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ORIGINAL RESEARCH ARTICLE

Population genetics and conservation of the threatened southeastern beach mouse (*Peromyscus polionotus niveiventris*): subspecies and evolutionary units

Jacob F. Degner · I. Jack Stout · James D. Roth · Christopher L. Parkinson

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Abstract We investigated genetic diversity within the southeastern beach mouse (SEBM-*Peromyscus polionotus niveiventris*) and also tested the hypothesis that the subspecies recognition of *P.p. niveiventris*, based on size and color differences, is congruent with this taxon representing a discrete evolutionary lineage. We used ten polymorphic microsatellite loci and mitochondrial cytochrome-*b* gene DNA sequences to investigate genetic diversity and population structure within the SEBM, and to determine the level of divergence between the SEBM and the nearest known inland subspecies of the oldfield mouse (*Peromyscus polionotus rhoadsi*). Moderate genetic distances were observed between the SEBM and the inland oldfield mouse based on microsatellite data, with F_{ST} values ranging from 0.11 to 0.22 between these taxa. Additionally, mitochondrial DNA haplotypes of the SEBM formed a distinct monophyletic group relative to haplotypes sampled from *P. p. rhoadsi*. Based on previous estimates of rates of mitochondrial DNA evolution in rodents, we inferred that Pleistocene sea-level fluctuations are likely responsible for the historical isolation of the SEBM lineage from mainland *P. polionotus*. Our data demonstrate the genetic distinctiveness of the SEBM, justifying the current subspecies designation for the SEBM and its continued protection under the United States Endangered Species Act. We classify the Cape Canaveral and Smyrna Dunes Park populations of SEBM as a single evolutionary significant unit. The two known extant allopatric populations of the SEBM showed some differentiation in microsatellite frequencies and were moderately reciprocally distinguishable based on assignment to distinct genetic clusters by a Bayesian admixture procedure. These results justify the classification of these two extant SEBM populations as distinct management units that should be independent targets of management and conservation attention.

Keywords Biogeography · Endangered species · Evolutionarily significant units · Management units · Microsatellites · *Peromyscus polionotus niveiventris*

J. F. Degner · I. J. Stout · J. D. Roth · C. L. Parkinson (✉)
Department of Biology, University of Central Florida, 4000 Central Florida Blvd., Orlando, FL 32816, USA
e-mail: cparkins@mail.ucf.edu

Introduction

Identifying units of conservation is controversial and a methodological consensus has not been reached (Moritz 2002; Gompert et al. 2006). Morphologically-defined taxa continue to be the fundamental biological units for comparative studies across fields of biology, including conservation. As a result, conservation efforts world-wide revolve around the protection of species, subspecies, or discrete population segments that have been defined primarily on the basis of morphological distinctiveness. The reliance on taxon definitions for conservation is particularly the case in the United States where species are afforded conservation attention via the Endangered Species Act (ESA). Be they units of analysis in scientific studies or units of conservation in environmental policy, it is crucial that the operational units used accurately reflect natural (i.e., evolutionary) groupings. That is, it is important that these units represent groups of organisms which have been reproductively isolated long enough to

develop unique adaptive potential, and ideally, individuals are more closely related within units than between units (e.g., within versus between species or subspecies). The use of species as units of analysis is often overly broad or even inaccurate, particularly in taxonomic groups where informative morphological taxonomic characters are rare or homoplastic.

When systematic revisions of species or subspecies defined by morphology are performed using alternative methods (e.g., molecular data), it is not uncommon that minor morphological differences observed in nominal taxa, such as color or size, are found to be poor indicators of discrete and exclusive evolutionary lineages (Burbrink et al. 2000; Fritz et al. 2005). Color has been shown to be polymorphic in a variety of organisms, including many species of frogs (Hoffman and Blouin 2000), fishes (Olendorf et al. 2006), and mammals (Hoekstra and Krenz 2005). While some studies have supported neutral or weak selection maintaining color variation (e.g., O'Hara 2005; Hoffman et al. 2006) others have suggested that color polymorphisms are under selection and may be poor indicators of shared evolutionary ancestry (Kettlewell 1955, 1956, Hadley et al. 1988; Hoekstra and Krenz 2005; Hoekstra et al. 2006). Recently, Hoekstra et al. (2006) determined coat color in beach mice (*Peromyscus polionotus* spp.) from the Gulf Coast of Florida is under selection for crypsis. A single amino acid substitution in the *Mc1r* gene increases the frequency of light morphs in coastal populations compared to inland conspecifics.

Recent studies have dramatically increased our ability to identify meaningful conservation units in cases where clear morphological demarcations do not exist to define discrete evolutionary lineages. In such cases, genetic variation has proved invaluable in the clarification of conservation units. Moreover, as threatened and endangered (T&E) taxa are impacted by landscape fragmentation, a transparent understanding of genetic diversity in T&E populations is crucial (Frankham et al. 2002). Most T&E populations have reduced levels of heterozygosity and have an increased probability of extinction (Spielman et al. 2004 and references therein). Thus, knowledge of population genetic structure within T&E species arms conservation authorities with added data enabling strategic and well-informed management decisions that may effectively conserve biological diversity.

Ryder (1986) introduced the concept of the ''evolutionarily significant unit'' (ESU) for prioritizing taxa for conservation, and the concept of ESUs has gained widespread use in the literature. There is controversy, however, over what exactly an ESU represents and if the concept of an ESU should continue to bias conservation strategies (Paetkau 1999; Crandall et al. 2000; Kizarian and Donnely 2004). Moritz (1994) proposed a clear and stringent set of criteria for the definition of an ESU by introducing the concept of reciprocal monophyly stating, ''ESUs should be reciprocally monophyletic for mtDNA and show significant divergence of allele frequencies at nuclear loci.'' However, Moritz (1994) recognized this criterion as potentially overly stringent, and suggested groups that did not show reciprocal monophyly, but did show significant allele frequency divergence, could also be considered ESUs. This ESU qualification has generally been accepted both by the scientific community and regulatory agencies. There are, however, notable exceptions (Paetkau 1999; Crandall et al. 2000; Kizarian and Donnely 2004), and arguments have been made that the inclusion of an ESU *sensu* Moritz (1994) in ESA legislation is problematic (Pennock and Dimmick 1997; Dimmick et al. 1999) and that this definition overlooks nested units of diversity (Paetkau 1999; Crandall et al. 2000; Kizirian and Donnelly 2004), thus negatively impacting the conservation of overall diversity. Many of these problems appear to be overcome with the recent more holistic approach of including both genetic and ecological exchangeability data in defining conservation units (Crandall et al. 2000; Fraser and Bernatchez 2001; Rader et al. 2005).

