DIVERSITY AND CONSERVATION GENETICS OF THE MEXICAN BEADED LIZARD (*HELODERMA HORRIDUM*)

THESIS

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ABSTRACT

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The Mexican beaded lizard (*Heloderma horridum*), one of only two venomous lizards in the world, is distributed throughout central and western Mexico. There are four currently recognized subspecies including: *H. h. horridum*, *H. h. charlesbogerti*, *H. h. alvarezi*, and *H. h. exasperatum* and all are now considered threatened. Due to the lack of verifiable locality data and ambiguities in diagnostic characters, it is often difficult to determine subspecific identity among captive individuals. Because uncertainties exist, genetic analyses of mitochondrial DNA (mtDNA) and nuclear microsatellites were used to define the patterns of genetic variation and population structure for the captive populations of *H. horridum* in order to make a genetically effective management program. Both categories of markers revealed a high level of genetic differentiation indicating significant population structure. All phylogenetic results converge toward a similar evolutionary hypothesis, with five mtDNA groups present among two major clades. Clustering obtained from microsatellites is not congruent with the mtDNA phylogenies, but is consistent with the unification of the subspecies *H. h. charlesbogerti* and *H. h. alvarezi*. The most conservative management plan would be to preserve six populations of *H. horridum* until the taxonomy can be revised.

CHAPTER I.

INTRODUCTION

The Mexican Beaded Lizard (Heloderma horridum)

The family Helodermatidae consists of the only two extant venomous lizards in the world, the Gila monster *Heloderma suspectum* (Cope 1869) and the Mexican beaded lizard *Heloderma horridum* (Wiegmann 1829). The two species that encompass this family represent a unique lineage whose venom has a profound impact on the medical field for the treatment of type 2 diabetes. Due to significant habitat destruction and the popularity of recreational herptofaunal collections, most populations of *H. horridum* have declined (Johnson & Ivanyi 2004). Currently, both species of Helodermatids are protected throughout their ranges by various federal and state laws, as well as international treaties (Campbell & Lamar 2004). *Heloderma horridum* is listed in the Convention on International Trade of Endangered Species (CITES) under Appendix II, the International Union for the Conservation of Nature (IUCN) Red List for Threatened Species, and the Mexican authorities consider the lizard threatened (Johnson & Ivanyi 2004). Because only the lizards of the family Helodermatidae possess venom and because the venom has a direct human benefit, conservation efforts are needed to avoid the risk of extinction. The purpose of this paper is to quantify the existing genetic diversity and recommend management strategies to effectively maintain the genetic diversity of captive *H. horridum.*

Heloderma horridum is a robust lizard, averaging one meter in length and weighing approximately two kilograms. These lizards have cylindrical bodies with along tail encompassing at least 65% of their body, which is used for fat storage. The Mexican beaded lizard is found in dry scrub and a light woodland habitat throughout central and western Mexico and spends approximately 90% of their life in burrows (Campbell & Lamar 2004). Beck and Lowe (1994) state that one of the difficulties in studying *Heloderma* is that its metabolic rate is the lowest of any squamate reptile. The diet of Helodermatid lizards consists of prey items such as eggs, small mammals and ground nesting birds, which do not require immobilization through venom injection; therefore, venom use is considered a defense mechanism rather than a means of obtaining prey.

The Mexican beaded lizard was originally classified in 1829 by Wiegmann as *Trachyderma horridum*, and then re-classified in 1834 as *Heloderma horridum*. *Heloderma horridum* translated means "beaded skinned terrible one", referring to the skin underlain by osteoderms. There are currently four known subspecies of *H. horridum*. In 1956, Bogert and Martin del Campo published a monumental work on the genus, describing three subspecies of *H. horridum* including: *H. h. horridum, H. h. alvarezi,* and *H. h. exasperatum*. A fourth subspecies, *H. h. charlesbogerti,* was identified by Campbell and Vannini (1988).

The four subspecies occupy distinct ranges (Fig. 1). *Heloderma horridum horridum* is found in the Pacific drainages of Mexico from Sinaloa to Chiapas. The adults are mostly dark brown with distinct yellow bands and tend to become paler with age. *Heloderma horridum alvarezi* is found in central Chiapas and extreme western Guatemala. The adults found in this area are mostly dark brown or slate gray, with pale

markings that are reduced. *Heloderma horridum exasperatum* is found in southern Sonora and northern Sinaloa. The individuals found in this area often have the pale coloration exceeding that of the dark and may be slightly pink. *Heloderma horridum charlesbogerti* is found in the Rio Motagua Valley and adjacent foothills of eastern and southeastern Guatemala. The adults have the normal coloration, with a dark brown body and yellow bands (Campbell & Lamar 2004).

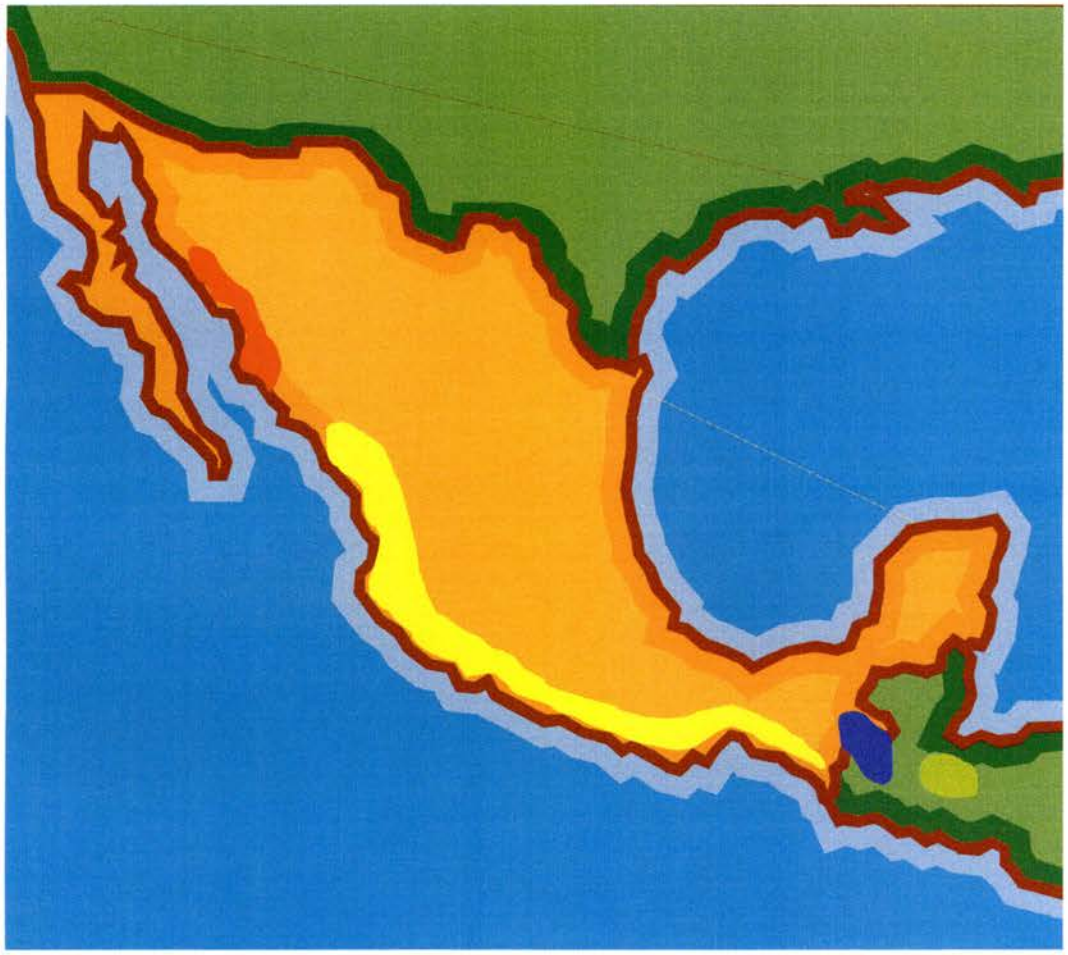


Figure 1. Current distribution for subspecies of *H. horridum* in western Mexico and Guatemala. (Yellow= *H. h. exasperatum*, Red= *H. h. horridum*, Blue= *H. h. alvarezi*, and Green= *H. h. charlesbogerti*).

Recently, the need of conservation for the threatened *H. horridum* has renewed interest for various management strategies. In an attempt to conserve this threatened

species, the first North American Beaded Lizard Studbook was published in 1995. The studbook is a compilation of information about the captive specimens of *H. horridum* and is used to aid in breeding management. At that time, all captive individuals were identified as belonging to one of the four subspecies based on morphological characteristics. Due to the lack of verifiable locality data for captive specimens and the overlap in diagnostic morphological characters, taxonomically grouping the captive individuals proved difficult; therefore, genetic methods were utilized. Because of inconsistencies with the preliminary phylogenetic results compared to the current taxonomy, all of the individuals were assigned a numerical value (1-6) instead of their respective trinomial. These preliminary findings using mitochondrial DNA (mtDNA) did not support the current subspecific taxonomy, but are currently being used to make breeding decisions for captive specimens (S. Davis & M. R. J. Forstner, unpublished data). Because of ambiguities in the morphological diagnosis of the four subspecies of *H. horridum*, and in order to preserve the true biodiversity of these subspecies, genetic conservation efforts must be employed to first define the genetic variation across the range of the species and second, to develop a more informed breeding design derived from that variation.

Conservation Genetics

Currently, we are in the midst of the most rapid extinction event since the demise of the species living during the Cretaceous (O'Brien 1994). To mitigate the factors threatening rare species, conservation biology has adopted an integrative approach that employs the principles of systematics, ecology, and evolutionary biology. An important

goal of this multi-disciplinary field is to maintain diversity at the molecular, species, community, and ecosystem levels, by first identifying possible conservation units at different levels of taxonomic hierarchy (M. Ruokonen, unpublished data). Additionally, conservation of intraspecific variation is a useful way to conserve variation among populations (Rhymer & Simerloff 1996) and is an important goal in the efforts to preserve biodiversity (Chambers & Bayless 1983). Investigations into certain endangered species have shown that the molecular genetic results contradict that of taxonomic distinctions based on phenotypic descriptions (O'Brien & Mayr 1991). Unfortunately, outdated taxonomic classifications based on overall similarity in morphological characters continue to be the bases for management and eligibility for protection, which undoubtedly hamper current conservation efforts (Avise 1989; Daugherty et al. 1990; May 1990; O'Brien & Mayr 1991; Mishler 1995).

