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Ancient DNA sheds light on the funerary practices of late Neolithic collective burial in southern France

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The Aven de la Boucle (Corconne, Gard, southern France) is a karst shaft used as a collective burial between 3600 and 2800 cal BCE. The site encompasses the skeletal remains of approximately 75 individuals comprising a large majority of adult individuals, represented by scattered and commingled remains. To date, few studies have explored the potential of ancient DNA to tackle the documentation of Neolithic collective burials, and the funerary selection rules within such structures remain largely debated. In this study, we combine genomic analysis of 37 individuals with archaeo-anthropological data and Bayesian modelling of radiocarbon dates. Through this multidisciplinary approach, we aim to characterize the identity of the deceased and their relationships, as well as untangle the genetic diversity and funerary dynamics of this community. Genomic results identify 76% of male Neolithic individuals, suggesting a marked sex-biased selection. Available data emphasize the importance of biological relatedness and a male-mediated transmission of social status, as the affiliation to a specific male-lineage appears as a preponderant selection factor. The genomic results argue in favour of 'continuous' deposits between 3600 and 2800 BCE, carried out by the same community, despite cultural changes reflected by the ceramic material.

1. Introduction

The use of collective burial (i.e. the successive gathering of several individuals within the same funerary structure over time) is an emblematic phenomenon of the second half of the Neolithic period throughout western Europe. In southern France, it became widespread during the second half of the fourth millennium BCE, which corresponds to the transition from the broad Chasséen culture to one of the various cultures that emerged at the regional level at the end of the Neolithic period (i.e. the Ferrières culture). Identifying the social motivation underlying the use of collective burial practices remains challenging for several reasons. For example, the dichotomy between megalithic monuments and cavities gave rise to debates surrounding the reasons for these distinct choices in funerary locations. Since the natural settings of certain regions within southern France permit both types of structures to coexist, the choice of location calls for arguments other than environmental opportunism and could be connected to cultural preferences and traditions [1–4]. Frequently used over several generations, collective burials are generally seen to include individuals from the same

community, symbolizing collective ancestry without emphasizing individual identities. However, it is also suggested that such burials could have included individuals from various families, thus serving to strengthen sociopolitical ties between distinct local groups [5,6]. Here, we propose a multidisciplinary approach combining archaeological, radiocarbon and genomic data from the Aven de la Boucle natural cavity to document for the first time the dynamics of the use of a Neolithic collective burial in France.

The Aven de la Boucle is located near Corconne (Gard) in southern France and was used as a collective burial from the second half of the fourth millennium BCE (figure 1, electronic supplementary material, figure C1). The group of the deceased is represented by scattered remains across the funerary space, with few anatomical connections [7–10]. The archaeo-anthropological study of the Aven de la Boucle assemblages demonstrated the primary deposition of the dead inside this cavity, with marked selection according to the age at death of the individuals, displayed by the massive exclusion of children.

The quantitative analysis of skeletal parts identified a minimum number of individuals approximating 75, based on calcaneus bones (MNI by exclusions, electronic supplementary material, figure C2), with evidence of the retrieval of some skeletal parts by the living [7–11]. Indeed, voluminous pieces such as coxal, cranial remains and long bones account for approximately 30 to 45 individuals (electronic supplementary material, figure C2). Two stratigraphic horizons are superposed and distinguished through archaeological material, notably ceramic elements. At the base, the first corresponds to a transitional stage from the Final Chasséen to the Ferrières cultures, also referred to as the 'Recent Neolithic' or Pre-Ferrières. The second belongs to the Ferrières culture *sensu stricto* [10]. These funerary occupations, whether continuous or not, took place as the cavity was accessible through the rearranged joint. This passage was condemned after its use for funerary purposes, and the cavity remained closed until a vertical shaft opened accidentally. Therefore, the cavity was reoccupied sporadically during the Fontbouisse period at the end of the third millennium BCE and the final stage of the Bronze Age (BA) (Final Bronze Age II; approx. 1200–900 BCE) [12]. The succession of these main Neolithic horizons raises the questions of continuity versus discontinuity of communities using the cavity and the evolution of funerary rites at the end of the Neolithic in eastern Languedoc. At the genomic scale, extensive documentation on the end of the Neolithic period is still lacking in France. Recent genome-wide studies revealed 39 individuals for the whole French territory, spanning approximately 3600 to 2000 BCE [13–17]. Despite the heterogeneity of cultural contexts and the variability of population dynamics documented during this time transect in the modern-day French territory, no local or microregional focus has been conducted thus far.

Our multidisciplinary study was articulated around several lines of research. First, we aimed to document the biological identity of the deceased and to discuss the entwinement of the genomic results and archaeo-anthropological observations. Second, we questioned the potential of combining ancient DNA studies and radiocarbon dating to decipher the dynamics of sepulchral cavity use, notably by addressing the question of continuity versus discontinuity regarding deceased deposits. In a third line of research, we also addressed the genomic results obtained at the population level by examining how the individuals represented at the Aven de la Boucle fit into the regional and pan-European picture.

2. Results

(a) Overview of the Aven de la Boucle dataset

In this study, we present genome-wide data for 37 individuals from the Aven de la Boucle, including 10 previously sequenced individuals from this cavity [18]. Among the 37 individuals analysed, 27 are directly dated, we report 17 new radiocarbon dates, complementing 10 dates previously reported [18] (figure 1b; electronic supplementary material, table S3). The dataset is represented by 34 Neolithic individuals (3800–2900 BCE) and three BA individuals (electronic supplementary material, tables S1–S3).

For palaeogenomic analyses, we sampled 41 left petrous bones to retrieve bone powder from which DNA was extracted. Libraries were then constructed, and post-mortem deamination patterns consistent with ancient DNA origin were examined. The first screening via shallow shotgun sequencing allowed us to select libraries from 39 individuals who passed quality filtering (electronic supplementary material, table S2, mapped reads and more than 0.1% of endogenous DNA). Ten libraries with low proportions of endogenous DNA content (0.3–13.5%) were enriched for 1.2 million single-nucleotide polymorphisms (SNPs) using targeted in-solution capture ('1240 k' SNP capture [19]), while 29 libraries with satisfactory proportions of endogenous DNA content (10.3–71.3%) were selected for whole-genome shotgun sequencing. After excluding one individual (BOU26) with a coverage of 0.04×, we obtained genome-wide data for nine samples with a mean coverage between 0.08× and 2.7× as well as 29 partial genomes with a coverage between 0.05X and 0.68X (electronic supplementary material, tables S1 and S2). We estimated potential contamination of the nuclear genome by examining the heterozygosity of polymorphic sites on the X chromosome in males. Assuming a contamination threshold of approximately 5%, we excluded one individual (BOU43) from downstream analyses [20].

