1 **Ancient DNA reveals differences in behaviour and sociality between brown**

2 **bears and extinct cave bears.**

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34 Running title: ***Investigating bear behaviour using ancient DNA***

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44 **ABSTRACT**

45 Ancient DNA studies have revolutionised the study of extinct species and populations, providing

46 insights on phylogeny, phylogeography, admixture and demographic history. However, inferences

47 on behaviour and sociality have been far less frequent. Here, we investigate the complete

48 mitochondrial genomes of extinct Late Pleistocene cave bears and middle Holocene brown bears

49 that each inhabited multiple geographically proximate caves in northern Spain. In cave bears, we

50 find that, although most caves were occupied simultaneously, each cave almost exclusively

51 contains a unique lineage of closely related haplotypes. This remarkable pattern suggests extreme

52 fidelity to their birth site in cave bears, best described as homing behaviour, and that cave bears

53 formed stable maternal social groups at least for hibernation. In contrast, brown bears do not

54 show any strong association of mitochondrial lineage and cave, suggesting that these two closely

55 related species differed in aspects of their behaviour and sociality. This difference is likely to

56 have contributed to cave bear extinction, which occurred at a time in which competition for caves

57 between bears and humans was likely intense and the ability to rapidly colonise new hibernation

58 sites would have been crucial for the survival of a species so dependent on caves for hibernation

59 as cave bears. Our study demonstrates the potential of ancient DNA to uncover patterns of

60 behaviour and sociality in ancient species and populations, even those that went extinct many tens

61 of thousands of years ago.

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66   **INTRODUCTION**

67   Behaviour and sociality represent key mechanisms allowing populations to rapidly adapt to

68   changing environments, to better exploit available resources, and also to resist pressures such as

69   predation or climatic extremes that may negatively affect survival probability. Conversely, some

70   behaviours could be maladaptive in certain contexts, particularly when populations are exposed to

71   new and/or rapidly changing selective pressures, and may ultimately lead to population or even

72   species extinction. Ancient animal remains can hold information on their behaviour and sociality.

73   Spatial and temporal patterns of association among individuals can be investigated using standard

74   paleontological and isotopic methods, and their relatedness can – at least in principle – be

75   determined using ancient DNA approaches. The later, however, may represent a considerable

76   technical challenge, as advanced DNA degradation will complicate recovery of suitable data that

77   allows fine-scale resolution of genetic relationships among sufficient numbers of individuals to

78   achieve statistical power.

79

80   Bears that lived in Eurasia during the Pleistocene represent a group that may be amenable to

81   behavioural investigations using ancient DNA. Two major species (or species complexes) were

82   widespread and sympatric in Pleistocene Eurasia: brown bears (*Ursus arctos*), that survived

83   through the last glacial maximum (LGM) and are currently widespread across the entire Holarctic

84   region; and the cave bear (*Ursus spelaeus* complex), an iconic representative of the Pleistocene

85   megafauna, that went extinct prior to the LGM (Pacher & Stuart 2009; Stiller *et al*. 2010; 2014).

86   For cave bears in particular, their habit to hibernate in caves has resulted in assemblages

87   consisting of the bones of thousands of individuals at some sites, providing the opportunity to

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88   investigate uniquely well-defined fossil populations, deposited within an environment that

89   enhances DNA preservation (Hofreiter *et al*. 2015). Although ancient brown bear remains

90   typically occur at a much lower frequency in caves in comparison to cave bears, comprehensive

91   palaeontological surveys of some caves have produced sufficient samples for population-level

92   analysis (e.g. in Kurten 1968).

93

94   The factors that drove the cave bear to extinction have been subject to considerable study and

95   discussion (Kurten 1968, Grayson & Delpech 2003; Pacher & Stuart 2009; Stiller *et al*. 2010). In

96   agreement with palaeontological data, genetic studies of cave bears have found high genetic

97   diversity and a large and constant population size until 50,000 yBP, followed by a decrease until

98   its ultimate extinction around 24,000 yBP (Pacher & Stuart 2009; Stiller *et al*. 2010; 2014). Thus,

99   the onset of decline of cave bear populations would have started around 25,000 years before the

100   LGM, and is therefore not associated with any periods of substantial climatic change in Europe

101   (Stiller *et al*. 2010; 2014). Brown bears, in contrast, show no evidence of population size changes

102   coinciding with the cave bear population decline (Stiller *et al*. 2010). It has been argued that

103   human activities played a major role in cave bear extinction (Grayson & Delpech 2003; Knapp *et*

104   *al*. 2009; Münzel & Conard 2004; Bon *et al*. 2011; Stiller *et al*. 2014). However, explanations of

105   why human activities could have so profoundly affected cave bear populations and not brown

106   bear populations remain elusive. Differences in behaviour between the two species may have

107   played a role, but identifying such differences is challenging because many aspects of cave bear

108   behaviour remain uncertain. For example, paleontological studies of some cave bear caves have

109   identified multiple depressions (hibernation beds or *bauges*, as described by Koby in 1953) in the

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110   cave floor that are thought to have been formed by hibernating bears. While this suggests

111   communal hibernation, it is uncertain whether these were social or even family groups, or rather

112   random assemblages of individuals forced together through competition for hibernation sites.

113   Although genetic data could allow testing of such hypotheses, only a few studies have examined

114   the population structure of cave bears at a local – i.e. individual cave – scale (Orlando *et al.* 2002;

115   Richards *et al.* 2008; Hofreiter *et al.* 2004; Bon *et al.* 2011). Moreover, these studies were all

116   based on short mtDNA fragments, which does not allow fine scale resolution of the genetic

117   relationship between individuals.

