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Genetic connectivity and diversity of pygmy rabbits (*Brachylagus idahoensis*) in southern Wyoming

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The pygmy rabbit (*Brachylagus idahoensis*), a leporid of the Great Basin and southern Wyoming, resides in habitats dominated by big sagebrush (*Artemisia tridentata*). Because of the patchy distribution of mature big sagebrush in southern Wyoming, we expected pygmy rabbits to exhibit genetic attributes associated with genetic isolation: high levels of spatially structured differentiation. We also predicted some reduction in genetic diversity in the peninsular Wyoming portion of the geographic range, relative to its Great Basin core. We used 14 microsatellite loci to compare genetic attributes between geographically distinct pygmy rabbit populations, and a subset of these microsatellite loci to compare with those of 2 sympatric cottontails (*Sylvilagus* spp.), both presumptive habitat generalists. Pygmy rabbits displayed moderate genetic diversity that was lower than that reported from locations near the core of the geographic range (Idaho and Montana). We observed only low levels of genetic differentiation in pygmy rabbits among sampling sites within Wyoming. Similarly, we observed low levels of differentiation in one species of cottontail sympatric with pygmy rabbits; however, the other species of sympatric cottontail displayed levels of differentiation congruent with those of populations at panmixia. Isolation-by-distance was the dominant genetic pattern observed, although examination of our data suggested that a 4-lane highway (Interstate 80) might affect gene flow measurably. In the recent evolutionary past, habitat connectivity and dispersal capacity for pygmy rabbits have been high enough to maintain gene flow among sites across southern Wyoming. Conservation of the species should focus on maintaining the connectivity among preferred habitats: old stands of big sagebrush.

Key words: *Brachylagus idahoensis*, genetic diversity, habitat specialization, isolation-by-distance, microsatellite DNA, population genetics, pygmy rabbit, Wyoming

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The rarity of a species and its ecological specialization interact to determine extinction risk (Davies et al. 2004). A precursor to extinction of any large population is always a period of rarity. However, species that are specialists are often intrinsically uncommon, inasmuch as the resources that they require are limited across the landscape (Harcourt et al. 2002). The relationships among habitat loss, habitat specialization, and extinction risk have been demonstrated for diverse taxa, including birds (Owens and Bennett 2000), bats (Safi and Kerth 2004), and coral-reef fishes (Munday 2004).

Across a heterogeneous landscape, connectivity of a preferred habitat and the dispersal capacity of a species that occupies it determine the genetic connectivity of the species (Neaves et al. 2009; Schwartz et al. 2009). Habitat generalists disperse more often and farther distances than specialists (Swihart et al. 2003), which results in greater gene flow between geographic groups of generalists. Thus, one can infer how a species perceives habitat heterogeneity from patterns of genetic structure. One such pattern is that of isolation-by-distance, wherein geographic proximity is positively correlated with genetic similarity (Kimura and Weiss 1964; Wright 1943). Alternative or confounding patterns may include isolation-by-barrier: mountain ranges, rivers, and arid zones have figured prominently in such findings (Frantz et al. 2010). We begin by assuming that connectivity between areas of suitable habitat has important implications for genetic structure and the population genetic landscape of a species can provide insights into dispersal abilities (Storfer et al. 2007).

The pygmy rabbit (*Brachylagus idahoensis*) is a small leporid of the North American Great Basin, extending north into Washington and east into southern Wyoming. This species associates closely with mature big sagebrush (*Artemisia tridentata*); the underlying mechanisms are thought to be physical cover from predators (Gabler et al. 2001; Katzner and Parker 1997) and an almost complete dietary reliance on big

sagebrush during winter (Green and Flinders 1980). Thus, the species is generally regarded as a specialist for mature big sagebrush. Further, because pygmy rabbits are obligate burrow users that construct their own burrows (Heady and Laundré 2005), certain soil characteristics are preferred or required (Himes and Drohan 2007; Weiss and Verts 1984). It is unclear which attributes of big sagebrush are obligatory and which are facultative for pygmy rabbits. However, habitats in which pygmy rabbits are typically observed are specific; namely, tall, old stands of big sagebrush, with deep, soft soils (Weiss and Verts 1984).

Warheit (2001) found that levels of genetic diversity were similar among pygmy rabbit populations from Idaho, Montana, and Oregon, but lower in the isolated Columbia Basin population in Washington. The genetic losses and geographic isolation of the Washington population led to its listing under the Endangered Species Act (United States Fish and Wildlife Service 2003). At much smaller scales, low levels of genetic substructure have been reported among sites $\leq$13 km distant and moderate genetic structure between sites approximately 20–30 km apart (Estes-Zumpf et al. 2010). Accordingly, genetic consequences of habitat or geographic range fragmentation seem possible. Until now, no studies have described the genetic diversity and genetic connectivity of the species in the Wyoming portion of its geographic range.

Cottontail rabbits (*Sylvilagus*) are ubiquitous across temperate North America and the several species vary in their geographic extent and degree of habitat specialization. Mountain cottontails (*S. nuttallii*) and desert cottontails (*S. audubonii*) are widely distributed habitat generalists. Their geographic ranges are broadly sympatric and overlap with that of the pygmy rabbit (Fig. 1). Orr (1940) described mountain cottontails as living largely in sagebrush found on rocky hills and canyons, and preferring areas containing dense woody cover. Conversely, desert cottontails associate with less woody cover and occupy open fields, desert, and brushy areas.

