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Validating a Target-Enrichment Design for Capturing Uniparental Haplotypes in Ancient Domesticated Animals

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ABSTRACT

In the last three decades, DNA sequencing of ancient animal osteological assemblages has become an important tool complementing standard archaeozoological approaches to reconstruct the history of animal domestication. However, osteological assemblages of key archaeological contexts are not always available or do not necessarily preserve enough ancient DNA for a cost-effective genetic analysis. Here, we develop an in-solution target-enrichment approach, based on 80-mer species-specific RNA probes (ranging from 306 to 1686 per species) to characterise (in single experiments) the mitochondrial genetic variation from eight domesticated animal species of major economic interest: cattle, chickens, dogs, donkeys, goats, horses, pigs and sheep. We also illustrate how our design can be adapted to enrich DNA library content and map the Y-chromosomal diversity within *Equus caballus*. By applying our target-enrichment assay to an extensive panel of ancient osteological remains, farm soil, and cave sediments spanning the last 43 kyrs, we demonstrate that minimal sequencing efforts are necessary to exhaust the DNA library complexity and to characterise mitogenomes to an average depth-of-coverage of 19.4 to

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2003.7-fold. Our assay further retrieved horse mitogenome and Y-chromosome data from Late Pleistocene coprolites, as well as *bona fide* mitochondrial sequences from species that were not part of the probe design, such as bison and cave hyena. Our methodology will prove especially useful to minimise costs related to the genetic analyses of maternal and paternal lineages of a wide range of domesticated and wild animal species, and for mapping their diversity changes over space and time, including from environmental samples.

1 | Introduction

Animal domestication marked a pivotal shift in human history by providing reliable sources of meat, milk and eggs, which enhanced nutrition and food security (Vigne 2011). Additionally, it enabled the production of wool and hides for clothing, facilitated transportation and labour through traction animals, and offered companionship, significantly shaping early human societies and economies (Zeder 2012, Larson and Burger 2013). Domesticated animals are integral to our modern societies, with a combined weight representing no less than ~58% of the total mammal biomass, and an estimated 70 billion chickens are killed every year (FAO stats). The process of domestication has led to the extraordinary biocultural diversity of domesticated breeds living on the planet today and has received extensive scholarly attention from across an array of scientific disciplines, including archaeology, genetics and behavioural sciences (Larson et al. 2012; Librado et al. 2021; Frantz et al. 2020; Daly et al. 2021; Bessa Ferreira et al. 2023; Nawroth et al. 2023). The population decline, and sometimes even extinction, of wild progenitors and multiple landraces to the benefit of a handful of over-dominant industrial breeds, combined with the difficulty of tracking markers of early husbandry practices in the archaeological record, has, however, limited our capacity to reconstruct the entire history of animal domestication (Diaz-Maroto et al. 2021).

The recent revolution of next-generation sequencing (Metzker 2010) along with improved techniques to extract and manipulate ancient DNA molecules preserved in archaeological bone assemblages (Orlando et al. 2021) has made it increasingly feasible to use genetic information to predict phenotypes of key agronomical relevance that do not otherwise survive in the fossil record. This revolution, in turn, helped gain finer resolution into the history of the biological transformations underlying domestication (Loog et al. 2017). Genome-wide patterns of DNA variation have also helped track changing reproductive practices, such as inbreeding through close-kin mating (Daly et al. 2021) and shortened generation times (Librado et al. 2024) to identify domestication ongoing at a time when no obvious morpho-anatomical change could be detected in the animal skeleton. Additionally, charting changes in effective population sizes and population movements over time has resolved long-debated questions regarding the location and timing of domestication for several species, such as horses (Librado et al. 2021, 2024) and chickens (Peters et al. 2022). The power of archaeogenomics for improved understanding of animal domestication is immense (Frantz et al. 2020) but limited by the availability of well-preserved osseous remains that harbour sufficient amounts of ancient DNA molecules from the animal species of interest (Dabney et al. 2013).

Ancient DNA molecules directly preserved in sediments may offer an alternative to, and complement, studies based on skeletal assemblages. First reported within Late Pleistocene permafrost contexts (Willerslev et al. 2003), ancient DNA molecules are now known to potentially be maintained in environmental sediments for over the entire domestication timeline and up to 2 million years (Kjaer et al. 2022), provided favourable preservation conditions. Their characterisation through the amplification and sequencing of mini-barcodes is problematic due to the extensive fragmentation altering DNA molecules post-mortem (Ziesemer et al. 2015). Although it allows for the identification of entire communities present in a given sample, shotgun sequencing is economically restrictive and associated with extreme computational running times (Pedersen et al. 2015). Target-enrichment approaches, which focus sequencing efforts on a subset of DNA markers of interest (Kozarewa et al. 2015) have, thus, provided a cost-effective alternative for tracking population turnovers (Vernot et al. 2021) and changes in the faunistic composition of cave environments over tens of thousands of years, from only a few grams of sediments (Slon et al. 2016). This approach was recently applied to Canadian permafrost sediments from the Klondike goldfields of Yukon to identify diverse metagenomic spectra of Pleistocene fauna (Murchie et al. 2022).

One common limitation of such hybridisation-based genome reduction techniques pertains to probe design and synthesis, which entail significant costs that are commensurate with the number of loci targeted. In this study, we developed a probe assay for characterising the diversity of maternal lineages present in a given environmental sample for the most economically important domesticated animals: cattle, chickens, dogs, donkeys, goats, horses, pigs and sheep. While encompassing the entire range of mitochondrial variation reported in these species, our design limits the total number of probes required by targeting mitochondrial haplotypes, rather than genotypes (Mathieson et al. 2015), within 80-mer RNA oligonucleotides. Given that mitochondrial DNA is present in hundreds to thousands of copies per cell, focusing on this marker also increases the chances of post-mortem preservation and detection (Pääbo et al. 2004).

We first demonstrated the performance of our approach using 35 ancient specimens preserved in a range of environments and spanning the last ~36,000 years (Table S1). We further validated our assay by retrieving mitochondrial DNA signatures of farm animals from modern soil sediments ('La petite ferme de Portet', Toulouse, France), as well as Holocene rock shelter sediments (Abrigo de la Malia, Spain) and Late Pleistocene coprolites (40,000–43,000 BP; Cassenade site, France) (Table S1). Our assay also enables the characterisation of paternal patterns of DNA variation in horses and can be easily extended to any

other domesticated species for which worldwide panels of Y-chromosomal haplotypes are available.

2 | Material and Methods

2.1 | Probe Design

We screened the literature to collect an extensive representation of full mitogenome sequences from a total of eight species of domesticated animals, namely cattle (*Bos taurus*), chickens (*Gallus gallus*), dogs (*Canis familiaris*), donkeys (*Equus asinus*), goats (*Capra aegagrus hircus*), horses (*Equus caballus*), pigs (*Sus scrofa domesticus*) and sheep (*Ovis aries*) (Table S1). We then added the mitochondrial haplotypes from three equid and three canid outgroups (*Equus hemionus*, $n = 1$; *E. africanus somaliensis*, $n = 1$; *E. hydruntinus*, $n = 1$; *Canis latrans*, $n = 5$; *C. himalayensis*, $n = 2$ and *C. lupus signatus*, $n = 2$, respectively) to the *E. asinus* and *C. familiaris* data sets to increase the chances of detecting unreported divergent genetic variation. Given that domesticated animals are generally managed in close proximity to human settlements and that the coprolites analysed may have contained human DNA, we also added to this dataset 476 full mitogenome sequences from hominins, including 433 *Homo sapiens sapiens* with a worldwide distribution, 34 *Homo sapiens neanderthaliensis*, 7 *Homo altaiensis*, and 2 *Homo heidelbergensis*. As one of our long-term objectives is to apply our technology to sedimentary cores from Pyrenean lakes, where brown bears (*Ursus arctos*) were recently reintroduced but lived until 2004 (Palazón 2017), we also included a total of 409 bear mitochondrial sequences.

