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FAST TRACK

Legacy lost: genetic variability and population size of extirpated US grey wolves (*Canis lupus*)

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Abstract

By the mid 20th century, the grey wolf (*Canis lupus*) was exterminated from most of the conterminous United States (cUS) and Mexico. However, because wolves disperse over long distances, extant populations in Canada and Alaska might have retained a substantial proportion of the genetic diversity once found in the cUS. We analysed mitochondrial DNA sequences of 34 pre-extermination wolves and found that they had more than twice the diversity of their modern conspecifics, implying a historic population size of several hundred thousand wolves in the western cUS and Mexico. Further, two-thirds of the haplotypes found in the historic sample are unique. Sequences from Mexican grey wolves (*C. l. baileyi*) and some historic grey wolves defined a unique southern clade supporting a much wider geographical mandate for the reintroduction of Mexican wolves than currently planned. Our results highlight the genetic consequences of population extinction within Ice Age refugia and imply that restoration goals for grey wolves in the western cUS include far less area and target vastly lower population sizes than existed historically.

Keywords: ancient DNA, genetic diversity, glacial refugia, Mexican wolves, mitochondrial DNA, predator control

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Introduction

Intense predator eradication programs combined with habitat loss and fragmentation have caused the decline of the grey wolf (*Canis lupus*) throughout most of its historic range in North America and its near extinction in the cUS (Boitani 2003). A predicted outcome of such population declines is the loss of genetic diversity that, in the extreme, can lead to a decrease in individual fitness and evolutionary potential, as well as an increased risk of population extinction (Frankham *et al*. 2002). Species characterized by limited mobility and strong population genetic structure are more likely to suffer a substantial loss in genetic diversity as a result of range reductions (Taylor *et al*. 1994; Bouzat *et al*. 1998; Wisely *et al*. 2002). In contrast, species with high mobility can potentially exchange genes across large geographical areas and consequently, their eradication over a limited area may not result in a significant loss of unique genetic diversity.

This optimistic scenario seems probable for the North American grey wolf because Canadian and Alaskan populations, currently numbering 60–70 000 individuals (Boitani 2003), persisted during the extermination program which eradicated wolves from the southern part of their historic geographical range in North America by the early to mid 1900s (Boitani 2003; Fig. 1). Wolves are highly mobile predators; dispersal distances of several hundred kilometers are common and record movements of ~1000 km have been observed (Fritts 1983; Wabakken *et al*. 2001). As a result, only limited genetic differentiation generally has been reported for wolves across large geographical regions (Wayne *et al*. 1992; Roy *et al*. 1994; Vilà *et al*. 1999). Therefore, considering the large population size of extant North American wolves and the high mobility of wolves in general, we expected that the genetic diversity of the extirpated (historic) cUS population was largely a subset of that currently existing in Canada and Alaska. If so, the historic cUS population potentially could be restored through natural and planned reintroductions (Smith 1997).