(b) Genetic sex determination and uniparental markers

We confidently identified 8 female individuals (XX) and 28 male individuals (XY) (electronic supplementary material, table S4, figure C10). For two additional individuals, genetic sex determination provided ambiguous results (BOU29, Y reads ratio 0.0287–0.0299, most likely female; BOU21 Y reads ratio 0.0564–0.0585, most likely male). In addition, individual BOU12 displayed a Y/autosome ratio of 0.84, while the X/autosome ratio was within the expected range for a male individual, and X-contamination was low. Therefore, it is likely that this individual carried an XYY karyotype (electronic supplementary
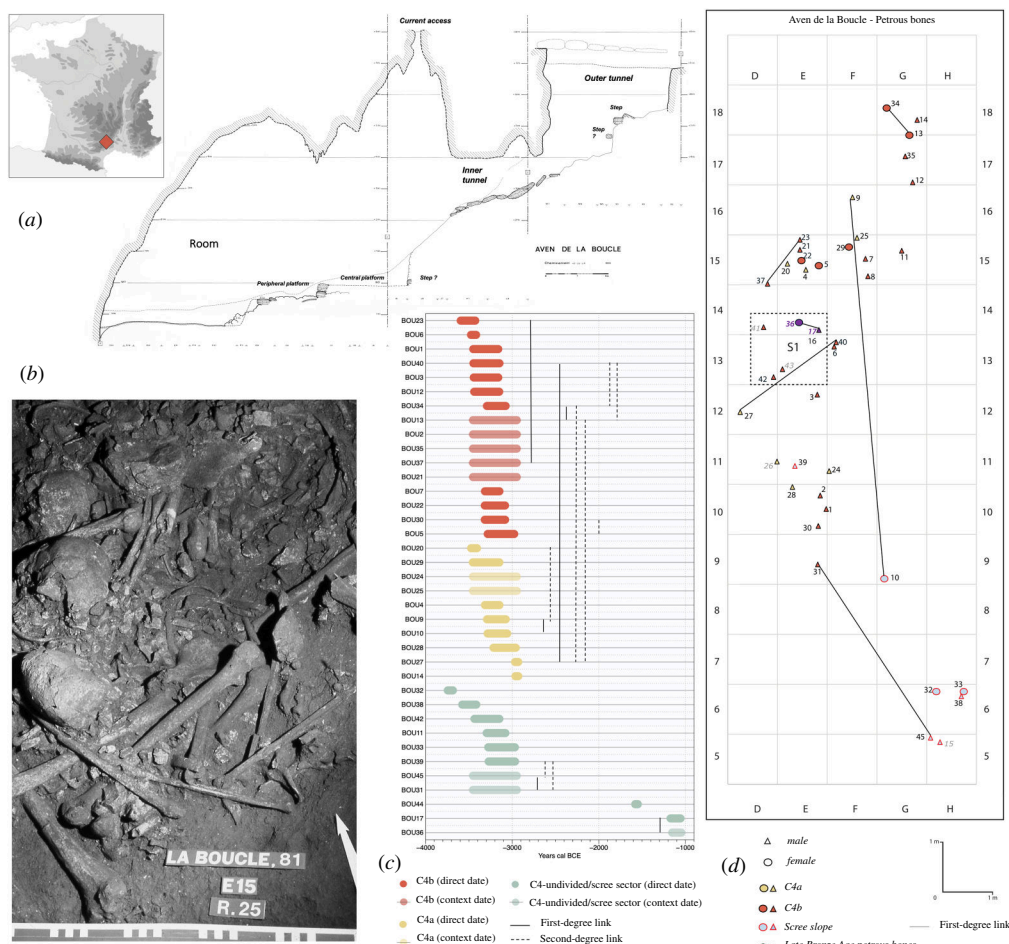
Figure 1. Geographical and chronological description of the Aven de la Boucle. (*a*) Location of the site in modern-day France and longitudinal section plan of the cavity. (*b*) Skull and long bone alignments gathered in bundles near the west wall of the cavity, photo: H. Duday, 1981. (*c*) Chronological distribution of samples displaying directly dated individuals and individuals dated through contextual date. Biological relations between individuals are indicated by the full lines (first degree) and the dashed lines (second degree), and colours indicate stratigraphic attributions (electronic supplementary material, tables S1, S3 and S8). (*d*) Plan and location of sampled petrous bones, samples without genomic results are marked in grey, grids indicate the denomination of excavated sectors within the cavity, colours indicate stratigraphic attributions and first-degree relations are signalled by full lines.

material, table S4, figure C10). This type of chromosomic anomaly is known as 47,XYY syndrome [21]. With a prevalence of one in every 1000 births, it is a relatively rare type of trisomy that can affect male individuals without phenotypic implications. It can also manifest as symptoms such as tall stature, learning disability and infertility.

Uniparentally inherited markers were identified using mitochondrial and Y chromosome data (electronic supplementary material, tables S6–S8). The Aven de la Boucle individuals carry mitochondrial sub-haplogroups H ($n = 13$), K ($n = 8$), U ($n = 9$), T ($n = 4$), X2b ($n = 2$) and V ($n = 1$), which were previously reported among Neolithic groups from western Europe and represent maternal ancestors of both Anatolian farmers and western European hunter–gatherers (electronic supplementary material, tables S6–S8). Indeed, six individuals carried haplogroups derived from U5b, and three individuals carried haplogroups U2e1c1, U4 and U8, which are likely to be attributable to the Mesolithic ancestry of western Europe [22–24].

Neolithic male individuals were found to carry Y chromosome haplogroups derived from I2a1 ($n = 4$), H2a1 ($n = 2$), G2 ($n = 19$) and R1b1b ($n = 1$). Except R1b1b, such haplogroups are relatively common in western European Neolithic communities from both continental and Mediterranean expansion routes (i.e. France [15,16,18,25,26], Switzerland [17], Germany [26–29] and the Iberian Peninsula [13,14,28,30–33]). Although rare in Neolithic communities from western Europe, the haplogroup R1b1b carried by BOU38 (3626–3369 BCE) has been previously identified in western Mediterranean Early Neolithic communities from Els Trocs, Cueva de Chaves and Grotta Continenza [19,32,34]. The high frequency of the G2a lineage (73%) is similar to what has been previously described for the contemporary cave of Les Treilles (90% of the G2a lineage [25]; Aveyron, France). This may reflect a general predominance of this haplogroup in southwestern France at the end of the Neolithic [31,35]. BOU44 and BOU17, dated to the second half of the second millennium BCE, both carry haplogroup R1b1a1b, consistent with previous records regarding the genetic impact of Pontic steppe herders [27].