Independent multiple sequence alignments (MSA) were built for each of the 8 species of domesticated animals (plus their outgroups) and hominins using MAFFT version 7.453 (Nakamura et al. 2018), and manually curated with AliView version 1.28 (Larsson 2014) and Seaview version 4.7 (Gouy et al. 2010). A maximum likelihood (ML) tree was then generated using IQ-Tree version 2.0.3 (Minh et al. 2020), with 1000 bootstrap pseudoreplicates (option-b 1000) for each data set and visualised in iTOL version 6.7 (Letunic and Bork 2024). The resulting tree topologies were manually compared with those published to confirm the haplogroup structure within each species group. MSAs were used to identify those biallelic Single Nucleotide Polymorphisms (SNPs) private to each individual haplogroup, which were used to prioritise probe design.

In the first step, we defined a set of 90 bp long primary probes centred on every SNP defining a haplogroup, considering as candidate probes all reported haplotypes starting with 45 and 44 nucleotides prior to the underlying SNP position. This first step was necessary to account for the possible presence of deletions in specific MSA regions. DNA variation consisting of gaps larger than three nucleotides and/or present only once was disregarded. In the second step, we defined a set of 80-mer candidate probes by further trimming each individual 90-mer symmetrically from both ends. Those probes showing %GC values within the 0.33–0.6 range were retained. In the case where two or more probes were available for the target SNP, the one showing the highest entropy value, indicative of greater information content, hence, sequence complexity, was preferentially selected (otherwise, the selection was random), delivering the final set of 80-mer probes. Our procedure resulted in a final set of 306–1686 probes per species group (Table S2). The sites overlapping with probes in each MSA were sub-selected for ML tree reconstruction in IQ-Tree, following the same procedure as above, to confirm their capacity to accurately identify the entire range of DNA variation.

A total of 18,780 probes targeting Y-chromosomal variation in horses were designed by applying the same procedure to an MSA containing 403 horse Y-chromosomal sequences (272 ancient and 131 modern; Fages et al. 2019; Librado et al. 2021, 2024), with the exception that 60-mers (adapted from Cruz-Dávalos et al. 2017) were considered instead of 80-mers. The mitochondrial and Y-chromosomal probe sequences were then sent to Arbor Biosciences, Ann Arbor, USA, for production as two independent sets of RNA baits without any additional filtering. These sets are provided as Files SX and SY.

2.2 | Sample Materials

In order to test the performance of our target-enrichment assay to retrieve a full range of mitochondrial haplotypes, we selected a total of 19 ancient equine samples (17 of which were previously characterised; Librado et al. 2021), and four ancient samples for the following species: chickens, donkeys, goats and sheep (Table S1). We also collected six soil samples (hereafter referred to as Soil1, Soil2, etc.) from 'La petite ferme de Portet', a small farm located in Toulouse, France, where 12 goats, 11 sheep, 2 oxen, as well as 1 donkey, 1 horse, and multiple chickens were resident (Figure 3). Around 1–2 g of the surface soil samples were collected into sterile 5 mL tubes with sterile scalpels from different parts of the farm (Figure 3). Sample tubes were then immediately wiped with DNA Away and were then wrapped in paper soaked in DNA Away before packing them in a sterile plastic bag. The samples were stored and transported in a portable freezer to the lab where they were kept at −20°C temperature before DNA extraction. These soil samples provided positive controls for testing our approach to environmental samples.

We also accessed 21 coprolite samples from Cassenade (hereafter referred to as CASDO08-CASDO25, CASDO27-CASDO28 and CASDO30-CASDO31), a site located in southwestern France, in the municipality of Saint-Martin-des-Combes (Discamps et al. 2019). These hyena coprolites were recovered in a layer of radiocarbon dated to 40,000–43,000 BP. Faunal material from this layer mostly belonged to *Equus ferus*, representing ~35% of the identifiable remains, Bovinae (24%) and *C. crocuta* (17%), alongside 12 other species (Discamps et al. 2019; Ruebens et al. 2024). These provided an additional control to test the capacity of our assay to retrieve genuine faunal genetic signatures. Finally, we collected 37 sediment samples from the rock shelter Abrigo de la Malia, Tamajón, Spain (Sala et al. 2024), spanning a total of 5 stratigraphic layers from 35 ka to the Holocene. Samples were collected by discarding the first centimetre of the vertical surface exposed to open air with a first disposable scalpel and using a second sterile disposable scalpel to transfer 2–2.5 g of unexposed sediment into sterile 5 mL tubes. The tube's external surfaces were immediately rinsed with DNA Away before being placed into individual sterile bags wrapped in paper soaked in

DNA Away to avoid any contamination while carrying the tubes back to the laboratory.

2.3 | DNA Extraction and Library Preparation

For archaeological bone and coprolite samples, ancient DNA extraction and DNA library construction were carried out in the ancient DNA facilities of the Centre for Anthropobiology and Genomics of Toulouse (CAGT), France. The methodology largely followed the procedures fully described by Seguin-Orlando et al. (2021) and Librado et al. (2021). For bones, a total of 100–590 mg of osseous material was powdered using the Mixel Mill MM200 (Retsch) Micro-dismembrator. For coprolites, a piece of 150–350 mg was cut off from the inner part of the sample using an Argofile diamond wheel and was powdered manually using a sterile mortar. In both cases, the powder obtained worked as starting material for DNA extraction using the Y2 procedure from Gamba et al. (2016) designed to maximise the recovery of the shorter DNA fragments. DNA extracts were treated with the USER enzymatic mix to limit the impact of post-mortem DNA damage on downstream sequence analyses before double-stranded DNA libraries were constructed using the triple indexing protocol from Fages et al. (2019), in which two internal indexes are added on both template ends during adapter ligation, plus one external index during PCR amplification. Amplified DNA libraries were purified using AMPure XP magnetic beads (Beckman Coulter) with a 1:1 bead–to–buffer ratio and were quantified using Qubit (ThermoFisher Scientific), and size profiled using the TapeStation instrument 4200 (Agilent). Finally, 20–50 DNA libraries, each carrying each of the three indexes only once, were pooled for low-depth sequencing either on the Illumina MiniSeq instrument at the CAGT, France (paired-end mode, 2×80, for bone samples) or on a NovaSeq X instrument at SciLifeLab, Sweden (paired-end mode, 2×150, for coprolites). The sediment samples from Abrigo de la Malia and the soil samples from 'La petite ferme de Portet' were treated applying the same protocol to 180–350 mg of soil/sediment as starting material in a lab dedicated to processing environmental samples at CAGT. These samples were also sequenced on the Illumina MiniSeq instrument at the CAGT, France (paired-end mode, 2×80).