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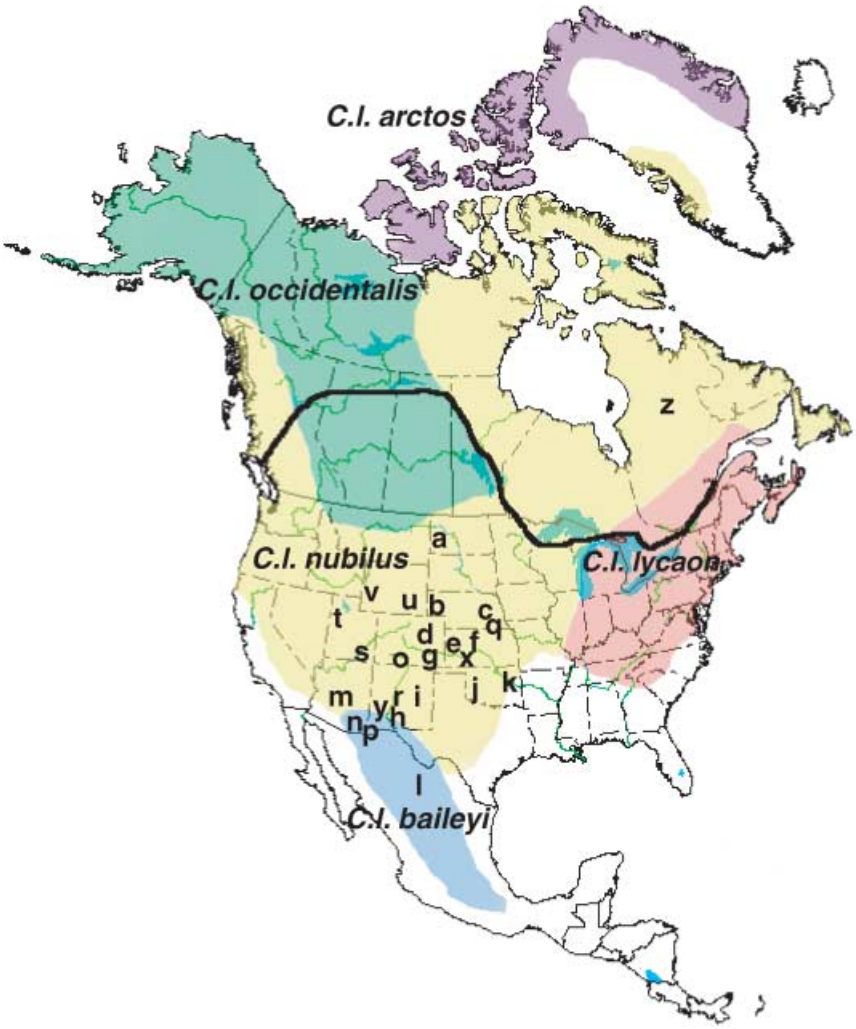

Fig. 1 Historic distribution of the grey wolf (*Canis lupus*) in North America and the five recently defined subspecies it comprises (Nowak 1995). Solid line marks the northern limit of eradication (Boitani 2003). Approximate localities of historic specimens included in this study are indicated as letters (see Table 1).

To test this expectation, we sequenced part of the mitochondrial control region of 34 museum specimens obtained 90–150 years ago from the historic range of the Mexican wolf (*C.l. baileyi*) and *C.l. nubilus*, the subspecies of wolf common in the interior cUS (Nowak 1995, Table 1, Fig. 1). We compared these sequences with a wider sampling of grey wolves and coyotes (*C. latrans*) (Vilà *et al*. 1999; Sharma *et al*. 2003), totaling 303 wolves from Eurasia and 96 from North America (Table 2).

Materials and Methods

Samples. Thirty-four historic wolf specimens collected in 1916 and earlier, including eight Mexican wolves, were obtained from the National Museum of Natural History (USNM), Smithsonian Institution, Washington D.C. (Fig. 1, Table 1).

Molecular methods. DNA was extracted from bone samples (tooth roots or rami) by phenol-chloroform extraction as in Leonard *et al*. (2000). Two blank (no bone powder) extractions were processed concurrently throughout all steps of the protocol to serve as negative controls. About 425 bp of mitochondrial control region I was amplified in three overlapping regions using primers Thr-L 5′-GAA TTC CCC GGT CTT GTA AAC C-3′ and dogdl5 5′-CAT TAA TGC ACG ACG TAC ATA GG-3′ (yielded PCR products of 207–227 bp in length); primer dogdl1g 5′-GTG CTA TGT CAG TAT CTC CAG G-3′ and dogdl2 5′-GCA AGG GTT GAT GGT TTC TCG-3′ (235 bp); and primer dogdl4 5′-GCA TAT CAC TTA GTC CAA TAA GGG-3′ and DLH-can 5′-CCT GAG GTA AGA ACC AGA TG-3′ (150 bp) as in Leonard *et al*. (2002). The set-up for polymerase chain reaction (PCR) amplification of DNA was performed in a separate lab exclusively devoted to ancient DNA extraction and each reaction consisted of 3–6 μL of extract and 25 ng BSA, 1X buffer, 0.1 mM dNTPs, 2.5 U AmpliTaq Gold (Perkin-Elmer), 2.5 mM Mg + +, 2.5 μM each primer in 25 μL, as in Leonard *et al*. (2002). The product of each successful reaction was either directly sequenced or used as a source for reamplification, which was then sequenced.

