

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

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75

76 **Abstract**

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

88

89

90 **Main Text**

91 Dire wolves (*Canis dirus*) were large (~68 kg) wolf-like canids and among the most common
92 extinct large carnivores of the American Late Pleistocene megafauna¹. Dire wolf remains are
93 present in the North American paleontological record from at least ~250,000 to ~13,000 years
94 ago, at the end of the Pleistocene, particularly in the lower latitudes² (Fig. 1A). Other canid
95 species present in Late Pleistocene North America include the slightly smaller gray wolf (*C.*
96 *lupus*), the much smaller coyote (*C. latrans*), and the dhole (or Asiatic wild dog; *Cuon alpinus*),
97 though dire wolves appear to be more common overall¹. For example, >4,000 individuals have
98 been excavated in California's fossil-rich Rancho La Brea tar seeps alone, where they
99 outnumber gray wolves more than 100-fold^{3,4}.

100

101 Despite the abundance of dire wolf fossils, the origin, taxonomic relationships, and ultimate
102 driver of their extinction remain unclear. Dire wolves are generally described as a sister species
103 to⁵⁻⁸, or even conspecific with the gray wolf⁹. The leading hypothesis to explain their extinction
104 is that, due to their larger body size than gray wolves and coyotes, dire wolves were more
105 specialized for hunting large prey, and were unable to survive the extinction of their megafaunal
106 prey (e.g.¹⁰⁻¹²). To test this hypothesis, we performed geometric morphometric analyses of
107 >700 specimens. Our results indicate that although dire wolves and gray wolves species can be
108 differentiated, their morphology is highly similar (Supplementary Information; Fig. 1B;
109 Supplementary Fig. 1-6). Although this morphometric similarity may partly be driven by
110 allometry (Supplementary Information; Fig. 1B), the lack of distinctiveness between gray wolves
111 and dire wolves has been interpreted as a result of a close evolutionary relationship^{7,9}.
112 Alternatively, a competing hypothesis maintains that these morphological similarities are the
113 result of convergence, and that dire wolves instead are a species belonging to a separate
114 taxonomic lineage (classified in the monotypic genus *Aenocyon*; "terrible or dreadful wolf"¹³).

115

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

126

127 Analyses of the dire wolf *COL1* sequence suggested that they were not closely related to gray
128 wolves, coyotes, African wolves (*C. anthus*), and dogs (*C. familiaris*) (Supplementary Fig. 7).
129 These data, however, could not confidently resolve the relationships between more distantly
130 related canids due to a lack of lineage-specific amino acid changes among these species¹⁴.
131 Phylogenetic analyses of the mitochondrial genomes indicated that dire wolves form a well-

132 supported monophyletic group that is highly divergent from gray wolves and coyotes
133 (Supplementary Fig. 10; see Supplementary Data 13 and Supplementary Table 2 for a list of
134 the 13 species used in this analysis), contradicting recent paleontological analyses⁵⁻⁷ (Figure
135 1B). Canid mitochondrial phylogenies, however, may not represent the true species evolutionary
136 relationships since both admixture and incomplete lineage sorting have been shown to affect
137 canid phylogenetic topologies^{15,16}.

138

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

155

156 We investigated canid phylogenetic relationships in greater detail using a range of species tree
157 analyses^{17,18} and D-statistics (Supplementary Information). These approaches produced
158 concordant trees that support the monophyly of three primary lineages: dire wolves, African
159 jackals, and a clade comprising all other extant wolf-like canids (Fig. 2A; Supplementary Fig. 11-
160 15). Although our species tree analyses provided equivocal results regarding the relationships
161 among these lineages, gray wolves (genus *Canis*) are more closely related to dholes
162 (Supplementary Fig. 21) (genus *Cuon*), African wild dogs (genus *Lycaon*) (Supplementary Fig.
163 19) and Ethiopian wolves (Supplementary Fig. 22) than to either dire wolves or African jackals
164 (both genus *Canis*). This finding is consistent with previously proposed designations of genus
165 *Lupulella*¹⁹ for the African jackals and *Aenocyon*¹³ for dire wolves.

