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Genetic Diversity in Captive Lineages of the Endangered Puerto Rican Boa, *Chilabothrus inornatus*

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Abstract

The endemic endangered Puerto Rican boa (*Chilabothrus inornatus*) has faced a variety of perils in its natural habitat, making it a species of concern in recent decades. While information regarding genetic diversity in wild populations of this species is finally being accrued, the viability and relative genetic diversity contained in captive populations is undetermined, which hampers efforts to develop a captive species management plan. Here that analysis is provided using an 1100bp fragment of the mitochondrial cytochrome *b* gene as well as five nuclear microsatellite loci. Samples were acquired that represent fifty boas from captive populations in the United States, a collection which included accessions from public and private collections. Relatedness among individuals was determined as well as overall genetic diversity in the captive population. These data were then compared to that obtained from wild populations across the island of Puerto Rico to determine genetic diversity in the captive population relative to wild lineages. A surprisingly low level of inbreeding and magnitude of genetic diversity has been conserved in the captive linages of *C. inornatus*. Genetic diversity parameters measured in captive lineages are on par with those found in wild populations. These results inform current breeding strategies as well as offer additional information that will facilitate continuation of captive conservation work.

1. Introduction

Chilabothrus inornatus (formerly *Epicrates inornatus*)[1] is a species of snake belonging to the Boidae family (Figure 1). This species is listed as federally endangered by the United States Fish and Wildlife Service (USFWS) and is protected under the Endangered Species Act of 1973. Collecting and hunting of this species is prohibited by the Puerto Rican government[2]. The inability to legally harvest new members of the species leads to the need for a well-informed management strategy of captive populations. Although many of these protections have been in place for several decades, little is actually known about this species' biology and ecology.

C. inornatus belongs to a genus comprised of 13 species, which diversified on islands of the West Indies[3]. The species evolved on the smallest of the four main Greater Antilles islands- Puerto Rico[4]. It is the largest native snake species on the island, reaching lengths up to two meters and a mass of up to six kilograms[5]. The species' nocturnal nature, crypsis, and tendency to reside primarily in heavily forested terrain makes it a difficult subject to study[6].

Figure 1. Adult (left) and juvenile (right) wild Puerto Rican Boas from Arecibo, Puerto Rico. © RG Reynolds.

Fortunately, the collection of knowledge on this species has increased in recent years[4]. Several studies have been able to accrue biological information on *C. inornatus* such as genetic diversity, spatial ecology, role in their ecosystem, habitat requirements, and diet[6,7,8]. Subsets of the population have notably been observed regularly congregating at the opening of bat caves, oftentimes catching their meals in midair[6]. This species is threatened by collection for traditional medicine and the pet trade, as well as suburban traffic (cars and people), and invasive predators including cats and mongooses[4,9]. A five-year evaluation conducted by the USFWS completed in 2011 determined that the species should be kept at the "endangered" classification owing to lack of knowledge and data regarding the species. The report encouraged the collection of additional information on the species so that research may accrue a better understanding of its ecological status and thus implement better conservation strategies[10].

The ability to analyze a species' genetic information, and to use this information in making informed conservation decisions, has proven to be a successful and increasingly vital component of species conservation. The implementation of molecular genetic data in captive breeding programs of endangered species has proven thus far to be a very helpful conservation strategy[11]. Oftentimes captive populations of a species are limited in numbers, leading to concerns about inbreeding depression and other related issues. By analyzing genetic data, it is possible for biologists to determine relatedness between individuals in captive collections, and to determine the most beneficial pairings for breeding programs. Avoiding breeding among kin is one of the most effective means of conserving genetic diversity in captive species[12]. Molecular data has also been found to be superior to pedigree information alone in making these decisions, particularly for endangered boid snakes[11].

The first genetic study of *C. inornatus*, conducted by Puente-Rolón, Reynolds, and Revell[13], focused on wild specimens. The study was able to ascertain the extent of genetic variation in wild populations at a coarse scale and suggest conservation strategies based on these results. In addition, a total of 48 microsatellite loci have been developed for this species using next-generation sequencing techniques, and 23 of these have been fully described and shown to be polymorphic in the species[3]. Additional unpublished genetic data exist for over 250 wild boas from Puerto Rico (Dr. Graham Reynolds), which provide a more complete picture of the extent of genetic diversity across the island.

