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# Relationships and Genetic Purity of the Endangered Mexican Wolf Based on Analysis of Microsatellite Loci

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**Abstract:** *The Mexican wolf (Canis lupus baileyi), an endangered subspecies of gray wolf, was native to parts of Mexico and the southwestern United States. Currently, only a few individuals, if any, exist in the wild, so planned reintroduction programs must use captive-raised wolves. In only one captive population, however, designated the certified lineage, are all the founders (n = 4) known to be obtained from a wild population of Mexican wolves. Two captive populations were founded from individuals of uncertain ancestry and have not been included in the species survival plan. To preserve genetic diversity and reduce inbreeding so that fitness will be maintained, it would be desirable to include these two captive populations in the breeding program if it could be shown that they were derived from a wild population similar to the certified lineage. We compared allele frequencies of 10 hypervariable microsatellite loci in Mexican gray wolves with those found in a sample of 42 domestic dogs, 151 northern gray wolves, and 142 coyotes to determine if uncertified Mexican wolves had specific markers from these animals. We analyzed pairwise genetic distance measures to demonstrate that the three captive populations of Mexican gray wolves were closely related to each other and distinct from dogs and northern gray wolves. The three captive populations are genetically more similar to each other than to any other population of dog or wolf-like canid, and they shared alleles that were rare in other canids. The genetic distance between them is similar to that between closely spaced populations of northern gray wolves. As a group, moreover, they are the most genetically distinct population of North American gray wolf. Therefore, the three captive populations could potentially be interbred to augment the genetic diversity of the certified lineage. Source individuals for reintroduction should be derived from the captive Mexican wolf population rather than populations of captive or wild northern gray wolves.*

Relaciones y Pureza Genética del Lobo Mexicano Basadas en el Análisis de Loci Microsatélites.

**Resumen:** *El lobo Mexicano (Canis lupus baileyi) nativo de algunas partes de México y el suroeste de los Estados Unidos, es una subespecie del lobo gris que se encuentra en peligro de extinción. En la actualidad solo unos cuantos organismos (y posiblemente ninguno) viven en estado salvaje, de tal forma que los programas de reintroducción tienen que hacerse con lobos mantenidos en cautiverio. Sin embargo, en una sola población cautiva de linaje certificado se encuentran todos los organismos fundadores (n = 4) conocidos por haber sido obtenidos de una población silvestre de lobos mexicanos. Dos poblaciones cautivas fueron fundadas con individuos de incierta procedencia y no han sido incluidas en el plan de supervivencia de especies. Para preservar la diversidad genética y reducir la consanguinidad de tal forma que la buena condición de la población se mantenga, sería deseable incluir estas dos poblaciones cautivas en el programa de reproducción siempre y cuando se demuestre que los organismos provienen de una población silvestre similar a la línea certificada. Con la finalidad de determinar si los lobos mexicanos sin certificar presentaban marcadores específicos comparamos las frecuencias alélicas de 10 loci microsatélites hipervariables en lobos grises mexicanos con aquellos obtenidos de una muestra de 42 perros domésticos, 151 lobos grises del norte y 142 coyotes. Se*

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empleó el análisis de comparaciones pareadas de las medidas de distancia genética, para demostrar que las tres poblaciones cautivas de lobos mexicanos se encuentran estrechamente relacionadas entre sí y distanciadadas de cualquier otra población de perros o cánidos relacionados con lobos, así como para demostrar que comparten alelos raros en otros cánidos. La distancia genética entre ellos es similar a las encontradas para poblaciones cercanas de lobo gris del norte. Como grupo, estos animales además conforman la población de lobos grises genéticamente más distintiva de norteamérica. Por lo tanto, las tres poblaciones cautivas pueden ser potencialmente apareadas entre sí para aumentar la diversidad genética del linaje certificado. Los individuos empleados para la reintroducción deberán provenir de las poblaciones cautivas de lobos mexicanos, en lugar de aquellas cautivas o silvêtres de lobos grises del norte.

## Introduction

The Mexican gray wolf (*Canis lupus baileyi*) once inhabited the southwestern United States and Mexico, extending as far south as the Isthmus of Tehuantepec (Fig. 1). Encroachment by European-style farming and animal husbandry, together with predator-control programs, led to the decline of this once-numerous subspecies of gray wolf. By 1940 the last populations of wild Mexican wolves in the U.S. had been eradicated, and by the mid-1960s a few isolated populations in the Sierra Madre Occidental (Chihuahua and Durango) were the last known to exist. Presently, apart from occasional unconfirmed reports of wolf sightings within the Mexican Sierra Madre Occidental, none appears to have survived in the wild. The Mexican gray wolf is now protected within the U.S. under the Endangered Species Act, having been recognized as the most endangered of the gray wolf sub-

species in North America (McBride 1980; Brown 1983; Bednarz 1988; Ginsberg & Macdonald 1990).

In 1984 the U.S. Fish and Wildlife Service initiated a captive-breeding program for the Mexican wolf in order eventually to reintroduce the subspecies into part of its historic range. Three wild-caught individuals from Durango and Chihuahua (northwestern Mexico) and the offspring of a pregnant female were used to establish the captive colony, designated the certified lineage. The relationship between the founders is uncertain because two of the adult wolves may have been related as mother and offspring (Bednarz 1988; Shields et al. 1988; Siminski 1993). Ideally, to avoid inbreeding depression (Laikre & Ryman 1991) and to increase the amount of genetic variation preserved, additional unrelated Mexican wolves are needed for the captive-breeding program. Only two other captive populations of Mexican wolves have been established. One is designated the

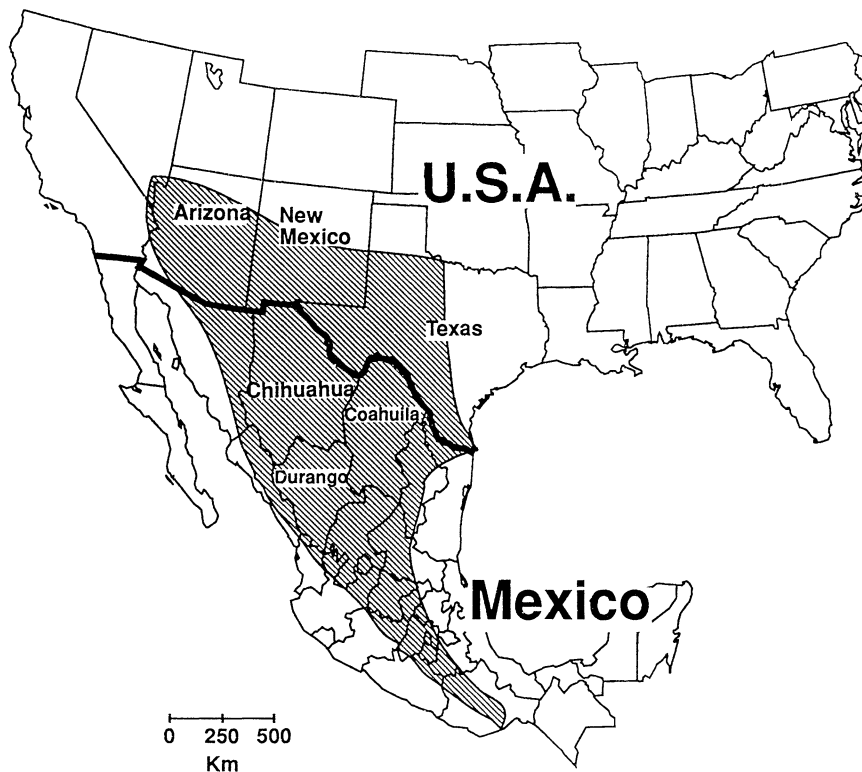


Figure 1. Historic geographic range (shaded area) of the Mexican wolf, modified from Carbyn (1987).

