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Evolutionary history of the extinct wolf population from France in the context of global phylogeographic changes throughout the Holocene

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Abstract

Phylogeographic patterns in large mammals result from natural environmental factors and anthropogenic effects, which in some cases include domestication. The grey wolf was once widely distributed across the Holarctic, but experienced phylogeographic shifts and demographic declines during the Holocene. In the 19th–20th centuries, the species became extirpated from large parts of Europe due to direct extermination and habitat loss. We reconstructed the evolutionary history of the extinct Western European wolves based on the mitogenomic composition of 78 samples from France (Neolithic–20th century) in the context of other populations of wolves and dogs worldwide. We found a close genetic similarity of French wolves from ancient,

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medieval and recent populations, which suggests the long-term continuity of maternal lineages. MtDNA haplotypes of the French wolves showed large diversity and fell into two main haplogroups of modern Holarctic wolves. Our worldwide phylogeographic analysis indicated that haplogroup W1, which includes wolves from Eurasia and North America, originated in Northern Siberia. Haplogroup W2, which includes only European wolves, originated in Europe ~35 kya and its frequency was reduced during the Holocene due to an expansion of haplogroup W1 from the east. Moreover, we found that dog haplogroup D, currently restricted to Europe and the Middle East, was nested within the wolf haplogroup W2. This suggests European origin of haplogroup D, probably as a result of an ancient introgression from European wolves. Our results highlight the dynamic evolutionary history of European wolves during the Holocene, with a partial lineage replacement and introgressive hybridization with local dog populations.

KEYWORDS

ancestry replacement, ancient DNA, dietary shift, diversity loss, domestic dog lineages, grey wolf, local extinction

1 | INTRODUCTION

Climatic and environmental changes have been major factors influencing species ranges, genetic diversity and demographic processes. Population range shifts including repeated contractions to refugial areas during glacial cycles have been shown to be drivers of genetic diversity and phylogeographic patterns observed today in various plant and animal species (Hewitt, 2000; Provan & Bennett, 2008). Population range shifts resulted in changes in frequency of genetic lineages and in some cases in complete lineage replacements. Such patterns were observed for example in woolly mammoths (*Mammuthus primigenius*; Palkopoulou et al., 2013), collared lemmings (*Dicrostonyx* spp.) (Brace et al., 2012; Palkopoulou et al., 2016), cave bears (*Ursus spelaeus*; Münzel et al., 2011) and common voles (*Microtus arvalis*; Baca et al., 2020). In some species, especially those from megafaunal communities, extreme range contractions and demographic declines during the Pleistocene/Holocene transition resulted in extinctions. Around 90 genera of large mammals (>44 kg) become extinct due to climate changes, human activities or a combination of both factors (Koch & Barnosky, 2006). Few large carnivores in the Holarctic survived the Late Pleistocene megafaunal extinctions, and the survivors experienced complex evolutionary histories, which often share similar patterns with the extinct species (e.g. Barlow et al., 2018; Barnett et al., 2020; Liu et al., 2018; Lucena-Perez et al., 2020; Rey-Iglesia et al., 2019; Salis et al., 2021). Factors shaping evolutionary histories of megafaunal species and contributing to either extinction or survival remain unclear (Lorenzen et al., 2011; Meltzer, 2020). For the extant large carnivore species, knowledge of the long-term dynamics of demographic and phylogenetic changes is important to correctly identify current threats to their survival.

Current distribution ranges and phylogeographic patterns in large mammals can be heavily modified as a result of anthropogenic pressures such as habitat modifications and direct hunting, which have increased in intensity in the past few centuries (e.g. Dufresnes et al., 2018; Yackulic et al., 2011). Another important anthropogenic effect on large mammals was domestication of some species, which had a profound effect on phylogeographic patterns in their wild relatives through introgressive hybridization (e.g. Pilot et al., 2019; Scandura et al., 2011). Therefore, reconstruction of evolutionary histories in extant species should take into account both long-term climate-driven range shifts and recent anthropogenic effects.

The grey wolf (*Canis lupus*) is one of the few Holarctic large carnivores that survived the Late Pleistocene megafaunal extinctions and intense hunting pressure in the past few centuries, as well as the only large carnivore that became domesticated. The species had a continuous distribution across the Holarctic until about 200–300 years ago (Boitani, 2003; Dufresnes et al., 2018; Leonard et al., 2005). Despite the capability to maintain this wide geographic range and adapt to diverse habitats, the species has experienced a gradual population decline since the Late Pleistocene (Fan et al., 2016; Loog et al., 2020), involving local extinctions, bottlenecks and phylogeographic shifts (Bergström et al., 2022; Koblmüller et al., 2016; Leonard et al., 2007; Pilot et al., 2010, 2014; Segawa et al., 2022). Both mitochondrial and nuclear genomic data suggest that all modern wolf populations worldwide originate from a single expansion event from Siberia that occurred at the end of the Last Glacial Maximum (Bergström et al., 2022; Loog et al., 2020). This was preceded by an earlier expansion event from Siberia to Europe between 50 and 35 kya (Bergström et al., 2022). Genomic analyses of ancient wolves from different regions in the Holarctic revealed multiple extinct lineages displaying

considerable morphological diversity (Loog et al., 2020; Ramos-Madrugal et al., 2021; Segawa et al., 2022; Skoglund et al., 2015; Thalmann et al., 2013), implying that these lineages have been replaced by the modern wolf lineage that expanded globally. These ancestry replacements were incomplete, and most modern Eurasian wolves probably retain small fractions of local Pleistocene ancestry (Bergström et al., 2022). Nevertheless, only two modern wolf populations, Indian and Himalayan, display clear phylogenetic distinctiveness and may represent relics of earlier genetic diversity surviving until present (Hennelly et al., 2021; Wang et al., 2020, 2021). With the exception of these two lineages, modern wolves do not display reciprocal monophyly of geographically distinct populations at mitochondrial genomes (e.g. Koblmüller et al., 2016), while Eurasian and North American wolves form two monophyletic clades at nuclear genomes (Fan et al., 2016; Sinding et al., 2018). Within modern European wolves, two mitochondrial haplogroups have been identified: haplogroup 1 (hereafter referred to as haplogroup W1) predominating among modern wolves, and haplogroup 2 (hereafter W2) being frequent in ancient samples (radiocarbon-dated to 10–45 thousand years ago (kya); Germonpré et al., 2009) and decreasing in frequency over time (Pilot et al., 2010).

The long-term wolf population decline increased in rate in the past few centuries due to extermination by humans and habitat loss and was associated with reductions in geographic range. This process was particularly intense in Western Europe, where the species became locally extinct by the end of the 19th century, with the exception of the Iberian Peninsula and Italy, resulting in drastic loss of genetic diversity (Dufresnes et al., 2018; Salado et al., 2022). In contrast, in Eastern Europe, where demographic declines were less severe, genetic diversity remained stable throughout the late 19th and 20th centuries (Dufresnes et al., 2018). Very little is known about the genetic variability of European wolf populations before the 19th century, but it is expected that a large proportion of genetic variation present in Europe throughout the Holocene could have been maintained until the last several hundred years (Dufresnes et al., 2018).

The timing of the modern wolf lineage expansion from Siberia, which started at about 25 kya and reached Europe about 23.5 kya (Loog et al., 2020), coincided with the early stages of domestication of the species. Although estimates of the domestication timing vary between studies, in the recent literature they have been narrowed down to 15–40 kya (Freedman et al., 2014; Skoglund et al., 2015; Wang et al., 2016). There is no consensus regarding the geographic region of wolf domestication, but some studies proposed Europe as either the only (Thalmann et al., 2013) or one of the two domestication regions (Frantz et al., 2016). Multiple studies consistently show the expansion of Asian dogs to Europe, which either brought the first domesticated canids to the continent or displaced the earlier native dog lineages (Frantz et al., 2016; Pilot et al., 2015; Wang et al., 2016). The oldest canid remains in Europe widely accepted as dogs, from Bonn-Oberkassel (Germany) and Kesslerloch (Switzerland), were radiocarbon-dated to 14.2 kya and 12.2 kya,

respectively (Boudadi-Maligne et al., 2012; Janssens et al., 2018; Napierala & Uerpmann, 2012). However, considerably older specimens, pre-dating the Beringian wolf expansion to Europe, for example canids from Předmostí (Czech Republic) dated to 28.5 kya have been proposed to represent domestic dogs (Prassack et al., 2020). Therefore, in Europe dogs co-existed with wolves for at least 14 kya, and both canids could be linked by gene flow throughout this period.

Similar as in the case of wolf lineages, prehistoric dog lineages are thought to be partially replaced by expanding dog populations from the east (e.g. Bergström et al., 2020; Frantz et al., 2016; Ollivier et al., 2018; Pilot et al., 2015). This was likely associated with the Neolithic transition in Europe 9–6 kya (Ollivier et al., 2018) and thus considerably postdated the earliest widely accepted dog specimens in Europe, which either originated from an earlier expansion from Asia or potentially from the local domestication in Europe. Mitochondrial genomes of modern domestic dogs fall into six distinct clades (haplogroups A–F), which also encompass most of the variation found in ancient dogs (Baumann et al., 2021; Thalmann et al., 2013). These six clades fall within different parts of the wolf phylogeny, which contrasts with the pattern observed in nuclear genomes, where modern dogs are reciprocally monophyletic with the Eurasian wolf clade (Fan et al., 2016). This discordance suggests that some of the mitochondrial variation in dogs could result from introgression from local wolf lineages. Accordingly, nuclear genomic data showed that domestic dogs have two sources of ancestry in East Asian and West Eurasian wolves, suggesting either an independent domestication in both regions or introgression from local wolves (Bergström et al., 2022). Currently, however, little is known about the local interactions between wolf and dog lineages following the appearance of domestic dogs in different parts of the wolf range, which could result in gene flow and introgression.

