Morphometric and Molecular Analysis of the *mabouia- brooki haitianus* Complex (Sauria: Gekkonidae) at the Western-Central Region of Puerto Rico.

by

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Morphometric and Molecular Analysis of the *mabouia- brooki haitianus* Complex (Sauria: Gekkonidae) at the Western-Central Region of Puerto Rico

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Under the supervision of Professor Jaime Acosta

and Professor Carlos Ríos Velázquez

at the University of Puerto Rico- Mayagüez Campus

The genus *Hemidactylus* belongs to the family Gekkonidae, a group of nocturnal lizards of African origin. Several species from this genus have arrived to the Western Hemisphere and have spawned many studies because the time and means of arrival are still uncertain. Of all the species, only two can be found in the Island, *Hemidactylus brooki-haitianus* and *H. mabouia*, living in sympatry. Historically, *Hemidactylus brooki-haitianus* has been the most widely spread of the two species and can be found throughout the Island. The only reports of *H. mabouia* are from Isabela, Aguirre, and the satellite islands of Vieques, Culebra, and Mona. Throughout the current research, the genus *Hemidactylus* was studied from morphologic and molecular points of view because both species have similar morphologic characters that make their identification harder. Various locations at the Western-Central region of the Island were studied with the purpose of finding an alternative method to identify each species and to report if a change in the distribution of the species has occurred in the last decades.

Geckoes collected were analyzed morphologically and molecularly using traditional taxonomic methods and *in silico* mitochondrial cytochrome *b* gene comparisons, respectively. Gecko DNA used for molecular studies was extracted from the distal portion of the lizard's tail.

All geckoes classified using morphological traits as well as molecular approaches were *H. mabouia*. In this study no *H. brooki-haitianus* individuals were found suggesting that a change in the distribution of both species has occurred in Puerto Rico during the last 25 years.

Análisis Morfométrico y Molecular del Complejo *mabouia- brooki haitianus* (Sauria: Gekkonidae) en la Región Oeste-Central de Puerto Rico

Alberto José González-Negrón

Bajo la supervisión del Profesor Jaime Acosta

y del Profesor Carlos Ríos Velázquez

en la Universidad de Puerto Rico- Recinto de Mayagüez

El género *Hemidactylus* pertenece a la familia de los Gekkonidos, un grupo de lagartos nocturnos de origen africano. Varias especies de este género han llegado al hemisferio occidental y han engendrado muchos estudios ya que el tiempo y los medios de su llegada permanecen inciertos. De todas las especies, solamente dos pueden ser encontradas en la Isla viviendo en simpatría. Históricamente *Hemidactylus brooki-haitianus* ha sido la especie de mayor dispersión y puede ser encontrada a través de la Isla. Los únicos reportes de *H. mabouia* son de Isabela, Aguirre y las islas satélites, Vieques, Culebra e Isla de Mona. A lo largo de esta investigación se estudió el genero *Hemidactylus* desde puntos de vista morfológicos y moleculares ya que ambas especies poseen características morfológicas similares que hacen difícil su clasificación. Varias localidades en la región oeste central de la Isla fueron estudiadas con el propósito de encontrar un método alterno para identificar las especies y reportar cambios en la distribución de las especies en las últimas décadas.

Los geckos colectados fueron analizados morfológicamente y molecularmente usando métodos taxonómicos tradicionales y comparaciones *in silico* del gen mitocondrial del citocromo *b* respectivamente. El ADN usado en estudios moleculares fue extraído de la porción distal del rabo de los lagartos.

Todos los geckos clasificados usando rasgos morfológicos y también moleculares fueron *H. mabouia*. En este estudio ningún individuo de *H. brooki-haitianus* fue encontrado sugiriendo que ha ocurrido un cambio en la distribución de ambas especies en Puerto Rico durante los pasados 25 años.

Dedicatory

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Chapter 1

Introduction and literature review

Introduction and literature review

The genus *Hemidactylus* is part of a group of nocturnal lizards from the family Gekkonidae. Although the genus is of African Origin, it is accepted that *H. haitianus* has been one of the naturalized invaders of the genus in the Caribbean. Kluge (1969) considered it a separate subspecies of *H. brooki* along with *H. brooki leightoni* and *H. palaichtus*, and it was elevated to the species status, *H. haitianus*, in 1996 by Powell *et al*. On the other hand, *H. mabouia* is considered an invasive species throughout its range in the neotropics, the Cape Verde Islands (Jesús *et al.*, 2001), and the Seychelles (Powell *et al.*, 1998). This idea is supported by scattered reports of its almost incidental, but growing, presence in the New World, including Puerto Rico (Kluge, 1969; Rivero, 1998). Most of all, it is considered a non-native due to the small morphological differences present in all the invasive populations, supporting the idea that *H. mabouia* from Africa and elsewhere should remain as a single species (Kluge, 1969). Nonetheless, this particular geographical distribution has spawned different studies including human-mediated dispersal, ecology, natural history, karyotype comparisons, and mitochondrial DNA analysis (Powell *et al.*, 1998). A few of these works deal with heterospecific interactions between *H. mabouia* and other *Hemidactylus* species (Hanley, 1998; Petren, 1998; Meshaka, 2000).

The commonly accepted distribution of the genus has always been an issue of debate in Puerto Rico. Of all the *Hemidactylus* species currently reported in the Americas, *H. mabouia* and *H. haitianus* are known to occur in Puerto Rico, in sympatry, with the latter being historically more widely distributed, found anywhere at elevations

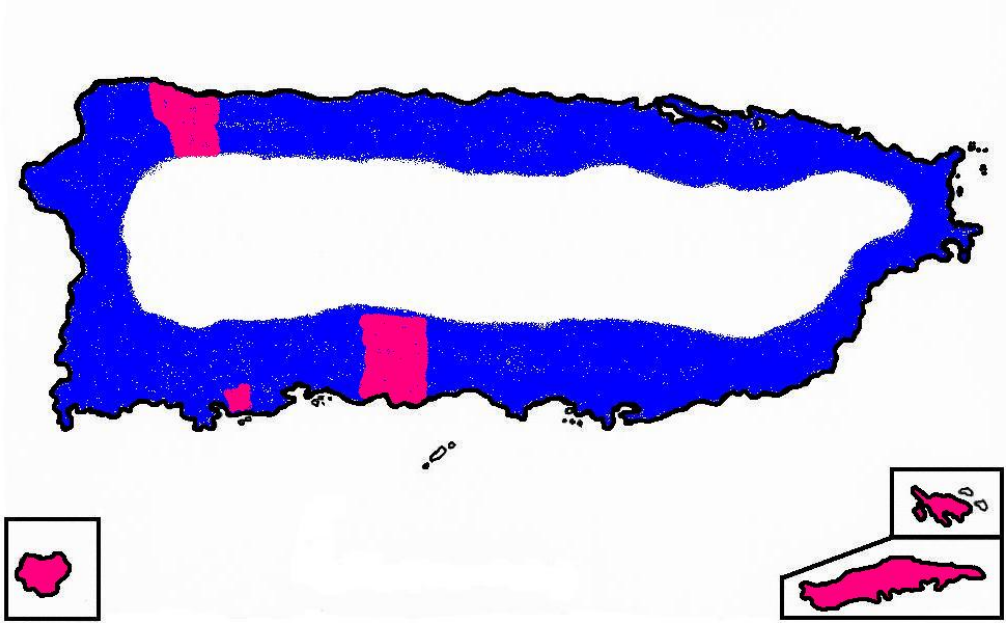

Figure 1.1. Distribution of the genus *Hemidactylus* in Puerto Rico (Rivero, 1998). *H. brooki-haitianus* (blue area) and *H. mabouia* (red area).

lower than about 600 m (Rivero, 1998). The reports of *Hemidactylus mabouia* have been limited to Isabela (northwestern Puerto Rico) and a single specimen known from Aguirre in Ponce, along with other common incidental sightings on Mona, Vieques, and Culebra islands (Rivero, 1998). There have also been a few scattered reports of *H. mabouia* from the southwest (Isla Cueva, La Parguera) and the north (Sabana Seca) of Puerto Rico (Mayer, 1999). Presumably, the descendants of *Hemidactylus brooki* that colonized the New World have had enough time to adapt successfully to this new environment without any other *Hemidactylus* competitor. This may explain why the intrusion of *H. mabouia* seems to be causing a shift in the gecko distribution. This situation is comparable with the intrusion of *H. mabouia* in places where other exotic *Hemidactylus* geckoes had arrived first, like *H. turcicus* and *H. garnotii* in Florida (Meshaka, 2000). Meshaka also

demonstrated how the abundance of *H. mabouia* in the Everglades National Park suddenly increased from 1991-92 to 1995-96, while at the same time the population of *H. garnotii* decreased in some areas. Jesús *et al.* (2001) suggested the same interaction between *H. mabouia* and the endemic *H. bouvieri* in Cape Verde Archipelago. This situation may be similar in Puerto Rico, since *H. haitianus* is considered a separate, New World species, different from *H. brooki*, its African ancestor, which does not coexist with *H. mabouia* in Africa according to Kluge (1969).

Species identification using morphological characteristics. As mentioned before, Powell *et al.* (1996) considered *H. haitianus* a different species from *H. brooki* using morphological traits as the base for his study. One of the problems is that geckoes from the genus *Hemidactylus* are extremely similar to each other. Almost every morphological characteristic used to identify one species, is used to identify another, for example the number of scansors and tubercles (Kluge, 1969). The problem in using morphological characters that are so similar is that in closely related species, like in *H. mabouia* and *H. haitianus*, the degree of overlap for almost every characteristic makes it nearly impossible to accurately identify a species. In Puerto Rico, the two species of this genus are very difficult to differentiate morphologically. The traditional morphological characters used to classify them are: body length, number of dorsal tubercles, number and position of scansors (lamellae), number of preano-femoral pores in males, and the number of tail rings (Kluge, 1969; Rivero, 1998; Spawls, 2002). Coloration patterns are not used since these geckoes have the ability to change skin color.

Hemidactylus haitianus is considered the smaller of the two species present in Puerto Rico. Kluge (1969) reported that *H. haitianus* reaches a maximum of 15 cm (including tail) and 6.8 cm from snout to vent (SVL). The distance from tip of snout to eye is roughly equal to the distance from eye to external auditory meatus (EAM) (Spawls, 2002). Kluge (1969) also noticed that *H. haitianus* shows 5 cheek tubercles, which are a series of scales between the anterior margin of the EAM and the posterior-most extreme of the angle of the mouth. The body is covered with granules or scales, among which are enlarged, keeled or smooth tubercles (Spawls, 2002). The tail is not constricted at the base and there are usually 6 to 8 rows of pointed tubercles on the tail or at least at its base. The inner-most digit is normal in length and the claw is free and angled upward. Also, the fingers and toes are not webbed at the base as in other members of this group of lizards.

The males of *H. haitianus* present 20 to 46 preano-femoral pores in a continuous row. There are enlarged tubercles strongly keeled in 14 to 25, more or less regular, longitudinal rows (Spawls, 2002), between the scales at the dorsum of the animal. Traditionally, the most common characteristic used in the classification of the two *Hemidactylus* in Puerto Rico is the length and number of scansors or lamellae in the hind feet, specially the fourth digit. The scansors are modified scales in the digits that attach to irregular surfaces (like walls and trees). As shown in Figure 1.3, *H. haitianus* presents 4 to 6 scansors beneath the first digit and 5 to 9 beneath the fourth digit. Kluge (1969) and Rivero (1998) called this feature “lamellae reaching the base of the digit” because the scansors reach the palm of the hind feet.

