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Investigating kinship of Neolithic post-LBK human remains from Krusza Zamkowa, Poland using ancient DNA

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ABSTRACT

We applied an interdisciplinary approach to investigate kinship patterns and funerary practices during the middle Neolithic. Genetic studies, radiocarbon dating, and taphonomic analyses were used to examine two grave clusters from Krusza Zamkowa, Poland. To reconstruct kinship and determine biological sex, we extracted DNA from bones and teeth, analyzed mitochondrial genomes and nuclear SNPs using the HID-Ion AmpliSeq™ Identity panel generated on Illumina and Ion Torrent platforms, respectively. We further dated the material (AMS ^{14}C) and to exclude aquatic radiocarbon reservoir effects, measures of carbon and nitrogen stable isotopes for diet reconstruction were used. We found distinct mitochondrial genomes belonging to haplogroups U5b2a1a, K1c and H3d in the first grave cluster, and excluded maternal kin patterns among the three analyzed individuals. In the second grave cluster one individual belonged to K1a4. However, we could not affiliate the second individual to a certain haplogroup due to the fragmented state of the mitochondrial genome. Although the individuals from the second grave cluster differ at position 6643, we believe that more data is needed to fully resolve this issue. We retrieved between 26 and 77 autosomal SNPs from three of the individuals. Based on kinship estimations, taking into account the allelic dropout distribution, we could not exclude first degree kin relation between the two individuals from the second grave cluster. We could, however, exclude a first degree kinship between these two individuals and an individual from the first grave cluster. Presumably, not only biological kinship, but also social relations played an important role in the funerary practice during this time period. We further conclude that the HID-Ion AmpliSeq™ Identity Panel may prove useful for first degree kin relation studies for samples with good DNA preservation, and that mitochondrial genome capture enrichment is a powerful tool for excluding direct maternal relationship in ancient individuals.

1. Introduction

The attitude toward kinship reflected in the funeral ritual of early agricultural populations has rarely been examined using empirical data. One of the characteristic traits of the earliest Neolithic societies in Europe was the practice of burying the deceased within the settlement, which is well known from the Linear Pottery Culture (LBK) from the 6th–5th millennium BC [1,2]. However, this custom is much older and goes back to Neolithic and Chalcolithic communities of the Middle East and Anatolia who

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buried their dead not only at the settlements but also within the houses [3,4]. A close spatial relationship between burials and between burials and individual buildings is often interpreted as a reflection of close kinship relations structuring the early farming societies. The post-LBK Late Lengyel culture, one of latest representatives of the Early Neolithic tradition in Central Europe (known also as the Brześć Kujawski culture [5], Late Band Pottery culture (phase II–III) [6] or Brześć Kujawski Group of the Lengyel culture [7]), inhabited the northern part of today's Poland roughly between 4500 and 3800 cal. BC. According to local chronological scheme, this period fits into the Middle Neolithic. The post-LBK Late Lengyel culture is well known for large, densely occupied and multi-phase settlements consisting of trapezoidal long houses, and nearby graves sometimes richly furnished [8,9]. At the classical stage of the development of Late Lengyel societies the burial ritual was governed by the rule of placing the bodies of the deceased in a crouched position, with the head towards the south – men were usually buried on their right side, and women on their left [10,11]. A typical form of burial was inhumations in single pit graves, in most cases located in a random manner within a cemetery.

One of the prime examples of post-LBK Late Lengyel population settlements in Poland is the site Krusza Zamkowa, located in the region of Kujawy (Fig. 1). The first excavations at this site took place in 1973–1974 and 1976–1977, and led to the discovery of human graves along with settlement features [12]. During the second excavation, conducted in 2013, two additional human graves were found along another close-by settlement feature. The clustering of graves at Krusza Zamkowa site suggested that human remains from double burials or buried in close proximity could be biologically related, particularly with regard to the graves of adults and children [13].

Our understanding of funerary rituals of past populations, which may reflect kinship relations, can be improved by anthropological examinations, including biological traits and health status, and taphonomic investigation of, for example, arrangement of the deceased in the grave [14]. It should be emphasized though that the methods based solely on archaeological and anthropological assessments are usually insufficient in establishing biological kinship. However, methods for improvement of the identification of biological kin relations in archaeological context have been suggested in recent years [15]. The most direct and precise method to test biological affinity among individuals in past populations is based on ancient DNA (aDNA) investigations. Biparentally inherited markers such as short tandem repeats (STR) have been used [16,17] however, due to fragmented nature of aDNA this method could be inefficient [18]. More often, haploid molecular markers involving fragments of mitochondrial DNA (mtDNA) and/or Y chromosomal markers are usually used to trace maternally or paternally related individuals [19–25]. However, uniparental markers, such as mtDNA and the Y chromosome can only exclude direct maternal and paternal relationship due to their mode of inheritance and lack of ability to uniquely identify related individuals. In other words, two unrelated individuals can for instance carry the same mt haplogroup (hg) just because the hgs exist in moderate frequencies among humans. Thus, genetic data, together with other bio-archaeological characteristics can reveal uniparental and autosomal kin relations.

