IMMUNOLOGICAL SYSTEMATICS OF PRAIRIE DOGS

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Abstract.—Relationships within the genus *Cynomys* were investigated using immunoelectrophoretic characters. A method is presented for coding these characters, by which numerical phylogenies can be constructed. No intraspecific differences were observed within *Cynomys*. Within the white-tailed group, *C. leucurus* and *C. parvidens* are antigenically similar, whereas *C. gunnisoni* appears more distantly related, although retaining many of the white-tailed characteristics. In the black-tailed group, *C. ludovicianus* and *C. mexicanus* appeared antigenically similar, but differentiated in two characters. The white-tailed and black-tailed groups are distinct lineages. The relationships of these two lineages to the *Spermophilus* species examined is unresolved.

Currently the genus *Cynomys* is classified in two subgenera, five species, and two subspecies. The genus ranges from extreme southern Saskatchewan to the valleys of the Sierra Madre Oriental of north-central México and from southwestern Utah to the eastern edge of the Great Plains (Hall, 1981). There have been several studies of the systematics of this group (Black, 1963; Bryant, 1945; Chesser, 1983a, 1983b; Hollister, 1916; Nadler et al., 1971; Pizzimenti, 1975, 1976a, 1976b; Pizzimenti and Nadler, 1972), but relationships of members of *Cynomys* to each other and to other sciurids are still problematic.

We investigated intrageneric relationships of *Cynomys* by immunoelectrophoresis. This technique has been used taxonomically to a limited degree in mammals, e.g., mink (Baranov et al., 1976, 1978) and bats (Haiduk, 1983). Some immunological work has been done on sciurids (Ellis and Maxson, 1980; Gerber and Birney, 1968; Hight et al., 1974) but ours is the first comprehensive immunoelectrophoretic study of *Cynomys*. Analyses were performed on the five species of *Cynomys* as well as a select group of related sciurids in order to evaluate the phylogenetic relationships among prairie dogs. An additional goal of this study was to develop a method for coding immunogenetic characters that is tractable for numerical phylogenetic analyses.

METHODS AND MATERIALS

Prairie dogs were either shot or live-trapped. Blood samples were taken and prepared as described by McCullough (1985). However, blood from *C. parvidens* was taken from the femoral vein and these animals were subsequently released. Serum samples were stored in liquid nitrogen and then recentrifuged upon return to the laboratory.

Antisera were generated against *C. ludovicianus* and *C. leucurus* in young male New Zealand white rabbits. These two species were chosen as they represent sufficiently divergent taxa within the genus to help make the proper interpretation of results possible. The method of antibody production and immunoelectrophoretic techniques followed Graber and Williams (1955) and Williams (1971) as modified by Diffley and Honigberg (1977), Haiduk (1983), and McCullough (1985). The antigens were electrophoresed (1% agarose in 0.5 M sodium barbital solution, pH 8.6) at 4°C for approximately 3–4 hours. Current remained constant at 15 mA per plate and a continuous buffer was used.