Aragón lineage, established at Parque Zoológico De San Juan De Aragón in Mexico City in 1985 and founded by two or three animals of poorly documented origin (Hedrick 1995). One of the male founders was captured near Chihuahua, Mexico, and may have been mated with a domestic dog. Moreover, careless management of this lineage resulted in the inclusion of a domestic dog-northern gray wolf hybrid that was allowed to mate and sire more than 20 pups. But these offspring were presumably eradicated during a disease outbreak in the group. The other population, designated as the Ghost Ranch lineage, was established at the Arizona-Sonora Desert Museum near Tucson, Arizona, and founded by two individuals. The male founder was bought as a pup by a tourist in the town of Yécora, Sonora (Mexico), and was later donated in 1961 to the Arizona-Sonora Desert Museum. The female founder was captured from the wild in Pack Canyon at the Tumacacori Mountains in southern Arizona in 1959, not far from the Mexican border. The wild-caught founders of both uncertified captive populations could conceivably have been domestic dogs or dog-gray wolf or coyote-gray wolf hybrids. In the United States over 10,000 wolf-dog hybrids are estimated to exist (J. Joslin, personal communication), although they may have been much less common in the 1960s. Hybridization between gray wolves and coyotes (*Canis latrans*) has been documented recently in eastern Canada and Minnesota by means of molecular-genetic techniques (Lehman et al. 1991; Roy et al. 1994). Moreover, gray wolves are frequently obtained as pets from private breeders and may not necessarily be derived from wolves living in the region where they are sold.

The certified and Ghost Ranch lineages of Mexican wolves were previously analyzed for allozyme and mitochondrial DNA (mtDNA) polymorphisms (Shields et al. 1988). The results of these analyses showed that, compared with a limited sample of gray wolves and domestic dogs, Mexican wolves have a unique mitochondrial haplotype. This result was confirmed by a much larger worldwide mtDNA study of gray wolves (Wayne et al. 1992). But no allozyme alleles were found specific to gray wolves or dogs that would allow putative Mexican gray wolves to be distinguished from other subspecies of gray wolf or wolf-dog hybrids (Ferrell, et al. 1978; Shields et al. 1988). Allozyme variability in general is low in gray wolves, and populations seem to differ little in allele frequency (Ferrell, et al. 1978; Shields et al. 1988; Kennedy et al. 1991; Wayne et al. 1991).

Unfortunately, because mtDNA is inherited maternally, mtDNA polymorphisms are useful only in establishing the species origin of female founders. Consequently, nuclear DNA polymorphisms are necessary to establish the purity and distinctiveness of founders from both sexes within the captive populations. Although mtDNA analysis of the Ghost Ranch lineage has estab-

lished that they all have a Mexican wolf haplotype, uncertainty exists primarily about the origin of the male founder (Shields et al. 1988). The Aragón lineage has not been studied by means of molecular-genetic techniques.

Recently, nuclear loci have been identified that are highly polymorphic and potentially allow the identification of population-specific polymorphisms (Bruford & Wayne 1993; Queller et al. 1993). These loci, often termed microsatellites, consist of a variable number of tandem repeats of short sequences and evolve through the gain or loss of repeat units rather than sequence substitutions. Because microsatellite loci have mutation rates as high as  $1 \times 10^{-3}$  per generation and because drift is proportional to the product of population size and mutation rate, analysis of microsatellite loci may reveal differentiation among small, isolated populations that cannot be distinguished by analysis of other, more slowly evolving loci (Bruford & Wayne 1993). Allozymes, for example, have mutation rates several orders of magnitude lower than microsatellite loci (Nei 1987).

Because of the uncertainty concerning the origin of the Aragón and Ghost Ranch lineages, we initiated a genetic study of Mexican gray wolves in captivity to determine if uncertified Mexican wolves showed evidence of hybridization with domestic dogs or coyotes and were genetically similar to certified Mexican gray wolves. We surveyed variation at 10 microsatellite loci in Mexican wolves from the certified Aragón and Ghost Ranch lineages. We compared the microsatellite polymorphisms in these wolves with those found in a sample of 151 gray wolves, 142 coyotes, 40 red wolves (Roy et al. 1994), and a sample of 42 dogs representing different breeds. Our results suggest that the two uncertified captive populations of Mexican wolves do not have alleles otherwise specific to domestic dogs or coyotes but rather share alleles found in certified Mexican wolves. The two putative captive populations of Mexican wolf are unlikely to have been founded by a simple cross between a gray wolf and a domestic dog or coyote but likely represent the descendants of pure Mexican gray wolves. We cannot eliminate the possibility however, that these two captive populations originated from other North American gray wolves or a dog whose offspring had backcrossed to wild wolves for several generations.

## Methods

### Samples

We obtained samples from three different lineages of Mexican wolves; the certified lineage (21 of 92 living animals), the Aragón lineage (8 of 18 living animals), and the Ghost Ranch lineage (10 of 18 living animals). Our sample of the certified wolves includes two of the four founders and some of the first-generation descendants of

the unsampled founders. The genealogy of the uncertified lineages is uncertain, and we have no samples of the founding individuals (Hedrick 1995). We also obtained 42 samples of domestic dogs representing different dog breeds. For comparison with gray wolves and coyotes we used the data presented in Roy et al. (1994) from 151 gray wolves, 142 coyotes, 40 red wolves, and 20 golden jackals. Coyotes were represented by samples from six populations, gray wolves by samples from seven populations, and red wolves by samples from a captive population founded by 14 wild-caught individuals. Five of the gray wolf populations were from areas where molecular-genetic data suggest that no hybridization occurs between gray wolves and coyotes (nonhybridizing; Lehman et al. 1991; Roy et al. 1994), and two populations were from areas where hybridization between the two species has occurred (hybridizing). We treat the two groups separately because of the potential effects hybridization may have on allele frequencies and the levels of heterozygosity of gray wolves (Roy et al. 1994). Genotype and allele frequencies of coyotes seem unaffected by hybridization, suggesting that gene flow between the two species is unidirectional (Lehman et al. 1991; Roy et al. 1994). DNA was obtained from all the samples following standard procedures of extraction (Sambrook et al. 1989).