We used landscape genetic theory to form hypotheses about genetic attributes of the pygmy rabbit in the Wyoming portion of its range. Specifically, we predicted that, relative to sympatric cottontails, pygmy rabbits would exhibit high genetic differentiation (isolation-by-distance or isolation-by-barrier), consistent with infrequent dispersal across unsuitable habitats. The Green River and Interstate Highway 80 separated some sampling sites, and we looked for evidence of these hypothesized barriers in pairwise tests. Additionally, because the geographic range of pygmy rabbits in Wyoming is peninsular, connected by a habitat isthmus to the Great Basin core of the geographic range to the west, we expected that gene diversity would be higher in the western than eastern parts of the Wyoming geographic range.

Materials and Methods

Study area.—The study area encompassed the Wyoming portion of the geographic range of the pygmy rabbit (41.65–42.78°N, 107.06–110.76°W; Table 1). This area is broadly defined as sagebrush steppe, characterized by big sagebrush and other drought-resistant shrubs (Knight 1994). The dominant shrub species at all of our capture locations was big sagebrush, although codominant shrub species varied. We captured pygmy rabbits at 5 sites (n = 5 sites; Fig. 1). The westernmost site and the site closest to the Great Basin core of the geographic range of the pygmy rabbit was Fossil Butte National Monument (FB). The northernmost site, Scab Creek Road (SCR), approached the northern limit of the pygmy rabbit range in Wyoming. The 3rd site was located in the vicinity of South Pass (SP), near the center of the Wyoming portion of the pygmy rabbit geographic range. The easternmost sites (S80 and N80) were approximately 10 km apart, separated by Interstate 80, and approached the eastern limit of the geographic range of the species. At 5 sites we captured >1 cottontail (*Sylvilagus*). For cottontails, FB was the westernmost site. Located along the Green River and abutting a campground, Slate Creek (SC) was 60 km east of FB. Three *Sylvilagus* sites were located slightly east of the known geographic range of the pygmy rabbit. Agate Flat (AF) was the northernmost site and contained large outcrops of weathered granite. The 2 remaining *Sylvilagus* sites were close together: Mineral-X Road (MX) was on the eastern edge of the Great Divide Basin and North Platte (NP) was located along the river of the same name, south of the Seminoe Reservoir. Sites where we captured pygmy rabbits were separated by 10–253 km; those for *Sylvilagus* by comparable distances, 27–307 km (Table 1; Fig. 1).

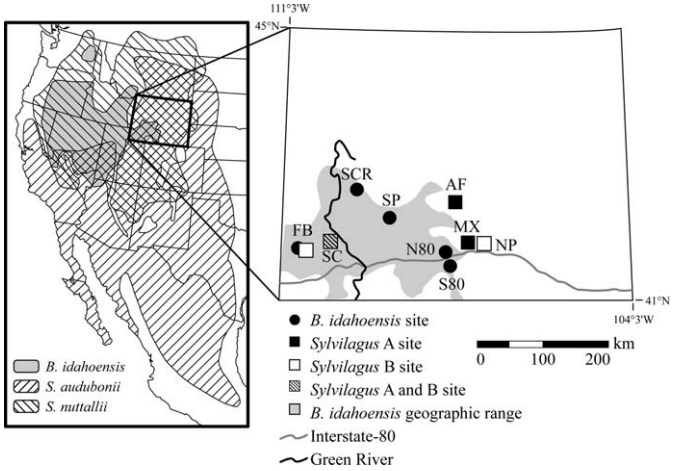

Fig. 1.—Geographic range of the pygmy rabbit (*Brachylagus idahoensis*), desert cottontail (*Sylvilagus audubonii*), and mountain cottontail (*S. nuttallii*) in North America, based on Patterson et al. (2007). Inset shows the Wyoming portion of the geographic range of the pygmy rabbit and sites (codes in Table 1) from which tissue samples from all 3 species were collected during 2008–2009.

Tissue sample collection.—We captured rabbits using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin) wrapped in burlap. We tested various attractant types and combinations: no bait, olfactory lure (cottontail trapping scent [Kishel's, Butler, Pennsylvania] or apple juice), and food bait (apples, canned green beans, or cabbage). We placed traps by burrow entrances of pygmy rabbits and under

Table 1.—Sampling sites, site codes, location, and sample sizes for a genetic study of the pygmy rabbit (*Brachylagus idahoensis*) and 2 species of *Sylvilagus* across southern Wyoming, 2008–2009.

Site	Site code	Latitude (°N)	Longitude (°W)	Elevation (m)	*n*		
						Sylvilagus	
					Pygmy rabbit	A	B
Fossil Butte National Monument	FB	41.83	110.76	2,049	16	0	14
Slate Creek	SC	41.98	110.05	1,945	1	4	2
Scab Creek Road	SCR	42.78	109.59	2,236	15	0	0
South Pass	SP	42.33	108.97	2,189	5	0	0
North of Interstate 80	N80	41.73	107.80	2,187	6	0	0
South of Interstate 80	S80	41.65	107.75	2,157	9	0	0
Agate Flat	AF	42.51	107.65	1,926	0	9	0
Mineral-X Road	MX	41.98	107.36	2,015	0	6	0
North Platte River	NP	41.88	107.06	1,954	0	0	5

shrubs near pellet piles. During winter, we also placed traps along runways created by pygmy rabbits in snow. To capture *Sylvilagus* we placed traps in areas that had high densities of pellets. For each capture, we recorded the location to within 7 m, which was later used in the isolation-by-distance analyses. With a leather punch, we took a 4-mm-diameter tissue sample from both ears of each rabbit. Tissue samples were placed in vials containing desiccant until they could be frozen. Animal handling protocols followed guidelines of the American Society of Mammalogists (Sikes et al. 2011) and were approved by the University of Wyoming Animal Care and Use Committee (Understanding the impact of fire on pygmy rabbit distribution, abundance, and movement, approved November 2007; Development of survey protocols for pygmy rabbit in the presence of cottontails, approved April 2008).