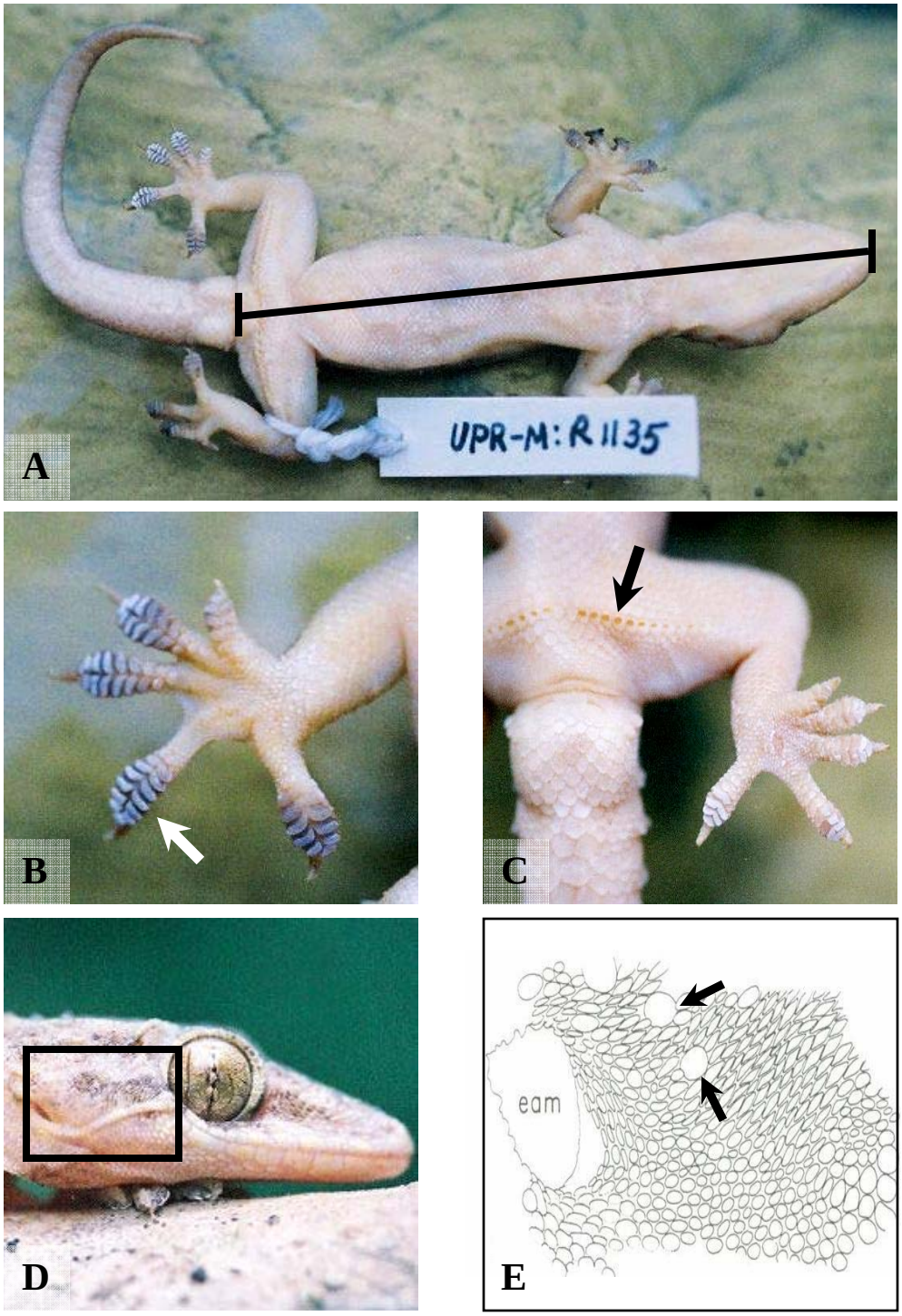

Figure 1.2. Morphological characteristics used for the identification of *Hemidactylus* species. A. Example of the snout to vent length (SVL) measurement. B. Position and number of scansors from the hind feet. C. Preano-femoral pores present on males. D. Cheek tubercles. E. Picture of cheek tubercles modified from Kluge (1969).

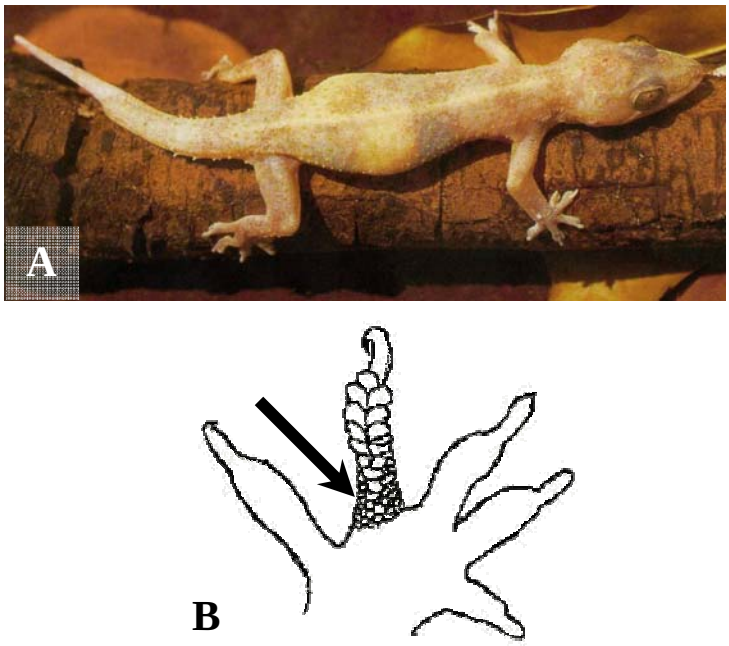

Figure 1.3. *Hemidactylus brooki-haitianus*. (A. Whole animal picture from Rivero, 1998. B. Lamellae of the fourth digit from the hind legs reaching the base of the digit modified from Kluge, 1969).

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On the other hand, *Hemidactylus mabouia* is considered larger than its counterpart. It reaches approximately 16 cm in body length (tail included) and 7 cm SVL. Unlike *H. haitianus*, the distance from the tip of the snout to the eye in *H. mabouia* is greater than the distance from the eye to ear opening. This species typically has only 2 cheek tubercles between the anterior margin of the EAM and the posterior-most extreme of the angle of the mouth. Its body is covered with granules or scales, the tail is not constricted at the base and the digits are not webbed. *Hemidactylus mabouia* has 6 rows of pointed tubercles on the tail or at least at its base. The males present between 20 and 40 preano-femoral pores in a continuous row (Spawls, 2002).

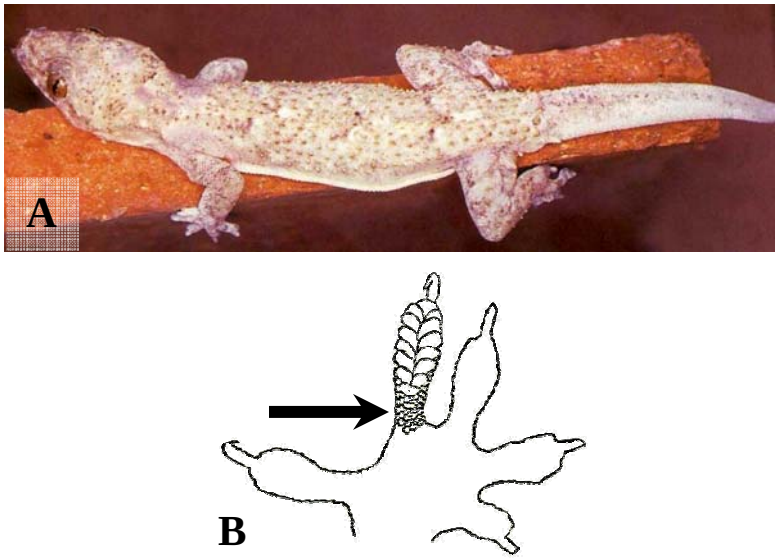


Figure 1.4. *Hemidactylus mabouia*. (A. Whole animal picture from Rivero, 1998). B. Lamellae of the fourth digit from the hind legs not reaching the base of the digit modified from Kluge, 1969).

Many of the characteristics mentioned tend to overlap, making the process of identification difficult and somewhat ambiguous. Kluge (1969), Rivero (1998), and Spawls (2002), all have used the position of the scansors as their main morphological trait to distinguish between species in their works.

Molecular analysis as a taxonomical tool. Molecular methods and bioinformatics are widely used by scientists around the world to study the patterns of evolutionary history recorded in the DNA sequences (Sarre, 1995). The result of a combination of different tools and techniques can give us valuable information about the evolutionary past of species, genetic variability, gene flow, and phylogeography within others (Avise *et al.*, 1994; Glenn *et al.*, 2002; Sato *et al.*, 1999). Researchers have been finding novel genes that can help study relationships within and among species. As a result of these findings,

new genes have been discovered and associated with species identification. Furthermore, these genes have proved to be of valuable importance in forensic sciences, as well as for the food industries and in environmental protection (Branicki, 2003; Holland *et al.*, 1999; Wilson *et al.*, 1995). As shown in Figure 1.5, genes such as cytochrome *b* (cyt *b*), control region (D-loop), and the 12S of the rDNA found in the mitochondrial DNA (mtDNA) are commonly used for molecular studies (Kocher *et al.*, 1989; Parson *et al.*, 2000). Studies regarding the family Gekkonidae involved sequencing of the cytochrome *b* gene and 12S rDNA for *Hemidactylus* species in Africa (Jesús *et al.*, 2001). These genes also have been used in studies with another genus of Gekkonid lizards (Sarre, 1995). Jesús *et al.* (2001) and Moritz *et al.* (1993) are the only studies, to present date, dealing with *Hemidactylus* geckoes, and thus they are the base for all comparisons. It is important to study species from a molecular level in order to understand the mechanisms and processes by which evolutionary changes are made.

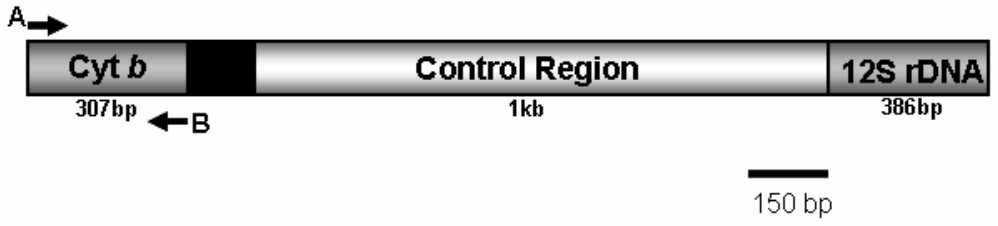


Figure 1.5. MtDNA genes and regions used for the molecular analysis. The arrows represent the primers and the direction of the amplification. (A) L14841 and (B) H15149 (Kocher *et al.*, 1989; Glenn *et al.*, 2002).

Mitochondrial DNA analysis. The mtDNA is a small, circular molecule that is present in abundant quantities in each cell. Regardless of its smaller size as compared to genomic DNA, it codes for at least 40 genes and half of them are part of the ribosomal DNA or the transfer RNA (Hartl, 1998). Both, rDNA and tRNA, are used in the mitochondria for protein synthesis. The remaining genes code for proteins used in electron transport such as the cytochromes. Another characteristic of the mtDNA is that it is maternally inherited. This feature may represent a problem when constructing gene trees because it will reflect a single genealogical process (Hartl *et al.*, 1997; Hedrick, 1985). Also, mtDNA does not undergo recombination.

Many scientists take for granted that the process of recombination accelerates the formation of beneficial genes. It is known that the rate of substitution in the mtDNA is 5 to 10 times greater than nuclear genes. The reason in part is because mtDNA polymerase lack proofreading, unlike nuclear DNA polymerase. This is the reason why mtDNA evolves faster than nuclear DNA even when it does not undergo recombination (Hartl, 1998; Avise *et al.*, 1994). The main reason to use mtDNA molecular studies is because it is present in a large number of copies per cell, making possible the amplification of any particular gene by means of Polymerase Chain Reaction (PCR), even if a small amount of sample is taken. Furthermore, the cytochrome *b* gene studied has been shown to possess enough variability among species of the genus *Hemidactylus*, making the process of species identification easier.

