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

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Dire wolves are considered to be one of the most common and widespread large carnivores in Pleistocene America[1], yet relatively little is known about their evolution or extinction. Here, to reconstruct the evolutionary history of dire wolves, we sequenced five genomes from sub-fossil remains dating from 13,000 to more than 50,000 years ago. Our results indicate that although they were similar morphologically to the extant grey wolf, dire wolves were a highly divergent lineage that split from living canids around 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 grey 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 grey wolves, coyotes and dholes evolved in Eurasia and colonized North America only relatively recently.

Dire wolves (*Canis dirus*) were large (around 68 kg) wolf-like canids and among the most common extinct large carnivores of the American Late Pleistocene megafauna[1]. Dire wolf remains are present in the North American palaeontological record from at least around 250,000 to about 13,000 years ago, at the end of the Pleistocene epoch, particularly in the lower latitudes[4] (Fig. 1a). Other canid species that were present in Late Pleistocene North America include the slightly smaller grey wolf (*Canis lupus*), the much smaller coyote (*Canis latrans*) and the dhole (or Asiatic wild dog; *Cuon alpinus*), although dire wolves appear to have been more common overall[1]. For example, more than 4,000 individuals have been excavated from California's fossil-rich Rancho La Brea tar seeps alone, where they outnumber grey wolves more than 100-fold[5,6].

Despite the abundance of dire wolf fossils, their origins, taxonomic relationships and ultimate driver of their extinction remain unclear. Dire wolves are generally described as a sister species to[7–10], or even conspecific with, the grey wolf[11]. The leading hypothesis to explain their extinction is that, owing to their larger body size compared with grey wolves and coyotes, dire wolves were more specialized for hunting large prey and were unable to survive the extinction of their megafaunal prey[12–14]. To test this hypothesis, we performed geometric morphometric analyses of more than 700 specimens. Our results indicate that although specimens of dire wolves and grey wolves can be differentiated, their morphology is highly similar (Fig. 1b, Supplementary Information, Supplementary Figs. 1–6 and Supplementary Data 3–12). Although this morphometric similarity may be driven in part by allometry (Fig. 1b, Supplementary Information), the lack of distinctiveness between grey wolves and dire wolves has been interpreted to be 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 that belongs 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), dating to between 12,900 and more than 50,000 years ago, that possessed sufficient endogenous DNA to obtain both mitochondrial genomes (between around 1× and 31× coverage) and low-coverage nuclear genome sequences (approximately 0.01× to 0.23× coverage) using hybridization capture or shotgun sequencing methods (Supplementary Information). All of these samples displayed molecular damage profiles that are typical of ancient DNA (Supplementary Figs. 8, 9). Although we did not successfully sequence DNA from dire wolf

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A list of affiliations appears at the end of the paper.