(c) Radiocarbon dating

Regarding radiocarbon dating, 25 individuals ranged between approximately 3800 and 2900 BCE and confirmed that the collective burial was mainly active during the second half of the fourth and beginning of the third millennium BCE, which
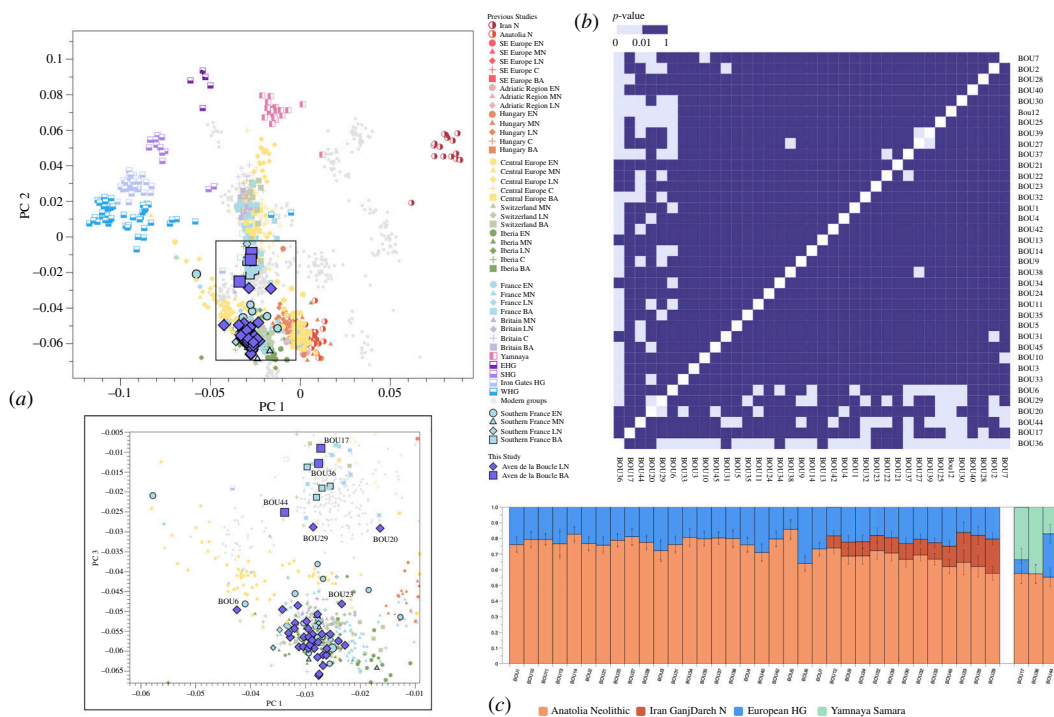
Figure 2. Population genetics analyses. (*a*) PCA presents the genetic variability of previously published ancient individuals from Eurasia and 37 individuals from the Aven de la Boucle collective burial (newly reported and published) projected onto modern populations from western Eurasia. (*b*) Heatmap of pairwise qpWave clustering of the Aven de la Boucle individuals. Pairs represented by light purple squares correspond to *p* values below 0.01 and were rejected based on [37]. Matrix details are provided in electronic supplementary material, table S9. (*c*) qpAdm analysis of BOU individuals using Anatolian Neolithic, European_HG, Iranian Neolithic or Yamnaya Samara groups as ancestral sources (electronic supplementary material, tables S12, S13 and S15).

broadly corresponds to the bounds of the Ferrières culture in the region [36]. In addition, five undated individuals can be attributed to this chronological range because they are involved in first- or second-degree biological kinship with directly dated individuals (figure 1*c*). In the scree slope sector and within *Sondage* 1 (electronic supplementary material S1; figure 1*d*), remains can be attributed to sporadic use of the cavity during the BA. Thus, individual BOU44 ranged from approximately 1600 to 1500 BCE, corresponding to the Middle BA, whereas individual BOU17 was directly dated to the Late BA, between approximately 1200 and approximately 1000 BCE (electronic supplementary material, table S8; figure 1*c*).

(d) Integration of the Aven de la Boucle group into macro-regional variability

To explore genetic variation among our individuals, we constructed a principal components analysis (PCA) based on a set of modern-day Eurasians, on which we projected the Aven de la Boucle samples along with previously published ancient individuals (figure 2). The Neolithic group of the Aven de la Boucle falls within the general variability of western European Neolithic individuals and, more specifically, with previously reported individuals from France and the Iberian Peninsula dated from the fifth to the late third millennium. Nevertheless, we detected two outliers, BOU20 and BOU29, which displayed a very distinct shift upwards in PC2 variation from the main cluster. These individuals cluster with previously reported Bell Beaker individuals from Central Europe and the Iberian Peninsula as well as Early to Middle BA samples from France, despite being contemporaneous with the rest of the Neolithic group from this sepulchral cavity [13,15,16,38].

The results of qpWave analysis supported the outlier status of Neolithic individuals BOU20 and BOU29 as well as individual BOU6, who carry additional WHG ancestry, as already described (electronic supplementary material, table S9) [18]. The remaining Neolithic individuals formed a clade with a common genetic background (figure 2*b*; electronic supplementary material, table S9). At the regional scale, qpWave results highlight the genetic clustering of the Aven de la Boucle Neolithic group with southern France individuals dating from the late fourth to the mid-third millennium and lacking steppe ancestry (electronic supplementary material, table S9, figure C20). Conversely, outlier individuals BOU20 and BOU29, as well as the three BA individuals, cluster with Bell Beaker (La Fare, Grotte des Tortues, Grotte Basse de la Vigne Perdue [13,16]) and BA individuals from southern France (Villard, Le Pirou, St Eugène, Quinquiris [15,38]). This clustering was also supported by the pairwise outgroup-f3 test of the f3 form (Mbuti; individual 1; individual 2) (electronic supplementary material, table S10, figure C16).

