

Taxonomic status of the Davis Mountains cottontail, *Sylvilagus robustus*, revealed by amplified fragment length polymorphism

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The Davis Mountains cottontail, *Sylvilagus robustus*, is morphologically different from the eastern cottontail, *S. floridanus*, but previous genetic analysis of mitochondrial DNA data did not recover 2 genetically distinct groups. Our study used a nuclear DNA fingerprinting technique, amplified fragment length polymorphism (AFLP), to test the hypothesis that *S. robustus* is genetically distinct from *S. floridanus*. We tentatively considered any individual collected at an elevation >1,400 m as *S. robustus* and later confirmed our identifications with morphological or genetic data, or both. Principal component and discriminant function analyses of 6 previously published cranial measurements confirmed morphological distinctiveness. For genetic analyses we analyzed 273 AFLP fragments from 20 individuals of *S. robustus* and compared them to 16 *S. floridanus*, 4 *S. audubonii*, and 1 *S. obscurus*. Results from phylogenetic and population genetic analyses suggest a significant lack of gene flow between the 2 species. Together, these data support recognition of *S. robustus* as a separate species. DOI: 10.1644/09-MAMM-A-382.1.

Key words: amplified fragment length polymorphism (AFLP), Davis Mountains cottontail, incomplete lineage sorting, *Sylvilagus floridanus*, *Sylvilagus robustus*

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The Davis Mountains cottontail, *Sylvilagus robustus* (Bailey, 1905), is a rare, endemic species that inhabits piñon–oak–juniper woodlands at elevations above 1,400 m near the tops of various mountains from southeastern New Mexico to Northern Coahuila, Mexico, including the Guadalupe Mountains in New Mexico and Texas; the Davis, Chinati, and Chisos mountains in Texas; and the Sierra del Carmen and Sierra de la Madera in Coahuila, Mexico (Raun 1965; Schmidly 1977, 2004; Fig. 1; Appendix I). Initially, Bailey (1905) placed *robustus* as a subspecies of *Lepus pinetis* (= *Sylvilagus nuttallii*), based on specimens collected in Jeff Davis County, Texas, at an elevation of 1,800 m. This taxon was reassigned to the *S. floridanus* group and elevated to species level by Nelson (1909) without explicit justification. Furthermore, Bailey proposed that 2 other species of rabbits that occur at high elevations in the southwestern United States, *S. cognatus* and *S. f. holzneri*, were close relatives of *S. robustus*. Although their distributions are geographically discontinuous (Hall 1981), Nelson (1909) proposed that intergradation might occur. Based in part on a morphologically intermediate specimen from the Guadalupe Mountains, Hall and Kelson (1951) synonymized *S. robustus* with the eastern cottontail, *S. floridanus*. *S. floridanus* is widely distributed from southern Canada into central and northwest-

ern South America, and 34 subspecies, including *S. f. robustus*, have been described (Chapman et al. 1980). *S. f. robustus* has remained as a subspecies in most succeeding works (Hall 1951, 1981; Hall and Kelson 1959). However, Davis (1960) recognized *S. robustus* as a separate species, but Davis and Schmidly (1994) placed it back as a subspecies of *S. floridanus* based on nominal cranial differences.

These “nominal” differences were further investigated by Ruedas (1998), who analyzed 26 cranial and mandibular characters and differences in the shape of the 2nd and 3rd upper premolars (P2 and P3) and the 3rd lower premolar (p3) from *S. nuttallii pinetis* and 4 subspecies of *S. floridanus*, *S. f. chapmani*, *S. f. cognatus*, *S. f. holzneri*, and *S. f. robustus*. His principal component analysis based on continuous morphological characters showed a cluster of *S. f. robustus* separate from any of the other 3 *S. floridanus* subspecies. When Ruedas (1998) compared the group mean of the 26 measurements in separate univariate analyses of variance, individuals of *S. robustus* had a significantly larger greatest length of skull,



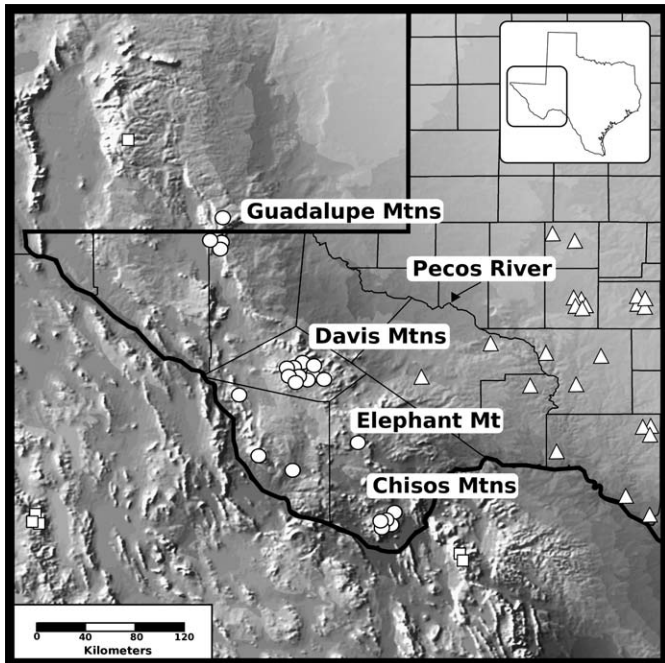


FIG. 1.—Specimen records of *Sylvilagus floridanus* and *S. robustus* in and around the Trans-Pecos region of Texas. Identifications were based on verified museum records (Appendix I). *S. floridanus* = triangles, *S. robustus* = circles, and unconfirmed specimens of *Sylvilagus floridanus/robustus* = squares. Map adapted from www.nationalatlas.gov.

condylopremaxillary length, breadth of rostrum, interbasiocapital length, width of auditory bullae, and mastoid breadth.

Ruedas (1998) also reported 3 discrete cranial characters that could be used to separate *S. f. robustus* and *S. f. chapmani*: *S. f. chapmani* had 1 basisphenoid foramen and a tympanic process, whereas *S. f. robustus* had 2 basisphenoid foramina and lacked a tympanic process. The mental foramen also was longer in *S. f. robustus* and located on the dorsal aspect of the mandible but was shorter and located on the labial aspect of the mandible in *S. f. chapmani*. Additionally, Ruedas (1998) reported that *S. f. robustus* and *S. f. chapmani* were distinguishable by discrete dental characteristics. On P2 the external anterior reentrant enamel fold was deeper in *S. f. robustus* than that of *S. floridanus*; and the main anterior reentrant was convoluted with only a thin layer of enamel in *S. f. robustus*, whereas *S. floridanus* exhibited a smooth main anterior reentrant with thick enamel.

Based on the compiled data from his morphometric analysis, Ruedas (1998) concluded that sufficient differences existed between *S. f. robustus* and the other *S. floridanus* subspecies to justify elevating *S. f. robustus* to species status. Schmidly (2004) and Hoffmann and Smith (2005) both followed this recommendation, although Schmidly (2004) indicated the need for a molecular approach to provide a more definitive answer to this vexing taxonomic question.

