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Phylogeography of the Turks Island Boa, *Chilabothrus chrysogaster*

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Abstract

The Turks Island Boa is the oldest cladogenically distinct lineage of the extant Lucayan boas. It is represented by two subspecies- The Turks Island Boa (*Chilabothrus chrysogaster chrysogaster*) found in the Turks and Caicos Islands, and the Inagua boa (*Chilabothrus chrysogaster relicquus*) found in the Bahamas. The Turks Island Boa is extensively studied in Southern Caicos, especially on Big Ambergris Cay. The Turks Island Boa is found on both the Turks and Caicos banks, which are separated by the ~20km Turks Island Passage and have never been connected. A study from 2011 suggests that there is shallow genetic divergence across the two banks, finding a maximum of seven mutational steps between populations of boas on the two banks (Reynolds 2012). However, that study was limited by having only three samples from the Turks Bank and using only a fraction of a mitochondrial locus. In 2022, a new population of boas was discovered on the Turks Bank, which afforded the opportunity to gain greater insight into population genetic diversity and divergence across the Turks and Caicos banks. I sequenced 30 individuals at an 1077bp mitochondrial locus, including 12 new individuals from the Turks bank. I then aligned these new sequences with an expanded dataset from Reynolds et al. (2011), and conducted phylogeographic analysis using the package ape in R. I found conclusive evidence of a distinct Turks Bank lineage of boas that is 1.58% divergent from the Caicos Bank. But I also found that there is a nearly equal divergence across the Caicos Bank, suggesting that genetic divergence is not exclusive to each bank. These data are particularly important as re-introduction programs are designed for boas in the Turks and Caicos Islands.

Introduction

Phylogeography

Phylogeography is defined as a field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species. (Riddle 2019) The basis of phylogeography as a discipline comes from mammalian studies. One of the key early findings of these studies was the high frequency of “cryptic” lineages, which are distinct evolutionary lineages imbedded within a morphologically conservative species. This indicated a gap between biodiversity discovery at this scale and operationalization of new knowledge. This gap holds important implications for conservation management. These authors offer the following fall-throughs for phylogeography. First, it requires that hundreds of samples are processed which is cost prohibitive. Second, any further sequencing than mtDNA would require a better-quality sample of fresh DNA. This will slow the process of creating a larger genome-scale sequence library. Third, the creation of a genomic lab would either be extremely expensive and time consuming or would require the outsourcing of almost all protocols to commercial companies. Fourth, the analysis of genomic data is challenging and requires advanced bioinformatic skills to generate custom coding. The ideal next decade includes the creation of labs which would begin the training cycle of including more students and postdocs who would then go on to find more remarkable and useful insights into diversity and population dynamics. Mitochondrial DNA is a unique way to study population genetics because it is maternally inherited and not subject to large amounts of recombination. (Ballard 2005) Phylogeography has also led to the development of coalescent theory which is a way that researchers determine whether populations have been isolated, and if so, for how long. Walker et al used phylogeography to show matrilineal lineages within freshwater and terrestrial turtles in the Southeastern United States. This study supports the theory that species have deep phylogeographic structures. Many of the widely distributed turtle species showed deep matrilineal separations on a regional scale. These splits are not automatically equated to deep separations in population trees. The southeastern US possesses 2 major water drainage systems: the Atlantic and the Gulf drainage, which have extremely distinct faunal regions. Using divergence within the matrilineal line to map unique populations, we can see the divide between Atlantic and Gulf area testudines. Considerations for conservation that arise from phylogeographic studies includes the knowledge of which major centers of biogeographic diversity should be considered in biome-based conservation efforts. This can serve to enhance the effectiveness and impact of conservation programs by providing a more concise action plan.

Island Archipelagos as Models for Diversification

Island archipelagos are important models for understanding diversification due to their usual support of taxonomically diverse and species-rich lineages. One notable example is the Philippines Island (Brown et al. 2009). The Philippines has become a natural laboratory for the study of geographic impacts on production, partitioning, and maintenance of biodiversity. Wallace established a position called the Wallace Line

which marked an abrupt faunal change on the Philippine archipelago. This line was later modified slightly by another researcher.