All antigen samples contained approximately 50 mg protein/ml. Serum samples were not diluted as dilution caused some precipitation lines to disappear. Before heterologous samples were run, homologous samples were referenced to detect any differences between age groups, sexes, or geographic areas. If no intraspecific differences were noted, heterologous samples were run. Absorption tests also were conducted to verify the homogeneity of antigenic reactivity both intra- and interspecifically. Antisera were absorbed with the antigens used to produce them. In this study, immunoelectrophoresis represents a qualitative analysis; differences were noted as the presence, absence, or relative mobility of particular precipitation lines (Haiduk, 1983). Finally, outgroup comparisons were made using the serum antigens from the following genera: *Marmota* (*M. flaviventris*); *Spermophilus* (*S. mexicanus, S. tridecemlineatus*); and *Ammospermophilus* (*A. leucurus*).
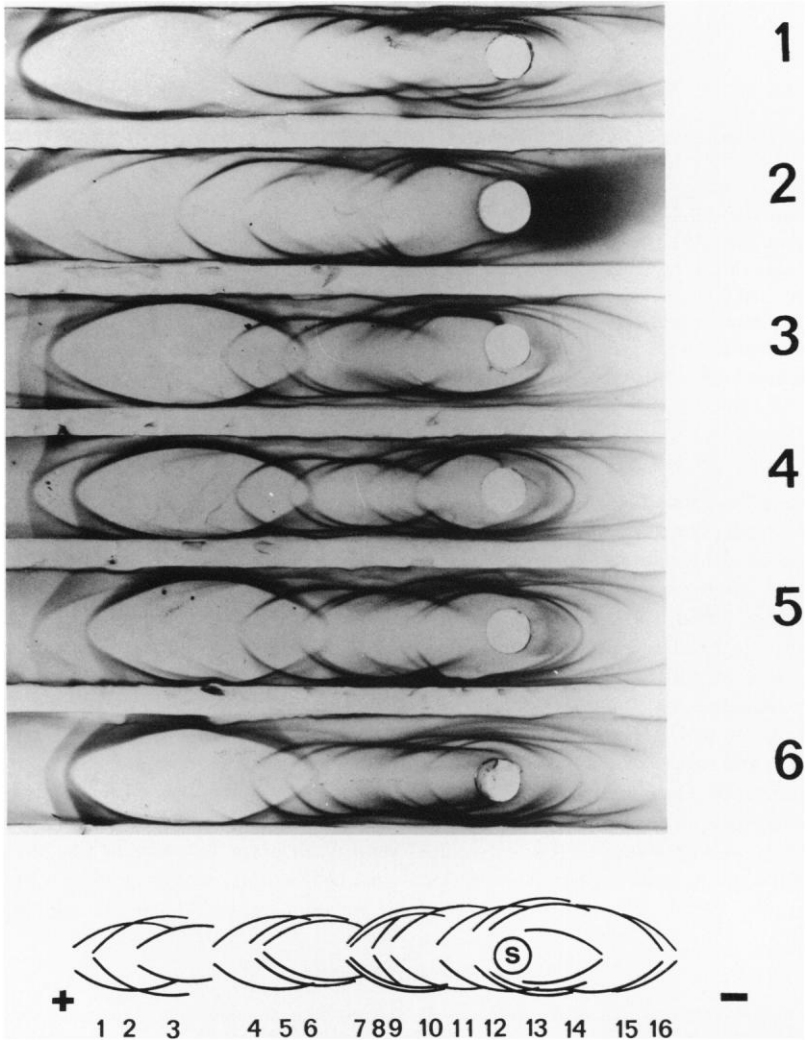
FIG. 1.—Photograph of a representative immunoelectrophoretic gel. Some precipitation bands may not be visible. Antigen samples are of various members of the genus *Cynomys* run against antiserum to *C. ludovicianus*. Sample numbers refer to the species: 1–2, *C. leucurus*; 3–5, *C. mexicanus*; 6, *C. ludovicianus*. The diagram below the photograph represents an approximation of a typical gel and the numbering pattern of precipitation bands (− = cathode, + = anode, S = sample well).

These comparisons were run to ascertain primitive character states for the group and to develop a proper phylogeny using immunoelectrophoretic data.

Precipitation bands represent areas of antigenic recognition and therefore are indicative of close antigenic relationships. Absence of precipitation bands is interpreted as lack of antigen recognition and hence reflects a dissimilarity of antigenic relationship. Mobility differences of precipitation bands indicate that, within a "recognized" antigen, modifications sufficient to alter the electrical charges have occurred. Thus the presence-absence data likely are more effective in inferring phylogenetic relationships among the less closely related taxa in the study, and the mobility data are more appropriate for resolving relationships among the more closely related taxa. We performed separate analyses for presence-absence and mobility data for constructing phylogenies of *Cynomys* and outgroup taxa. Data for the presence or absence of reactions against antisera to *C. ludovicianus* and to *C. leucurus* were combined to give a total of 20 binary presence-absence characters used in the analysis.

TABLE 1.—*Discrete immunoelectrophoretic differences between species of the genus* Cynomys *and outgroup taxa based upon responses to antisera from either* C. ludovicianus *or* C. leucurus. *Numerals refer to precipitation bands depicted in Fig. 1. A minus sign denotes a missing band or bands.*