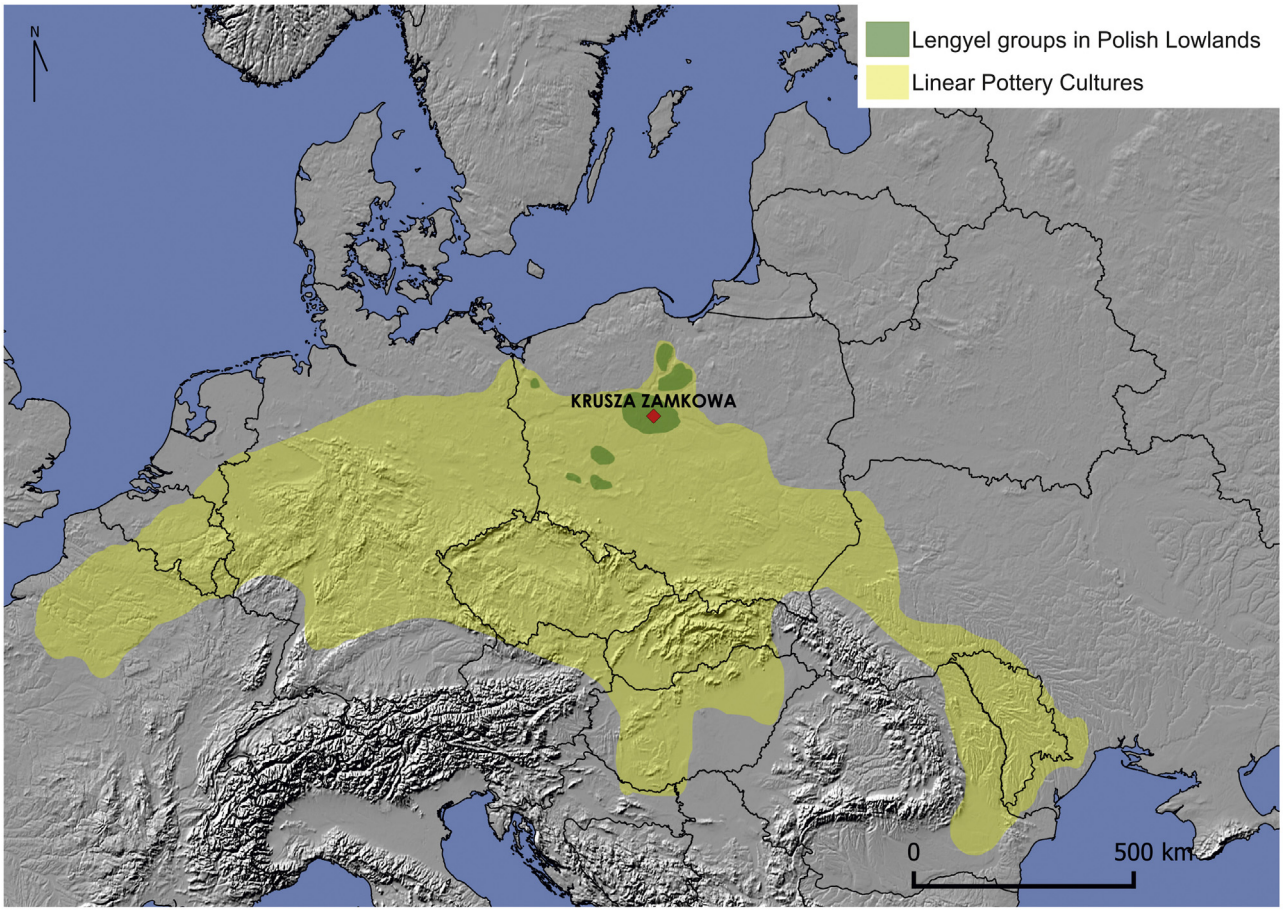

Fig. 1. Geographical distribution of European Linear Pottery Culture and Krusza Zamkowa burial site.

The aim of our study was to investigate possible kinship among five individuals from Krusza Zamkowa. The discoveries of human remains belonging to two adults and three children from two grave clusters pose an interesting question about the biological relationship among the individuals. To gain direct insight into the kinship relations, we used complete mitochondrial genome sequences and the HID-Ion AmpliSeq™ Identity Panel, both obtained through applying Next Generation Sequencing (NGS) methods.

2. Materials and methods

2.1. Archaeological samples

We investigated human remains from five individuals that were excavated at the archaeological site of Krusza Zamkowa in 1977 and 2013. The locations of the graves are presented in Fig. 2. In 1977, two rich interments were discovered containing the human remains of KZ4 and KZ5, commonly called the graves of "princesses" [13,26]. The burial pits were abutted along the longer walls. In the larger pit, a richly equipped adult individual (KZ4) was buried. A smaller pit contained a child (KZ5) with equally rich offerings. The grave goods included a bracelet and belts consisting of beads made of shells (up to 2295 pieces) and copper, accompanied by decorated pauldrons made of animal bones, and various copper and calcite adornments [12]. In 2013, the next three human remains were discovered in one double and one single burial, located within the limits of an abandoned house (Fig. 2). The double burial, in a rectangular pit with curved corners, comprised two skeletons KZ1 (adult) and KZ3 (child). The second pit, with an irregular outline, contained the remains of KZ2 individual (child) (Fig. 3). The distance between the burials discovered in 1977 and 2013 was about 25 m.

2.2. Radiocarbon dating and stable isotope analyses

Collagen samples from the KZ1 and KZ2 skeletons were AMS ^{14}C-dated at the Poznań Radiocarbon Laboratory, in Poznan, Poland. Details concerning the chemical pre-treatment of the samples are described in Supplementary materials A. The procedures of CO_2 production, graphitisation, and AMS ^{14}C measurements are presented in [27,28]. The skeletal remains of individual KZ4 were conventionally ^{14}C-dated in Radiocarbon Dating Laboratory of the German Academy of Sciences of Berlin in the 1970s [10]. The methods of sample pre-treatment and radiocarbon measurements were not reported, we can only assume that they were the same as in other analyses made in this laboratory at the time e.g. [29]. Carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope analyses were not included in this case.

All ^{14}C dates were calibrated with OxCal 4.2 [29] using the IntCal 13 curve [30]. To test whether KZ1, KZ2 and KZ4 are from the same time period an independent-samples *t*-test was conducted using CALIB 7.0.4 [31].