DNA extraction and amplification.—We extracted DNA from an ear tissue sample using a DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, California) following the recommended protocol. We assessed 15 microsatellite loci for amplification in pygmy rabbits ($n = 3$ individuals) and *Sylvilagus* spp. ($n = 4$ individuals), using loci that were previously developed for the pygmy rabbit (9 loci—Estes-Zumpf et al. 2008) and the European rabbit (*Oryctolagus cuniculus*; 4 loci from Rico et al. [1994] and 2 loci from Surridge et al. [1997]). A subset of the microsatellites developed for European rabbits had previously been shown to amplify in pygmy rabbits as well as desert cottontails (Estes-Zumpf et al. 2010; Surridge et al. 1997). Polymerase chain reactions (2.3–2.5 mM MgCl and 0.025–0.5 μM of both forward and reverse primers; see Thimmayya [2010] for final cocktails) and sequencing were performed at the Nucleic Acid Exploration Facility (University of Wyoming, Laramie, Wyoming). Initial testing and all subsequent amplification of microsatellite loci (10-μl reactions) and cytochrome-*b* segments (20-μl reactions) were performed on a PCT-100 thermal cycler (Global Medical Instrumentation, Albertville, Minnesota). Thermal cycler programs followed those in Rico et al. (1994), Surridge et al. (1997), and Estes-Zumpf (2008) for microsatellites. Microsatellite fragment analysis and cytochrome-*b* sequencing were conducted on a 3130*xl* 16-capillary Genetic Analyzer (Applied Biosystems, Carlsbad, California). We replicated each microsatellite locus for every individual ≥ 3 times and scored microsatellites using Peak Scanner version 1.0 (Applied Biosystems). In cases where inconsistencies in microsatellite length were found between the 3 replicates, an additional replicate was conclusive.

Microsatellite analyses.—For each locus at each sampling site, we calculated expected heterozygosity (H_E) as Nei's unbiased gene diversity, which considers sample size (Nei 1987), and observed heterozygosity (H_O) using Microsatellite Toolkit for Excel (Park 2001). We used FSTAT version 2.9.3.2 (Goudet 1995, 2002) to calculate allelic richness—number of alleles per locus—which is adjusted for sample size. We used Micro-checker version 2.2.3 (Oosterhout et al. 2004; Shipley 2003) to test for the presence of null alleles at each locus at each sampling site because we were using microsatellite loci that had been developed for other species (Chapuis and Estoup 2007). Using GENEPOP on the Web version 4.0.10 (Raymond and Rousset 1995; Rousset 2008), we tested for Hardy–Weinberg equilibrium at each locus at each sampling site (option 1: Hardy–Weinberg exact tests, suboption 3: probability test) and for linkage disequilibrium at each locus across sites (option 2: linkage disequilibrium, suboption 1: probability test). We set $\alpha = 0.05$ and adjusted for multiple comparisons using a false discovery rate correction (Benjamini and Hochberg 1995) for all probability tests.

In order to evaluate population differentiation, we used 3 genetic metrics. We calculated global and pairwise F_{ST} before (Weir 1996) and after correcting for null alleles using FreeNA (Chapuis and Estoup 2007), denoting F_{ST} values after null-allele correction as F_{ST}^{ENE}. Additionally, we calculated Jost's D_{est} (Jost 2008), a measure similar to F_{ST} that accounts for private alleles across all loci and sites, as well as for pairwise comparisons using the program SMOGD (Crawford 2010). In most cases, these 3 differentiation metrics were similar, therefore we used only F_{ST} and D_{est} in the isolation-by-distance analyses. Isolation-by-distance across sites was tested using a Mantel test (generating a Spearman's ρ) for pairwise F_{ST} or D_{est} values against pairwise sampling site distances. We did not use $F_{ST}/(1 - F_{ST})$ (Rousset 1997), because all F_{ST}

Table 2.—Microsatellite loci, allele size range (base pairs), and number of alleles per locus for the pygmy rabbit (*Brachylagus idahoensis*) and 2 species of *Sylvilagus* across southern Wyoming, 2008–2009. A dash (—) indicates no amplification under tested conditions.