Here, we investigated the mitogenomic composition of the extinct French wolf population from the Mesolithic to modern times in the context of mitogenomic variation in other European wolf populations as well as ancient and modern domestic dogs. The aim of the study was to reconstruct the phylogeographic changes in European wolves throughout the Holocene and understand their interactions with domestic dogs following their appearance in Europe. Moreover, we assessed the genetic variability and population structure in the extinct French population in order to understand whether and to which extent the current recolonization of the country from the east and the south may enable the recovery of the past genetic variation. We hypothesized that (1) the extinct French wolf population will show signatures of a population turnover (mtDNA lineage exchange over time) as a result of the expansion of wolves from Siberia into Europe at the end of the Pleistocene (Bergström et al., 2022; Loog et al., 2020); (2) Western European wolves will show evidence of mtDNA haplotype sharing with domestic dogs resulting from both ancient and recent admixture events (Bergström et al., 2020; Pilot et al., 2019); (3) The extinct Western European wolves represented genetic diversity that is largely absent from the contemporary European populations.

2 | MATERIALS AND METHODS

2.1 | Samples and DNA extraction

Grey wolf samples from France ($N=107$) and Switzerland ($N=8$) from ancient/medieval (Mesolithic-Middle Ages; >700years ago) and historical (19th–20th century) periods were obtained from museum collections, research institutes and private owners (Table S1, Figure 1b). DNA extraction was performed using two methods: (1) de Bruyn et al. (2014), (2) Rohland et al. (2018) at dedicated ancient DNA laboratories of the School of Life Sciences, University of Lincoln (1) and Museum and Institute of Zoology, Polish Academy of Sciences (2). Detailed DNA extraction procedures are described in Supplemental Information.

Ten individuals had atypical morphological features, which could indicate that they are either domestic dogs or wolf-dog hybrids: M17 had an unusual spotted black and grey coat (Figure S1c) and M14, M35 and M90 had yellow coat colour (Figures 1d, S1a, S1j; Tables S1,

S2). Six other individuals (M16, M18, M26, M27, M41 and M112) had black coat colour (Figures 1c, S1). Black coloration in wolves is associated with a dominant variant of *CBD103* gene that encodes a beta-defensin protein, which was shown to be acquired by wolves as a result of ancient hybridization with domestic dogs (Anderson et al., 2009). The measurements of the carnassial tooth P4 for four specimens for which the skulls were available showed that three of them clustered with domestic dogs and the fourth one had intermediate values between wolves and dogs (Figure S2). Individuals with the atypical features described above could be either dogs or hybrids, or originate from more distant hybridization events.

2.2 | DNA sequencing and sequence analyses

Fragments of mtDNA D-loop were amplified from DNA extracts using primers and PCR conditions from Dufresnes et al. (2018) to check the success of DNA extraction. D-loop fragments were

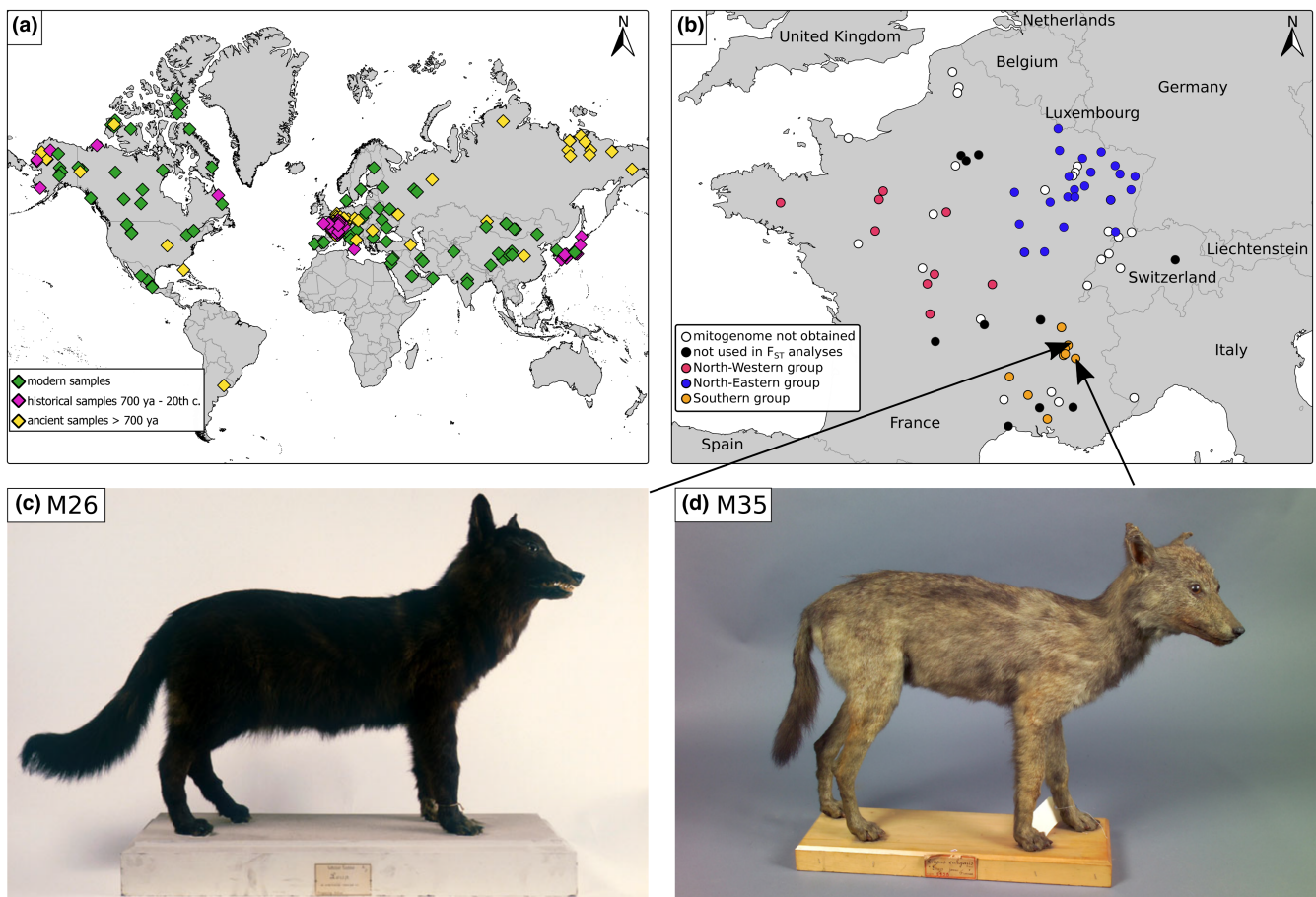


FIGURE 1 Maps of approximate locations of samples used in this study and photographs of atypical samples. (a) Location of samples from this study and from the literature that were used in phylogenetic analyses; (b) Location of samples from this study. Samples for which mitogenome sequence was not obtained are represented by white circles. Historical samples divided into three geographical regions used in genetic distance analysis (North-Western France, North-Eastern France and Southern France; Tables S8, S9) are represented by pink, blue and yellow circles, respectively. Ancient/medieval samples not used in the genetic analyses of the historical population are represented by black circles. Maps were constructed using QGIS v. 3.16.4 (<https://qgis.org/>) and Natural Earth data (<https://www.naturalearthdata.com/>); (c) Black individual M26 (haplogroup W1); (d) Individual M35 with mtDNA haplotype from dog haplogroup A.

successfully amplified in 72 (65%) samples. In the mitogenome analyses, we included these 72 samples and 18 samples for which D-loop amplification failed, but which were important to the study (e.g. the oldest samples). Because the majority of endogenous aDNA is very short, we expected that for some of these samples the library preparation would be successful even though D-loop amplification products were not obtained. The selected 90 DNA extracts were used to prepare double-indexed libraries for Next Generation Sequencing (NGS) on Illumina following the Meyer and Kircher (2010) protocol with a few modifications (described in the SI).

DNA libraries were enriched for the mitochondrial genome using home-made baits (described in the SI) and the protocol from Horn (2012). Enriched libraries were pooled, quantified using NEBNext® Library Quant Kit for Illumina® (NEB) and sequenced on NextSeq 500 (Illumina) using three NextSeq 500/550 Mid Output v2.5 (2×75 cycles) kits.

Raw reads were trimmed, filtered and collapsed using Adapter Removal v. 2 (options `-minlength 30 -trimms -trimqualities -collapse`) (Schubert et al., 2016). Following these QC procedures, reads were mapped to the CanFam3 dog reference genome using *bwa aln* and filtered by *samtools* (command `samtools view -q 30`) (Li et al., 2009). PCR duplicates were removed using Picard MarkDuplicates (<https://broadinstitute.github.io/picard/>). Damage patterns of aDNA were assessed by MapDamage v. 2.0 (Jónsson et al., 2013), and quality scores of damaged sites were rescaled. Variant and mtDNA consensus were called using *samtools* and *bcftools* on the merged bam files from the same individual obtained from independent libraries. Only positions with at least 3X coverage were used for creating consensus sequences with a length of 16,729 bp.

2.3 | Phylogenetic and population genetic analyses

To evaluate the phylogenetic position of the newly sequenced French wolves, we carried out a phylogenetic analysis, which included previously published wolf and dog mitogenome sequences (Table S3, Figure 1a). After excluding sequences with more than 20% of missing data (after control region trimming), we aligned the remaining sequences with the sequences obtained in this study using MAFFT v. 7 (Kato et al., 2019). We removed sites in the alignment that were represented in less than 90% of samples as well as two codon positions adjacent to the excluded sites. The resulting alignment of a length 15,378bp was named 'dataset A'. Phylogenetic analysis was performed in MrBayes (Ronquist & Huelsenbeck, 2003) using substitution models defined by PartitionFinder2 (Lanfear et al., 2017; Table S4) and 15 mLn generations. The coyote sequence (*Canis latrans*, NC008093) was used as an outgroup. The first 25% of samples were discarded as burn-in. This analysis allowed us to reconstruct phylogenetic relationships of the French wolves studied with other wolf populations worldwide and identify individuals belonging to maternal lineages of domestic dogs.