Fueled in large part by the advent of molecular techniques, conservation biologists have been quick to recognize the utility of genetic data for the explicit purposes of objectively defining taxonomic relationships and the structure of populations (Avise 1989; Haig 1998). Conservation biologists utilizing genetic data are often interested in two general issues including 1) evolutionary distinctness of taxonomic units and 2) patterns of genetic variation within these taxonomic units (Rand 1996). Among the kinds of genetic data that are capable of resolving these issues, DNA sequence data and characterization of specific alleles found in the nuclear genome are most frequently used (Goldstein et al. 2000).

DNA sequencing has become a routine procedure since the development of the dideoxy chain termination method and has proven to be especially powerful when

combined with the analysis of various regions of the mitochondrial genome (Parker et al. 1998). Recently, mtDNA has become the most widely used marker for animal systematics due to its ease of isolation and interpretation (Avise et al. 1987). Mitochondrial DNA is small (15-17kb) and circular, and characterized by a matrilineal transmission and a rapid rate of evolution, making it an ideal marker for recovering intraspecific phylogenies (Brown et al. 1982; Parker et al. 1998).

A molecular phylogeny is a hypothesized evolutionary history based on the comparison of DNA or protein sequences. Soltis and Gitzendanner (1999) stated that phylogenetic classifications are essential to organize biodiversity in such a way as to set conservation priorities and develop informed conservation strategies. There are two general categories in which the methods for calculating phylogenetic trees can be grouped. These categories include discrete character methods and distance-matrix methods (Page & Holmes 1998). The least complex and most often used discrete character method is maximum parsimony. Maximum parsimony infers nucleotide sequences of the ancestral species and chooses a tree with the least number of mutational changes (Graur & Li 1999). In distance-matrix methods, relationships can be viewed by evolutionary distance. By counting the number of differences and dividing by the total number of operational taxonomic units (OTU's), the uncorrected distance can easily be calculated. Because evolutionary changes in sequences exist, a simple count of the differences between two sequences will underestimate how much evolution is occurring. Therefore, correction models have been developed to account for the differences. The algorithms for these models examine various possible topologies, but only one final tree

is produced. Therefore, it is easy to study the reliabilities of the trees produced by these methods (Sourdis & Nei 1988).

Nuclear genomes are much larger than mitochondrial genomes and contain both unique single copy regions and non-unique repetitive regions. These non-unique repetitive regions include mini- and micro- satellites, which are some of the most variable markers in the genome (Parker et al. 1998). Microsatellite analyses have become probably the most popular and powerful method for identifying highly polymorphic Mendelian markers (Scribner & Pearce 2000; Li et al. 2002). Each microsatellite locus consists of tandemly repeated units that are usually 2-6 base pairs long (Tautz 1989), and the variation in the number of repeats often underlies an abundance of distinguishable alleles within a population (Avise 2004).

Calculating the frequency of alleles found in the highly variable microsatellite loci greatly increases the detail with which populations can be described and differentiated. Additionally, allele frequencies can be used to assess levels of population structure and evolutionary processes (e.g., founder relationships, effective population size [N_e], and rates of gene flow), as well as estimating genetic variation within and among different hierarchical levels.

The genetic information revealed by such analyses is critical to making informed management decisions for the preservation and conservation of threatened and endangered species. In the last two decades molecular techniques, such as mtDNA sequencing and microsatellite analyses have provided important insight that has critically affected the management of endangered species (O'Brien 1994). Conservation biologists routinely use molecular techniques to assess genetic variability in terms of overall

heterozygosity, levels of population structure, and the genetic distinctiveness of taxonomic units (Avise 1995; Stockwell et al. 1996). Such techniques have been employed for numerous endangered specimens such as the Dusky Seaside Sparrow *Ammodramus maritimus nigrescens* (Avise & Nelson 1989) and the African black rhinoceros *Diceros bicornis* (Ashley et al. 1990).

The primary genetic goal of a captive breeding management program is to ensure that existing genetic variation be maintained in threatened and endangered species so that the possibility of recovery, adaptability, and persistence of these populations be preserved (Allendorf & Leary 1988; Hedrick 1996; Haig 1998). Factors such as inbreeding depression and genetic drift may cause genetic deterioration that can ultimately lead to extinction (Hedrick 1996). The loss of genetic variation resulting from inbreeding and genetic drift is of special concern for managing captive populations, which are often small and subdivided, making these populations more vulnerable to extinction. Alternatively, high levels of genetic variation may increase components associated with fitness (Allendorf & Leary 1988).

Before an effective management program for the preservation of threatened species can be designed, the specific status and genetic vulnerability of the populations must be assessed. Managers of captive populations directly oversee changes in genetic variation, population size, and population structure by determining which individuals reproduce, the rate at which they reproduce, and with whom they breed (Earnhardt et al. 2004). Pedigree analysis, which examines the genetic structure of a particular multigenerational population, may accurately model the changes in the genetic variation that will be essential for long-term conservation of the population (Lacy et al. 1995).

Identification of relatedness, as described from the pedigree analysis, is essential to prioritize breeding of individuals. By breeding individuals with the lowest relatedness, the retention of genetic variation is maximized.

Objectives

The purpose of this study is to provide genetic data relevant to the preservation of the genetic diversity in the threatened species *Heloderma horridum* by defining the genetic variation and delineating the relationships among subspecies based on two categories of molecular markers, mtDNA sequences and nuclear microsatellites. In addition, population genetic studies will be performed to quantify within- and among-population diversity. Using existing pedigree data for the captive population, the genetic contribution of founding individuals will be assessed, and these analyses will be useful for designing future breeding programs. Results from both phylogenetic reconstruction and population genetic analyses will be used to draw inferences on evolutionary relationships, captive population management, and provide insight relevant for conservation efforts. The goal is to maintain the genetic integrity of *H. horridum* while assuring viable populations for the future.

The research questions include: How is genetic variation distributed in captive populations of *H. horridum* based on the mitochondrial dataset? How is genetic variation distributed across microsatellite loci in captive populations of *H. horridum*? Is the genetic structure defined by the mitochondrial DNA congruent with the genetic structure revealed by the microsatellite allelic differences? What is the best approach for genetically informed management of the captive populations?

CHAPTER II.

MATERIALS AND METHODS

Taxon Sampling

Blood and tissue samples were obtained from captive specimens of *H. horridum* and *H. suspectum* from many public institutions, as well as the specimens held in the private sector in the United States, Canada, and Mexico. DNA was isolated from the blood and tissue using a Qiagen DNeasy extraction kit as directed by the manufacturer's instructions.

Mitochondrial DNA Analyses

The polymerase chain reaction (PCR) was used to amplify the mtDNA gene fragment ND4 to Leucine. The ND4 to Leucine gene region was chosen because it is characterized by rapidly accumulating silent substitutions and transitions, which make it an ideal marker for analyzing populations within a species (Avise 2004). PCR reactions included 35.25 μl ddH_2O, 5.0 μl 10x buffer, 5.0 μl $MgCl_2$, 2.0 μl dNTPs, 1.0 μl of both forward and backward primers, 0.5 μl of genomic DNA and 0.5 μl Taq polymerase. The primers used to amplify the mtDNA gene fragment were ND4 (CACCTATGACTACCAAAAGCTCATGTAGAAGC) and Leucine (CATTACTTTTACTTGGATTTGCACCA), with the internal primers HF1

(AACTATGAACGAACAAAAGCCGAAC) and HR1 (AGTGTTCGGCTTTTTGTTCGTTC). PCR amplification was performed on the MJ Research PTC-200, which consisted of 40 cycles of denaturing at 95°C, annealing at 50°C and extension at 72°C. The samples were held at 4°C until the results of the PCR were visualized on a 1% agarose gel using gel electrophoresis. The Marligen Rapid PCR Purification System, as well as the Agencourt AMPure PCR Purification kit, was then used to remove excess reagents from the PCR product. Cycle sequencing was conducted according to Beckman-Coulter specifications using 5.0 μl purified PCR product, 5.0 μl ddH_2O, 2.0 μl primer, and 8.0 μl DTCS QS master mix. The MJ Research PTC-200 performed 25 cycles of 96° C for denaturing, 50° C for annealing, and 60° C for extension. The Agencourt CleanSeq protocol was followed on the Biomek 3000 (Beckman-Coulter, Fullerton, CA) to remove unincorporated ddNTPs from the cycle sequencing reaction. The CEQ 8800 (Beckman-Coulter, Fullerton, CA) DNA sequencer was used to assay clean cycle sequence products.

The resulting mtDNA sequences were aligned and visually confirmed in Sequencher (GeneCodes Corp.). Unique haplotypes were identified in MacClade 4.05 (Maddison & Maddison 2000) and neutrality of the mitochondrial dataset was tested using Tajima's test of neutrality (Tajima 1989) in Arlequin 2.000 (Schneider et al. 2000). Mitochondrial gene diversity was estimated by calculating the number of haplotypes, haplotype diversity, and nucleotide diversity using Arlequin 2.000 (Schneider et al. 2000).

The unique haplotypes were imported into PAUP* v.4.0b10 (Swofford 2002) and a full heuristic search for the most parsimonious tree was executed. The tree was rooted

with *H. suspectum* defined as the outgroup. All uninformative characters were excluded and considered unordered and equally weighted. Character state optimization was set to ACCTRAN, the starting trees were acquired by stepwise addition beginning with a random seed, the branches were swapped using the tree-bisection reconnection (TBR) algorithm, and the saving multiple trees (MulTrees) option was in effect.

Unlike parsimony, neighbor joining (NJ) analyses do not assume that the evolutionary rate is constant or that lineages have diverged at equal amounts. Neighbor joining consisted of two main steps that were repeated until a tree was obtained. The first step consisted of choosing a pair of taxa to be joined. In the second step, distances from the new node to all other nodes were inferred (Bruno et al. 2000). MacClade 4.05 (Maddison & Maddison 2000) was used to assess the nucleotide composition and to determine the transition/transversion ratio. Based on these parameters, a bootstrap analysis was performed with 1,000 replicates and a topology including bootstrap values was obtained.

