[Supplementary material]

Kinship practices in Early Iron Age South-east Europe: genetic and isotopic analysis of burials from the Dolge njive barrow cemetery, Dolenjska, Slovenia

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Ancient DNA analysis: technical report

Introduction

All of the surviving burials from the barrow cemetery at Dolge njive were analysed for aDNA, comprising seven individuals from Barrow 1 and one individual each from Barrows 2 and 3. Analysis was undertaken at the Harvard Medical School ancient DNA facilities. Powdered samples were obtained in a clean dedicated room. DNA was extracted using a

method that is optimised to retain small DNA fragments (Dabney *et al*. 2013; Rohland *et al*. 2018). Between one and four double-stranded libraries were built for each sample with the enzyme Uracil-DNA Glycosylase (UDG) to remove characteristic ancient DNA damage and thereby greatly reduce the rate of damage-induced errors (Rohland *et al*. 2015). Libraries were enriched both for sequences overlapping mitochondrial DNA (Fu *et al*. 2013) and for sequences overlapping about 1.2 million nuclear targets (Fu *et al*. 2015; Mathieson *et al*. 2015), and then sequenced on Illumina instruments. The paired-end reads obtained from sequencing were merged and aligned to the mitochondrial DNA reference sequence RSRS (Behar *et al*. 2012) and human genome reference sequence (version hg19) using BWA (version 0.6.1) (Li & Durbin 2010), following previously published protocols (e.g. Mathieson *et al*. 2015). Mitochondrial haplogroups were determined based on the consensus sequence using Haplogrep2 (Weissensteiner *et al*. 2016). We assessed ancient DNA authenticity based on consistency with the mitochondrial DNA consensus using contamMix (Fu *et al*. 2013) and to the consensus sequence on the X chromosome in males using ANGSD. For population genetic analysis, we represented each targeted single nucleotide polymorphism (SNP) by a randomly selected sequence (for SNPs covered at least once). Table S1 presents the results by individual libraries, while Table S2 presents the results by sample (where we merge data for multiple libraries).

Kinship analysis

We performed kinship analysis, using the software READ (Relationship Estimation from Ancient DNA; Kuhn *et al*. 2018). Our results highlight several biological relationships among the individuals from Barrow 1:

First degree relatives:

- I22935 and I5686
- I22935 and I5685
- I22935 and I5684
- I22935 and I22934
- I5684 and I5686
- I5684 and I5685
- I5685 and I5686
- I22934 and I5686
- I22934 and I5684

- I22934 and I5685

Second degree relatives:

- I23971 and I5685
- I23971 and I5684
- I23971 and I5686
- I23971 and I22934
- I23971 and I22935

Third degree relatives:

- I5687 and I5685
- I5687 and I5686
- I5687 and I5684
- I5687 and I22935

Phenotypes

We used the HIrisPlex SNPs panel (Walsh *et al*. 2013; Chaitanya *et al*. 2018), which analyses 41 SNPs, to predict eye and hair colour, and skin pigmentation. We were able to predict eye colour for four individuals:

- I5684 has a substantial probability of having blue eyes (76 per cent)
- I5685 has a substantial probability of having blue eyes (64 per cent)
- I5686 has a substantial probability of having blue eyes (76 per cent)
- I5687 has a substantial probability of having blue eyes (78 per cent)

It was not possible to predict hair or skin colour with any confidence due to the absence of data from too many essential SNPs.

Population genomics analysis

In order to carry out population genomics analysis we used the database provided by the David Reich Laboratory (which curates previously published data from many sources), and which is available at: https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-genotypes-present-day-and-ancient-dna-data

The comparative dataset comprises different types of data based on capture and shotgun sequencing. The shotgun sequencing data are in many cases derived from low coverage

genomes (from 0.1 to 0.5X). This means that the recovered sequences are randomly distributed across the genome and not targeted as in capture analysis.
Of the first degree relatives, we only use individual I5685 for the following analyses (except the PCA), as this individual has the best coverage of the group comprising I5685, I5687, I23971, I22933 and I22936.