Vestal (2005) also compared the morphology between *S. robustus* and *S. floridanus* and used mitochondrial sequences of the control region and cytochrome *b* to address the question

of genetic divergence of the 2 species. Her morphological analysis of *S. robustus* and *S. f. chapmani* agreed with the work of Ruedas (1998) in that *S. robustus* had a larger mean value for the cranial measurements, but she found that the discrete cranial characters did not distinguish the 2 species reliably. Her divergence values between specimens of *S. robustus* and *S. f. chapmani* were low (1.64% cytochrome *b* and 3.7% control region), and reciprocal monophyly was not recovered in either gene (Vestal 2005).

A lack of reciprocal monophyly in a mitochondrial DNA (mtDNA) data set can have a number of causes, and thus the use of mtDNA alone may not be a reliable method for assessing species boundaries (Funk and Omland 2003; Rubinoff and Holland 2005). Studies have documented mtDNA introgression and pseudogenes in a variety of taxa (Bensch and Akesson 2005; Mendelson and Simons 2006; Meudt and Clarke 2007; Triant and DeWoody 2007), indicating that caution is warranted when basing conclusions on mtDNA data alone. To avoid these potential pitfalls data from the nuclear genome also should be used (Mendelson and Simons 2006). One such source of nuclear data, with high amounts of variation, is amplified fragment length polymorphisms (AFLP—Vos et al. 1995). This technique uses restriction enzymes to cut the entire genome into fragments that can be used as an anonymous multilocus DNA profile (Bensch and Akesson 2005).

Amplified fragment length polymorphism methods have been shown to give taxonomic discrimination across congeneric species and at finer evolutionary levels (Bensch and Akesson 2005; Egger et al. 2007; Ogden and Thorpe 2002; Sullivan et al. 2004). They therefore appear to constitute a very powerful technique for addressing questions of species boundaries (Kingston and Rosel 2004; McDonough et al. 2008). AFLPs are especially well suited for resolving closely related and recently diverged taxa because they use random fragments from the nuclear genome, which mostly consists of nonprotein coding regions and is therefore highly variable (Mendelson and Simons 2006; Meudt and Clarke 2007). The AFLP technique has a high level of reproducibility among samples run at separate times or even in separate laboratories (Savelkoul et al. 1999). AFLP markers had an overall reproducibility error rate of <2.5% when tests were repeated on new extracts from the same individuals (Bensch and Akesson 2005; Mian et al. 2002). Furthermore, the probability that bands are homologous is greater at lower taxonomic levels (Meudt and Clarke 2007; Robinson and Harris 1999). Nearly all sequenced AFLP fragments were homologous using the criterion of high sequence identity (>95% [Roupe van der Voort et al. 1997] and >99% [Mendelson and Shaw 2005]).

We hypothesized that AFLP could help resolve the incongruity between morphological and mtDNA data for delineating *S. robustus* and *S. floridanus*. Based on the morphological differences, *S. robustus* was predicted to be genetically distinct from *S. floridanus*. The objective of this study was to use AFLP data to determine if nuclear divergence exists between populations of *S. robustus* and *S. floridanus*.

MATERIALS AND METHODS

To refine the distribution map of Schmidly (2004) we verified the identifications of cottontail specimens labeled as *S. robustus*, *S. floridanus*, or *S. f. robustus* from in and around the Trans-Pecos region of Texas in the Angelo State Natural History Collection (ASNHC), at Sul Ross State University (SRSU), and records from museum collections located using MaNIS (manisnet.org) and ARCTOS (arctos.database.museum/home.cfm) searches as of May 2009. Specimens were verified subsequently using a combination of morphological measurements (Ruedas 1998; Schmidly 2004; Vestal 2005) and capture locality (Hall 1981). These verified records and the specimens examined by Schmidly (1977) were used for the distribution map (Fig. 1; Appendix I).

Taxonomic sampling.—A total of 55 individuals of *Sylvilagus* were used in morphological or AFLP analyses, or both: *S. audubonii* ($n = 4$), *S. floridanus* ($n = 25$), *S. obscurus* ($n = 1$), and *S. robustus* ($n = 25$; Appendix I). We tentatively considered any cottontail collected at an elevation $> 1,400$ m as *S. robustus* (Schmidly 1977; Schmidly 2004) and later confirmed our identification with morphological or genetic data, or both. Specimens were obtained from fieldwork and the following museum collections: ASNHC, SRSU, Museum of Southwestern Biology (MSB), and Museum of Texas Tech University, Natural Science Research Laboratory. In situations where animals were euthanized in the field, guidelines of the American Society of Mammalogists were followed (Gannon et al. 2007).

Tissues were sampled from 16 individuals of *S. floridanus* representing 5 subspecies, including *S. f. alacer*, *S. f. chapmani*, *S. f. llanensis*, *S. f. mearnsi*, and *S. f. orizabae* (Chapman et al. 1980). Tissues from 20 *S. robustus* were obtained from many locations in Jeff Davis and Brewster counties in Texas and from one individual from Eddy County in New Mexico. Members from 2 other recognized species, *Sylvilagus audubonii* (desert cottontail) and *Sylvilagus obscurus* (Appalachian cottontail), also were included to serve as outgroups for this study based on their close phylogenetic relationship to *S. floridanus* (Halanych and Robinson 1997).

Morphological analysis.—Standard measurements, including total length, tail length, hind-foot length, and ear length, were recorded from museum specimen tags when available: *S. robustus* ($n = 15$) and *S. floridanus* ($n = 9$; only from Texas localities, Appendix I). Some specimens were found as roadkills and had fragmented skulls, greatly reducing sample sizes for the remaining morphological analyses. Six cranial measurements, originally defined by White (1987) and again by Ruedas (1998), also were recorded if the characters were intact on the specimens: *S. robustus* ($n = 10$) and *S. floridanus* ($n = 9$). Cranial measurement included greatest length of skull, condylopremaxillary length, breadth of rostrum, interbasiooccipital length, width of auditory bulla, and mastoid breadth. The means from 3 replicate measurements were used for statistical analyses including a principal component analysis. Factor scores from the principal component analysis then were used in subsequent multivariate analyses because the direct measurements did not meet the assumptions of

TABLE 1.—*AseI* primers (5'-GATGAGTCCTGAGTAATNNN-35') used with *EcoRI* primer (5'-ACTGCGTACCAATTCAT-3') for amplified fragment length polymorphism analysis of *Sylvilagus* with the number of fragments scored and percentage of polymorphism for each primer.