This archipelago is representative of high amounts of endemic land vertebrate diversity, which leads to the question of what fundamental processes are present to fuel diversification and adaptive radiation. It is crucial to consider a multi-faceted model of biodiversity which would include things such as sea-level changes, patterns of diversification, geological mechanisms, and ecological features of the present landmass (Brown et al. 2009)

PAIC (Pleistocene Aggregate Island Complex) Diversification Model is a model which organizes species distribution by biogeographical sub-provinces which correspond to Pleistocene land connections. The land connections were established by tracing underwater bathymetric contours. Some islands within the Philippines are surrounded by deep channels and have never been connected via land-bridges to other PAICs. These deep-water islands hold restricted range endemics despite close proximity to large islands. There are a handful of testable predictions that have been derived from the development of PAICs including the following:

1. widespread species may exhibit greater among PAIC than within PAIC genetic variation.
2. fragmentation of populations within PAICs may result in greater variation within PAICs.
3. lineages within a PAIC tend to display monophyly.

This model has provided a plethora of testable hypotheses which have inspired new ways of testing hypotheses concerning evolutionary diversification on the archipelago. The Turks and Caicos Banks are 150-million-year-old sediment and limestone platforms. They have remained near the ocean's surface due to reef building and various components have emerged and sunk throughout time with both banks becoming fully emerged in the last 8-15 thousand years. The banks are separated by the Turks Island Passage and have never been joined (Reynolds 2011). This island set up is arguably similar to the model Philippines Archipelago in terms of diversification methods and adaptive radiation. It is likely that both the Turks and the Caicos banks were emergent as recently as 8000 years ago and these super-island complexes had low topographic relief. The only expected barriers to gene flow between boas were salinas, which are brackish wetland areas. This event explains much of the low-level of divergence within a bank. Frequent dispersal across the Turks Passage is unlikely, so one existing explanation for shallow divergence is that a colonization event was recent and either naturally or human-mediated.

Chilabothrus chrysogaster

The genus *Chilabothrus* has 14 species, all of which are found on islands of the Greater Antilles and Lucayan Archipelago (Reynolds et al. 2023). The latter region is a chain of islands stretching from just east of southern Florida south and east nearly to Hispaniola (**Fig. 1)**. These islands are politically administered by the Commonwealth of the Bahamas and the Turks and Caicos Islands Government. Remarkably, five species

of boas are known from this archipelago, including the Turks and Caicos Boa (*Chilabothrus chrysogaster*).

The Turks Island Boa (**Fig. 2**) is a medium to small-sized member of the genus *Chilabothrus*. The body shape is slender in most individuals, and there is significant sexual size dimorphism with females growing to much more robust proportions than males. Juveniles are orange to reddish and have dark gray dorsal markings, they transition to a gray color as they age in a process known as ontogenetic color change. At least 6 color patterns have been recognized within this species: “single row of spots or saddles (single); dorsolateral paired spots (pair); 2 or 4 dorsolateral stripes (stripe); little to no pattern present on ≥ 2/3 of the body length (little); stripes interrupted by paired spots or areas with no pattern (broken stripe); mixture of single, double, and saddle spots (mixed)” (Reynolds et al. 2020). The striped morphs of *C. chrysogaster* are restricted to the Caicos bank and occur at 15% within the Big Ambergris Cay population. *Chilabothrus chrysogaster* can occupy a range of habitats like closed-canopy tropical dry forests on North Caicos to xeric scrub vegetation on Big Ambergris Cay. They generally prefer rocky areas with dense vegetation and protective rock coverings. Large females are often found at the base of bushes. These snakes are largely nocturnal and are more active during warmer periods, and feed almost exclusively on lizards, with an occasional bird taken (Reynolds et al. 2023). *Chilabothrus chrysogaster* is the oldest cladogenically separate lineage of extant Lucayan boas, they diverged around 5 million years ago (Reynolds 2012). The species is represented by two subspecies: the Inagua boa (*C. chrysogaster relicquus*) found on the Inagua Bank in the Bahamas, and the Turks Island boa (C. c. chrysogaster) found on the Turks and Caicos banks (Reynolds 2012). The latter subspecies is extensively studied on the Caicos Bank, while nearly nothing is known about the former (Reynolds et al. 2023). The species is considered Near Threatened on the IUCN Red List (Reynolds and Buckner 2021) and is at risk owing to habitat loss and the introduction of damaging invasive predators such as cats.