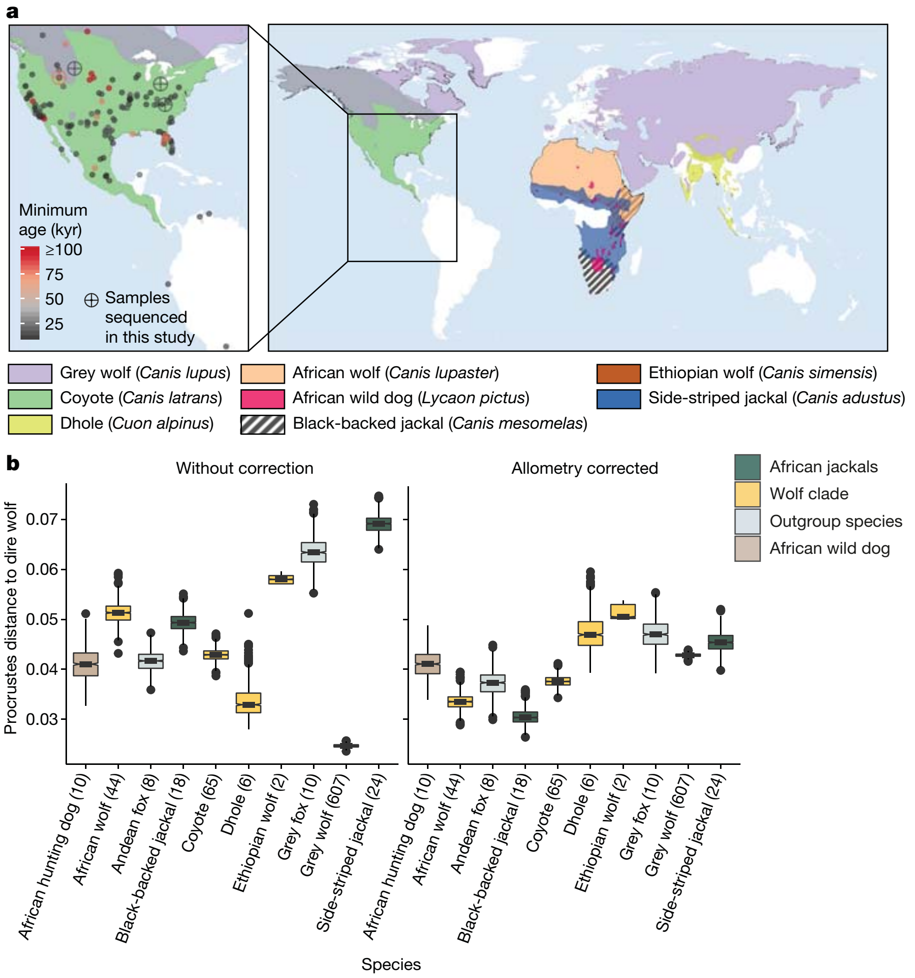
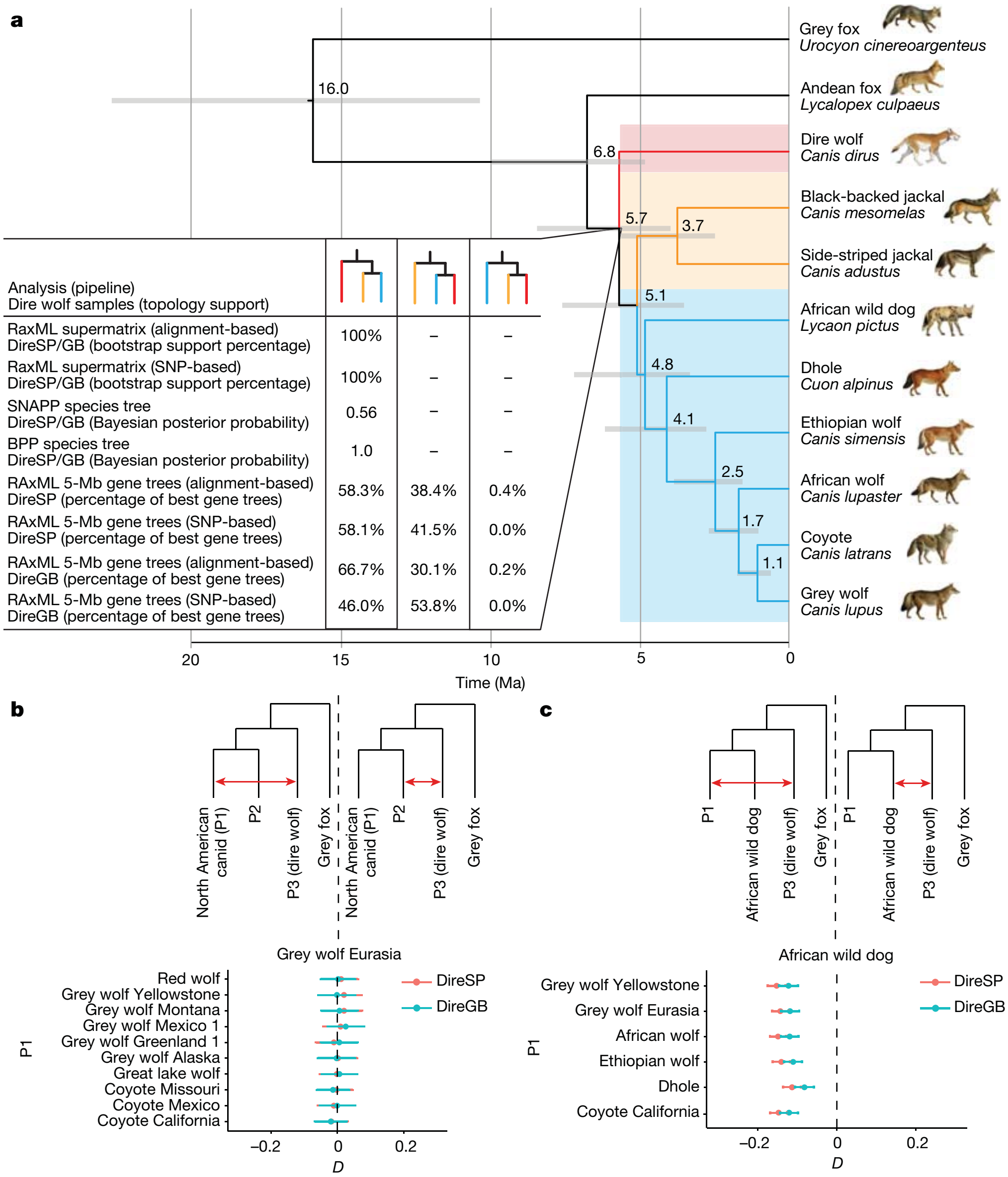


