Interdisciplinary analyses of Bronze Age communities from Western Hungary reveal complex population histories

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Abstract

In this study we report 21 ancient shotgun genomes from present-day Western Hungary (3530 – 1620 cal BCE), from previously understudied Late Copper Age Baden, and Bronze Age Somogyvár-Vinkovci, Kisapostag, and Encrusted Pottery archaeological cultures. Our results indicate the presence of high steppe ancestry in Somogyvár-Vinkovci culture that was replaced by the Kisapostag group having an outstandingly high (up to ~47%) Mesolithic hunter-gatherer ancestry, despite this component being thought to be highly diluted by the time of the Early Bronze Age. The Kisapostag population was also the genetic basis of the succeeding community of the Encrusted pottery culture. We also found an elevated hunter-gatherer component in a local Baden culture associated individual, but no connections were proven to the Bronze Age individuals. The hunter-gatherer ancestry in Kisapostag is likely derived from two main sources, one from a Funnelbeaker or Globular Amphora culture related population and one from a previously unrecognised source in Eastern Europe. We show that this ancestry not only appeared in various groups in Bronze Age Central Europe, but also made contributions to Baltic populations. The social structure of Kisapostag and Encrusted pottery cultures is patrilocal, similarly to most contemporaneous groups. Furthermore, we developed new methods and method standards for computational analyses of ancient DNA, implemented to our newly developed and freely available bioinformatic package. By analysing clinical traits, we found carriers of aneuploidy and inheritable genetic diseases. Finally, based on genetic and anthropological data, we present here the first female facial reconstruction from the Bronze Age Carpathian Basin.

Significance

The hunter-gatherer ancestry we recovered in this study promotes the rethinking of the survival dynamics of Mesolithic populations, especially in the region of East-Central Europe. Despite strong genetic ties of patrilocal populations recovered at Balatonkeresztúr site toward groups of completely different social structure, high flexibility in social organisation can be assumed during the Bronze Age of the region. The newly presented bioinformatic tools ease the routine analysis of clinical and phenotype traits and help a yet underresearched part of the field. Reconstruction of a mass grave and a burial along with a forensic facial reconstruction bring closer past populations to our understanding of the prehistory.

Introduction

Several studies addressed major population historical events in Prehistoric Europe regarding pre-Neolithic hunter-gatherers (HG)[1–3], their assimilation to early European farmers during the Neolithic era[2,4–6], and the appearance, expansion and admixture of steppe ancestry between the Eneolithic / Late Copper Age and the dawn of Early Bronze Age[4,7–9]. It is necessary to understand the roots of the European gene pool, but there are only a few studies available that uncover regional interaction or social stratification using kinship analyses[10–12], especially in the region of today's Hungary concerning Copper (~4500-2700 BCE) and Bronze (~2700-800 BCE) Ages. A number of cultural transformations occurred in the Carpathian Basin, often as a result of population changes and genetic influxes. These, however, sparsely covered intensive european HG introgression into early european farmer (EEF) or steppe ancestry groups[6], (besides one such case from today's Romania[13]) despite the known HG presence in the region at the beginning of the Neolithic[5,14]. In contrast, ancient populations from other parts of Europe, such as Scandinavia[2,15–17], today's Poland[18] or Iberia[19] show much higher and much later introgression of HG ancestry. Later on, at the beginning of the 3rd millennium BCE, the appearance of steppe related ancestry shaped the regional genetic landscape extensively, founding the modern day European genetic makeup[4,5,7,8,14,20–22].
Besides monitoring population events, archaeogenetics opens a new window to study health qualities of ancient populations that may lead to a better understanding of the background of recent genetics related diseases. Studies that aimed to uncover variants under selective pressure in Homo sapiens populations, such as Lactase persistence (LCT) or Human Leukocyte Antigen (HLA) genes and pigmentation markers are beginning to thrive[23–26]. However, variants for rare genetic diseases or aneuploidies are sparsely checked on ancient datasets, except for a few cases, such as the study of the Suontaka grave[27].

Our study aimed to make a transect analysis on a single site presenting understudied archaeological assemblies, applying population genetic analyses, isotope analyses, phenotype (pigmentation) and clinical variant analyses. Moreover we present a series of bioinformatic tools for kinship, ploidy and variant analyses implemented in a new bioinformatic package for archaic DNA analysis. We analysed the archaeological finds from Balatonkeresztúr-Réti-dűlő site in Western Hungary (Transdanubia), where - among others - Bronze Age assemblies and human remains were found during roadwork in 2003. Three Bronze Age archaeological horizons were distinguished based on ^{14}C dates: the Somogyvár-Vinkovci culture (~2500-2200 BCE, n=1), Kisapostag culture (~2200–1900 BCE, n=11) and the Encrusted pottery culture (~1900–1450 BCE, n=8) that are referred to as Bk-I, II and III phases in this study, respectively (Table 1, Supplementary Information section 1). All three cultural horizons have only a limited number of inhumation remains: this study presents the first validated Somogyvár-Vinkovci culture associated individual from Hungary, while the Kisapostag and Encrusted pottery cultures have been mainly characterised by cremation burials so far. The cultural connection system of the Kisapostag has been explained with various traditions[28,29], along with its strong connection to the Encrusted Pottery[30]. The archaeological origin of Kisapostag culture is enigmatic, multiple theories arose to explain its possible connections: The pottery decoration technique observed in Kisapostag originated either from Corded Ware in the Middle Dnieper region (Ukraine) or epi-Corded Ware groups (northern Carpathians), *e.g.* Chłopice-Veselé (Slovakia). The latter option is also supported by inhumation practises and the burial positions[28,31–35]. However, local development of communities with eastern (Makó–Kosihy–Čaka) or southern (Somogyvár–Vinkovci) origins, as well as western and southwestern connections (with the Litzenkeramik or Guntramsdorf-Drassburg group in eastern Austria, Slovenia, western

Croatia) were also raised in archaeological literature[36–38]. Besides, Bell Beaker influence was mentioned based on the craniometry data (so-called *Glockenbecher* or brachycranic skull type[29,39,40]).
In order to provide additional proxy to population ancestry of the region one further Late Copper Age individual from a multiple grave of the Baden culture (~3600-2800 BCE) excavated at site Balatonlelle-Rádpuszta, ~30 km away from Balatonkeresztúr was added to our dataset. Our data highlight not only detailed population events in a microregion, but also reveal hidden processes that formed the genetic landscape of East-Central Europe at the beginning of the Bronze Age.

Results

We shotgun sequenced genomes of 21 individuals yielding between 0.008x and 2.1x average genomic coverage. We also sequenced reads of a capture set consisting 3000 nuclear SNPs (single nucleotide polymorphisms, see Methods), and whole mitochondrial DNAs (mtDNAs) of all individuals. The shotgun and the capture sequenced samples ultimately resulted in an average ~144k SNPs/individual using the 1240k SNP panel for genotype calling[26], see Materials and Methods and Supplementary Tables 4 and 7. We utilised STR (Short Tandem Repeat) analysis of the Y chromosome to ascertain direct paternal kinship (Supplementary Table 3). Furthermore, by using all known biological and archaeological details, we reconstructed the face of individual S13 (from phase Bk-II), see Supplementary Information section 4. The bioarchaeological analyses were completed with ^{14}C dating and $^{87}Sr/^{86}Sr$ isotope analyses, the latter is routinely used to trace individual mobility[41].