The oldfield mouse (*Peromyscus polionotus)* is a monogamous, burrow-building species distributed throughout sandy habitats in the extreme southeastern United States of America. Sixteen subspecies have been defined based on pelage and morphological differences (Hall 1981). Beach forms of *Peromyscus polionotus* occur on the dune systems of the Atlantic and Gulf Coasts and are nominally referred to as ''beach mice.'' Due in part to extensive urban development of coastal habitats, six of seven extant beach subspecies are federally listed as threatened or endangered, and one Atlantic coast subspecies is already believed to be extinct (Ehrhardt 1978; Humphrey and Barbour 1981; Humphrey 1992). The southeastern beach mouse (SEMB, *Peromyscus polionotus niveiventris*) is the largest-bodied subspecies and historically occupied a geographically isolated range along barrier islands and mainland beaches of the east coast of central and south Florida (Stout 1992). Originally described by Chapman (1889; see also Osgood 1909), the SEBM is distinguished from other subspecies by overall size and pelage characteristics (Hall 1981). The historical range of this subspecies once spanned 281 kilometers of coastline. Due to extensive development of Florida's east coast and loss of coastal habitat, this range was

reduced to 64 km of coastline in the northernmost part of its historic range by 1993 (Hall 1981; U.S Fish and Wildlife Service 1993). Since 1993, the range has continued to contract and the only known persistent populations occur within the Merritt Island complex in east-central Florida from Cape Canaveral Air Force Station to Smyrna Dunes Park (approximately 56 km of coastline; Fig. 1.) Several individuals have been captured recently at Pelican Island National Wildlife Refuge, but their abundance and distribution there are unknown (J. Van Zant, I. J. Stout, J. D. Roth, C.L. Parkinson, unpublished data). The SEBM was federally listed under the ESA with Threatened status in 1989.

We used ten microsatellite loci, together with sequences of the complete mitochondrial cytochrome-*b* gene, to assess genetic diversity, genetic structure, and demographic patterns within the SEMB. Our sampling included individuals from essentially all portions of the known extant range of the SEBM, including the isolated Smyrna Dunes Park population and the Cape Canaveral Air Force Station. This extensive sampling facilitates a nearly complete survey of the genetic diversity, variability, and structure of the SEBM. In addition, we compared the SEBM with the inland *P.p. rhoadsi*, hereafter referred to as the oldfield mouse, to assess the evolutionary distinctness and validity of the subspecies status of the SEBM.