Ex situ conservation, or the preservation of species in captivity, is a common component of species' conservation plans, and many public and private breeders construct pedigrees of the species to facilitate informed stud (breeding) decisions. A fact not well appreciated (and never characterized) until the present study is that a fairly large number of captive *C. inornatus* are kept in public and private collections in the mainland US. These are found within zoos and aquaria (public) and snake breeder collections (private). Importantly, there is no known registry or studbook for endangered *C. inornatus* kept in captivity, and no genetic studies have been conducted on the captive population. This study involved the documentation and characterization of captive breeding of *C. inornatus* in the US, and it is the first genetics study of captive lineages of this species. Further, this study is the first to use the newly-developed microsatellite library[3] on this species. A study of this nature will provide valuable information regarding current genetic diversity found in captive populations as well as how best to maintain that diversity by determining which individuals might be best paired in future breeding endeavors. A comparison of captive-versus-wild genetic diversity also allows the assessment of the extent of genetic diversity that is being preserved in captivity. The goal of these

analyses is to provide information to better facilitate the management of this species and to minimize potential for inbreeding, while encouraging the creation of a genetically-informed studbook or other captive breeding management database for public and private breeders.

2. Methods

2.1 Sample Collection

I attempted to secure samples representing the breadth of the US captive population of Puerto Rican Boas. Numerous private breeders and zoos were contacted and information on whether they keep or breed this species and samples of their specimens were requested. Individuals with knowledge of the whereabouts of captive animals were also contacted in order to track down which collections might contain *C. inornatus*. Dr. Peter Tolson of the Toledo Zoo (Table 1) and Mr. Jeff Murray (a knowledgeable private breeder) were of crucial assistance in this regard, and they contributed a great deal of assistance in identifying public and private breeders, respectively. When requesting samples from zoos, the submission of research justification forms and narratives were often required, while private breeders were assured that the information from the study would be both anonymized and made available to them. Samples were mailed to UNC Asheville in the form of dry freshly-shed skins sealed in plastic bags.

Table 1. Holdings of *C. inornatus* in public zoos in the US.

Institution	Males.Females
Hogle Zoo	0.1
Akron Zoo	0.2
Sacramento Zoo	1.0
Gul Shores Zoo	0.1
Disney (Orlando)	2.1
Brookfield Zoo	2.0
Abilene Zoo	1.0
Indian River Zoo	0.1
Drehler Park Zoo	1.0

In total, shed skins from 50 captive individuals were obtained; two from the Hogle Zoo in Utah, one from the Alabama Gulf Coast Zoo, and 46 from private breeders. Each individual was assigned a number 1-50. There are several other zoos with this species, though no zoo has more than three individuals and they are not actively bred at any at present. Most zoos either declined to submit samples, did not want to wait for animals to shed, or required conditions that were beyond my ability to accede to, including elaborate research proposals. It is unknown how many private breeders declined to share samples, but information gathered over the course of this study suggests that stock is exchanged among private breeders and thus a relatively small number of samples should yield a good representation of the diversity in these collections. Extracted DNA from these sheds was accomplished using the Wizard SV© Genomic DNA Purification System[14] and associated *Quick protocol* steps, with additional digestion time (18 hours minimum). Shed skin samples and extracted DNA products at were stored at -80°C.

2.2 mtDNA Nucleotide Sequencing

For each of the 50 DNA samples, the mitochondrial DNA gene cytochrome B (*CYTB*) was amplified via polymerase chain reaction (PCR). Reactions of 25 µl were prepared with a master mix composed of 10.4 µL H_2O, 5 µL PCR buffer, 2.5µL $MgCl_2$, 1.5µL dNTPs, 1.25µL each of forward and reverse primers, and 0.125µL of *Taq* DNA Polymerase for each sample. All PCR reactions were performed in SimpliAmp® Thermocyclers (Applied Biosystems©, CA). PCR products were visually checked via agarose gel electrophoresis post-stained with ethidium bromide for approximately 45 minutes, finding that 43 of the 50 samples amplified successfully. PCR products from these 43 samples were then sent to the Genomics Sciences Laboratory at North Carolina State University for clean-up and sequencing in both directions.