Primer	No. fragments scored	% of fragments polymorphic
<i>AseI</i> -TGT	48	87.5
<i>AseI</i> -TGG	34	70.6
<i>AseI</i> -TGA	43	76.7
<i>AseI</i> -TAT	43	86.0
<i>AseI</i> -TAG	44	95.5
<i>AseI</i> -TCTG	29	69.0
<i>AseI</i> -TCAG	15	80.0

normality. Multivariate analysis of variance (MANOVA) was used to test the difference in the centroid means of the 2 clusters. Discriminant function analysis with jackknife (Lance et al. 2000) was used to determine how often the individuals could be classified into the groups correctly. All statistical analyses were performed in SYSTAT version 11.0 (SYSTAT Software, Inc., San Jose, California). Three discrete characters described by Ruedas (1998), number of basisphenoid foramina, presence or absence of a tympanic process, and the shape and location of the mental foramen, were evaluated if they were intact (*S. robustus*: $n = 19$ or 20 ; *S. floridanus*: $n = 14$ or 15). All individuals with a total length less than 375 mm were considered juvenile in age and were excluded from the analysis because Hall (1981) reports the range in total length for *S. floridanus* to be 375–463 mm.

Amplified fragment length polymorphism (AFLP) analysis.—Whole genomic DNA was isolated from either frozen liver or muscle tissue, liver stored in lysis buffer, or liver in 95% ethanol, using a DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) following the manufacturer's protocol. The DNA then was diluted to approximately 50 ng/ μ l by adding sterile TE (0.01 M Tris Base and 0.001 M ethylenediaminetetraacetic acid, pH 8.0).

The AFLP procedure followed Vos et al. (1995) as modified by Phillips et al. (2007). Samples (200 ng) were digested with *EcoRI* and *AseI* (New England BioLabs, Ipswich, Massachusetts) and ligated to appropriate adapters. One of the 2 *AseI* adaptor sequences was written erroneously in table 1 of Phillips et al. (2007). The correct *AseI* adaptor sequences are: 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'. Diluted ligation products were used to amplify fragments preselectively using *EcoRI*-C and *AseI*-T primers (Table 1). The number of fragments was further reduced by selective polymerase chain reaction amplification using the primer combinations *EcoRI*-CAT and *AseI*-TNN or *AseI*-TNNN (where N represents any base; Table 1). The reaction products were separated on a Beckman-Coulter CEQ8000 DNA analysis system (Beckman-Coulter, Inc., Fullerton, California).

Initially, only *AseI*- +3-base pairs (bp) primers were used because studies have shown that some +2-bp primers produced too many bands to score and some +4-bp primers did not generate sufficient polymorphisms (Robinson and Harris

TABLE 2.—Descriptive statistics for body measurements of *Sylvilagus robustus* and *S. floridanus*.

Measurement (length in mm)	<i>S. robustus</i> (n = 15)			<i>S. floridanus</i> (n = 9)		
	\bar{X}	SD	Range	\bar{X}	SD	Range
Total	425	25	381–471	404	13	382–420
Tail	55	10	30–71	48	7	40–60
Hind foot	101	4	93–106	94	5	83–102
Ear	74	4	68–86	60	2	57–64

1999). However, in situations where +3-bp primers resulted in an excess of fragments of what could be accurately scored, we also used selective primers with a 4-base extension. To generate at least 200 polymorphic fragments, as recommended by Bonin et al. (2007), we used 7 primer combinations (having both +3-bp and +4-bp selective nucleotides; Table 1).

Beckman-Coulter CEQ8000 DNA analysis system software, version 9.0 (Beckman-Coulter, Inc., Fullerton, California) sized the fragments produced by the AFLP method to an internal 400-bp size standard and assigned fragments to 1-bp size bins for ease of scoring. All binned fragments then were evaluated by eye, and only unambiguous fragments were included in the data set (Bonin et al. 2005). Scoring consisted of evaluating a fragment for presence (1) or absence (0). All individuals were scored in random order to avoid bias.

Population analysis.—From the binary data matrix a genetic distance similarity matrix was generated using GenAlEx version 6.1 software (Peakall and Smouse 2006). Patterns of divergence among all species were visualized with a principal coordinate analysis by plotting the first 2 eigenvectors using SYSTAT version 11.0 (SYSTAT Software, Inc., San Jose, California). A separate analysis including only *S. robustus* and *S. floridanus* was used to examine the separation among populations without having the presence of outgroups affect the analysis. This subset of the data also was used in an analysis of molecular variance (AMOVA) in GenAlEx version 6.1 to compare the variation between populations to the variation within populations. Typically F_{ST} values are reported as a measure of interpopulation genetic differentiation; however, Andrade et al. (2007) recommend using Φ_{PT} , which is analogous to F_{ST} , but is more appropriate for a binary AFLP data set. Φ_{PT} values were calculated ($\alpha = 0.05$) in GenAlEx version 6.1 with 1,000 permutations. The program AFLP-SURV version 1.0 (Vekemans 2002) was used to convert the binary data matrix (excluding outgroups) to a genetic distance matrix, using Nei–Li gene divergence index, to calculate an F_{ST} value ($\alpha = 0.05$) with 1,000 permutations (Nei and Li 1979). The raw data matrix also was used in the program STRUCTURE version 2.2 (Falush et al. 2003, 2007; Pritchard et al. 2000) to estimate the highest degree of genetic structure between the populations. In STRUCTURE, 5 independent runs were performed for each assumed number of populations (K), 1–5. The “no admixture” model was used, and length of the burn-in period was set at 30,000 followed by 100,000 iterations. The modal value ΔK was calculated using the average log likelihoods as an estimate of the number of

TABLE 3.—Descriptive statistics for cranial measurements of *Sylvilagus robustus* and *S. floridanus*. Abbreviations are as follows: greatest length of skull (GLS), condylopremaxillary length (CONDL), breadth of rostrum (BROSTR), interbasiooccipital length (INTBOC), width of auditory bulla (WIDBULL), and mastoid breadth (MASTOID).

Measurement (mm)	<i>S. robustus</i> (n = 10)			<i>S. floridanus</i> (n = 9)		
	\bar{X}	SD	Range	\bar{X}	SD	Range
GLS	75.03	1.31	72.94–76.91	70.26	2.70	66.96–74.88
CONDL	66.57	0.59	65.72–67.71	61.57	2.39	58.86–65.61
BROSTR	20.35	0.79	19.2–21.71	19.17	0.79	18.06–20.06
INTBOC	20.75	0.85	19.80–21.62	19.50	1.33	17.48–21.98
WIDBULL	11.05	0.44	10.04–11.53	10.08	0.57	9.23–10.83
MASTOID	31.99	1.18	30.23–34.41	29.25	0.61	28.20–30.27

populations. Evanno et al. (2005) found that this value best detected the real number of groups in their simulations. However, ΔK cannot find the best K if $K = 1$, therefore we used the $L(K)$ to evaluate $K = 1$.

Phylogenetic analysis.—The original binary matrix including all species was used to construct phylograms using neighbor-joining (Saitou and Nei 1987) and parsimony analyses as implemented in PAUP* version 4.0b10 software (Swofford 2001). Some have questioned whether parsimony is an appropriate method for use with AFLP data (Robinson and Harris 1999), but because no consensus exists on which is the most appropriate and effective method, we used both neighbor-joining and parsimony (Sullivan et al. 2004). All fragments were weighted equally, and both phylograms were rooted with *S. audubonii* and *S. obscurus*. Statistical support for both phylogenetic analyses was provided by 1,000 bootstrap pseudoreplicates, and only statistically significant values (>70%) were reported (Felsenstein 1985; Hillis and Bull 1993). Finally, inter- and intraspecific Nei–Li genetic distances were calculated in PAUP* (Swofford 2001) and averaged.