2.4 | Hybridisation Capture

A total of 4–23 uniquely triple-indexed libraries were mixed at an equimolar concentration within 7 pools, each exceeding 100 ng. Samples were pooled based on the target animal species, with each pool containing four samples initially. This strategy resulted in 5 pools for Horse samples ($n = 20$) and one pool for every other species of interest. After the successful capture of 4 sample pools, we pooled 6 sediment samples (including extraction blanks) from Abrigo de la Malia. We further increased the number of samples in a pool to 15 in an experiment that is not included here and is part of another study. Finally, we combined DNA from 23 coprolite samples in one pool. These pooling strategies were simultaneously applied to mitochondrial as well as Y-chromosomal probes. The pools were purified and concentrated to 10 μL final volume using Min-Elute columns (QIAgen) before closely following the myBaits Manual version 5.03 provided by Arbor Biosciences for carrying out target enrichment. In brief, we prepared for each pool a 20 μL volume of hybridisation mix comprising 4.4 μL of baits, 9.25 μL of Hyb N, 3.5 μL of Hyb D, 0.5 μL of Hyb S, 1.25 μL of Hyb R and 1.1 μL of nuclease-free sterile water. This mix was incubated for 10 min at 60°C. A volume of 5 μL of blocker mix consisting of 2.5 μL each of Block O and C and 0.5 μL of Block X was prepared before being mixed with 7 μL of pooled DNA libraries for 5 min incubation at 95°C, followed by 5 min at 55°C. A total of 18.5 μL of hybridisation mix was added and incubated at 55°C for 24 h in a thermocycler.

This step was followed by purification through streptavidin-coated magnetic beads (Dynabeads, Invitrogen), eluted in a final volume of 30 μL of total elute that was split in half to carry out two independent amplification reactions using Accuprime enzyme and IS5 and IS6 primers (500 nM each; Meyer and Kircher 2010). PCR conditions consisted of 5 min denaturation at 95°C, followed by 14 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 68°C and ended with a final elongation for 5 min at 68°C. Parallel amplification reactions were pooled and purified on MinElute columns (QIAgen), and eluted in a final volume of 10 μL EB. A total of 7 μL of such purified amplifications were used in a second hybridisation capture round, using the same conditions as above, except that only 1.1 μL of baits was used while preparing the hybridisation mix. The 30 μL eluate resulting from Dynabeads purification was halved, and one half was PCR amplified for 8 cycles, and purified using AMPure XP beads as described above. Library profiles and concentrations were estimated using the TapeStation 4200 instrument (Agilent) and Qubit HS dsDNA assay (Invitrogen), before pooling at an equimolar concentration for sequencing on the Illumina MiniSeq instrument (paired-end mode, 2×80).

2.5 | Sequence Data Analysis

Raw sequence FASTQ files were demultiplexed on the basis of the two internal indices using AdapterRemoval2 (Schubert et al. 2016; –-barcode-mm-r1 1 --barcode-mm-r2 1 --minlength 25 --trimns --trimqualities --minadapteroverlap 3 --mm 5), which generates 'collapsed' single reads for read mates showing significant sequence overlap, as well as 'collapsed truncated' reads, if collapsed but low base quality scores at read ends require further trimming, and 'paired' read for those remaining read mates. Shotgun and captured sequenced data were compared using the same number of collapsed reads, randomly down-sampling for the condition associated with the greatest number of reads. Reads were aligned against the reference genomes corresponding to the species of interest using Bowtie2 version 2.3.5.1 (Langmead and Salzberg 2012), with the parameters recommended by Poullet and Orlando (2020), through the Paleomix version 1.2.13.2 pipeline (Schubert et al. 2014). Final BAM read alignment files was filtered for PCR duplicates and minimal mapping quality Phred scores of 25. Post-mortem DNA damage signatures were assessed using mapDamage version 2.2.1 (Jónsson et al. 2013) (Figure S1).

The following statistics were calculated for the shotgun and post-capture libraries samtools version 1.10 (Li et al. 2009) and bedtools version 2.29.2 (Quinlan and Hall 2010) commands (e.g.,

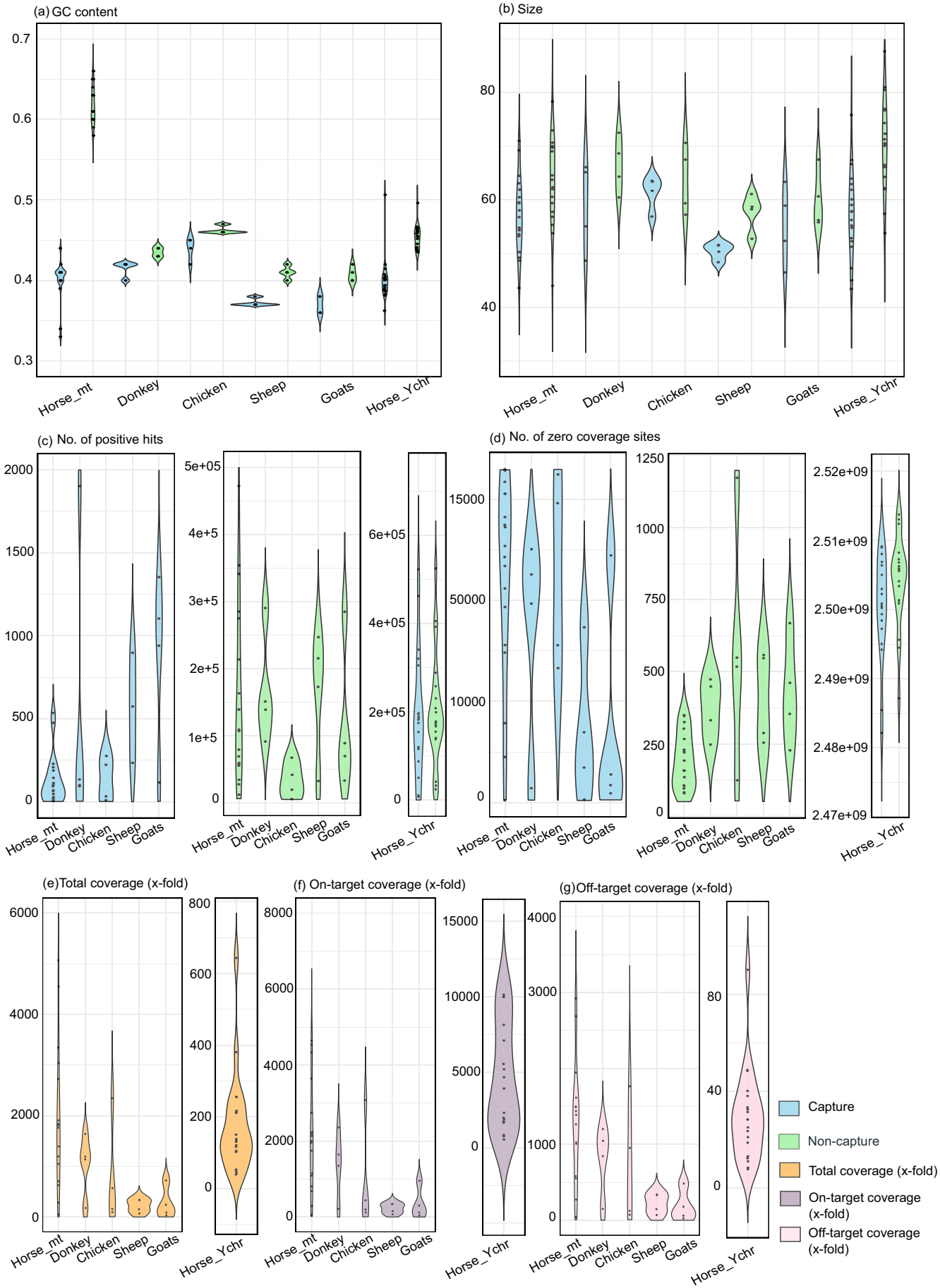

FIGURE 1 | Legend on next page.