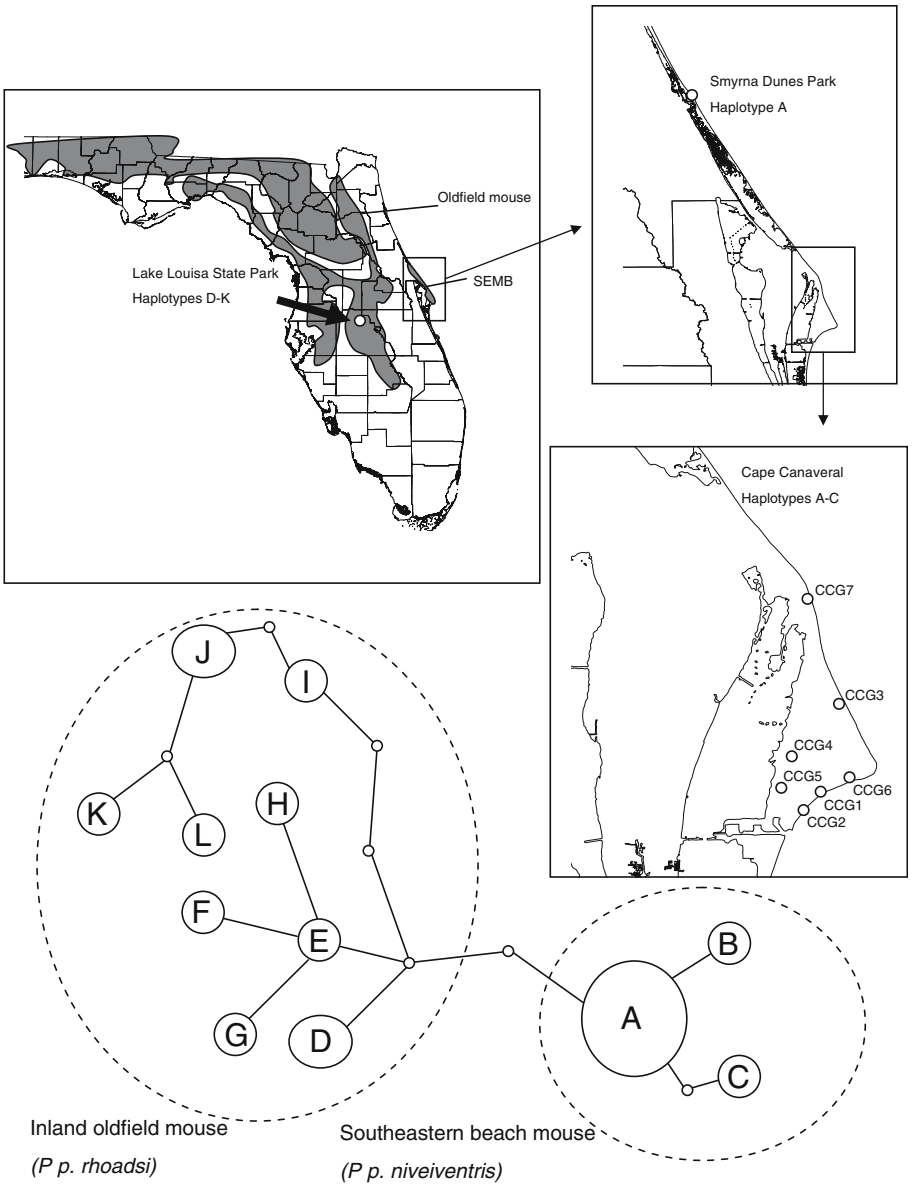

Fig. 1 The current range of the southeastern beach mouse (*Peromyscus polionotus niveiventris*) and the inland oldfield mouse (*Peromyscus polionotus rhoadsi*). Sampling localities for the oldfield mouse (Lake Louisa State Park) and the SEBM (Cape Canveral grids 1–7, and Smyrna Dunes Park), observed mitochondrial haplotypes (per locality or group of localities), and statistical parsimony haplotype network are additionally shown. In the haplotype network, each indicated step (circle) represents single nucleotide differences in the cytochrome-*b* gene. The size of the circle is scaled to represent the relative frequency of that haplotype in the total sample, and the smallest circles represent inferred unsampled haplotypes

Methods

Field tissue collection

Tissue samples from the SEBM were obtained from eight discrete sampling localities located on the Merritt Island complex in 2004 (Fig. 1). Tissue samples from the inland oldfield mouse were obtained from Lake Louisa State Park (in 2004 and 2005), the inland population nearest to the sampling locations of SEBM (Fig. 1). Specimens were captured using Sherman live traps spaced approximately 15 meters apart. Scissors sterilized with ethanol wipes were used to clip a small portion of skin (2–4 mm) from the tail of each new capture. Immediately, Kwik Stop styptic powder was applied to the wound and pressure was applied until minor bleeding had completely subsided. Mice were marked with a metal ear tag, weighed, sexed, and released at the point of capture. Tail tips were placed in 95% ethanol and transported to the University of Central Florida for genetic analysis.

DNA isolation and microsatellite genotyping

Whole genomic DNA was isolated from tail tips (approximately 30 samples per grid, Table 1) using Qiagen DNeasy tissue purification kits (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocols. A total of 10 microsatellite loci were amplified and scored per individual. Both alleles (diploid co-dominant autosomal markers) were amplified per individual using the polymerase chain reaction (PCR), and the PCR products were sized using automated capillary gel-electrophoresis on a Beckman-Coulter CEQ 8000 Genetic Analysis System (Beckman-Coulter, Fullerton, CA). CEQ 8.0 software was used to automate allele sizing (based on comparisons with Size Standard 400; Beckman-Coulter), although each chromatogram was also manually reviewed for accuracy. PCR reactions were carried out in 10 μl volumes. Each 10 μl reaction contained 1 μl 10× PCR buffer (Sigma, St Louis Mo), 0.3 Units of taq polymerase (Sigma), 1–10 ng template DNA, 0.2 μM of both forward and reverse primer for one of the loci: pml-11, pml-02, pml-06 (Chirhart et al. 2000), PO-25, PO-105, PO-71, PO3-68, PO3-85 (Prince et al. 2002), PPA-01, or PPA-46 (Wooten et al. 1999) and 0.8 mM (combined) DNTPs. Final MgCl concentrations and thermal cycling parameters varied depending on optimal conditions for each primer pair. Forward primers were labeled with WellRED fluorescent dyes D2-PA, D3-PA, or D4-PA (Proligo, Boulder, Colorado). Negative controls were run with each PCR set to control for contamination. Loci PO-25 and PO-71 were amplified jointly; for this combined reaction the concentration of both PO-71 primers was increased twofold.

Capillary electrophoresis of microsatellite PCR product for each individual was performed on the Beckman-Coulter CEQ 8000. Loci pml-06, PO3-68, PO3-85, and pml-02 were amplified separately; PCR product was combined, into a single well on a 96 well PCR plate, in the ratio of 1:1:5:10 by volume, and ethanol precipitated to remove non-DNA PCR components. Ethanol precipitated DNA was dried in a vacuum centrifuge and suspended in 20 μl of deionized formamide with Size Standard 400 (Beckman-Coulter; 0.2 μl per well) and separated on the CEQ 8000 according to slightly modified manufacturers protocols. Likewise, for each individual, loci ppa-46, PO-105, and the combined PCR reaction of loci PO-71 and PO-25 were run together in the ratio of 1.25:1.25:5. Loci ppa-01 and pml-11 were electrophoresed jointly in the ratio of 5:1.25. If problems occurred for individual loci (e.g. non amplification, dye signal out of readable range, extraneous PCR amplification), the problem locus for that individual was re-amplified and run on the capillary electrophoresis system independently of other loci.

Table 1 Population, sample size (n), number of alleles per locus (A), allelic richness compensating for sample size, average observed (H_O) and expected (H_E) heterozygosity for each sampling location. Lake Louisa State Park samples represent inland oldfield mouse (*Peromyscus polionotus rhoadsi*): all other sampling locations represent the southeastern beach mouse (*Peromyscus polionotus niveiventris*)

