Diversity and Distributions, (Diversity Distrib.) (2012) 1–14

BIODIVERSITY RESEARCH

# Taxonomy versus phylogeny: evolutionary history of marsh rabbits without hopping to conclusions

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## ABSTRACT

**Aim** To evaluate whether population genetic structure reflects taxonomic recognition of the endangered Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*) and the two mainland subspecies.

**Location** Southeastern United States.

**Methods** We inferred phylogenetic relationships, population structure and genetic diversity within *S. palustris* using a mitochondrial gene (cytochrome *b*) and 10 microsatellite loci.

**Results** The cytochrome *b* sequence data revealed taxonomy-phylogeography incongruence, and microsatellite data revealed moderate structure ($F_{ST}$ = 0.22) with two genetic clusters recovered: one grouping the western Lower Keys, and the second grouping the eastern Lower Keys together with the mainland. Furthermore, island genetic diversity was not reduced relative to mainland populations (cyt *b*: $\pi$: $t = -0.6952$, $P = 0.5651$; *h*: $t = -1.2053$, $P = 0.4305$; microsatellite: $H_E$: $t = -4.1201$, $P = 0.1313$; *AR*: $t = -2.3113$, $P = 0.2441$).

**Main conclusions** The taxonomy-phylogeny disparity reveals unknown aspects of the evolutionary history including an absence of contemporary dispersal barriers between the mainland subspecies and a more recent Lower Keys isolation than originally thought. Moreover, diversity patterns indicate that undocumented man-mediated transfers may contribute to current genetic structure between eastern Lower Keys and the mainland. Although subspecies designations were not confirmed, these findings support recognition of western Lower Keys populations as a distinct population segment under the Endangered Species Act.

## Keywords

**Distinct population segment, evolutionary history, genetic structure, island subspecies, Lower Keys marsh rabbits, subspecies.**

## INTRODUCTION

A fundamental goal of biology is to understand how evolutionary history influences patterns of genetic variation within a species. In an attempt to account for organisms that exhibit regional variants, subspecies classifications are often used to partition observed variation across taxa such as mammals (e.g. *Peromyscus polionotus,* Hall, 1981), birds (e.g. *Buteo lineatus,* Clark & Wheeler, 1987), amphibians (e.g. *Acris crepitans,* Conant & Collins, 1998), reptiles (e.g. *Rhinocheilus lecontei,* Grismer, 1990) and arthropods (e.g. *Limenitis arthemis,* Mullen *et al.*, 2008). Although accounting for morphological variation across a species range may be practical, from a conservation genetics perspective, subspecific groups should be distinguishable based in part on genetic differentiation (Zink, 2004; Frankham *et al.*, 2010). However, recent analyses of molecular data 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 panthers, *Puma concolor,* actually represent a single evolutionary lineage. Similar examples of misclassified subspecies abound (e.g. Burbrink *et al.*, 2000; Manier, 2004; Hull *et al.*, 2008).



DOI: 10.1111/j.1472-4642.2012.00915.x
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A Journal of Conservation Biogeography
Diversity and Distributions

An ongoing challenge of subspecies classification occurs in island populations. Because island populations may differ morphologically from their mainland counterparts, these populations are frequently considered members of distinct subspecies or even species (e.g. Gonzalez *et al.*, 1996; Pergams & Ashley, 1999; Furness *et al.*, 2010). This assumption of differentiation is plausible given that water represents a barrier to gene flow for many terrestrial, non-volant organisms. Such disruption in genetic exchange can result in divergence of allele frequencies among populations, with continued isolation ultimately leading to separate evolutionary trajectories (e.g. Funk *et al.*, 2007; Wilson *et al.*, 2009). Accordingly, studies have shown that some island populations have differentiated from other islands and/or mainland populations (e.g. Estoup *et al.*, 1996; Paetkau *et al.*, 1998; Degner *et al.*, 2007; Duffie *et al.*, 2009; Barry & Tallmon, 2010). However, whether isolation and morphological variation can be regarded as indicators of island population divergence is unclear.

The island chain of the Florida Lower Keys has multiple morphologically recognized endemic subspecies, thereby providing an excellent model system to explore the interface of island – mainland evolutionary history and taxonomy. The Florida Lower Keys were formed by the deposition of sand banks on the Southwestern end of an ancient coral reef about 125 thousand years ago (ka) when South Florida was submerged below sea level (Shinn, 1988). Currently, the Lower Keys are separated from the Upper and Middle Keys by an 11-km biogeographical break known as the Moser Channel (Shinn, 1988). It is hypothesized that during the Last Glacial Maximum (40–12 ka), species were able to colonize the exposed South Florida plateau but later became isolated due to sea level rise approximately 10 ka (Lazell, 1984). It is also hypothesized that unique ecological circumstances led to the differentiation of many currently recognized subspecies, such as the Key deer (*Odocoileus virginianus clavium*), the silver rice rat (*Oryzomys palustris natator*), the Torch Key raccoon (*Procyon lotor incautus*) and the Lower Keys marsh rabbit (*Sylvilagus palustris hefneri*).

In this study, we sought to uncover the population genetic structure of the marsh rabbit (*S. palustris*) species complex, of which the Lower Keys subspecies, *S. p. hefneri*, is of particular conservation interest. The mainland subspecies, *Sylvilagus palustris paludicola* and *S. p. palustris* as described by Nelson (1909), occupy the Southeast coastal plains and the Florida peninsula (Fig. 1) and were documented based on differences in molariform row length and ventral guard hair colour (Lazell, 1984). In addition to confinement to the Lower Keys (Fig. 1), taxonomic designation of *S. p. hefneri* was based on shorter molariform tooth row, high and convex frontonasal profile, broad cranium, elongated dentary symphysis, pelage coloration and body size, as it is the smallest of the three marsh rabbit subspecies (Lazell, 1984; USFWS, 2007a). Furthermore, differences in fecundity between *S. p. paludicola* and *S. p. hefneri* have been reported (3.7 litters/year in *S. p. hefneri* as opposed to 5.7–6.9 litters/year in *S. p. paludicola*, Holler & Conaway, 1979; Forys & Humphrey, 1996). Whether these morphological and physiological differences among *S. palustris*

