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IS LEPROSY SPREADING AMONG NINE-BANDED ARMADILLOS IN THE SOUTHEASTERN UNITED STATES?

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ABSTRACT: In the United States, nine-banded armadillo (*Dasypus novemcinctus*) populations are derived from two sources: (1) a continuous range expansion from Mexico led to western populations, some of which, particularly along the western Gulf Coast and west side of the Mississippi River delta, exhibit persistently high rates of leprosy infection, and (2) a small group of animals released from captivity in Florida gave rise to eastern populations that were all considered leprosy free. Given that western and eastern populations have now merged, an important question becomes, to what extent is leprosy spreading into formerly uninfected populations? To answer this question, we sampled 500 animals from populations in Mississippi, Alabama, and Georgia. Analyses of nuclear microsatellite DNA markers confirmed the historic link between source populations from Texas and Florida, but did not permit resolution of the extent to which these intermediate populations represented eastern versus western gene pools. Prevalence of leprosy was determined by screening blood samples for the presence of antibodies against *Mycobacterium leprae* and via polymerase chain reaction amplification of armadillo tissues to detect *M. leprae* DNA. The proportion of infected individuals within each population varied from 0% to 10%. Although rare, a number of positive individuals were identified in eastern sites previously considered uninfected. This indicates leprosy may be spreading eastward and calls into question hypotheses proposing leprosy infection is confined because of ecologic constraints to areas west of the Mississippi River.

Key words: Armadillo, *Dasypus novemcinctus*, leprosy, *Mycobacterium leprae*, population genetics.

INTRODUCTION

Aside from humans, nine-banded armadillos (*Dasypus novemcinctus*) are the only other free-ranging vertebrates known to harbor naturally occurring infections of *Mycobacterium leprae*, the causative agent in producing leprosy. Within the United States, armadillo populations are derived from two sources. First, armadillos crossed the Rio Grande into southeast Texas sometime in the 1820s, with populations spreading rapidly north and east since (Taulman and Robbins, 1996). Second, releases of a limited number of captive animals occurred in south-central Florida in the 1920s (Humphrey, 1974). Descendents of these animals established populations that subsequently spread north and west. Western and eastern populations have now merged, probably in eastern Mississippi or western Alabama in the 1980s (Taulman and Robbins, 1996), to form a continuous distribution across much of the southern United States.

This biogeographic history appears linked with patterns of leprosy infection. Early histopathologic studies and later serologic analyses both identified infected animals at multiple locations in Louisiana and Texas, in populations clearly derived from western sources (Truman, 2008). In contrast, virtually no infected animals were found east of the Mississippi River (Howerth et al., 1990; Truman, 2008), perhaps because of the absence of the disease among the small number of founding individuals from which these populations were derived. Now that eastern and western populations have merged, it seems possible that leprosy could spread eastward into previously uninfected populations.

However, not all western populations of armadillos exhibit leprosy infection. Mirroring the pattern of human infection, most cases of leprosy in armadillos appear confined to a region along the western Gulf Coast and west side of the Mississippi River (Truman, 2005). Truman (1996, 2005) argued this might be due to certain ecologic features that restrict leprosy to these areas. The exact nature of these features remains unknown, but might include 1) appropriate low-lying, humid soils to facilitate survival of *M. leprae* outside a host; 2) high densities of armadillos; and/or 3) other environmental agents that affect either disease transmission or susceptibility. If true, this ecologic-constraints hypothesis predicts no eastward spread of leprosy because the necessary conditions do not typically exist east of the Mississippi River. In contrast, epidemic models (Scholl et al., 1995) predict a steady spread of leprosy as infected animals disperse eastward.

The purpose of the present study was to sample armadillo populations at several locations east of the Mississippi River to determine whether leprosy has spread in that direction. These results are then discussed in light of the ecologic-constraints versus epidemic models of leprosy distribution.