Population	n	A	Allelic richness	H_O	H_E
Cape Canaveral Grid 1	31.0 ± 0.0	8.9 ± 0.99	8.03 ± 0.87	0.72 ± 0.07	0.76 ± 0.06
Cape Canaveral Grid 2	34.0 ± 0.0	8.8 ± 1.08	7.66 ± 0.93	0.75 ± 0.07	0.73 ± 0.07
Cape Canaveral Grid 3	26.2 ± 0.2	8.8 ± 1.27	7.96 ± 1.10	0.71 ± 0.06	0.76 ± 0.6
Cape Canaveral Grid 4	32.0 ± 0.0	7.6 ± 0.92	6.82 ± 0.81	0.72 ± 0.09	0.72 ± 0.07
Cape Canaveral Grid 5	30.0 ± 0.0	8.6 ± 1.14	7.70 ± 0.93	0.69 ± 0.07	0.74 ± 0.06
Cape Canaveral Grid 6	44.9 ± 0.1	9.1 ± 1.20	7.83 ± 0.94	0.71 ± 0.08	0.74 ± 0.06
Cape Canaveral Grid 7	42.1 ± 0.4	8.7 ± 0.93	7.29 ± 0.78	0.69 ± 0.09	0.75 ± 0.06
Smyrna Dunes Park	18.5 ± 0.2	4.2 ± 0.42	4.17 ± 0.42	0.59 ± 0.07	0.56 ± 0.07
Lake Louisa	23.5 ± 0.4	11.7 ± 1.11	11.00 ± 0.95	0.81 ± 0.03	0.88 ± 0.01

To confirm homology among microsatellite loci, the DNA sequence for each locus was determined and compared to previously published data (particularly the non-repetitive regions flanking the microsatellite repeats). PCR products of two individuals for each microsatellite locus were purified using the GeneCleanIII kit (BIO101, Irvine, California). Purified PCR products were then cloned using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, California). Multiple positive clones were grown overnight and plasmid DNA was isolated using the Qiagen Qiaquick miniprep kit. Cloned DNA fragments were sequenced using M13 reverse primers on the Beckman-Coulter CEQ 8000 Genetic Analysis System according to manufacturer's protocols.

The complete DNA sequence of the mitochondrial cytochrome-*b* gene (1139 bp) was obtained for six to fourteen individuals from each discrete population: Cape Canaveral Air Force Station, Smyrna Dunes Park, and Lake Louisa State Park. The cytochrome-*b* gene was amplified as in Herron et al. (2004). Positive PCR products were purified as above and directly sequenced using the Beckman-Coulter CEQ 8000 Genetic Analysis System.

Statistical analysis

An intraspecific haplotype network was constructed using the algorithm of Templeton et al. (1992). Haplotype connections were made under a 95% connection limit. Statistical parsimony was implemented in TCS 1.21 (Clement et al. 2000). Additionally, phylogenetic relationships among *P. polionotus* haplotypes were assessed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian Markov chain Monte Carlo (MCMC). Phylogenetic analyses were rooted using *P. maniculatus, P. melanotis, P. leucopus*, and *P. gossypinus* cytochrome-*b* sequences obtained from Genbank (accession numbers: DQ385633, DQ385627, DQ000483, DQ385625 respectively). MP and ML methods were implemented in PAUP* v 4.0b10 (Swofford 2002). Nodal support for ML and MP analyses were assessed using nonparametric bootstrapping of 2000 pseudo-replicates of the original sequence alignment. Bayesian phylogenetic analysis was implemented in MrBayes v 3.1 (Ronquist and Huelsenbeck 2003). For Bayesian and ML analysis, ModelTest 3.0 (Posada and Crandall 1998, 2001) was used to select a model of DNA evolution using Akaike Information Criterion. The General Time Reversible Model with a gamma distributed among-site rate variation (GTR + γ) was preferred. Prior parameter distributions were set to their default values. Four MCMC chains were run starting from different random trees, and parameters were sampled every 100 generations. Each MCMC run was 5 million generations although the first two hundred thousand generations were discarded as burn in. A 50% majority rule consensus phylogram was constructed from posterior distribution of trees in the four MCMC runs after burn in.

Both observed and expected levels of heterozygosity and Hardy-Weinberg Equilibrium expectations per microsatellite locus and per sampling locality were determined using ARLEQUIN version 3.000 (Schneider et al. 2005). In ARLEQUIN, standard errors and significance levels were calculated with a Markov Chain using 100,000 steps. Allelic richness, compensating for sample size effects, was calculated in FSTAT v. 2.9.3 based on a minimum sample size of 18 (El Mousadik and Petit 1996, Goudet 1995). A test for the significance of regional differences among expected heterozygosities and allelic richnesses was performed using a Wilcoxon's signed-ranks test for which the data need not be normally distributed.

Allelic richness and heterozygosity at microsatellite loci were compared among sampling localities (Table 1). The null hypothesis that each population pair had identical allele frequencies was tested for all population comparisons by the method of Raymond and Rousset (1995). The degree of genetic differentiation between all pairs of sampling localities was measured by pairwise F_{ST} (Weir and Cockerham 1984) calculated from raw allelic data in GENEPOP. A Mantel test was conducted, using the web-based program IBDWS (Jensen et al. 2005), to test for a correlation between microsatellite-based genetic distance and geographic distance (i.e. isolation by distance).

A Bayesian admixture procedure (STRUCTURE v2.1; Pritchard et al. 2000) was used to identify the number of genetically distinct clusters (K) in the entire microsatellite data set. This procedure introduces population structure to the data that minimizes Hardy Weinberg or linkage disequilibrium, and produces an estimate of the log probability of the data $\Pr(X|K)$ for a specified value of K. Often, the value of $\Pr(X|K)$ estimated by STRUCTURE continues to increase with increasing values of K, rendering its maximum a poor criterion for determining the ''best'' estimate of K. An alternative measure, essentially a second order derivative of $\Pr(X|K)$, ΔK, has been shown to successfully identify the highest level of meaningful population structure under a wide variety of simulation scenarios (Evanno et al. 2005). Thus, we used the modal value of ΔK to determine the number of clusters that best explain the highest level of population structure in our data.