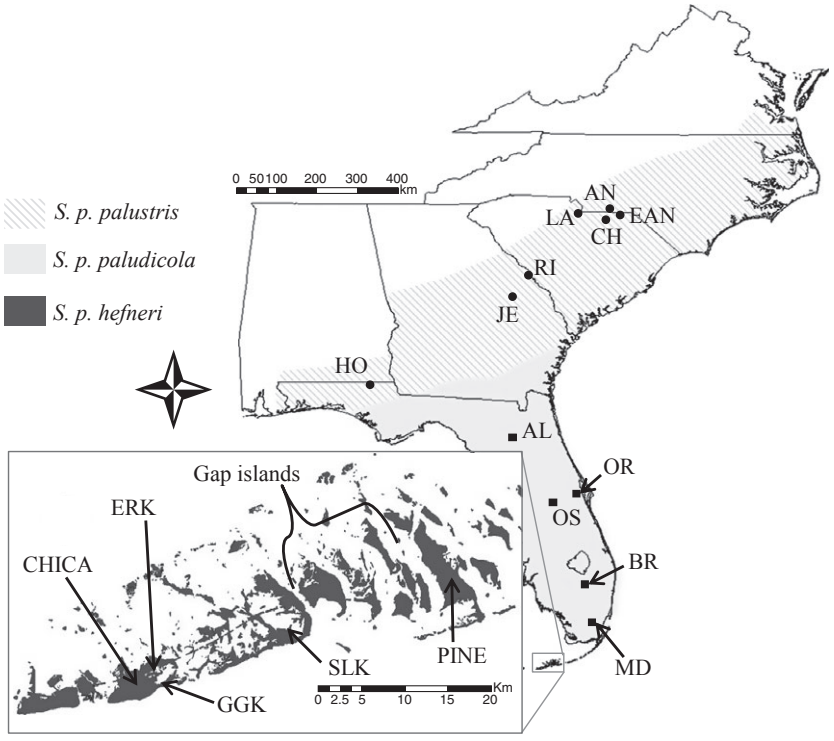


Figure 1 Geographical distribution of *Sylvilagus palustris* subspecies and sampling locations. Inset: Sampling locations of *S. p. hefneri* in the Lower Keys. Population abbreviations defined in Table 1.

Table 1 Location information of *Sylvilagus palustris* samples

| Location | ID | Latitude | Longitude | Sample size | Samples used | |
|---|---|---|---|---|---|---|
| | | | | | Cyt *b* | msat |
| *Sylvilagus palustris palustris* | | | | | | |
| Anson Co., NC | AN | 34.983 | −80.116 | 1 | 1 | 0 |
| East Anson Co., NC | EAN | 34.869 | −79.892 | 1 | 1 | 0 |
| Lancaster Co., SC | LA | 34.925 | −80.815 | 1 | 1 | 0 |
| Chesterfield Co., SC | CH | 34.743 | −80.169 | 1 | 1 | 0 |
| Richmond Co., GA | RI | 33.456 | −81.963 | 19 | 11 | 19 |
| Jefferson Co., GA | JE | 32.976 | −82.331 | 2 | 2 | 0 |
| Holmes Co., FL | HO | 30.883 | −85.657 | 1 | 1 | 0 |
| *Sylvilagus palustris paludicola* | | | | | | |
| Alachua Co., FL* | AL | 29.651 | −82.325 | 1 | 1 | 0 |
| Orange Co., FL | OR | 28.366 | −80.880 | 22 | 17 | 22 |
| Osceola Co., FL | OS | 28.137 | −81.445 | 1 | 1 | 0 |
| Broward Co., FL | BR | 26.332 | −80.623 | 17 | 14 | 17 |
| Miami-Dade Co., FL† | MD | 25.433 | −80.479 | 3 | 3 | 0 |
| *Sylvilagus palustris hefneri* | | | | | | |
| Big Pine Key, FL | PINE | 24.702 | −81.376 | 6 | 6 | 6 |
| Sugarloaf Key, FL | SLK | 24.587 | −81.664 | 5 | 0 | 5 |
| East Rockland Key, FL | ERK | 24.631 | −81.533 | 7 | 0 | 7 |
| Geiger Key, FL | GGK | 24.574 | −81.666 | 14 | 0 | 14 |
| Boca Chica Key, FL | CHICA | 24.573 | −81.692 | 48 | 9 | 48 |

Sampling location, sampling identification (ID), geographical coordinates (in decimal degrees), sample size and samples used per locality for cytochrome b (cyt *b*) and microsatellites (msat) are shown.

*Florida Museum of Natural History Catalog No. 1178.

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

subspecies are reflective of genetic differentiation remains to be tested.

In order to determine whether island and mainland marsh rabbit subspecies represent separate evolutionary lineages, we examined genetic differentiation among the three recognized *S. palustris* subspecies and characterized levels of genetic variation between island and mainland populations. Based on the previously published differences in morphological and biological traits, we first hypothesized that the three *S. palustris* subspecies were genetically differentiated and would comprise three distinct evolutionary lineages. Within the same hypothesis of differentiation but on a smaller scale, we expected that populations on eastern Lower Keys islands will be differentiated from populations on islands further west. This is based on previous findings by Crouse *et al.* (2009) who used mitochondrial DNA markers and identified a partition between the eastern Lower Keys (Big Pine Key) and the western Lower Keys (Boca Chica Key and Sugarloaf Key), separated by a gap of islands historically inhabited by *S. p. hefneri* but that currently contain no rabbits (Fig. 1) (Lazell, 1989; Crouse *et al.*, 2009). Second, we hypothesized that *S. p. hefneri* would exhibit typical island population traits (Frankham, 1997), by having smaller effective population sizes and decreased genetic diversity in comparison to mainland populations of *S. p. paludicola* and *S. p. palustris*. To address these hypotheses, we collected samples from throughout the range of *S. palustris* and sequenced the mitochondrial cytochrome *b* gene to evaluate phylogenetic relationships among the three subspecies. In addition, we genotyped island and mainland populations using 10 polymorphic microsatellite loci to evaluate population structure, current patterns of gene flow, contemporary effective population sizes and levels of genetic diversity. The results of this study are discussed with insights into factors influencing mainland and island differentiation and diversity patterns, as well as implications for the management of these island populations.