Turks and Caicos Banks

The Turks Island boa is found on 10 islands on the Caicos bank, an archipelago of dozens of islands stretching about 122 km across. The species is extremely well-studied on the island of Big Ambergris Cay, towards the southeastern edge of the bank. This island likely contains the last ecologically-intact population of the species, with population density estimates on Big Ambergris Cay of just under 10,000 individuals (Reynolds and Davis, unpublished). At least one documented population, on South Caicos, has been extirpated.

The Turks Bank is separated from the Caicos Bank by a deep water channel, the Turks Island Passage (**Fig. 3)**. This ~30km stretch of water is over 2,000m deep, and indicates that the Turks and Caicos banks have never been physically joined to each other or any other land mass. Boas were historically recorded from the main island of Grand Turk, although that population is now functionally extirpated (Reynolds et al. 2023). In 2009, RG Reynolds and M Niemiller discovered a new population on the tiny

island of Gibbs Cay. This prompted a study (Reynolds et al. 2011) to examine genetic divergence across the banks, which used a <700 bp region of the mitochondrial locus cytochrome B (as well as a few nuclear loci) to estimate diversity and divergence within the species. That study found shallow genetic divergence and suggested that the species be considered as a single evolutionarily significant unit (ESU) which includes a number of insular populations which are assumed to be genetically interchangeable (Reynolds et al 2012).

Importantly, in March 2022, RG Reynolds discovered a second, and much larger, population of boas on the Turks Bank. East Cay is located about 9km southeast of the main island of Grand Turk and is an uninhabited, ecologically intact island. Few visitors go there, as it requires sailing directly into the trade winds, and is considered remote. In fact, most visitors to the island are involved in searching for lost bales of narcotics that frequently wash ashore. The island has a robust food chain, with abundant arthropods, lizards, and hence, a dense population of boas.

The discovery of this new population, as well as improved sampling and sequencing methods, prompts a necessary revisit to estimating diversity and divergence in the Turks Island Boas.

Methods

Fieldwork

In March 2023, I accompanied the Reynolds Lab to Big Ambergris Cay, Turks and Caicos Islands.

While on the island I had the opportunity to work on two different projects. The first of which is a long-term radiotelemetry survey of boa habitats on the island. This project has been the topic of research for two former students of Reynolds Lab and has included 4 cohorts of snakes. All individuals are large female who are chosen due to their large size which allows for the implantation of a radio transmitter and battery. The March cohort had been captured and transmitters installed in August 2022. Each afternoon (15:00) and evening (19:00) we would track the 10 snakes using a Radio Telemeter. The radio tracking units are each tuned to a specific frequency which is associated with an individual snake. We recorded the resulting GPS location for each snake twice daily as well as a physical description of their location (in a hole, under rocks, out and on the move, in a bush). We also recorded the time elapsed for ten pulses of the telemeter which correlates to the internal temperature of each snake. Some snakes tended to stay in the same location regardless of time of day or temperature while some wandered over a good distance each day. The snakes were much more active at night, and it was not unusual to see them on the move after dark. The resulting GPS data can be used to create range maps for individual snakes. We

can also use it to compare movement patterns during different seasons and identify conservation methods to preserve their habitats.