Fig. 1 | Map of dire wolf remains and morphological differentiation with wolf-like canids. a, Right, map representing the geographical range of the canid species investigated in this study. The data (shape file) for this plot were obtained from the IUCN Red List database[36] and plotted using R[37]. Left, map representing the distribution of sites in the Americas where dire wolf remains (*Canis dirus*) have been identified (Supplementary Data 1, 2). Coloured circles represent the locations and approximate ages 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 distances between the combined mandible and M^1 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 (±1.5× the interquartile range) and dots represent outliers.

specimens from the La Brea tar seeps, one specimen did contain type-I collagen (*COL1*) that was suitable for sequencing using palaeoproteomic methods (Supplementary Data 1 and Supplementary Information).

Analyses of the dire wolf *COL1* sequence suggested that they were not closely related to grey wolves, coyotes, African wolves (*Canis lupaster*) or dogs (*Canis familiaris*) (Supplementary Fig. 7). These data, however, could not confidently resolve the relationships between more distantly related canids owing to a lack of lineage-specific amino acid changes among these species[16]. Phylogenetic analyses of the mitochondrial genomes indicated that dire wolves form a well-supported monophyletic group that is highly divergent from grey wolves and coyotes (Supplementary Fig. 10; see Supplementary Data 13 and Supplementary Tables 2–4 for a list of the 13 species and their coverage used in this analysis), contradicting recent palaeontological analyses[7–9] (Fig. 1b). Canid mitochondrial phylogenies, however, may not represent the true evolutionary relationships of the species as both admixture and incomplete lineage sorting have been shown to affect canid phylogenetic topologies[3,17].

To resolve the phylogenetic relationships of dire wolves, we analysed the nuclear genomic data of the dire wolves together with previously published genomic data from eight extant canids: grey wolf, coyote, African wolf, dhole, Ethiopian wolf (*Canis simensis*), African wild dog (*Lycaon pictus*), Andean fox (*Lycalopex culpaeus*) and grey fox (*Urocyon cinereoargenteus*—an outgroup). Of these species, the geographical ranges of grey wolves, coyotes, dholes and grey foxes overlapped with that of dire wolves during the Pleistocene epoch (Fig. 1a). We also generated new nuclear genome sequences for a grey wolf from Montana and the two endemic African jackals—the black-backed and side-striped jackal (*Canis mesomelas* and *Canis adustus*, respectively)—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 nuclear sequence alignments of 70 kb to 28 Mb (depending on overall coverage for each dire wolf genome; Supplementary Tables 5, 7) confirmed a distant evolutionary relationship between dire wolves and the other wolf-like canids (Fig. 2a, Supplementary Fig. 11 and Supplementary Figs. 15, 16). This analysis, however, could not definitively resolve whether dire wolves were the



