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Evidence for two subspecies of Gunnison's prairie dogs (*Cynomys gunnisoni*), and the general importance of the subspecies concept

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ABSTRACT

Accurate taxonomy is essential for conservation, but subspecies-level systematics can be hampered both by a lack of consensus on what constitutes a subspecies and by discordance among data types (e.g., genetics vs. morphology). Here we provide a framework for evaluating subspecies using multidimensional criteria, and suggest that taxa must satisfy multiple criteria to qualify as subspecies. As a case study, we use the Gunnison's prairie dog (*Cynomys gunnisoni*), a species for which there has been disagreement regarding the existence of subspecies due to inconsistent application of criteria for defining subspecies. To explicitly test the hypothesis that two subspecies exist, we generated five predictions that could be evaluated with genetic data, while also using morphological and ecological criteria. We sampled 838 Gunnison's prairie dogs from across the species range and performed a series of genetic analyses using 16 microsatellite and two mitochondrial loci (cytochrome b and the control region). We compared subspecies morphology and quantitatively evaluated whether abiotic and biotic habitat characteristics encountered by each subspecies differed. Genetic results from all five predictions supported the existence of two distinct subspecies within the confines of a proposed revision in the boundary between subspecies. The subspecies differed marginally in morphology and significantly in their habitats, suggesting ecological differentiation. Our results, which are in line with historical descriptions of morphologically distinct subspecies, suggest the subspecies should be recognized. This work provides support for the utility of integrating multiple data and analysis types to inform systematics and conservation.

1. Introduction

In recent years, there have been tremendous advances in our knowledge of interspecific phylogenies (e.g., Bininda-Emonds et al., 2007; Wiens et al., 2012; McCormack et al., 2013a,b), bolstered by an increase in computing power and tools available for generating and analyzing large datasets (Zwickl, 2006; Kubatko et al., 2009; O'Meara, 2010; McCormack et al., 2013a,b). Despite these advances, we lag in our ability to accurately characterize taxa at lower levels such as subspecies. This difficulty partly results from the lack of universal acceptance of a single species concept (Mayr, 1963; Nixon and Wheeler, 1990; Crandall et al., 2000; Cohan, 2001; Templeton, 2001). Consequently, subspecies recognition is often based on inconsistently applied criteria such as concordance in multiple, independent, genetically-based traits (Ball and Avise, 1992), geographic and phylogenetic separation but reproductive compatibility (O'Brien and Mayr, 1991), and differences in morphology, behavior, life history or ecology (Haig et al., 2006).

Subspecies taxonomy is biologically meaningful because subspecies are unique evolutionary lineages (Lidicker, 1962; Smith and Patton, 1980), and divergence between subspecies is not qualitatively different than between species. For instance, divergence occurs along a continuum in reproductive isolation (Gill, 1984; Patten et al., 2004; Bímová et al., 2011) and genetic differentiation (Hey and Pinho, 2012). Despite the practical challenges, identification of subspecies is of theoretical importance due to its influence in contemporary questions in evolutionary biology

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(e.g., ecological divergence arising in subspecies, Wang et al., 1997; molecular adaptation to climate in subspecies, Wilson et al., 2012), and in the application of conservation (e.g., the Endangered Species Act provides for protection of subspecies) and management strategies (Gutiérrez and Helgen, 2013). In mammalian systematics, subspecies rank is commonly used to define groups of populations that are geographically separated, are morphologically distinct, and have unique evolutionary potential (Grinnell, 1935; Lidicker, 1962; Smith and Patton, 1980).

The accuracy of taxonomic assignment depends, in part, on the breadth of data available for assessing whether or not distinct groups exist (Haig et al., 2006). Unfortunately, studies differ in their application of criteria (Carstens et al., 2013), and taxonomic schemes are often based on few characters (e.g., only morphology) or on data with insufficient resolution (e.g., allozymes) for the taxonomic level of interest (Pizzimenti, 1976; McCullough, 1991; Ramey et al., 2005). This inconsistency makes it difficult to evaluate whether taxonomy is accurate, and results in controversy when management decisions are made. Ideally, taxonomy should be based on objective criteria and data for multiple biological properties that can be replicated across different investigators (Haig et al., 2006) and analyses (Carstens et al., 2013). When multiple criteria are evaluated, subspecies can be definable, defendable, and identifiable as distinct entities (Garcia-Moreno et al., 1996; Roemer and Wayne, 2003; King et al., 2006; Hafner and Smith, 2010). In this paper, we provide an example of the utility of using multiple types of data and analyses to inform taxonomy by explicitly testing the hypothesis that there are two genetically, morphologically, and ecologically distinct subspecies of Gunnison's prairie dogs.

Prairie dogs (genus *Cynomys*) are social, semi-fossorial rodents in the family Sciuridae that inhabit the grasslands and shrublands of western North America. Populations of all five species of prairie dogs have declined precipitously over the last century as a consequence of introduced sylvatic plague, eradication campaigns and habitat loss from land conversion (Van Putten and Miller, 1999; Miller and Cully, 2001; Hoogland, 2006). Increasingly, colonies occur within a complex landscape matrix (Sackett et al., 2012) and experience metapopulation dynamics largely driven by local extirpations from plague (Roach et al., 2001) and recolonization from nearby colonies (Sackett et al., 2013).

Gunnison's prairie dogs (*Cynomys gunnisoni*, GUPD; Baird, 1855) occur in areas of Arizona, Colorado, New Mexico and Utah (Fig. S1). As a result of morphological variation (Hollister, 1916; Pizzimenti, 1975) and geographic separation, scientists historically recognized two subspecies: *C. g. gunnisoni* and *C. g. zuniensis* (Hollister, 1916; Aldous, 1935; Longhurst, 1944; Lechleitner, 1969; Pizzimenti and Hoffman, 1973). The subspecies differ in ways that may influence viability and persistence: *C. g. gunnisoni* is restricted to a smaller geographical area and occupies more fragmented habitat than *C. g. zuniensis* (Seglund and Schnurr, 2009). Large areas of plague extirpation have occurred in both *C. g. gunnisoni* (Lechleitner et al., 1962, 1968; Cully et al., 1997) and *C. g. zuniensis* (Wagner et al., 2006), but because *C. g. gunnisoni* populations are more fragmented, the resulting decline of connectivity among colonies may reduce recolonization probability (Sackett et al., 2013). Differences in the abundance, distribution, and the connectivity among colonies across the landscape between the two subspecies led the U.S. Fish and Wildlife Service (USFWS) to conclude that *C. gunnisoni* was a candidate for listing as a federally protected species within a portion of its range they defined as 'montane' (USFWS, 2008), although the subspecies is currently not listed.