The second project I was able to work on was the collection of individual snakes to determine population size via mark recapture data as well as collection of body measurements to better understand the body morphology of this species. Each evening around 19:00 hours, researchers would go to the south end of the island which contains a handful of large rock piles where boas are known to spend time at after dark. We would walk and look for boas using head lamps and handheld flashlights. Once a snake was spotted and captured, it was placed in a cloth pillowcase and given a unique letter-number combination to allow us to release them at the same location they were caught. GPS coordinates were recorded at each point of capture. Some snakes were caught at ruins on the north end of the island or in roadways across the island, but the majority came from the rock piles on the south side. The following morning consisted of data collection. Each snake was weighed and had body measurements including snout-vent length and a handful of head measurements recorded. Then I was able to assist with inserting a unique passive integrated transponder, or PIT tag, which is a unique identifier similar to microchips used in domestic animals. If a PIT tag was already present, new body measurements were taken and included with data from the previous capture. Pictures of new individuals were taken against a white background to see their dorsal patterning. This data can later be used to visualize body morphology as well as estimate population density using mark-recapture equations.

While the samples I collected were not included in the following methods, they will be useful for future studies of this species.

Sample Collection and DNA Extraction

Samples of *C. chrysogaster* were collected by RG Reynolds between December 2007 and March 2022. Diurnal surveys included turning over and replacing cover objects. Nocturnal surveys were conducted by walking through suitable habitats with headlamps and flashlights. Samples were preserved in 95% ethanol and stored at -20 degrees C. Tissue was obtained by either clipping the distal 3-4 mm of the tail or clipping 3-4 ventral scales (Reynolds et al. 2011). Tails were sanitized using sterile wipes prior to and after clipping and an antiseptic adhesive was applied to prevent infection.

Previous study of divergence across the Turks and Caicos banks only had access to three DNA samples from the Turks Bank (Reynolds et al. 2011). Importantly, this current study is using three additional samples collected from Gibbs Cay as well as nine samples collected from East Cay in 2022, for a total of 15 samples from the Turks Bank. I also obtained additional DNA from 18 individuals from Big Ambergris Cay that were archived in the Reynolds Lab freezer.

I extracted whole genomic DNA from these 30 tissue samples using the Wizard SV DNA purification system (Promega, Madison, Wisconsin) according to the manufacturer’s protocol.

DNA Sequencing

The polymerase chain reaction (PCR) was used to amplify ~1100 base-pairs of cytochrome B (CytB) which is a portion of the mitochondrial genome. The CytB fragment was amplified using primers from Reynolds et al. (2013). Polymerase chain reactions were done in SimpliAmp® thermocycler under the following conditions: denaturation at 94 degrees C for 5 minutes, followed by 35 cycles at 94 degrees C for 30 seconds, 52 degrees for 30 seconds, 72 degrees C for 1 minute, and a final extension at 72 degrees C for 5 minutes. The reaction was then halted and held at -4 degrees C until it was removed from the cycler. Polymerase chain reaction products were visualized for amplification by gel electrophoresis. Successful PCR products were sent to the Genomic Sciences Laboratory at North Carolina State University, Raleigh, NC to be purified and sequenced in both directions on a Sanger sequencer. I then assembled contiguous sequences and checked each contig using Geneious® 10.2.1 (Biomatters, Auckland, New Zealand).

Alignment and Phylogeographic Analyses

I took the 30 sequences I generated and aligned them with an existing unpublished dataset of CYTB sequences from the species to produce a final alignment. All alignments were done in Geneious using the ClustalX algorithm. This final alignment included 57 individuals from the following locations: *Caicos Bank*: Big Ambergris Cay (n=26), North Caicos (n=11), Providenciales (n=4), Middle Caicos (n=1); *Turks Bank*: Gibbs Cay (n=6), East Cay (n=9). I exported the alignment as a fasta file, then loaded this into R version 4.2.3 using the ape package and the function *read.dna* (Paradis and Schliep 2019). I then created an external datafile with a vector of population labels for each sample. I added these population labels using the *rownames* function, which produced a data frame with each sequence and a vector of labels of island population names. I used the *haplotype* function in the R package pegas (Paradis 2009) to collapse each sequence into mitochondrial haplotypes, or groups whose sequences showed evidence of recent maternal ancestry. I then created a haplotype network using the pegas function haploNet. I then colored each haplotype by population and sized each haplotype by the number of sequences it contained using the pegas function haploFreq. I plotted the network using the function *prNet*, and I used the *replot()* function to make the plot interactive so that I could drag haplotypes around to make the plot easier to interpret. I then created a heatmap of genetic distance measures with a dendrogram on each side using the function *heatmap* in ape.