To exclude the impact of aquatic radiocarbon reservoir effects e.g. [32] on ^{14}C dates, a diet reconstruction was performed based on the measurements of carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope ratios in the bone collagen. The isotopic analyses were conducted in the Institute of Geological Sciences of the Polish Academy of Sciences in Warsaw (for details see Supplementary materials A). The end-point values for terrestrial and freshwater types of human diet were calculated. As the δ^{13}C and δ^{15}N ranges for local fauna are unknown we used the data published for terrestrial herbivores and omnivores from a Late Lengyel settlement in Racot, located in the neighbouring Wielkopolska region [33]. In the case of freshwater fish, we used values available for a medieval settlement in Kałdus (11th–13th c. AD), located to the north-east of Kujawy and Middle Neolithic (c. 4400/4300–3000/

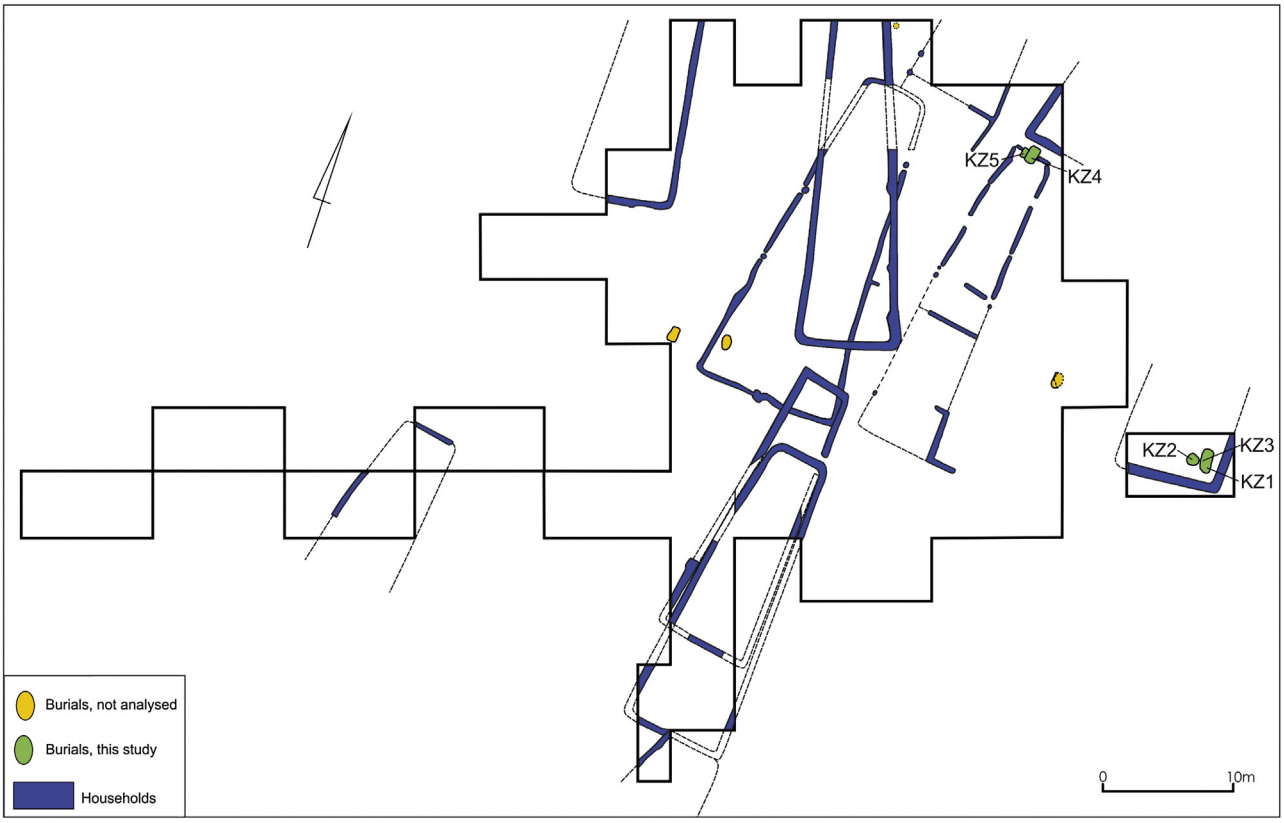

Fig. 2. Localisation of graves in the Krusza Zamkowa burial site.