RESULTS

Morphology.—External measurements for *S. robustus* ($n = 15$) were larger than for *S. floridanus* ($n = 9$) for each measurement (Table 2). The average for each cranial measurement was larger in *S. robustus* than in *S. floridanus*, but overlap was observed in the ranges for each character except for the condylopremaxillary length (Table 3). Based on a plot of the first 2 axes of the principal component analysis (Fig. 2), *S. robustus* and *S. floridanus* formed 2 clusters. MANOVA revealed a significant difference between the 2 a priori groups (Wilks' lambda = 0.211, $F_{5,13} = 9.743$, $P < 0.001$). The discriminant function analysis correctly identified 100% of the individuals. When jackknife was applied, 84% of the individuals were identified correctly. The discrete cranial characters (number of basisphenoid foramina, tympanic process, and mental foramen shape) did not separate *S. robustus* from *S. floridanus*. Only about one-half of the specimens of *S. floridanus* had all *floridanus*-like characters,

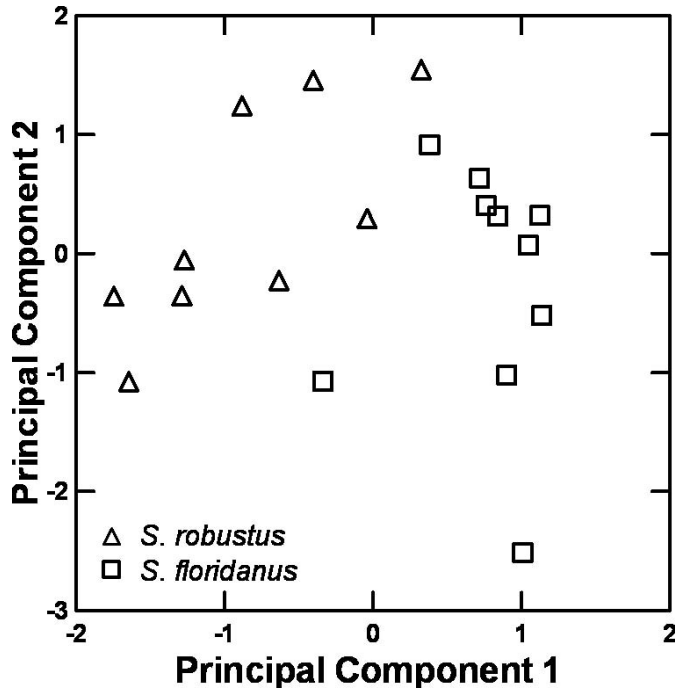


FIG. 2.—Principal component analysis (PCA) of *Sylvilagus robustus* ($n = 10$) and *S. floridanus* ($n = 9$) based on 6 cranial measurements.

and none of the specimens of *S. robustus* had all *robustus*-like characters. Of the individuals of *S. robustus* examined, 2 of 19 were *robustus*-like for the basisphenoid character, 15 of 19 were *robustus*-like for the tympanic process character, and 14 of 20 were *robustus*-like for the mental foramen character. Of the individuals of *S. floridanus*, 14 of 15 were *floridanus*-like for the basisphenoid character, 13 of 14 were *floridanus*-like for the tympanic process character, and only 8 of 15 were *floridanus*-like for the mental foramen character.

Amplified fragment length polymorphism (AFLP).—A total of 7 different primer combinations were used to score 273 AFLP fragments (including outgroup taxa), of which 83.9% were polymorphic (Table 1). In the principal coordinate analysis 4 distinct clusters of individuals emerged, consistent with their initial identifications (Fig. 3A). These results confirm the 1st record (TK78740) of *S. robustus* from Elephant Mountain. When the 2 outgroup species were removed, 230 AFLP fragments (68.3% polymorphic) were used to generate a 2nd principal coordinate analysis (Fig. 3B). In this reduced data set clear separation of *S. robustus* from *S. floridanus* still was apparent. We observed no evidence of divergence among the different mountain populations of *S. robustus* because in no instance did all individuals from a single mountain range form a distinct group.

The AMOVA indicated that 72% of the total genetic diversity resided among individuals within populations and 28% between *S. robustus* and *S. floridanus*. Estimated population divergence using both statistics, $\Phi_{PT} = 0.278$ ($P = 0.001$) and $F_{ST} = 0.1728$ ($P = 0.000$), suggested significant lack of gene flow between *S. robustus* and *S. floridanus*. When only *S. robustus* and *S. floridanus* were used in STRUCTURE, the clustering

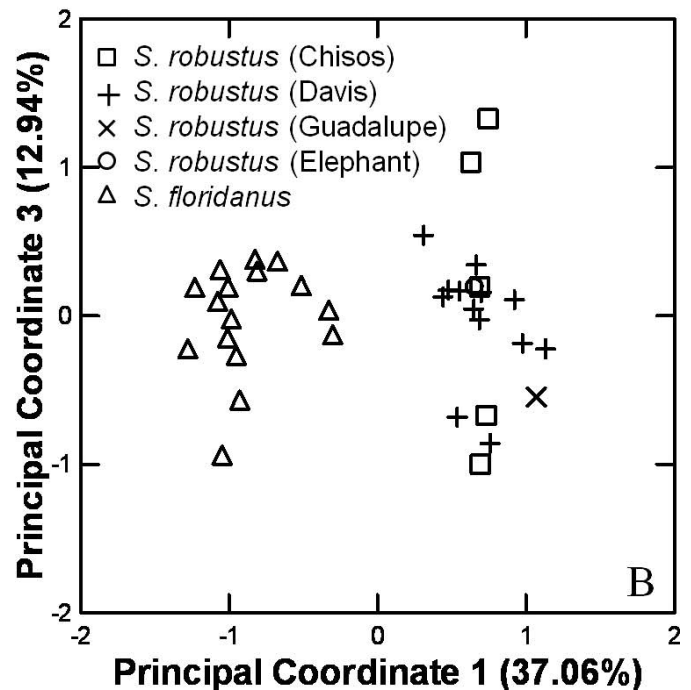
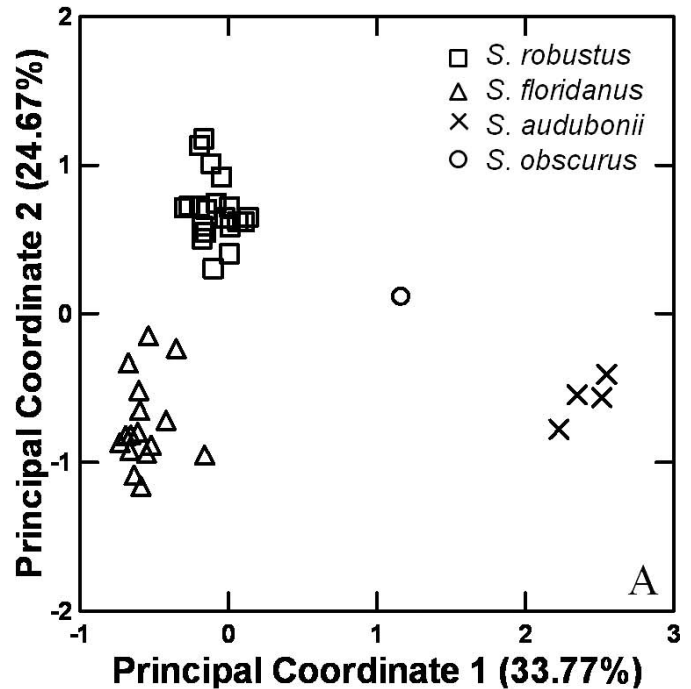


