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TAXONOMY VERSUS PHYLOGENY:
PHYLOGEOGRAPHY OF MARSH RABBITS WITHOUT HOPPING TO CONCLUSIONS

by

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B.S. Florida Atlantic University, Boca Raton, FL, 2006

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biology
in the College of Sciences
at the University of Central Florida
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ABSTRACT

Subspecific taxonomic designations solely based on morphological characters can often lead to erroneous assumptions about the evolutionary history of populations. This study sought to investigate evolutionary questions and conservation implications associated with morphological subspecific designations of island populations. To this end, I focused my attention on the Lower Keys of Florida, a unique chain of islands with well-known geologic history and rich in endemic, endangered subspecies. I employed genetic analyses to evaluate historical variation and contemporary restriction of gene flow between the endangered Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*) and its sister mainland taxa. A Bayesian phylogeny using 1063 base pairs of the mitochondrial cytochrome *b* gene did not recover reciprocal monophyly of the three named subspecies, and a 95% statistical parsimony haplotype network showed haplotypes being shared among subspecies. Furthermore, clustering analyses using 10 microsatellite loci identified a break within the Lower Keys, separating the western Lower Keys from the island of Big Pine Key. Surprisingly, Big Pine Key grouped with mainland populations and exhibits higher genetic diversity than the western Lower Keys islands. These unexpected findings suggest either a stepping-stone colonization pattern or recent gene flow between the mainland and Big Pine Key via natural dispersal or undocumented man-mediated transfers. Although these results suggest that subspecies designations within *S. palustris* are unwarranted, this study supports the designation western Lower Keys population as a discrete unit of conservation with regard to both DPS and ESU criteria. The importance of using several lines of evidence to uncover the evolutionary history of populations and implications for the conservation of island populations are discussed.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF FIGURES	vii
LIST OF TABLES	x
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: METHODS	8
Sample Collection	8
Laboratory Methods	11
DNA Extraction	11
Mitochondrial DNA Cytochrome <i>b</i> (cyt <i>b</i>) Amplification and Sequencing	11
Microsatellite Development and Genotyping	13
Statistical Analyses	16
Genetic Differentiation of <i>Sylvilagus palustris hefneri</i>	16
Genetic Diversity in Island and Mainland Populations	18
Genetic Differentiation between BPK and Western Lower Keys	19
CHAPTER 3: RESULTS	20
Genetic Differentiation of <i>Sylvilagus palustris hefneri</i>	20
Genetic Diversity in Island and Mainland Populations	31
Genetic Differentiation between BPK and Western Lower Keys	32

CHAPTER 4: DISCUSSION.....	34
Taxonomic and Conservation Implications.....	41
CHAPTER 5: CONCLUSION.....	44
LIST OF REFERENCES.....	45

LIST OF FIGURES

Figure 1. Map of the Florida Keys divided into Upper, Middle and Lower Keys. The Moser Channel, better known as the Seven-Mile Bridge, is an 11-km break separating the Lower Keys from the Middle and Upper Keys.	4
Figure 2. Geographic distribution of <i>Sylvilagus palustris</i> subspecies and sampling locations. Inset: Distribution of <i>S. p. hefneri</i> sampling locations in Big Pine Key (BPK), Sugarloaf Key (SLK), Geiger Key (GGK), East Rockland Key (ERK) and Boca Chica Key (BCK)	6
Figure 3. Schematic representation of amplification of cytochrome <i>b</i> gene in <i>Sylvilagus palustris</i> . The full gene was amplified in smaller, overlapping fragments.	12
Figure 4. Phylogenetic relationships among haplotypes of <i>Sylvilagus palustris</i> . Subspecies abbreviated as follows: <i>S. p. hefneri</i> =Hef, <i>S. p. paludicola</i> =Pld and <i>S. p. palustris</i> =Pls. Locations refer to counties. Nodes with closed and open circles represent posterior probabilities >95% and >90%, respectively. Bayesian tree was rooted using cyt b sequences from <i>S. floridanus</i> and <i>S. aquaticus</i> (the <i>S. floridanus</i> branch is longer than shown in this figure).....	22
Figure 5. Statistical parsimony haplotype network of <i>Sylvilagus palustris</i> haplotypes. Each circle represents a unique haplotype, with the size of the circle scaled to represent the frequency of the haplotype. Each black dot represents a single nucleotide change between haplotypes. Subspecies color-coding shown in legend.	23
Figure 6. Geographic distribution of inferred <i>Sylvilagus palustris</i> haplotypes. Haplotype 1 shared between <i>S. p. palustris</i> and <i>S. p. paludicola</i> . Haplotype 29 shared between <i>S. p. paludicola</i> and <i>S. p. hefneri</i>	24

Figure 7. Plot of $F_{ST} / (1 - F_{ST})$ over geographic distance (km) among all sampling localities of <i>Sylvilagus palustris</i> (except G GK, SLK and ERK; see text for explanation). No significant correlation was found between genetic and geographic distance as confirmed by a Mantel test ($P = 0.21$, after 30,000 randomizations). Population abbreviations defined in Table 1.....	26
Figure 8. Estimation of genetic clusters within <i>Sylvilagus palustris</i> . Both the method of Evanno et al. (2005; line, left axis) and the log likelihood (diamonds, right axis) identified the highest level of population structure at $K=2$	27
Figure 9. Membership coefficients of <i>Sylvilagus palustris</i> individuals as estimated by an admixture model in STRUCTURE. Estimation of genetic clusters at larger scales did not require the inclusion of location information (A and B). Addition of location information was necessary at the smallest scale for the identification of genetic clusters (C). Population abbreviations defined in Table 1.	28
Figure 10. Output from GENELAND showing three major clusters of <i>Sylvilagus palustris</i> populations at the largest scale. Lighter colors denote high probability of belonging to each cluster. Cluster A corresponds to the population in Georgia (RI). Cluster B groups OR, BR and BPK together. Cluster C groups the western Lower Keys (BCK, ERK, G GK and SLK).....	30
Figure 11. Estimates of mitochondrial (A & B) and microsatellite (C & D) diversity for island (dark gray) and mainland (light gray) populations of <i>Sylvilagus palustris</i> . Intermediate coloration of BPK bar is to represent its grouping with the mainland populations in clustering analyses. Allelic richness was rarefied to five (5) samples. Error bars represent standard deviation and numbers above bars represent sample size. Welch's Two-sample T-test used to compare island vs. mainland genetic diversity; P -values shown in graphs.....	32

Figure 12. Close-up of the separation within *Sylvilagus palustris hefneri* in the Lower Keys. The lighter color shows the probability of belonging to the BPK cluster.....33

LIST OF TABLES

Table 1. Location information of <i>Sylvilagus palustris</i> samples. Sampling locations, sampling location identifications (ID), geographical coordinates in decimal degrees and total samples used per locality	10
Table 2. Primers used for amplification of overlapping cyt <i>b</i> fragments in <i>Sylvilagus palustris</i> . Annealing temperatures (T_a) and fragment sizes (bp) included.	12
Table 3. Primer information for microsatellite amplification in <i>Sylvilagus palustris</i> . Microsatellite markers used, primer sequences, annealing temperatures (T_a), allelic size range, repeat motif, maximum number of allele and references.	15
Table 4. Pairwise matrix of genetic distances (F_{ST} , above diagonal) and geographic distance (km, below diagonal) between populations of <i>Sylvilagus palustris</i> . Values in bold are significant after 10,000 permutations and adjustment for multiple comparisons. Population abbreviations defined in Table 1.	26

CHAPTER 1: INTRODUCTION

A major goal of evolutionary biology is to understand patterns of genetic variation within species that inhabit wide geographic ranges. In an attempt to account for species that exhibit regional variants, subspecies classifications are often used to partition observed variation across taxa such as mammals (*Peromyscus polionotus*, Hall 1981; *Cynopterus nusatenggara*, Kitchener & Maharadatunkamsi 1996), birds (*Buteo lineatus*, Clark & Wheeler 1987; *Somateria mollissima*, Furness *et al.* 2010), amphibians (*Acris crepitans*, Conant & Collins 1998), reptiles (*Gallotia galloti*, Gonzalez *et al.* 1996; *Rhinocheilus lecontei*, Grismer 1990) and arthropods (*Limenitis arthemis*, Mullen *et al.* 2008). The basis for subspecies designations, however, remains vague. The earliest criterion for the designation of subspecies is the 75% rule, which states that 75% of a population must lie outside 99% of the range of other populations for a given defining character or characters (Amadon 1949; Mayr 1969). The main concern with such broad criteria is that the characters used in the description of subspecies may be arbitrary. The recent inclusion of molecular data into subspecies designations has revealed that many currently recognized subspecies do not represent distinct evolutionary lineages. For example, Culver *et al.* (2000) demonstrated that the 15 recognized North American subspecies of *Puma concolor* actually represent a single evolutionary lineage. Similar studies of misidentified subspecies abound (*e.g.* Burbrink *et al.* 2000; Hull *et al.* 2008; Manier 2004)

An ongoing challenge of subspecies identification occurs in island populations. Because islands by definition occupy areas outside the mainland, and because island populations may differ morphologically from their mainland counterparts, these populations are frequently considered

members of distinct subspecies or species (*e.g.* Furness *et al.* 2010; Gonzalez *et al.* 1996; Pergams & Ashley 1999). This assumption of differentiation is plausible given that gene flow among islands and between islands and the mainland is often minimal. Such disruption in genetic exchange can result in divergence of allele frequencies among population, with the ultimate outcome of continued isolation leading to separate evolutionary trajectories (Barr *et al.* 2008; Funk *et al.* 2007; Hitchings & Beebee 1997; Postma *et al.* 2009; Wilson *et al.* 2009). Accordingly, studies have shown that some island populations have differentiated from other islands and/or mainland populations (*e.g.* Barry & Tallmon 2010; Degner *et al.* 2007; Duffie *et al.* 2009; Estoup *et al.* 1996). However, island population differentiation is dependent upon many factors such as geological and colonization events, as well as the dispersal ability of a species (*e.g.* Graziotin *et al.* 2006; Heaney *et al.* 2005; Paetkau *et al.* 1998; Steinfartz *et al.* 2009; Yeung *et al.* 2009)

Proper recognition of evolutionary lineages in island populations is particularly important because these populations tend to be more prone to extinction than their mainland counterparts (Frankham 1998). The natural genetic isolation of island populations of non-volant species can result in the overall loss of genetic diversity through genetic drift (Couvét 2002; Frankham 1997; Ingvarsson 2001; Palstra & Ruzzante 2008; Vila *et al.* 2003), which may have short-term costs in the form of inbreeding depression as well as compromising the ability of populations to adapt to new environmental conditions (Frankham 1998; Frankham 2005; Lynch *et al.* 1995; Willi *et al.* 2006; Wright *et al.* 2008). More recently, anthropogenic pressures such as habitat loss, fragmentation and introduction of exotic species have exacerbated this extinction threat by further disrupting connectivity and reducing population sizes. As a result, isolated populations often require active

management in order to minimize extinction risks (Fahrig & Merriam 1994; Templeton *et al.* 1990). Regrettably, a lack of understanding regarding the evolutionary history of island populations not only affects conservation priorities, but can also hinder management strategies such as reintroductions and translocations.

The island chain of the Florida Lower Keys provides an excellent model system to explore the interface of island population evolutionary history, subspecies designations and conservation efforts. The Florida Keys are divided into the Upper, Middle and Lower Keys (Figure 1). The Upper and Middle Keys are composed of Key Largo limestone. This sedimentary layer is the exposed remnant of an ancient coral reef that formed when South Florida was submerged in sea water about 125,000 years before present (YBP, Shinn 1988). Deposition of sand banks on the southwestern end of the reef formed the Lower Keys that are now separated from the Upper and Middle Keys by an 11-km biogeographic break known as the Moser Channel (Shinn 1988)(Figure 1). During the Last Glacial Maximum (40,000-12,000 YBP), species were able to colonize the exposed South Florida plateau but later became isolated due to sea level rise approximately 10,000 YBP (Lazell 1984). It is hypothesized that unique ecological circumstances led to the differentiation of currently recognized endemic subspecies, such as the Key deer (*Odocoileus virginiana clavium*), the silver rice rat (*Oryzomys palustris natator*) and the Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*).