Despite the historical taxonomic legacy, there is currently no consensus among biologists about whether there are two subspecies. More recent studies on morphology (Pizzimenti, 1975) and genetics (Pizzimenti, 1976; McCullough, 1991; Hafner et al., 2005) have produced equivocal results, although these studies were limited in scope and data resolution. The lack of agreement also reflects differences among biologists in interpretation of existing data (Pizzimenti, 1975), and variation among biologists in opinion about the criteria for delineating subspecies (Online Appendix). Resolving the debate is important because both subspecies are subject to management actions that include relocation efforts. Failure to accurately recognize different taxa may result in moving prairie dog subspecies outside their native range, potentially compromising the establishment of relocated animals or eroding local adaptation. In this study, we drew from multiple subspecies definitions to generate explicit predictions for the hypothesis that there are two distinct subspecies of Gunnison's prairie dog (*C. g. gunnisoni* and *C. g zuniensis*), and then evaluated whether genetic, morphological and ecological evidence align with the predictions.

2. Materials and methods

2.1. Study locations, sample and DNA collection

For our analyses, we use the ecological delineation depicted by the USFWS (2008; Fig. S1). The 2008 finding represents a collection of the best available knowledge of the subspecies, although they are referred to there as 'montane' and 'prairie' forms rather than subspecies. In addition to this "previously delineated subspecies" comparison—which differs from the historically recognized subspecies range—we reanalyze data based on a simpler linear boundary, which we refer to as the "revised subspecies boundary" (see results) and which more closely matches historical descriptions. We sampled prairie dogs from 48 sites spanning the GUPD range in New Mexico, Colorado, Arizona and Utah from 2008 to 2010 (Fig. S1, Table S1). Prairie dogs were trapped from May–September in 39 of the 48 sites, and tissue samples were collected from animals in a relocation holding facility from six additional colonies. Tissues were also obtained from one control effort (DN) and from animals killed on roads at two sites (PEFO, RM). At each of the 39 trapped colonies, 24–68 Tomahawk traps were pre-baited with a corn-oat-barley mixture for at least five days with the traps held open to acclimate prairie dogs to the traps and bait. After pre-baiting, traps were baited, set, and checked every 1–2 h depending on daytime temperatures. Prairie dogs were trapped for 1–2 weeks at each site by targeting active burrows with one to four traps (Hoogland, 1995).

Prairie dog trapping and processing were conducted in accordance with a protocol approved by the Colorado Division of Wildlife's and University of Colorado's Institutional Animal Care and Use Committees and are described in detail therein (protocols 05-2008 and 1004.09, available on request). Captured prairie dogs were anesthetized with 1–4% isoflurane in oxygen using a vaporizer to control the dosage (Heath et al., 1997). Tissue for DNA analysis was collected using a 2-mm diameter ear punch (Braintree Scientific) and stored frozen in a solution of EDTA-DMSO until DNA extraction. Animals were weighed and measured (total length and tail length), and their sex was determined. After processing, animals were allowed to recover from anesthesia in traps and returned to their capture locations.

DNA from prairie dogs was extracted using a Qiagen DNeasy tissue kit, and 838 individuals were genotyped at 16 microsatellite loci (following Jones et al., 2005 and Sackett et al., 2010; Table S2) and two mitochondrial genes: cytochrome b and d-loop (following Oshida et al., 2001 and Harrison et al., 2003; Table S2). The program jModelTest (Guindon and Gascuel, 2003; Posada, 2008) was used to determine the nucleotide substitution model for each mitochondrial gene. Microsatellite loci were examined for null (non-amplifying) alleles in the program Micro-checker (van Oosterhout et al., 2004) and linkage disequilibrium using Genepop software (Rousset, 2008).

2.2. Genetic variation

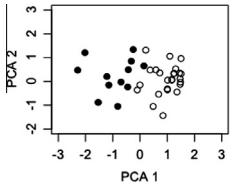
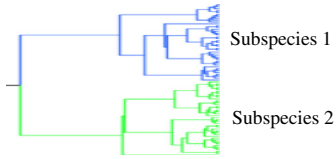
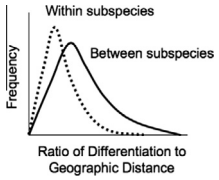
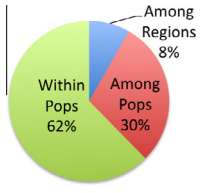
We explicitly evaluate the hypothesis that there are two subspecies using five testable predictions drawing on widely used criteria for assessing genetic differentiation (Table 1). Although subspecies are not necessarily expected to satisfy all five criteria—due to ongoing gene flow and incomplete reproductive isolation—these criteria provide a useful, albeit conservative, starting point for evaluating subspecies. First, genotypes should separate into two distinct groups with little overlap. Second, there should be geographic separation between the genetically defined groups. Third, phylogenetic analysis of mtDNA should reveal separation of individuals into distinct clades by subspecies (but see Degnan, 1993; Hickerson et al., 2006; Toews and Brelsford, 2012). Fourth, analysis of genetic differentiation as a function of geographic distance should demonstrate higher divergence for comparisons of colonies comprising different subspecies than between colonies of the same subspecies. Finally, analysis of molecular variance (AMOVA) should partition a significant amount of variation between subspecies, and genetic differentiation between subspecies should be significant. All of these criteria have been used for deciphering taxonomic distinction in published studies (Proudfoot et al., 2006; Phillimore et al., 2008; Tsang et al., 2008; Karberg and Gale, 2010; Hey and Pinho, 2012). Additional methodological details are provided in the online Supporting Information.

To evaluate prediction 1, genetic composition of colonies was assessed using a Principal Component Analysis (PCA) of genotypes across the 16 microsatellite loci, performed in the ade4 package (Chessel et al., 2004; Dray and Dufour, 2007) for R (The R Foundation for Statistical Computing, http://www.r-project.org/). Because PCA performs poorly with missing data, we removed individuals with fewer than 50% of loci genotyped ($N = 44$). With the 'between' function, we performed 10,000 randomizations of individuals between subspecies. We assessed statistical significance of the differentiation between: (1) the previously delineated subspecies and (2) the proposed revised subspecies, by counting the proportion of times that observed differentiation was less than that generated by randomization.

To evaluate prediction 2, we performed a Bayesian assignment analysis using microsatellite genotypes in Structure (Pritchard et al., 2000), a program that assigns individuals into one of K clusters based on linkage disequilibrium. We allowed K to vary from 1 to 15 (an upper bound much greater than the expected number of subspecies). We determined the most parsimonious number of genetic clusters (Pritchard et al., 2000; Evanno et al., 2005) using four simulations per K. Simulations used a burn-in of 250,000 generations followed by 750,000 iterations. We removed individuals missing genotypes at >50% of loci. We supplemented this analysis with an inference of the optimal number of populations implemented in Structurama (Huelsenbeck et al., 2011), using both fixed priors for k and priors following a gamma distribution (more details in online Supporting Information). Because the scale of interest in this study was the population, not the individual, we assessed only the proportion of membership of each population in K genetic clusters.