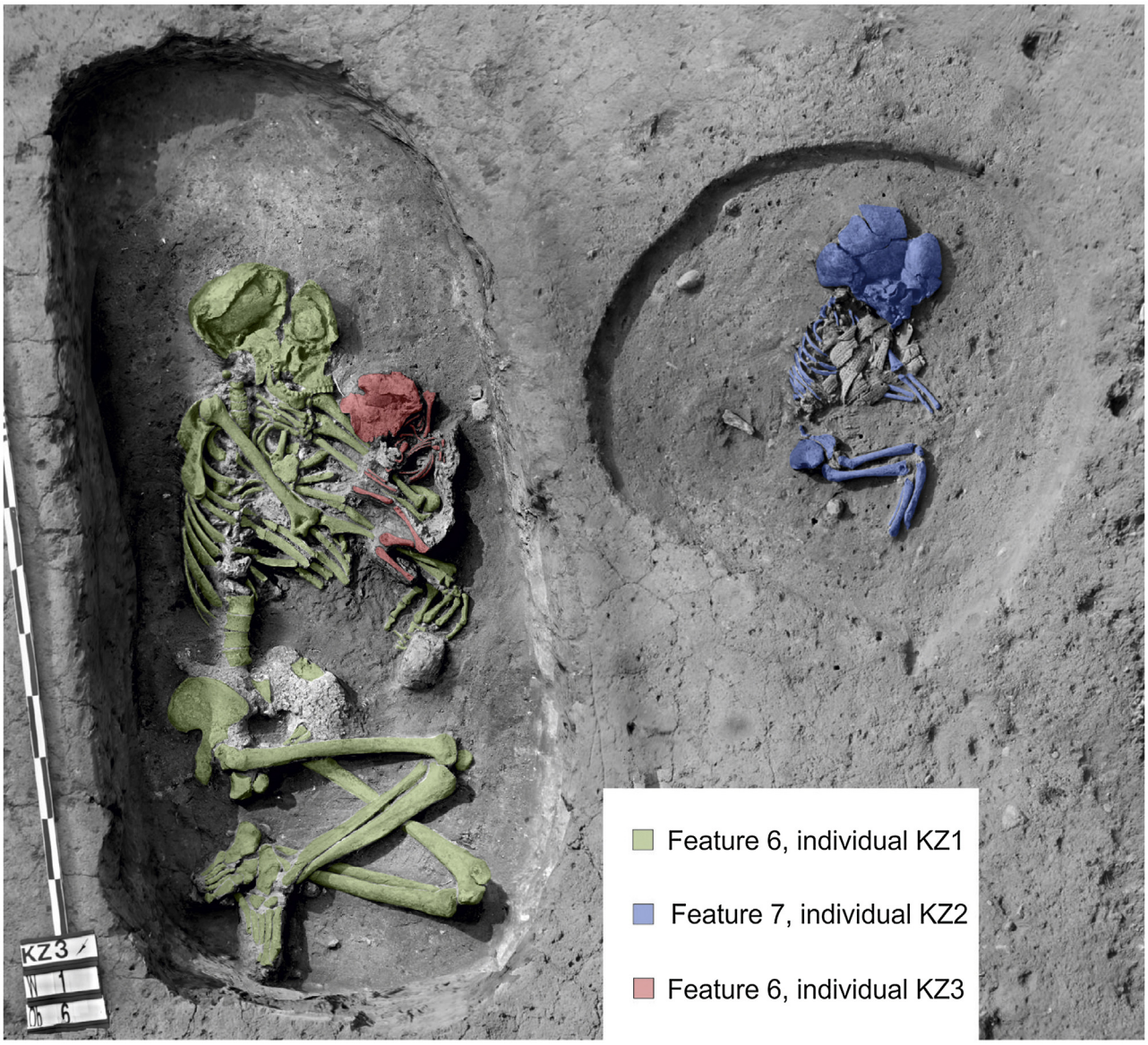

Fig. 3. In situ ortophotograph of three individuals (KZ1, KZ2 and KZ3) from Krusza Zamkowa archaeological site.

2900 cal. BC) settlements in Šventoji in Lithuania [34]. Following the generally accepted practice, a diet-to-consumer shift value between humans and animals consumed by them was assumed to 1‰ for $\delta^{13}C$ and 3.5‰ for $\delta^{15}N$ as in e.g. [35].

2.3. Anthropological analysis

The age-at-death and morphological sex of the individuals were determined through standard methods applied in physical anthropology. The age of the subadults was assessed according to the tooth development and eruption [36], and the age of the adult individuals was estimated on the basis of changes on the surface of the pubic symphysis and the degree of tooth wear [36,37]. Sex assessment of adults was based on morphological features of the skull and pelvis [36,37]. The taphonomic analysis was based on the current status of the skeletal remains and in-situ documentation [14]. For the details of the methods used in the anthropological analysis, including an assessment of stress markers and health status, see Supplementary materials B.

2.4. Ancient DNA analysis

2.4.1. Samples

Ancient DNA analyses were performed for all five individuals. The three skeletons excavated in 2013 (KZ1, KZ2 and KZ3) had been cut from the ground directly at the archaeological excavation site, and transferred to the Department of Human Evolutionary Biology, Faculty of Biology, Adam Mickiewicz University in Poznan (AMU) in a block of sediment. Bone samples for aDNA extraction were collected before washing the skeletons and prior to any anthropological analyses. Two other skeletons excavated in 1977 (KZ4 and KZ5) were stored in the Osteological Collection at AMU under the supervision of the Department of Human Evolutionary Biology, Institute of Anthropology.

All necessary precautions against DNA contamination were used during the collection of samples, including wearing face masks and gloves. For the adult skeletons (KZ1 and KZ4), two intact teeth, in a perfect state of preservation, were extracted directly from the mandible. For the three infant skeletons (KZ2, KZ3 and

KZ5), two deciduous teeth, a femur and two permanent teeth were collected, respectively. All the bone material was secured in sterile string bags and stored at −80 °C. Detailed information about the samples is presented in Supplementary Table S1.

2.4.2. Ancient DNA extraction

DNA extraction and library preparation was conducted in the sterile aDNA laboratory dedicated exclusively to aDNA studies, at the Faculty of Biology, AMU. The lab work was performed keeping all precaution procedures against modern DNA contamination, as previously described [38]. Prior to DNA extraction, the samples were cleaned with 5% NaOCl and rinsed with sterile water. After cleaning, the samples were UV irradiated for at least two hours per each side. Following UV irradiation, roots of teeth or fragments of bones were drilled with the use of Dremel® drill bits and ~250 mg of bone powder was collected to sterile tubes (2 ml). DNA was extracted using a silica-based method [39] modified by [40]. For each individual at least two independent DNA extracts were obtained.

2.4.3. Library preparation and illumina sequencing

Illumina shotgun sequencing was used to all samples excluding KZ5 because of a relatively poor preservation of aDNA. Twenty microliters of DNA extract was converted into a blunt-end Illumina library, according to the method presented by Meyer and Kircher [41], skipping the initial nebulization step, due to the fragmented nature of ancient DNA. Genomic libraries were enriched by setting up six PCR reactions for each library. Amplification was performed in 25 μl by mixing 3 μl of the DNA library template with 12.5 μl of 1 x AmpliTaq Gold® 360 Master Mix (Life Technologies), 0.5 μl of PCR primer IS4 (10 μM) and 0.5 μl of indexing primer (10 μM) [42]. Primer sequences used in the study are presented in Supplementary Tables S2–S4. One negative control reaction was included in each amplification. The thermocycling conditions were as follows: initial denaturation at 94 °C for 12 min, 12–16 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 10 min [42]. All six PCR reactions per individual were pooled and purified with AMPure® XP Reagents (Agencourt-Beckman Coulter) according to the manufacturer's protocol. The concentrations of the libraries and DNA fragment length distributions were established with the use of High Sensitivity D1000 Screen Tape assay on 2200 TapeStation system (Agilent). DNA libraries with different indexes were pooled in equimolar amounts and sequenced on Illumina's HiSeq2500 (125 bp pair end, each library on 1/10 lane) at the SNP & SEQ technology platform in Uppsala, Sweden.

