Analysis of ancient human mitochondrial DNA from Verteba Cave, Ukraine: insights into the Late Neolithic-Chalcolithic Cucuteni–Tripolye culture

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Abstract Verteba Cave (VC) in western Ukraine dates to the Eneolithic period (c. 5500 YBP), and contains the largest collection yet found of human skeletal remains associated with the Cucuteni–Tripolye culture. The subsistence economy of this people was based on agropastoralism, and included some of the largest and densest Middle Neolithic settlement sites in all of Europe. To understand further the evolutionary history of the Tripolye people, we examined population genetics patterns in mitochondrial DNA from ancient human remains excavated from VC chambers. From five commingled and secondary burial sites within the cave, we obtained 368 bp mtDNA HVR1 sequences from 22 individuals assignable to eight haplogroups: H (three haplotypes), HV (two haplotypes), W, K, and T. Overall nucleotide diversity is low ($\pi = 0.00621$). The two largest samples, from Chamber G3 and Site 7, were significantly differentiated with respect to haplotype composition: G3 ($n = 8$) is dominated by haplotype W ($\pi = 0$), whereas Site 7 ($n = 15$) is dominated by H haplotypes ($\pi = 0.00439$). Tajima’s $D$ as an indication of population expansion was not significantly negative for the complete sample ($D = –1.37$) or for sites G3 ($D = –0.973$) and 7 ($D = –1.35$), which were analyzed separately. Individuals from the Tripolye culture buried at VC c. 5500 YBP had predominantly haplogroup H and related haplotypes. This contrasts with predominantly haplogroup U individuals in preEneolithic peoples from the same area, which suggests lack of genetic continuity in a site that has been dated to the Mesolithic. Peoples of the Tripolye culture are more closely related to other early European farmers than to Mesolithic hunter-gatherers and/or pre-Eneolithic cultures.

Key words: mtDNA, ancient DNA, Tripolye, Eneolithic, Ukraine

Introduction

Profound cultural transitions accompanied the Neolithic and Bronze Age in Europe. A continuing question of debate among researchers is how migrations from West Asia or the Pontic-Caspian steppe affected the genetic composition of modern-day Europe (Haak et al., 2010; Bollongino et al., 2013; Allentoft et al., 2015; Brandt et al., 2015; Jones et al., 2015; Mathieson et al., 2015). A key question in this debate posits whether these cultural changes were the results of movements of people (demic diffusion model), or the movement of ideas and artifacts (cultural diffusion model) (Ammerman and Cavalli-Sforza, 1984). That is, were these large-scale migrations a process of cultural diffusion, with little or no genetic admixture among early Neolithic farmers and Mesolithic hunter-gatherers? Or, is a model of demic diffusion (Ammerman and Cavalli-Sforza, 1984; Cavalli-Sforza, 2000; Pinhasi and von Cramon-Taubadel, 2009; Haber et al., 2016) more appropriate, whereby different regions of Europe were more or less affected by admixture with early farmers, and later steppe herders during the Bronze Age?

The transition to farming from a foraging lifestyle first appeared in the Near East c. 10500 years before present (YBP) in modern-day southeastern Anatolia and Syria. Archaeologists have described two major and contemporane-
ous routes of expansion, namely the Continental (Danubian) and Mediterranean routes. By 9500 YBP, farming spread into parts of Central Europe through the migration of peoples associated with the Linear Pottery culture (or Linear-bandkeramik, LBK). These LBK cultures originated in Hungary and Slovakia (the Carpathian Basin) and then spread rapidly as far as the Paris Basin and Ukraine. A lingering question among archaeologists has always been whether these first farmers were descendants of local hunter-gatherers or whether they migrated from the Near East. Paleogenetic studies have generally suggested these early farmers were migrants, though in some places peoples continued to admix after the adoption of agriculture (Sampietro et al., 2007; Lacan et al., 2011a, b; Hervella et al., 2012; Brandt et al., 2015).

Southeastern Europe (SE Europe) has not been as extensively investigated as southwestern Europe, Central Europe, or southern Scandinavia, in terms of their ancient DNA variation. In contrast to Central Europe, the area of what is modern Ukraine saw the adoption of agriculture late. Although features of the Neolithic package are visible in Ukraine as early as 8500–7500 YBP [we use calBP (calibrated before present) to indicate radiocarbon dating], agriculture was not adopted as a primary subsistence economy until the Eneolithic or Chalcolithic period (c. 6500 YBP) (Zvelebil and Dolukhanov, 1991). Whereas Central Europe saw mostly demic diffusion (Pinhasi and von Cramon-Taubadel, 2009), SE Europe seems to have adopted agriculture through innovative subsistence strategies as a result of transfer of ideas, with little genetic influence and genetic continuity from the Mesolithic to the Neolithic (Richards et al., 2002). Therefore, the Neolithic transition occurred at a slower pace, thus perhaps shaping the genetic composition of this region differently than in other parts of Europe.