Another topology was derived using a NJ algorithm under the maximum likelihood settings using the evolutionary model obtained through Modeltest 3.7 (Posada & Crandall 1998). Modeltest selected the lowest Akaike Informative Criterion (AIC), as the model of DNA evolution that is most likely to fit the data. Maximum likelihood (ML) is an optimality approach. Similar to maximum parsimony, ML evaluated topologies from different trees and chose the best one according to an optimality criterion, the likelihood score. The likelihood score is usually evaluated by the logarithmic transformation, the log likelihood ($\ln L$). The topology that had the highest likelihood value was chosen to be the maximum likelihood tree (Graur & Li 1999).

Because of complexity and number of parameters, not all evolutionary models obtained from Modeltest can be implemented in MRBAYES (Huelsenbeck & Ronquist 2001). Therefore, the model with the lowest AIC that MRBAYES would allow was accepted. MRBAYES used MCMC, and a variant called Metropolis coupled Markov chain Monte Carlo $(MC)^3$ to approximate the posterior probabilities of trees. Using the default temperature parameter, one cold and three incrementally heated chains were run for one million generations, which allowed sufficient time for burnin to occur and the likelihoods to stabilize. The trees were sampled every 1,000 generations, and the results of the first 25,000 samples were discarded. A 50% majority rule consensus tree was created from the Bayesian results.

To exam the distribution of genetic variation, an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was implemented. An AMOVA partitions the total variance into separate components, each of which describes the amount of total variance at distinct hierarchical levels (Excoffier et al. 1992). Ratios of the variance components were then used to estimate the Φ-statistics, analogous to the hierarchical F-statistics (Excoffier et al. 1992). AMOVAs were conducted to assess the percent variation within populations and among the groups defined from the mtDNA phylogenies. The proportion of variance due to population subdivision was measured by F_{ST} values based on genetic distances between sequences of mtDNA haplotypes.

Microsatellite Analyses

Nuclear DNA variation was characterized at four microsatellite loci with di-nucleotide repeats (Table 1) specifically developed for *Heloderma* (Feltoon et al., in

review). PCR reactions were set up in 10.0 μl volumes, with 7.1 μl ddH_2O, 1.0 μl $MgCl_2$, 1.0 μl 10X buffer, 0.4 μl dNTPs, 0.125 μl of both the reverse and labeled forward primers, 0.05 μl *Taq* polymerase, and 0.2 μl of genomic DNA. Amplifications were performed on the MJ Research PTC-200, which consisted of 40 cycles of denaturing at 95°C, annealing at 50°C and extension at 72°C. PCR products were separated on the CEQ 8800, and alleles were compared with a standard sequence to determine size differences. Departures from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested across all loci using Arlequin 2.000. To minimize type-I errors, the probability values were adjusted for multiple simultaneous table-wide tests using the sequential Bonferroni adjustments (Rice 1989). Microsatellite gene diversity was quantified by calculating the number of alleles per locus, observed heterozygosity, and unbiased gene diversity using Arlequin 2.000.

A Bayesian algorithm was used to identify the number of genetically homogenous groups of individuals (K=the number of groups) among the ancestral individuals using the software program Structure 2.1 (Pritchard et al. 2000). Nonancestral individuals were excluded from this analysis because they could potentially overestimate admixture. In addition, Structure computed the likelihood that a given genotype originated in each population. Based on the likelihood scores, individuals of unknown origin were placed in their respective populations. Most parameters were left at their default values, with admixture assumed for the ancestry model and allele frequencies considered correlated. The burnin length was 50,000, and the simulation was run 300,000 times after the burnin to get the parameter estimates. The range of possible Ks tested was from one to seven with 10 runs carried out for each K. Using the log likelihood scores obtained from

Structure, an ad hoc statistic was plotted following the method developed by Evanno and colleagues (Evanno et al. 2005).

AMOVAs were conducted to estimate the genetic variance within populations and among the groups obtained from Structure. Genetic differentiation comparing pairs of populations was quantified by estimation of pairwise fixation indices based on variance in allelic frequencies using the θ estimates of Weir and Cockerham (1984).

Table 1. Primer sequences and characteristics of four variable microsatellite loci in *Heloderma horridum.*

Locus	Primer sequence	Repeat motif	No. of alleles	Size range
HELO-6.A6F	GATCAGGAGAATCAGGAGGTG	$(GT)_{17}$	12	179-219
HELO-6.A6R	GGCAGAGAAAACCAGTGTGTC			
HELO-G1582F	TGAGAAGAGGTTGTCTGTATCTC	$(CA)_{10}$	6	108-120
HELO-G1582R	TAGATACATAAATGCAGGCGCT			
HELO-12G7F	TGGTGCTTTCCGACTTC	$(TG)_8$	5	208-218
HELO-12G7R	CATGATAATTGGGTGTTACTG			
HELO-5B11F	CCTTGCCATTACTTGCTTT	$(TG)_{11}$	4	150-164
HELO-5B11R	TCCCCCTCCCTTTCTT			

Comparing mtDNA and Microsatellites

In addition to the AMOVAs previously executed, AMOVAs were also executed in Arlequin 2.000 to test alternative hypotheses of the hierarchical subdivision of populations resulting from the mtDNA and microsatellite analyses.

CHAPTER III.

RESULTS

Mitochondrial DNA Analyses

A total of 719 bp of the ND4 to Leucine gene fragment were amplified. Among the 180 individuals analyzed, 59 unique haplotypes were identified. A total of 49 *H. horridum* haplotypes were identified, which ranged from 2 to 14 haplotypes per population. The mean haplotype diversity was 0.7000 (range: 0.4167- 1.000), whereas mean nucleotide diversity ranged from 0.0014 to 0.0075 (Table 2). The mtDNA dataset had no significant departures from neutrality, according to Tajima's test of neutrality (Tajima's D= 1.7480, P= 0.96).

The topologies obtained from maximum parsimony, neighbor joining, maximum likelihood, and Bayesian analyses are shown in figures 2-5. For ease of discussion, clades have been labeled 1-6 as specified in the *H. horridum* studbook. Based on their mtDNA haplotype composition, captive *H. horridum* formed five distinct clades that were well supported (Figs. 2-5).

A total of 562 uninformative characters were excluded from the parsimony analysis, leaving 157 informative characters. A 50% majority rule consensus tree was obtained under maximum parsimony settings (Fig. 2).

The Hasegawa-Kishino-Yano (HKY85) correction model was applied to the neighbor joining (NJ) distance method to account for among-site rate variation. The

topology obtained from the HKY85 correction model applied to the NJ distance method is shown in Fig. 3, with bootstrap values shown on the clades.

Using the parameters designated from Modeltest, a maximum likelihood analysis was performed and the phylogram was bootstrapped (Fig. 4). The transversion model (TVM) of evolution with a gamma-distributed rate heterogeneity model and an estimated proportion of invariable sites was the most appropriate model of evolution for these data according to Modeltest 3.7. The parameters for the model included set base frequencies (A=0.3259, C=0.2541, G=0.1126, and T=0.3074), the proportion of invariable sites (i) equal to 0.5421, and a gamma distribution (g) with a shape parameter equal to 1.1592.

The (TVM) + i + g model chosen by ModelTest was too complex to be implement in MRBAYES. Therefore, the GTR + i + g, which allows all six possible substitution types to have different rates, was implemented. The parameters for the model included set base frequencies (A=0.3269, C=0.2524, G=0.1155, and T=0.3053), the proportion of invariable sites (i) equal to 0.53, and a gamma distribution (g) with a shape parameter equal to 1.1021. The frequencies of the bipartitions are shown on the clades of the topology created in MRBAYES (Fig. 5).

An AMOVA performed among the two major clades evident from the mtDNA phylogenies (types 1, 2, and 3 compared to 4, 5, and 6) revealed a $\Phi_{CT} = 0.6104$ ($P<0.0001$). The overall level of genetic differentiation within populations was high, as revealed by a $\Phi_{ST} = 0.3631$ ($P< 0.0001$). Significant heterogeneity ($F_{ST} = 0.9740$, $P< 0.0001$) between populations in mtDNA haplotype frequencies was also detected (Table 4).