118

119   In this study, we investigate complete mitochondrial genome sequences generated from the

120   subfossil remains of multiple cave bears and brown bears from several caves in the North of

121   Spain (Fig. 1). Four of the cave bear caves are located in close proximity (within a radius of

122   10km) within the Serra do Courel mountains (NW Spain), while the fifth one is located 450 km

123   away in Navarra (NE Spain). The brown bear caves are also in close proximity (within a radius of

124   50km). In all cases, there are no apparent topographic barriers separating caves from one another.

125   Thus, for such large bodied and presumably highly mobile mammals as cave bears and brown

126   bears, movement between these caves would, in general, not have represented any significant

127   challenge. In cave bears, we find that, even though caves were occupied simultaneously, each

128   cave almost exclusively contained a unique clade of closely related haplotypes. This remarkable

129   pattern suggests that cave bears returned to the cave where they were born and formed stable

130   maternal social groups for hibernation. In brown bears, however, no such pattern is found

131   suggesting greater flexibility with regard to hibernation site in this closely related species. We

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132   discuss the implications of these behavioural differences for the extinction of the cave bear, in

133   addition to the wider potential of ancient DNA for the study of behavioural ecology, sociality, and

134   extinction.

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137   **MATERIALS AND METHODS**

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139   *Methods overview*

140   We generated mitogenome sequences of cave bears and brown bears from their skeletal remains

141   found in the caves shown in Figure 1. These sequences were used alongside published sequences

142   obtained from GenBank to compare the maternal relatedness of individuals occurring within

143   caves with that occurring among caves using haplotype network analysis, phylogenetic analysis

144   and trait-phylogeny association tests. Finally, the ages of individuals were estimated using a

145   combination of ^{14}C and molecular dating. In particular, we investigated whether the occupation of

146   caves was likely simultaneous, or instead temporally separated.

147

148   All but one of the novel Spanish bear mitogenome sequences reported here were obtained in a

149   single experiment (we refer to as Experiment 1) that used hybridisation capture to enrich

150   sequencing libraries for mtDNA prior to high-throughput sequencing. The details of Experiment 1

151   are reported below. A single Spanish cave bear sequence (sample E-VD-1838), in addition to

152   sequences from seven bears from elsewhere in Europe, were obtained in separate experiments

153   that are described in Section 1 of the Supporting Information.

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Sampling locations

156 The focal specimens used in this study were excavated in caves within karstic systems in the

157 north-west of Spain, and were identified morphologically as either *U. spelaeus* or *U. arctos.* All

158 of these sites represent natural accumulations and none of the remains are in archaeological

159 context. Individual samples originated from different individual animals, identified based on age,

160 sex or spatial distribution of the remains. Initially, specimens from 19 cave sites were

161 investigated. These comprised 85 individuals from nine caves containing cave bear remains, and

162 24 individuals from ten caves containing brown bear remains. Many of these failed initial

163 screening to identify samples that were likely permit recovery of the complete mitogenome

164 sequence (see below), which limited sampling to five brown bear caves and five cave bear caves

165 (shown in Fig. 1). Full details of the caves and samples investigated are provided in Section 2,

166 Tables S1 & S2, and Fig. S1 of the Supporting Information.

167

DNA extraction and sample screening

169 All pre-amplification aDNA analyses were performed in dedicated aDNA laboratories at the

170 University of York (UK) or at the University of Potsdam (Germany). The compact part of bones,

171 either femur, tibia, ribs, skull fragments or teeth, were utilised for DNA extraction. Prior to

172 extraction, samples were UV irradiated for 10 minutes on each side and disposable cutting disks

173 attached to a rotating electric drill were used to remove the outermost bone surface. For each

174 sample, around 250 mg of cleaned bone was ground to powder using ceramic mortar and pestles.

175 DNA extraction followed the protocol of Rohland *et al.* (2010).

8

176

177   DNA extracts were screened for likely presence and quality of endogenous DNA by attempting to

178   PCR amplify 104bp and 126bp fragments of the mitochondrial control regions of cave bears and

179   brown bears, respectively, using the primers described in Hofreiter *et al*. (2004) and a novel

180   brown bear primer, UaF7 (5'-TCGTGCATTAATGGCGTG-3'). Amplification was assessed using

181   agarose gel eletrophoresis and the authenticity of amplification products verified by Sanger

182   sequencing, carried out in both directions using an ABI 3130XL at the Sequencing Service SAI

183   (Servicios Centrais de Investigacion, University of A Coruña, Spain), followed by BLAST

184   alignment of the consensus sequences.

185

186   ***Sequencing library generation and hybridisation capture***

187   We generated individually barcoded Illumina sequencing libraries using 20µl of those extracts for

188   which short-amplicon PCR had previously been successful, following the protocol described in

189   Meyer & Kircher (2010) with the following modifications. First, the filtration step between the

190   blunt end repair and the adapter ligation was substituted by heat inactivation of the enzymes

191   (Bollongino *et al.* 2013; Fortes and Paijmans 2015), in order to reduce the loss of short DNA

192   fragments. Second, we used a double index barcoding system in which both the P5 and P7

193   adapters include a molecular barcode specific for each sample (Kircher *et al.* 2011; Fortes and

194   Paijmans 2015). This facilitates the identification of chimeric molecules that could be formed

195   during PCR amplification of the captured products. Library indexing and amplification involved

196   4 replicate parallel PCRs, each using 15 cycles, which were then pooled and purified using silica

197   columns (Qiagen, France). The resulting cave bear and brown bear libraries were quantified using

198 a Nanodrop Spectrophotometer (Thermo Scientific) and pooled, respectively, in equimolar

199 quantities at a final concentration of 2 ng in 520 µl for hybridisation capture.

