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Prevalence of zoonotic *Bartonella* among prairie rodents in Illinois

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Bartonella is a genus of gram-negative bacteria that includes a variety of human and veterinary pathogens. These pathogens are transmitted from reservoirs to secondary hosts through the bite of arthropod vectors including lice and fleas. Once in the secondary host, the bacteria cause a variety of pathologies including cat-scratch disease, endocarditis, and myocarditis. Reservoirs of these bacteria are numerous and include several species of large mammals, mesocarnivores, and small mammals. Research on reservoirs of these bacteria has focused on western North America, Europe, and Asia, with little focus on the eastern and central United States. We assessed the prevalence of zoonotic *Bartonella* species among prairie-dwelling rodent species in the midwestern United States. Tissue samples (n = 700) were collected between 2015 and 2017 from five rodent species and screened for the presence of *Bartonella* DNA via PCR and sequencing of two loci using *Bartonella*-specific primers. *Bartonella* were prevalent among all five species, with 13-lined ground squirrels (*Ictidomys tridecemlineatus*) serving as a likely reservoir of the pathogen *B. washoensis*, and other rodents serving as reservoirs of the pathogens *B. grahamii* and *B. vinsonii* subsp. *arupensis*. These results demonstrate the value of studies of disease ecology in grassland systems, particularly in the context of habitat restoration and human–vector interactions.

Key words: *Bartonella*, *Ictidomys*, *Microtus*, prairie, reservoir, vector, *Zapus*

Bartonella is a genus of bacteria of increasing research interest due to the emergence of numerous zoonotic diseases associated with several of its members (Karem et al. 2000; Breitschwerdt et al. 2010; Angelakis and Raoult 2014). *Bartonella* species are obligate intracellular parasites that infect the endothelial lining of blood vessels and erythrocytes (Pulliainen and Dehio 2012). Recently, several new species of *Bartonella* have been identified, (Sato et al. 2013; Li et al. 2015; McKee et al. 2017) including pathogenic species (Eremeeva et al. 2007; Vayssier-Taussat et al. 2016). Additionally, while infections have historically been considered rare, diagnoses of both human and non-human *Bartonella* infections have increased (Breitschwerdt et al. 2010; Chomel and Kasten 2010). Concurrently, studies on identification of potential reservoirs of *Bartonella* are lacking, particularly in prairie ecosystems (Jardine et al. 2005; Buffet et al. 2013).

Bartonella are transmitted both horizontally and vertically. Horizontal transmission occurs primarily through blood-feeding arthropods (Billeter et al. 2008) including biting flies (Dehio et al. 2004), sand flies (Ready 2013), fleas (Chomel et al. 1996; Stevenson et al. 2003), and lice (Alsmark et al. 2004; Bonilla et al. 2009). The role of ticks as vectors of *Bartonella* is poorly understood, with studies identifying the presence of pathogenic *Bartonella* species in ticks (Adelson et al. 2004; Kim et al. 2005; Nelder et al. 2016), but conflicting evidence on the competency of ticks as vectors (Angelakis et al. 2010; Reis et al. 2011; Müller et al. 2016). Comparatively, vertical transmission has been identified in both vectors (Halos et al. 2004; de Bruin et al. 2015; Leulmi et al. 2015) and reservoirs (Kosoy et al. 1998; Cevidanes et al. 2017; Tołkacz et al. 2018). However, this transmission is not universal, with several species showing no evidence of vertical transmission (Bown et al. 2004; Bouhsira et al. 2013; Morick et al. 2013).

Of the 35 currently recognized species of *Bartonella*, many are known to be pathogenic to cattle, companion animals (Breitschwerdt and Kordick 2000; Breitschwerdt et al. 2010), and humans (Gutiérrez et al. 2015; Silaghi et al. 2016). In humans, these species include the causative agents of Carrion's disease, cat-scratch disease, and multiple agents of myocarditis, endocarditis, and tumorigenesis in immunocompromised individuals (Karem et al. 2000; Pulliainen and Dehio 2012). Although it is not understood if *Bartonella* are pathogenic to rodents (Lei and Olival 2014; Kim et al. 2016; Malania et al. 2016), at least five species isolated from rodents are known to

be pathogenic to humans (Silaghi et al. 2016), and several rodents have been implicated as reservoirs of *Bartonella* species (Buffet et al. 2013). These rodent species cover a wide taxonomic breadth, including members of Cricetidae (Kosoy et al. 2004; Bai et al. 2011), Muridae (Ellis et al. 1999; Mediannikov et al. 2005; Kosoy et al. 2010), and Sciuridae (Kosoy et al. 2003; Stevenson et al. 2003; Bai et al. 2008a, 2008b).