Following the establishment of farming communities in the Balkan Peninsula, a series of complex societies formed, culminating in large settlements. By 6500 YBP, agriculture had reached Eastern Europe, in the form of the Cucuteni–Trypillian (C-T) complex in the area of present-day Moldova, Romania, and Ukraine. This culture spanned close to 2000 years and influenced much of SE Europe and the Baltic regions. It is known for elaborate anthropomorphic and animal figurines, as well as distinct, elegantly painted pottery. Around 5000 YBP, these societies began to change, with the large settlements being abandoned, and archaeological evidence suggesting contact with nomadic steppe populations from the East.

We address the complex process of Neolithisation (Ammerman and Cavalli-Sforza, 1984) in SE Europe by examining an Eneolithic (Chalcolithic) Cucuteni–Tripolye site from Ukraine. For brevity, we will use the term Tripolye, as it is known in Ukraine. Tripolye culture (7100–5000 calBP) is defined as Eneolithic based on the presence of copper artifacts and the onset of metallurgy, and ends at the beginning of the Bronze Age (Ledogar et al., 2019). The Tripolye culture occupied a large area from the Carpathian Mountains in the west to the Dnieper River in the east, and extended as far south as the Black Sea and north to Kiev (Figure 1). Relative and absolute chronologies divide the Tripolye culture into several phases (Table 1), which normally accompany changes in pottery manufacture and decoration (Ryzhov, 2012). The people associated with this culture are known as Trypillians.

In the present study, we investigate human remains found at a single Tripolye site known as Verteba Cave (VC), with human and faunal remains dating to the Eneolithic (Ryzhov,
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2012; Kadrow and Pokutta, 2016). VC has been excavated as an archaeological site since the 1820s, though more intensive excavation has been ongoing since 1996 under the direction of coauthor M. Sokhatsky (Borschiv Regional Museum of the Ukrainian Ministry of Culture and Arts). Verteba is a gypsum cave located in western Ukraine, in the boreal forest-steppe zone of the East European Plain. It is one of many vast underground cave systems in the region.

Interpretations surrounding the use of the cave during these periods vary. Some believe the cave was used as a temporary shelter, while increasing archaeological evidence suggests use as a ritual site or a mortuary function (Kadrow and Pokutta, 2016; Ledogar, 2017). There is also evidence to support the idea that individuals buried in the cave, which are largely secondary in nature, are victims of warfare or sacrifice, due to the high frequency of blunt force trauma (Anthony, 2007; Madden et al., 2018). The cave contains the largest accumulation of human remains associated with the Tripolye culture found to date. Very few Tripolye culture human remains exist, making the cave one of the most important sites for the investigation of the diet, health, pathology, and population history of Trypillian peoples (Karsten et al., 2014, 2015).

Using data obtained from the mitochondrial HVR-I region, we ask several interrelated questions about individuals buried at VC. First, is there evidence for a maternal genetic continuity, and thus a degree of cultural diffusion, with local Mesolithic hunter-gatherers, as suggested in several recent studies? If there is no evidence for this, how are these individuals related to earlier farmer groups from SE or Central Europe? Is there any indication of a steppe influence for the Trypillian peoples? And lastly, can we infer something about the collapse of the Trypillian people from maternal lineages? Several studies (Nikitin, 2011; Nikitin et al., 2010, 2017) have briefly addressed two of these three questions, indicating a link to early Neolithic farmers with some possibility of a link with Mesolithic hunter-gatherers. However, in those studies, the sample sizes tended to be smaller and the human remains used came from only a single chamber. Here, we analyze mitochondrial (mt) DNA data from several chambers in different locations found throughout the cave in an attempt to answer these questions.

### Materials and Methods

#### Samples

Human remains for DNA analyses come from several excavation sites located within VC. VC is a mortuary site located outside the modern village of Bilche Zolote, Ternopil Oblast, Ukraine (Figure 1). Most samples date to the Tripolye CII period (c. 5500 YBP) based exclusively on associated pottery found in the same cultural layer as the human remains. Nikitin et al. (2010, 2017) and Ledogar et al. (2019) radiocarbon dated human and animal remains, as well as pottery sherds from Verteba, and found that the dates correspond to transitional phases in pottery decoration, with peak activity placed c. 5500 calBP; although some remains date to before or after this date. All skeletal samples excavated within VC are commingled and individual burials are difficult to identify. Therefore, in order to avoid sequencing the same individual twice, one of us (J.K.) collected second right metacarpal bones for analysis from a single chamber (Site 7). These samples were collected over several excavation field seasons (2008–2014) by J.K. The remains were initially kept at the University of Wisconsin-Oshkosh, and later transferred to Kitasato University for processing. R.W.S. then collected bone and teeth samples on site and in situ using sterile sampling methods (wearing coveralls, gloves, and facemask) from four additional chambers (20, G1, G2, G3) during field seasons 2015–2016. We attempted to collect samples from these other chambers that did not overlap elements from a single individual and we are confident this is the case for sites 20, G1, and G2 as samples were collected from different levels and from different parts of the site; however, site G3 had several skeletal elements within a small chamber and thus these samples could derive from a single individual or only a few individuals—though they may likely be members of the same family. Samples from sites 20, G1, G2, and G3 were deposited into sterile bags on site and are now stored at the University of Vienna, Vienna, Austria under the curation of R. Pinhasi. A total of 63 skeletal elements were analyzed for this study.