200

201 Hybridisation capture was carried out using 244k DNA SureSelect™ microarrays (Agilent,

202 Boblingen, Germany) with 2-fold tiling and 60bp probes. Separate arrays were used for the cave

203 bear and brown bear library pools, with probes based on published mitogenome sequences of a

204 Western European cave bear (EU327344, Bon *et al.* 2008) and brown bear (EU497665, Bon *et al.*

205 2008), respectively. Hybridisation capture followed the protocol of Hodges *et al.* (2009) with one

206 modification. After the initial round of capture enrichment, library pools were amplified using

207 primers IS5 and IS6 (Meyer & Kircher 2010) in 12 parallel PCRs and the resulting products were

208 subjected to a second round of capture enrichment, as described in Fortes & Paijmans (2015).

209

210 ***DNA sequencing and data processing***

211 100bp single-end sequencing of mtDNA enriched library pools was carried out on a single lane of

212 an Illumina HiSeq2000 instrument at the Danish National Sequencing Centre in the University of

213 Copenhagen. The resulting BCL files were converted to fastq format using the Illumina base-

214 calling pipeline (Illumina Pipeline v1.4). The program Cutadapt v1.3 (Martin, 2011) was then

215 used to trim any P7 adapter sequences occurring at the 3' ends of reads, and a custom script used

216 to identify and discard any reads that did not contain the appropriate P5 index, and then trim the

217 index sequence from the remaining reads. Following this procedure, any reads < 25 bp were also

218 discarded. The resulting cave bear and brown bear reads were then mapped to their respective

219 reference mitogenome sequences used for capture probe design, using bwa-0.5.9 (Li & Durbin

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220 2009) with seeding disabled, as suggested by Schubert *et al.* (2012). The alignment was sorted,

221 filtered for minimum mapping quality (-q 30) and PCR duplicates removed using samtools (Li *et*

222 *al.* 2009). The Mpileup tool in samtools 0.1.19-44428 was used to generate consensus sequences

223 and to call polymorphic positions, using the -s option to specify a haploid genome. In order to

224 prevent miscalling of polymorphic sites resulting from the presence of postmortem molecular

225 damage to the ancient templates, the terminal five nucleotides at both 5' and 3' read ends were

226 excluded from SNP calling, and for sites covered by less than 3 reads the bases were only called

227 when all reads had the same nucleotide. All polymorphic sites identified in the vcf file were

228 further checked by eye on Tablet version 1.13.05.02 (Milne *et al.* 2013). Read depth and coverage

229 were determined using GATK (MacKenna *et al.* 2010). The presence of molecular damage

230 characteristic of aDNA was confirmed using the software MapDamage (Ginolhac *et al.* 2011).

231

232 ***Phylogenetic and network analysis***

233 Only those novel sequences that provided > 70% total coverage of the mitogenome were used in

234 subsequent analyses. Novel Spanish sequences were aligned along with seven novel sequences

235 from ancient bears found elsewhere in Europe and 174 published mitogenome sequences from

236 cave bears, brown bear and polar bears using the program MUSCLE (Edgar & Robert 2004) with

237 default settings. A repetitive section of the d-loop was removed from the alignment as this was

238 not recovered in many ancient samples and even when present could not be aligned

239 unambiguously. All subsequent analyses used this alignment or subsamples of it.

240

241 To investigate the phylogenetic relation of Spanish cave bear and brown bear haplotypes to those

242   occurring elsewhere in their respective distributions, we conducted phylogenetic analysis of the

243   complete alignment under maximum likelihood (ML) using RAxML-HPC2 8.2.3 (Stamatakis,

244   2014) on the CIPRES Portal (Miller *et al.* 2010) using the American black bear (*U. americanus*)

245   as outgroup. The ML tree was estimated under the GTR+G model and clade support assessed by

246   500 bootstrap replicates using the GTR+CAT model.

247

248   Networks of Spanish cave bear and brown bear haplotypes were then generated using the median-

249   joining algorithm implemented in the program NETWORK (fluxus-engineering.com, Bandelt *et*

250   *al.* 1999). To avoid any confounding effects of missing data on haplotype identification, all

251   alignment columns containing missing data and/or alignment gaps were removed for network

252   analysis.

253

254   We then investigated the strength of association of mitochondrial lineage and cave using trait-

255   phylogeny association tests that account for phylogenetic uncertainty in the software BaTS

256   (Parker *et al.* 2008). If mitochondrial phylogeny and cave are strongly associated, then the

257   inferred number of changes in cave occupation across the phylogeny should be fewer than for a

258   random prediction with no such association. We generated a Bayesian posterior sample of trees in

259   BEAST v. 1.8.2 (Drummond *et al.* 2012), and then randomised the assignment of individuals to

260   caves in order to generate a null distribution of the number of changes in cave occupancy when

261   phylogeny and cave show no association. This strength of association was then tested by

262   comparing this null distribution to the observed number of changes occurring across the posterior

263   sample of trees using the parsimony score (PS) statistic (Slatkin & Maddison 1989). PS is a

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264   discrete metric and therefore models changes in cave occupation occurring across the phylogeny

265   as discrete events.

266

267   To generate the posterior sample of trees used in trait-phylogeny association tests, the program

268   PartitionFinder (Lanfear *et al.* 2012) was first used to select appropriate partitions and

269   substitution models within each alignment (details in Section 2 of the Supporting Information,

270   results in Tables S5 & S6, Supporting Information). BEAST analyses involved a coalescent

271   Bayesian Skyline population model with unlinked substitution and strict clock models for each

272   partition. Non-zero variation in substitution rates was rejected by preliminary runs using relaxed

273   clock models. No clock calibrations were applied, and instead the substitution rate of the fastest-

274   evolving partition was fixed to 1 and substitution rates for the remaining partitions estimated

275   relative to the latter partition within open uniform priors between 0–2. MCMC chains ran for

276   sufficient length to achieve convergence and sufficient sampling of all parameters (ESS > 200)

277   after removal of burn-in, as verified in the program TRACER (Rambaut *et al.* 2014).

