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Research Paper

Title: Maternal relationships within an Iron Age burial at the High Pasture Cave, Isle of Skye, Scotland

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Declaration of interest: none.

Highlights

- Rare Iron Age cave burial assemblage of an adult female with foetal remains
- First report of complete ancient mitochondrial DNA sequences from foetal-aged bone fragments
- Shot-gun sequencing and mitochondrial genome analysis for maternal lineage identification
- Female could be excluded as being the mother of one the infants

Abstract

Human remains from the Iron Age in Atlantic Scotland are rare, which makes the assemblage of an adult female and numerous foetal bones at High Pasture Cave, on the Isle of Skye, particularly noteworthy. Archaeological evidence suggests that the female had been deposited as an articulated skeleton when the cave entrance was blocked off, marking the end of use of the site. Particularly intriguing is the deposition of disarticulated remains from a foetus and perinate close to the adult female, which opens the possibility that the female might have been the mother of both of the infants. We used shotgun genome sequencing in order to analyse the mitochondrial genomes of all three individuals and investigate their maternal relationship, and we report here, for the first time, complete ancient mitogenomes from foetal-aged bone fragments. While we could not exclude the possibility that the female was the mother of, or maternally related to, the foetus, we could definitely say that she was not the mother of the perinate buried alongside her. This finding is contrary to the standard archaeological interpretation, that women in such burials most likely died in childbirth and were buried together with their foetuses.

Keywords: ancient DNA, foetus, mitochondrial DNA, shot-gun sequencing

Introduction

High Pasture Cave (Uamh an Ard Achadh) is situated in the Parish of Strath on the Isle of Skye in Scotland. The wider environment surrounding the site revealed a diverse archaeological landscape of monuments and sites relating to prehistoric and historic periods, including a number of funerary monuments and hut-circles, combined with well-preserved field systems (Birch and Wildgoose, 2013). Radiocarbon dates of samples from different layers within the cave, and from the complex deposits outside the cave entrance, indicate periodic activity at High Pasture Cave from the 19th century cal. BC to cal. AD 1st century (Birch and Wildgoose, 2013). This, and the diversity of small finds, indicates a complex occupation history with several phases of intense activity during the Iron Age (Birch and Wildgoose, 2013).

Stonework found in the surface trench was associated with a series of entrance arrangements leading down a stone stairwell into the cave, whose system provided direct access to an underground stream. Although the archaeological material found in the cave indicated a fairly typical domestic assemblage (comprising bone, stone and iron tools, ceramics, and residues of metalworking), their depositional context, including the restricted morphology of the cave, raises questions about this interpretation. A well-preserved faunal assemblage was recovered, including animal and fish bones, and shellfish, as well as charred plant remains. Among the

animal bones, a high percentage were assigned to domesticated pig, which suggests a feasting deposit (Livarda and Madgwick, 2017), and the bones displayed evidence of unusual butchery practices, such as deliberate division of skeletons into left and right parts, and unusual cut-marks across several ribs (Drew, 2005). The majority of the remains found at this site displayed distinct ritual aspects, such as the burial of animals after butchery, and the presence of both disarticulated human remains and complete human inhumations, including foetal and infant burials (Birch and Wildgoose, 2013; Livarda and Madgwick, 2017).

A century or so after the stairwell was back-filled, with the passage being deliberately blocked with boulders and sediments, a final act of closure at High Pasture Cave was carried out (Birch & Wildgoose, 2013). Human remains from three individuals were deposited into the top of the blockage (**Figure 1**), consisting of an adult female, aged 25–40 years and 1.55m tall, together with two infants: one foetus that died between 12 and 26 weeks of gestation, and one perinate that died during the last month of foetal development or during the first two weeks of life. The woman had been laid out in the top of the backfilled stairwell that originally provided access to the natural cave below, and large stones had been placed on top of her. The remains were badly fragmented in the head, chest, abdominal area, and the right upper and lower arm, and left lower arm regions, consistent with blunt force trauma from the stones. This happened either at, or around, the time of deposition, precluding any chance to ascertain if the injuries were the cause of her death.