166

167 To assess the timing of divergence among the major wolf-like canid lineages we performed a
168 molecular clock analysis based on two fossil calibrations using MCMCtree²⁰. Although the dire
169 wolf sequences are low coverage and include post-mortem damage, extensive simulations
170 indicated this is unlikely to affect the time of divergence estimates inferred by MCMCtree
171 (Supplementary Information; Supplementary Fig. 17). This analysis confirmed that the initial
172 divergences of the three primary wolf-like canid lineages occurred rapidly, contributing to the
173 poor resolution of the tree as a result of incomplete lineage sorting (Fig. 2A). The dire wolf
174 lineage last shared a common ancestor with extant wolf-like canids ~5.7 million years ago (95%

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

177
178 Given the propensity for sympatric canid species to interbreed^{15,21,22}, we tested for genomic
179 signals of admixture between extant North American canids and dire wolves using D statistics²³
180 (Supplementary Information) in a data set that included 22 modern North American gray wolves
181 and coyotes, three ancient dogs²⁴⁻²⁶, and a Pleistocene wolf²⁷ (Supplementary Data 13).
182 Specifically, we computed statistics of the form D (outgroup [gray fox]; dire wolf; North American
183 canid [gray wolf or coyote], African wolf/Eurasian wolf) and found no significant excess of
184 shared derived alleles between dire wolves and any extant North American canid (Fig. 2B;
185 Supplementary Fig. 18; Supplementary Data 14). This result indicates that the dire wolves
186 sequenced in this study did not possess ancestry from gray wolves, coyotes, or their recent
187 North American ancestors. Although we cannot exclude the possibility that some unsampled
188 canid population has some dire wolf hybrid ancestry, the lack of signal of hybridization in our
189 broad set of genomes suggests that admixture is unlikely to have occurred. While we did not
190 find evidence of recent admixture, we did find that African wild dogs share fewer derived alleles
191 with dire wolves than with gray wolves, coyote, African wolves, dhole, or Ethiopian wolves (Fig.
192 2C; Supplementary Fig. 20; Supplementary data 15). This indicates that an episode of ancient
193 admixture between the ancestor of dire wolves and the ancestor of wolves, coyotes, and dhole
194 occurred at least ~3 million years ago (based on the lower bound of the 95% HPD on the age of
195 their common ancestor; Fig. 2A), which may have contributed challenges resolving the
196 branching order of the basal wolf-like canid lineages (Fig. 2A).

197
198 Hybridization is common among wolf-like canid lineages when their ranges overlap. For
199 example, modern gray wolves and coyotes hybridize readily in North America (e.g.,²¹).
200 Genomic data also suggest gene flow occurred between dholes and African wild dogs during
201 the Pleistocene¹⁵, millions of years after their divergence. Consequently, our finding of no
202 evidence for gene flow between dire wolves and gray wolves, coyotes, or their common
203 ancestor, despite substantial range overlap with dire wolves during the Late Pleistocene
204 suggests that the common ancestor of gray wolves and coyotes probably evolved in
205 geographical isolation from members of the dire wolf lineage. This result is consistent with the
206 hypothesis that dire wolves originated in the Americas^{1,4,28,29}, likely from the extinct
207 Armbuster's wolf (*C. armbusteri*⁵).

208
209 Long term isolation of the dire wolf lineage in the Americas implies that other American fossil
210 taxa, such as the Pliocene *C. edwardii*, a proposed relative of the coyote⁵, may instead belong
211 to the dire wolf lineage. Thus, the diversification of the extant wolf-like canids likely occurred in
212 parallel outside of the Americas, and perhaps began earlier than hypothesized. The living *Canis*
213 species may have descended from Old World members of the extinct genus *Eucyon*, which first
214 appeared in the fossil record of Africa and Eurasia at the end of the Miocene (see³⁰).
215 Geographic isolation since the late Miocene is consistent with our molecular estimates for the
216 age of the dire wolf lineage, and may have allowed dire wolves to evolve some degree of
217 reproductive isolation prior to the Late Pleistocene North American arrival of gray wolves,
218 coyotes, dholes, and *Xenocyon* (another extinct wolf-like canid).