### Microsatellite Analysis

We surveyed 10 GT(n) microsatellite loci identified from a domestic dog genomic library and known to be polymorphic in wolf-like canids (Ostrander et al. 1993; Gottelli et al. 1994; Roy et al. 1994). Simple sequence alleles from genomic DNA were detected by a standard protocol for microsatellite amplification and detection (Amos et al. 1993; Choudhary et al. 1993; Roy et al. 1994). Briefly, we end-labeled one primer of the pair with [ $\gamma$ - $^{32}$ P]ATP (Amersham) and catalyzed the reaction with  $T_4$  polynucleotide kinase (Sambrook et al. 1989). Amplification through PCR was achieved after 28 cycles with denaturation at 94°C for 45 seconds, annealing at 50–55°C for 45 seconds and extension at 72°C for 60 seconds, except for the final extension that lasted 5 minutes, in a 25- $\mu$ l reaction volume using 50 ng of target DNA, 2 mM  $MgCl_2$ , and 0.8 U Taq DNA polymerase (Promega). Three  $\mu$ l of each product were then mixed with the same volume of formamide loading dye, heated to 94°C for 5 minutes and loaded onto a 6% sequencing gel containing 50% w/v urea. An M13 sequence was run adjacent to the samples as an absolute size marker.

### Data Analysis

Measures of genetic variability of the 10 loci were calculated using the BIOSYS program (Swofford & Selander 1981). Genetic polymorphism for each population was measured as the mean number of alleles per locus (allelic

diversity,  $A$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) from Hardy-Weinberg assumptions (Nei 1978, 1987). Deviations from Hardy-Weinberg equilibrium were tested using the chi-square test (Hartl & Clarke 1989). Because a chi-square test may not be appropriate if some genotypes frequencies are low, we grouped genotypes into three classes for each locus; homozygotes for the most common allele, common/rare heterozygotes, and other genotypes (Swofford & Selander 1981).

We analyzed differences in allele frequency between lineages of Mexican wolves using three approaches. First, we summarized the proportion of unique alleles in all three captive populations compared with species that were thought possibly to serve as founding stock (gray wolf, coyote, and domestic dog). We also simulated founder events by sampling two gray wolves from a single population randomly and computed the number of unique alleles they had when compared with the entire sample of dogs. We repeated the procedure 1000 times and did the same using a dog and a gray wolf as founders; we then compared the mean number of unique alleles for both simulations using a Mann-Whitney  $U$  test. More generally, we tested the likelihood of various founding scenarios by randomly sampling 1000 times two gray wolves, a gray wolf and a domestic dog (gray wolf-dog), or a gray wolf and coyote (gray wolf-coyote), and by assessing the alleles they shared with both lineages of uncertified Mexican wolves. We compared these distributions with that obtained from the analogous sampling of two certified Mexican wolves.

Second, we used multi-dimensional scaling to summarize allele frequency variation over all microsatellite loci surveyed. We used linear multi-dimension scaling of a Pearson correlation matrix of allele frequencies because it summarizes allele frequency variation on two dimensions and makes few assumptions about the distribution of the data (Borg 1981). The stress related to fitting the correlation into two dimensions was examined from the resulting Shepard diagram (Shepard 1962). All these statistical calculations were performed with the SYSTAT<sup>TM</sup> program (Wilkinson et al. 1992). Finally, we computed Nei's unbiased genetic distance (1978) and used a neighbor-joining clustering algorithm to generate relationship trees (Saitou & Nei 1987; Jin & Nei 1991). This approach offers a contrast to the MDS analysis, which does not assume a specific evolutionary model. We describe both approaches here because the evolutionary model most applicable to microsatellite data is uncertain (Estoup et al. 1995; Goldstein et al. 1995).

### Results

The 10 microsatellite loci we surveyed in Mexican wolves were much less polymorphic than in other wolf-

**Table 1.** Number of alleles, expected heterozygosity ( $H_E$ ) and observed heterozygosity ( $H_O$ ) of the 10 microsatellite loci surveyed in three lineages of Mexican wolves and in 42 breeds of domestic dog.

Locus	Lineage	Sample size	Number of alleles	$H_E$	$H_O$
204	Certified	20	4	0.682	0.650
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	2	0.325	0.375
	Dogs	37	7	0.635	0.432*
123	Certified	21	2	0.511	0.571
	Ghost Ranch	10	2	0.189	0.200
	Aragón	8	2	0.525	0.625
	Dogs	36	6	0.764	0.583*
2	Certified	21	2	0.483	0.476
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	2	0.500	0.750
	Dogs	40	8	0.797	0.400*
344	Certified	21	2	0.455	0.476
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	1	0.000	0.000
	Dogs	40	4	0.229	0.125*
213	Certified	21	2	0.136	0.143
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	1	0.000	0.000
	Dogs	32	7	0.825	0.469*
250	Certified	21	2	0.508	0.714
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	1	0.000	0.000
	Dogs	41	9	0.830	0.512
172	Certified	21	3	0.330	0.381
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	1	0.000	0.000
	Dogs	41	3	0.095	0.049*
109	Certified	21	3	0.553	0.619
	Ghost Ranch	10	2	0.505	0.000*
	Aragón	8	2	0.458	0.375
	Dogs	38	6	0.729	0.447*
225	Certified	21	2	0.093	0.095
	Ghost Ranch	10	2	0.337	0.200
	Aragón	8	2	0.400	0.500
	Dogs	40	5	0.575	0.475
377	Certified	21	3	0.617	0.905*
	Ghost Ranch	10	1	0.000	0.000
	Aragón	8	2	0.325	0.375
	Dogs	39	8	0.682	0.513*

\*Loci deviating from Hardy-Weinberg equilibrium ( $p < 0.05$ ).

like canids (Tables 1 & 2). The average number of alleles per locus ranged from  $1.3 \pm 0.2$  for the Ghost Ranch lineage to  $2.5 \pm 0.2$  for the certified lineage. Northern gray wolves showed a significantly greater number of alleles per locus, averaging  $4.5 \pm 1.1$  in nonhybridizing popula-

tions and  $6.4 \pm 0.7$  in hybridizing populations (Table 2). The mean number of alleles in other wolf-like canids has been shown to range from  $2.4 \pm 0.3$  for the endangered Ethiopian wolf to  $5.9 \pm 0.7$  for the coyote (Table 2; Gottelli et al. 1994; Roy et al. 1994). Mean expected heterozygosity values for the certified ( $0.437 \pm 0.061$ ) and Aragón ( $0.253 \pm 0.072$ ) populations were lower than that found in nonhybridizing ( $0.620 \pm 0.070$ ) and hybridizing ( $0.713 \pm 0.039$ ) populations of gray wolves (Table 2). The heterozygosity of the Ghost Ranch lineage ( $0.103 \pm 0.058$ ) was dramatically lower than that of the gray wolf populations, reflecting monomorphism at 7 of 10 microsatellite loci (Table 1).

Two loci showed significant deviations from Hardy-Weinberg expectations in Mexican wolf population (Table 1) ( $p < 0.05$ , chi-square test with pooling). The certified lineage showed deviations from Hardy-Weinberg expectations in 1 of 10 loci (locus 377), the Ghost Ranch deviated in 1 of 10 loci (locus 109), and dog breeds in 8 of 10 loci (loci 204, 123, 2, 344, 213, 172, 107, 377). Such deviations might be expected considering the departures from random breeding that have occurred in dog breeds and in captive populations. Moreover, our dogs represent individuals from different breeds and hence are not samples from a single breeding population.

