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Authors: Crouse, Amanda L., Honeycutt, Rodney L., McCleery, Robert A., Faulhaber, Craig A., Perry, Neil D., et al.

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# Population Structure of the Lower Keys Marsh Rabbit as Determined by Mitochondrial DNA Analysis

AMANDA L. CROUSE, *College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4461, USA*

RODNEY L. HONEYCUTT, *Natural Science Division, Pepperdine University, Malibu, CA 90263-4321, USA*

ROBERT A. MCCLEERY,<sup>1</sup> *Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843-2258, USA*

CRAIG A. FAULHABER, *Department of Wildland Resources, Utah State University, Logan, UT 84322-5230, USA*

NEIL D. PERRY, *Utah Division of Wildlife Resources, Cedar City, UT 84270-0606, USA*

ROEL R. LOPEZ, *Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843-2258, USA*

**ABSTRACT** We used nucleotide sequence data from a mitochondrial DNA fragment to characterize variation within the endangered Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*). We observed 5 unique mitochondrial haplotypes across different sampling sites in the Lower Florida Keys, USA. Based on the frequency of these haplotypes at different geographic locations and relationships among haplotypes, we observed 2 distinct clades or groups of sampling sites (western and eastern clades). These 2 groups showed low levels of gene flow. Regardless of their origin, marsh rabbits from the Lower Florida Keys can be separated into 2 genetically distinct management units, which should be considered prior to implementation of translocations as a means of offsetting recent population declines. (JOURNAL OF WILDLIFE MANAGEMENT 73(3):362-367; 2009)

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**KEY WORDS** Florida Keys, genetic, marsh rabbit, mitochondrial DNA, population structure, *Sylvilagus palustris hefneri*.

The Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*) is listed as a federally endangered subspecies by the United States Fish and Wildlife Service (USFWS 1991). The smallest of the 3 subspecies of marsh rabbit, the Lower Keys marsh rabbit, is distinguished from marsh rabbits occupying northern mainland Florida, USA (*S. p. palustris*), and the remainder of the state and the Upper Keys (*S. p. paludicola*) by distinct cranial characteristics, including a shorter molariform tooth row, a broader skull with a higher, more convex frontonasal profile, and a longer dentary symphysis (Lazell 1984). Although marsh rabbits are found throughout the southeastern United States, the historic range of the Lower Keys marsh rabbit is limited exclusively to the Lower Florida Keys, extending from Big Pine Key to Boca Chica Key and possibly Key West (Faulhaber et al. 2007; Fig. 1). Like the Key deer (*Odocoileus virginianus clavium*) and the silver rice rat (*Oryzomys palustris natator*), marsh rabbits were probably isolated from the mainland of Florida as ocean levels rose and separated the Keys approximately 10,000 years ago (Lazell 1989). Historically, marsh rabbits likely occupied all islands in the Lower Keys with suitable habitat (Forys 1995, Forys et al. 1996, USFWS 1999). Nonetheless, their restricted range, coupled with loss and fragmentation of habitat by commercial and residential development in the Keys, has led to the decline of marsh rabbits in the Lower Keys. A population viability analysis carried out by Forys (1995) projected that, under current conditions, the subspecies could become extinct in as little as 50 years.