Principal component analysis
The PCA is built by projecting the ancient DNA data onto a set of modern European and Near Eastern populations. Here we project available data for Iron Age populations in Western Europe (Gamba *et al*. 2014; Allentoft *et al*. 2015; Schiffels *et al*. 2016; Martiniano *et al*. 2016; Damgaard *et al*. 2018; Mathieson *et al*. 2018; Antonio *et al*. 2019; Jarve *et al*. 2019; Olalde *et al*. 2019; Saag *et al*. 2019; Sikora *et al*. 2019; Brunel *et al*. 2020; Fernandes *et al*. 2020; Marcus *et al*. 2020; Margaryan *et al*. 2020).
Our samples from Dolge njive, as well as others samples from Slovenia, plot in an intermediate position between Western European samples (UK, France, Spain) and Southern European samples (Italy) (Figure S1). However, one individual (I22936) seems to have more affinity with Southern European samples (Italy, Eastern Mediterranean or Punic samples from Sardinia), but this outlying position potentially reflects the limited coverage (24864 SNPs among the 600 000 SNPs targeted).

Affinities among the Dolge njive samples
We performed a qpWave analysis as implemented in ADMIXTOOLS (Patterson *et al*. 2012) to determine if individuals from Dolge njive can be defined as a clade (following Fernandes *et al*. 2020). For this test, we used the following set of populations: Mbuti.SDG, EHG, Russia_Afanasievo, Turkey_N, Germany_EN_LBK, WHG, Russia_Samara_EBA_Yamnaya. Outliers are defined when $p < 0.1$.
Our results highlight that all individuals from the Dolge njive site form a clade, including individual I22936 who is an apparent outlier on the PCA (and who was buried in a separate barrow from the biologically related individuals in Barrow 1). This is consistent with their outlying position in the PCA being due to limited data size.

Affinities with ancestral populations
We performed a qpAdm analysis as implemented in ADMIXTOOLS (Haak *et al*. 2015) to investigate the percentage of several ancestries in the individuals from Dolge njive. Here we

estimate the relative proportions of EEF, Yamnaya (Steppe) and Western Hunter Gatherer ancestries. We performed this test on each sample.