Archaeological and anthropological evaluation of samples

We included only one juvenile individual (BAD002) from the site of Balatonlelle belonging to the early phase of Baden culture, ^{14}C dated between 3530-3370 cal. BCE (95.4% CI). From the main site of this study called Balatonkeresztúr Réti-dűlő we sampled and sequenced an overall 20 individuals named from S1 to S45, skipping numbers that does not belong to the Bronze Age horizon or weren't suitable for genetic testing. One male individual (S9) that could be ordered to Bk-I by ^{14}C data, has a very long (ultradolichocran) skull type, which differentiates him from most individuals in Bk-II and Bk-III who have a very short (brachycranic) skull type[42] (Table 1). The male dominance (~78%) in Bk-II and Bk-III suggests distinctive funeral treatment for males and females. Bk-II phase is represented by 4 juveniles (a 7-8 years old child and three 16-19 years old teenagers) and 7 adults (30+ years olds). They are spatially distributed into grave groups of A and B with two further separate inhumations (Table 1, Supplementary Information Fig. S.1.2.1). Most of the burials contained no remaining grave goods except for small copper jewellery in S10 and S13, and some shell fragments in S45. Radiocarbon dates place these inhumations to ~2200-1770 cal BCE (95.4% CI), however, with Bayesian analysis using the OxCal software[43] the timespan of the Bk-II burials can be reduced to ~2050-1940 cal BCE with a 84.4% CI, whereas only two graves (individuals S10 and S11) were possibly slightly earlier (Supplementary Information section 1.8). The lack of children at Bk-II is parallel to other archaeological sites as this phenomenon is common in most periods, that can be traced back to different skeletal taphonomy or burial practises to adults[41], while the reason for the absence of young adults (~20-30 year olds) is unknown. Bk-III is represented by a single mass grave with skeletal remains of 8 people of various ages that turned out to be an unusual find in a period when the cremation practises and single inhumations were common, in ~1870-1620 cal BCE (95.4% CI). For detailed description of the sites and burials, see Supplementary Information section 1.

Uniparental genetics and kinship analyses

First glimpse on uniparental (maternal and paternal) lineages not only provide rough estimates on genetic composition, but are inevitable to assess kinship and social structure for the studied population. Additionally, we performed phylogenetic analysis by using MrBayes software[44] on mitochondrial DNA to see the phylogeographic affinities of the studied individuals. According to our results, Bk-II mostly shows mtDNA connections to the region of present-day Poland and its surroundings, whereas Bk-III has a more diverse maternal composition, see Supplementary Information section 2.1. Most male individuals in Bk-II and Bk-III

belong to the Y chromosome haplogroup I2a-L1229, except for two haplogroups R1b-Z2103 (Table 1). Similar phylogeographic analysis to the mitochondrial DNA can be performed on the paternal lineages as well using STR markers. Network analysis (Supplementary Information section 2.2) narrowed down regional Y-chromosomal affinities to the North European plain and indicated continuity between Bk-II and Bk-III. Uniparental makeup shows a patrilocal social structure that is similar to previously reported Bronze Age findings[10,12,45]. Results are highly similar to previous observations on Encrusted Pottery culture's population at the Jagodnjak site, Croatia[11]. Inferring kinship relations were based on READ[46] and our newly developed method called Modified Pairwise Mismatch Rate (*MPMR*, Supplementary Information section 2.3). The kinship network (Fig.1.a, Fig.1.d) of Bk-II approximately follows the distribution of individuals in A-B grave groups (Fig.1.b), which were likely established along family relationships and chronology. Individuals buried in the Bk-III mass grave only show a few blood relations: a half-brother, a father-son and a dizygotic twin, to our knowledge the latter is the oldest detection of such a kinship relation. None of the distant inhumations (S10, S45) show biological relationship to any other individuals up to second degree. For further details, see Supplementary Information section 2 and Supplementary Tables 1-3.

Table 1
Summary of the investigated samples. MtDNA and ChrY denote mitochondrial haplogroup and Y chromosome haplogroup, respectively. In column "Kinship" 1st and 2nd mean the degree of relations. For the feature, grave ID and details on newly reported ^{14}C *dates see the Supplementary Table 1.*

Group	ID	Grave group	cal BCE date (95.4% CI)	Age	Sex	MtDNA	ChrY	Kinship
Baden	BAD002		3530-3370	8-9	M	K1a4a1	I-M170	
Bk-I: Somogyvár - Vinkovci culture	S9		2560-2290	35-40	M	K1a3a	R1a-V2670	
Bk-II: Kisapostag or Early Encrusted Pottery culture	S1	A	2120-1880	40+	M	V	I2a-L1229	2nd to S2
	S2	A	2120-1880	30-35	M	U5a2b1a	I2a-L1229	2nd to S1
	S4	B		17-19	M	H10a1	I2a-L1229	1st to S8
	S5	A		16-18	M	T1a4	I2a-L1229	1st to S6 & S11
	S6	A	2030-1770	17-18	M	T1a4	I2a-L1229	1st to S5 & S11
	S7	A	2120-1880	35-50	F	V		
	S8	B		30-40	M	T2b	I2a-L1229	1st to S4
	S10		2140-1940	7-8	M	K1a4a1g	I2a-L1229	
	S11	B	2200-1980	34-43	M	T2b	I2a-L1229	1st to S5 & S6
	S13	B	2120-1890	35-45	F	J2b1		
	S45		2200-1980	45-55	M	U5a1g	I2a-L1229	
Bk-III: Transdanubian Encrusted Pottery culture	S14	Mass Grave B-938		7-8	F	H10a1		
	S15			21-23	M	U4b1b1	I2a-L1229	2nd to S17
	S16		1890-1640	35-44	M	T2g2	I2a-L1229	
	S17		1870-1540	26-35	M	U5b1b1+@16192	I2a-L1229	1st to S19; 2nd to S15
	S18			3-4	M	U4a2	R1b-Z2103	
	S19			9-10	M	T2b	I2a-L1229	1st to S17
	S20			1.5-2	M	K1a+195	R1b-Z2103	1st to S21
	S21			1.5-2	F	K1a+195		1st to S20