In STRUCTURE, we set most parameters to their default values as suggested by the user manual. We chose a model allowing admixture and correlated allele frequencies between populations. We let α, the degree of admixture, be inferred from the data. The parameter of the distribution of allele frequencies (λ) was set to one. The first 100,000 generations of data were discarded as burn-in, and data were collected for 1,000,000 generations thereafter. A visual inspection of $\Pr(X|K)$ plotted against the number of generations, and consistency (i.e., convergence) across runs, supported 100,000 generations as more than a sufficient amount of burn-in. For each value of K (1–6), twenty independent STRUCTURE runs were conducted to obtain precise estimates of the variance among runs (as these pooled data were used to calculate ΔK). To determine the behavior of $\Pr(X|K)$ beyond this K ($K > 6$), three runs were carried out for each value of K up to $K = 10$. For the K that was determined to be the best-fit, membership coefficients of each individual in each of the population clusters were plotted.

Results

Polymorphism, genetic variation and heterozygosity within the SEBM

The DNA sequence obtained for each microsatellite locus was compared to previously published *Peromyscus polionotus* sequences obtained from Genbank. In all cases, the regions flanking the microsatellite repeat motif were identical to previously published data, suggesting that we had succeeded in amplifying homologous microsatellite loci in this study.

Microsatellite genotypes and allele frequencies were determined for a total of 305 individuals, from nine trapping localities, at ten microsatellite loci (Table 1). All loci were found to be polymorphic. Generally, heterozygosities conformed to Hardy–Weinberg Equilibrium (HWE) expectations (Table 1). After sequential Bonforoni correction for multiple comparisons, there were five significant deviations from HWE. Each significant deviation from HWE was due to a heterozygote deficiency, and six out of nine sampling localities exhibited lower than expected average heterozygosity (Table 1). Two sampling localities, Cape Canaveral Grid (CCG) 6 and CCG7, deviated from HWE at two loci. At CCG4 and Lake Louisa, the PO-25 locus was significantly heterozygote deficient. All other significant deviations were not repeated across multiple sampling localities or loci. As the majority of populations adhered to HWE for any given locus, all loci and populations were included in subsequent analyses.

Estimates of expected heterozygosity and allelic diversity were significantly higher for the inland population of oldfield mouse compared to the SEBM (Wilcoxon's signed-ranks test; $P = 0.0006$ and $P = 0.0001$, respectively). For the SEBM, the expected proportions of heterozygous individuals (mean $H_E = 0.74$) for all grids on Cape Canaveral were very similar, but significantly higher than heterozygosities for the animals at Smyrna Dunes Park ($H_E = 0.56$; $P = 0.003$). Also, the allelic richness per locus was significantly higher for the grids on Cape Canaveral compared to the Smyrna Dunes Park (Table 1; $P = 0.002$).

Unique cytochrome-*b* sequences were deposited in Genebank under accession numbers EF216336-EF216347. A single cytochrome-*b* haplotype was observed in all seven individuals sequenced for the Smyrna Dunes Park population (labeled haplotype A). This haplotype was also found in the Cape Canaveral population, along with two other rare haplotypes (B, C; Figs. 1 and 2). The fourteen individuals sequenced for cytochrome-*b* from the inland population of oldfield mouse yielded nine observed haplotypes, none of which were shared with the SEBM. However, given that 64% of oldfield mouse haplotypes were unique, it is likely that additional un-sampled haplotypes exist. Kimura 2-parameter pairwise sequence divergences between haplotypes within the SEBM were at most 0.2%, while pairwise sequence

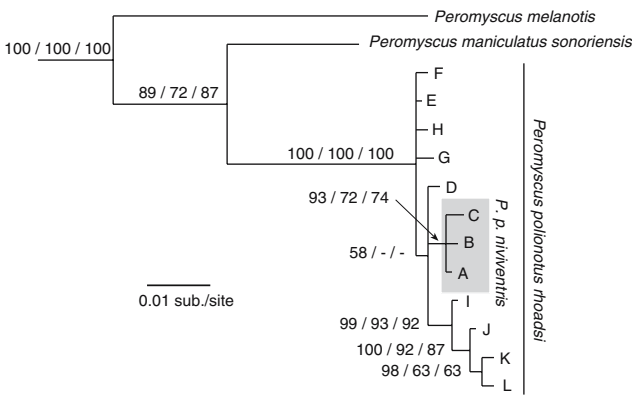

Fig. 2 Inferred phylogenetic relationships among haplotypes of SEBM (*Peromyscus polionotus niveiventris*) and the inland oldfield mouse (*Peromyscus polionotus rhoadsi*). *Peromyscus maniculatus, melanotis, leucopus*, and *gossypinus* were used to root this phylogeny (*leucopus*, and *gossypinus* are not shown). Nodal support displayed is Bayesian posterior probability/ML bootstrap support/MP bootstrap support, dash (–) indicates lower than 50%. SEBM haplotypes are shaded, while all other haplotypes were sampled from the inland oldfield mouse

divergence between haplotypes within the oldfield mouse were from 0.1 to 0.9%. Sequence divergence between haplotypes of the oldfield mouse and the SEBM ranged from 0.3 to 1.0%. The intraspecific haplotype network as well as Bayesian, MP, and ML phylogenetic analyses supported the monophyly of SEBM haplotypes (Figs. 1 and 2). Utilizing four *Peromyscus* species as outgroups (*maniculatus, melanotis, leucopus*, and *gossypinus*) the SEBM and the inland oldfield mouse haplotypes were not reciprocally monophyletic (Fig. 2). The 50% majority rule phylogram indicates that the SEBM haplotypes are nested within the inland oldfield mouse haplotypes. Although, the nodal support values are very low for this clade.

Genetic structure (microsatellites)

An exact test of genetic differentiation (Raymond and Rousset 1995) revealed that allele frequencies were significantly different for all pairwise population comparisons. Generally, the level of differentiation for sampling localities within Cape Canaveral was slight (yet significant), with pairwise F_{ST} values ranging from 0.001 to 0.03 (Table 2). Pairwise F_{ST} values between Cape Canaveral and Smyrna Dunes Park populations were much larger (0.11–0.15). Pairwise F_{ST} values between Cape Canaveral and the oldfield mouse ranged from 0.11 to 0.13. The greatest genetic distance was observed between oldfield mouse and the Smyrna Dunes Park population of SEBM ($F_{ST} = 0.22$). A Mantel test showed that geographic distance and genetic differentiation (based on microsatellite data) were positively correlated (Fig. 3), implying some effect of geographic distance in genetically isolating populations.