To date, many studies have used PCR techniques to obtain the desired mtDNA amplicons with the help of conserved primers developed by Kocher *et al*. (1989). These primers have been used to study mtDNA variability among vertebrates, such as mammals

(Kocher *et al.*, 1989), birds (Sato *et al.*, 1999), and reptiles (Glenn *et al.*, 2002; Jesús *et al.*, 2001; Sarre *et al.*, 1995). Sato *et al*. (1999) used these primers to study relations between the 14 species of Darwin's Finches in the Galapagos Archipelago.

Glenn *et al*. (2002) studied reptiles and found a non-coding sequence in the Control Region of the mtDNA of two species of crocodiles in North America. Other studies deal with members of the Gekkonid family. Sarre *et al*. (1995) studied mtDNA variation among populations of geckoes from the genus *Oedura* and argued about the concept of metapopulations over a fragmented habitat.

Past systematic work on the genus *Hemidactylus* involved taxonomic reviews and morphometric analysis species inhabiting restricted geographic regions (Kluge, 1969; Powell *et al*. 1996). None has addressed the genus in the Western Hemisphere and the only molecular data bearing on relations between *Hemidactylus* have been from Jesús *et al*. (2001). They studied the cytochrome *b* gene of four *Hemidactylus* species, including *H. brooki* and *H. mabouia*, from Cape Verde Archipelago, in the northern region of Africa. These data are the only observations available for comparisons among populations that are close to the original localities of this group of lizards and populations in the Americas. Throughout the present work, Jesús *et al*. (2001) data will be considered as derived from the ancestral population or control group. The current work is one of the first studies done in the Caribbean dealing with molecular analysis of the reptiles and the first molecular work involving the genus *Hemidactylus*. Others have conducted works on behavioral or ecological observations (Meshaka *et al.*, 1994a, b: Meshaka, 2000) and parasitological observations (Martínez *et al.*, 2003) of *Hemidactylus* in the Western

Hemisphere. Data gathered will help future workers to identify or understand the origin, dispersion, and time and means of arrival of these geckoes to the West Indies.

***In silico* analysis of the genus *Hemidactylus*.** *In silico* analysis implies work or analysis using computers and the software or programs available at resources, such as the websites of the National Institutes of Health (NIH). November, 1988, at NIH, by an act of the U.S. Congress, marked the birth of a new branch of computational studies in the biological sciences, better known today as bioinformatics (Korf, 2003). One of the first visions of this science was to create nucleic acid and protein banks that could store large numbers of sequences from all over the world, so they could be used by the scientific community to compare molecular data from virtually any place (Altschul *et al.*, 1997). Months later the National Center for Biotechnology Information (NCBI) announced the development of a computer program named BLAST (Basic Local Alignment Search Tool), one of the most used bioinformatics tools around the world (Altschul *et al.*, 1990) (Figure 1.6). BLAST is a computer software that identifies similarities between biological sequences, within DNA, RNA, or proteins. One of the most important features of BLAST is its reliability from the statistical point of view (Korf, 2003).

Bioinformatics as a true powerful research tool is still growing and each day new programs for *in silico* analysis are being developed. Some of the simplest programs are designed to convert any biological sequence to its reverse complement; much needed to clean, align, and compare sequences. More sophisticated programs such as CLUSTAL W (Thompson *et al.*, 1994) are used to align multiple sequences (more than two sequences at once) to study similarities among them. This analysis is very helpful since it can reveal

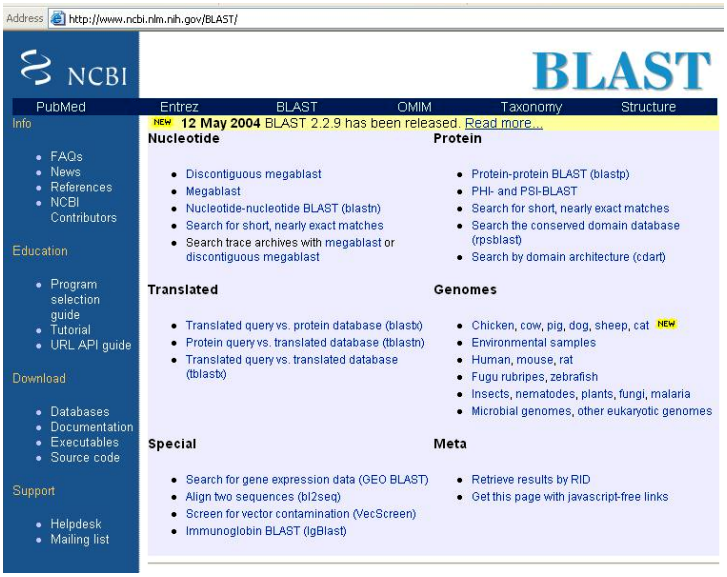


Figure 1.6. BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST/). This program is linked to the NCBI homepage. Tools like nucleotide and protein search, protein translation, and sequence alignment can be accessed from this webpage.

conserved regions (regions that share the same bases among every organism) as well as variable regions. Much of the information hidden in these regions is important because it may hold the elements necessary to identify the sources of variability between species or among populations of the same species (Korf, 2003). Data obtained from the analyses can also be used to develop new molecular tools, such as to design PCR primers needed to amplify any portion of any gene, or amplicons that may be specific to one group of organisms. Other programs search throughout the sequence to find specific locations where a restriction enzyme may cut the DNA fragment, and therefore produce a particular or unique restriction pattern. This experimental procedure is called Restriction Fragment Length Polymorphism (RFLP), in which the pattern of fragments produced when the restriction enzyme cuts the sequence at a specific location may be different between species depending on the nucleotide sequence. All the data produced by the

software must be validated by actually performing an enzymatic digestion using the fragment under study. If this *in silico* restriction site analysis is proven to work, it may hasten the process of identifying specific restriction enzymes for almost any organism.

As mentioned throughout this chapter, the purpose of this study is to use genetic tools as a way to discriminate closely related species. Then, compare the results with results from other parts of the world, in order to understand the evolutionary pathways these species, *Hemidactylus brooki-haitianus* and *H. mabouia*, have followed. This study will provide possible answers to many ecological questions, such as if a shift in the distribution of *Hemidactylus* populations occurred during the last 25 years in Puerto Rico.

Chapter 2

Morphometric analysis of the *Hemidactylus* geckoes

Summary

Geckoes of the genus *Hemidactylus* have been the focus of many ecological studies. One of the reasons is their poorly understood arrival to the Western Hemisphere. Of all the species reported for the Americas, only *Hemidactylus brooki-haitianus* and *H. mabouia* are known to Puerto Rico, living in sympatry. The first of the two species was considered very common and more widely distributed than the latter. Reports of *H. mabouia* were scattered, limiting its distribution to Isabela, Ponce, and the satellite islands of Mona, Vieques and Culebra. The aim of this portion of the study was to determine the present distribution of the genus on the Western-Central region of Puerto Rico by performing morphometric analysis. Geckoes were collected at night from 19 municipalities at the Western-Central region of Puerto Rico. Geckoes were held in captivity while performing morphological analysis, and released at the same location they were collected after the measuring process was completed. The results demonstrated that all individuals collected at the study sites were indeed *H. mabouia*. This results contrast with the commonly accepted distribution of the *Hemidactylus* geckoes in Puerto Rico, suggesting that a shift in the lizard's populations has occurred during the last two decades.

Introduction

Of all the *Hemidactylus* species inhabiting the New World, only *H. brooki-haitianus* and *H. mabouia* are reported from Puerto Rico. The latter of the two species is considered an invasive species throughout its range in the neotropics, Cape Verde Archipelago (Jesús *et al.*, 2001), and the Seychelles (Powell *et al*. 1998). The commonly accepted distribution of these lizards established *H. brooki-haitianus* as the dominant and more widely spread species on Puerto Rico, where it could be found at elevations lower than 600m (Rivero, 1998). Reports of incidental sightings of *H. mabouia* were limited to Isabela, Ponce, and the satellite islands of Mona, Vieques, and Culebra. There have been also a few scattered reports of *H. mabouia* from the southwest (Isla Cueva, La Parguera) and the north (Sabana Seca) of Puerto Rico (Mayer, 1999).

In a survey conducted in 2000, it was found that most of the geckoes observed haphazardly at various locations in Puerto Rico were, in fact, *H. mabouia* (pers. obs.). Only three individuals of *H. haitianus* were observed during the survey. This shows that *H. mabouia* is present over a larger area than the one reported by Rivero (1998) and also suggests that the distribution of *H. haitianus* on Puerto Rico is different from the commonly accepted pattern.

One of the problems with the genus *Hemidactylus* is the classification of its species since species like *H. brooki-haitianus* and *H. mabouia* are very similar to each other. Morphological traits used in the identification of the species tend to overlap, making the process of classification nearly impossible (Spawls, 2002). This is why many scientists have used the location or distribution of the geckoes in Puerto Rico for their

identification (Rivero, 1998). *Hemidactylus mabouia* is considered the larger of the two geckoes found in Puerto Rico (Kluge, 1969: Rivero, 1998). It reaches approximately 16 cm in body length and 7 cm from snout to vent (SVL). It only presents 2 cheek tubercles between the anterior margin of the external auditory meatus (EAM) and the posterior most extreme of the angle of the mouth (Kluge, 1969). In general, the digits are not webbed, the tail is not constricted at the base, and the entire body is covered with granules or scales. Males of the species present between 20 and 40 preano-femoral pores across the hind legs. Each of the authors mentioned before have used the position of the scansors or lamellae from the fourth digit of the hind feet as the main morphological trait to distinguish between species. This feature, as they called it, is: "lamellae not reaching the base of the digit". Scansors are enlarged scales underneath the digit that helps the geckoes to walk on vertical surfaces, such as walls.

To date, works on *Hemidactylus* are based in morphological analysis, including the elevation of H. *brooki-haitianus* to species status of *H. haitianus* (Powell *et al*., 1996). Other studies are based on ecological observations (Meshaka *et al.*, 1994a,b) and new reports of mites found in the Americas, using *H. mabouia* as host (Martinez *et al.*, 2003). Meshaka (2000) considered *H. mabouia* an invasive species and reported an increase on this gecko's population in the Everglades Park. Meshaka, Powell, and Rivero have also noticed the changes in distribution of *H. mabouia* in Puerto Rico (personal communication) but no official reports regarding this matter have been made.

This study seeks to identify specimens of *Hemidactylus* geckoes from localities at the Western-Cental region of the Island using morphological and molecular approaches. Samples from this study will be used for later mitochondrial DNA and *in silico* analysis.

Experimental procedures

Sampling. Geckoes were collected from 20 municipalities at the Western-Central region of Puerto Rico as shown in Figure 2.1. All specimens were gathered at night near buildings or houses between 19:00 and 21:00. The collection site coordinates were taken using a Garmin® eTREX Vista GPS (Global Positioning System) unit. Captured geckoes were kept in plastic enclosures for a 2 or 3 days period for the morphometric analysis procedure. None of the geckoes was sacrificed during the study and each gecko was released at the exact location in which it was collected.

Haphazard collection sites at Mayagüez. To determine the presence of *Hemidactylus* species throughout one of the sample sites, samples from 6 different localities were taken from Mayagüez. Following the same sampling procedure described before, geckoes were classified using morphological approaches.

Morphometric analysis. Complete body length and SVL were measured using a Vernier® Caliper. Scales and scansors counts were measured directly from the organism. Pictures of the geckoes and the lamellae were taken with a Canon® EOS Rebel 2000 camera using a Sigma 105mm f/2.8 EX MACRO 1:1 lens. Individuals collected were compared with specimens from the Herpetological Collection of the University of Puerto Rico, Mayagüez Campus (Table 2.1).