219

220 Despite their overall phenotypic similarities, gray wolves and coyotes survived the Late
221 Pleistocene megafaunal extinctions while dire wolves did not. One possible reason may be that
222 both gray wolves and coyotes possessed greater morphological plasticity and dietary flexibility,
223 thus allowing them to avoid extinction and become the dominant terrestrial predators in North
224 America^{12,31}. This scenario is supported by the date we obtained from the DireGWC specimen
225 (12,820-12,720 calBP), which suggests that dire wolves survived until at least the Younger
226 Dryas cold reversal, a period that also witnessed the latest known dates for other specialized
227 North American mega-carnivores such as the American lion (*Panthera atrox*) and giant short-
228 faced bear (*Arctodus simus*)^{32,33}. Alternatively, gray wolves and coyotes may have survived as a
229 result of their ability to hybridize with other canids. Through adaptive introgression with dogs,
230 North American gray wolves are known to have acquired traits related to coat color, hypoxia,
231 and immune response^{34,35}. Specifically, enhanced immunity may have allowed gray wolves to
232 resist novel diseases carried by newly arriving Old World taxa. Since our results demonstrate
233 that dire wolves did not derive any ancestry from other wolf-like canid species, it is plausible that
234 reproductive isolation prevented dire wolves from acquiring traits that may have allowed them to
235 survive into the Holocene.

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238 **References**

- 239 1. Dundas, R. G. Quaternary records of the dire wolf, *Canis dirus*, in North and South
240 America. *Boreas* **28**, 375–385 (1999).
- 241 2. Meachen, J. A., Brannick, A. L. & Fry, T. J. Extinct Beringian wolf morphotype found in the
242 continental U.S. has implications for wolf migration and evolution. *Ecol. Evol.* **6**, 3430–3438
243 (2016).
- 244 3. Leonard, J. A. *et al.* Megafaunal extinctions and the disappearance of a specialized wolf
245 ecomorph. *Curr. Biol.* **17**, 1146–1150 (2007).
- 246 4. Kurtén, B. & Anderson, E. *Pleistocene mammals of North America*. (Columbia University
247 Press, 1980).
- 248 5. Tedford, R. H., Wang, X. & Taylor, B. E. Phylogenetic Systematics of the North American
249 Fossil Caninae (Carnivora: Canidae). *Bull. Am. Mus. Nat. Hist.* **325**, 1–218 (2009).
- 250 6. Prevosti, F. J. Phylogeny of the large extinct South American Canids (Mammalia,
251 Carnivora, Canidae) using a ‘total evidence’ approach. *Cladistics* **26**, 456–481 (2010).
- 252 7. Zrzavý, J., Duda, P., Robovský, J., Okřinová, I. & Pavelková Řičánková, V. Phylogeny of