To reconstruct the population dynamics of European wolves, we performed a coalescence analysis in BEAST v. 1.10.4 (Drummond

et al., 2012). For this purpose, we created a dataset (named 'dataset B') that excluded sequences from dog haplogroups and wolf sequences outside Europe and outside the Modern Holarctic Wolf clade (MHW, see Results). We used six substitution models estimated by PartitionFinder2 (Table S4), a strict clock with tip calibration and a Skyline model that was chosen as the best model by marginal likelihood estimation (MLE). We ran two MCMC simulations through 100 mLn generations with a sampling frequency of 10,000 steps. MLE analysis was run using path/stepping-stone sampling with chain length of 1 mLn. For each parameter, we checked mixing and convergence by accessing if the ESS values were above 100 and visually inspected the trace plots using TRACER v.1.6 (Rambaut et al., 2014). The phylogenetic trees were summarized in TreeAnnotator 1.10.4 with 10% burn-in. Skygrid analysis was performed in TRACER. We also performed separate analyses in BEAST that included either sequences from Haplogroup W1 (dataset C) or W2 (dataset D). We used six substitution models defined by PartitionFinder2 (Table S4), strict (dataset C) or uncorrelated relaxed (dataset D) clock, 50–100 mLn generations and Skygrid population models as chosen by MLE analysis.

We constructed the temporal statistical parsimony network using TempNet (Prost & Anderson, 2011) to illustrate the relationships between mitogenome haplotypes of European wolves in four time periods: (1) Late Pleistocene, (2) early Holocene-Middle Ages, (3) 19th–20th century and (4) contemporary and to assess whether there was genetic continuity in European wolf populations since the Late Pleistocene.

To confirm the phylogenetic positions of the dog haplogroups relative to wolves from different geographic regions (see Results), we reconstructed a phylogeny based on a 533bp D-loop fragment, which allowed us to include additional West Asian wolves for which mitogenomes were unavailable. Of 121 haplotypes included in this analysis, 38 haplotypes were from West Asian individuals. This analysis was performed in MrBayes for 10 mLn generations with a substitution model set to `lst nst=mixed` and a coyote sequence used as an outgroup. The first 25% of samples were discarded as burn-in.

To assess phylogenetic relationships between the historical French wolf population and other historical European populations, we carried out a separate analysis using a shorter D-loop fragment (201bp) available from an earlier study (Dufresnes et al., 2018). The dataset included 41 haplotypes found in historical populations across Europe (Dufresnes et al., 2018) and 67 D-loop sequences obtained in this study, collapsed into 22 haplotypes. We created a haplotype network in PopArt v. 1.7 (Leigh & Bryant, 2015) using the median-joining model. Standard genetic diversity indices for individuals from historical European populations were calculated in DNASP (Librado & Rozas, 2009).

To assess genetic differentiation in historical French wolves, we divided them into three geographic regions (Southern, North-Eastern and North-Western; Figure 1b). The division into northern and southern regions reflects the division between lowlands in the north and mountainous areas in the south. Such an environmental gradient was shown to lead to genetic differentiation in other wolf

populations (e.g. Pilot et al., 2006). It is reflected in the current recolonization patterns, with southern France being recolonized by the Italian wolf population through the Alps, and northern France being recolonized from Germany. The division between the north-eastern and north-western regions is based on the discontinuity in the historical sample distribution. This discontinuity may coincide with the location of large cities, Paris, Auxerre and Bourges, which existed since at least Roman times and where wolves could have been extirpated from earlier than other regions. For each region, we calculated pairwise population F_{ST} in Arlequin v. 3.5.2.2 (Excoffier & Lischer, 2010) based on either mitogenome or D-loop data (218bp fragment).

2.4 | Approximate Bayesian Computation Analyses (ABC)

To test the demographic scenarios that were detected using Skygrid or Skyline population models, we performed the ABC analyses for three datasets: (1) European samples from haplogroup W1, (2) European samples from haplogroup W2 and (3) European samples from both haplogroup W1 and W2. For each dataset, we compared three different scenarios: (A) constant population size, (B) population decline and (C) bottleneck. Analyses were performed in BaySICS v. 1.9.7.9.5 software (Sandoval-Castellanos et al., 2014). We set the generation time of the species to 3 years (Koblmüller et al., 2016) and mutation rate to 2% per million years following Aggarwal et al. (2003). Other parameters such as transition/transversion bias, gamma shape parameter and nucleotide frequency were set based on calculations in MEGA X (Kumar et al., 2018). We ran the 60–200 thousand simulations and compared different scenarios using Bayes Factors (BF) for every pair of scenarios and a set of summary statistics (SuSt): nucleotide diversity, haplotype diversity and Tajima's D (Mourier et al., 2012). The consistency of the model likelihoods and BFs was assessed by applying the model choice procedure with 20 different acceptance proportions, from 0.0025% to 0.5%.

2.5 | Phylogeographic analyses

To investigate the geographic origin of two distinct wolf lineages found in Europe (W1 and W2), we carried out the phylogeographic analyses in RASP (Yu et al., 2015, 2020) and BayesTraits (Pagel & Meade, 2021). For the RASP analysis, we used the set of phylogenetic trees that were constructed using 235 wolf mitogenome sequences, excluding sequences from dog haplogroups (dataset E). Trees were constructed in BEAST using six partitions (Table S4), uncorrelated relaxed clock, Skygrid model and 300 mLn generations. Sequences used in RASP analyses were divided into seven geographic regions: Beringia, North America, Japan, South Asia, Central North Asia, Middle East and Europe. We used 9000 trees after removing the first 1000 as a burn-in and performed S-DIVA

reconstruction with maximum areas set to seven. For BayesTraits analysis, we used the majority rule consensus tree obtained by summarizing trees in TreeAnnotator with 10% burn-in. We used the geographic model with a set of geographic coordinates for each terminal node. For sequences without detailed coordinates, we used the approximate location. The analysis was run for 1,010,000 MCMC generations with a sampling frequency of 1000. These analyses allowed us to reconstruct the distribution of ancestral populations of wolf mtDNA haplogroups, which provided novel information regarding the evolutionary history of Eurasian wolves.

2.6 | Stable isotopes analysis

To reconstruct the diet of wolves from the extinct French population, we performed the stable isotope analyses. Isotopic composition of carbon and nitrogen was measured from collagen of 21 canid bone samples (Table S5) at the OEA Laboratories Ltd, UK. Eight samples contained insufficient collagen concentrations for the analyses. For the remaining 13 samples, we used the δN^{15} and δC^{13} values to reconstruct the diet with the IsoSource (Phillips & Gregg, 2003). Mean values were used for the samples with more than one measurement.

We combined the isotopic values from potential prey species gathered from the literature (Table S6) into larger groups to avoid overrepresentation of prey species with similar isotopic ranges. For historical samples, we used five groups of modern herbivores from Central-Eastern Europe: European hare, beaver, wild boar, roe deer and large ungulates (red deer, moose and bison). Older prey samples were clustered into six groups: ungulates (roe deer, red deer, moose, aurochs, bison and horse), leporids, caprine, cattle, pig and wild boar. For samples A8 and A13, we performed an additional analysis that included the reindeer and bison from the Magdalenian period, to include prey species that were within the range of the analysed wolf samples. We used mean values from prey groups corrected for the trophic enrichment factors of $1.3 \pm 0.6\%$ δC^{13} and $4.6 \pm 0.7\%$ δN^{15} , following Pilot et al. (2012). For historical samples analysed here and modern samples from the literature, we accounted for the changes in the anthropogenic CO_2 emission and corrected the δC^{13} values following the formula from Feng (1998). This analysis allowed us to reconstruct the diet of each individual assessed and to identify individuals with diet composition corresponding to domestic rather than wild canids.

3 | RESULTS

Consensus mtDNA genome sequence successfully obtained from 78 (87%) samples was called with the average genome coverage of 195X by mapping NGS reads to the CanFam3 dog reference genome. The remaining 12 samples were removed from further analyses due to low coverage and missing data (Table S1). The final dataset included only one sample from Switzerland; therefore, we refer to the analysed dataset as 'French wolves'.

Analysis of damage patterns typical for aDNA showed that average C>T substitution on 5' end was 15.2% for ancient/medieval sequences (>700 years ago), and 4.5% for historical samples (Figure S3). One Bronze Age sample (A109) showed a very low C>T substitution rate (1.5%), indicating either contamination or an incorrect sample age (which was obtained from the context rather than direct radiocarbon dating). Moreover, the sample age estimated by molecular dating was later than the Bronze Age (median 1202 years BP). For these reasons, this sample was excluded from the BEAST and phylogeographic analyses that used the sample dating (see discussion in SI).

3.1 | Phylogeny

We analysed phylogenetic relationships between the newly sequenced French wolves and worldwide wolves and domestic dogs sequenced in earlier studies (Table S3) in MrBayes. The reconstructed phylogenetic tree showed early branching of Tibetan wolves, Indian wolves, Late Pleistocene canids from Europe, Siberia and North America, recently extinct Japanese wolves and dog haplogroup F. This was consistent with earlier studies, which showed similar topology with Tibetan/Indian modern wolves, Late Pleistocene Eurasian and North American canids as well as Japanese wolves forming distinct clades at the root of the trees (Baumann et al., 2021; Loog et al., 2020; Thalman et al., 2013). The largest clade, labelled as MHW (Modern Holarctic Wolves), included all modern wolves except the Tibetan and Indian lineages (Figures 2, S4). MHW clade included two main haplotype groups (haplogroup W1 and W2). Haplogroup W1 consisted of haplotypes from modern, historical and ancient/medieval wolves from Eurasia and North America (Figure 2) as well as four dog haplogroups (A, B, C and E). Haplogroup W1 was identified based on the mitogenomic phylogeny reconstructed for European wolves only, where it formed a monophyletic clade (Figure S5), but was either monophyletic (BEAST analysis, Figure 3c) or contained a polytomy (MrBayes analysis, Figure 2) when wolves from other continents were included. Haplogroup W2 was monophyletic in all analyses and consisted of European wolf haplotypes from contemporary, historical and ancient/medieval populations (Figure 2), as well as dog haplogroup D. A small clade containing wolves from Europe and the Middle East had different clustering patterns in trees constructed using different methods and sample sets (Figures 2, 3c) and therefore was not classified into either haplogroup.

Haplogroup W2 predominated in ancient/medieval French wolves (56% samples) but was rare among historical French wolves from the 19th to 20th century (22% samples). When considering the frequency of haplogroups in the entire European population, we found that haplogroup W2 was dominant in the Late Pleistocene (22–12 kya) and the Holocene (10 kya–850 years ago), occurring in 64% and 75% of samples, respectively. In historical times, its frequency was reduced to 22%.