	Pygmy rabbit		*Sylvilagus* A		*Sylvilagus* B	
Locus	Size range	No. alleles	Size range	No. alleles	Size range	No. alleles
A124[a]	207–217	3	202	1	211–223	6
A133[a]	201–207	4	203–209	4	201–207	4
D118[a]	276–292	5	238	1	244–278	10
D126[a]	176–188	4	175–192	6	173–179	4
sol08[b]	123–131	4	121–143	9	121–123	3
sol30[b]	315–329	6	145–166	10	168–170	2
sol44[c]	207–213	4	191–217	13	203–217	6
A10[a]	209–233	5	—		—	
A121[a]	193–206	4	—		—	
A2[a]	116–132	5	—		—	
D103[a]	113–133	4	—		—	
D121[a]	237–245	3	—		—	
sol28[d]	169–171	2	—		—	
sol33[e]	212–214	3	—		—	
sol03[b]	—		228–252	11	250–252	2

[a] Developed for pygmy rabbits (Estes-Zumpf et al. 2010).
[b] Developed for European rabbits (*Oryctolagus cuniculus*—Rico et al. 1994); previous amplification in pygmy rabbits and desert cottontails (Surridge et al. 1997).
[c] Developed for European rabbits; amplification in desert cottontails (Surridge et al. 1997) and pygmy rabbits (Estes-Zumpf et al. 2010).
[d] Developed for European rabbits (Rico et al. 1994); amplification in pygmy rabbits (Surridge et al. 1997).
[e] Developed for European rabbits; amplification in desert cottontails, but not pygmy rabbits prior to this study (Surridge et al. 1997).

values were low, so this adjustment had little effect. We also tested for isolation-by-distance at the level of the individual using the $\hat{a}$ statistic of Rousset (2000). We examined the effect of using only individuals that were >10 km apart to calculate the slope, but because this did not affect the outcome meaningfully, we kept all pairwise individual distances in our analyses. All isolation-by-distance tests were done in GENEPOP on the Web version 4.0.10 (option 6: F_{ST} and other correlations, suboption 9: isolation-by-distance [using Isolde] or suboption 5: analysis of isolation-by-distance between individuals).

We implemented a Bayesian clustering protocol in Program *Structure* version 2.3.1 (Pritchard et al. 2000) to examine whether pygmy rabbits of each taxon were genetically structured across southern Wyoming. We used the admixture model and tested for 1–5 subpopulations (K = 1–5). We used the methods outlined in Pritchard et al. (2007) and Evanno et al. (2005) to determine the number of genetic clusters for pygmy rabbits and *Sylvilagus*.

Results

In Program *Structure*, the high likelihood and large ΔK when $K = 2$ suggested that we sampled 2 genetic clusters of *Sylvilagus* (Thimmayya 2010). The estimated membership of individuals was always >99% to 1 group, indicating that the 2 clusters were highly distinct. Group membership was the same for all individuals within a sampling site, except for SC where both lineages occurred syntopically. AF and MX individuals were all assigned to *Sylvilagus* A, whereas individuals from FB and NP, the 2 farthest sites from each other, were assigned to *Sylvilagus* B. Available cytochrome-*b* sequence data did not allow us to assign these 2 clusters to individual species (*S. nuttallii* and *S. audubonii*, GenBank accession numbers: JF269237–JF269258 [Thimmayya 2010]).

Table 3.—Measures of genetic diversity based on microsatellite loci for the pygmy rabbit (*Brachylagus idahoensis*) and 2 species of *Sylvilagus* across southern Wyoming, 2008–2009. 95% *CI* = 95% confidence interval.

	Site[a]	*n*	A_r[b]	H_E[c] (95% *CI*)	H_O[d]	*P*-value[e]
Pygmy rabbit	FB	16	3.1	0.59 (0.56–0.61)	0.54	0.15
	N80	6	3.0	0.61 (0.58–0.63)	0.54	0.47
	S80	9	2.8	0.54 (0.51–0.57)	0.51	0.46
	SCR	15	3.1	0.57 (0.55–0.60)	0.53	0.002[f]
	SP	5	3.1	0.60 (0.58–0.68)	0.57	0.41
Sylvilagus A	AF	9	4.8	0.82 (0.78–0.86)	0.69	0.0001[f]
	MX	6	4.7	0.79 (0.74–0.84)	0.69	0.03
	SC	4	4.2	0.73 (0.67–0.80)	0.75	0.87
Sylvilagus B	FB	14	2.4	0.63 (0.58–0.67)	0.52	<0.001[f]
	NP	5	1.9	0.40 (0.32–0.48)	0.43	0.59
	SC	2	2.1	0.50 (0.41–0.59)	0.44	0.90

[a] Site codes (Table 1).
[b] Allelic richness (Goudet 2002).
[c] Expected heterozygosity expressed as Nei's unbiased gene diversity (Nei 1987).
[d] Observed heterozygosity.
[e] Chi-square (χ^2) test for Hardy–Weinberg equilibrium.
[f] $\alpha = 0.05$ after false discovery rate correction (Benjamini and Hochberg 1995).

We captured pygmy rabbits (n = 52) at 6 sites, *Sylvilagus* A (n = 19) at 3 sites, and *Sylvilagus* B (n = 21) at 3 sites (Table 1). Pygmy rabbits occurred over a broader range of elevations than *Sylvilagus*; *Sylvilagus* A and B occurred over similar elevation ranges.

Pygmy rabbits.—We were able to amplify 14 microsatellite loci, all of which were polymorphic with 2–6 alleles/locus ($\bar{X}$ = 4.0; Table 2). Mean allelic richness across all sites was 3.0 alleles/locus and variation was low among sites (Table 3). All loci were at linkage equilibrium and were at Hardy–Weinberg equilibrium except for A10 and A2 at SCR. The departure of A2 and A10 from Hardy–Weinberg equilibrium led to an overall departure across loci at SCR, likely due to null alleles. There was also evidence of null alleles at locus A124 at SCR. Pygmy rabbits showed moderate levels of H_E ($\bar{X}$ = 0.58) and H_O ($\bar{X}$ = 0.54) across loci and sampling locations (Table 3). Overlapping 95% confidence intervals (95% *CI*s) indicated that H_E was similar across most sites, except for S80, which exhibited H_E lower than that for N80 or SP.

