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Genetic Variation and Structure in Contrasting Geographic Distributions: Widespread Versus Restricted Black-Tailed Prairie Dogs (Subgenus *Cynomys*)

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Abstract

Species of restricted distribution are considered more vulnerable to extinction because of low levels of genetic variation relative to widespread taxa. Species of the subgenus *Cynomys* are an excellent system to compare genetic variation and degree of genetic structure in contrasting geographic distributions. We assessed levels of genetic variation, genetic structure, and genetic differentiation in widespread *Cynomys ludovicianus* and restricted *C. mexicanus* using 1997 bp from the cytochrome *b* and control region (n = 223 *C. ludovicianus*; 77 *C. mexicanus*), and 10 nuclear microsatellite loci (n = 207 and 78, respectively). Genetic variation for both species was high, and genetic structure in the widespread species was higher than in the restricted species. *C. mexicanus* showed values of genetic variation, genetic structure, and genetic differentiation similar to *C. ludovicianus* at smaller geographic scales. Results suggest the presence of at least 2 historical refuges for *C. ludovicianus* and that the Sierra Madre Occidental represents a barrier to gene flow. Chihuahua and New Mexico possess high levels of genetic diversity and should be protected, while Sonora should be treated as an independent management unit. For *C. mexicanus*, connectivity among colonies is very important and habitat fragmentation and habitat loss should be mitigated to maintain gene flow.

Resumen

Las especies de distribución restringida pueden ser consideradas más vulnerables a la extinción debido a la presencia de niveles bajos de variación genética, en contraste con los niveles de variación

presentes en especies de amplia distribución. Las especies del subgénero *Cynomys* son un sistema excelente para comparar la variación genética y el grado de estructura genética en distribuciones geográficas contrastantes. Evaluamos los niveles de variación genética, de estructura genética y de diferenciación genética en una especie de distribución amplia *Cynomys ludovicianus* y una especie de distribución restringida *C. mexicanus* utilizando 1997 pb del citocromo b y la región control (n = 223 *C. ludovicianus*; 77 *C. mexicanus*) y diez loci de microsatélites nucleares (n = 207 y 78, respectivamente). La variación genética en ambas especies fue alta y la estructura genética en *C. ludovicianus* fue mayor que la de la especie de distribución restringida. *C. mexicanus* presentó valores de variación genética, estructura genética y diferenciación genética similares a los que se han observado en *C. ludovicianus* a escala geográfica local. Los resultados sugieren la presencia de al menos dos refugios históricos para *C. ludovicianus* y que la Sierra Madre Occidental representa una barrera al flujo génico. Las poblaciones de Chihuahua y Nuevo México presentaron altos niveles de diversidad genética y deben protegerse, mientras que la población de Sonora debe ser tratada como una unidad de manejo independiente. Para *C. mexicanus* la conectividad entre colonias es muy importante y la fragmentación y pérdida de hábitat deben ser mitigadas para mantener el flujo génico entre colonias.

Subject areas: Population structure and phylogeography, Conservation genetics and biodiversity
Key words: Conservation genetics; *Cynomys ludovicianus*; *Cynomys mexicanus*; microsatellites; mitochondria; phylogeography

Patterns of genetic variation and structure between widespread and endemic taxa have been the main focus of many population and conservation genetics studies (Hamrick and Godt 1996; Gitzendanner and Soltis 2000; Broadhurst and Coates 2002; Coates et al. 2003; Eguiarte et al. 2013; Hobbs et al. 2013; Blair et al. 2014). Nevertheless, this approach has been seldom used in the study of mammals (Moraes-Barros et al. 2006; Campbell et al. 2007; Blair et al. 2014). Evolutionary trajectories of species with restricted geographic ranges resemble those of small populations. In this regard, species with restricted ranges are often found in small and isolated populations that possess low levels of genetic variation due to the ongoing effects of genetic drift and inbreeding. This in turn could increase their risk of extinction (Broadhurst and Coates 2002; Coates et al. 2003; Frankham et al. 2004).

Previous empirical studies in plants and animals have not reached a consensus on whether restricted species possess lower levels of genetic variation than widespread taxa (Gitzendanner and Soltis 2000; Coates et al. 2003; Hobbs et al. 2013; Blair et al. 2014). This relates to the heterogeneity of their life histories, because levels of genetic variation depend not only on the actual population size, but also on the complex demographic historical patterns, adaptation, natural selection, and reproductive ecology (Hamrick and Godt 1996; Gitzendanner and Soltis 2000; Kelley et al. 2000; Broadhurst and Coates 2002; Hinten et al. 2003; Boessenkool et al. 2007; Raduski et al. 2010; Bock et al. 2012; Hobbs et al. 2013).

In general terms, species with restricted distributions are expected to show lower levels of genetic structure under an isolation-by-distance model, as the different populations would be seldom or never far away (Coates et al. 2003). Genetic structure is associated with the breeding system, dispersal capacity, and historical isolation, among other factors (Broadhurst and Coates 2002; Moraes-Barros et al. 2006; Campbell et al. 2007; Hedrick 2011). Consequently, the degree of historical isolation and gene flow between populations of widespread taxa varies considerably, and even restricted species, depending on their evolutionary history, can show deep phylogeographic divergence (especially if they are habitat specialists—Moritz 1999).

In this context, restricted species face a higher extinction risk than their widespread congeners, and conservation action should focus on the maintenance and restoration of microevolutionary processes that determine the distribution of genetic variation (Moritz 1999; Frankham et al. 2004). Phylogeography is crucial to understanding the dynamics of species distributions, their genetic variation and structure, and the factors that influence them (Rodríguez-Sánchez et al. 2010). Therefore, phylogeography is of major importance for conservation and management of endangered species.

Black-tailed prairie dogs (Subgenus *Cynomys*) are an illustrative system for the study of genetic variation and genetic structure in both widespread and restricted species. Black-tailed (*Cynomys ludovicianus*) and Mexican (*C. mexicanus*) prairie dogs are associated with the arid grasslands of North America because they are keystone species and "ecosystem engineers" that depend on open grasslands for their survival (Slobodchikoff et al. 2009; Martínez-Estévez et al. 2013). Currently, *C. ludovicianus* is the species with the widest range and can be found in the Great Plains of North America, from southern Canada to northern Mexico. On the other hand, *C. mexicanus* is endemic to Mexico and inhabits valleys within a 477 km^2 region in central Mexico (Scott-Morales et al. 2005; Slobodchikoff et al. 2009). McCullough and Chesser (1987) assessed allozyme diversity in both species and determined low genetic differentiation among populations of *C. mexicanus*. Genetic variation levels in *C. mexicanus* were high and similar to those reported by Chesser (1983) for populations of *C. ludovicianus* separated by long geographic distances in New Mexico. Gene flow between populations of *C. mexicanus* was high and similar to that reported by Chesser (1983) for colonies of *C. ludovicianus* located in close proximity. Nevertheless, the results from these analyses were based on a single type of low-resolution molecular marker (14 allozyme loci) and on a limited sample size for both species (29 samples from *C. mexicanus*, and 15 samples from *C. ludovicianus* from 3 colonies each).