## METHODS

### Study species and conservation status

*Sylvilagus palustris* is a small to medium sized cottontail, largely confined to marshy habitats and adjacent ecotones (Chapman & Willner, 1981). Interestingly, *S. palustris* and its sister species, *S. aquaticus*, are the only rabbits known to have the ability to swim (Chapman & Willner, 1981). In the Lower Keys, increasing development has resulted in a drastic drop in population size (around 200–300 *S. p. hefneri* rabbits remained as of 1995, although this number may have been an underestimation; Forys & Humphrey, 1996) and in the listing of *S. p. hefneri* as an endangered subspecies since 1990 (USFWS, 2007a). An updated distribution of *S. p. hefneri* (Faulhaber *et al.*, 2007) showed that the largest number of

occupied patches occurred on Big Pine Key (PINE), Boca Chica Key (CHICA) and Sugarloaf Key (SLK) (Fig. 1). All populations of *S. p. hefneri* have been declining steadily since 1988, but in Sugarloaf Key and especially in Big Pine Key such declines have been more drastic (USFWS, 2007b). In the Everglades, road surveys indicate that small mammal populations, including rabbits, have been experiencing drastic declines since 2003 (Dorcas *et al.*, 2012) which appear to be correlated with time and space by the invasion of Burmese pythons (*Python molurus bivittatus*).

### Sample collection

We collected 150 marsh rabbit samples from 17 localities dispersed throughout the range of the three subspecies (Fig. 1, Table 1). All 26 tissue samples of *S. p. palustris* were donated by hunters during the 2008–2009 hunting season. Harvested samples consisted of ear clips placed in individual 50 ml tubes containing anhydrous calcium sulphate for preservation at room temperatures. Samples were sent to the University of Central Florida within 5 days of collection for analysis. Forty ear-punch and hair follicle samples of *S. p. paludicola* were collected from live-trapped individuals from December 2008 through July 2009. Museum skins from 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. Of the 80 samples of *S. p. hefneri*, 68 consisted of hair follicles collected from live-trapped individuals during the summer of 2008 by Texas A&M University and the U.S. Fish and Wildlife Service (hereafter USFWS), and 12 consisted of road kill tissue samples collected and donated by the USFWS. All live trapping was carried out for a minimum of 7 days per location. Trapping continued daily until 15–30 rabbits were caught, although the endangered populations of *S. p. hefneri* sometimes had lower yields. Rabbits were immediately released after processing at the site of capture.

We experienced some species specific problems that caused us to have uneven sampling numbers among populations that need further explanation. Specifically, microsatellite data were unobtainable from museum specimens owing to DNA degradation. Therefore, not all samples for which we acquired sequence data could be used to collect microsatellite data. Furthermore, for most of the samples from the Lower Keys we acquired hair follicles (owing to USFWS constraints on sampling from the endangered Lower Keys populations) in which to extract DNA. The limited amount of DNA extracted from follicles was primarily utilized to amplify the microsatellite loci, which often led to depletion of the sample. Some Lower Keys samples consisted of road kill tissue collected and donated by the USFWS (these road kills were collected by the USFWS over a period of 2 years – the six samples from Big Pine Key included every rabbit the USFWS was able to obtain from Big Pine Key in 2 years of continuous sampling). These road kills comprise the samples in which we were able to obtain both microsatellite and cyt *b* data. Although some sample sizes are low, sample sizes are sufficient for the analyses we conducted (see below).

### DNA extraction

DNA from ear punches and hunter-harvested tissues was extracted using a standard phenol-chloroform protocol (Sambrook & Russell, 2001). A Qiagen DNeasy tissue purification kit (Qiagen, Valencia, CA, USA) was used for museum samples and hair follicles following the recommendations of Mullen & Hoekstra (2008) with a few modifications: during the elution step, water was used instead of buffer AE to avoid interference with PCR reactions. Also, water was preheated to 70°C and was allowed to incubate on the membrane for 5 min prior to elution. Finally, elution was repeated twice using 50 μl per 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.

### mtDNA amplification and sequencing

In order to obtain historical patterns of variation, the mitochondrial cytrochome *b* gene (cyt *b*) was amplified and sequenced. Amplification of the cyt *b* gene from low quality samples such as road kills, museum skins and hair was performed using primer combinations to amplify smaller, overlapping sequences (Table S1, Fig. S1). DNA amplifications consisted of 20 μl reactions containing 30 ng of genomic DNA, 0.5 μM of each primer, 2 μl of 10X PCR buffer, 2.5 mM of $MgCl_2$, 200 μM of each dNTP and 1 unit of Taq polymerase. Thermal cycling consisted of 95°C for 4 min, followed by 40 cycles of 30 s at 95°C, 30 s at the annealing temperature (see Table S1) and 45 s at 72°C, then a final extension cycle at 72°C for 7 min. Amplified sequence products were cleaned with Exo-SAP-IT (USB Affymetrix, Santa Clara, CA, USA) or NucleoSpin Extract II spin columns (Macherey-Nagel, Bethlehem, PA, USA) and sequenced in both directions in an ABI 3730 DNA analyser (Applied Biosystems, Carlsbad, CA, USA) at the Nevada Genomics Center (NGC; Reno, NV, USA) and the University of Arizona Genetics Core (UAGC; Tucson, AZ, USA). The number of samples used per location is specified in Table 1.

### Microsatellite development and genotyping

A summary of microsatellite primers used is available in Table S1. Four microsatellite loci originally isolated for *Oryctolagus cuniculus* (Sol08, Rico *et al.*, 1994; Sat8, Mougel *et al.*, 1997; D1L5G7, Korstanje *et al.*, 2001) and *Sylvilagus floridanus* (Sfl011, Berkman *et al.*, 2009) were cross-amplified in *S. palustris*. Six additional loci specific for *S. palustris* were developed using an enrichment protocol from Hoffman *et al.* (2003) summarized here. First, about 40 ng of genomic DNA was cut into smaller pieces using a degenerate oligonucleotide-primed PCR (DOP-PCR) and then enriched using

5′-biotinylated, 3′-amino modified $(GATA)_8$ or $(CA)_{15}$ primers. Enriched product was then cloned using a TOPO TA Cloning Kit (Invitrogen, Grand Island, NY, USA). Colonies were plucked using sterile tips, placed in 100 μl of $H_2O$ and boiled for 10 min to release the plasmid. Positive colonies were screened using the T3/T7 procedure outlined by Cabe & Marshall (2001). Amplifications for all microsatellites were performed in 10 μl reactions containing 5 ng of template DNA, 1 μl of 10X PCR buffer, 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.125 μM of M13-tagged forward primer and 0.5 μM of reverse primer, 0.5 μM of fluorescently labelled M13 primer and 1 unit of Taq polymerase. PCR amplifications used the standard touchdown protocol preloaded in a BioRad MyCycler thermalcycler (Bio-Rad Laboratories, Hercules, CA, USA). Cycles started with a denaturing step for 4 min at 95°C, followed by 15°C touchdown cycles of 95°C for 30 s, annealing temperature ($T_a$, see Table S1) decrease by 0.5°C/cycle for 30 s and 72°C for 45 s. After the final touchdown cycle, 30 additional cycles were performed with a $T_a$ of 45°C with a final extension of 7 min. PCR products were visualized on a 2% agarose gel and then genotypes were determined with a CEQ 8000 DNA analyser (Beckman Coulter, Indianapolis, IN, USA).