FIGURE 1 | Enrichment capture vs shotgun comparison performed after normalisation of sequencing efforts. (a) average GC content (percentage, max = 1), (b) average size of recovered sequences, (c) Number unique positive hits (mapping quality > 25), (d) number of sites with zero coverage, enrichment fold increase in (e) total coverage (X-fold), (f) on-target coverage (X-fold) and (g) off-target coverage (X-fold).

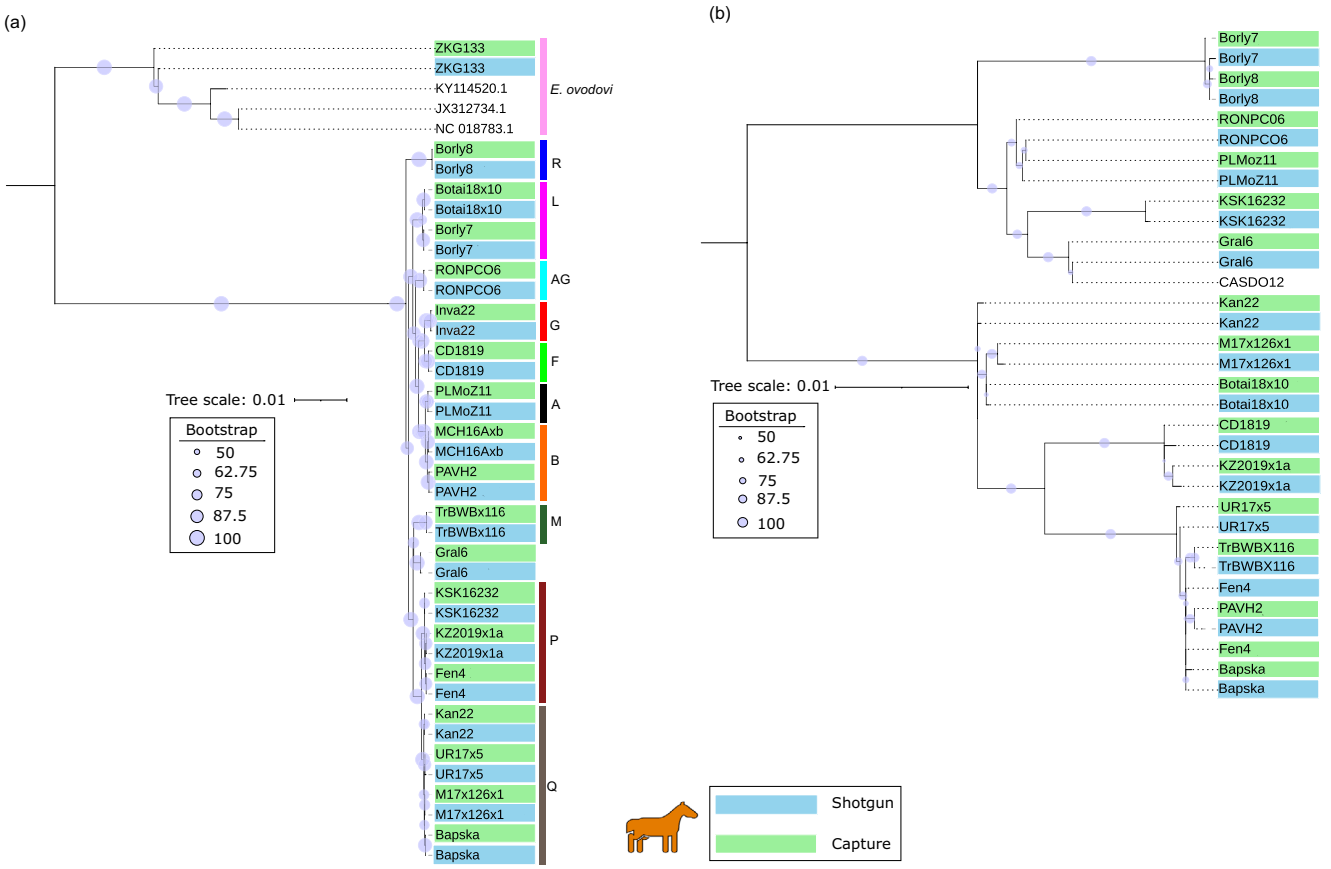

FIGURE 2 | IQ-TREE maximum likelihood (ML) phylogenetic reconstructions. (a) Mitochondrial and (b) Y chromosomal sequences recovered from shotgun and captured samples occupied identical phylogenetic placements on the *Equus caballus* phylogenetic tree (each coloured bar indicates an individual haplogroup).

view, depth) in combination with in-house Perl scripts: total number of target hits, total number of zero coverage hits, size of mapped reads, on-target and off-target coverage, and %GC content for each sample set (Figure 1, Tables S3 and S4). The extent of daisy chaining (Cruz-Dávalos et al. 2017) was investigated by following the drop of sequence coverage with increasing distances from the closest probe (Figure S2a). We also examined whether the depth of coverage per position was a function of the number of probes for that species (Figure S2b), and whether the number of reference vs. alternate alleles sequenced at each polymorphic site for a given sample was highly skewed, indicating the presence of a single haplotype, or balanced, indicating the coexistence of several haplotypes (Table S5).

Saturation curves were generated using the preseq (Daley et al. 2014) c_curve command with sequence number increments of 1000. These curves were used to assess the fraction of new, unique high-quality alignments that would be gained from increasing sequencing efforts; hence, to estimate the minimal sequencing effort that would have delivered 95% of the total number of high-quality alignments characterised in each sample (Figure S3).

Consensus mitochondrial sequences were called following Librado et al. (2021) and using Bcftools (Danecek et al. 2021), with the mpileup command (considering base phred quality scores of at least 30), followed by a normalisation step through the norm command (−c x -d all), and flagging sites showing FMT/GQ ratio < 3 and DP < 5 for final filtering with the filter command. The resulting VCF file was indexed using tabix version 1.7-41-g816a220 (Li 2011), and the genome sequence was converted into fasta format using the vcf_to_fasta command from the PALEOMIX pipeline (Schubert et al. 2014). FASTA sequences obtained from captured and uncaptured libraries were added to the MSA representative of the global mitogenome diversity of each respective species (Figure S4), using MAFFT version 7.453 (Nakamura et al. 2018), or restricted to a subselection including an outgroup and pairs of shotgun and captured haplotypes (Figure 2a,b). Maximum likelihood trees were constructed using IQ-Tree version 2.0.3 (Minh et al. 2020), with