FIG. 3.—Principal coordinate analyses (PCoAs) based on amplified fragment length polymorphism (AFLP) data. A) Four species of *Sylvilagus* based on 273 AFLP fragments. B) All *S. floridanus* and *S. robustus* from each of the mountain ranges based on 230 AFLP fragments. Numbers in parentheses refer to the percentage of variance explained by the principal coordinates. The 1st and 3rd axes are shown, although the 1st and 2nd explained a similar amount of variation (57.43%).

program suggested either 2 ($P(K|X) = 0.46$) or 3 clusters ($P(K|X) = 0.54$). The 3rd cluster consisted of 2 of the 5 *S. robustus* from the Chisos Mountains (ASK 6332 and ASK 6334—Lee 2009). The modal value of ΔK was 2, indicating

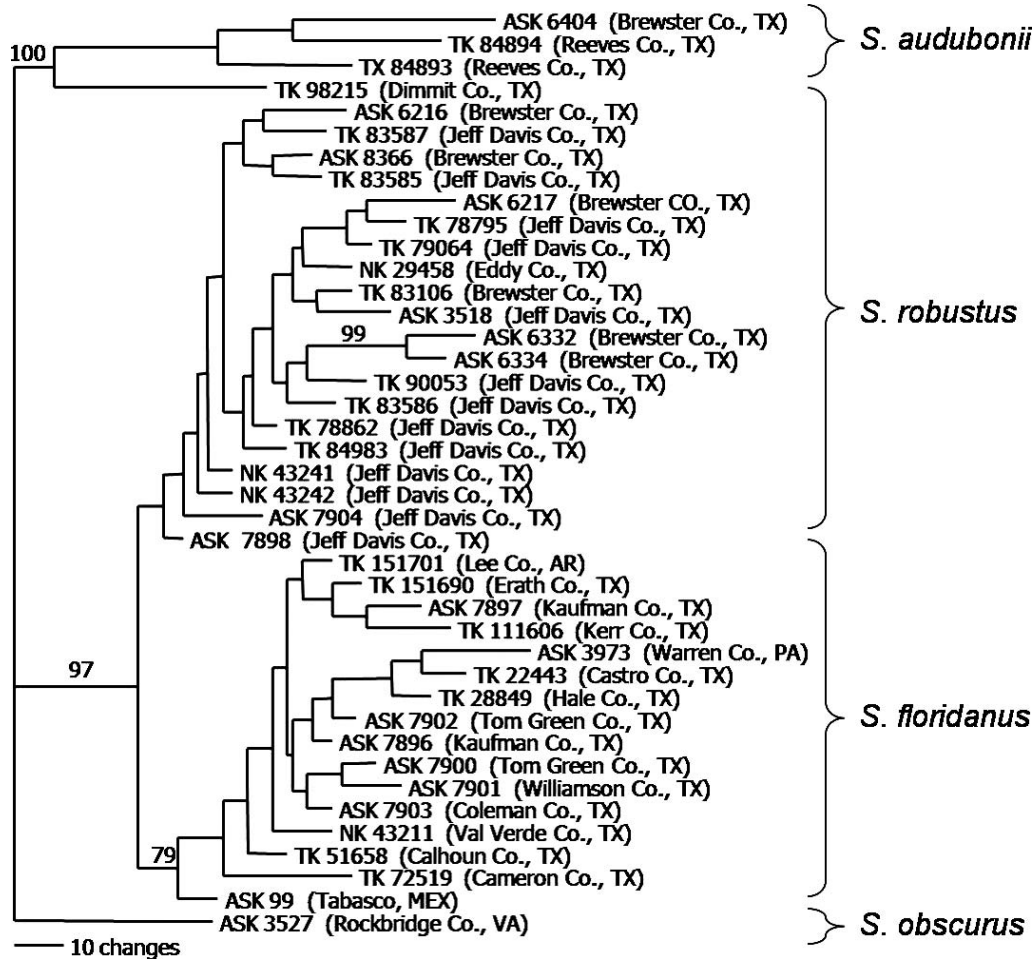


FIG. 4.—Parsimony phylogram of 37 individuals of *Sylvilagus* using 175 parsimony-informative amplified fragment length polymorphism fragments. Numbers above the nodes are bootstrap values > 70% provided by 1,000 bootstrap replicates.

that 2 populations best explained the data. None of the criteria for estimating *K* suggested that *K* = 1.

When either the distance or parsimony criterion was used, analysis of AFLP fragments produced reciprocally monophyletic groups of *S. robustus* and *S. floridanus*. The *S. floridanus* cluster was supported by a significant bootstrap value (79%). However, the monophyly of *S. robustus* was not strongly supported (<50%). Although the relationships among individuals at the tips of the clusters differed in neighbor-joining and parsimony analyses, they did not do so significantly (Fig. 4). As in the principal coordinate analysis, no divergence was observed among *S. robustus* representing different mountain populations. Two of the 5 *S. robustus* from the Chisos Mountains (ASK 6332 and ASK 6334) had high bootstrap support in both analyses, not surprising considering these individuals formed the 3rd cluster suggested by the STRUCTURE analysis. In both the neighbor-joining and parsimony analyses *S. f. orizabae* from Tabasco, Mexico (ASK 99), was the most basal within the *S. floridanus* group, but this did not have high bootstrap support. The average Nei–Li genetic distance between *S. floridanus* and *S. robustus* (2.42%) was greater than the distance within each species (1.69% and 1.71%; Table 4).

DISCUSSION

de Queiroz (1998, 2007) has proposed a unification of species concepts that separates the theoretical concept of species from various operational criteria used for recognizing them. In his view species are separately evolving metapopulation lineages that can be recognized by 1 or more secondary properties including reproductive isolation, phenetic distinctiveness, and reciprocal monophyly of mitochondrial and nuclear data sets. These secondary properties then become lines of evidence for lineage separation rather than defining properties of species. For recent studies of mammalian diversity (Reeder et al. 2007), degree of genetic differentia-

TABLE 4.—Average Nei–Li genetic distances (%) between *Sylvilagus* species and within each species based on 273 amplified fragment length polymorphism fragments.