Among these potential reservoirs, only the black-tailed prairie dog (*Cynomys ludovicianus*) is primarily associated with prairie habitat. Prairie habitats are of increasing interest with regard to disease reservoirs and transmission. With as much as 99% of historical tallgrass prairies in the United States converted for human use (Samson and Knopf 1994), a great effort has focused on prairie restoration (Ruiz Jaen and Aide 2005; Wortley et al. 2013). In many cases, these restorations lie within close proximity to urban and suburban areas (Fischer et al. 2013; Klaus 2013). While there are numerous benefits to such restoration, one downside is increased contact between humans and both vectors and reservoirs of disease due to incidental and recreational contact (Miller 2006; Tóth et al. 2009). This contact creates an increased potential for zoonotic transmission. As such, studies of rodent communities in restored prairie ecosystems are of high consequence for the identification of potential disease reservoirs.

In this study, we determined if zoonotic *Bartonella* were present among prairie-dwelling rodents in a restored tallgrass ecosystem in a suburban area of the Midwest region of the United States. We also assessed the temporal dynamics of prevalence of those *Bartonella* among rodents.

Materials and Methods

Study site and sample collection.—We collected samples at the Distillery Road Conservation Area in Boone County, Illinois (42.2589°N, 88.9095°W). This site is a ~30-ha tallgrass prairie restoration in a suburban area managed by the Boone County Conservation District (BCCD) that was previously used for crop agriculture. Prairie restoration efforts began in 2003, with various patches undergoing restoration through 2010. Management efforts include seeding with native grasses and forbs, invasive species removal, and whole site controlled burns on a 3-year cycle (BCCD 2018).

Two 0.6-ha trapping grids were established in 2015 (40 m × 150 m) in separate patches of the restoration. The first was placed in a patch restored in 2003, and the second in a patch restored in 2008. The grids were separated by ~250 m of contiguous prairie habitat. On each grid, we established 60 trap stations (four rows of 15 traps) separated by 10 m. We placed either a Sherman live trap (H. B. Sherman Co., Tallahassee, Florida) or modified Fitch trap (Rose 1973) at each station in an alternating manner. We conducted trapping on 2 consecutive days each week from mid-May to mid-September from 2015 to 2017. Traps were baited with commercial squirrel and critter blend (Kaytee Products, Chilton, Wisconsin) between 1,600 and 1,800 h and checked the next morning between 0600 and 0800 h. This maximized the likelihood of catching both diurnal and nocturnal species.

For each captured individual, we recorded species, age, sex, reproductive condition, and mass. Animals were examined for ticks, and collected ticks were placed in 70% ethanol solution for later DNA extraction. The distal phalanx of the second lateral digit of the hind foot was removed using sterile scissors and was also stored in 70% ethanol for later DNA extraction. Toe clips were chosen over ear punches or tail clips due to the small size of the pinna of *Ictidomys* and *Microtus* species, and the tendency of the skin of *Ictidomys* tails to detach when handled. Retroorbital bleeding was not considered due to the need to anesthetize animals and the inclusion of inexperienced undergraduate students in sampling. In addition, toe clips provided an opportunity to differentiate between recaptured and new animals. Toe clipping was superior to the use of ear tags due to the incidence of ear tag loss in burrowing species and the lack of a need for individual identification. After we completed tissue sampling, animals were retained in hand to ensure bleeding had ceased and were released at the site of capture within 5 min of initial handling. Protocols for animal handling and tissue sampling were approved by the Illinois Department of Natural Resources and the BCCD. No animal care and use committee existed at the institution where this research was initiated, but all methods complied with guidelines of the American Society of Mammalogists (Sikes et al. 2016).

DNA extraction.—Prior to extraction, tissue samples were removed from ethanol, dried, and diced using a sterile scalpel blade to maximize surface area. Tissue samples were then extracted using a standard ammonium acetate procedure with ethanol precipitation (Supplementary Data SD1). Following extraction, DNA was reconstituted and stored in 50 µl of Tris-EDTA buffer.