Resulting sequence reads were cleaned and aligned in Geneious® 10.1.1 (Biomatters©, New Zealand). For each sequence read a contig was created, comprised of the forward and reverse sequencing reads, then checked by eye for errors or gaps. The consensus sequence from each contig was then aligned with others using the ClustalW[15] algorithm in Geneious. To infer the number of haplotypes present in captive lineages of the Puerto Rican Boa, the alignment was exported as a *fasta* file, then inferred haplotypes in the online program FaBox[16]. These haplotypes were then aligned with haplotypes from across the native range of the species (Dr. Graham Reynolds, unpublished data) and inferred a maximum-likelihood phylogenetic gene tree in Geneious using the RaxML algorithm[17]. To visualize diversity and relationships of haplotypes in the captive lineages, a haplotype network was created in the program Arlequin v3.5[18]. The haplotype file was imported and from it a minimum-spanning tree was inferred. This tree was visualized using the program Hapstar[19]. Summary statistics were calculated in Arlequin v3.5: N: number of individuals sampled, S: number of segregating sites, *n*: nucleotide variability, h: haplotype diversity, and π: nucleotide diversity.

2.3 Microsatellite Fragment Analysis

I began by testing 17 of the available microsatellite markers developed in 2014[3] (M1, M2, M3, M4, M5, M8, M10, M11, M12, M13, M14, M20, M30, M32, M33, M45, M47) on a subset of my samples. A M13 technique was used, whereby each PCR reaction contained the primer pair with an "M13" extension, a 19-base pair sequence tag (5'-CACGACGTTGTAAAACGAC -3') that allowed for ligation of a third forward primer 5' labeled with one of two fluorophores (6-FAM or HEX). Touchdown PCR (TD-PCR) on SimpliAmp® thermocyclers was used for amplification of all primers, consisting of denaturation at 95° C for 5 min; 10 cycles at 95° C for 20 s, 60°-50° C for 60 s, and 72° C for 40 s, stepping down 1° C from 60° to 50° C; 20 cycles at 95° C for 20 s, 48° C for 20 s, and 72° C for 40 s; and a final extension at 72° C for 10 min. Each PCR product was checked with the aforementioned gel electrophoresis technique. Consistent PCR products were obtained for nine primers (M1, M2, M3, M4, M11, M12, M20, M30, M32), which I then ran on all 43 of the 50 DNA samples that worked for the mtDNA PCR. Microsatellite PCR products were then multiplexed using the two fluorophores and sent these samples to the Genomics Sciences Laboratory at NCSU (four primers). Data obtained from Genomics Sciences Laboratory unfortunately lacked distinct ladder peaks and was therefore unusable. Remaining primer products were sent to Massachusetts General Hospital CCIB DNA Core facility at MIT in Cambridge, MA for fragment analysis via capillary electrophoresis on an ABI 3730XL automated sequencer. Data from one microsatellite locus (M11) failed to express peaks and was unusable. Therefore, five microsatellite loci yielded usable information for this study. Alleles were binned and called using the microsatellite extension in the program Geneious, using a range of fragment sizes based on the known repeat motifs.

Summary statistics were calculated with the *adegenet* package[20] in R v3.4.1[21]: number of alleles (NA), observed heterozygosity (HO) and expected heterozygosity (HE). An inbreeding coefficient was calculated ($F_{bar} \sim F_{IS}$) across all samples from captive specimens, using *adegenet*. Genetic groupings were estimated within the captive lineages using first a principal components analysis implemented in R, followed by attempting to find axes of discrimination among genetic clusters using a discriminant analysis of principal components analysis[22] (DAPC) in the *adegenet* package in R.

3. Results

I found that a total of only 13 individuals of *C. inornatus* currently reside in US public collections (zoos). Further, these individuals are largely derived from the same stock originally bred at the Toledo Zoo in the 1980's by Dr. Tolson. There was no recordkeeping on private breeding stock shared with me, thus I was unable to quantify the number of individuals in private collections, though I estimate it is between 100-200 reproductively mature individuals based on information from Mr. Murray.

3.1 mtDNA Analysis

A total of 39 of the 50 *CYTB* samples produced clean sequence data and were used in subsequent analyses. Samples that did not were likely due to the fact that sheds must be fresh and dry to obtain quality DNA. These trimmed and aligned sequences consisted of 1096 base pairs each, encompassing nearly the entire coding region of the cytochrome *b* gene. These sequences collapsed into 13 unique haplotypes among the US captive Puerto Rican boas. The distribution of haplotype alleles in public and private collections were determined and arranged in a haplotype network (Figure 2).

