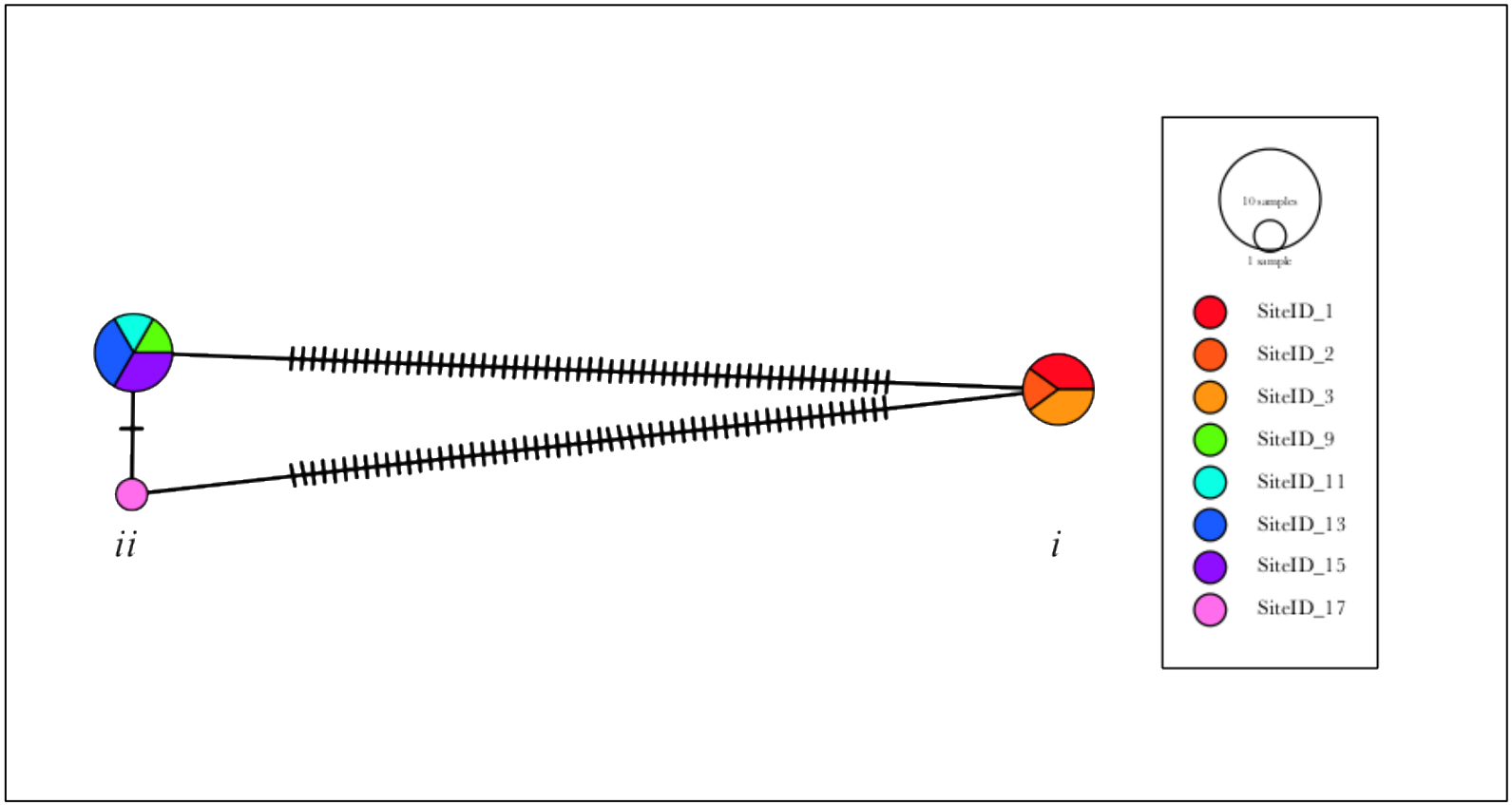

Figure 4.1 *Rickettsia* spp. *gltA* gene minimum spanning network

Minimum spanning network built using 463 bp fragments of the *gltA* gene for *Rickettsia* spp. Colors used in sites identification are the same used in Fig. 3.1 (see also Fig. 3.2 and Fig. 3.3 to compare diversity and distribution in iguanas and their tick parasites). Haplogroup *i* was identified as *R. tamurae,* and both haplotypes in *ii* were identified as *R. bellii*.

CHAPTER V

INBREEDING DEPRESSION AND ITS CORRELATION WITH POPULATION SIZE AND DYNAMICS IN A NATURAL SYSTEM: INSIGHTS FROM *CYCLURA CARINATA* IGUANAS

Abstract

Long-term persistence of small and isolated populations has often been associated with the inevitable and progressive decay of their genetic variability. Yet, many natural populations of threatened species seem to thrive despite their reduced size and isolation, defying the theoretical expectation of inbreeding depression. In such cases demographic history and the architecture of the genetic load are considered deterministic factors regulating populations' susceptibility to inbreeding depression and local extinction, especially under regimes of random mating. In this study I investigate the mechanisms favoring heterozygous genotypes in a small isolated and natural population of the critically endangered *Cyclura carinata* iguanas in the Turks and Caicos Islands. I tested the specific hypothesis that soft-selection, the component of natural selection associated with competition, is largely responsible for the expression of inbreeding depression. If heterozygote excess reflects a competitive advantage for outbred individuals then small populations should be relatively robust. On the contrary, if observed attrition results from hard-selection, i.e., the expression of those genetic factors that, when combined, are responsible for individuals' deaths (lethal equivalents), then these small populations are

more likely ephemeral, requiring relatively frequent recolonization. Using polymorphic microsatellites I found indirect evidence suggesting that small natural populations can maintain significant levels of segregating genetic load. Under regimes of random mating this load can be exposed to natural selection through increased competition, as populations approach carrying capacity. However, quantification of the relative roles of hard and soft-selection affecting the population dynamics of these iguanas was not entirely feasible. Hence, the genetic resilience of small isolated populations of this species remains unclear.

Introduction

Many natural populations, especially in threatened species, are small and isolated. In the majority of cases their persistence is dependent on anthropogenic activities affecting them (Pimm et al. 2014). Even populations sheltered from human disturbances can go locally extinct due to environmental stochasticity and genetic problems (Lande 1988; Frankham 2005). Indeed, population size and structure play critical roles shaping their dynamics and evolutionary trajectories.

Theory predicts that geographically isolated and small populations should be affected by the predominant effect of random genetic drift (Hartl and Clark 1997). Because of their reduced size, individuals within populations are also more likely to mate with related partners, even under regimes of random mating. Finally, even unrelated individuals are more likely to share identical by state alleles due to the effect of genetic drift. In combination these processes contribute to the progressive loss of genetic variation at the population level, the loss of individual variability due to the overall increase in homozygosity, and a potential fitness loss (Mitton 1993).

The correlation between fitness and genetic variability is well documented. A number of studies have now positively correlated heterozygosity with different fitness components such as viability (Zouros et al. 1983; Mitton and Jeffers 1989), growth rate (Pierce and Mitton 1982; Koehn and Gaffney 1984), physiological efficiency (Garton 1984; Danzmann et al. 1987), and fecundity (Rodhouse et al. 1986; Gajardo and Beardmore 1989). More recently, numerous researchers have demonstrated the fitness advantages of heterozygosity in populations facing abrupt climate and environmental changes (Armbruster and Reed 2005; Gregory et al. 2006; Fox and Reed 2011; Forcada and Hoffman 2014; Vranckx et al. 2014). Finally, the genetics of fitness traits and the mechanisms underlying their reduction due to increased homozygosity are becoming increasingly clear. Studies on inbreeding depression, the reduced fitness in offspring of related mates, and heterosis, the burst in fitness triggered by intercrossing isolated lines, suggest dominance and overdominance as the most likely mechanisms to explain the generally higher fitness of heterozygous genotypes over homozygous ones (Sommer 2005; Charlesworth and Willis 2009).

Yet, susceptibility to inbreeding depression varies markedly among species and some taxa seem unaffected by it (Laws and Jamieson 2011). Many threatened natural populations, despite their reduced size, isolation, and low genetic variation, can persist seemingly unaffected by phenotypic costs usually associated with inbreeding depression, and can maintain a high density of individuals (Keane et al. 1996; Keller and Arcese 1998; Hansson et al. 2007; Nichols et al. 2014). In such cases demographic history and the architecture of the genetic load are considered discriminating factors regulating

populations' susceptibility to fitness loss, and possibly extinction (Glémin et al. 2003; Glémin 2003; Chapman et al. 2009).

The term genetic load, introduced in the mid twentieth century (Haldane 1949), actually refers to a biological abstraction. Hypothesizing the existence of a "best" possible genotype within a specific population, any genotype differing from this optimum should display reduced fitness proportional to its genetic load. In nature, and particularly in recombining species (Dobzhansky 1970; Ridley 2003), there is no one best genotype, but at any given time "better" genotypes are expected to have higher fitness compared to those with a higher genetic load (O'Grady et al. 2006; Fox et al. 2008; Agrawal 2010; Ho and Agrawal 2012). To better understand how genetic load varies according to population size and history it is useful to further divide it into *drift load* and *segregating load* (Glémin 2003; Chapman et al. 2009). The former describes the proportion of the genetic load associated with deleterious mutations, arguably of small effect, that are fixed in a population, while the latter refers to those deleterious mutations still segregating in a population (Haag and Roze 2007). In large, randomly mating populations any significant deviation from the random assortment of gametes results in increased homozygosity and, generally, inbreeding depression. In other words, due to random mating and selection acting against inbreeding, large populations are expected to maintain low levels of fixed drift load while harboring substantial segregating load in the form of deleterious and recessive mutations because these are not expressed in heterozygotes (Lohr and Haag 2015). Inversely, populations that stay small and isolated for prolonged periods of time are expected to have purged a large proportion of strongly deleterious and lethal segregating mutations while increasing the overall proportion of drift load (Willi et al.

2006; Agrawal and Whitlock 2012; Lohr and Haag 2015). These theoretical expectations have recently been observed in natural populations of *Daphnia magna* where genetic load, inbreeding depression, and heterosis vary significantly with population size (Lohr and Haag 2015). The same study also indicates that in long standing small populations, where drift load is expected to be high, inbreeding is unlikely to further depress individuals' fitness due to the overall lack of segregating deleterious mutations (Lohr and Haag 2015).

Nonetheless, many studies have now shown that inbreeding depression is widespread among taxa, even within small populations (Chapman et al. 2009; Szulkin et al. 2010; Neaves et al. 2015). However, the detection of inbreeding depression in small longstanding wild populations is complex, and the mechanisms contributing to its expression are unclear (Hedrick 1994; Hansson and Westerberg 2002; Hansson and Westerberg 2008; Szulkin et al. 2010). One hypothesis is that intense competition could help expose more of the segregating load to selection, thereby contributing to the elimination of harmful mutations in small populations (Laffafian et al. 2010; Ho and Agrawal 2012; Lohr and Haag 2015).

Wallace and Christiansen introduced a model of density dependent selective pressure according to which reproductive success and recruitment to adulthood are hampered by the relative fitness and density of specific genotypes. They called this model *soft-selection* (Wallace 1968; Christiansen 1975; Wallace 1975). In other words, reduced viability or fertility is dependent on the fitness of other local genotypes. This model is alternative, but not mutually exclusive, to the *hard-selection* model, where viability is dependent on the expression of one lethal equivalency (Agrawal 2010). A lethal

equivalent is represented by those genetic factors that, when combined together and exposed to natural selection, are responsible for individual deaths (Allendorf et al. 2012).

Meagher and colleagues (2000) documented the cost of competition to the reproductive success of inbred wild mice reared in semi-natural conditions. Specifically, the authors provided evidence that inbred males sired considerably fewer offspring than outbred individuals when competition was high (Meagher et al. 2000). Consistent with the competition hypothesis, Ho & Agrawal (2012) found some evidence to support the idea that intraspecific competition, measured as density of individuals in an experimental population of *Drosophila* spp., makes selection softer (Ho and Agrawal 2012). The soft and hard-selection models and their influence on the evolution of natural populations are, arguably, consistent with the distinction between drift load and segregating load. Small, natural populations that have been accumulating substantial fixed load are more likely to be influenced by the soft-selection mechanism. This theoretical model provides a framework to investigate the relative importance of competition to the expression of inbreeding depression in small, wild and isolated populations.

Distinguishing between hard and soft-selection is of great interest, especially in species and populations threatened with extinction and for which inbreeding depression has been reported. If competition is responsible for exposing the genetic load to selection, then populations are potentially robust and not in imminent risk of extinction. In contrast, if inbreeding depression reflects the expression of lethal genotypes, then small populations may be ephemeral and should be targeted with specific and immediate conservation programs. Thus important questions to ask are: what proportion of population attrition is attributable to soft-selection in natural populations? Where is the

threshold beyond which the expression of lethal equivalents should lead a population to extinction?

Cyclura carinata are critically endangered iguanas endemic to the Turks and Caicos Islands (TCI; Gerber 2004). This species offers a unique opportunity to test the relative importance of hard and soft-selection in the wild. First, previous research provided strong evidence that survivorship and dominance in at least one population of these iguanas are influenced by inbreeding depression (Berk 2013; see below). Second, the natural variation in population size across islands in the TCI offers the unique opportunity to compare how different demographic characteristics, such as population size, may be affecting recruitment and the expression of inbreeding depression. Third, because of their Critically Endangered status, some *C. carinata* populations have been monitored for over 20 years, and a fairly complete dataset of individuals, and detailed demographic characteristics for many populations already exists. Finally, several translocation experiments were initiated in 2002 and newly established populations have been monitored since the translocation events. Translocated populations, hence, represent a further opportunity to investigate the relative role of hard and soft selection regulating population dynamics (see Materials and Methods for more details on the species, sampling sites and translocations).

