

Dire wolves were the last of an ancient New World canid lineage

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Summary Paragraph

Dire wolves are considered one of the most common and widespread large carnivores in Pleistocene America¹, yet relatively little is known about their evolution or extinction. To reconstruct the evolutionary history of dire wolves, we sequenced five genomes from sub-fossil bones dating from 13,000 to over 50,000 years ago. Our results indicate that though they were similar morphologically to the extant gray wolf, dire wolves were a highly divergent lineage that split from living canids ~5.7 million years ago. In contrast to numerous examples of hybridization across Canidae^{2,3}, there is no evidence for gene flow between dire wolves and either North American gray wolves or coyotes. This suggests that dire wolves evolved in isolation from the Pleistocene ancestors of these species. Our results also support an early New World origin of dire wolves, while the ancestors of gray wolves, coyotes, and dholes evolved in Eurasia and only colonized North America relatively recently.

Main Text

Dire wolves (*Canis dirus*) were large (~68 kg) wolf-like canids and among the most common extinct large carnivores of the American Late Pleistocene megafauna¹. Dire wolf remains are present in the North American paleontological record from at least ~250,000 to ~13,000 years ago, at the end of the Pleistocene, particularly in the lower latitudes⁴ (Fig. 1A). Other canid species present in Late Pleistocene North America include the slightly smaller gray wolf (*C. lupus*), the much smaller coyote (*C. latrans*), and the dhole (or Asiatic wild dog; *Cuon alpinus*), though dire wolves appear to be more common overall¹. For example, >4,000 individuals have been excavated in California's fossil-rich Rancho La Brea tar seeps alone, where they outnumber gray wolves more than 100-fold^{5,6}.

Despite the abundance of dire wolf fossils, the origin, taxonomic relationships, and ultimate driver of their extinction remain unclear. Dire wolves are generally described as a sister species to⁷⁻¹⁰, or even conspecific with the gray wolf¹¹. The leading hypothesis to explain their extinction is that, due to their larger body size than gray wolves and coyotes, dire wolves were more specialized for hunting large prey, and were unable to survive the extinction of their megafaunal prey (e.g.¹²⁻¹⁴). To test this hypothesis, we performed geometric morphometric analyses of >700 specimens. Our results indicate that although dire wolves and gray wolves species can be differentiated, their morphology is highly similar (Supplementary Information; Fig. 1B; Supplementary Fig. 1-6; Supplementary Data 3-12). Although this morphometric similarity may partly be driven by allometry (Supplementary Information; Fig. 1B), the lack of distinctiveness between gray wolves and dire wolves has been interpreted as a result of a close evolutionary relationship^{9,11}. Alternatively, a competing hypothesis maintains that these morphological similarities are the result of convergence, and that dire wolves instead are a species belonging to a separate taxonomic lineage (classified in the monotypic genus *Aenocyon*; "terrible or dreadful wolf"¹⁵).

To resolve the evolutionary history of dire wolves, we screened 46 sub-fossil specimens for the presence of preserved genomic DNA (Supplementary Data 1). We identified five samples from Idaho (DireAFR & DireGB), Ohio (DireSP), Tennessee (DireGWC), and Wyoming (DireNTC) ranging in age from 12,900 to >50,000 years before present, that possessed sufficient endogenous DNA to obtain both mitochondrial genomes (between ~1x and 31x coverage) and low-coverage nuclear genome sequences (~0.01x to 0.23x coverage) using hybridization capture or shotgun sequencing methods (Supplementary Information). All of these displayed molecular damage profiles typical of ancient DNA (Supplementary Fig. 8-9). Although we did not successfully sequence DNA from the La Brea tar seeps dire wolf specimens, one specimen did contain type I collagen (*COL1*) suitable for sequencing using paleoproteomic methods (Supplementary Data 1; Supplementary Information).

Analyses of the dire wolf *COL1* sequence suggested that they were not closely related to gray wolves, coyotes, African wolves (*C. anthus*), and dogs (*C. familiaris*) (Supplementary Fig. 7). These data, however, could not confidently resolve the relationships between more distantly related canids due to a lack of lineage-specific amino acid changes among these species¹⁶.