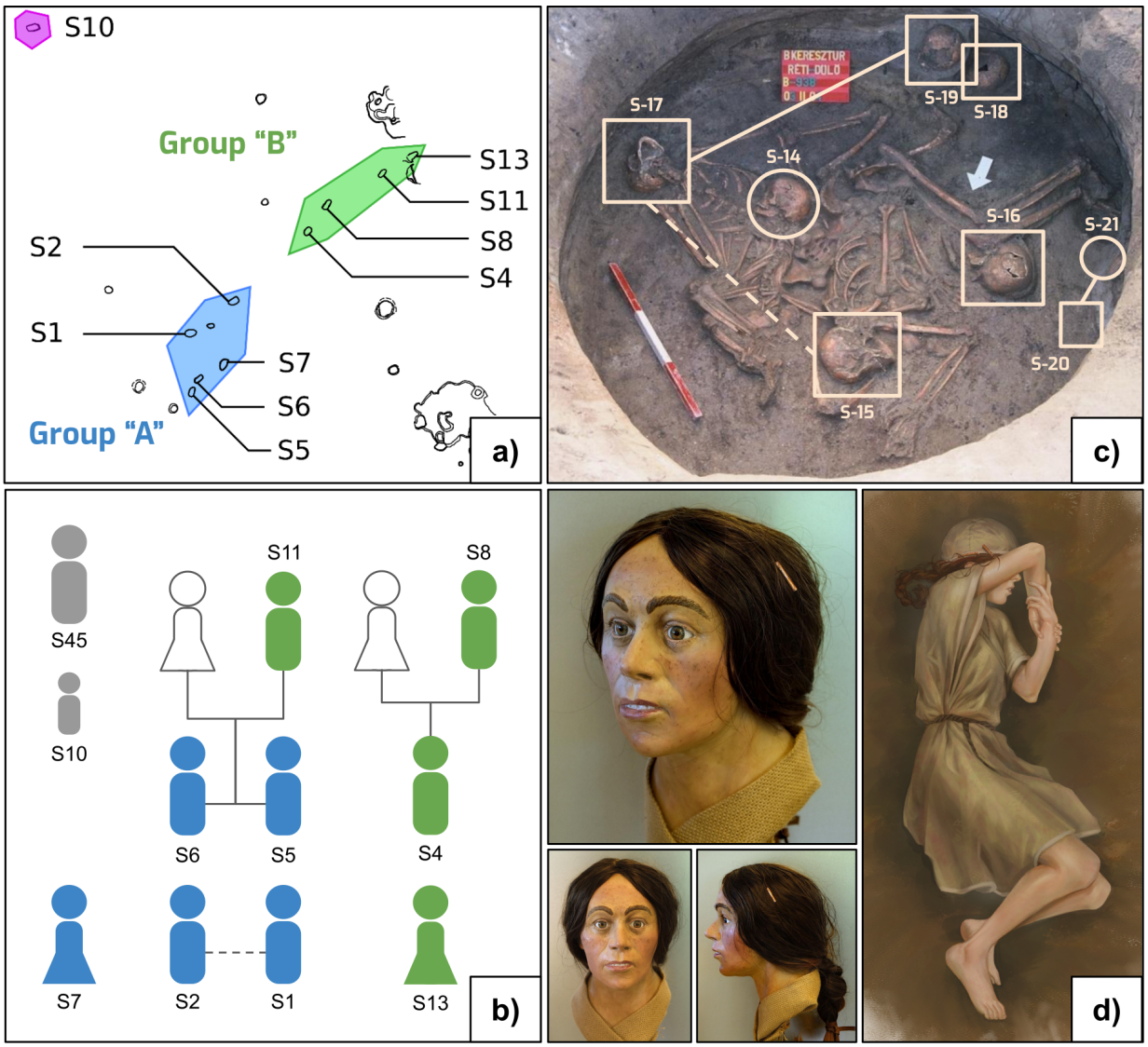

Fig. 1 **Kinship connections of Bk-II and Bk-III, reconstruction of individual S13.**
a) Distribution of graves of Bk-II: individuals S1, S2, S5, S6 and S7 belong to grave group 'A' highlighted in blue, whereas individuals S4, S8, S11 and S13 belong to grave group 'B' highlighted in green. Individuals S10 and S45 (not shown) placed separately from grave groups. b) Kinship network of Bk-II individuals, where colours denote the corresponding grave groups in figure 'a'. The dashed line between individuals S1 and S2 represent an undirected second degree relationship. c) Kinship relations of Bk-III projected onto the photo of the mass grave (obj. B-938). Brackets denote males, circles females, solid lines first degree and dashed lines second degree relationships. d) Forensic facial and burial reconstruction of individual S13.

Genetic disorders and pigmentation

Investigating genetic disorders in archaic datasets can be useful to improve our knowledge on the history of health and medicine, and also highlights the overall genetic health of past populations. Genetic disorders, if accompanied with severe phenotypic anomalies, could also explain unusual burial practices, for example as it was described in cases of dwarfism in Byzantine era[47], or in the case of the Suontaka burial[27]. Therefore, we analysed the ploidy of the autosomes not only for genetic sex determination, but to recover possible aneuploidies resulting in serious health related traits, such as Turner or Down syndrome[48]. For inferring pigmentation of the studied individuals, we used the HIrisPlex[49] system supplemented with further variants

obtained from SNPedia database[50]. Finally, we created our own disease panel described further below and in Supplementary Information section 3.

Aneuploidies

The abnormal number of chromosomes result in a few well known diseases which we tested thoroughly. We developed a new method called Z-score Adjusted Coverage (*ZAC*) for ploidy estimates by using a set of reference genomes. Our method can estimate ploidy for samples as low as 0.008x average genomic coverage, enabling genetic sex determination and aneuploidy assessment for all of our samples (Supplementary Information section 3.1). As a result, we found one individual, S10 - the only child burial in Bk-II - with XYY gonosomal genotype, described as Jacob's syndrome. This syndrome is relatively frequent (~0.1%) in today's populations. In most of the cases it remains silent but occasionally comes with a wide scale of symptoms, mainly behavioural disorders[51].

Mitochondrial DNA diseases

We examined the clinical significances of the polymorphisms that can be found in the mtDNA by using the *mitopathotool* software on the *AmtDB* database[52], and found that individual S1 (40+ years old male from Bk-II) had one of the defining mutations (T14484C, 48x coverage) of Leber's hereditary optic neuropathy (LHON) causing complete vision loss in ~50% of males between 20-40 years of age, rarely accompanying by other neuropathies[53].