278   LOGCOMBINER was used to remove pre-burn-in trees prior to trait-phylogeny association tests.

279

280   ***Dating of cave lineages***

281   Thirty-nine samples were directly ^{14}C dated and 2-sigma calibrated using OxCal 4.2 online

282   (accession date: 07/07/2015), based on the IntCal-13 curve (Reimer *et al*. 2013). For samples that

283   lacked ^{14}C dates, or were beyond the range of ^{14}C dating, we estimated their ages using a

284   Bayesian phylogenetic approach in BEAST (Shapiro *et al.* 2011). Phylogenetic age estimation

285   was conducted individually for each undated cave bear and brown bear based on ^{14}C dated

13

286 representatives of their respective clades. We additionally tested the reliability of this procedure

287 using a crossvalidation method, in which the age of each ^{14}C dated sample was estimated and

288 compared to its original ^{14}C age. Due to the large number of individual analyses required, we a

289 custom Perl script was used to automate the generation of BEAST input files. In each analysis,

290 the posterior distribution of the tip date of the undated sample was sampled within an open

291 uniform prior between 0 (present day) and one million years, both of which represent implausible

292 extremes for the ages of these samples, while fixing the ages of ^{14}C dated samples to the mean

293 calibrated date. Substitution rates for all partitions were estimated within open uniform priors

294 between $0–5 \times 10^{-7}$ substitutions site^{-1} year^{-1}. Other details of the BEAST analyses were as

295 described above. Finally, we generated fully sampled calibrated phylogenies of the cave bear and

296 brown bear clades by fixing tip dates to either mean calibrated ^{14}C ages or median phylogenetic

297 age estimates.

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299

300 **RESULTS**

301

302 *DNA sequences*

303 PCR screening resulted in successful amplification of mitochondrial control region fragments in

304 57 out of 85 cave bear extracts and 23 out of 24 brown bear DNA extracts (details in Table S2,

305 Supporting Information), which were then subjected to hybridisation capture enrichment and

306 high-throughput sequencing. Mapping of sequence reads to their respective reference

307 mitogenome sequences resulted in consensus sequences of 26 cave bears and 15 brown bears that

14

308   were > 70% complete and used for further analysis (details in Table S4, Supporting Information).

309   All datasets showed molecular damage patterns characteristic of ancient DNA (Figs. S2 & S3,

310   Supporting Information). For cave bears, we added the sequence from an additional shotgun-

311   sequenced individual (Section 1, Supporting Information) and previously published sequences

312   from four other individuals from the focal caves, bringing the total number of Spanish cave bears

313   analysed to 31.

314

315   Phylogenetic analysis supported the inclusion of these Spanish cave bear and brown bear

316   sequences within the Western European *U. spelaeus* cave bear clade and the Western European

317   brown bear clade 1 (Fig. S4, Supporting Information), identified by previous phylogeographic

318   studies (Hirata *et al.* 2013; Stiller *et al.* 2014). Spanish cave bear and brown bear haplotypes were

319   unique compared to all previously published haplotypes of conspecific bears occurring elsewhere

320   in their respective distributions.

321

322   ***Association of mitochondrial DNA and cave***

323   Network analysis of Spanish cave bear haplotypes revealed close relationships between

324   haplotypes found within the same cave (Fig. 2a). Most caves contain multiple unique haplotypes

325   that are separated from each other by single nucleotide mutations. For example, Eirós and

326   Amutxate caves each contain two unique haplotypes differing from one another by a single

327   nucleotide mutation. Similarly, five unique and closely related haplotypes were found in A Ceza

328   cave, but with the addition of a more divergent haplotype found in a single A Ceza individual

329   (sample C7) that is shared with individuals from Arcoia and Liñares. An additional unique

15

330   haplotype was found in Liñares cave that differs from this shared haplotype by a single nucleotide

331   mutation. Even considering the occurrence of a single haplotype that is shared among three caves,

332   an overall pattern of separation of haplotype clusters into caves is clear and obvious. Trait-

333   phylogeny association tests further confirmed this pattern, showing fewer observed changes in

334   cave occupation than expected by random (observed mean 5.9, null mean 18.0, p < 0.001),

335   indicating a strong association of Spanish cave bear mitochondrial lineages with particular caves.

336

337   In contrast, an obvious segregation of mitochondrial haplotypes among different caves was not

338   observed in middle Holocene Spanish brown bears (Fig. 2b). Haplotypes are widely shared

339   among caves, with the exception of Pena Paleira, which contains three unique haplotypes, but

340   these are not closely related. Trait-phylogeny association tests found the observed number of

341   changes in cave occupation to not differ significantly from random (observed mean 6.5, null

342   mean 8.2, p = 0.08), indicating a lack of statistically significant association between

343   mitochondrial lineage and cave in these middle Holocene Spanish brown bears.

344

345   The association of mitochondrial haplotype lineage and cave revealed by network analysis for

346   Iberian cave bears, but not for Iberian Holocene brown bears, is also evident from the time-

347   calibrated phylogenies of their respective clades (Figs. 3 & 4). In addition, the broader geographic

348   sampling of cave bear haplotypes in this analysis reveals that Spanish haplotypes as a whole are

349   not monophyletic, with some cave linages sharing more recent common ancestry with haplotypes

350   found in France and/or Germany.

351

352 ***Dating***

353 ^{14}C ages spanned a range of > 40,000 to 28,251 yBP for cave bears and 41,201 to 2,520 yBP for

354 brown bears (Table S3, Supporting Information).

355

356 Crossvalidation testing of the phylogenetic age estimation procedure resulted in 95% highest

357 posterior densities (HPDs) that included the actual ^{14}C age for all brown bears and all but one

358 cave bear. Median estimated ages were also very close to the known age in most cases (Figs. S5

359 & S6, Supporting Information). These results support the reliability of this approach in estimating

360 the ages of samples without ^{14}C dates. Furthermore, age estimation for undated samples produced

361 unimodal posterior estimates that are consistent with other sources of age information, where

362 available, such as samples that were outside the range of ^{14}C dating and those dated by amino acid

363 racemisation (Table S7, Supporting Information).