MATERIALS AND METHODS

Field work

Samples were collected from May 2005 to July 2005 at the five locations shown in Figure 1, with follow-up sampling at two sites (YZ and RS) during the summer of 2006. Yazoo National Wildlife Refuge (YZ; 33°05′N, 90°59′W) had a soil profile and proximity to infected populations across the Mississippi River such that both ecologic-constraints and epidemic hypotheses predicted high levels of leprosy infection. St. Catherine's Creek National Wildlife Refuge (SC; 31°22′N, 91°26′W), like Yazoo, was in close proximity to populations exhibiting high levels of infection (Truman, 2005, 2008) but possessed a different soil profile. Thus, the ecologic-constraints hypothesis predicted little occurrence of leprosy, whereas the epidemic hypothesis predicted the opposite. Stimpson Wildlife Sanctuary (ST; 31°23′N, 87°51′W) and DeWayne Hayes Recreational Area (DH; 33°36′N, 88°28′W) were located at roughly the same latitudes as St. Catherine's Creek and Yazoo, respectively, but 241–338 km farther east. Soil conditions at both sites led to the prediction from the ecologic-constraints hypothesis that no leprosy should be present. However, the epidemic hypothesis predicted leprosy could occur, with prevalence dependent on the extent to which each population had contact with western-derived individuals. Samples from Riverside (RS; 32°54′N, 88°11′W) were pooled as part of the DeWayne Hayes data in 2005, but were collected and analyzed separately in 2006. Finally, Pinebloom Plantation (PB; 31°43′N, 84°38′W) was located so far east and had a soil profile such that both hypotheses predicted no occurrence of leprosy there.

Basic methods for capturing live animals followed previously published protocols (McDonough and Loughry, 2005). Upon capture, two ear notches were collected: one was preserved in 100% ethanol for genetic screening, the other in 70% ethanol for polymerase chain reaction (PCR) analyses to detect *M. leprae* DNA in putatively infected animals. In addition, we clipped the end of one toenail to obtain a small blood sample, collected onto Nobuto blood strips (Advantec, Dublin, California, USA).

We also sampled dead armadillos. These included all the animals from Pinebloom and Riverside (in 2006), 34 of the animals from DeWayne Hayes, and fresh road-kills from a variety of locations. In these cases, in addition to collecting ear and blood samples, we also collected the spleen from each animal, preserving it in 100% ethanol.

Genetic analyses

To further evaluate the epidemic hypothesis, ear samples were screened for genetic markers that might discriminate between western and eastern source populations and allow estimates of gene flow. Previously collected samples from Welder Wildlife Refuge (WR; 28°07′N, 97°22′W) and Tall Timbers Research Station (TT; 30°38′N, 84°13′W) were used as representatives of western and eastern gene pool sources, respectively,