The Bayesian admixture procedure implemented in STRUCTURE (Pritchard et al. 2000) showed large incremental increases in the likelihood [Pr($X|K$)] as the number of genetic clusters in the model increased from 1 to 4. Thereafter, there continued to be slight increases in the likelihood as the number of clusters used in the model increased to $K = 10$ (Fig. 4A). Following the method of Evanno et al. (2005), we determined the distribution of ΔK to have a strong modal value at $K = 2$ (Fig. 4A) indicating that the highest level of population structure exists between two genetic clusters. The membership coefficients of each individual in these clusters, along with the corresponding collecting localities, are shown in Fig. 4B. The separation of these two genetically-defined population-clusters clearly corresponds to the separation of the SEBM and the oldfield mouse. Furthermore, the continued large incremental increases in likelihood up to $K = 4$ suggest that secondary levels of structure exist below the level separating the SEBM and the oldfield mouse, within the SEBM. Allowing for an additional genetic cluster ($K = 3$) clearly separates the Smyrna Dunes population from the Cape Canaveral population of SEBM. Using a critical membership coefficient of 90% for inclusion in a cluster, the majority of SEBM individuals from New Smyrna and the inland oldfield mouse are assigned correctly. We found that 19 of 19 individuals genotyped from New Smyrna were included in the 'New Smyrna cluster' and 24 of 25 individuals genotyped from the inland oldfield mouse fall in the 'oldfield mouse cluster'. Adding this additional cluster causes the individuals genotyped from Cape Canaveral to show more mixed ancestry. At a 90% critical value, 19 of 231 individuals that were captured at Cape Canaveral were included in the 'New Smyrna cluster,' and only 45 of 231 individuals fall exclusively into the 'Cape Canaveral cluster.' The admixture seen at the 'Cape Canaveral cluster' is almost exclusively between the two populations of SEBM with 229 of 231 individuals having combined membership coefficients of greater than 90% in the

Table 2 Pairwise matrix of genetic distances F_{ST} (Weir and Cockerham 1984) below diagonal and geographic distances (km) above diagonal between all pairs of sampling localities

	CCG1	CCG2	CCG3	CCG4	CCG5	CCG6	CCG7	NS	LL
CCG1	–	1.65	8.53	4.86	2.67	2.34	17.10	78.33	114.61
CCG2	0.011	–	9.99	5.75	2.13	3.94	18.29	78.89	114.04
CCG3	0.004	0.008	–	5.10	9.21	6.94	8.90	71.33	117.77
CCG4	0.019	0.029	0.025	-	4.42	4.62	12.64	73.60	113.30
CCG5	0.009	0.014	0.009	0.016	–	4.61	17.02	77.12	112.52
CCG6	0.008	0.012	0.001	0.019	0.012	–	15.68	77.70	115.49
CCG7	0.018	0.028	0.008	0.032	0.017	0.017	-	62.57	114.83
SDP	0.136	0.108	0.107	0.15	0.109	0.108	0.114	–	106.11
LL	0.111	0.122	0.117	0.131	0.124	0.128	0.122	0.215	–

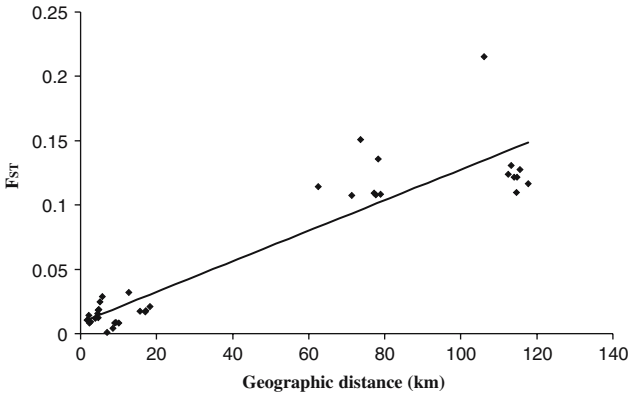

Fig. 3 Plot of F_{ST} values (based on microsatellite data) versus geographic distance among all sampling localities indicating evidence for isolation by distance. F_{ST} values are plotted against corresponding straight-line geographic distances (d) between sites. The equation of the best fit line (shown) is $F_{ST} = 0.0012d + 0.0086$ (Mantel Test, $r = 0.92$, one-sided $P = 0.006$ from 1000 randomizations)

two SEBM clusters (Fig. 4C). Adding a fourth genetic cluster ($K = 4$) increases the likelihood, but does not, however, separate individuals according to discrete sampling location (*i.e.*, at $K = 4$, two genetic clusters completely overlap geographically).

Discussion

Does *Peromyscus polionotus niveiventris* represent a distinct evolutionary lineage?

Our analysis comparing the SEBM and the oldfield mouse demonstrates significant divergence between these taxa based on both mitochondrial and microsatellite data. Analyses of mitochondrial cytochrome-*b* haplotypes suggest that the SEBM represents a monophyletic lineage, and shares no haplotypes with the oldfield mouse, although it is likely that unsampled haplotypes exist in the oldfield mouse, and these could correspond to SEBM haplotypes. The sequence divergence between haplotype groups representing these two taxa is low (0.3–1.0% Kimura two-parameter corrected pairwise divergence). This observed level of sequence divergence in the cytochrome-b gene is typical of intra-specific variation in other *Peromyscus* species, and is markedly lower than the typical level of divergence observed between sister-taxa (Bradley and Baker 2001, 2006).