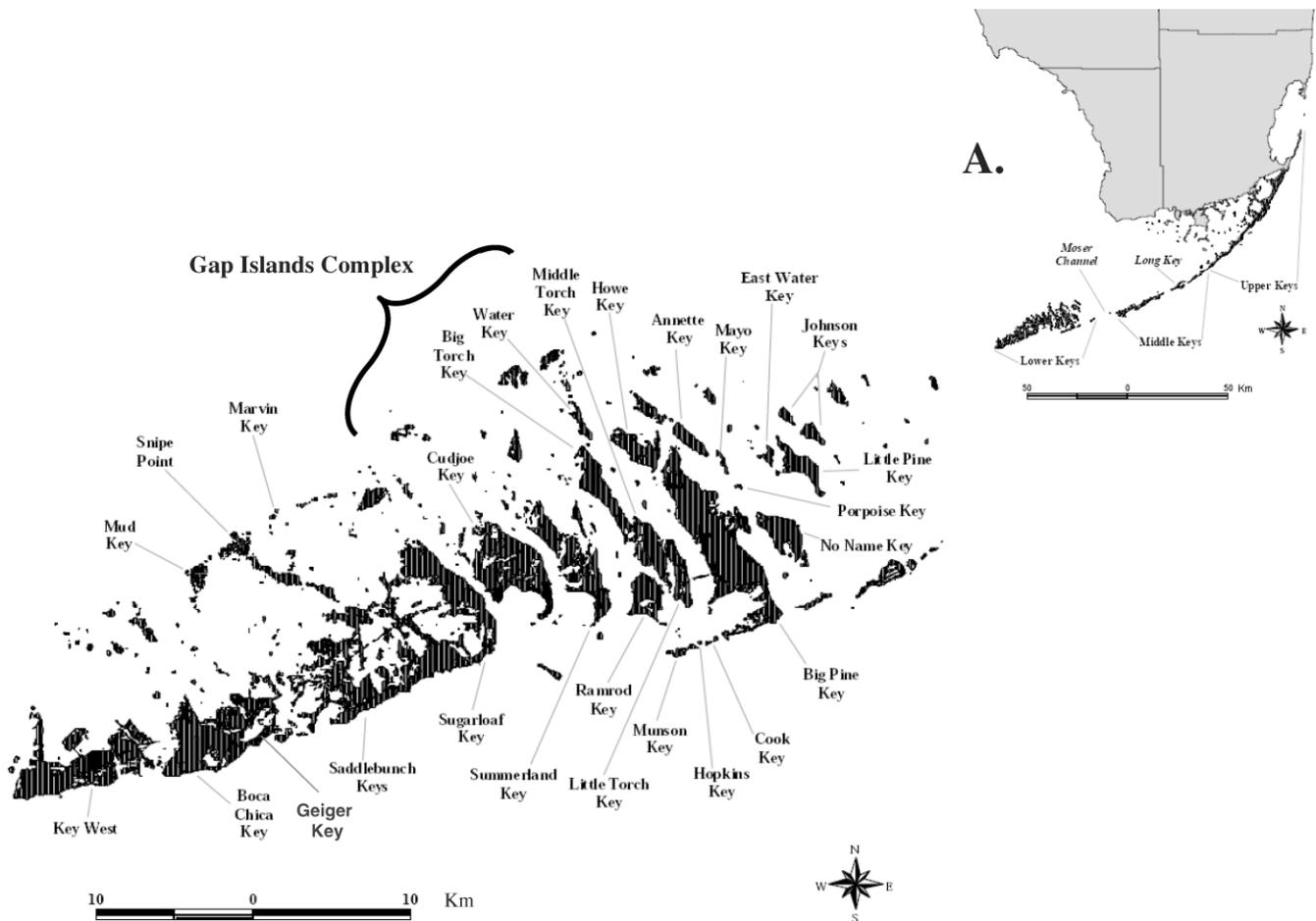
There are few remaining areas of the marsh rabbit's preferred habitat (coastal marsh) >5 ha, and most of these patches of habitat are surrounded by urban development

(Forys and Humphrey 1999b). In the island habitat of the Lower Keys, water also serves as the decisive barrier between most populations. These landscape features have combined to create small subpopulations of marsh rabbits that are likely isolated from each other (Forys et al. 1996).

This level of patch isolation is the most critical threat that faces the continued existence of the Lower Keys marsh rabbit and should play a substantial role in determination of population structure (Johst et al. 2002). The serious nature of this problem is realized by the fact that such isolation may already be occurring in an area known as the Gap Islands Complex (Faulhaber 2003). This group of islands, located in the center of the marsh rabbit's range (extending from Middle Torch Key to Cudjoe Key; Fig. 1), contains patches of suitable marsh rabbit habitat, yet the only recorded rabbit sightings were on Cudjoe Key several decades ago (Faulhaber et al. 2007).