### Phylogenetic reconstruction and divergence times

To determine whether *S. palustris* subspecies represent distinct evolutionary lineages (Hypothesis 1), we tested for historical restriction of gene flow evidenced by reciprocally monophyletic groups of *S. p. palustris*, *S. p. paludicola* and *S. p. hefneri*. All cyt *b* sequences from each individual from which sequence data were obtained (see Table 1) were edited using Sequencher *v.* 4.8 (Gene codes, Ann Arbor, MI, USA), aligned in Mega *v.* 4.0 (Kumar *et al.*, 2004) using Clustal and checked for possible misalignments by eye. We inferred phylogenetic relationships and lineage divergence times within *S. palustris* using a Bayesian approach in the program Beast *v.* 1.6.1 (Drummond & Rambaut, 2007) and a maximum likelihood (ML) approach in the program PhyML (Guindon *et al.*, 2010). Cytochrome *b* sequences obtained from GenBank for *S. floridanus* (GenBank No. AY192724) and *S. aquaticus* (GenBank No. AY292726) 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, 2004). The model of evolution chosen (HKY + G + I; see Results) was used for both the Bayesian tree and the ML tree.

With regard to the Beast analysis, we initially ran two separate analyses to determine whether our data conformed to a strict clock model versus a relaxed clock model. Using Bayes factors, we compared the two models using Tracer *v.* 1.5 (Rambaut & Drummond, 2007). This analysis indicated that the simpler strict clock model was appropriate (2LogBF = 16.13). We added a temporal component to the analysis using the divergence time estimates (date ± standard deviation) of *S. floridanus*/*S. palustris* (5.33 ± 0.82 Ma) and *S. aquaticus*/*S. palustris* (2.18 ± 0.47 Ma) estimated from 7 genes, including cyt *b*, by Matthee *et al.* (2004). Thus, using *S. floridanus* and *S. aquaticus* as outgroups with node dates and SD as described above, we ran four independent runs from random starting trees for 50 million generations and sampled every 1000 generations with a Bayesian skyline coalescent for the tree prior. We ran the analyses with a Yule process speciation prior, and a normal distribution prior was placed on the dating calibration points as these dates were mean estimates of Matthee *et al.* (2004). We removed the first 25% generations as burn-in and the remaining samples were combined to summarize the posterior distribution of dates on the maximum clade credibility tree. For the ML tree, transition/transversion ratio, proportion of invariable sites and Gamma shape parameter was estimated from the data. The analysis was set to improve tree topology using both a Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) algorithm and to select the best tree based on the data. Nodal support was then analysed using 1000 bootstrap replicates. In order to further investigate relationships among cyt *b* sequences, we 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).

### Genetic differentiation and gene flow

We also tested for genetic differentiation at the population level using microsatellite data obtained from locations where five or more individuals were collected (Table 1). To check for possible scoring errors due to null alleles and allelic dropout, we used Micro-Checker *v.* 2.2. We 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; Rousset, 2008) and we applied a Bonferroni correction to account for multiple comparisons (Rice, 1989). We estimated global and pairwise genetic distances based on allelic state ($F_{ST}$) in SPAGeDi *v.* 1.3 (Hardy & Vekemans, 2002). For pairwise $F_{ST}$ comparisons and genetic diversity estimates, the population from Geiger Key was removed due to lack of statistical independence (see clustering analysis results). Also, because an unbalanced sample size may result in the inability to detect structure (Ryman *et al.*, 2006), we decided to take the conservative approach and remove the smaller populations of East Rockland and Sugarloaf Key and only use the Boca Chica Key samples given the sufficient sample size of this population ($n = 48$). However, Big Pine Key was included in all differentiation and diversity analyses despite low sample size ($n = 6$) because we had hypothesized that this population was differentiated from populations in the western Lower Keys (Crouse *et al.*, 2009). To test whether increases in geographical distance would also result in increased genetic differentiation (i.e. isolation by distance), we performed a Mantel test of genetic distance over geographical distance in the program IBDWS *v.* 3.16 (Jensen *et al.*, 2005). Due to the linear

arrangement of populations in this study, untransformed geographical distances were used for this correlation analysis.

As a final analysis to address Hypothesis 1, we used the microsatellite data to test for evidence of genetic structuring between island and mainland, and among islands by determining the partitioning of genetic units throughout the range of *S. palustris*. For this purpose, we analysed all sampled locations using two Bayesian clustering analyses that differ in their basic model assumptions. First, we 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 to 9. Because Structure is designed to only find the highest level of population structuring, we hierarchically tested for substructuring within each inferred cluster until all structure levels were found (see Degner *et al.*, 2010). For all Structure analyses, 20 independent runs at each possible *K* were conducted with Markov chain Monte Carlo (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, $\Delta K$. Second, we used a spatially explicit model with uncorrelated allele frequencies in the R package Geneland *v.* 3.2 (Guillot *et al.*, 2005) to determine whether spatial information would change inferences obtained solely from genetic data. Because *S. palustris* is a mobile species, we introduced 0.1 decimal degrees of spatial coordinate uncertainty (equivalent to approximately 11.1 km). As with Structure, we 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, we 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 and effective population size in island and mainland populations

To evaluate whether island populations harbour lower levels of genetic diversity than mainland populations (Hypothesis 2), we compared estimates of genetic diversity using cyt *b* and microsatellites between island and mainland populations. Because all clustering analyses group all western Lower Keys populations together suggesting lack of independence (see Results), the island populations were limited to Boca Chica Key and Big Pine Key. We estimated nucleotide diversity ($\pi$) 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, we estimated average expected heterozygosity ($H_E$) using the program Genepop *v.* 4.0 (Raymond & Rousset, 1995; Rousset, 2008) and allelic richness ($AR$) using the program Fstat *v.* 1.2 (Goudet, 1995). To test for statistically significant differences in genetic diversity levels between island and mainland populations, we implemented a Welch's two-sample t-test in the program R *v.* 2.11.1 (R Development Core Team, 2006).