Previous research on *C. carinata* iguanas on Little Water Cay (LWC from hereafter; Fig. 5.1) indicates that this population might be suffering from inbreeding depression (Berk 2013). Specifically, the author found adult iguanas to be significantly more heterozygous than hatchlings on LWC, suggesting differential viability penalizing more homozygous individuals. It was estimated that the intensity of selection acting on

homozygous genotypes is responsible for ≈ 60% of the attrition in this population (Berk 2013). Moreover, among adults a positive and significant correlation between standardized heterozygosity and fitness proxies, i.e., Heterozygosity Fitness Correlations (HFCs hereafter), was found (Berk 2013). Interestingly, the author failed to identify any significant departure from random mating expectations, indicating that another mechanism besides inbreeding, perhaps associated with individuals' competition and the soft selection hypothesis, may be responsible for the documented patterns.

In the present study I evaluate the hypothesis that soft-selection is the driving mechanism in generating patterns of inbreeding depression in *C. carinata* iguanas on LWC. To test this hypothesis I compare evidence of inbreeding depression from LWC with that estimated for iguanas on Big Ambergris Cay (BAC hereafter), a large population assumed at or near carrying capacity. I further compare the genetic characteristics of these two populations with that of four recently translocated populations of iguanas: Bay Cay (BC hereafter) and Middle Cay (MC hereafter), sourced with adults translocated from LWC in 2002, and French Cay (FC hereafter) and Six Hills Cay (SHC hereafter), sourced with adults translocated from BAC in 2002 and 2003, respectively (see Material and Methods for details). Evidence consistent with the soft-selection hypothesis would come from reduced, if any, evidence of inbreeding depression in the larger population of BAC, as well as in the translocated populations that are yet to reach carrying capacity and for which competition is assumed to be lower.

Materials and Methods

Study System

Cyclura carinata is endemic to the TCI, 900 km southeast of Florida and 150 km north of Hispaniola (Fig. 5.1). The archipelago has over 200 islands, ranging in size from less than 1 ha up to 13,500 ha. All islands are low-lying, composed of limestone and sand, with subtropical dry-forest and scrub habitats. The only population of *C. carinata* residing outside of the TCI is found on Booby Cay, a small island on the east side of the Mayaguana Bank (Bryan et al. 2007; Welch et al. in review). The IUCN Red List of Threatened Species lists the species as critically endangered (Gerber 2004), and CITES lists it in Appendix I (Gerber and Pagni 2012). The primary threats to Turks and Caicos Rock Iguanas are feral mammals (Gerber and Iverson 2000). Because of their smaller size, relative to most other *Cyclura* species, and their naïve behavior towards introduced mammalian predators, populations of *C. carinata* iguanas are particularly sensitive to extirpation (Iverson 1978; Iverson 1979). Thirteen island populations went extinct in the second part of the 20th century, all involving introduced mammals (Gerber and Iverson 2000; Gerber 2004). The presence of cats, dogs, goats, cattle, rats and donkeys are strongly correlated with the species decline (Gerber and Iverson 2000; Gerber 2004).

Sampling sites

Samples used in this project were collected from two large natural populations (LWC and BAC), and four populations recently established by translocation (BC, MC, FC and SHC) using founders from LWC and BAC (see below for more details).

Little Water Cay has a surface of ≈ 100 ha with approximately 70% of this surface constituting desirable habitat for *C. carinata* iguanas. The population size (N) on

LWC is estimated at ≈ 2000 individuals (Iverson 1979; Welch et al. 2004; Gerber 2007). From late March till early May, i.e., during the mating season, *C. carinata* iguanas present an activity peak. Males increasingly become interested in females and fiercely compete for access to areas with highest female density (Iverson 1979). Females compete for access to nesting grounds. After laying their clutch in May/June, threatening or aggressive nest defense displays towards potential intruders have been recorded (Iverson 1979). Mate selection has been shown to have a very high fitness cost in other iguana species (Vitousek et al. 2007) and dense populations may experience an especially high fitness cost.

Genetic data (Welch et al. 2004; Bryan et al. 2007; Welch et al. in review) compounded with documented changes in sea level (Lighty et al. 1982; Fairbanks 1989; Toscano and Macintyre 2003) suggest that, assuming a generation time of 14 years as suggested in Iverson (1979) and Gerber (2004), some populations in the TCI have been isolated for at least 1000 generations. Although this might be true, LWC has recently been colonized by cats from a nearby larger island due to a storm induced sandbar now connecting LWC with an adjacent cay (Gerber 2007). Sandbar connections have likely been formed multiple times over millennia and their impact on the overall genetic homogeneity of iguana populations is hard to assess. A recent analysis conducted at both mitochondrial and nuclear DNA suggests that these sandbar connections are likely to facilitate gene flow only among geographically close populations (Welch et al., in review). Cats on LWC were first sighted in the year 2000 (Gerber 2007). Given the threat represented by these predators, the presence of rats, and the relative robustness and large iguana population on the island, LWC was selected in 2002 as a source population for a

translocation project. Fifty-eight and 18 adults, in equal sex ratio, were collected in January 2002 and translocated to BC and MC (4 ha and 1 ha respectively). Five months after relocating adults on recipient islands, female iguanas were already laying eggs, and the first hatchlings were observed in August 2002 (Gerber 2007). Populations on BC and MC have been systematically visited since the translocation event and iguanas hatched on these cays have been sampled in 2002, 2003, 2004, 2005, 2007, 2009 and 2015. Upon capture by hand or noose, iguanas were individually marked with colored beads (Rodda et al. 1988). Morphological measurements such as body mass (BM_g), snout vent length (SVL_{mm}), and head width (HW_{mm}) were collected for each specimen using pesola scales, calipers, and rulers. Additionally, animals were sexed using cloacal probes (Dellinger and Hegel 1990). For each individual, 0.2 – 1.0 ml of blood was drawn via the ventral coccygeal vein using heparinized syringes. Blood samples were stored in cryotubes containing an equal or greater amount of SDS lysis buffer (0.1M Tris-HCl pH 8.0, 0.1M EDTA, 0.01M NaCl, 0.5% SDS) at ambient temperature prior to long term storage at -80 ºC. The sampling procedure was the same across all islands.

Big Ambergris Cay is about 400 ha, with N estimated at ≈ 15,000 individuals (Gerber 2004; Welch et al. 2004; Gerber 2007). This island is located on the opposite side of the Caicos Bank from LWC with ≈ 85 Km of open water separating them (see Fig. 5.1). Previous genetic analyses showed that LWC and BAC have independent dynamics with virtually no gene flow (Welch et al in review). Privately owned, BAC is currently undergoing development which started in 1998 (Gerber 2007). Due to this threat and its large population size BAC too was selected as a source for a translocation, and in 2002 and 2003 a total of 144 individuals were translocated to the recipient islands

of FC (12 ha) and SHC (4 ha), respectively. A total of 175 adults from BAC were sampled between 2002 and 2003 and analyzed in this study. This sample includes the 82 adults that were translocated to FC in January of 2002, and the 62 adult individuals that were moved to SHC in February of 2003 (Figure 5.1). The translocated populations on FC and SHC have been systematically visited since being founded, and iguanas hatched on these cays have been sampled in 2002, 2003, 2004, 2005, 2007, 2009 and 2015.

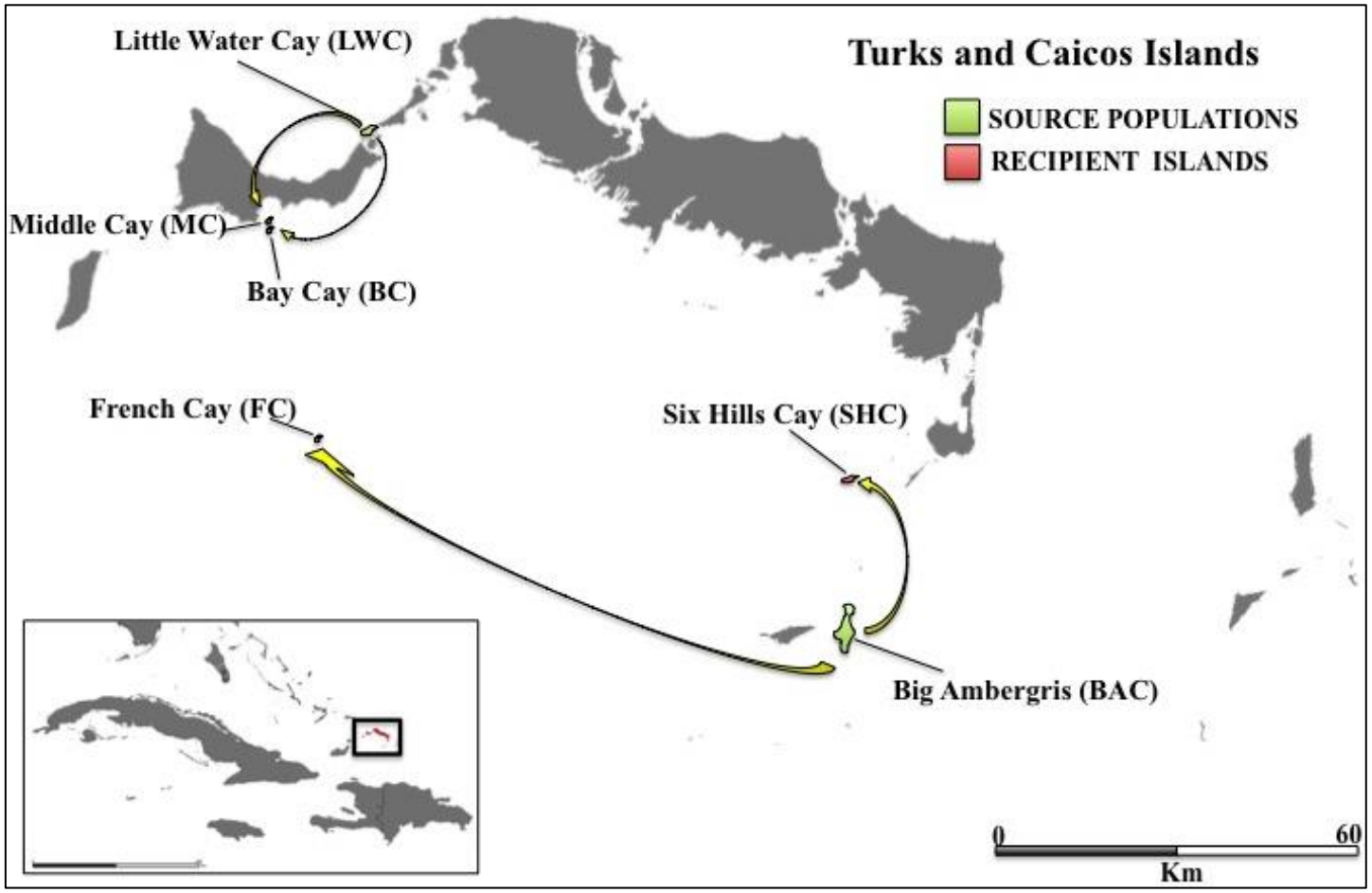

Figure 5.1 Map of the Turks and Caicos

Insert: Geographic location of the Turks and Caicos Islands (in red within square) in the Atlantic Ocean. **Main**: Map of the Turks and Caicos Islands. The 2 populations used as source for translocation (LWC and BAC) are highlighted in green. The 4 recipient islands (MC, BC, SHC and FC) are shown in red.

DNA Extraction and Marker Amplification

Approximately 20 µl of blood have been used to extract genomic DNA using a Promega™-Maxwel-16 machine and proprietary chemistry (Promega, Madison, WI). Polymorphic microsatellite loci were used to calculate year-by-year diversity indexes and to estimate correlations between genotype and morphological features known to be good fitness proxies in iguanas (Iverson 1979; Christian and Waldschmidt 1984; Alberts et al. 2002; Perry and Garland 2002). Although researchers have raised questions about the actual number of markers necessary to detect significant genotype-fitness correlation a recent case study demonstrated that as little as 11 loci can produce estimates of HFCs as accurate as large panels of SNPs (Forstmeier et al. 2012; Miller et al. 2014; Miller and Coltman 2014). I focused my attention on 24 microsatellites that have been used in previous studies of *C. carinata* and demonstrated to be polymorphic (Welch et al. 2004; Welch et al. 2011; Welch et al. in review; Berk 2013; Tab. 5.2).

Two or three primer PCR (Schuelke 2000) was performed in 10 µL volumes with ≈ 10 ng DNA, 2 mM $MgCl_2$, 30 mM tricine (pH 8.4) KOH, 50 mM KCl, 100 µM of each dNTP, 200 nM of reverse primer and M-13 (CACGACGTTGTAAAACGAC) forward primer labeled with fluorescent dye (HEX, NED, FAM, VIC or PET), between 40 and 150 nM forward primer with the M-13 extension, and 0.4 U of Taq DNA polymerase. Table 4.2 shows loci characteristics and annealing temperatures used during PCR. For some loci touchdown PCR (Don et al. 1991) profiles were set with an initial denaturation period of 5 min at 94 ºC followed by 10 touchdown cycles with 30 sec at 94 ºC, 30 sec at annealing temperature and 30 sec at 72 ºC. In touchdown protocols the initial annealing temperature is 10 ºC above the final annealing temperature. In each successive PCR cycle

the annealing temperature drops by 1 ºC. The remaining 25 cycles had thermal cycling profiles of 30 sec at 94 ºC, 30 sec at 52 ºC, and 30 sec at 72 ºC. A final elongation phase of 7 min at 72 ºC completed the PCR cycle profiles. Fragment analysis was performed on an Applied Biosystems 3730*xl* DNA Analyzer Instrument (ABI, Foster City, CA) at the Arizona State University DNA Laboratory using LIZ-500 as size standard (GeneScan – 500 LIZ Size Standard -Applied Biosystems). Genotypes were manually scored using PEAK SCANNER version 1.0 (Applied Biosystems).