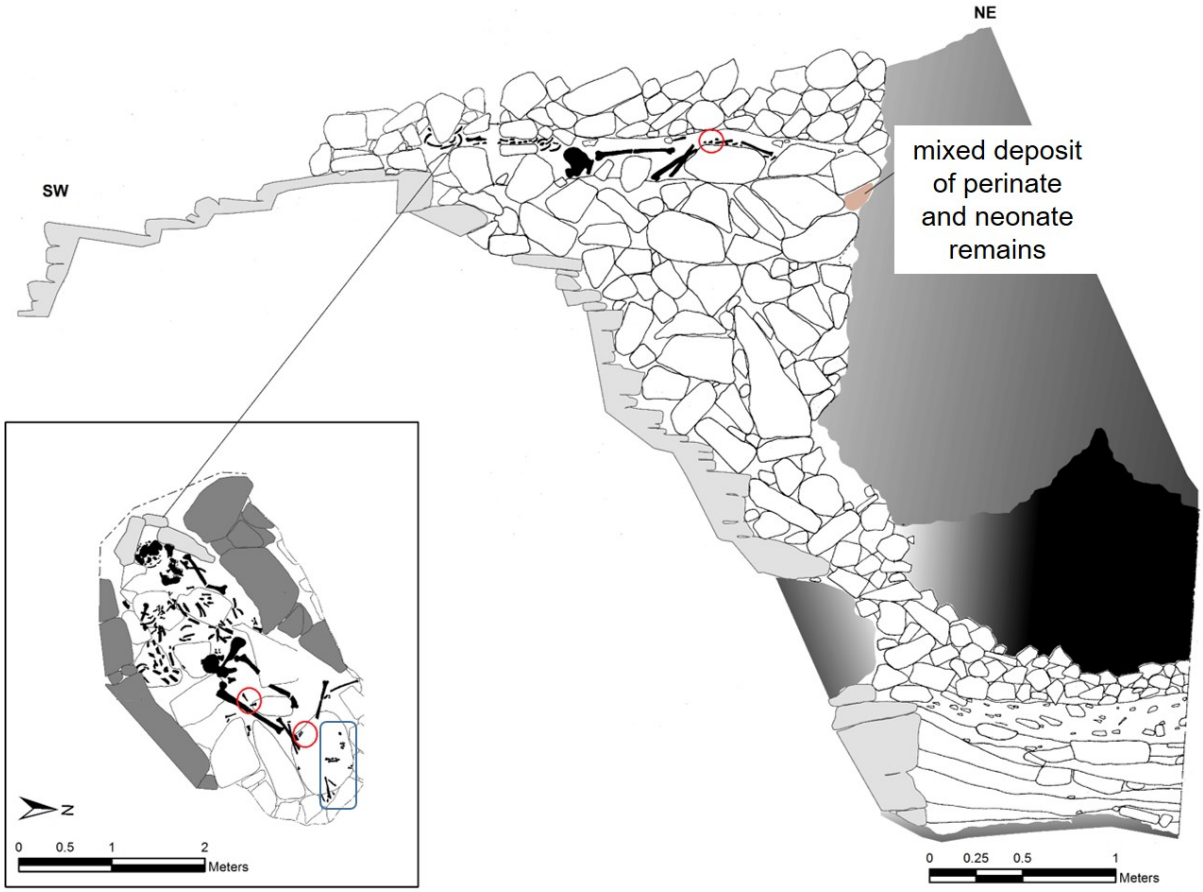

Figure 1. Burial schematic at High Pasture Cave, showing the position of the human remains within the stairwell blockage and, inset, the location of the adult, infant and animal bones. The remains of the foetus and perinate are indicated by red circles, with the foetal bones closer to the female's pelvis than the perinate. The blue rectangle shows the location of the foetal pig and perinatal dog, and also included a mixed deposit of perinate and neonate remains, as shown in the section of the stairwell.

Bones of the foetus were recovered from between the knees of the woman, while two small bundles of bones by her feet contained the skeletal elements from the perinate, and the remains of a foetal pig and a perinatal dog (**Figure 1**). The reasons for the segregation of the bones from the human foetus and perinate, and piglet and puppy are difficult to explain, but this may have formed a major part of the overall internment process. Although the relationship between the infant remains and the adult woman in the burial were uncertain, radiocarbon dates indicate that all three are roughly contemporary. The adult female yielded a radiocarbon date of cal. AD 28–230 (2σ; SUERC-14946), which corresponds to the Iron Age in this region. The infant remains were radiocarbon dated to cal. AD 63–219 (SUERC-66371) for the foetus, and cal. AD 76–228 (SUERC-66372) for the perinate (**Figure 2**). The majority of the other human bones (including infants and foetuses) found at this site (**Figure 1**), belonged to different contexts, and were dated as being older than the here presented analysed individuals

(Supplementary Information and **Table S1**). Although one perinate (D) bone, found in the west wall of the stairwell (**Figure 1**), was roughly contemporaneous with the three individuals studied here (**Table S1**), it only consisted of a proximal right humerus, and the entire sample had been used for radiocarbon dating as part of an earlier study (Birch and Wildgoose, 2013; Shapland et al., 2016), and so was unavailable for DNA analysis.