Table 1 Sample information for museum specimens. All samples are from the National Museum of Natural History, Smithsonian Institution, Washington D.C., U.S.A. (USNM). Subspecies are as identified in museum collection

Sample	Haplotype	Museum number	Subspecies	Year	Locality	Map
JAL 471	lu33[s]	USNM 95752	*C.l. baileyi*	1898	Sierra Madre, Chihuahua, Mexico	l
JAL 473	lu33[s]	USNM 98311	*C.l. baileyi*	1899	Colonia Garcia, Chihuahua, Mexico	l
JAL 474	lu60	USNM 98313	*C.l. baileyi*	1899	Colonia Juarez, Chihuahua, Mexico	l
JAL 475	lu32	USNM 15278	*C.l. baileyi*	*	Tanks, Arizona	m
JAL 476	lu33[s]	USNM 58393	*C.l. baileyi*	1894	Ft. Bowie, Arizona	n
JAL 477	lu47[s]	USNM 3188	*C.l. baileyi*	1857	Fort Massachusetts, New Mexico	o
JAL 478	lu32	USNM 3191	*C.l. baileyi*	1856	Fort Massachusetts, New Mexico	o
JAL 545	lu33[s]	USNM 224484	*C.l. baileyi*	1916	Cloverdale, New Mexico	p
JAL 515	lu32	USNM A2568	*C.l. nubilus*	1856	Platt River, Colorado	d
JAL 516	lu48	USNM 36126	*C.l. nubilus*	1892	Bent County, Colorado	g
JAL 517	lu38	USNM A11591	*C.l. nubilus*	*	Kansas	x
JAL 518	lu28	USNM A11592	*C.l. nubilus*	*	Fort Harker, Kansas	f
JAL 519	lu49	USNM 137132	*C.l. nubilus*	1872	Grove City, Kansas	e
JAL 520	lu38	USNM 139156	*C.l. nubilus*	1872	Grove City, Kansas	e
JAL 521	lu50[s]	USNM A884	*C.l. nubilus*	*	Fort Kearny, Nebraska	c
JAL 522	lu28	USNM A885	*C.l. nubilus*	*	Fort Kearny, Nebraska	c
JAL 523	lu38	USNM A2611	*C.l. nubilus*	*	Platt River, Nebraska	q
JAL 524	lu50[s]	USNM 118692	*C.l. nubilus*	1902	Guyo Canyon, New Mexico	y
JAL 525	lu28	USNM 168344	*C.l. nubilus*	1910	Santa Rosa, New Mexico	i
JAL 526	lu50[s]	USNM 168345	*C.l. nubilus*	1910	Santa Rosa, New Mexico	i
JAL 527	lu51[s]	USNM 221674	*C.l. nubilus*	1916	Carthage, New Mexico	h
JAL 528	lu28	USNM 69486	*C.l. nubilus*	1894	Medora, North Dakota	a
JAL 529	lu50[s]	USNM 147703	*C.l. nubilus*	1906	Witchita Forest Reserve, Oklahoma	j
JAL 530	lu52	USNM 196943	*C.l. nubilus*	1902	Afton, Oklahoma	k
JAL 539	lu50[s]	USNM 224478	*C.l. youngi*	1916	Haynes, New Mexico	r
JAL 540	lu28	USNM 221829	*C.l. youngi*	1916	La Sol, Utah	s
JAL 541	lu50[s]	USNM 221961	*C.l. youngi*	1916	Box Elder County, Utah	t
JAL 542	lu53	USNM 224000	*C.l. youngi*	1916	Heart Draw, Utah	s
JAL 543	lu28	USNM 208508	*C.l. youngi*	1915	Laramie, Wyoming	u
JAL 544	lu28	USNM 210179	*C.l. youngi*	1915	Wagon Creek, Wyoming	v
JAL 480	lu32	USNM 210059	*C.l. labradorius*	1912	Porcupine, Labrador	z
JAL 557	lu54	USNM 23140	*C.l. labradorius*	1882/3	Labrador Peninsula	z
JAL 558	lu32	USNM 23138	*C.l. labradorius*	1882/3	Labrador Peninsula	z
JAL 559	lu54	USNM 23137	*C.l. labradorius*	1882/3	Labrador Peninsula	z

*specimen accessioned before 1893; [s]southern clade mitochondrial DNA haplotype, see Fig. 2.