Figure 1 Map of the greater Caribbean region, showing location of the Lucayan Archipelago as well as the Bahamas and Turks and Caicos Islands (adapted from Reynolds et al. 2023)

Figure 2 A female Turks and Caicos Boa, *Chilabothrus chrysogaster*, emerging from a rock on Big Ambergris Cay. Photo by author

Figure 3 Map of the Turks and Caicos Islands (adapted from Reynolds et al. 2022), showing locations of the sampled populations. Blue islands represent those with documented populations of *C. chrysogaster* (after Reynolds et al. 2023).

Results

I created a final alignment of 57 individual sequences of mitochondrial DNA from *Chilabothrus chrysogaster*. This alignment consisted of 1,077 base pairs of cytochrome B, which spans the entire coding region of this gene. These 57 sequences collapsed into 17 haplotypes (Table 1). Five of these haplotypes (I-V) are exclusive to the Turks Bank, while the remaining 12 are exclusive to the Caicos Bank. Gibbs Cay consists of a single haplotype, while East Cay has four, and no haplotypes are shared on these islands. On the Caicos Bank, North Caicos has five haplotypes, Providenciales has two, Middle has one (there was only 1 sample), and Big Ambergris Cay has four. None of the haplotypes are shared among islands. This indicates that there is no gene flow between islands or across banks.

The maximum genetic distance between haplotypes across the banks is 17 mutational steps, a 1.58% divergence, while the maximum genetic distance within the Caicos Bank is 16 mutational steps, a 1.49% divergence (Fig. 4). A dendrogram of these genetic divergences reveals a distinct separation between the Turks and Caicos Banks (Fig. 5). We see two reciprocally monophyletic lineages, one for each bank.

Within-bank diversity on the Turks Bank is relatively low, while within-bank diversity on the Caicos Bank is nearly as high as between banks (Fig. 5). The low diversity seen in the Turks Bank comes from the small island size found there. Small populations experience a higher level of genetic drift which removes alleles, this results in reduced diversity.

Table 1 Quantification of haplotype groups and their locations within the Turks and Caicos Banks. Numbers in columns are the number of boas that belong to each haplotype group.

	Turks Bank		Caicos Bank			
Haplotype	East Cay	Gibbs Cay	BAC	Middle Caicos	North Caicos	Providenciales
I	6	0	0	0	0	0
II	1	0	0	0	0	0
III	1	0	0	0	0	0
IV	1	0	0	0	0	0
V	0	6	0	0	0	0
VI	0	0	0	0	1	0
VII	0	0	0	0	7	0
VIII	0	0	0	0	1	0
IX	0	0	0	0	1	0
X	0	0	0	0	1	0
XI	0	0	0	0	0	1
XII	0	0	0	0	0	3
XIII	0	0	0	1	0	0
XIV	0	0	15	0	0	0
XV	0	0	8	0	0	0
XVI	0	0	2	0	0	0
XVII	0	0	1	0	0	0