Table 2.1

List of Specimens

Hemidactylus brooki-haitianus

UPRM-R166	Mayagüez, PR	February, 1975
UPRM-R189	Mayagüez, PR	April, 1956
UPRM-R191	Sábana Grande, PR	March, 1972
UPRM-R1081	Sábana Grande, PR	October, 1981
UPRM-R1082	Sábana Grande, PR	October, 1981
UPRM-R1135	Aguadilla, PR	February, 1983

Hemidactylus mabouia

UPRM-R160	Isabela, PR	March, 1974
UPRM-R161	Mona Island, PR	May, 1973
UPRM-R162	Mona Island, PR	May, 1973
UPRM-R163	Mona Island, PR	May, 1973
UPRM-R271	Mona Island, PR	April, 1979
UPRM-R272	Culebra, PR	February, 1979
UPRM-R273	Culebra, PR	February, 1979

Results

New Distribution of *Hemidactylus mabouia*. A total of 20 municipalities were sampled. As shown in Figure 2.1, colored areas represent the study sites. This figure along with Table 2.2 describes the number of specimens gathered at each study site. All the geckoes collected were identified as *H. mabouia*, using the position of the lamellae from the fourth digit of the hind legs as the main taxonomical feature. A total of 69 organisms were collected, from which 17 specimens were used for further mtDNA analysis in order to corroborate the morphological results using molecular approaches.

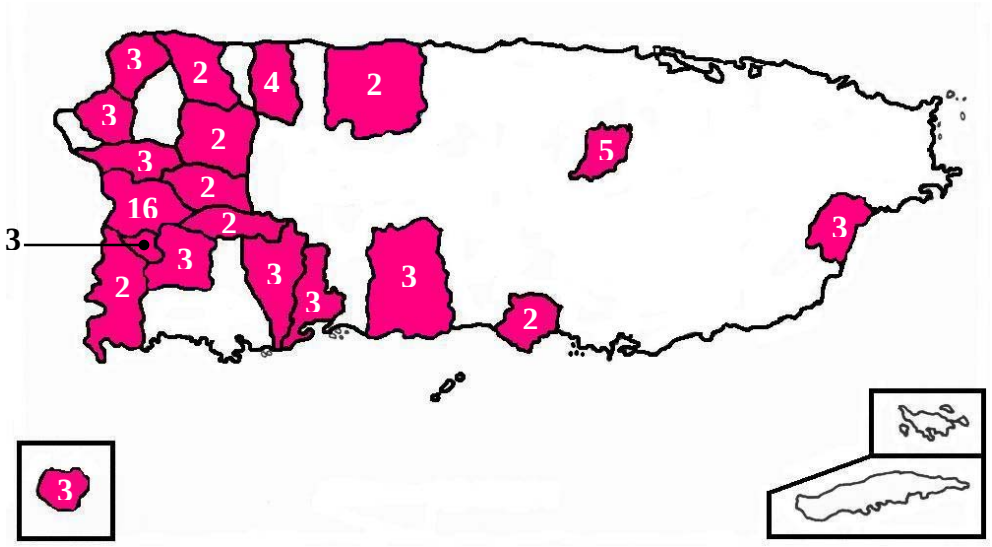


Figure 2.1. New distribution of *Hemidactylus mabouia*. Municipalities studied were colored and the numbers represent sample size. Classification based on morpholocical traits.

Table 2.2

Sampling sites and their respective GPS coordinates.

Municipality	Samples	GPS Coodinates	
Aguada	3	18°39.00 N	67°20.07 W
Aguadilla	3	18°48.12 N	67°14.67 W
Añasco	3	18°28.91 N	66°13.24 W
Arecibo	2	18°45.67 N	66°73.59 W
Cabo Rojo	2	18°23.45 N	66°46.55 W
Camuy	4	18°47.62 N	66°65.13 W
Guayanilla	3	18°02.11 N	66°79.22 W
Humacao	3	18°15.02 N	65°82.17 W
Homigueros	3	18°14.21 N	67°12.74 W
Isabela	2	18°50.62 N	67°01.23 W
Las Marías	2	18°25.39 N	66°15.81 W
Maricao	2	18°18.23 N	66°97.93 W
Mayagüez	16	(see Table 2.3)	
Naranjito	5	18°54.30 N	66°24.95 W
Ponce	3	18°09.67 N	66°62.79 W
San Germán	3	18°13.73 N	66°03.75 W
San Sebastián	2	18°33.55 N	66°97.82 W
Santa Isabel	2	18°01.23 N	66°37.63 W
Yauco	3	18°03.70 N	66°85.04 W
Mona Island	3	18°05.22 N	67°56.33 W

Haphazard collection sites at Mayagüez. Table 2.3 shows the location as well as the number of samples and the GPS coordinate. All geckoes collected at these sites were also identified as *H. mabouia*. No specimens of *H. brooki-haitianus* were found at the sample sites.

Table 2.3

Samples of *H. mabouia* from various localities at Mayagüez.

Locality	Samples	GPS	
Barrio Rio Hondo	3	18°10.53 N	67°07.96 W
Urb. Valle Hermoso	3	18°09.20 N	67°08.82 W
Barrio París	2	18°12.18 N	67°08.20 W
Alturas de Mayagüez	3	18°13.86 N	67°09.61 W
Quebrada Grande	3	18°10.59 N	67°07.30 W
University of Puerto Rico	2	18°20.31 N	67°14.00 W

Morphometric analysis of the *Hemidactylus* geckoes. Samples collected from 20 municipalities were identified using the traditional morphological characteristics. All geckoes presented the *H. mabouia* digit type of "lamellae not reaching the base of the digit, as shown in Figure 2.2. A total of 13 specimens from the Herpetological Collection of the University of Puerto Rico at Mayagüez were studied. Table 2.1 shows the collection number, collection date, and locality for the studied specimens. Of the six specimens of *H. brooki-haitianus* studied, at least one (UPRM-R1135 from Aguadilla, PR), showed no similarity with the others. As shown in Figure 2.3, the scansors from the fourth digit of the hind feet, from picture D, does not reach the base of the digit or palm.

Table 2.4

Morphological comparison of the *mabouia-brooki* complex by Spawls (2002).

Feature	*H. mabouia*	*H. brooki*
Body length	16 cm	15 cm
Snout vent length (SVL)	7 cm	6.8 cm
Cheek tubercles	2	5
Tail tubercles	6 rows	6-8 rows
Preano-femoral pores	20-40	20-46
Lamellae from the first digit	5-6 pairs	4-6 pairs
Lamellae from the fourth digit	6-11 pairs	5-9 pairs

The morphometric measurements taken indicated that all geckoes collected for this study were *H. mabouia*. The body length of the geckoes varied with the organism age. Most were medium-size geckoes, measuring from 10-16 cm ($\bar{x}$ = 14.8 cm) of total body length and from 4.5- 6.5 cm ($\bar{x}$ = 6.1 cm) SVL. All geckoes presented 2 cheek tubercles and from 6- 9 ($\bar{x}$ = 7.2) rows of tail tubercles. As shown in Figure 2.2, geckoes presented a hemidactylid digit type whose enlarged subdigital lamellae fail to reach the origin of the digit or palm. The lamellae are arranged in pairs, and so, each measurement represents one pair of scansors. Geckoes presented 5- 6 ($\bar{x}$ = 5.2) subdigital lamellae on the first toe and 7-8 ($\bar{x}$ = 7.4) on the fourth toe.

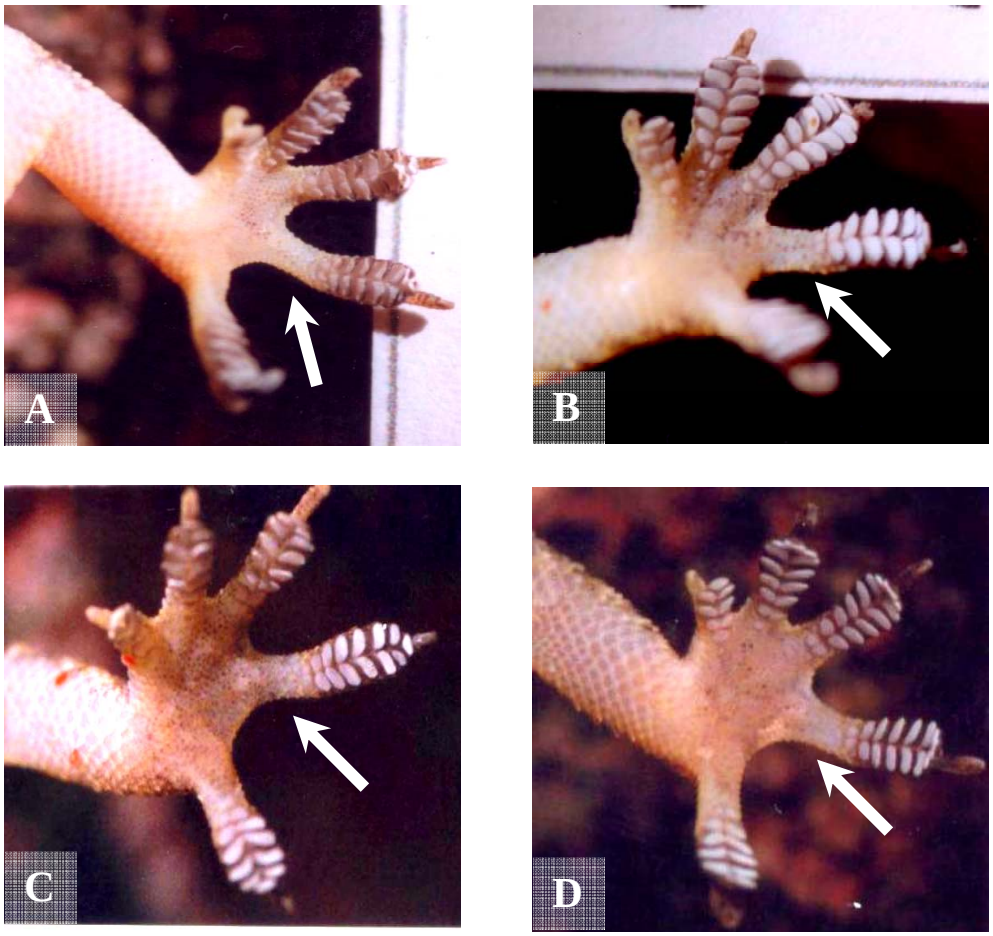


Figure 2.2. Pictures of the lamellae from the hind legs of *Hemidactylus* samples. The criteria used for the classification is the position of the scansors or enlarged scales from the fourth digit (from left to right). As seen the lamellae does not reach the base of the digit or palm. Specimens from: Mayagüez (A), Ponce (B), Isabela (C), and San Germán (D). All samples were used in further mtDNA analysis.