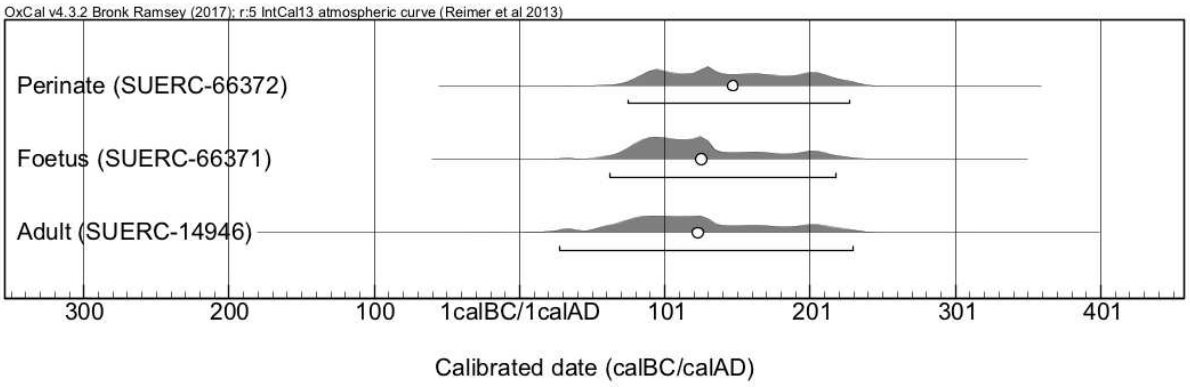

Figure 2. Radiocarbon dates for the human remains analysed from the High Pasture Cave stairwell, calibrated using OxCal v.4.3.2 (Bronk Ramsey, 2017) and the most recent calibration curve IntCal 13 (Reimer et al., 2013).

Several of the bones from the extremities of the articulated adult woman were missing, suggesting that excarnation was the most likely pre-treatment of the body prior to final deposition (Birch and Wildgoose, 2013). This may also have been the case with the foetus, perinate, foetal piglet and perinatal puppy, as no cut-marks were noted on any of the bone elements, making it unlikely that the bodies were cut up or dissected but rather were left to decompose naturally. This type of funerary tradition would have provided the opportunity for disarticulated human remains to be recovered for curation, use, or deposition, and disarticulated human remains have indeed been recovered from a large number of Iron Age settlement sites (Tucker, 2010). Therefore, it is possible that the remains of either the human foetus or perinate were curated and only interred after the woman had died.

There does not appear to have been a tradition of formal burial of the dead throughout the majority of the Iron Age in Atlantic Scotland, and it has frequently been posited that an archaeologically 'invisible' form of disposal of the dead, such as exposure, must have been practiced (Armit and Ginn, 2007). Consequently, human remains from this period are rarely recovered; on Skye, for example, just one other Iron Age site has produced human remains – Fiskavaig, a rock-shelter with evidence of Middle Iron Age occupation, from which a single perforated fragment of adult human cranium was recovered in 2009 (Birch 2009). However,

144 distinctive and specific burials for women, foetuses and infants during the Iron Age in Britain,
145 including Scotland, were rather common (Finlay, 2000; Armit and Ginn, 2007). The general
146 archaeological interpretation has been that the women must have died in childbirth and were
147 thus buried together with their foetuses (Millet and Gowland, 2015). However, to our
148 knowledge, this hypothesis has not, to date, been analysed genetically. In order to test this
149 assumption, we used whole mitochondrial genome (mitogenome) data to analyse the maternal
150 genetic relationship of the adult female to both the foetus and the perinate.

151

Material and Methods

Sampling and DNA extraction from ancient remains

We sampled a petrous bone from the female adult, and two infant bone fragments from the perinate (ischium) and the foetus (scapula) for genetic analysis. We carried out the sampling at the Ancient DNA Facility at the University of Huddersfield under dedicated clean-room conditions. Laboratory researchers wore full body suits, hairnets, gloves and face masks throughout the drilling, extraction and library preparation processes. We constantly cleaned all tools and surfaces with LookOut® DNA Erase (SIGMA Life Sciences), as well as with bleach, ethanol and long exposures to UV light.