Across sampling sites, the global F_{ST} = 0.015 (95% *CI*: 0.001–0.031), did not differ when corrected for null alleles (global F_{ST}^{ENE} = 0.021, 95% *CI*: 0.008–0.037). Across loci and sampling sites, global D_{est} = 0.028 (95% *CI*: 0.001–0.055). All pairwise F_{ST} values had 95% *CI*s that included 0, except for the one corresponding with 2 sites located the farthest apart, FB and S80 (Table 4). Pairwise D_{est} values

TABLE 4.—Three pairwise genetic measures used to examine population differentiation based on microsatellite loci for the pygmy rabbit (*Brachylagus idahoensis*) and 2 species of *Sylvilagus* across southern Wyoming, 2008–2009.

Species	Between sites[a]		Distance (km)	Pairwise F_{ST}[b]	Pairwise F_{ST}^{ENE}[c]	Pairwise D_{est}[d]
Pygmy rabbit	N80	S80	10	0.003	0.010	<0.001
	SCR	SP	71	0.016	0.025	0.032
	N80	SP	117	<0.001	0.002	<0.001
	S80	SP	127	0.028	0.036	0.031
	SCR	FB	143	0.008	0.010	0.011
	FB	SP	159	0.005	0.010	0.008
	N80	SCR	188	<0.001	0.004	<0.001
	S80	SCR	198	0.022	0.029[e]	0.037
	N80	FB	248	0.029	0.028	0.031
	S80	FB	253	0.040[e]	0.046[e]	0.057[e]
Sylvilagus A	AF	MX	64	<0.001	0.010	0.058[e]
	SC	AF	206	0.035	0.051[e]	0.198[e]
	SC	MX	223	0.024	0.027	0.113[e]
Sylvilagus B	SC	FB	62	<0.001	0.008	0.007
	SC	NP	248	0.099	0.114	0.034
	FB	NP	307	0.084[e]	0.104[e]	0.059[e]

[a] Site code (Table 1).
[b] Weir (1996).
[c] F_{ST} corrected for the presence of null alleles (Chapuis and Estoup 2007).
[d] Jost (2008).
[e] 95% confidence interval did not include 0.

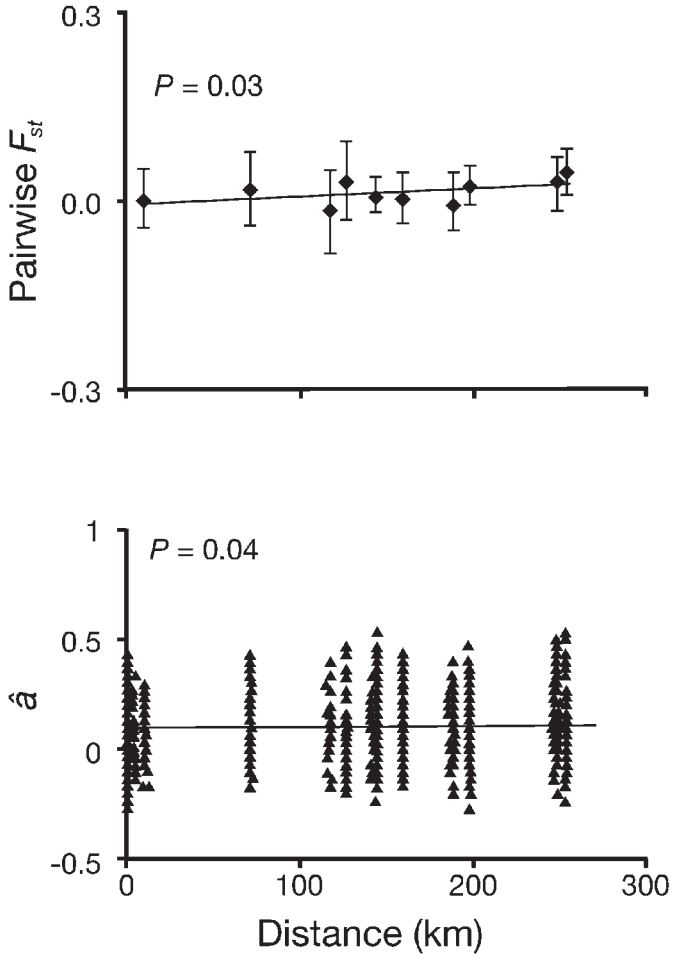

FIG. 2.—Isolation-by-distance analyses for the pygmy rabbit (*Brachylagus idahoensis*) across southern Wyoming, 2008–2009. Top one-half shows isolation-by-distance between sites with pairwise F_{ST} values regressed on Euclidean distance. Bottom figure shows isolation-by-distance between individual animals, regressing $\hat{a}$ on Euclidean distance.

followed the same trend as pairwise F_{ST} values, indicating genetic differentiation between only FB and S80. After correcting for null alleles, pairwise F_{ST}^{ENE} values again indicated genetic differentiation between FB and S80, and also between SCR and S80. We observed a weak isolation-by-distance effect at the site level, pairwise $F_{ST} = -0.003 + 0.0001$d (Spearman's ñ = 0.714, $P = 0.03$; Fig. 2) and pairwise $D_{est} = -0.0006 + 0.0001$d (Spearman's ñ = 0.794, $P = 0.005$), where d = straight-line distance (km) between sampling sites. Isolation-by-distance for pairs of distances between individual animals, rather than between sites, produced a similar outcome, $\hat{a} = 0.088 + 0.0001$d (Spearman's ñ = 0.76, $P = 0.04$; Fig. 2), where d is defined as for site pairs.