Nuclear variants with clinical significance

We also examined the nuclear genomes to find regions with clinical significance. Since a complete panel for determining disease susceptibility only exists in commercial DNA kits with non-available descriptions similar to the 1240k panel, we created our own SNP calling panel focusing on various conditions including amyotrophic lateral sclerosis, Alzheimer disease, autism, Crohn's disease, diabetes, lactose intolerance, mental disorders, Parkinson disease, schizophrenia and ulcerative colitis. For this study we used a ~5k set of clinically significant SNPs, which were marked as "pathogenic" or "likely pathogenic" in the ClinVar database[54], by ignoring deletion, duplication and copy number variants, as well as SNPs with questionable (signed as "reported", "conflicting reports", *etc.*) contribution to diseases. The exact method of calling variants can be found at Supplementary Information section 3.2.2. Both the tool and the ~5k set are built in the PAPline package. We also created a bioinformatic tool that rolls up variant information from input data, which is available in our *PAPline* package (see the *PAPline* chapter for details). After running the panel, we excluded low coverage transitional variants from the final evaluation due to the possible presence of DNA damage. We only made exceptions, when skeletal features supported the presence of the low quality variant or when more than one sample possessed the same allele. Nevertheless, we listed all alternate variant hits in Supplementary Table 6. We are aware that low coverage data is not sufficient for firm conclusions, however, the aim was more of a technical description of such analyses. Here we summarise only a few mentionable results of the run, but for the detailed discussion see Supplementary section 3.2. Lig4 syndrome is a transitional mutation (rs104894421[55]) induced disease with skeletal abnormalities[55], for which individuals S15 and JAG93 from Jagodnjak site of Encrusted pottery culture[11] both provided a single read hit. Albeit we excluded the Jagodnjak group from our analyses for the lack of UDG treatment, meaning that both hits could be false positives, individual S15 possesses the distinguishing skeletal features of this disease, increasing the possibility of the actual presence of this allele in the Encrusted pottery population. Another ambiguous, but possible hit is rs121434442 in individual S6, which SNP is the causative factor for hereditary spastic paraplegia[56]. This disease is mostly recognised by the muscle stiffness in lower limbs causing movement restrictions: individual S11, father of S6 show signs of a limb condition that may be linked to this disease. Finally, autism 15 susceptibility signature transversional variant (rs7794745)[57] was present in individuals S6 and S45. Severe bruxism on the upper front teeth of S45 (Supplementary figure S.3.2.2.4) suggests compulsive behaviour that occurs frequently among people with autism spectrum disorder[57,58]. While this condition itself could be linked to some profession related abrasion, the physical features completed with genetic data and the distinguished burial treatment (Supplementary figure S.1.5.11) speak for a decent possibility for the actual onset of symptoms.

Pigmentation

According to our results based on a final set of 58 SNPs, the pigmentation patterns highly differ between horizons, as Bk-I mostly possesses variants for light pigmentation, blue eyes and blonde hair, while Bk-II is more similar to populations of Neolithic Europe of darker colouration[23,24] (Fig.1.c), although some variants for lighter pigmentation exist within this group too. Members of Bk-III on the other hand show a wide range from dark to light pigmentation tones and even the presence of variants for red hair (Supplementary Table 5, Supplementary Information section 3.2.1).

Whole genome composition and genetic ancestry

Balatonkeresztúr site samples

To get a general overview of the autosomal composition of our samples, we performed Principal Component Analysis (PCA) with *smartpca* software[59] based on 590k nuclear SNPs[60] and ADMIXTURE[61] analyses based on the 1240k SNP set[60]. According to PCA (Fig.2.a) Bk-I is clearly separated from Bk-II and Bk-III, where Bk-II has a strong shift towards European hunter-gatherers[60] overlapping with only a fraction of known ancient samples[60] and Bk-III. *Admixture* analyses (Fig.2.b) for assessing genetic components revealed ~17% HG, ~40% EEF, and ~43% steppe ancestry for Bk-I, similar to average Bronze Age Europeans[4,7,8,14,22] (Supplementary Table 9, 12-16; Supplementary Information sections 5.2, 5.5.2, and 5.6). According to *qpAdm*[62], Bk-I is most likely the ~1:2 mixture of a Vučedol culture associated individual (Croatia_EBA_Vucedol_3, ~38±4%), and a mostly steppe characteristic source. This steppe source can be best modelled as a Srubnaya/Alakul culture related population (Russia_Srubnaya_Alakul.SG, ~62±4%), in line with archaeological observations[63]. However, this high proportion of steppe ancestry is likely derived from a previously unsampled group in Eastern Europe, maybe in the vicinity of the Baltics (for details, see Supplementary Information section 5.6.2.1). Bk-II comprises a unique makeup of ~42% HG, ~41% EEF, and ~17% steppe ancestries. *qpAdm* analysis revealed most plausible sources as a Sweden_FBC (Funnelbeaker culture) related population and Ukraine_EBA with almost equal contribution (Supplementary Information section 5.6.2.2), however, both populations are likely only an approximation for the actual ancestry of Bk-II, which we discuss further below. Bk-III shows a slight shift in ancestry composition from Bk-II with ~29% HG, ~46% EEF, and ~25% steppe ancestries. *qpAdm* analyses uncovered that the main ancestry component for Bk-III is Bk-II (~60±8%), while “dilution” of Bk-II to Bk-III is mostly driven by contact with various local populations, genetically best represented by later Transdanubian Hungary_LBA or Serbia_Mokrin_EBA_Maros (Maros culture) groups (Supplementary Information section 5.6.2.3, Supplementary Table 15).