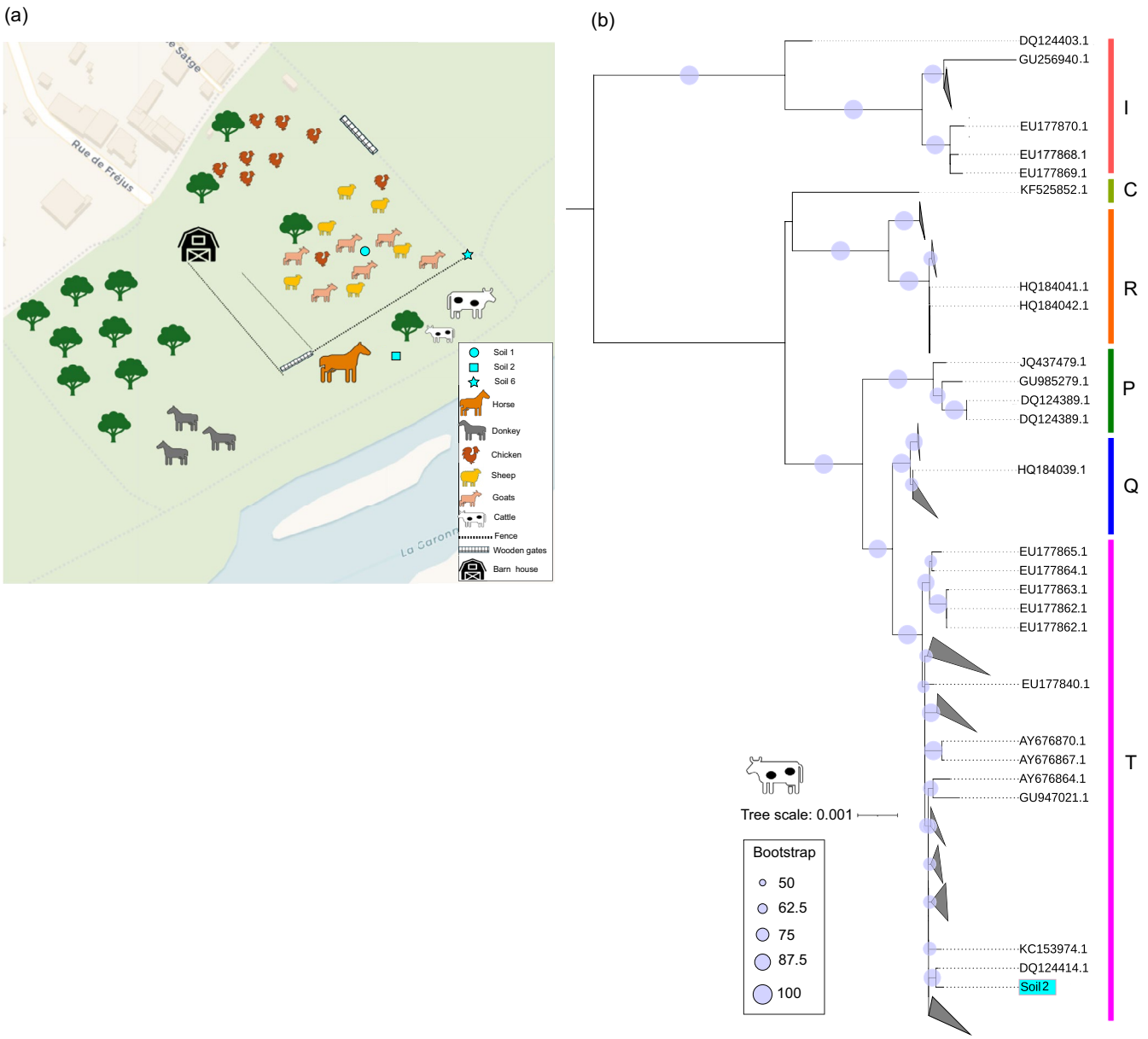

FIGURE 3 | Assay validation using soil samples from 'La petite ferme de Portet', Toulouse, France. (a) Schematic design of animal arrangements on site. The Soil1 sample was taken from the area mainly grouping sheep and goats, versus horses, donkeys and cattle for the Soil2 sample. Soil6 was taken from near the fences. (b) ML phylogenetic reconstruction of cattle mitochondrial variation, including the mitogenome recovered from the Soil2 sample, which clusters within haplogroup T, especially in sub-haplogroup T3, which is the most common haplogroup of domestic cattle in Europe (each coloured bar indicates an individual haplogroup).

1000 bootstrap pseudo-replicates (option-b 1000) and were visualised in iTOL version 6.7 (Letunic and Bork 2024).

Although no hyena sequences were considered while designing probes, we aligned the coprolite sequence data against a complete mitogenome from the *C. crocuta* species (Accession Nb. NC_020670.1) and carried out phylogenetic reconstruction based on all complete mitochondrial sequences available from NCBI. As 24% of the faunal material in the layer from which hyena coprolites were recovered belonged to Bovineae, we hypothesised that hyenas could have preyed upon a bovine. Hence, we also aligned coprolite sequence data against a complete mitogenome of European *Bison bonasus* (NC 014044.1). We further carried out phylogenetic reconstruction including published mitogenomes of other members of the genera Bison (*B. priscus*, *B. bison* and *B. schoetensacki*) and Bos (*B. taurus*, *B. mutus* and *B. grunniens*).

Horse Y-chromosome sequence data were processed as described by Librado and colleagues (Librado et al. 2021, 2024), except that (1) base quality scores within individual read alignments were not downscaled according to the presence of post-mortem DNA damage and (2) alignment termini were not trimmed. The number of sites associated with no coverage was estimated relative to the total number of sites overlapping the list of '*single copy*' Y-chromosomal loci identified by Felkel et al. (2019), representing a total of 5,834,017 bases from 751 contigs. Y-chromosomal phylogenies were constructed from pseudo-haploidised data,

following the procedures from Librado et al. (2021), disregarding sites covered in less than 10% of the samples and using IQ-Tree v2.0.3 (Minh et al. 2020), as described above.

3 | Results

3.1 | Probe and Study Design

We assembled an extensive sequence data set of complete mitochondrial genomes spanning worldwide patterns of genetic variation in eight animal species, with individual haplotypes ranging from 217 (goats, *C. aegagrus hircus*) to 1958 (dogs, *C. familiaris*) per species (Table S2). These sequences were complemented with those from outgroups to maximise the chances of probe annealing to divergent sequences not yet characterised amongst the ancient and modern individuals presently sequenced. Combined with maximum likelihood (ML) phylogenetic reconstruction, multiple sequence alignments per species were used to identify diagnostic SNP markers for haplogroup assignment, representing a total of 5 (donkeys) to 38 (horses) haplogroups per species. Encompassing the whole mitochondrial diversity range of ancient and modern horses resulted in the identification of 20 new haplogroups for horses, complementing the 18 previously described on the basis of modern DNA variation alone (Achilli et al. 2012). Although the data sets included a large number of sequences, our probe design procedure focused on diagnostic SNP markers only, leaving a reasonable number of 80-mer RNA probes for synthesis (i.e., 306–1686 per species; Table S2), which minimises production costs.

We next subjected a range of 35 ancient biological remains, including 1 museum specimen and 34 archaeological bones and teeth, to our mitochondrial capture assay, following two rounds of probe-to-library annealing in order to assess the probe's capacity to enrich divergent mitochondrial haplotypes. The sample panel included 18 horses, 1 *Equus ovodovi*, 4 chickens, 4 donkeys, 4 goats and 4 sheep from across Europe, Russia, Central and Southwest Asia, and South America, dating back from the Late Pleistocene (horse sample Gral6, from Igue du Gral, France) to the 18–20th century (chicken sample OL2347) (Table S1). The performance of our capture assay was assessed relative to the sequence data obtained following shotgun DNA sequencing of the same DNA libraries, following the normalisation of sequencing efforts. More specifically, we assessed mitochondrial coverage (on- and off-target, and number of sites remaining uncovered), enrichment folds, and possible shifts in the size and base compositional profiles of endogenous DNA following previous work (Suchan, Chauvey, et al. 2022; Suchan, Kusliy, et al. 2022).