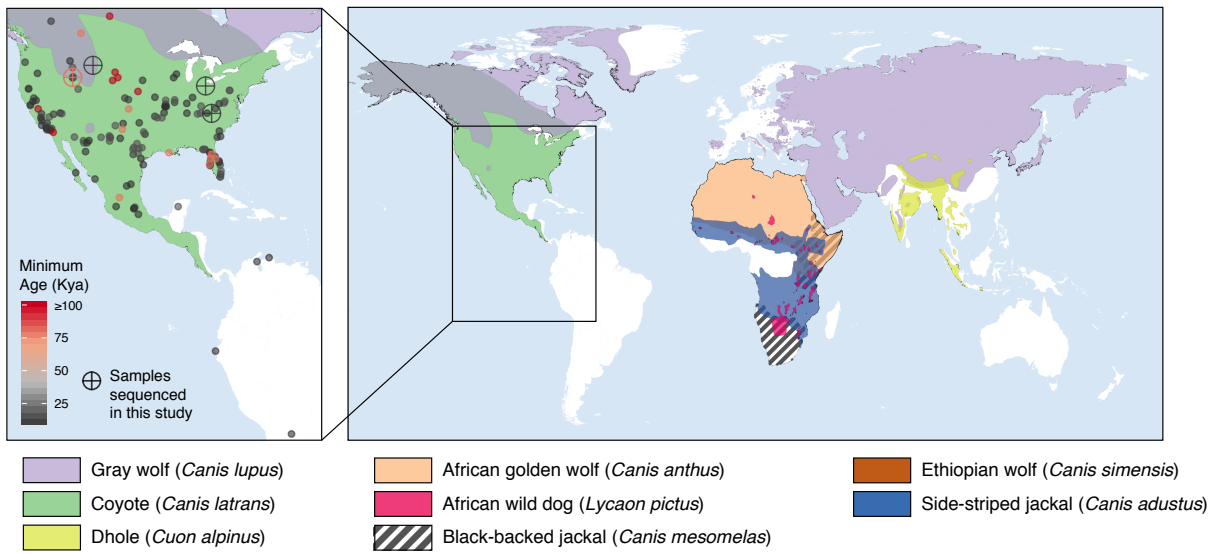
- 253 the Caninae (Carnivora): Combining morphology, behaviour, genes and fossils. *Zool. Scr.*
254 **47**, 373–389 (2018).
- 255 8. Álvarez-Carretero, S., Goswami, A., Yang, Z. & Dos Reis, M. Bayesian Estimation of
256 Species Divergence Times Using Correlated Quantitative Characters. *Systematic Biology*
257 vol. 68 967–986 (2019).
- 258 9. Goulet, G. D. Comparison of temporal and geographical skull variation among Nearctic
259 modern, Holocene and Late Pleistocene gray wolves (*Canis lupus*)(and selected *Canis*).
260 (1993).
- 261 10. Graham, R. W. & Mead, J. I. Environmental fluctuations and evolution of mammalian
262 faunas during the last deglaciation in North America. *North America and adjacent oceans*
263 *during the last deglaciation* 371–402 (1987).
- 264 11. Barnosky, A. D. The late Pleistocene event as a paradigm for widespread mammal
265 extinction. *Mass extinctions: processes and evidence* 235–254 (1989).
- 266 12. DeSantis, L. R. G. *et al.* Causes and Consequences of Pleistocene Megafaunal Extinctions
267 as Revealed from Rancho La Brea Mammals. *Curr. Biol.* **29**, 2488–2495.e2 (2019).
- 268 13. Merriam, J. C. Note on the systematic position of the wolves of the *Canis dirus* group.
269 *Bulletin of the Department of Geology of the University of California* **10**, 531–533 (1918).
- 270 14. Buckley, M., Harvey, V. L. & Chamberlain, A. T. Species identification and decay
271 assessment of Late Pleistocene fragmentary vertebrate remains from Pin Hole Cave
272 (Creswell Crags, UK) using collagen fingerprinting. *Boreas* **46**, 402–411 (2017).
- 273 15. Gopalakrishnan, S. *et al.* Interspecific Gene Flow Shaped the Evolution of the Genus
274 *Canis*. *Curr. Biol.* **28**, 3441–3449.e5 (2018).
- 275 16. Koepfli, K.-P. *et al.* Genome-wide Evidence Reveals that African and Eurasian Golden
276 Jackals Are Distinct Species. *Curr. Biol.* **25**, 2158–2165 (2015).
- 277 17. Bryant, D., Bouckaert, R., Felsenstein, J., Rosenberg, N. A. & RoyChoudhury, A. Inferring
278 species trees directly from biallelic genetic markers: bypassing gene trees in a full

- 279 coalescent analysis. *Mol. Biol. Evol.* **29**, 1917–1932 (2012).
- 280 18. Yang, Z. The BPP program for species tree estimation and species delimitation. *Current*
281 *Zoology* vol. 61 854–865 (2015).
- 282 19. Geraads, D. A revision of the fossil Canidae (Mammalia) of north-western Africa.
283 *Palaeontology* **54**, 429–446 (2011).
- 284 20. Yang, Z. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**, 1586–
285 1591 (2007).
- 286 21. vonHoldt, B. M. *et al.* Whole-genome sequence analysis shows that two endemic species
287 of North American wolf are admixtures of the coyote and gray wolf. *Sci Adv* **2**, e1501714
288 (2016).
- 289 22. vonHoldt, B. M. *et al.* A genome-wide perspective on the evolutionary history of enigmatic
290 wolf-like canids. *Genome Res.* **21**, 1294–1305 (2011).
- 291 23. Patterson, N. *et al.* Ancient admixture in human history. *Genetics* **192**, 1065–1093 (2012).
- 292 24. Sinding, M.-H. S. *et al.* Arctic-adapted dogs emerged at the Pleistocene-Holocene
293 transition. *Science* **368**, 1495–1499 (2020).
- 294 25. Ní Leathlobhair, M. *et al.* The evolutionary history of dogs in the Americas. *Science* **361**,
295 81–85 (2018).
- 296 26. Frantz, L. A. F. *et al.* Genomic and archaeological evidence suggest a dual origin of
297 domestic dogs. *Science* **352**, 1228–1231 (2016).
- 298 27. Skoglund, P., Ersmark, E., Palkopoulou, E. & Dalén, L. Ancient wolf genome reveals an
299 early divergence of domestic dog ancestors and admixture into high-latitude breeds. *Curr.*
300 *Biol.* **25**, 1515–1519 (2015).
- 301 28. Nowak, R. M. North American Quaternary Canis, Monograph of the Museum of Natural
302 History, The University of Kansas. (1979).
- 303 29. Nowak, R. M. Wolf evolution and taxonomy. *Wolves: Behavior, ecology, and conservation*
304 239–258 (2003).