Data Analysis

Molecular Diversity

Number of alleles and their respective frequencies were estimated for each marker using a function developed in R (Appendix C.1 for R-code). Null alleles, large allele dropout and/or allele misclassifications are potential sources of bias while genotyping individuals, and tend to reduce within population diversity (Chapuis and Estoup 2007). Although previously used and assessed in another work with *C. carinata* (Berk 2013; Welch et al., 2011; Welch et al. in review), I estimated the frequency of null alleles at every locus using the EM algorithm by Dempster, Laird & Rubin (1977) within the software GENPOP v4.2 (Raymond and Rousset 1995; Rousset 2008). Such estimates were calculated on adult samples from the source population of LWC. I further used ARLEQUIN v3.5 (Excoffier et al. 2010) to calculate locus by locus diversity indexes such as expected and observed heterozygosity (H_e and H_o).

Table 5.1 Molecular marker information for loci used in this study.

Name	Reference	Tag	T ºC	Size Range	N_a	H_o	H_e
CIDK113	(Welch et al. 2011)	VIC	52	338-348	4	0.476	0.423
CIDK144	(Welch et al. 2011)	VIC	54	198-207	3	0.189	0.199
CIDK163	(Welch et al. 2011)	FAM	52	343-353	4	0.393	0.361
CIDK177	(Welch et al. 2011)	FAM	52	240-247	4	0.065	0.071
CIDK184	(Welch et al. 2011)	NED	53	320-323	4	0.371	0.370
Z151	(An et al. 2004)	NED	52	209-221	3	0.377	0.394
D1	(Lau et al. 2009)	VIC	52	183-203	4	0.431	0.471
Z65	(An et al. 2004)	VIC	52	169-175	4	0.405	0.401
Z106	(An et al. 2004)	PET	52	173-177	3	0.058	0.056
D9	(Lau et al. 2009)	VIC	td52	132-153	5	0.650	0.705
D11	(Lau et al. 2009)	FAM	54	157-173	4	0.429	0.433
D107	(Lau et al. 2009)	VIC	52	265-281	4	0.291	0.315
D110	(Lau et al. 2009)	FAM	52	215-243	5	0.397	0.418
D111	(Lau et al. 2009)	VIC	50	167-207	6	0.632	0.646
D130	(Lau et al. 2009)	FAM	td52	280-288	3	0.388	0.346
D137	(Lau et al. 2009)	VIC	54	221-242	5	0.686	0.651
D140	(Lau et al. 2009)	VIC	52	234-254	6	0.449	0.451
C6	(Lau et al. 2009)	FAM	52	131-139	2	0.565	0.453
C103	(Lau et al. 2009)	VIC	52	206-210	2	0.553	0.450
C113	(Lau et al. 2009)	VIC	td52	204-225	5	0.600	0.621
CCSTE02	(Rosas et al. 2008)	VIC	52	277-293	4	0.479	0.470
CIDK101	(Welch et al. 2011)	FAM	57	261-265	3	0.317	0.427
CIDK109	(Welch et al. 2011)	VIC	57	360-363	2	0.008	0.008
F478	(An et al. 2004)	NED	54	203-209	4	0.265	0.287

Name, reference, colored tag used (Tag), annealing temperature (T ºC), size range, number of alleles (N_a) observed heterozygosity and its standard error (H_o) and expected heterozygosity and its standard error (H_e). Summary statistics are based on the total number of sampled iguanas.

LWC Population Dynamics

One of the main assumptions in this study is that the population on LWC is currently at carrying capacity and, hence, adult individuals are experiencing intense competition. Along with this assumption, I anticipated that population size and density on LWC have been stable for many generations. If this holds true, I can expect an equal probability for molecular markers to present heterozygosity excess or deficit (Piry et al. 1999). I used polymorphic microsatellites to estimate significant differences in the expected and the observed number of loci showing a heterozygosity excess or deficit with the program BOTTLENECK v1.2.02 (Piry et al. 1999). The program computes observed and expected values of heterozygosity assuming the population is at mutation-drift equilibrium. Two alternative models of microsatellite evolution are considered for this calculation: the Infinite Allele Model–IAM (Maruyama and Fuerst 1985) and the Stepwise Mutation Model–SMM (Cornuet and Luikart 1996). Significant variations from expected genotypic frequencies are calculated using a Wilcoxon signed rank test and by comparing the allele frequency distribution with the L-shape distribution expected under equilibrium (Luikart and Cornuet 1998; Piry et al. 1999).

Population size and Heterozygosity Fitness Correlations

To investigate how population size may contribute in generating heterozygosity fitness correlations I compared patterns of HFCs from LWC with those in the larger population of iguanas on BAC. Two mechanistic explanations can be invoked to explain patterns of HFCs: overdominance (the direct positive effect of analyzed markers on individuals' fitness), and associative overdominance (the fitness variation among

genotypes generated by the statistical association between neutral loci, such as microsatellites, and loci directly under selection; Szulkin et al. 2010). Associative overdominance, also dubbed local effect, is the most likely mechanisms to explain patterns of HFCs detected using microsatellites, since these markers are often regarded as non-coding polymorphic loci (David et al. 1995; David 1998). Statistically, the number of markers linked to a particular locus is considerably smaller than the unlinked ones. Therefore, only specific population dynamics, such as reduced population size, population admixture, and bottlenecks, are likely to generate HFCs due to local effect (Szulkin et al. 2010). I first estimated effective population size (N_e) on both LWC and BAC. Effective population size is defined as the number of individuals an idealized population, sensu Wright-Fisher (Crow 2010), needs in order to maintain the same level of genetic variability of the population under investigation. Estimates of this quantity can be inferred using individual genotypes and the software N_E-ESTIMATOR v2 (Do et al. 2014). This program takes advantage of single sampling events, multi-locus genotypes, and three different algorithms to estimate N_e: the Linkage Disequilibrium, or LD method (Waples and Do 2008); the Heterozygous Excess, or Het_{ex} method (Pudovkin et al. 1996; Zhdanova and Pudovkin 2008; Pudovkin et al. 2010); and the Co-ancestry method (Nomura 2008). The LD method is based on the Burrow's Δ estimates of LD, which can account for small sample sizes and alleles with low frequency to produce a correlation coefficient for each locus and allele in the sample. The correlation coefficients are then used to estimates N_e values (Waples and Do 2008). The Het_{ex} is based on the excess heterozygous offspring expected from the mating between parents with differences in allele frequencies (Pudovkin et al. 1996). The last method is based on the estimates of a

molecular co-ancestry parameter. This parameter estimates the effective number of breeders by looking at the average probability that alleles at loci in two individuals at generation *t* are coming from the same individual in generation *t-1* (Nomura 2008). For each methodology, the program allows to filter out of the analysis loci with allele frequencies lower than a certain threshold. We used the three default values of 0.05, 0.02 and 0.01 to run the estimates of population size.

Heterozygosity fitness correlations are widespread in nature, although the proportion of variance in fitness traits explained by multi locus heterozygosity (MLH) is typically low, ranging from values of 0.07% to 3.3% (Balloux et al. 2004; Slate et al. 2004; Chapman et al. 2009; Szulkin et al. 2010). Significant correlations are more likely to be detected when sample sizes and population context are favorable (Szulkin et al. 2010). I used genotyped adults collected on BAC to calculate three different heterozygosity indexes: StHe–standardized heterozygosity (Coltman et al. 1999), HL–homozygosity by loci (Aparicio et al. 2006), and IR–internal relatedness (Amos et al. 2001). These three measures account for a variety of bias that could affect individuals' multi-locus heterozygosity estimates. For example, StHe takes into account biases introduced by missing genotypes at certain loci and is equal to: StHe = proportion of heterozygous typed loci/mean heterozygosity of typed loci (Coltman et al. 1999). The HL index accounts for locus specific allele variability in its contribution to estimates of homozygosity: $HL = (\sum E_h)/(\sum E_h + \sum E_j)$, with E_h and E_j being the expected heterozygosity at loci for which an individual is homozygous ($_h$) or heterozygous ($_j$) (Aparicio et al. 2006). Finally, the IR index is an estimate of heterozygosity that weighs the typing of individuals using the relative frequency of alleles at different loci: $IR = (2H-\sum f_i)/(2N-$

$\sum f_i$), with H being the number of homozygous loci, N the total number of loci, and f the frequency of the i-th allele in the genotype (Amos et al. 2001). All these measures have been used in the vast majority of HFCs studies as well as in previous studies specifically involving *C. carinata* iguanas, making further comparisons easier to do. I used the R package Rhh (Alho et al. 2010) to calculate these three indexes. Morphometric values (body mass in grams, BM_g; snout-vent length in millimeters SVL_{mm}; head-width in millimeters HW_{mm}) were then regressed on the predictor variables, i.e., heterozygosity estimates. To account for natural male-female sexual dimorphism I standardized morphometric values calculating their z-scores (Appendix C.2 for R-code), forcing a mean of zero and a standard deviation of one (Sokal and Rohlf 1995; see also Berk 2013).

Population dynamics on translocated islands

Population size is an important factor in regulating the loss of genetic variability through the action of genetic drift. Translocated adult iguanas became reproductively active a few months after the translocation event (Gerber 2007). Rather than the census population size, differences in N_e are expected to influence the strength of genetic drift and the expression of inbreeding depression (Crow and Kimura 1970). Hence, estimating a parameter such as N_e is important for conservation practices and inferences of local evolutionary dynamics. To estimate contemporary N_e in the translocated cays I used N_E-ESTIMATOR v2 (Do et al. 2014).

According to the original hypothesis, as population size increases towards carrying capacity we can expect competition to start affecting individual fitness by favoring individuals with less of the segregating load expressed, i.e., with greater

heterozygosity. To test this hypothesis I measured levels of heterozygosity within the founder individuals translocated to BC, MC, FC and SHC and compared those values with that of individuals born and collected on the translocated islands in the following years. Comparing excess or lack of heterozygous genotypes in individuals born after the translocation event should help understand the relative impact of hard and soft-selection on population fitness. If hard-selection is affecting recruitment, then we could expect heterozygous genotypes to be immediately favored. On the contrary, if soft-selection is responsible for patterns of heterozygous excess one should expect such an effect to manifest later on, as the population reaches carrying capacity.

I estimated the relative excess of homozygosity and/or heterozygosity by calculating locus specific and average F_{is} values and their significance in GENPOP v4.2 (Raymond and Rousset 1995; Rousset 2008). I set the program to run 50000 dememorization steps, 100 batches, and 10000 independent iterations. I used the *shapiro.test* function in R to test for normality of F_{is} values and I then compared significant differences in mean F_{is} values across all time points. I specifically focused on differences between founders and samples collected on BC and MC across years using an ANOVA and a Tukey-HSD test.

Mating dynamics

One of the primary causes of inbreeding depression is, indeed, inbreeding or the non random association of gametes at reproduction (Charlesworth and Willis 2009). Mating dynamics in *C. carinata* on LWC, as suggested in Berk (2013), seem to indicate the independent assortment of gametes. In this study I investigate mating dynamics in source as well in translocated populations looking for significant excess or lack of

heterozygous genotypes using the F_{is} statistic as a proxy for genomic inbreeding (David et al. 2007). F_{is} estimates can sometime be biased upward due to genotyping mistakes or the presence of null alleles. I therefore paired such estimates with the information provided by the g_2 statistic developed by David and colleagues and implemented in the program RMES (David et al. 2007). This method, rather than looking at average differences between expected and observed values in heterozygosity, estimates the relative excess of double heterozygotes across all loci, or identity disequilibrium (Weir and Cockerham 1973; David et al. 2007).

Results

Number of genotyped individuals

All islands, including the source populations, have been visited since the original translocation producing a dataset of marked and recaptured individuals spanning a time frame of thirteen years. Samples were collected during every visit. Table 5.2 shows a summary of the total number of captured individuals on the translocated islands while Figure 5.2 gives the distribution of newly captured iguanas by year on every translocated island.

A total of 124 individuals from LWC, including the 58 adults translocated to BC in 2002, the 18 individuals translocated to MC in 2002, and an additional 48 adults resident on LWC collected in 2002/2003, were genotyped at 24 microsatellites. I genotyped a total of 106 individuals hatched and collected on BC and a total of 56 hatched and collected on MC (Tab. 5.3). I further genotyped 175 individuals collected on BAC. Included in this sample from BAC are the 82 adults translocated to FR in 2002, the 60 individuals translocated to SHC in 2003, plus an additional 33 resident adults

collected on BAC in 2003. Finally, I genotyped a total of 64 individuals hatched and collected on FC and 59 hatched and collected on SHC (Table 5.3).

Table 5.2 Summary of individuals sampled for all translocated populations in this study

Island	Number of founders	Number of individuals hatched and collected on translocated islands
BC	58	126
MC	18	64
FC	82	92
SHC	60	115

Island name, number of adults used as founders for translocations, and number of individuals hatched and collected on translocated islands. BC = Bay Cay; MC = Middle Cay; FC = French Cay, SHC = Six Hills Cay.