Analysis (pipeline) Dire wolf samples (topology support)			
RaxML supermatrix (alignment-based) DireSP/GB (bootstrap support percentage)	100%	–	–
RaxML supermatrix (SNP-based) DireSP/GB (bootstrap support percentage)	100%	–	–
SNAPP species tree DireSP/GB (Bayesian posterior probability)	0.56	–	–
BPP species tree DireSP/GB (Bayesian posterior probability)	1.0	–	–
RAxML 5-Mb gene trees (alignment-based) DireSP (percentage of best gene trees)	58.3%	38.4%	0.4%
RAxML 5-Mb gene trees (SNP-based) DireSP (percentage of best gene trees)	58.1%	41.5%	0.0%
RAxML 5-Mb gene trees (alignment-based) DireGB (percentage of best gene trees)	66.7%	30.1%	0.2%
RAxML 5-Mb gene trees (SNP-based) DireGB (percentage of best gene trees)	46.0%	53.8%	0.0%

Fig. 2 | Relationships among living and extinct wolf-like canids. **a**, Time-scaled nuclear phylogeny generated in MCMCtree based on the best-scoring maximum likelihood species tree topology obtained from BPP and SNAPP. Values associated with nodes are mean age estimates (millions of years before present) and bars represent 95% highest posterior densities. The inset table shows the 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 using 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 in some analyses as they form a monophyletic clade (for example, Supplementary Figs. 12, 13, 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 5-Mb blocks across the genome. *Z* values of $|Z| > 3$ 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* were not significantly different from 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, grey wolf and coyotes and the lineage of the dire wolf (consistently non-zero values of *D* regardless of P1). P1, P2 and P3 in the tree schematic represent genomes that are used in the admixture test.

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 Tables 6–8 and Supplementary Figs. 11–16). Although our species tree analyses provided equivocal results regarding the relationships among these lineages, grey wolves (genus *Canis*) are more closely related to African wild dogs (genus *Lycaon*) (Supplementary Fig. 19), dholes (genus *Cuon*) (Supplementary Fig. 21) 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 the genera *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 Bayesian clock-dating analysis using MCMCtree[21]. Although the dire wolf sequences are low coverage and include post-mortem damage, extensive simulations indicated that this is unlikely to affect the time of divergence estimates inferred by MCMCtree (Supplementary Information, Supplementary Tables 9–11 and Supplementary Fig. 17). This analysis confirmed that the initial divergences of the three primary wolf-like canid lineages occurred rapidly, which contributes 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 around 5.7 million years ago (95% highest posterior density (HPD), 4.0–8.5 million years ago) (Fig. 2a), followed by the divergence of African jackals around 5.1 million years ago (95% HPD, 3.5–7.6 million years ago) (Fig. 2b).

Given the tendency 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*-statistics[23] (Supplementary Information) on a dataset that included 22 modern North American grey wolves and coyotes, three ancient dogs[24–26] and a Pleistocene wolf[27] (Supplementary Data 13). Specifically, we computed statistics of the form *D*(outgroup (grey fox); dire wolf; North American canid (grey 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 and Supplementary Data 14). This result indicates that the dire wolves sequenced in this study did not possess ancestry from grey 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 a hybridization signal in our broad set of genomes suggests that admixture is unlikely to have occurred. Although we did not find evidence of recent admixture, we did find that African wild dogs share fewer derived alleles with dire wolves than with grey wolves, coyotes, African wolves, dholes or Ethiopian wolves (Fig. 2c, Supplementary Fig. 20 and 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 dholes occurred at least around 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 to the challenge of 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 grey wolves and coyotes hybridize readily in North America[2]. Genomic data also suggest that gene flow occurred between dholes and African wild dogs during the Pleistocene epoch[3], millions of years after their divergence. Consequently, our finding of no evidence for gene flow between dire wolves and grey wolves, coyotes or their common ancestor—despite substantial range overlap with dire wolves during the Late Pleistocene—suggests that the common ancestor of grey 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], and probably belonged to the same lineage as the extinct Armbruster's wolf (*Canis armbrusteri*)[7].