Phylogenetic analyses of the mitochondrial genomes indicated that dire wolves form a well-supported monophyletic group that is highly divergent from gray wolves and coyotes (Supplementary Fig. 10; see Supplementary Data 13 and Supplementary Table 2-4 for a list of the 13 species and their coverage used in this analysis), contradicting recent paleontological analyses⁷⁻⁹ (Figure 1B). Canid mitochondrial phylogenies, however, may not represent the true species evolutionary relationships since both admixture and incomplete lineage sorting have been shown to affect canid phylogenetic topologies^{3,17}.

In order to resolve the phylogenetic relationships of dire wolves, we analyzed our dire wolves' nuclear genomic data with previously published genomic data from eight extant canids: gray wolf, coyote, African wolf, dhole, Ethiopian wolf (*C. simensis*), African wild dog (*Lycaon pictus*), Andean fox (*Lycalopex culpaeus*), and gray fox (*Urocyon cinereoargenteus*; an outgroup). Of these species, the geographical ranges of gray wolves, coyotes, dholes, and gray foxes overlapped with that of dire wolves during the Pleistocene (Fig. 1A). We also generated new nuclear genome sequences from a gray wolf from Montana and from the two endemic African jackals, the black-backed and side-striped jackal (*C. mesomelas* and *C. adustus*, respectively), in order to ensure representation of all extant members of the "wolf-like canid" clade (comprising *Canis*, *Lycaon*, *Cuon*, and their extinct relatives) (Supplementary Data 13). Supermatrix analyses, based on 70 Kb to 28 Mb nuclear sequence alignments (depending on overall coverage for each dire wolf genome, see Supplementary Table 5 & 7) confirmed a distant evolutionary relationship between dire wolves and the other wolf-like canids (Fig. 2A; Supplementary Fig. 11; Supplementary Fig. 15-16). This analysis, however, could not definitively resolve whether dire wolves were the basal members of the wolf-like canid clade, or the second lineage to diverge after the common ancestor of the two African jackals.

We investigated canid phylogenetic relationships in greater detail using a range of species tree analyses^{18,19} and D-statistics (Supplementary Information). These approaches produced concordant trees that support the monophyly of three primary lineages: dire wolves, African jackals, and a clade comprising all other extant wolf-like canids (Fig. 2A; Supplementary Table 6-8; Supplementary Fig. 11-16). Although our species tree analyses provided equivocal results regarding the relationships among these lineages, gray wolves (genus *Canis*) are more closely related to African wild dogs (genus *Lycaon*) (Supplementary Fig. 19), dholes, (Supplementary Fig. 21) (genus *Cuon*), and Ethiopian wolves (Supplementary Fig. 22) than to either dire wolves or African jackals (both genus *Canis*). This finding is consistent with previously proposed designations of genus *Lupulella*²⁰ for the African jackals and *Aenocyon*¹⁵ for dire wolves.

To assess the timing of divergence among the major wolf-like canid lineages we performed a molecular clock analysis based on two fossil calibrations using MCMCtree²¹. Although the dire wolf sequences are low coverage and include post-mortem damage, extensive simulations indicated this is unlikely to affect the time of divergence estimates inferred by MCMCtree (Supplementary Information; Supplementary Table 9-11; Supplementary Fig. 17). This analysis confirmed that the initial divergences of the three primary wolf-like canid lineages occurred rapidly, contributing to the poor resolution of the tree as a result of incomplete lineage sorting (Fig. 2A). The dire wolf lineage last shared a common ancestor with extant wolf-like canids ~5.7

million years ago (95% HPD=4.0-8.5 million years ago; Fig. 2A), followed by the divergence of African jackals ~5.1 million years ago (95% HPD=3.5-7.6 million years ago; Fig. 2B).