#### Ancient DNA extraction

Prior to extraction, samples were wiped with a 10% bleach solution, rinsed in DNA-free water, and UV irradiated in a cross-linker for 30 minutes on each side before drying. DNA extraction was carried out with ~100–300 mg of bone powder using a modified silica-column-based protocol (Yang et al., 1998). First, the surface of the bone was cleaned using a diamond drill bit at low speed. Then, we either used the drill bit to obtain powder, or powdered the bone in a mixer mill (ShakeMaster Auto v. 2.0; BioMedical Science Inc.). For samples VC001–VC035 and VC046–VC063, we used the following protocol: bone powder was incubated for 24 h at 55°C followed by 24 h at 37°C in 2 ml tubes with 1 ml of lysis buffer in final concentrations of Tris–HCl (pH 7.4), 20 mM; 0.7% Sarkosyl NL; 0.5 M EDTA (pH 8.0), 47.5 mM; 0.65 U/ml Proteinase K; with shaking at 300 rpm in a Thermomixer (Thermomixer Comfort Eppendorf). Samples were then centrifuged at 13000 rpm for 10 min and the supernatant was removed. Fresh lysis buffer (1 ml) was then added to the pellet, vortexed, and the incubation and centrifugation steps were repeated.

The second supernatant was then transferred to an Amicon Ultra-4 Centrifugal Filter Unit 30K (Merck), diluted with 3 ml of TE, and centrifuged at ~2500 rpm until a final concentration of ~100 μl was obtained. This volume was then transferred to a silica column (MinElute PCR Purifica-

### Table 1. Chronology for Cucuteni-Tripolye period

| Phase | Date          |
|-------|---------------|
| A     | 5100/5000–4700/4600 calBP |
| BI    | 4700/4600–4400/4300 calBP |
| BI/II | 4400/4300–4200/4100 calBP |
| BII   | 4200/4100–3900 calBP |
| CI    | 3900–3450/3350 calBP |
| CII   | 3450/3300–3000/2900 calBP |
tion Kit, Qiagen) and purified according to manufacturer’s instructions, except at the final step adding TWEEN 20 (at 0.05% final concentration) to 60 μl EB buffer preheated to 60°C.

For samples VC036–VC044, our second protocol followed the first with the following modifications based on a ‘predigestion’ step recommended in Gamba et al. (2016). This protocol was used in an effort to increase DNA yield from degraded samples. It has been shown that a predigestion step generally improves endogenous DNA from degraded archaeological bone (Damgaard et al., 2015). Fresh lysis buffer was added to the powder and incubated in a Thermomixer for 1 h at 56°C, shaking at 1200 rpm. After 1 h, the sample was centrifuged for 2 min at 13000 rpm and the supernatant was discarded. Fresh lysis buffer was then added to the pellet and incubated at 56°C for 1 h followed by 37°C overnight, shaking at 1200 rpm. After ultrafiltration in an Amicon Ultra-4 Centrifugal Filter Unit 30K, the sample was transferred to a silica column (MiniElute PCR Purification Kit) with the following modifications: after adding the PB buffer, the centrifugation speed was reduced to 8000 rpm and the elution step included a 5 min incubation at room temperature.

Contamination controls
To control for contamination, all pre-PCR procedures were conducted in a controlled-access, positive-pressure laboratory with HEPA-filtered air that is used exclusively for ancient DNA analysis at Kitasato University. Disposable protective clothing was worn during all sampling and extraction procedures. Pipettes with aerosol-resistant tips were used. The lab is cleaned prior to all procedures with DNA-Off (Takara, Japan), and exposed to UV irradiation for at least 2 hours after each procedure. All PCR reactions and post-PCR procedures were performed in a separate laboratory. The movement of laboratory materials and personnel was always unidirectional, from the ancient to modern facilities. The mtDNA of all researchers with access to the clean lab was transferred to a silica column (MiniElute PCR Purification Kit) with the following modifications: after adding the PB buffer, the centrifugation speed was reduced to 8000 rpm and the elution step included a 5 min incubation at room temperature.