Mirroring these findings, outgroup-f3 statistics of the form f3 (Mbuti; *Test*, BOU), using various western European Neolithic groups between approximately 5600 and 2500 BCE as *Test*, permitted us to explore the geographical affinities of the Aven de la Boucle group and outlier individuals with contemporaneous western European Neolithic individuals (electronic supplementary material, table S11, figure C19). The results indicate that the Aven de la Boucle Neolithic group has local variability, as it shows the highest genetic affinities with neighbouring groups originating from collective burials at Les Peirières (2900–2650 BCE [15]) and Collet Redon (3600–3400 BCE [13]). The Neolithic outlier individuals BOU20 and BOU29 display lower f3 values and

therefore lower genetic affinities with local groups. Individual BOU20 shares more affinities with contemporaneous individuals from Murcia, Spain (2900–2600 BCE), and BOU29 shares the highest genetic affinities with the late Neolithic individual TGM008 from Tangermünde, Germany [13,26].

We used qpAdm to explore sources of genetic ancestries in the Aven de la Boucle community. First, we modelled the Neolithic individuals as a two-way mixture of Anatolia farmers and European HG ancestries (electronic supplementary material, table S12). As previously reported, the Neolithic individuals from the Aven de la Boucle carry various amounts of the European HG genetic component (14.7–36.2%; electronic supplementary material, table S12), consistent with heterogeneous HG legacy and heterogeneous admixture processes between human groups throughout the Neolithic [16,18,26]. Drawing from the observations reported in previous studies on the differential distribution of various types of HG and early farmer ancestries among western European Neolithic groups, we explored alternative models. We aimed to trace the presence of so-called Magdalenian-related ancestry, as previous studies highlighted the persistence of this type of genetic component among South-western European Neolithic groups. Notably, we sought to test whether an additional GoyetQ2 component could be a candidate for explaining the outlier status of BOU29 and BOU20 [15,18,31,32]. We found no evidence of additional Goyet-Q2-like ancestry, as all Neolithic individuals can be modelled with Oberkassel ancestry only (formal Villabruna ancestry [39]; electronic supplementary material, table S14, Model 3). In another test, we modelled the Aven de la Boucle Neolithic group by adding 'Iran_Ganj_Dareh_Neolithic' as a third source of the ancestral component to the first model (electronic supplementary material, table S15, Model 4). This additional genetic component was previously highlighted in several recent studies, appearing sporadically alongside the main Neolithic ancestry component in the Anatolian and Levantine groups from the sixth millennium BCE as well as within European Neolithic communities mainly stemming from the western Mediterranean [31,35,37]. This additional source seems to slightly improve the fit of the model, notably for BOU29 (p = 0.984886). We note that several individuals can be modelled as a three-way mixture of European HG, Anatolia and Iran Neolithic ancestries (n = 12, 7.7–19.9% Iran_Ganj_Dareh_N ancestry). This could therefore indicate that an additional ancestral component maximized in Iranian Neolithic individuals from Ganj Dareh could have contributed to the genetic ancestry of the Aven de la Boucle individuals to various degrees (electronic supplementary material, table S15; figure 2c). Individuals BOU20 and BOU29 carry the highest levels of this type of genetic ancestry, as they can be modelled with 19.9 and 21.9% of Iran_Ganj_Dareh ancestry, respectively. This point is also reflected in the results of the outgroup-f3 statistics of the f3 (Mbuti; Ind, Iran_Ganj_Dareh) form, with BOU20 and BOU29 displaying the highest f3 values among the groups (electronic supplementary material, figure C18).

It has been hypothesized that farmers spreading towards Europe along the Mediterranean were initially characterized by subtle variation in Near Eastern ancestry with different proportions of Levantine/Western Caucasus components as opposed to Western Anatolian ancestry. However, we cannot exclude the possibility that this genetic component was implemented in the western Mediterranean gene pool through exchanges with later groups originating from the Near East [31].

(e) Bronze Age individuals

The three BA samples originating from the Aven de la Boucle correspond to one female (BOU36) and two male individuals (BOU17 and BOU44) belonging to the Y chromosome haplogroup R1b1a1 (electronic supplementary material, tables S4 and S7), which has been linked to the dispersion of Pontic steppe herders who moved westwards at the end of the Neolithic Age/beginning of the BA [13,15,16,27]. The projection of the three new BA samples from the Aven de la Boucle collective burials on the PCA overlapped with previously published Early to Late BA samples from France and southwestern Europe and clustered more specifically with Early to Late BA individuals from southern France (Quinquiris, Le Pirou, Grotte des Tortues, Saint Eugène; figure 2a). Using qpAdm, we were able to model these individuals as a three-way mixture of Anatolia farmers (Anatolia_N), European HG and steppe-related (Russia_Yamnaya_Samara) ancestry (electronic supplementary material, table S13, qpAdm Model 2, figure 2c). The three individuals exhibited medium to low amounts of steppe-related ancestral components, with estimates ranging between 17 and 43% of Yamnaya_Samara ancestry. These observations fit previous records of the local impact of steppe migrations, as lower ancestry proportions were reported for southwestern Europe late Neolithic and BA communities [13,40].

(f) Biological relatedness, funerary and social functioning of the Neolithic community

The genetic sex analysis highlighted a strong under-representation of female individuals, as only 8 were identified as opposed to 26 male individuals, providing a sex ratio significantly imbalanced in favour of male individuals representing 76% of the Neolithic group (p = 0.0432; electronic supplementary material, tables S1 and S4, figure C10). The morphoscopic examinations of coxal bones also suspected a preponderance of male subjects, although proper quantification could not be performed.

Among male individuals, the analysis of uniparentally inherited markers underlines the over-representation of the Y chromosome haplogroup G2a2 (n = 19/26 males; 73%). Conversely, mitochondrial haplogroups exhibit a more diverse distribution, as frequently observed among Neolithic groups, which is often interpreted as evidence of female mobility and the practice of female exogamy in a patrilocal system (electronic supplementary material, tables S6 and S7 [41]).