PCR products were purified using the UltraClean kit (MoBio). Products were cycle-sequenced in either a 9600 (Perkin-Elmer) or Primus 96 plus (MWG-Biotech) PCR instrument with BIG dye terminator (Perkin-Elmer, Boston, Massachusetts) chemistry. Cycle sequencing conditions for the 9600 followed manufacturer protocol. Cycle sequencing conditions for the Primus 96 plus were as recommended by MWG-Biotech. Sequences were then separated on an ABI automated sequencer 377 following the manufacturer's protocols. The same primers that were used for PCR were used for the sequencing reactions.

Ancient DNA precautions and replication. Precautions for the analysis of low copy DNA include nucleic acid isolation in a separately designated ancient DNA facility and replications as in Leonard *et al*. (2002). All pre-PCR work was done in a dedicated ancient DNA facility separated from the PCR amplification laboratory by five floors and two corridors. The ancient DNA lab used ultraviolet lights to inactivate DNA, had an independent air handling system with hepa filters on all vents, and was under positive air displacement. Two PCR blanks and both extraction blanks were run with each PCR reaction to monitor for contamination. To detect contamination, we used three different sets of PCR primers to produce overlapping sequences that could be compared. Further, 26 fragments were replicated twice; nine fragments were replicated three times; and one fragment was replicated four times. Four of the haplotypes found in ancient samples had also been independently observed in modern specimens

Table 2 Distribution of North American grey wolf mitochondrial control region haplotypes. The number of individuals with each haplotype, and the total and unique number of haplotypes at each locality are shown. Numbers in bold indicate historic specimens

	Alaska									
Haplotype	Kenai	Denali	Anaktuvuk	Alberta	Inuvik	Yukon	Montana	Labrador	*C.l. baileyi*	*C.l. nubilus*
lu11					2					
lu28	2	3 + 3*		3	1					**7**
lu29	2				3	1*				
lu30		1	7			1*				
lu31		4	4		4	1*	1 + 4*			
lu32		1		7	22 + 3*			**2** + 3*	**2**	**1**
lu33									**4** + 6*†	
lu37		1								
lu38		1			4					**3**
lu47									**1**	
lu48										**1**
lu49										**1**
lu50										**6**
lu51										**1**
lu52										**1**
lu53										**1**
lu54								**2**		
lu60									**1**	
lu61					1					
N	4	14	11	10	40	3	5	7	14	22
Different haplotypes	2	6	2	2	7	3	1	2	4	9
Unique haplotypes	0	1	0	0	1	0	0	1	3	6

*sequences previously published (Vilà *et al*. 1999). †from the captive breeding program. Sequences have been submitted to GenBank, accession numbers: AY812730–AY812741.

suggesting that polymerase error was not the origin of these haplotypes. Two of the novel historic haplotypes were found in multiple specimens.

Estimation of the total number of haplotypes. To assess if the number of haplotypes observed in the modern and in the historic samples are significantly different, we implemented a resampling/permutation procedure. We divided our sample in two random groups of the same size as the modern and historic samples 1000 times and counted in how many of these the difference in diversity (number of haplotypes) in the two groups was similar or larger to the difference observed in the real sample.

To estimate the number of haplotypes that were present in historic and modern American wolves, we constructed rarefaction curves plotting the cumulative number of haplotypes found with increasing sample size. The total number of haplotypes was estimated as the asymptote of this curve (Kohn *et al*. 1999; Eggert *et al*. 2003). Because the sampling order has a strong influence on the shape of the curve, we randomly sampled our data sets 1000 times without replacement and generated 1000 rarefaction curves using Microsoft EXCEL 2000. The asymptote (a) for each curve was calculated from the function $y = (ax)/(b + x)$, where y equals the cumulative number of haplotypes, x is the number of individuals sampled and b is the rate of decline in the value of the slope (Kohn *et al*. 1999; Eggert *et al*. 2003). The values of a and b were estimated through least-squares fit of the randomized data sets to the non-linear model in the program MATHEMATICA (Wolfram Media, Champaign, IL). The standard error (SE) for the parameter estimates was obtained from the 1000 pseudo-replicates, and the 95% confidence interval corresponds to the 0.025 and 0.975 percentiles.