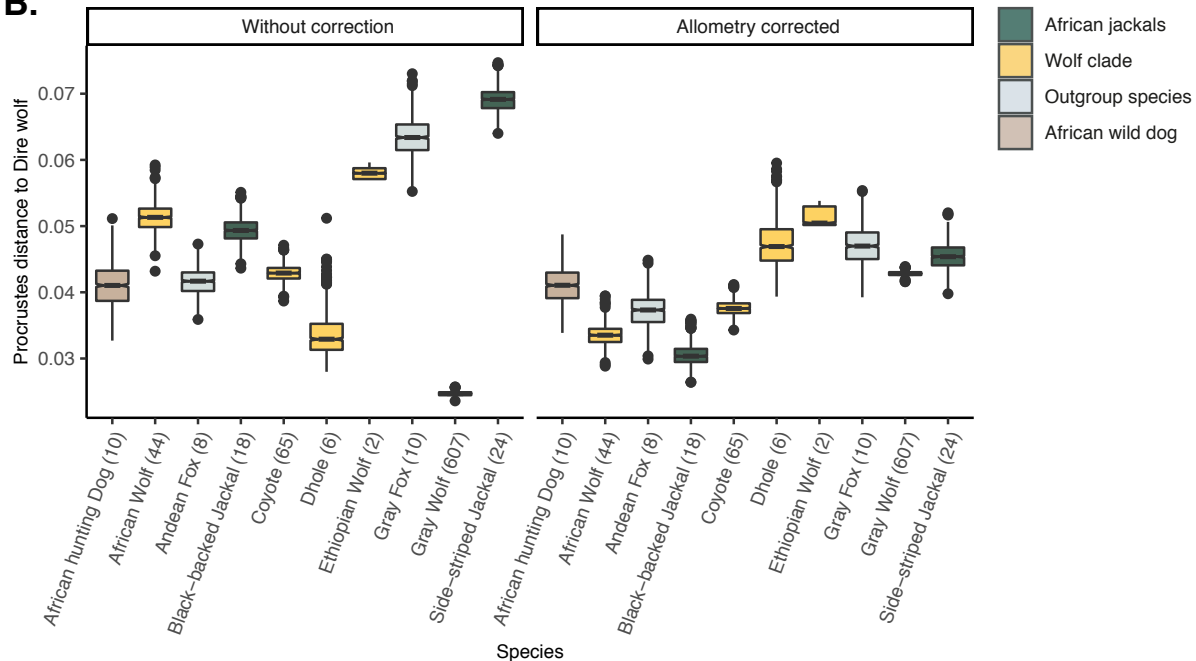
- 305 30. Sotnikova, M. & Rook, L. Dispersal of the Canini (Mammalia, Canidae: Caninae) across
306 Eurasia during the Late Miocene to Early Pleistocene. *Quat. Int.* **212**, 86–97 (2010).
- 307 31. Van Valkenburgh, B. & Hertel, F. *The decline of North American predators during the late*
308 *Pleistocene*. (4320).
- 309 32. Cooper, A. *et al.* PALEOECOLOGY. Abrupt warming events drove Late Pleistocene
310 Holarctic megafaunal turnover. *Science* **349**, 602–606 (2015).
- 311 33. Schubert, B. W. Late Quaternary chronology and extinction of North American giant short-
312 faced bears (*Arctodus simus*). *Quat. Int.* **217**, 188–194 (2010).
- 313 34. Schweizer, R. M. *et al.* Natural Selection and Origin of a Melanistic Allele in North American
314 Gray Wolves. *Mol. Biol. Evol.* **35**, 1190–1209 (2018).
- 315 35. Anderson, T. M. *et al.* Molecular and evolutionary history of melanism in North American
316 gray wolves. *Science* **323**, 1339–1343 (2009).