Genotyping of Microtus *species.*—To verify the identity of *Microtus* species, we genotyped individuals at a microsatellite locus of the *avpr1a* gene using established methods (Fink et al. 2006; Henterly et al. 2011). This method distinguishes species on the basis of the number of repeats with *M. pennsylvanicus* producing a 200–300 bp product and *M. ochrogaster* producing a 600–800 bp product.

Identification of Bartonella *presence.*—We screened rodent DNA samples for the presence of *Bartonella* species via PCR of the 16-23S intergenic spacer (IGS) region using the methods of Jensen et al. (2000). Primers in our study were designed to specifically target and amplify a portion of the 16S–23S IGS region of medically relevant *Bartonella* species. The forward and reverse primers were 5′-CTCTTTCTTCAGATGATGA TCC-3′ and 5′-AACCAACTGAGCTACAAGCCCT-3′, respectively. Targets were amplified in a 50 µl reaction and each PCR contained a sample run with sterile water rather than sample DNA to act as a negative control. Each DNA sample was run two to three times to verify amplification. Products were then loaded onto a 2% agarose gel as described above and electrophoresed for 60 min at 100 V in 1× TBE buffer before visualization on a UV light box. PCR products between 150 and 250 bp were identified as positive.

Any specimens that successfully amplified for the IGS region were further screened via nested PCR of a portion of the citrate

synthase gene (gltA) using the methods of Bai et al. (2016). Targets were amplified in a 50 µl reaction and each PCR contained a sample run with sterile water rather than sample DNA to act as a negative control. Products were loaded onto a 2% agarose gel and electrophoresed for 90 min at 100V in 1× TBE buffer. A specimen was considered positive for *Bartonella* if an ~350 bp product was identified.

Bartonella *species identification.*—Due to the lack of reliability of PCR-only methods in identifying *Bartonella*, and the desire to identify isolates to species, a multilocus sequencing approach was performed as recommended by Kosoy et al. (2018). To do this, amplicons were gel-extracted using the method of Sun et al. (2012) and sequenced in both directions (Eurofins MWG). Resulting sequences were aligned using BioEdit version 7.0.5 and sequence identity was determined using BLAST (Altschul et al. 1990). A minimum sequence similarity of 96% was required to identify gltA sequences to species (La Scola et al. 2003). The gltA sequence identity was also used for species identification if an IGS sequence was most similar to an unidentified *Bartonella*, or IGS sequences similarity was below 96%. Upon successful species identification, unique sequences were deposited in GenBank (accession numbers: MK984778–MK984795).

Statistical analyses.—We used chi-square tests to determine if abundance of rodent species were consistent among years during the study. For each species, we calculated the average infection rate within and across years and used the Clopper–Pearson method to determine the 95% confidence interval of those rates. A logistic regression model was fit to the data (JMP version 10; SAS Institute Inc. 2012) to determine which variables (species, year, species × year) explained the prevalence of *Bartonella*.

Results

Over the 3 years, we captured and sampled 700 individuals representing five species: 13-lined ground squirrel, *Ictidomys tridecemlineatus*; meadow jumping mouse, *Zapus hudsonius*; deer mouse, *Peromyscus maniculatus*; meadow vole, *M. pennsylvanicus*; and prairie vole, *M. ochrogaster*. Following microsatellite genotyping, it was confirmed that the majority of animals trapped were *M. pennsylvanicus* ($n = 275$) followed by *I. tridecemlineatus* ($n = 193$) and *Z. hudsonius* ($n = 176$). Two other species were much less abundant: *M. ochrogaster* ($n = 34$) and *P. maniculatus* ($n = 22$). Three of the species had consistent abundances across years: *I. tridecemlineatus* ($\chi^2_2 = 0.69$, $P = 0.707$), *M. ochrogaster* ($\chi^2_2 = 2.17$, $P = 0.337$), and *M. pennsylvanicus* ($\chi^2_2 = 5.29$, $P = 0.071$). Both *P. maniculatus* ($\chi^2_2 = 19.73$, $P < 0.001$) and *Z. hudsonius* ($\chi^2_2 = 14.33$, $P < 0.001$) exhibited temporal fluctuations during the study.