To evaluate prediction 3, we used mtDNA to infer phylogenetic relationships among sampled GUPD individuals. To do so, we used Bayesian inference implemented in MrBayes version 3.04 (Huelsenbeck and Ronquist, 2001) through the CIPRES Science

Table 1
A list of the testable predictions of the hypothesis that there are two genetically distinct subspecies, a brief summary of the test, and a visualization of hypothetical data that would support recognition of distinct subspecies.

Prediction	Test	Visualization
(1) Genotypic separation of putative subspecies into two separate "clouds" of points	Plot of principal component scores for populations based on individual genotypes	
(2) Spatial segregation of the two different genotype groups that conforms to the expected putative subspecies distributions	Mapping the geographic location of populations assigned into one of two groups	
(3) Monophyly of one or both putative subspecies at mtDNA sequences	Phylogenetic analysis of individuals sampled across the range of the two putative subspecies	
(4) Higher differentiation for comparisons between subspecies than within subspecies for a given geographic separation of colonies	Compare distribution of differentiation corrected for geographic distance between colonies for all comparisons within and between subspecies	
(5) Relatively large and significant fraction of the total variation partitioned between subspecies	Hierarchical AMOVA	

Gateway (Miller et al., 2010). We concatenated cytochrome b and d-loop sequences and condensed our dataset into unique haplotypes using MacClade version 4.08 (Maddison and Maddison, 2003). We allowed the two genes to evolve independently, with different mutation rates and models, and partitioned codons to allow mutation rate to vary across codon position. We used a white-tailed prairie dog (*C. leucurus*), the sister taxon of GUPD, haplotype to root the tree. The program was run with 8 chains for 5 million generations, with a burn-in of 7500, and trees were sampled every 5000 generations.

To evaluate prediction 4, we examined the degree of pairwise genetic differentiation between colonies. Differentiation was estimated for microsatellites using F_{ST} in Genepop Version 4.0 (Rousset, 2008) and standardized in Genodive (Meirmans and Van Tienderen, 2004) to prevent confounding diversity and differentiation (Hedrick, 2005) and to control for unequal sample sizes. For mtDNA, F_{ST} was estimated (Weir and Cockerham, 1984) in Arlequin version 3.1 (Excoffier et al., 2005); calculations allowed 15% missing data for both mtDNA and microsatellites. We tested for isolation by distance using a Mantel test on the relationship between (1) linearized F_{ST} values for microsatellites, and (2) raw F_{ST} values for mtDNA on the log of geographic distance. Finally, we compared the ratio of F_{ST} to log (distance) between colonies within subspecies and between subspecies (both delineations) using unpaired tests for differences in means.

To evaluate prediction 5, we performed a series of AMOVAs in Arlequin on (1) microsatellite allele identity (Weir and Cockerham, 1984), (2) microsatellite repeat number (Slatkin, 1995), and (3) mtDNA to determine how genetic diversity was distributed within and between the previously delineated subspecies. AMOVAs were then repeated with the revised subspecies delineation. In order to maximize the amount of variation between subspecies (i.e., to determine that we were accurately revising the subspecies delineation), we repeated the AMOVAs removing one, two, or three admixed colonies at a time and placing them in the other subspecies. Finally, average pairwise differentiation between subspecies was estimated by calculating F_{ST} as above, but by pooling all individuals within subspecies. We also estimated the degree of gene flow between subspecies by calculating F_{ST}-derived N_m (Slatkin, 1995) for microsatellite and mtDNA. We used the observed geographic distribution of microsatellite and mitochondrial diversity to revise the geographic boundary between subspecies.

2.3. Morphological variation

Morphological analyses have been performed more thoroughly in other studies (Hollister, 1916; Pizzimenti, 1975), so assessing morphological variation between subspecies was not a central focus of this study. Nonetheless, we present results from our field measurements of body size (mass, body length, and tail length). To determine whether there were differences in body size between subspecies, using the revised boundary, we performed general linear mixed modeling using the lme4 package for R (Bates et al., 2012). First, we estimated the degree of sexual dimorphism for each measure of body size. We initially modeled all individuals together, but because the sexes were significantly different in all models, and because prairie dogs are sexually dimorphic (Hoogland, 2003), we chose to model the sexes separately. In each model, body size was the dependent variable and subspecies and sampling date were fixed effects, and population was a random effect. Sampling date was included in the model because size varies throughout the year (Hoogland, 2003), although not always consistently (e.g., weight varies with reproductive state, precipitation patterns, and food availability). An interaction between subspecies and date was allowed to account for potential bias in sampling, and if significant, *a posteriori* tests were performed with each independent variable separately. We repeated these modeling steps using mass, body length excluding tail, and tail length as the measures of body size. Our models were based on field measurements of 268 *C. g. zuniensis* males and 278 females, and 121 *C. g. gunnisoni* males and 130 females.

2.4. Ecological variation

All site locations were verified using Google Earth version 4.3 (Google Inc., Mountain View, CA) to ensure that data extracted in ArcMap corresponded to the actual sampling locations. Ecological characteristics of sampled colonies were first analyzed in ArcGIS version 10 (ESRI, Redlands, CA) using land cover data at 30 m resolution from the Southwest Regional Gap Analysis Project (SWReGAP, Lowry et al., 2005) and climate data at 800 m resolution from the Worldclim database (Hijmans et al., 2005). We compared ecological characteristics between subspecies using the revised boundary. Associations between subspecies and land cover type were evaluated with a chi-square test, repeating for three hierarchical classifications of land cover provided in the SWReGAP database.

Next, we performed ecological niche modeling for each subspecies separately to infer occupancy of different niches under current climatic conditions using R, QGis (www.qgis.org) and Diva-Gis (Hijmans et al., 2001). Additional occurrence records for each subspecies were collated from the Global Biodiversity Information Facility (GBIF, www.gbif.org). To reduce error, each record was verified by comparing coordinates with site locality descriptions, and records were removed if they were duplicate occurrences, if there was potential for the records being subspecies hybrids, or if they were of uncertain subspecies identity. This search yielded 131 unique collecting localities for *C. g. zuniensis* and 169 unique localities for *C. g. gunnisoni*, including the 48 sampling locations from the present study.