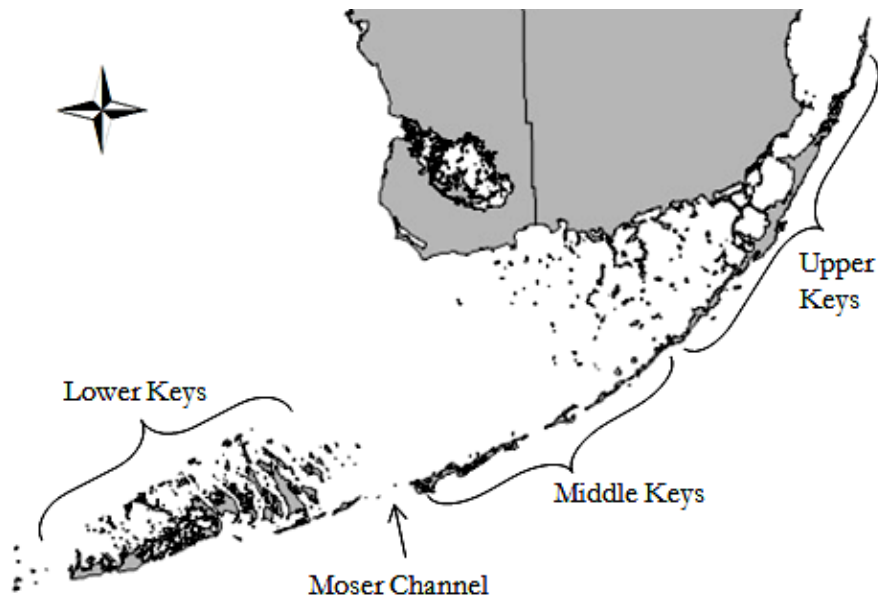


Figure 1. Map of the Florida Keys divided into Upper, Middle and Lower Keys. The Moser Channel, better known as the Seven-Mile Bridge, is an 11-km break separating the Lower Keys from the Middle and Upper Keys.

Increasing development of the Lower Keys has been detrimental to many endemic populations both directly (through destruction and fragmentation of habitat) as well as indirectly (through the introduction of invasive predators, Forsy & Humphrey 1996). These anthropogenic pressures have resulted in the Lower Keys marsh rabbit, *Sylvilagus palustris hefneri*, being listed as an endangered subspecies since 1990 (USFWS 2007b). In addition to *S. p. hefneri*, two other subspecies marsh rabbits (both found in the mainland) are currently recognized: *S. p. palustris* and *S. p. paludicola* (Figure 2). Taxonomic designation of *S. p. hefneri* is based on morphological criteria; it differs from the other two subspecies in pelage coloration, in cranial morphology (Lazell 1984) and in size, as it is the smallest of the three marsh rabbit subspecies (USFWS 2007b). Dispersal of *S. p. hefneri* typically consists of mature males emigrating up to two kilometers from the natal nest through areas with

adequate ground coverage (Forys & Humphrey 1996), which makes island-mainland dispersal unlikely. As of 1995, the population of *S. p. hefneri* was estimated to be between 100 and 300 individuals (Forys & Humphrey 1996). An updated distribution of *S. p. hefneri* (Figure 2) (Faulhaber *et al.* 2007) showed that the largest number of occupied patches occurred on Big Pine Key (BPK), Boca Chica Key (BCK), and Sugarloaf Key (SLK). All populations of *S. p. hefneri* have been declining steadily since 1988, but in SLK and especially in BPK such declines have been more drastic (USFWS 2007a) potentially due to the damaging effects of Hurricane Wilma.

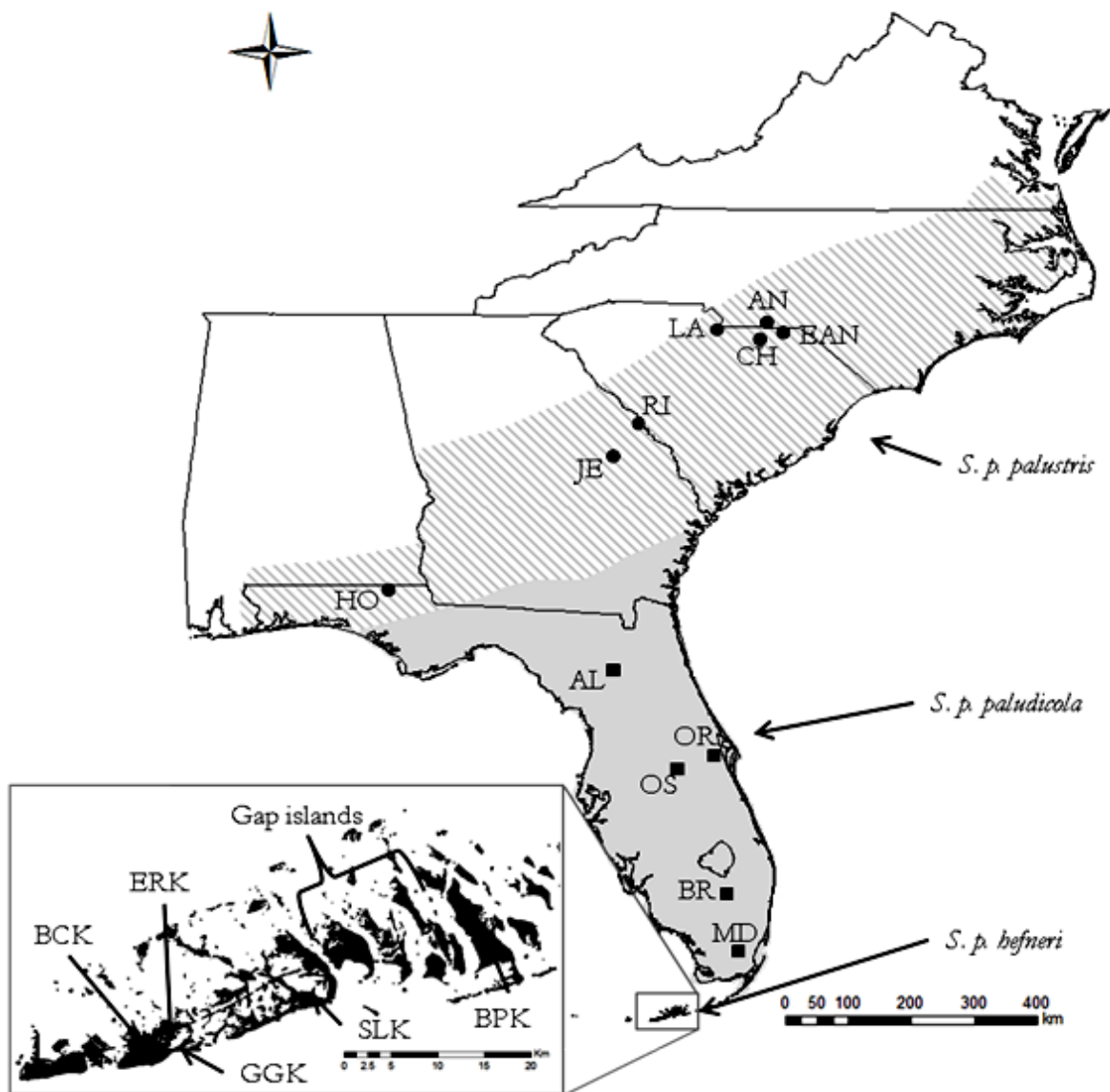


Figure 2. Geographic distribution of *Sylvilagus palustris* subspecies and sampling locations. Inset: Distribution of *S. p. hefneri* sampling locations in Big Pine Key (BPK), Sugarloaf Key (SLK), Geiger Key (GGK), East Rockland Key (ERK) and Boca Chica Key (BCK)

In order to determine whether *S. p. hefneri* represents a separate evolutionary lineage and to aid in conservation efforts, I sought to examine the genetic differentiation of *S. p. hefneri* and characterize levels of genetic variation between island and mainland populations. I first hypothesized that *S. p.*

hefneri was genetically differentiated from the mainland and was therefore a distinct evolutionary lineage, which would be evidenced by a well-supported monophyletic group in mitochondrial genes and significant differences in nuclear allele frequencies. I based this hypothesis on the previously mentioned morphological differences and on reported differences in fecundity (3.7 litters/year in *S. p. hefneri* as opposed to 5.7 - 6.9 litters/year in *S. p. paludicola*, Forsy & Humphrey 1996; Holler & Conaway 1979). Second, because island populations generally exhibit lower levels of genetic diversity than mainland populations (Frankham 1997), I expected that *S. p. hefneri* would be genetically depauperate in comparison to mainland populations of *S. p. paludicola* and *S. p. palustris*. Finally, I hypothesize that populations on BPK will be differentiated from populations on SLK and islands further west. This hypothesis is based on a previous study by Crouse *et al.* (2009) that used mitochondrial DNA markers and identified a partition between the eastern Lower Keys (BPK) and the western Lower Keys (BCK and SLK), separated by a gap of islands that appear to have been historically inhabited by *S. p. hefneri* but currently contain no rabbits (Figure 2) (Crouse *et al.* 2009; Lazell 1989).

To address these hypotheses, I collected samples from the *S. palustris* range and sequenced the mitochondrial gene cytochrome *b* (cyt *b*) to construct a Bayesian phylogeny and a haplotype network to investigate historical genetic divergence among the three subspecies. In addition, I genotyped island and mainland populations using 10 polymorphic microsatellite loci to evaluate population structure, current patterns of gene flow and levels of genetic diversity. The results of this study are discussed with insights into colonization and differentiation patterns, as well as implications for the management and conservation of these island populations and other endangered insular species.

CHAPTER 2: METHODS

Sample Collection

Marsh rabbits (*Sylvilagus palustris*) are small to medium sized cottontails, with reddish-brown coat, small, slender feet and short, broad ears (Chapman & Willner 1981). Unlike other cottontails, the abdominal area and underside of the tail is gray in color instead of white. Also, unlike other *Sylvilagus* species, *S. palustris* is confined solely to marshy habitats. Early accounts estimate that home ranges seem to be small (~200 yards in extend, Blair 1936). *S. palustris* and its sister species, *S. aquaticus*, are the only rabbits known to have the ability to swim. I collected 150 marsh rabbit samples from throughout the range of the three subspecies (Figure 2, Table 1). All 26 tissues from *S. p. palustris* were donated by hunters during the 2008-2009 hunting season. Hunted-harvested samples consisted of ear clips placed in a 50mL tube containing anhydrous calcium sulfate for preservation at room temperatures. Samples were sent to our lab at the University of Central Florida within five days of collection. Forty samples of *S. p. paludicola* were live-trapped (see below) from December 2008 through July 2009. Four additional samples of *S. p. paludicola*, one from Alachua County, FL (AL) and three from Miami-Dade County, FL (MD), were provided by the Florida Museum of Natural History. The 80 *S. p. hefneri* samples consisted of 68 live-trapped individuals collected during the summer of 2008 by the U.S. Fish and Wildlife Service (hereafter the USFWS) and 12 road kill tissue samples collected and donated by the USFWS.

All live trapping was carried out for a minimum of seven days per location. Trapping would continue until 15 – 30 rabbits were caught, although these numbers were allowed to be lower (as low as five samples) for the endangered populations of *S. p. hefneri*. Trapping was discontinued if less than two rabbits were caught within seven days. Double-door Tomahawk Live traps (Tomahawk Live Trap Co., WI) were baited with cut apples and carrots and placed in natural vegetation tunnels in the evening and checked at dawn for the presence of rabbits. To minimize stress and the possibility of injury, rabbits were handled either with a bag made of cloth or with a cloth device modified from the design of Koprowski (2002), which allowed handling by a single investigator. Tissue from live-trapped individuals consisted of ear punches and hair follicles for *S. p. paludicola*, or just hair follicles for *S. p. hefneri*. Ear punches were placed in 1.5mL tubes, and hair follicles were placed in bags, both containing anhydrous calcium sulfate. In addition, a patch of hair was cut to identify recaptures. Rabbits were immediately released after processing on the site of capture.