We analysed the genetic relatedness between all Neolithic individuals and identified 16 pairs of biologically related individuals to the first-, second- and third-degree relationship (electronic supplementary material, table S8, figures C11–C15; figure 3). Interestingly, biological relatedness involves both male and female individuals despite the marked male-biased selection identified. The only two related female individuals, BOU13 and BOU34, were found in close proximity in the northern part of the cavity. These individuals carry haplogroup H4a and share second-degree relations with several individuals.
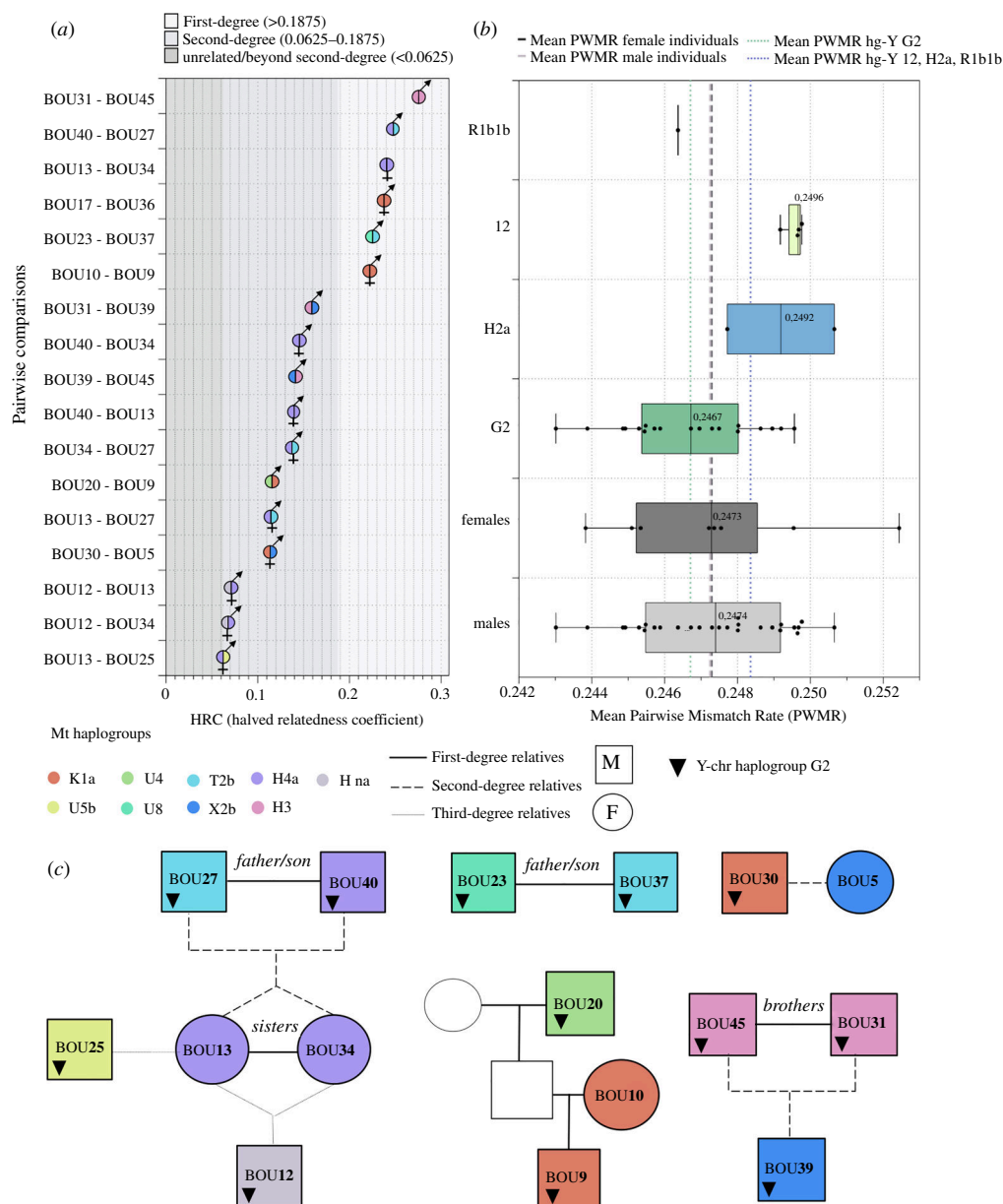
Figure 3. Biological relatedness analysis. (*a*) First-, second/third-degree relations using TKGWV2 results. For each pair, mt haplogroups are indicated by the colour. (*b*) Mean pairwise mismatch rate extracted from READ for the 34 Neolithic individuals, colours indicate Y chromosome haplogroups for male individuals (*c*) Pedigrees reconstructed for 16 individuals involved in biological relatedness, first-degree relations are represented by full lines and second-degree by dashed lines (electronic supplementary material, table S8, section V).

Combining these observations with sex and uniparental marker attributions, different pedigree scenarios were explored and are described in the electronic supplementary material (figure 3; electronic supplementary material, figures C11–C15). Although we cannot infer precise pedigrees with a satisfactory resolution, the results of biological relatedness and pairwise outgroup-f3 analysis are consistent with a close-knit family group using the cavity as a collective burial between approximately 3600 and approximately 2900 BCE (electronic supplementary material, tables S8 and S10, figure C15). Moreover, the anthropological study of the Aven de la Boucle collective burial identified a high frequency of an anatomical variation called *patella emarginata*. Indeed, 75% of the patellae display pronounced emargination of the upper lateral edge, equal to or exceeding a length of 10 mm (electronic supplementary material, figure C5). Interestingly, out of 11 individuals exhibiting an emargination exceeding 16 mm in length, 8 individuals were associated with the C4a unit (electronic supplementary material, figure C5). Given the unusually high frequency of this anatomical variation, the hypothesis of a high level of biological proximity between the deceased was originally proposed [7,8,10].