The sampling regimes for modern and museum specimens differed as the modern populations were represented by many samples per locality whereas only one or two historic samples per locality were obtained. A second set of randomizations was generated limiting the maximum sample size of each population to four individuals. The total number of haplotypes obtained in random samples of 34 individuals with and without this restriction was compared and because the results were very similar (average of 1000 pseudo-replicates differed by less than 7%), all subsequent analyses were conducted on the randomizations without restriction on the sample size per population.

To assess if the rarefaction approach allows for reliable estimates of the number of haplotypes in the population, we simulated the random sampling of 30 or 90 individuals from a population containing 15 or 30 haplotypes. Because the frequencies of the haplotypes found in the historic samples were uneven, we assumed that the relative frequencies of the haplotypes in the simulated population were equally skewed. Therefore, for the simulations where the total number of haplotypes in the population was set equal to 15, we assumed that each haplotype was present with arbitrary relative frequencies similar to those in our historic data set: 7, 6, 5, 4, 3, 3, 3, 2, 2, 2, 1, 1, 1, 1, 1. For the simulated population containing 30 haplotypes, this series was repeated twice. The random sampling was repeated 1000 times for each condition (30 or 90 individuals, and 15 or 30 haplotypes), generating the corresponding 1000 accumulative curves, and the asymptote for each one of them was estimated as described above. The average of the asymptotes, corresponding to the estimated number of haplotypes in the simulated population, was close to the true number (15 or 30, depending on the simulation) and was always included in the 95% confidence intervals in all conditions (for 15 haplotypes and 90 samples: mean = 16.3, ± SE = 1.5; for 15 haplotypes and 30 samples: 17.4 ± 5.5; for 30 haplotypes and 90 samples: 32.1 ± 4.5; for 30 haplotypes and 30 samples: 37.3 ± 17.3). Consequently, the hyperbolic function described above provides a good estimate of the total number of haplotypes in the population, even if the variance in the haplotype frequencies is large. An exponential function (Eggert *et al*. 2003) was also tested, but the asymptote estimates failed to converge and very poor estimates were obtained.

Estimation of population size. We used estimates of θ based on the nucleotide diversity (Tajima 1996; Rozas & Rozas 1999) instead of coalescent-based estimates (Roman & Palumbi 2003) because North American wolves have a polyphyletic origin from multiple invading Eurasian wolf lineages (Vilà *et al*. 1999). Nevertheless, coalescent-based estimates for the historic specimens, estimated assuming fluctuations in population size using the program FLUCTUATE (Kuhner *et al*. 1998) gave similar values.

The estimates of past population size based on θ are dependent on the substitution rate. This rate was estimated according to the divergence between wolves and coyotes (see Results) using the sequences in Vilà *et al*. (1999) plus those reported here, and the standard error was calculated with 1000 bootstrap pseudo-replicates with the program MEGA3 (Kumar *et al*. 2004). The model of sequence evolution used was Tamura-Nei with a gamma shape parameter $a = 0.152$, as estimated using PAUP*4.0b10 (Swofford 2002).

This same model of evolution was used to assess the degree of differentiation between the historic and modern North American wolves by using Φ_{ST} and evaluating the significance with 1000 permutations as implemented in ARLEQUIN 2.000 (Schneider *et al*. 2000).

Phylogenetic analyses. The phylogenetic trees were based on analysis of 423–429 bp of mitochondrial DNA control region sequence from a worldwide sample of grey wolves and constructed in PAUP* 4.0b10 (Swofford 2002) using a HKY model of sequence evolution with a proportion of invariable sites I = 0.5882 and a gamma shape parameter $a = 0.5722$ as estimated in MODELTEST version 3.04 (Posada & Crandall 1998). The phylogenies were rooted with a previously published coyote sequence (Vilà *et al*. 1999). In addition to neighbour-joining we also constructed trees using unweighted maximum parsimony and maximum likelihood (quartet puzzling) algorithms. All methods provided similar tree topologies and the southern clade was supported in all cases (see Results and Discussion).