We decontaminated all of the bone surfaces by UV radiation for 30 minutes on each side, followed by cleaning with 5µm aluminum oxide powder using a compressed air abrasive system. For the adult sample, we excised the densest part of the petrous bone (Pinhasi et al., 2015) using a circular saw. We obtained bone powder by crushing this excised petrous portion, and the complete bone fragments from the two infants, in a MixerMill (Retsch MM400). We extracted DNA from 150mg of the petrous powder and 50–100mg of each infant bone, following the protocol by Yang et al. (1998), with modifications by MacHugh et al. (2000). We included blank controls throughout the extractions, library preparation and amplification reactions to monitor for possible modern DNA contamination.

Library preparation and sequencing of ancient DNA

We constructed next-generation sequencing libraries from the ancient DNA extracts using the method described in Meyer and Kircher (2010), with modifications outlined in Gamba et al. (2014) and Martiniano et al. (2014). Briefly, the changes were as follows: we used T4 DNA polymerase buffer (Thermo Scientific) instead of Tango buffer in the blunt-end repair step, and we heat inactivated *Bst* polymerase activity by incubating the libraries for 20 minutes at 80°C. We set up indexing amplifications using Accuprime *Pfx* Supermix (Life Technology), primer IS4 (0.2µM), a specific indexing primer (0.2µM) and 3µl of sample library, with a total reaction volume of 25µl. We performed all DNA purification steps using the QIAQuick MinElute purification kit (Qiagen) following the manufacturer's protocol, with the modification of adding 0.05% Tween 20 (Fisher BioReagents) to the elution buffer.

We prepared single-end libraries for both of the infant samples. We ran amplification under the following thermal cycling conditions: 5 minutes at 95°C; 11 cycles of 15 seconds at 95°C,

30 seconds at 60°C and 30 seconds at 68°C; with a final extension step of 5 minutes at 68°C. We purified the resulting amplification product using the QIAQuick MinElute purification kit (Qiagen).

As the adult was part of an earlier study, four dual-index libraries (Kircher et al., 2012) were made from this individual. We set up amplification using AmpliTaq Gold (5U/µl), Thermopol reaction buffer (10x), dNTPs (10mM each), both indexing primers (10µM each) and 10µl DNA sample library. The following thermal cycling steps were used for amplification: 12 minutes at 95°C; 10 cycles of 20 seconds at 95°C, 30 seconds at 60°C, and 40 seconds at 72°C; with a final extension step of 5 minutes at 72°C. We purified the amplification product using the QIAQuick MinElute PCR purification kit. For the second amplification round, we used Accuprime *Pfx* SuperMix (Life Technology), together with primers IS5 (10µM), IS6 (10µM) and 2.5µl of sample library. We carried out amplification under the following thermal cycling conditions: 30 seconds at 98°C; 10 cycles of 20 seconds at 98°C, 30 seconds at 60°C, and 40 seconds at 72°C; with a final extension of 5 minutes at 72°C. We purified the resulting amplification product as previously described.

We quantified all amplification reactions using a Qubit® ds-DNA High Sensitivity assay kit on the Qubit® 3.0 Fluorometer, and additionally we checked the quality of each library using an Agilent 2100 Bioanalyzer High Sensitivity DNA kit prior to pooling equimolarly for next-generation sequencing (NGS) purposes. For the adult, we sent four dual-indexed libraries for 100-bp (base-pair) single-end sequencing on a HiSeq2500 (NBAF Liverpool, United Kingdom), whereas we assigned the infant remains one single-indexed library each, and sent them both for 100-bp paired-end sequencing on a HiSeq4000 (Macrogen, South Korea).

Analysis of ancient sequence data

We trimmed the NGS reads and aligned to the human mitochondrial genome revised Cambridge Reference Sequence (rCRS, NC_012920; Andrews et al., 1999) and the human reference genome (UCSC hg19). We trimmed the reads using cutadapt (v.1.13; Martin, 2011), allowing a minimum overlap of 1bp between read and adapter (adapter sequence: AGATCGGAAGAGCACACGTCTGAA-CTCCAGTCAC), discarding reads shorter than 30bp. We used BWA aln (v.0.7.12-r1039; Li and Durbin, 2009) to map reads to both the rCRS and hg19, filtering by base quality 15, and disabling seed length, as recommended for ancient DNA data (Schubert et al., 2012), with a maximum edit distance of 0.01 and maximum number of gap opens set to 2. We used SAMtools v.1.3 to sort and filter reads, and to remove PCR duplicates, and assessed DNA damage patterns using mapDamage 2.0 (Jónsson et al., 2013) and bamdamage (Malaspinas et al., 2014). We used picard tools (v.2.18,