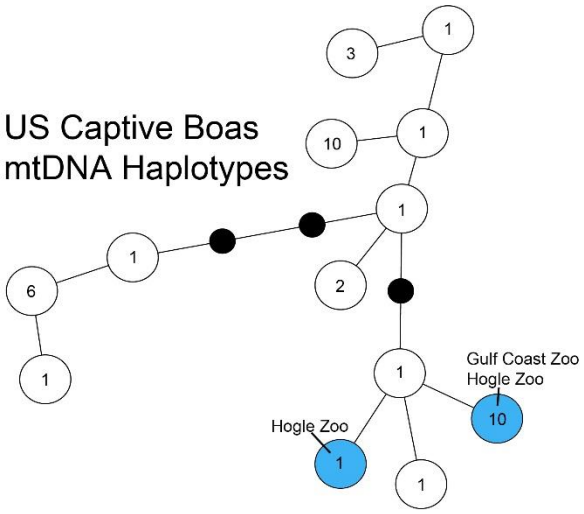

Figure 2**.** Haplotype network of captive boas.

Figure 2. Haplotype network constructed using data from captive boa lineages. The number of individuals of each haplotype is indicated within each haplotype circle. Black circles represent un-sampled mutational steps. Haplotypes found in public collections are indicated in blue.

Data on wild *C. inornatus* provided by Reynolds were used for a side-by-side comparison of genetic diversity found in wild and captive populations (Table 2). Another haplotype network constructed using the previously unpublished haplotypes from wild populations shows the relation of captive haplotypes to wild haplotypes (Figure 3). Surprisingly, captive haplotypes are found distributed widely within the network of wild haplotypes.

Table 2. mtDNA summary statistics retrieved from captive population and four wild Puerto Rican populations[13].

mtDNA	N	S	*n*	*h*	π
Captive	39	9	13	0.85	0.0025
Agrodel cave	7	10	3	0.57	0.0016
Mata de Platano Cave	21	13	2	0.81	0.0037
Dorado Beach	9	2	2	0.5	0.0006
Rio Encantado	15	27	9	0.93	0.005

N: number of individuals sampled, S: number of segregating sites, *n*: nucleotide variability, h: haplotype diversity, π: nucleotide diversity.

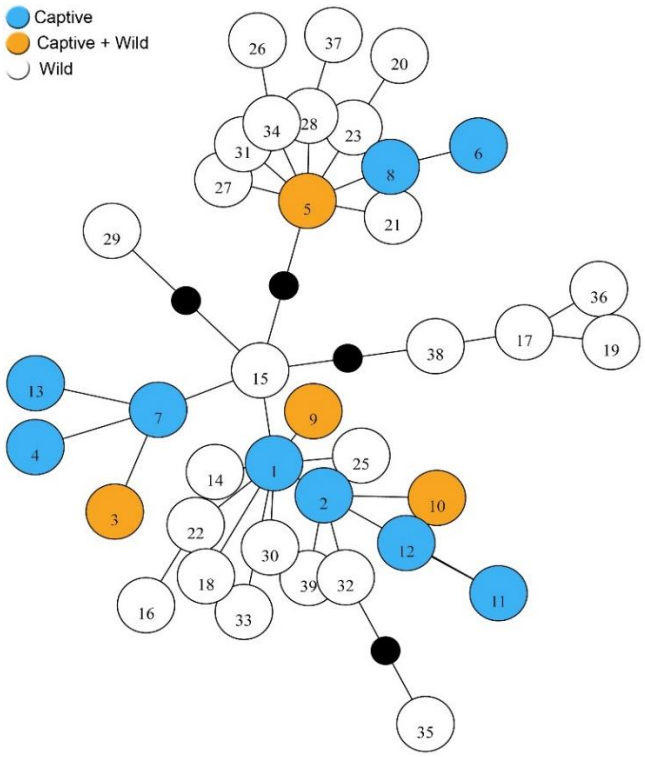

Figure 3**.** Integrated haplotype network of captive and wild boas.

Figure 3 Haplotype network made with data from this study alongside data from 178 wild boas (Reynolds, unpublished). Haplotype designations are indicated within each haplotype circle. Black circles represent un-sampled mutational steps.

3.2 Microsatellite Analysis

A total of 43 samples were genotyped for microsatellite analysis. Five of the primers yielded reliable peaks for allele-calling in Geneious. Using the *adegenet* package in R, summary statistics were calculated for each of the five loci, some of which haven't been published and thus are characterized here for the first time (Table 3).