Lastly, to test whether island populations harboured smaller effective population sizes ($N_e$) compared to the mainland, we estimated $N_e$ from Boca Chica Key and the mainland populations using two methods. First, we used an approximate Bayesian computation (*ABC*) approach in the online program OneSamp (Tallmon *et al.*, 2008) with priors for effective population size set from 2 (minimum) to 500 and 5000 (maximum). Second, we estimated $N_e$ based on an analysis of sibship assignment implemented in the program Colony (Wang, 2009) using a full-likelihood approach with medium length runs. Unfortunately, the population in Big Pine Key could not be included in the analysis because the sample size would impede reliable results.

## RESULTS

### Phylogenetic reconstruction and divergence times

A total of 1063 bp of the cyt *b* gene were successfully amplified from 69 samples used for phylogenetic analysis. From these 69 samples, we uncovered 33 unique haplotypes (GenBank No. JQ955688 - JQ955720) defined by 63 variable sites, 44 of which were parsimony informative across all samples including all three subspecies. The final tree chosen was under the HKY + G + I model of DNA evolution (Hasegawa *et al.*, 1985). Although the phylogeny provided support for *S. palustris* samples as a monophyletic group relative to outgroup taxa, there was very low support overall for most of the ingroup clades (Fig. 2). In addition, well-supported clades (> 95% posterior probability, > 70% ML bootstrap support) showed a mixture of haplotypes from all three subspecies indicating lack of historical differentiation. Although nodal support differed at one key node (Node 2, Fig. 2), overall tree topology was similar between Bayesian tree and the ML tree. The strict clock phylogenetic tree indicated that all marsh rabbit divergence dates (age ± 95% confidence limits) occurred in the Pleistocene epoch, with the most basal divergence (Node 1, Fig. 2) estimated to be 356 (199–549) thousand years ago (ka). Internal branches were estimated to have originated 155 (62–284) ka (Node 2), 135 (64–229) ka (Node 3) and 52 (19–104) ka (Node 4).

Detailed information about cyt *b* haplotype relationships was obtained in the 95% statistical parsimony haplotype network (Fig. 3). In correspondence with the phylogeny, shared haplotypes occurred 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 Big Pine Key (*S. p. hefneri*) were also found in *S. p. paludicola* individuals (Haplotype 29) or grouping with mainland haplotypes (Haplotype 21) in all analyses (Figs 2 & 3, Fig. S2). Haplotype 30 was the only haplotype unique to *S. p. hefneri*, and it was also the most frequent because it included all individuals

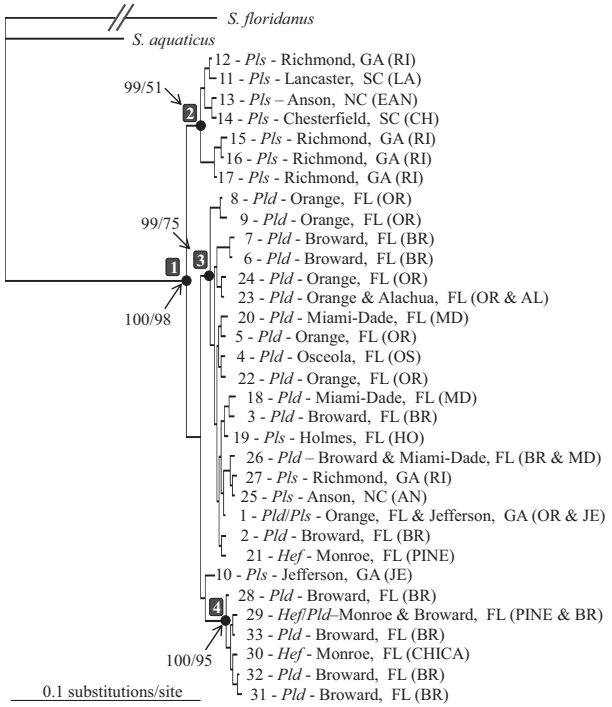


Figure 2 Phylogenetic relationships among haplotypes of *Sylvilagus palustris*. Subspecies abbreviated as follows: *S. p. hefneri* = *Hef*, *S. p. paludicola* = *Pld* and *S. p. palustris* = *Pls*. Locations refer to counties. Nodes with closed circles display posterior probability/bootstrap value, both shown as percentages. Shaded numbers next to nodes are used for reference in the text. Phylogenetic tree was rooted using cyt *b* sequences from *Sylvilagus floridanus* and *S. aquaticus* (the *S. floridanus* branch is longer than shown in this figure).

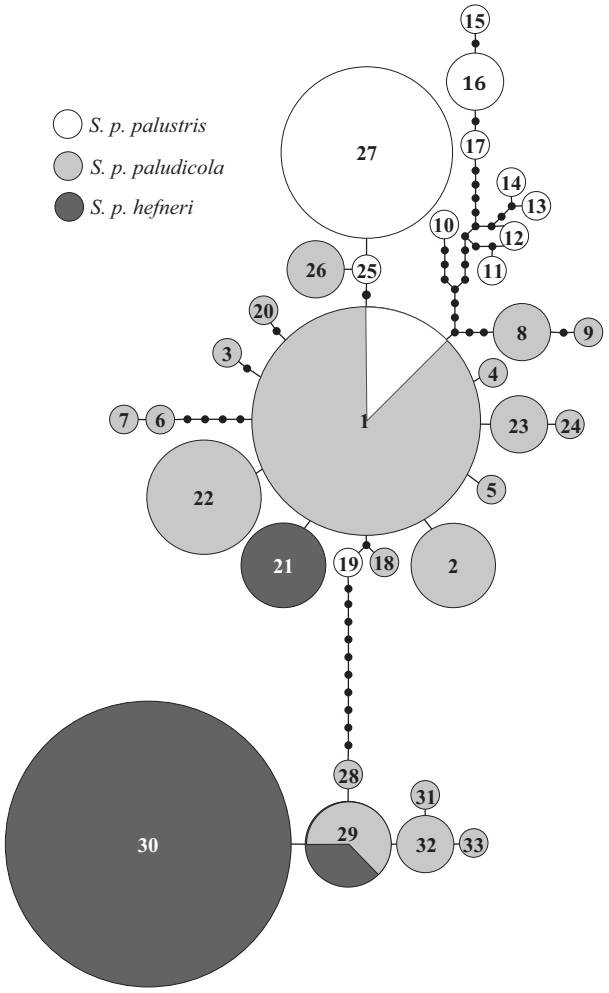


Figure 3 Statistical parsimony haplotype network of *Sylvilagus palustris* haplotypes. Each circle represents a unique haplotype, with the size of the circle scaled to represent haplotype frequency. Each black dot represents a single nucleotide change between haplotypes. Subspecies color-coding shown in legend.

from Boca Chica Key and one individual from Big Pine Key. The overall pattern revealed by the haplotype network suggests incomplete segregation of haplotypes, supporting the results from the Bayesian phylogeny.