PCR amplification and direct sequencing
The hypervariable region 1 (HVR-I) of the mitochondrial D-loop (368 bp, nucleotide positions 15999–16366) was amplified with three sets of overlapping primers (Adachi et al., 2004, 2013, 2014) (Table 1). This region also includes part of the tRNAPro gene, although none of our samples exhibited mutations in this region, and thus this did not affect our results. PCR amplification was carried out using 2 μl extract in a 50 μl reaction mixture containing Ex Taq Hot Start (TaKaRa, Japan), 10× Ex Taq buffer (containing MgCl2), 2.5 mM dNTPs, and 10 μM of each primer. PCR conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 55°C and 61.5°C for Primer Set 1 and Primer Sets 2/3, respectively, annealing and extension temperature for 5 min 30 sec, and a hold at 4°C. PCR products were visualized on 2% agarose gel containing ethidium bromide, and purified using the MiniElute PCR Purification Kit. Amplification products were sequenced using Applied Biosystems Big Dye protocols and analysed on an Applied Biosystems 3130 Genetic Analyzer at Kitasato University.

Data analysis
Sequences were visually inspected, corrected, and compared against the revised Cambridge Reference Sequence (rCRS; Andrews et al., 1999) using DNASTAR Lasergene software (SeqMan Pro). A maximum-parsimonious phylogenetic network was constructed manually. HVR-I haplotypes were classified into possible haplogroups and sub-haplogroups using MITOMAP (Lott et al., 2013). Although we understand that the resolution of haplotype motifs could be resolved using HVR-II, most of the haplotypes defined in this study would not be improved with the analysis of HVR-II as our intention is to define broader haplogroups to compare with Mesolithic and Neolithic peoples, and therefore our results are generally unaffected by not including data from HVR-II. We generated population genetic statistics, number of sequence types, number of polymorphic sites, nucleotide diversity (π), and Tajima’s D using the software DnaSP 5 (Librado and Rozas, 2009).

Results
We began with 63 specimens (53 bones and 10 teeth) from VC (Figure 1), from which we made 156 DNA extractions. Of these, we were successful in PCR amplification of 38 specimens with three overlapping primer sets each (Table 2). We sequenced all 114 amplicons using the Sanger method on an ABI 3130 Genetic Analyzer, and obtained contigs based on all three amplicon sequences from 34 of the 38 specimens. We removed 6 specimens where the amplicon sequences were mutually incompatible, so as to produce a final set of 28 specimens with consensus sequences over a

| Primer set | Primer   | Primer sequence                  | Nucleotide position | Product size (bp) |
|------------|----------|----------------------------------|---------------------|-------------------|
| 1          | L15998   | 5’-CCATTAGCACCACCAAGCTA-3’       | 15980–16161         | 182               |
|            | H16142   | 5’-ATGTACTACAGGTGGTCAG-3’        |                     |                   |
| 2          | L16120   | 5’-TTACTGCGAGCCACCATGAA-3’       | 16101–16258         | 158               |
|            | H16239   | 5’-TGCGTTTGAGGACTGCATTG-3’       |                     |                   |
| 3          | L16208   | 5’-CCCCATGCCTCAAAGCAG-3’         | 16190–16384         | 195               |
|            | H16367   | 5’-CTGAGGGGGGTCATCATC-3’         |                     |                   |
Eight distinct HVR-1 sequences were found in the 28 specimens. We also include the sequence types for two of us (K.W. and R.S.) who worked on DNA extraction. K.W. and R.S. sequence types were not identical to the eight sequence types, except for Seq type II from one bone specimen that could not eliminate the possibility that it is contamination (Table 4). This sequence assemblage, the possibility exists that only 22 individuals are present in our dataset. This reasoning is based on the following: we know that all of the samples from Site 7 are different individuals (each is represented by a single right second metacarpal bone, \( n = 15 \)); the samples from the ‘undefined chamber’ were also two right metacarpal bones \( (n = 2) \); samples from sites G2 and 20 derive from different layers and are not in close approximation to each other, i.e. sites are spatially separated \( (n = 3) \); and site G3 contains different skeletal elements that may come from a single individual, though we observe two different haplotypes, so it is possible that \( n = 2 \) rather than \( n = 8 \). This gives us a minimum of 22 individuals in our dataset for population genetic analyses.

We tested for nucleotide diversity (\( \pi \)) and Tajima’s \( D \) (Table 4). A significantly negative Tajima’s \( D \) would suggest that a population has experienced a demographic expansion.
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Table 4. Genetic diversity statistics for Verteba Cave

| Name of population   | Size | Number of sequence types | Number of segregating sites (S) | Nucleotide diversity (π) | Tajima’s D |
|----------------------|------|--------------------------|---------------------------------|-------------------------|------------|
| Verteba Cave         | 22   | 7                        | 14                              | 0.00621                 | -1.36690   |
| Site 7               | 14   | 4                        | 8                               | 0.00439                 | -1.34944   |
| Chamber G3           | 5    | 2                        | 2                               | 0.00217                 | -0.97256   |
| Haplogroup W of Site G3 | 4  | 1                        | 0                               | 0                       | 0          |

(Tajima, 1989); otherwise, there is no evidence of departure from constant population size. Tajima’s D values of VC specimens were negative (−1.36690) but not significantly different from zero (P = 0.078). Several values for nucleotide diversity were inferred for the different Seq types (apart from Seq Type II) (Table 4) and show relatively low diversity, although the sample sizes for each are small.