364

365 Age estimates for cave bears (Fig. 5a) are compatible with the contemporaneous existence of the

366 A Ceza, Amutxate, Arcoia and Liñares mitochondrial lineages. Although phylogenetic age

367 estimates are associated with substantial uncertainty, the 95% HPDs of age estimates for these

368 four caves show considerable overlap and median estimated ages are broadly comparable with

369 each other, and with ^{14}C dated samples. The simultaneous occupation of these caves is also

370 supported by ^{14}C dating of other specimens not included in this study (Pérez-Rama *et al.* 2011). In

371 contrast to these caves, the Eiros mitochondrial lineage appears to have existed more recently and

372 potentially without temporal overlap with those from other caves, although we do find slight

373 overlap of Eiros ^{14}C dates and HPDs from other caves in some cases (Fig. 5b). Generally younger

17

374   ^{14}C dates of Eirós in comparison to the other caves have also been reported previously, however, a

375   single specimen was dated to more than 40,000 yBP (Pérez-Rama *et al.* 2011), and may therefore

376   have existed contemporaneously with individuals from other caves. Unfortunately, this sample

377   failed to yield any usable DNA and so its phylogenetic relation to more recent Eirós cave bears

378   remains unknown. Caves containing brown bear remains were almost certainly inhabited

379   simultaneously. ^{14}C ages and a single phylogenetic estimate indicate temporal overlap in the

380   habitation of these five caves between approximately 10,000 and 6,500 yBP (Fig. 5b).

381

382

383   **DISCUSSION**

384

385   ***Evidence for homing behaviour***

386   Cave bears and brown bears that died in caves in the north of Spain show remarkably contrasting

387   patterns of mitochondrial haplotype segregation. While no significant association of

388   mitochondrial haplotypes and cave is found in middle Holocene brown bears, in the case of Late

389   Pleistocene cave bears each cave contains, almost exclusively, a unique clade of closely related

390   haplotypes. This structure exists despite caves being located in close geographic proximity and

391   being inhabited simultaneously. We therefore interpret this as evidence of homing behaviour in

392   cave bears. This scenario would involve a single intermixing cave bear population within which

393   individuals – both males and females – returned to their native caves annually for hibernation,

394   that is, the cave in which their mother hibernated and also gave birth, as demonstrated by the

395   large amounts of perinatal individuals in the sites (Torres *et al.* 2002; Pérez-Rama *et al.* 2011).

18

396 Such homing behaviour does not exclude mating between bears from different caves, but would

397 have sorted the mitochondrial lineages by caves. In contrast, the lack of association between

398 mitochondrial haplotype and cave in middle Holocene brown bears rejects this type of homing

399 behaviour in this closely related species. This is further supported by studies of extant brown bear

400 populations which show greater flexibility with regard to hibernation site than inferred here for

401 cave bears (e.g. in Naves & Palomero 1993).

402

403 Evidence suggests that cave bears hibernated communally (e.g. Philippe & Fosse 2003). Homing

404 behaviour would therefore result in non-random groups of close maternal relatives assembled at

405 each cave. Thus, this behaviour can be further considered as a form of sociality. The temporal

406 stability of these social groups is demonstrated by the observation of multiple unique haplotypes

407 within caves that differ from their nearest relative by a single nucleotide substitution (Fig. 2).

408 This suggests that within-cave haplotype variability is the result of nucleotide mutations that

409 occurred during the period of cave occupation, most likely over thousands of years. A stepwise

410 pattern of haplotype variability within caves has previously been reported for short cave bear

411 control region sequences from the Ach valley, south-western Germany (Hofreiter *et al.* 2007),

412 which in light of our finding suggests the potential for similar homing behaviour in that

413 population. The temporal stability of cave occupation by cave bears is further demonstrated by

414 two morphologically distinct cave bear forms that each occupied separate caves located only a

415 few kilometers apart in Austria. These morphotypes sort into respective, genetically divergent

416 mitochondrial clades. Despite their close proximity, a previous study found no evidence of

417 haplotype exchange between caves even though simultaneous occupation over thousands of

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418   years, implying both site fidelity and reproductive isolation (Hofreiter *et al.* 2004). In the case of

419   Spanish cave bears, however, we consider reproductive isolation unlikely due to a lack of any

420   obvious morphological separation and relatively low levels of haplotype divergence between

421   caves. Our preferred alternative, a single population with homing behaviour, makes specific

422   predictions about patterns of nuclear DNA divergence among caves, and obtaining such data

423   would be a valuable direction for future cave bear research.

424

425   Although we found a clear association of mitochondrial lineage and cave in Spanish cave bears,

426   the association is not perfect. Specifically, we found a single haplotype that is shared among three

427   caves: Liñares, A Ceza and Arcoia. This shared haplotype is common among Liñares individuals,

428   and separated from a second Liñares haplotype by a single nucleotide mutation. In the second

429   cave, A Ceza, the shared haplotype is considerably diverged from other haplotypes within that

430   cave. In the third cave, Arcoia, both samples investigated have the shared haplotype. These later

431   samples are the remains of juvenile individuals and no other cave bear remains have been found

432   in this cave, raising the possibility that these juveniles (and potentially the A Ceza individual

433   carrying the same haplotype) originate from Liñares. Regardless of the origin of this shared

434   haplotype, while this pattern does imply some degree of movement between caves, the overall

435   evidence for homing behaviour is clear and substantial. An ability to disperse and occupy other

436   caves is further indicated by the sister group relationship found between Eirós cave haplotypes

437   and a haplotype from Chauvet cave in France, two caves that were occupied simultaneously (see

438   Table S3, Supporting Information; Bon *et al.* 2008; 2011). Thus, the Eirós haplotype lineage may

439   be the result of long distance dispersal by female bears from distant caves, rather than movement

20

440   among localised Spanish caves, which is also consistent with the apparent temporal separation of

441   this lineage from the other Spanish caves.