Bartonella was identified in all five captured species. After fitting a logistic regression model ($\chi^2_9 = 43.54$, $P < 0.0001$), log ratio tests indicated there was no effect of sampling year on the prevalence of *Bartonella* ($\chi^2_1 = 2.481$, $P = 0.1152$). However, there was an effect of species ($\chi^2_4 = 29.709$, $P = 0.0001$) on prevalence, and an interaction effect between species and year ($\chi^2_4 = 12.538$, $P = 0.0138$). Infection rates were similar among most species, with the exception of *I. tridecemlineatus*, which exhibited a rate that was 1.8–2.3 times that of other species (Table 1). There was no consistent pattern across species with regard to infection rates over time (Fig. 1). *Zapus hudsonius* exhibited little variation during the study. By comparison, *I. tridecemlineatus* and *M. ochrogaster* exhibited modest interannual variation and *M. pennsylvanicus* experienced a sharp decline in infection rate during the study. While *P. maniculatus* exhibited a high infection rate in 2015 ($n = 17$); small sample sizes in 2016 ($n = 4$) and 2017 ($n = 1$) resulted in no observed infections.

Eight *Bartonella* genotypes were identified from IGS amplicons. Three genotypes were most similar to a *B. grahamii* reference genome (accession: CP001621; 95.06–96.89% similarity; Fig. 2). All specimens with these genotypes shared a common gltA genotype that was most similar to the same *B. grahamii* reference genome (accession: CP001621; 99.21% similarity). A fourth IGS genotype was most similar to a reference sequence for an uncultured *Bartonella* species (accession: KX267694; 97.33% similarity). This genotype was found in *M. pennsylvanicus* and *P. maniculatus* harboring one of two gltA genotypes. Each of those gltA genotypes was most similar to a reference sequence from *B. vinsonii* subsp. *arupensis* (accession: FJ946836; 96.99–97.27% similarity; Fig. 2). The remaining four IGS genotypes were found in *I. tridecemlineatus* and were highly similar to a reference sequence for ***B. washoensis*** (accession: AB674251; 98.24–99.41% similarity). Ground squirrels with those four IGS genotypes contained one of four gltA genotypes, all of which were most similar to a reference sequence from *B. washoensis* (accession: DQ834440; 97.87–99.73% similarity; Fig. 2).

Discussion

Numerous studies have identified the role of rodent species as reservoirs of pathogenic *Bartonella* strains (Kosoy et al. 2012; Buffet et al. 2013; Gutiérrez et al. 2015). However, these studies were mainly focused in western North America, Europe, and Asia. As a result, several widespread North American species have not been assessed as potential reservoirs of *Bartonella*, including prairie-dwelling rodents in the midwestern United States. This study identified at least one likely reservoir of these bacteria, *I. tridecemlineatus*, while also identifying several other candidates, and adds to an expanding understanding of the prevalence of pathogenic *Bartonella* species among small mammals (Buffet et al. 2013). Our results also suggest

Table 1.—Overall *Bartonella* infection rates and confidence intervals (*CI*) for the five rodent species tested during 2015–2017 in Illinois.

Species	No. individuals	Infection rate (%)	95% *CI* (%)
Ictidomys tridecemlineatus	193	39.38	32.44–46.65
Microtus ochrogaster	275	17.65	6.76–34.53
Microtus pennsylvanicus	34	18.91	14.46–24.0
Peromyscus maniculatus	22	18.18	5.19–40.28
Zapus hudsonius	176	21.59	15.76–28.41