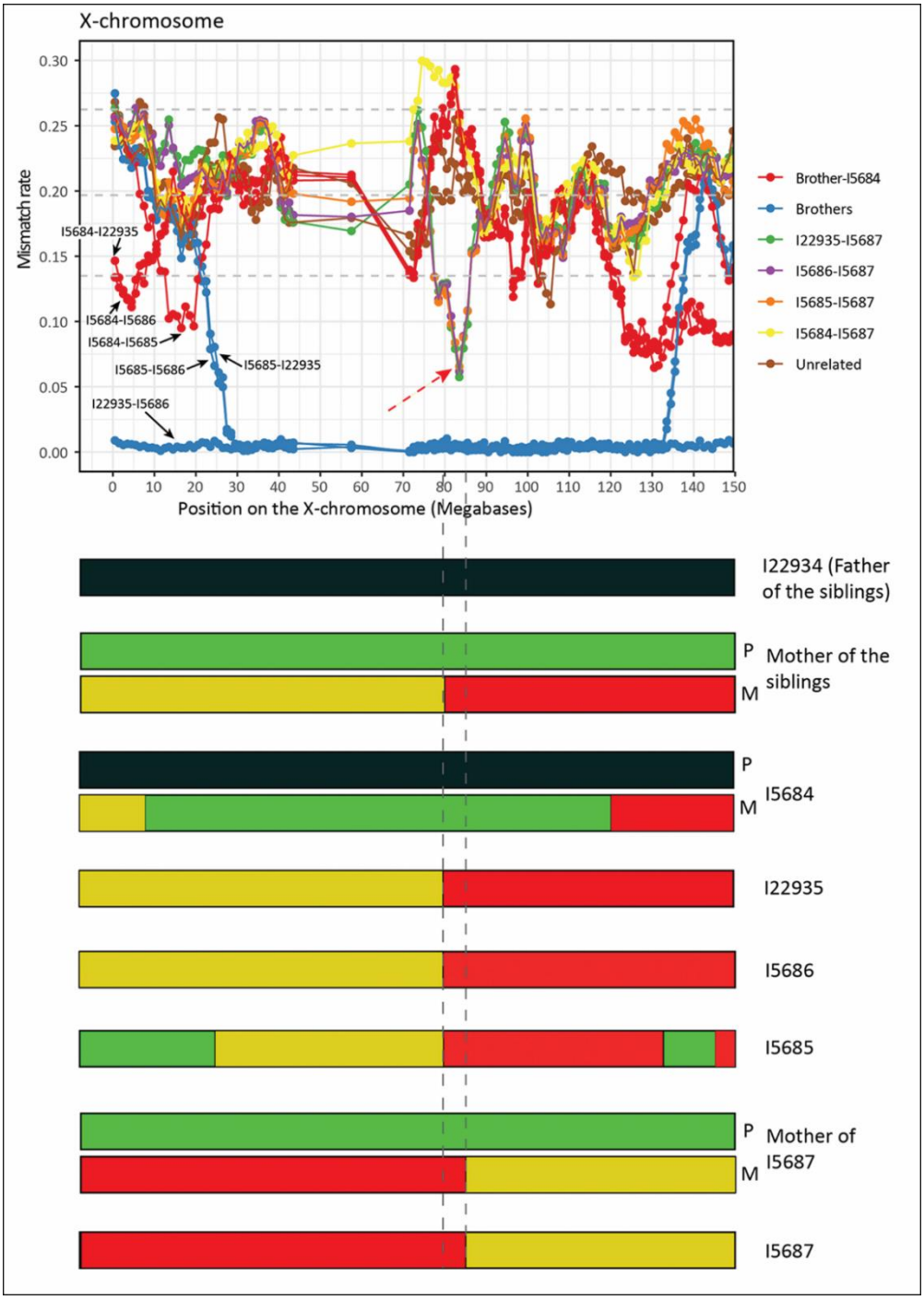

Figure S1. PCA with Iron Age samples available in Europe (figure by the authors).

Our results indicate that individuals from Dolge njive seem quite homogeneous in terms of ancestry (Figure S2; note that two individuals (I22933 and I23971) are not plotted as the model does not fit (p value <0.05)).