442

443   ***Wider implications***

444   Homing behaviour has wider implications for species survival and conservation. For example, in

445   extant black bears (*Ursus americanus)*, it has been discussed as a potential problem for

446   repopulation programs, as both females and males are able to track back to their home area after

447   being captured by humans and released several kilometres away (Beeman & Pelton, 1976; Rogers

448   & Lynn 1986; Clark *et al.* 2002). The same effect has been observed in Asian black bears (*Ursus*

449   *thibetanus*), where genetic studies showed that 63% of the translocated bears migrate back to

450   their original sites (Mukesh *et al.* 2015). Other well known examples include anadromous fishes,

451   whose ability to return to breeding sites is affected by anthropogenic disruption of freshwater

452   river systems (e.g. Pess *et al.* 2014), and similarly in marine turtles, where anthropogenic coastal

453   development threatens habitats used for egg deposition (e.g. Wallace *et al.* 2011). Although

454   ancient DNA provides the potential to investigate such behavioural patterns in species that have

455   already gone extinct, behavioural inferences based on ancient DNA have been rare (notable

456   examples are Huynen *et al.* 2010; and Allentoft *et al.* 2015). Our study clearly demonstrates the

457   potential utility of ancient DNA in the study of behavioural ecology by revealing evidence of

458   homing behaviour in extinct cave bears, and furthermore, through comparison with a closely

459   related extant species, we have also uncovered clues on the potential causes of cave bear

460   extinction.

461

462 The role of humans in the extinction of the cave bear has been debated (Grayson & Delpech

463 2003; Munzel & Conrad 2004; Knapp *et al.* 2009; Bon *et al.* 2011; Stiller *et al.* 2014), but

464 explanations that also account for the survival of the sympatric brown bear have remained

465 elusive. It is likely that the high dependence of cave bears on their native caves would have made

466 them more sensitive to human competition for caves for several reasons. First, as noted

467 previously (Grayson *et al.* 2003; Stiller *et al.* 2010), the generally high dependence of cave bears

468 on caves for hibernation would have brought them into severe competition with humans (both

469 Neanderthals and modern humans). Second, their tendency to come back to the same cave site

470 would have made them comparatively predictable prey, which fits to the growing evidence of

471 cave bear hunting, again by both Neanderthals and modern humans (Munzel & Conrad 2004;

472 Wojtal *et al.* 2015). And third, this homing behaviour would have prevented a rapid

473 recolonisation of empty caves from neighbouring populations. Overall, these factors could have

474 contributed to the extinction of the cave bear as modern human populations expanded from

475 Eastern to Western Europe, indeed, advancing in the same direction as the subsequent cave bear

476 extinction. This is in agreement with recent studies that have questioned the relative contribution

477 of Pleistocene climatic changes to cave bear extinction, and suggested instead a major impact of

478 human activities (Knapp *et al.* 2009; Bon *et al.* 2011; Stiller *et al.* 2014). Finally, the lack of

479 evidence of homing behaviour to their maternal caves in Spanish brown bears, a species that lived

480 in widespread sympatry with cave bears but survived the human expansion into Western Europe,

481 further implicates this behaviour as a factor in the extinction of the cave bear.

482

483

22

484 **ACKNOWLEDGMENTS**

493

494

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661

662

663   **DATA ACCESSIBILITY**:

664   DNA sequences from the cave and brown bears obtained in this study are deposited in Genbank

665   with accession numbers: XXXXX. DNA sequence alignment has been deposited in Dryad with

666   accession number XXXXXX

667

668   **AUTHORS CONTRIBUTIONS:**

669   G.G.F, A.B, A.G and M.H designed and conceived of the study; G.G.F, A.B and I.N.M.

670   performed molecular work; G.G.F, A.B, B.K and D.F, performed NGS data processing and

671   statistical analysis; A.G, A.G.V, A.C.P, S.C, T.J.T, J.E.O, C.F and G.R collected and identified the

672   ancient remains. G.G.F, A.B, and M.H drafted the manuscript with input from A.G and A.G.V. All

673   authors gave final approval for publication.