	<i>S. audubonii</i> (n = 4)	<i>S. floridanus</i> (n = 16)	<i>S. robustus</i> (n = 20)	<i>S. obscurus</i> (n = 1)
<i>S. audubonii</i>	2.34			
<i>S. floridanus</i>	5.28	1.71		
<i>S. robustus</i>	4.93	2.42	1.69	
<i>S. obscurus</i>	5.64	5.31	4.85	0.0

tion, especially at the cytochrome-*b* locus (Baker and Bradley 2006; Bradley and Baker 2001), has been used as an indicator of lineage separation. The greater the time since 2 lineages have been separated, the more secondary properties one will observe. Thus, species in early stages of taxonomic divergence will not exhibit all secondary properties. The more lines of evidence in support of lineage separation, the more confident one is in declaring populations to be distinct species. In situations of allopatric distributions of sister taxa, researchers have been using this approach for defining species for many years and continue to do so (Baker et al. 2009; de Queiroz 2007). The populations of cottontails in our study exhibit at least 2 of these secondary properties, reciprocal monophyly of nuclear AFLP data and morphological distinction. Wiens (2004) emphasized the role that ecological factors can play in allopatric speciation. Although different ecological requirements of *S. robustus* and *S. floridanus* have been suggested (Nelson 1909; Ruedas 1998; Schmidly 1977), they have not yet been demonstrated clearly because the 2 species can be separated geographically by a shared intolerance of desert habitats rather than each having unique preferences.

We propose that the taxonomic status of *S. robustus* be retained at the species level. Average genetic distances (based on AFLP data) between *S. robustus* and *S. floridanus* (2.42%) were lower than comparisons between all other *Sylvilagus* species pairs (4.84–5.64%), indicating that they have diverged more recently. The divergence we observed between *S. robustus* and *S. floridanus* was only marginally higher than that seen within *S. audubonii*.

Morphologically, *S. robustus* could be distinguished from *S. floridanus* using a principal component analysis of the cranial measurements (Fig. 2), as also proposed by Ruedas (1998), and the discriminant function analysis showed individuals can be placed in the correct groups despite the relatively low level of genetic divergence. Although the ranges for most measurements overlapped (Table 3), averages for each of these 6 measurements in *S. robustus* were larger than in *S. floridanus*, which agrees with results from Ruedas (1998) and Vestal (2005). However, as Vestal (2005) and this study concluded, the discrete cranial characters of Ruedas (1998) were found to be too variable to identify *S. robustus* and *S. floridanus* reliably. Additional morphological distinctiveness was shown by Debelica and Thies (2009) who describe identifying characteristics of the hair that can distinguish *S. robustus* and *S. floridanus* reliably.

Although reciprocal monophyly was not observed in the mitochondrial data set of Vestal (2005), our STRUCTURE analysis described 2 populations, and phylogenetic analyses of AFLP data indicated the presence of 2 monophyletic lineages (Lee 2009; Fig. 4) corresponding to *S. robustus* and *S. floridanus*. Incongruence of mitochondrial and nuclear AFLP data is consistent with examples of isolation by adaptation, as reviewed by Nosil et al. (2009). Elevated nuclear diversification that is not mirrored by mitochondrial divergence is emerging as a common pattern in many taxa. Nosil et al. (2009) described a variety of models to explain the

mechanisms that could yield genomic differentiation that occurs faster than neutral expectations. One possibility is that divergent selection at a particular locus could extend to linked regions termed “genomic islands,” and these regions may grow by allopatric, ecological, or structural models. At this point it is impossible to determine how the variation recovered by AFLP is distributed across the genome. Future work is necessary to determine the likelihood that isolation by adaptation is responsible for the patterns observed in *Sylvilagus*. Other possible explanations for the absence of mitochondrial reciprocal monophyly are mitochondrial introgression, incomplete lineage sorting, or amplification of a nuclear mitochondrial insert (Funk and Omland 2003).

Mitochondrial introgression (or mitochondrial capture—Weckstein et al. 2001) could have resulted from historical interbreeding between *S. robustus* and *S. floridanus*. However, if this occurred, it must have been followed by complete replacement of the mitochondrial genome of 1 species by that of the other because of the absence of 2 distinct sets of haplotypes (representing the divergent haplotypes of the 2 species prior to introgression) within either *S. robustus* or *S. floridanus*. For example, complete replacement has been documented in hybridizing *Geomys* (Jones et al. 1995).

Lack of mtDNA monophyly also can be caused by incomplete lineage sorting (Avice et al. 1990). All recently diverged population lineages pass through a stage where the stochastic loss of haplotype lineages by genetic drift has not yet occurred. Although it is expected that mtDNA will reach reciprocal monophyly prior to nuclear DNA because of the lower effective population size for mtDNA, this might be true only when comparing single locus sequences (or a small subset of loci). The large numbers of polymorphic nuclear markers (such as can be generated using AFLP or microsatellites) can reveal monophyletic clades within 10,000–20,000 years during which a population is isolated (Albertson et al. 1999; Goldstein et al. 1999; Nosil et al. 2009). Isolation of populations of *Sylvilagus* in Trans-Pecos Texas might have occurred following the latest Wisconsin pluvial as recently as 11,500 years ago (Wells 1977), resulting in retention of ancestral polymorphism in the mitochondrial data set.

Nuclear mitochondrial inserts (Numts) are copies of mtDNA that have been inserted into the nuclear genome (Bensasson et al. 2001). Numts typically are slower evolving and thus will reach reciprocal monophyly later than their mitochondrial counterparts. Preferential amplification of Numts over mtDNA is possible and could go unnoticed in the absence of frameshift mutations and stop codons. If Vestal et al. (2005) amplified a slower-evolving Numt, and true mtDNA haplotypes from *S. robustus* and *S. floridanus* are divergent, preferential amplification of the Numt must have occurred because divergent haplotypes were not observed as would be expected if both the mtDNA and Numt DNA were coamplified and sequenced. Although the likelihood of this is probably quite low, it cannot be discounted.

As the forested lowlands in the Trans-Pecos of Texas became drier beginning approximately 10,000 years ago,

many mammals in this region probably went locally extinct or survived in refuges at higher elevations (Schmidly 1977; Stangl et al. 1994; Wells 1977). In the case of *Sylvilagus* it is possible that the associated habitat changes, especially in the lower-elevation Pecos Plain region (Schmidly 1977), separated populations of the common ancestor of *S. floridanus* and *S. robustus*. Populations in the more-mesic habitats to the east would have been subject to different evolutionary fates than those that survived at higher elevations in the west. Another result of these vegetation changes could have been the isolation of populations in the high-elevation habitat fragments. Some species, such as the southern red-backed vole (*Myodes gapperi*) in the southern Appalachians (Browne and Ferree 2007) and the Mexican spotted owl (*Strix occidentalis*) in southeastern Arizona (Barrowclough et al. 2006), also exist in high-elevation fragmented habitats or “sky islands,” but have significant genetic structure. Our AFLP analyses of the putative sky island populations of *S. robustus* (Davis, Chisos, and Guadalupe mountains) do not reveal genetic structure, indicating that these populations are either not truly isolated (with gene flow currently among them) or that insufficient time has elapsed since isolation for the effects of genetic drift or selection to be detected by examination of AFLP data. We suspect the latter because neither *S. floridanus* nor *S. robustus* are known from the desert lowlands surrounding the high-elevation sites. However, as recently as 1,000–2,500 years ago the piñon–juniper woodland habitats, which may be preferred by *S. robustus*, are thought to have been more extensive (Bryant 1977), creating the potential for gene flow. Thus, it seems probable that the mountain populations of *S. robustus* became isolated only very recently relative to the separation of *S. floridanus* and *S. robustus*.