Despite their importance for grassland conservation, prairie dog populations have faced a severe reduction and fragmentation of their distribution. *C. ludovicianus* currently occupies only approximately 2% of its historical distribution, while *C. mexicanus* is found in 26% of its smaller historical distribution (Scott-Morales et al. 2005; Slobodchikoff et al. 2009). Although the IUCN lists *C. ludovicianus* as a species of least concern for conservation, it is regarded as threatened within Mexico. *C. mexicanus* is considered as an endangered species by Mexican law, CITES (Appendix I; www.cites.org) and the IUCN (Semarnat 2010; Cites 2013; IUCN 2014).

Phylogenetically, *C. ludovicianus* and *C. mexicanus* are sister species, and evidence from the fossil record and molecular analyses is consistent with the hypothesis of the origin of *C. mexicanus* from a relict population of *C. ludovicianus*, diverging 20 000–40 000 years ago (McCullough and Chesser 1987; Goodwin 1995; Harrison et al. 2003). Both species are highly social and live in associations called colonies that are composed of social groups called coteries. Each coterie consists of several related adult females, 1 or 2 unrelated adult males, and their progeny. Females are philopatric and dispersal is male-biased (Ceballos and Wilson 1985; Hoogland 1996, 2013; Slobodchikoff et al. 2009).

The aim of this study is to assess patterns of genetic variation, genetic differentiation, and genetic structure in a restricted species (*C. mexicanus*) and a closely related widespread species (*C. ludovicianus*). For this analysis, we use concatenated sequences of the control region (*CR*) and cytochrome *b* (*cyt-b*) of the mitochondrial DNA (mtDNA), and 10 nuclear microsatellite loci. We predict that, at the species level, widespread *C. ludovicianus* will show higher levels of genetic variation, higher genetic differentiation among sites and higher genetic structure than restricted *C. mexicanus*. Furthermore, we also predict that given the biological similarities between these species, *C. mexicanus* will show overall values of genetic differentiation among colonies similar to those reported between colonies of *C. ludovicianus* within regions (i.e. between colonies from Janos, Chihuahua—Castellanos-Morales et al. 2014). Finally, we discuss the implications of our results for the conservation of each species.

Materials and Methods

Sample Collection

Given the broad distribution of *C. ludovicianus*, and to exclude possible influences on genetic variation such as strong differences in climates, soils, and seasonality that are faced by populations from outside Mexico, we analyzed populations of *C. ludovicianus* from the southern part of their range. These populations inhabit arid short-grass prairies that are ecologically similar to the area occupied by *C. mexicanus*. Castellanos-Morales et al. (2014) obtained samples from 161 black-tailed prairie dogs (*C. ludovicianus*) from 13 colonies located in Janos, Chihuahua (Chi), Mexico in 2007. Between 2009 and 2013, we obtained additional samples from 152 prairie dogs of both species: 74 samples from *C. ludovicianus* from Sonora (Son), Colorado (CO), and New Mexico (NM), and 78 samples from *C. mexicanus* from 6 colonies throughout its distribution (Figure 1). Several family groups within each colony were identified and 1 or 2 members from each family group were captured following the method described in Castellanos-Morales et al. (2014) and Sackett et al. (2012). Capture and nonlethal sampling was performed following the American Society of Mammalogists (Sikes et al. 2011) and Secretaria del Medio Ambiente y Recursos Naturales guidelines for ethical animal experimentation.

Samples consisted of 1 mm of fresh tissue from the tip of the tail, and 2 mm ear punches (Braintree Scientific) for the prairie dogs from Colorado. Tissue was obtained from the tip of the tail by making a clear cut using sterile surgical scissors. The injury was treated to prevent infection and the prairie dog was released at capture site. Tissue was deposited in a 2-mL Eppendorf tube containing 90% ethanol. All samples were maintained at –80 °C until DNA extraction.

DNA Extraction/PCR Amplification

Total genomic DNA was extracted from tissue samples with a Qiagen Blood and Tissue Kit (QIAGEN Sample & Assay Technologies, Hilden, Germany). mtDNA *cyt-b* sequences were obtained using primers L14725 (5′-TGAAAAAYCATCGTTGT-3′) and H15915 (5′-TCTTCATTTYWGGTTTACAAGAC-3’) (Harrison et al. 2003),

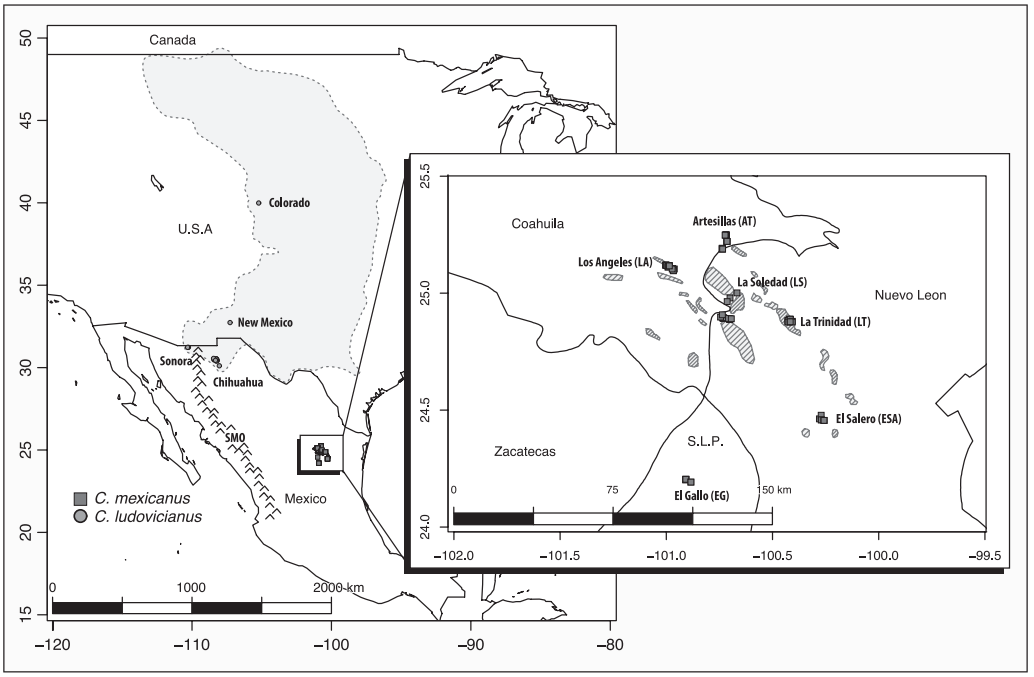

Figure 1. Spatial location of the sampled areas within the distribution of *C. ludovicianus* (gray dots) and *C. mexicanus* (gray squares). The figure shows the distribution of black-tailed prairie dogs (*C. ludovicianus*) as a light gray polygon demarcated by a dashed line, and the distribution of Mexican prairie dogs (*C. mexicanus*) is depicted as hatched polygons. Location of the Sierra Madre Occidental (SMO) is shown as a barrier separating colonies from Sonora and the rest of *C. ludovicianus* distribution.

following the conditions reported by Castellanos-Morales et al. (2014). PCR products were sequenced with an ABI 3730xl sequencer (Applied Biosystems) at the High Throughput Genomics Center (UWHTSeq FinchLab; www.htseq.org) using primers L14725, L14935 and L15955 to cover the 1140 bp of the *cyt-b* gen (Harrison et al. 2003). *CR* sequences were obtained for all samples using primers L15933 (*5′*-CTCTGGTCTTGTAAACCAAAAATG-3′) and H637 (*5′*-AGGACCAAACCTTTGTGTTTATG-3′) (Oshida et al. 2001), following the conditions reported by Ochoa et al. (2012). The *CR* was sequenced using primers L15933 (Oshida et al. 2001) and CR1F (Ochoa et al. 2012) to increase the quality of the reads and to cover 857 bp. In addition, we amplified and sequenced nuclear introns BGN (Chen et al. 1999) and CHRNA (Lyons et al. 1997) for 5 individuals of each species. However, sequences for these nuclear markers were monomorphic, and were discarded from the analysis.