2.4.4. Mitochondrial DNA enrichment by hybridization capture and ion Torrent PGM sequencing

Mitochondrial DNA capture was performed on KZ3 and KZ5 individuals with the use of commercially biotinylated probes for human mtDNA (MYbaits®), supplied by MYcroarray® (Ann Arbor, MI, USA; www.mycroarray.com). Prior to the hybridization, the DNA libraries (each ~100 ng) were concentrated to dryness using a Speedvak concentrator (Savant) and resuspended in 6.8 μl of ddH_2O. Two rounds of enrichment were conducted according to the manufacturer's protocol (version 2.3.1). Hybridization reactions were carried out at 60 °C for 24 h in a final volume of 30 μl. Captured targets were recovered with Dynabeads® MyOne Streptavidin C1 magnetic beads (Invitrogen), followed by bead-bait binding and washing according to the manufacturer's recommendations. There were two rounds of capture enrichment. After the first round, post captured amplification was carried out in five separate reactions per sample, each containing: 10 μl of 2x HiFi HotStart ReadyMix (KAPA), 1 μl of PCR primer IS5 (10 μM), 1 μl of PCR primer IS6 (10 μM), 2 μl of H_2O and 6 μl of bead-bound DNA library. The thermal profile used was: initial denaturation at 98 °C for 2 min, 12 cycles of 98 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 5 min. Post captured amplification, after the second round of enrichment, was carried out also in five separate reactions per sample, each containing: 10 μl of 2x HiFi HotStart ReadyMix (KAPA), 1 μl of PCR primer PISI (10 μM), 1 μl of PCR primer AIS4 (10 μM), 2 μl of H_2O and 6 μl of bead-bind DNA library. Primers PISI and AIS4 (presented in Supplementary Table S4) were designed in order to allow sequencing of blunt-end Illumina libraries on Ion Torrent PGM system. The thermocycling conditions were as above except for the primer annealing temperature (57 °C). After post capture amplification, five separate PCR reactions per sample were pooled and purified using MinElute spin columns (Qiagen), and eluted in 22 μl following manufacturer's protocol.

Enriched and indexed libraries were pooled in equimolar concentrations and adjusted to a final concentration of 20 pM for sequencing with the Ion Torrent Personal Genome Machine (Ion PGM) system (Ion Torrent, Thermo Fisher Scientific Inc.) at the Molecular Biology Techniques Laboratory, Faculty of Biology, AMU. Pooled libraries were subjected to emulsion PCR and enrichment with positive ion sphere particles (ISPs) using the Ion Torrent One Touch System II and the Ion One Touch 200 template kit v2 DL according to the manufacturer's recommendations. Sequencing was performed on the Ion 318™ Chip Kit v2 with the use of 520 flows and the Ion PGM Hi-Q sequencing kit v2.

2.4.5. SNP genotyping on Ion PGM

In order to trace possible kinship and test if any of the analyzed individuals were males we used the HID-Ion AmpliSeq™ Identity panel to genotype 90 autosomal SNPs and 34 upper Y clade SNPs. Multiplex PCR reactions were set up for KZ1, KZ4 and KZ5 samples, each containing: 4 μl of 5x Ion AmpliSeq™ HiFi mix, 10 μl of HID-Ion AmpliSeq™ Identity Panel, and 6 μl of aDNA extract. Three to four separate multiplex PCR reactions were set up per individual. Thermocycling conditions consisted of enzyme activation at 99 °C for 2 min, 21 cycles of 99 °C for 15 s, 60 °C for 4 min, 10 °C hold. Sequencing libraries were prepared with the use of Ion AmpliSeq™ Library kit 2.0 (Life Technologies) and barcoded with Ion Xpress™ Barcode adapters 1–16 (Life Technologies). Prior to the ligation of the barcoded adapters, partial digestion of primer sequences was conducted by adding 2 μl of FuPa Reagent (Life Technologies) following the manufacturer's instructions. Thermal profile for digestion was as follows: 50 °C for 10 min, 55 °C for 10 min, 60 °C for 20 min, 10 °C hold (up to one hour). Ligation of barcoded adapters to the amplicons was carried out according to the manufacturer's protocol and followed by purification using AMPure XP® Reagents (Agencourt-Beckman Coulter). The concentrations of the libraries were measured by quantitative PCR (qPCR) using the Ion Library Quantitation Kit (Life Technologies). Sequencing libraries were pooled in equimolar amounts and subjected to emulsion PCR and enrichment using the Ion Torrent One Touch System II and the Ion One Touch 200 template kit v2 DL following the manufacturer's instructions. Sequencing was performed on the Ion Torrent PGM using Ion 314™ Chip Kit v2 and the Ion PGM Hi-Q sequencing kit v2.

2.4.6. Bioinformatic analyses

Illumina's HiSeq2500 shotgun sequencing data was processed using a custom analytical pipeline [42]. Read pairs were merged, requiring an overlap of at least 11 bp and summing up base qualities, and the adapters were removed using MergeReadsFastQ_cc.py [41]. Merged reads were mapped as single-end reads against the human reference genome (UCSC Genome Browser hg19) using BWA aln version 0.7.8 [43] with the non-default parameters -l 16500 -n 0.01 -o 2 -t 2.

Ion Torrent PGM data from the mtDNA enriched libraries was processed using a pipeline adjusted specifically for those reads. The fastx_barcode_splitter.pl and fastx_trimmer scripts (http://hannonlab.cshl.edu/fastx_toolkit/) were used to demultiplex the reads by barcode, using a one mismatch threshold. Cutadapt v1.8.1 [44] was then used to trim adapters using a maximum error rate of 0.33 (−e 0.3333), and to remove short (−m 35), long (−M 110) and low-quality sequences (−q 20), for a total of five passes (−n 5). The filtered reads were checked with FastQC v 0.11.3 [45] before being mapped against the rCRS using TMAP v3.4.1 [46] with the following options: −g 3 -M 3 -n 7 -v stage1 –stage-keep-all map1–seed-length 12–seed-max-diff 4 stage2 map2–z-best 5 map3–max-seed-hits 10.

PCR duplicate reads with identical start and end coordinates, for both Illumina and PGM data, were collapsed into consensus sequences using FilterUniqueSAMCons.py [41]. Misincorporation patterns were assessed using mapDamage v2.0.5 [47].

The resulting sequence assembly was visualized using Biomatters IGV software v2.3.66 [48]. ANGSD v0.910 [49] was used to build the consensus sequence; only reads with mapping score of 30, a minimum base quality of 20 and a minimum coverage of three times were used. Due to the high DNA fragmentation of KZ5 we used additional criteria for determining the polymorphisms. G to A and C to T mutations were only kept if they were present in reads from both the positive and the negative strand. We also excluded C to T or G to A polymorphisms if they were situated at the read termini (in the last 5 bp).