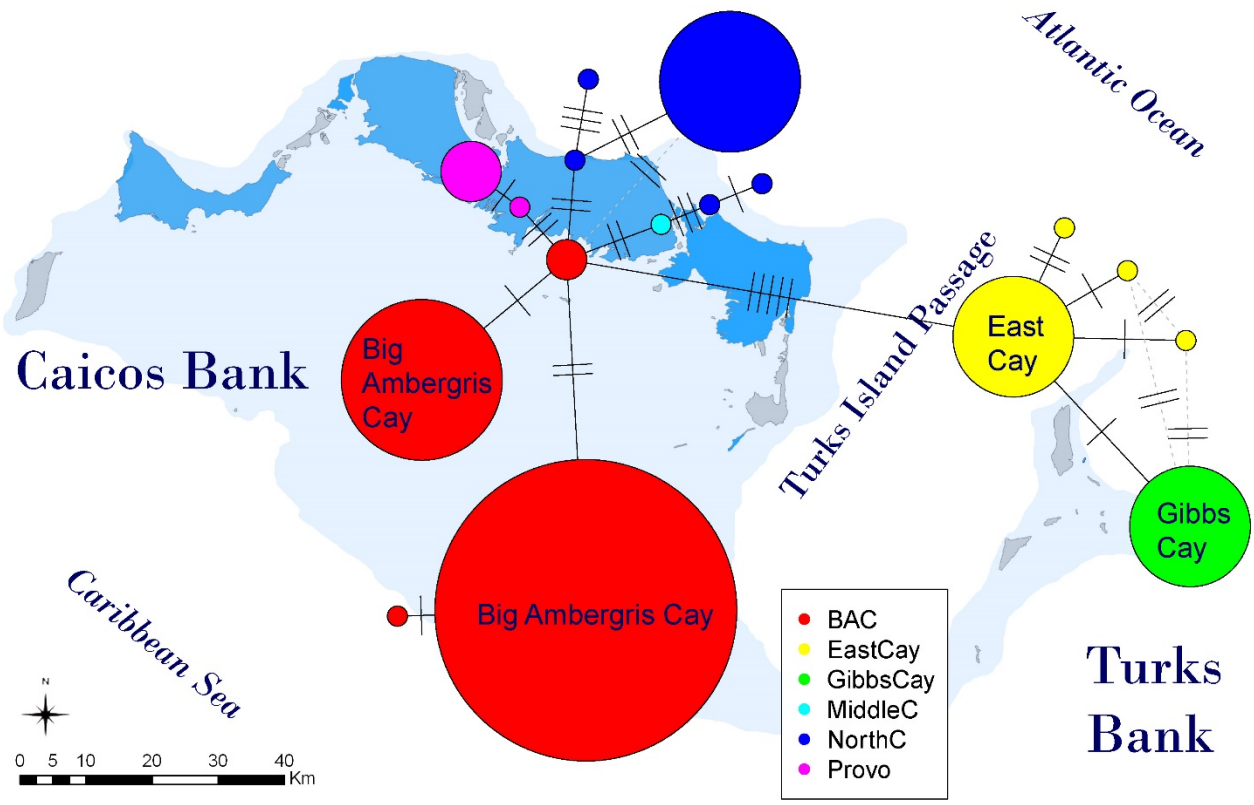

Figure 4 Haplotype network overlaid onto a map of the Turks and Caicos Islands. Size of the circle is based on number of individuals within the haplotype group and hash marks represent number of mutations between groups.