Discussion

In this study we have attempted to answer a number of related questions surrounding Trypillian population history using data gleaned from maternal ancestry. We include a minimum of 22 individuals buried at VC, an Eneolithic site associated with the Tripolye culture. Although the data are limited, our findings may offer some insight into aspects of Trypillian people’s genetic affiliation with other Neolithic groups.

To address the complexities associated with the transition to farming (or the process of Neolithisation), a wealth of ancient mtDNA data has been amassed from the Late Mesolithic to the Late Bronze Age (Brandt et al., 2015; Haak et al., 2015). To explain these genetic changes in various regions of Europe, it is important to fully understand the genetic substratum spanning the Mesolithic–Neolithic transition. Studies have suggested that the maternal signature of local hunter-gatherer groups in many parts of Europe is homogeneous, with a relatively small population size and haplogroups dominated by lineage haplogroup U, such as U2, U4, U5a, U5b, and U8 (Haak et al., 2015; Mathieson et al., 2015, 2018). In contrast, Neolithic mtDNA arrived in Central Europe c. 8000 years BP along with cultures associated with LBK farmers, comprising largely haplogroups N1a, T2, K, J, HV, V, W, and H. These lineages replaced local signatures of haplogroup U from peoples associated with Mesolithic cultures, over large areas of Central Europe.

We first explored whether individuals buried at VC are more closely related to earlier Neolithic farmers from Central Europe, or perhaps have some connection with local hunter-gatherers, thus emphasizing the role of cultural diffusion in the adoption of agriculture. mtDNA haplogroup data for modern and ancient populations in Europe and West Asia have shown there was a discontinuity between late hunter-gatherers and early farmers, and later extant European populations, in most locations throughout Europe. An exception has been in SE Europe and the Baltic where cultural diffusion may have played a larger role (Jones et al., 2017).

The VC haplotype distribution indicates common haplotypes among Eurasian populations (Richards et al., 2002). These include haplogroups H, T, K, and W. The majority of haplotypes occur in haplogroup H, which is the most common haplogroup among modern-day Europeans and peoples of the West Asia, accounting for around 40% in Europeans (Van Oven and Kayser, 2009), including approximately 44% of modern Ukrainians (Malyarchuk and Derenko, 2001). This suggests a possible continuity between the Tripolye and modern Ukrainians. The VC haplotypes included one T2b individual (Table 3), which is a possible marker of Anatolian expansion (Brandt et al., 2015) that has also been found at high frequency in the Carpathian Mountains (Nikitin et al., 2009). In Nikitin et al. (2009), an individual from Bilche Zolote was found to have haplogroup T2b. Bilche Zolote is only 3 km from the VC site, indicating some degree of local continuity with the Trypillian people.

Previous ancient DNA studies showed that hunter-gatherers before 6500 YBP in Europe commonly had haplogroups U, U4, U5, and H, whereas hunter-gatherers after 6500 YBP in Europe had a lower frequency of haplogroup H than before (Brandt et al., 2015). Haplogroups T and K appeared in hunter-gatherers only after 6500 YBP, indicating a degree of admixture in some places between farmers and hunter-gatherers. Farmers before and after 6500 YBP in Europe had haplogroups W, HV*, H, T, K, and these are also found in individuals buried at VC (Deguilloux and Mendisco, 2013). Therefore, our data point to a common ancestry with early European farmers.

Our data may suggest a degree of population replacement from the Mesolithic to the Neolithic. Mathieson et al. (2018) analyzed a number of Neolithic Ukrainian samples (petrous bone) from several sites in southern, northern, and western Ukraine, dating to c. 8500–6000 YBP, and found exclusively U (U4 and U5) mtDNA lineages. It should be noted that ‘Neolithic’ in this context does not mean the adoption of agriculture, but is simply coincident with a change in material culture. They also analyzed several Trypillian individuals from VC (different samples than those included in this study), among whom they found a wider diversity of mtDNA lineages, including H5a, HV, and T2b. One individual (I3151) had haplotype U8b1b. This finding is similar to an earlier study by Nikitin et al. (2017), who analyzed the same samples for only mtDNA variation. Although found as early as the Paleolithic, haplotype U8b1 has also been discovered in Neolithic Anatolian farmers (Mathieson et al., 2015). In the analysis by Mathieson et al. (2018), the individual with U8b1b showed little evidence that their genome-wide variation was more similar to earlier Neolithic or Mesolithic groups, displaying a mixture of mostly Balkans Neolithic with some contribution from western hunter-gatherers, Ukraine Neolithic, and steppe Yamnaya. In fact, the Trypillians in Mathieson et al. (2018) had up to 80% Neolithic
Anatomol ancestry. Based on these data, combined with our preliminary results, it appears the Trypillians were very much a distinct people with ancestral roots tied to early Neolithic groups from Anatolia.