We also aimed to examine the performance of our capture assay on non-skeletal remains, including Late Pleistocene coprolites (from the Cassenade cave, France), modern farm soils (from 'La petite ferme de Portet', Toulouse, France), and Holocene cave sediments (from Abrigo de la Malia, Spain). As such samples may hold DNA from a range of hominins, we extended our probe design to a total of 476 complete mitochondrial sequences from modern and ancient hominins (including 34 Neanderthals, 7 Denisovans, and 2 *H. heidelbergensis*). As one of our long-term objectives intends to apply these probes on samples collected from lakes in the Pyrenees mountains where brown bears (*U. actos*) were recently reintroduced but lived until 2004 (Palazón 2017), we also included 409 complete mitochondrial sequences of brown bears (Table S2). This resulted in the addition of 1686 and 1647 80-mer RNA oligonucleotides to our probe panel, respectively, targeting 428 and 497 diagnostic SNP markers defining 67 and 11 main mitochondrial haplogroups (File SX). Finally, we also applied our probe design procedure to a Y-chromosomal sequence data set consisting of 272 ancient and 131 modern horses, to target 2270 diagnostic SNP markers for haplogroup assignment from 18,780 individual probes (File SY). This was carried out as a proof of principle to assess the capacity of our approach to also retrieve nuclear sequence data.

3.2 | Assay Validation

All of the 35 ancient biological samples tested returned sufficient sequence data following both shotgun and mitochondrial capture (Figure 1; Tables S3 and S4). Post-capture sequence alignments showed the hallmark of post-mortem DNA degradation following USER treatment (Rohland et al. 2015; Orlando et al. 2021), including an over-representation of cytosines at the reference genome position preceding read starts, and rampant C → T nucleotide mis-incorporations at read termini (Figure S1). These results confirm that the capture assay designed in this study succeeded in retrieving authentic ancient DNA sequence data from the full range of samples examined.

While shotgun sequencing largely failed to characterise mitochondrial genome sequences (only 2 out of 35 samples returned average depth-of-coverage ≥ 5×; Table S3), the average depth of coverage obtained post-capture was at least 19.4× and reached up to 2003.7× (median = 583.0×; Table S4), despite similar sequencing efforts (Figure S2). As a result, the number of mitochondrial sites associated with no sequence data considerably dropped post-capture, effectively allowing for the characterisation of pseudo-complete mitochondrial genome sequences (Figure 1). While on-target regions showed a greater coverage than off-target regions, off-target mitochondrial regions showed non-zero coverage post-capture due to daisy chaining reactions during probe-to-library annealing. This corresponds to enrichment fold between 41.3× and 10,020.2× (median = 1348.8×), restricting the analysis to those high-quality unique alignments identified (Figure 1). Patterns of depth-of-coverage variation indicated that positions located 125 nucleotides away from the closest probe still showed at least 50%–80% of the average depth-of-coverage measured on-target (Figure S2a). This suggests a significant range for daisy chaining, in line with previous reports using 60-mer RNA oligonucleotides showing inflated coverage relative to shotgun sequencing 100 nucleotides away from the closest probe (Cruz-Dávalos et al. 2017).

Interestingly, positions targeted by more than one probe did not show a greater depth of coverage on average than those targeted with one single probe (Figure S2b), suggesting that most of the DNA mitochondrial templates present in the individual libraries were successfully enriched and sequenced. In fact, saturation curves revealed that 95% of all unique high-quality alignments would have been characterised by more limited sequencing efforts (from 36.2% to 88.9% of the current sequencing effort; median = 55.0%). Combined with the fact that pools of 4–23 DNA

libraries were co-captured, our approach to characterising complete mitochondrial genomes translates into minimal extra experimental costs, corresponding to approximately 60–130 euros per sample depending on the number of libraries in a pool, if sequenced with Miniseq. These costs would be further reduced by using other DNA sequencing instruments, e.g., NovaSeq X 10B-25B.

We noticed that the capture assay resulted in shifting significantly both the size distribution and the %GC content of those endogenous DNA templates sequenced upwards (Table S3). This is in line with previous reports (Cruz-Dávalos et al. 2017; Cruz-Dávalos et al. 2018; Suchan, Chauvey, et al. 2022, Suchan, Kusliy, et al. 2022) and confirms that size and higher %GC content are among the factors enhancing the stability of probe-to-library annealing reactions in the stringency parameters of our experiments. Finally, we explored the distribution of reads supporting the presence of different alleles at those SNP markers diagnostic for haplogroups. We observed that virtually all such sites showed a majority of reads (≥95%) sharing the same allele (Table S5), which indicates that only one single mitochondrial genome was sequenced per individual, as expected.

Combined, our results establish the performance of our DNA capture assay in characterising the pseudo-complete mitochondrial genome sequence across a wide range of domesticated animal species. We also observed that shotgun sequence data occupied identical phylogenetic placements (Figure 2, Figure S4), and that the samples captured in this study could be successfully assigned to a full range of haplogroups. For example, the two clades structuring worldwide patterns of donkey mitochondrial diversity were retrieved (Figure S4a), haplogroup E for chickens (Figure S4b), as well as mitochondrial haplogroups A and B for sheep (Figure S4c), A and C for goats (Figure S4d), and no less than 10 haplogroups for horses (Figure 2a).

Similar performance was observed using the Y-chromosome probe set for horses (Table S4), including enrichment-folds between 513.1-fold and 10,134.7-fold, increased sequence coverage ON-target propagating away from probes due to daisy chaining reactions, and significant shifts towards DNA templates of longer sizes and greater %GC content (Figure 1). In ML phylogenetic reconstructions, the capture sequence data also grouped together with the Y-chromosomal haplotypes previously characterised through shotgun sequencing with considerably larger sequencing efforts (Librado et al. 2021, 2024) (Figure 2b). The ML tree of Y-chromosomal variation also spanned the whole range of haplogroups examined, including those segregating prior to domestication, confirming the suitability of our approach to retrieve the whole range of paternal genetic diversity in horses.

3.3 | Soil, Sediment and Coprolite Samples

We next applied our capture assay to more complex material, including soil, sediment, and coprolites, to assess its potential for retrieving genuine genetic information from environmental samples (Table S3). Six soil samples were collected from the farm 'La petite ferme de Portet', focusing on specific enclosures in which various farm animals were kept. Soil samples Soil3, Soil4 and Soil5 showed extreme PCR inhibition even after 10–50× dilutions, hence, were not considered for analysis. Two of the 6 soil samples (Soil1 and Soil2) returned sequence data aligning with the species kept nearby, including goat and sheep mitochondrial DNA for Soil1 (average depth of coverage = 53.9× and 567.6×, respectively), and horse (Figure S5) and cattle (Figure 3a) mitochondrial DNA for Soil2 (average depth of coverage = 10.5× and 19.2×, respectively). Sample Soil6 did not yield mitochondrial genome data with reliable coverage (< 5×), and was, thus, not included in the analysis.

No sequence data were obtained when enriching DNA libraries for Y-chromosomal targets, in line with the horse, kept in the enclosure being a draft mare. Additionally, the horse mitochondrial data retrieved post-capture were assigned to haplogroup A (Figure S5). Although horse mitochondrial diversity poorly segregates with breeds, and haplogroup A is not found in high frequency across Eurasia (Achilli et al. 2012), it has been reported in a variety of breeds, including draft horses such as Percherons, suggesting that the DNA signature retrieved most likely originated from the animal kept in the enclosure (Figure 3 and Figure S5).