We assembled the sequences with *Consed* 6.0 (Ewing et al. 1998; Gordon et al. 1998), and polymorphism was checked manually. We performed a BLAST search in GenBank to corroborate correspondence of our sequences with previously posted *cyt-b* and *CR* data. Records from 8 haplotypes (JQ885584–JQ885591) obtained from 157 *cyt-b* sequences of *C. ludovicianus* from Chihuahua were taken from Castellanos-Morales et al. (2014) and 149 of these samples were amplified for the *CR*. In addition, we downloaded from GenBank sequences for the sister genus *Xerospermophilus* [*X. spilosoma* (*CR*: DQ106857, DQ106858; *cyt-b*: AF157885, AF157911) and *X. perotensis* (*CR*: JQ326958, JQ326959; *cyt-b*: AF157840, AF157948)] and the sister subgenus *Leucocrossuromys* [*Cynomys gunnisoni* (*CR*: GU453240, GU453337; *cyt-b*: AF157923, AF157930)] to be used as outgroups. We aligned all sequences by hand using BioEdit v. 7.1.3 (Hall 1999) and concatenated both regions of the mtDNA genome with DnaSP v5 (Librado and Rozas 2009).

We amplified by PCR 10 nuclear microsatellite loci (A2, A8, A101, A104, A119, C116, D1, D2, D115, and D120; Jones et al. 2005), using the conditions reported by Castellanos-Morales et al. (2014) in 10 µL reaction volumes. We sent PCR products for genotyping with an ABI 3730xl sequencer (Applied Biosystems) to UIUC Core Sequencing Facility at the University of Illinois (unicorn.biotec.illinois.edu). We obtained genotypes for 160 *C. ludovicianus* individuals from Chihuahua from Castellanos-Morales et al. (2014), and reamplified 10% of these samples to standardize allele reads. In addition, we re-amplified all microsatellite loci for 20% of the samples to control for genotyping error. We visualized the fragments in Peak Scanner software v1.0 (Applied Biosystems). We performed null allele analyses with MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) and FreeNA (Chapuis and Estoup 2007). We tested Hardy–Weinberg equilibrium and linkage disequilibrium with Arlequin v3.5 (Excoffier and Lischer 2010).

Data Archiving

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses in Dryad and GenBank (accession numbers KP217107–KP217141).

Genetic Diversity

We estimated standard measures of genetic variation for mtDNA sequences for each population and species [number of segregating sites (S), haplotype number (h), haplotype diversity (Hd), and nucleotide diversity (π)] with DnaSP v5 (Librado and Rozas 2009). For microsatellite loci, we obtained measures of genetic variation for each population and species [allelic richness (A), observed heterozygosity (H_O), and genetic diversity (H_E)] with Arlequin v3.5 and GENODIVE 2.0b21 (Meirmans and Van Tienderen 2004). As suggested by Gitzendanner and Soltis (2000), we compared measures of genetic diversity obtained for both species using a Wilcoxon-signed rank test, which is a nonparametric test, using the R Stats package for R v 3.0.2 (R Development Core Team 2013).

Species Evolutionary Relationships

We constructed a gene genealogy (Posada and Crandall 2001) for the mtDNA sequences using the Maximum-Likelihood method with the approximate likelihood ratio test and 1000 bootstrap (BS) replicates implemented in PhyML 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010), and using the substitution model (HKY+Γ+I) determined by jModelTest 0.1.1 (Posada 2008). To explore the relationships between haplotypes within each species, we constructed a median joining network with Network 4.6.1.1 (Fluxus-engineering 2014) using the least cost criterion and the default parameters. We only included variable sites in the analysis. We used the MP option to clean up the network and used the shortest tree.

Genetic Structure

To determine the presence of overall genetic differentiation within species, we estimated F_{ST} for mtDNA and R_{ST} for nuclear microsatellites (Weir and Cockerham 1984; Holsinger and Weir 2009) for each species with Arlequin v3.5 for comparison with previous reports. Nevertheless, genetic differentiation measures have shown a dependency on the amount of within population variation, especially for microsatellite data. Therefore, we also estimated Hedrick's standardized G_{ST} (G''_{ST} Meirmans and Hedrick 2011) for nuclear microsatellite loci using GENODIVE 2.0b21. This measure is corrected by the maximum heterozygosity and provides an unbiased estimate (Meirmans and Hedrick 2011).

To determine the presence of genetic clusters within each species for mtDNA, we performed 2 independent runs on Bayesian Analysis of Population Structure (BAPS) v5.3 (Corander et al. 2004, 2008) with K = 10 and 20 repetitions using the method of "clustering for linked loci". To account for genetic structure and gene flow between populations for microsatellite loci, we used Structure 2.2 (Pritchard et al. 2000) implementing the model with admixture and uncorrelated allele frequencies without using the sampling locations as a prior. We used the uncorrelated allele frequencies prior, which is appropriate for populations that are not extremely closely related, and populations with different allele frequencies (Pritchard et al. 2000). We expect the allele frequencies among species to depart considerably because these species have allopatric distributions, and diverged 40 000–20 000 years ago (McCullough and Chesser 1987; Goodwin 1995; Harrison et al. 2003). We performed an initial run with Markov chain Monte Carlo (MCMC) resampling using 250 000 steps after a burn-in of 50 000 steps and with 5 repetitions for each K (number of clusters), where K = 1 to 20 to determine the necessary run length for the $ln(P)$ to converge across repetitions. Accordingly, we performed 2 independent runs with MCMC resampling using 500 000 steps after a burn-in of 100 000 steps and, 15 repetitions for each K, and K = 1 to 20. We determined the most appropriate value of K following the value of $ln(P)$. We selected the value with the best posterior probability and the smallest variance between repetitions (Pritchard et al. 2000). Microsatellite amplification for the individuals from New Mexico was not successful and reported data from Colorado was obtained using a partially overlapping set of microsatellite loci. Therefore, in this analysis we included

only allelic data from individuals from Chihuahua and Sonora from *C. ludovicianus* and all individuals from *C. mexicanus*.

To analyze how genetic variation is distributed within each species, we used Arlequin v3.5 to conduct a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992), which considered the genetic clusters defined by BAPS v5.3 for mtDNA and Structure 2.2 for nuclear microsatellite data. To determine the genetic relationship between colonies within each species, we also estimated pairwise F_{ST} for mtDNA and pairwise R_{ST} and G''_{ST} (Weir and Cockerham 1984; Holsinger and Weir 2009; Meirmans and Hedrick 2011) for nuclear microsatellite loci using Arlequin v3.5 and GENODIVE 2.0b21. We used the Geographic Distance Matrix Generator version 1.2.3 (Ersts 2011) to obtain a matrix of linear geographic distances between colonies for each species. Finally, we tested isolation by distance through a Mantel test, using 9999 permutations with the R ade4 library (Dray and Dufour 2007), using the linearized F_{ST} for mitochondrial sequences, and linearized R_{ST} and G''_{ST} for nuclear microsatellites.