http://broadinstitute.github.io/picard) to add read groups to the sequencing reads aligned to hg19, and assessed the sequencing quality using FastQC (v.0.11.5; Andrews 2010) and qualimap (v.2.2.1; Okonechnikov et al., 2015). We used samtools mpileup and bcftools (v.1.3) to create a consensus FASTQ file of the alignment to the rCRS, and SEQTK (v.1.2-r102-dirty) to convert the FASTQ file into FASTA format. We used schmutzi (Renaud et al., 2015) to detect possible modern mitochondrial contamination, and we assigned haplotypes using Haplogrep 2 (Weissensteiner et al., 2016) and confirmed them manually using the Integrative Genomics Viewer (IGV v.2.4.13; Robinson et al., 2011; Thorvaldsdóttir et al. 2013). The only difference in the treatment of the paired-end reads compared to single-end reads was our use of AdapterRemoval (v.2.1.7; Schubert et al., 2016), which trimmed the adapters from the read pairs and merged the reads.

Mitochondrial haplotype calling for ancient samples

We created the mitochondrial genome consensus sequences using SAMtools mpileup and BCFtools. As the coverage of the foetus and perinate mitogenomes was very low (see Results section), we also used GATK (v.3.7) to call the variants against the rCRS for the two infant samples. This approach allowed a maximum output of variants in order to determine the mitochondrial haplogroup of the studied individuals, and also allowed us to compare both methods. All other bioinformatic steps were the same for both read types.

We called the mitochondrial haplotypes of the newly sequenced modern samples using HaplotypeCaller, with 'Downsampling' set to 250, 'Ploidy' set to 100 and 'Standard Call Confidence' set to 30, and, for SNP filtering, we set the 'Minimum Coverage Filter' to 2. In order to filter the mutations and heteroplasmies, we used bcftools v.1.3 (Li and Durbin, 2009; Li, 2011). We considered mutations with a read frequency ≥0.7 as variants, whilst mutations with an allele frequency >0.3 and <0.7 were considered heteroplasmies. We checked all heteroplasmies manually in Geneious® (version 6.1.8). We excluded variants at positions around 302-315 including 309.1C(C) and 315.1C, AC insertions and deletions at 515-522, 16182C, 16183C, and 16193.1C(C) from analyses. We checked the final haplotype classifications with HaploGrep 2 (Weissensteiner et al., 2016) and converted these into FASTA files with HaploFasta.

Modern mitochondrial genomic sequences and SNP data

We sequenced a total of 13 modern samples belonging to H7a1 (Germany, *n* = 9; Scotland, *n* = 3; Wales, *n* = 1) to accompany the published data (Denmark, 15; England, 2; Finland, 1;

Germany, 1; Italy, 3 [including 1 Sardinian]; Russia, 2; Scotland, 1; United Kingdom, 1; USA, 2; unknown geographic origin, 9; Bulgaria ancient, 1) (**Table S2**; **Figure S1**). The four newly generated British samples originated from a selection of samples collected by BritainsDNA (now owned by Source BioScience). An overall mitochondrial SNP (single nucleotide polymorphism) dataset of anonymised samples was available to us, also from BritainsDNA/Source, consisting of 5,337 British and Irish individuals. We used these SNP data for mitochondrial haplogroup frequency estimates.

Analysis of mitochondrial DNA data

We carried out polymerase chain reactions (PCR) for modern samples in the modern genetics lab at the University of Huddersfield. To amplify the complete mitogenome (16,568 bp), we used two fragments for each sample following the method in Brandini et al. (2017). We purified the PCR products using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) with an elution volume of 50µl. We assessed the DNA concentration of each fragment using 1µl of the purified PCR product using a Qubit® 3.0 fluorometer. After quantification, we pooled both fragments equimolarly with a final volume of 40µl and a concentration of 1ng/µl. We then sent the samples for sequencing at the Earlham Institute (Norwich, UK) on a MiSeq250 (PE-Nano x1). We used EAGER (Peltzer et al., 2016) for assessment of quality control, removal of duplicates, mapping and SNP calling, using the following settings: AdapterRemoval for the removal of adapters and read merging; bwa-mem to align sequences to the rCRS (Andrews et al., 1999); and removal of duplicates using DeDup. We aligned published sequences to the rCRS using Sequencher™ version 5.1 (*http://www.genecodes.com*), and converted these to variants using mtDNAGeneSyn (Pereira et al., 2009). We verified haplogroup classifications using Haplogrep 2 (Weissensteiner et al., 2016). We also used HaploGrep2 to call haplogroups from the SNP data of the 5,337 British and Irish individuals.