Program *Structure* indicated that pygmy rabbits from the 5 sampling sites comprised 1 genetic cluster. The highest log-likelihood value was for $K = 1$, indicating that the model for 1 genetic cluster of pygmy rabbits was most supported. Additionally, although the ΔK value was the highest for $K = 2$, all values for ΔK were small ($\Delta K < 2$). The estimated membership of individuals in different groups was symmetrical for $K = 2$–5 (Pritchard et al. 2007), indicating no population substructure across the range of pygmy rabbit sites we sampled (Fig. 3).

Sylvilagus A.—We were able to amplify 8 microsatellite loci for *Sylvilagus* A and all were at linkage equilibrium. All loci were polymorphic with 4–13 alleles/locus ($\bar{X} = 8.8$ alleles/locus), except for A124 and D118, which were monomorphic (Table 2). Loci A133, sol03, and sol30 were not at Hardy–Weinberg equilibrium at AF; all other loci were at Hardy–Weinberg equilibrium within a site. Again, there was evidence that the departure from Hardy–Weinberg equilibrium at AF was due to the presence of null alleles. Allelic richness was similar across sites with a mean of 4.6 alleles/locus (Table 3). H_E ($\bar{X} = 0.78$) and H_O ($\bar{X} = 0.71$) were high across sampling sites and loci. H_E was similar across sampling sites.

Global F_{ST} (0.016, 95% *CI*: −0.023–0.057) and F_{ST}^{ENE} (0.027, 95% *CI*: −0.003–0.058) were similar and did not differ from 0. Across loci and sampling sites, $D_{est} = 0.123$ (95% *CI*: 0.001–0.245). Pairwise F_{ST} values were similar between all site pairs and did not differ from 0 (Table 4). However, the pairwise F_{ST}^{ENE} value for SC-AF indicated population differentiation between these 2 sites. Additionally pairwise D_{est} values indicated differentiation between all sites sampled. There was no evidence of isolation-by-distance at the site (pairwise F_{ST} Spearman's $\rho < 0.03$, $P = 0.50$; pairwise D_{est} Spearman's $\rho < 0.03$, $P = 0.50$) or individual (Spearman's $\rho < 0.05$, $P = 0.93$) levels of analysis.

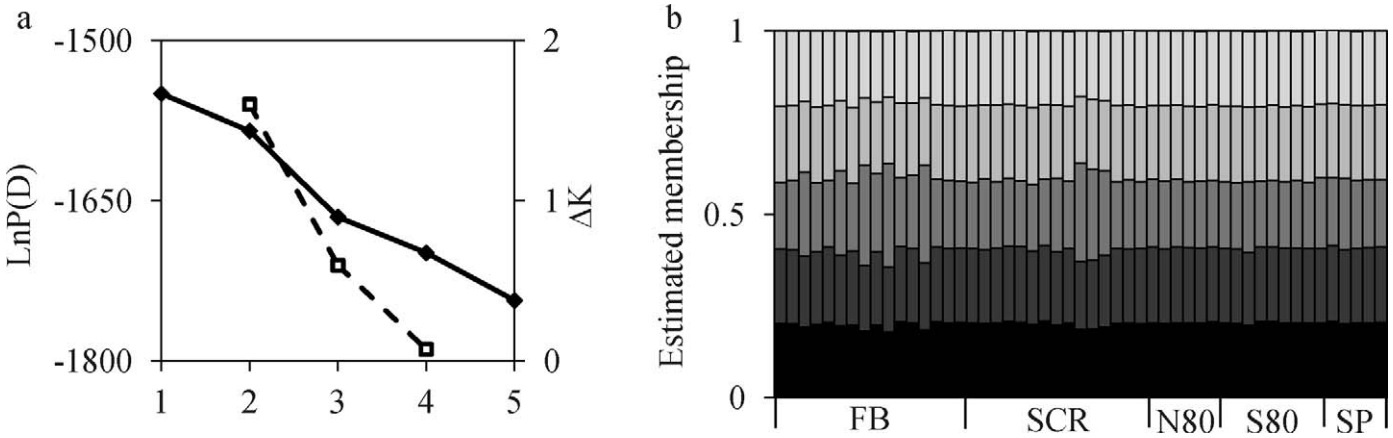

FIG. 3.—Results from Program *Structure* evaluating population structure of the pygmy rabbit (*Brachylagus idahoensis*) across southern Wyoming, 2008–2009. A) The correct number of populations into which the populations are sorted is 1, for tested values of $K = 1$–5. B) Estimated membership for individuals to each population, denoted by shades of gray (site codes in Table 1), for $K = 5$. The estimated membership in different populations was proportionally similar for each individual pygmy rabbit, indicating that the correct number of populations was 1.