### Genetic differentiation and gene flow

Our population level analyses used locations where five or more samples were collected. Therefore, we only obtained genotypes from a total of 138 individuals from eight sampling localities (Table 1). All microsatellites used were polymorphic, with the number of alleles ranging from three to 13 across all populations. 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 a 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 among island populations ($F_{ST}$ = 0.11). Pairwise $F_{ST}$ values ranged from 0.071 (between BR and OR) to 0.367 (between RI and Boca Chica Key). While all pairwise combinations with Boca Chica Key have significantly high $F_{ST}$ values, all mainland-Big Pine Key population pairs have non-significant $F_{ST}$ values (Table 2). However, this may be an artefact of sample size differences between mainland and Big Pine Key as such unbalanced comparisons may decrease statistical power to detect differentiation (Ryman *et al.*, 2006). In addition, no significant association of genetic differentiation over geographical distance was found over all sampled population (Mantel $r$ = 0.4778, $P$ = 0.2123).

With 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) (Fig. S3). Interestingly, these major genetic clusters did not reveal a clean split between island and mainland populations or between mainland subspecies. Instead, the genetic clusters group all mainland populations together with Big Pine Key, while all western Lower Keys islands formed the second

Table 2 Pairwise matrix of genetic distances ($F_{ST}$, above diagonal) and geographical distance (km, below diagonal) between populations of *Sylvilagus palustris*

| | CHICA | PINE | BR | OR | RI |
|---|---|---|---|---|---|
| CHICA | – | **0.265** | **0.270** | **0.268** | **0.367** |
| PINE | 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 | |

Values in bold are significant after 10,000 permutations and adjustment for multiple comparisons. Population abbreviations defined in Table 1.

cluster (Fig. 4a). These results confirm reduced genetic exchange between the western islands and Big Pine Key. Analyzing STRUCTURE within the Big Pine Key-mainland group resulted in two additional clusters, this time separating the northern most population in Georgia (RI, *S. p. palustris*) from the Florida populations OR, BR and Big Pine Key (Fig. 4b). No substructure was found in the western island group. The addition of spatial information in the model using GENELAND did not change the overall structuring of populations, as the same clusters (western Lower Keys, Big Pine Key-BR-OR and RI) were recovered (Figs S4 & S5).

### Genetic diversity and effective population size

Mitochondrial diversity estimates in Boca Chica Key were zero because only a single haplotype was uncovered (Table 3). Conversely, Big Pine Key yielded three haplotypes despite having only six individuals sampled, resulting in mitochondrial diversities similar to those found in the mainland (Table 3). Overall, average nucleotide diversity ($\pi$) in the mainland populations was slightly higher than in the islands (0.0072 vs. 0.0039). Similar results were found with haplotype diversity ($h$), which averaged 0.816 in the mainland and 0.367 in the island populations. Likewise, microsatellite variation was slightly higher in the mainland than in the island populations, with heterozygosity ($H_E$) in the mainland averaging 0.537 as opposed to 0.355 in the islands, and with allelic richness in the mainland almost twice as high as that in the island populations (4.602 vs. 2.133). However, island genetic diversity using all estimates was not significantly lower than genetic diversity of mainland populations as shown by Welch's two-sample $t$-tests (for $\pi$: $t = -0.6952$, $P = 0.5651$; for $h$: $t = -1.2053$, $P = 0.4305$; for $H_E$: $t = -4.1201$, $P = 0.1313$; for $AR$: $t = -2.3113$, $P = 0.2441$).

Effective population size estimates using *ABC* and sibship analysis were comparable (Table 3). For the *ABC* approach, using smaller maximum population priors of 500 resulted in more accurate results as credibility limits were smaller. Although the population in Boca Chica Key has lower $N_e$ than mainland population, the difference is not significant given the overlap in credibility limits with mainland populations.

## DISCUSSION

Subspecies designations as taxonomic units have been widely used in species showing morphological variants, especially when such morphologically different populations are isolated. In this study we employed genetic analyses to evaluate patterns of differentiation and genetic diversity in the endangered insular Lower Keys marsh rabbit (*S. p. hefneri*) and its mainland sister-subspecies. Contrary to our expectations of monophyletic lineages comprised of subspecific taxa and microsatellite data reflecting restricted gene flow among subspecific groups, we did not find genetic distinction among morphologically defined taxonomic units. Moreover, the island population from Big Pine Key displayed high levels of genetic diversity and clustered with mainland populations, suggesting either higher levels of gene flow with mainland than with neighbouring island populations, or a more recent splitting from the mainland than previously recognized. Our

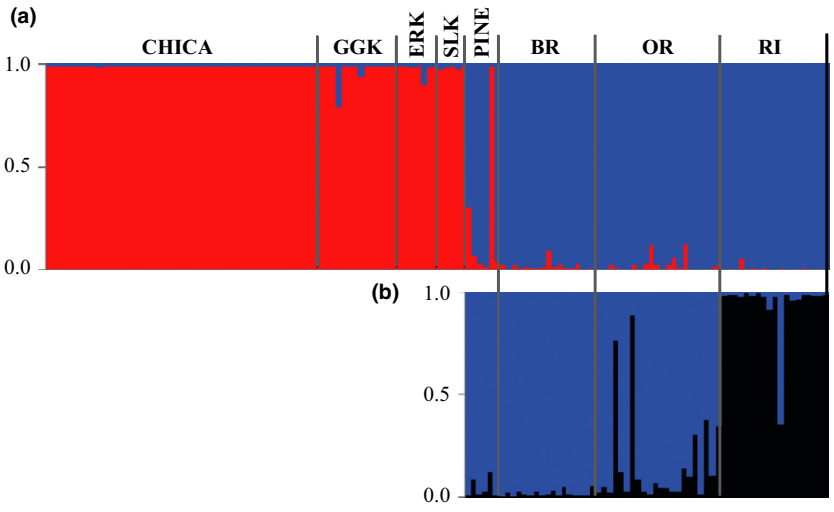


Figure 4 Membership coefficients of *Sylvilagus palustris* individuals as estimated by an admixture model in STRUCTURE. Population abbreviations defined in Table 1.