We compared ecological niches between subspecies utilizing multiple methods. First, we extracted the climate conditions and elevation at each collecting event, performed a PCA, and executed a randomization test on each of the first three principal components (which together explained >89% of the variation) and compared observed differences to randomized differences. Second, we used a combination of niche modeling and niche similarity tests in MaxEnt (Phillips et al., 2006) and ENMTools (Warren et al., 2010) to test for niche overlap in environmental space.

Finally, we tested for niche similarity between subspecies using ENMTools' background test, which determines whether niches are differentiated when accounting for different conditions given that the subspecies are nearly geographically allopatric. To do this, we imported MaxEnt rasters representing logistic suitability scores per pixel for both subspecies and measured niche similarity using Schoener's D (Schoener, 1968) and the I statistic (Warren et al., 2008). The background test compares niche similarity scores from models of a focal subspecies to models based on the background environment of the other subspecies. It does so by performing 100 random draws of a set of points from the background (defined here as a rectangular area with a 1° buffer around the subspecies distribution) of the comparison subspecies, with the number of points equal to the number of that subspecies' occurrences. We determined whether the actual niche similarity score was within the middle 95% of the randomized scores, which dictates accepting the null hypothesis of no difference in niche.

3. Results

3.1. Genetic variation

In total, we sampled 856 individuals from 48 colonies (mean = 17.5 per colony): 34 colonies from *C. g. zuniensis* (269

males, 280 females and 8 of unknown sex) and 14 colonies from *C. g. gunnisoni* (121 males, 130 females and 48 of unknown sex), using the revised boundary (Fig. A1, Table A1; online Appendix). Omitting populations with fewer than 5 individuals did not affect our conclusions (not shown). Amplification of mtDNA resulted in 929 bp of the cytochrome b gene and 1113 bp of the control region for 838 individuals. Cytochrome b best fit the HKY + γ mutation model including invariant sites, and d-loop best fit the GTR + γ mutation model. There were 150 unique haplotypes belonging to GUPD. The mean number of microsatellite alleles per locus was 10.1 for *C. g. zuniensis* and 8.7 for *C. g. gunnisoni*; average observed heterozygosity was 0.543 for *C. g. zuniensis* and 0.527 for *C. g. gunnisoni*. There was no evidence of null alleles or linkage disequilibrium between markers. There were 49 microsatellite alleles found only in *C. g. zuniensis* (3 of which were at frequency >0.05) and 24 alleles found only in *C. g. gunnisoni* (3 of which were at frequency >0.05).

Prediction 1: The PCA of genotypes depicted three distinct groups of colonies corresponding to the two subspecies and two outlier populations located within one kilometer of each other. One of these populations (RM) consisted of two road kill animals, and the other (TESW) comprised 16 individuals; both populations were characterized by unusually low genetic diversity (observed heterozygosity <0.2, allelic richness <2). Regardless of whether these populations were included in the PCA, randomization tests demonstrated that the previously delineated subspecies were not significantly different ($p > 0.1$). However, with a revised linear subspecies boundary (corresponding to the original range defined by Hollister (1916)), there was significant separation of genotypically distinct subspecies ($p = 0.009$; Fig. 1).

Prediction 2: The Evanno et al. (2005) criterion for identifying cluster number demonstrated the highest degree of support for two microsatellite genotype clusters (Fig. A2), which partially corresponded to the previously delineated subspecies (Fig. 2). However, six colonies placed in *C. g. gunnisoni* with the previous delineation (VADO, CBAR, FUEN, BBM, VCNP and SYWS) clustered instead with *C. g. zuniensis* populations, congruent with the revised boundary. Three colonies (DCB, TPRR, and ENSP) were considered admixed (containing genes from both subspecies) because at least 30% of individuals assigned to the alternative cluster or were assigned at $Q < 0.7$ (Fig. 2). All admixed colonies were on the boundary between subspecies. Changing input parameters in Structure did not alter the conclusions reached from the main analysis. Structurama was unable to consistently resolve the number of clusters, but most commonly found four or five clusters (Fig. A3). Although the inferred groupings in Structurama did not coincide with the two subspecies, the eight populations in northeastern Colorado always clustered together to the exclusion of the remaining populations.

Prediction 3: Bayesian phylogenetic analysis recovered three monophyletic groups with strong nodal support (Fig. A4). One clade contained the 14 *C. g. gunnisoni* colonies present in Colorado