Given the propensity for sympatric canid species to interbreed^{2,3,22}, we tested for genomic signals of admixture between extant North American canids and dire wolves using D statistics²³ (Supplementary Information) in a data set that included 22 modern North American gray wolves and coyotes, three ancient dogs²⁴⁻²⁶, and a Pleistocene wolf²⁷ (Supplementary Data 13). Specifically, we computed statistics of the form D (outgroup [gray fox]; dire wolf; North American canid [gray wolf or coyote], African wolf/Eurasian wolf) and found no significant excess of shared derived alleles between dire wolves and any extant North American canid (Fig. 2B; Supplementary Fig. 18; Supplementary Data 14). This result indicates that the dire wolves sequenced in this study did not possess ancestry from gray wolves, coyotes, or their recent North American ancestors. Although we cannot exclude the possibility that some unsampled canid population has some dire wolf hybrid ancestry, the lack of signal of hybridization in our broad set of genomes suggests that admixture is unlikely to have occurred. While we did not find evidence of recent admixture, we did find that African wild dogs share fewer derived alleles with dire wolves than with gray wolves, coyote, African wolves, dhole, or Ethiopian wolves (Fig. 2C; Supplementary Fig. 20; Supplementary data 15-16). This indicates that an episode of ancient admixture between the ancestor of dire wolves and the ancestor of wolves, coyotes, and dhole occurred at least ~3 million years ago (based on the lower bound of the 95% HPD on the age of their common ancestor; Fig. 2A), which may have contributed challenges resolving the branching order of the basal wolf-like canid lineages (Fig. 2A).

Hybridization is common among wolf-like canid lineages when their ranges overlap. For example, modern gray wolves and coyotes hybridize readily in North America (e.g.,²). Genomic data also suggest gene flow occurred between dholes and African wild dogs during the Pleistocene³, millions of years after their divergence. Consequently, our finding of no evidence for gene flow between dire wolves and gray wolves, coyotes, or their common ancestor, despite substantial range overlap with dire wolves during the Late Pleistocene suggests that the common ancestor of gray wolves and coyotes probably evolved in geographical isolation from members of the dire wolf lineage. This result is consistent with the hypothesis that dire wolves originated in the Americas^{1,6,28,29}, likely from the extinct Armbruster's wolf (*C. armbrusteri*⁷).

Long term isolation of the dire wolf lineage in the Americas implies that other American fossil taxa, such as the Pliocene *C. edwardii*, a proposed relative of the coyote⁷, may instead belong to the dire wolf lineage. Thus, the diversification of the extant wolf-like canids likely occurred in parallel outside of the Americas, and perhaps began earlier than hypothesized. The living *Canis* species may have descended from Old World members of the extinct genus *Eucyon*, which first appeared in the fossil record of Africa and Eurasia at the end of the Miocene (see³⁰). Geographic isolation since the late Miocene is consistent with our molecular estimates for the age of the dire wolf lineage, and may have allowed dire wolves to evolve some degree of reproductive isolation prior to the Late Pleistocene North American arrival of gray wolves, coyotes, dholes, and *Xenocyon* (another extinct wolf-like canid).

Despite their overall phenotypic similarities, gray wolves and coyotes survived the Late Pleistocene megafaunal extinctions while dire wolves did not. One possible reason may be that both gray wolves and coyotes possessed greater morphological plasticity and dietary flexibility, thus allowing them to avoid extinction and become the dominant terrestrial predators in North America^{14,31}. This scenario is supported by the date we obtained from the DireGWC specimen (12,820-12,720 calBP), which suggests that dire wolves survived until at least the Younger Dryas cold reversal, a period that also witnessed the latest known dates for other specialized North American mega-carnivores such as the American lion (*Panthera atrox*) and giant short-faced bear (*Arctodus simus*)^{32,33}. Alternatively, gray wolves and coyotes may have survived as a result of their ability to hybridize with other canids. Through adaptive introgression with dogs, North American gray wolves are known to have acquired traits related to coat color, hypoxia, and immune response^{34,35}. Specifically, enhanced immunity may have allowed gray wolves to resist novel diseases carried by newly arriving Old World taxa. Since our results demonstrate that dire wolves did not derive any ancestry from other wolf-like canid species, it is plausible that reproductive isolation prevented dire wolves from acquiring traits that may have allowed them to survive into the Holocene.