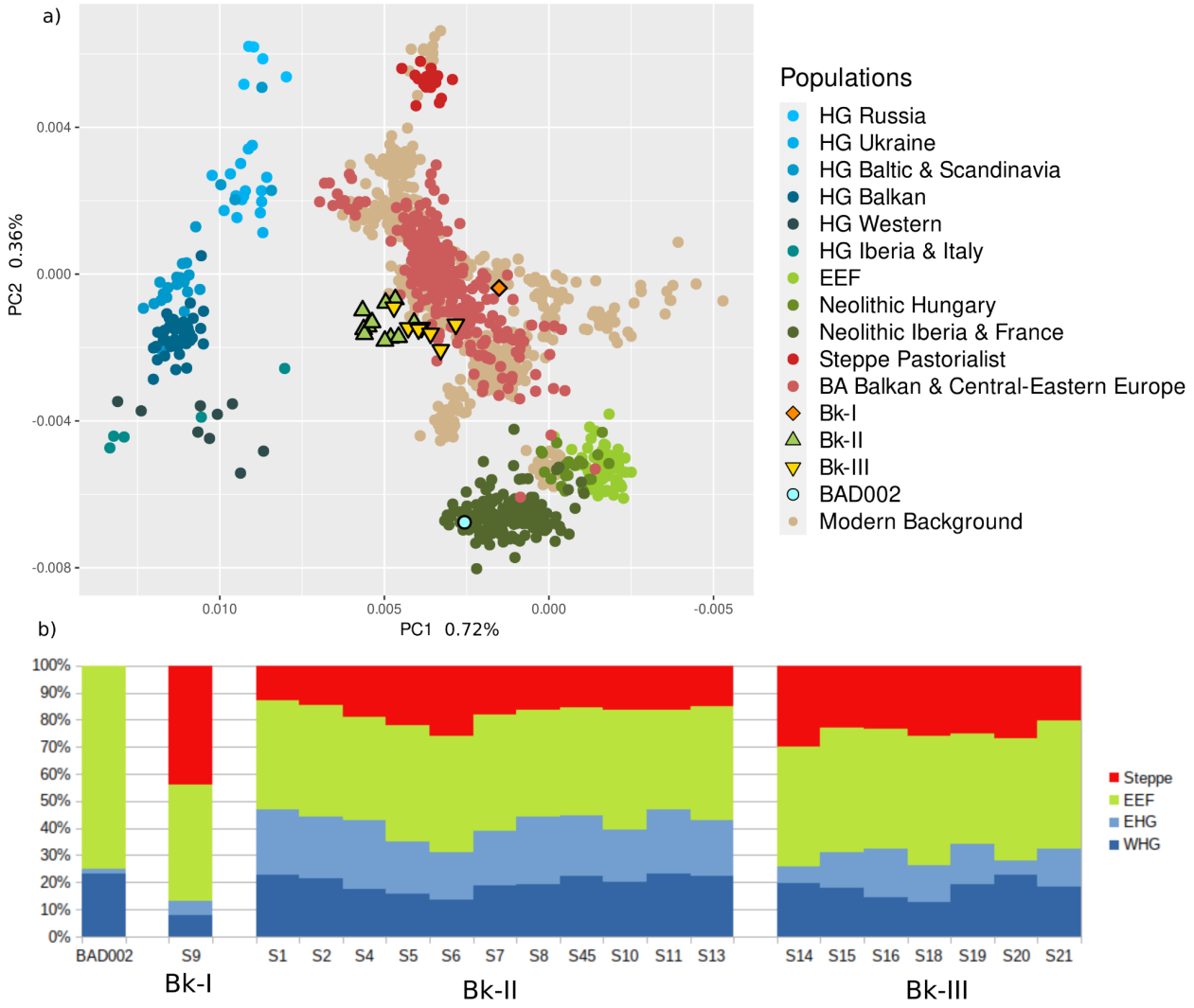

Fig. 2 ***Basic genetic composition of the investigated samples.***
a) Principal Component Analysis based on 590k SNPs calculated by the smartpca software[59], where Bk-II (marked with green triangles) clearly separated from all other archaic Central-Eastern European populations. b) The admixture proportions of the BAD002, Bk-I, Bk-II, and Bk-III samples, where the percentage of steppe ancestry is showed with red, early European farmer (EEF) with light green, Eastern hunter-gatherer (EHG) with light blue, and Western hunter-gatherer (WHG) with dark blue colour (supervised Admixture analyses).

Genetic outliers from previous studies and the origin of HG ancestry in Bk-II

We aimed to investigate further the exact composition of HG ancestry in Bk-II. *qpAdm* analysis of the basic composition resulted in EEF ~40±2%, EHG ~39±3%, WHG (Western HG) ~13±2.7%, Caucasus HG ~8±2%, *p*=0.0917 in par with *Admixture* analysis, pointing towards a rather EHG characteristic composition. Next, we performed an *f4* test in the form of f4(test HG, Serbia_IronGates_Mesolithic, Bk-II, Mbuti.DG)[62], to see which HG population relates the best with the Bk-II samples. The aim of this analysis was to detect different HG ancestry contributions besides Iron Gates HG, which has knowingly contributed to Neolithic European populations in the study region and witnessed an intermediate composition between the WHG and EHG[5]. Contrary to the *Admixture* and *qpAdm* results, this test revealed that Bk-II has an excess HG ancestry mainly from WHG groups or other mixed characteristic HGs (Croatia_mesolithic, Poland_BKG_o2.SG (Brześć Kujawski Group outlier) or KO1 (Körös culture outlier HG)), but only marginal relations with the EHG (Lithuania_Mesolithic) populations (for detailed results, see Supplementary Information section 5.3). Surprisingly, none of these HG populations with mixed characteristics (and neither Iron Gates) have enough

EHG component to explain the ancestry of the Bk-II samples. The *f4* test also revealed that the Bk-II and Bk-III populations differed significantly from other EHG characteristic populations, such as Russian, Ukrainian, younger Baltic or Scandinavian HG-s, although we can see some weak connections to to older (down to sixth millennium BCE) Lithuanian HG-s. These results may reflect the population turnover at the sixth millennium BCE in the Baltics[2], suggesting that this EHG ancestry is related to Lithuania_Mesolithic. On the other hand, *qpAdm* always provides negative weights for this component when we try to model Bk-II as a combination of WHG (Loschbour_WHG), EHG (Lithuania_Mesolithic), EEF (Turkey_N) and Yamnaya (Russia_EBA_Samara_Yamnaya), suggesting that Lithuania_Mesolithic is not a good proxy for the actual EHG component.

To infer the timing of HG admixture, we used the *DATES*[64] analysis. This test revealed that the HG ancestry in Bk-II resulted from three independent admixture events: one from Iron Gates HG at the beginning of the Neolithic (similar to other populations at that time), one from a WHG characteristic source around the turn of the fourth and third millennium BCE, and an EHG characteristic source around the second half of the third millennium BCE (for details, see Supplementary Information section 5.4). Summarising these results, we conclude that the EHG characteristic source of the Bk-II individuals does not exist in the current database.

We were interested in whether other populations carry this peculiar HG ancestry, to see which region it might originate from. To achieve this, we did a literature search to select individuals with high levels of HG ancestry, who were genetic outliers in their cultural or geographical or temporal context, in order to assess whether they are related to our Bk-II group. Selection was based on previous observations and HG ancestry differences within groups using the results of the *Admixture* analysis (Supplementary Information section 5.2.2). Then, to reveal similar patterns of HG ancestry, we ran *f3* statistics in the form of f3(test HG, test population, Mbuti.DG) on all of the groups (obtained from AADR[60] database, listed in Supplementary Table 8). Subsequent euclidean distance based clustering of *f3* values revealed a number of outliers and even whole populations belonging to the same subcluster as Bk-II (Fig.3, Supplementary Information section 5.5). Accordingly, the earliest signs of such HG ancestry appeared among various Neolithic groups from Western Europe (in line with characteristically high WHG ancestry among Megalithic, Globular Amphora or Funnelbeaker cultures' population) and from Eastern Europe (Bulgaria and Ukraine). Individuals with this ancestry predating Bk-II with only a few generations appeared in Czechia, Northern Hungary, Eastern Germany and Western Poland, indicating the Kisapostag associated population's arrival to Transdanubia on a Northern route, in line with observations of Freilich et al.[11]. Many contemporaneous populations to Bk-II and Bk-III from the British Isles to today's Poland, down to today's Serbia have outliers with Bk-II-like genomic composition, mostly overlapping with known Kisapostag and Encrusted pottery contact regions (Fig. 3). Interestingly, at the end of the second millennium BCE many Baltic groups appear to be highly similar to Bk-II, indicating long term success of this ancestry outside the Carpathian Basin. Notably, in the vicinity of Prague many pre- and post-Bk-II outlier appears along with the archaeological presence of Kisapostag culture, including the Tollense group, which also originates from the region of Bohemia according to isotopic evidence[65], suggesting a local reservoir of the population. While the appearance of Bk-II ancestry in the Baltics could be connected to this reservoir, especially in the light of the mobility of Tollense group, the ^{14}C date of Lithuania_LN_o around 2000 BCE suggest that the population was likely prevalent in nearby unsampled regions of Eastern Europe. Taking into consideration all of the genetic parallels, their dates and geographic locations, one plausible scenario is that the EHG characteristic core of Bk-II (which ultimately could be best modelled as Ukraine_EBA by composition) moved northward from the region of today's Eastern Romania, Moldavia or Western Ukraine, subsequently mixed with Funnel Beaker culture (FBC) or Globular Amphora culture (GAC) related populations and split into two groups, one taking a route to Transdanubia and one to further North. These results are highly in par with Mittnik et al.[2], who suggested population replacement at the end of the second millennium BCE in the Baltic region from a nearby, unsampled region by a population of considerably higher steppe, EEF and WHG ancestry, than the prevailing ones. This idea is also supported directly by the phylogenetic tree of mtDNA U4b1b1 (Supplementary Information section 2.1), however, further data is needed from Eastern Europe to firm this hypothesis.