Haplogroup W was also observed in several specimens deriving from Site G3 (Table 3). Although we are unsure if all of these haplogroups come from a single or multiple individuals, this observation is interesting in that it is relatively rare and isolated among Neolithic samples. It has, however, been found in samples dating to the Bronze Age (Wilde et al., 2014). Wilde et al. (2014) found haplogroup W present in two samples from the Early Bronze Age associated with the Yamnaya and Usatovo cultures. The Usatovo culture (c. 3500–2500 BC) was found in Romania, Moldova, and southern Ukraine. It was a conglomeration of Tripolye and North Pontic steppe cultures. Therefore, this individual could link the Trypillian peoples to the Usatovo peoples and perhaps to the greater Yamnaya steppe migrations during the Bronze Age that lead to the Corded Ware culture (Kristiansen et al., 2017).

VC contains archaeological evidence for the Tripolye cultural complex, including implements for agrarian cultivation, including grain processing. Based on the material culture, the immensity of certain settlements (some housing up to 10000 people; Müller et al., 2016), as well as the deterioration in biological health resulting from grain consumption, it is clear the subsistence economy of the Trypillians was based on agriculture. Previous studies report that modern hunter-gatherers do not indicate a signal of demographic expansion in mismatch distribution and/or Tajima’s D test, but farmers tend to show expansion based on increasing numbers of individuals living in sedentary conditions (Watson et al., 1996; Oota et al., 2002). To investigate overall demography in VC, we examined population genetic statistics. There was no evidence that the Trypillian people (at least their maternal lineages) experienced any demographic expansion, at least during the Late Neolithic (Table 4); although we find a negative value for Tajima’s D when analyzing all of the sampled individuals (n = 22), it is not significant.

However, we are unable to treat our sample population as being deposited at or around the same time period. Although most individuals included in this study come from Site 7, which has been firmly established to date to c. 5500 calBP based on ceramics and radiocarbon dating (Nikitin et al., 2010; Ledogar et al., 2019), other chambers in the cave are not as confidently dated. For example, Chamber G3 had very few artifacts from the Tripolye culture. Given this caveat, we analyzed separately individuals buried in Site 7 (n = 15) and found a negative Tajima’s D that was not significant (−1.34944; P = 0.085).

An explanation for our observed population size stability as seen in Tajima’s D might be due to sampling strategy and the temporal component of ancient DNA sites. If a population migrates in low numbers into a new environment, such as the case with early migrating farmers from Anatolia, we would slowly see an increase in the population as they become increasingly sedentary over time. If we were to sample from this site and test for demographic expansion, we would most likely see that reflected in a statistic such as Tajima’s D. As the population increases and resources reach an upper limit, groups would begin to split and settle into new locations in close geographic proximity. Sampling individuals from each of these new sites, we would expect to see the maintenance of population stability since the groups, though genetically related, are spread out and thus would maintain population equilibrium with bidirectional migration. If, at some point in time, these sites again become aggregated because of increased population size, and we were to sample from this new, larger archaeological site, we would again witness demographic expansion simply because the overall population size has increased. Therefore, farmer populations as a whole (over the course of the Neolithic) generally see a trend for increased population expansion as local villages turn into larger settlements (as could be the case for peak occupation at VC, where nearby settlements tended to be large). However, if we sample from each of those localities over time, as perhaps we are seeing with our results when we include samples from all sites (chambers), then we do not see demographic expansion, but rather maintenance of population size over time.

Archaeologically, it has been documented that Tripolye settlements began to disappear at the beginning of the Bronze Age. The reasons for this vary, but could be influenced by their interaction with steppe groups from the east. Of the possibilities for settlement abandonment is warfare. It has been well documented at VC that interpersonal violence was a common phenomena (Anthony, 2007; Madden et al., 2018). Madden et al. (2018) found a high degree of trauma-related cranial injuries among Trypillian burials. It is believed these individuals were killed by an outside raiding group and were later buried by members of the Tripolye culture.

The Trypillians were one of the last of the ‘Old Europe’ cultures that lived along the shores of the Danube River. By 5800 calBP, many of these Neolithic Danubian cultures were wiped out after the arrival of pastoralists from the steppe (Anthony, 2007). It is believed that by 5300 calBP, the Trypillians were in conflict with members of the Usatovo culture to the south, no longer benefiting from trade relationships across the forest–steppe boundaries.