Each Mexican wolf lineage has some unique alleles as well as shared alleles that differentiate them from domestic dogs, other gray wolves, and coyotes. (Fig. 2, Appendix; Roy et al. 1994). Allele *G* at locus 172 is an example of a diagnostic allele because it is common in all three Mexican wolf lineages but absent from domestic dogs and rare in coyotes (1.5%) and other gray wolves (8.9%). Another allele common to all three lineages of Mexican wolves is allele *D* at locus 204 (Fig. 2). This allele is also common in Arctic and Northwest gray wolves but rare in domestic dogs and absent from coyotes. Of those tested, only two dogs, a Siberian Husky and a Rottweiler, are heterozygous for the *D* allele. Other alleles found frequently in the three Mexican wolf lineages but rarely or much less frequently in dogs, coyotes, or gray wolves included allele *I* at locus 213, allele *C* at locus 225, allele *C* at locus 109, and allele *G* at locus 250 (Fig. 2, Appendix).

Other alleles served to distinguish one or two of the Mexican wolf lineages from other canids. Allele *K* at locus 200 is fixed in Ghost Ranch gray wolves, common in Aragón wolves (63%), but found in only one of 151 gray wolves and in three of 142 coyotes (Fig. 2; Appendix). Allele *K* was not found in the domestic dog sample. At locus 377 the *R* and *S* alleles are unique to certified Mexican wolves. Also at this locus the *B* allele is fixed in the Ghost Ranch lineage and common in the Aragón population (81%). This allele is otherwise found only in an Alaskan Husky and is rare in other gray wolves and in coyotes.

**Table 2.** Allelic diversity (mean number of alleles per locus), mean expected ( $H_E$ ), and observed ( $H_O$ ) heterozygosity for 10 microsatellite loci surveyed in Mexican wolves and other wolf-like canid populations.\*

Population	Mean sample size per locus	Allelic diversity	Mean heterozygosity	
			$H_E$	$H_O$
Mexican wolf				
Certified	20.9 $\pm$ 0.1	2.5 $\pm$ 0.2	0.437 $\pm$ 0.061	0.503 $\pm$ 0.079
Ghost Ranch	10.0 $\pm$ 0.0	1.3 $\pm$ 0.2	0.103 $\pm$ 0.058	0.040 $\pm$ 0.027
Aragón	8.0 $\pm$ 0.0	1.6 $\pm$ 0.2	0.253 $\pm$ 0.072	0.300 $\pm$ 0.090
Comparison populations				
Gray wolf				
Nonhybridizing (4 populations)	16.8 $\pm$ 3.6	4.5 $\pm$ 1.1	0.620 $\pm$ 0.070	0.528 $\pm$ 0.067
Hybridizing (2 populations)	19.9 $\pm$ 1.4	6.4 $\pm$ 0.7	0.713 $\pm$ 0.039	0.562 $\pm$ 0.043
Coyote (6 populations)	17.0 $\pm$ 3.1	5.9 $\pm$ 0.65	0.675 $\pm$ 0.035	0.583 $\pm$ 0.061
Red wolf				
Captive	29.9 $\pm$ 1.0	5.3 $\pm$ 0.8	0.548 $\pm$ 0.072	0.507 $\pm$ 0.082
Domestic dogs (42 breeds)	38.4 $\pm$ 0.9	6.3 $\pm$ 0.6	0.616 $\pm$ 0.081	0.401 $\pm$ 0.055
Golden jackal (1 population)	16.4 $\pm$ 0.7	4.8 $\pm$ 0.8	0.520 $\pm$ 0.103	0.412 $\pm$ 0.095

\*Standard error is averaged over all loci. Sources: Gottelli et al. 1994; Roy et al. 1994.

Comparison of the alleles found in domestic dogs, coyotes, and northern gray wolves shows that 16–20% of alleles are unique to each species (Table 3). Eleven and 16 alleles are found in domestic dogs and coyotes, respectively, that are not present in northern gray wolves. But the number of unique alleles increases with the number of individuals sampled (Roy et al. 1994). Therefore, to determine the number of unique alleles expected for a more limited sampling of a larger population, as occurred in the founding of the three Mexican wolf lineages, we performed simulation samplings of two individuals. The mean number of unique alleles after 1000 simulations in which two gray wolves from the same population were picked at random and compared with all the domestic dogs was 4.73 ( $\pm$  2.37). In contrast, a significantly smaller mean value of 2.92 ( $\pm$  1.72) unique alleles was expected when one wolf and one dog were drawn in 1000 trials and their alleles compared with domestic dogs ( $p < 0.001$ ,  $Z = 47.87$ , Mann-Whitney  $U$  test). The Aragón and Certified lineages have five and nine unique alleles, respectively, when compared with domestic dogs, indicating a greater level of distinction than expected if the population were founded by a domestic dog–gray wolf cross. The Ghost Ranch lineage has only three unique alleles when compared with domestic dogs, but the Ghost Ranch population has very low allelic diversity (only 3 of 10 loci are polymorphic), and unique alleles present in the founding individuals were likely lost due to drift in a small population. In fact, 23% of alleles in the Ghost Ranch lineage are unique compared with domestic dogs, whereas in simulations

the expected percentage of unique alleles in a gray wolf–domestic dog cross is 12.4%. In our simulation only 5.2% of 1000 random samples of a gray wolf and a domestic dog had greater than 23% unique alleles, compared with our entire sample of dogs.

More generally, we tested by simulation the likelihood of northern gray wolves, and their crosses with dogs and coyotes, as founder stock for the uncertified Mexican wolves. Despite being more limited in variability than outbred canid populations, the allele sampling distribution created by random samples of two certified Mexican wolves had many more alleles in common with the uncertified Mexican wolves than alternative sampling distributions (Fig. 3). Twenty-three percent of 1000 random samples of two certified wolves had 13 (81%) or greater of the alleles found in Aragón wolves, whereas none of 1000 samples of other gene pools had this value or greater. Because the Aragón wolves have two alleles not found in the certified wolves, it is not possible for all alleles to be shared in any sample of two certified wolves. Similar results were obtained in simulations involving comparisons with Ghost Ranch wolves (Fig. 3). Thirteen percent of 1000 samples of two certified Mexican wolves had 11 (85%) of the alleles found in the Ghost Ranch wolves, whereas only 0.3 and 0.1% of gray wolf and gray wolf–dog samples, respectively, had this value. These simulations show that the founders of the uncertified Mexican wolves were likely drawn from a gene pool similar to that which founded the certified lineage rather than to one involving a gray wolf–dog or –coyote cross.