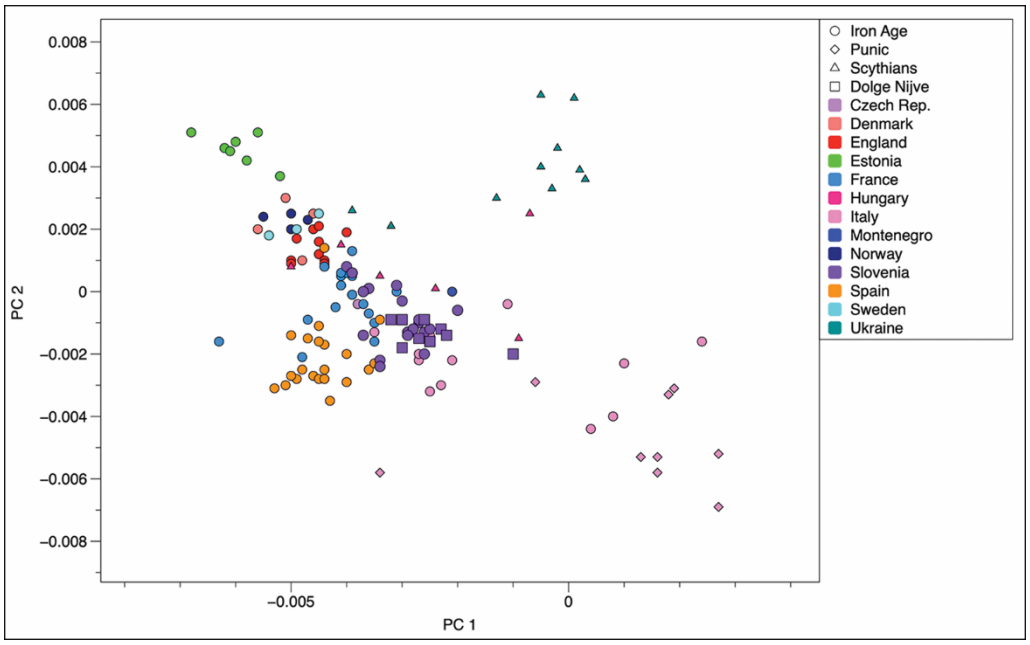

Figure S2. Percentage of Neolithic, WHG and Steppe Ancestry in samples from Dolge Njive (figure by the authors).

Affinities with contemporaneous populations

We computed an outgroup-f3 statistic (Raghavan *et al*. 2014) as implemented in ADMIXTOOLS in the form (Mbuti; Individual from Dolge njive, Iron Age*) where we tested the individuals from Dolge njive against the available Iron Age groups. This statistic allows us to explore the affinities between two groups: a high f3 means that the groups are close and a low f3 means that the groups show few affinities.

Our results show that the individuals from Dolge njive are closer to other samples from Slovenia than from other parts of Europe, with the exception of two outliers: one from Spain and one from England.

Data availability

The raw data are available as aligned sequences (bam files) through the European Nucleotide Archive under accession number PRJEB58709.

Multi-isotope analysis

Isotope analyses were conducted on the bones and teeth of the individuals buried in Barrow 1 to explore evidence of heterogeneity within the diet and potential differences in geographical location within the group during their childhoods (Table S3).

Dietary reconstruction

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) collagen isotope analyses are commonly used to reconstruct the protein contribution to the diet (Ambrose & Norr 1993; Keller & Schoeninger 2007). Nitrogen isotopes track nitrogen pathways within the local ecosystem, reflect the trophic level of the consumer, and detect whether the diet is composed of primarily terrestrial or aquatic resources (Schoeninger *et al*. 1983; Hedges & Reynard 2007). Carbon isotopes track carbon pathways through plants into the food chain reflecting the contribution of C_3 or C_4 vegetation within the diet and can also be used as a means of differentiating between terrestrial and marine food chains (Lee-Thorp *et al*. 1989; Richards & Hedges 1999; Richards 2003; Tafuri *et al*. 2009). Carbonate carbon isotopes ($\delta^{13}C_{CARB}$), which are extracted from the mineral component, can further elucidate the carbon component of the diet, as, unlike carbon isotopes from bone and dentine collagen, $\delta^{13}C_{CARB}$ reflects the whole diet, including carbohydrates and lipids (Loftus & Sealy 2012; Katzenberg & Waters-Rist 2018). For an overview of the principles behind dietary reconstruction using stable isotopes and reviews of previous research within this field, see Hedges & Reynard (2007); Makarewicz & Sealy (2015); and Katzenburg & Waters-Rist (2018).

By sampling skeletal elements, with different turnover rates, within the same skeleton it is possible to explore variations in diet over an individual's lifetime (Reitsema & Vercellotti 2012). Once formed, teeth do not undergo significant remodelling and so preserve isotopic information on dietary input during the formation of the tooth, i.e. during childhood and adolescence (Fuller *et al*. 2003; Beaumont *et al*. 2013). The mid-shaft femur gradually remodels over an individual's lifetime and thus isotope values represent a much longer period, reflecting an average protein consumption throughout adulthood (Hedges *et al*. 2007). The rib collagen is constantly remodelling (up to 7.7 per cent a year for adults), and so will reflect a relatively short period of dietary consumption prior to death (Parfitt 2002).