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B.



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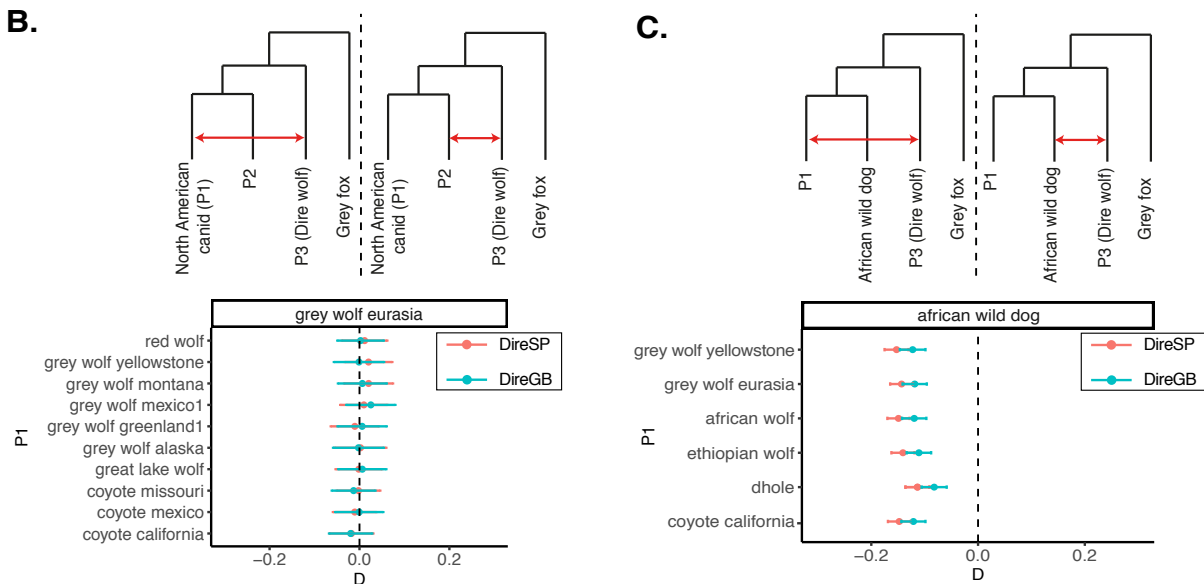
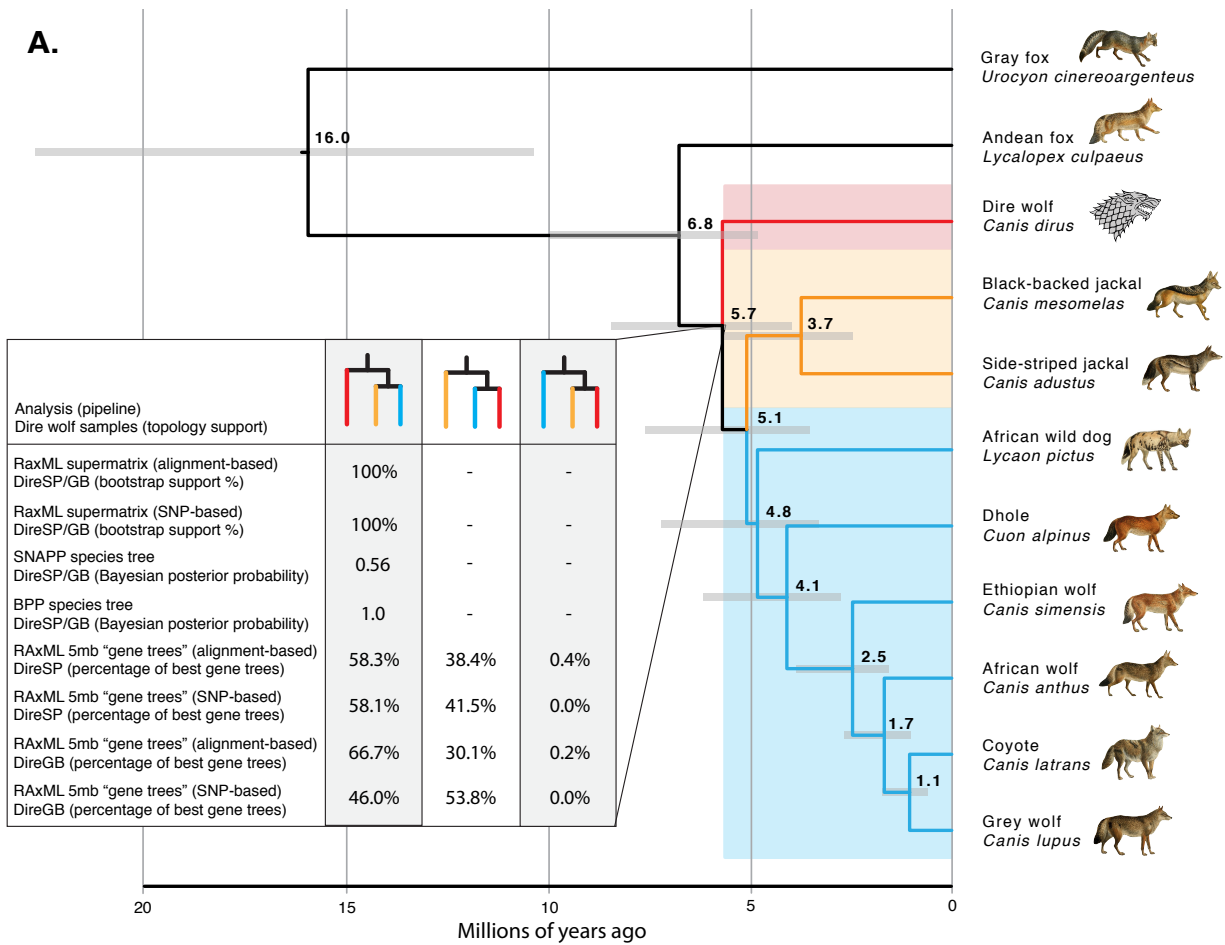
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Figure 1. Map of dire wolf remains and morphological differentiation with wolf-like