674

675

676   **FIGURE LEGENDS**

677

678 **Figure 1**. Map of Northern Spain showing locations of the caves investigated in this study.
679 Circles represent sites with cave bears. Squares are sites with brown bears. Colours are consistent
680 with Fig. 2.
681
682 **Figure 2**. Haplotype networks of A. Iberian cave bears and B. Iberian brown bears, coloured
683 according to the cave in which that haplotype was found (indicated next to each network). Circles
684 are sized relative to haplotype frequency. Dashes along edges indicate single nucleotide
685 mutations.
686
687 **Figure 3.** Time calibrated phylogeny of the Western European *U. spelaeus* cave bear clade. The
688 lower scale shows kyBP. Branch labels indicate posterior clade probabilities ≥0.95, except for
689 terminal tip clades where labels have been removed for simplicity. Nodes are centered on the
690 median estimated divergence time and bars show the 95% HPD. Circles next to taxon names
691 indicate Iberian cave bears and are coloured according to cave (consistent with Fig. 2). The *U.*
692 *ingressus* clade that is sister to the *U. spelaeus* clade and was utilised for molecular dating is
693 shown collapsed for simplicity.
694
695 **Figure 4.** Time calibrated phylogeny of the Western European brown bear clade. The lower scale
696 shows kyBP. Branch labels indicate posterior clade probabilities ≥0.95. Circles next to taxon
697 names indicate Iberian brown bears and are coloured according to cave (consistent with Fig. 2).
698 Two additional representatives of the West European brown bear clade, from Austria (sample
699 Uap) and Bulgaria (GenBank Accession AP012591), were analysed and found to form a well
700 supported sister lineage to the clade shown here that diverged an estimated 68,401 yBP ago (95%
701 HPD 50,409–92,631 yBP). This lineage is not shown in order to better visualise divergence times
702 among Iberian brown bear haplotypes.
703
704 **Figure 5.** Time lines of A. Iberian cave bear and B. Iberian brown bear sample ages. Time in yBP
705 is shown on the Y axes. Each point indicates the estimated age of an individual bear. Black points
706 are median phylogenetic age estimates and red points are mean calibrated ^{14}C ages. Error bars
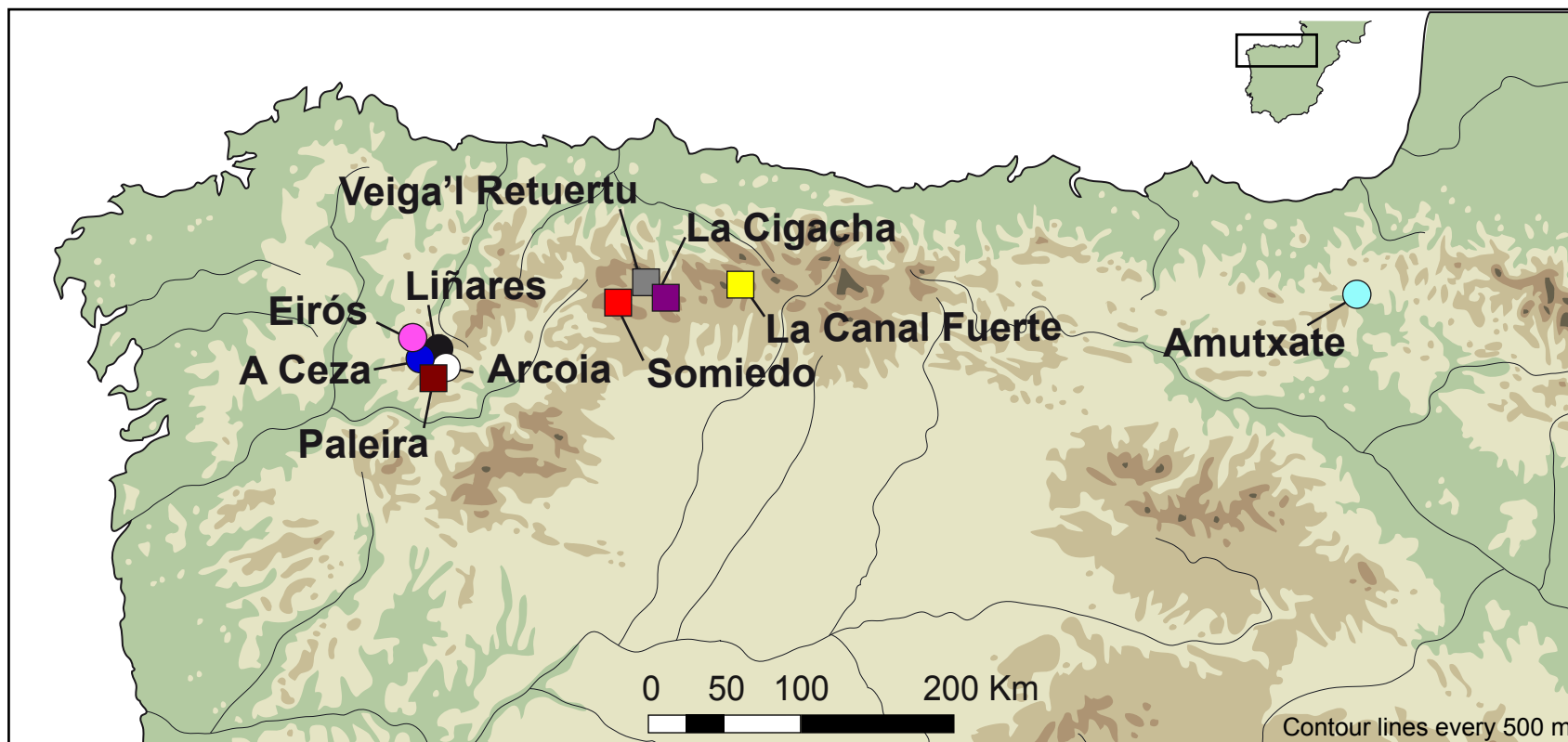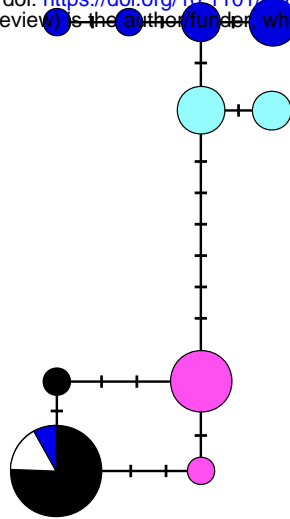707 show 95% HPD and calibrated ^{14}C uncertainty for phylogenetic age estimates and ^{14}C ages,
708 respectively.

28

Figure 1.