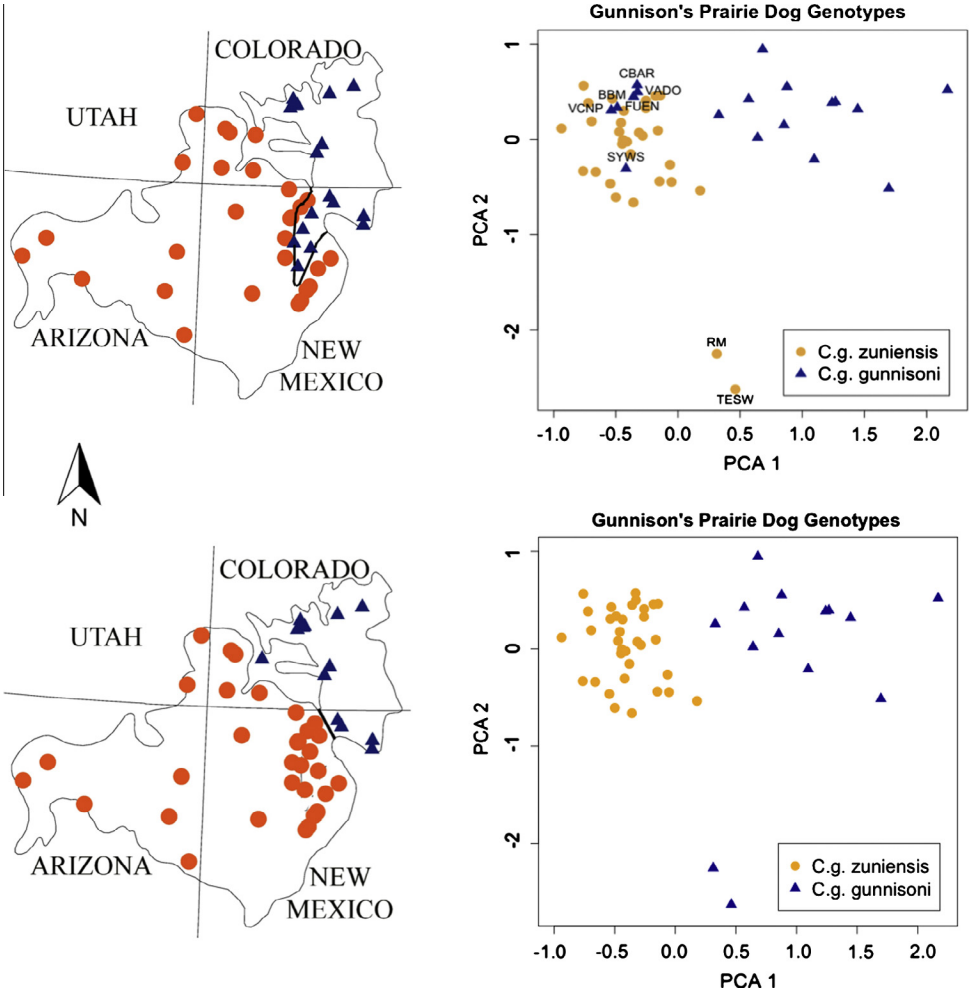


Fig. 1. Upper panel: Map showing the geographic distribution of the two described Gunnison's prairie dog subspecies (left) and the corresponding PCA of microsatellite genotype similarity of colonies (right). Lower panel: Map showing a hypothesized new (and simpler) geographic distribution of the subspecies (left) and the corresponding PCA of microsatellite genotypes (right). Populations re-classified under a different subspecies under the new delineation are labeled in the upper panel.

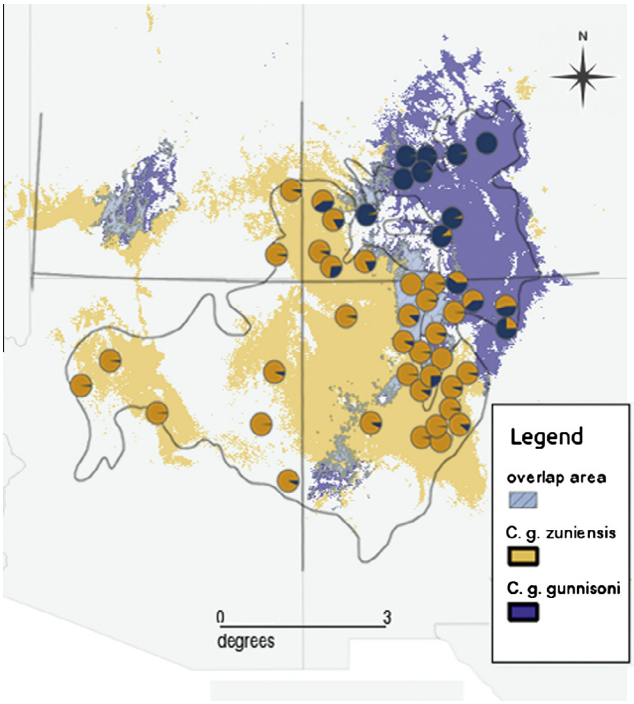


Fig. 2. Map of population composition of Structure-inferred microsatellite clusters, superimposed on MaxEnt-based predicted distribution of both subspecies using current climate and landcover data.

and far northern New Mexico; the other two clades corresponded to *C. g. zuniensis* and were more geographically widespread (Fig. 3). One haplotype sampled from two individuals from a single locality (HMSW) was not included in any of the three clades with confidence (i.e., posterior probabilities for placement of these individuals was low), but usually fell within one of the *C. g. zuniensis* clades. Removing this haplotype from the analysis resulted in higher support for nodes. Three colonies along the subspecies contact zone (DCB, HMSW and TPRR) contained haplotypes from multiple mitochondrial clades (Fig. 3).

Prediction 4: Pairwise F_{ST} values among colonies averaged 0.292 for microsatellites and 0.692 for mtDNA, and were higher between subspecies (0.574 μsat, 0.808 mtDNA) than within subspecies (0.382 μsat, 0.639 mtDNA), using the revised boundary. The ratios of F_{ST} to geographic distance were also higher between than within subspecies ($p \ll 0.001$, Fig. A5). With microsatellite markers, a Mantel test indicated marginally significant isolation by distance across the range of GUPD ($r = 0.0929$, $p = 0.087$); the pattern was stronger with mtDNA ($r = 0.2091$, $p = 0.001$).

Prediction 5: The initial AMOVA using microsatellite allele identity indicated that only 3.21% of the microsatellite variation was attributed to differences between the previously delineated subspecies; similar results were obtained with microsatellite repeat number (Table 2). With mtDNA, 11.20% of the variation was apportioned between subspecies. However, when the subspecies map was redrawn to reflect a linear boundary, more than twice as much variation was distributed between subspecies (8.21% for microsatellite allele size and 33.22% for mtDNA; Table 2; Fig. A6). Removing 1–3 admixed colonies at a time and placing them in the other subspecies did not consistently increase the amount of variation apportioned between subspecies. Pooling all individuals within each subspecies also produced significant differentiation between subspecies (μsat-derived linear $F_{ST} = 0.1170$, $p \ll 0.001$; mtDNA-derived $F_{ST} = 0.3622$, $p \ll 0.001$). Corrected pairwise sequence difference between subspecies, using the revised boundary, was 7.339. With microsatellite DNA, we estimated 2.136 effective migrants per generation between subspecies, and with mtDNA, there were 0.881 effective migrants per generation (both estimates derived from F_{ST}, individuals pooled). The average pairwise mtDNA sequence divergence between subspecies was 1.07% (Table A3).

3.2. Morphological variation

The general linear mixed models revealed significant differences in body size between subspecies in tail length, and marginally significant differences in body mass and body length without the tail. In line with our understanding of sexual dimorphism in prairie dogs (Hoogland, 2003), we observed a significant effect of sex on body size ($p < 0.05$) in all but one model (body length among *C. g. gunnisoni*), with males larger than females. Interestingly, this