Among the 78 newly sequenced French samples, we identified 50 haplotypes. Most of them were unique and not reported before in

other wolf populations worldwide. One haplotype from the Neolithic (sample A14) clustered with haplotypes found in Mesolithic France and Belgium (Figures 2, S4). None of the nine haplotypes of the ancient/medieval French wolves was shared with historical or modern wolves, although some of them clustered with haplotypes of French wolves from the end of 19th century. For example, specimens A10 (Roman Age) and M34 (19th century) clustered together, and specimens A5 (Iron Age) and A11 (10th–11th centuries) clustered with several French wolves from the 19th and 20th centuries.

Five samples from which we obtained mitogenomes came from black individuals (Tables S1, S2), suggesting their possible hybrid origin, but only one of them, individual M112, clustered within dog haplogroup A. Six other (non-black) French canid samples clustered within dog haplogroups A and C. Two of them, M58 and M35 (dog haplogroup A), have been suspected to be domestic dogs: M35 had a yellow coat colour, and M58 was found close to a human dwelling. Three other samples that had atypical coat colours: M17 (spotted grey and black), M14 and M90 (yellow) clustered with wolf haplogroups W1 and W2. An ancient wolf sample (1.7 kya) from Germany reported in Prassack et al. (2020) was sister to the dog haplogroup D. This dog haplogroup was nested within wolf haplogroup W2 comprising European wolves only, suggesting that it originated in Europe. However, only five mitogenome sequences of West Asian wolves were available and could be included in our analysis. To reduce the possible bias associated with underrepresentation of West Asian haplotypes, we carried out an additional analysis using D-loop sequences, which included 38 haplotypes from West Asia. In consistency with the mitogenome analysis, the D-loop analysis showed that dog haplogroup D clustered with European wolves from haplogroup W2 (Figure S6), while all haplotypes from other parts of the world, including West Asia, were placed outside haplogroup W2.

3.2 | Population dynamics of European wolves

The coalescence analysis carried out in BEAST for mitogenome sequences of European wolves estimated the divergence time of haplogroups W1 and W2 at 44,092 years BP (95% highest posterior density, HPD: 56,112–33,707 years BP, node A in Figure S5). The Most Recent Common Ancestor (MRCA) of the haplogroup W1 was estimated at 37,355 years BP (95% HPD: 49,175–28,078 years BP; node B in Figure S5), and the MRCA of the haplogroup W2 was estimated at 34,949 years BP (95% HPD: 44,408–27,437 years BP; node C in Figure S5).

Effective population size (N_e) of European wolves from the MHW clade (consisting of haplogroups W1 and W2) reconstructed using the BEAST's Skyline model was relatively stable during the last 40 kya until a sharp decline that has started about 1 kya and continued until present (Figure 4a). A similar pattern was inferred from the Skygrid analysis focussed on all wolves from Eurasia and North America (Figure 4b). The analysis of this dataset also showed an increase in N_e between 30 and 10 kya, which was not observed when analysing the European wolves only (Figure 4b). Analyses focussed

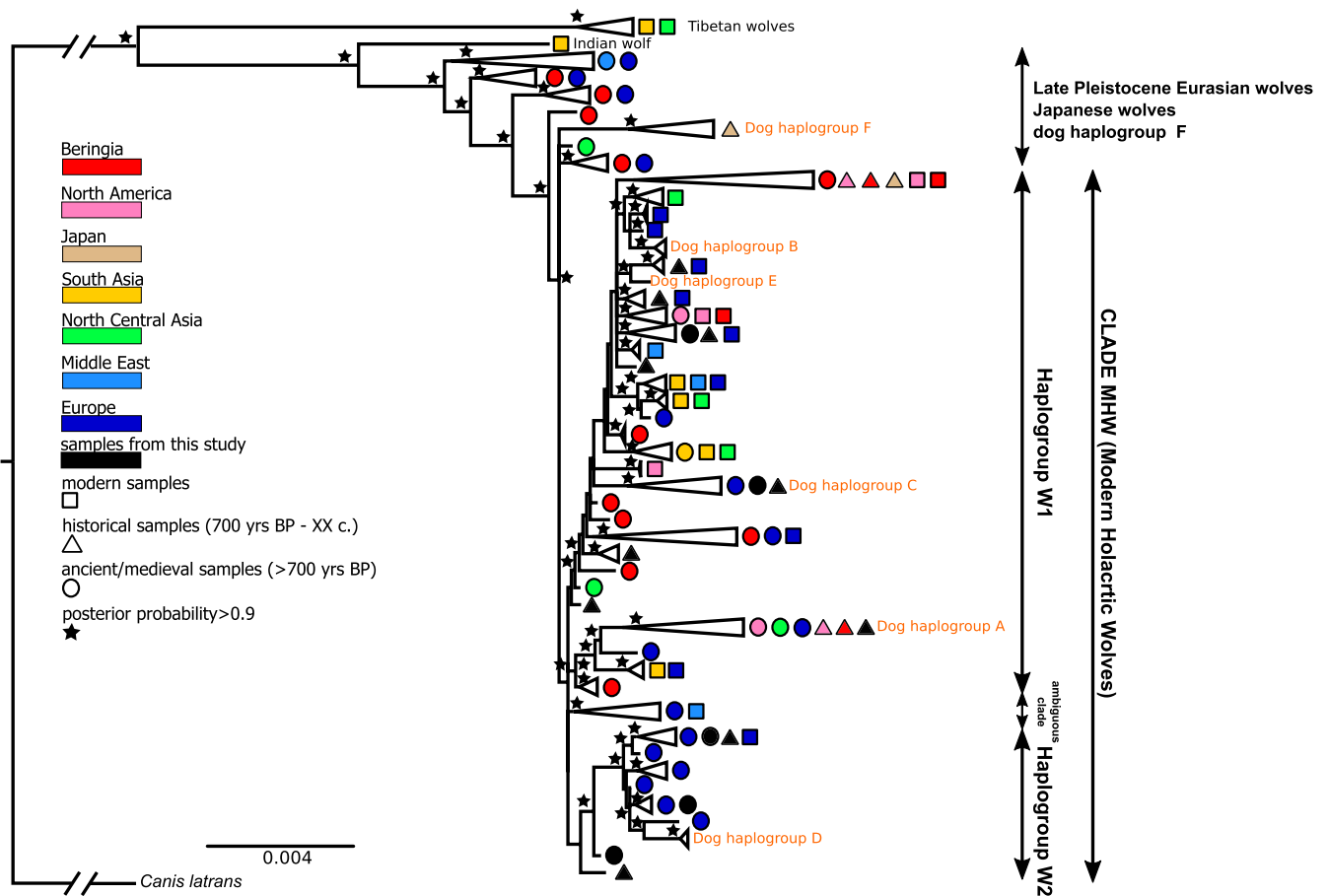


FIGURE 2 Bayesian phylogeny of mitogenome haplotypes (15,378 bp) from ancient/medieval, historical and modern canids specimens reconstructed using MrBayes. Geographic distribution of haplotypes is colour coded. 'Ambiguous clade' represents haplotypes that cluster with either Haplogroup W1 or W2 depending on the sample set and analysis performed and therefore could not be classified into any of these haplogroups. Detailed descriptions of haplotypes and the fully expanded tree are shown in the Supplemental Information (Tables S1, S3 and Figure S4, respectively).

on European wolf haplotypes from haplogroup W1 and W2 separately showed less pronounced declines in N_e (Figure S7). For haplogroup W2, the signal of decline depended on the population model used. In the most supported Skygrid model, a scenario with constant population size could not be excluded due to wide 95% HPD intervals (Figure S7B). However, when the Skyline model was used, a more pronounced N_e decline was observed (Figure S7C). It is worth mentioning that 95% HPD intervals of the reconstructed effective population size changes were generally wide in all analyses.

The model choice of the ABC analyses, regardless of the dataset and the number of statistical groups, showed that the most probable scenario is the constant population size (scenario A) with model likelihood > 0.7 for each analyses and BF of at least 7 but in one case as high as 309. These results indicate that we cannot exclude a constant population size model for the European wolf population. This is not entirely consistent with the results obtained by BEAST analyses (see Figures 4 and S7), which show the constant population size until about 1 kya followed by a decline. Therefore, the results from both methods should be treated with caution, and a constant population size model for the European wolf population cannot be excluded.

Loss of haplotype diversity in European wolves was clearly shown on the temporal network of haplotypes (Figure S8). All haplotypes present in Western Europe in the Late Pleistocene and early Holocene were absent from modern European populations. Only one haplotype was shared between the 19th-century wolves and modern times. It was present a wolf from 1848 CE from the French Alps and contemporary wolves from the French Alps, Italy and Belarus.

Genetic variability estimates for the historical European populations inferred based on a short D-loop fragment were consistent with those based on the whole mitogenomes. The historical European population showed moderate-to-high genetic variation in the D-loop region (overall $H_d = 0.83$, $\pi = 1.163\%$, Table S7). The lowest values of genetic diversity indices were observed in Italy, the Iberian Peninsula and Poland (Table S7). Ancient/medieval and historical French wolf populations showed similar estimates of genetic diversity. More than half of the 22 haplotypes identified in European wolves living in the 19th and early 20th century were found in France (Figure 5, Table S7).