Carbon and nitrogen stable isotope data were produced from the bulk collagen of long bones (principally mid-shaft femur), ribs and dentine (principally permanent first molars; dentine samples were removed from the apex (approximately the final 2mm) of tooth roots, reflecting the isotopic composition of diet and metabolic condition of an individual in the final stages of the tooth's development (i.e. late childhood/early adolescence)). Where available, all three elements were sampled from each skeleton, as a means of detecting inter- and intra-individual isotopic heterogeneity. All samples included in this study produced a >1% collagen yield and had C:N ratios between 2.9 and 3.5, indicative of well-preserved collagen (Van-Klinken

1999). Bone and dentine collagen was extracted using a modified Longin method (Longin 1971; Brown *et al*. 1988). The isotopic analyses were carried out at the University of Bradford Light Stable Isotope Facility using the protocol outlined in Nicholls *et al*. (2020). To explore the subtle differences in individual $\delta^{13}C$ isotope ratios further, dental enamel samples were taken for carbonate analysis (France & Owsley 2015). Enamel samples rather than bone apatite was chosen to minimise potential diagenetic contamination (Snoeck *et al*. 2015). For both the $\delta^{13}C_{CARB}$ and $\delta^{18}O_{CARB}$ analyses, tooth enamel was removed using dental burs and these were taken from the same teeth as those sampled for dentine collagen. The resultant powder was then agitated in NaOCl using a whirl mixer and then left for 30 minutes, to remove any remaining organic material. Samples were then centrifuged and rinsed three times in deionised water, before an acetic acid solution was added and the samples left for 10 minutes to remove any diagenetic carbonate. A further four rinses were carried out with deionised water before each sample was frozen and freeze dried. Approximately 2mg of enamel powder was reacted with 100% phosphoric acid at 70°C for 60 minutes in a Thermo Delta V connected to a Gasbench II, to produce CO2. The precision of this analysis is also ±0.2. The analysis was undertaken at the University of Bradford Light Stable Isotope Facility. Internal standards were run alongside samples to monitor measurement variation. These Internal standards were Merck (pure calcium carbonate, carbon: −34.45, oxygen: +13.35 VSMOW) and OES (ostrich eggshell, carbon: −10.72, oxygen: +25.45 VSMOW).

Childhood location and mobility

Strontium and oxygen isotopes vary depending on the local geology and climate (Bentley & Knipper 2005; Britton *et al*. 2009; Montgomery 2010). Carbonate oxygen isotopes ($\delta^{18}O_{CARB}$) largely reflect local water sources derived from meteoric water (rain and snow), or from recycled water (lakes, wells or springs) (Sponheimer & Lee-Thorp 1999; Bentley & Knipper 2005). Isotope ratios of strontium directly represent the geographic area of food production/acquisition and, thus, the mobility of dietary resources or their consumers, or both (Budd *et al*. 2000; Montgomery *et al*. 2007). They are dependent upon the variation in strontium isotope composition of local rocks and geology, with the addition of local rainfall and terrestrial aerosols (Budd *et al*. 2000; Montgomery *et al*. 2007; Montgomery 2010). Thus, analyses of these isotopes in dental enamel, which does not remodel, can be used as a means of determining childhood origins and residential mobility that may have occurred during formation of the tooth (Evans *et al*. 2006; Prowse *et al*. 2007).

Carbonate oxygen isotopes ($\delta^{18}O_{CARB}$) were measured alongside carbonate carbon ($\delta^{12}C_{CARB}$) using the method outlined above. Strontium analysis was undertaken at the Durham University Earth Sciences Department using the protocol outlined by Gron and colleagues (2016).

The area surrounding the Dolge njive cemetery, which lies approximately 155m above sea level, is geologically complex. If the interred individuals inhabited the hillfort, at least four different geological formations surrounded them. These include marls, sandstones and quartzite marls (Miocene Age); limestones and calcirudite (Miocene Age); coarse-grained dolomite (Triassic); and other marls and limestones (Cretaceous) (Dular & Tecco Hvala 2007: 44–64; Geološki zavod Slovenije/Geological Survey of Slovenia). There are also Quaternary and Holocene clay deposits to the south of the barrows in the vicinity of Dolge njive itself, derived from erosion products from the Triassic and Cretaceous deposits (as above) in the Bela Cerkev and Draga areas.