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Author contributions

A.R.P., K.J.M., A.M., R.K.W., G.L., L.A.F.F., and A. Cooper conceived of the project and designed the research; A.R.P. and K.J.M. coordinated the sample collection efforts with input from R.K.W., G.L., L.A.F.F., K.M.H., and A. Cooper; A.R.P., K.J.M., A.H.-B., J.M., C.A., J.C.C., A.E., J.K., A.L., A.O., S.P., B.W.S., M.T., M.J.C., M.-M.H.S., M.T.P.G., A.C.S., B.S., B.V.V., R.W.K., and A. Cooper provided and/or collected samples; A.R.P., K.J.M., K.W., A.M., C.S., J.H., A.J., A.T.S., P.B., and H.H. conducted the genomic laboratory work; K.J.M., A.M., G.G., G.L., L.A.F.F. and A. Cooper conducted the analyses of the genomic data; S. A.-C., A.H.-B., J.M., C.A., and A.E. conducted the morphological analyses; A.R.P., K.J.M., A.M., S.A.-C., B.V.V., R.K.W., G.L., L.A.F.F. and A. Cooper wrote the paper with input from all other authors.

Data availability

The reads generated for this study have been deposited at the European Nucleotide Archive (ENA) with project number PRJEB31639. The accession number for the publicly available genomes used in this study can be found Supplementary Table 2 and Supplementary Data 13. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021930. Ancient collagen consensus sequences for the dire wolf can be found in Supplementary Data 17. 2D mandibular and dental shape (Geomorphometric data) were deposited on Dryad (DOI: <https://doi.org/10.5061/dryad.63xsj3v16>).

Competing Interests

The authors declare no competing interests.

Additional Information

Supplementary Information is available for this paper. Correspondence and requests for materials should be addressed to Laurent Frantz (laurent.frantz@gmail.com), Angela Perri (angela.r.perri@durham.ac.uk) or Kieren Mitchell (kieren.mitchell@adelaide.edu.au).