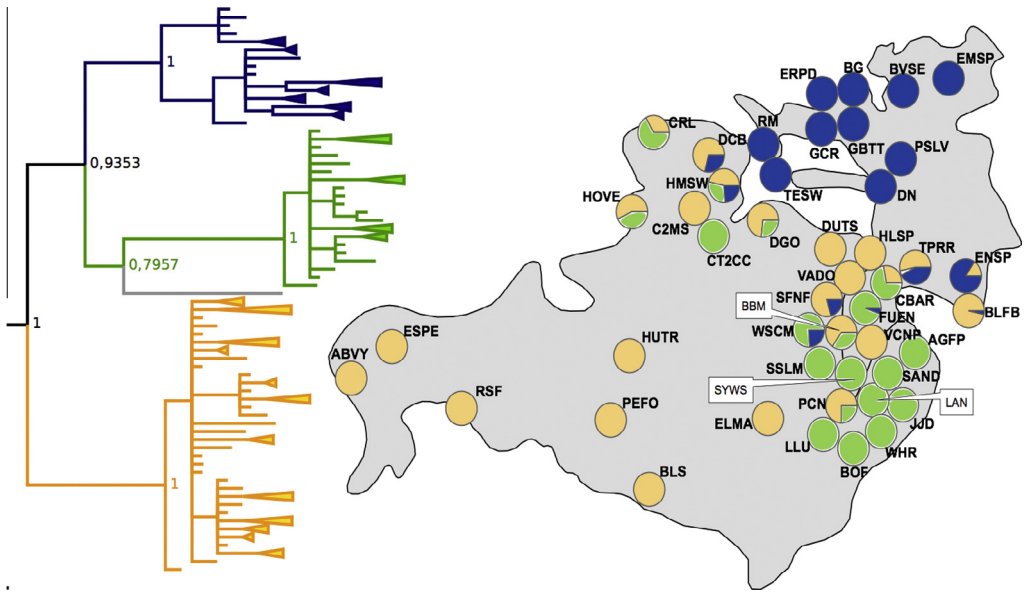


Fig. 3. Left: Inferred phylogenetic relationships among 150 unique mtDNA haplotypes from 763 prairie dogs. The gray taxon was not consistently placed in any clade. Right: Geographic distribution of the three mtDNA clades; dark blue represents *C. g. gunnisoni* and both light colors correspond to *C. g. zuniensis*. *C. leucurus* outgroup not included for clarity.

Table 2
Percentage of variation attributed to differences between subspecies, among populations, and among individuals.[a]

	Microsatellite allele sizes		Microsatellite repeat number		mtDNA	
	Old subspecies	New subspecies	Old subspecies	New subspecies	Old subspecies	New subspecies
Between subspecies	3.21	8.21	4.21	9.05	11.20*	33.22
Among populations	24.72	22.28	24.40	21.65	65.37	47.21
Among individuals	72.07	69.51	71.39	69.31	23.43	19.57

[a] AMOVAs were conducted in Arlequin and significance was assessed by permutation test. All were highly significant ($p < 0.001$) except where noted by an asterisk ($p < 0.05$).

difference was more pronounced in *C. g. zuniensis* (males were 104 g heavier) than in *C. g. gunnisoni* (males 50 g heavier): sexual dimorphism was more prominent in *C. g. zuniensis* than in *C. g. gunnisoni*. A similar pattern of increased dimorphism among *C. g. zuniensis* was observed for other measures of body size (Table A4; R code, Online Appendix).

For male prairie dog weight, there was no effect of subspecies: Although *C. g. zuniensis* males tended to be heavier (mean 718 g; Table A4) than *C. g. gunnisoni* males (mean 648 g), the difference was not significant ($\chi^2 = 1.47_{(df=1)}$, $p = 0.225$); date also had no linear effect on mass ($\chi^2 = 0.12_{(df=1)}$, $p = 0.729$). Among females, the best model recovered marginally significant effects of subspecies ($\chi^2 = 3.13_{(df=1)}$, $p = 0.077$) and date ($\chi^2 = 3.70_{(df=1)}$, $p = 0.055$) on weight. For male body length (excluding tail), the best model revealed a marginally significant effect of subspecies ($\chi^2 = 3.67_{(df=1)}$, $p = 0.056$) and significant effect of date ($\chi^2 = 5.10_{(df=1)}$, $p = 0.0239$) on body length, with *C. g. zuniensis* males longer than *C. g. gunnisoni*. Among females, the best model revealed a marginally significant effect of subspecies ($\chi^2 = 2.93_{(df=1)}$, $p = 0.087$) and a significant effect of date ($\chi^2 = 12.98_{(df=1)}$, $p < 0.001$) on body length. Finally, for prairie dog tail length, highly significant differences in tail length existed between subspecies for both sexes ($\chi^2 > 19_{(df=1)}$, $p \ll 0.001$; Fig. A7), and date had no effect ($\chi^2 < 0.09_{(df=1)}$, $p > 0.7$).

3.3. Ecological variation

With the exception of elevation and one temperature measure, ecological differences were always greater between the revised subspecies (data reported here) than the previously proposed subspecies (data available on request). Colonies of the two subspecies were found at significantly different elevations (mean *C. g. zuniensis* = 1962 m, mean *C. g. gunnisoni* = 2507 m, $F_{(1,46)} = 42.26$, $p \ll 0.001$), and there was isolation-by-elevation even when controlling for geographic distance (microsatellites: $r = 0.2125$, $p = 0.001$; mtDNA: $r = 0.1257$, $p = 0.009$). When incorporating occurrence records from GBIF, PCA separated climate data into axes representing precipitation (PC axis 1), temperature (PC axis 2), and seasonal variation in both (PC axis 3). The randomization test demonstrated that subspecies differed significantly in the climate of their habitats ($p \ll 0.001$ for all three axes, Figs. A7–A9). If elevation was removed from the climate data, similar results were obtained (not shown), likely due to the close relationship between elevation and temperature.

Land cover was significantly associated with subspecies identity at three levels of hierarchical classification. At the coarsest scale, land cover in the range of GUPD was classified as either forest/woodland, grassland/shrubland, or semi-desert (Table A5). *C. g. gunnisoni* was found more often, and *C. g. zuniensis* less often, in grassland/shrubland than expected by chance ($\chi^2 = 7.88_{(df=2)}$, $p < 0.025$). After dividing each land cover type into sub-classifications two times, the association between land cover and subspecies persisted. At the intermediate classification, *C. g. gunnisoni* colonies were found more often in 'Western North American grassland/shrubland' and less often in 'Western North American cool temperate woodland and scrub' than expected by chance ($\chi^2 = 10.23_{(df=4)}$, $p < 0.05$). At the finest classification scale, *C. g. gunnisoni* was found more often than expected in 'Southern Rocky Mountain montane grassland and shrubland' and less often than expected in 'Rocky Mountain Two-needle piñon-juniper woodland' ($\chi^2 = 16.88_{(df=9)}$, $p = 0.05$).

Biomod2 showed that MaxEnt performed the best among all model approaches, with TSS and AUC scores consistently the highest across all three model runs with three separate background layers (MaxEnt *C. g. gunnisoni* AUC = 0.98, and *C. g. zuniensis* = 0.914). For binary presence/absence maps we used an equal test sensitivity and specificity criterion (0.311 for *C. g. gunnisoni* and 0.265 for *C. g. zuniensis*). Niche model binary results from MaxEnt modeling supported the idea that subspecies are nearly allopatric with minimal areas of potential overlap (Fig. 2), some of which corresponded to regions of genetic admixture. Niche similarity scores for the two subspecies were 0.293 for Schoener's D and 0.537 for the I-statistic. Background tests showed that the comparison of *C. g. zuniensis* occurrences to *C. g. gunnisoni* background had minimum scores of Schoener's D of 0.36 and 0.66 for the I-statistic. Comparing these minimum scores to the actual values (above) indicated that the two subspecies are significantly niche differentiated even when accounting for the environmental background characteristics. However, the reciprocal comparison of *C. g. gunnisoni* occurrences to *C. g. zuniensis* background was not significantly different (minimum Schoener's D 0.257, I-statistic 0.524).