We assessed the genetic differentiation between historical French wolves from three geographic regions (Southern,

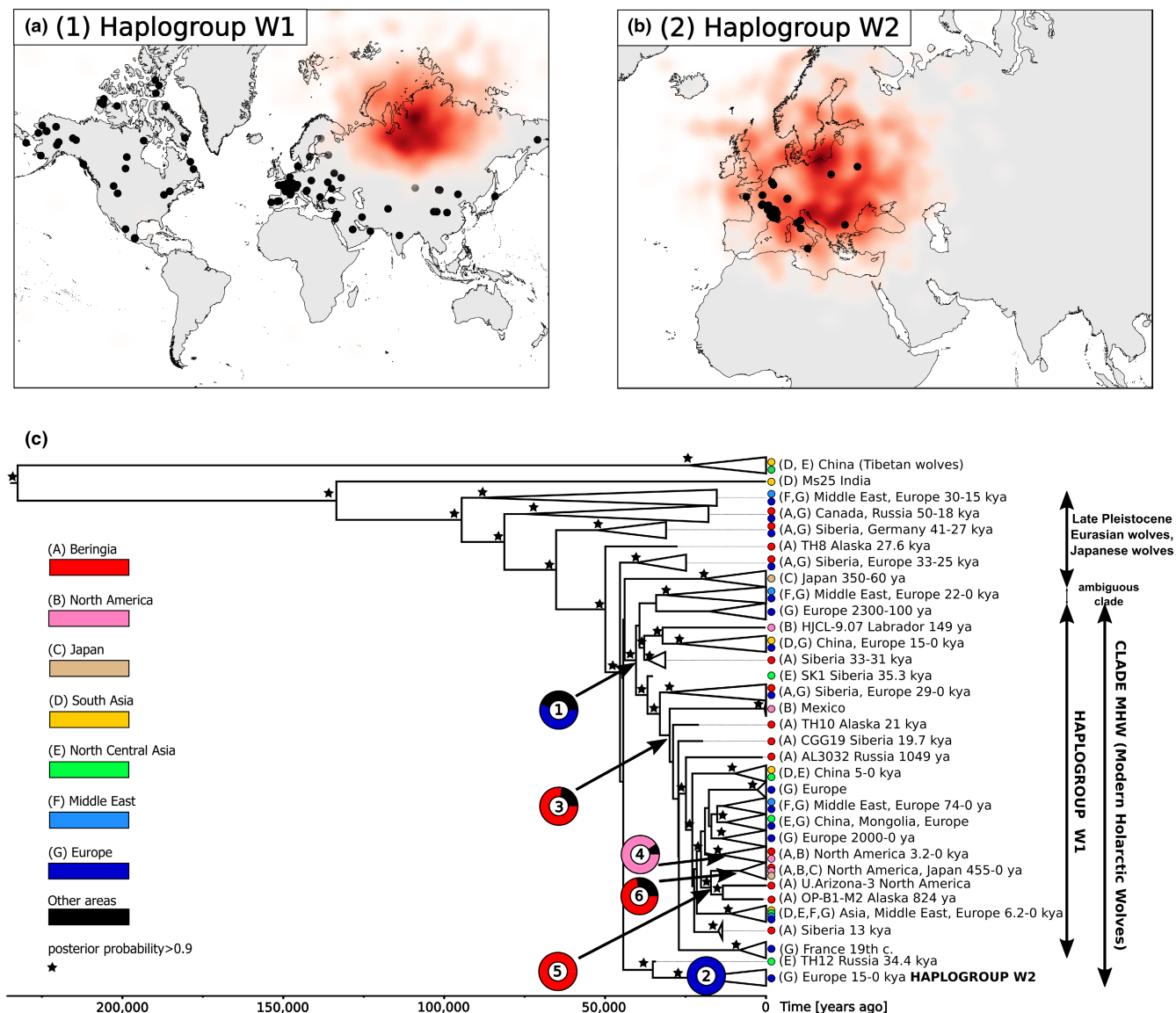


FIGURE 3 Results of phylogeographic analyses in BayesTraits and RAPS. (a, b) Geographic ranges of the ancestral nodes estimated using a geographic model in BayesTraits. Red colour represents the heatmap constructed from posterior distribution of estimated geographic coordinates for ancestral nodes. Black points are locations of the wolf samples that belong to the respective clade. Numbers in parentheses correspond to node numbers of a tree in the lower panel. Maps were constructed using QGIS v. 3.16.4 (<https://qgis.org/>) and Natural Earth data (<https://www.naturalearthdata.com/>). (c) Bayesian phylogeny constructed in BEAST for grey wolves only (excluding the dog haplogroups) with geographic distributions of the ancestral populations estimated in RASP. The reconstruction of ancestral populations is shown for only six (1–6) ancestral nodes. Posterior probabilities for node support above 0.9 are marked by asterisks. Geographic regions are colour and letter coded, and regions with probability < 0.3 are hidden and included in the category 'other areas' (black colour). Individual sequences are coded by the sample name, origin and sample age. Collapsed clades are named by the origin and sample age range. Fully expanded tree with estimation for each node is presented on [Figure S12](#).

North-Eastern and North-Western; [Figure 1](#)) using pairwise F_{ST} calculated based on either mitogenome or D-loop data. Mitogenome data ([Table S8](#)) showed that the North-Eastern population was differentiated from both Southern ($F_{ST}=0.53$) and North-Western population ($F_{ST}=0.42$), while there was no differentiation between two latter regions ($F_{ST}=0$). D-loop data showed similar results ([Table S9](#)). We also assessed the genetic differentiation between historical French wolves and modern European and Middle Eastern populations. Mitogenome data showed that Southern and North-Western

historical French wolves had the lowest genetic distance from modern populations from Balkans and Eastern Europe (F_{ST} : 0.08–0.14). Analyses based on the D-loop dataset (which contained a considerably larger number of samples from European populations available from earlier studies) indicated that historical French wolves were generally less differentiated from other historical European/Middle Eastern populations (F_{ST} : 0.00–0.63; mean F_{ST} : 0.10, 0.17, 0.47 for Southern, North-Western and North-Eastern France, respectively) than from modern populations (F_{ST} : 0.09–0.95, mean F_{ST} : 0.51, 0.57

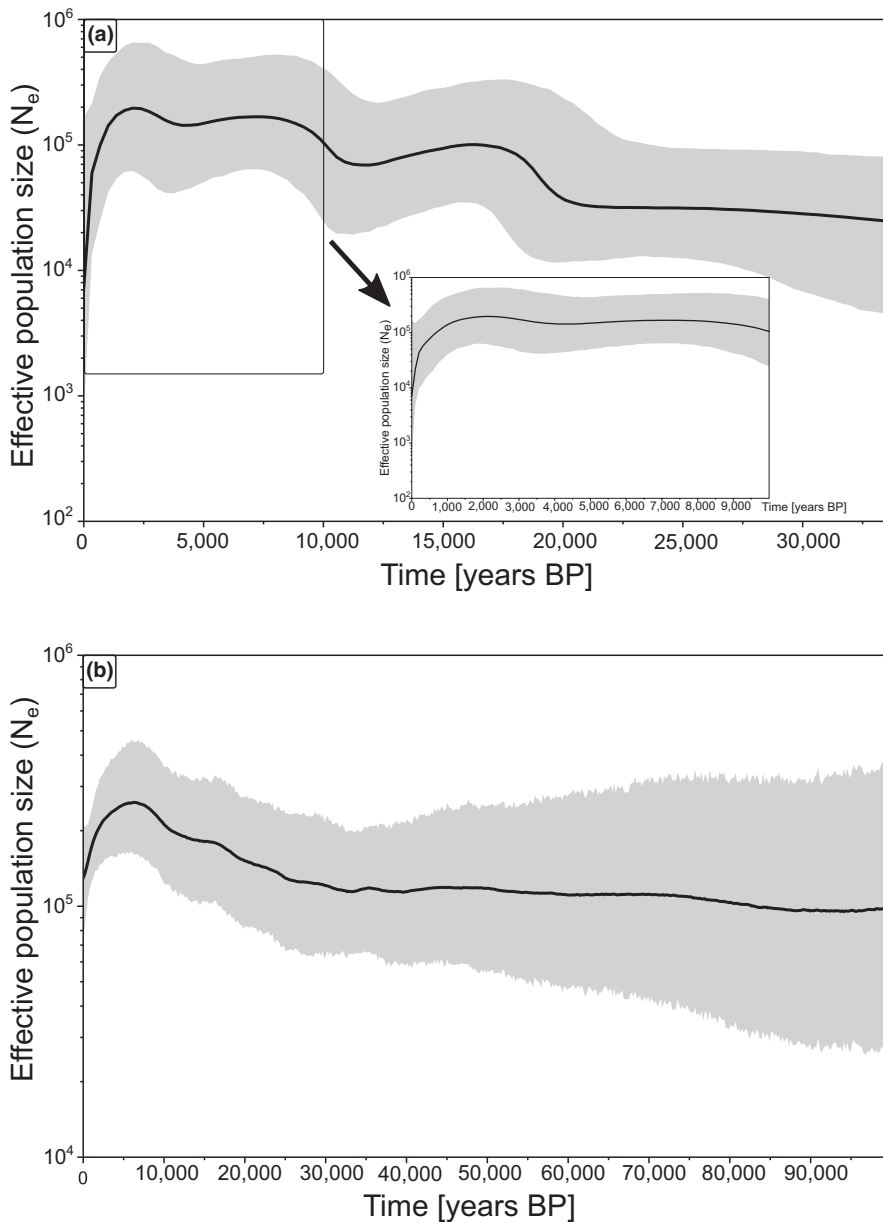


FIGURE 4 Temporal changes in effective population sizes (N_e) in European (a) and Holarctic wolves (b). A—Skyline plot for European wolves (Haplogroups W1 and W2), B—Skygrid plot for wolves from Eurasia and North America (dataset E). The line represents the median, and the grey area represents 95% HPD intervals.

and 0.60 for Southern, North-Western and North-Eastern France, respectively). This may result from lower genetic diversity in modern populations, which leads to higher F_{ST} values. The French populations showed the lowest significant F_{ST} in pairwise comparisons with historical populations from Italy, Central-Eastern Europe and Switzerland (Table S9).