Sylvilagus B.—We were able to amplify the same 8 loci in *Sylvilagus* B as in *Sylvilagus* A under the same reaction conditions. In contrast to *Sylvilagus* A, all loci were polymorphic with 2–10 alleles/locus ($\overline{X} = 4.6$ alleles/locus; Table 2). All loci were at Hardy–Weinberg equilibrium within each site, except for sol08 at FB. The apparent departure from Hardy–Weinberg equilibrium at this locus and site was attributable to the presence of null alleles. There was physical dependence between sol03 and sol30 at all sites, so we removed sol30 from analyses of population differentiation and isolation-by-distance. Across all loci and sampling sites, allelic richness was low ($\overline{X} = 2.1$ alleles/locus; Table 3), and H_E ($\overline{X} = 0.51$) and H_O ($\overline{X} = 0.46$) were moderate. H_E was higher at FB than at NP, although FB-SC and NP-SC had similar levels of H_E, indicated by overlapping 95% *CI*s.

Global F_{ST} (0.059, 95% *CI*: 0.018–0.101) and F_{ST}^{ENE} (0.080, 95% *CI*: 0.025–0.142) values were similar and indicated low levels of genetic differentiation. However, the D_{est} value indicated there was no genetic differentiation among sites ($D_{est} = 0.156$, 95% *CI*: −0.020–0.332). There was no genetic differentiation between SC-FB or SC-NP, indicated by the 95% *CI*s of pairwise F_{ST} values that included 0 (Table 4). Pairwise F_{ST} values indicated moderate differentiation between the sites separated by the greatest geographic distance, FB-NP. Pairwise F_{ST}^{ENE} and D_{est} values followed the same trend as pairwise F_{ST}. There was no evidence of isolation-by-distance at the site (pairwise F_{ST} Spearman's $\rho < 0.03$, $P = 0.50$; pairwise D_{est} Spearman's $\rho = 0.06$, Mantel $P = 0.20$) or individual (Spearman's $\rho < 0.05$, $P = 0.61$) levels.

DISCUSSION

Contrary to our expectations, measures of genetic connectivity for pygmy rabbits, a habitat specialist, were similar to those of both species of *Sylvilagus*, habitat generalists. Observed levels of population substructure were low for pygmy rabbits across the Wyoming portion of the range. Pygmy rabbits did exhibit a statistically stronger isolation-by-distance effect than either species of *Sylvilagus*; however, this could be attributable to the small sample sizes for both *Sylvilagus* which weakened our comparisons.

Allelic richness and heterozygosity of pygmy rabbits were moderate across our sample sites. In Wyoming ($H_E = 0.58$), pygmy rabbits exhibited H_E 18% lower than that in Montana ($H_E = 0.71$, mean across sampling sites [Estes-Zumpf 2008]). Similarly, examination of our data showed H_E 20% lower than that in Idaho ($H_E = 0.73$, mean across sampling sites, 12 loci from our study in Wyoming were also used in the study in Montana and Idaho [Estes-Zumpf 2008]). Allelic richness was lower for pygmy rabbits in our study (range of site values: 2.8–3.1) than in Idaho (range of site values: 4.3–5.6 [Estes-Zumpf et al. 2010]). As expected, H_E, H_O, and allelic richness of *Sylvilagus* A were higher than those of pygmy rabbits in our study. However, for *Sylvilagus* B, all measures were lower than those of pygmy rabbits.

Gene flow among geographic groups of pygmy rabbits across Wyoming has precluded even moderate levels of population genetic structure. This was evident by the values of the 3 global and pairwise genetic measures we used, as well as the results obtained using Program *Structure*. Although all 3 global measures indicated that pygmy rabbits in Wyoming were not panmictic, Wright (1978) stated that F_{ST} values < 0.05 indicate little differentiation. All of our global and pairwise measures of differentiation were <0.05, and some $F_{ST} < 0.02$, indicating that sampling sites were well connected through gene flow over the periods that microsatellites evolve. As we expected, *Sylvilagus* A showed less population differentiation across the sampled area than the pygmy rabbit, indicated by F_{ST} and F_{ST}^{ENE} values suggesting panmixia. However, the global D_{est} value indicated differentiation in *Sylvilagus* A similar to that of pygmy rabbits and all global D_{est} values indicated differentiation between sites. The pattern in the various differentiation measures was the opposite for *Sylvilagus* B as that for *Sylvilagus* A. *Sylvilagus* B had global F_{ST} and

F_{ST}^{ENE} values that indicated slight population differentiation and a D_{est} value that indicated no differentiation. Again, broad 95% *CI*s were associated with the differentiation values for both *Sylvilagus* spp. due to small sample sizes, which weakened statistical comparisons.

Greater isolation-by-distance in a habitat specialist species than in an otherwise similar generalist species has been reported for various species (kestrels [Alcaide et al. 2009] and bees [Zayed et al. 2005]), and we expected such differences between pygmy rabbits and cottontails. However, for pygmy rabbits, the only sites exhibiting significant pairwise genetic structure were about 200 km apart. Estes-Zumpf et al. (2010) reported levels of differentiation (pairwise F_{ST}: 0.01–0.14) in Idaho ranging from similar to those we observed to 3.5 times our observed values. This was true despite the much greater distances between sites in our study ($\leq$253 km) than in theirs ($\leq$32 km). The reason for the much higher levels of genetic differentiation in their study than in ours is unclear.