Results

Genetic Diversity of Mitochondrial Data

We obtained a total of 300 concatenated sequences, including the *cyt-b* and the control (*CR*) regions (Table 1). Sequences were 1997 bp long and showed a total of 55 variable sites (48 parsimony informative). For *C. ludovicianus*, we obtained 223 sequences with 37 segregating sites (including 149 *cyt-b* sequences taken from Castellanos-Morales et al. (2014) that were amplified for the *CR*). For *C. mexicanus*, we obtained 77 sequences with 18 segregating sites.

We found a total of 19 mitochondrial haplotypes in *C. ludovicianus* (CL1–CL19). Only 1 of these haplotypes (CL12) was shared between Chihuahua and New Mexico (NM), while the rest were private to each site within the distribution area of *C. ludovicianus* (Supplementary Table S1). For *C. mexicanus*, we found a total of 16 haplotypes (CM1–CM16). One haplotype was widespread throughout the species distribution. Two haplotypes were shared between colonies and 13 were private to each colony within the distribution of this species.

Mitochondrial genetic variation was higher for widespread *C. ludovicianus* than for restricted *C. mexicanus*, but the difference was not significant ($P = 0.831$ for H_d, and $P = 0.522$ for π). Within *C. ludovicianus*, levels of nucleotide diversity per site varied from 0 in Sonora to 0.0084 in NM (Table 1), while levels of nucleotide diversity per colony within each site varied from 0.0002 to 0.0065 in Chihuahua, and from 0 to 0.0011 in Colorado (Table 1; Supplementary Table S2). Within *C. mexicanus*, nucleotide diversity per colony ranged from 0.0003 to 0.0018 (Table 1).

Genetic Diversity of Nuclear Data

We obtained genotypes for 10 nuclear microsatellite loci for 285 samples, 207 from *C. ludovicianus* (including 160 genotypes taken from Castellanos-Morales et al. 2014), and 78 samples from *C. mexicanus* (Table 1). All microsatellite loci were polymorphic and within Hardy–Weinberg equilibrium. No signals of linkage disequilibrium among them or null alleles were detected. We found a total of 80 alleles (4–10 alleles per locus). About 54 alleles were shared between species; 11 alleles were private to *C. ludovicianus* and 15 alleles were private to *C. mexicanus* (Supplementary Table S3).

Nuclear genetic diversity was higher for restricted *C. mexicanus* than for widespread *C. ludovicianus* but the difference was not significant ($P = 0.197$ for H_E) (Table 1). For *C. ludovicianus*, expected heterozygosity was higher in Chihuahua (0.53) than Sonora (0.49). Expected heterozygosity for the colonies within each site ranged from 0.45 to 0.62 in Chihuahua and 0.5 to 0.56 in Sonora. For *C. mexicanus*, expected heterozygosity ranged from 0.52 to 0.66 (Table 1; Supplementary Table S2).

Species Evolutionary Relationships

According to the mitochondrial gene genealogy, each species forms a well-defined clade (Figure 2). Within *C. ludovicianus*, 2 distinct maternal lineages can be distinguished. One clade (the southern clade) is found in Chihuahua, Sonora, and NM, while the other clade (the south-central clade) is distributed in Chihuahua, NM, and Colorado. Haplotypes found in *C. mexicanus* form a single maternal lineage.

Results from the median joining network were consistent with the gene genealogy. Within *C. ludovicianus*, the haplotype network showed a clear geographic structure, with the presence of closely related haplotypes in Colorado (Figure 3a). The haplotype found in Sonora was related to haplotype from NM and Chihuahua. For *C. mexicanus*, there was no clear geographic structure. The most frequent haplotype (CM3) represented the center of the network, with many derived haplotypes that were private to different populations (Figure 3b).

Genetic Structure

Both species showed significant ($P < 0.05$) levels of genetic differentiation, and genetic differentiation across the sampled range was higher for *C. ludovicianus* ($F_{ST} = 0.448$ for mtDNA; $R_{ST} = 0.228$, $G''_{ST} = 0.504$ for nuclear microsatellites) than for *C. mexicanus* ($F_{ST} = 0.203$ for mtDNA; $R_{ST} = 0.127$, $G''_{ST} = 0.362$ for nuclear microsatellites). Furthermore, the genetic differentiation found throughout the range of *C. mexicanus* was similar to the genetic differentiation estimated for colonies in the region of Chihuahua for *C. ludovicianus* ($F_{ST} = 0.259$ for mtDNA; $R_{ST} = 0.128$, $G''_{ST} = 0.289$ for nuclear microsatellites) (Table 1).

The analysis performed with BAPS v5.3 for mtDNA separated the 2 species with $K = 5$ ($ln(P) = -1961.2125$). The analysis assigned individuals from *C. ludovicianus* to 4 genetic clusters, and *C. mexicanus* to only 1 cluster. Within *C. ludovicianus*, individuals from Colorado and Sonora were assigned to their own clusters. Individuals from Chihuahua and NM were assigned to 2 codistributed clusters (Figure 4a).

To detect substructure within *C. mexicanus*, we performed 2 additional independent runs with BAPS v5.3 following the same strategy as before, but including only mitochondrial sequences from this species. The best K was 3 ($ln(P) = -344.1436$). From the 77 *C. mexicanus* mitochondrial sequences included in the analysis, 66.3% were assigned to 1 cluster, while 24.1% were assigned to another cluster with presence in 2 colonies (LA and LS) and 9.6% (all from LT) were assigned to a third cluster (Figure 4b).

To determine if genetic substructure within *C. mexicanus* is comparable to substructure in *C. ludovicianus* at a local scale, we also performed an additional BAPS analysis including sequences obtained from 13 colonies sampled in Chihuahua. The best K was $K = 5$ ($ln(P) = -558.8862$). From the 149 sequences included in the analysis, 39.2% were assigned to cluster 1, 25.2% to cluster 2, 29.5% to cluster 3, 0.7% to cluster 4, and 5.4% to cluster 5, with individuals from mixed colonies assigned to each cluster (Supplementary Figure S4).

Table 1. Genetic diversity data obtained from mtDNA sequence data and 10 nuclear microsatellite loci for black-tailed prairie dogs (*Cynomys ludovicianus*) and Mexican prairie dogs (*C. mexicanus*). Site, number of sampled colonies (no. Colonies), number of sequences per site (*N*), number of segregating sites (S), number of haplotypes (h), haplotype diversity (H_d), and nucleotide diversity (π) for mtDNA. Number of individuals genotyped (*N*), allelic richness (A), observed heterozygosity (H_O), expected heterozygosity (H_E), genetic structure (R_{ST}), and Hedrick's standardized genetic structure (G''_{ST}) for microsatellite data. Range values for individual colonies are shown in parentheses when available, detailed data is available in Supplementary Table S2.