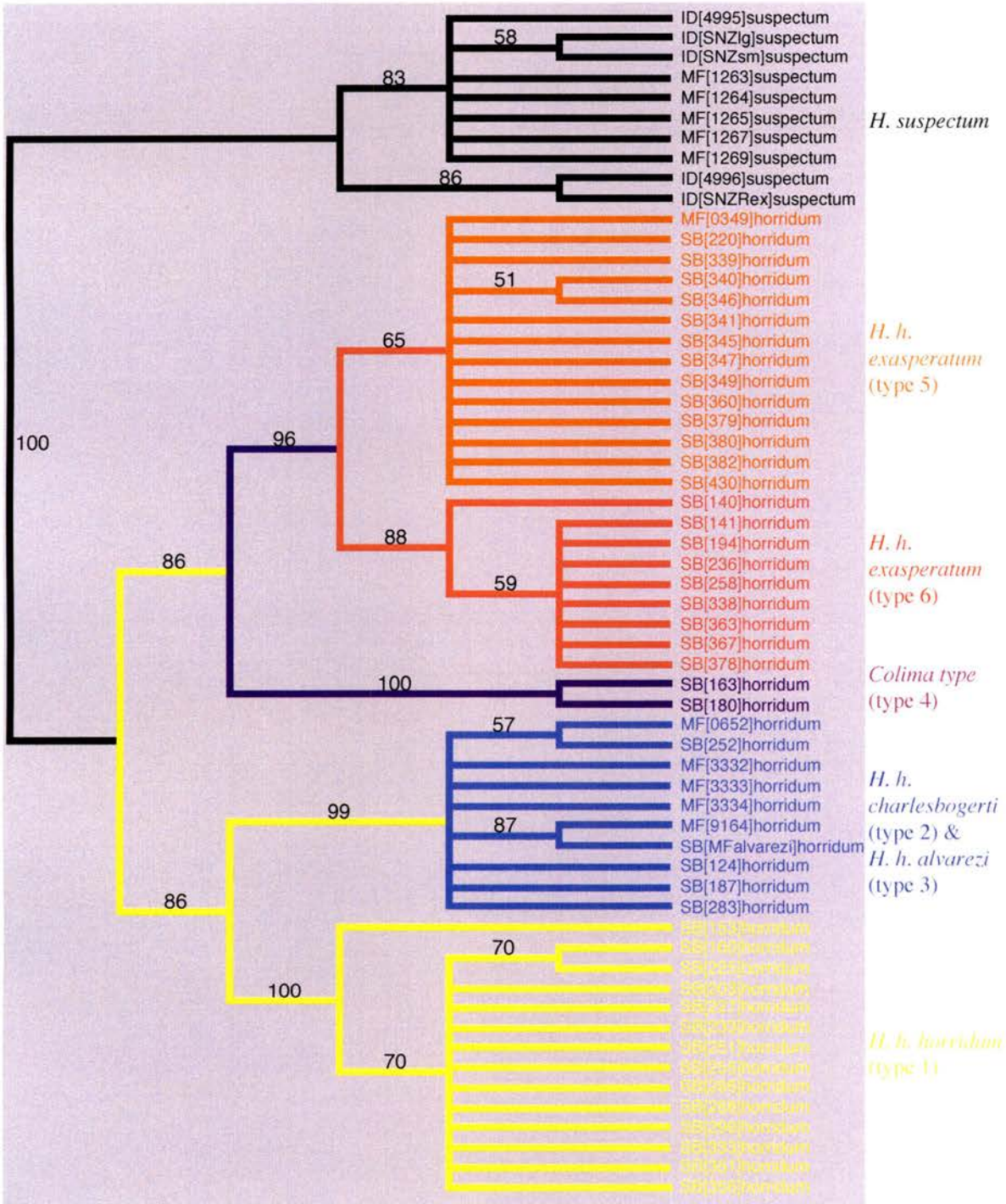


Figure 2. The 50% majority rule consensus tree obtained from a full heuristic search. The numbers on the clades are the frequencies of the bipartitions. The specimens used to create this tree are haplotypes. The remaining specimens, with identical sequences as those above, can be found in Appendix 2.

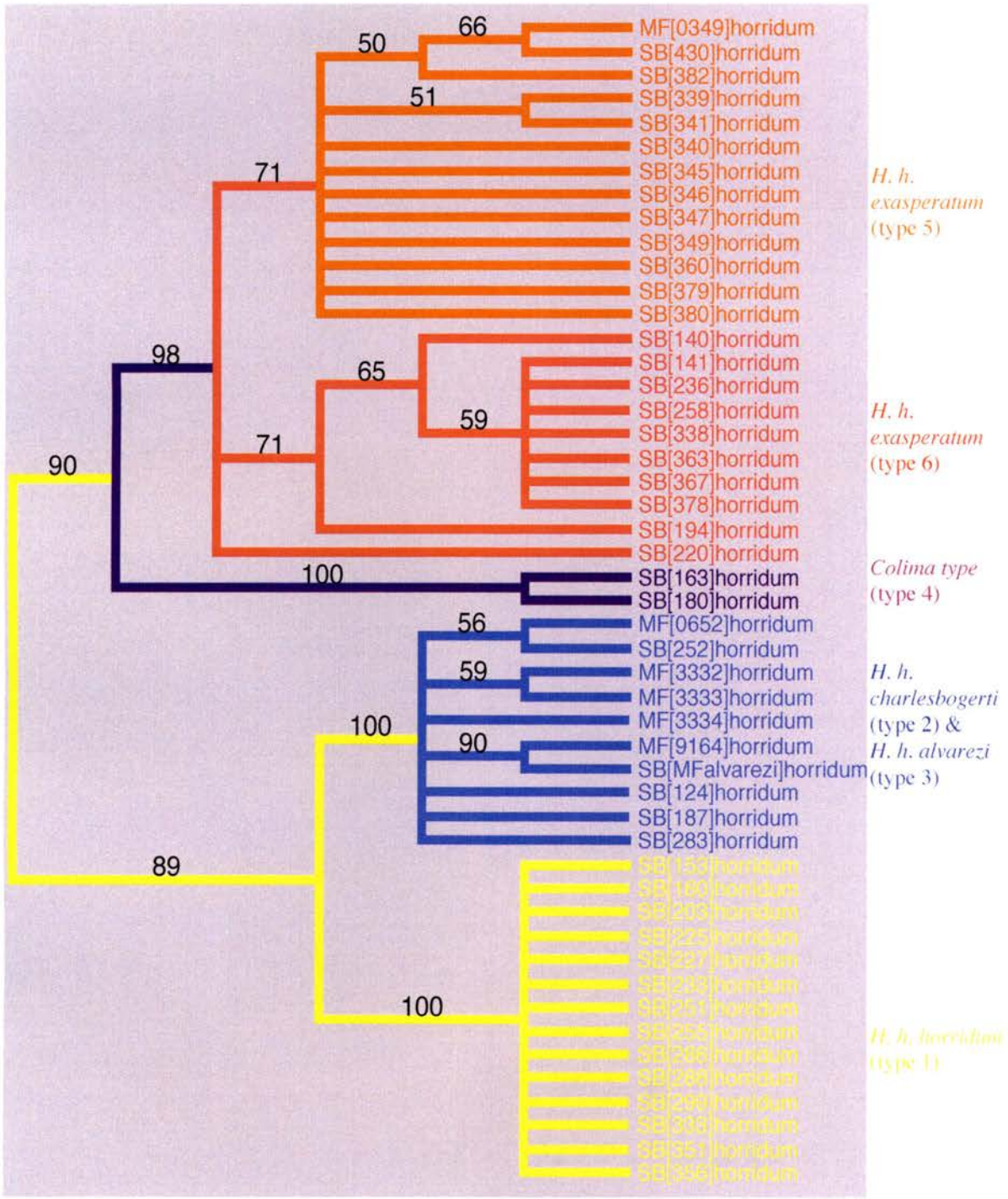


Figure 3. This is the cladogram obtained from the HKY85 correction model applied to the NJ distance method. The numbers on the clades are bootstrap values, which are indicative of support. The specimens used to create this tree are haplotypes. The remaining specimens, with identical sequences as those above, can be found in Appendix 2.

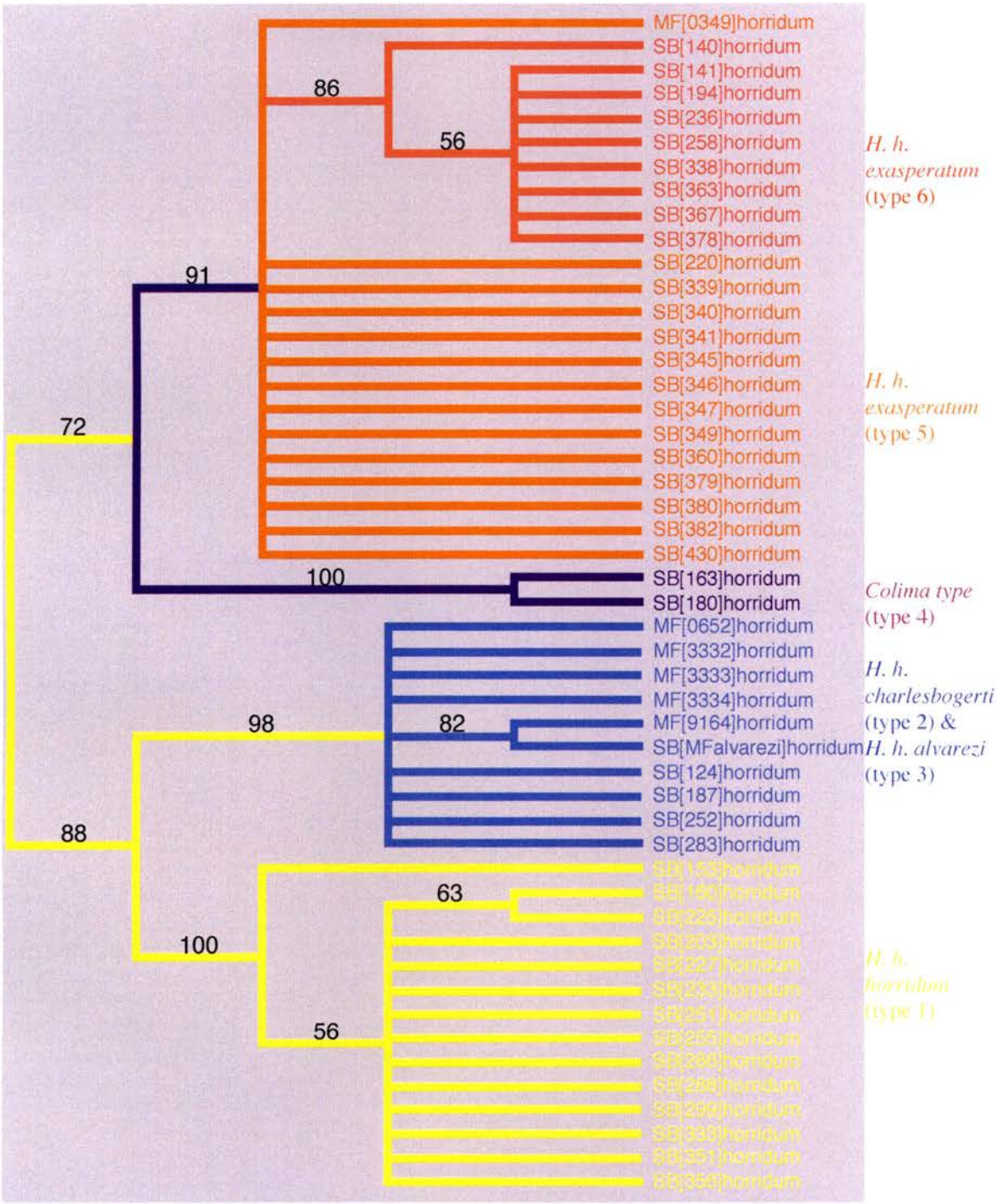


Figure 4. Maximum likelihood cladogram obtained from a NJ algorithm using a (TVM) + i + g model of evolution. The specimens used to create this tree are haplotypes. The remaining specimens, with identical sequences as those above, can be found in Appendix 2.