A. cave bears

- ● Liñares
- ● A Ceza
- ● Eirós
- ● Amutxate
- ○ Arcoia

B. brown bears

- ● Veiga'l Retuertu
- ● Canal Fuerte
- ● Paleira
- ● Somiedo
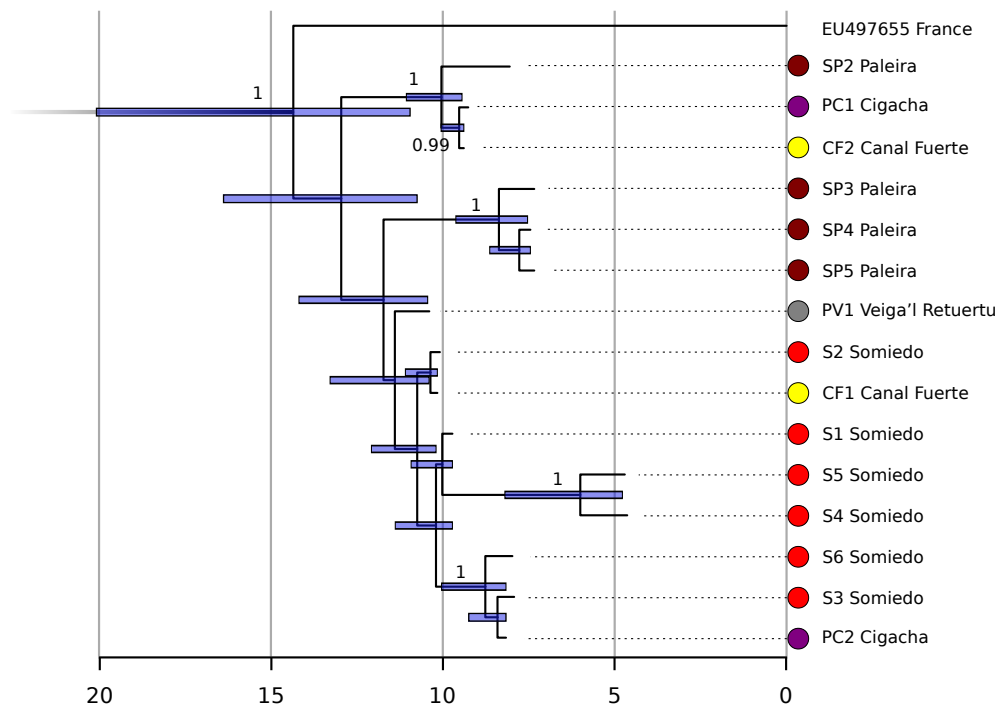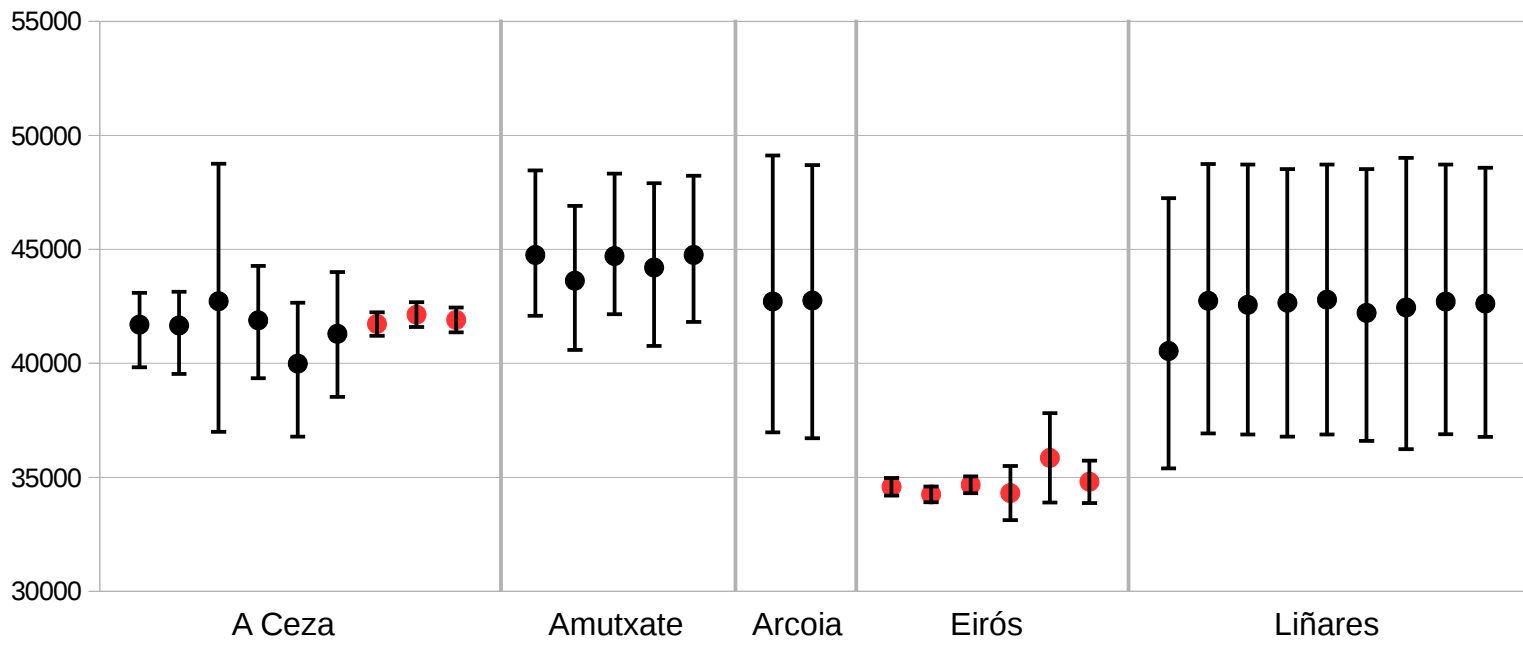- ● Cigacha
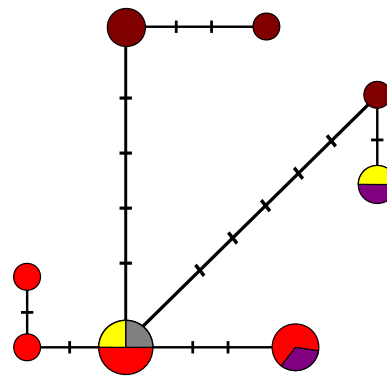
Figure 2.

Figure 3.

Figure 4.

Figure 5.