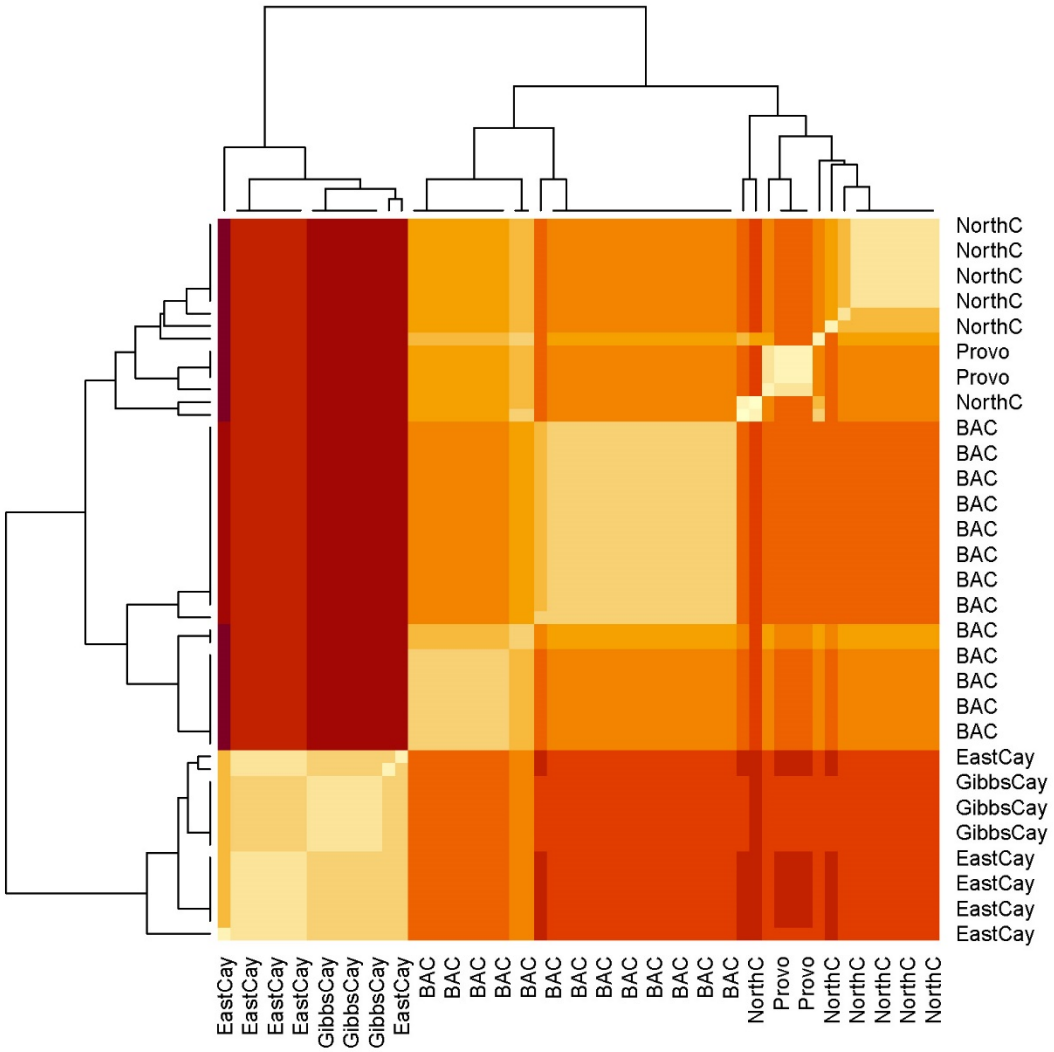

Figure 5 Heatmap of pairwise of genetic distances among sequences of *Chilabothrus chrysogaster* organized by island of origin.

Discussion

Genetic Divergence

The magnitude of genetic divergence within the Caicos Bank is equivalent to the magnitude of genetic divergence between the Turks Bank and the Caicos Bank. The Turks Bank does have a unique maternal lineage. The diversity of present lineages is higher within the Caicos Bank (eleven lineages) than on the Turks Bank (six lineages). When looking at key physical differences between the Turks Bank and the Caicos Bank, we see that the islands on the Turks Bank are much smaller. Having small islands means that the populations on these islands are also smaller and thus subject to high

levels of genetic drift and allele loss. This creates lower diversity which is in line with our findings. It is likely that as we find and include new populations of boas from the Turks Bank, we will not see a dramatic increase in Turks Bank diversity.

The diversity we see in between the two banks is expected based on the PAIC diversification model. Using this model, we would expect to see greater species variation between the two banks than within a single bank. The Caicos Bank (**Fig 4**) has the higher divergence (16 mutational steps) of the two banks, but it is still less divergent than the variation across the banks (17 mutational steps). We also expect to see monophyly within each bank (**Fig 5**). Each bank has a lineage that is reciprocally monophyletic. The Turks Bank boas share a most recent common ancestor with each other more recently than they share one with any of the Caicos Bank boas. This finding also reinforces the finding that there is no recent gene flow between banks.