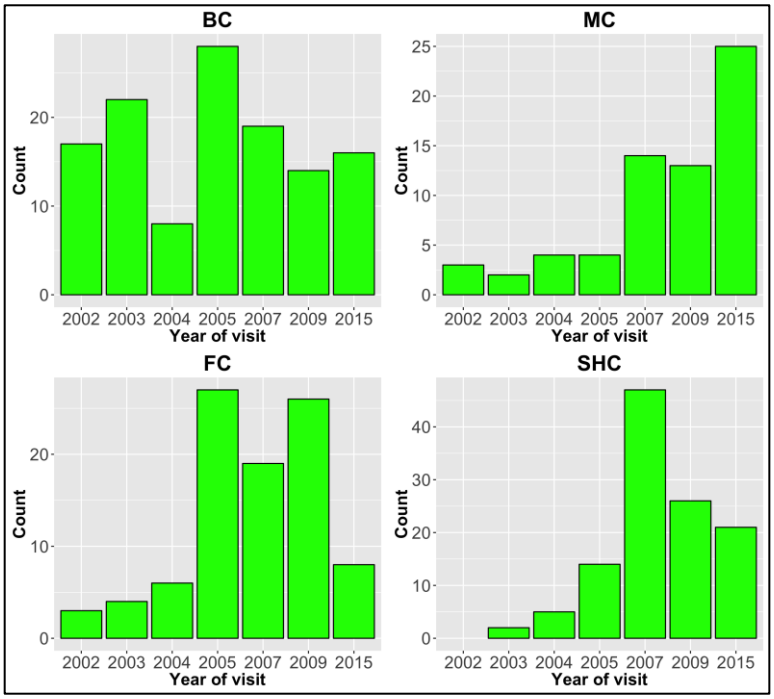

Figure 5.2 Capture profiles over years for all translocated populations in this study

Capture profile since the translocation event without including the adults used as founders. The x-axis shows the different sampling years. The y-axis shows number of sampled individuals. Green bars represent newly marked individuals sampled each visit; blue bars represent recaptured individuals. BC = Bay Cay; MC = Middle Cay; FC = French Cay, SHC = Six Hills Cay.

Molecular diversity

Dakin and Avise (2004) suggest caution when genotyped loci present evidence of null alleles with a frequency > 0.2 (Dakin and Avise 2004). Some of the microsatellites showed evidence of high frequency of null alleles on both LWC and BA. To assess their impact throughout the rest of the analyses I proceeded to calculate mean H_o, H_e and F_{is} on LWC and BAC, respectively, including and excluding loci with high null alleles' frequency. I compared the means in the different treatments through an analysis of variance. Including or excluding loci with putative presence of null alleles did not significantly affect following measurements. I hence decided to use all loci throughout the rest of the study (Null alleles ANOVA results presented in Appendix C.3, Tab. C.1). General information about loci and their diversity values are presented in Table 5.1.

Table 5.3 Number of genotyped individuals

Island	N	Notes
LWC	124	48 adults resident on LWC, 58 adults translocated to BC, and 18 adults translocated to MC
BC	106	Individuals hatched and collected on BC
MC	56	Individuals hatched and collected on MC
BAC	175	33 adults resident on BAC, 82 adults translocated to FC, and 60 adults translocated to SHC
FC	64	Individuals hatched and collected on FC
SHC	59	Individuals hatched and collected on SHC

Island name, number of genotyped individuals, and notes for all the samples genotyped in this study.

LWC Population Dynamics

No signs of a recent population bottleneck were detected on LWC indicating the population size on this island has been stable for many generations. The allele frequency distribution was L-shaped as it would be expected under mutation-drift equilibrium. The Wilcoxon signed rank test found no significant deviation from expectation under the IAM (p-val, two tails for heterozygosity excess and deficiency = 0.178) while the SMM model resulted in a significant heterozygosity deficiency (p-val, two tails for heterozygosity excess and deficiency = 0.0004). Although this latter result could be indicative of a demographic change on LWC, the SMM has often been considered too simplistic to explain patterns of microsatellite evolution (Calabrese et al. 2001; Estoup et al. 2002; Sainudiin et al. 2004; Wu and Drummond 2011).

Effective Population Size and Heterozygosity Fitness Correlations

Estimates of N_e, and their confidence intervals, calculated using three different frequencies for screening out rare alleles are presented in Table 5.4. Big Ambergris Cay, consistent with the estimates of population size, shows larger values of N_e. No significant correlations were found between any of the multi-locus heterozygosity indexes, calculated using the Rhh package, and standardized morphological traits collected from 175 adult individuals sampled on Big Ambergris Cay (Fig. 5.7 for details).

Population Dynamics on Translocated Islands

Estimates of effective population size in the four translocated islands are summarized in Tabs. 5.5. The analysis of variance performed on mean F_{is} values for samples collected across years on Bay Cay was significant ($F_{7,155} = 2.115$, $p = 0.045$). To

further disentangle significant differences in means across years I performed a Tukey-HSD test and found a significant increase in heterozygosity only between founders and individuals sampled in 2004 (Fig. 5.3 and Appendix C.4, Fig. C.1). The ANOVA performed on samples from Middle Cay also produced significant results ($F_{7,120} = 7.416$, $p = 2.13e^{-7}$). The Tukey-HSD post-hoc analysis on MC identified multiple significant pairwise comparisons between founder iguanas and samples collected in different years (Fig. 5.4 and Appendix C.4, Fig. C.2). The analysis of variance performed on individuals hatched and sampled on French Cay were significant as well ($F_{4,192} = 4.492$, p-val = 0.0003). The Tukey HSD post-hoc analysis on FC samples also identified multiple significant pairwise differences (Fig. 5.5 and Appendix C.4, Fig. C.3). Finally, neither the ANOVA ($F_{5,107} = 0.973$, p-val = 0.438) nor the post-hoc Tukey HSD analysis of SHC samples revealed any significant difference (Fig. 5.6 and Appendix C.4, Fig. C.4).

Table 5.4 LWC and BAC effective population size comparison

Cays	LD-N_e	0.050	0.020	0.010
LWC (n =124)	**N_e**	217.5	153.9	139.9
	95% pCI	140.2-423.9	114.1-224.5	106.1-196.4
	Jacknife	112.8-902.4	95.5-315.7	90.6-258.2
BAC (n = 175)	**N_e**	419.8	507.0	484.5
	95% pCI	243.5-1171.9	281.7-1771.0	274.1-1539.3
	Jacknife	168.7-∞	199.0-∞	197.2-∞

Cay and number of individuals used in the analysis. Effective population size (N_e) and confidence interval using a parametric test (95% pCI) and Jacknife. The parameter N_e is estimated using the Linkage Disequilibrium method (LD-N_e). The comparison is between Little Water Cay and Big Ambergris Cay.

Table 5.5 Effective population size estimates on translocated cays

Cays	LD-N_e	0.050	0.020	0.010
BC (n = 164)	**N_e**	29.5	43.0	42.7
	95% pCI	25.7-33.8	38.2-48.6	38-48.2
	Jacknife	20.7-41.9	32.3-57.2	32.3-57.2
MC (n = 74)	**N_e**	26.8	28.4	29.4
	95% pCI	21.9-33	23.5-34.5	24.6-35.5
	Jacknife	18.5-40.2	19.9-42	21.7-41
FC (n = 146)	**N_e**	59.7	59.3	62.2
	95% pCI	49.1-73.5	49.8-71.4	52-75.1
	Jacknife	46.1-79	47.6-75	50.4-77
SHC (n = 119)	**N_e**	104.3	118.6	105.9
	95% pCI	78.2-147.7	88.6-169.6	81.4-144.6
	Jacknife	66.2-195.2	76.6-217.8	71.4-177.5

Cay and number of individuals used in the analysis. Effective population size (N_e) and confidence interval using a parametric test (95% pCI) and Jacknife. The parameter N_e is estimated using the Linkage Disequilibrium method (LD-N_e). The comparison is among translocated populations.

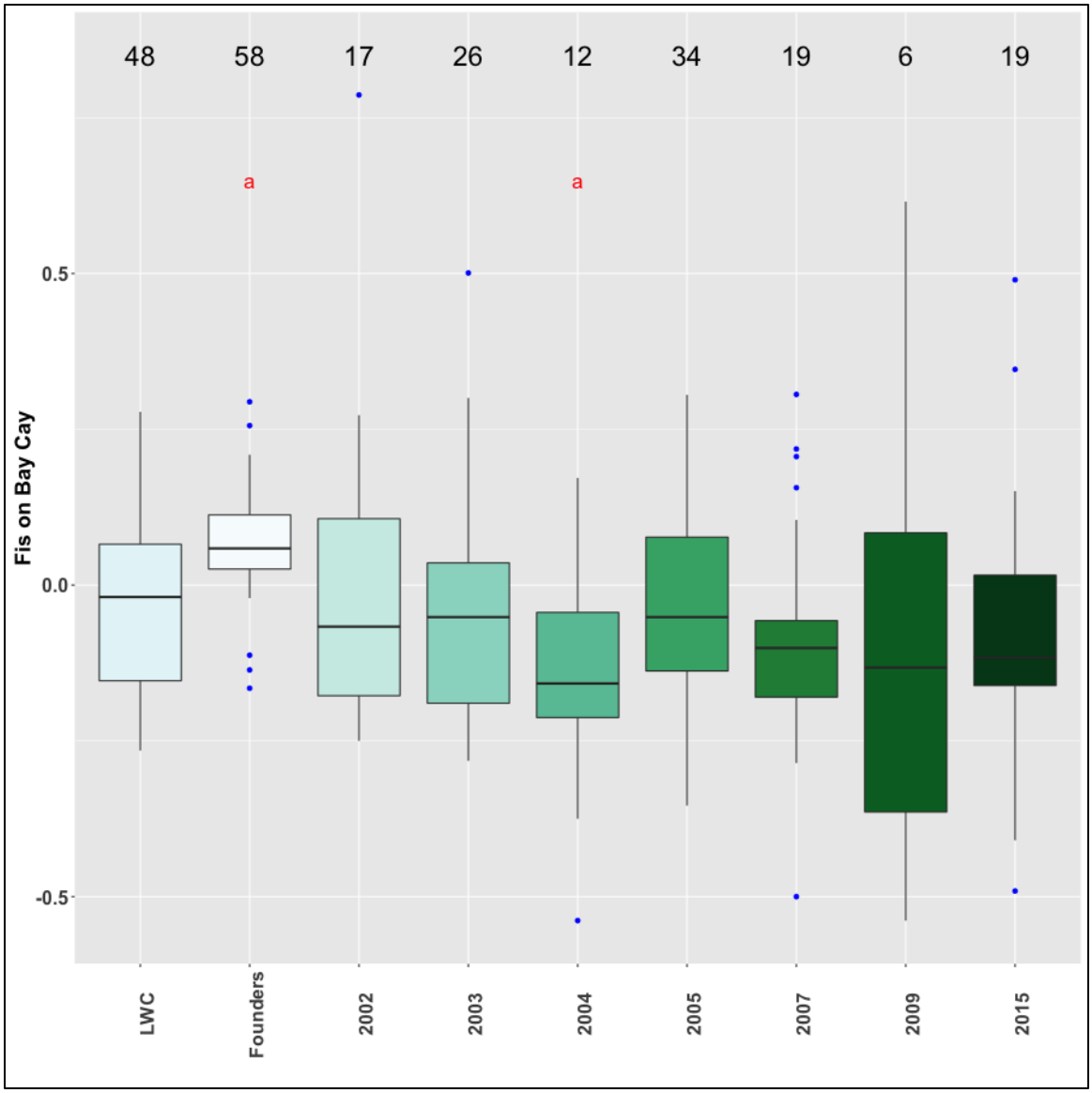

Figure 5.3 F_{is} comparison across years – Bay Cay (BC)

F_{is} values (y-axis) estimated at 24 microsatellite loci for each sampling year (x-axis) since translocation on Bay Cay. Values are represented in the form of boxplots. Black bars within each boxplot represent median F_{is} values. Lower and upper whiskers extending from each boxplot represent the lowest and upper value within 1.5 * IQR (Inter Quartile Range). Values falling outside the whiskers are considered outliers and are represented as blue dots. Pairwise significant differences between mean F_{is} values among groups are indicated by red letters. Boxplots sharing the same letter are significantly different ($p < 0.05$) from each other in Tukey-HSD test (see also Appendix C.4 Fig. C.1). Numbers above each boxplot represent the sample size used to estimate F_{is} values. The leftmost boxplot is the F_{is} value calculated on the source population of LWC using 48 adults.