The Bayesian admixture procedure implemented in STRUCTURE (Pritchard et al. 2000), based on microsatellite data, clearly separated the oldfield mouse from the SEBM (Fig. 4B). Using a 90% membership coefficient cutoff for inclusion in a cluster, all individuals of oldfield mice genotyped fall in the 'oldfield mouse cluster' ($n = 25$) and 247 of 250 individuals of SEBM fall into the 'SEBM cluster.' The remaining three individuals of SEBM could represent individuals sharing recent co-ancestry (i.e., within the last several generations) or this result could be the explained by genotyping errors (e.g., PCR contamination). Regardless of these three outliers, the STRUCTURE analysis demonstrates undeniable allelic differentiation between the two taxa. Estimates of heterozygosity and allelic diversity were significantly higher for the inland population of oldfield mouse, compared to the SEBM (Table 1). These trends of reduced genetic diversity in the range-restricted SEBM are consistent with founder effects and/or genetic drift in smaller populations of the SEBM.

Several studies have employed rates of molecular evolution to estimate divergence times in rodents (Smith and Patton 1993; Lessa and Cook 1998; Jaarola and Searle 2002; Brunhoff et al. 2003; Van Zant 2006). Incorporating the broadest consensus of evolutionary rate estimates across studies provides a range of 2–10% per Myr (Jaarola and Searle 2002, Brunhoff et al. 2003, Van Zant 2006). Applying this broad range of estimated evolutionary rates to our estimate of net nucleotide substitutions per site from the cytochrome-*b* data (Da = 0.23%) yields an estimated range of 23,000–115,000 years ago for the divergence between the SEBM and the inland oldfield mouse. While the absence of a single reliable taxon-specific mutation rate limits the precision of our estimates of time-since-divergence, applying this very broad range of potential rates clarifies the probable causes of isolation of these two taxa.

Florida's geology has changed dramatically in response to variable sea levels during the Pleistocene Epoch (1.8 MYA to 10,000 YA, Webb 1990). These dynamics have undoubtedly resulted in major changes in distribution of viable habitat for *P. polionotus*. Oldfield mice and beach mice inhabit sandy upland soils where water-tables are low enough year-round to support dry burrows several feet below ground (Gentry and Smith 1968). Throughout the Pleistocene, sea-levels rose and fell such that shorelines and dune systems on the Atlantic coast of the Florida peninsula dramatically advanced and receded on the order of hundreds of kilometers. During periodic glacial minima, the Florida peninsula was restricted to what is now the Lake Wales Ridge and associated uplands. During glacial maxima, eastern shores of the Florida peninsula were farther east, and the total land area of the Florida peninsula was much greater than at present (Webb 1990). Our data superimposed on the historical

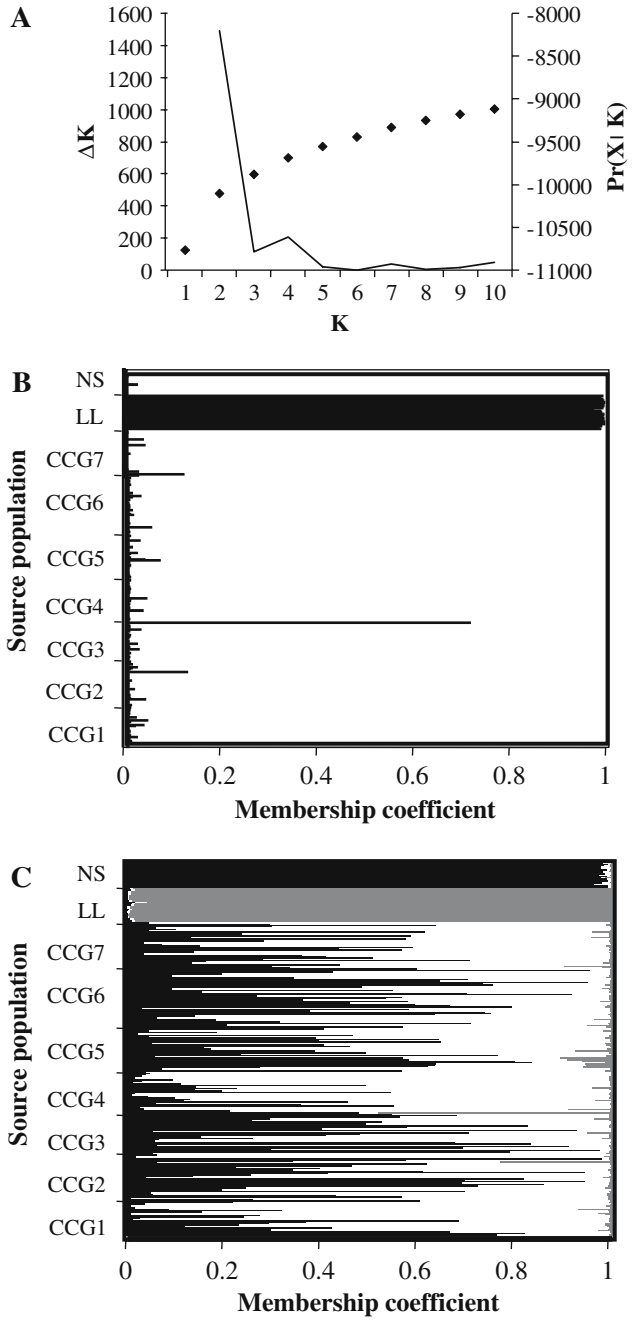

Fig. 4 The results of the analyses of population structure using the method of Pritchard et al. (2000), implemented in the program STRUCTURE. Data shown here represents the pooled results of 3–20 independent MCMC runs (see text for details). (**A**) Plot of the number of genetically discrete populations (K) versus the two optimality criteria: the raw average Ln likelihood indicated by diamonds and scaled to the right vertical axis, and ΔK (described in Evanno et al. 2005) indicated by solid line and scaled to the left vertical axis. The confidence intervals among 3–20 runs were too narrow to be visible if displayed graphically. (**B**) Estimated membership coefficients based on admixture analyses for $K = 2$ genetically defined populations for each individual sampled. Vertical axis labels indicate the source population of each individual plotted, and the horizontal axis indicates the membership coefficient in the 'oldfield mouse' genetic cluster. (**C**) Estimated membership coefficients based on admixture analyses for $K = 3$ genetically defined populations for each individual sampled. Vertical axis labels indicate the source population of each individual plotted, and the horizontal axis indicates the membership coefficient in each of the 'New Smyrna cluster' (black bars), 'oldfield mouse cluster' (grey bars), and 'Cape Canaveral cluster' (white bars)

biogeography of the region support a scenario in which the most recent Pleistocene fluctuations in sea levels caused the isolation of SEBM from ancestral inland populations. Perhaps an ancestral SEBM population was isolated on the Atlantic Coastal Ridge or on upland habitat islands such as exist today and has subsequently remained isolated, leading to the evolutionary lineage that is now known as *P. p. niveiventris*.