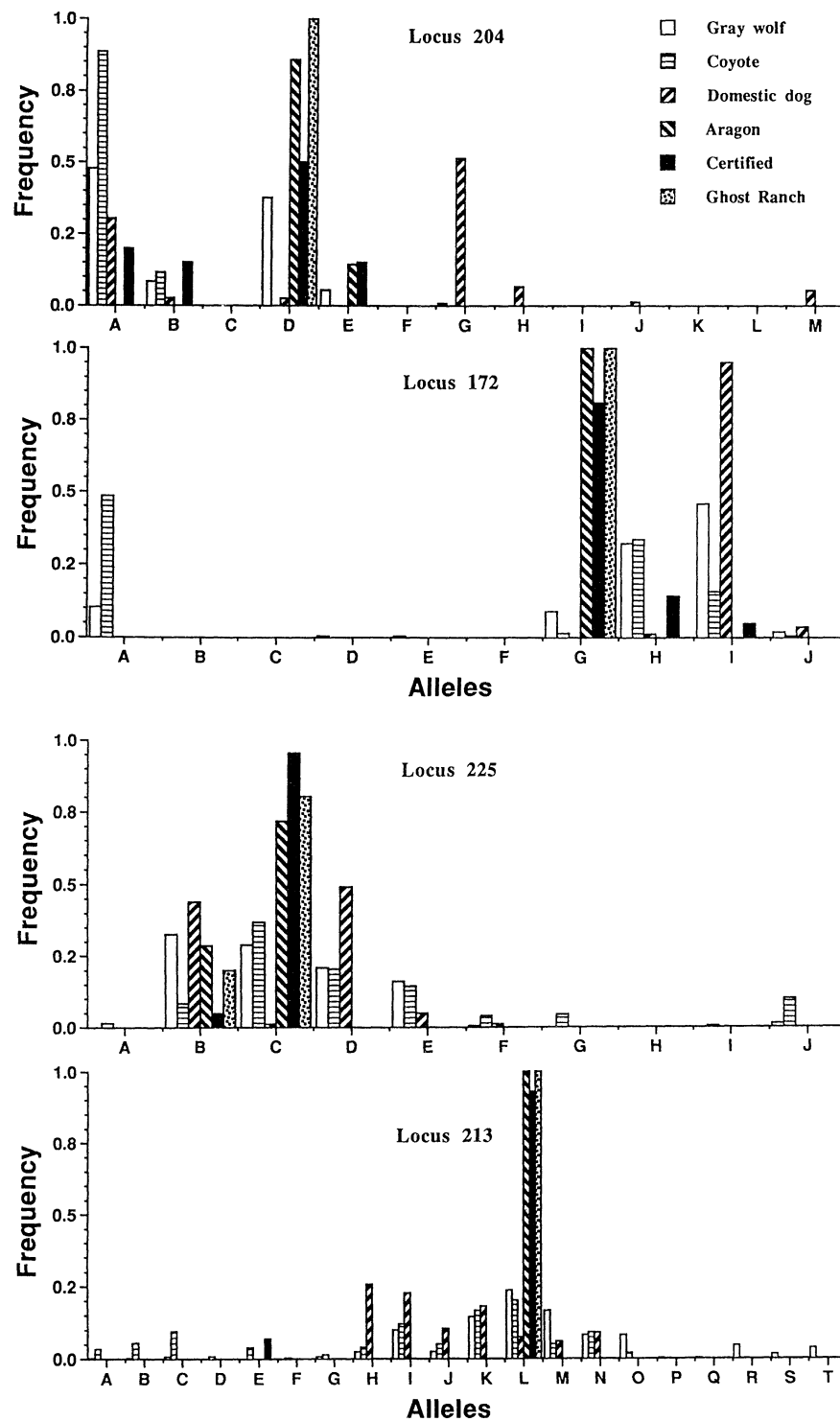


Figure 2. Frequency histograms for 6 of 10 microsatellite loci typed in three lineages of Mexican wolves, domestic dogs, and northern gray wolves (see Appendix).

We did not find any coyote or domestic dog marker alleles in uncertified wolves, but few marker alleles would be expected. The average domestic dog in our sample has  $0.95 (\pm 0.83)$  unique alleles in comparison with nonhybridizing gray wolves. Similarly, an average coyote has  $1.55 (\pm 0.96)$  alleles not present in our sample of nonhybridizing gray wolves. Only one allele otherwise

unique to coyotes (allele E, locus 213) was found in the certified Mexican wolves (Fig. 2, Appendix). The presence of this marker allele in the certified lineage that is rare in coyotes (4.0%) was unexpected but may reflect a parallel mutation rather than interspecific hybridization. Parallel evolution of alleles may be a common occurrence in the evolution of microsatellite loci because of



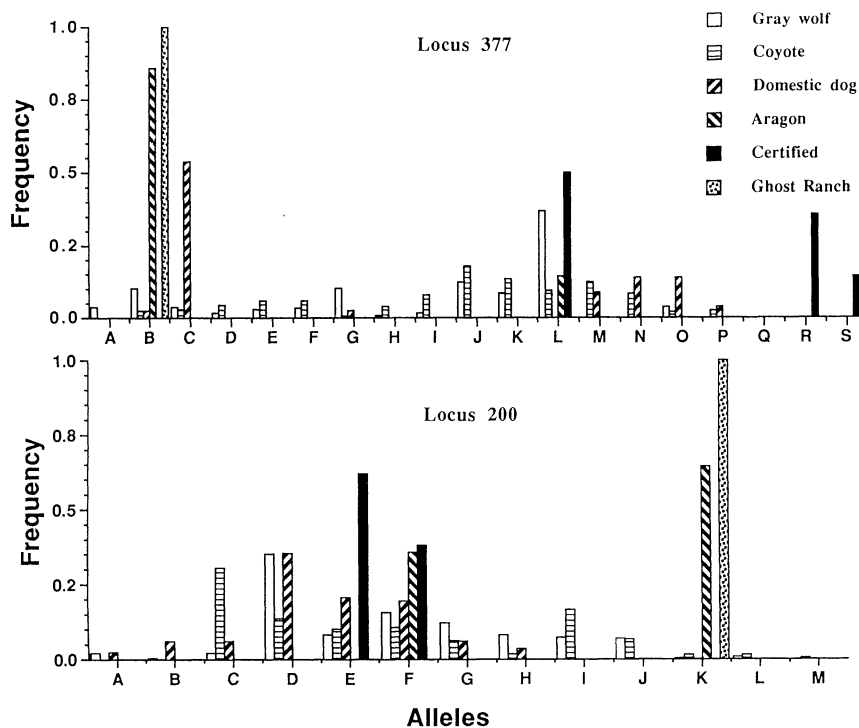


Figure 2 (continued)

their high mutation rates (Garza et al. 1995; Goldstein 1995; Slatkin 1995). None of the 11 alleles unique to domestic dogs or the 16 alleles unique to coyotes relative to gray wolves was found in any uncertified Mexican wolf (Table 3). Considering both the large proportion of certified Mexican wolf alleles in the uncertified lineages and the absence of specific markers of dogs or coyotes in these two populations, our results support the theory that the uncertified lineages were not founded simply by a domestic dog or coyote-gray wolf cross.

We cannot eliminate the possibility that some of the founding wolves were from Canada, Alaska, or the few northern United States where gray wolf populations have become established. The certified lineage of Mexican wolves has three alleles not found in northern gray wolves, and the other two Mexican wolf lineages have

no unique alleles relative to gray wolves. In the Aragón and Ghost Ranch lineages, however, two alleles are fixed or very common that are extremely rare in coyotes, dogs, or other gray wolves (locus 200, allele K; and locus 377, allele B; Fig. 2). The presence of these alleles that are rare in other canids indicates a common population origin for both uncertified lineages.