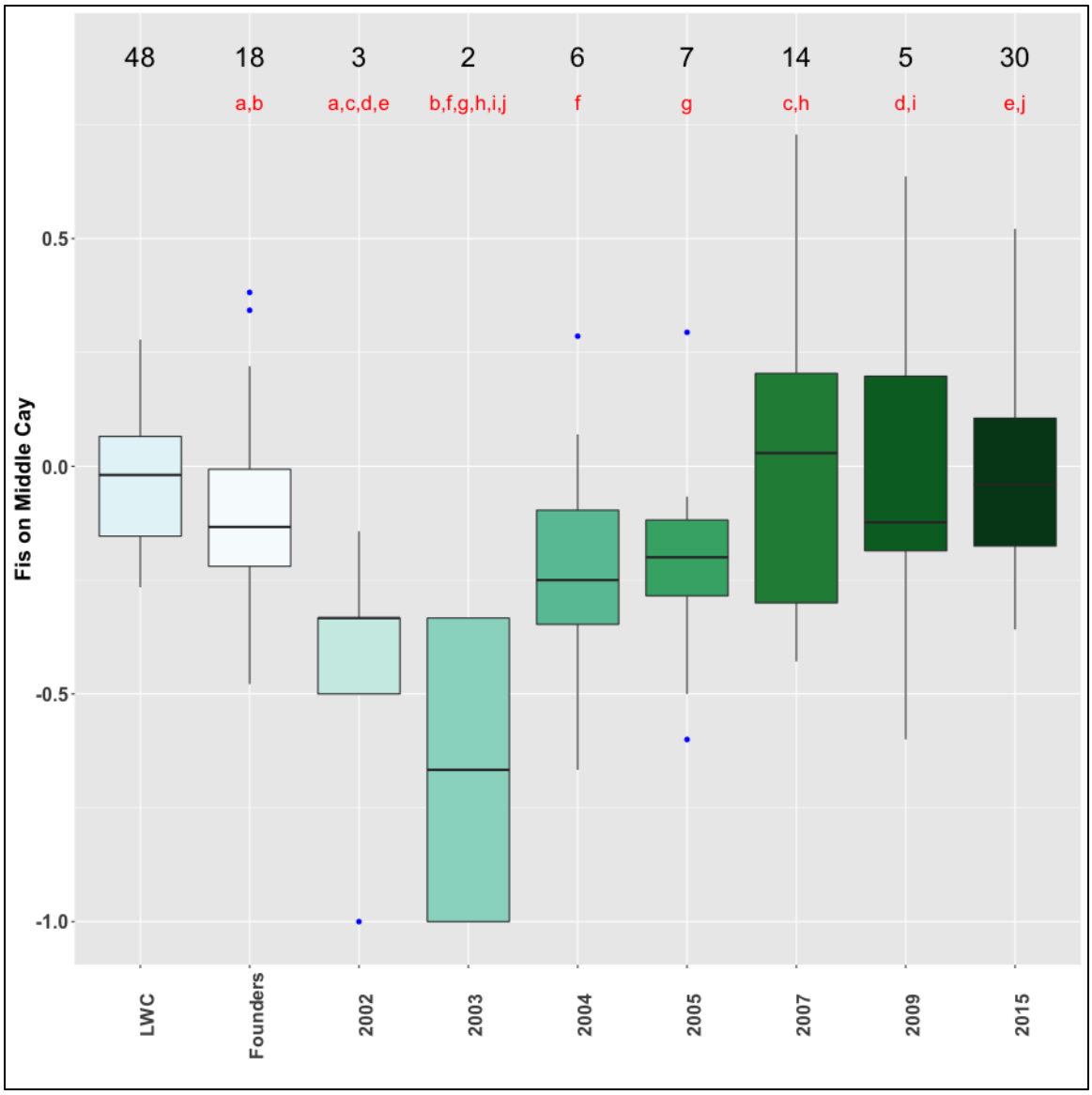

Figure 5.4 F_{is} comparison across years – Middle Cay (MC)

F_{is} values (y-axis) estimated at 24 microsatellite loci for each sampling year (x-axis) since translocation on Middle Cay. Values are represented in the form of boxplots. Black bars within each boxplot represent median F_{is} values. Lower and upper whiskers extending from each boxplot represent the lowest and upper value within 1.5 * IQR (Inter Quartile Range). Values falling outside the whiskers are considered outliers and are represented as blue dots. Pairwise significant differences between mean F_{is} values among groups are indicated by red letters. Boxplots sharing the same letter are significantly different ($p < 0.05$) from each other in Tukey-HSD test (see also Appendix C.4 Fig. C.2). Numbers above each boxplot represent the sample size used to estimate F_{is} values. The leftmost boxplot is the F_{is} value calculated on the source population of LWC using 48 adults.

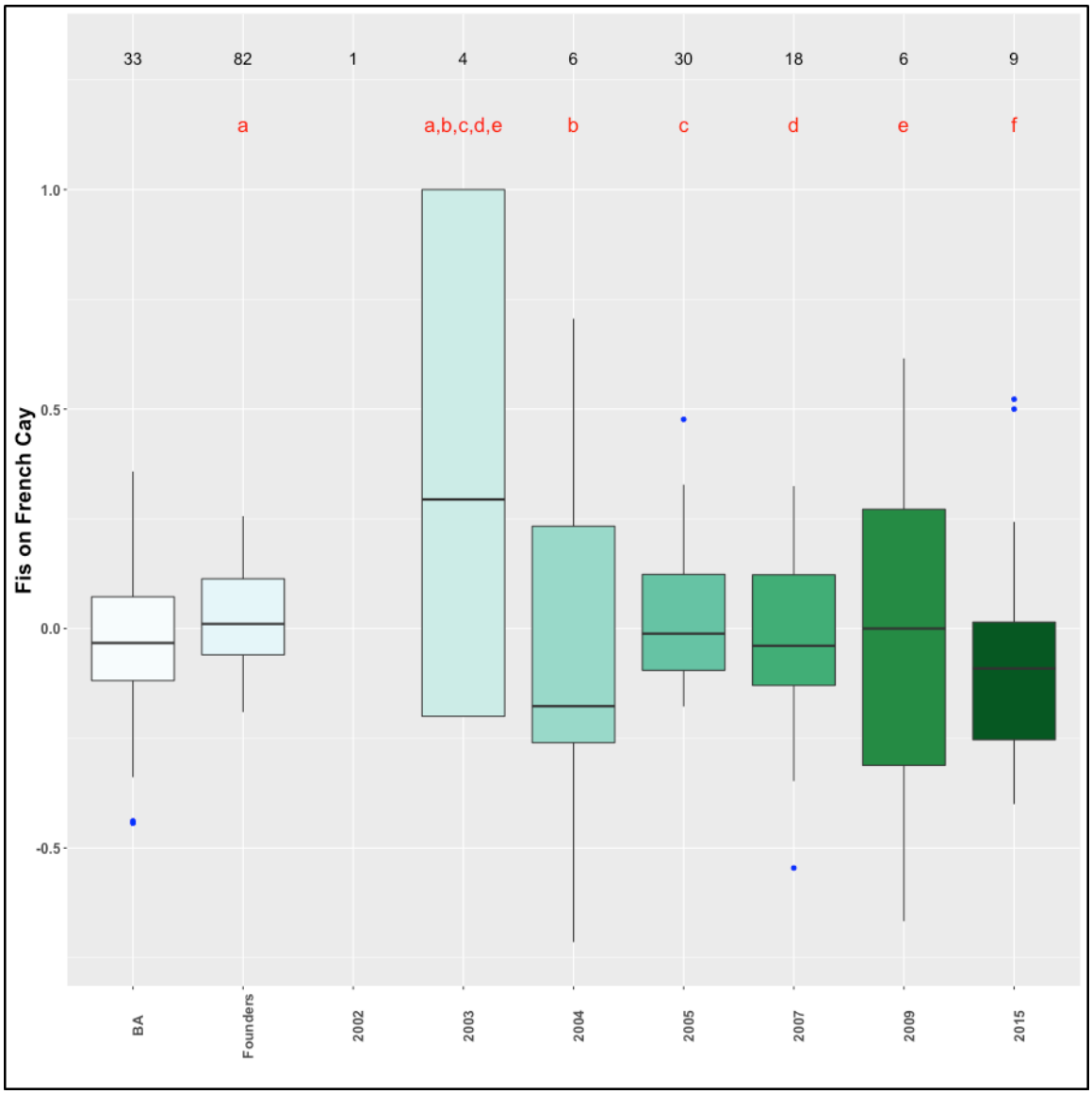

Figure 5.5 F_{is} comparison across years – French Cay (FC)

F_{is} values (y-axis) estimated at 24 microsatellite loci for each sampling year (x-axis) since translocation on French Cay. Values are represented in the form of boxplots. Black bars within each boxplot represent median F_{is} values. Lower and upper whiskers extending from each boxplot represent the lowest and upper value within 1.5 * IQR (Inter Quartile Range). Values falling outside the whiskers are considered outliers and are represented as blue dots. Pairwise significant differences between mean F_{is} values among groups are indicated by red letters. Boxplots sharing the same letter are significantly different ($p < 0.05$) from each other in Tukey-HSD test (see also Appendix C.4 Fig. C.3). Numbers above each boxplot represent the sample size used to estimate F_{is} values. The leftmost boxplot is the F_{is} value calculated on the source population of BAC using 33 adults.

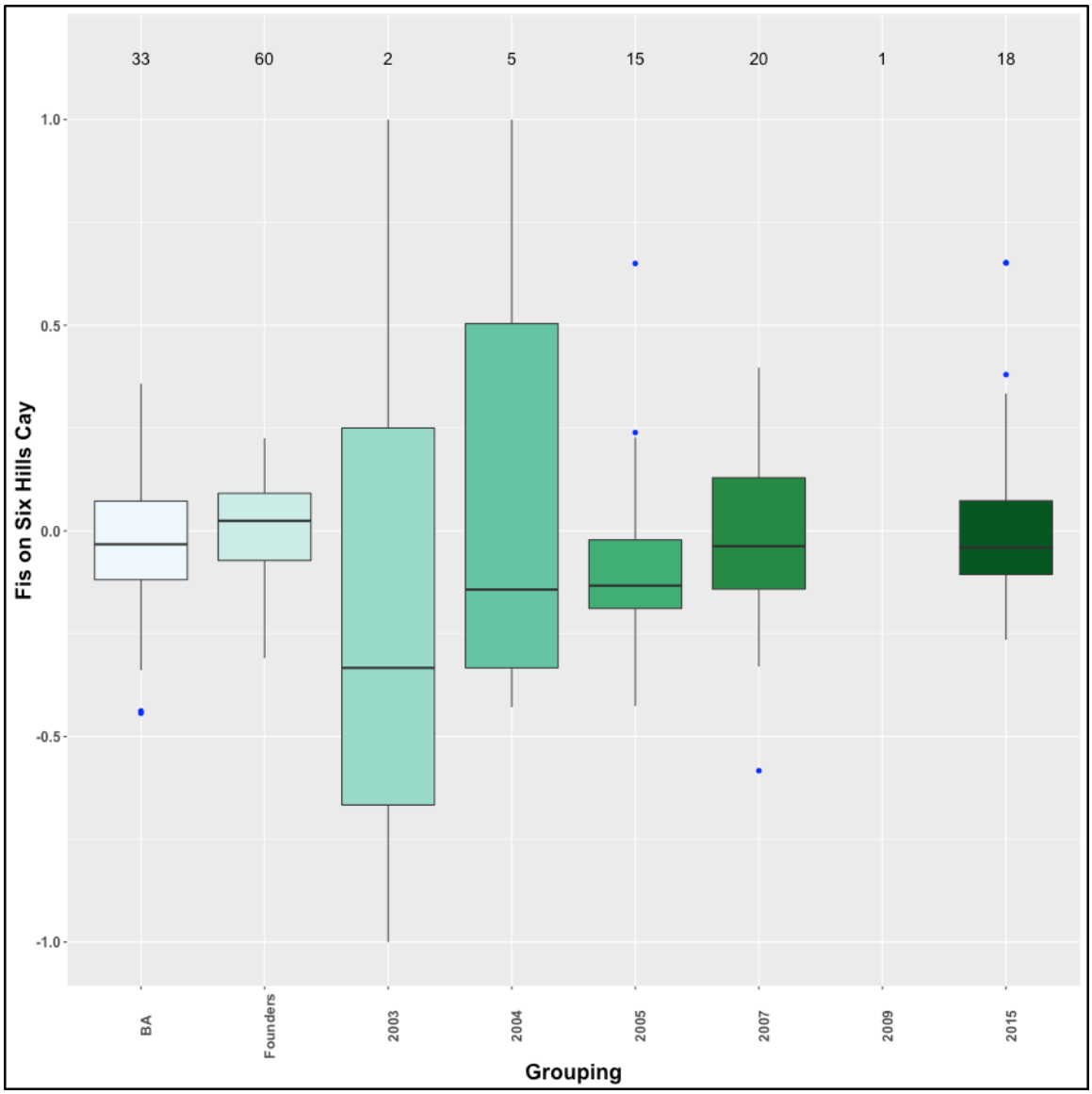

Figure 5.6 F_{is} comparison across years – Six Hills Cay (SHC)

F_{is} values (y-axis) estimated at 24 microsatellite loci for each sampling year (x-axis) since translocation on French Cay. Values are represented in the form of boxplots. Black bars within each boxplot represent median F_{is} values. Lower and upper whiskers extending from each boxplot represent the lowest and upper value within 1.5 * IQR (Inter Quartile Range). Values falling outside the whiskers are considered outliers and are represented as blue dots. I found no significant pairwise significant differences between mean F_{is} values among groups (see also Appendix C.4 Fig. C.4). Numbers above each boxplot represent the sample size used to estimate F_{is} values. The leftmost boxplot is the F_{is} value calculated on the source population of BAC using 33 adults.