More strikingly, ML phylogenetic placement indicated that the cattle, goat and sheep mitochondrial sequences belonged to haplogroup T3, A and B respectively, which correspond to the three most prominent mitochondrial haplogroups for these species in Europe (Figure S4) (Ginja et al. 2019; Yi et al. 2022; Machová et al. 2022). Furthermore, the read distribution at SNP markers diagnostic for haplogroup assignment indicated that only one mitochondrial haplotype was sequenced, which suggests that the DNA detected originated from only one individual or relatives within the same maternal kin, or from multiple unrelated individuals sharing the same haplogroup. Regardless, our combined results demonstrate that our capture assay can successfully detect mitochondrial DNA from farm animals in fresh soil sediments.

Only a subset of samples representative ($n = 5$) of the cave stratigraphy amongst the 37 cave sediments collected at Abrigo de la Malia were subjected to capture, with a single sample (AMN21) delivering sufficient sequence data mapping against some of the mitochondrial genomes of the domesticated animal species investigated here (Table S3). This sample belonged to the most recent, Holocene, stratigraphic layer and may include reworked sediments from the 19th century CE, as indicated by the discovery of metal nails during excavation. The mitochondrial sequence was characterised by an average 5.4× depth of coverage and belonged to Sheep haplogroup B (Figure S4c), the most common in western Europe (Machová et al. 2022), confirming possible DNA sources dating from the Cardial Neolithic expansion from Anatolia to the modern period.

One Late Pleistocene coprolite sample (CASDO12) from the Cassenade cave returned substantial mitochondrial and Y-chromosomal sequence data (Tables S3 and S4) with typical signatures of ancient DNA post-mortem damage (Figure S1f), leading to an average depth of coverage of 485.0× (mtDNA) and 0.6× (on-target Y-chromosomal coverage), compatible with ML phylogenetic reconstruction. While the mitochondrial data did not cluster with any previously reported haplotype (Figure S5),

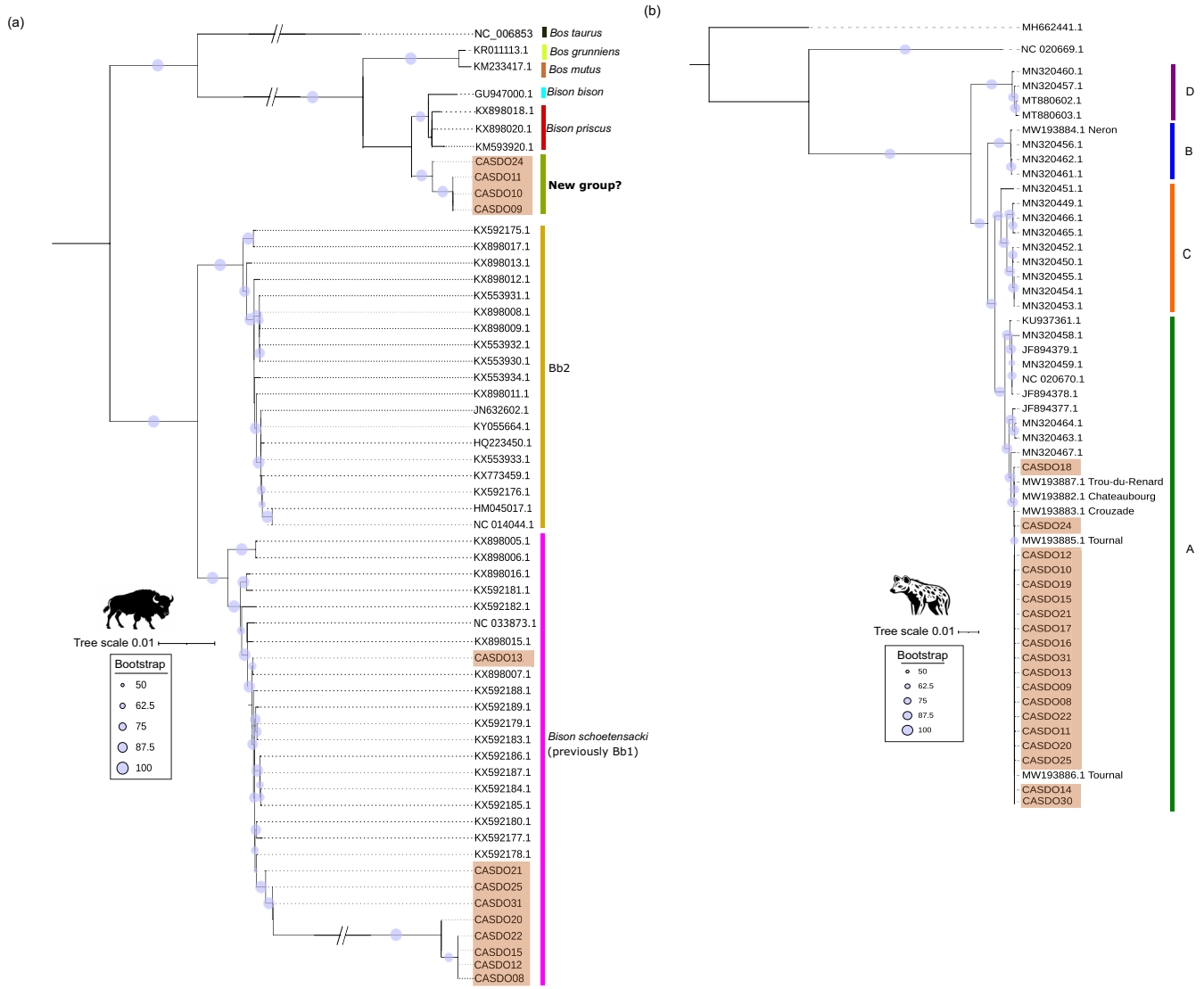

FIGURE 4 | Assay validation using cave hyena coprolite samples from Cassenade cave, France. (a) Maximum likelihood (ML) phylogenetic reconstruction of *Bison* and *Bos* mitochondrial variation, including the mitochondrial sequence data retrieved from 13 Cassenade coprolites. (b) ML phylogenetic reconstruction of *Crocuta crocuta* mitochondrial variation, using *Proteles cristata* as an outgroup, and including the mitochondrial sequence data retrieved from 19 Cassenade coprolites (each coloured bar indicates an individual haplogroup).

the Y-chromosomal data co-clustered with those from another Late Pleistocene horse excavated at Igue-du-Gral, France, located ~218 km away from Cassenade. This suggests that the hyena individual who defecated the coprolite preyed upon an individual belonging to a now-extinct local lineage of wild horses.

Interestingly, we mapped the coprolite data against the complete mitochondrial sequence from a *B. bonasus* individual (NC 014044.1) and retrieved an average depth of coverage greater than 5× for a total of 13 samples. ML phylogenetic reconstruction revealed that these samples belonged to two different clusters, with nine specimens clustering with the Bb1 haplogroup, alongside samples from both Western and Eastern Europe (Massilani et al. 2016) (Figure 4a). This group exclusively comprises members of the extinct *B. bonasus* population that has been suggested to represent a different species (*B. schoetensacki*) (Palacio et al. 2017). The other 4 bison mitogenomes characterised grouped within a clade composed of both *B. priscus* and *B. bison* haplotypes and were distant from both the extant and extinct *B. bonasus* previously sequenced. This is suggestive of another extinct mitochondrial lineage of European bison.