3.3 | Distribution of ancestral wolf populations

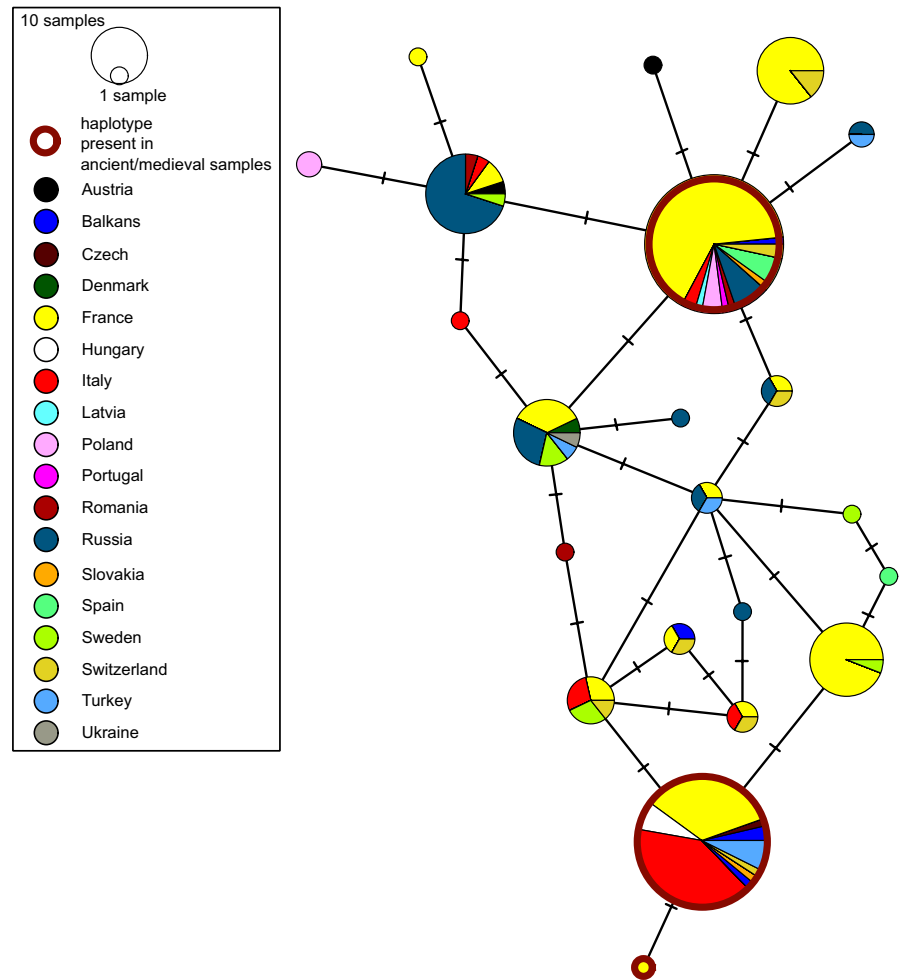
To reconstruct the phylogeographic changes associated with the temporal changes in frequencies of haplogroups W1 and W2 in Europe, we estimated the distribution of ancestral wolf populations using S-Diva in RASP and BayesTraits. The ancestral distribution of the Haplogroup W1 was inferred to be either in North-Western Siberia, around Yamal Peninsula (BayesTraits; Figure 3a) or Europe (S-Diva,

Figure 3c). Both S-Diva and BayesTraits analyses showed that the most probable distribution of the ancestral population of the haplogroup W2 was Europe (Figure 3b, c). To test the accuracy of these analyses, we reconstructed the distribution of the ancestral population of North American wolves, which are known from previous studies to have colonized the continent from North-Eastern Asia via Bering Land Bridge (Ersmark et al., 2016; Loog et al., 2020). In agreement with these studies, the estimated ancestral distributions of clades including North American wolves were identified as North America, Beringia or Northern Eurasia (nodes 3–6 in Figure 3c; Figure S9, see SI).

3.4 | Diet reconstruction

Isotopic values of 13 French canids analysed, which dated between the Iron Age and early 20th century, ranged from 7.3‰ to 11.11‰ for

FIGURE 5 Haplotype network of historical European wolves constructed for a 201 bp control region fragment in PopArt. Network is coloured by the haplotype distribution.



δN^{15} and from -21.94% to -19.15% for δC^{13} (Table S5). These values were within the range of modern and ancient European wolves and dogs (Figure 6). We reconstructed the diet of each individual using a dietary mixing model in IsoSource and isotopic values of either modern or ancient individuals representing potential herbivore prey species, taken from the literature (Table S6). For most historical wolves (19th–20th centuries), the inferred diet included mainly large ungulates (red deer, bison, moose; 34–95%), followed by roe deer and wild boar (11–34%). Smaller herbivores—beaver and hare accounted for less than 30% of the diet in each individual (Table S5, Figure S10). The only exception was individual M102, for which wild boar and beaver were identified as the main food source (51% and 48%, respectively). Two individuals (M58 and M74) with mtDNA haplotypes falling within dog haplogroups (Figures 2, S4) had similar diets as other historical French wolves, consisting mostly of wild ungulates.

The ancient and medieval wolves analysed had a more diverse diet. For three individuals (A5, A11 and A94), large ungulates (roe deer, red deer, moose, aurochs, bison and horse) were the main food sources (41–99%, Table S5, Figure S11), with other prey species accounting for less than 20% of their diet. For the specimen A7, leporids were the most common prey (53%), followed by large ungulates (42%). For two individuals (A12 and A92), cattle was the main food source (57–63%), with large wild ungulates being the second

most common prey (30–36%). Both these individuals had mtDNA haplotypes falling within dog haplogroup C (Figure 2), and A92 also clustered with dogs based on the morphological measurements (see Supplemental Information and Figure S2); therefore, these individuals could be domestic dogs or wolf-dog hybrids.

Low δN^{15} values for individuals A8 and A13 placed them outside the range of prey values used in the initial analysis (Figure S11), which resulted in the lack of available solution until a very high tolerance parameter was used (1.7–1.9). This suggested that in the initial set the main sources of their diet were not included. Therefore, we carried out an additional analysis, adding reindeer and bison from the Magdalenian period with lower δN^{15} values, closer to the A8 and A13 samples. These ungulates were not included in the original analysis, because we focussed there on prey contemporaneous to the analysed samples. This new analysis suggested that A8 and A13 individuals also fed with large ungulates, but different species than other ancient/medieval wolves (Table S5, Figure S11).

4 | DISCUSSION

Reconstructing the evolutionary histories of extant species is crucial to understand factors shaping their distribution, diversity and

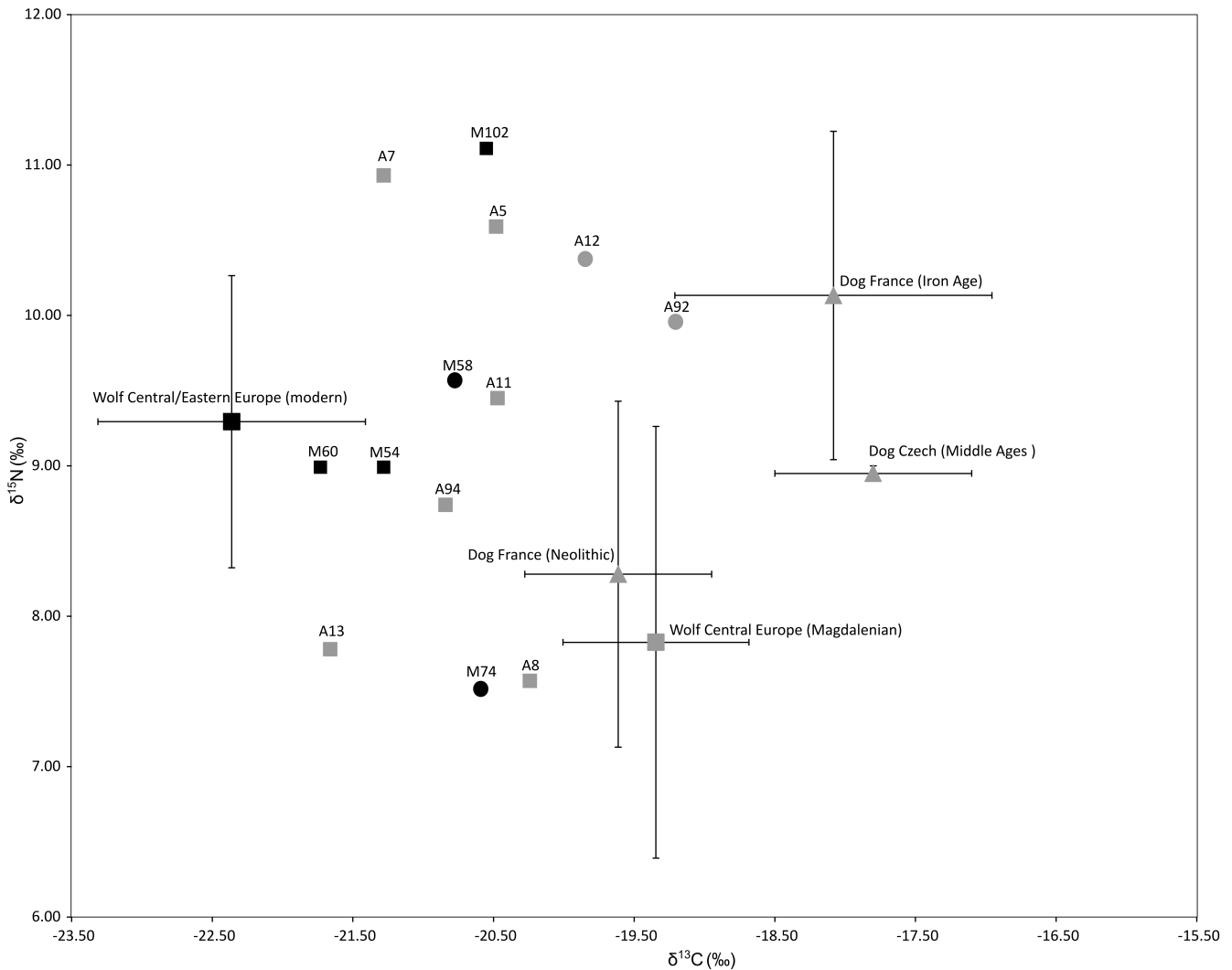


FIGURE 6 Isotopic values of French samples plotted with wolves and dogs from literature. Wolf samples are shown as rectangles and dog samples are marked with triangles, while samples of uncertain identification are shown in circles. Black colour represents historical (19th–20th centuries) and modern samples. Grey colour represents ancient/medieval samples. For previously published samples, mean and standard deviation of the group is shown.

demography. Knowing these factors is important to identify potential threats that can lead to species extinction and to prevent it by the management practices. Analyses of past populations using aDNA have been shown to be especially important in resolving the questions of evolutionary biology that could have not been answered by analyses of contemporary populations alone (Orlando et al., 2021 and references therein). We showed that like in other mammalian species (e.g. Kotlík et al., 2018; Palkopoulou et al., 2016) population range expansion in wolves caused substantial changes in the phylogeographic patterns. We also showed that hybridization between wolves and domestic dogs played an important role in shaping the evolutionary history of both canids, which was also the case of other species with domesticated relatives, for example wild boars vs domestic pigs (Frantz et al., 2019; Scandura et al., 2011), European wildcats vs domestic cats (Mattucci et al., 2019) and wild vs domestic sheep (Cao et al., 2021). Our study also pointed out that anthropogenic factors had a profound effect on wolf populations.