Table 1. Location information of *Sylvilagus palustris* samples. Sampling locations, sampling location identifications (ID), geographical coordinates in decimal degrees and total samples used per locality

Location	ID	Latitude (°)	Longitude (°)	Sample Size
<i>Sylvilagus palustris palustris</i>				
Anson Co., NC	AN	34.983	-80.116	1
East Anson Co., NC	EAN	34.869	-79.892	1
Lancaster Co., SC	LA	34.925	-80.815	1
Chesterfield Co., SC	CH	34.743	-80.169	1
Richmond Co., GA	RI	33.456	-81.963	19
Jefferson Co., GA	JE	32.976	-82.331	2
Holmes Co., FL	HO	30.883	-85.657	1
<i>Sylvilagus palustris paludicola</i>				
Alachua Co., FL*	AL	29.651	-82.325	1
Orange Co., FL	OR	28.366	-80.880	22
Osceola Co., FL	OS	28.137	-81.445	1
Broward Co., FL	BR	26.332	-80.623	17
Miami-Dade Co., FL†	MD	25.433	-80.479	3
<i>Sylvilagus palustris hefneri</i>				
Big Pine Key, FL	BPK	24.702	-81.376	6
Sugarloaf Key, FL	SLK	24.587	-81.664	5
East Rockland Key, FL	ERK	24.631	-81.533	7
Geiger Key, FL	GGK	24.574	-81.666	14
Boca Chica Key, FL	BCK	24.573	-81.692	48

*Florida Museum of Natural History Catalog No. 1178

†Florida Museum of Natural History Catalog No. 1579, 1649, 1650

Laboratory Methods

DNA Extraction

DNA from hunter-donated tissue, road kills and ear punches was extracted using a standard phenol-chloroform extraction (Sambrook & Russell 2001). Qiagen DNeasy tissue purification kit (Qiagen Inc., Germany) was used for museum samples and hair follicles following the recommendations of Mullen and Hoekstra (2008) with a few modifications: During the elution step, 50 μ L of water was used instead of buffer AE to avoid interference with PCR reactions. Also, water was preheated to 70°C prior to elution and was allowed to incubate for five minutes after addition to the membrane before centrifugation. Finally, elution was repeated twice using 50 μ L for each elution to ensure maximum recovery of DNA. For samples consisting only of hair, a minimum of six follicles were used per sample for DNA extraction.

Mitochondrial DNA Cytochrome *b* (cyt *b*) Amplification and Sequencing

Amplification of the entire cyt *b* gene (1140 bp) from low quality samples such as road kills, museum skins and hair was performed using multiple primers that would amplify smaller, overlapping sequences (Table 2, Figure 3). DNA amplifications consisted of 20 μ L reactions containing 30ng of genomic DNA, 0.5 μ M of each primer, 2 μ L of 10X PCR buffer, 2.5mM of MgCl₂, 200 μ M of each dNTP and 1 Unit of Taq polymerase. PCR protocols consisted of 95°C for 4 minutes, followed by 40 cycles with 30 seconds at 95°C, 30 seconds at the annealing temperature (see Table 2), and 45

seconds at 72°C, then a final extension cycle at 72°C for 7 minutes. PCR products were cleaned with Exo-SAP-IT (USB Affymetrix, CA) or NucleoSpin Extract II spin columns (Macherey-Nagel, PA) and sequenced in both directions in an ABI 3730 DNA analyzer (Applied Biosystems, CA). Mitochondrial sequences from each individual were edited using SEQUENCHER *v. 4.8* (Gene codes, MI). Sequence alignment was performed in MEGA *v. 4.0* (Kumar *et al.* 2004) using CLUSTAL and checked for possible misalignments by eye.

Table 2. Primers used for amplification of overlapping cyt *b* fragments in *Sylvilagus palustris*. Annealing temperatures (T_a) and fragment sizes (bp) included.

Locus	Primer sequence (5'-3')	T_a (°C)	Fragment size (bp)
EAH 532 (F)	CATCGTGTGTTTTCAACTATAAGAACC	50	396
EAH 533 (R)	ACTGCGAATAGCAGGATAATG		
EAH 534 (F)	TCCGACACACTTACAGCCTTC	50	473
EAH 535 (R)	TCTGAAGGAATCCCTGATGG		
EAH 536 (F)	TCTTTTATCAGCCATCCCCTA	55	398
EAH 537 (R)	GAATGGCGTAGGCGAATAGA		
EAH 538 (F)	CTGGGATTCTCGCCCTAA	55	393
EAH 539 (R)	GGTGAAGTAGAGGATGGATGC		
EAH 540 (F)	CCATATATCCAAACAACGCAGT	55	249
EAH 541 (R)	GGCCAGGGTAATGAATTATACTACT		

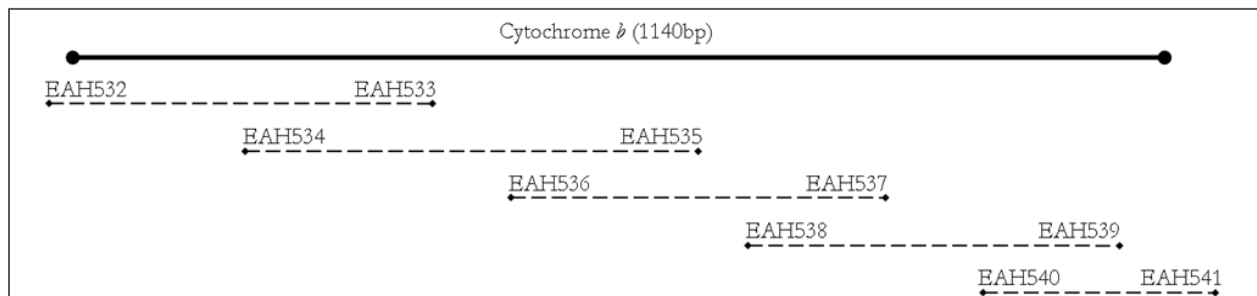


Figure 3. Schematic representation of amplification of cytochrome *b* gene in *Sylvilagus palustris*. The full gene was amplified in smaller, overlapping fragments.

Microsatellite Development and Genotyping

Microsatellite primers were obtained from a variety of sources (Table 3). Four microsatellite loci originally isolated for *Oryctolagus cuniculus* (Korstanje *et al.* 2001; Mougél *et al.* 1997; Rico *et al.* 1994) and *Sylvilagus floridanus* (Berkman *et al.* 2009) were cross-amplified in *Sylvilagus palustris*. To obtain additional loci for *S. palustris*, I employed an enrichment protocol summarized in Hoffman *et al.* (2003). First, about 40ng of genomic DNA was cut into smaller pieces using a degenerate oligonucleotide-primed PCR (DOP-PCR). The DOP-PCR product was then enriched using 5'-biotynilated, 3'-amino modified (GATA)₈ or (CA)₁₅ primers. Hybridized product was separated using streptavidin-coated magnetic beads as explained in Ardren *et al.* (2002) and the enriched genomic library underwent a second round of DOP-PCR. Enriched product was then cloned using the TOPO TA Cloning Kit from Invitrogen (Invitrogen, CA). Colonies were plucked using sterile tips, placed in 100µl of H₂O and boiled for 10 minutes to release the plasmid. Positive colonies were screened using the T3/T7 procedure outlined by Cabe & Marshall (2001). Out of all positive clones from which primers were designed, six were polymorphic and in Hardy-Weinberg Equilibrium (HWE) for a total of 10 polymorphic loci used in this study (Table 3).

Amplifications for all microsatellites were performed in 10µL reactions containing 5ng of template DNA, 1µL of 10X PCR buffer, 2.5mM of MgCl₂, 200µM of each dNTP, 0.125uM of M13-tagged forward primer and 0.5µM of reverse primer, 0.5µM of fluorescently-labeled M13 primer and 1 Unit of Taq polymerase. PCR amplifications used the standard touchdown protocol preloaded in a BioRad MyCycler thermalcycler (Bio-Rad Laboratories, CA). Cycles started with a denaturing step

for 4 minutes at 95°C, followed by 15°C touchdown cycles of 95°C for 30 seconds, annealing temperature (T_a , see Table 3) decrease by 0.5°C/cycle for 30 seconds, and 72°C for 45 seconds. After the final touchdown cycle, 30 additional cycles were performed with a T_a of 45°C with a final extension of 7 minutes. Annealing temperatures for each primer pair can be found in Table 3. PCR products were visualized on a 2% agarose gel and then genotypes were determined on a CEQ 8000 DNA analyzer (Beckman Coulter, CA). To check for possible scoring errors due to null alleles and allelic dropout, I used MICRO-CHECKER *v.* 2.2. I also checked all 10 loci for deviations from HWE and linkage equilibrium (LE) using the Fisher's exact test employed in GENEPOP *v.* 4.0 (Raymond & Rousset 1995) and I applied a Bonferroni correction to account for multiple comparisons (Rice 1989).

Table 3. Primer information for microsatellite amplification in *Sylvilagus palustris*. Microsatellite markers used, primer sequences, annealing temperatures (T_a), allelic size range, repeat motif, maximum number of allele and references.

Locus	Primer sequence (5'-3')	T_a	Allele Size Range (bp)	Repeat motif in clone	N_A	Reference
sol08	F-GGATTGGGCCCCTTTGCTCACACTTG R-ATCGCAGCCATATCTGAGAGAACTC	58	138-144	(TG) ₁₉ (N) ₁₅ (TG) ₅	4	Rico et al. 1994
Sat8	F-CAGACCCGGCAGTTGCAGAG R-GGGAGAGAGGGATGGAGGTATG	53	124-128	(CT) ₁₄ (GT)TT(GT) ₅	3	Mougel et al. 1997
D1L5G7	F-GGCCTCATATCACGTAACATCC R-GCCATCTTTGCTTCCCTGAGT	55	355-381	(CA) ₁₂ (GA) ₁₂	13	Korstanje et al. 2001
Sfl011	F-GCACAGCAGCATATTCCATGC R-CCATGAATCAATACAGGTTAATGCC	62	205-233	(GT) ₁₈	12	Berkman et al. 2009
Spal003	F-CAGCCATCTGGGGTGTAAG R-CAAATTGATCAAAGCAATGTGA	53	224-248	(GATA) ₆ (GACA) ₂	3	This study
Spal004	F-GCATTTTGGAGAAAACCAACA R-TGTCCTTTTATTTCTGTTTTCAGT	53	196-232	(CTAT) ₆ -imperfect	9	This study
Spal019	F-GGCGGCGGTTCACTTTAT R-ATATGGACGATCCCACCAGA	56	155-179	(CTAT) ₁₃ -imperfect	7	This study
Spal017	F-TCATTTGATTTTAAAGAGGACAAGAAA R-GGGCATTTGGACATTTGAAAC	52	167-195	(GATA) ₁₆ -imperfect	8	This study
Spal033	F-ATGCTTGTGCCTCTCAAGGT R-AGTCCTGGCTGTTAGGGACA	56	314-350	(CTAT) ₃ -(CTAT) ₄ -	4	This study
Spal038	F-TGTGTCAAGAAACAGCCATAGAA R-TTCCTTCAGAAATTTCAACTCCAA	55	325-335	(GT) ₁₅ -(GC) ₂ -(GC) ₃	6	This study

Statistical Analyses

Genetic Differentiation of *Sylvilagus palustris hefneri*

To determine whether *S. p. hefneri* is genetically differentiated from mainland subspecies (Hypothesis 1), I first looked for evidence of historic restriction of gene flow evidenced by a well-supported monophyletic *S. p. hefneri* group with respect to the other two subspecies. To this end, I inferred phylogenetic relationships among unique haplotypes within *S. palustris* using a Bayesian Markov Chain Monte Carlo (MCMC) in the program MRBAYES *v.* 3.1 (Ronquist & Huelsenbeck 2003). Cytochrome *b* sequences obtained from Genbank for *S. floridanus* (Genbank No. AY192724.1) and *S. aquaticus* (Genbank No. AY292726.1) were used as outgroup taxa. The best-fit model of nucleotide substitution was selected based on Akaike Information Criteria (AIC) in the program MRMODELTEST *v.* 2.2 (Nylander *et al.* 2004) for all possible partitions. These partitions included (i) the unpartitioned dataset; (ii) first codon position; (ii) second codon position; (iii) first and second codon position; and (iv) third codon position. Separate trees were generated for all possible partitions using the following parameters: four MCMC chains were run for 5 million generations with parameters sampled every 100 generations with a burn in of 5000 generations. The phylogeny reported was selected based on the partitions chosen according to Bayes factors (Kass & Raftery 1995). In order to further investigate relationships among cyt *b* sequences, I employed an algorithm by Templeton *et al.* (1992) to construct an intraspecific haplotype network under a 95% connection limit in TCS *v.* 1.21 (Clement *et al.* 2000).