In an attempt to test for the presence of hyena DNA within the Cassenade coprolites, we mapped the sequence data generated post-capture against the complete mitochondrial genome sequence of an extinct cave hyena (*C. crocuta*; NC020669.1). This returned 19 positive coprolites (> 5× coverage), with haplotypes sequenced at an average depth of coverage ranging between 5.2× and 365.6× (Table S6), and DNA reads showing typical signatures of post-mortem DNA damage (Figure S1g). In ML phylogenies, all of the recovered haplotypes clustered within members of haplogroup A (Figure 4b), which was previously identified in coprolite specimens from other Late Pleistocene caves in southeastern France (i.e., Chateaubourg, La Crouzade, Le Figuier, Tournal, and Trou-du-Renard) (Elalouf et al. 2021), all located within ~200–500 km from Cassenade.

Combined, the *B. bonasus* and *C. crocuta* sequence data retrieved from Late Pleistocene coprolite demonstrate the performance of our capture assay to detect the presence of mitochondrial DNA from species that were not included during probe design. This most likely results from a combination of daisy chaining reactions and the presence of probes from relatively close species, such as *B. taurus* for bison (1.65 million years divergence; Zeyland et al. 2012), and the wide range of animal groups represented during design, since the divergence of canids and hyenas is estimated to be ~53.9 million years ago (Yang et al. 2020). The versatility of our capture assay to enrich for DNA material from outside the strict panel of species included during probe design is also confirmed by the sequence data generated on one sample from a now-extinct equine species (specimen ZKG133 from *E. ovodovi*), a member of the stenonine lineage that diverged from the caballine horse lineage approximately 4.5 million years ago (Orlando et al. 2013). The data collected were indeed sufficient to characterise a pseudo-complete mitochondrial haplotype at an average depth of coverage of 197.9× (Table S3), unambiguously grouping with the other species members previously sequenced (Figure 3a) (Vilstrup et al. 2013; Cai et al. 2022).

4 | Discussion

In this study, we developed a set of 80-mer RNA probes for characterising the mitochondrial DNA variation in eight domesticated animal species, representing those of most important economic interest. Our capture assay demonstrates excellent performance across a full array of ancient and modern samples, including hair, teeth, bones, coprolites, soil, and sediments. With enrichment folds typically ranging between 100- and 1000-fold (but reaching 10,020× in one Bronze Age horse specimen dating back to around 1950 BCE; Table S3), the experimental procedures described allow for an economical retrieval of genetic data from samples whose endogenous DNA preservation levels may be limited. We recommend that no more than 0.5–1 million read pairs are produced and up to 23 DNA libraries are pooled to characterise pseudo-complete mitochondrial sequences at minimal production costs.

The sequence data collected confirmed the phylogenetic placement of all specimens previously analysed with more extensive sequencing efforts. These were selected to span the wide range of mitochondrial haplogroup diversity in the various species tested. Additionally, we used the horse as a model to demonstrate that our probe design and methodological framework could achieve similar performance when targeting Y-chromosomal haplogroup variation. The retrieval of divergent Y-chromosomal haplotypes from the metagenomic content preserved in Late Pleistocene coprolites establishes that our approach could indeed detect the presence of genetic diversity that no longer segregates in modern populations, virtually leading to the possibility of monitoring both paternal and maternal contributions to animal domestication. These findings echo those reported by Vernot et al. (2021), who designed a capture assay for the specific retrieval of mitochondrial and nuclear DNA from the traces of genetic material left by ancient human individuals, and even extinct hominins, in cave sediments (Vernot et al. 2021). With the exception of the most recent stratigraphic layer which delivered a sheep mitogenome belonging to haplogroup B, the cave sediments sampled at Abrigo de la Malia returned negative results. Considering the positive results obtained in even older coprolite and osseous remains, we believe that the failure to detect any of the targeted species in pre-Holocene cave sediments at Abrigo de la Malia likely reflects the severely compromised DNA preservation in sediments (Haile et al. 2007; Slon et al. 2017), rather than methodological issues.

In the last decade, ancient DNA analyses have considerably advanced our understanding of animal domestication. These approaches have revised the number and location of domestication centres (e.g., in horses; Gaunitz et al. 2018; Librado et al. 2021, 2024), identified signatures of reproduction control in contexts pre-dating the emergence of standard skeletal markers of domestication (e.g., in goats; Daly et al. 2021), revealed unexpected population turnovers at continental-wide scales (e.g., in dogs, Ní Leathlobhair et al. 2018, and; in pigs, Frantz et al. 2020), and started unveiling global trends of loss in genetic diversity (e.g., in the last 200 years in horses; Fages et al. 2019). Most of the work thus far relied on the characterisation of ancient (mito)genomes through shotgun sequencing, limiting genetic analyses to the subset of archaeological remains showing the best DNA preservation levels. The approach presented here holds the potential to requalify a significant subset of samples for genetic analyses (important from contexts generally associated with poor DNA preservation, such as the Fertile Crescent at the dawn of domestication). By retrieving DNA signatures from environmental and/or metagenomic samples, our approach also opens for a more systematic analysis of the sediments excavated, which could help map the distribution of both maternal and paternal lineages on site and their long-term maintenance, or replacement, following important changes of activity that may otherwise be tractable in the material culture. Such an approach targeting within-archaeological site patterns of genetic diversity in domestic animal species (Perry and Makarewicz 2019) will provide finer-grained resolution into the husbandry practices developed locally by various pastoral groups, thereby advantageously complementing the macroscale approaches that have dominated the DNA scientific literature thus far (Frantz et al. 2020). This approach may also benefit ongoing research aimed at retracing the history of animal resource exchange across trans-continental scales by charting the distribution of the various genetic lineages from archaeological and/or lake sediments through space and time. In addition to retrieving genetic data from the intended species of interest, our assay also delivered high-quality mitochondrial haplotypes from some of their phylogenetic relatives, such as an extinct equid, bison, and cave hyena. The extent to which other taxonomic groups than those currently identified could be detected remains to be tested. For now, our results, however, demonstrate that applying our capture assay to coprolites can at least partially help reconstruct the range of dietary preferences of species such as cave hyenas.

Author Contributions

K.D.M. and L.O. designed and conceived the study. E.D., J.L.G., O.E., H.P., H.C., N.M.G., S.C., C.B., S.B., J.C.-M., D.A.-C., M.F., E.B., L. C., E.R., T.N., R.Z., S.S., E.V., R.B., M.M., P.M.F., R.K., A.N., A.A.V., P.K., J.-L.H., J.B., J.W., M.M.-d.H., M.N., W.T., A.B., R.D., N.B., B.A., S.S., G.M., P.R.Š., M.B., T.T.V., N.A., J.-C.C., M.B.-M., A.P., N.S.B., B.S., C.L.R. and S.C. provided samples. D.W.G.S., V.E.M., K.G.D., D.G.B.,

L.F., G.L. and L.O. provided curated mitochondrial and Y-chromosomal sequence alignments. K.D.M., O.L., A.S.-O., O.E., L.D., L.T.-C., L.C., G.T. and S.S. performed lab work, with input from L.O., K.D.M. and L.O. analysed data. K.D.M. and L.O. prepared the figures and tables. L.O. wrote the manuscript with input from K.D.M. and all co-authors.

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Disclosure

Benefit Sharing Statement: A research collaboration was developed with scientists from the countries providing genetic samples; all collaborators are included as co-authors. Individuals providing farm soil samples have been mentioned in acknowledgements.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Sample metadata and shotgun as well as captured sequences were submitted to the European Nucleotide Archive under bioproject PRJEB79254. Mitochondrial and Y-chromosomal probes are available as Files SX and SY in Dryad at doi:10.5061/dryad.612jm64cr (https://datadryad.org/stash/share/TFOa09rBQ6hsOxNSllwUSjSB3yMb3nnhXhdvGKA9-ng).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.