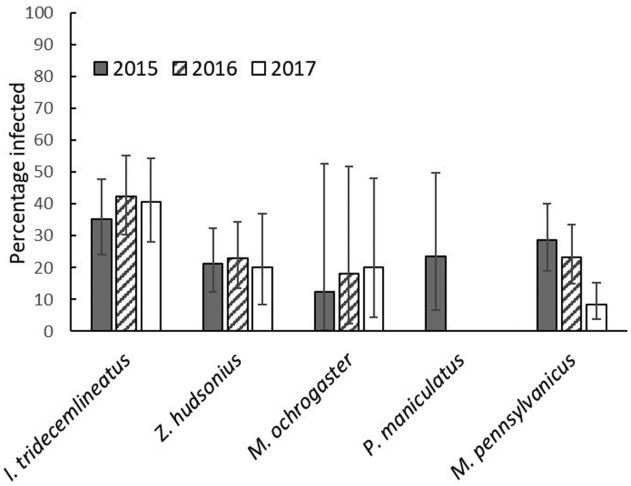

Fig. 1.—Percentage (±95% *CI*) of captured individuals (n = 700) that tested positive for *Bartonella* between 2015 and 2017. Five species of rodents were tested for *Bartonella* via PCR of the intergenic spacer region using *Bartonella*-specific primers: 13-lined ground squirrels (*Ictidomys tridecemlineatus*; n = 193), meadow jumping mice (*Zapus hudsonius*; n = 176), prairie voles (*Microtus ochrogaster*; n = 34), deer mice (*Peromyscus maniculatus*; n = 22), and meadow voles (*Microtus pennsylvanicus*; n = 275).

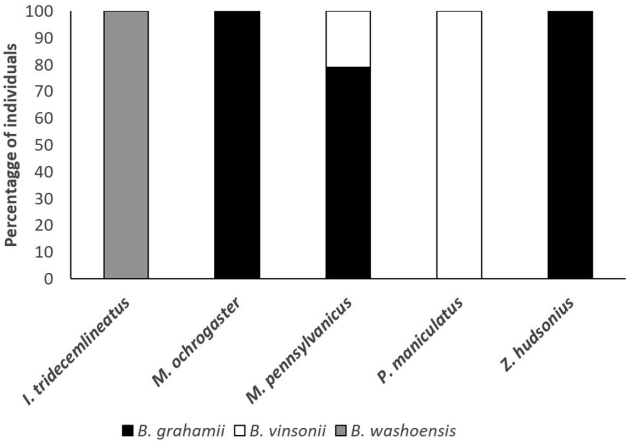

Fig. 2.—Distribution of *Bartonella* species identified among DNA collected from rodent tissues. *Bartonella* species were identified by sequencing of two loci using species-specific primers. Most rodent species harbored only a single species of *Bartonella*. *Microtus pennsylvanicus* harbored two species with 79% of specimens containing *B. grahamii* and 21% containing *B. vinsonii* subsp. *arupensis.*

that habitat restoration efforts in human-dominated landscapes may have the unintended consequence of increasing the likelihood of zoonotic disease transmission to humans and companion animals.

Infection rates exhibited substantial variation among species and were highest among *I. tridecemlineatus*. Of the species of ground squirrels sampled elsewhere, albeit mostly with small sample sizes, all show evidence of *Bartonella* infection (Stevenson et al. 2003; Inoue et al. 2011; Kosoy et al. 2012; Ziedins et al. 2016). Most of this research has focused on Richardson's ground squirrel (*Urocitellus richardsonii*) in Nevada and Saskatchewan (Jardine et al. 2005; Kosoy et al. 2003) with the latter study demonstrating a nearly 50% infection rate. While our estimated infection rate in *I. tridecemlineatus* (39.38%) is lower than that identified by Kosoy et al. (2003) for *U. richardsonii*, it is similar to the 37% Jardine et al. (2006) estimated for adult *U. richardsonii* ($\chi^2 = 0.636$, $P = 0.425$). These data suggest that *I. tridecemlineatus* may represent a reservoir of a known human pathogen in the midwestern United States.

Due to small sample sizes and inconsistent capture frequency across years, it is not possible to make similar assertions about the reservoir status of *P. maniculatus* in the midwestern United States. However, the small number of *P. maniculatus* that tested positive in our study (four out of 22) all harbored *B. vinsonii arupensis*. This supports the findings of other studies that have identified *P. maniculatus* as a likely reservoir of *B. vinsonii arupensis* (Bai et al. 2011; Buffet et al. 2013; Rubio et al. 2014).