Second, I tested for genetic differentiation at the population level using microsatellite data. Here, I limited the analyses to locations where five or more individuals were collected (Table 1). First, I estimated global and pairwise genetic distances based on allelic state (F_{ST}) in SPAGEDi *v. 1.3* (Hardy & Vekemans 2002). To test whether increases in geographic distance would also result in increased genetic differentiation (*i.e.* isolation by distance, or IBD), I performed a Mantel test of genetic distance over geographic distance in the program IBDWS *v. 3.16* (Jensen *et al.* 2005). Due to the linear arrangement of populations in this study, untransformed geographic distances were used for this correlation analysis.

As a final analysis to address Hypothesis 1, I looked for evidence of current gene flow between island and mainland by determining the genetic structuring of populations throughout the *S. palustris* range. For this purpose, I used two Bayesian clustering analyses that differ in the type of location information that can be incorporated. First, I implemented an admixture model with correlated allele frequencies that did not incorporate any prior location information in STRUCTURE *v. 2.3* (Pritchard *et al.* 2000). The number of possible clusters, K , was allowed to vary from 1 – 9. Because STRUCTURE is designed to only find the highest level of population structuring, I hierarchically looked within each inferred cluster until all structure levels were found (see Degner *et al.* 2007). The datasets used for some of these hierarchical analyses were small and therefore required addition of location information. For all STRUCTURE analyses, 20 independent runs at each possible K were conducted with MCMC parameters set to 300,000 iterations with a burn in period of 10,000. The highest level of population structuring was determined using the Evanno *et al.* (2005) criterion, ΔK .

Because the STRUCTURE model is not designed to incorporate spatial information, I used a spatially explicit model with uncorrelated allele frequencies in the R package, GENELAND *v. 3.2* (Guillot *et al.* 2005) to make sure that spatial information would not change inferences obtained solely from genetic data. I introduced 0.1 decimal degrees of spatial coordinate uncertainty as suggested by the user manual for vagile organisms. As with STRUCTURE, I first included all populations to detect any major clusters and then repeated the analysis hierarchically at smaller scales to identify any further substructure. For all analyses, I conducted 10 runs for 300,000 iterations each, and parameters were sampled every 100 iterations after a burn in of 2000 iterations. The best run for each analysis was chosen based on mean posterior density.

Genetic Diversity in Island and Mainland Populations

To determine whether island populations harbor lower levels of genetic diversity than mainland populations (Hypothesis 2), I estimated nucleotide diversity (π) and gene diversity (h) for each population using the cyt *b* dataset in the program DNASP *v. 5.0* (Librado & Rozas 2009). Using the microsatellite dataset, I averaged the observed (H_O) and expected (H_E) heterozygosities using the program GENEPOP *v. 4.0* (Raymond & Rousset 1995). Finally, I used FSTAT *v. 1.2* (Goudet 2002) to calculate the allelic richness (AR) of each population, which employs a rarefaction method to account for differences in sample sizes when looking at allelic counts. To test for statistically significant differences in genetic diversity levels between island and mainland populations, I implemented a Welch's Two-Sample T-Test in the program R *v. 2.11.1* (R Development Core Team 2006). It was also important to determine whether populations have undergone any recent

population bottlenecks that could affect current levels of genetic diversity. Recent bottlenecks can be detected when observed heterozygosities are higher than expected because the number of alleles can decrease much faster than heterozygosity levels (Cornuet & Luikart 1996). For this purpose, all study populations were tested for heterozygote excess in the program BOTTLENECK *v. 1.2.02* (Piry *et al.* 1999). A Two-Phase Model (TPM) was used as it better fits microsatellite evolution (Dirienzo *et al.* 1999). All other parameters were left to the default settings, with 1000 replications assuming 70% stepwise mutation model and 30% infinite allele model. Because we are using fewer than 20 microsatellite loci, a Wilcoxon signed rank test was used to assess significance (Piry *et al.* 1999).

Genetic Differentiation between BPK and Western Lower Keys

To determine whether populations in BPK were genetically differentiated from populations in the Western Lower Keys (Hypothesis 3), I checked for evidence of restricted gene flow within populations sampled only in the Lower Keys (Figure 2). To this end, I repeated the Bayesian clustering analyses implemented in both STRUCTURE *v. 2.3* (Pritchard *et al.* 2000) and GENELAND *v. 3.2* (Guillot *et al.* 2005). The parameters for these island-level analyses remained unchanged from the full-scale analyses described above.

CHAPTER 3: RESULTS

Genetic Differentiation of *Sylvilagus palustris hefneri*

I successfully amplified 1063 bp of the *cyt b* gene from 69 samples used for phylogenetic analysis. I found a total of 33 unique haplotypes defined by 63 variable sites, 44 of which were parsimony informative across all samples including all three subspecies. Using the Bayes factors criterion, the final tree chosen was the unpartitioned dataset under the Hasegawa-Kishino-Yano + gamma + invariant sites (HKY+G+I) model of DNA evolution (Hasegawa *et al.* 1985). Although the phylogeny provided support for the *S. palustris* samples as a monophyletic group relative to outgroup taxa, there was very low support overall for most of the in-group clades (Figure 4). In addition, well-supported clades (>95% posterior probability) showed a mixture of mainland and island haplotypes, suggesting lack of historical differentiation. Detailed information about haplotype relationships was obtained in the 95% statistical parsimony haplotype network (Figure 5). Unexpectedly, shared haplotypes occurred not only within populations of the same subspecies, but also among subspecific groups. Haplotype 1, which was identified as the most likely ancestral haplotype given its outgroup weight (Clement *et al.* 2000), was found in populations of both *S. p. paludicola* and *S. p. palustris*. Interestingly, two haplotypes found in BPK were also either found in *S. p. paludicola* (Haplotype 29) or grouped with mainland haplotypes (Haplotype 21) in all analyses (Figure 5 and Figure 6). Only one haplotype (Haplotype 30) was endemic to *S. p. hefneri*. This haplotype (Haplotype 30) was also the most frequent because it included all individuals from BCK and one individual from BPK

(Figure 5 and Figure 6). The patterns revealed by the haplotype network suggest incomplete segregation of haplotypes, supporting the results from the Bayesian phylogeny.

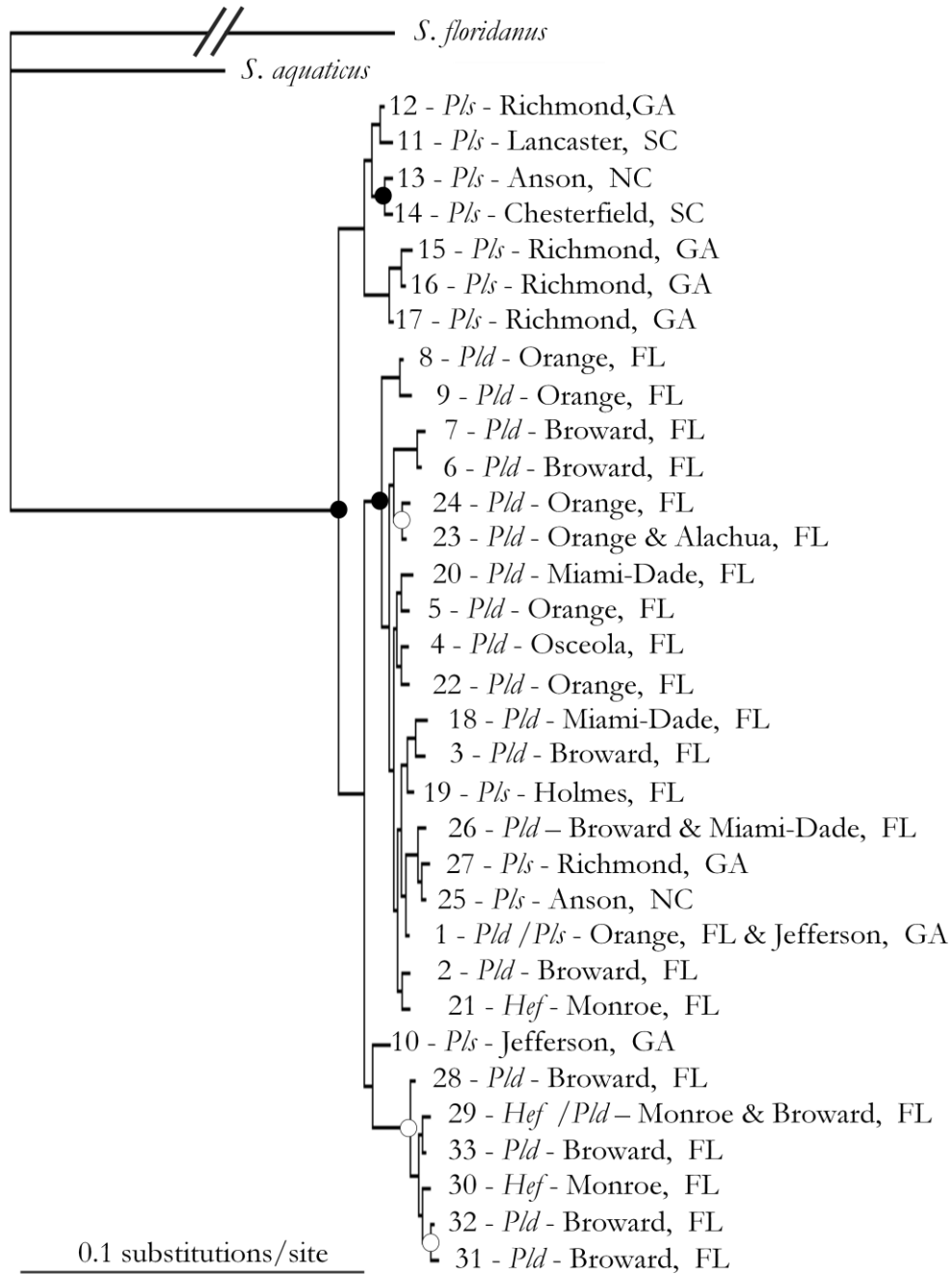


Figure 4. Phylogenetic relationships among haplotypes of *Syphilagus palustris*. Subspecies abbreviated as follows: *S. p. hefneri* = *Hef*, *S. p. paludicola* = *Pld* and *S. p. palustris* = *Pls*. Locations refer to counties. Nodes with closed and open circles represent posterior probabilities >95% and >90%, respectively. Bayesian tree was rooted using cyt b sequences from *S. floridanus* and *S. aquaticus* (the *S. floridanus* branch is longer than shown in this figure).