Site	No. colonies	*mtDNA* sequences						Nuclear microsatellites					
		N	S	*h*	H_d	π	F_{ST}[a]	*N*	*A*	H_O	H_E	R_{ST}[a]	G''_{ST}[a]
Chihuahua[b]	13	149	28	12	0.829 (0.44–0.82)	0.0026 (0.0002–0.006)	0.259	161	3.5 (1.6–3.3)	0.431 (0.31–0.55)	0.532 (0.45–0.62)	0.128	0.289
New Mexico[c]	1	5	21	4	0.900	0.0084	NA	NA	NA	NA	NA	NA	NA
Sonora	2	59	0	1	0.000	0.0000	0	42	5.5 (4–4.6)	0.437 (0.44–0.47)	0.492 (0.50–0.56)	0.112	0.302
Colorado[d]	3	10	4	3	0.511 (0–1)	0.0009 (0–0.0011)	NA	510	9.3	NA	0.633	NA	NA
C. ludovicianus	**19**	**223**	**37**	**19**	**0.854**	**0.0054**	**0.448**	**207**	**6.5**	**0.432**	**0.524**	**0.228**	**0.504**
Artesillas	1	16	3	4	0.691	0.0006	NA	16	4.2	0.528	0.539	NA	NA
El Gallo	1	5	5	2	0.400	0.0014	NA	5	2.8	0.365	0.522	NA	NA
El Salero	1	5	1	2	0.400	0.0003	NA	5	3.2	0.465	0.639	NA	NA
Los Angeles	1	23	4	3	0.695	0.0013	NA	24	4.3	0.48	0.566	NA	NA
La Soledad	1	18	5	5	0.556	0.0008	NA	18	5.2	0.637	0.656	NA	NA
La Trinidad	1	10	7	5	0.844	0.0018	NA	10	3.8	0.581	0.634	NA	NA
C. mexicanus	**6**	**77**	**18**	**16**	**0.778**	**0.0016**	**0.203**	**78**	**7**	**0.509**	**0.591**	**0.127**	**0.362**

[a] F_{ST} and G''_{ST} values for Chihuahua and Sonora were obtained considering the genetic structure between colonies within each site.

[b] *Cyt-b* sequences for 157 individuals were taken from Castellanos-Morales et al. (2014), and 149 of these samples were amplified for the *CR* for the current analysis. Microsatellite genotypes for 160 individuals were obtained from Castellanos-Morales et al. (2014), and one additional sample was genotyped for the current analysis.

[c] Samples from New Mexico could not be amplified for microsatellite loci because of low DNA quality.

[d] Data for 11 nuclear microsatellite loci from 510 prairie dogs from 9 colonies located in Boulder, Colorado were reported in Sackett et al. (2012).

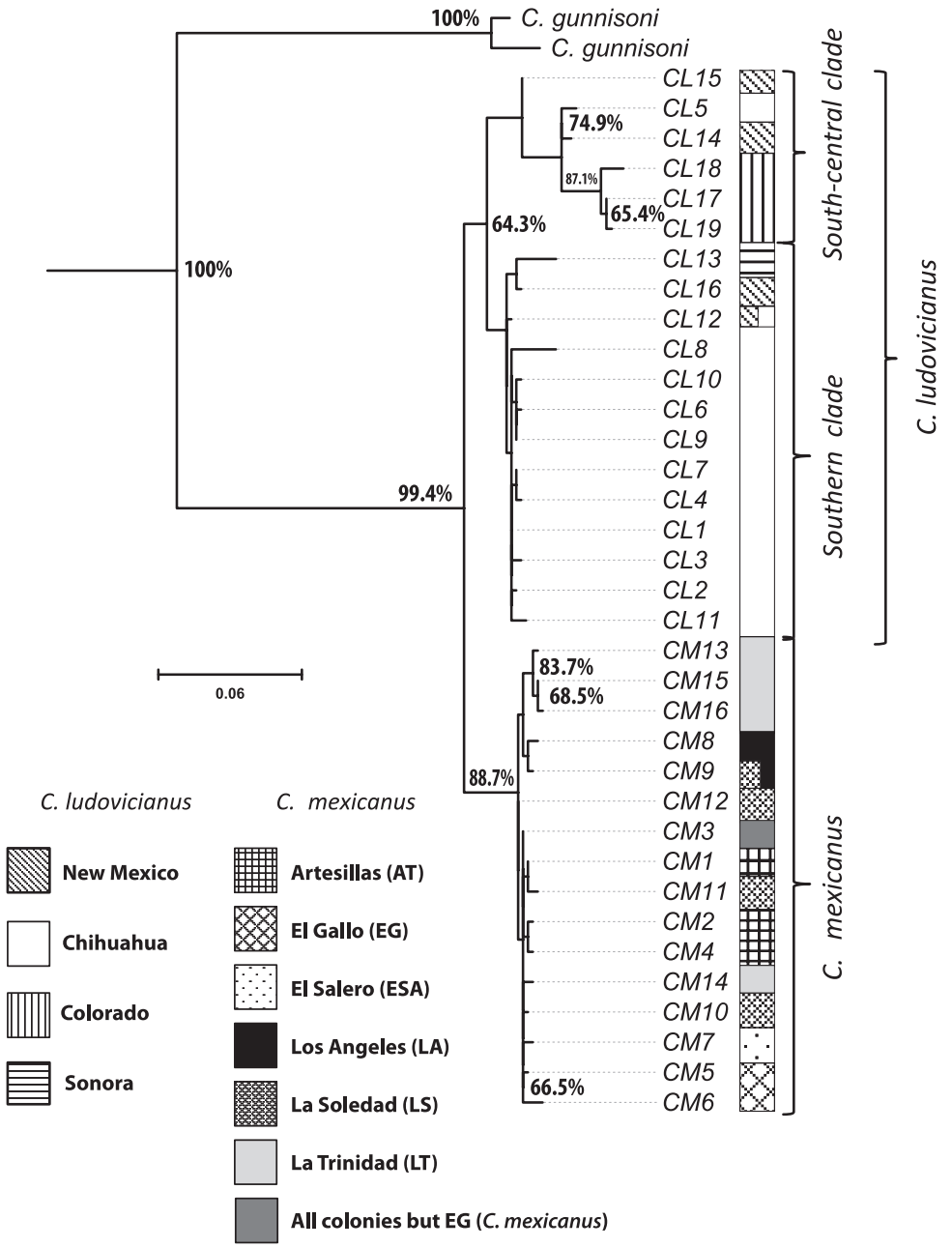

Figure 2. Maximum-likelihood gene genealogy for concatenated Control Region and Cytochrome *b* sequences from *Cynomys ludovicianus* and *C. mexicanus* showing bootstrap values higher than 60%. Color codes on the right side of the gene genealogy represent sampling site as depicted in the internal legend. Haplotypes CL1–CL19 represent the 19 haplotypes found in *C. ludovicianus* from Chihuahua (CL1–CL12), Sonora (CL13), New Mexico (CL14–CL16), and Colorado (CL17–CL19). Haplotypes CM1–CM16 represent the haplotypes found in *C. mexicanus* from 6 colonies. Sequences from representatives of the sister subgenus *Leucocrossuromys* (*C. gunnisoni*), and from the sister genus *Xerospermophilus* (*X. spilosoma* and *X. perotensis*; 100% bootstrap value not shown in the figure) obtained from GenBank were used as outgroups to root the gene genealogy.