Table 3. Summary statistics and additional characterizations for new and previously published microsatellite loci used to genotype samples in this study.

Locus	Repeat Motif	Size range	N_A	H_O	H_E
M1 (new)	$(AAAGGC)^n$	255-303	9	0.79	0.83
M2	$(AAGGAG)^n$	410-458	8	0.9	0.83
M3	$(AAGGAG)^n$	222-258	7	0.77	0.81
M4 (new)	$(ATATAC)^n$	252-277	5	0.74	0.71
M11	$(ATAGG)^n$	301-326	6	0.79	0.8

N_A is the number of alleles observed per locus; H_0 and H_E are observed and expected heterozygosity, respectively.

A t-test in R determined that the observed heterozygosity was not significantly lower than the expected heterozygosity (t = -0.07, df = 4, P = 0.52), which suggests no signature of inbreeding and therefore no apparent dramatic loss of heterozygosity in the captive population. I was also able to further estimate the extent of inbreeding in the captive population (Figure 4).

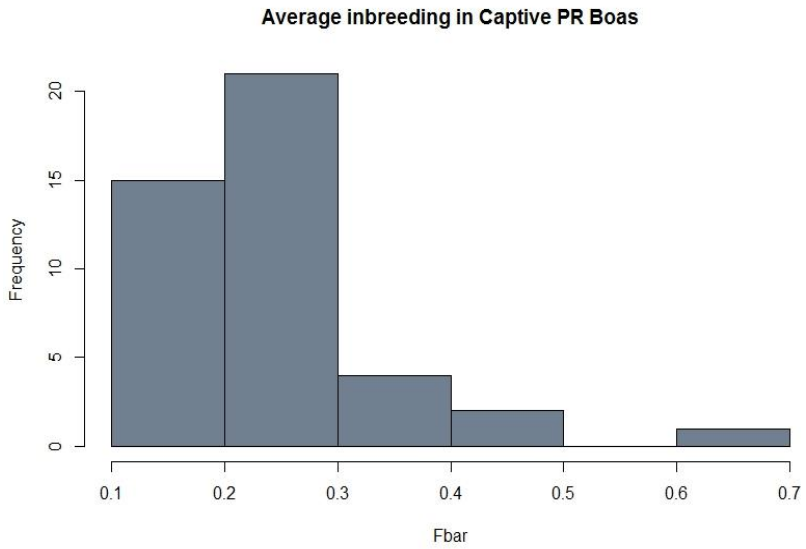

Figure. 4. Average Inbreeding in Captive Boas.

Figure 4 The majority of individuals in this study are not closely related: individuals found at lower values are less closely related whereas boas with a large signature of inbreeding are found closer to Fbar = 1.0

Only two of the individuals in this study (#36 and #43) are highly inbred, at the level of a consanguineous backcross (Fbar > 0.5). Twenty-four boas are about as related as siblings (in close range of Fbar > 0.25), and about a quarter of the individuals are less related to one another than siblings. A multivariate principal component analysis (PCA) showed spreading along the first two axes, suggesting a range of relatedness among genotypes (Figure 5).

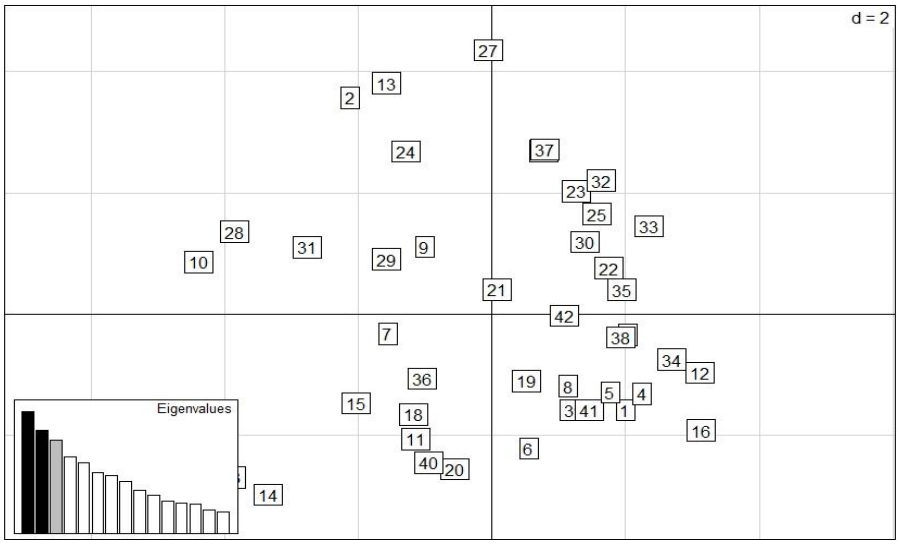

Figure 5. PCA visualization of genetic distance.