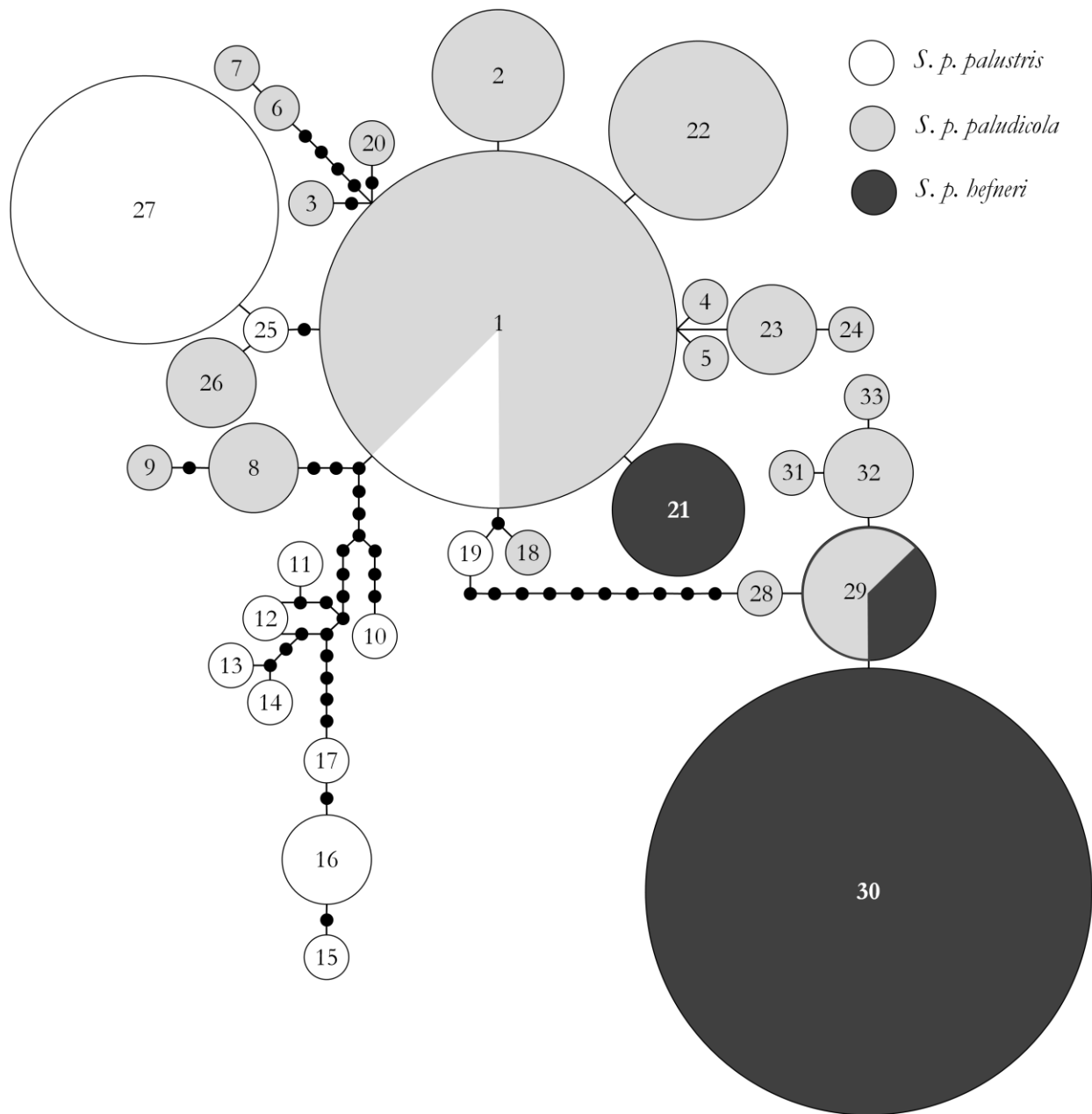


Figure 5. Statistical parsimony haplotype network of *Sylvilagus palustris* haplotypes. Each circle represents a unique haplotype, with the size of the circle scaled to represent the frequency of the haplotype. Each black dot represents a single nucleotide change between haplotypes. Subspecies color-coding shown in legend.

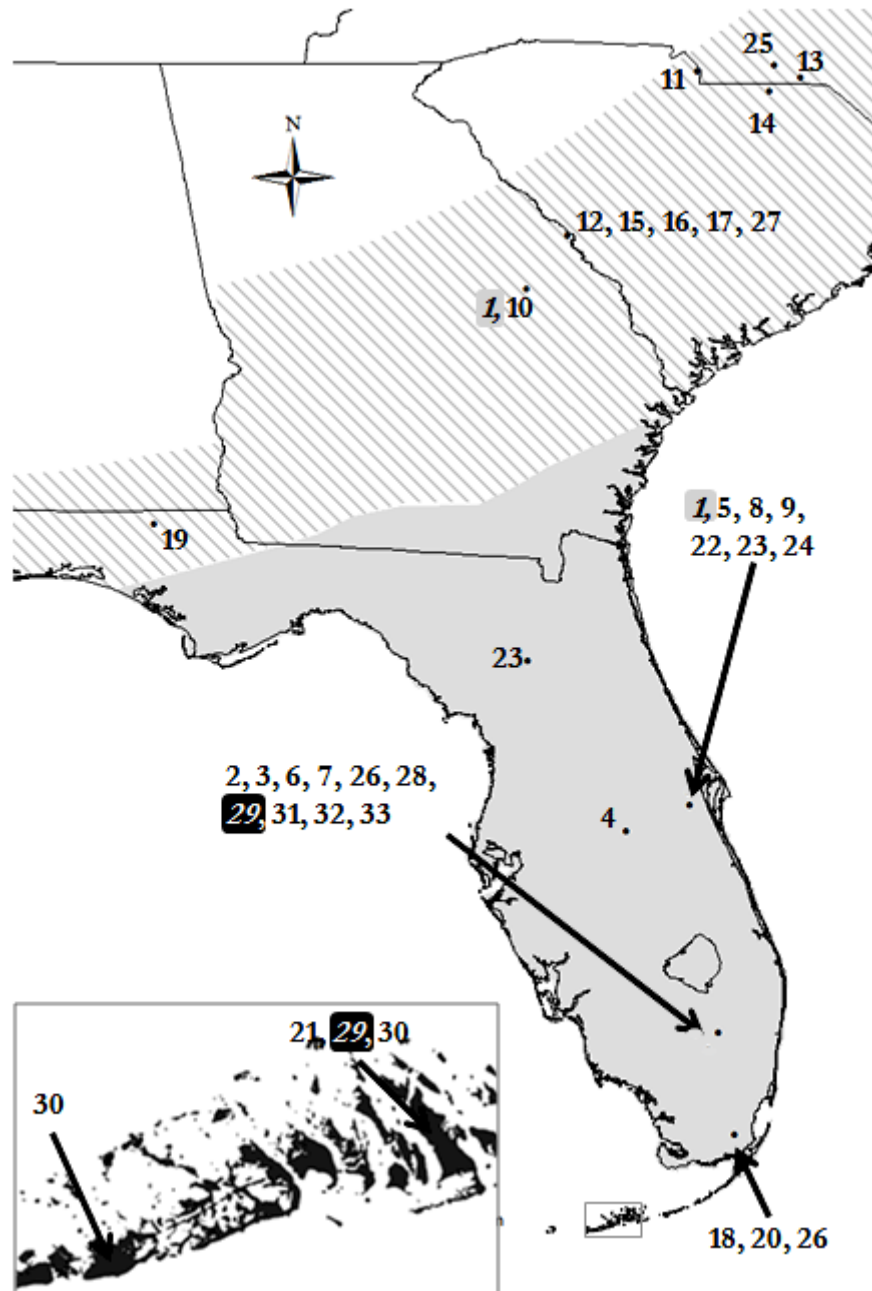


Figure 6. Geographic distribution of inferred *Sylvilagus palustris* haplotypes. Haplotype 1 shared between *S. p. palustris* and *S. p. paludicola*. Haplotype 29 shared between *S. p. paludicola* and *S. p. beffneri*.

For population level analyses, genotypes were obtained for a total of 138 individuals from eight sampling localities (Table 1). There was no evidence of scoring errors due to stuttering, null alleles or allelic dropout as verified by the program MICROCHECKER. Additionally, there was no significant deviation from expected heterozygosities, and therefore all populations conformed to HWE and LE expectations after Bonferroni correction for multiple comparisons.

Overall, differentiation was high among all sampled populations with a global F_{ST} of 0.22. Moreover, genetic differentiation was moderate within island populations ($F_{ST} = 0.11$), even after the most distant island population in BPK was removed ($F_{ST} = 0.06$). For pairwise F_{ST} comparisons (and genetic diversity estimates below), the population from GSK was removed due to close proximity to BCK and lack of statistical independence. In addition, populations from ERK and SLK were removed due to their small sample sizes. However, BPK was left in all analyses despite low sample sizes because previous studies suggested that this population might be differentiated from populations in the western Keys (Crouse *et al.* 2009). Pairwise F_{ST} values ranged from 0.071 (between BR and OR) to 0.367 (between RI and BCK). While all pairwise combinations with BCK have significantly high F_{ST} values, mainland-BPK population pairs have relatively low and non-significant F_{ST} values (Table 4). No significant association of genetic differentiation over geographic distance was found over all sampled population (Figure 7).

Table 4. Pairwise matrix of genetic distances (F_{ST} , above diagonal) and geographic distance (km, below diagonal) between populations of *Sylvilagus palustris*. Values in bold are significant after 10,000 permutations and adjustment for multiple comparisons. Population abbreviations defined in Table 1.

	BCK	BPK	BR	OR	RI
BCK	-	0.265	0.270	0.268	0.367
BPK	36.73	-	0.126	0.135	0.267
BR	282.97	246.51	-	0.071	0.204
OR	522.64	488.44	225.77	-	0.104
RI	1087.51	1051.05	799.37	573.48	-

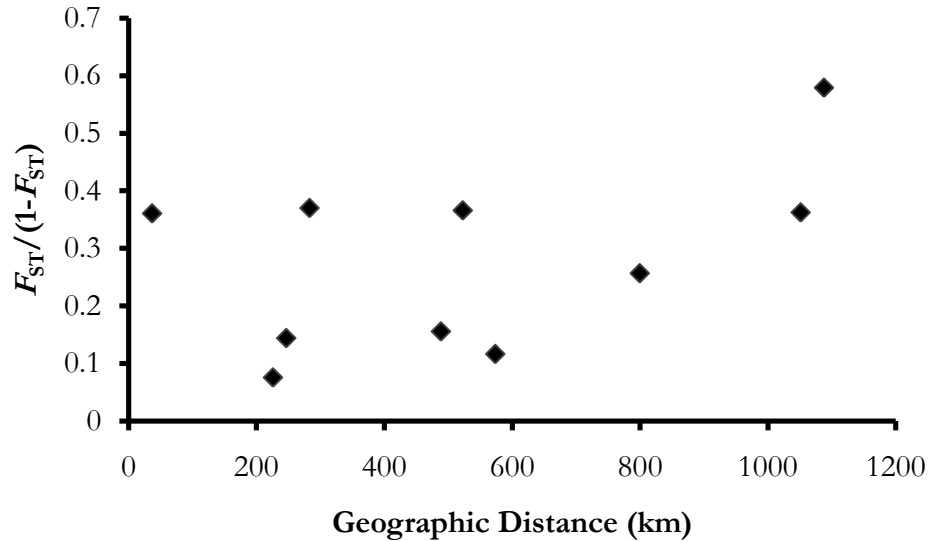


Figure 7. Plot of $F_{ST} / (1 - F_{ST})$ over geographic distance (km) among all sampling localities of *Sylvilagus palustris* (except G GK, SLK and ERK; see text for explanation). No significant correlation was found between genetic and geographic distance as confirmed by a Mantel test ($P = 0.21$, after 30,000 randomizations). Population abbreviations defined in Table 1.