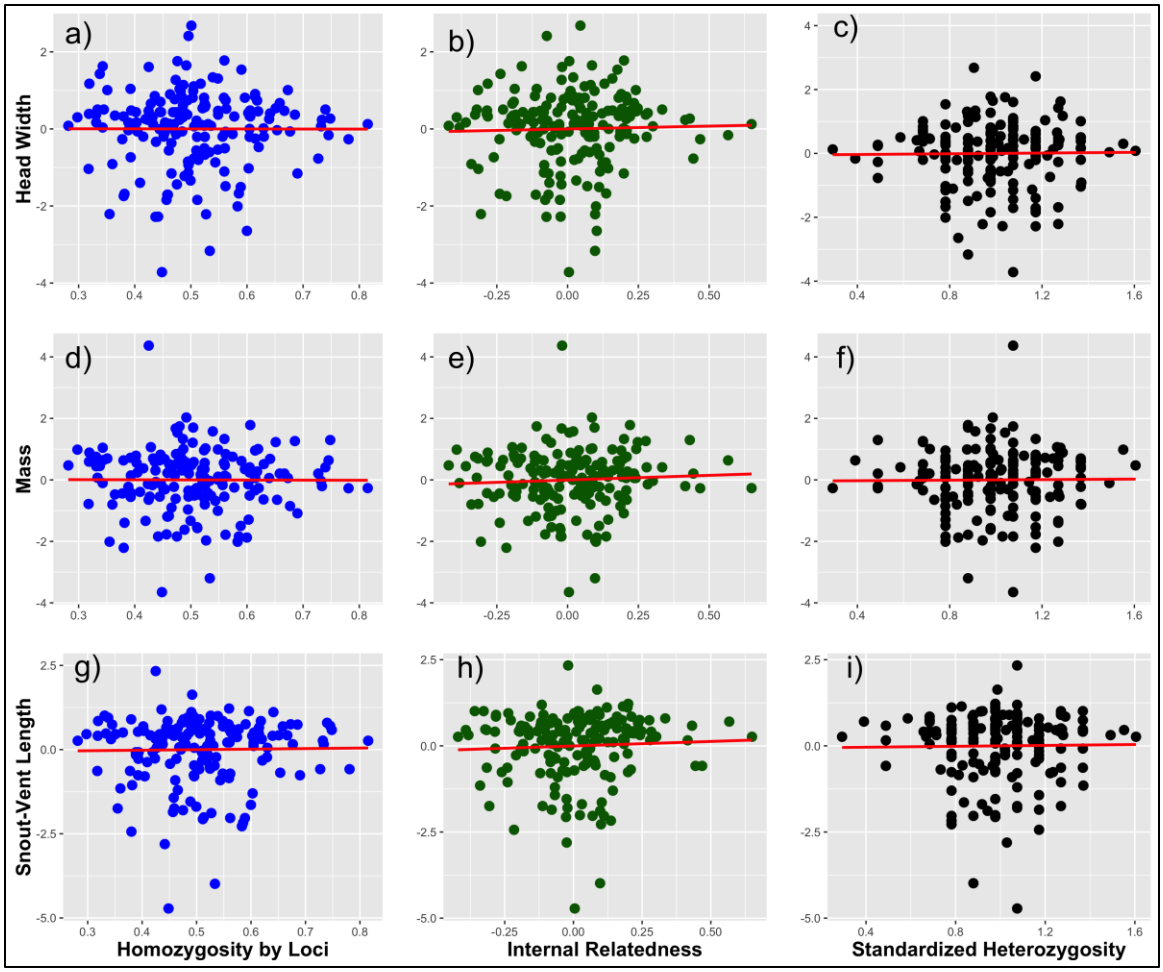

Figure 5.7 Heterozygosity fitness correlation in adults from Big Ambergris Cay

Heterozygosity fitness correlations in 175 adults collected from Big Ambergris. Each graph represents a correlation between a morphological measurement and a measure of multilocus heterozygosity (see Material and Methods for details). **a)** Head Width with Homozygosity by Loci: $r^2 = 0.000$, $p = 0.983$; **b)** Head Width with Internal Relatedness: $r^2 = 0.0007$, $p = 0.715$; **c)** Head Width and Standardized Heterozygosity: $r^2 = 0.0001$, $p = 0.869$; **d)** Mass with Homozygosity by Loci: $r^2 = 0.000$, $p = 0.957$; **e)** Mass with Internal Relatedness: $r^2 = 0.003$, $p = 0.459$; **f)** Mass and Standardized Heterozygosity: $r^2 = 0.000$, $p = 0.898$; **g)** Snout Vent Length with Homozygosity by Loci: $r^2 = 0.0002$, $p = 0.832$; **h)** Snout Vent Length with Internal Relatedness: $r^2 = 0.002$, $p = 0.519$; **i)** Snout Vent Length with Standardized Heterozygosity: $r^2 = 0.0002$, $p = 0.851$.

Discussion

The study of inbreeding depression and its evolutionary consequences in natural populations poses a series of problems. Even the simple characterization of mating strategies (i.e., inbreeding VS outbreeding) can be problematic in non-model organisms (Waser 1993). Historically, estimates of inbreeding depression have generally been obtained using individuals with known pedigree information (Crnokrak and Roff 1999; Pemberton 2004), or measuring fitness performance in controlled crosses between outbred and inbred lines (Crnokrak and Barrett 2002). These approaches are not feasible when analyzing wild populations due to problems associated with the biology of the analyzed species, the presence of migrants, the absence of precise pedigree information, possible multiple paternity, sampling bias and complex biogeographic histories (Charlesworth and Charlesworth 1987; Coltman and Slate 2003; Chapman et al. 2009). Moreover, analyzing the intensity of intraspecific density dependent selection can be biased by the confounding effect of growth and age as well as spatial heterogeneity (He and Duncan 2000; Pujol and McKey 2006). Microsatellite markers made the investigation of inbreeding depression and its evolutionary consequences in the wild somewhat easier. Still, studies accounting for the combined effect of inbreeding and genetic drift in natural populations are scant and mostly focused on plants, invertebrates, and other species with short generation time and amenable to laboratory experimentation (Pujol and McKey 2006; Wright et al. 2008; Pekkala et al. 2012; Pekkala et al. 2014; Lohr and Haag 2015). This study is an attempt to indirectly describe the evolutionary consequences of population size and isolation to the expression of inbreeding depression in a long lived, critically endangered iguana. I found circumstantial, non-definitive

evidence supporting the original hypothesis that soft-selection plays a role in generating the observed patterns of inbreeding depression in the population of *C. carinata* iguanas on Little Water Cay.

Berk (2013) found significant evidence of inbreeding depression in *C. carinata* iguanas on Little Water Cay. The author found strong evidence of selection favoring heterozygotes in the transition from juvenile stage to adulthood (Berk 2013). Even within the adult age class the author showed a significant positive correlation between heterozygosity and a fitness proxy (Berk 2013). Yet, no significant departure from random mating expectations was detected (Berk 2013). These results, compounded with the species behavior and the population's history, led to the formulation of the hypothesis that natural selection may be largely influencing recruitment by conferring a competitive advantage to slightly outbred individuals, soft-selection, rather than directly acting on unfit genotypes, hard-selection.

The first evidence in support of the soft-selection hypothesis comes from the analysis of HFCs in the adult iguana population of Big Ambergris Cay. I failed to find any significant correlation between morphological traits and multilocus heterozygosity calculated using 24 microsatellites and 175 adults sampled from BAC (Fig. 5.6). Big Ambergris Cay is larger than LWC (≈ 400 ha and ≈ 100 ha, respectively) and sustains a larger population of iguanas ($N \approx 15{,}000$ and $N \approx 2{,}000$, and $N_e \approx 450$ and $N_e \approx 150$, respectively; see Materials and Methods and Tab. 5.4). Despite differences in surface area and population size, both islands present similar adult iguana densities (Gerber 2004; Welch et al. 2004; Gerber 2007; Gerber and Pagni 2012). Intense intraspecific competition is known to affect populations and their individuals in a variety of ways,

from morphology and physiology to life history traits. Vervust and colleagues showed the differential cost of intraspecific competition in two island populations of the Mediterranean lizard *Podarcis sicula* varying in density (Vervust et al. 2008). Specifically, the authors found that intraspecific competition was the most likely explanation for the increased occurrence of non-lethal injuries in these lizard, and that the frequency of toe amputation could be used as an indirect predictor of the intensity of intraspecific competition on those two islands (Vervust et al. 2009). On oceanic islands where population density of the passerine species *Zosterops lateralis chlorocephalusi* is high, intense intraspecific competition has been used to explain the dominance hypothesis, according to which traits conferring a competitive advantage in social dynamics, like larger body size, are more likely to be selected for (Robinson-Wolrath and Owens 2003). Moreover, intraspecific competition is also known to play a role in determining how small genomic differences can be translated into fitness differences (Meagher et al. 2000; Haag et al. 2002; Yun and Agrawal 2014; but see also Pilakouta et al. 2015). The lack of significant heterozygosity fitness correlations on BAC adults suggests that variation in population size, and therefore in N_e, could increase the overall fitness stress caused by density dependent processes (Weiner 1985; Yun and Agrawal 2014). This result also led me to speculate on the possible mechanism generating the heterozygosity fitness correlation on LWC. As Little Water Cay is a smaller island I expected inbreeding to be greater compared to BAC (Wright et al. 2008; Frankham et al. 2010). When a fraction of the population is experiencing a higher rate of consanguineous mating, a correlation across loci is generated such that heterozygosity (or homozygosity) at selected markers should reflect a genome wide state, i.e., identity disequilibrium

(Szulkin et al. 2010). In such cases, inbreeding can be regarded as the main cause of HFCs. Yet, in Berk (2013) and in the present study I failed to find any significant evidence of deviation from random mating expectations on LWC (N = 124, F_{is} = -0.030, p-val = 0.931; g_2 = 0.006, s.d. = 0.008, p-val = 0.179) and on BAC (N = 175, F_{is} = -0.030, p-val = 0.965; g_2 = 0.0001, s.d. = 0.005, p-val = 0.477). An alternative hypothesis to explain patterns of HFCs without involving consanguineous mating is the local-effect hypothesis (David et al. 1995). According to this hypothesis HFC patterns observed are generated by polymorphic fitness genes in physical linkage with one or few markers under investigation (Szulkin et al. 2010). Once again, in order to produce a correlation between loci across the genome certain population dynamics are required. For example, the long-term action of genetic drift, of particular interest in small and isolated populations, could create substantial identity disequilibrium (ID; the correlation in homozygosity or heterozygosity across loci) and linkage disequilibrium (LD; the non random association of alleles in gametes) across loci even under regimes of random mating (Szulkin et al. 2010). Natural populations of iguanas in the Turks and Caicos Islands, including the one on Little Water Cay, are characterized by a high degree of isolation (Welch et al. in review, but see Material and Methods). Moreover, the data suggest no recent dramatic variations in population size, suggesting that the population on LWC has most likely been constant in size for at least 1000 generations (Berk 2013; Welch et al. in review). Finally, estimates of effective population size indicate that the population on LWC is approximately three times smaller than the one on Big Ambergris. Hence, LWC should in theory be more likely to accumulate significant ID due to increased opportunities for inbreeding, and LD due to fewer recombination events each

generation. The former should generate HFCs due to universal effects while the latter could explain HFCs in the adult population through local effects.

Additional support for the soft-selection hypothesis comes from the comparison of juvenile growth rates between LWC and the translocated populations on BC and MC. Juvenile iguanas on Bay and Middle Cays showed significantly higher growth rates than juveniles on LWC. The same was observed for juveniles on FC and SHC when compared to juveniles on BAC (Gerber, personal communication). Particularly, juveniles on Middle Cay grew up to three times faster than on the source population (Gerber, personal communication). Population specific increased growth rate, and, in general, increased rates of morphological differentiation can be explained via ecological opportunity (Nosil and Reimchen 2005; Harmon et al. 2008; Kassen 2009). Ecological opportunity happens when individuals in a population experience reduced selective pressures due to reduction in competition and/or predation (Parent and Crespi 2009; Losos 2010).

Although the aforementioned circumstantial evidence points toward the soft-selection hypotheses, the analysis of heterozygosity over the years in translocated populations does not support this hypothesis. For example, after correcting for multiple comparisons, the Tukey-HSD test on mean F_{is} values in Bay Cay revealed a significant increase in the relative proportion of heterozygous genotypes between founder individuals and iguanas sampled in 2004 (see Fig. 5.3). The prediction of the soft selection hypothesis is increased homozygosity in early generations relative to that observed in the source populations. No other significant departures from expected heterozygosity were detected, but the overall trend points towards a general increase in heterozygosity. Although a general increase in heterozygosity would be expected on the

translocated island as population size and density increase, the significant increase of heterozygosity after only two years since translocation could be explained by hard selection acting against homozygous individuals expressing lethal equivalents. On Middle Cay too I found some significant differences between founders and individuals hatched in the following years (see Fig. 5.4). Unfortunately no significant conclusions can be drawn due to low sample sizes of the sampling seasons involved in the pairwise comparison (specifically, years 2002 and 2003 only have sample sizes of 2 and 3, respectively). Data obtained from translocated populations sourced using adult iguanas from BAC do not reveal any significant trend. Significant differences in sampling years on FC are potentially affected by sampling errors, while no major differences were found on SHC. In all instances, and particularly on BC and MC, estimates of effective population size on translocated islands (see Tab. 5.5) indicate that, despite the rapid increase in number of individuals (Gerber, personal communication), these populations are effectively small and prone to the deleterious influence of genetic drift in the generations to come.

I found two major sources of bias that could have affected my results and that warrant further investigation in the future. Iverson (1979) estimated an average generation time for *Cyclura carinata* iguanas of 14 years. This suggests that adult iguanas on the translocated islands may just now be reaching levels of competition intense enough to see a significant and stronger signal at the genetic level. Increased juveniles growth rate on translocated populations from either source islands (Gerber, personal communication) mitigates this "generation" problem. Due to increased growth rate, iguanas on translocated islands could reach sexual maturity and start reproducing earlier

than on the source populations. In addition to this, analyses of local reproductive dynamics on translocated islands (results not shown), and the low estimates of effective population size, indicate that only a fraction of the founding iguanas used for translocation may be reproductively successful. Therefore a more long-term monitoring of the translocated populations, along with a direct quantitative estimate of population density and adult interactions, may uncover a stronger influence of competition. An additional source of bias is limited sample sizes across different age classes to analyze genetic patterns in more details on the translocated islands. In conclusion, I found very limited support for the original hypothesis of "*soft selection*" as the main driver of the pattern of heterozygosity fitness correlation detected on the island Little Water Cay.

CHAPTER VI

FINAL SUMMARY

In this dissertation I examined the population dynamics of endangered and critically endangered species of iguanas in the genus *Cyclura,* and tried to elicit important conservation and evolutionary aspects pertaining the long-term persistence of their natural populations.