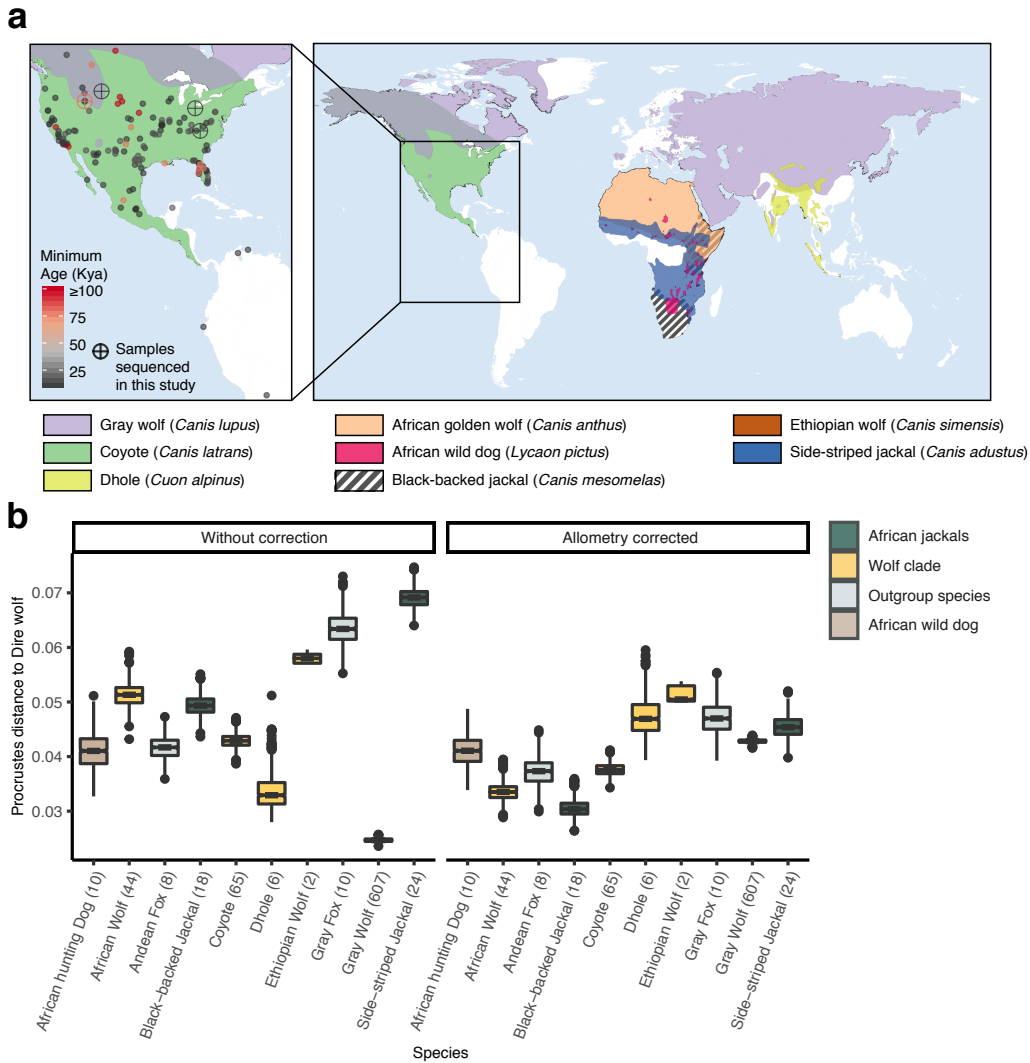


Figure 1. Map of dire wolf remains and morphological differentiation with wolf-like canids

a. Right: map representing the geographic range of the canid species investigated in this study. The data (shape file) for this plot was obtained from the IUCN red list database³⁶ and plotted using R³⁷. Left: map representing the distribution of sites in the Americas where dire wolf remains (*Canis dirus*) were identified (Supplementary Data 1-2). Colored circles represent the location and approximate age of the remains, with crossed circles representing the five samples from Idaho (2), Ohio (1), Tennessee (1), and Wyoming (1) that yielded sufficient endogenous DNA to reconstruct both mitochondrial genomes and low-coverage nuclear genome sequences.

b. Procrustes distance between the combined mandible and M1 shape of dire wolf and other extant canid species. Pairwise procrustes distances were calculated by superimposing landmarks from molar and mandibular shapes between pairs of specimens and by computing the square root of the squared differences between the coordinates of corresponding landmarks, with and without correction for allometry (Supplementary Information). The centre of the box represents the median, the box bounds represent the quartiles, the whiskers represent maximum and minimum values ($\pm 1.5 \times \text{Interquartile Range [IQR]}$) and dots represent outliers.