Long-term isolation of the dire wolf lineage in the Americas implies that other American fossil taxa, such as the Pliocene *Canis edwardii*, a proposed relative of the coyote[7], may instead belong to the dire wolf lineage. Thus, the diversification of the extant wolf-like canids probably 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 epoch[30]. Geographical 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 before the arrival of grey wolves, coyotes, dholes and *Xenocyon* (another extinct wolf-like canid) in North America during the Late Pleistocene.

Despite their overall phenotypic similarities, grey wolves and coyotes survived the Late Pleistocene megafaunal extinctions whereas dire wolves did not. One possible reason may be that both grey 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 that we obtained from the DireGWC specimen (12,820–12,720 calibrated years before present), 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, grey wolves and coyotes may have survived as a result of their ability to hybridize with other canids. Through adaptive introgression with dogs, North American grey wolves are known to have acquired traits related to coat colour, hypoxia and immune response[34,35]. Specifically, enhanced immunity may have allowed grey wolves to resist diseases carried by newly arriving Old World taxa. Because 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 epoch.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-03082-x.

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Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The reads generated for this study have been deposited in the European Nucleotide Archive (ENA) (project number PRJEB31639). The accession numbers for the publicly available genomes used in this study can be found in Supplementary Table 2 and Supplementary Data 13. The mass-spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (PXD021930). Ancient collagen consensus sequences for the dire wolf can be found in Supplementary Data 17. Two-dimensional mandibular and dental shape (geometric morphometric) data have been deposited in Dryad (https://doi.org/10.5061/dryad.63xsj3v16).

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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 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. and A. Cooper; A.R.P., K.J.M., A.H.-B., J.M., C.A., J.C.C., A.E., P.G., J.K., A.L., A.T.O., S.P., B.W.S., M.T., M.J.C., M.-H.S.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., R.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., K.M.H., and A.E. conducted the morphological analyses; A.R.P., K.J.M., A.M., S.A.-C., B.V.V., K.M.H., R.K.W., G.L., L.A.F.F. and A. Cooper wrote the paper with input from all other authors.

Competing interests The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-020-03082-x.
Correspondence and requests for materials should be addressed to A.R.P., K.J.M. or L.A.F.F.
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☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	TpsDig 2.3 software was used for digitising landmarks in morphometric analysis
Data analysis	Morphometric analyses: Morpho R package (v2.8); Shapes R package (v1.2.5); Geomorph R package (v3.2.0); Geiger R package (v2.0.6.4); Phytools R package (v0.6-99). Proteomics analyses: PEAKS (v7.5); MAFFT (v7.123b); MrBayes (v3.2.6). Genomic analyses: BWA (mem and aln mode; v0.7.17); Picard (v1.137); MapDamage (v2.0); GATK (v4.0.11.0); ANGSD (v0.931-2-gfd2a527); RAxML (v8.2.9); htsbox (v-r327); AMAS (v0.94); PartitionFinder (v2); MrBayes (v3.2.1); BedTools (v2.17.0); BPP (v4.0.4); DISCOVISTA(https://github.com/esayyari/DiscoVista); mcmc3r; BEAST (v2.5.1); BCFtools (v0.1.17); eig-utils (https://github.com/grahamgower/eig-utils); PAML (v4.9); Phyclust R package (v0.1-28); mcmc3r (https://github.com/dosreislab/mcmc3r); ADMIXTOOLS (v3);

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The genomic data (reads) generated for this study have been deposited at the European Nucleotide Archive (ENA) with project number PRJEB31639: https://www.ebi.ac.uk/ena/browser/view/PRJEB31639.