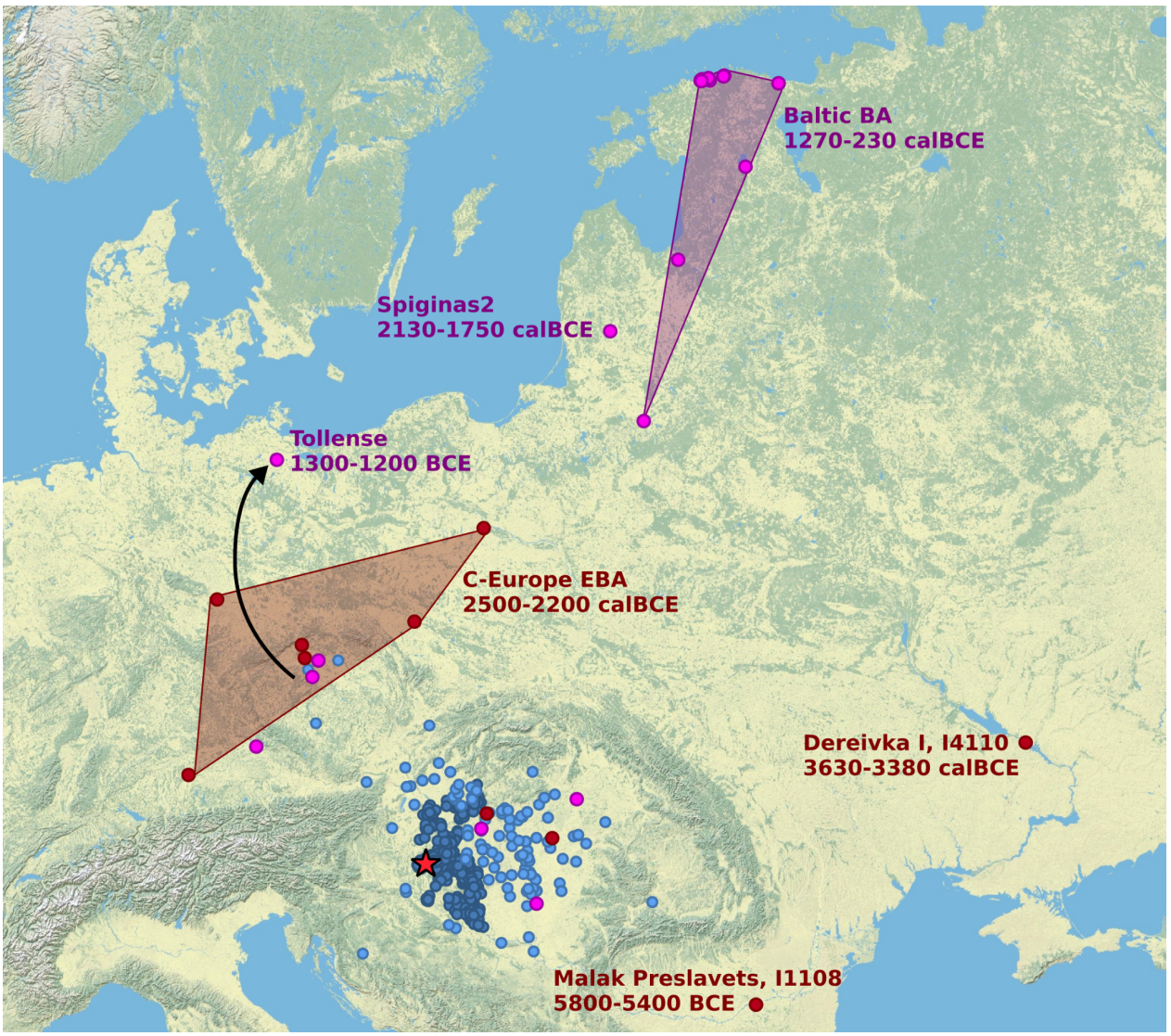

Fig. 3 ***Map of East-Central Europe with sites and genetic parallels of Kisapostag/Encrusted pottery culture.***
The map shows the site of Balatonkeresztúr (red star), Kisapostag and Encrusted pottery culture archaeological sites (dark blue circles), their archaeological connections based on pottery and metal finds (light blue circles) after Kiss[36]. Red and purple circles represent individuals that are connected to Bk-II individuals by HG ancestry. Also, red circles are preceding, pinks are succeeding or contemporaneous to Bk-II horizon.

Isotope analyses

We took samples from molars of individuals and measured the ratio of $^{87}Sr/^{86}Sr$ isotopes to evaluate whether individuals were born in the area of their burial. According to the results (Fig. 4), almost all samples share the same pattern that blends well with local values, which indicates that none of the studied individuals are first generation occupants. It is, however, interesting how the M3 molar values for individuals S15, S16 and S17 (all from the mass grave) differ from the others. While these values are not out of local isotope ratio diversity, strongly suggest some movement within the region. This movement could have occurred at the same time for these individuals, as the stronger the divergence from the majority, the younger the individual was at the time of death. It is particularly interesting how individual S15 shows the highest divergence from the others, as this individual had severe complications for walking due to hip dysplasia (see also Supplementary Information section 3.2.2). Moreover, since M3 values show divergence, but their M1 are not, these

individuals likely grew up in the vicinity of the site, while they spent many years away from it and then returned to the same place where they died and was buried.