Results and Discussion

We found nine mtDNA haplotypes in 22 historic cUS *Canis lupus nubilus* but only three of these haplotypes were present in modern North American wolves (lu28, lu32 and lu38), and the remaining six were novel (lu48, lu49, lu50, lu51, lu52, and lu53) (Fig. 1, Tables 1 and 2). A ubiquitous (lu32) and a unique haplotype (lu54) were found in four historic Labrador, Canada wolves. In eight historic Mexican wolves we observed four haplotypes: four individuals had haplotype lu33 found previously in extant Mexican wolves from the captive breeding program, two individuals had haplotype lu32, a ubiquitous North American grey wolf haplotype, and the remaining two had unique haplotypes lu47 and lu60 (Tables 1 and 2). Haplotype lu60 was only one base pair different from a sequence found in a Mexican coyote and we assume it was derived from interspecific hybridization (Lehman *et al*. 1991; Wayne & Jenks 1991). In total, we found 13 haplotypes in 34 historic specimens and 10 haplotypes in 96 modern North American wolves. The diversity in the historic sample was significantly higher than in the modern sample (resampling test, $P = 0.002$). Nine and six haplotypes were unique to the historic and modern samples, respectively (Table 2).

The validity of ancient sequences is supported by their comparison to those from modern wolves. First, except for the haplotype lu60 (see below), none of the historic sequences were dramatically different from those found in extant wolves (Fig. 2). Second, no transversions were observed separating historic and modern sequences and third, the spectrum of variable sites was similar in both. We found 21 variable sites in the eight unique grey wolf-like historic haplotypes. Fifteen of these sites were also variable in the sample of modern wolves and showed the same transition substitution. Six other transitions were also

observed. Four of these were C-T transitions at the following alignment positions: base pair (bp) 113 (lu49), 138 (lu51), 150 (lu49) and 363 (lu50) and two were A-G transitions at the following alignment positions: bp 261 (lu48) and bp 278 (lu51). The electropherograms were unambiguous. The mutation at bp 363 was observed in six different individuals, and the mutations at bp 138 and 278 were confirmed by direct sequencing of multiple PCR products from the same specimen. Finally, the presence of lu33, the haplotype found in extant Mexican wolves, in four historic Mexican wolves, as well as the closely related haplotype lu47 in a fifth historic Mexican wolf sample, supports the validity of our results (Fig. 2).

The high diversity of historic wolf sequences suggests that the mtDNA diversity of the eradicated western cUS grey wolf population was more than twice that of the extant population. Modern wolves are a depauperate subset of the historic population. We estimate the total number of haplotypes for the modern and historic wolf populations as the average value of the asymptote in rarefaction curves resulting from 1000 randomizations of the data set (Lehman & Wayne 1991; Kohn *et al*. 1999). For the historic sample, the average asymptote corresponds to 20.8 haplotypes (SE = 6.3; 95% CI = 13.7–37.8). For the current sample, the estimated maximum number of haplotypes was 9.9 (SE = 1.0; 95% CI = 8.2–12.1), a value that was significantly lower than that for the historic sample (95% confidence intervals do not overlap). The same calculation, based on a random sample of modern individuals of the same size as the historic sample, provides a similar number of haplotypes of 9.2 (SE = 2.5, 95% CI = 6.1–15.8) and was significantly different from the average value for the historic sample (Fig. 3). Significant differentiation between historic and modern sample was observed, with a Φ_{ST} value of 0.025 ($P = < 0.001$). The large number of haplotypes in the historic population suggests that despite high mobility, more than 50% of the haplotypes that were present in the historic wolf population were lost during the last century.

We estimated female effective population size from the expression $\theta = 2N_{ef}\mu$ where θ is a measure of haplotype diversity, N_{ef} is the female effective population size and μ is the mutation rate (Vilà *et al*. 1999; Girman *et al*. 2000). The estimated values of θ are 0.008 and 0.013 for current and historic North American grey wolves, respectively. We used a value of μ based on a divergence time between coyotes and grey wolves of 2 million years (Nowak 2003) and a generation time of 3 years (Mech & Seal 1987), which yields a value of μ equal to 1.14×10^{-7} substitutions/site/generation (S.E. = 3.6×10^{-8}). Consequently, assuming that reproduction is equal among the sexes, that 60% of adults reproduce and that 50% of the population are adults (as in Vilà *et al*. 1999), the value of θ for historic western cUS and Mexico wolves implies a census size of approximately 380 000 individuals (95% confidence interval considering the S.E. of the substitution rate: 290 000–560 000). This estimate of population size depends on the model chosen to estimate theta as well as the demographic parameters used to infer population size from θ. Consequently, this exercise provides only a rough estimate of actual past population size. Moreover, this population estimate, although large, likely represents an underestimation because it assumes identical contributions to reproduction by all females. Considering that our estimate only refers to the population size in part of the North American geographical range of the grey wolf and that it is likely to be an underestimate, it is compatible with the estimate of Seton (1929) of about two million wolves in North America. Minimally, the value of θ from the historic sample implies that at least several hundred thousand wolves once inhabited the western cUS and Mexico whereas fewer than a thousand exist there today.