Although determination of the current distribution of populations of *S. robustus* was not a primary objective of this study, through our genetic analyses of vouchered museum specimens and field collection we confirmed that they remain present in the Davis Mountains and Guadalupe Mountains and are not extirpated from the Chisos Mountains as suggested by Ruedas (1998). In addition, our study is the 1st to confirm the presence of *S. robustus* at Elephant Mountain (Brewster County). Records exist for *S. robustus* in the Sierra de la Madera from Coahuila, Mexico (Baker 1956—reported as *S. f. nelsoni*), and a population apparently still exists there (C. Jones, Texas Tech University, pers. comm.), but specimens were not available to include in this analysis. We know of no new specimens from the Chinati Mountains (Presidio County, Texas) since 1948; thus the current status of potential populations of *S. robustus* in the Chinati Mountains remains unresolved. Based on our verification of museum records, we located only 4 sites from west of the Pecos River where *S. floridanus* occurs (Pecos and Terrell counties). We found no evidence of sympatry or syntopy between *S. robustus* and *S. floridanus*. The closest verified occurrence of the 2 species is 80 km between *S. robustus* at Elephant Mountain and *S. floridanus* 39 km southwest of Fort Stockton.

In conclusion, examination of nuclear AFLP data supports the hypothesis that gene flow is not occurring between *S.*

robustus and *S. f. chapmani*. Therefore, these findings further corroborate the designation of *S. robustus* as a distinct species. A complete understanding of the evolution of *S. robustus* will require examination of its relationship to 2 other rabbits that occur on mountains in the southwestern United States, *S. cognatus* and *S. f. holzneri*. It also will be important for future studies to document the current distributional limits of both *S. robustus* and *S. floridanus* to verify the apparent allopatric distribution of these 2 species.

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APPENDIX I

Species, locality, tissue, and catalog number for specimens used in the morphological analysis (M), principal component analysis (P), and amplified fragment length polymorphism analysis (A), or to plot species occurrence in Fig. 1 (D). Specimens from the following institutions are included: University of Michigan Museum of Zoology (UMMZ); Museum of Vertebrate Zoology, University of California at Berkeley (MVZ); Louisiana State University Museum of Zoology (LSUMZ); United States National Museum of Natural History (USNM); Texas Cooperative Wildlife Collection, Texas A&M University (TCWC); Museum of Natural History, University of Kansas (KU); Sul Ross State University (SRSU); Museum of Southwestern Biology, University of New Mexico (MSB, NK); Natural Science Research Laboratory, Texas Tech University (TTU, TK); and the Angelo State Natural History Collections (ASNHC, ASK).

Sylvilagus audubonii.—USA: Texas; Brewster Co., Big Bend National Park, 5 mi N Persimmon Gap on 385 ASNHC13518 ASK6404 (A). Dimmit Co., Chaparral Wildlife Management Area TTU98010 TK98215 (A). Reeves Co., Sandia Springs (MLP-14) TTU81105 TK84893 (A), TTU81106 TK84894 (A).

Sylvilagus floridanus.—Mexico: Zacatecas; Tabasco, 3.8 km SW Ruinas Acalan ASNHC2330 ASK99 (A). USA, Arkansas; Lee Co., 3.5 mi S Marianna TK151701 (A). Pennsylvania; Warren Co., Spring Creek, Cemetery Rd. ASNHC10465 ASK3973 (A). Texas: Calhoun Co., Guadalupe Delta Wildlife Management Area 14R 3155306 175241 TTU75189 TK51658 (P, A). Cameron Co., Las Palomas Wildlife Management Area, Longoria East Unit TTU77168 TK72519 (A). Castro Co., Glen Merrit Farm, Playa Lake TTU40768 TK22443 (A). Coleman Co., US HWY 67, 4 mi W of Coleman County Line, 31.79943°N 99.78810°W, 536 m ASNHC13509 ASK7903 (A). Concho Co., 4.5 mi S, 11 mi W Paint Rock ASNHC10646

ASK4579 (M, P). *Coryell Co.*, Fort Hood Military Installation Training Area 53, 14R 616071 3469130 ASNHC9601 ASK3897 (M, P). *Crockett Co.*, 5 mi S, 5 mi E Iraan TTU58331 (D); 7.1 mi N, 1.1 mi E Ozona ASNHC10447 (D); 25 mi SE Ozona, J. Baggett Ranch TCWC22790–22792 (D). *Erath Co.*, 5 mi S Stephenville TK151690 (A). *Glasscock Co.*, 7 mi N Garden City, Carter Ranch ASNHC662 (D); 9 mi S Stanton, Thomasson's Ranch TTU2091 (D). *Hale Co.*, 15–20 mi NNW of Lubbock near Cotton Center on FR2528 TTU43218 TK28849 (A). *Irion Co.*, 1.4 mi E Ranch Road 72, US 67 ASNHC9489 (D); 11 mi N, 2 mi W Mertzon ASNHC7911 (D); 11.4 mi N, 4 mi W Mertzon, RR411 ASNHC12841 (D); 11.5 mi N, 20 mi W Mertzon ASNHC7909, 7910 (D); 2.6 mi N, 11.1 mi W Mertzon, Big Hollow Drainage ASNHC9487, 12845 (D); 3 mi N, 9 mi W Mertzon ASNHC12842–12844 (D), ASNHC12859 (D); 3.0 mi W Mertzon ASNHC12846 (D); 4.8 mi, N, 1 mi W Mertzon, Lopez Creek Drainage ASNHC9478 (D, M, P) ASNHC9479–9485 (D), ASNHC10455 (D); 5 mi N, 2.3 mi W Mertzon, Lopez Creek drainage ASNHC9475 (D); 5.4 mi N, 13 mi W Mertzon ASNHC9488 (D). *Kaufman Co.*, 2 mi E of Prairieville ASNHC13510 ASK7896 (P, A), ASNHC13511 ASK7897 (P, A). *Kerr Co.*, Kerr Wildlife Management Area 14R 0452122 3330856 TTU98381 TK111606 (A). *Pecos Co.*: 23 mi SW McCamey, McKensy Ranch ASNHC925, 926, 928, 929, 931–934, (D); 24 mi SW Ft. Stockton ASNHC935 (D, M), ASNHC936–939 (D), ASNHC940 (D, M). *Terrell Co.*, 31 mi N, 14 mi E Dryden ASNHC10448 (D); 20 mi S of Sheffield TTU93424 (D). *Tom Green County*, 5 mi N of San Angelo, ASU farm ASNHC867 (M, P); US HWY 67 W of Twin Buttes, 31.38877°N 100.57523°W, 606 m ASNHC13512 ASK7900 (A); US HWY 67 W of Twin Buttes, 31.39836°N 100.57523°W, 613 m ASNHC13513 ASK7902 (P, A). *Val Verde Co.*: 10 mi SE Del Rio on RT 227 28199MSB (D); Devil's River State Natural Area ASNHC10792–10795 (D), ASNHC11050, 11051 (D), ASNHC11052 ASK4951 (D, M, P), ASNHC11053 ASK4947 (D, M), ASNHC11062 (M), ASNHC11063–11065 (D); Long Point, Amistad National Research Area TCWC31151, 31152 (D); 29°53.771N 101°44.430W MSB85714 (D), MSB85715 NK43211 (D, A). *Williamson Co.*, 10 mi E Georgetown, 30.63772°N 101.537°W, 613 m ASNHC13514 ASK7901 (A).