At the largest scale, *i.e.*, all populations included, the Bayesian algorithm in STRUCTURE identified $K=2$ as the highest level of genetic structure using the method of Evanno et al. (2005) (Figure 8).

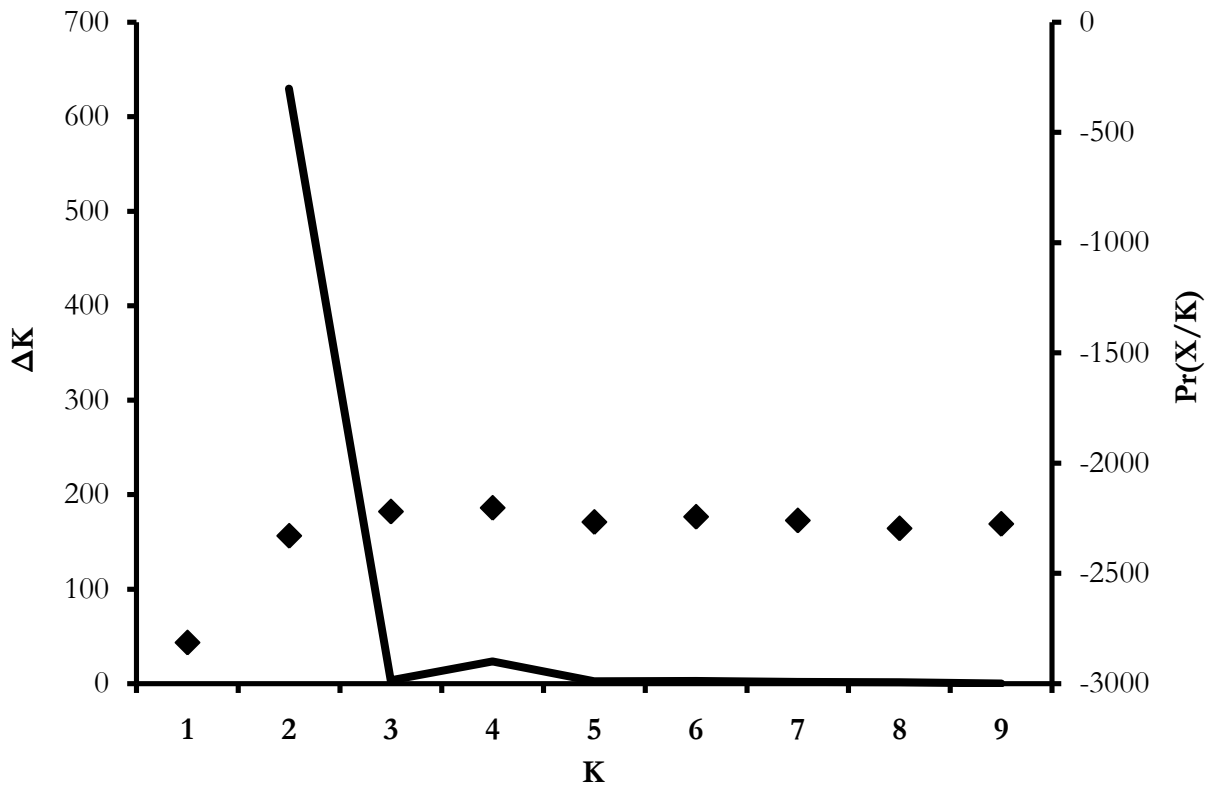


Figure 8. Estimation of genetic clusters within *Syphilagus palustris*. Both the method of Evanno et al. (2005; line, left axis) and the log likelihood (diamonds, right axis) identified the highest level of population structure at $K=2$.

Surprisingly, the genetic clusters did not split island populations from the mainland. Instead, the genetic clusters group all mainland populations together with BPK, while BCK, GGK, SLK and ERK formed the second cluster (Figure 9). However, only one individual from BPK was included in the island cluster. Repeating the analysis within the BPK-mainland group resulted in two additional clusters, this time separating the northern most population in Georgia (RI) from the Florida populations OR, BR and BPK. No further substructure was found within this new cluster except when including prior location information, in which case each cluster corresponded to each

population. No substructure was found in the island group containing BCK, GGK, ERK and SLK, even when location information was included.

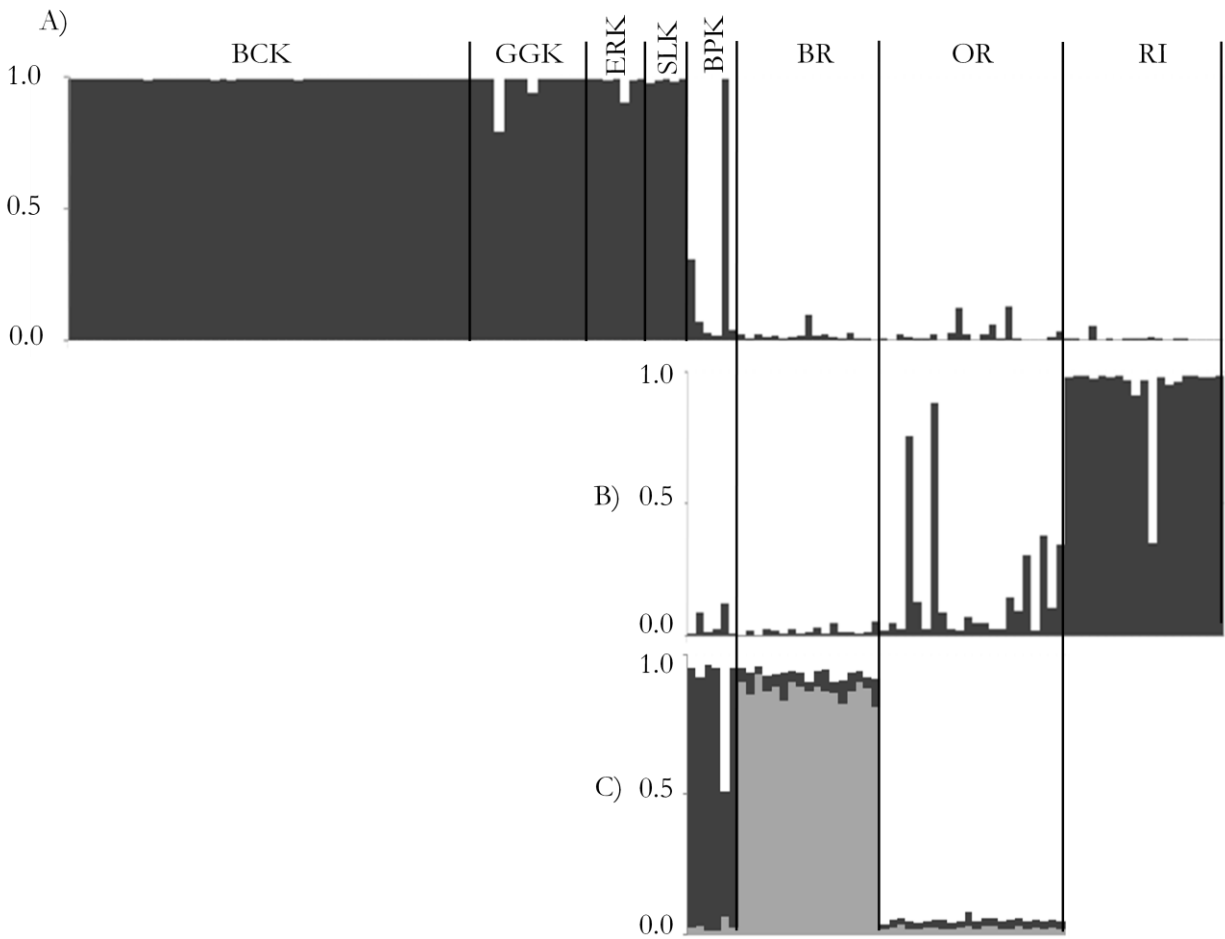


Figure 9. Membership coefficients of *Syvilagus palustris* individuals as estimated by an admixture model in STRUCTURE. Estimation of genetic clusters at larger scales did not require the inclusion of location information (A and B). Addition of location information was necessary at the smallest scale for the identification of genetic clusters (C). Population abbreviations defined in Table 1.

The addition of spatial information in the model as determined with GENELAND did not change the overall structuring of populations. The highest mean posterior density at the largest scale identified three main clusters (Figure 10): The first cluster separated the population in Georgia (RI) from the rest of the populations (Figure 10A). The second cluster grouped BPK together with the two Florida mainland populations (Figure 10B), while the third cluster grouped the rest of the islands (BCK, GGK, ERK, SLK; Figure 10B and Figure 12). No further substructure was found within any of these clusters.

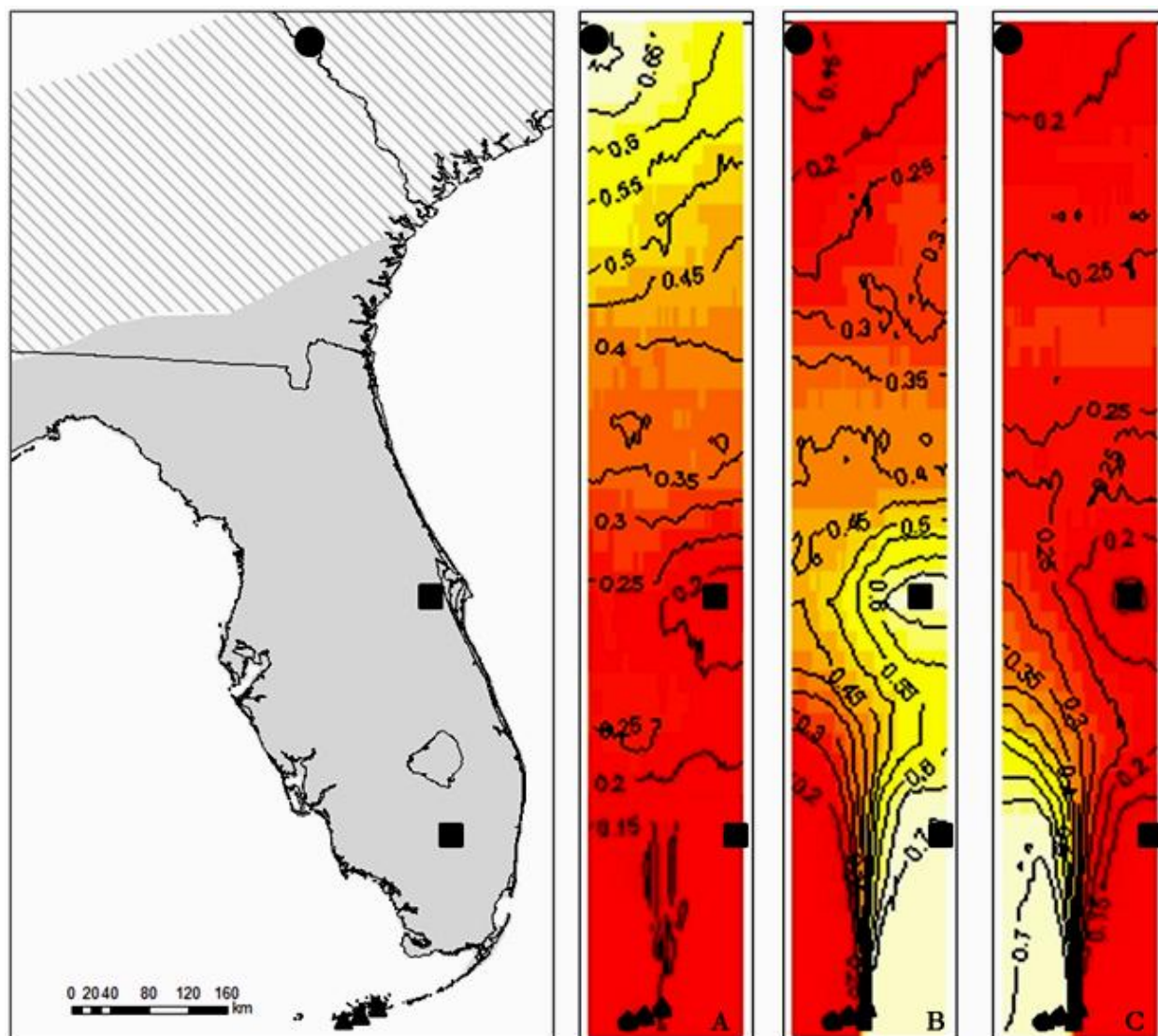


Figure 10. Output from GENELAND showing three major clusters of *Syphilagus palustris* populations at the largest scale. Lighter colors denote high probability of belonging to each cluster. Cluster A corresponds to the population in Georgia (RI). Cluster B groups OR, BR and BPK together. Cluster C groups the western Lower Keys (BCK, ERK, G GK and SLK)

Genetic Diversity in Island and Mainland Populations

Overall, mitochondrial diversity was highly variable among all populations (Figure 11A & B). Cytochrome *b* diversity estimates in BCK were zero because I only uncovered a single cyt *b* haplotype in this population. BPK, however, yielded three haplotypes despite having only six individuals sampled, resulting in nucleotide and gene diversities similar to those found in the mainland. At the microsatellite level, island heterozygosity was not significantly lower than mainland populations (Figure 11C & D); however, allelic richness estimates in BCK were significantly lower than those found in the mainland (Figure 11D). Island population genetic diversity estimates were not significantly lower than genetic diversity of mainland populations as shown by Welch's two-sample t-tests (Figure 11). Overall, there was no strong signal of recent population bottleneck as evidenced by ambiguous Wilcoxon signed ranked tests using TPM or other models of microsatellite mutation.