Phylogenetic tree reconstruction

We placed all newly generated sequences in a maximum-parsimony phylogenetic tree, constructed by compiling published (n = 38) and unpublished (n = 15; 13 modern and the two ancient) complete mitogenome data (**Table S2**; **Figure S1**). We also included data generated at Huddersfield as part of a larger British Isles study. We used mtPhyl v.4.015 (Eltsov and Volodko, 2011; *https://sites.google.com/site/mtphyl/home*) to reconstruct the phylogeny following the classification of PhyloTree build 17 (*http://www.phylotree.org/tree/index.htm*), and using the molecular clock developed by Soares et al. (2009) to date nodes. We calculated maximum-likelihood (ML) estimates assuming the HKY85 mutation model with gamma-

distributed (32 categories) rates (plus invariant sites) and two partitions: coding region (positions 00577–16023) and control region (positions 16024–00576) with baseml v.4.7 from PAML package (Yang, 1997). We then converted the distances into years using a mutation rate of one substitution in every 3,624 years (Soares et al. 2009), further accounted for purifying selection. We obtained coalescent time estimates using rho (ρ) and sigma (σ) values exported from the in-house developed founder analysis software, applying the same clock. All ancient samples (*n* = 3) were excluded from the age calculations.

Results

We successfully extracted and sequenced DNA from all three ancient samples. The endogenous DNA content of all individuals was quite low, at 11.9%, 8.0% and 7.6% for the adult, perinate and foetus respectively. The nuclear genome of the adult female was sequenced to a coverage of 0.28x, whilst her mitochondrial genome was sequenced to a coverage of 58.07x. For the two infants, the nuclear genome coverage was 0.014x for the perinate and 0.006x for the foetus, with mitochondrial genome coverages of 4.14x and 2.24x, respectively. Due to the low coverage of the nuclear genomes of the foetus and the perinate, and the extremely low number of overlapping SNPs retrieved (<10,000), no meaningful autosomal analysis was possible. The genome-wide data of the adult female is currently being further analysed in a broader Iron Age study (in preparation) and, since the focus of the present study is the maternal relationship of the individuals analysed, and the coverage of the genome-wide data retrieved from the two infant remains was too low to be useful, we do not present any genome-wide analysis here.

Contamination estimation

Contamination estimates ranged from 0.165% to 0.185% for the adult female, and 0 to 0.005% for both infants. We observed no exogenous contamination in extraction or library blanks, supporting the authenticity of the results, and there were no matches between the mitochondrial haplotypes from the three ancient samples and those of the researchers.

Genetic sex identification

We identified the genetic sex using the tool published by Skoglund et al. (2013). The osteological determination that the adult was female was supported by the genetic sex identification. We were unable to assign the genetic sex for the two infants due to their low genomic coverage.

Mitochondrial haplotype determination

To securely determine mitochondrial haplotypes for the infants, we compared the results from two bioinformatic approaches (that is, GATK Variant Call *vs.* calling the consensus sequence, as discussed in the Methods section), and we assigned the same haplotypes to each individual using both methods. Due to the low coverage in both infants' mitochondrial genomes, the

haplotypes initially generated presented a number of ambiguous base calls (**Table 1**). In order to reduce the number of ambiguities, and thus simplify any haplotype comparison, we checked each one of the ambiguous positions manually, and made a decision by combining phylogenetic and molecular criteria. We provide a thorough overview of the rationale behind every mutation disregarded in **Table S3**. Although the haplotypes finally reconstructed could still contain some errors (**Table 1**), they show that only one of the infants, the foetus, shares haplogroup (H7a1b) with the adult female and, could, therefore, possibly be related to her. We assigned the perinate to haplogroup J1c3, thus excluding a direct maternal kinship.

Table 1. Comparison of mutations scored for all three ancient mitochondrial genomes using Haplogrep 2.