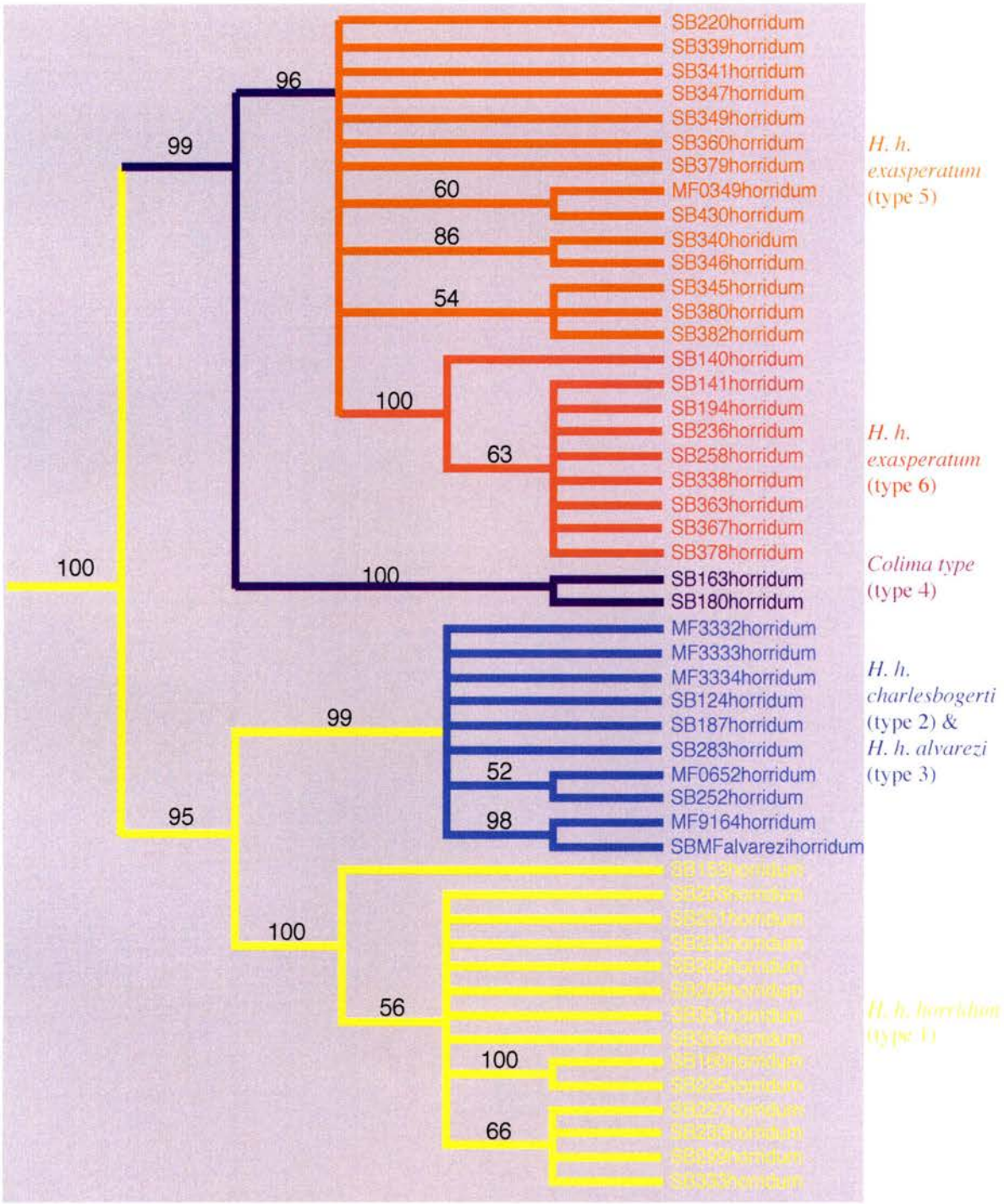


Figure 5. This figure illustrates the consensus tree obtained from 1,000,000 generations sampled every 1,000 generations in MRBAYES. The frequencies of the bipartitions are on the clades and range from 97-100. The specimens used to create this tree are haplotypes. The remaining specimens, with identical sequences as those above, can be found in Appendix 2.

Table 2. Summary of variation at mtDNA and four microsatellite loci from captive *H. horridum*: number of haplotypes (*n*), haplotype diversity (*h*), nucleotide diversity (π), number of alleles per locus (*A*), allele size range (*AR*), observed heterozygosity (H_O), expected heterozygosity (H_E). * N, null allele.

	H. h. horridum (type 1)	*H. h. charlesbogerti* (type 2)	*H. h. alvarezi* (type 3)	Colima type (type 4)	*H. h. exasperatum* (type 5)	*H. h. exasperatum* (type 6)
mtDNA						
n	14	3	3	2	14	9
h	0.7019	0.4167	1.0000	0.6667	0.6478	0.7667
π	0.0018	0.0014	0.0075	0.0016	0.0019	0.0021
6.A6						
A	4	3	2	2	3	7
AR	179-199	195-205	193-N*	179-191	179-185	179-219
H_O	0.3044	0.4444	monomorphic	0.3333	0.2963	0.5000
H_E	0.4966	0.5490	monomorphic	0.6000	0.4500	0.6051
G1582						
A	5	2	1	3	2	5
AR	108-118	114-116	114	108-120	110-116	108-118
H_O	0.6522	0.4444	monomorphic	0.6667	0.3704	0.7692
H_E	0.5903	0.5229	monomorphic	0.6000	0.3913	0.6769
12G7						
A	4	2	1	2	1	3
AR	208-216	214-218	N*	208-214	216	214-218
H_O	0.5652	0.3333	monomorphic	0.3333	monomorphic	0.5385
H_E	0.6232	0.5294	monomorphic	0.6000	monomorphic	0.5415
5B11						
A	4	1	1	3	2	4
AR	150-162	162	162	150-160	162-N*	158-164
H_O	0.5000	monomorphic	monomorphic	0.6667	monomorphic	0.4615
H_E	0.5909	monomorphic	monomorphic	0.7333	monomorphic	0.5569

Microsatellite Analyses

The number of alleles per locus averaged 7.75, with 5-12 alleles per locus. The gene diversity varied from 0.5000 to 0.9846. The observed heterozygosity for each locus in each population varied from minimal to moderate. The null hypothesis of HWE was rejected at the 5% level in every case following the Bonferroni sequential adjustment for multiple tests. The null hypothesis for linkage disequilibrium was rejected following the Bonferroni sequential adjustment in all cases except for linkage between the first and third alleles (6.A6 and 12G7) in the Colima type (type 4) with a P=0.0450 and the third and fourth alleles (12G7 and 5B11) in *H. h. exasperatum* (type 6) with a P=0.0461 (Table 2).

To identify genetically homogenous groups of individuals (*K*), a Bayesian clustering approach was applied to the genotypes occurring across the four microsatellite loci. Plotting the ΔK showed a clear peak at $K= 3$, which is the most hierarchical level of structure for the scenarios tested. Assuming three clusters, Structure then assigned each type designation (1-6) into each of the three clusters (Table 3).

A $\Phi_{CT} = 0.3257$ ($P< 0.0001$) was obtained from a hierarchical AMOVA performed using the 3 clusters (populations 1 and 4 compared to 2, 3 and 6 compared to 5) identified by Structure (Table 4). The overall level of genetic differentiation within populations for the microsatellite loci was pronounced ($\Phi_{ST} = 0.4899$, $P< 0.0001$; $\theta= 0.4940$, $P< 0.0001$).

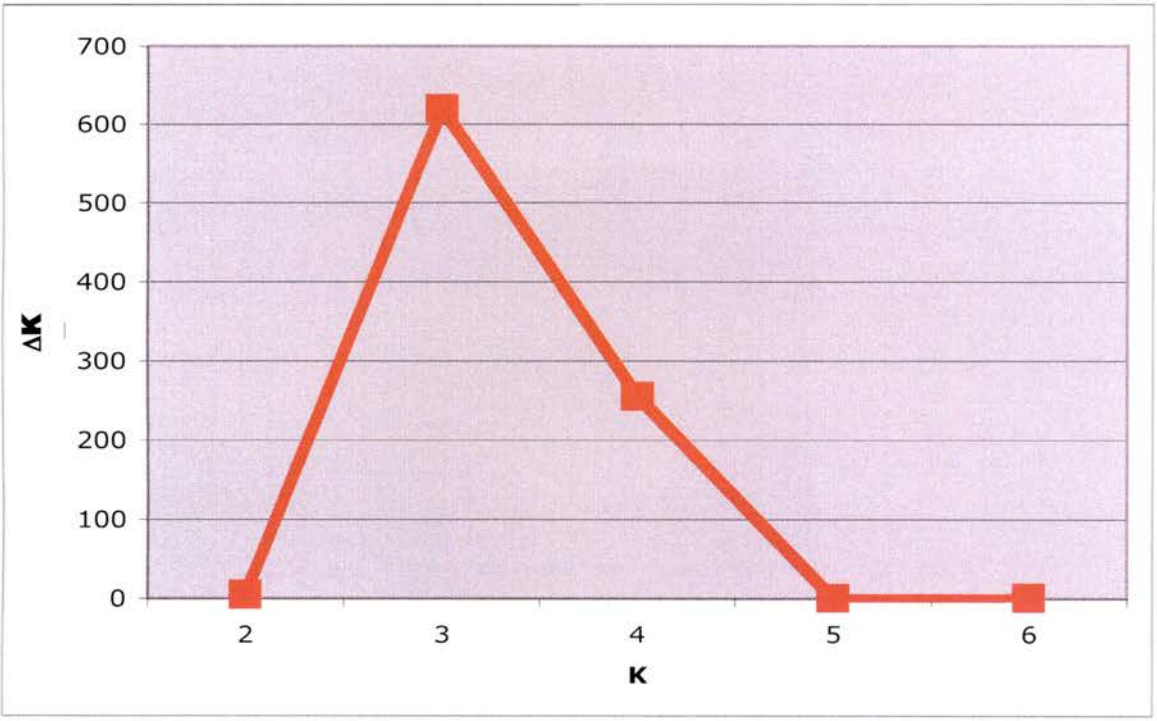


Figure 6. The ΔK estimated from the log likelihood scores obtained from Structure versus the number of possible populations tested. Where the graph peaks at K=3 indicates that the genetic variation across the four microsatellite loci can best be explained assuming three hierarchical clusters.