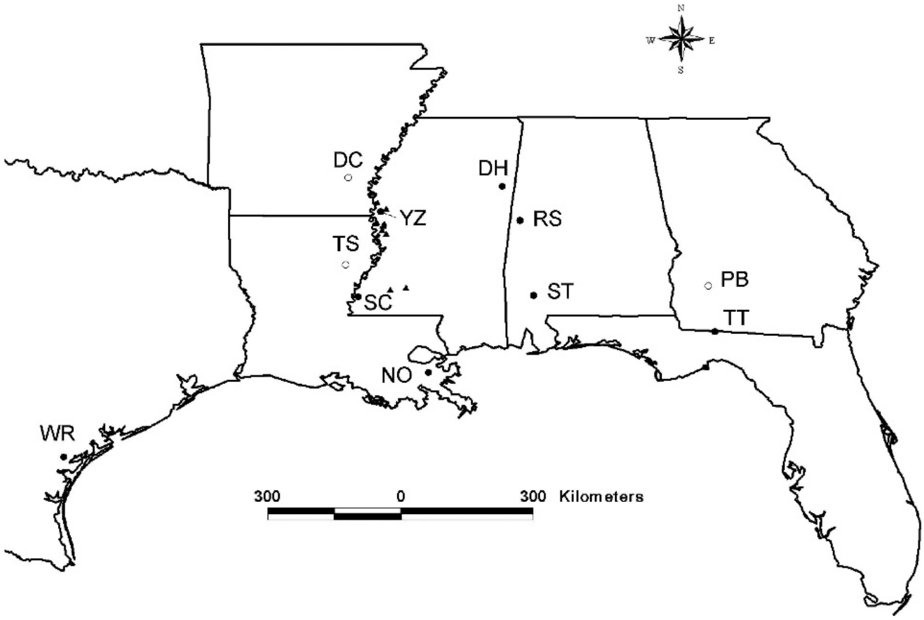

FIGURE 1. Map of sampling localities. DH = DeWayne Hayes Recreational Area (Columbus, Mississippi, USA), PB = Pinebloom Plantation (Albany, Georgia, USA), RS = Riverside (Riverside, Alabama, USA), SC = St. Catherine's Creek National Wildlife Refuge (Natchez, Mississippi, USA), ST = Stimpson Wildlife Sanctuary (Jackson, Alabama, USA), and YZ = Yazoo National Wildlife Refuge (Hollandale, Mississippi, USA). In addition to these main sampling localities, a few (n=12) fresh road-killed animals were collected in various parts of Mississippi. These individuals are shown as ▲. Also shown are the locations of reference populations from just west of the Mississippi River presenting high prevalence of leprosy infection (DC = Desha County, Arkansas, USA; and TS = Tensas National Wildlife Refuge, Louisiana, USA) and sources of genetic samples used to represent a western (WR = Welder Wildlife Refuge, Sinton, Texas, USA), eastern (TT = Tall Timbers Research Station, Tallahassee, Florida, USA) and intermediate (NO = New Orleans, Louisiana, USA) population. Populations used for genetic analyses are indicated by ●.

whereas samples from the Tulane University Museum of Natural History (NO; 29°50′N, 90°00′W) were included as an additional intermediate population (Fig. 1).

Twenty individuals from each sampled population (n=7; note Pinebloom was not included because of its close proximity to Tall Timbers and that samples from DeWayne Hayes and Riverside were pooled) were randomly selected and genotyped for five of the nuclear microsatellite loci (*Dnov1*, *Dnov6*, *Dnov7*, *Dnov16*, and *Dnov24*), previously identified by Prodöhl et al. (1996). Loci were amplified by PCR using an end-labeled reverse primer with fluorescein. Two microliters of PCR product from each individual were subsequently loaded onto an 8% acrylamide gel (Bio-Rad, Hercules, California, USA). DNA fingerprints were visualized with the FMBIO fluorescent imaging system (Hitachi Software Engineering America, Ltd., South San Francisco, California, USA). Allele numbers were determined with a fluorescently labeled ladder (Promega Corporation, Madison, Wisconsin, USA), using the FMBIO ANALYSIS 8.0 image analyzer program.

Intrapopulation genetic variation was estimated by observed (Ho) and expected (He) heterozygosities (unbiased estimate, Nei, 1978), using GENETIX 4.04 (Belkhir et al., 2001). We estimated the number of alleles, independent of the sample size (hereafter called allelic richness), using an adaptation of the rarefaction index of Hurlbert (1971), as implemented in FSTAT 2.9.3 (Goudet, 1995). Because one locus exhibited a serious Hardy–Weinberg disequilibrium in six populations (see Results), measures of intrapopulation genetic diversity were averaged over the four remaining loci.

We tested for linkage disequilibrium between all pairs of loci in each population using exact tests as implemented in GENEPOP 3.3 (Raymond and Rousset, 1995). Departure from Hardy–Weinberg equilibrium was tested using permutation procedures of GENETIX for each locus in each population and across all loci. Deviations from Hardy–Weinberg proportions were quantified by the unbiased estimator of Wright's inbreeding coefficient (F_{IS}), calculated according to Weir and Cockerham (1984).

Differentiation between all pairs of populations was assessed using a log-likelihood *G*-based exact test (Goudet et al., 1996). Tests computed for each locus were combined in a global test by use of Fisher's method (Manly, 1985). Pairwise levels of differentiation (F_{ST}) were calculated using the Weir and Cockerham (1984) estimators. These analyses were conducted using Genepop. In addition, a neighbor-joining tree (Saitou and Nei, 1987) was constructed from chord distances (*Dc*) among populations (Cavalli-Sforza and Edwards, 1967).

Leprosy screening

Some animals from Yazoo were captured and transferred to the animal colony maintained by the National Hansen's Disease Program at Louisiana State University (LSU). For these animals, whole blood was collected by venipuncture and the serum was harvested after centrifugation in serum separation tubes (BD-Vacutainer, Franklin Lakes, New Jersey, USA) and stored frozen until use. For all other animals, whole blood collected on Nobuto strips was air dried and held at room temperature before the serum was extracted by immersion in phosphate buffered saline at pH 7.2 for 3 hr. Both regular sera and Nobuto-eluted whole blood showed equal proficiency among test animals in an earlier pilot study.

Serum or eluted whole blood was tested in an enzyme-linked immunosorbent assay (ELISA) for immunoglobulin M (IgM) antibodies to the species-specific paragangliomas-1 (PGL1) antigen of *M. leprae* using the procedure of Truman et al. (1986). The synthetic neoglycoconjugate antigen, natural disaccharide octyl bovine serum albumin (ND-O-BSA), was used in all studies and was supplied by Dr Patrick Brennan (Colorado State University, Fort Collins, Colorado, USA) through contract with the National Institute of Allergy and Infectious Disease. Samples were tested in triplicate at a 1:35 dilution, and all positive or equivocal reactions reassessed three times to confirm consistency.