Sample	Haplogroup	Final Haplotype[a]	Initial Haplotype[b]	Gaps[c]
Adult female	H7a1b	*263G 750G* **1393A** *1438G* **1719A** *4769G* **4793G** *8860G 15326G* **16261T** 16519C	-	-
Foetus	H7a1b	*263G 750G* **1393A 1719A** *4769G* **4793G** (7025G) *8860G* **16261T** 16519C	263G 633M 750G 911K 1393A 1719A 4769G 4793R 5239K 6272R 6359M 6842Y 7022K 7025R 8576K 8860G 10674K 10750R 11629R 12308R 12766Y 12822R 12864K 12873W 13207W 14864K 15601K 16261T 16519C	1438G 15326G
Perinate	J1c3	*73G* **185A 228A** *263G* **295T 462T 489C** *750G 2706G* **3010A 10398G 11251G** *11719A* **12612G 13708A 13934T** *14766T* **14798C** *15326G* **15452A 16069T** 16126C	73G 152Y 185A 228A 263G 295T 462T 489C 539Y 750G 2706G 3010A 3276R 4892M 6243R 9233Y 9477R 9814Y 10398G 10567W 11251R 11719A 12210W 12612G 12835R 12998Y 13708A 13934T 14766T 14798C 15326G 15452A 15854W 16022Y 16069T 16126C	-

[a] *Italics*: changes *vs.* the rCRS up to the H2 root; **bold**: H7 to H7a1b diagnostic mutations in the case of the adult and foetus, and JT to J1c3 diagnostic mutations in the case of the perinate; the dubious possible mutation seen in the foetus is shown in parentheses.
[b] underlined: ambiguous positions
[c] gaps: positions seen in the adult, but not covered in the foetal mitogenome

Discussion

Here we report for the first time complete ancient mitogenomes from foetal-aged bone fragments. Previous studies have used PCR-based methods to determine the genetic sex of neonates (Faerman et al., 1998; Waldron et al., 1999; Irish et al., 2008), but no mitogenomes or genome-wide data have been published to date. Using next-generation sequencing to generate whole mitogenomes results in a more detailed analysis of the haplotype than a PCR-based approach, where typically only fragments of the non-coding region are amplified. Although we generated whole mitogenomes from the two infants, we were unable to determine the molecular sex of either the perinate or the foetus due to their low nuclear genome coverage.

Mitochondrial genomes are used to trace the female line of descent as they are passed on from mother to child without recombination. The only changes that occur over generations are naturally-occurring mutations, and these can be used for phylogenetic reconstructions over wide geographic areas and long timespans. Both the female and the foetus belong to the very distinctive and uncommon (among present-day populations) haplogroup H7a1b. In contrast, the perinate belongs to haplogroup J1c3, and so can be ruled out as a child of the adult female. In addition, as we saw no other defining mutations, we can say that the mitogenome of this individual is basal to J1c3. For more details about haplogroup J1c, see Supplementary Information.

The main defining mutations of haplogroup H7a1b are the transitions at positions 4793, 1719, 16261 and 1393, which define H7, H7a, H7a1 and H7a1b, respectively. These are all found in both individuals (shown in bold in **Table 1**). After all ambiguities were assessed (**Table S3**), the sequence of the foetus displayed a single plausible difference compared to the adult female, the A7025G transition (**Table 1**). The 7025 transition defines subclade H5a5, but has never been observed in any H7 sequence to date. Further sequencing would be necessary to securely define the base read at position 7025 for the foetal sequence. However, based on the current data, we must recognise that, although the foetus and the adult female share the same rare haplogroup, they might not share the same haplotype (if a mutation at np 7025 is accepted).

In our modern mtDNA SNP database, haplogroup H occurs in 41% of all modern British Isles mitochondrial lineages (2247 out of 5537). However, haplogroup H7 is only a minor European subclade, which is found at 1.6% (85 out of 5337) in our British Isles dataset, while its nested subclade, H7a1b is even rarer, and is found at 0.7% (13 out of 1926) in Scotland, 0.3% (1 out of 374) in Ireland and 0.2% in England (4 out of 2304), while it has not been detected in Wales (0 out of 733). For more details about haplogroup H7, see Supplementary Information. The

subhaplogroup of H7a1b dates to *c*. 4300 years [2400–6300] and is seen in modern-day Scotland and England, but also in Denmark, Finland and Sardinia (Behar et al., 2012; Li et al., 2014; Raule et al., 2014). The apparently wide geographic distribution and young age of H7a1b suggest that it may have been introduced into Britain more recently than the arrival of the Bell Beaker settlers, *c*. 4450 years ago, when the genome-wide pattern of the British gene pool began to take on its modern form (Olalde et al. 2018) – possibly in the Bronze Age or in the Iron Age itself. The two individuals from High Pasture Cave are, nevertheless, the earliest known H7 sequences from north-west Europe (**Figure S1**). The nodal haplotype represented by the female, and possibly by the foetus, has survived in modern-day Scotland and Denmark.