Grey wolves can readily cross the cUS-Canadian border and have recently dispersed into Montana, Idaho and North Dakota from Canada (Boitani 2003). Further, rates of gene flow among extant grey wolf populations are high (Roy *et al*. 1994; Vilà *et al*. 1999). Currently, the Rocky Mountain chain provides a corridor for dispersal of grey wolves into the cUS (Boyd *et al*. 1995; Forbes & Boyd 1996; Boitani 2003). Therefore, barriers to dispersal do not seem to have limited the recent migration of grey wolves between the western cUS and Canada. To explain the presence of so many unique haplotypes in the historic sample, we suggest that the North American population had not attained genetic homogeneity across its range since the last glacial maximum about 18 000 years ago. The spread of boreal forest in Canada began with the retreat of the glacial ice sheets about 12 000 years ago and reached its current distribution about 8000 years ago (Lessa *et al*. 2003). The area south of the ice sheets in the southern cUS and Mexico was likely a refugium for grey wolves during the last glaciation and consequently served as a source of grey wolf colonists for deglaciated Canada. As observed in Europe, populations of plants and animals in deglaciated areas have reduced levels of variation and contain fewer mitochondrial haplotypes (Hewitt 2002). Therefore, an unfortunate consequence of southern cUS and Mexico being a late Pleistocene refugium is that the eradication of grey wolves from these areas disproportionately affected the current genetic diversity of North American grey wolves. For example, had the extinction occurred over a similar area in Alaska, as represented by Denali National Park, Kenai Peninsula and Anaktuvuk populations, only one haplotype would have been lost, and that haplotype (lu37) was only found in a single individual (Table 2). Consequently, with regard to genetic diversity, refugial populations should have high priority for conservation (Taberlet & Cheddadi 2002; Tzedakis *et al*. 2002).

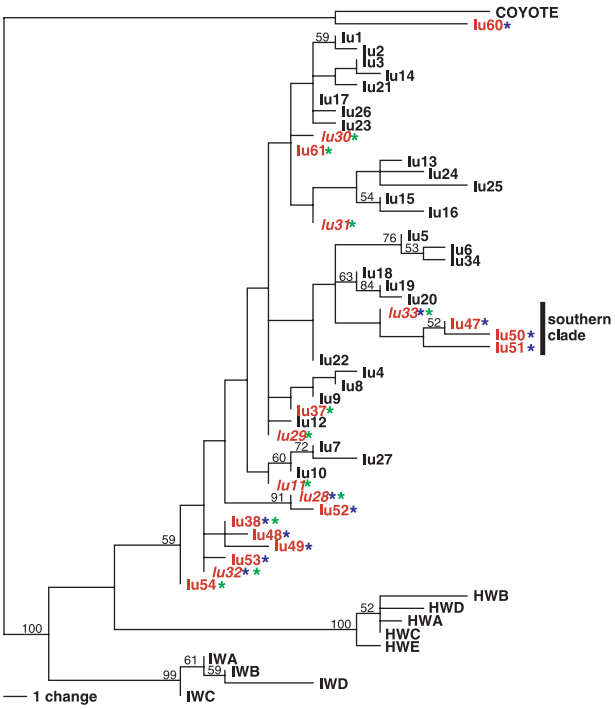

Fig. 2 Neighbour-joining tree of a worldwide sample of grey wolf haplotypes. The American wolves are in red (described in Table 2), and the Eurasian wolves are in black (Vilà *et al.* 1999; Sharma 2003; Indian wolves are labelled IW and HW). Blue asterisk indicates historic North American grey wolf haplotypes and green asterisk indicates modern North American grey wolf haplotypes. Clades supported in more than 50% of 1000 bootstrap pseudo-replicates are identified with the bootstrap support near nodes.