Table 3. Bayesian partitioning of each pre-defined population's genome within 154 *H. horridum* based on the microsatellite loci as obtained from Structure.

Population	1	2	3
***H. h. horridum* (type 1)**	0.028	0.960	0.012
***H. h. charlesbogerti* (type 2)**	0.009	0.006	0.985
***H. h alvarezi* (type 3)**	0.015	0.015	0.969
Colima (type 4)	0.005	0.979	0.016
***H. h. exasperatum* (type 5)**	0.982	0.009	0.009
***H. h. exasperatum* (type 6)**	0.049	0.117	0.834

Comparing mtDNA and Microsatellites

Because the hierarchical subdivisions resulting from mtDNA and microsatellite analyses were not congruent, additional AMOVAs were executed to test the alternative hypotheses on the alternate marker (Table 4). The level of genetic differentiation among the three clusters identified from the microsatellite analysis (types 1 and 4 compared to 2, 3, and 6 compared to 5) using the mtDNA haplotypes resulted in a $\Phi_{CT} = 0.4028$ (P= 0.1290), whereas an AMOVA among the two groups obtained from the mtDNA phylogenies (types 1, 2, and 3 compared to 4, 5, and 6) using the microsatellite dataset revealed a $\Phi_{CT} = 0.0148$ (P= 0.2952).

Table 4. Hierarchical analysis of molecular variance (AMOVA) based on mtDNA and microsatellite data.

		Partioning of variance:				
			among			
	marker	**among**	**populations**	**within**		**Weir's**
Structure	**type**	**groups**	**within groups**	**populations**	**F_{ST}**	**θ**
mtDNA defined	mtDNA	61.04	36.31	2.64	0.974	--
mtDNA defined	microsatellite	1.48*	48.03	50.49	--	0.494
Msat defined	mtDNA	40.28*	57.09	2.63	0.974	--
Msat defined	microsatellite	32.57	18.44	48.99	--	0.494

*, non-significant p-value

CHAPTER IV.

DISCUSSION

This study was undertaken to provide a reliable, genetically based management strategy for the captive *H. horridum* population, which was necessary due to the lack of verifiable locality data and the overlap in diagnostic morphological characters. Captive populations often serve as sources of reintroduction, as well as genetic reservoirs for the future (Frankham et al. 2002). Because of space limitations in zoological facilities, most captive populations are reduced in size, thereby increasing the risk of extinction. Maintaining genetic diversity is essential for the long-term viability of small populations, especially if these populations are to be used for reintroduction to the wild. In order to develop and initiate guidelines for the preservation of captive *H. horridum*, quantification of the existing genetic variation was conducted. Two categories of genetic markers (mitochondrial DNA sequencing and microsatellite analyses) were used to delineate and prioritize groups of *H. horridum* for conservation.

All of the phylogenetic analyses of mtDNA data assemble toward a similar evolutionary hypothesis. The notable results to be extracted from the topologies include: *H. h. charlesbogerti* (type 2) and *H. h. alvarezi* (type 3) are combined into one clade, the *H. h. exasperatum* clade is divided into two sister taxa (types 5 and 6), and individuals collected from Colima (type 4) form a distinct clade. Additionally, two major branches are present where *H. h. horridum* (type 1) is a sister taxon to the Guatemalan subspecies

H. h. charlesbogerti (type 2) and *H. h. alvarezi* (type 3), and the individuals from Colima (type 4) are in a monophyletic group with the *exasperatum* types (5 and 6).

Levels of mtDNA diversity were relatively high, with a mean haplotype diversity in all populations greater than 0.4167. Considerable genetic differentiation and heterogeneity was seen within populations of *H. horridum*, as seen from both the Φ_{ST} and F_{ST} values.

The microsatellite clustering results contrasted with the number of groups evident from the mtDNA, although similar trends were seen. Based on the number of genetically homogenous groups identified from the microsatellites across four loci (Fig. 6), *H. h. horridum* (type 1) and the Colima type (type 4) form a cluster, *H. h. charlesbogerti* (type 2), *H. h. alvarezi* (type 3), and *H. h. exasperatum* (type 6) are in a cluster, and *H. h. exasperatum* (type 5) form a group (Table 2).

The gene diversities across all 4 loci (Table 2) are considerably higher than averages of gene diversity for critically endangered vertebrates, including the North Atlantic right whale (Waldick et al. 2002), Galápagos penguin (Akst et al. 2002), and Delmarva fox squirrel (Lance et al. 2003). In these endangered species, the mean gene diversity ranged from 0.038 to 0.457. The genetic variance seen across the microsatellite loci within populations of *H. horridum* was further supported by a θ=0.494 estimate of genetic differentiation. An AMOVA showed a significant component of genetic variance among the clusters produced from the microsatellite data (Table 4).

AMOVAs testing the two hierarchical levels of population structure were executed using the dataset of the alternate marker. Both tests of genetic differentiation were non-significant, with the mtDNA groups tested on the microsatellite dataset

accounting for only 1.48% of the total genetic variance. The collective results of the AMOVAs indicate that most of the genetic variation is best explained by highly structured population differentiation rather than groups of populations.

In analyses of mtDNA and microsatelllites, subspecies *H. h. charlesbogerti* (type 2) and *H. h. alvarezi* (type 3) consistently showed no genetic differentiation. Although Campbell and Lamar (2004) state that the distribution among these southernmost subspecies is isolated by some 230 km of mountainous terrain, the Zacapan region, which includes the Rio Motagua Valley where *H. h. charlesbogerti* occur, is known as a historically important dispersal corridor through which many species have gained access to by way of Pacific drainages. Lack of genetic differentiation may be a result of high levels of gene flow among the ranges of these two subspecies. Additionally, it is possible that the lack of genetic differentiation among these two subspecies may be due to insufficient time since divergence. All of the individuals sampled for these populations were wild-caught, so the results are expected to accurately reflect that of the ancestral populations; therefore, it is possible that these two subspecies may need to be taxonomically revised. Alternatively, the sample sizes were very small within both populations, which may fail to indicate some attributes that are truly polymorphic. Undersampling of individuals within these subspecies may have biased the analyses toward the recognition of one population instead of two (Sites & Crandall 1997).

In addition to grouping the subspecies *H. h. charlesbogerti* (type 2) and *H. h. alvarezi* (type 3) together, microsatellite analyses also placed the *H. h. exasperatum* type (type 6) with that cluster. Although there was significant support for this group based on the four microsatellite loci (Table 2), this hypothesis places the northernmost population

(type 6) with the southernmost populations (types 2 and 3), which is not well supported by the geographical boundaries in Mexico. A series of mountains, dormant and active volcanoes, collectively known as the trans-Mexican volcanic belt, extends 900 km from west to east from Jalisco to southern Veracruz, which is uninhabitable by *H. horridum* and most likely acts as a barrier to gene flow between the populations. Furthermore, these results are not congruent with the mtDNA phylogenies. One factor that can potentially cause discrepancies in patterns of population differentiation is the differential mutation rates between markers. The high mutation rate in microsatellites could have resulted in the accumulation of alleles that are identical by state, but are not identical by decent, which would have contributed to the reduction in the extent of population differentiation (Lu et al. 2001).

Microsatellite analyses cluster the subspecies *H. h. horridum* (type 1) with the Colima type (type 4) individuals. Although this grouping is not supported by the mtDNA phylogenies, incongruence of these two markers is valuable because it may reveal important evolutionary processes. Considering the sympatric distribution of these two populations throughout parts of Jalisco and Colima, gene flow via male-mediated dispersal is the most likely scenario accounting for the lack of genetic differentiation seen across the four microsatellite loci.

Members of a subspecies are not reproductively isolated; however, they will normally be allopatric and exhibit recognizable phylogenetic partitioning (O'Brien & Mayr 1991). Furthermore, "evidence for phylogenetic distinction must normally come from the concordant distributions of multiple, independent genetically based traits" (Avise & Ball 1990). A recommendation for the subspecies classification of the

exasperatum type (type 5) comes from the congruent evidence found from mtDNA and microsatellites. Although the majority of the individuals analyzed from this population were wild-caught, there are no known localities recorded. The geographic locality of this group is needed to formally define this unit as a subspecies according to the general definition proposed by O'Brien and Mayr (1991).

The preservation of existing genetic variation requires delineating the extent of genetic differentiation within and among populations (Haig 1998). Fortunately, because of the existing high levels of genetic variation evident from the mtDNA and microsatellites, more management options are available to conserve this variation in captive *H. horridum.* Based on the cumulative results from phylogenetic and microsatellite analyses, four management options have been proposed varying in the number of populations to be managed.

The most conservative approach to effectively managing the existing genetic variation would be to breed the existing nominal taxa (as designated 1-6). The recognition of six distinct groups is neither supported by the genetic results or the current taxonomy. However, attempting to capture each of the six evolutionary taxa incorporated from the current taxonomy and the mtDNA phylogenies is a way of maximizing or maintaining intraspecific variation until further resolution is obtained.

If additional founder individuals of *H. h. charlesbogerti* (type 2), *H. h. alvarezi* (type 3), and Colima type (type 4) are unavailable to supplement the population sizes, hard decisions will have to be made as to how to preserve the unique alleles present in the populations. One method for preserving unique alleles is seen in the classic example involving the Dusky Seaside Sparrow *Ammodramus maritimus* (Avise & Nelson 1989).

The Dusky, whose population size was formerly in the thousands, was listed as endangered in 1966. By 1980, only 6 birds (all male) could be found in existence. As a last resort, 5 of the birds were brought into captivity and hybridized with Scott's Seaside Sparrow (*A. m. peninsulae*) in an effort to preserve the Dusky genes. Hybridization, in this way, may be a strategy to conserve rare genomes (Soltis & Gitzendanner 1999).