Tissues (ear or spleen) from animals deemed to be positive for PGL1 IgM antibodies in the ELISA were examined with PCR to confirm presence of *M. leprae* DNA following the methods of Williams et al. (1990). Automated DNA sequencing was performed by the LSU Genelab facility to confirm identity as *M. leprae.*

Population data analyses

Using data collected in 2005, we compared the number of armadillos observed per hour of observation at each site to determine whether prevalence was related to population density (Truman et al., 1991). Pair-wise chi-square tests were used to compare leprosy prevalence between populations. We used previously published prevalence data (Truman, 2005) from two populations west of the Mississippi River, Tensas National Wildlife Refuge (TS; 31°57′N, 91°23′W), Louisiana (18/77, 23% animals leprosy-positive), and Desha County (DC; 33°44′N, 91°16′W), Arkansas (9/42, 21% animals positive; Fig. 1) to compare prevalence between eastern and western populations. Because leprosy prevalence in these western populations was similar, we combined data from them for comparison with the eastern populations.

RESULTS

Population density

There were no obvious demographic differences among the sampled populations, but significantly more armadillos were observed per hour at Yazoo than at other sites where such data were available (Table 1, analysis of variance $F_{3,57}=69.47$, $P<0.0001$; post-hoc pair-wise comparisons were significant for Yazoo versus each other site, but there were no significant differences in pair-wise comparisons among the other three sites).

Intrapopulation genetic diversity

The loci *Dnov1*, *Dnov6*, *Dnov7*, *Dnov16*, and *Dnov24* revealed 10, 8, 4, 4, and 5 alleles, respectively. Indices of intrapopulation genetic diversity are presented in Table 2. Overall, intrapopulation genetic diversity declined slightly from west to east, as did allelic richness. We found no evidence for genotypic disequilibrium, as only four of the 70 tests performed (population–loci pair combinations) were statistically significant at the 0.05 level, and none remained significant after sequential Bonferroni correction (Ta-

TABLE 1. Prevalence of leprosy infection and other features of the nine-banded armadillo populations sampled.[a]

Population	No. sampled				No. of days (hours) of sampling	Density (No. of animals observed per hour)	No. infected (% positive)[b]
	Adults		Juveniles				
	Males	Females	Males	Females			
DeWayne Hayes Recreational Area	36	20	4	1	8 (37.77)	1.08±0.88	2 (3%)
Riverside (2006)	32	35	0	1	NA	NA	7 (10%)
Pinebloom Plantation	24	33	5	3	NA	NA	0
St. Catherine's Creek NWR	29	38	0	3	21 (134.80)	0.99±0.42	0
Stimpson Wildlife Sanctuary	30	33	2	5	14 (89.00)	1.04±0.44	1 (1%)
Yazoo NWR (2005 + 2006)	72	72	6	4	18 (88.25)	3.23±0.56	9 (6%)
Roadkills	7	3	1	1	NA	NA	0

[a] NA = not applicable; NWR = national wildlife refuge; PCR = polymerase chain reaction.

[b] All infected animals tested positive in both serologic and PCR analyses.

ble 3). Thus, the five loci can be considered statistically independent. However, we did find strong evidence for Hardy–Weinberg disequilibrium at one locus (*Dnov16*). Indeed, 11 of the 35 exact tests performed were significant at the 0.05 level, and six tests remained significant after Bonferroni correction (Table 3). These six tests corresponded to a strong heterozygote deficiency (F_{IS} from 0.743 to 1.000) at locus *Dnov16*. This may be due to the presence of null alleles in high frequencies. Hence, locus *Dnov16* was not included in subsequent population genetic analyses.

Population differentiation

All population pairs were significantly differentiated, even after Bonferroni correction. Levels of differentiation measured by F_{ST} values ranged from 0.030 between populations NO and ST to 0.232 between populations TT and DH. However, the tree constructed from genetic distances between populations did not show a marked population structure (Fig. 2). Interestingly, populations from Welder in Texas and Tall Timbers in Florida were relatively close to each other, even though they were geographically the most widely separated. Apart from this peculiar population pair, other populations clustered according to their geographic proximity (Fig. 2).