To assess the parental relatedness of the deceased individuals deposited in the Aven de la Boucle, we analysed runs of homozygosity through hapROH (electronic supplementary material, table S16, figure C21 [42]). Three individuals displayed long ROH (BOU31, BOU28 and BOU35), whose length and distribution could suggest that their parents were related as second cousins. These observations aside, the overall limitation of long ROH could indicate that this funerary group represents a population in which close kin unions were limited. The pattern of ROH distribution observed at the Aven de la Boucle echoes those of contemporaneous Neolithic groups and individuals reported in western Europe (electronic supplementary material, table S16, figure C21).
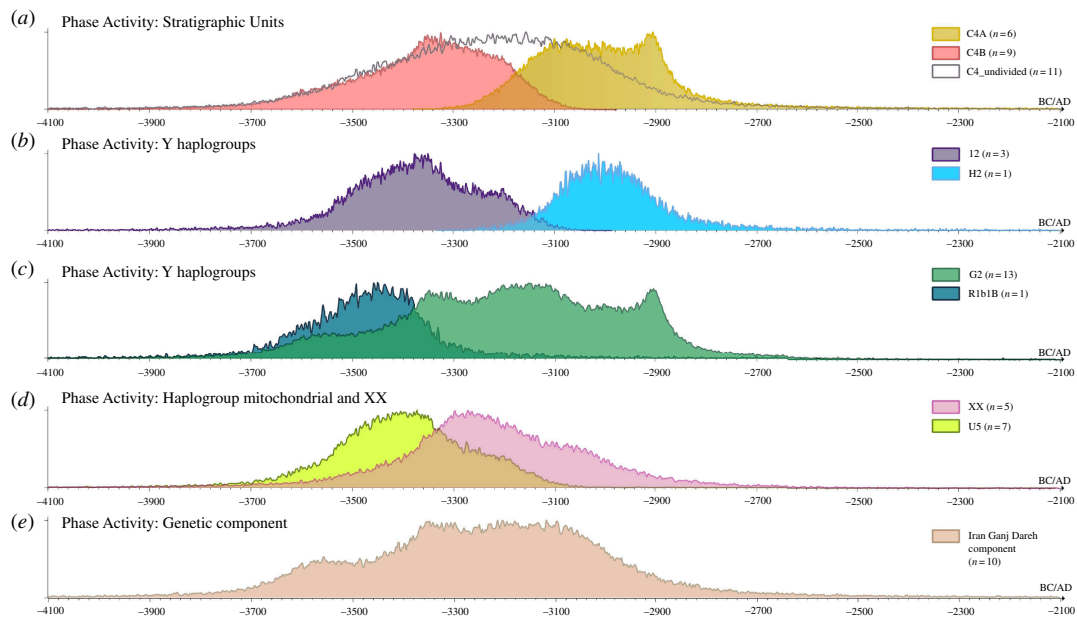
Figure 4. Bayesian modelling of the chronological phases of the Aven de la Boucle collective burial. The diagram illustrates the *a posteriori* chronology described by activity curves (probability densities of events within the different phases and clusters; electronic supplementary material, tables S17 and S18, section III).

To confirm female exogamy practices within this Neolithic community, we tested whether the few female individuals buried at the Aven de la Boucle had fewer close relatives at the site than male individuals (figure 3*b*; electronic supplementary material, table S8, figure C15; approach described in [31]). The observed mean pairwise mismatch rates between male and female individuals are highly similar; therefore, biological relatedness between Neolithic individuals is unlikely to be sex biased (figure 3*b*). We tested for a significant difference in genetic proximity between male and female individuals with f3 statistics for the f3 (Mbuti; female, female), f3 (Mbuti; female, male) and f3 (Mbuti; male, male) forms (electronic supplemenatry material, figure C17). We found no significant difference in f3 values between the female–female and male–male groups (*p*-value = 0.13) or between the female–female and female–male groups (*p*-value = 0.146). This does not support a significant difference in genetic proximity between females with other female individuals and males with either male or female individuals (electronic supplemenatry material, figure C17). These observations contrast with the female exogamy signal expressed by uniparental markers and could be linked to the biased selection of individuals who were granted access to the collective burial.

(g) Chronological modelling

We conducted Bayesian modelling of the radiocarbon dates obtained for 26 Neolithic individuals analysed on the genomic scale (figure 4; electronic supplementary material, tables S17 and S18, section III). We examined the chronological distribution of different clusters of individuals defined according to stratigraphic attributions. The *a posteriori* modes of phases beginning and ending [43] were retained here as relevant to describe the phases of site utilization (electronic supplementary material, table S18; [44]).

Both models constructed place the C4b unit ('Recent Neolithic'/Pre-Ferrières/Final Chasséen Culture) between 3600 and 3185 BCE, with a marked density of activity at approximately 3350 BCE and the C4a unit (Ferrières) between 3140 and 2880 BCE, and with a plateau of activity between approximately 3100 and 2910 BCE (figure 4).

The combined observations of stratigraphic attribution and radiocarbon dating of cranial remains therefore reveal no clear break between the first ('Recent Neolithic'/Final Chasséen Culture, C4b unit) and second phases of occupation (Ferrières culture, C4a unit). To an extent, these observations could argue in favour of the continuous use of collective burial throughout the cultural transition reflected in the archaeological deposits rather than a hiatus between distinct phases.

We also examined the traces of activity *a posteriori* of different clusters of individuals defined according to genetic criteria, i.e. males versus females, males carrying different Y chromosome haplogroups, mitochondrial haplogroup U5 or 'Iran Ganj Dareh' ancestry (electronic supplementary material, tables S1, S6, S7, S15 and C1, figures C8 and C9). From these models, several observations and hypotheses can be discussed. Traces of Y chromosome haplogroup I2 and mt haplogroup U5 are strongly correlated with each other (results of pairs overlap correlation—hereafter res.OCPairs: 0.91) and with the activity of the C4b unit (res.OCPairs: 0.86 and 0.82, respectively) but not with C4a (res.OCPairs: 0.14 and 0.13, respectively; figure 4; electronic supplementary material, figure C9, table C1). Moreover, several individuals belonging to the Y chromosome haplogroup G2 cluster contribute to the beginning of the C4b phase (res.OCPairs: 0.59, electronic supplementary material, figure C9, table C1), but while the activity traces of haplogroup I2 disappear after 3100 BCE, the chronological distribution of individuals carrying G2 is wider and vastly extends to the C4a unit (res.OCPairs: 0.57; electronic supplementary material, figure C9, table C1). Indeed, if we consider the distribution of the cluster formed by individuals carrying the G2 paternal lineage, as well as the female individual cluster (XX), they both significantly contribute to the C4a unit (and secondarily individuals carrying Y-H2 at the end of the phase). In addition, individuals who can be modelled with 'Iran Ganj Dareh' ancestry (GD; electronic supplementary material, table S15) are correlated with the distribution of the G2 cluster (BOU12, BOU23 and BOU30) and the

whole female cluster (res.OCPairs: 0.91 and 0.84, respectively; electronic supplementary material, figure C9, table C1). These individuals appear to contribute equally to the C4b and C4a units (res.OCPairs: 0.67 and 0.49, respectively). They appear at the beginning of C4b, and even as early as the late Chasséen, during the first half of the fourth millennium, if we consider the genetic profile and date of BOU32 (electronic supplementary material, tables S3, S17 and S18).