The conservation of five populations is another management option. In this scenario, breeding would occur only in like type-specimens, with types 2 and 3 one population. Although the delineation of five populations is not congruent with the current taxonomy, it is supported by mtDNA phylogenies. Microsatellites analyses additionally show no genetic differentiation between *H. h. charlesbogerti* (type 2) and *H. h. alvarezi* (type 3). Similar results were found in a study involving the African black rhinoceros (*Diceros bicornis*). Low mtDNA variability in the two subspecies (*minor* and *michaeli*) lead Ashley and colleagues to suggest that the two subspecies should be a single population (Ashley et al. 1990). Additionally, combining the two populations would increase the breeding population size and immediately increase the number of founders for the population (Table 3).

The least conservative management plan would be the conservation of only two groups, as identified by the hierarchical mtDNA lineages. Individuals from types 1, 2, and 3 would be managed as one taxonomic unit, and individuals of types 4, 5, and 6 would be managed as another. Conserving the three groups identified from the hierarchical genetic structuring of the microsatellites is another possible management option. Managing these three groups, however, would neglect any results obtained

involving mtDNA. More importantly, these management options are only recommended for extreme situations of population decline.

Based on the cumulative data generated in this study, the distribution of genetic variation is not congruent with the current taxonomy. The results of this study re-classifies the previously recognized subspecies *H. h. charlesbogerti* and *H. h. alvarezi* as one taxonomic unit and recommends that individuals representing the lineage of the *H. h. exasperatum* (type 5) be classified as a new subspecies. The most conservative management approach would be the continued preservation of each of the six nominal subspecies. Future investigation into the systematics of this species is essential. Understanding the evolutionary history of *H. horridum* would provide further insights into mechanisms and processes accountable for their variability, as well as continuing to aid in conservation management.

APPENDIX 1

Current inventory of the samples used to evaluate the taxonomy of *Heloderma horridum.* Given below are the studbook number that corresponds to the specimen, the MF number or catalog number, and the subspecies defined in the current *Heloderma horridum* studbook.

SB#	MF #	Subspecies
SB#009	MF#5772	Unknown
SB#058	MF#5773	Unknown
SB#079	MF#7868	Unknown
SB#080	MF#7869	Unknown
SB#086	MF#7867	Unknown
SB#087	MF#7873	Unknown
SB#088	MF#7871	Unknown
SB#089	MF#9220	*H. h. horridum*
SB#124	MF#9236	*H. h. charlesbogerti*
SB#133	MF#9127	*H. h. exasperatum A*
SB#140	MF#9210	*H. h. exasperatum B*
SB#141	MF#9193	*H. h. exasperatum B*
SB#142	MF#9209	*H. h. horridum*
SB#152	MF#9165	*H. h. horridum*
SB#153	MF#7876	Unknown
SB#157	MF#9168	*H. h. horridum*
SB#158	MF#9166	*H. h. horridum*
SB#160	MF#9207	*H. h. horridum*
SB#163	MF#0249	Colima type
SB#164	MF#9122	Colima type
SB#175	MF#9686	*H. h. exasperatum A*
SB#176	MF#9226	*H. h. exasperatum B*
SB#178	MF#9200	*H. h. horridum*
SB#180	MF#9136	Colima type
SB#183	MF#0221/9155	*H. h. horridum*
SB#185	MF#9132	*H. h. horridum*
SB#187	MF#0283	*H. h. alvarezi*
SB#190	MF#9137	*H. h. exasperatum A*
SB#193	MF#9171	*H. h. horridum*
SB#194	MF#9169	*H. h. exasperatum B*
SB#200	MF#9140	*H. h. horridum*
SB#203	MF#9231	*H. h. horridum*
SB#204	MF#9201	*H. h. horridum*
SB#206	MF#9202	*H. h. charlesbogerti*
SB#207	MF#9671	*H. h. horridum*
SB#208	MF#9216	*H. h. horridum*
SB#210	MF#9149	*H. h. horridum*
SB#211	MF#9142	*H. h. horridum*
SB#212	MF#9163	*H. h. horridum*
SB#213	MF#9233	*H. h. horridum*
SB#218	MF#9173	*H. h. horridum*
SB#219	MF#9190	*H. h. horridum*
SB#220	MF#9135	*H. h. exasperatum A*
SB#221	MF#9234	*H. h. exasperatum A*
SB#222	MF#9683	*H. h. charlesbogerti*
SB#223	MF#9684	*H. h. charlesbogerti*
SB#224	MF#9685	*H. h. charlesbogerti*
SB#225	MF#9208	*H. h. horridum*
SB#227	MF#9196	*H. h. horridum*

SB#	MF #	Subspecies
SB#233	MF#9160	*H. h. horridum*
SB#234	MF#9235	*H. h. charlesbogerti*
SB#235	MF#9211	*H. h. charlesbogerti*
SB#236	MF#9204	*H. h. exasperatum B*
SB#237	MF#9139	*H. h. exasperatum A*
SB#238	MF#9672	*H. h. horridum*
SB#239	MF#9673	*H. h. horridum*
SB#240	MF#9170	*H. h. exasperatum A*
SB#241	MF#9212	*H. h. horridum*
SB#243	MF#9227	*H. h. exasperatum A*
SB#250	MF#0317	*H. h. horridum*
SB#251	MF#0318	*H. h. horridum*
SB#252	MF#0350	*H. h. charlesbogerti*
SB#255	MF#9176	*H. h. horridum*
SB#256	MF#9178	*H. h. exasperatum A*
SB#257	MF#9161	*H. h. horridum*
SB#258	MF#9213	*H. h. exasperatum B*
SB#259	MF#7564	Unknown
SB#260	MF#7565	Unknown
SB#267	MF#9229	*H. h. exasperatum A*
SB#282	MF#9167	*H. h. horridum*
SB#283	No accession #	Unknown
SB#284	MF#9199/9674	*H. h. exasperatum B*
SB#285	MF#9198	*H. h. exasperatum B*
SB#286	MF#9197	*H. h. horridum*
SB#288	MF#7865	Unknown
SB#290	MF#9675	*H. h. horridum*
SB#294	MF#9129	*H. h. horridum*
SB#295	MF#9130	*H. h. horridum*
SB#299	MF#9214	*H. h. horridum*
SB#300	MF#9215	*H. h. exasperatum A*
SB#302	MF#9172	*H. h. exasperatum A*
SB#303	MF#9676	*H. h. horridum*
SB#310	MF#9195	*H. h. exasperatum A*
SB#311	MF#9185	*H. h. exasperatum A*
SB#313	MF#9192	Hybrid
SB#319	MF#9128	*H. h. horridum*
SB#320	MF#9131	*H. h. horridum*
SB#321	MF#9154	*H. h. horridum*
SB#322	MF#5777	Unknown
SB#326	MF#9138	Unknown
SB#327	MF#9153	Unknown
SB#333	MF#9144	*H. h. horridum*
SB#334	MF#9148	*H. h. exasperatum A*
SB#335	MF#9177	*H. h. exasperatum B*
SB#336	MF#9189	*H. h. exasperatum A*
SB#337	MF#9180	*H. h. exasperatum A*
SB#338	MF#9183	*H. h. exasperatum B*
SB#339	MF#9677	*H. h. exasperatum A*

SB#	MF #	Subspecies
SB#340	MF#9181	*H. h. exasperatum A*
SB#341	MF#9678	*H. h. exasperatum A*
SB#342	MF#9679	*H. h. exasperatum A*
SB#343	MF#9179	*H. h. exasperatum A*
SB#344	MF#9680	*H. h. exasperatum A*
SB#345	MF#9191	*H. h. exasperatum A*
SB#346	MF#9182	*H. h. exasperatum A*
SB#347	MF#9205	*H. h. exasperatum A*
SB#348	MF#9206	*H. h. exasperatum A*
SB#349	MF#0238	*H. h. exasperatum A*
SB#350	MF#0234	*H. h. exasperatum A*
SB#351	MF#0224/5778	Unknown
SB#352	MF#9162	*H. h. exasperatum A*
SB#353	MF#7875	Unknown
SB#354	MF#7877	Unknown
SB#355	MF#7874	Unknown
SB#356	MF#9228	*H. h. horridum*
SB#360	MF#7866	Unknown
SB#362	MF#7872	Unknown
SB#363	MF#9221	*H. h. exasperatum B*
SB#364	MF#9223	*H. h. exasperatum B*
SB#365	MF#9222	*H. h. exasperatum A*
SB#366	MF#9224	*H. h. exasperatum B*
SB#367	MF#9225	*H. h. exasperatum B*
SB#374	MF#0316	Hybrid
SB#375	MF#9133	Hybrid
SB#378	MF#9188	*H. h. exasperatum B*
SB#379	MF#9187	*H. h. exasperatum A*
SB#380	MF#9186	*H. h. exasperatum A*
SB#381	MF#9184	*H. h. exasperatum A*
SB#382	MF#9174	*H. h. exasperatum A*
SB#383	MF#9175	*H. h. exasperatum B*
SB#384	MF#9134	Hybrid
SB#393	MF#9217	*H. h. horridum*
SB#396	No accession #	*H. h. horridum*
SB#397	MF#9194	*H. h. horridum*
SB#399	MF#9218	*H. h. horridum*
SB#406	MF#9687	*H. h. exasperatum A*
SB#411	MF#9143	*H. h. horridum*
SB#420	MF#9147	*H. h. horridum*
SB#421	MF#9146	*H. h. exasperatum A*
SB#430	No accession #	*H. h. exasperatum A*
SB#432	MF#7864	Unknown
SB#434	MF#9125	*H. h. horridum*
SB#444	MF#9123	*H. h. horridum*
SB#465	MF#9230	*H. h. horridum*
SB#467	MF#9232	*H. h. exasperatum B*
SB#482	MF#9203	*H. h. charlesbogerti*
SB#490	MF#9151	Unknown