Chapter II focused strictly on the conservation of critically endangered *Cyclura cychlura cychlura*. I started from ecological data suggesting the demographic cohesion of these iguanas across their range on Andros Island, the Bahamas, and tested the hypothesis that demographic cohesion corresponds to reproductive cohesion. In order to test this hypothesis I used a suite of 16 polymorphic microsatellites to genotype individuals sampled across the species range. Contrary to the original hypothesis and expectations, I found significant restriction in gene flow and I was able to characterize three distinct genetic clusters. Even more interestingly, the three identified clusters seem to be largely concordant with the presence of major water channels dividing the island in different landmasses and previously thought not to represent significant barriers to gene flow. The data suggest that from a conservation perspective it would be inadvisable and potentially dangerous to treat iguanas on Andros as belonging to a single large panmictic population. Moreover, I found that iguanas sampled from the central region of the island had greater genetic diversity compared to those from the other two identified clusters, suggesting that

this population may be of greater importance for preserving the overall taxon genetic diversity. Luckily, the Bahamas National Trust recently expanded the boundaries of the Westside National Park on Andros and clusters identified in this study are currently legally protected (Bahamas National Trust 2012).

Chapter III, expanded from conservation issues to also include considerations on the evolutionary and ecological mechanisms that contributed to the current patterns of genetic variability and distribution in a multi-taxa system in the Bahamas. Specifically, I investigated the geographic diversity and distribution of mtDNA in endemic Rock Iguanas and their tick ectoparasites in the Bahamas. I was motivated by two major questions: can *Cyclura* iguanas and their parasites be used to investigate local patterns of multispecies distribution? Is vicariance the main driver of current biodiversity patterns in the investigated taxa? The specific hypothesis tested in this study is that the dispersal of *Amblyomma* ticks in the Bahamas is "vertical", i.e. limited by the dispersal of the iguana hosts. Alternatively, ticks' distribution could be limited by ecological factors such as the presence of other appropriate hosts to complete different stages of their life-cycle, or they could disperse "horizontally" across multiple islands via other hosts such as birds. I found compelling evidence to support the original hypothesis that dispersal mechanism of the parasitic taxon (i.e. *Amblyomma*) is largely vertical and that a recent vicariant event is potentially responsible for current patterns of diversity. At the same time patterns of diversity and gene flow on the host species measured using microsatellite markers also suggest that historical levels of gene flow may have been low even prior to the vicariant event. In chapter IV I analyze the possible presence of *Rickettsia* superparsites within *Amblyomma* ticks. I identified a potentially dangerous species of *Rickettsia* in ticks

parasitizing iguanas within the Exuma Islands chain. Given the strong interest associated with the pet trade of these lizards, the documented presence of pathogenic bacteria in ticks parasitizing them, and the fact that many other species of *Cyclura* outside the Bahamas carry *Amblyomma* ticks it is of paramount importance to further investigate prevalence of infections and possibly co-infection with other bacteria.

Finally, the V data chapter of this dissertation intertwines conservation and evolutionary aspects. In this study I investigated the mechanisms favoring heterozygous genotypes in a small isolated and natural population of the critically endangered iguanas *Cyclura carinata*. I tested the specific hypothesis that "soft selection", the component of natural selection associated with competition, is largely responsible for the expression of inbreeding depression. If heterozygote excess reflects a competitive advantage for outbred individuals, then small populations should be more robust than expected assuming that heterozygous excess results from expressing lethal equivalents, i.e., hard-selection. On the contrary, if observed attrition rates due to inbreeding depression result from hard-selection then these small populations are more likely ephemeral, and require relatively frequent recolonization. Using polymorphic microsatellites I found indirect evidence suggesting that small natural populations can maintain significant levels of segregating load. Under regimes of random mating this load can be exposed to natural selection through increased competition, as populations approach carrying capacity. However, quantification of the relative roles of hard and soft-selection affecting the population dynamic of these iguanas was not feasible. Hence, the genetic resilience of small isolated populations of this species remains unclear.

The data presented in this work shall be used and built-upon to foster conservation strategies for these endangered and critically endangered iguanas. For example, the results presented in this dissertation together with that of other researchers (Iverson 1978; Iverson 1979; Malone et al. 2003; Welch et al. 2004; Gerber 2007; Gerber and Pagni 2012; Carreras De León 2015; Aplasca et al. 2016) indicate that maintaining locally pristine habitat conditions may be key for the survival of these species. Despite the ability of some of these iguanas to actively disperse away from their nests, natural geographic barriers, combined with competition and patterns of local adaptation, suggest that *Cyclura* iguanas may not be able to readily colonize new areas. This could represent a problem for the long-term survival of many iguana populations if the urbanization process in West Indies and the invasion of human-commensal species were to continue. Moreover, although the success of some head-starting and captive breeding programs has been incredibly high, these practices are usually long and can be hard to reintroduce animals in the wild (García et al. 2007; Pérez-Buitrago et al. 2008).

Among the others, translocation of iguanas on islands that are sheltered from human influences has been proven a successful strategy (Knapp and Malone 2003; Gerber et al. 2004; Gerber 2007). Yet, the data presented in my work suggest that despite the unquestionable short-term benefit of this conservation strategy, its long-term success may be jeopardized by the insurgence of genetic hurdles. Therefore, a continuous monitoring at both the ecological and genetic level on translocated populations is warranted and a deeper understanding of the mechanisms affecting long-term population persistence of these iguanas is vital.

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APPENDIX A

R CODE TO ESTIMATE BAYESIAN DEVIANCE

Bayesian Deviance

The following R code has ben used to calculate the Bayesian deviance from the results obtained using BAYESASS 3.0 (Wilson and Rannala 2003). A more detailed explanation of the code is available in Patrick G. Meirmans (2013) and Faubet et al. (2007).

```
># calculateDeviance.R; Patrick G. Meirmans (2013) Non-convergence in Bayesian
  estimation of migration rates, Molecular Ecology Resources; Supplementary Material
># This script will calculate the Bayesian Deviance from the output of BayesAss
># For more information on the use of the deviance, see Faubet et al. (2007)
># To use this script, run BayesAss version 3 with the -t flag to produce a trace-file
># Then set the working directory of R to the folder with the output from BayesAss
># Change this value to the actual burnin used for your MCMC run
>burnin = 1000000
># Change this value to the actual sampling interval used for your MCMC run
>sampling.interval = 2000
># Read the data from the trace file
>trace=read.table("BA3trace.txt", header=TRUE)
># Plotting the likelihoods is always a good idea
>plot(trace$State,trace$LogProb, xlab="State", ylab="LogProb", type="l", lwd=2,
  col="firebrick3", font.lab=2)
># Draw a vertical line to indicate the end of the burnin abline(v=burnin, col="grey70",
  lty=2)
># Calculate the deviance
>range = (trace$State > burnin & trace$State %% sampling.interval == 0)
>D = -2*mean(trace$LogProb[range])
># Print the result to the console
>print(D)
```

APPENDIX B

R CODE TO RUN PARALLEL STRUCTURE

Parallel Structure code

The following R code has been used to run the STRUCTURE analysis taking advantage of parallel computing capabilities in computer with multiple cores. The code can be implemented via the package *ParallelStructure* (Besnier and Glover 2013). I run the analysis relying on the mclapply() function from the *Parallel* package that can be accessed only running R from the MacOS Shell.

```
> library(ParallelStructure)
> library(parallel)
> STR_path <- '/Applications/Structure.app/Contents/Resources/Java/bin/'
> parallel_structure(structure_path=STR_path, joblist='job_list1.txt', n_cpu=5,
  infile='FigginsiInput_Structure12loci.txt', outpath='Results_12loci/', numinds=192,
  numloci=12, printqhat=0, plot_output=1, onerowperind=1)
```

APPENDIX C

R CODE TO CALCULATE ALLELE FREQUENCY, Z-SCORES, AND TO USE RMARK. NULL ALLELE ANOVA OUTPUT, TUKEY-HSD OUTPUT AND *RMARK* MODELS

Allele Frequency Function

The following R function was used to calculate and graph multi locus allele frequency. MyData should be an object of class *data.frame* containing individuals ID in the first column and genotypes in the following columns. This function produces a graphic representation of allele frequencies. It also produces an OUT file with the numeric values of allele frequencies. It furthers creates a data frame with genotypes that can be used directly with the *mlh* function from the Rhh package.

```
> AllFreqWGraph <- function (MyData) {
 OUT<<-NULL
 MyData.1<-MyData[,-1]
 L<-ncol(MyData.1)
 locus_positions<-(2*(unique(round((1:(L-2))/2)))+1)
 lnames<-colnames(MyData.1)
 for (x in locus_positions) {
  alleles<-c(MyData.1[,x],MyData.1[,x+1])
  alleles2<-as.data.frame(table(alleles))
  if (alleles2[1,1] == 0 ) {
   alleles3<-alleles2[-which(alleles2[,1]==0),]
   alleles4<-cbind(alleles3,alleles3[,2]/sum(alleles3[,2]))
  } else {
   alleles3<-alleles2
   alleles4<-cbind(alleles3,alleles3[,2]/sum(alleles3[,2]))
  }
  output<-cbind(x,lnames[x],alleles4)
  OUT <<- rbind(OUT,output)
 }
 colnames(OUT) <- c("Number","Locus","allele","count","frequency")
 Allelefreqs<-OUT[,-1]
 locName<-as.vector(unique(OUT$Locus))
 par(mfrow = c(2, 2))
 for (x in locName){
  Locus<-Allelefreqs[which(Allelefreqs[,1]==x),]
  plot(as.vector.factor(Locus[,2]), Locus[,4], xlab="",
ylab="Frequency",main=paste("Locus_",Locus[1,1]),pch=21,
bg = "blue",cex=1, cex.axis=.7, las=2)
 }
```

```
  par(mfrow = c(1, 1))
  par(no.readonly = T)
  MyDataMLHready<<-NULL
  if (length(which(OUT[,5]==1))>0) {
   to_remove<-c(OUT[which(OUT[,5]==1),2])
   print(to_remove)
   rem<-((to_remove)*2)
    if (length(rem) > 1) {
     loc_remove_mlh<-c((rem),((rem)+1))
     print(loc_remove_mlh)
     MyDataMLHready<<-MyData[,-loc_remove_mlh]
    } else {
     loc_remove_mlh<-c(rem,rem+1)
     print(loc_remove_mlh)
     MyDataMLHready<<-MyData[,-loc_remove_mlh]
    }
   print(OUT)
   print(MyDataMLHready)
  } else {
   MyDataMLHready<<-MyData
   print(OUT)
   print("There are no monomorphic loci!")
   print(MyDataMLHready)
  }
}
```

Z-Score function

This function was used to standardize morphometric values to be used in HFCs calculations. X is a vector containing individuals morphometric values.

```
>z_score <- function (x)  {
  x.mean <- mean(x)
  x.sd <- sd(x)
  x.z <- (x - x.mean)/x.sd
  return(x.z)
}
```

Null allele ANOVA

The following table shows the details for the ANOVA comparison between estimates of mean H_o, H_e and F_{is} with and without loci with putative high frequency of null alleles.

Table C.1 ANOVA table

	LWC				BA			
	w	**w/o**	**F**	**p-val**	**w**	**w/o**	**F**	**p-val**
H_o	0.394	0.384	0.034	0.855	0.426	0.419	0.013	0.909
H_e	0.394	0.386	0.021	0.886	0.430	0.413	0.093	0.762
F_{is}	-0.030	-0.024	0.062	0.805	-0.030	-0.044	0.518	0.476

w = with null alleles; **w/o** = without null alleles; F test and its significance. Mean values for observed and expected heterozygosity (H_o and H_e) and fixation index (F_{is}) are presented.

Tukey-HSD tests

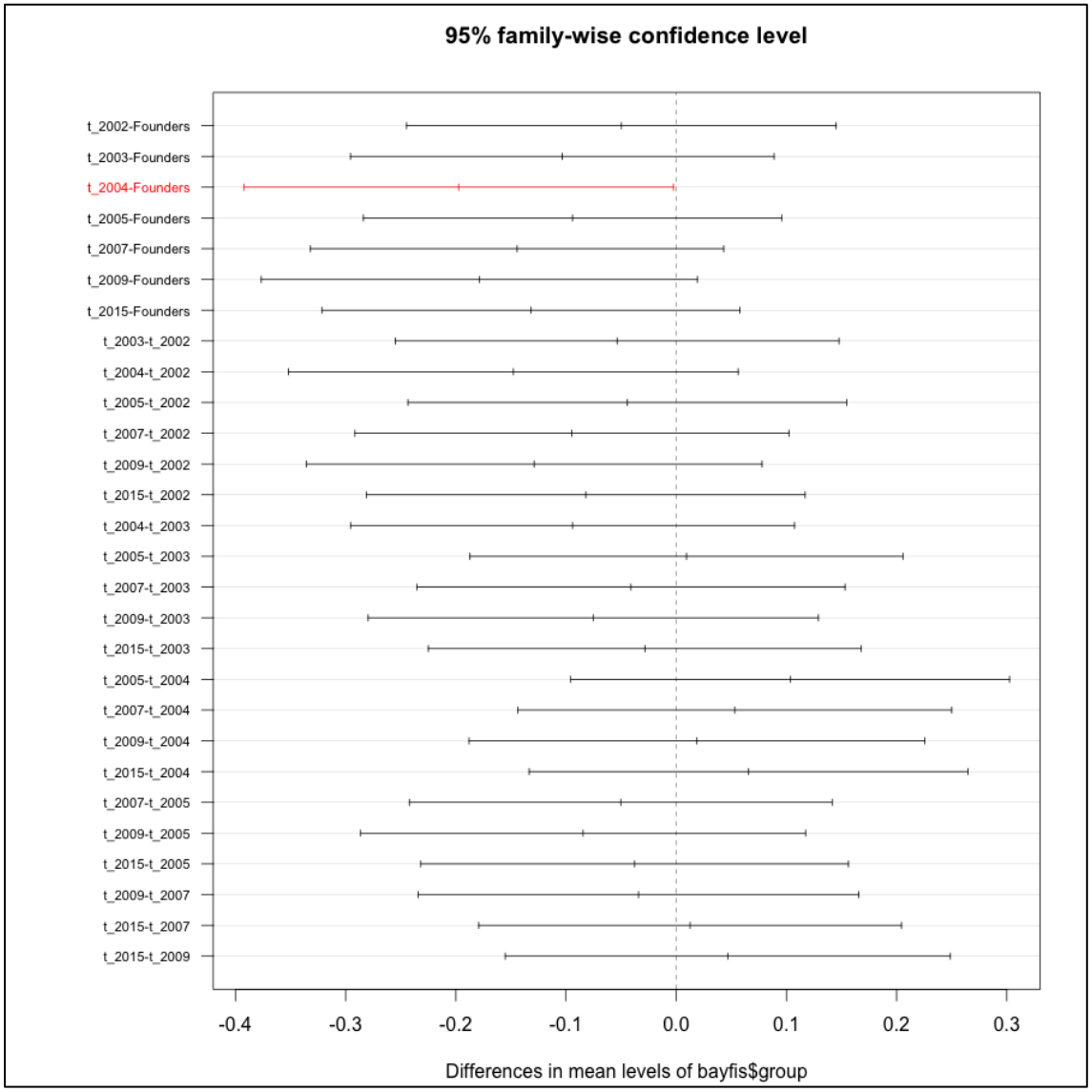


Figure C.1 Tukey-HSD in mean F_{is} values across years – Bay Cay

Post-Hoc Tukey-HSD test to compare differences in mean F_{is} values across years for samples collected on Bay Cay. Significant differences after Bonferroni correction for multiple comparisons are highlighted in red.