Mitochondrial haplotypes were defined for each individual using HAPLOFIND [50], a web application focused on the mtDNA complete sequence annotation based on PhyloTree Build 17 phylogenetic tree [51]. The mutations reported as missing or unexpected were visually inspected in the original binary alignment map (BAM) files to double-check if they could be results of misincorporations in low coverage regions.

Sequence reads obtained through HID-Ion AmpliSeq™ Identity panel were mapped to the reference human genome (build 19) and used to create BAM files that were subsequently analyzed with the HID_SNP_Genotyper (v.4.2) plug-in with low stringency settings. We observed randomly distributed homopolymer sequencing errors typical for Ion Torrent sequencing system as mentioned by [52–54], however these errors did not cause false-positive base calls, since each base position was covered with enough depth. Allelic dropout (P) was estimated for each SNP locus following [55] using the formula: $P(\text{false homozygote}) = (K) \times (K/2)^{n-1}$, where K is the average of the observed fraction of false homozygotes and n is the number of replicates.

2.4.7. Kinship and molecular sex estimations

Maternal kinship estimations were performed using the mitochondrial genome dataset. We investigated relatedness within the grave cluster of KZ1, KZ2 and KZ3, and within the second grave cluster involving KZ4 and KZ5, and then between the individuals from both clusters.

Autosomal kin relations were analyzed based on SNPs recovered from the HID panel assay. The likelihood ratio (LR) was calculated for parent-child kinship scenario. Kin relations were tested between pairs of individuals: KZ4 vs. KZ5, KZ1vs. KZ4, and KZ1 vs. KZ5. We estimated LR as the probability of the parent–child scenario is true to the probability unrelated is true [p(scenario)/p(unrelated)]. In forensics, issuing paternity reports require LR values higher than 10^4 [56]. LR > 1 favors hypotheses which confirm tested pedigree scenario as probable. Relatedness coefficients and scenario probability computations given different combinations of genotypes were calculated by custom script written in Python 3.5 using the cited sources and equations [57]. The population frequencies for the SNP alleles used were taken from the 1000 Genome Project, genome assembly: GRCh37.p13 [58] reflecting the present-day European population. In the LR calculations we took into account the allelic drop-out estimations, and therefore we add a probability that an observed homozygote genotype is in reality a heterozygote genotype [P(false homozygote) Eq. (1)] (for details see Supplementary Table S5). This probability depended on the successful number of typings of the homozygote genotype.

For molecular sex determination based on sequence data (Illumina), we used the ratio of reads mapped to Y and X chromosomes (R_y) as described in [59]. The analysis was restricted to the sequence reads with mapping qualities of at least 30. To determine molecular sex, we also took into account the Y chromosomal genotype calls from HID panel assay.

3. Results

3.1. Dating and stable isotopes analyses

3.1.1. Absolute age of human remains

The results of ^{14}C-dating and carbon and nitrogen stable isotope analyses are presented in Table 1. There was no significant difference between the AMS ^{14}C-dates for the individuals KZ1 (mean 4226 ± 74 cal. BC) and KZ2 (mean 4211 ± 78 cal. BC) and t(1) = 0.0088496, p = 0.05. Hence, within the accuracy limits of the method it is plausible that the adjacent burials of KZ1 and KZ2 were contemporaneous. In the case of the individuals KZ4 and KZ5, we could not test the temporal distance between the times of their death, as only KZ4 was ^{14}C-dated. However, the result of the t-test indicates that there is no significant difference among the radiocarbon ages of KZ1, KZ2, and KZ4 individuals (mean 4162 ± 86 cal. BC); t(1) = 0.3818198, p = 0.05.

3.1.2. Potential aquatic radiocarbon reservoir effects

The end-point values for terrestrial diet, based to 100% on proteins of terrestrial wild and domesticated animals were

Table 1
Description of analyzed individuals.

Ind.	Feature	Sample	Age at death	Sex			mtDNA haplotype	Absolute age	δ^{13}C (‰)	δ^{15}N (‰)
				Morph.	Mol.	Arch.				
KZ1	6/2013	teeth	20–25	Female	Female	Female	U5b2a1a	5375 ± 35 BP, 4332–4066 cal. B.C. (95.4%)	−19	7
KZ2	7/2013	bone	2	n.d.	Female	Female	K1c	5370 ± 40 BP, 4331–4057 cal. B.C. (95.4%)	−18.4	12.1
KZ3	6/2013	teeth	0–0.6	n.d.	n.d.	n.d.	H3d	n.a.	n.a.	n.a.
KZ4	392	teeth	35–45	Female	Female	Female	K1a4	5330 ± 65 BP, 4329–3997 cal. B.C. (95.4%)	n.a	n.a.
KZ5	412	teeth	6	n.d.	Female	Female	n.d.	n.a.	n.a.	n.a.

Ind.–individual; n.d.–not detectable; n.a.–not analyzed.

calculated to a range from −20.9 to −18.6‰ for $\delta^{13}C$ and 7.7 to 11.8‰ for $\delta^{15}N$. In case of pure freshwater diet the end-point values were estimated to a span from −27.2 to −20.6 ($\delta^{13}C$), and from 7.3 to 16.1 ($\delta^{15}N$). The results received for the individuals KZ1 and KZ2 indicate no considerable input of freshwater food in their diets. In case of the individual KZ1 low $\delta^{15}N$ values (Table 1) point to a rather herbivorous diet and relatively small amounts of consumed animal proteins. We found a noticeable high $\delta^{15}N$ signal (12.1‰) in the bone collagen of the individual KZ2, which likely results from breastfeeding of this child. Hence, we can disclaim any significant offset of the radiocarbon ages of individuals KZ1 and KZ2 caused by consumption of aquatic foodstuffs.

3.2. Anthropology and taphonomy

The five examined skeletons seem to represent primary burials. In-situ documentation shows that the skeletons are relatively complete with only minor bone displacements.