As the geology of the immediate area is so geologically diverse, $^{86}Sr/^{87}Sr$ values obtained for individuals buried at Dolge njive probably represent an average of the isotope ratios consistent with these bedrocks and deposits. The $^{86}Sr/^{87}Sr$ values of 0.709–0.7101 are higher than might be expected from chalk or limestone geologies, and may have been influenced by the fluvial deposits, or could indicate resources coming from further afield (Bentley & Knipper 2005; Evans *et al*. 2006; Montgomery *et al*. 2007; Montgomery 2010). Without any baseline data reflecting potential local values of biologically available strontium (e.g. from plant, soil or faunal samples) and drinking water, it is difficult to speculate further on potential differences in childhood mobility between the individuals from Barrow 1.

Osteological analysis: methods

Preservation and completeness were recorded for each skeleton from Dolge njive. Preservation relates to the condition of the bone (which was frequently affected by weathering), post-mortem breaks and loss of the cortical surface. Completeness was recorded as a percentage of the skeleton present for analysis. The preservation and completeness in this population severely impacted the osteological analysis, particularly as the ossa coxae were poorly preserved in this population (Nicholls 2017). As all individuals were adult, no methods for the analysis of non-adults are discussed here.

For adults, biological sex was assessed using the pelvis and skull, using the following traits: greater sciatic notch, pre−auricular sulcus (Walker 2005), ventral arc, sub-pubic concavity, ischiopubic ramus (Phenice 1969; Klales *et al*. 2012), nuchal crest, supra-orbital margin,

glabella, mastoid process, mental eminence (Buikstra & Ubelaker 1994; Walker 2008), parietal eminences, posterior zygomatic arch, the shape of the orbits and orbital margins, gonial angle, and presence or absence of gonial flaring (Brothwell 1981: 59–61; İşcan & Steyn 2013: 160–69).

Adult age was estimated using the pubic symphysis, auricular surface, cranial suture closure and dental wear (Brothwell 1981; Meindl & Lovejoy 1985; Brooks & Suchey 1990; Buckberry & Chamberlain 2002). Due to poor levels of preservation, age estimates relied upon dental wear; however, it must be noted that there are no published, calibrated, population-specific standards for Slovenia and there are not enough well-preserved juvenile jaws to calibrate dental wear for the region; thus, age estimates are very approximate. Each individual was placed into an age category for analysis and interpretation (Table S4).

Concordance with the excavation record

Table S5 provides a concordance between the burial numbers used in this paper and the context and small finds numbers used in the original excavation archive.