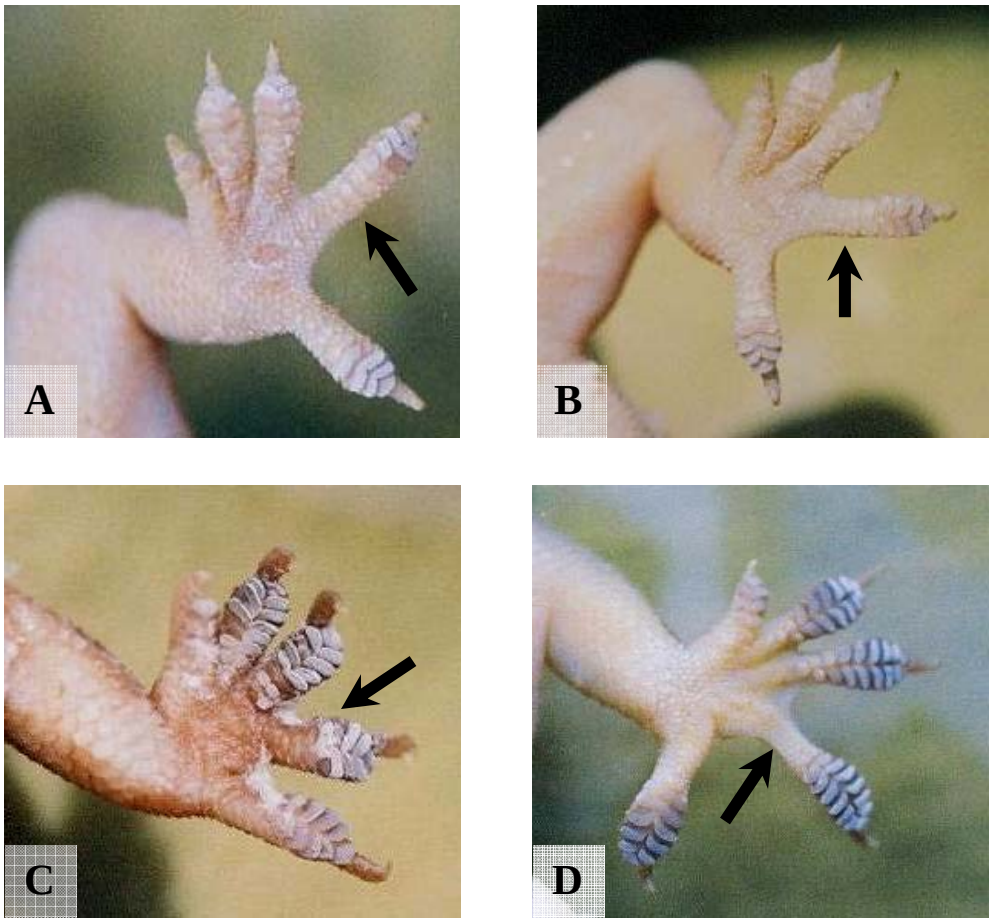


Figure 2.3. Pictures of the lamellae from the hind legs of *Hemidactylus brooki-haitianus* specimens from the University of Puerto Rico Collection at Mayagüez Campus. Specimens: UPRM-R189 from Mayagüez (A), UPRM-R191 from Sábana Grande (B), UPRM-R1082 from Sábana Grande (C), UPRM-R1135 from Aguadilla (D). As seen, the lamellae (scales from the fourth digit from left to right) reach the base of the digit or palm, except specimen D.

Discussion

Morphometric analysis performed on *Hemidactylus* samples was used to determine the current distribution of the genus on Puerto Rico. Although we studied 20 municipalities, no specimens of *H. brooki- haitianus* were found. Each location where *H. mabouia* specimens were collected represents new records for its distribution on Puerto Rico. Previous sightings only located *H. mabouia* at Isabela and Ponce (in the mainland) and from Mona, Vieques, and Culebra Islands. Geckoes collected at Naranjito and Maricao demonstrated that geckoes could be found at altitudes higher than 600 m, contrasting the commonly accepted altitudinal distribution of the genus. Geckoes collected from vegetation or rural areas were also identified as *H. mabouia*.

Our findings revealed a shift in the distribution of both geckoes. Traditionally, in Puerto Rico the dominant species was *H. brooki-haitianus*, but as in the case of Everglades Park (Meshaka, 2000), *H. mabouia* populations have increased over the years. Presumably, *H. brooki-haitianus* is not as abundant as it used to be 25 years ago. Our data supports the observations made by Kluge (1969) and Meshaka (2000), in which they observed a decrease in the population of geckoes that competed against *H. mabouia*.

To avoid sampling bias, we collected geckoes haphazardly from more than one location at the one municipality, and also from urban and rural areas as well. Our results showed that *H. mabouia* was widely distributed across Mayagüez. We took samples as distant as possible [Valle Hermoso (close to Hormigueros), Alturas de Mayagüez (close to Añasco), and Quebrada Grande (near the Cerro las Mesas)]. Also, with this procedure

we avoided the bias of sampling only urban sites. Geckoes found in vegetation (at trees and under rocks) were identified as *H. mabouia*.

The specimens studied from the Herpetological Collection of the University of Puerto Rico at Mayagüez showed that *H. brooki-haitianus* was present on Puerto Rico, at least until 1981. Interestingly, specimen UPRM-R1135 collected at Aguadilla on February, 1983, fails to be classified as *H. brooki-haitianus* (as shown in Figure 2.3, picture D). This specimen presents morphological traits similar to *H. mabouia*. These data supports the idea the *H. mabouia* increased its populations over the years and that it is possible that its growing presence may have passed unnoticed.

Future studies regarding the present status of *H. brooki-haitianus* should be done in rural areas, including Natural Reserves such as for example Susúa Forest and Guánica Forest. This kind of study will help determine if the presence of *H. mabouia* caused a displacement of *H. brooki-haitianus* to low or non habited areas.

Chapter 3

Molecular analysis of mitochondrial cytochrome *b* gene as a taxonomic tool.

Summary

The geckonid lizards of the genus *Hemidactylus* have been the focus of many ecological and biogeographical studies in the Americas, due to their origin, and their poorly understood arrival to the Western Hemisphere. Of all the *Hemidactylus* species reported in the Americas, only *H. mabouia* and *H. brooki-haitianus* are known to occur in sympatry in Puerto Rico. One of the major problems with this group of lizards is the controversy concerning their taxonomic identity. To date, all the descriptions are based only on morphological characters. The main focus of this study is to determine if the mtDNA cytochrome *b* gene can be used to distinguish between the two species of *Hemidactylus* currently recorded in Puerto Rico. Geckoes from the Central and Western regions of Puerto Rico were collected for this study. Sequences of conserved regions of mtDNA cytochrome *b* gene were amplified using total DNA extracted from the distal portion of the lizard's tail. The resulting amplicons were sequenced and then analyzed *in silico* using available data bases such as BLAST. The 310 bp amplified DNA-sequenced-fragment of the captured geckoes suggests that all specimens belong to *H. mabouia*. These data contrast with the reported distribution of this lizard in Puerto Rico 25 years ago, when *H. brooki-haitianus* was the dominant species.

Introduction

Geckoes of the genus *Hemidactylus*, a group of nocturnal lizards, have been the focus of many ecological and biogeographical studies due to their origin and poorly understood Western Hemisphere arrival. Of all the species present in the Americas, only *Hemidactylus brooki-haitianus* and *Hemidactylus mabouia* are reported to occur in Puerto Rico. Many recent studies have dealt with *H. mabouia* since it is considered an invasive species throughout its range in the neotropics, Cape Verde Archipelago (Jesús *et al.*, 2001), and the Seychelles (Powell *et al*. 1998). This invasion hypothesis is supported by reports of its presence in the New World, including Puerto Rico (Kluge, 1969: Meshaka *et al.*, 1994a, b; Meshaka 2000; Rivero, 1998). Most of all, it is considered a non-native due to the small differences present in all invasive populations. This fact supports the idea that *H. mabouia* from Africa and elsewhere should remain a single species (Kluge, 1969). Reports of *H. mabouia* in Puerto Rico have been limited to Isabela (northwestern part of Puerto Rico). Also, single specimens were collected from Ponce, and other incidental sightings reported from the satellite islands of Mona, Vieques, and Culebra.

Mitochondrial DNA has proven to be an important tool in phylogenetic studies with geckonid lizards (Carranza *et al.*, 2002; Jesús *et al.*, 2001; Sarre, 1995) and also has been validated in its use for species identification studies (Branicki, 2003; Wilson *et al*., 1995). Few researchers deal with molecular aspects of *Hemidactylus*. Moritz *et al.* (1993) described patterns of variation in chromosomes, mitochondrial DNA, and allozymes for two parthenogenic geckoes (*Lepidodactylus lugubris* and *Hemidactylus*

garnotii) and one sexual species (*H. frenatus*) from a group of Pacific Ocean islands. They analyzed the distribution of genetic variation and the events of colonization. Jesús *et al.* (2001) tested for genetic diversity between individuals of *Hemidactylus brooki*, *H. mabouia*, and *H. bouvieri* from different islands of the Cape Verde Archipelago, by extracting genomic DNA from preserved museum specimens and conducting *in silico* analysis of sequences obtained from individuals in a Data Banks. All data available for the *Hemidactylus* geckoes from these works are limited to the Northwestern region of Africa. There are few systematic works performed on the genus at the Caribbean (Powell *et al.*, 1996) and none has addressed molecular data bearing on relationships between species reported from either the Caribbean, or Puerto Rico *per se*. Data gathered herein will help future systematic studies and also will clarify the origin, time, and means of arrival of this group of nocturnal lizards to the West Indies.

The aim of this work was to study the genus *Hemidactylus* using molecular approaches, such as cytochrome *b* gene as an alternative tool to identify the two species found in Puerto Rico. Both *Hemidactylus* are extremely similar, from a morphological point of view, making the identification process difficult and somewhat ambiguous. At the same time, any change in the geographical distribution of the species will be documented in order to establish a shift in the distribution of the geckoes.

Experimental procedures

Sampling. Geckoes were collected from 20 municipalities at the Western-Central region of Puerto Rico, as shown in Figure 3.1. All specimens were gathered at night near buildings or houses between 19:00 and 21:00. Captured geckoes were kept in plastic enclosures for a 2 or 3 days period for the process of identification of the species and genetic material extraction. None of the geckoes were sacrificed during the study and each gecko was released at the exact location from which it was collected.

Distribution of *Hemidactylus mabouia*. DNA samples of 43 of the geckoes were taken and stored at -20°C for further analysis. PCR was performed to amplify the cytochrome *b* gene from the mtDNA. As shown in Figure 3.1, a total of 17 geckoes were collected from 5 municipalities [Arecibo (2), Isabela (2), Mayagüez (8), Ponce (3), and San Germán (2)] and sequenced to perform *in silico* analysis. Morphologic identification was performed on every gecko.

DNA extraction. Total DNA was extracted from the distal portion of the lizard's tail using the FastDNA® Kit from Q-Biogene with the help of the FastPrep® Instrument or cell disruptor. A second extraction was performed to clean the DNA sample following the phenol-chloroform extraction and ethanol precipitation protocol (Sambrook *et al.*, 1989). The FastDNA® Kit, along with the disruptor, produces PCR grade purified genomic DNA. The FastPrep® Instrument is a physical extraction method that works shaking sample tubes up and down with a twisting motion at very high speeds to break the tissue.

The kit also incorporates a chemical extraction method. It includes Lysing Matrix, a mixture of detergent and salts, which contributes to inactivate nucleases during the lysing step to prevent the DNA fragmentation.

Total DNA extraction procedure was performed as follows. Approximately 25 to 30 mg of tissue cut from the distal portion of the tail was stored in a 1.5 ml microcentrifuge tube. Tissue samples were washed with alcohol, and carefully inspected to remove mites that could be attached between scales. Tissue was cut into small pieces using a sterile spatula or scalpel to avoid contamination. The sample was transferred to 2 ml tube containing Ceramic Spheres and Garnet Matrix from the FastDNA® Kit. Then, 0.500 ml of the sample specific Cell Lysis Solution (CLS-TC) designed for animal and tissue DNA extraction was added. The 1.5 ml microcentrifuge tube which contained the sample was washed with another 0.500 ml of CLS-TC solution and poured into a 2 ml tube. Samples were placed in the FastPrep® Instrument and were homogenized twice for 40 seconds at a speed of 5.0. Samples were left for a 3 to 4 minute period to rest (this allowed the sample to chill).