The skeletons KZ1, KZ3, and KZ2, from the block of sediment, reflected an in-situ arrangement (Supplementary materials B). The KZ1 (female aged 20–25 years) and KZ3 (infant aged c. 6 months) individuals were buried in one pit. The woman was placed on her left side, with the right upper limb extended forward, and the left bent, and with legs flexed (Fig. 3). The infant was put on his/her right side with flexed legs. Bone arrangement indicates rather a simultaneous double burial, because the female skeleton seems undisturbed by placing the infant's remains. The infant was laid down on the female's left and right upper limbs at the level of her chest. The skeleton KZ2 from the adjacent grave belongs to a 2-year-old child, buried on her (see Sex determination Section) left side, with arms extended forward, and legs flexed. The original position of the other set of pit burials, skeleton KZ4 and KZ5 could be observed only on photographs and drawings (Supplementary materials B). KZ5, a girl (see Sex determination section) aged 6 years, was laid down in a similar position to KZ2. KZ4, a female who died at the age of 35–45 years, was placed on her left side, with arms and legs flexed. Both burial clusters can possibly contain close family members given the ages of the adults and children, and the burial positions.

3.3. Ancient DNA

3.3.1. Authenticity of the results

The mitochondrial sequences displayed the expected misincorporation patterns for endogenous ancient DNA with deaminated cytosine residues accumulated towards the end of the molecules. The average percentage of deamination rates at the first base were between 27.4% and 32.5% of 5′ end (C to T transition) and between 12.9% and 61.4% at the 3′ end (G to A transition) (Supplementary Fig. S1). No exogenous contaminations in the extraction blanks and negative controls that were set up during amplifications of DNA libraries were found.

3.3.2. Sex determination

Sex determination based on the ratio of reads mapping to X and Y chromosomes was successful in three out of four specimens sequenced on the Illumina high-throughput platform. We determined the molecular sex for the two adults (KZ1 and KZ4) and for one infant (KZ2) (Table 1; Supplementary Table S1). All estimated R_Y values were below 0.016 (0.0007 in KZ1, 0.0004 in KZ2 and 0.0005 in KZ4) showing that these individuals are females. We were unsuccessful in establishing molecular sex of the KZ3 infant due to its low preservation of DNA and low number of sequence reads mapping to X and Y chromosome. None of the Y-SNP loci was detected using the HID-Ion AmpliSeq™ Identity panel, as opposed to approximately 30–80% successful genotypings from the autosomal SNPs, which additionally supports that KZ1, KZ4 and KZ5 were females.

3.3.3. Mitochondrial genome data and maternal kinship

We successfully retrieved complete or nearly complete mtDNA genomes from all five analyzed individuals. DNA libraries sequenced on Illumina HiSeq2500 generated complete mitochondrial genomes for samples KZ1, KZ2, and KZ4 (Supplementary Table S1). For these three samples we obtained 99.7%–99.9% of the mitochondrial genome, with depth of coverage ranging from 10x to 37x. The two rounds of enrichment using in-solution mtDNA capture and IonTorrent sequencing for KZ3 yielded 37x depth of coverage and nearly complete mtDNA genome (99.98%). For the sample KZ5, we also carried out capture enrichment, retrieving 47.9% of the mitochondrial genome at an average of 6.4x coverage.

The consensus sequences of the mitochondrial genomes were used to assign haplotypes and KZ1, KZ2, KZ3 and KZ4 were established to belong to U5b2a1a, K1c, H3d and K1a4 respectively (Table 1). Due to the fragmented state of the mitochondrial genome, KZ5 could not be affiliated to a certain haplogroup. However, we could find 11 sites where KZ5 display a derived allele compared to rCRS. First we investigated the allele state at these 11 positions in the other samples. KZ1, KZ2, KZ3 displayed the ancestral allele at 4, 1 and 5 of these positions. The allele state of KZ4 only differed from KZ5 at nucleotide position 6643. Therefore, we further looked at the polymorphic positions seen in the other samples (the polymorphic positions in addition to the 11 polymorphic sites found in KZ5). We investigated for how many of these positions that KZ5 had enough coverage, and differed from the other samples. KZ5 displayed the ancestral state at 9, 1 and 5 of the derived positions seen in KZ1, KZ2 and KZ3. Thus, in all, KZ5 differs from KZ1, KZ2 and KZ3 at 13, 2 and 10 positions. No more different substitution states were found between KZ4 and KZ5. Detailed information on mitochondrial data involving SNPs against rCRS are included in Supplementary Table S1. Complete mitochondrial genomes were deposited in GenBank under accession numbers KX450777-KX450780.

3.3.4. Kinship determination based on SNPs from the HID-Ion AmpliSeq™ Identity panel

Y chromosomal and autosomal SNPs were genotyped using the HID-Ion AmpliSeq™ Identity panel for KZ1, KZ4 and KZ5. We retrieved different numbers of total SNP genotype calls from the individuals (for details see Appendix F, Supporting information). Among the 90 autosomal SNPs included in the panel we were able to obtain 73, 77 and 26 genotype calls for KZ1, KZ4 and KZ5, respectively. None of the Y chromosomal SNP were successfully genotyped for any of analyzed individuals.

We estimated allelic dropout from the genotype calls and the risk of not detecting a heterozygous genotype (P false homozygote). Each locus varied in a number of successful replicates, thus if a homozygote was typed once, the probability that it is truly a heterozygote was 0.21, whereas if we typed it 4 times, [P(false homozygote)] was 0.00024. The probabilities were calculated for each locus and they were further used in the LR estimations. For parent-child relations among KZ1 vs. KZ5 and KZ1 vs. KZ4, the obtained LR values were 0.0. In the case of KZ4 vs. KZ5, where only 24 SNPs loci were analyzed, the LR was 89.9 (Supplementary Table S7).

4. Discussion

4.1. Temporal distance between burials

Radiocarbon datings confirmed that burial KZ1 and KZ2 might be contemporaneous. Although the temporal distance between

individuals KZ1 and KZ3 was not determined by ^{14}C dating, the taphonomic observations indicate that these were primary burials. From the bone arrangement it appears that the individuals KZ1 and KZ3 were buried at the same time, as the child (KZ3) was put on the arm of the female (KZ1), and the position of the bones of the latter seems undisturbed. For the individuals KZ4 and KZ5 taphonomic data cannot yield information about the relative chronology of the burials, because the bodies were laid in separate burial pits. However, based on the stylistics and structure of the grave goods of individual KZ4 and KZ5 [12], it is plausible that they were buried more or less at the same time.