**Table 3** Genetic diversity and effective population size estimates of mainland and island populations of *Sylvilagus palustris*

| | Mitochondrial diversity | | | | | Microsatellite diversity | | | $N_e$ estimates | |
|---|---|---|---|---|---|---|---|---|---|---|
| Location | *n* | No. of haplotypes | No. of segregating sites | $\pi$ (SD) | *h* (SD) | *n* | *AR* (SD) | $H_E$ (SD) | $N_{e\ (ABC,\ 500)}$ | $N_{e\ (SA)}$ |
| Mainland | | | | | | | | | | |
| RI | 11 | 5 | 22 | 0.0098 (0.0014) | 0.709 (0.137) | 19 | 2.983 (1.265) | 0.519 (0.278) | 27.56 (19.48–58.33) | 19 (10–44) |
| OR | 17 | 7 | 10 | 0.0022 (0.0006) | 0.794 (0.078) | 22 | 3.231 (1.368) | 0.555 (0.287) | 40.11 (27.92–87.20) | 27 (15–52) |
| BR | 14 | 10 | 28 | 0.0096 (0.0010) | 0.945 (0.045) | 17 | 2.991 (1.329) | 0.536 (0.314) | 24.00 (18.09–47.42) | 25 (13–54) |
| Island | | | | | | | | | | |
| PINE | 6 | 3 | 14 | 0.0079 (0.0016) | 0.733 (0.155) | 6 | 2.529 (1.475) | 0.398 (0.354) | – | – |
| CHICA | 9 | 1 | 0 | 0 | 0 | 48 | 1.7359 (0.412) | 0.312 (0.189) | 14.42 (10.48–21.94) | 14 (8–30) |

Genetic diversity shows number of individuals used (*n*), number of haplotypes, number of segregating sites, nucleotide diversity ($\pi$), haplotype diversity (*h*), allelic richness (*AR*, rarefied to five samples), heterozygosity ($H_E$) and standard deviation (SD) for each estimate. Effective population results show estimates based on approximate Bayesian computation using a max prior of 500 ($N_{e\ ABC,\ 500}$) and based on sibship analysis ($N_{e\ SA}$) with 95% credibility limits.

study presents a cautionary tale with regard to subspecies designations based solely on morphological variation and presumed isolation.

The Southeastern United States is a region marked by phylogeographical breaks that result in distinctive genetic patterns within species occupying this area. By comparing the phylogeographical signature of co-distributed taxa, Soltis *et al.* (2006) were able to identify patterns associated with geographical barriers such as the Apalachicola River and the Appalachian mountains. These patterns may be generally associated with Pleistocene glacial cycles, but some of these discontinuities may have arisen at even earlier times (i.e. in the Pliocene). With regard to our study, not only are the actual lineage splits within *S. palustris* far more recent (Node 1 = 356 ka, Fig. 2), but also the alleged geographical split between *S. p. palustris* and *S. p. paludicola* does not coincide with any known geographical break in this region. Moreover, many *S. palustris* cyt *b* haplotypes sampled from a single location grouped in separate, well-supported clades, potentially indicating contemporary intraspecific introgression of lineages that may have diversified during glacial-caused habitat shifts, but did not occur long enough to create subspecific taxonomic units. Hence, the current absence of dispersal barriers among mainland taxa likely explains the lack of contemporary genetic differentiation between the recognized mainland *S. palustris* subspecies.

In the case of island populations, where surrounding water is often a sharp dispersal barrier for low-vagility species, time since isolation is a critical factor influencing the degree of genetic divergence. For example, Heaney *et al.* (2005) determined that mammal populations occupying Philippine islands that formed a single land mass during the Pleistocene showed nearly absent levels of differentiation relative to islands that were separated during the same time period. Similarly, Pulvers & Colgan (2007) found that genetically similar species and subspecies of fruit bats *(Meloncyteris)* occupied islands in northern Melanesia that were connected by land bridges between 18 and 125 ka. Clearly, the last time populations were in contact is of greater importance than merely contemporary isolation. Indeed, it is possible that an uncertainty in the time of Lower Keys isolation may have contributed to an expectation of differentiation of *S. p. hefneri*. 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 with final isolation occurring only 2 ka. Another hypothesis based on coral settlement patterns suggests that final Florida Keys isolation occurred around 6.5 ka (Shinn, 1988). Phylogeographical concordance among taxa provides an avenue to disentangle these conflicting geological hypotheses. Consistent with a recent separation between mainland and islands, previous studies on Lower Keys species such as the Key deer (*O. v. clavium*) and the silver rice rat (*O. p. natator*) found low levels of historical divergence between mainland and island mitochondrial haplotypes (Ellsworth *et al.*, 1994; Gaines *et al.*, 1997). Interestingly, however, these studies

found that both Key deer and silver rice rat populations were defined by a unique island haplotype, which is not what we found in *S. p. hefneri* as the Big Pine Key population shared haplotypes with the mainland.

In addition to presumed isolation, differences in coloration, body size and cranial morphology were of paramount importance in the designation of subspecific groups within *S. palustris*. However, morphological variation can be confounded by plastic responses to the environment. It has been shown, for instance, that coloration differences in snakes do not indicate genetic divergence (e.g. Burbrink *et al.*, 2000). Likewise, experimental studies have found that changes in food type can cause changes in skull shape in rodents (Kiliaridis *et al.*, 1985; Myers *et al.*, 1996). It is also well established that island environments, latitude and elevation gradients can affect biological traits such as cranial morphology (Grieco & Rizk, 2010), body size (Bergmann, 1847; Van Valen, 1973) and litter size (Barkalow, 1962; Conaway *et al.*, 1974; Adler & Levins, 1994). A combination of these environmental factors may be responsible for morphological variation in the absence of genetic differentiation within *S. palustris*, and it highlights the uncertainty in using morphological variation as the sole criterion in subspecies designations.