Comparisons of allele frequencies suggest that the Mexican wolf lineages are more similar to each other than to other gray wolves or domestic dogs. Similarity in allele frequencies across the 10 microsatellite loci may be summarized using a multi-dimensional scaling analysis (Fig. 4; Borg 1981). This two-dimensional representation of allele frequency variation shows that the Mexican wolves are nearest neighbors and are distinct from gray wolves, coyotes, and domestic dogs. Although the

**Table 3.** Number and proportion (in parentheses) of unique alleles observed between each of the three captive Mexican wolf populations, certified and uncertified Mexican wolf populations combined, and several North American wolf-like canids.\*

Population (total alleles)	Mexican wolves				Dogs	Gray wolf	Coyote
	Aragón	Ghost	Certified	Combined			
Aragón (16)	—	4 (0.25)	2 (0.09)	—	5 (0.31)	0 (0.00)	2 (0.12)
Ghost (13)	1 (0.08)	—	2 (0.15)	—	3 (0.23)	0 (0.00)	1 (0.08)
Certified (25)	11 (0.44)	14 (0.56)	—	—	9 (0.36)	3 (0.12)	4 (0.16)
Combined (27)	—	—	—	—	10 (0.37)	3 (0.11)	4 (0.15)
Dogs (63)	52 (0.82)	53 (0.84)	47 (0.75)	46 (0.73)	—	11 (0.17)	10 (0.16)
Gray wolf (95)	79 (0.83)	82 (0.86)	73 (0.77)	71 (0.75)	43 (0.45)	—	19 (0.20)
Coyote (92)	78 (0.85)	80 (0.87)	71 (0.77)	69 (0.75)	39 (0.42)	16 (0.17)	—

\* Pair comparisons in this table are not symmetrical. The values indicate the number (and proportion) of unique alleles of the species (or population) listed in the left column compared with those listed in the top row.

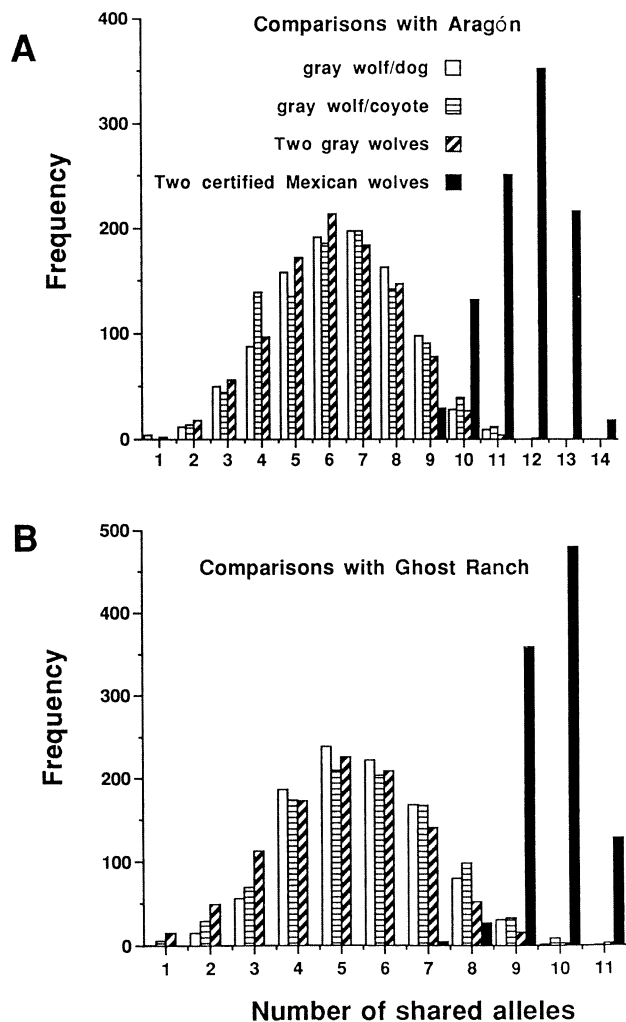


Figure 3. Frequency histograms showing alleles shared with Aragón (a) or Ghost Ranch wolves (b) in 1000 sampling trials of two canids (one gray wolf and a dog, one gray wolf and a coyote, two gray wolves, or two certified wolves).

allele frequencies of the captive populations of Mexican wolf were probably severely influenced by founder composition and drift in the small captive populations, they show a striking allele frequency similarity to each other and are distinct from other wolf-like canids.

These conclusions are supported by Nei's genetic distance values (Table 4, Fig. 5). In general, the Mexican wolves showed smaller distances among themselves than when compared with any other population, ranging from 0.091 between the Aragón and Ghost Ranch lineage to 0.318 between the certified and Ghost Ranch lineage. In comparison, the distance between Mexican wolves and domestic dogs ranges from 1.055 to 1.176, and from  $0.591 \pm 0.037$  to  $0.700 \pm 0.041$  between Mexican wolves and other gray wolf populations (Table 4). A neighbor-joining relationship tree based on Nei's genetic

distance clusters the three lineages of Mexican wolves together as the most distinct grouping of gray wolves (Fig. 5). This grouping is supported in 99% of 1000 bootstrap replications of our data (Felsenstein 1985). Coyote populations also formed a distinct cluster with the captive population of red wolves associated with them. Previous microsatellite analysis suggested that red wolves may have acquired genetic similarity to coyotes through interspecific hybridization (Roy et al. 1994). Although the coyote-red wolf and Mexican wolf groupings were also found in distance-Wagner and UPGMA trees, associations of the gray wolf and domestic dog populations varied, reflecting the small internodal distances between them.

## Discussion

The three captive populations of Mexican wolves were established with different founders at different times. None of the surveyed individuals from the uncertified lineages had domestic dog or coyote marker alleles, and both populations had a large proportion of alleles in common with certified wolves, suggesting that they were not simply founded by a cross between a gray wolf and a domestic dog or coyote. For example, both uncertified and certified lineages have alleles that are fixed or at high frequency that are not found or are very rare in domestic dogs or coyotes (allele *G*, locus 172; allele *D*, locus 204; allele *L*, locus 213; and allele *C*, locus 225; Fig. 2, Appendix). Moreover the uncertified lineages share alleles *B* and *K* at loci 377 and 200, respectively, which are absent or very rare in domestic dogs, gray wolves, and coyotes. At locus 204 the *D* allele found in Mexican wolves is known only from a Siberian Husky and a Rottweiler, and at 377 the *B* allele found in uncertified wolves is present only in an Alaskan Husky and is rare in other gray wolves. Huskies are recently derived from gray wolves and continue to be interbred with them, so they might be expected to share a greater proportion of alleles with gray wolves. Finally, simulations of founding events indicate that the uncertified lineages have an allelic distribution closer to that of certified Mexican wolves, as might be expected if they were founded from the same ancestral population (Fig. 3).