Taxon	C. *ludovicianus* antiserum	C. *leucurus* antiserum
C. *ludovicianus*	none	slow 1, 2, 5, 16, −3, −one central band
C. *mexicanus*	medium 1, fast 7	medium 1, slow 2, 5, 16, −3, −7 −one central band
C. *gunnisoni*	fast 1, 2, 16, −one central band	−3, −6, −12
C. *leucurus*	fast 1, 2, 5, 16, −6, −two central bands	none
C. *parvidens*	fast 1, 2, 5, 6, 16, −one central band	none
A. *leucurus*	medium fast 1, 2, −six bands	fast 1, 2, −five bands
M. *flaviventris*	very fast 1, 2, −seven bands	very fast 1, 2, −seven bands
S. *mexicanus*	fast 1, 2, −four bands	−three bands
S. *tridecemlineatus*	fast 1, 2, −four bands	−three bands

Our data show that *Marmota* is considerably less similar to *Cynomys* than is *Ammospermophilus* or *Spermophilus*; thus, the character states exhibited by *Marmota* were used to root Wagner trees, using *Ammospermophilus* and *Spermophilus* as additional outgroups following the method of Maddison et al. (1984). A Wagner parsimony tree (Farris, 1970) was constructed using the WAGNER78 package written by J. S. Farris. The program was run five times, each time changing the order of taxon entry (except *Marmota*) to explore the possibility of alternate tree topologies being produced under the parsimony criterion.

Data from mobility differences of precipitation bands were analyzed separately, in an analogous manner. Character state trees were defined as linear, with intermediate protein mobilities assigned to intermediate positions on the trees. Order analogy between relative mobility and character state tree position was assumed because amino acid substitution may affect mobility additively (Nei, 1971). Characters were coded by the additive binary method (Sokal and Sneath, 1963). Each species therefore was coded positive for the mobility value it exhibited, as well as for those mobility values considered more primitive for that particular precipitation band. Two of these bands had three states present; thus seven binary characters were available for this analysis. *Marmota* was excluded from the analysis of mobility data because the absence of a large number of precipitation bands for this species would have severely restricted the number of characters available in the analysis, and because *Ammospermophilus* was shown by the analysis of presence-absence data to be an acceptable outgroup for the *Cynomys* and *Spermophilus* portion of the data.

Specimens examined.—All specimens collected are deposited in The Museum, Texas Tech University. All specimens of *C. parvidens* were released after capture. *Cynomys ludovicianus*—Texas: Motley Co., 11 mi E Matador (10); 9 mi E 8 mi S Matador (25); Colorado: Boulder Co., Township 1 S Range 69 W Section 13 (2). *C. mexicanus*—México: Nuevo León, 1 km S Providencia (8); Coahuila, 3.7 km S Los Colonias (9); 6 km W Gomez Farias (10); 8 km W Los Angeles (1). *C. gunnisoni gunnisoni*—New Mexico: Colfax Co., 3 mi S Eagles Nest (6). *C. g. zuniensis*—New Mexico: Sandoval Co., 7.5 mi S Cuba (15). *C. leucurus*—Colorado: Mesa Co., 1 mi SE Orchard Mesa (18); Montrose Co., 2 mi E Cimarron (15); Gunnison Co., 21 mi E Cimarron (7). *C. parvidens*—Utah: Iron Co. (10); Garfield Co. (10). *Ammospermophilus leucurus*—California: Riverside Co., 15.3 mi NE Mecca on Box Canyon road (1). *Marmota flaviventris*—Colorado: Ouray Co., 7 mi S Ridgway on Hwy 50 (1). *Spermophilus mexicanus*—Texas: Motley Co., 9 mi E 8 mi S Matador (1). *S. tridecemlineatus*—Texas: Lubbock Co., T. T. U. Campus (1).

RESULTS

Immunoelectrophoresis of ingroup and outgroup taxa resulted in 16 consistently recognizable bands and several "central" bands (Fig. 1). Distinct immunoelectrophoretic subgeneric and interspecific differences were evident within the genus *Cynomys* (Table 1). No intraspecific differences were found in any species.