After the tissue was processed, sample tubes were removed from the instrument and centrifuged for 15 min at 12,000 rpms. Then, 600 µl of the supernatant were transferred to a clean 1.5 ml microcentrifuge tube and each sample tube was labeled. Afterwards 600 µl of the Binding Matrix were added to the new tube containing the sample and mixed gently by inversion. Samples were incubated at room temperature for 5 min and then centrifuged for 5 min at 12,000 rpms. The supernatant was discarded. At this point, the DNA remained attached to the Binding Matrix. The pellet was resuspended in 500 µl of Salt Ethanol Wash (SEW-M) solution and centrifuged for 2 minutes. The

supernatant was discarded and the SEW-M solution excess was removed by performing a 20 seconds "quick spin" and residual solution was removed by pipeting.

The most important and critical step in the protocol was the DNA elution. For a successful elution, 100 µl of DNA Elution Solution (DES) or ultra pure water were added to the sample. The pellet was resuspended and incubated for 3 min at room temperature. Samples were centrifuged for 1 min at 12,000 rpms and the supernatant carefully transferred to a new 1.5 ml microcentrifuge tube, previously labeled. To check the results of the extraction 4 µl of DNA sample were electrophoressed in a 0.8 % agarose gel. The ladder λ H*ind* III was used as a molecular marker (New England Bio Labs, Inc).

DNA extraction from formalin fixed specimens. Samples from the Herpetological Collection of the University of Puerto Rico at Mayagüez were studied in order to isolate total DNA for further mtDNA analysis. A portion of the tail from an *H. brooki-haitianus* specimen (UPRM-R191 from Sábana Grande, PR) was used in the study. The extraction followed the procedure described by Fang *et al.* (2002) and Shedlock *et al.* (1997). These protocols for DNA extraction incorporated serial alcohol dehydration and critical point drying techniques to remove formalin from the fixed tissue.

PCR amplification. Mitochondrial DNA was amplified in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems). The PCR reaction was performed using a final concentration of the corresponding 1X Buffer, 3 mM of $MgSO_4$, 0.25 mM of each dNTP, Dimethyl Sulfoxide (DMSO) at 1% of total reaction volume, and 1 pmol / µl of primers from Kocher *et al*. (1989). Primers letters L [L14841 (5'-AAAAAGCTTCCATCCAAC

ATCTCAGCATGATGAAA-3')] and H [H15149 (5'-AAACTGCAGCCCCTCAGAAT GATATTTGTCCTCA-3')] refers to the light and heavy strands, and the numbers refers to the position of the 3' base of the primer in the complete human mtDNA. $Vent_R^{®}$ DNA Polymerase, isolated from the archaea *Thermococcus litoralis*, was used at 0.025 units per 30 µl PCR reactions.

Parameters for PCR were an initial denaturation of 3 min at 95°C, then 30 cycles of 95°C for 1 min of denaturation, 62°C for 0.5 min of annealing and 72°C for 1 min of extension, and a final extension of 10 min at 72°C. To check the PCR results, 4 µl of the final product were electrophoressed in a 1.2% agarose gel. The 100 bp Ladder (New England Bio Labs, Inc.) was used as molecular marker.

DNA purification. The mtDNA amplicons were extracted from the agarose gel and purified using StrataPrep® DNA Gel Extraction Kit from Stratagene, Inc. A total of 20 µl of PCR product were electrophoressed in a 1.2% agarose gel. The fragments were cut directly from the agarose and placed in 1.5 ml microcentrifuge tubes. The rest of the procedure was done by following the manufacturer's specifications.

Amplicons were eluted by adding 15 µl of elution buffer directly on top of the fiber matrix on the microspin cup and tubes were incubated at room temperature for 5 minutes. Samples were centrifuged for 30 seconds. Then, another 15 µl of elution buffer were added as described before and incubated for 5 minutes at room temperature. Samples were centrifuged for 30 seconds at 12,000 rpms and the microspin cup was discarded. The 300bp purified amplicons were stored in the 1.5 ml microcentrifuge tube at -20°C.

DNA sequencing and *in silico* analysis. Purified amplicons were sent to the Molecular Resource Facility (MRF) at the University of Medicine and Dentistry of New Jersey for sequencing. Samples were submitted in a labeled 1.5 ml microcentrifuge tube. Each sample tube contained 10 µl of PCR template per reaction at a 10 ng/µl concentration, along with 5 µl of each primer per reaction at a 3 pmol/µl concentration. DNA samples were sequenced using ABI BigDye Terminator chemistry on an ABI 3100 Genetic Analyzer.

In order to observe variable and conserved regions, DNA sequences were aligned using the multiple sequence program CLUSTAL W (http//:www.ebi.ac.uk/cgi-bin/clustalw/) (Thompson *et al.*, 1994). The search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find similarities between the produced sequence and data from species of the genus *Hemidactylus* available at the Data Banks.

Results

New distribution of *Hemidactylus mabouia*. Figure 3.1, shows a total of 17 geckoes collected from 5 municipalities [Arecibo (2), Isabela (2), Mayagüez (8), Ponce (3), and San Germán (2)] and sequenced to perform *in silico* analysis.

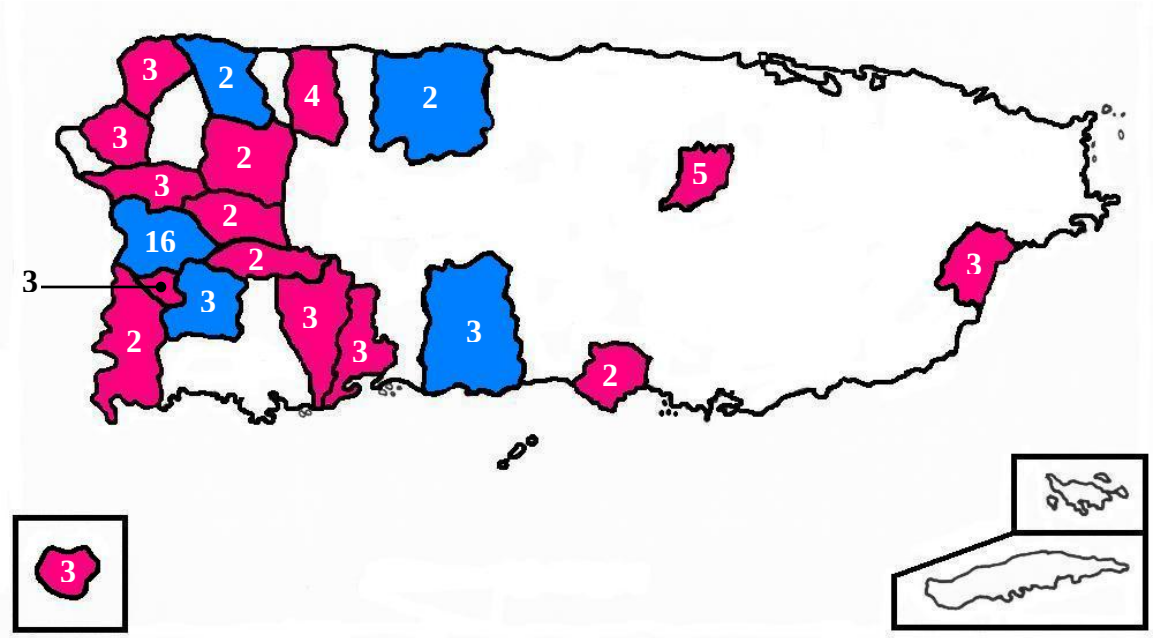


Figure 3.1. New distribution of *Hemidactylus mabouia* using molecular approaches. Municipalities studied are colored. Municipalities in blue color were sampled and DNA was sequenced. Numbers represents sample size at each location.

Total DNA extraction and PCR amplification agarose gels. After extracting total DNA from the 43 geckoes, 4 µl of the product was electrophoressed in a 0.8% agarose gel. The total DNA extraction for geckoes collected from Arecibo, Isabela, Camuy, Ponce, Naranjito, and Mayagüez are shown in Figure 3.2. The average size of total DNA extracted ranged approximately from 500bp to more than 25Kbp.

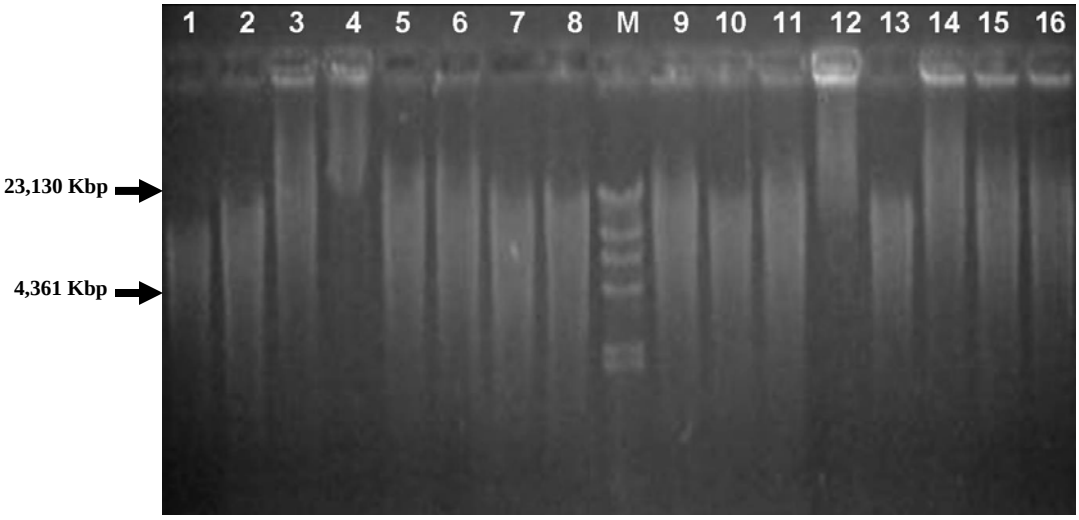


Figure 3.2. Total DNA extractions in 0.8% agarose gel. As shown in the gel, samples of high molecular weight were obtained from the lizard's tail. The Ladder λ *Hind* III (M) was used between samples 8 and 9. Samples shown are from Arecibo (1,2), Isabela (3,4), Camuy (6 to 8), Naranjito (9 to 11), and Mayagüez (12 to 16).

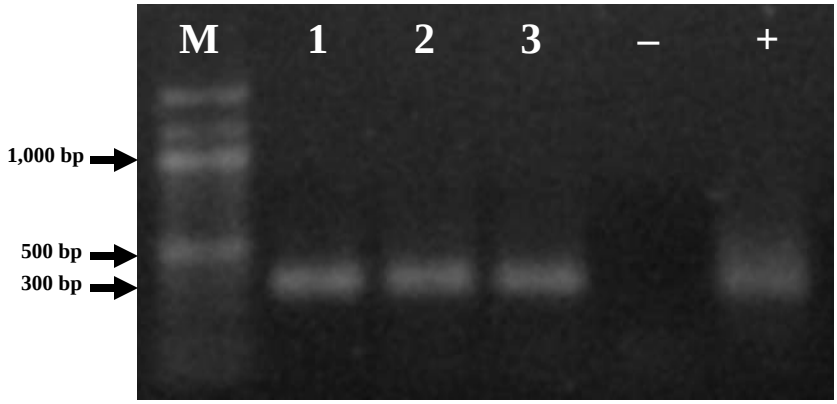


Figure 3.3. Cytochrome *b* mtDNA gene PCR product electrophoresis. The size of the amplicons is approximately 300bp. Sample order: 100 bp Ladder (M) followed by samples from Ponce (1), San Germán (2), and Mayagüez (3). Negative control (–) contains PCR reagents and primers mixture, but DNA template was not added. Positive control (+) contains a DNA sample of a previously sequenced specimen of *Hemidactylus mabouia* from Isabela.

Amplicons of the cytochrome *b* gene were electrophoressed in a 1.2% agarose gel using 100 bp Ladder as molecular marker. All samples produced a single band of the expected size, approximately 300 to 310bp (Figure 3.3). DNA fragments were cut from the 1.2% agarose gel and purified using StratPrep®DNA Gel Extraction Kit. Fragments were processed for sequencing and further *in silico* analysis.