Perhaps as important as the issue of the purity of the founders for each captive colony is their overall genetic similarity and distinction. Multi-dimensional scaling analysis and genetic distance phenograms show that the three captive lineages are closely related and are as similar in allele frequencies to each other as are populations of North American gray wolves (Fig. 4). Also, the three captive lineages are the most distinct grouping of North American wolves, supporting their designation as an endangered subspecies. Therefore, the overall genetic similarity of the three Mexican wolf captive populations to

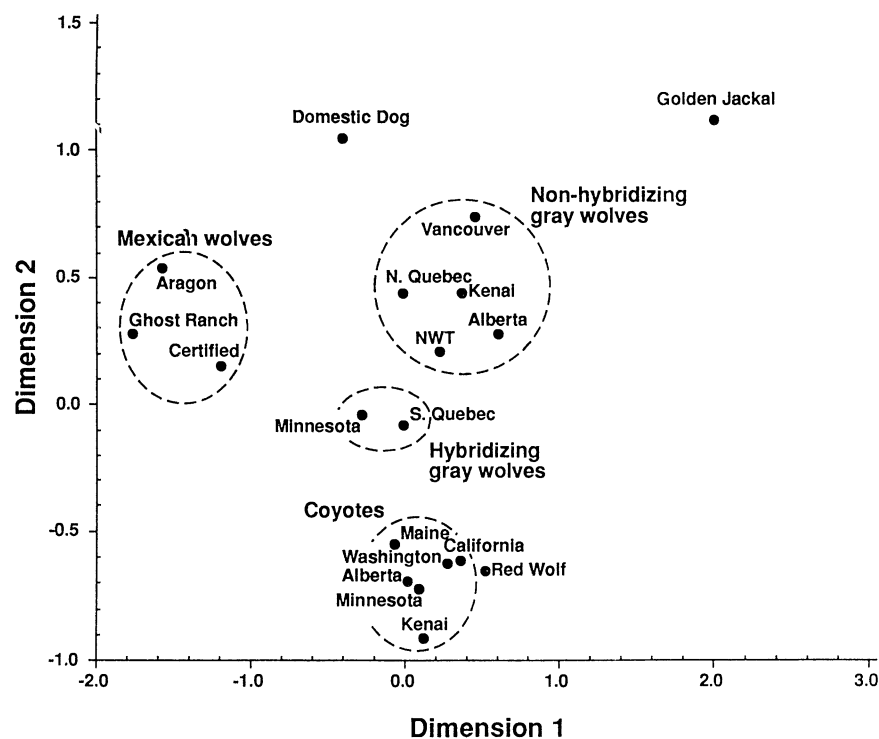


Figure 4. Multi-dimensional scaling analysis of allele frequency data of 10 microsatellite loci in several species of wolf-like canids. Mexican wolves, coyotes, gray wolves, domestic dogs, and golden jackals are the comparison species in this analysis. Coyote and gray wolf populations are indicated by state or province.

each other, and their level of genetic distinction from other North American wolves, argue that they are derived from a similar source population and should potentially be interbred. Moreover, because of their genetic uniqueness, captive Mexican wolves should be used as a source for reintroduction to the southwestern U.S., if possible, rather than northern gray wolves.

The three captive lineages do not overlap completely in the diversity of alleles they contain. For example, the *R* and *S* alleles at locus 377 are unique to the certified

lineage, and the *B* allele at this locus is fixed in the Ghost Ranch lineage and common in the Aragón population (86%) but is not found in the certified wolves. Similarly, the *K* allele at locus 200 is common to the Aragón and Ghost Ranch lineages but is not found in the certified wolves (Fig. 2). These results suggest that the genetic variation contained within each lineage does not constitute a subset of the variation found in the others. Therefore, a careful pedigree analysis of the three colonies should be undertaken so that future breeding plans

Table 4. Mean ( $\pm$  SD) Nei's unbiased (1978) genetic distance between Mexican wolf, gray wolf, domestic dog, and coyote populations.\*

	Mexican wolves			Coyote	Gray Wolf	Red wolf	Dog
	Aragón	Ghost Ranch	Certified				
Aragón	—	0.091 ( <i>n</i> = 1)	0.255 ( <i>n</i> = 1)	0.733 $\pm$ 0.022 ( <i>n</i> = 6)	0.667 $\pm$ 0.028 ( <i>n</i> = 7)	1.550 ( <i>n</i> = 1)	1.119 ( <i>n</i> = 1)
Ghost Ranch		—	0.318 ( <i>n</i> = 1)	0.737 $\pm$ 0.016 ( <i>n</i> = 6)	0.700 $\pm$ 0.041 ( <i>n</i> = 7)	1.456 ( <i>n</i> = 1)	1.176 ( <i>n</i> = 1)
Certified			—	0.641 $\pm$ 0.034 ( <i>n</i> = 6)	0.591 $\pm$ 0.037 ( <i>n</i> = 7)	0.956 ( <i>n</i> = 1)	1.055 ( <i>n</i> = 1)
Coyote				0.190 $\pm$ 0.053 ( <i>n</i> = 15)	0.515 $\pm$ 0.151 ( <i>n</i> = 42)	0.338 $\pm$ 0.052 ( <i>n</i> = 6)	0.762 $\pm$ 0.153 ( <i>n</i> = 6)
Gray wolf					0.346 $\pm$ 0.138 ( <i>n</i> = 21)	0.518 $\pm$ 0.161 ( <i>n</i> = 7)	0.672 $\pm$ 0.135 ( <i>n</i> = 7)
Red wolf						—	0.874 ( <i>n</i> = 1)
Dog							—

\*The average intrapopulation Nei's distance for the coyote and gray wolf is given on the diagonal.

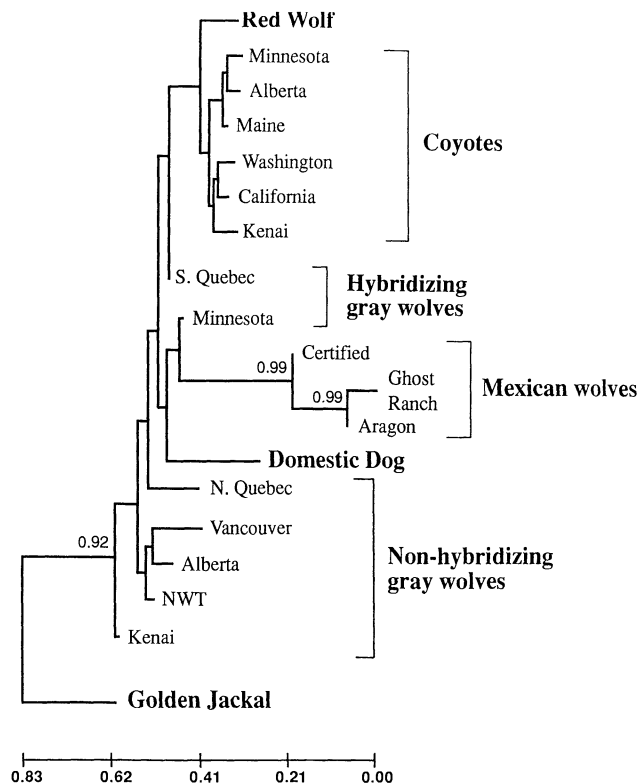


Figure 5. Neighbor-joining tree of wolf-like canid populations based on Nei's (1978) genetic-distance statistic. The fraction of nodes supported in 1000 bootstrap replications of our data are indicated if the value is over 50%. NWT = Northwest Territory.

best preserve the allelic diversity present in all three captive populations of Mexican wolves. In addition, loss of alleles and heterozygosity in the Aragón and Ghost Ranch lineages suggest that effective population sizes are small enough that genetic variability is being lost. Interbreeding of the three lineages might better preserve allelic diversity that may be important to individual fitness (Allendorf 1986) and alleviate the risk of inbreeding depression (Laikre & Ryman 1991). Reports of anatomical defects in the Aragón wolves and cryptorchidism in the certified lineage (D. Parsons, personal communication) highlight the possibility that inbreeding depression is already affecting captive Mexican wolves (Roelke et al. 1993).