Despite long-term population decline in the global wolf population (e.g. Bergström et al., 2022; Fan et al., 2016; Loog et al., 2020; Pilot et al., 2014), the local French population maintained relatively high genetic diversity before the human-mediated eradication. However, genetic diversity drastically dropped during the 20th century, shortly before the extinction of the French and other Western European populations (Dufresnes et al., 2018).

4.1 | Demographic changes in the European wolf population

Our results showed that the European wolf population experienced genetic diversity loss and changes in the mtDNA haplogroup frequencies during the Holocene. Although our reconstruction of demographic patterns suggests that the population decline started no later than 1 kya, the strongest genetic diversity loss

occurred during the last 200 years and was associated with the local extinctions in Western Europe. In the 19th and early 20th century, the wolf genetic diversity in most European countries was still relatively high and most haplotypes at that time were shared between regions (Dufresnes et al., 2018). French wolves from that period carried diverse haplotypes, which represented both main haplogroups identified in the grey wolf mitochondrial phylogeny and were shared with wolves from the Iberian and Apennine Peninsulas as well as Eastern Europe. Earlier genomic studies showed that the Iberian and Apennine wolf populations experienced long-term isolation and bottlenecks (Pilot et al., 2014; Silva et al., 2020), with the divergence time between them estimated at about 10.3 kya and limited gene flow since that time (Silva et al., 2020). Our results suggest that the isolation of the Southern Peninsular regions was either incomplete or more recent than previously estimated.

Although the direct persecution and habitat loss in the last few centuries considerably contributed to the demographic decline and genetic diversity loss in European wolves, our reconstruction of the temporal changes in N_e suggests that the decline has already started around 1 kya. Earlier studies point to an even earlier onset of the decline, for example a study of modern European populations based on genome-wide SNP data showed gradual decrease in N_e since about 60 kya (Pilot et al., 2014), the analyses of whole genomes showed that Eurasian wolf populations experienced declines around 30–20 kya (Bergström et al., 2022; Fan et al., 2016; Freedman et al., 2014), while mitochondrial genomes point out that the decline in the global wolf population started around 50 kya (Loog et al., 2020). Differences in these estimates may be caused by the use of different datasets and differences in estimates of mutation rates and divergence times. Here, we reconstructed N_e changes in the European population based on European wolves from the MHW clade only, while samples representing older lineages were excluded. We also excluded the sequences which were placed within the dog haplogroups (Figures 2, S4), for example TH14, TU14, TU11 and CGG16 from Loog et al. (2020). Our BEAST analysis with Skygrid reconstruction for the global wolf population, including extinct lineages, showed an earlier onset of the decline at around 7–5 kya (Figure 4b) and increase in N_e (around 30–10 kya, Figure 4b) not observed in the Skyline reconstruction for European wolves. The observed increase in N_e could be driven by the increasing population differentiation between North American and European wolves, which would have explained why is not observed when analysing the European dataset only. However, the wide 95% HPD intervals of reconstructed N_e changes do not allow us to draw conclusions about the demographic changes with high certainty. Our analyses using Approximate Bayesian Computations showed that we cannot reject the constant population size model for European wolves, although such result may be caused by difficulties to detect moderate bottlenecks using mtDNA with simulation analyses (Mourier et al., 2012, see detailed discussion in SI). Therefore, it may be important to further assess the demographic changes in European wolves using nuclear genomic data.

4.2 | Partial replacement of mitochondrial lineages in Europe

Our analyses suggest that the haplogroup W2 of the MHW clade originated in Europe around 35 kya, that is prior to the estimated time of immigration of Siberian wolves to Europe about 23.5 kya (Loog et al., 2020). Haplogroup W2 may therefore represent the population that existed in Europe prior to that event and was not completely replaced by Siberian wolves, but experienced a demographic decline. Partial ancestry replacement is supported by the observed changes in the haplogroup frequencies over time. Haplogroup W2, which was dominant in Europe since the Late Pleistocene, became much less frequent between the early Holocene and 20th century and was observed in only 20% of analysed samples from 19th to 20th centuries. These results are consistent with those from the whole-genome study on ancient wolves, showing that the ancestry replacement resulting from the expansion of Siberian wolves after the Last Glacial Maximum was incomplete and a small proportion of older ancestry has persisted in Europe until present (Bergström et al., 2022). Our reconstruction of the ancestral distribution suggested that the haplogroup W1 originated from North-Western Siberia, in or near the Yamal Peninsula (Figure 3a), while an earlier study pointed to Beringia as the source of the expanding modern wolf population (Loog et al., 2020). This discrepancy may result from the poor sample coverage of Siberia in both our and earlier studies, and therefore, more intense sampling from this region is required to establish the precise geographic source of this expansion.

4.3 | Population history of French wolves

Recent population history of French wolves is similar to other Western European populations. In the 19th and 20th centuries, European wolf populations declined dramatically as a result of human persecution and the species was extirpated from most of western and northern Europe (Baillon, 2016; Krofel et al., 2017). The Alpine wolf population reportedly disappeared at the beginning of the 20th century (Valière et al., 2003 and references therein), although a recent study showed that a few individuals survived in the French Alps up to the mid-20th century (Dufresnes et al., 2019). The youngest samples from our dataset are dated to 1944 CE (Central France) and 1954 CE (Southern France), which is consistent with Baillon (2016), who reported that wolves in France were observed until the 1970s.

Analyses of the genetic distances based on a D-loop fragment showed that historical French populations were generally less differentiated from other historical European/Middle Eastern populations than from modern populations (Table S9). These results indicate that in the past European wolf populations were more connected and less structured than nowadays, which is consistent with previous results (Dufresnes et al., 2018). High differentiation between north-eastern and southern/north-western populations shown by the pairwise F_{ST} (Tables S8, S9) suggest limitations in gene flow between southern and north-eastern France

in the past. Accordingly, current wolf colonization from Italy to France occurs mostly through the Alps (Fabbri et al., 2014; Valière et al., 2003), while north-eastern France may be recolonized from both Germany and Italy. However, the genetic distance estimates for the historical French population may be affected by the sample size; therefore, more extensive sampling will be needed to corroborate our findings.

We found that the French population before eradication had high genetic diversity, with haplotype diversity of 0.67 and 0.76 in ancient/medieval and historical populations, respectively, and nucleotide diversity of about 1% in both periods (Figure 5, Table S7). Although none of the haplotypes that were present in France between early Holocene and 11th century was shared with the historical population, the close genetic similarity between samples from Iron Age, Roman Age and Medieval periods with samples from 19th–20th centuries suggest that some maternal lineages in the French population maintained continuity from the Middle Holocene to the period directly preceding human-mediated eradication. The only haplotype that is shared between the historical French wolves and modern wolves that recolonized the country is typical of the Alpine population. Italian wolves have one predominating mtDNA haplotype, which as a result of northward expansion of this population through the Alps is currently spreading in southern France (Dufresnes et al., 2019; Fabbri et al., 2014; Randi, 2011). This Italian/Alpine haplotype was present in a wolf M27 from 1848CE and closely related haplotypes were found in wolves M60 (1900CE) and A109 (age unknown—see discussion in SI), all of which originated from Southern France, near the Alpine region. These results suggest that in historical times, the distribution of what is now the Alpine population was wider and not restricted to the mountainous region, which is consistent with the findings of Dufresnes et al. (2018), who showed that haplotypes from the Alpine population were present in eastern and northern Europe as well as in the Middle East. Genetic similarity between the historical French wolves and contemporary individuals may also suggest that the current wolf expansion from Italy through the Alps may enable a partial recreation of the gene pool of the original French population. Recent studies of the Iberian wolves showed that despite the population expansion and stable population size in the last decades, the mitochondrial genetic diversity is still declining (Salado et al., 2022). Therefore, although the wolf population in France is in recovery, the close genetic monitoring of the expanding populations is important, as this population may still be of conservation concern.

4.4 | Diet composition of French wolves

Diet reconstruction based on stable isotopes showed that French wolves in both ancient/medieval and historical (19th–20th century) times fed mostly on large ungulates, similar to modern European wolves (Newsome et al., 2016; Pilot et al., 2012). Few individuals showed a high proportion of leporids (rabbits and hares) in their

diet, which is not typical to wolves and suggests that these individuals had a different ecological niche. A study of Magdalenian canids showed that a high proportion of small mammals like ground squirrels (*Spermophilus major*) and hares are characteristic for a fox-like niche (Baumann et al., 2020). However, all the samples except A8 (which failed the sequencing) were confirmed to be grey wolves or dogs based on their mtDNA. This shows that diet composition alone is not a good criterion for species identification.

One of the historical samples, M102, had a different diet from contemporaneous individuals, with a higher proportion of beaver and wild boar. M102 was the only historical sample from central France subject to stable isotope analysis, while the other analysed historical samples originated from eastern France; therefore, differentiation in diet may result from differences in habitat types and the composition of ungulate communities. Similar differences in diet composition between regional populations were reported for contemporary Eastern European wolves, for example northern subpopulations showed higher proportions of moose, hare and beaver in their diet, compared with southern subpopulations that showed a higher proportion of wild boar in their diet (Pilot et al., 2012).