Reduction in effective population size and genetic diversity in island populations relative to their mainland counterparts has been documented in many studies (e.g. Frankham, 1997; Eldridge *et al.*, 1999; Boessenkool *et al.*, 2007). This reduction is expected to occur owing to inbreeding and genetic drift as a result of founder effects. Although the population in Boca Chica Key did not show significantly lower effective population sizes, it did conform to expectations of reduced genetic diversity particularly at the mitochondrial level. The population in Big Pine Key, however, did not match these genetic diversity trends. Recently, increased genetic diversity has been documented in island populations that underwent recent expansions and/or additional founder events, as was the case for some populations of the Galapagos marine iguana, *Amblyrhynchus cristatus* (Steinfartz *et al.*, 2009). Although testing for effective population size in Big Pine Key was not possible due to small sample size, population expansion is not likely to account for increased diversity in Big Pine Key as that population has reportedly been declining (USFWS, 2007b). This suggests that the Big Pine Key population may be the result of a large founding population, or that it may have experienced multiple founding events.

Multiple founding events or a large founding population would not only explain the increased diversity in Big Pine Key, but also its genetic clustering with mainland populations (Fig. 4, Fig. S4). The grouping of Big Pine Key with mainland populations contradicts patterns found in silver rice rats and in Key deer, as Big Pine Key populations for those species had significantly divergent allele frequencies compared to the mainland (Banks, 2001; Crouse, 2005). Hence, the Big Pine Key – mainland grouping is unique to *S. palustris* with regard to other Lower Keys endemic species. A mainland-island stepping stone colonization involving a large founding population in Big Pine Key and a subset of those migrating to islands further west could explain this pattern. Yet, the fact that similarly distributed species support vicariant colonization makes stepping stone colonization for *S. palustris* less probable. An alternative explanation is recent dispersal from the mainland into Big Pine Key. However, natural dispersal at high frequencies is highly unlikely because (1) distance from the mainland to Big Pine Key is at least approximately 45 km and it is reported that marsh rabbits swim mostly as a means to escape predation (USFWS, 2007b), and (2) dispersing over roads would expose marsh rabbits to avian predators and vehicular traffic. Still, it is possible that dispersal has been facilitated via undocumented, man-mediated translocations. Translocations of lagomorphs for economic and gaming reasons are known to have occurred historically (Suchentrunk *et al.*, 2006; Masseti & De Marinis, 2008). Furthermore, these actions are not limited to lagomorphs as genetic evidence of recent, undocumented translocations has been found in other taxa (e.g. Hoffman & Blouin, 2004). Although validating this scenario is problematic without any documentation, it is still a strong alternative due to lack of support for the two previous natural explanations and the fact that co-distributed taxa do not exhibit the same pattern. Increasing genetic sampling from the Middle and Upper Keys and from other areas within South Florida will help resolve among these and other possible scenarios that may explain this unique pattern of genetic variation.

## Taxonomic conclusions and conservation implications

Based on the overall lack of historical divergence among the three *S. palustris* subspecies, we suggest all three subspecies be synonymized. We do, however, suggest affording the western Lower Keys populations conservation priority. Since 1978, the Endangered Species Act 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. 'Significance' refers to habitat use that is atypical for the taxon and 'discreteness' can be determined by looking at patterns of gene flow using genetic data (Policy regarding recognition of DPS, 1996). Although all Lower Keys populations meet the 'significance' criterion based on confinement to insular habitats, the 'discreteness' criterion is only met by the western Lower Keys populations. Here, 'discreteness' is based on lack of contemporary gene flow with the mainland (and *eastern* Lower Keys) populations as indicated by the microsatellite data. Assessment of discreteness of the Big Pine Key population remains a challenge because of the potential that assisted genetic exchange may have occurred with mainland populations, thereby altering the natural history of this population. Future reintroduction and translocation practices

will depend on the ultimate goal of island population conservation. If the goal is to maintain genetic distinctiveness of island populations, then only individuals from Boca Chica Key, Geiger Key and East Rockland Key should be used as sources for future population founding events on currently uninhabited islands. Alternatively, because the population in Big Pine Key is not genetically differentiated from the mainland, then individuals from the mainland could be used to supplement and increase these populations in Big Pine Key. Translocation from Big Pine Key or from the mainland to the western Lower Keys is not recommended to avoid compromising the 'discreteness' of the western island populations. Any reintroduction and translocation strategy, however, should only be part of a larger effort to restore degraded native habitat.

## ACKNOWLEDGEMENTS

This project received financial support from the U.S. Fish and Wildlife Service and the University of Central Florida. Animal handling was carried out under the UCF Institutional Animal Care and Use Committee permit no. 08-09W. We thank Chris Parkinson, Jack Stout and Jane Waterman for their support and for providing crucial field equipment and transportation. Sarah May, Gina Ferrie, Tyler Hether, Sara Williams, Nancy Gillis, Haakon Kalkvik, Allyson Fenwick, Genevieve Metzger, Marybeth Osbourne, Juan Daza, Greg Territo, Ocean Cohen and James Angelo provided invaluable assistance in numerous ways. We are thankful to all the people and institutions that assisted in sample collection through field access and assistance: The Florida Museum of Natural History, James Roth, Helen Mojorox, Chad Anderson, Monica Folk, Tom O'Neil, Marsha Ward, Missy Juntunen, Daniel Mitchell, Susana Fernandes, Iker Tursi and Angela Tursi. Collection in the northern ranges and completion of this project would not have been possible without the efforts and generosity of Jackie Burris, Jimmy Richardson, Chuck Terry, Wendell McInnis, Pete Newsome, Scott Wilson, Jeff Grimes and Jerry Newman. We are thankful to Anne Morkill, Jason Schmidt and Angela Dedrickson for indispensible assistance for collection in the Lower Keys.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Schematic representation of cyt *b* gene amplification in smaller, overlapping fragments in *Sylvilagus palustris*.

**Figure S2** Geographical distribution of inferred *Sylvilagus palustris* cytochrome *b* haplotypes.

**Figure S3** Estimation of genetic clusters within *Sylvilagus palustris*.

**Figure S4** Output from GENELAND showing three major clusters of *Sylvilagus palustris*.

**Figure S5** Close up of the separation within *Sylvilagus palustris hefneri* in the Lower Keys.

**Table S1** Primers used for amplification of overlapping cyt b fragments and for microsatellite amplification in *Sylvilagus palustris*.

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Author Contributions: R.T., P.H. and E.H. conceived the ideas; R.T. collected the data; R.T. and E.H. analysed the data; and R.T. and E.H. led the writing.

Editor: Jeremy Austin