To bolster small subpopulations and to reintroduce marsh rabbits to suitable habitat patches, the USFWS initiated translocations in January 2002 (Faulhaber et al. 2006). These efforts were undertaken with no information on the genetic structure of the population. Although information on ecology and behavior of marsh rabbits was available, no studies of genetic variation had been conducted. As with other studies of threatened and endangered populations (Awise 1989, Kalinowski et al. 1999, Malone et al. 2003), a genetic survey of the Lower Keys marsh rabbit can provide meaningful data on the structure of local populations and presence or absence of barriers to gene flow between existing populations. Such information is fundamental to management of this endangered subspecies, and it can be used to identify source populations for future translocation of individuals to sites where marsh rabbits have become rare. The objective of this study was to provide preliminary information on the genetic

<sup>1</sup> E-mail: [bmc@tamu.edu](mailto:bmc@tamu.edu)



**Figure 1.** A) Southern tip of peninsular Florida, USA and the Florida Keys archipelago. The Keys are divided into 3 sections based on geological composition: Upper Keys, Middle Keys, and Lower Keys (isolated from Upper and Middle Keys by the 11-km Moser Channel). B) Map of the current range (Nov 2001–Jul 2004) of the Lower Keys marsh rabbit, from Big Pine Key and its surrounding islands to Boca Chica Key, Florida, USA. The Gap Islands Complex is an area uninhabited by marsh rabbits, although it contains patches of suitable habitat (adapted from Faulhaber 2003).

structure of marsh rabbits in the Lower Keys through an analysis of mitochondrial DNA variation.

## STUDY AREA

The Florida Keys archipelago extends in a narrow arc (6.4-km average width) for 240 km to the south and west of mainland Florida, USA. Florida Bay, 60 km of shallow open water, separates the islands from the mainland. The Keys were divided into 3 sections based on geological composition: the Upper Keys, Middle Keys (177 km in length), and Lower Keys (64 km in length), which were effectively isolated from Middle and Upper Keys by the 11-km Moser channel (Fig. 1). The Lower Keys marsh rabbit is endemic to the Lower Keys where it occurs in freshwater wetlands dominated by saw grass (*Cladium jamaicense*) and brackish wetlands dominated by gulf cord grass (*Spartina spartinae*), salt-marsh fringe-rush (*Fimbristylis castanea*), marsh hay cord grass (*S. patens*), sea-shore dropseed (*Sporobolus virginicus*), and buttonwood trees (*Conocarpus erectus*).

## METHODS

We collected hair samples from 59 Lower Keys marsh rabbits for a population survey conducted from November

2001 through July 2004 at the following localities: Boca Chica ( $n = 19$ ), Sugarloaf ( $n = 13$ ), Saddlebunch ( $n = 16$ ), Big Pine ( $n = 9$ ), and Geiger ( $n = 2$ ) Keys (Fig. 1; Faulhaber 2003, Faulhaber et al. 2007). Our survey constituted the most comprehensive trapping of Lower Keys marsh rabbit ever undertaken (Faulhaber et al. 2007). However, it is important to note that samples sizes were limited by the sparse and scattered distribution of this endangered species. We saturated known rabbit habitat patches with traps and took hair samples from every captured rabbit. In addition to collecting samples from rabbits captured in the Lower Keys, we obtained one mainland individual from Lover's Key, located on the southwestern coast of Florida, which was in the range of the subspecies *S. p. paludicola* (Chapman and Willner 1981, Lazell 1984).

We placed unbaited Tomahawk Traps (Tomahawk Live Traps, Tomahawk, WI) lined with grasses in vegetation tunnels and covered traps with vegetation to simulate a natural passageway. In addition, we placed drift fences made of chicken wire next to traps in an attempt to funnel rabbits into the traps. Prior to analysis, we stored all hair samples in either 15 mL of lysis buffer (Longmire's solution; Longmire et al. 1997) or plastic bags. We trapped and handled rabbits

in a humane manner following guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998) as approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocol No. 2001-109).