TABLE 2. Genetic polymorphism in seven populations of nine-banded armadillos.[a]

Population	n	Allelic richness	Observed heterozygosity (Ho)	Gene diversity (He)
DeWayne Hayes	20	3.77	0.63	0.57
New Orleans	20	3.95	0.79	0.69
St. Catherine's Creek	19	4.29	0.55	0.62
Stimpson	20	3.89	0.54	0.63
Tall Timbers	20	3.48	0.62	0.56
Welder	20	4.33	0.64	0.68
Yazoo	20	3.81	0.73	0.67

[a] Measures were averaged over four loci (*Dnov16* excluded).

Leprosy prevalence

A total of 500 armadillos were sampled (Table 1). No road-killed animals tested positive for leprosy, nor were any positive samples collected at Pinebloom or St. Catherine's, but each of the remaining three sites presented at least one leprosy-positive individual (Table 1). In all cases, infection was confirmed both serologically and with PCR. The prevalence presented for Yazoo is somewhat problematic because of fungal contamination on some of the Nobuto strips collected there. Consequently, a second estimate was obtained from the 71 animals (38 adult males and 33 adult females) relocated to LSU. Five (7.0%) of these animals were positive.

TABLE 3. Departures from Hardy–Weinberg proportions (F_{IS}) for seven populations of nine-banded armadillos at each of the five loci examined and their combination.

Population	*Dnov1*	*Dnov6*	*Dnov7*	*Dnov16*	*Dnov24*	All loci
DeWayne Hayes	−0.356[a]	0.069	0.043	0.887[b,c]	−0.068	−0.100
New Orleans	−0.197	−0.308[a]	0.000	0.900[b,c]	−0.083	−0.156[a]
St. Catherine's Creek	0.378[a]	−0.041	0.357	0.743[b,c]	−0.112	0.110
Stimpson	−0.206	0.293[a]	0.253	1.000[b,c]	0.179	0.143
Tall Timbers	−0.073	−0.136	−0.167	0.273	−0.088	−0.109
Welder	−0.143	−0.216	0.526	0.876[b,c]	0.089	0.056
Yazoo	−0.261	−0.039	−0.023	1.000[b,c]	−0.032	−0.094

[a] $P<0.05$.

[b] $P<0.001$.

[c] $P<0.05$ after Bonferroni correction.

Except for the small group of road-killed animals, all sampled populations exhibited significantly lower levels of leprosy prevalence in comparison with reference populations from west of the Mississippi River (Table 4). Leprosy prevalence at Riverside in 2006 was significantly higher than that observed in the Pinebloom and St. Catherine's samples, but otherwise there were no significant differences in prevalence among the sampled populations (Table 4).

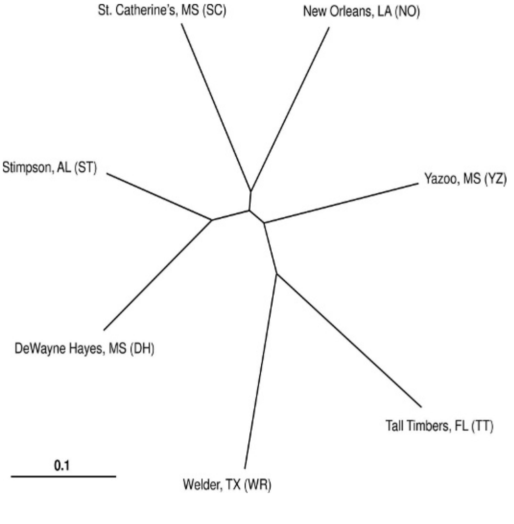

FIGURE 2. Neighbor-joining radial tree inferred from the Cavalli-Sforza and Edwards (1967) genetic distances between populations, as calculated from microsatellite data. Branch lengths are drawn proportionally to genetic distances between populations, expressed in units of expected numbers of mutations accumulated per locus.

DISCUSSION

The last extensive sampling for leprosy among armadillos east of the Mississippi River occurred in the late 1980s and found no evidence of infection based on histopathologic examination of nerve damage in ear tissues (Howerth et al., 1990). Although this method is less sensitive, the large sample size in that study argues against the possibility that infected animals were present and not detected, leading us to conclude that, historically, leprosy was not present in eastern populations. Our study indicates this is no longer true and provides a valuable update of current infection patterns.

Our data also provide some support for both hypotheses of leprosy occurrence in nine-banded armadillos. First, consistent with the ecologic-constraints hypothesis, no infected animals were found at St. Catherine's even though, like Yazoo, this population is located close to populations across the Mississippi River that exhibit substantial levels of infection (Fig. 1). Similarly, Yazoo, with its appropriate soil conditions, harbored a population with several infected individuals, although prevalence was significantly lower than in nearby populations to the west. In contrast, consistent with the epidemic hypothesis, both populations in eastern Mississippi/western Alabama contained at least one infected animal. Most remark-

TABLE 4. Chi-square values[a] for pair-wise comparisons of leprosy prevalence (proportion of infected individuals) among sampled populations of nine-banded armadillos.