Changes in prey availability and resulting shifts in diet have been suggested for wolf populations in the Late Pleistocene and during the Holocene (Germonpré et al., 2009; Leonard et al., 2007). For example, in the Late Pleistocene wolves from Yukon territory were primarily feeding on horses (*Equus* sp.) with the addition of woolly mammoth (*Mammuthus primigenius*) and reindeer (*Rangifer tarandus*), while contemporary Yukon wolves feed mostly on reindeer and moose (*Alces alces*; Landry et al., 2021). This shift in diet, resulting from the local extinction of horses and mammoths, was proposed to be coupled with the extinction and replacement of one of the Late Pleistocene wolf ecomorphs, which resulted in the genetic diversity loss (Leonard et al., 2007). Stable isotope and dental microwear analyses provide evidence for a similar shift in diet in European wolves (Flower et al., 2021; Flower & Schreve, 2014). Before the Last Glacial Maximum European wolves fed on larger prey such as horse, woolly rhinoceros (*Coelodonta antiquitatis*) and bison (Flower et al., 2021), whereas modern wolf diet is mostly dominated by medium-size wild ungulates: roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), and chamois (*Rupicapra rupicapra*) and larger ungulates: moose and red deer (*Cervus elaphus*; Newsome et al., 2016). The contraction of geographic ranges in herbivore species, for example aurochs (*Bos primigenius*), European bison (*Bison bonasus*) and moose (Crees et al., 2016) during the Holocene (Iron Age–Roman Age) predates the estimated time of the wolf population decline in this study. Diet reconstruction for the analysed samples showed that in ancient times (Iron Age–Roman Age) and the recent historical period (19th–20th centuries) large ungulates were the main source of the wolf diet (Table S5). Our analyses were, however, not precise enough to detect changes in the composition of hunted species through time due to small sample sizes and overlapping ranges of isotopic values for different ungulate species.

4.5 | Origin of dog mtDNA haplogroups

Dog haplogroup D, which currently has a restricted distribution in Europe and the Middle East (Duleba et al., 2015), clusters within the wolf haplogroup W2 (Figures 2, S6). We showed that this wolf haplogroup occurs in European wolves only and originated in Europe in the Late Pleistocene (27–44 kya). The conclusion that the distribution range of haplogroup W2 was restricted to Europe could have been affected by the underrepresentation of mitogenome sequences of wolves from West Asia relative to Europe. However, we confirmed this conclusion in an additional analysis using D-loop sequences, where 31% of haplotypes included originated from West Asia. The European origin of the wolf haplogroup W2 implies that dog haplogroup D originated in Europe as well, either as a result of local domestication of European wolves or introgression of mtDNA haplotypes from European wolves into local dog populations. This contradicts previous studies suggesting that dog haplogroup D appeared in Europe via expansion from South-East Asia (Duleba et al., 2015) or human-mediated introduction from the Near East in the Neolithic (Ollivier et al., 2018). The spatio-temporal patterns of haplogroup D distribution are consistent with the latter hypothesis, as the oldest dog remains representing haplogroup D come from Iran (7.9 kya), followed by slightly younger remains from Southern and Central Europe (7.4–4.3 kya; Frantz et al., 2016; Pionnier-Capitan, 2010). Moreover, Ollivier et al. (2018) showed that the observed frequencies of haplogroup D after the Neolithic transition could have arisen due to genetic drift only if the frequency of this haplogroup in Southern Europe was very high (above 41%) in the pre-Neolithic period. This is inconsistent with the lack of pre-Neolithic records of this haplogroup in Europe. However, conclusions based on the age of the remains should be treated with caution, as new findings may revise previous hypotheses. For example, archaeological data previously suggested that dog haplogroup A arrived in Europe no earlier than in the Bronze Age (Frantz et al., 2016), but more recent discoveries showed that haplogroup A was already present in Iberia in the Mesolithic (Pires et al., 2019) and in the Apennine Peninsula in the Late Pleistocene (25 kya; Ciucani et al., 2019). Moreover, Ollivier et al. (2018) stated that based on their analyses they cannot reject the hypothesis that haplogroup D emerged in Europe and changed frequency to the level observed in the post-Neolithic period due to drift alone, when considering whole Europe as a single region.

The hypothesis of the Neolithic expansion of dogs from the Near East is consistent with a recent finding based on the nuclear genomic data that European Neolithic dogs show the east-west cline of genomic ancestry related to Near Eastern dogs (Bergström et al., 2020). That study concluded that human Neolithic expansion from the Near East to Southern Europe affected the gene pool of European dogs and the observed cline reflects admixture of the European Mesolithic dogs with dogs brought by Near Eastern Neolithic farmers (Bergström et al., 2020). Our results do not contradict this finding, but show that dog haplogroup D did not originate from the Neolithic expansion of Near Eastern dogs and had local origins in Europe. We also found that haplogroup D is closely related to

canids from Germany and Switzerland dated to 15–14 kya (Figures 2, S4), which were hypothesized to be the representatives of an early wolf domestication stage (Baumann et al., 2021), further supporting local domestication or introgression from wolves carrying the wolf haplogroup W2 to dogs in Europe. This is consistent with the finding that East Asian and West Eurasian wolves constitute two ancestry sources of domestic dogs, which implies either two domestication sources or introgression from local wolves from West Eurasia to domestic dogs originating from East Asia (Bergström et al., 2022).

Haplotypes of seven French wolf samples and one sample from Czech Republic (Y37, see Discussion in SI) fall within dog haplogroups A and C (Figures 2, S4). This result may suggest that either: (1) these individuals are domestic dogs or F1 hybrids, (2) these individuals carry dog haplotypes because of introgression of dog mtDNA lineages into the wolf gene pool, or (3) the dog haplogroups A and C originated from the introgression of mtDNA lineages of European wolves to the domestic dog gene pool. For four samples carrying dog haplotypes (A12, A92, M58 and M74), we obtained dietary reconstruction based on isotopic analyses. Both historical samples, M58 and M74, had very similar isotopic signatures to the wolf samples from the same period, with large ungulates inferred as the main food source (Table S5). Therefore, these two individuals were more likely wolf-dog hybrids rather than domestic dogs, or carried dog haplotypes because of an earlier introgression event. Morphological analyses of the sample M74 showed its intermediate position between wolves and dogs (see Supplemental Information), which suggests that this individual is most probably a wolf-dog hybrid.

For the older samples—A12 and A92—the most common prey was cattle, while other ancient/medieval wolves analysed were shown to feed mostly on large wild ungulates. The high proportion of domestic animals in the diet of A12 and A92 individuals in combination with their mitochondrial lineages falling into the dog haplogroups and morphological measurements of A92 also falling into the dog cluster indicate that these individuals were domestic dogs. The remaining French canids most likely carried dog haplotypes as a result of past hybridization followed by introgression to the wolf gene pool (scenario 2). Unlike the case of dog haplogroup D, there is no evidence for the origin of haplogroups A and C from European wolves, which makes scenario 3 (which assumed that dog haplogroups A and C originated from the introgression of European wolf haplotypes to the domestic dog gene pool) unlikely.

The occurrence of haplogroups A and C in ancient dogs from France is consistent with previous studies, which showed that these were the dominant dog haplogroups in Portugal (Pires et al., 2019) and Italy (Koupadi et al., 2020) throughout the Holocene, although in Bulgarian dogs haplogroup B, instead of C, was the second most dominant haplogroup (Yankova et al., 2019). Due to the small sample size, we cannot make assumptions about the changes in dog haplogroup frequency in France, but it is worth mentioning that samples from haplogroup C are older (Iron Age and 13th–14th century) than samples from haplogroup A (19th century), which is consistent with general pattern of increase in the frequency of haplogroup A over time in Eurasian dogs (Koupadi et al., 2020).

Five French canids included in our phylogenetic analyses had black coat coloration and therefore were suspected to have a hybrid origin (see Anderson et al., 2009). One individual, M112, was most likely a domestic dog, as both phylogenetic and morphological analyses clustered it with domestic dogs (Tables S1, S2, Figure S2). Other four black individuals (M16, M26, M27 and M41) carried mtDNA haplotypes typical of wolves, which did not fall into any of the dog haplogroups. These individuals could be hybrids originating from dogs in a paternal line, or their black coat colour could have resulted from introgression following an earlier hybridization event. However, the deletion associated with black coat colour was recently found in a 14,000 years old wolf from Siberia, suggesting that Pleistocene wolves could have been the ultimate source of this trait (Bergström et al., 2022). Therefore, black colouration in wolves is not necessarily an indicator of dog ancestry and nuclear DNA analyses are required to identify possible signatures of dog admixture.

5 | CONCLUSIONS

Our results suggest that despite the demographic changes and decline in N_e in European wolves, there was a genetic continuity in the French wolf lineages throughout the Holocene, and prior to the human-mediated eradication the French population was characterized by high genetic diversity. Current recolonization of France by Alpine wolves that show a genetic similarity with the historical French population gives a chance of at least partial restoration of the gene pool of the original population.

Our worldwide phylogeographic analysis indicated that mitochondrial haplotypes of wolves that lived in Europe throughout the Holocene cluster into two main haplogroups. One of them (W2) has been restricted to Europe and originated in this continent, while the second one (W1) has a Holarctic distribution and its inferred place of origin was north-western Siberia. Haplogroup W1 has increased in frequency in Europe during the Holocene, which is consistent with the recent inference of an incomplete ancestry replacement on this continent following expansion of Siberian wolves during the Last Glacial Maximum (Bergström et al., 2022). The reasons for the population decline and ancestry replacement in the Holocene are yet to be discovered, but ecological changes may be the main factor, as suggested for the North American population. Our results also suggest that one of the main dog haplogroups originated in Europe as a result of local domestication or introgression from European wolves. The evolutionary history of European wolves during the Holocene thus involved a long-term demographic decline, partial lineage replacement and gene flow with domesticated lineages.

BENEFIT-SHARING STATEMENT

A research collaboration was developed with scientists who provided genetic samples; all collaborators are included as co-authors.

The results of the research have been shared with the broader public via public databases.

AUTHOR CONTRIBUTIONS

M.P. and A.S. designed the study; K.D. and F.P. performed research, K.D. analysed data; A.S., G.L., F.B., E.B., E.C.-B., S. Frère, S. Fourcas, A.G., Ch.G., A.L., W.M., R.P., S.P. and J.-H. Y. provided samples; A.S., M.P. and G.L. acquired funding for the project; K.D. and M.P. wrote the manuscript with input from all co-authors.

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CONFLICT OF INTEREST STATEMENT

This research was funded by the National Federation of Hunters, France. One of the study authors, Gérard Lang, is a member of this association. However, the study does not express any opinions about the positive or negative effects of hunting and does not make any recommendations about the management of wolves.

DATA AVAILABILITY STATEMENT

Raw reads from this study were deposited at the NCBI SRA under accession no. PRJNA921966. Mitochondrial genome consensus sequences obtained in this study were deposited at the GenBank under accession nos. OM743353–OM743430 (Doan et al., 2023).

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