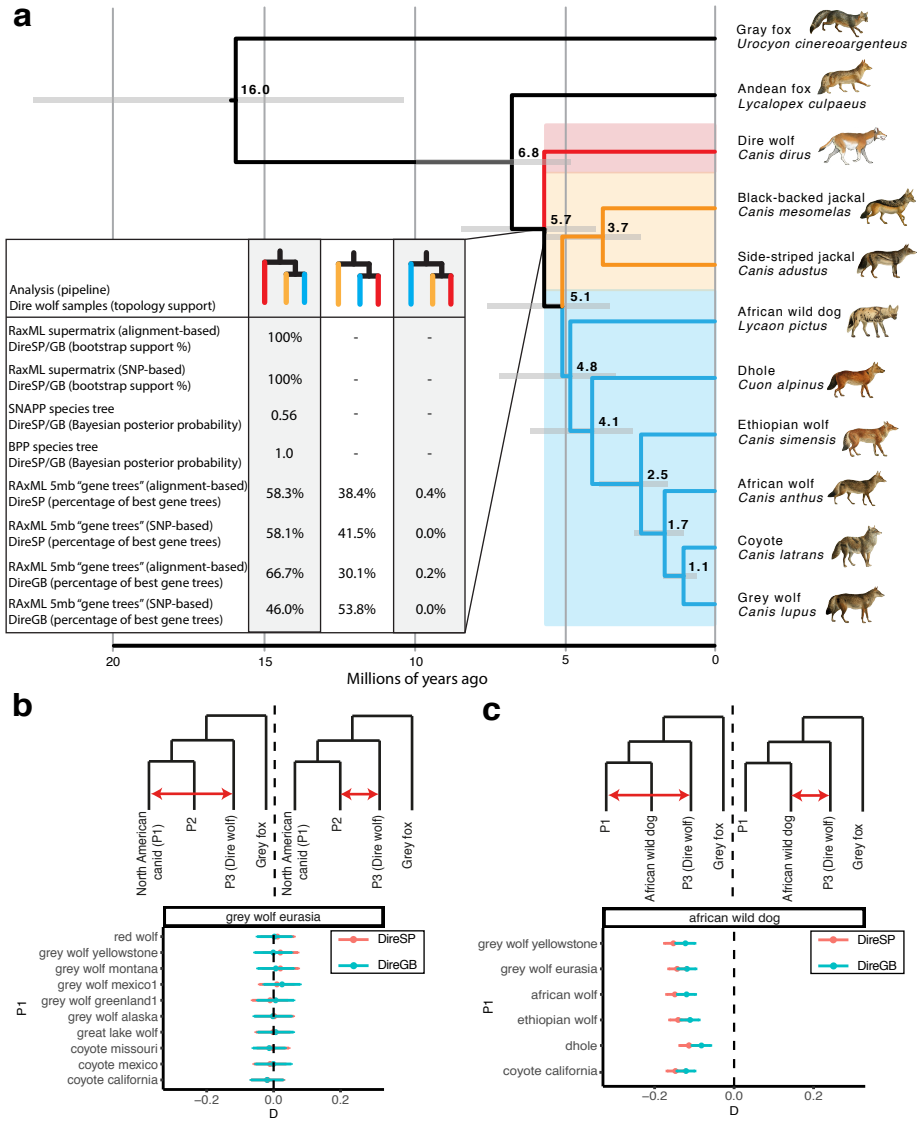


Figure 2. Relationships among living and extinct wolf-like canids **a.** Time-scaled nuclear phylogeny generated in MCMCtree based on the best species tree topology obtained from BPP and SNAPP. Values associated with nodes are mean age estimates (millions of years before present) while bars represent 95% Highest Posterior Densities. The inset table shows levels of support for the three possible arrangements of the dire wolf (red), the African jackals (orange), and the remaining wolf-like canids (blue) that we obtained under different analytical frameworks when including either one or both of our two highest coverage dire wolf samples (DireSP and DireGB). Although only one dire wolf branch is depicted in the tree, multiple dire wolf individuals were included they formed a monophyletic clade (Supplementary Fig. 12-13, and 15). **b.** Results of D statistics used to assess the possibility of gene flow between the dire wolf and extant North American canids. Each dot represents the mean D calculated along the genome and the error bar represents 3 standard deviations computed using a weighted block jackknife procedure over 5Mbp blocks across the genome. Z value $|Z| > 3$ were considered significant. These plots show that the dire wolf genomes do not share significantly more derived alleles with extant North American canids compared to Eurasian wolves (values of D not significantly different to zero),

suggesting that no hybridization occurred between the dire wolf and the ancestor of extant North American canids. Non significant D-statistics were also obtained using an alternative reference genome and using the African wolf as P2 (Supplementary Fig. 18 and Supplementary Data 14).

c. Results of D statistics showing the existence of an ancient gene flow event between the ancestor of the dhole, Ethiopian wolf, African wolf, gray wolf and coyotes and the lineage of the dire wolf (consistently non-zero values of D regardless of P1).