We used a Genra Puregene™ DNA isolation kit (Genra Systems, Minneapolis, MN) to extract DNA from individual hair samples. We amplified with polymerase chain reaction (PCR; Saiki et al. 1988) a 763-base-pairs (bp) fragment of mitochondrial DNA (mtDNA), which we later sequenced. The fragment contained predominantly the control region (488 bp), a noncoding region of the mitochondrial genome that is associated with transcription and replication (Clayton 1991), a portion of the cytochrome b gene (141 bp), and 2 transfer ribonucleic acid (tRNA) genes (134 bp; tRNA<sup>Thr</sup> and tRNA<sup>Pro</sup>). We used PCR primers designed to prevent amplification of tandem repeats that are located in the second half of the control region in lagomorphs; these repeat regions are inappropriate for population-level comparisons because they vary within individuals (Casane et al. 1997). We used the primer set L15774 (5'-TGAATTGGAGGACAACCAGT-3') and H16498 (5'-CCTGAAGTAGGAACCAGATG-3') for amplification of the fragment (Shields and Kocher 1991), as well as an additional internal set of primers, L15934 (5'-CCCTGGTCTTGTAAGCCAGAAATGG-3') and H16431 (5'-GGGCGGGTTGCTGGTTTCACG-3'; Litvaitis et al. 1997) for sequencing. We conducted PCR amplifications in 50- $\mu$ L reactions with the following components and concentrations: 2  $\mu$ L DNA, 25.7  $\mu$ L ddH<sub>2</sub>O, 5  $\mu$ L 10X PCR buffer (Takara Shuzo, Shiga, Japan), 2.5  $\mu$ L 10X bovine serum albumin, 4.5  $\mu$ L of 10mM deoxyribonucleotide triphosphates (Takara Shuzo), 5  $\mu$ L each of forward and reverse primers (2 pmol/ $\mu$ L), and 1.5 U *Taq* polymerase (Takara Shuzo). We performed amplifications in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) with the amplification protocol consisting of an initial denaturation cycle at 95° C for 4 minutes, followed by 35 cycles of 30 seconds at 95° C, 1 minute at 40° C, 2 minutes at 72° C, and a final extension cycle of 10 minutes at 72° C. We visualized PCR products on a 1% agarose and Tris-Borate EDTA gel matrix. We purified amplified products by treatment with a combination of Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP; USB, Cleveland, OH).

We sequenced each PCR fragment in both the forward and reverse directions on an ABI 3100 automated sequencer (Applied Biosystems Inc. [ABI], Foster City, CA) with the use of Big Dye termination chemistry (ABI) and a GeneAmp® PCR System 2700 Thermal Cycler (ABI). We aligned all sequences with the Sequencher 4.2 software program (Gene Codes Corporation, Ann Arbor, MI) and submitted all sequences of mitochondrial haplotypes to GenBank under accession number EF062313–EF062318.

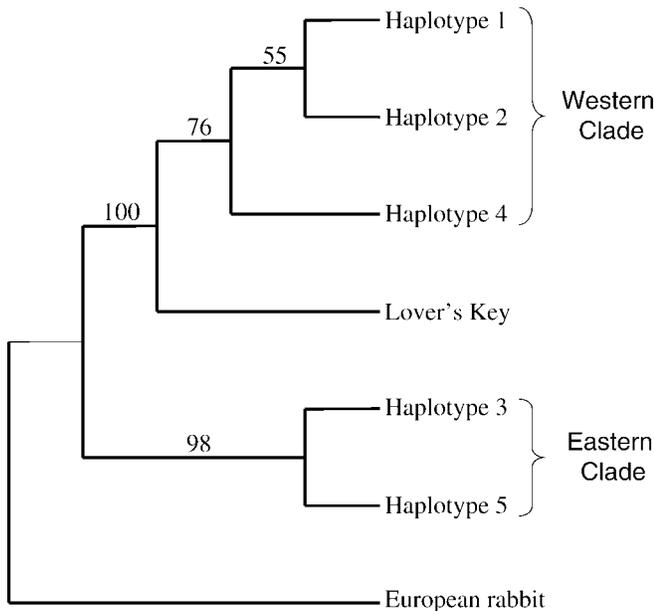
We used the DnaSP version 4.0.6 software package (Rozas et al. 2003) to estimate several population statistics, including nucleotide diversity ( $\pi$ ), haplotypic diversity ( $h$ ),