Figure 5 Analysis performed via eigenvalue decomposition and illustrates the degree of variation found when focusing on parameters of maximum diversity between individuals. In this instance, each number represents an individual boa and the spacing between each is correlated to overall genetic distance.

To examine whether distinct genetic clusters exist among captive *C. inornatus*, a Discriminant Analysis of Principal Components (DAPC) was conducted. This multivariate analysis method is designed to ascertain clusters of genetically related individuals without any prior knowledge on existing groupings. The appropriate number of PCA axes to retain was estimated using an optimization simulation within *adegenet*, finding that retaining 6-10 PCA axes maximized retention of variance without over parameterizing the model. This species is best represented by four separate genetic clusters (Figure 6).

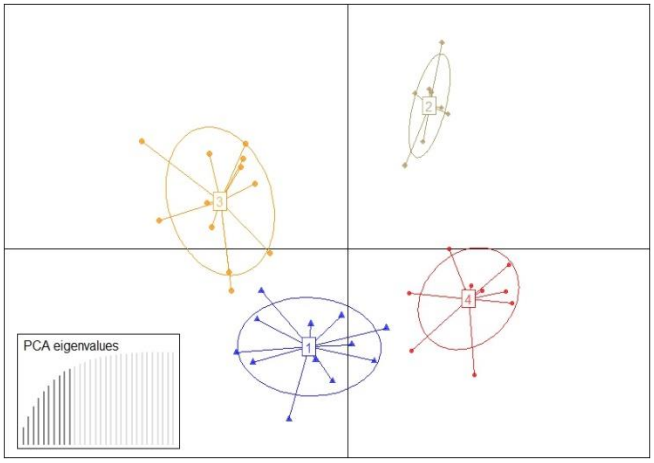

Figure 6. Graphical representation of the DAPC results.

Figure 6 DAPC results with retention of 10 PCA axes. Four distinct clusters are present in the captive population.

Jeff Murray and colleagues are in possession of boas in clusters 1-4, the boas from Hogle Zoo are in cluster 1, and the boa from the Gulf Coast Zoo is in cluster 3. The difference in lineage between the publicly owned boas shows that at least one of these institutions did not acquire their *C. inornatus* from the stock originally bred at the Toledo Zoo in the 1980's by Dr. Tolson. An individual genotype cluster probability graph shows the cluster membership of each individual boa (Figure 7). It is apparent that all but one of the forty-three individuals sampled belong distinctly to one cluster. Boa #23 is split evenly between clusters 1 and 3, indicating a cross between parents representing the two groups. The existence of distinct clustering of genotypes among captive snakes indicates that either these lineages are being deliberately maintained as distinct, or that perhaps each of these four clusters is primarily owned by a different breeder, or group of breeders, and are therefore somewhat or entirely reproductively isolated from one another.

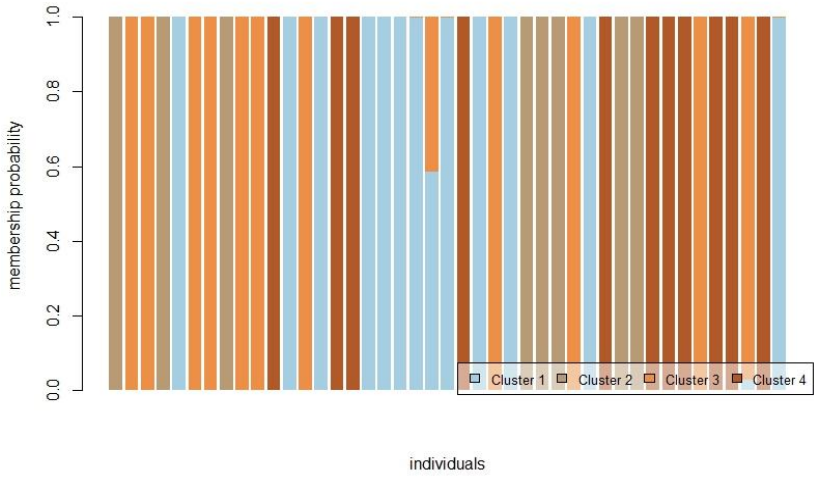

Figure 7. Individual genotype cluster probabilities.