canids **A.** Right: map representing the geographic range (obtained from IUCN, <https://www.iucnredlist.org>) of the canid species investigated in this study. Left: map representing the distribution of sites in the Americas where dire wolf remains (*Canis dirus*) were identified (Supplementary Table 1). 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

330 squared differences between the coordinates of corresponding landmarks, with and without
331 correction for allometry (Supplementary Information).



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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

337 support for the three possible arrangements of the dire wolf (red), the African jackals (orange),
338 and the remaining wolf-like canids (blue) that we obtained under different analytical frameworks
339 (Supplementary Information) when including either one or both of our two highest coverage dire
340 wolf samples (DireSP and DireGB). Although only one dire wolf branch is depicted in the tree,
341 multiple dire wolf individuals were included they formed a monophyletic clade (Supplementary
342 Fig. 12-13, and 15). **B.** Results of D statistics used to assess the possibility of gene flow
343 between the dire wolf and extant North American canids. Each dot represents the mean D
344 calculated along the genome and the error bar represents 3 standard deviations. These plots
345 show that the dire wolf genomes do not share significantly more derived alleles with extant
346 North American canids compared to Eurasian wolves (values of D not significantly different to
347 zero), suggesting that no hybridization occurred between the dire wolf and the ancestor of
348 extant North American canids. Non significant D-statistics were also obtained using an
349 alternative reference genome and using the African wolf as P2 (Supplementary Fig. 18 and
350 Supplementary Data 14). **C.** Results of D statistics showing the existence of an ancient gene
351 flow event between the ancestor of the dhole, Ethiopian wolf, African wolf, gray wolf and
352 coyotes and the lineage of the dire wolf (consistently non-zero values of D regardless of P1).