Another explanation for the sudden collapse of Tripolye culture may be an early form of plague, recently documented, that was widespread from Siberia to the Baltic at c. 5000 YBP (Rasmussen et al., 2015; Andrades Valtueña et al., 2017; Rascovan et al., 2019). Neolithic communities contracting this early form of the plague would have devastated Tripolye mega-sites, thus creating a demographic collapse that we are only glimpsing in our population genetic analyses. To get a better understanding of the demographic collapse in Tripolye society, we will need to obtain genome-wide data to further explore how these early agropastoralists eventually declined or were replaced by steppe nomads from the east.

Conclusions

In this study, we have shown that mtDNA diversity during the Eneolithic at the VC site is closely related to early European farmers and that the represented haplogroups are qual-
itatively different from the mtDNA haplotypes found during the Mesolithic and Early Neolithic at other Ukrainian sites. Although based on a single locus, this may suggest some population transition by newly migrating farmers who replaced or, in some cases, admixed with local groups during the Mesolithic or Early Neolithic. Archaeologically, we also know that the Trypillians seemed to have disappeared at the beginning of the Bronze Age. Although we do not know the exact cause of the abandonment of Trypillian sites, several scenarios could account for this, including conflict with steppe groups to the south and east, or the spread of disease in the form of plague. Additional material will be needed to understand genome-wide variation of the Trypillians and how nuclear diversity changed during the rise and fall of the Cucuteni–Tripolye culture.

Declarations

Ethics approval and consent to participate
The human skeletal material used in this study comes from an archaeological site that is dated to 5500 YBP. Additionally, all material was used with permission from the Director of the Borshchiv Regional Museum, M. Sokhatsky, the co-author of this paper.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request. Nucleotide sequences have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC336799–LC336806.

Competing interests
The authors declare that they have no competing interests.

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Author’s contributions
H.O., M.O., and R.W.S. designed the study. K.W., R.W.S., and T.G. performed laboratory analysis. K.W., D.W. and K.K. performed data analysis. M.S. provided samples. R.W.S., J.K. and H.O. wrote the paper along with input from all authors.