Our data indicate that the SEBM fits a majority of the criteria for classification as an ESU. This taxon shows strong nuclear allelic differentiation (compared to the inland oldfield mouse), and is monophyletic based on current mitochondrial DNA data. Further, the morphological distinctions by which the SEBM was originally defined (overall larger size and lighter coat color) is consistent with this taxon showing ecological non-exchangeability (see Crandall et al. 2000). Coat color, in particular, may be a specific adaptation for crypsis in coastal habitats (Hoekstra et al. 2006), and the differences observed in the SEBM may be indicative of a unique adaptive potential or evolutionary trajectory distinct from the inland oldfield mouse. Based on our limited sampling of *P. polionotus* populations, we can not thoroughly evaluate the reciprocal monophyly of the SEBM relative to other *P. polionotus* subspecies, although this work is currently underway (J. Van Zant, I. J. Stout, J. D. Roth, C.L. Parkinson, unpublished data). Our phylogenetic analyses suggest that the inland oldfield mouse and the SEBM are currently not reciprocally monophyletic, albeit with low nodal support. However, all the evidence taken together supports the recognition of SEBM as an ESU.

Genetic evidence for multiple conservation units within the SEBM

The STRUCTURE analyses conducted to determine the optimal number of genetically-definable populations clearly indicated that the highest level of genetic structure occurred between the SEBM and the oldfield mouse (Fig. 4A). Based on a model assuming two genetically-defined populations ($K = 2$), the vast majority of individuals had over 90% membership coefficient in either the SEBM cluster or the oldfield

mouse cluster (Fig. 4B). The STRUCTURE analyses also showed a large increase in likelihood going from $K = 2$ to $K = 3$ (Fig. 4) suggesting two genetically distinct clusters within the SEBM, one composed of individuals from Cape Canaveral, and one of individuals from Smyrna Dunes Park, although more individuals belonging to these clusters showed significant mixed membership between these two clusters (Fig. 4C). Pairwise F_{ST} values calculated from microsatellite data, however, showed similar levels of divergence between the two subspecies (SEBM and the inland oldfield mouse; $F_{ST} = 0.11–0.22$) compared to the divergence observed between populations of SEBM ($F_{ST} = 0.11–0.15$).

The STRUCTURE analysis using $K = 3$ assigned all individuals captured at New Smyrna to the 'New Smyrna' cluster. However, 19 individuals captured at Cape Canaveral were also assigned to this 'New Smyrna' cluster (Fig. 4C). One might take this mixed cluster as evidence that the New Smyrna population represents merely a subset of the genetic diversity seen at Cape Canaveral; however, each of these discrete populations of the SEBM contained unique (i.e., endemic) microsatellite alleles. Mitochondrial cytochrome-*b* haplotypes also appear to show different relative frequencies between the two populations, although our sampling of mitochondrial haplotypes is insufficient to precisely characterize these differences. Lower values of heterozygosity, haplotype diversity, and allelic richness observed in the smaller Smyrna Dunes Park population (relative to the Cape Canaveral population) may be causally related to the elevated impact of founder effects and/or drift in this smaller and isolated population. From a conservation perspective, we recommend that these two populations (Smyrna Dunes Park and Cape Canaveral) be managed separately in order to maintain local genetic diversity based on evidence of unique microsatellite alleles observed in both populations.

Conservation and management

In the Alabama beach mouse (*P. p. ammobates*), Swilling and Wooten (2002) found that 55% of mice remained philopatric, while 45% dispersed greater than one home range from their natal site, with the average dispersal distance being only ~160 m. This limited dispersal capability agrees with our demonstration that geographic distance is a contributing factor acting to isolate sub-populations of the SEBM within the Merritt Island complex, with F_{ST} increasing with geographic distance between sampling localities. To conserve the high levels of polymorphism observed within the SEBM Cape Canaveral population, habitat connectivity should be maintained so that individual populations of beach mice are not isolated and subjected to elevated levels of inbreeding and the increased effects of genetic drift.

Overall heterozygosity and allelic richness in the SEBM were significantly lower than values observed in our sample of the inland oldfield mouse. Additionally, the New Smyrna population of the SEBM showed significantly lower heterozygosity and allelic richness compared to the Cape Canaveral population. These findings suggest that genetic diversity is diminished in the SEBM (especially in the New Smyrna population). Higher levels of heterozygosity, allelic diversity, and numbers of alleles per locus suggest that the SEBM population inhabiting Cape Canaveral is larger (consistent with the geographic area) and more genetically diverse than the Smyrna Dunes Park population. These data, in turn, suggest that the Cape Canaveral population is exceedingly important for the sustained survival of the SEBM and would be the best candidate for a source population for potential reintroduction programs. Each smaller peripheral population, however, is important for maintaining the level of genetic diversity within this evolutionarily distinct subspecies. We found unique alleles in both New Smyrna Park and Cape Canaveral, as well as strong evidence for overall genetic differentiation between SEMB populations (based on STRUCTURE results), suggesting both populations contain endemic patterns of genetic diversity. Therefore we suggest treating these populations as two separate conservation management units while striving to conserving SEMB throughout its entire current range. Collectively, our results support the continued recognition of the SEBM as a unique taxon and the importance of its protection under the United States Endangered Species Act.

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