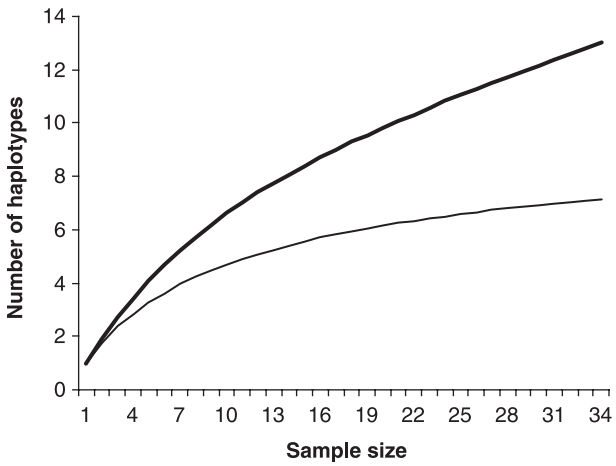

Fig. 3 Accumulative number of haplotypes found with increasing sample size for historic museum wolves (dark continuous line) and modern wolves (light continuous line) in North America (average estimated from 1000 randomizations).

A phylogenetic analysis of grey wolf haplotypes worldwide (Fig. 2) shows that current and historic North America (red) haplotypes are not monophyletic and are grouped with those from Europe and Asia (black). However, a monophyletic group exclusive to North American is defined by haplotype lu33 and historic haplotypes lu47, lu50 and lu51 (southern clade, Fig. 2). Haplotypes lu33 and lu47 are found only in samples of current and historic Mexican wolves whereas haplotypes lu50 and lu51 are found in historic samples of grey wolves from Utah to Nebraska and are intermixed with haplotypes common in northern grey wolves (Fig. 1, Table 2). The divergence within this southern clade ranges from two to six substitutions (0.5–1.4%) and although bootstrap support is weak in the neighbour-joining tree, the clade was present in the maximum likelihood tree and 99% of the most parsimonious trees. The wide distribution of the southern clade suggests that gene flow was extensive across the recognized limit of the subspecies (Fig. 1). Currently, captive populations of Mexican wolves are being used as a source for reintroduction to one locality along the Arizona-New Mexico border (USFWS 1998) and original plans include areas only within the supposed historic range in the US (Fig. 1). Our results suggest that Mexican grey wolves or a mix of Canadian and Mexican grey wolves could be introduced to a wider area of the cUS to mimic past intergradation. The larger Canadian grey wolves might be introduced to forested and mountainous areas similar to their natal habitat whereas the smaller (Nowak 1995), more arid adapted Mexican wolf might be introduced to more open habitats. Given the genetic evidence of intergradation of Northern and Mexican wolf-related haplotypes, ecological rather than genetic heritage should guide reintroduction. In highly mobile species, large zones of intergradation may characterize subspecies boundaries and provide important sources for adaptive evolution (Arnold 1997).

The large historic population size of about 380 000 grey wolves implied by the genetic data provides a striking contrast to restoration goals in the western cUS. Currently, US Fish and Wildlife restoration targets for the grey wolf are 30 packs totalling 300 individuals in Western States and 100 wolves in the range formerly inhabited by the Mexican wolf (USFWS 2003). Reintroduction of wolves into Yellowstone National Park has had substantial impacts on ungulate populations resulting in restoration of overgrazed plant communities as well as changes in numerical abundance of smaller carnivores and their prey (Smith *et al.* 2003). Therefore, we suggest restoration goals might be reconsidered so as to better restore wolves to past population sizes and enable them to significantly influence the dynamics of the Rocky Mountain ecosystem. The Mexican wolf can be an integral part of this reintroduction plan. However, rather than following carefully delineated subspecific boundaries based on historic and morphologic data, the genetic evidence suggests a southern clade of

haplotypes extended into the central plains and intergraded with haplotypes characteristic of Northern populations. Our genetic results provide a wider geographical mandate for reintroduction and suggest admixture was a characteristic of past populations that might enhance the adaptive potential of reintroduced stocks. The tragedy of the extermination program, supported by Federal and State bounties, cannot be undone because Mexico and the southern US represented a unique refugium for genetic biodiversity. However, the evolutionary dynamics and ecosystem properties that once existed in the refugium could yet be restored through carefully planned and more extensive reintroductions of top predators such as the grey wolf.

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