number of segregating sites ( $S$ ), average number of nucleotide substitutions/site between populations ( $D_{xy}$ ), and average number of nucleotide differences between populations. Haplotypic diversity indicates how closely 2 haplotypes are related. We obtained estimates of  $F_{ST}$  (Hudson et al. 1992) and  $\gamma_{ST}$  ( $\gamma_{ST}$ ; Nei 1982) with DnaSP (Rozas et al. 2003). Both  $F_{ST}$  and  $\gamma_{ST}$  measure amount of genetic variation contained in a subpopulation ( $S$ ) as compared to the total population ( $T$ ). Values range from zero to one; values closer to one indicate high amounts of differentiation between populations. We used estimates of  $\gamma_{ST}$  (Nei 1982) and  $F_{ST}$  (Hudson et al. 1992) to provide an estimate of gene flow ( $N_f m$ ) between populations. We used an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to calculate significance of haplotypic variation between populations. We employed this analysis to specifically test for distinctions between 2 major geographic locations in the Lower Keys (i.e., E and W of the Gap Islands Complex). We used ARLEQUIN version 2.0 (Schneider et al. 2000) to perform an AMOVA based on  $\Phi$ -analogues of Wright's (1965)  $F$ -statistics. This program estimates proportion of genetic variation present at different hierarchical levels based on haplotype distribution and pairwise distance. We tested for significance in ARLEQUIN by means of a nonparametric permutation of haplotypes among both populations and groups (10,000 permutations).

We used the maximum parsimony method for phylogenetic reconstruction in PAUP\*4.0b10 (Swofford 2002) to determine relationships among mitochondrial haplotypes. We employed the exhaustive search option to evaluate all possible trees and determined support for particular groupings with a bootstrap procedure (branch and bound option with 1,000 replications; Felsenstein 1985). We obtained GenBank sequences (accession no. NC001913) from the European rabbit (*Ortholagus cuniculus*), which was designated as an out-group. We also used PAUP to calculate estimates of Tamura–Nei genetic divergence based on a gamma shape parameter or  $\alpha = 0.05$ .

## RESULTS

The MtDNA sequences, derived from 59 Lower Keys marsh rabbits, revealed 5 unique haplotypes, defined by  $\geq 1$  single-base transition substitutions at 23 varying sites. In addition, we observed a sixth haplotype for the individual from Lover's Key located along the southwestern coast of Florida. These haplotypes represented 2 distinct clades (phylogenetic groups) within the Lower Keys (western clade and eastern clade; Fig. 2) separated by 19 nucleotide substitutions. Haplotypic diversity ( $h \pm SD$ ) was low ( $66 \pm 3\%$ ), and haplotypes were distributed unevenly, with most samples represented by 3 types (Table 1). Nucleotide diversity ( $\pi \pm SD$ ) over all Lower Keys sites was  $0.018 \pm 0.005$ , and values for the eastern and western clades were  $0.001 \pm 0.000$  and  $0.003 \pm 0.001$ , respectively. As indicated by the AMOVA, 98% of observed variation was between the 2 major groups (e.g., western and eastern clades) identified by the phylogeny (Fig. 2), whereas variation



**Figure 2.** Phylogenetic tree of mitochondrial haplotypes for the Lower Keys marsh rabbit produced with maximum parsimony. The western clade refers to all trap sites west of the Gap Islands Complex; the eastern clade is composed of all samples from Big Pine Key, Florida, USA (Nov 2001–Jul 2004). Numbers along lineages are bootstrap values (1,000 replication; 100 random additional search). Overall tree length was 184 with a consistency index equal to 0.989. Lover's Key is from the southwestern coast of Florida.