To exclude any offset of radiocarbon ages caused by consumption of marine or freshwater foodstuffs we made an attempt to reconstruct the diet of individuals KZ1 and KZ2. Because lakes and rivers contain dissolved ancient carbonates which are ^{14}C-free, consumption of freshwater organisms by humans can lead to spurious apparent ages and this is known as Freshwater Reservoir Effect or FRE [32,60]. Although, consumption of freshwater foodstuffs in Late Lengyel populations seemed plausible, our results do not show an offset of the radiocarbon ages caused by FRE for KZ1 and KZ2. We found mainly herbivorous diet of KZ1, since the isotopic values were close to the ones obtained for sheep/goat and pig from Racot [33]. Moreover, the most reasonable explanation of noticeable high $\delta^{15}N$ signal in the bone collagen of the individual KZ2 is breastfeeding, which leads to metabolic fractioning between the infant and mother/wet-nurse and elevated nitrogen isotope ratios [61].

4.2. Kinship relations

All of the analyzed specimens from Krusza Zamkowa represented different mitochondrial subhaplogroups (U5b2a1a, K1c, H3d, and K1a4). Although KZ5 only yielded half a mitochondrial genome, and consequently had no haplogroup affiliation, the ancestral state at 13, 2 and 10 nucleotide positions compared to KZ1, KZ2 and KZ3, still clearly indicate a different maternal origin for this individual. Due to the fragmented state of mtDNA of KZ5 we would not exclude maternal relation between KZ4 and KZ5. Although they differ at position 6643, we believe that more data is needed to fully resolve this issue. The inferred haplogroups have previously been found in LBK and post-LBK populations [62–65]. Based on the mitochondrial data we can thus exclude first degree relations such as mother-child or half-siblings (the same mother of each individual) among KZ1, KZ2, KZ3 and KZ4. The analyzed individuals were females and thus paternal relationship could not be investigated using Y chromosomal data. LR estimates based on autosomal SNPs confirmed the mitochondrial results by excluding a first degree relationship among KZ1 vs. KZ4 and KZ1 vs. KZ5 analyzed with this method. The LR values were higher than 1 (LR = 89.9) only in the case of KZ4 and KZ5 for a parent-child level. However, we retrieved only 24 SNPs which could be used to calculate LR, thus more data will be needed to fully resolve this issue.

We were not able to test other pedigree scenarios such as full-sibling or half-sibling, since at least 127 SNPs are needed to recover reliable results from full-sibling scenario and 491 SNP loci are required to conduct reliable second degree relative studies [66].

Notably, the lack of maternal kin relations between some of the individuals was unexpected, particularly with regard to the woman and the child from the double grave (KZ1 and KZ3) and the child from the adjacent grave (KZ2). According to [21] adult females and children buried in one burial pit, are very often termed as mothers and offspring. We presume that this inference is mostly viewed from the perspective of present-day culture as well as contemporary family models and funeral rituals, where relatives are usually buried together. Studies of a parent-child kinship of an adult and a child from one grave have brought so far inconclusive results for prehistoric burials. For instance, kinship was both, denied and confirmed in late Neolithic multiple burials from Eulau (Germany) [19]. The lack of maternal kinship among buried individuals was similar to the situation observed by [67] in early LBK population from Karsdorf (Germany), where no evidence for maternal kin relations was revealed among the individuals buried in close proximity in particular households (LBK settlement features). We do not exactly know what rules, other than biological kinship, may govern social divisions of people in a given society. Several alternative forms of non-biological relatedness have been discussed recently [15]. Presented concepts particularly concern social kinship which could be considered in a combination with biological relatedness [21] or associated with socioeconomic organizations and termed as e.g. practical or fictive kin relations [4,68]. For instance, Pilloud and Larsen [4] performed the studies of Neolithic Çatalhöyük archaeological site in Anatolia and assumed that households could have played a role as places of non-biologically related people grouped according to their “practical kin” connected to specific activities such as herding animals, planting crops or for social reasons involving inheritance of the lands and possessing properties. Concerning Krusza Zamkowa, we cannot exclude the presence of some specific social kinship, reflected in funeral practices. However, due to the small number of excavated human remains our inference on alternative forms of non-biological kin relations is currently limited.

4.3. Sex determination

Anthropological analyses revealed that KZ1, like KZ4, were likely females. The morphological sex of the children (KZ2, KZ3 and KZ5) could not be estimated as there are no acceptable standards for determining sex of subadults on the basis of the skeletal features [36]. The Illumina sequencing data obtained for KZ1, KZ2, KZ3 and KZ4 as well as the absence of any successful Y chromosome genotypes from the HID-Ion AmpliSeq™ Identity Panel (for KZ1, KZ4 and KZ5) confirmed that the two adults (KZ1 and KZ4) were females and further reveal that two of the children (KZ2 and KZ5) also belong to the female sex. Unfortunately, there was not enough data to assign the biological sex of the KZ3 child. Sex assignments of all analyzed individuals were in accordance with archaeological estimations based on the artifacts found in the burial pits. The grave goods included bracelets and belts consisting of beads made of shells, pauldrons made of animal bones, and other adornments being rather female attributes [12]. Moreover, we found that all these females were buried on their left side with heads directed toward the south. This is in agreement with previous findings that females and males from Late Lengyel populations were buried in a different manner, with females buried as described above [10,11]. Only the KZ3 infant was buried on his/her right side, however, the sex of this child could not be determined due to poor preservation of DNA.

5. Conclusions

We show that, contrary to intuitive interpretation, in post-LBK Late Lengyel individuals buried together in one grave or in graves with close proximity to each other shared no first degree kinship. Our data show that HID-Ion AmpliSeq™ Identity panel is rather inefficient in the case of samples with poorly preserved DNA, however more ancient samples need to be tested to fully resolve this issue. We also point to the importance of interdisciplinary approach, involving molecular, anthropological and archaeological investigations to infer about kinship and burial rites in past populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2016.10.008.

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