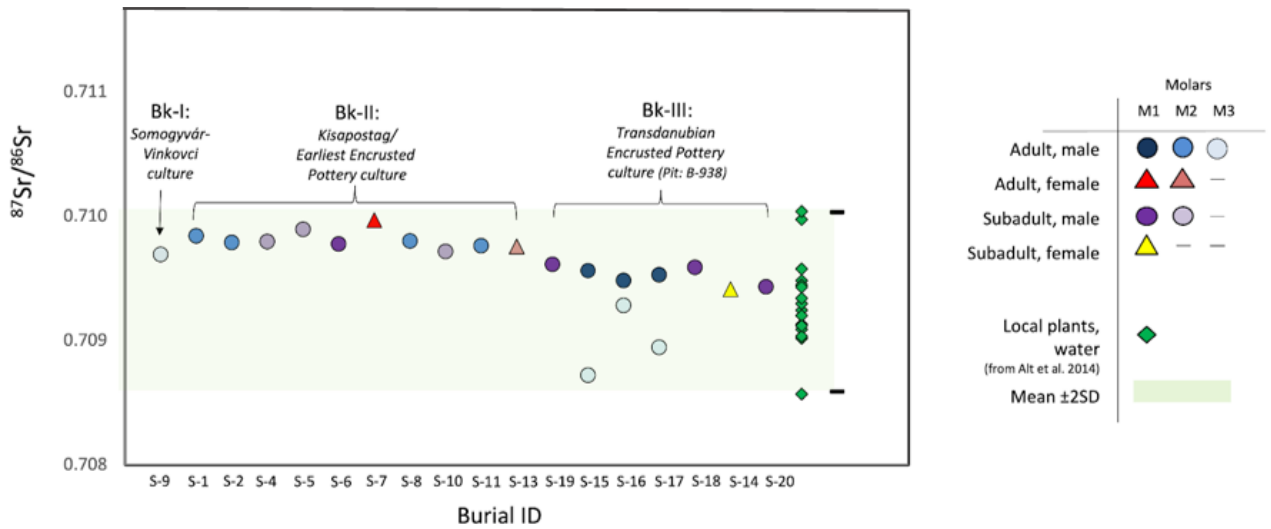

Fig. 4. ***^{87}Sr/^{86}Sr isotope data from the Balatonkeresztúr site.***
Samples were taken from dental enamel (first, second and third molars) to evaluate whether individuals were born in the area, or grew up in a geologically distinct region. All of the samples are consistent with previously published plant and water ^{87}Sr/^{86}Sr ratio (green diamonds) data collected from the southern portion of Lake Balaton[41]. For further data see Supplementary Information section 1.9.

A Late Copper Age outlier individual from Balatonlelle site

We included in this study a Late Copper Age individual, BAD002, from Balatonlelle site because of his high HG genomic ancestry component. Mitogenome of BAD002 (K1a4a1) shows affinity to Iberian Bell Beaker culture associated individuals (Supplementary Information Fig. S.2.1.1). His Y chromosomal haplogroup belongs to I-M170. Compared to known Neolithic and Copper Age populations in the Carpathian Basin[6], BAD002 has higher HG component (~34%), and he also lacked steppe related ancestry. Therefore, on the genomic PCA BAD002 relates with Iberian and French Neolithic individuals. According to our ancestry estimates, France_MontAime_MLN.SG describes best the BAD002 individual, however, other Western European sources, such as Spain_EN can not be excluded (for details, see Supplementary Information section 5.6.1). Pigmentation pattern of BAD002 shows resemblance to average Neolithic Europeans. The foreign cultural traits of the boy's jewellery is in line with his outlier genetic composition in the study region[66]. Notably, further tests (outgroup *f3*-statistics and *qpAdm*) excluded contribution of BAD002 to Bk-II (Supplementary Information section 5). Therefore we conclude that this individual testifies large-scale migration in the Copper Age, providing research questions for future studies.

PAPline

We introduce our newly developed, freely available bioinformatic package, named *PAPline* (Performing Archaeogenetic Pipeline), written in *linux bash, R,* and *python v3.8.10* programming languages. One can use this package primarily to analyse next generation sequencing data of archaeogenomic samples, supplemented by tools, including ploidy test, MPMR kinship analysis and clinical variant test. The standalone tools and the core workflow of the first version of *PAPline* is available at https://github.com/gerberd-workshop/papline. In the future *PAPline* is aimed to be compared to the EAGER[67] and the Paleomix[68] pipelines, for detailed description visit the github page.

Discussion

The Carpathian Basin was inhabited by the Baden cultures' population at the end of the Copper Age. Their genetic composition was represented by an EEF and – compared to the previous Neolithic populations of the region[6] – a slightly increased HG genetic component. Here, we demonstrated that in the early phase of this culture, a Western European group appeared in Transdanubia, diversifying our previous knowledge about the region's Late Copper Age.

The Carpathian Basin experienced the influx of steppe-related genetic ancestry from the Late Copper Age[5,8]. This transformation was already detectable at the Bronze Age genetic picture of the Balatonkeresztúr-Réti-dűlő site as well, where we could examine multiple populations. The earliest Bronze Age horizon Bk-I (representative of the Somogyvár-Vinkovci culture) is best described by the mixture of local (Vučedol) and a high steppe ancestry population from Eastern Europe that was replaced by the Kisapostag culture associated group of Bk-II likely around the 23-22th century BCE. According to our results, the Bk-II population had an outstandingly high HG genetic ancestry level, compared to other Bronze Age groups of the region. This can be traced back to two main sources, one to a WHG, and one to an EHG characteristic population, best modelled as FBC/GAC and Ukraine_EBA, however, likely both are only proximate to the actual source, which are yet to be described. The Y chromosome haplogroup I2a-L1229 can be linked to the FBC/GAC component, although this exact same subgroup only appears first in Bk-II and related groups. The calculated admixture dates suggest the presence of a highly EHG characteristic population in Eastern Europe as late as the beginning of the Bronze Age. This EHG component shows the closest resemblance to Lithuanian Mesolithic individuals, however the best proxy for this population is probably missing from the published database opening research question for future studies.
Following the formation of the population represented here by Bk-II, it contributed to various populations in Central-Eastern Europe, whose genetic legacy persisted mostly in the region of today's Hungary and Czechia at least until the end of the Bronze Age, and even to the end of the first millennium BCE in the Baltic region. This study do not disclaim any of the archaeological theories regarding the origin of Kisapostag culture[28,29,36–38], as the EHG core of the Kisapostag associated group fits really well with the Middle Dnieper origin, while further adaptation of cultural elements during their arrival and during their occupation in Transdanubia is plausible. The latter idea is further supported by the $^{87}Sr/^{86}Sr$ isotope ratio data (representing through nutrition the bioavailable Sr in the area where people lived in a certain age interval) which shows local isotope ratios for both sexes in both Bk-II and Bk-III. These results place back the time of their arrival with a few generations, meaning that local and southern impact of cultural traits could explain the culture's archaeological heterogeneity.

Bk-III was the direct descendant of Bk-II, forming cultural (Encrusted pottery) and genetic continuity for hundreds of years at the studied site. Observable dilution of HG ancestry in Bk-III compared to Bk-II can be connected to continuous female-biased admixture with nearby communities according to our and previous genetic[11] and archaeological[28,69] evidence.

In both periods, the homogeneity of paternal lineages suggest a patrilocal residence system, similarly to previously described social organisations[10,11]. However, $^{87}Sr/^{86}Sr$ isotope data shows local values for both sexes, which along with similar genomic makeup of females and males suggest exogamy most probably between villages of the same population. The overlap between outlier parallels of Bk-II/III and archaeological contact regions is also noteworthy, as it signalises smaller scale migrations of Kisapostag/Encrusted pottery individuals or groups along trading networks, mobility possibly connected to wandering merchants.

Notably, none but one (mtDNA haplogroup U5a1g) of the uniparental lineages are the same at the haplogroup level with the individuals from the Croatian Encrusted Pottery culture Jagodnjak site, despite high similarities in cultural traits, social structure and genomic composition of the communities[11], suggesting a regionally patrilocal, clan-like superfamily structure of Kisapostag and Encrusted Pottery groups. This finding is particularly interesting in the light of a strikingly different social structure observed among the 2100-1800 BCE Mokrin individuals[12], which Maros culture group nonetheless shows extensive amounts of admixture related to the groups of the Kisapostag/Encrusted pottery culture.