Finally, one potential argument against the use of the Aragón lineage in the captive breeding program is that they have a mitochondrial haplotype common in northern gray wolves rather than having the characteristic Mexican gray wolf genotype found in the Ghost Ranch and certified lineages (S. Fain, personal communication). But well-sampled populations of northern gray wolves commonly have more than one mtDNA haplotype (Wayne et al. 1992), and the historic population of Mexican wolves may have had several. Previous mtDNA anal-

ysis of wolf-like canids from southeastern Texas caught in 1975 found that both Mexican and northern gray wolf haplotypes existed in this region (Wayne & Jenks 1991). A molecular-genetic analysis of museum skins of Mexican wolves from throughout their historic range would better demonstrate if northern gray wolf haplotypes were abundant in historic Mexican wolf populations.

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## Appendix

Allele frequencies of domestic dog and North American gray wolves, Mexican wolves, and coyotes.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Certified Mexican wolves*																				
109 (102)	—	0.619	0.238	—	—	0.143	—	—	—	—	—	—	—	—	—	—	—	—	—	—
123 (102)	—	—	—	—	0.524	—	—	0.476	—	—	—	—	—	—	—	—	—	—	—	—
172 (114)	—	—	—	—	—	—	0.810	0.143	0.048	—	—	—	—	—	—	—	—	—	—	—
200 (173)	—	—	—	—	0.619	0.381	—	—	—	—	—	—	—	—	—	—	—	—	—	—
204 (160)	0.200	0.150	—	0.500	0.150	—	—	—	—	—	—	0.929	—	—	—	—	—	—	—	—
213 (106)	—	—	—	—	0.071	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
225 (122)	—	0.048	0.952	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
250 (90)	—	—	—	—	0.452	—	0.548	—	—	—	—	—	—	—	—	—	—	—	—	—
344 (115)	0.333	0.667	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
377 (96)	—	—	—	—	—	—	—	—	—	—	—	0.500	—	—	—	—	—	0.357	0.143	—
Aragón Mexican wolves																				
109	—	—	0.286	—	—	0.714	—	—	—	—	—	—	—	—	—	—	—	—	—	—
123	—	—	—	—	0.500	—	—	0.500	—	—	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—
200	—	—	—	—	—	0.357	—	—	—	—	0.643	—	—	—	—	—	—	—	—	—
204	—	—	—	0.857	0.143	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
213	—	—	—	—	—	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—
225	—	0.286	0.714	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
250	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—
344	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
377	—	0.857	—	—	—	—	—	—	—	—	—	0.143	—	—	—	—	—	—	—	—
Ghost Ranch Mexican wolves																				
109	—	0.600	0.400	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
123	—	—	—	—	0.100	—	—	0.900	—	—	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—
200	—	—	—	—	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—	—
204	—	—	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
213	—	—	—	—	—	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—
225	—	0.200	0.800	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
250	—	—	—	—	—	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—
344	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
377	—	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Dogs																				
109	0.026	0.410	—	0.077	0.205	0.244	0.038	—	—	—	—	—	—	—	—	—	—	—	—	—
123	—	—	—	—	0.149	0.027	0.365	0.270	0.149	0.041	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	0.012	0.952	0.036	—	—	—	—	—	—	—	—	—	—
200	0.024	0.061	0.061	0.354	0.207	0.195	0.061	0.037	—	—	—	—	—	—	—	—	—	—	—	—
204	0.303	0.026	—	0.026	—	—	0.513	0.066	—	0.013	—	—	0.053	—	—	—	—	—	—	—
213	—	—	—	—	—	—	—	0.258	0.227	0.106	0.182	0.076	0.061	0.091	—	—	—	—	—	—
225	—	0.439	0.012	0.488	0.049	0.012	—	0.131	0.298	0.048	0.119	0.048	0.036	—	—	—	—	—	—	—
250	—	—	—	0.024	—	0.083	0.214	—	—	—	—	—	0.012	0.085	—	—	—	—	—	—
344	—	0.878	0.024	—	—	—	—	—	—	—	—	—	0.087	0.138	0.138	0.038	0.013	—	—	—
377	—	0.025	0.538	—	—	—	0.025	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix Continued

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Gray wolves																				
109	0.020	0.290	0.119	0.266	0.198	0.091	0.012	—	0.004	—	—	—	—	—	—	—	—	—	—	—
123	—	—	—	0.004	0.535	0.094	0.134	0.059	0.051	0.102	0.008	0.012	—	—	—	—	—	—	—	—
172	0.103	—	—	0.005	0.005	—	0.089	0.322	0.458	0.019	—	—	—	—	—	—	—	—	—	—
200	0.022	0.004	0.022	0.352	0.083	0.157	0.122	0.083	0.074	0.070	0.004	0.009	—	—	—	—	—	—	—	—
204	0.479	0.083	—	0.376	0.054	—	0.008	—	—	—	—	—	—	—	—	—	—	—	—	—
213	0.004	0.004	0.008	—	—	—	0.008	0.025	0.100	0.025	0.146	0.237	0.167	0.083	0.083	0.004	0.004	0.046	0.017	0.038
225	—	0.326	0.288	0.208	0.161	0.004	—	—	—	0.013	—	—	—	—	—	—	—	—	—	—
250	—	—	—	—	0.056	0.138	0.267	0.254	0.138	0.078	0.009	0.013	—	—	—	0.004	0.039	—	0.004	—
344	0.608	0.233	0.021	0.042	0.087	0.004	0.004	—	—	—	—	—	—	—	—	—	—	—	—	—
377	0.038	0.102	0.038	0.017	0.030	0.034	0.102	0.008	0.017	0.123	0.085	0.369	—	—	0.038	—	—	—	—	—
Coyotes																				
109	0.044	0.184	0.058	0.330	0.150	0.141	0.005	0.029	0.034	0.019	0.005	—	—	—	—	—	—	—	—	—
123	—	—	—	0.022	0.052	0.151	0.246	0.392	0.065	0.065	0.009	—	—	—	—	—	—	—	—	—
172	0.485	—	—	—	—	—	0.015	0.337	0.158	0.005	—	—	—	—	—	—	—	—	—	—
200	—	—	0.306	0.136	0.102	0.107	0.063	0.019	0.165	0.068	0.015	0.015	0.005	—	—	—	—	—	—	—
204	0.886	0.114	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
213	0.035	0.056	0.096	0.010	0.040	0.005	0.015	0.040	0.121	0.051	0.167	0.202	0.051	0.091	0.020	—	—	—	—	—
225	0.015	0.084	0.366	0.203	0.144	0.040	0.045	—	0.005	0.099	—	—	—	—	—	—	—	—	—	—
250	—	—	—	0.047	0.084	0.068	0.142	0.174	0.237	0.132	0.053	0.047	0.011	—	0.005	—	—	—	—	—
344	0.578	0.359	0.010	—	0.031	0.021	—	—	—	—	—	—	—	—	—	—	—	—	—	—
377	—	0.025	0.030	0.045	0.059	0.059	0.005	0.040	0.079	0.178	0.134	0.094	0.124	0.084	0.020	0.025	—	—	—	—

\*The size in base pairs of the smallest allele in certified Mexican wolves is given in parentheses.