within populations (e.g., each of the 5 sampling sites) and among populations within groups was 2.2% and 0.20%, respectively. Haplotype 6 from Lover's Key grouped with the haplotypes of the western clade, differing from the other haplotypes by an average of 0.4% or 3 substitutions (Fig. 2). Genetic variation was partitioned among the 2 groups, with  $F_{ST} = 0.98$  (Hudson et al. 1992) and  $\gamma_{ST} = 0.92$  (Nei 1982; both calculated in DnaSP [Rozas et al. 2003]). Gene flow between the 2 major groups was low based on both measures ( $\gamma_{ST}$ ,  $N_{fm} = 0.04$  [Nei 1982];  $F_{ST}$ ,  $N_{fm} = 0.01$  [Hudson et al. 1992]).

## DISCUSSION

One of the few genetic studies on rabbits of the genus *Sylvilagus* that is comparable to our study was conducted by Litvaitis et al. (1997) on rabbits from the northeastern United States. Variation in a 376-bp fragment of the mitochondrial DNA, containing 310 bp of the control region and 66 bp of the tRNA<sup>Pro</sup> gene, was examined for both the New England cottontail (*S. transitionalis*) and the eastern cottontail (*S. floridanus*). Throughout the regions examined for each species, nucleotide diversity was considerably higher than we observed for the Lower Keys marsh rabbit. For instance, nucleotide diversity within populations of the New England and Appalachian populations was 0.198 and 0.177, respectively, whereas it was 0.018 throughout all localities we examined for the Lower Keys marsh rabbit. Unlike both New England cottontail and eastern cottontail, which showed high within-group variation and low between-group variation, localities of Lower Keys marsh rabbit grouped into 2 main haplogroups within

**Table 1.** Number and frequency of mitochondrial DNA haplotypes occurring at localities where we sampled rabbits in the Lower Keys, Florida, USA (Nov 2001–Jul 2004).

Trapping locations	Haplotypes (n)				
	1	2	3	4	5
Eastern clade					
Big Pine Key			8		1
Western clade					
Sugar Loaf Key	5	8			
Saddle Bunch Key	16				
Geiger Key		2			
Boca Chica Key	2	15		2	
Totals	23	25	8	2	1

the Lower Keys. These 2 groups of Lower Keys marsh rabbit have a greater nucleotide divergence (3.2%) than seen between populations of the New England cottontail that were once considered different species (*S. transitionalis* and *S. obscurus*).

Based on the phylogenetic analysis, haplogroups of marsh rabbits in the Lower Keys are clearly separated geographically, with an eastern clade (trap sites E of the Gap Island,  $n = 9$ ) and a western clade (all trap sites W of the Gap Island complex,  $n = 50$ ; Fig. 2). Patterns of strong genetic differentiation indicated little gene flow between these eastern and western localities, which are separated by a region unoccupied by marsh rabbits, the Gap Islands Complex. Small sample size from the eastern Lower Keys is due to reduced densities of this endangered species, with small numbers of individuals confined to habitat patches (Faulhaber et al. 2007). Although samples sizes are low, the genetic distinction between these 2 regions is congruent with some morphological evidence. For instance, Lazell (1989) noted variation in pelage color between marsh rabbits captured on Big Pine Key (E of Gap Islands) and those captured west of the Gap Island Complex.

There are a number of possible explanations for the patterns of strong genetic differentiation we observed. For instance, variation in pelage and genetic divergence between the clades suggests a historic separation, possibly caused by currents, depths, and widths of channels formed during the last ice age when the Florida Keys were created. Such barriers to dispersal could have limited the degree to which individuals were exchanged between the eastern and western groups, resulting in changes in haplotype frequency. It is also possible that the Gap Island Complex never contained substantial habitat suitable for marsh rabbits, thus presenting a barrier to dispersal between these 2 regions. Previous studies within the Gap Island Complex have only identified small disjunct patches of suitable marsh rabbit habitat (Faulhaber 2003).