Population[b]	TS + DC	YZ-all	YZ-LSU	DH-all	RS	PB	SC	ST	RK
TS + DC	—	—	—	—	—	—	—	—	—
YZ-all	15.20***	—	—	—	—	—	—	—	—
YZ-LSU	6.70**	0.002	—	—	—	—	—	—	—
DH-all	11.08***	0.02	0.07	—	—	—	—	—	—
RS	3.67*	0.81	0.14	0.29	—	—	—	—	—
PB	15.52***	2.62	2.97	3.31	5.15*	—	—	—	—
SC	16.48***	2.83	3.20	3.56	5.51*	NC[c]	—	—	—
ST	14.15***	1.29	1.52	1.88	3.47	0.001	0.001	—	—
RK	2.71	0.10	0.16	0.19	0.53	NC	NC	0.81	—

[a] * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

[b] Full names for all populations are given in Figure 1. DH-all includes data from DeWayne Hayes and Riverside collected in 2005 and 2006; YZ-LSU refers to 71 animals relocated from Yazoo to the Hansen's Disease research colony at Louisiana State University.

[c] NC = not calculated because two cells contained zeros.

able were the data from Riverside, where prevalence mirrored that found 240 km to the west at Yazoo. This is the first report of any leprosy-infected armadillos occurring at substantial distances east of the Mississippi River and indicates leprosy is spreading eastward. Thus, if ecologic constraints do influence the occurrence of leprosy, our results suggest they are not as restrictive as previously supposed (Truman, 1996, 2005).

Of course, the two hypotheses are not mutually exclusive, so both likely play a role. For example, environmental conditions in eastern populations might limit leprosy prevalence to levels much lower than those seen west of the Mississippi River, but infection could nonetheless persist because of dispersal of infected animals into these areas. Such a scenario is supported by data indicating armadillo populations are relatively fluid and that some individuals move considerable distances in short periods of time (Taulman and Robbins, 1996; Bond et al., 2000; Gammons, 2006; McDonough et al., 2007). Alternatively, waves of infection may have spread rapidly through the entire range of *D. novemcinctus* in the United States, with pockets of infection persisting in local areas containing appropriate environmental conditions (Brooks, pers. comm.). Additional sampling will be required to evaluate these possibilities.

Further sampling will be necessary to address other questions raised by our findings. For example, populations in central Mississippi need to be examined to determine whether there is a continuous distribution of infection across the state or if Stimpson and DeWayne Hayes/Riverside represent isolated eastern pockets of infected animals. Similarly, the prevalence of leprosy at Riverside suggests populations in central and eastern Alabama should be screened to ascertain whether the disease has spread even farther to the east.

Unfortunately, genetic analyses were of limited value in understanding the patterns of infection we report. The recent founding of US populations seems to have resulted in high levels of uniformity at both protein (Ramsey and Grigsby, 1985; Moncrief, 1988; Huchon et al., 1999) and molecular genetic levels (Loughry et al., 1998; Huchon et al., 1999), which prevent easy discrimination among them. Ideally, analyses of gene flow could provide important insights. For example, the epidemic hypothesis would be strengthened if we could show that animals at Stimpson and Riverside possess genetic markers shared by infected populations to the west. However, the limited number of loci we examined did not permit such fine-scale assessment.

Nevertheless, our genetic data did retrieve the historic pattern of colonization in confirming that founding populations from Florida were derived from individuals of western origin in Texas. Moreover, the pattern of intrapopulation genetic diversity was consistent with the colonization history of the sampled area. It is also noteworthy that populations from Mississippi (Yazoo and St. Catherine's) and Louisiana (New Orleans) had a genetic diversity comparable to that of the reference source population from Texas (Welder). Two alternative scenarios might explain these observations. The first is that there has been no erosion of genetic diversity following colonization toward the northeast from the region around Welder. The second envisions an initial decrease in genetic diversity following this colonization, but which was later counterbalanced by an increase in diversity because of the meeting of populations coming from the east after their introduction in Florida. Both additional sample sites and more molecular markers (such as those now available because of the sequencing of the *D. novemcinctus* genome; Chang and Adams, 2008) are required to evaluate these alternatives and allow more precise determination of gene flow among US armadillo populations.

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