Geomorphometric data (2D) was deposited on Dryad with DOI: https://doi.org/10.5061/dryad.63xsj3v16
The mass spectrometry proteomics data are available via ProteomeXchange with identifier PXD021930 - and consensus sequence of the dire wolf can be found in Supplementary Data 17.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Describe the first genome sequence of dire wolves obtained from sub-fossils.
Research sample	Sub-fossils of dire wolves from various locations in the US. Detailed information about the samples and their locations are available below, in the supplementary information and in Supplementary Data 1
Sampling strategy	All sub-fossils which possessed enough bone material were sampled and analysed in this study (see Supplementary Data 1)
Data collection	DireAFR: A.R.P sampled the original specimen. AJ extracted DNA from the sample and created libraries for high-throughput DNA sequencing on a Illumina Hiseq 4000. DireNTC: A. Cooper sampled the original specimen. H.H. and K.J.M. extracted DNA from the sample and created libraries for high-throughput DNA sequencing on an Illumina NextSeq. DireGB: K.J.M. sampled the original specimen. P.B. and K.J.M. extracted DNA from the sample and created libraries for high-throughput DNA sequencing on an Illumina HiSeq X Ten. DireSP: A. Cooper sampled the original specimen. A.T.S. and H.H. extracted DNA from the sample and created libraries for high-throughput DNA sequencing on an Illumina HiSeq X Ten. DireGWC: B.W.S. sampled the original specimen. C.S. extracted DNA from the sample. ArborScience created the libraries for high-throughput DNA sequencing on an Illumina Hiseq 4000.
Timing and spatial scale	Sample age range from >50,000 years to ~11,000 years
Data exclusions	No data was excluded from the analysis
Reproducibility	Detailed description of all experimental work is available in the supplementary information. All museum codes from which samples were taken are also detailed. No-template ("blank") controls were processed alongside all samples. These samples failed to amplify efficiently and no reads sequenced from no-template libraries could be unambiguously mapped, indicating that background and cross-contamination were minimal.
Randomization	No experiment requiring randomization was conducted in this study
Blinding	No experiment requiring randomization was conducted in this study

Did the study involve field work? ☐ Yes ☒ No

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Materials & experimental systems

n/a	Involved in the study	
☒	☐	Antibodies
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☒	☐	Animals and other organisms
☒	☐	Human research participants
☒	☐	Clinical data

Methods

n/a	Involved in the study	
☒	☐	ChIP-seq
☒	☐	Flow cytometry
☒	☐	MRI-based neuroimaging

Palaeontology

Specimen provenance	DireAFR: American Falls Reservoir, Idaho, USA. DireNTC: Natural Trap Cave, Wyoming, USA. DireGB: Gigantobison Bay (48001), American Falls Reservoir, Idaho, USA. DireSP: Sheriden Pit, Ohio, USA. DireGWC: Guy Wilson Cave, Tennessee, USA. No permits were necessary as all specimens were already collected and accessioned in museum collections. Samples were taken with permission from the organisations holding the collections.
Specimen deposition	DireAFR: Specimen accessioned as IMNH 255/8007 at the Idaho Museum of Natural History. DNA accessioned as AJ66 at the PalaeoBarn (University of Oxford) DireNTC: Specimen accessioned as KU48130 at the University of Kansas Museum. DNA accessioned as ACAD5529 at Australian Centre for Ancient DNA (University of Adelaide) DireGB: Specimen accessioned as IMNH 48001/52 at the Idaho Museum of Natural History. DNA accessioned as ACAD18742 at Australian Centre for Ancient DNA (University of Adelaide) DireSP: Specimen accessioned as VP1737 at the Cincinnati Museum Centre. DNA accessioned as ACAD1735 at Australian Centre for Ancient DNA (University of Adelaide) DireGWC: Specimen accessioned as MMNHC 0013 at the McClung Museum, University of Tennessee. DNA accessioned as RW001 at UCLA.
Dating methods	DireAFR: N/A DireNTC: Pretreated with HCl-NaOH-HCl rinses and ultrafiltration (>30kDa) then radiocarbon dated at the Oxford Radiocarbon Accelerator Unit (OxA-37752; 19970 ± 110 BP) DireGB: N/A DireSP: N/A DireGWC: Submitted to DirectAMS for radiocarbon dating and underwent standard gelatin extraction procedures (D-AMS 26659; 10944 ± 22 years BP)

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