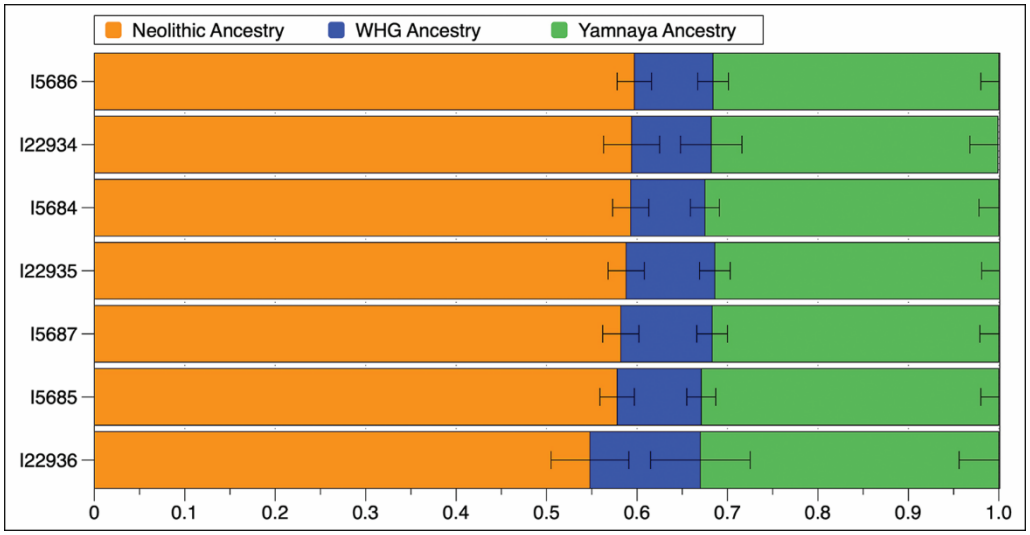

Figure S3. Top: Pairwise allelic mismatch rate values across sliding windows of 10 Mb on the X-chromosome, moving by 1 Mb each step. The red dotted arrow indicates a shared DNA segment between I5687 and the three brothers (I22935, I5686 and I5685). Bottom: A model of DNA sharing on the X-chromosome between the four siblings (I5684, I22935, I5686 and I5685) and their parents that fits the mismatch rate patterns. We also include I5687 and his mother under the scenario in which I5687 is a maternal cousin of the four siblings. Different colours represent DNA segments inherited from different ancestors (figure by the authors).

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SUPPLEMENTARY TABLES

Table S1. Genetic data by library information.

Library ID	Genetic individual ID	Raw shotgun sequences	Percent mapping to human genome	Raw sequences for capture experiment	mtDNA average coverage	Sequences aligning to mtDNA post-duplication-removal	Sequences passing filters and covering targeted nuclear SNPs before duplication removal	Sequences passing filters and covering targeted nuclear SNPs after duplication removal	Average coverage on targeted autosomal SNPs	Percentage of cytosine-to-uracil deamination in final nucleotide
S22933.Y1.E1.L1	I22933	301664	1.8	35401024	52	16329	12338281	377361	0.31	12.3
S22934.Y1.E1.L1	I22934	339023	1.8	36650351	80	25109	13439229	146531	0.12	10.8
S22935.Y1.E1.L1	I22935	344758	2.7	33741712	180	48730	13683391	1263341	1.01	8.5
S22936.Y1.E1.L1	I22936	326898	0.054	7455206	30	8444	1178292	47739	0.039	14.0
S23971.Y1.E1.L1	I23971	164242	12	27142873	54	16831	9848872	1167775	0.98	12.6
S5684.E1.L1	I5684	–	–	–	–	–	–	–	–	–
S5684.E2.L1	I5684	172131	53	784460	134	42606	5988289	3300388	–	9.4
S5684.E2.L2	I5684	180287	53	181086	38	10889	6306172	4802036	–	9.9
S5685.E1.L1	I5685	–	–	–	–	–	–	–	–	–
S5685.E2.L1	I5685	197163	58	822774	140	43145	5851388	4224926	–	9.8
S5685.E2.L2	I5685	222915	57	250513	52	14306	5758608	4970279	–	10.4
S5687.E1.L1	I5687	–	–	–	–	–	–	–	–	–
S5687.E2.L1	I5687	229376	69	393479	101	28335	6711816	5152435	–	10.2
S5687.E2.L2	I5687	213652	66	328537	87	24133	5749346	5192989	–	10.4
S5688.E1.L1	I5687	–	–	–	–	–	–	–	–	–

Table S2. Genetic data by sample information.