It is perhaps necessary to consider the division of C4b into two stages, the earliest more strongly anchored in the late Chasséen and the next marked by the contribution of individuals with uniparental markers related to Mesolithic ancestry (Y chromosome haplogroup I2 and mt haplogroup U5), whose visibility fades afterwards (figure 4; electronic supplementary material, figures C8 and C9, table C1).

3. Discussion and perspectives

A major challenge in the study of collective burials and commingled assemblages is to decipher the pre-established rules of selection and identify the biological and/or social principles according to which an individual is entitled to access the grave. The Aven de la Boucle sepulchral cavity is one of the most thoroughly documented among the fourth millennial collective burials in the Languedoc region. The assemblage that characterizes this site, in which two funerary sequences are discriminated by material culture, is also a unique opportunity to address the notions of cultural versus biological continuity or discontinuity during the late Neolithic period.

The genomic results highlighted a marked sex-biased selection, manifested by the over-representation of male individuals deposited inside the cavity. Within collective burials, morphological sex is frequently inaccessible or unreliable due to commingling and fragmentation of pelvic bones. Therefore, ancient DNA is an important contribution in this regard as reflected in this study. This marked imbalance in sexual representation argues in favour of the presence of a male-biased selection that could have been added to the selection of adults previously identified [9,10]. A differential preservation of less robust craniofacial blocks with a female deficit (through fragmentation or the action of water) appears highly unlikely as the Neolithic levels of the cavity have yielded a high number of much more fragile human bone pieces, such as hyoid bones and ossified thyroid cartilage [9].

In the light of the secondary gestures, the cranial remains represent a sub-sample of the dead population (56%, electronic supplementary material). Therefore, several questions can be raised regarding the under-representation of female individuals. First, this biased representation could illustrate that the primary deposition inside the cavity was preferentially granted to male individuals, whereas only a limited number of female individuals were allowed or chose to be included in the collective burial. A second hypothesis to explain the over-representation of males could be that cranial and coxal remains belonging to female individuals were preferentially removed from the cavity after decomposition occurred and moved elsewhere. Within the ethnographic record, the practice of temporary access to a collective funerary structure is, for instance, attested in West Sumba, where individuals awaiting their own funerary monument will temporarily be granted access to a collective burial without properly fitting the selective patterns [45]. Occurrences of temporary burials are also found among the Merina of the Antananarivo region in Madagascar, where remains can be temporarily placed within a collective tomb before the traditional exhumation of the bones is performed during the *famadihana* ceremonies [46–48]. At the Aven de la Boucle, the hypothesis of temporary female burials would presuppose that those bones remained identifiable, even after skeletonization. Considering these observations, the living would have had to recover both craniofacial blocks and coxal bones from a large number of women. Biased selection in favour of male subjects could therefore be more likely than selective recovery of many female remains after decomposition.

Among the Neolithic community, the selection of the deceased could therefore include age at death and biological sex criteria, but not in a totally fixed and strict manner insofar as a minority of female and immature individuals still accessed to the cavity.

Kinship ties, both biologically and socially defined, are often a strong candidate to explain the successive gathering of the deceased in collective burials. At the Aven de la Boucle, the biological proximity of the deceased suspected based on non-metric traits echoes the patterns of biological relatedness highlighted by genomic results. The over-representation of male individuals, as well as the predominance of the G2a2 paternal lineage, which is also the only lineage represented among closely related individuals, could indicate that selection criteria regarding access to the cavity were also anchored in a patrilineal descent [49]. These observations are somewhat reminiscent of the selection pattern described in the group buried inside the Treilles I cave, dated to 3000 BCE and located approximately 70 km away from the Aven de la Boucle. This sepulchral cave also contains the remains of predominantly male individuals, most of which were found to carry the Y chromosome lineage G2a [25]. Similar patterns have been reported recently in more geographically distant megalithic collective contexts, notably in the British Isles, and have been interpreted as relating to the transgenerational appropriation of burial grounds by patrilineal structured communities [50,51].

Furthermore, Bayesian modelling of the chronological distribution of the cranial remains indicates that the use of this collective burial started in a 'Recent Neolithic' (pre-Ferrières) phase corresponding to the C4b unit and included a group dominated by male individuals characterized by higher paternal lineage diversity. Without a clear hiatus separating phase, the results indicate that the use of the cavity as a collective burial continued during a period associated with the Ferrières and corresponding to the C4a unit. However, during this phase, a modification of the funerary functioning, or at least of the group using the cavity, is perceived as the G2 paternal lineage becoming largely dominant and almost exclusive. This finding appears to be correlated with the inclusion of several female subjects and the importance of close biological relatedness. Overall, these observations could illustrate a phase during which the male-mediated transmission of social status is more exclusive than during the previous phase. The affiliation with a specific male lineage appears to become a preponderant selection factor that

would not be incompatible with the inclusion of certain women tied in this filiation system. These observations might also indicate that women could choose their burial location [52].

However, both available materials and funerary gestures are important biases to consider, as they undoubtedly impede the ability to make a clear representation of the full living community or communities involved in the utilization of this sepulchral cavity. This representation bias has to consider both the retrieval of bone pieces and funerary selection employed by the living.

In southern France, genomic studies of late Neolithic megalithic tombs are scarce and have been restricted to a small number of individuals per site, with a focus on population genetics and migration dynamics. The present study represents the first extensive archaeogenomic documentation of a collective burial in France and a mean to explore the funerary selection processes from a different perspective. Additional data retrieved from different contexts, notably dolmens, would therefore be instrumental for properly comparing groups displaying different expressions of collective burial within a restricted region.

4. Material and methods

(a) Archaeological context and anthropological observations

The Aven de la Boucle is a natural cavity located near Corconne in the Gard region in southern France. The excavations were conducted under the supervision of H. Duday and S. Cours between 1974 and 2002. The oldest archaeological deposits, attributed to a transitional stage postdating the late Chasséen stage (also known as the 'Recent Neolithic' or Pre-Ferrières), correspond to stratigraphic unit C4b. A second group of deposits, corresponding to the C4a unit, represents a later stage of this late Neolithic horizon and can be attributed to the Ferrières culture. Archaeo-anthropological analysis revealed that approximately 75 individuals remained, with a massive under-representation of immature individuals; quantitative osteology revealed a notable deficit of the largest bones (craniomaxillofacial blocks, long bones of the limbs) (electronic supplementary material, figure C2).