Sequencing and *in silico* analysis results. To determine the molecular identity of each processed DNA, samples were checked for sequencing errors using the computer software CHROMAS Version 1.45 in a process described as gene cleansing, then the sequences were analyzed using BLASTN software. As shown in Table 3.1, the range for the score number values is between 519 and 650 bits. A higher score number indicates greater similarity to the sequence from the Data Bank. The raw score is shown without units, and the normalized score is followed by "bits". The expected E-values or the

number of alignments expected at random given the size of the search space were between e-150 and e-179. The lower the E-value, the less likely this is a random similarity (Korf *et al*., 2003). As described in column 4 of Table 3.1, the number of shared nucleotides (nucleotide-nucleotide search or BLASTN), also known as percent identity, varied from 97% to 99%. The best match for each sample was the certified specimen of *H. mabouia* (accession # U69833).

Table 3.1

Results for the *in silico* analysis using BLASTN search tool.

Sample/location		Score	Identities	Percent	E-value
A1	Mayagüez	519 bits (262)	291/298	97%	e-150
A2	Mayagüez	553bits (279)	285/287	99%	e-160
A3	Ponce	632bits (319)	325/327	99%	0.0*
A4	Ponce	632bits (319)	325/327	99%	0.0*
A5	Ponce	617bits (311)	317/319	99%	e-179
A6	Arecibo	553bits (279)	285/257	99%	e-160
A7	Arecibo	561bits (283)	292/295	98%	e-162
A11	Isabela	587bits (296)	302/304	99%	e-170
A12	Isabela	569bits (287)	293/295	99%	e-165
A15	San Germán	650bits (328)	331/332	99%	0.0*
A16	San Germán	632bits (319)	325/327	99%	0.0*
A21	Mayagüez	632bits (319	325/327	99%	0.0*
A22	Mayagüez	559bits (302)	308/310	99%	e-174
A24	Mayagüez	609bits (307)	316/319	98%	e-177
A25	Mayagüez	603bits (304)	319/324	98%	e-179
A29	Mayagüez	617 bits (311)	317/319	99%	e-179
A30	Mayagüez	617 bits (311)	317/319	99%	e-179

* E-value is smaller than e-180

Nucleotide analysis using BLAST. The results of the BLAST report are summarized in Table 3.1. This section shows the results obtained from the sequence comparisons between the obtained samples and specimen information from the Data Banks. Figure 3.4 shows graphical results from the sequence comparison output derived from the program. The submitted sequence is shown as a red thick line with numbers indicating the sequence length. The next 3 red lines represent the "best hit" or nearest match. All 3 results correspond to *H. mabouia* specimens. The score value for the search was 553 bits and the expected or E-value was e-160. The green lines represent the portion of the nucleotide sequence that sample A2 from Mayagüez shared with *H. brooki* from the Data Bank.

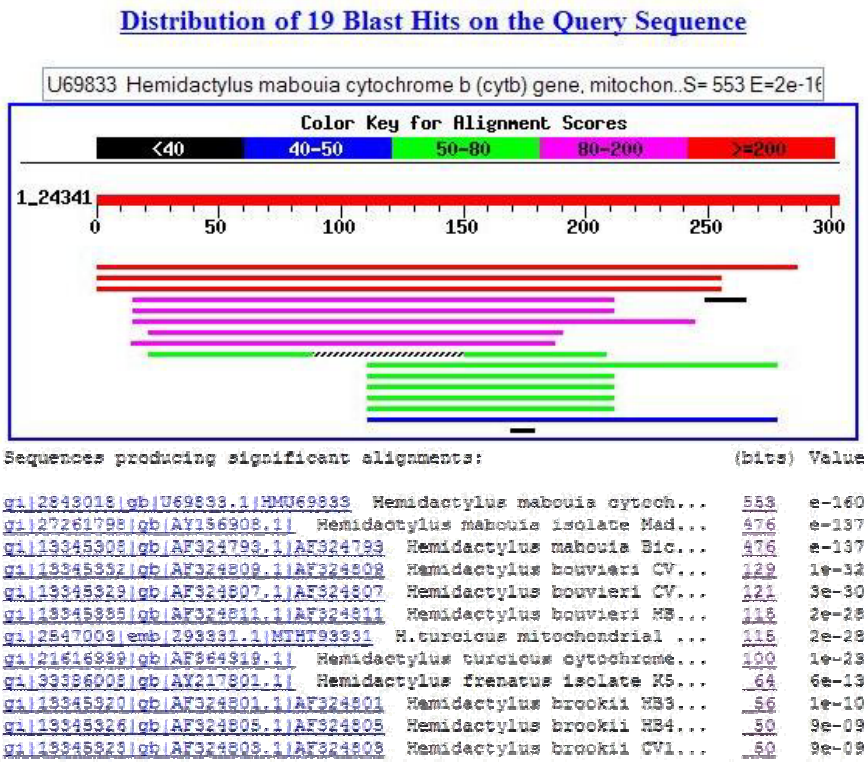


Figure 3.4. BLAST results for sample A2 (*Hemidactylus mabouia*) collected at Mayagüez, showing a high nucleotide identity with *H. mabouia* from the data bank. Analyzed with BLASTN (http//:www.ncbi.nlm.nih/BLAST/).

Table 3.2 shows the accession number, species, author, and sample location of the specimens from Data Banks, similar to sample A2 from Puerto Rico.

Table 3.2

Specimen information for BLAST results derived from Figure 3.4.

Accession #	Species	Author	Location
U69833	*H. mabouia*	Campbell, 1997	Thesis from Canada
AY156908	*H. mabouia*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324793	*H. mabouia*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324809	*H. bouvieri*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324807	*H. bouvieri*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324811	*H. bouvieri*	Jesús *et al.*, 2001	Cape Verde, Africa
AF364319	*H. turcicus*	Carranza *et al.*, 2002	North Africa
AY217801	*H. frenatus*	Whiting *et al.*, 2003	sub-Sahara, Africa
AF324801	*H. brooki*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324805	*H. brooki*	Jesús *et al.*, 2001	Cape Verde, Africa
AF324803	*H. brooki*	Jesús *et al.*, 2001	Cape Verde, Africa

The following sets of alignments compare complete *Hemidactylus* cytochrome *b* sequences. Figure 3.5 shows a set of data revealed from the alignment results between *H. mabouia* (A2 sample) and *H. mabouia* from Data Bank (accession # AF324793) using the "*align two sequences tool*" (bl2seq). The sequences were 98% identical and shared 252 nucleotides. The sequences only differed in 4 nucleotides.

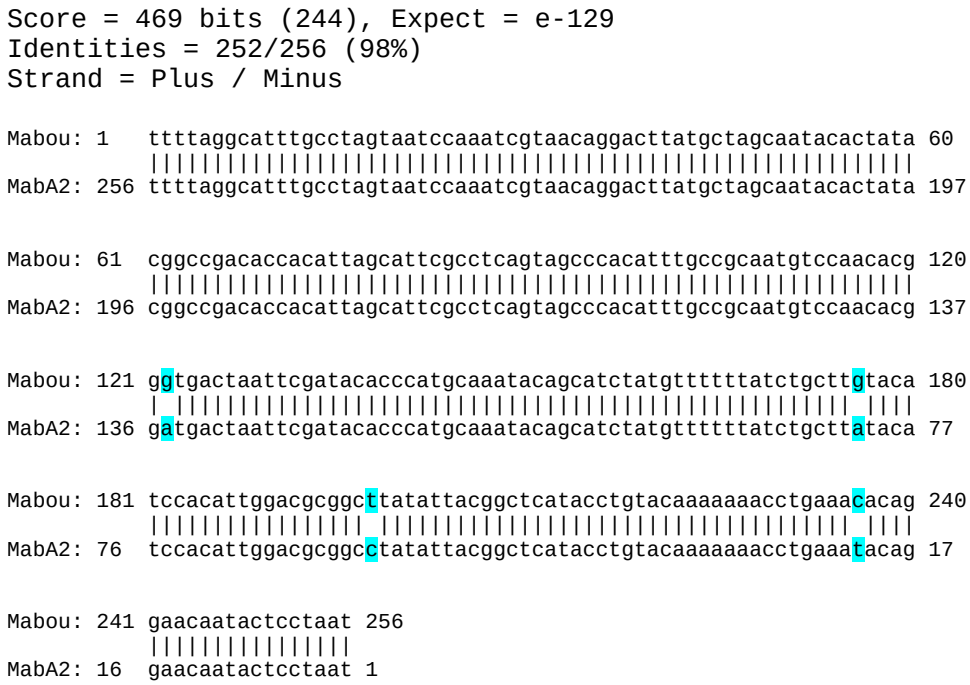


Figure 3.5. Results of cytochrome *b* gene sequence alignment between sample A2 from Mayagüez and *Hemidactylus mabouia* (accession # AF324793) from Africa. Changes in nucleotide sequences are in blue color.

To determine how different is the cytochrome *b* gene of the two *Hemidactylus* species found in Puerto Rico, a DNA sequence of *H. brooki* available at Data Banks was aligned with sequences from this study. As Figure 3.6 shows, the sequences are only 75% identical, due to differences in at least 62 nucleotides.

```
Score = 121 bits (63), Expect = 7e-25
Identities = 187/249 (75%)
Strand = Plus / Minus

Brook: 12  ggctcactactaggcatatgcctgattatgcagctaacaacagggctatttctagcaata 71
           ||||||||  |||||||  |||||  | ||  ||  |   ||||||  ||  | |||||||||
MabA2: 263 ggctcacttttaggcatttgcctagtaatccaaatcgtaacaggacttatgctagcaata 204

Brook: 72  cattacaccgccgacacaacgttagccttttcatcagtcgtccacatctgtcgcgacgta 131
           ||  ||  ||  ||||||||  ||  |||||  ||   | |||||  | ||||||  ||  |||  | ||
MabA2: 203 cactatacggccgacaccacattagcattcgcctcagtagccacatttgccgcaatgtc 144

Brook: 132 caacacgggtgactaatccgaaacacccatgccaacggcgcatcaatgttcttcatttt 191
           ||||||||  ||||||||  |||  ||||||||||  ||     |||||  |||||  ||  ||  |
MabA2: 143 caacacggatgactaattcgatacacccatgcaaatacagcatctatgtttttttatctgc 84

Brook: 192 atctacctgcacattggccgaggcctctactacggatcctacttatacaaagagacgtga 251
           | |||  |  ||||||||  ||  |||||  ||  |||||  ||  |||  |  ||||||  |  ||  |||
MabA2: 83  ttatacatccacattggacgcggcctatattacggctcatacctgtacaaaaaaacctga 24

Brook: 252 aacacagga 260
           ||  ||||||
MAbA2: 23  aatacagga 15
```

Figure 3.6. Results of cytochrome *b* gene sequence alignment between sample A2 from Mayagüez and *Hemidactylus brooki* (accession # AF324803) from Africa. The sequences differ in 62 nucleotides (shown in green color).

Sequences analyzed using CLUSTAL W. To determine the nucleotide similarity between sequenced samples, data were analyzed using CLUSTAL W multiple sequences alignment research tool. Sequences were aligned and compared among them. A total of 266/ 307 nucleotides were shared between every sequence (Figure 3.7).