353 354 **Acknowledgement**

355 We thank the staff at the Carnegie Museum of Natural History, Cincinnati Museum Center,
356 Danish Zoological Museum, Harrison Zoological Museum, Harvard Museum of Comparative
357 Zoology, Idaho Museum of Natural History, Institute of Archaeology (Russian Academy of
358 Sciences), Institute of Systematics and Animal Ecology (Russian Academy of Sciences),
359 Institute of Zoology (Chinese Academy of Sciences), Instituto de Conservação da Natureza e
360 das Florestas, Kansas Museum of Natural History, La Brea Tar Pits and Museum, Ludwig
361 Maximilian University, McClung Museum, Museum of the Institute of Plant and Animal Ecology
362 (Russian Academy of Sciences), Museum National d'Histoire Naturelle, National Museums
363 Scotland, Natural History Museum London, Naturalis Biodiversity Center, Naturhistorisches
364 Museum Bern, Smithsonian National Museum of Natural History, Swedish Naturhistoriska
365 Riksmuseet, United States Bureau of Reclamation, University of California Museum of
366 Paleontology, University of Texas at El Paso, University of Washington Burke Museum, and the
367 Zoological Institute (Russian Academy of Sciences; state assignment № state assignment №
368 AAAA-A19-119032590102-7) for access to specimens in their care. We also thank T. Barnosky,
369 S. Bray, A. Farrell, R. Fischer, A. Harris, J. Harris, A. Henrici, P. Holroyd, R. MacPhee, T.
370 Martin, A. Philpot, J. Saunders, J. Southon, G. Storrs, G. Takeuchi, X. Wang and C. Widga for
371 their assistance. A.M. used computational and storage services associated with the Hoffman2
372 Shared Cluster provided by UCLA Institute for Digital Research and Education's Research
373 Technology Group. DireGWC was sequenced using the Vincent J. Coates Genomics
374 Sequencing Laboratory at UC Berkeley, supported by NIH S10 OD018174 Instrumentation
375 Grant. Further, the authors would like to acknowledge the assistance of the Danish National
376 High-Throughput Sequencing Centre, BGI-Europe, the Garvan Institute of Medical Research
377 and the Australian Cancer Research Foundation (ACRF) Cancer Genomics Facility for
378 assistance in Illumina and BGISEQ500 data generation. A.R.P. was supported by a Marie Curie
379 COFUND Junior Research Fellowship (Durham University). A.M. was supported by an NSF
380 grant (award number: 1457106) and the QCB Collaboratory Postdoctoral Fellowship (UCLA).

381 L.A.F.F., J.H., A.H-B. and G.L. were supported either by a European Research Council grant
382 (ERC-2013-StG-337574-UNDEAD) or Natural Environmental Research Council grants
383 (NE/K005243/1 and NE/K003259/1), or both. K.S. was supported by a grant from Barrett, the
384 Honors College at Arizona State University. A.T.O. was supported by the Strategic Initiative
385 Funds, Office of the President, Arizona State University to the Institute of Human Origins' DNA
386 and Human Origins at Arizona State University project. L.A.F.F. was supported by a Junior
387 Research Fellowship (Wolfson College, University of Oxford) and L.A.F.F. and A.Carmagnini
388 were supported by the Wellcome Trust (210119/Z/18/Z). S.G. was supported by
389 Carlsbergfondet grant CF14–0995 and Marie Skłodowska-Curie Actions grant 655732-
390 WhereWolf. M.T.P.G was supported by ERC Consolidator grant 681396-Extinction Genomics.
391 B.S. and J.K. were supported by IMLS MG-30-17-0045-17 and NSF DEB-1754451. A.H-B. was
392 supported by the Leverhulme Trust. A.Cooper, K.J.M., and H.H. were supported by the
393 Australian Research Council. A.S. and G.G. were supported by Australian Government
394 Research Training Program Scholarships. A.T.L. is supported by the Peter Buck Postdoctoral
395 Fellowship from the Smithsonian Institution's National Museum of Natural History. Finally, we
396 thank the editors, Larisa DeSantis and the other reviewers for their helpful comments.
397

398 **Author contributions**

399 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
400 designed the research; A.R.P. and K.J.M. coordinated the sample collection efforts with input
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402 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.,
403 R.W.K., and A. Cooper provided and/or collected samples; A.R.P., K.J.M., K.W., A.M., C.S.,
404 J.H., A.J., A.T.S., P.B., and H.H. conducted the genomic laboratory work; K.J.M., A.M., G.G.,
405 G.L., L.A.F.F. and A. Cooper conducted the analyses of the genomic data; S. A.-C., A.H.-B.,
406 J.M., C.A., and A.E. conducted the morphological analyses; A.R.P., K.J.M., A.M., S.A.-C.,
407 B.V.V., R.K.W., G.L., L.A.F.F. and A. Cooper wrote the paper with input from all other authors.
408

409 **Data availability**

410 The reads generated for this study have been deposited at the European Nucleotide Archive
411 (ENA) with project number PRJEB31639. Geomorphometric data and collagen sequence were
412 deposited onto a Dryad archive (TBD).