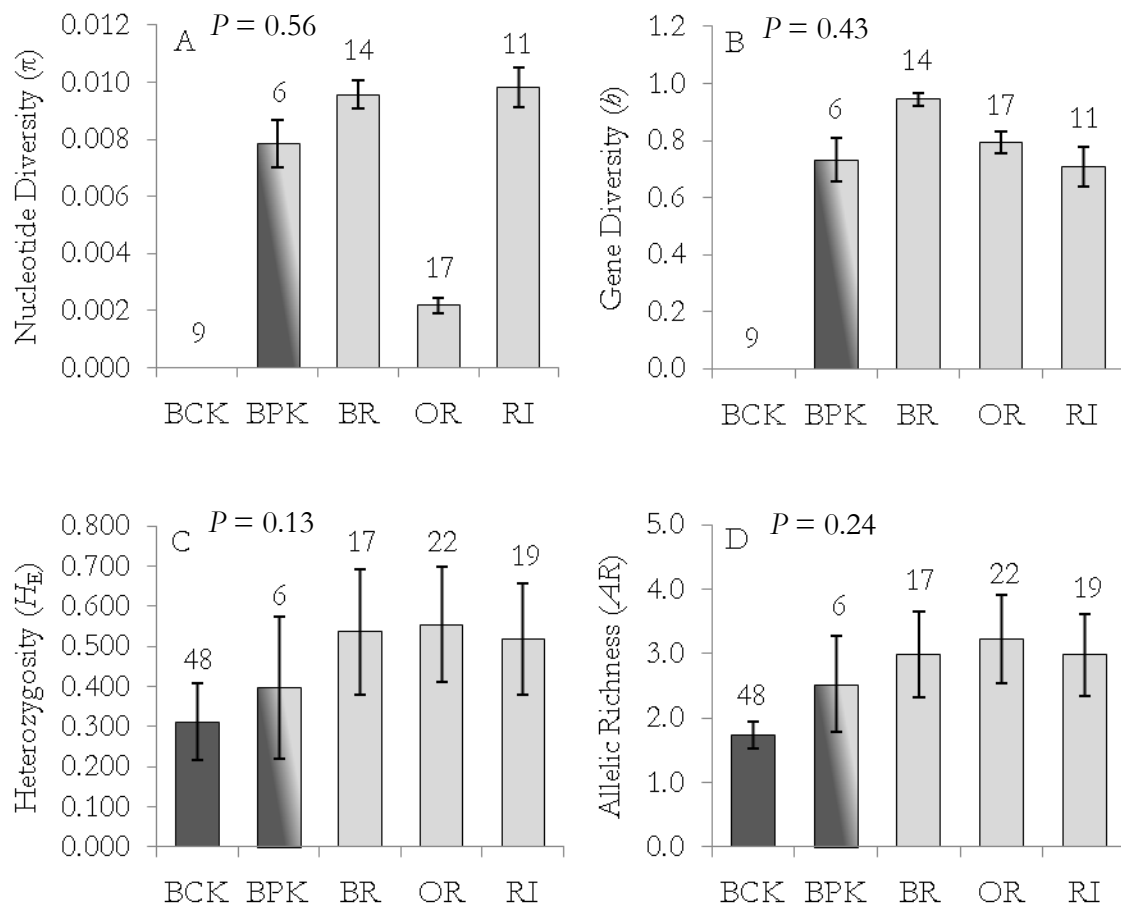


Figure 11. Estimates of mitochondrial (A & B) and microsatellite (C & D) diversity for island (dark gray) and mainland (light gray) populations of *Sylvilagus palustris*. Intermediate coloration of BPK bar is to represent its grouping with the mainland populations in clustering analyses. Allelic richness was rarefied to five (5) samples. Error bars represent standard deviation and numbers above bars represent sample size. Welch's Two-sample T-test used to compare island vs. mainland genetic diversity; P -values shown in graphs.

Genetic Differentiation between BPK and Western Lower Keys

The Bayesian algorithm in both STRUCTURE and GENELAND found a clear split between BPK and the Western Lower Keys (only GENELAND results are shown, Figure 12), suggesting lack of recent

genetic exchange between the Western populations and the populations in BPK. These results corroborate previous findings of genetic differentiation within the Lower Keys (Crouse *et al.* 2009).

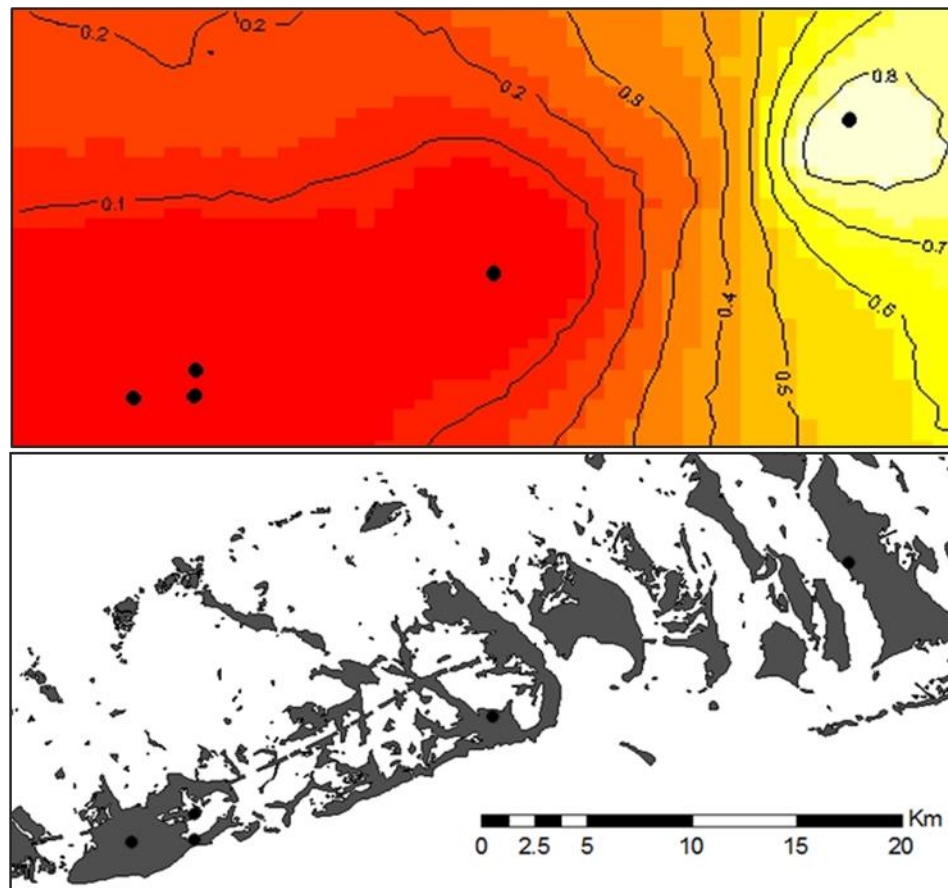


Figure 12. Close-up of the separation within *Sylvilagus palustris hefneri* in the Lower Keys. The lighter color shows the probability of belonging to the BPK cluster.

CHAPTER 4: DISCUSSION

In this study, I employed genetic analyses to evaluate historical variation and contemporary patterns of gene flow between the island endemic Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*) and its mainland sister taxa. Contrary to my expectations, the genetic data did not support previous conclusions based on morphological data with regard to the evolutionary history of the marsh rabbit species complex, as there was no genetic distinction among subspecific units. Moreover, the population from BPK not only displayed high levels of genetic diversity despite the small sample size but it also clustered with mainland populations suggesting higher levels of gene flow with mainland populations than neighboring island populations. These findings contradict my first hypothesis of genetic differentiation. Hence, the genetic data do not support subspecies designations within *S. palustris*.

These results raise the question as to why three different subspecific groups were originally recognized. The two mainland subspecies, described by Nelson (1909), differ in molariform row length and ventral guard hair color (Lazell 1984). The presumed range split between *S. p. palustris* and *S. p. paludicola* coincides roughly with the Suwannee Straits. This barrier separating peninsular Florida from the mainland, however, occurred between the Late Cretaceous and Middle Miocene (Randazzo 1997) and a genetic signal of this break between the two subspecies was not detected (Figure 4 and Figure 5). The genetic data do indicate a pattern of northward expansion from central Florida, evidenced by i) the most ancestral haplotype being present in central Florida and comprising many Florida individuals (Haplotype 1, Figure 5) and by ii) slightly lower genetic diversity in the northern

population (RI, Figure 11). Such a pattern of northward expansion concurs with hypothesized patterns of refugial migrations proposed in other species (see Soltis *et al.* 2006 for a review). Regardless of the migration patterns that may have occurred in the mainland, the genetic data do not support the splitting of the mainland populations into two subspecies.

With regard to the Lower Keys populations, I also recovered a surprising genetic pattern. Despite island confinement and differences in morphological characters (body size, fecundity, cranial morphology and coat color), no historical genetic differentiation in *S. p. hefneri* was detected given the lack of monophyly (Figure 4) and presence of shared haplotypes (Figure 5) with the mainland subspecies. The absence of genetic differentiation of island populations suggests that, if the populations are truly isolated, then there has not been enough time for complete lineage sorting. The time of isolation of the Lower Keys remains controversial as the past 10,000 years are characterized by a complex geologic history of sea level fluctuations. Whereas Lazell (1984) hypothesized that the Lower Keys have been isolated for about 10,000 years, Fairbridge (1974) advocates a model of unstable sea level fluctuations during the past 10,000 years, with final isolation of the Lower Keys occurring as recently as 2,000 years ago. Another hypothesis of Florida Keys isolation suggests that the pattern of coral settlement indicates the most recent Florida Keys isolation occurred around 6,500 year ago (Shinn 1988). The surprising genetic pattern begs the question of how the genetic patterns in *S. p. hefneri* match similarly distributed species. Previous studies on other Lower Keys subspecies such as the key deer (*Odocoileus virginianus clavium*; Ellsworth *et al.* 1994a) and the silver rice rat (*Oryzomys palustris natator*; Gaines *et al.* 1997) also found a single, unique mitochondrial haplotype endemic to island populations that had low levels of divergence from mainland haplotypes. Also,

somewhat similar to *S. p. hefneri*, recent genetic differentiation was found using allozyme variation in *O. v. clavium* (Ellsworth *et al.* 1994b) and microsatellite data in *O. p. natator* (Wang *et al.* 2005). The phylogenetic concordance of all three of these species support an interpretation that the time of Lower Keys isolation was too recent for complete lineage sorting to occur.