One of the testable predictions from this model states that fragmentation within a PAIC may result in greater variation within that PAIC. This is applicable to the Turks and Caicos Boa because we see a similar fragmentation with the population scattered across island groups on two different banks. Moving forward with interspecies research, it would be interesting to consider other testable predictions regarding endemic species in the Philippine Islands and how applicable they are to other island groups such as the Turks and Caicos.

Molecular Phylogenies

As we move forward with conservation of *Chilabothrus chrysogaster*, we can use molecular phylogenies to contribute to a more accurate targeting of resources as well as a basis of information on population statuses (Moritz 1995). Describing biodiversity is the foundation of conservation. Using mtDNA in conjunction with known geographic parameters, we are able to divide populations into management units (MU) which are sets of the population that are currently demographically independent and evolutionarily significant units (ESU) which are historically isolated sets of the population. These units together encompass evolutionary diversity of a particular species (Moritz 1995). Using these definitions, I would tentatively define each haplotype group as a MU because the differences seen here are defined only with differences in allele frequencies. I would go on to define the haplotype groups found on each bank (Turks Bank and Caicos Bank) as two potential ESUs. ESUs are good subjects for long term conservation efforts because the groups are larger and less subject to genetic drift.

Obviously, the use of molecular analysis can be beneficial in defining conservation units as well as making assumptions about population processes over time (Moritz 1995). Mitochondrial DNA can provide valuable insight into a snapshot of evolutionary time. Seeing haplotype groups and maternal lineages can show greater evolutionary trends. On the other hand, looking exclusively at molecular processes can be misleading in regard to current population changes. Thus, it is important to also generate data that can be used to compare populations in the future against the current

population in order to better understand population processes such as genetic drift. It is my hope that by sequencing and helping to build the gene bank of the Turks and Caicos Boa, that I can help future researchers better quantify genetic relationships within this species.

Conservation Implications

As seen in Figure 4, the Caicos Bank has a much higher level of diversity within its population of Turks and Caicos Boa. The Caicos also has more large islands which can support large populations of boas. Large populations experience less genetic drift and retain more alleles which results in a greater level of diversity. The largest historical population present in the Turks was likely found on Grand Turk but has since been extirpated. Factors with negatively impact boa populations include human development, domestic animals and roadway mortality. Because *C. chrysogaster* populations look very different on each bank, conservation efforts should reflect this. Conserving diversity is important within the Caicos Bank because the Turks Bank is predominately small islands which do not support large amounts of diversity. In locations such as Big Ambergris Cay, conservationists work with island managers to irradicate rats, cats, and other domestic animals. They also advocate for reduction and caution surrounding new developments to protect the existing habitats and home ranges of the boa. These researchers work with grants such as the Darwin Initiative to fund their research and planning for future conservation efforts.

Population genetics is an important first step in conservation assessments and allows for better guidelines in resulting conservation strategies. For conservation biology to be effective in setting priorities and managing guidelines, an objective point of view is needed (Crozier, 1997). It is important to use a multifaceted approach to both define new species as well as identify species of conservation importance.

The development of new phylogenetic measurements to define species boundaries and justify conservation of said species has given rise to two main types of reasoning for marking species of high conservation worth (Crozier, 1997). The first position is the moral position which justifies each species as having the right to exist and thus be conserved. The contrasting position is called the utilitarian position, where the point of view is that humans derive material benefit from the existence of other species. It is my opinion that these two positions both have merit within the conservation biology realm. The moral position is interesting because it assigns a right to exist to a non-human species. Having every species worthy of conservation, however, is not practical. With current species guidelines, species can be separated by a single geographic barrier or a single gene mutation. This calls for the need to look holistically at species definitions as well as the necessity of the utilitarian conservation position. This states that human survival is enhanced by the existence of large numbers of species based on potential resources humans could gather from certain species. This has led to the field of bioprospecting in which scientists survey land for species that

humans could use for novel biological research. As such, the diversity of Nature has inherent value in a variety of contexts.

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