Genetic structure analysis performed with Structure 2.2 for nuclear microsatellite data also separated the 2 species. This analysis also defined 5 clusters (Figure 4c), but the clusters were different from those recovered by mtDNA. Within *C. ludovicianus*, Sonora and Chihuahua were separated while Chihuahua consisted of 2 genetic clusters, as previously reported by Castellanos-Morales et al. (2014). Within *C. mexicanus* we observed 2 clusters that separated individuals from 1 colony (LA) and detected admixture in 2 individuals from distinct colonies: LS and ESA.

AMOVA results for *C. ludovicianus* mtDNA population structure given by BAPS v5.3, apportioned a high proportion of genetic variation among clusters, followed by within-colony variation (Table 2). Population structure obtained with Structure 2.2 using nuclear microsatellite loci allocated similar percentages of variation among clusters and within colonies. For *C. mexicanus*, the results for AMOVA analyses based on the clustering given by BAPS v5.3 for mtDNA sequences and Structure 2.2 for nuclear microsatellite were consistent. Both apportioned the highest percentage of variation within colonies. The AMOVA performed for mtDNA sequences

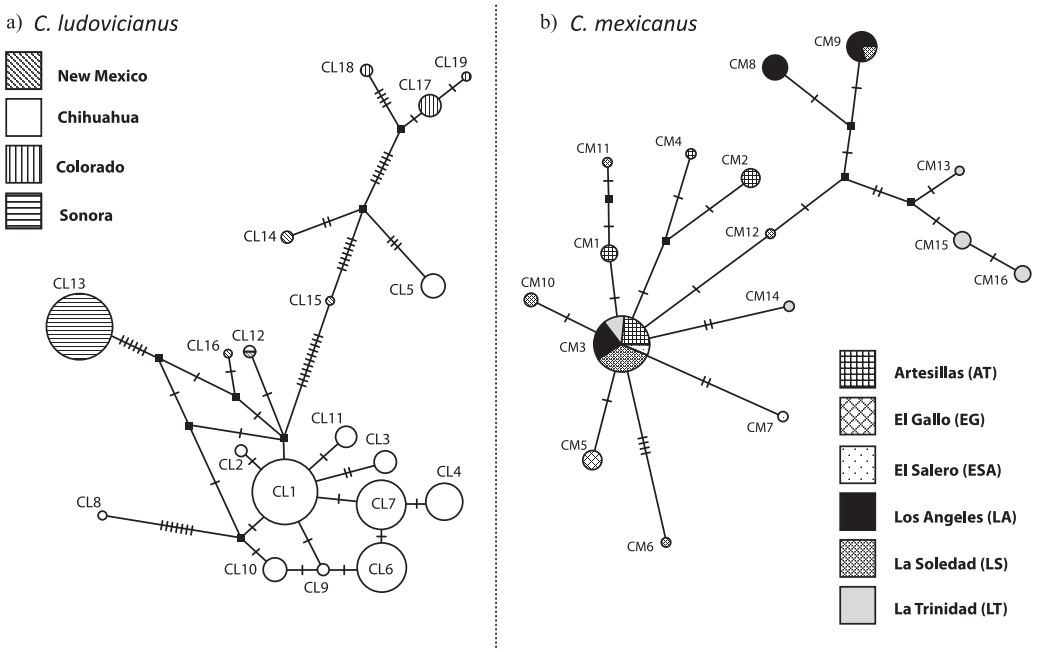

Figure 3. Median joining networks constructed for (**a**) *C. ludovicianus* and (**b**) *C. mexicanus* mtDNA haplotypes, estimated with Network 4.6.1.1 considering only variable sites. The size of each pie represents the frequency of each haplotype; color represents sampling site as depicted in the gene genealogy.

and nuclear microsatellites defining each species as a group determined that the highest percentage of variation was found between species (Table 2).

Finally, Mantel tests indicated no isolation by distance (IBD) for *C. mexicanus* for mtDNA (r = 0.170, P = 0.274 using F_{ST}) or nuclear microsatellites (r = –0.05, P = 0.548 with R_{ST} and r = –0.061, P = 0.538 using G''_{ST}). For *C. ludovicianus*, results were nonsignificant for mtDNA (r = 0.388, P = 0.067 using F_{ST}), but there was strong IBD for nuclear microsatellite loci (r = 0.839, P = 0.006 for R_{ST} and r = 0.819, P = 0.002 using G''_{ST}) (Supplementary Table S5).

Discussion

The aim of this study was to examine patterns of genetic variation, genetic structure, and genetic differentiation in a widespread (*C. ludovicianus*) and a restricted species (*C. mexicanus*). High levels of genetic variation (H_d = 0.77 for mtDNA; H_E = 0.591 for nuclear loci) in *C. mexicanus* departed from our expectations. This can be explained by: 1) the maintenance of genetic variation associated with substructure within colonies and differential allele fixation within family groups promoted by female philopatry; 2) high levels of male-mediated gene flow between colonies within its distribution; 3) large population sizes; and 4) that *C. mexicanus* originated recently (20 000–40 000 years ago according to Goodwin 1995) from a widespread ancestor with high genetic variation.

In contrast, our results supported our prediction of higher genetic structure and higher genetic differentiation in the widespread *C. ludovicianus* because of its large range and the presence of large barriers to dispersal between regions within this range. Genetic variation, genetic structure, and genetic differentiation in *C. mexicanus* agreed with results reported by McCullough and Chesser (1987) based on allozymes. These authors found low genetic differentiation among populations, and levels of genetic variation similar to those reported for populations of *C. ludovicianus* separated by long geographic distances.

Furthermore, our results supported the prediction that *C. mexicanus* would show patterns of genetic structure similar to results reported among colonies of *C. ludovicianus* within regions (McCullough and Chesser 1987; Roach et al. 2001; Jones and Britten 2010; Magle et al. 2010; Sackett et al. 2012, 2013; Castellanos-Morales et al. 2014). Genetic structure and differentiation for both of these sister species are influenced by their complex social behavior (association into family groups where females are philopatric and dispersal is male-biased), limited dispersal capacity and their evolutionary history (a late Pleistocene origin from a relict population of *C. ludovicianus*) (McCullough and Chesser 1987).

Genetic Variation

Levels of genetic variation for both molecular markers in *C. ludovicianus* and *C. mexicanus*, at the species level, were within the range reported for other sciurids (for mtDNA, H_d = 0.288–0.953; Hoisington-Lopez et al. 2012; Liu et al. 2014; for nuclear microsatellite loci, H_E = 0.33–0.75; Haynie et al. 2003; Říčanová et al. 2011; Reid et al. 2010; Fitak et al. 2013). Furthermore, levels of genetic variation in *C. mexicanus* were inside the range reported for colonies of *C. ludovicianus* within different areas (H_d = 0–0.9 for mtDNA, and H_E = 0.53–0.63 for nuclear microsatellite loci; Roach et al. 2001; Jones and Britten 2010; Magle et al. 2010; Sackett et al. 2012, 2013; Castellanos-Morales et al. 2014). High levels of genetic variation in both species have been linked to their complex social system (female philopatry and male-biased gene flow), subdivided populations (McCullough and Chesser 1987; Slobodchikoff et al. 2009; Castellanos-Morales et al. 2014), and metapopulation structure (Roach et al. 2001; Sackett et al. 2013).