Our a priori hypothesis of genetic differentiation was based on previous documentations of morphological differences between mainland and Lower Keys samples. What could explain morphological differentiation in the absence of genetic divergence? The use of morphological traits for subspecies designations has been shown to be confounded by plastic responses to the environment (*e.g.* Paetkau *et al.* 1998; Yeung *et al.* 2009). Differences in isolation and latitude are known to have predictable effects on biological traits such as body size and reproduction. The “island rule” (Van Valen 1973), for instance, states that larger species tend to get smaller and small species tend to get larger in island ecosystems. Also, “Bergmann’s rule” (Bergmann 1847) states that individuals within species tend to be larger in cooler environments. Other biological traits such as litter size have been shown to be directly or indirectly affected by degree of isolation (in rodents, Adler & Levins 1994) or latitude (in lagomorphs, Barkalow 1962; Conaway *et al.* 1974). Although exceptions and variations to such trends have been found (see Ashton *et al.* 2000; Lomolino 1985; McNab 2010; Meiri *et al.* 2008), environmental variation and resource availability are known to affect biological traits, and yet, there is no evidence that these patterns are adaptive. The differences in body size and fecundity reported between *S. p. hefneri* and mainland subspecies could therefore be a result of differences in environmental variables associated with island environments and lower latitudes, and may not necessarily be representative of genetic adaptations.

Of the morphological differences reported in *S. p. hefneri*, only coat color and cranial morphology have the potential of representing local adaptation. Variations in coat color have been found to match the background of the surrounding habitat in mammals (see Caro 2005 for a review). However, even though coat color is known to have a genetic basis in rodents (Hoekstra 2006; Hoekstra *et al.* 2006), establishing the adaptive nature of color variations is still a challenging task (Wlasiuk & Nachman 2007). Cranial morphology has been essential to many evolutionary adaptations, including changes during the evolution of *Homo sapiens* (Lieberman *et al.* 2002). Yet, variation in cranial morphology within species has also been coupled with geographic variation (elevational gradients, Grieco & Rizk 2010; island environments, Pergams & Ashley 1999). More importantly, experimental studies have found that changes in food types can cause changes in skull shape in rodents (Kiliaridis *et al.* 1985; Myers *et al.* 1996). A recent study found that *S. p. hefneri* is a specialist feeder (Gordon 2010), primarily feeding on *Spartina spartinae* and *Borrchia frutescens*. It is therefore likely that the slight but significant differences in cranial proportions between island and mainland subspecies (Lazell 1984) represent plastic phenotypic responses to changes in food sources (or nutrients derived from those food sources) in the island environment. The absence of genetic divergence found in this study show that the differences in morphological traits could be a result of phenotypic plasticity rather than evolutionary changes.

My results also contradicted my hypothesis that island populations would harbor reduced genetic diversity relative to mainland populations. Isolation and restriction of gene flow are known to result in reduced genetic diversity and increased genetic divergence (Couvét 2002; Frankham 1997). Interestingly, only the population in BCK conformed to these expectations, as evidenced by low

levels of genetic diversity (Figure 11) as well as recent differentiation from the mainland (Table 4, Figure 9). Additionally, as I predicted based on previous studies (Crouse *et al.* 2009), gene flow is currently restricted between BPK and the western Lower Keys (Figure 12). Even though BPK harbors much smaller population sizes than BCK, BPK displayed higher levels of genetic diversity than BCK using both cytochrome *b* and microsatellite estimates of diversity (Figure 11). Additionally, as I predicted based on previous studies (Crouse *et al.* 2009), gene flow is currently restricted between BPK and the western Lower Keys (Figure 12). However, one unanticipated finding in this study is the grouping of BPK with mainland populations (Figure 9). Indeed, this result contradicts genetic patterns found in the silver rice rat (*O. p. natator*, Crouse 2005). Furthermore, genetic studies have found a unique mtDNA haplotype for Key Deer (*O. v. clavium*); although not divergent, the fact that this haplotype is not found on the mainland suggests a lack of recent gene flow (Ellsworth *et al.* 1994a). With regard to marsh rabbits, there are three possible explanations that could explain the grouping of BPK samples with mainland populations: 1) a stepping-stone island colonization pattern from the mainland (rather than vicariant isolation), 2) recent natural dispersal from the mainland to BPK, or 3) undocumented translocations from the mainland to BPK. These possibilities are discussed below.

1) A mainland-island stepping-stone colonization pattern would involve individuals from the mainland migrating in a southwest direction from island to island. Given that BPK is closer to the mainland than the western Lower Keys, it would then make sense for BPK to be less divergent than western Lower Keys and to have higher levels of genetic diversity. This scenario would also be supported by the absence of private alleles in the western Lower Keys: the allelic diversity we see in

the western Lower Keys is simply a subset of alleles already found in BPK and the mainland but with divergent frequencies, suggestive of founder events in the western islands. However, a recent study on *Oryzomys palustris natator* found all Lower Keys populations forming a monophyletic group and recent genetic differentiation from ten individuals sampled in the mainland (Crouse 2005). Because these two Lower Keys subspecies, *O. p. palustris* and *S. p. hefneri*, show different patterns of differentiation, the stepping-stone colonization scenario appears less likely.

2) The disparity in genetic divergence may have also been caused by recent gene flow from the mainland to BPK, implying that some *S. palustris* individuals have the ability to frequently disperse long distances over water or man-made bridges. This is highly unlikely because i) despite their ability to swim, it is reported that marsh rabbits take to water mostly as a means to escape (USFWS 2007b). The distance from the mainland to BPK is ~45km, and no marsh rabbits have been documented to swim over such long distances; ii) marsh rabbits are habitat specialists and favor dispersal in areas with vegetation ground cover for protection against predators (Forys & Humphrey 1996). Dispersing over man-made bridges and roads would expose marsh rabbits to avian predators and vehicular traffic, and iii) this would not explain why gene flow between BPK and the western Lower Keys is lower than it is between BPK and mainland. Comparatively, the western Keys are much closer and the intervening habitat within the Lower Keys is more suitable than between Lower Keys and the mainland.

3) Rejection of the stepping-stone colonization and natural dispersal alternatives suggests undocumented, man-mediated translocations may have taken place in this species. Historic

translocations of lagomorphs for economic and hunting reasons are known to have occurred (Alves *et al.* 2008; Suchentrunk *et al.* 2006) and the fact that *S. palustris* is a game species in areas where it is not endangered also supports this scenario. Other studies have also found genetic evidence of recent, undocumented translocations in other taxa (*e.g.* Hoffman & Blouin 2004), suggesting that these actions are not limited to lagomorphs. However, without any documentation it would be difficult to confirm this possibility. Moreover, although these results cannot address whether the western Lower Keys comprise a true Lower Keys lineage, my genetic data do indicate that only BPK populations have been compromised. It is necessary to revisit the phylogeography of other Lower Keys endemics to see how differentiation patterns match my results on *S. p. hefneri*. Such information would help disentangle between and among different scenarios of recent mainland (natural or artificial) gene flow or stepping-stone colonization alternatives.

Overall, this study demonstrates that it is important not to hop to conclusions based solely on isolation and a few differentiated characters, as these may lead to erroneous assumptions of divergence and misguided subspecific designations. A combination of many factors, such as geological events and dispersal ability of a species, and more recently the role of human actions can lead to very interesting and sometimes unpredictable patterns of geographical structuring of genetic variability and differentiation. The wide number of species endangered due to natural or anthropogenic isolation highlights the importance of considering different lines of geological, ecological and molecular evidence to elucidate population structure, genetic differentiation and gene flow, all of which are crucial for practical conservation purposes.

Taxonomic and Conservation Implications

Because my results suggest that subspecies designations within *S. palustris* are not warranted, it is important to discuss the conservation implications for the island populations. Since 1978, the Endangered Species Act (hereafter the ESA) has afforded protection to populations of terrestrial vertebrates below the species level that are recognized as “distinct populations segments” (DPS) to facilitate management of populations of conservation concern (Pennock & Dimmick 1997). To be recognized as a DPS, a population must be “discrete”, “significant” and endangered relative to other conspecific populations. The “discreteness” of a population can be determined by looking at patterns of gene flow using genetic data. Although determining “significance” can be more challenging, one of the factors considered is habitat use that is atypical for the taxon (Policy regarding recognition of DPS Policy 1996). The populations from the western Lower Keys meet both the “discreteness” and “significance” criteria based on lack of contemporary gene flow with the mainland (and BPK) and confinement to insular and isolated habitats. Moreover, these criteria of “discreteness” and “significance”, together with morphological variation, also fit Crandall’s criteria for recognition as an Evolutionary Significant Unit (ESU, Crandall *et al.* 2000) based on recent genetic (significant allele divergence) and ecological (inhabiting island habitats and potential morphological adaptations) differentiation. Therefore, this study supports the current designation (at least for the western Lower Keys) of *S. p. hefneri* as a discrete unit of conservation with regard to both DPS and ESU criteria.

Assessment of discreteness of the BPK population remains a challenge (Policy Regarding Recognition of DPS 1996). While this population meets the requirement for significance because it is confined to an island, the discreteness criterion is more difficult to establish because there is potential for recent genetic exchange to have occurred with mainland populations. Although the probability for this gene flow to have occurred naturally is low, further studies are needed to determine whether the patterns found in this study are a reflection of a stepping-stone colonization pattern or undocumented historical transfers from the mainland to BPK.

Future reintroduction and translocation practices will depend on the ultimate goal of island population conservation. If the goal is to maintain genetic distinctiveness, then only individuals from BCK, GCK and ERK should be used as sources for future translocation plans, including translocations to BPK. Further support for using these populations as founders in the Keys arises from the fact that a significant proportion of the *S. p. hefneri* population is found in the Naval Air Station, in BCK (USFWS 2007a). Translocation from BPK to the western Lower Keys is not recommended to avoid compromising the genetic uniqueness of the western islands. Alternatively, because the populations in BPK are not differentiated from the mainland, then individuals from the mainland could be used to supplement and increase these populations in BPK, which are in great decline due to habitat degradation. The translocation of mainland individuals to western islands, however, is debatable because these populations are fairly stable and compromising their genetic distinctiveness seems unwarranted at this point. Any reintroduction and translocation strategy implemented, however, should only be a small part of a larger effort to restore degraded native habitat. A population viability analysis conducted over a decade ago (Forys & Humphrey 1999)

indicated that management efforts focused on removal of exotic predators, particularly of feral and free-roaming domestic cats, would provide the highest chance of survival for these endangered populations. Accordingly, there are signs of successful breeding and population growth in BCK, GGK and ERK (Hughes, personal communication) due to ongoing efforts of habitat restoration via prescribed burning and exotic predator removal.

CHAPTER 5: CONCLUSION

My study adds to the growing body of literature confirming that subspecies designations are not always reflective of evolutionary history, even in the case of island populations. The results of my study demonstrates that assuming divergence based solely on isolation and a few differentiated characters may lead to erroneous conclusions regarding the distribution of variation within species. Although differentiation of island populations is often confirmed, the level of differentiation is dependent on the geologic processes, time since isolation and dispersal ability of the organisms present in the islands. Using different lines of evidence, such as ecological factors combined with genetic markers with different rates of evolution, can aid in the detection of population divergence at all levels, from historical to recent, from genetic to ecological, and from natural to anthropogenic. The broad applicability of these findings is based not only on the high proportion of endangered populations on natural islands, but also on the degree to which human-induced habitat loss and fragmentation have resulted in the isolation of many populations into island-like patches of habitat. As connectivity continues to become lost among populations, management of local populations receives more emphasis. Understanding the mechanisms driving the patterns of differentiation and how genetic diversity is distributed as a result of natural and/or anthropogenic causes will aid in the prioritization of conservation efforts, the preservation of evolutionary processes and thus, the preservation of biodiversity as a whole.

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