4. Discussion

There is evidence from multiple data types and analyses that supports recognition of two distinct subspecies of *C. gunnisoni* corresponding to a revised subspecies boundary (Figs. 1, A1). All five testable predictions demonstrate support for the subspecies hypothesis: Evidence from both microsatellite and mtDNA suggests that GUPD in the 'montane' region of their range in Colorado form a distinct group that also includes four colonies in New Mexico. These colonies, coinciding with *C. g. gunnisoni*, display a moderate degree of morphological differentiation and occur in environments that differ from the localities occupied by *C. g. zuniensis*. It is unknown whether the two subspecies are incipient species subject to divergent selection due to habitat characteristics or reflect a recent and expanding secondary contact zone that will ultimately dissolve into a single species through hybridization.

4.1. Genetic variation

Our analyses revealed three spatially disparate mitochondrial clades, one of which was restricted in distribution and corresponded to *C. g. gunnisoni* (Figs. 3 and 4, A3). The polyphyly of *C. g. zuniensis* mtDNA, and its discordance with nuclear microsatellite patterns, may arise for several reasons (Toews and Brelsford, 2012). First, it may be due to incomplete lineage sorting (the persistence of ancestral alleles in both populations), which is common in taxa that are recently diverged (Pamilo and Nei, 1988).

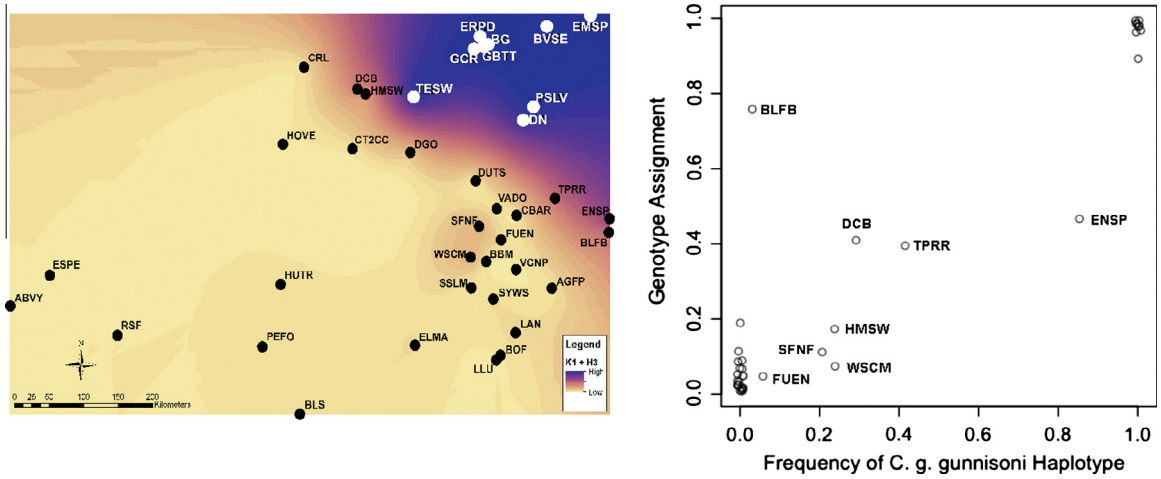


Fig. 4. Degree to which each sampled population genetically matches *C. g. gunnisoni*. Left: Map showing the sum of the proportion of membership in the *C. g. gunnisoni* microsatellite cluster (K1) and the frequency of *C. g. gunnisoni* mtDNA haplotypes (H3) in each population; darker blue signifies more strongly *C. g. gunnisoni*. Values in unsampled regions were generated using the kriging function in ArcMap. Right: Proportion of membership in K1 plotted as a function of the frequency of H3. Colonies along the contact zone have intermediate values for both measures and are labeled. Unlabeled values are 0,0 and 1,1 and were adjusted to show multiple points.

Non-monophyly in mitochondrial genes could also result from sex-biased dispersal (e.g., Cathey et al., 1998), which is documented in GUPD (Hoogland, 1999). The pattern may also be due to adaptive introgression of advantageous alleles (e.g., Alves et al., 2008), movement of the contact zone between subspecies (e.g., Krosby and Rohwer, 2009), or other reasons (e.g., Arntzen et al., 2009). Future research should attempt to construct phylogenies by using nuclear sequence data from multiple loci (e.g., Pagés et al., 2008), implementing multilocus inference methods (e.g., Larget et al., 2010), and explicitly modeling variation in the coalescent process (Carstens and Knowles, 2007).

Our Structure analysis, using the Evanno criterion for evaluating K, indicated the existence of two clusters that agreed with the revised subspecies designation, and PCA and AMOVAs supported the separation of colonies into these same two groups. If three groups are forced in the Structure analysis, they do not correspond to the mitochondrial clades, to any known ecological, geographical or morphological data, or to the other genetic data. The amount of molecular variance between subspecies is similar to or greater than variance among subspecies in other studies of ground squirrels, including those proposed for recognition as separate species (Hoisington-Lopez et al., 2012). The existence of unique alleles in each subspecies may offer a useful diagnostic tool for managers interested in classifying particular populations as one subspecies or the other.

Most colonies belonged to a microsatellite cluster that corresponded with its mitochondrial clade (e.g., colonies in Arizona contained the *C. g. zuniensis* mitochondrial clade and belonged to the *C. z. zuniensis* microsatellite cluster). In particular, colonies at the northeastern and southwestern range edges were unambiguously part of either *C. g. zuniensis* or *C. g. gunnisoni*. However, four colonies at the intersection of the subspecies' ranges (DCB, HMSW, TPRR, and ENSP) contained at least 30% of individuals (mtDNA haplotypes, μsat genotypes, or both) from both subspecies (Fig. 3); in addition, three colonies (SAM, ENSP and BLFB) belonged to a different microsatellite cluster (e.g., *C. g. gunnisoni*) than the corresponding mitochondrial clade (e.g., *C. g. zuniensis*). This admixture provides evidence of gene flow across the subspecies boundary, as expected for subspecies (or recently diverged species) that have not undergone complete reproductive isolation. Some species of prairie dogs disperse up to 6–10 km (Knowles, 1985; Garrett and Franklin, 1988); even if such long-distance dispersal events are infrequent (Hoogland, 1999), they could facilitate gene flow across the boundary.