Sylvilagus obscurus.—USA: *Virginia*; *Rockbridge Co.*, Goshen Wildlife Management Area ASK3527 (A).

Sylvilagus robustus.—USA, *New Mexico*, *Eddy Co.*: Guadalupe Mts., Dark Canyon, 32°5.901N 104°45.711W MSB89015 NK29458 (D, A). *Texas*, *Brewster Co.*, Big Bend National Park, Chisos Mts. ASNHC12239 (D), USNM108695 (D); Big Bend National Park, Basin Road, mile marker 2 ASNHC12937 (D); Big Bend National Park, Basin Road, mile marker 3 ASNHC12935 (D); Big Bend National Park, Basin Road, mile marker 4 ASNHC12936 ASK6332 (D, M, P, A); Big Bend National Park, Basin Road, 2.5 mi N of bear crossing sign near 4.5 mi mark on Rt. 16 ASK6334 (D, A); Big Bend National Park, Boot Springs, 2,072 m TCWC6042 (D); Big Bend National Park, Cherokee Springs, Pinnacle Mts. TCWC3654 (D); Big Bend National Park, Chisos Basin Campground Turnoff ASNHC12939 (D); Big Bend National Park, Emory Peak, 2,286 m

TCWC3657 (D); Big Bend National Park, Green Gulch, 1,524 m TCWC6207 (D), TCWC6210 (D); Big Bend National Park, Green Gulch, 1,554 m TCWC6209 (D); Big Bend National Park, Green Gulch Rd. ASNHC12940 (D); Big Bend National Park, Green Gulch Rd, 13R 668269 3241909 ASK6217 (D, A); Big Bend National Park, Green Gulch Rd, 13R 667290 3241116 ASNHC12941 ASK6216 (D, A); Big Bend National Park, Laguna Meadows Trail, 13R 664296 3236849, 1,987 m ASNHC13515 (D, A); Big Bend National Park, Lost Pine Trail, 1,981 m TCWC6208 (D); Big Bend National Park, Oak Creek, Chisos Mts. MVZ80518 (D); Big Bend National Park, Panther Pass ASNHC12942 (D); Big Bend National Park, Pine Canyon UMMZ90112 (D); Big Bend National Park, The Basin, Chisos Mts. TCWC3655, 3656 (D); Elephant Mountain Wildlife Management Area TTU83106 TK78740 (D, M, A). *Culberson Co.*: Frijole LSUMZ658 (D); Guadalupe Mts. National Park, Hunter Ranch, McKittrick Canyon KU84388 (D); Guadalupe Mts. National Park, The Bowl TTU19994 (D). *Jeff Davis Co.*: 1 mi S Rockpile Park ASNHC544, 545, 868 (D); 1 mi NW Ft. Davis UMMZ79381, 79382, 79383 (D); 2 mi N Rockpile Park ASNHC968 (D); 2 mi NW Ft. Davis UMMZ79380 (D); 2.2 mi NW Fort Davis KU147341 (D); Fort Davis USNM18261, 18262 (D); Fort Davis, 2 mi NW of Limpia Canyon KU147340 (D); 2 mi SW Rockpile Park ASNHC549–554 (D); 3 mi N Rockpile Park ASNHC546 (D); 3 mi NW Ft. Davis SRSU464 (D, M, P); 10 mi N Ft. Davis SRSU459 (D, M, P); 2 mi NW Ft. Davis TTU3876 (D); 5 mi E Mt. Livermore, 1,676 m UMMZ79379 (D); Davis Mountains MSB89142 (D), USNM109095 (D); Davis Mountains State Park ASNHC7912 ASK3518 (D, M, A); HWY 118N, ca. 6 mi NW Fort Davis, 1,571 m MSB82184 (D); HWY 118 near entrance to Davis Mountains State Park; ca. 3 mi NW Fort Davis MSB85726 NK43241 (D, A), MSB85727 NK43242 (D, A); Limpia Canyon, 1 mi N Ft. Davis TCWC2515, 2516 (D); Lower Madera Canyon, Old Hunsacker Ranch SRSU1937 (D, M, P), SRSU1938 (D, M); Mt. Livermore Preserve TTU81191 TK84983 (D, M, P, A), TTU101634 TK78795 (D, A), TTU101636 TK78862 (D, A), TTU101637 TK79064 (D, M, P, A), TTU101638 TK83585 (D, M, P, A), TTU101639 TK83586 (D, M, P, A), TTU101639 TK83587 (D, M, P, A), TTU101640–101641 (D), TTU101642 TK90053 (D, M, A); Near top of Mt. Locke, NW Ft. Davis SRSU1066 (D); Sawtooth Mountain ASNHC1135–1138 (D), SRSU1101 (D, M, P); SH 118 W McDonald Observatory, 13R 586254 3397151, 1,810 m ASNHC13516 ASK7898 (D, A); SH 118 W McDonald Observatory, 13R 588358 3395474, 1,856 m ASNHC13517 ASK7904 (D, M, A). *Presidio Co.*, 11 mi W of Valentine TTU93418–93420 (D); 35 mi S of Marfa USNM18212 (D); Chinati Peak, 2,347 m TCWC2517 (D).

Unconfirmed Sylvilagus floridanus/robustus.—*Mexico*, *Chihuahua*: 15.5 mi NE Santa Clara, W side Sierra El Nido MVZ128261 (D); Arroyo El Mesteño, Sierra El Nido MVZ124792 (D); Arroyo El Nido, 30 mi SW Gallego MVZ121726 (D); Cañon del Alamo, Sierra El Nido MVZ124793–124796 (D). *Coahuila*: 5 mi W Piedra Blanca, Sierra del Carmen MVZ116978 (D); 8 mi SW Piedra Blanca, Sierra del Carmen MVZ116979–116981 (D). USA: *New Mexico*, *Otero Co.*, Sacramento Mts. 1 mi N Cloudcroft; T15S, R12E MSB7189 (D).