The phylogenetic grouping of haplotype 6 from Lover's Key with haplotypes from the western clade (Fig. 2) is rather surprising, given its closer geographic proximity to the eastern clade. Marsh rabbits from Lover's Key, a locality near the southwest Florida mainland, are considered members of a different subspecies, *S. p. paludicola* (Chapman

and Willner 1981). The Lover's Key haplotype is divergent from the other western clade haplotypes, differing by 0.4%. Given the lack of information on patterns of haplotype diversity in mainland populations of marsh rabbits, especially populations in southern Florida, it is difficult to make any definitive statements regarding the Lover's Key haplotype. The western clade has an overall higher level of haplotype diversity compared to the eastern clade, perhaps as a result of larger historical populations in this area of the Lower Keys and the retention of more haplotype diversity, including haplotypes found in high frequency on the mainland. Alternatively, similarity in haplotypes between Lover's Key and the western clade may be the result of recent introductions of marsh rabbits from the mainland (*S. p. paludicola*) mediated by man. Nevertheless, based on the pelage (a noticeable dark pelage without reddish dorsal tint; Lazell 1984) of all captured rabbits, we have no reason to believe the marsh rabbits captured were not Lower Keys marsh rabbits.

Mitochondrial haplotype diversity in Lower Keys marsh rabbits is higher than that seen for 2 other species of mammals occurring in the Lower Florida Keys. Based on restriction site variation of the entire mitochondrial genome, Ellsworth et al. (1994) found one mitochondrial haplotype for the Florida Key deer that differed from mainland populations in South Florida by 0.14%. Examining one location Gaines et al. (1997) also found only one haplotype for the Florida Keys silver rice rat, which differed from mainland populations by approximately 2%. However, a more extensive study by Crouse (2005) found more haplotypes in the Lower Keys; yet the overall divergence among localities of the Lower Keys rice rat averages 0.5% in comparison to the 2.2% observed between the 2 groups of marsh rabbits in the Lower Keys. Therefore, both the Key deer and silver rice rat show a pattern more suggestive of one invasion in contrast to that observed for the marsh rabbit. Nonetheless, we recognize the limited scope of our study and recommend a more thorough examination of marsh rabbit populations from the mainland to test the possibility of 2 separate invasions of the Lower Keys by marsh rabbits from the mainland.

Regardless of the cause of the patterns of genetic differentiation seen in our study, the problem of limited gene flow between the populations has been exacerbated by human development in the Lower Keys. Development in the Lower Keys has increased the number of road-kills (USFWS 1999), predation by feral cats (Forys and Humphrey 1999a), and habitat loss (Forys et al. 1996) of mammals endemic to the Lower Keys and has almost certainly reduced survival of dispersing rabbits.

Moritz (1994) advocated using distinct separation of mtDNA haplotypes (termed reciprocal monophyly) to identify historically isolated populations that potentially have distinct evolutionary potential. Populations that do not show clear separation, yet are functionally independent due to limited gene flow, may be designated as management units, a still-relevant designator for conservation efforts. Our

data suggest that as a consequence of limited gene flow and separation of the 2 groups in the Lower Keys, marsh rabbits should be considered as 2 management units.

### Management Implications

One management strategy implemented to offset declining numbers of marsh rabbits in the Lower Keys is translocation of individuals to suitable habitat patches. Successful translocations have already occurred on Little Pine and Water Keys (Faulhaber et al. 2006), involving rabbits from source populations in both the eastern and western regions of the Lower Keys. Currently, Water Key in the Gap Island Complex has a population introduced from Boca Chica Key (western clade), and Little Pine Key in the eastern range of islands has an introduced population of rabbits from Boca Chica, Saddlebunch, Sugarloaf (all from the western clade) and Big Pine Keys (eastern clade; Faulhaber et al. 2006). Future translocations involving Lower Keys marsh rabbits should be initiated in a manner that avoids mixing of individuals from the 2 genetically distinct clades identified in our study. Furthermore, our results suggest a need for a management strategy that treats the eastern and western groups of Lower Keys marsh rabbits as separate management units.

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