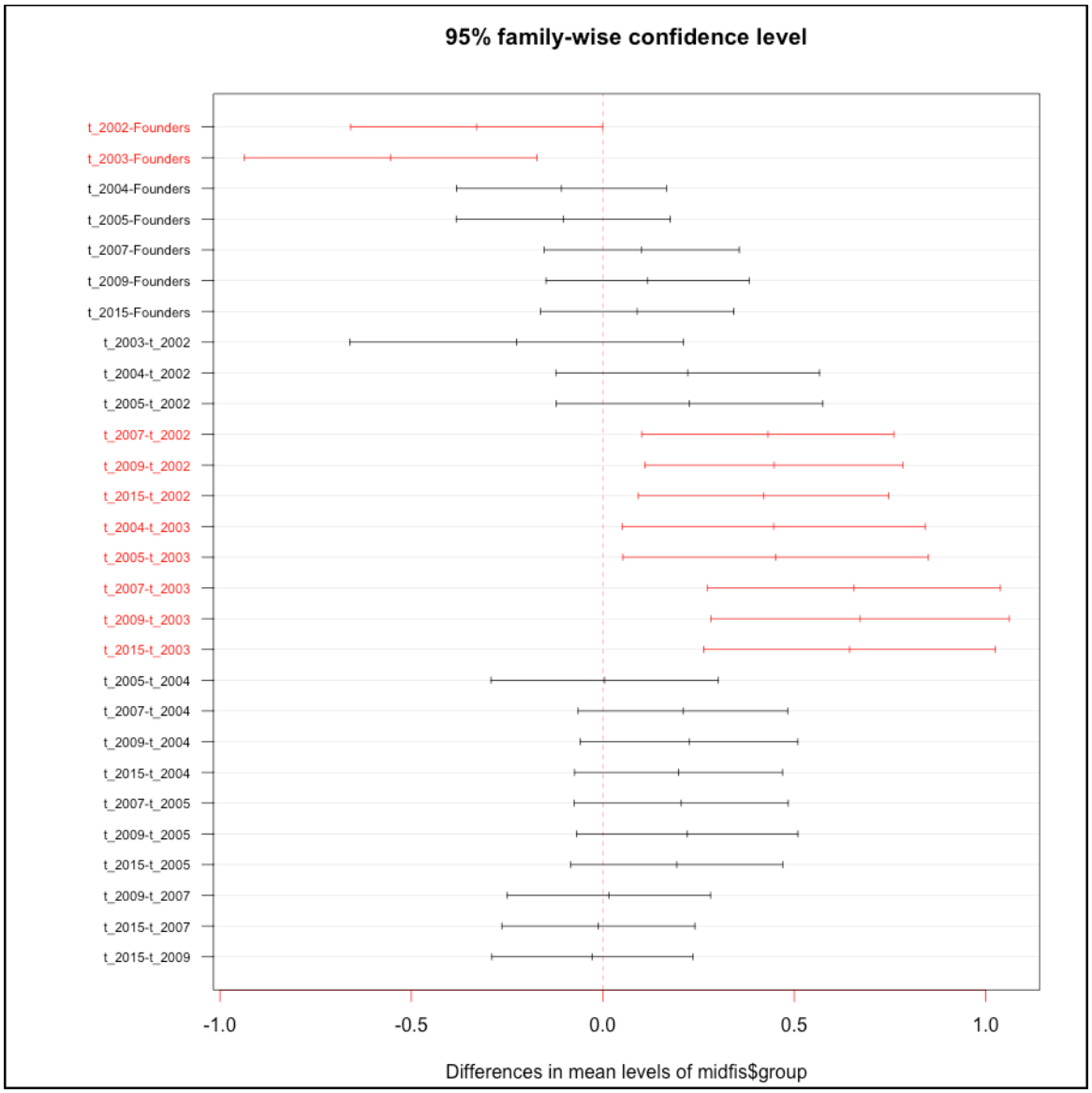
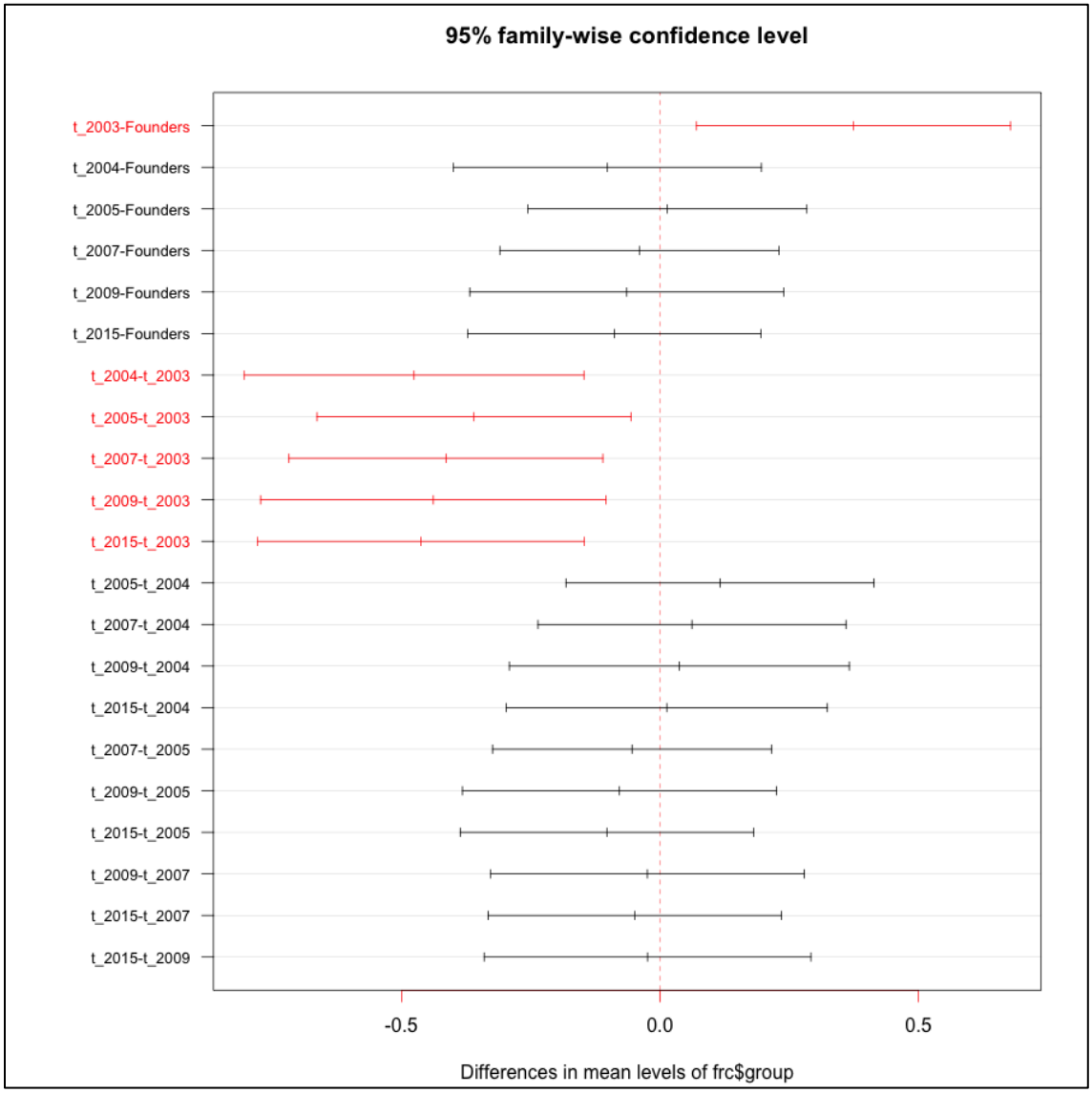


Figure C.2 Tukey-HSD in mean F_{is} values across years – Middle Cay

Post-Hoc Tukey-HSD test to compare differences in mean F_{is} values across years for samples collected on Middle Cay. Significant differences after Bonferroni correction for multiple comparisons are highlighted in red.

Figure C.3 Tukey-HSD in mean F_{is} values across years French Cay



Post-Hoc Tukey-HSD test to compare differences in mean F_{is} values across years for samples collected on French Cay. Significant differences after Bonferroni correction for multiple comparisons are highlighted in red.

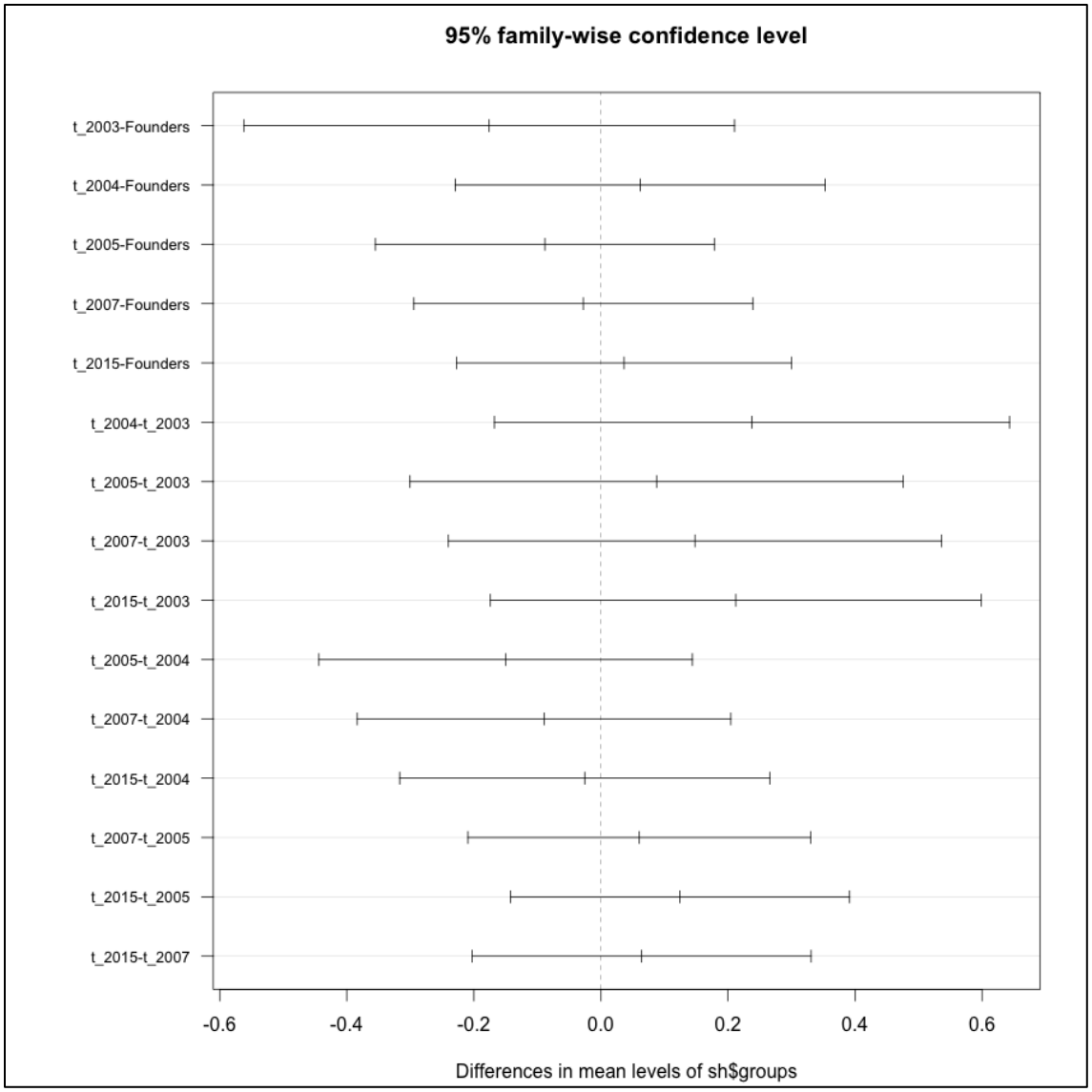


Figure C.4 Tukey-HSD in mean F_{is} values across years Six Hills Cay

Post-Hoc Tukey-HSD test to compare differences in mean F_{is} values across years for samples collected on Six Hills Cay. No significant differences were found after Bonferroni correction for multiple comparisons.