In the recent evolutionary past, the Wyoming portion of the geographic range of pygmy rabbits has apparently experienced sufficient gene flow to prevent even moderate genetic differentiation. We expected that because of the presumptive habitat specialization of pygmy rabbits, gene flow would be limited and genetic structure pronounced. However, examination of our data indicates only slight genetic structuring across linear distances of up to 250 km, which suggests that mature stands of big sagebrush have been contiguous enough in the ecological past, or the dispersal capacity of pygmy rabbits great enough through habitats not currently recognized as suitable, to facilitate high gene flow across the Wyoming portion of the geographic range. Median natal dispersal distances for pygmy rabbits are short (1.0 km for males and 2.9 km for females), although maximum natal dispersal distances can be greater (6.5 km for males and 11.9 km for females [Estes-Zumpf and Rachlow 2009]). Katzner and Parker (1998) reported a pygmy rabbit dispersal of 3.5 km, much of it through presumably unsuitable habitat. Although this rabbit was able to move through a suboptimal matrix, it was observed resting in isolated patches of sagebrush along the way. Genetic studies in Idaho indicated that roads and perennial streams were not barriers to gene flow for pygmy rabbits, although there was evidence that the combination of both a highway and a stream reduced gene flow between 2 sites (Estes-Zumpf et al. 2010). Additionally, the Snake River and the Snake River plain were considerable barriers to gene flow (Estes-Zumpf 2008). In our study, despite the presence of various roads, including Interstate 80, and perennial waterways, particularly the Green River, we found little evidence that these landscape features obstructed gene flow. One possible exception involves Interstate 80: the only pairwise comparisons that differed from panmixia were S80-FB and S80-SCR; N80, only 10 km distant from S80, but on the same side of the Interstate 80 as all other sites except S80, did not show differentiation from any sites. H_E was also lower at S80 than at N80, and S80 was the only site that showed evidence of reduced H_E relative to other sites. This would suggest that the combination of distance and barrier (Interstate 80) may have resulted in some reduction in gene flow.

Compared to populations near the core, populations at the edges of geographic ranges may experience reductions in effective population size (Vucetich and Waite 2003) and genetic diversity, and increased genetic differentiation (Eckert et al. 2008). Within Wyoming, we observed no reduction in genetic diversity with increasing distance from the Great Basin core of the geographic range. FB was the site closest to, and S80 the most distant from, the core of the geographic range of the pygmy rabbit; however, H_E was similar between these sites. The entire Wyoming portion of the geographic range of the pygmy rabbit is peninsular to the core (Fig. 1) and, although FB is the closest to the core, it still is peninsular. Conversely, in comparison with populations from Idaho and Montana (Estes-Zumpf 2008), we observed reduced H_E in Wyoming, expected for populations at the margin of a geographic range. Although the sampling sites in Montana and Idaho are arguably also on the margin of the geographic range, populations in these states are well connected to the core in multiple directions, whereas in Wyoming, they are connected only to the west by a narrow strip of suitable habitat along the Wyoming–Utah border.

Interestingly, pygmy rabbits displayed greater genetic connectivity within Wyoming than within Idaho (Estes-Zumpf 2008). This may reflect more intact habitats found in Wyoming compared to those in Idaho. The study area in Idaho included tilled croplands, a type of human-caused fragmentation rare in southern Wyoming. It is also possible that the low levels of differentiation we observed between our study sites had an evolutionary basis. Pygmy rabbits in Wyoming could be better adapted to patchy habitats, as is often found on the margins of a species' geographic range, than those in Idaho. At the edge of a geographic range, directional selection may be prevalent, as opposed to stabilizing selection, which may be more common near the core (Sexton et al. 2009). Although a complex optimization problem, dispersal strategy clearly varies across the geographic range of a species (Dytham 2009). Furthermore, at the margins of the geographic range, directional selection toward increased dispersal distances compared to those at the core has been demonstrated in species undergoing range expansion (Simmons and Thomas 2004). In Wyoming, pygmy rabbits could have a higher dispersal tendency than those nearer to the Great Basin core, allowing greater gene flow over highways and other small-scale human-caused fragmentation. In Idaho, nearer to the core, selection may have favored smaller dispersal distances, which could have led to pygmy rabbits being less adapted to cope with small-scale anthropogenic fragmentation than pygmy rabbits in Wyoming. The apparent genetic connectivity of pygmy rabbits in Wyoming, in conjunction with relatively low genetic diversity when compared to pygmy rabbits closer to the core of the geographic range, also could be a result of relatively recent range expansion into Wyoming. Such an expansion would be recent on an evolutionary time scale, and would have required centuries or millennia.

Although low genetic differentiation of pygmy rabbits in Wyoming showed high levels of gene flow among groups in recent evolutionary past, lower genetic diversity relative to that near the core of the geographic range suggested that pygmy rabbits in Wyoming have experienced isolation from populations outside of the state. Low genetic diversity is a conservation concern, inasmuch as populations with reduced genetic variation might suffer reduced adaptive capacity (Frankham et al. 2002). Although low genetic differentiation implied high rates of gene flow in the ecological past among pygmy rabbits in Wyoming, it is unclear whether this connectivity continues into recent decades. In Wyoming, conservation efforts for pygmy rabbits should strive to maintain the connectivity among habitats, as well as habitat area.

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