(b) Radiocarbon dating and chronological modelling

Four initial radiometric measurements obtained on charcoal attributed to the funerary occupation of the Aven de la Boucle between the thirty-seventh and twenty-fifth centuries BCE. During this study, 28 dates were obtained by AMS on human bone collagen (accelerator mass spectrometry). Among them, 26 refer to Neolithic collective burials and 2 to punctual introductions of decreased in the cave during the Middle and Final BA. We conducted Bayesian modelling of ^{14}C dates retrieved on petrous bones [53] (electronic supplementary material, section III). To do so, we first constructed different clusters of individuals defined according to stratigraphic attributions. We then constructed different clusters of dated individuals defined according to genetic criteria:

— males versus females;
— male individuals carrying different Y chromosome haplogroups (Y-G2, Y-H2, Y-R1b1b, Y-I2; electronic supplementary material, tables S6 and S18);
— individuals carrying mitochondrial haplogroup U5 (electronic supplementary material, tables S7 and S18); and
— individuals who we were able to model with 'Iran Ganj Dareh' ancestry (refer electronic supplementary material, table S15, qpadm model 4).

The models are built only from the 26 individuals dated to the Neolithic; indeed, those from the BA are too distant in time to constrain the earlier dates. Two Bayesian models were constructed, taking (BCL_M1) or not taking into account (BCL_M2) an individual (BOU32) whose ^{14}C date and stratigraphic position suggest that the cave was used for funerary purposes during the late Chasséen period.

Electronic supplementary material, table S18 presents a general overview of the clusters built for Bayesian modelling. Additional information is available in the electronic supplementary material, section III.

(c) Palaeogenomic analyses

All experiments were conducted in dedicated clean rooms of the ancient DNA facilities at the laboratory of Laboratory of PACEA, CNRS, University of Bordeaux. We targeted a large number of petrous bones available (n = 42) to maximize the achievement of usable genomic results and to extensively document the group(s) represented in this sepulchral cavity. We sampled 42 human petrous bones to retrieve between 100 and 200 mg of bone powder per sample. Steps of decontamination were conducted before sampling. Petrous bones were irradiated with ultraviolet light for 30 min on each side; bone surface was scraped on the area targeted for sampling, and tools were cleaned before drilling into the cochlea to retrieve bone powder [54]. All 42 bone powder samples originating from the Aven de la Boucle were processed according to a two-step extraction procedure and purification followed a silica-based method using a MinElute column (Qiagen) [15].

For each DNA extract, double-stranded libraries were produced according to a protocol adapted from [15,55], using a partial UDG treatment (UDG half) to remove deaminations while preserving ancient DNA damage patterns [56]. Libraries were pooled and sequenced on an Illumina NextSeq 500 at Institut de Recherche Biomédicale des Armées (Brétigny-sur-Orge, France) using a NextSeq 500/550 High Output Kit v. 2.5 (150 cycles).

Raw sequenced data were processed after demultiplexing through EAGER (v. 1.92.37) using the following steps [57]. Adaptor sequences were trimmed and processed into single reads with Clip&Merge, and trimmed sequences were then mapped to Human Reference Genome hs37d5 using BWA v. 0.7.12 [58,59]. Duplicate reads were removed with DeDup, and to observe characteristic ancient DNA damage patterns, we used mapDamage v. 2.06, excluding reads with a mapping quality <30 [60]. Sequencing results are presented in electronic supplementary material, table S2.

All our bam files were trimmed for two bases on each side using trimBam function from BamUtil package and were genotyped by using PileupCaller. We considered the human genome as pseudo-haploid, randomly calling one allele for each position according to the human origin (approx. 600 000 SNPs) and 1240k panels [28,61–63]. Individuals having at least 19 000 SNPs on the 1240k panels were considered for further analysis, and coverage information for the 37 individuals retained is given in electronic supplementary material, table S1.

We determined genetic sex using the method described in [64], based on the estimations of reads ratios mapping to X and Y chromosomes compared to reads mapping to the autosomes. We determined a threshold of Y ratio based on the method published by [64]. We used an upper threshold of 0.016 of the ratio of sequence mapping to the Y chromosome for females and a lower bound of 0.077 for males (electronic supplementary material, table S4). For the 10 capture samples, we also calculated the coverage for the X and Y chromosomes and compared to the relative coverage observed for the autosomes [65] (electronic supplementary material, table S4, figure C10).

We used the Analysis of Next Generation Sequencing Data (ANGSD) package to test the degree of heterozygosity of polymorphic sites on the X chromosome and estimate contamination levels in all male individuals [20]. Considering a contamination threshold of approximately 5%, we excluded one individual (BOU43–13.7%) of downstream analyses (electronic supplementary material, table S5).

Reads were mapped to the revised Cambridge Reference Sequence, and Variant Call Format (VCF) files were constructed using bcftools before being processed through HaploGrep 2 and Phy-Mer to determine mitochondrial haplotypes [66,67]. Mitochondrial sequences were also visually inspected through geneious to reinforce mitochondrial lineages attributions, presented in electronic supplementary material, tables S1 and S7.

For each male individual, Y chromosome haplotypes were inferred using both Yleaf software package (v. 2.1) [68] and a manual assignment through an in-house script. Results for the 28 male individuals are provided in electronic supplementary material, table S6.

Degrees of genetic relatedness between all individuals included in this study were estimated by applying a combination of four methods, TKGWV2, relationship estimation from ancient DNA (READ), lcMLkin and KIN to infer pairwise relationships up to the second and third degrees (electronic supplementary material, section V) [69–72].

We tested the overall mean relatedness for each Neolithic individual by extracting the pairwise mismatch rate (PWMR) from READ for each pair of individuals. We then calculated the mean PWMR per individual, considering each of the 33 pairs of individuals possible. We also confronted the mean PWMR obtained for all female individuals to the one calculated for all male individuals (electronic supplementary material, table S8, figure C15).

For population genetic analyses, we co-analysed our data with published ancient and modern individuals from Eurasia. To qualitatively explore the genetic variability of our dataset, we conducted a PCA using the HO dataset and the program smartpca (Eigensoft) [73]. PCA was constructed based on the genetic variation of 796 modern individuals from Eurasia on which ancient individuals (>10 000 SNPs) were then projected (lsqproject: YES, shrinkmode: YES; figure 2).

We assessed runs of homozygosity and levels of inbreeding in our samples using the 1000 genome as a reference panel, calling SNPs for each individual chromosome by chromosome, through hapROH [42]. We were able to retrieve data for all our samples (electronic supplementary material, table S16, figure C21).

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