Figure 7 Genotype probabilities for each captive boa represented by vertical bars, with boas #1 and #43 found first and last, respectively.

A side-by-side comparison of microsatellite characteristics of both wild and captive populations demonstrates the retention of genetic diversity in the captive lineages (Table 4). There is little discrepancy between the observed heterozygosity and F_{IS} between the two populations, which supports the previous finding of a lack of inbreeding in captive lineages.

Table 4. Microsatellite summary statistics for both captive and wild *C. inornatus* samples.

Population	N	N_A	H_0	F_{IS}
Captive	43	7	0.8	0.01
Agrodel	21	5	0.71	-0.02
Mata de Platano	7	4.7	0.61	0.2
Dorado Beach	9	4	0.63	0.09
Rio Encantado	15	6.3	0.89	-0.16

N: number of individuals, N_A: number of alleles, $N_{E:}$ effective number of alleles, H0: observed heterozygosity, F_{IS}: mean level of inbreeding

4. Discussion

This study attempted to characterize genetic diversity in US captive populations of the endangered Puerto Rican boa *C. inornatus*. Few US zoos have captive specimens, as I was only able to identify 13 specimens currently held in collections. However, many captive specimens exist in private breeder collections. From these public and private captive lineages, 50 samples were successfully obtained. Three of these samples were provided by public collections.

The original expectation was that little genetic diversity would exist in captive lineages. Surprisingly, my analyses found that 13 separate *CYTB* haplotypes exist within the 39 individuals that were successfully sequenced, suggesting high genetic diversity in captive lineages and likely within individual collections. The two most common haplotypes, both of which occurred in 10 individuals, were separated by five mutations. Given that fewer than 20 generations have likely elapsed since the founding of the US captive colonies, current captive stock represents a snapshot of genetic lineages currently found in the wild in Puerto Rico (Fig. 3). mtDNA data thus suggest that the original stock of females taken from Puerto Rico was obtained from a diverse number of regions on the island, and further, that quite a few females were used to found US captive populations. Using an already established map of haplotype occurrence in Puerto Rico,[13] I was able to ascertain from which regions the original imported stock was likely collected- finding that most females originate from the central and eastern Puerto Rican populations, rather than those in the west or south[13].

The close-relatedness of some of the individuals in this study, as shown by the microsatellite analyses, would generally hint at low genetic diversity. However, it seems as though keepers of this species have been very conscientious in breeding efforts to not only maintain lineages but genetic diversity within those. The degree of diversity seen in nuclear DNA indicates that a sizable number of males also made up the original imported stock. Four lineages, or genetic clusters, have been carefully maintained with only one boa in these results being a direct mix of two clusters. While the maintenance of these lineages is impressive, and in some circumstances beneficial, I recommend that the maintenance is unnecessary in this case, and that it would be most advantageous to freely interbreed among these lineages to further increase genetic diversity in individuals. While these lineages possess distinct genetic mutations, most if not all of these mutations are random rather than selected for adaptively. Therefore, the lineages are not so far evolutionarily separated as to encourage breeding them separately. This shift in strategy may help deter inbreeding depression generations down the line.

Both mtDNA and microsatellite analysis point at a well-maintained captive stock with an abundance of genetic diversity that is similar to that found in wild populations. The data generated in this study demonstrates that captive US lineages of Puerto Rican Boas are more diverse than expected, and that the captive population retains a higher-than-expected number of haplotypes and relatively low signature of loss of heterozygosity. Thus, the data generated in this study will allow for recommendations to breeders regarding genetically informed stud decisions and animal

sharing among breeding facilities. These data will also assist an in-preparation Species Survival Plan being developed by USFWS, Dr. Reynolds, and the North Carolina Zoo (Reynolds, pers. com.).

5. Acknowledgements and Funding

Mr. Jeff Murray was instrumental in helping to obtain samples from the private breeder community by sending samples from his own collection as well as posting messages on Facebook groups to encourage other breeders to contribute to the project. I am extremely grateful for his help. I am also grateful to Dr. Peter Tolson of the Toledo Zoo, who provided substantial information about where to locate captive specimens of this species in public collections. This project was funded by a University of North Carolina Asheville Summer Research Grant and the lab of Dr. Reynolds.

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