The fact that the two ancient individuals share the same rare haplogroup, and possibly the same haplotype, suggests that, even if they were not directly related, they belonged to a population that, similarly to other Iron Age populations, was most likely small and in-bred. The possibility that the two individuals might not share the same haplotype is potentially even more tantalizing, as it would imply that, although small and geographically isolated, this population carried a level of diversity (represented by the nodal haplotype +7025 mutation) that could have either arisen *in loco*, thus implying some level of prolonged isolation, or been introduced from a more diverse, and possibly larger, and interconnected, metapopulation.

Infants and young children were often distinguished in burial customs from the rest of society (Ucko, 1969). In early Iron Age Thrace, young or newborn children were sacrificed for rituals and it was only in the late Iron Age that newborn and young animals were used instead (Nekhrizov and Tzvetkova, 2010). In Roman and Anglo-Saxon periods in England, children who had not yet teethed were, instead of being cremated, buried within building walls or under buildings (Stoodley, 2000; Gowland et al., 2014). Also, in Roman Dorset and Buckinghamshire, women who died in pregnancy were only buried after the child had been removed from the womb (embryotomy surgery) (Gowland et al., 2014).

The separation of infant burials from the rest of the population appears to have been widespread across the Iron Age and into the Romano-British period (Redfern and Dewitte, 2011). In the British Iron Age, women who died in childbirth, as well as foetuses and young infants, were often given special burials, highlighting the importance of the link between woman and child (Millet and Gowland, 2015). Human remains of infants were often placed into cave systems, and the number and geographic spread of cave burials during this period in Scotland is relatively high – especially given that burials from this period are generally not abundant (Saville and Hallén, 1994), and that

the deposition of individual articulated skeletons in Iron Age Scotland is rare (Tucker, 2010). However, there is also evidence of infants being buried in unstable, abandoned buildings or disused settlement sites. For example, an excavation at the site of Howe in Orkney revealed an Iron Age burial of an adult male, aged 35–45 years, along with a perinate and an infant, all of whom had been placed within accumulated rubble in a midden in a disused yard (Armit and Ginn, 2007). The bones showed gnaw marks, and their layout has been interpreted as an informal burial; that is, they were deposited without ceremony (Armit and Ginn, 2007). Another example is the Iron Age site at the Knowe of Skea, also in Orkney, where over one hundred, mainly infant, bodies were found deposited in domestic rubble contexts (Armit and Ginn, 2007). Cremations and inhumations of adult women, alongside foetal/neonatal remains, are also known from Ireland, located in reused, separate burial areas (Finlay, 2000).

The context of the High Pasture Cave appears to mirror those burials in cave systems, with the adult female being deposited as an articulated skeleton just prior to the closure of the cave. However, the trauma evident on the female (whether inflicted before or after death), and the lack of any wider tradition of inhumation in the region, has led to the suggestion that this burial was a deviant one, or even a sacrifice connected to the closure of the cave stairwell, rather than a ceremonial burial of a mother and her children (Armit, in press). The disarticulated remains of the foetus and perinate appear to have been collected and purposefully deposited with her, raising several questions, especially now that our results show that we can positively exclude a direct maternal kinship for the perinate.

Conclusions

The results of our study show that one of the two infants buried alongside the adult female at High Pasture Cave was definitely not her child, whereas the foetus could not be excluded as being maternally related to her. This is an intriguing finding considering the common archaeological understanding of this type of female/child burial, where the infants are assumed to be the children of the women. So far, archaeological interpretations of these types of burial indicate either mother–child relationships, or a midwife buried in the same place as infants. In the case of High Pasture Cave, where the woman had potentially been crushed to death, archaeological questions still remain. Why might she have been chosen for this extremely unusual form of treatment before/after death? Why were the human bones of infants curated, collected, and deposited in the cave with her, and why was she buried with an unrelated child?

Our genetic analysis shows that a general assumption of mother–child relations within such burials can be misleading, and that genetic analysis can aid the analysis and interpretation of such archaeological contexts.

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Data availability

Sequencing data deposited in GenBank, accession number xxxx.

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