Contrary to our prediction, the restricted species (*C. mexicanus*) showed high levels of genetic variation. High genetic variation in species with restricted distributions has been attributed to a recent origin from a widespread ancestor (Coates et al. 2003), such as the suggested origin of *C. mexicanus* from a relict population of *C. ludovicianus* 20 000–40 000 years ago, followed by demographic

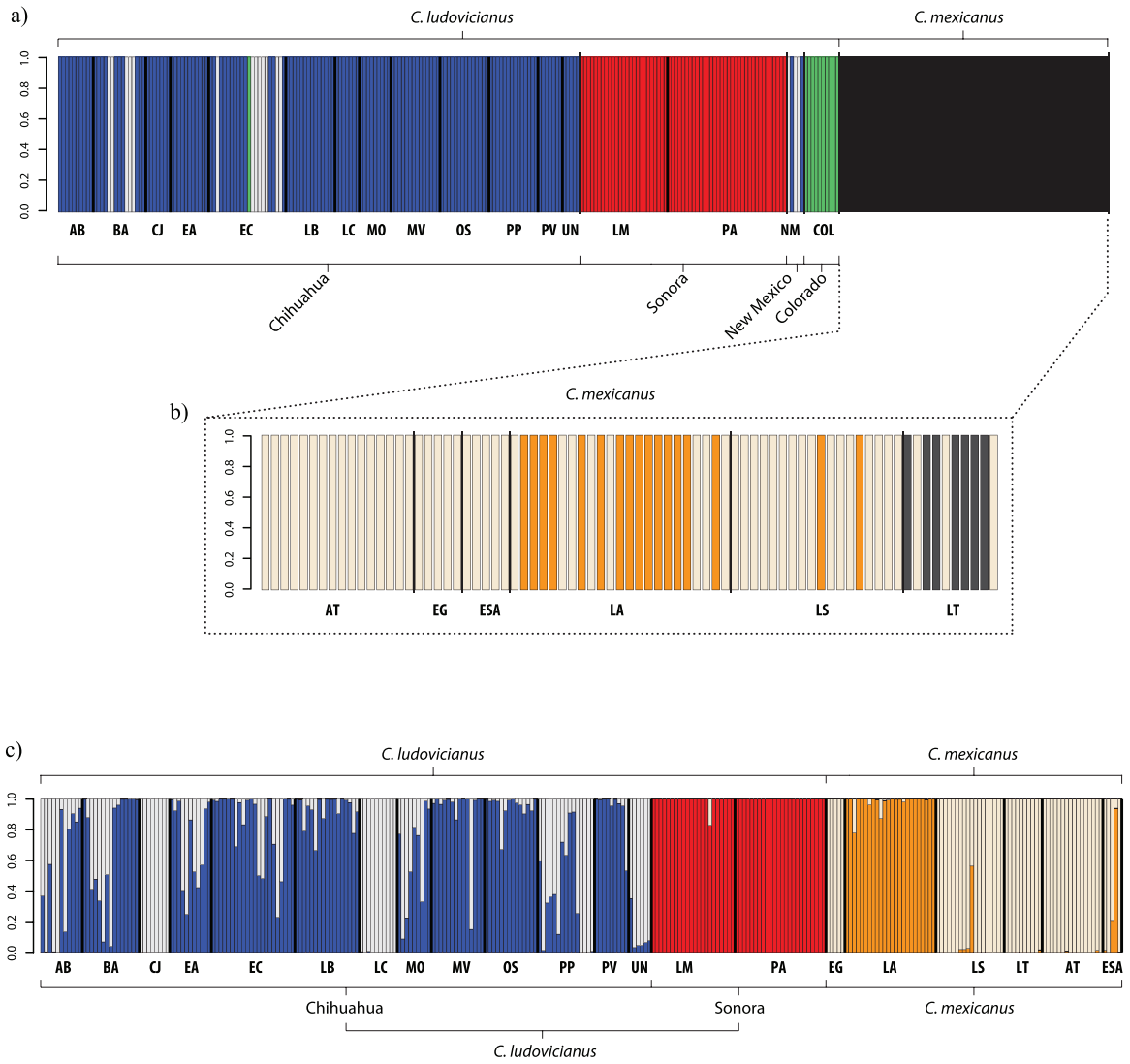

Figure 4. (**a**) Bayesian clustering analysis of mtDNA sequences from black-tailed (*C. ludovicianus*) and Mexican prairie dogs (*C. mexicanus*) as determined by BAPS v 5.3. resulting in $K = 5$. We detected 4 clusters within *C. ludovicianus* (cluster 1 = Chihuahua and NM; cluster 2 = Chihuahua and NM; cluster 3 = Sonora; and cluster 4 = Colorado), and one cluster for *C. mexicanus*. (**b**) BAPS analysis of mtDNA sequences for *C. mexicanus* resulted in 3 clusters (cluster 1 = all colonies; cluster 2 = LA and LS; and cluster 3 = LT). (**c**) Bayesian clustering analysis for nuclear microsatellite loci from both species as determined by Structure 2.2. ($K = 5$). Within *C. ludovicianus,* Sonora and Chihuahua were differentiated with substructure within Chihuahua ($K = 2$ as reported by Castellanos-Morales et al. 2014), and *C. mexicanus* individuals were assigned to two clusters (cluster 1 = all colonies but LA; and cluster 2 = LA and ESA). New Mexico and Colorado were not included in the microsatellites analysis.

expansion (McCullough and Chesser 1987; Goodwin 1995). In this regard, the presence of moderate genetic variation and closely related haplotypes in mtDNA may suggest the occurrence of a recent founder event or a bottleneck. Conversely, high genetic variation for nuclear microsatellite loci may indicate that the bottleneck associated with the speciation event was short and/or followed by demographic expansion.

Evolutionary Relationships

The gene genealogy depicted each species as monophyletic. The presence of 2 maternal lineages (south-central and southern clades) in *C. ludovicianus* may be evidence for at least 2 refugia or biogeographic barriers. Nevertheless, further sampling across the entire range will be needed to ascertain this. In addition, high levels of genetic variation in the southern area of the species distribution (Chihuahua and New Mexico) suggests that this is an area of older occupation and/or larger effective population size, and that *C. ludovicianus* underwent a south–north colonization process.

In *C. mexicanus*, the star shape of the haplotype network and the lack of a clear geographic structure support the idea of recent demographic expansion (Allendorf and Luikart 2007). In addition, the lack of geographic structure depicted for *C. mexicanus* was previously reported for the distribution of *cyt-b* haplotypes between colonies from *C. ludovicianus* in Chihuahua (Castellanos-Morales et al. 2014). These data support our prediction that *C. mexicanus* would show similar patterns of genetic structure as those reported between colonies of *C. ludovicianus* at local scales.