Our data represent the first large-scale assessment of *Bartonella* in any *Microtus* populations in North America. Baker (1946) previously identified the presence of *B. vinsonii vinsonii* in five out of 10 *M. pennsylvanicus* captured in Quebec, Canada. Since then, Inoue et al. (2009a) identified the presence of *B. grahamii* in three *M. ochrogaster* from South Dakota. Although our data indicate *Microtus* in the midwestern United States harbor *Bartonella* at lower levels than in those studies, it is difficult to compare prevalence rates because those studies had small sample sizes. In addition, studies with larger sample sizes conducted on European *Microtus* species found prevalence rates between 20% and 27.7% (Engbaek and Lawson 2004; Pawelczyk et al. 2004), which are in line with the rates in this study for *M. pennsylvanicus* (28.57% in 2015) and *M. ochrogaster* (20% in 2017). In our study, *M. pennsylvanicus* also demonstrated interannual variation in infection rate with an unexplained marked decrease in 2017. These results were verified by reperforming the PCR analysis. Without additional sampling, however, it is not possible to determine if the decrease was due to random fluctuation or unmeasured environmental factors.

We identified two species of pathogenic *Bartonella* in *Microtus* at our study site. *Bartonella grahamii* has been associated with cases of neuroretinitis (Kerkhoff et al. 1999) and cat-scratch disease (Oksi et al. 2013) in immunocompromised individuals. This is the first study to identify the presence of *B. grahamii* in *M. pennsylvanicus*. However, several European studies reported that *B. grahamii* is widespread among other *Microtus* species (Telfer et al. 2007; Tołkacz et al. 2018). Additionally, our findings of *B. grahamii* in *M. ochrogaster* support those of Inoue et al. (2009a). Collectively, these results suggest a role of North American *Microtus* in the enzootic cycle of *B. grahamii*.

We also identified the presence of *B. vinsonii* subsp. *arupensis* in *M. pennsylvanicus*, albeit at a lower prevalence (21% of infections) than *B. grahamii*. This is the first time *arupensis* has been identified in any *Microtus* species. This pathogen is more commonly associated with *Peromyscus* (Bai et al. 2011; Ziedins et al. 2016) but *arupensis* has been identified in other vole species in Alaska

(Matsumoto et al. 2010). Likewise, the closely related *B. vinsonii* subsp. *vinsonii* was first characterized in *M. pennsylvanicus* (Baker 1946). Further, the co-occurrence of infected *M. pennsylvanicus* and *P. maniculatus*, and the high degree of sequence similarity of *B. vinsonii* subsp. *arupensis* among rodent species in this study, suggests the high likelihood that this bacterium can be transmitted between reservoirs via arthropod vectors.

Zapus hudsonius is also of interest as a potential reservoir. This species exhibited the most consistent prevalence rates across the study and was also one of the most abundant species. No studies have focused on *Bartonella* prevalence in *Zapus* or any North American members of Dipodidae (Buffet et al. 2013). Only one study examined *Bartonella* in any member of Dipodidae and found infection rates ranging from 0% to 81% among jerboas in the Japanese pet trade (Inoue et al. 2009b). Our data indicate that *Z. hudsonius* harbor *B. grahamii* at similar or higher levels than *Microtus* species (Table 1). Such prevalence rates, combined with the wide geographic range of the species and its preference for grassland habitats, suggest *Z. hudsonius* may be a reservoir of *Bartonella* in these ecosystems. An increased focus on *Zapus* is warranted in the study of *Bartonella* and other vector-borne diseases.

Our results indicate that prairie systems, particularly prairie restorations, may play a substantial role in zoonotic transmission of *Bartonella*. Urban and suburban restorations are capable of supporting diverse and stable populations of small mammals. Many of these are potential reservoirs of pathogenic *Bartonella* and other vector-borne pathogens. In this study, all five rodent species at the site were infected with *Bartonella*, and all *Bartonella* species were known pathogens. In addition, these areas present contact points where reservoirs, vectors, and dead-end hosts, such as humans, comingle, permitting pathogen spillover (Alexander et al. 2018). This interaction is enhanced in recreation areas, such as mowed paths, where vectors are more likely to occur (Hahn et al. 2018), increasing the likelihood of disease transmission to people using these areas (McDaniel et al. 2018).

To understand more broadly how these prairie rodents function in harboring and transmitting pathogenic *Bartonella*, it is essential to conduct additional studies of disease prevalence in other grassland ecosystems. These studies are particularly valuable in the midwestern United States where these pathogens and their reservoirs are poorly researched. Moreover, it is necessary to assess the ability of these organisms to transmit the diseases to known and potential vectors.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Protocol for DNA extraction.

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