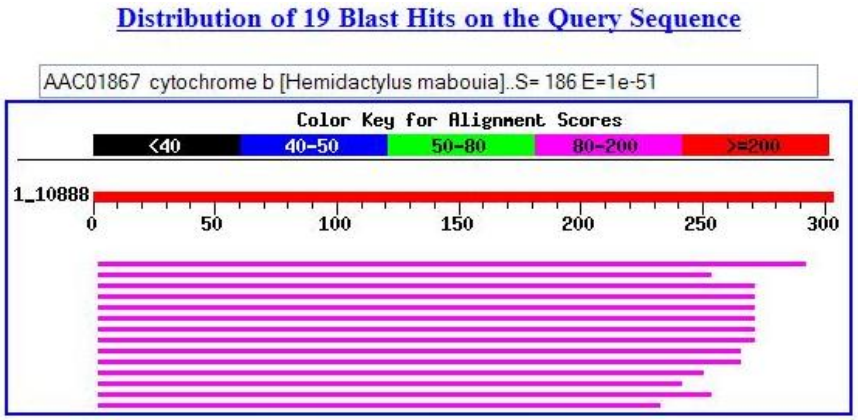
CLUSTAL W (1.82) multiple sequence alignment

```
MayaguezA2        ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
PonceA3           ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
PonceA4           ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
PonceA5           ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
AreciboA6         ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
AreciboA7         ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
IsabelaA11        ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
IsabelaA12        ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
SanGermanA15      ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
SanGermanA16      ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA21       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA22       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA24       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA25       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA30       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
MayaguezA29       ATTAGGAGTA TTGTTCCTGT ATTTCAGGTT TTTTTGTACA GGTATGAGCC GTAATATAGG 60
                  ********** ********** ********** ********** ********** **********

MayaguezA2        CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
PonceA3           CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
PonceA4           CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
PonceA5           CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
AreciboA6         CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
AreciboA7         CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
IsabelaA11        CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
IsabelaA12        CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
SanGermanA15      CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
SanGermanA16      CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA21       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA22       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA24       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA25       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA30       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
MayaguezA29       CCGCGTCCAA TGTGGATGTA TAAGCAGATA AAAAACATAG ATGCTGTATT TGCATGGGTG 120
                  ********** ********** ********** ********** ********** **********
```

Figure 3.7. Results of CLUSTAL W multiple sequence alignment analysis using samples gathered from the Western-Central region of Puerto Rico. The figure shows the first 120 nucleotides out of a 266 nucleotide shared sequence. The asterisks (bottom rows) represent repeated nucleotide throughout individuals. Specimens were identified as *Hemidactylus mabouia* using morphological and molecular approaches.

***In silico* analysis of the cytochrome *b* at amino acid level**. To validate the results, nucleotide sequences were translated to protein using another feature of the BLAST program: Translated query vs. Protein database (blastx). Here, sequences were analyzed to determine if the translated sequence corresponds to a functional cytochrome *b*, for which the original sequence coded. Figure 3.8 shows the results of blastx where the query or submitted sequence is similar to the cytochrome *b* of *Hemidactylus mabouia*.



Sequences producing significant alignments:		Score (bits)	E Value
gi|2843019|gb|AAC01867.1|	cytochrome b [Hemidactylus mabouia]	186	1e-51
gi|27261799|gb|AAN86051.1|	cytochrome b [Hemidactylus mabou...	164	3e-45
gi|13345336|gb|AAK19294.1|	cytochrome b [Hemidactylus bouvi...	159	1e-43
gi|13345324|gb|AAK19290.1|	cytochrome b [Hemidactylus brookii]	152	1e-41
gi|13345327|gb|AAK19291.1|	cytochrome b [Hemidactylus brookii]	150	5e-41
gi|13345321|gb|AAK19289.1|	cytochrome b [Hemidactylus brookii]	150	5e-41
gi|13345315|gb|AAK19287.1|	cytochrome b [Hemidactylus brookii]	150	5e-41
gi|13345318|gb|AAK19288.1|	cytochrome b [Hemidactylus brookii]	149	2e-40
gi|21616340|gb|AAM66186.1|	cytochrome b [Hemidactylus turci...	147	6e-40
gi|9246301|gb|AAF85985.1|	cytochrome b [Hemidactylus turcicus]	146	1e-39
gi|33386009|gb|AAP45103.1|	cytochrome b [Hemidactylus frena...	142	2e-38
gi|13345333|gb|AAK19293.1|	cytochrome b [Hemidactylus bouvi...	138	3e-37
gi|13345312|gb|AAK19286.1|	cytochrome b [Hemidactylus brookii]	136	1e-36

Figure 3.8. Results of the sequence translated to protein for sample A2 (*Hemidactylus mabouia*) collected at Mayagüez. The amino acid sequence corresponds to a cytochrome *b* of *H. mabouia* (accession # C01867) from Africa.

To compare the amino acid sequences it was necessary to translate the nucleotide sequence changing the search parameters. This search had to be done by changing the default genetic code [standard (1)] to genetic code for vertebrate mitochondrial [code (2)]. The results shown in Figure 3.9 point out that the 4 changes of nucleotides between sequences resulted in only one change of amino acid.

Amino acid sequence for sample A2 (*H. mabouia*) from Mayagüez

```
Query: 266 FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI 87
           FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI
Sbjct: 32  FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI 91

Query: 86  CLYIHIGRGLYYGSYLYKKTWNTGTMLL 3
           CLYIHIGRGLYYGSYLYKKTWNTGTMLL
Sbjct: 92  CLYIHIGRGLYYGSYLYKKTWNTGTMLL 119
```

Amino acid sequence for *H. mabouia* (accession #AF324793)

```
Query: 3   LGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFICFYI 182
           LGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFICLYI
Sbjct: 36  LGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFICLYI 95

Query: 183 HIGRGLYYGSYLYKKTWNTGTMLLILTMATAFVGYVL 293
           HIGRGLYYGSYLYKKTWNTGTMLLILTMATAFVGYVL
Sbjct: 96  HIGRGLYYGSYLYKKTWNTGTMLLILTMATAFVGYVL 132
```

Figure 3.9. Amino acid comparison between sample A2 (*H. mabouia*) from Mayagüez and *H. mabouia* from Data Bank (accession #AF324793). Results present the shared amino acid sequence highlighted in grey. The 4 amino acids containing the changes in nucleotide sequence are highlighted in blue with the amino acid shift in green.

The results shown in Figure 3.10 point out the differences among *H. mabouia* from Puerto Rico (Sample A2), *H. mabouia* (accession # AAC01867) from Africa, and *H. brooki* (accession # AAK19290) from Africa. The results were obtained using BLASTP search tool, after the nucleotide sequences were translated to amino acid sequences using the genetic code for vertebrate mitochondrial [code (2)].

Hemidactylus mabouia **cytochrome *b* (accession # AAC01867)**

```
        Length = 261
        Score = 187 bits (474), Expect = 5e-52
        Identities = 87/88 (99%), Positives = 88/88 (100%)

Sample A2: 1   FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI 60
           FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI
H.mabouia: 32  FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI 91

Sample A2: 61  CLYIHIGRGLYYGSYLYKKTWNTGTMLL 88
           CLYIHIGRGLYYGSYLYKKTWNTGTMLL
H.mabouia: 92  CFYIHIGRGLYYGSYLYKKTWNTGTMLL 119
```

Hemidactylus brooki **cytochrome *b* (accession # AAK19290)**

```
        Length = 101
        Score = 158 bits (400), Expect = 2e-43
        Identities = 71/88 (80%), Positives = 81/88 (92%)

Sample A2: 1  FGSLLGICLVIQIVTGLMLAMHYTADTTLAFASVAHICRNVQHGWLIRYTHANTASMFFI 60
              FGSLLG+CL++Q+ TGL LAMHYTADTTLAF+SV HICR+VQHGWLIR THAN ASMFFI
H. brooki: 3  FGSLLGMCLIMQLTTGLFLAMHYTADTTLAFSSVVHICRDVQHGWLIRNTHANGASMFFI 62

Sample A2: 61 CLYIHIGRGLYYGSYLYKKTWNTGTMLL 88
                +Y+HIGRGLYYGSYLYK+TWNTG +LL
H. brooki: 63 FIYLHIGRGLYYGSYLYKETWNTGVVLL 90
```

Figure 3.10. Amino acid comparison between sample A2 (*H. mabouia*) from Mayagüez, *H. mabouia* from Data Bank (accession # AAC01867), and *H. brooki* (accession # AAK19290) from Africa. Results present amino acids sequences that each species share with the samples from Puerto Rico. Amino acid changes were highlighted in blue (A2 vs. *H. mabouia*) and in green (A2 vs. *H. brooki*).

Discussion

In this study, nucleotide and amino acid sequences of the mtDNA cytochrome *b* gene were analyzed *in silico* and later used as a molecular approach that will help distinguish between the two species of *Hemidactylus* geckoes present in Puerto Rico. Results showed that genomic and mtDNA could be extracted from the tail of the lizard, without harming the animal. The reason for using the tail is that it can be regenerated by the animal. Furthermore, mtDNA genes could be amplified from the DNA samples. Our findings demonstrated that it is not always necessary to use the animals liver to extract and amplify mtDNA.

The cytochrome *b* gene analysis proved to be an affective tool to identify *Hemidactylus* species, not only because of the significant differences in nucleotide sequence between *H. brooki* (*H. brooki-haitianus* sequences were not available at Data Banks) and *H. mabouia*, but also for the amino acid sequences they code for. At nucleotide level the difference between *H. brooki* from Africa and the *H. mabouia* found in Puerto Rico was 62 nucleotides. At the other hand, *H. brooki* differs from its counterpart, *H. mabouia* from Africa, in 57 bases. These results were expected because changes in the genetic code between the *Hemidactylus* species are due to reproductive isolation since populations are distant from each other. This separation provokes a stop in gene flow, causing the mutations to accumulate in populations over time.

At a protein level the sequence of amino acids was affected only by 4 mutations in the cytochrome *b* gene sequence between specimens from Africa and Puerto Rico. Having in mind that more than one codon (a 3 nucleotide sequence) code for the same

amino acid (redundancy), the results showed that 3 of the 4 mutations follows that statement (neutral mutation). This is the case for the codon GGG found in the *H. mabouia* sequence from Africa, which codes for glycine. The codon sequence for *H. mabouia* from Puerto Rico is GGA, this nucleotide change does not represent a change in the amino acid sequence because it also code for glycine.

In the case of the second mutation, the shift in nucleotides changes the amino acid sequence. In this mutation the codon from the African specimen is TTG that code for phenylalanine (F). In contrast, the codon of *H. mabouia* from Puerto Rico is TTA, which codes for to leucine (L). This mutation directly affects the amino acid sequence but does not have the same effect over the protein. Presumably the amino acid change did not alter the protein structure and function significantly, because the mutation replaced a hydrophobic amino acid with another hydrophobic amino acid. Both, phenylalanine and leucine, are the most hydrophobic amino acids with values of 1.00 and 0.943, respectively (Black *et al.*, 1991). This is why this mutation was able to be fixed from one generation to another; without being harmful or lethal to the organism.

Regarding the isolation of total DNA from formalin fixed specimens, no successful extraction could be achieved. One of the probable causes for these results relies on the specimens handling and care procedures throughout the decades. At first, all specimens were preserved by injecting a formalin solution directly to the muscles and to the organs (Rivero, 1998). Then, formalin was removed and replaced with alcohol, to avoid human injuries caused by direct formalin exposure to living (respiratory) tissue. Tissues from specimens treated with this procedure suffered damages and DNA degradation, making the extraction protocol more difficult (Fang *et al.*, 2002; Shedlock *et*

al., 1997). In order to increase the potential of success of DNA extraction from formalin treated samples, larger tissue sample should be collected, possibly using whole preserved specimens.

Our findings support the hypothesis of no stable coexistence between the two *Hemidactylus* species. As stated by Kluge (1969) and Meshaka (1994), both species are ecologically analogous. Both species play similar roles in the environment, thus competing for the same resources, such as food and shelter. In other words, they occupy the same niche, so that the presence of both species over the same area results in the competitive displacement of the species with least aptitude. Although we can not state that *H. brooki-haitianus* is not present in Puerto Rico, no specimens of *H. brooki-haitianus* were found in this study. There is an evident increase in *H. mabouia* populations in Puerto Rico. In conclusion, this study indicates a significant change in the distribution of these species in Puerto Rico. All municipalities studied, with the exceptions of Isabela, Ponce, and Mona Island, are new localities for *H. mabouia* in Puerto Rico.

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