The relatively limited presence of female and children burials in both Bk-II and Bk-III periods may suggest distinctive treatment or another (here undiscovered) burial group for women and children at the same site. However, in other cemeteries of the culture, *e.g.* Ordacsehi and Bonyhád in Hungary, males, females, and children were buried close to each other, suggesting high variance of burial practises[33,34,70].

While low genomic coverage did not allow fine SNP recovery, we did find evidence for malignant variants within all of our tested groups, and undoubtedly showed the presence of LHON and Jacob's syndrome within Bk-II. While it only remains a possibility, the presence of autism risk factor in CNTNAP2 gene, signs for severe bruxism and distinctive burial treatment of individual S45 suggest the actual onset of symptoms. Additionally, the disease panel we created and made freely available, can be extended and used in future studies, providing insight into past population health qualities.

Considering the unstructured age and kinship distribution in the mass grave Bk-III compared to Bk-II, the coetaneous death of eight people at least, the absence of traumatic or ritual events on bones, and non-cremated nature of the burial all signals a sudden tragic event in the Middle Bronze Age period (Encrusted Pottery population), most likely an epidemic, as first suggested based on the anthropological analyses[71]. Careful burial positions also suggest that the deceased were buried by their own community. Interestingly, comparative $^{87}Sr/^{86}Sr$ isotope analyses on the first and third molar of the individuals in the BK-III mass grave indicate that subadult males – including a severely disabled individual (S15) with hip dysplasia – left their community for a while and then returned to their birthplace prior to their death, raising further questions for future studies on prehistoric lifeways and social organisations.

Materials and Methods

Isotope analyses

Radiocarbon dating was performed at the HEKAL AMS C-14 facility of the Institute for Nuclear Research in Debrecen, Hungary (see Supplementary Information section 1.8). $^{87}Sr/^{86}Sr$ isotope measurements were performed in the ICER Centre, Institute for Nuclear Research Debrecen, Hungary and at Quinnipiac and Yale University, Connecticut, USA (see Supplementary Information section 1.9).

Ancient DNA laboratory work

Petrous bones and teeth were taken from skulls for genetic investigation (Supplementary Table 1). Laboratory work was performed in a dedicated ancient DNA laboratory facility (Institute of Archaeogenomics, Research Centre for the Humanities, Eötvös Loránd Research Network, Budapest, Hungary). Each step was carried out in separate rooms under sterile conditions, during work protective clothing was used. Irradiated UV-C light, DNA-ExitusPlus™ (AppliChem) and/or bleach were applied for cleaning after and between work stages, and also, blank controls were utilised at all times.

Sample surfaces were cleaned by sandblasting and mechanically milled to powder. DNA extraction was performed according to Dabney et al. 2013[72] with minor changes according to Lipson et al. 2017[6]. DNA extraction success was verified by PCR using mtDNA primer pairs (F16209-R06348; F16045-R06240). Half-UDG treated libraries were used according to Rohland et al. 2015[73] with minor changes. Unique double internal barcode combinations were used for each library (Supplementary Table 1). Libraries were amplified with TwistAmp Basic (Twist DX Ltd) and purified with AMPure XP beads (Agilent). Then, concentration measurements were taken on Qubit 2.0 fluorometer, fragment sizes were checked on Agilent 4200 TapeStation System (Agilent High Sensitivity D1000 ScreenTape Assay).

Hybridisation capture method for mtDNA and 3k nuclear SNP was used besides whole genome shotgun, as described by Haak et al. 2015, Lipson et al. 2017 and Csáky et al. 2020[4,6,74]. Bait production was based on

Fu et al. 2016[1] and N. Rohland's personal communication, the oligos as a pool was ordered from CustomArray Inc. Both for shotgun and capture libraries, universal iP5 and unique iP7 indexes were used. Sequencing was done on Illumina MiSeq and NovaSeq platforms with custom setup and 150, 200 and 300 cycles, respectively.

Additionally, we investigated Y chromosome STR profiles (17 markers) with AmpFISTR® Yfiler® PCR Amplification Kit (Applied Biosystems), having one blank and one positive control at each reaction preparation. The workflow followed the recommended protocol except the PCR cycles were increased from 30 to 34 and reactions were halved in volume. Two repeats were done where at least 4 markers yielded results. Data analyses were carried out in GeneMapper® ID Software v3.2.1 (Applied Biosystems), results are summarised in Supplementary Table 3.

Bioinformatic analyses

Illumina sequencing paired-end reads were processed by the *PAPline* https://github.com/gerberd-workshop/papline. We used the GRCH37.p13 reference sequence to call the pseudohaploid genomes. For kinship inferences we applied the *READ* software[46] and a custom script (named *MPMR*, see Supplementary Information section 2.3 and Supplementary Table 2). MtDNA analyses included phylogenetic analyses using the *MrBayes* v3.2.6[44] and the *BEAST* v1.10.4[75] software and diversity tests using the *Popgenome*[76] *R* package, see Supplementary Information section 2.1. For Y chromosome haplogroup determination the *Yleaf* v1[77] software was applied. We used the *Network* v10.1.0.0 and *Network publisher* v2.1.2.5[78,79] programs for analysing the network of STR data, see Supplementary Information section 2.2. We discarded individuals S2, S4, S5, S6, S17 and S20 from the population genetic analyses due to low genomic coverages and/or being first degree relative of other samples. The Principal Component Analysis was made by the Eigensoft smartpca software[59] using the Human Origins Panel SNP set[62], for other analyses the 1240k array SNP set[26] was used for variant calling, for results, see Supplementary Table 7. For investigating ancestry estimates we used supervised admixture analysis calculated by the *ADMIXTURE* v1.3.0 software[61]. *f*-statistics and qpAdm were performed using the *admixr* v0.9.1[80] and the *admixtools* v2.0.0[62] *R* packages. The timing of the admixture events were inferred by using the *DATES* software[64].

Data availability

All studied data are cited in the article and/or Supplementary Information and tables. New sequencing data are deposited in the European Nucleotide Archive (ENA) under accession number PRJEB49524.

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Author contribution

D.G, V.K., A.Sz-N. conceived and designed the experiments. D.G. processed the sequencing data, created the *PAPline*, and performed downstream bioinformatic analyses. B.Sz. did all molecular laboratory work. O.Sz. created the mtDNA database for phylogenetic analyses. B.E. obtained Y chromosome STR data. B.Gy. and E.A. optimised genetic analyses. J.I.G., A.H., L.P. performed Sr isotope analysis. G.K., Sz.F., V. K. and M.B. evaluated the archaeological context. B.G.M., Á.K. and K.K. did the anthropological examination of

the remains. Á.K. made the facial reconstruction. V.Sz., I.M. and M.M. performed ^{14}C calibrations and modelling. B.G.M. sampled the remains. E.A., V.K. and A.Sz-N. jointly supervised the research and wrote the paper with D.G. All authors provide critical feedback for this study and contribute to the final manuscript.

Ethics declarations

The authors declare that they had requested and got permission for the destructive bioarchaeological analyses of the archaeological material in the study from the stakeholders, excavator and processor archaeologists.

Competing interests

The authors declare no competing interests.

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