In this study, samples homologous to the reference sample (the antigen that generated the antiserum) have the most precipitation bands, whereas heterologous samples have fewer. Those that are present, have equal mobility, and are not shared with any of the outgroups, are considered synapomorphies. Species presenting many bands are presumed more closely related to the reference species than those with few. Differences between the two subgenera of *Cynomys* were readily apparent. All members of the white-tailed group, *Leucocrossuromys*, have much faster
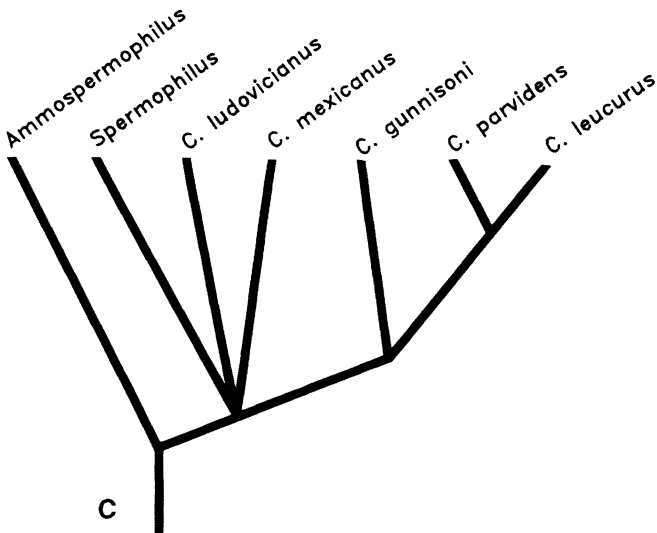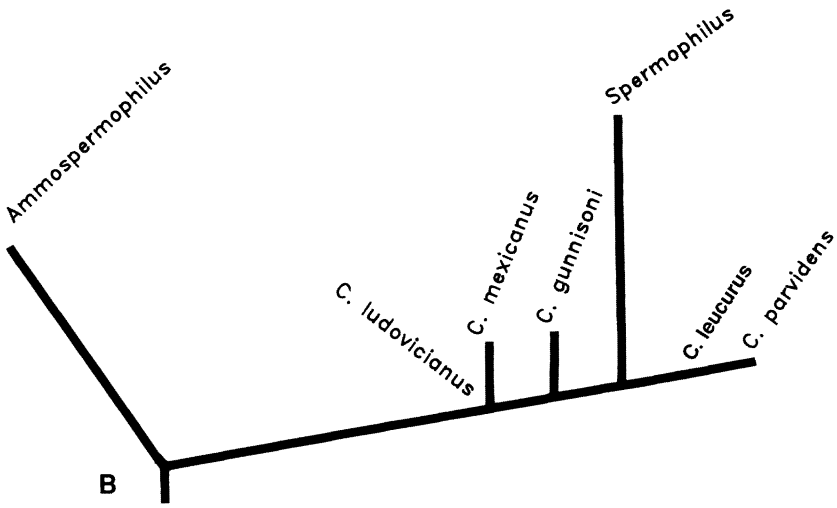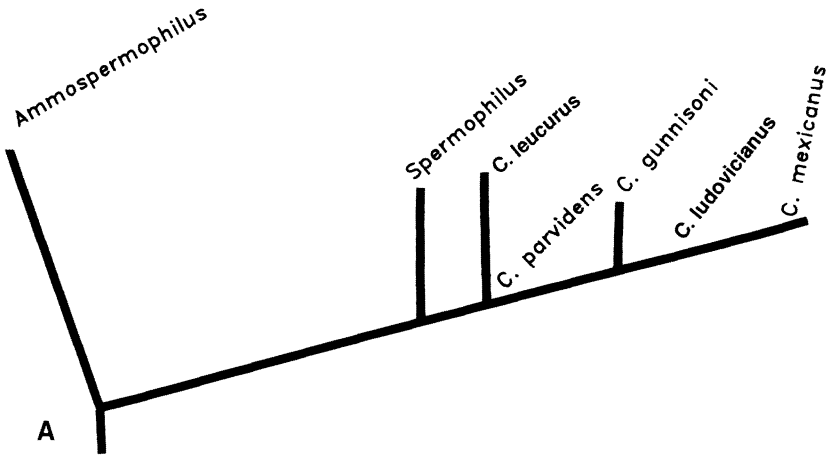
bands 1 and 2. These differences were consistent using either antiserum. For all other systems, results from the two antisera are reported separately (Table 1).

Within *Leucocrossuromys*, *C. leucurus* and *C. parvidens* appear antigenically very similar, varying in only two characters when challenged with *C. ludovicianus* antiserum and none when challenged with *C. leucurus* antiserum. *Cynomys gunnisoni* is distinct from *C. leucurus* and *C. parvidens* in two antigenic characters using *C. ludovicianus* antiserum, and three when using *C. leucurus* antiserum. There is a close relationship between *C. ludovicianus* and *C. mexicanus* as they differ in only two characters using either antiserum. *Marmota* appears the most divergent of the outgroup taxa analyzed, with *Spermophilus* being the most similar to *Cynomys*.