Genetic ID	I22933	I22934	I22935	I22936	I23971	I5684	I5685	I5686	I5687
Barrow	3	1	1	2	1	1	1	1	1
Burial	1	5	3b	1	2	1	3a	4	6
Skeletal element	tooth	tooth	tooth	bone	petrous	petrous	petrous	petrous	petrous
LibraryID(s)	S22933.Y1.E1.L1	S22934.Y1.E1.L1	S22935.Y1.E1.L1	S22936.Y1.E1.L1	S23971.Y1.E1.L1	S5684.E1.L1, S5684.E2.L1, S5684.E2.L2	S5685.E1.L1, S5685.E2.L1, S5685.E2.L2	S5686.E1.L1	S5687.E1.L1, S5688.E1.L1, S5687.E2.L1, S5687.E2.L2
Average coverage on targeted autosomal SNPs	0.309	0.117	1.006	0.039	0.985	10.206	13.245	6.419	19.544
Number of autosomal SNPs covered at least once	300267	127732	640596	44661	559866	1011266	1002893	871455	1006818
Genetic sex	Male	Male	Male	Male	Female	Female	Male	Male	Male
Y haplogroup in ISOGG v15.73 notation	R1b1a1b1a1a	R1b1a1b	R1b1a1b1a1a2b1	R1b1a1b	n/a (female)	n/a (female)	R1b1a1b1a1a2b1c2	R1b1a1b1a1a2b1	R1b1a1b1a1a2b1c2
mtDNA haplogroup from haplogrep	H5a6	H	H1e5a	H1ba	H1e5a	H1e5a	H1e5a	H1e5a	H1e5a
mtDNA match rate to consensus sequence from contamix	[0.972,0.990]	[0.986,0.999]	[0.993,1.000]	[0.980,0.998]	[0.977,0.994]	[0.98,0.996]	[0.981,0.997]	[0.969,0.991]	[0.99,1]
Damage rate in first nucleotide on sequences overlapping targets	0.123	0.108	0.085	0.14	0.126	..	..	0.086	..
Ratio of Y to Y+X sequences	0.395	0.39	0.41	0.399	0.014	..	..	..	..
X−chromosome contamination from ANGSD (95% CI truncated at 0)	[0.000,0.024]	n/a (too little data)	[0.002,0.011]	n/a (too little data)	n/a (female)	n/a (female)	[0.002,0.007]	[0.001,0.007]	[0.002,0.007]

Table S3. Isotope data for Barrow 1 at Dolge njive.

Burial	Element	$\delta^{13}C_{COL\ VPDB}$ ‰	$\delta^{15}N_{AIR}$ ‰	C: N	$\delta^{13}C_{CARB\ VPDB}$ ‰	$\Delta\ \delta^{13}C_{CARB-COL}$	$\delta^{18}O_{CARB\ VSMOW}$ ‰	Sr conc. ppm	$^{87}Sr/^{86}Sr$ norm
1	tooth	−14.8	8.9	3.2	−9.2	5.6	22.3	135	0.7095
1	long bone	−15.7	8.0	3.1					
2	tooth	−15.6	8.8	3.3	−7.9	7.7	22.0	165	0.7097
2	rib	−16.5	8.3	3.3					
2	long bone	−16.6	8.3	3.2					
3a	tooth	−15.8	8.9	3.2	−9.8	6.0	22.8	59	0.7100
3a	rib	−15.5	8.1	3.3					
3a	long bone	−15.1	8.5	3.1					
3b	tooth	−14.9	9.5	3.2	−8.5	6.4	21.8	121	0.7100
3b	rib	−15.0	8.6	3.2					
4	tooth	−13.6	9.5	3.2	−7.3	6.2	22.2	86	0.7101
4	rib	−16.3	9.0	3.5					
4	long bone	−14.7	9.1	3.2					
5	tooth	−15.9	8.7	3.2	−7.2	8.7	21.1	226	0.7092
5	rib	−15.3	7.9	3.2					
5	long bone	−15.3	8.3	3.2					
6	rib	−15.4	8.3	3.2					

Table S4. Age categories (following Buikstra and Ubelaker 1994).

Age category	Age range
Young adult	approx. 20–35 years
Middle adult	approx. 36–50 years
Mature adult	approx. 50+ years
Adult	approx. 18+ years

Table S5. Burial numbers with context and small find numbers

Burial	Context	Small find no.
Barrow 1		
1	1763	1708 (1775)
2	1746	1781
3a	1748	2603
3b	1748	2604
4	2644	2680a
5	2650	2900
6	2653	2903
Barrow 2		
1	n/a	2361/6
Barrow 3		
1	n/a	1883