Collectively, our genetic results support recognition of two genetically distinct subspecies with a boundary line between the two that is approximately linear (Fig. 1). The degree of sequence divergence between subspecies (1.07%) is analogous to the degree of differentiation at the same or similar loci between other currently recognized subspecies described within the family Sciuridae (Wettstein et al., 1995; Steppan et al., 1999; Oshida and Masuda, 2000; Oshida et al., 2000; Lance et al., 2003; Herron et al., 2005; Hoisington-Lopez et al., 2012). Moreover, mutation rates in cytochrome b within *Cynomys* are thought to be 5–10 times slower than in other members of the ground squirrel subfamily (Xerinae) (Nabholz et al., 2008), indicating that divergence times for a given degree of sequence divergence are older.

4.2. Morphological variation

The two subspecies displayed moderate differences in weight and body length, and highly significant differences in tail length, with *C. g. zuniensis* consistently larger and with more pronounced sexual dimorphism. There was a large amount of variation among populations and across dates in all size measures, likely due to local factors such as food availability and to seasonal weight gain (e.g., among juveniles) or loss (e.g., nursing females or breeding males). The degree of variability may have obscured a possible relationship between date and male weight, or the relationship may be nonlinear. Future studies should attempt to minimize variation due to sampling date and to assess bacular morphology. Our findings are in line with previous studies, which have documented differences between the subspecies in pelage (Hollister, 1916; Bailey, 1931), cranial morphology, hind foot length, number of tail vertebrae (Hollister, 1916), and bacular morphology (Pizzimenti, 1975).

A primary argument against subspecies recognition stems from Pizzimenti (1975), in which the author found that the slight morphological differentiation was less than that among other *species* of prairie dogs. We disagree with his conclusion for several reasons: (1) subspecies are expected to display less differentiation than species; (2) at least one of the 10 populations (El Rito, NM; located between CBAR and FUEN, Fig. S1) that Pizzimenti (1975) grouped within *C. g. gunnisoni*—the one with the largest sample size—most likely belongs instead to *C. g. zuniensis*, in light of the

revised subspecies boundary; (3) none of the morphological characters used in his study were sufficient to distinguish among prairie dog species (Pizzimenti, 1975, pg. 21); (4) in that study, sexes were pooled for all analyses, despite the existence of sexual dimorphism (pg. 3; Table 3, pg. 7); and 5) observed differences between subspecies (cranial morphology, Table 14, pg. 29) were either discounted as being unimportant (pg. 31) or were not discussed (e.g., bacular morphology, Fig. 14, pg. 51). The present study supports the morphological distinction between subspecies documented by other authors (Hollister, 1916; Bailey, 1931).

4.3. Ecological variation

In addition to isolation-by-distance, we observed isolation-by-habitat (Wagner and McCune, 2009) even when controlling for the effects of geographic distance. *C. g. gunnisoni* colonies existed at higher elevations and in colder sites than *C. g. zuniensis*. Ecological niche modeling demonstrated niche divergence between subspecies, supporting the idea that climate may be important in limiting distributional extents (Wagner and Drickamer, 2004). The asymmetrical niche differentiation observed with the background test is common in cases where one taxon may specialize more within an environmental matrix (e.g., *C. g. gunnisoni*) than the other taxon. The overall pattern is strongly suggestive of niche differentiation, particularly because this test, unlike niche identity tests—which do not consider the environmental context from which samples are drawn—are conservatively aimed at finding similarities rather than significant differences. Niche identity tests showed overwhelming support for niche differentiation (Fig. A9).

Abiotic habitat differences were paralleled by differences in plant communities between subspecies: While *C. g. zuniensis* occurred in habitats ranging from semi-desert to piñon-juniper woodland, *C. g. gunnisoni* colonies were restricted to montane grasslands. Prairie dogs are diet generalists and tend to eat whatever forage is available (Pizzimenti and Hoffman, 1973), but plant community structure may be important to prairie dog divergence if it influences the way sound from mating calls travels through the habitat (Boughman, 2002; Perla and Slobodchikoff, 2002). Temperature may contribute to evolutionary divergence of prairie dogs because the timing of estrus is related to the date of emergence from hibernation (Hoogland, 1997, 1998), which varies by elevation (Longhurst, 1944). It is unknown whether *C. g. gunnisoni* individuals possess physiological adaptations for living at high elevations; nonetheless, the association with habitat suggests that the *C. g. gunnisoni* subspecies has become specialized to a montane environment. Collectively, these results invoke an ecological explanation for the existence of subspecies.

4.4. Revision of subspecies boundary

We propose a revised, more parsimonious range map with an approximately linear subspecies boundary based on several lines of evidence: (1) the PCA of genotypes did not detect significant differences between the previously delineated subspecies, but did resolve significant differences between the revised subspecies; (2) the species comprises two microsatellite genotype clusters in agreement with other analyses; (3) the geographic distribution of mitochondrial clades provides support for a unique subspecies (*C. g. gunnisoni*) that exists in a more restricted distribution than previously thought (Fig. 1); and 4) the proportion of genetic variation attributed to between-subspecies differences was substantially higher with the revised than the previously delineated subspecies. Redefining the delimitation between GUPD subspecies better encapsulates the ecological and genetic diversity of the species, and is congruent with historical morphological descriptions of the subspecies (Hollister, 1916).

5. Conclusions

5.1. Conservation and management implications

Continued recognition of subspecies has important management implications. Recently, the USFWS determined that GUPD populations "located in central and southern Colorado and north-central New Mexico are warranted for protection under the Endangered Species Act" (USFWS, 2008). This declared region corresponds with the range of *C. g. gunnisoni*. The most important contemporary factor threatening prairie dogs is thought to be sylvatic plague (USFWS, 2008; Cully et al., 2010; but see Hoogland, 2006), which causes local extinctions throughout the range of *C. gunnisoni*. Extirpated colonies are recolonized predominantly by dispersal when nearby source colonies exist (Sackett et al., 2013), or from relocations implemented by managers. Our results suggest that the two subspecies are distinct biological entities, and that managers should consider subspecies identity when choosing source populations for relocation.

Subspecies are often defined solely based on one or few characteristics (e.g., geographical occurrence, pelage color), leading to doubts about the practical importance of subspecies. However, subspecies recognition has biologically relevant implications for taxa that possess geographically- or genetically-based intraspecific variation in pathogen susceptibility (Atkinson et al., 2000), physiological tolerance to environmental conditions (Henry et al., 2012), dispersal ability (Foote and Larkin, 1988) or other traits. In this paper, we have shown that subspecies can be quantitatively evaluated by using multiple data types and analyses to test the hypothesis that unique groups exist.

Data archiving

DNA sequences will be available on GenBank (Accessions TBD), and microsatellite genotypes and morphological data will be uploaded to Dryad (doi TBD pending acceptance).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.03.010.

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