Wagner analysis of the presence-absence data resulted in two equally parsimonious trees (Fig. 2A, B). In both, *Ammospermophilus* is sister to the *Spermophilus-Cynomys* group, and *C. parvidens* is sister to *C. leucurus*. In both trees seven homoplasies are found. An Adams-2 consensus tree (Adams, 1972) was synthesized from these two topologies (Fig. 2C), which shows that the presence-absence data fail to resolve the relationships among *Spermophilus* and the two subgenera of *Cynomys*.

With *Ammospermophilus* as the outgroup, *Spermophilus* and the five prairie dog species were analyzed using the mobility values for the five precipitation bands which all seven species had present. The resultant Wagner tree, which includes no homoplasies, contains two unresolved trifurcations (Fig. 3A). *Spermophilus* cannot be differentiated by these data from the most recent common ancestor of the two prairie dog clades. Similarly, the three white-tailed species are identical across the five diagnostic bands. The immunoelectrophoretic data offer no further resolution to the more basal trifurcation; however, the white-tailed prairie dogs may be further resolved. Using *Spermophilus* as the outgroup (the best estimate of the ancestral condition), and analyzing only the three *Cynomys* species in question, we were able to include data from another two precipitation bands not present in all taxa in the first analysis of mobility data (thus providing nine binary characters for analysis). This Wagner tree, which also contains no homoplasies, shows *C. gunnisoni* to be sister to the other two white-tailed species. Application of this information to the tree of Fig. 3A results in the final topology shown in Fig. 3B. The two black-tailed species form one clade, the three white-tailed species form another clade, and the relationship of these two clades to *Spermophilus* is unresolved.

DISCUSSION

Morphologically, *Cynomys* can be divided readily into the two subgenera *Leucocrossuromys* and *Cynomys* (Hollister, 1916). Within the subgenera, relationships among species are less clear (Chesser, 1983*b*; Hollister, 1916; Pizzimenti, 1975; Pizzimenti and McClenaghan, 1974). Antigenically, the species are more readily distinguished. Within the subgenus *Cynomys*, *C. mexicanus* and *C. ludovicianus* are very similar. The few differences between the two taxa lend support to the hypothesis that *C. mexicanus* may indeed be a relict population of *C. ludovicianus* (Hoffmann and Jones, 1970). Within the subgenus *Leucocrossuromys*, *C. leucurus* and *C. parvidens* appear the closest antigenically. Phylogenetically, *C. gunnisoni* appears to be the sister taxon to the *C. leucurus*-*C. parvidens* clade. Antigenically, the two subgenera *Leucocrossuromys* and *Cynomys* are distinct. There appear to be no intraspecific antigenic differences in any of the species examined, although electrophoretically they have been shown to be polymorphic (Chesser, 1983*a*; Nadler et al., 1971; Nichols and Nash, 1980).

C. gunnisoni is unique among prairie dogs in that it has a diploid karyotype of 40 chromosomes (Nadler et al., 1971). All of the other species have a diploid number of 50 (Nadler et al., 1971),