SB#	MF #	Subspecies
SB#511	MF#9145	Unknown
SB#516	MF#9219	*H. h. horridum*
SB#518	MF#9152	*H. h. horridum*
SB#522	MF#9150	*H. h. horridum*
SB#523	MF#9124	*H. h. horridum*
SB#526	MF#9126	*H. h. horridum*
SB#528	MF#9141	*H. h. horridum*
SB#579	MF#7870	Unknown
SB#600	MF#5764	Unknown
SB#602	MF#5775	Unknown
SB#608	MF#5776	Unknown
SB#657	MF#8189	Unknown
SB#SIZ	MF#9682	Unknown
00-0125-9711	MF#9164	*H. h. alvarezi*
MFalvarezi	No accession #	*H. h. alvarezi*
SB#3.3	No accession #	*H. h. exasperatum B*
SB#5.5	MF#9681	*H. h. exasperatum A*
To be assigned	MF#0247	*H. h. alvarezi*
To be assigned	MF#0349	Unknown
To be assigned	MF#0652	Unknown
To be assigned	MF#1366	Unknown
To be assigned	MF#3332	Unknown
To be assigned	MF#3333	Unknown
To be assigned	MF#3334	Unknown
To be assigned	MF#3335	Unknown
To be assigned	MF#3336	Unknown

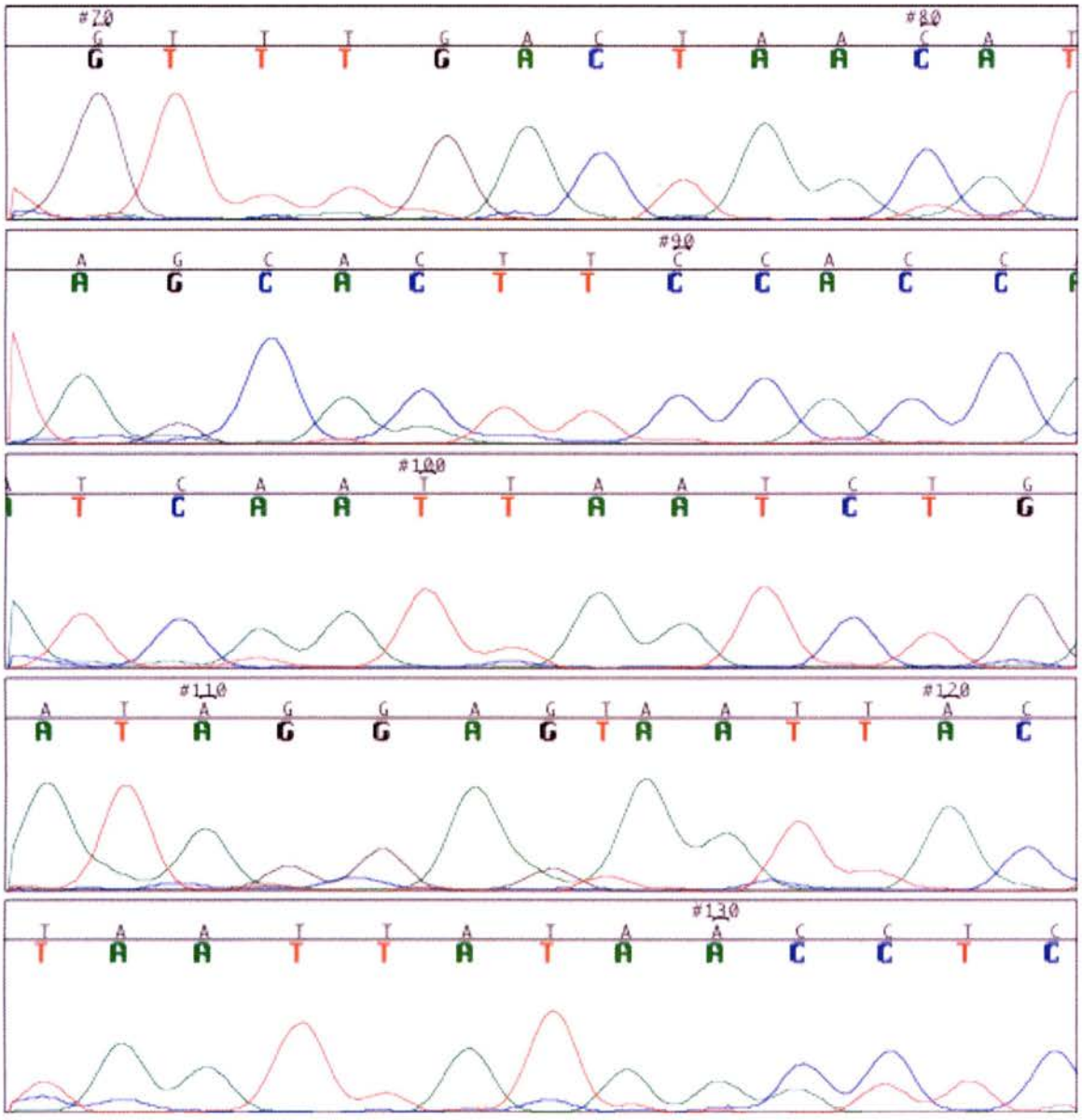
APPENDIX 2

All of the individuals with identical mtDNA sequences that are not listed in the haplotype trees (Figs. 2-5).

Individuals in haplotype tree	Individuals with identical mtDNA sequences
SB(124)horridum	SB206, SB222, SB224, SB234, SB235, SB482
SB(140)horridum	SB602, SB608
SB(141)horridum	SB079, SB080, SB176, SB284, SB285, SB3.3, SB322, SB378, SB383, SB490, SB579
SB(163)horridum	SB164
SB(203)horridum	SB204, SB250
SB(288)horridum	SB009, SB058, SB142, SB157, SB158, SB183, SB185, SB210, SB211, SB212, SB239, SB257, SB303, SB393, SB396, SB399, SB420, SB434, SB444, SB465, SB518, SB522, SB523, SB526, SB528, SBSIZ
SB(299)horridum	SB152, SB178, SB193, SB200, SB207, SB208, SB218, SB219, SB238, SB241, SB282, SB290, SB411, SB516
SB(340)horridum	SB365
SB(341)horridum	SB267, SB336, SB352, SB381
SB(345)horridum	SB311
SB(349)horridum	SB350
MF(0349)horridum	
SB(360)horridum	MF1366, SB086, SB087, SB088, SB133, SB221, SB237, SB240, SB243, SB256, SB259, SB260, SB300, SB302, SB310, SB334, SB337, SB342, SB343, SB344, SB348, SB354, SB355, SB362, SB406, SB421, SB432, SB5 5, SB511
SB(378)horridum	SB284
SB(430)horridum	SB190
MF(1263)suspectum	MF1278
MF(1264)suspectum	ID95238, MF1266, MF1270, MF1271, MF1274, MF1276, MF1277
MF(1267)suspectum	MF1268
MF(1269)suspectum	ID95235, MF1272, MF1273, MF1275
ID(4995)suspectum	ID3194
ID(4996)suspectum	ID8057

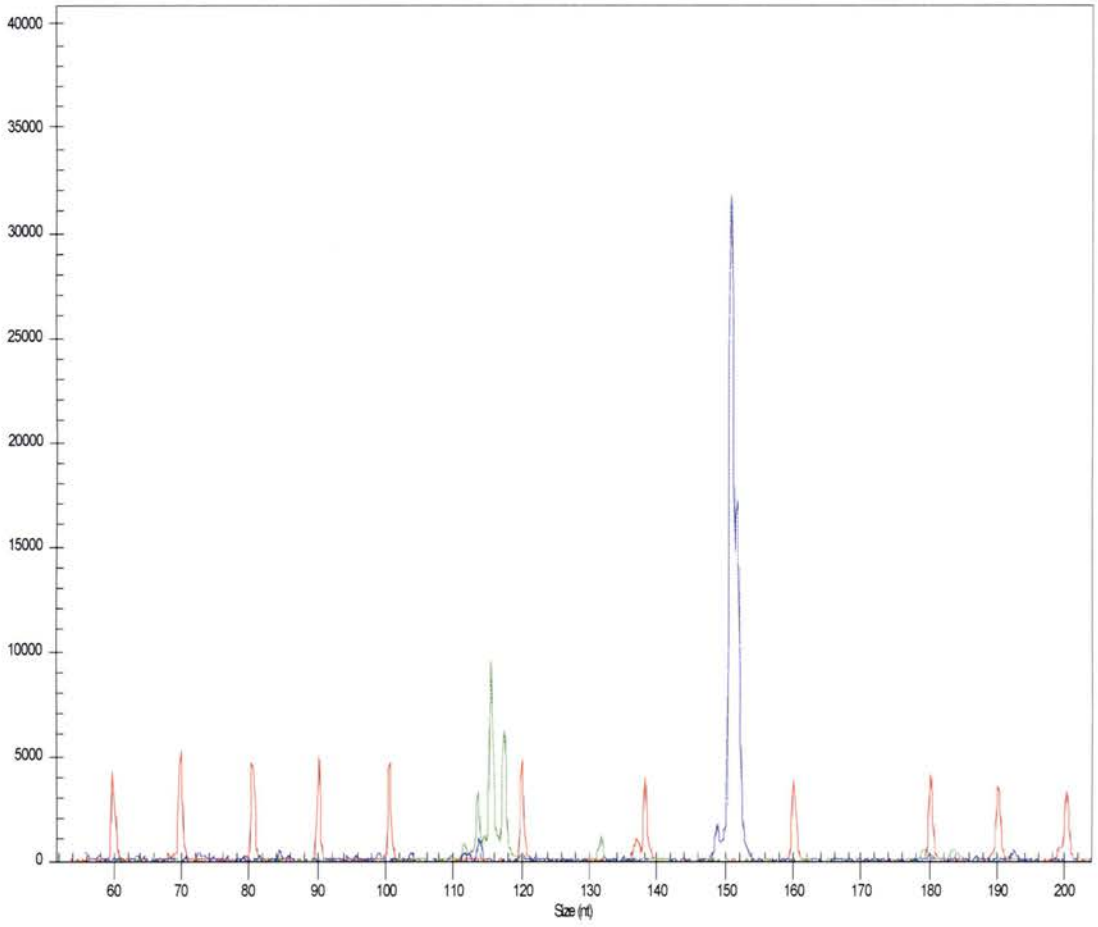
APPENDIX 3

Mitochondrial DNA sequence data were derived from chromatograms as seen in this sample showing a partial sequence of *Heloderma horridum.*


#70
#80
#90
#100
#110
#120
#130

APPENDIX 4

Alleles across four microsatellite loci were scored from peaks as seen in this sample showing two different loci.


40000
35000
30000
25000
20000
15000
10000
5000
0
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
Size (nt)

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