Genetic Structure in *C. ludovicianus*

That *C. ludovicianus* has higher genetic differentiation than *C. mexicanus* in part is the obvious consequence of the contrasting distribution area of each species (widespread *vs*. restricted). The

Table 2. Components of an analysis of molecular variance (AMOVA—Excoffier et al. 1992) of mtDNA and nuclear microsatellite loci for *C. ludovicianus* and *C. mexicanus* considering the genetic clusters obtained with BAPS v5.3 and Structure 2.2 within each species and considering each species as a genetic group

Source of variation	mtDNA AMOVA			Nuclear microsatellite loci AMOVA			
	C. ludovicianus[a]	*C. mexicanus*[b]	Species[c]	*C. ludovicianus*[d]	*C. mexicanus*		Species[g]
	Percentage of the variation	Percentage of the variation	Percentage of the variation	Percentage of the variation	Percentage of the variation		Percentage of the variation
Among clusters	68.9	32.3	55.7	50.0	–2.2[e]	—[f]	40.5
Among colonies within clusters	9.2	8.0	31.1	4.1	19.4[e]	18.0[f]	20.5
Within colonies	21.9	59.8	13.3	45.9	82.8[e]	82.0[f]	39.0
F_{SC}	0.294	0.117	0.700	0.082	0.189[e]	—[f]	0.345
F_{ST}	0.781	0.402	0.867	0.541	0.172[e]	0.180[f]	0.610
F_{CT}	0.689	0.323	0.557	0.500	–0.022[e]	—[f]	0.405

[a]According to BAPS v5.3 genotypic assignment for mtDNA sequences within C. *ludovicianus*. Group 1: Chihuahua + New Mexico; Group 2: Sonora; Group 3: Colorado.

[b]According to BAPS v5.3 genotypic assignment for mtDNA sequences within C. *mexicanus*. Group 1: LS, ESA, EGI, and AT; group 2: LA; Group 3: LS.

[c]For mtDNA, determining each species as a group. Group 1 (*C. ludovicianus*): Chihuahua, Colorado, New Mexico and Sonora; Group 2 (*C. mexicanus*): AT, EGI, ESA, LA, LS, and LT.

[d]According to Structure 2.2 genotypic assignment for 10 nuclear microsatellite loci within C. *ludovicianus*. Group 1: 10 colonies from Chihuahua (AB, BA, EA, EC, LB, MO, MV, OS, PP, and PV); Group 2: 3 colonies from Chihuahua (CJ, LC, and UN); Group 3: Sonora.

[e]According to Structure 2.2 genotypic assignment for 10 nuclear microsatellite loci within C. *mexicanus*. Group 1: AT, EGI, ESA, LS, and LT; Group 2: LA.

[f]For 10 nuclear microsatellite loci, considering all colonies for *C. mexicanus* as a single group. Group 1: AT, EGI, ESA, LA, LS, and LT.

[g]For 10 nuclear microsatellite loci, determining each species as a group. Group 1 (*C. ludovicianus*): Chihuahua and Sonora; Group 2 (*C. mexicanus*): AT, EGI, ESA, LA, LS, and LT.

distance between sampled sites (1.3–638.5 km) and the actual gaps in the distribution of C. *ludovicianus* promote genetic differentiation between areas. This pattern of genetic variation is consistent with demographic autonomy between populations (Avise 1995), which is further supported by the presence of IBD for microsatellite loci. Nevertheless, sampling across the entire range is needed to confirm this idea.

The assignment tests for mtDNA (BAPS v5.3) indicate a close genetic relationship between Chihuahua and NM and support the presence of contact between the south-central and southern clades or incomplete lineage sorting in this area. In contrast, the analyses performed with each molecular marker (BAPS v5.3 and Structure 2.2) separated Sonora, suggesting that the Sierra Madre Occidental represents an effective barrier to gene flow. At the local level, the 13 colonies from Chihuahua showed strong substructure (presence of several genetic clusters in this site) and high admixture between colonies (clusters did not show a geographic pattern). This pattern of genetic substructure within Chihuahua may relate to a large effective population size and high historical connectivity between colonies (Ceballos et al. 2010; Castellanos-Morales et al. 2014).

The distribution of variance resolved by the AMOVA for mtDNA is congruent with female philopatry, and matches our expectations because a widespread distribution will promote structuring between distant populations (Broadhurst and Coates 2002; Campbell et al. 2007). In contrast, nuclear microsatellite loci results are consistent with geographic isolation because of male-biased gene flow (10 km maximum dispersal; Hoogland 1996), and differential allele fixation within colonies (Dobson et al. 2004; Castellanos-Morales et al. 2014). Among-colony variation suggests high gene flow among colonies within each site, which is coherent with the metapopulation dynamic reported for colonies within an area (Roach et al. 2001; Antolin et al. 2006; Sackett et al. 2013).

Genetic Structure in *C. mexicanus*

Genetic structure found in C. *mexicanus* was consistent with values estimated between colonies of C. *ludovicianus* at different regions (F_{ST} = 0.259 in Chihuahua for mtDNA and R_{ST} = 0.112 in Sonora and 0.128 in Chihuahua for nuclear microsatellites), and agree with previous reports based on nuclear markers (McCullough and Chesser 1987). Assignment analyses for both mtDNA and nuclear microsatellite loci for C. *mexicanus* separated colony LA, suggesting that this colony located in the northwestern area of the species distribution range might be isolated. In addition, our results suggest high connectivity between colonies via male-biased dispersal, similar to what has been reported locally for C. *ludovicianus* (Roach et al. 2001; Jones and Britten 2010; Magle et al. 2010; Sackett et al. 2012; Castellanos-Morales et al. 2014).

The distribution of variance resolved by AMOVA at both markers is congruent with the recent origin of C. *mexicanus*, its restricted distribution and high gene flow between colonies. For mtDNA, population structure estimated by BAPS v5.3 partially explained diversity allocation between clusters. Assuming female philopatry, our results may indicate that time since the divergence of this species has not been enough to promote higher differentiation between intraspecific clusters through genetic drift. For nuclear loci, within-colony variation relate to differential allele fixation within coteries (Dobson et al. 2004; Castellanos-Morales et al. 2014). The variation apportioned between colonies suggests high gene flow, which is consistent with the restricted distribution of this species (McCullough and Chesser 1987).

Finally, the lack of IBD in C. *mexicanus* is coherent with the recent origin of this species and current gene flow because of a small distance between colonies (6.7 km to 80.8 km). This result is consistent with what has been reported locally for C. *ludovicianus* and has been associated with the effect of genetic drift, founder events and mutation (Chesser 1983; Castellanos-Morales et al. 2014).

Implications for Conservation

Previous studies have highlighted the importance of connectivity between colonies within geographically restricted areas of the widespread species (*C. ludovicianus*—Roach et al. 2001; Castellanos-Morales et al. 2014). However, high genetic structure between regions may indicate isolation between populations, and conservation management should be planned as to consider the microevolutionary processes that have driven this differentiation. In this sense, our data suggest that Chihuahua and NM might be important areas for conservation. These sites showed high levels of genetic variation (for both nuclear and mitochondrial markers), and the presence of 2 maternal lineages in this area. Nevertheless, a sampling scheme that includes the entire distribution of this species is needed to corroborate whether there is high genetic differentiation among regions and to assess levels of genetic diversity, especially for mtDNA, in other sites of the species range. In addition, colonies from Sonora should be considered as an independent management unit that should be conserved carefully (Moritz 1994). This conclusion relies on the uniqueness and the divergence of the haplotype present in Sonora, the presence of private alleles in the colonies located in this site, their high genetic variation for microsatellite loci, and their level of genetic differentiation.

Within *C. mexicanus*, colonies with high genetic variation (e.g., LS, AT, LA and LT) should receive long-term protection as reservoirs for genetic diversity. Furthermore, connectivity among colonies is important for the conservation of genetic diversity in this endemic species. Therefore, conservation management should mitigate habitat fragmentation and habitat loss that, together with long periods of drought, have reduced the populations of this key species of the arid grasslands of Mexico.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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