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FIG. 2.—A, B) Two equally parsimonious trees generated from a Wagner analysis of the presence-absence data of precipitation bands; C) Adams-2 consensus tree synthesized from trees A and B. The genus *Marmota* serves as the stem root.
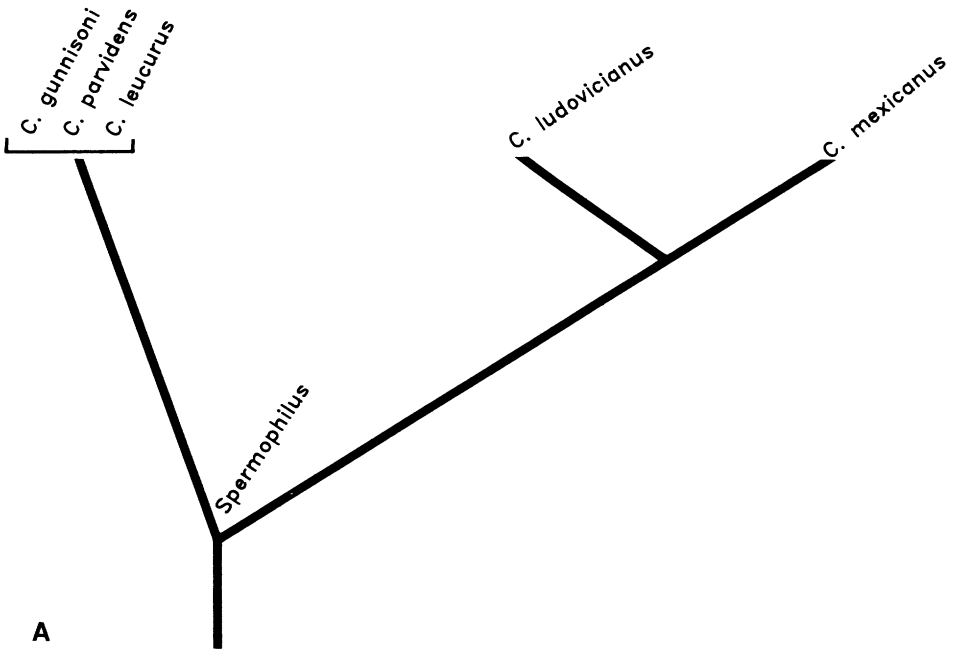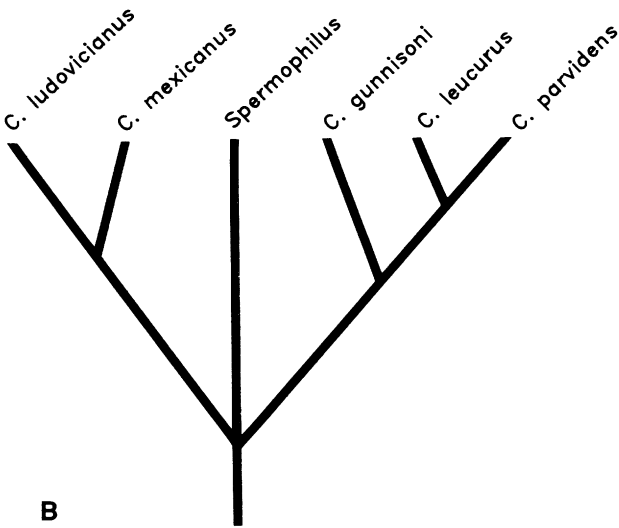
FIG. 3.—A) Wagner tree based on mobility data of precipitation bands with *Ammospermophilus* serving as the stem root; B) Phylogeny of *Cynomys* species based on both presence-absence and mobility data of precipitation bands.

except for limited populations of *C. leucurus* in west-central Colorado which have a diploid number varying from 48 to 51 (Pizzimenti, 1976a). It has been suggested that *C. gunnisoni* is the primitive species for the genus, assuming that a diploid number of 34–40 is the ancestral form as is found in the genus *Spermophilus* (Clark et al., 1971; Nadler et al., 1971). *Cynomys* is the only North American sciurid with a diploid number as high as 50 (Nadler and Harris, 1967). If $2n = 50$ was the primitive form one would expect more sciurids, especially *Spermophilus*-like taxa, to have higher diploid numbers. However, if the phylogeny based on Wagner analysis of the immunoelectrophoretic data is correct (Fig. 3B), an equally parsimonious explanation is that the primitive condition of $2n = 50$ was possessed by the ancestral *Cynomys* and the diploid number of 40 in *C. gunnisoni* represents a derived condition.

Immunoelectrophoretic results have been difficult for previous workers to interpret phylogenetically. Usually a subjective band-by-band analysis has been undertaken (Baranov et al., 1976, 1978; Haiduk, 1983; Mao and Chen, 1982). Using the Wagner tree analysis with the coding methodology described herein, a concise, reliable method of cladistical analysis of immunoelectrophoretic data is possible. This is the first documented use of this method of data coding in immunoelectrophoretic studies.

The results of this study do not deviate greatly from the current systematic understanding of prairie dogs. This study has documented the close relationship between the species *C. leucurus* and *C. parvidens*, as well as between *C. ludovicianus* and *C. mexicanus*. Also of interest is the relative position of *C. gunnisoni* to the remainder of the genus. Additional investigations utilizing various methodologies and a greater diversity of outgroup taxa are needed to further elucidate the relationships of the members of this genus to each other and to other North American ground squirrels.

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