References
Adachi N., Umetsu K., Takigawa W., and Sakaue K. (2004) Phylogenetic analysis of the human ancient mitochondrial DNA. Journal of Archaeological Science, 31: 1339–1348.
Adachi N., Sawada J., Yoneda M., Kobayashi K., and Itoh S. (2013) Mitochondrial DNA analysis of the initial Jomon phase excavated at the Yugura cave site, Nagano, Japan. Anthropological Science, 121: 137–143.
Adachi N., Umetsu K., and Shojo H. (2014) Forensic strategy to ensure the quality of sequencing of mitochondrial DNA in highly degraded samples. Legal Medicine, 16: 52–55.
Allentoft M.E., Sikora M., Sjögren K.G., Rasmussen S., Rasmussen M., Stenderup J., Damgaard P.B., Schroeder H., Ahlström T., Vinner L., Malaspina A.S., Margaryan A., Hāgham T., Chivall D., Lymnertu N., Harvig L., Baron J., Della Casa P., Daubrowski P., Duffy P.R., Ebel A.V., Epmihakoh A., Frei K., Furnaramek M., Gnäk T., Gromov A., Gronkiewicz S., Grue G., Hajdu T., Jarysz R., Khartanovich V., Khothklov A., Kiss V., Kolàr J., Kriska A., Lasak I., Longhi M., McClellan G., Merkevicius A., Merklyte I., Metspalu M., Mkrtchyan R., Moiseyev V., Paja L., Pálido G., Pokutta D., Pospieszynski L., Price T.D., Saag L., Sabin M., Shishлина N., Mrvčá K., Soenov V.I., Szeverényi V., Tóth G., Trifanová S.V., Varul L., Vicé M., Yevpikosoylov L., Zhitenev V., Orlando L., Sicheritz-Pontén T., Brunak S., Nielsen R., Kristiansen K., and Willerslev E. (2015) Population genomics of Bronze Age Eurasia. Nature, 522: 167–172.
Ammerman A.J. and Cavalli-Sforza L.L. (1984) The Neolithic Transition and the Genetics of Populations in Europe. Princeton University Press, Princeton, NJ.
Andrades Valtueña A., Mittern A., Key F.M., Haak W., Allmäe R., Belinskij A., Daubars M., Feldman M., Jankowiak A., Jankowiak I., Massy K., Novak M., Pitrengre S., Reinhold S., Söllner M., Sprou M.A., Szczeńśi-Nagy A., Tóvý M., Hansen S., Bos K.I., Stockhammer P.W., Herbig A., and Krause J. (2017) The Stone Age plague and its persistence in Eurasia. Current Biology, 27: 3683–3691.e1–e8.
Andrews R.M., Kubacka I., Chinery P.F., Lightowlers R.N., Turnbull D.M., and Howell N. (1999) Reanalysis and revision of the Cambridge Reference Sequence for human mitochondrial DNA. Nature Genetics, 23: 147.
Anthony D.W. (2007) The Horse, the Wheel, and Language: How Bronze Age Riders from the Eurasian Steppes Shaped the Modern World. Princeton University Press, Princeton, NJ.
Bollongino R., Nehlich O., Richards M.P., Orscheidt J., Thomas M.G., Sell C., Fajkosová Z., Powell A., and Burger J. (2013) 2000 years of parallel societies in Stone Age Central Europe. Science, 342: 479–481.
Brandt G., Szczeńśi-Nagy A., Roth C., Alt K.W., and Haak W. (2015) Human paleogenetics of Europe—the known knowns and the known unknowns. Journal of Human Evolution, 79: 73–92.
Cavalli-Sforza L.L. (2000) Genes, Peoples, and Languages. University of California Press, Berkeley and Los Angeles.
Damgaard P.B., Margaryan A., Schroeder H., Orlando L., Willerslev E., and Allentoft M.E. (2015) Improving access to endogenous DNA in ancient bones and teeth. Scientific Reports, 5: 11184.
Deguilloux M. and Mendisco F. (2013) Ancient DNA: a window to the past of Europe. Human Heredity, 76: 121–132.
Gamba C., Hanghö J., Guaitz C., Alfararhi A.H., Alquraishi S.A., Al-Rasheed K.A.S., Bradley D.G., and Orlando L. (2016) Comparing the performance of three ancient DNA extraction methods for high-throughput sequencing. Molecular Ecology Resources, 16: 459–469.
Haak W., Balanovsky O., Saneev J.J., Koshele S., Zaporozhchenko V., Adler C.J., Der Sarkissian C.S.I., Brandt G., Schwarz C., Nicklisch N., Dresely V., Fritsch B., Balanovskova E., Villems R., Meller H., Alt K.W., Cooper A., and the Genographic Consortium (2010) Ancient DNA from European early Neolithic farmers reveals their Near Eastern affinities. PLoS Biology, 8: e1000536.
Haak W., Lazaridis I., Patterson N., Rohland N., Mallick S., Llamas B., Brandt G., Nordenfelt S., Harney E., Stewartson K., Fu Q., Mittern A., Bautly E., Economou G., Francken M., Friedebich S., Pena R.G., Hallgren F., Khartanovich V., Khothklov A., Kunst M., Kuznetso P., Meller H., Chivall D., Lynnerup N., Harvig L., Baron J., Della Casa P., Daubrowski P., Duffy P.R., Ebel A.V., Epmihakoh A., Frei K., Furnaramek M., Gnäk T., Gromov A., Gronkiewicz S., Grue G., Hajdu T., Jarysz R., Khartanovich V., Khothklov A., Kiss V., Kolàr J., Kriska A., Lasak I., Longhi M., McClellan G., Merkevicius A., Merklyte I., Metspalu M., Mkrtchyan R., Moiseyev V., Paja L., Pálido G., Pokutta D., Pospieszynski L., Price T.D., Saag L., Sabin M., Shishлина N., Mrvčá K., Soenov V.I., Szeverényi V., Tóth G., Trifanová S.V., Varul L., Vicé M., Yevpikosoylov L., Zhitenev V., Orlando L., Sicheritz-Pontén T., Brunak S., Nielsen R., Kristiansen K., and Willerslev E. (2015) Population genomics of Bronze Age Eurasia. Nature, 522: 167–172.
Rasmussen S., Allentoft M.E., Nielsen K., Orlando L., Sikora M., Sjögren K.G., Pedersen A.G., Schubert M., Van Dam A., Kapel C.M.O., Nielsen H.B., Brunak S., Avetisyan P., Epimakhov A., Khalyapin M.V., Gnuni A., Kriiska A., Lasak I., Metspalu M., Moiseyev V., Gromov A., Pokutta D., Saag L., Varul L., Yepiskoposyan L., Sicheritz-Pontén T., Foley R.A., Lahr M.M., Nielsen R., Kristiansen K., and Willerslev E. (2015) Early divergent strains of *Yersinia pestis* in Eurasia 5,000 years ago. Cell, 163: 571–582.

Richards M., Macaulay V., Torroni A., and Bandelt H.-J. (2002) In search of geographical patterns in European mitochondrial DNA. American Journal of Human Genetics, 71: 1168–1174.

Ryzhov S.N. (2012) Relative chronology of the giant-settlement period BII-CI. In: Menotti F. and Korvin-Piotrovskiy A.G. (eds.), The Tripolye Culture Giant-Settlements in Ukraine: Formation, Development and Decline. Oxbow Books, Oxford, pp. 79–115.

Sampietro M.L., Lao O., Caramelli D., Lari M., Pou R., Martí M., Bertranpetit J., and Lalueza-Fox C. (2007) Palaeogenetic evidence supports a dual model of Neolithic spreading into Europe. Proceedings of the Royal Society B: Biological Sciences, 274: 2161–2167.

Tajima F. (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123: 585–595.

Van Oven M. and Kayser M. (2009) Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Human Mutation, 30: E386–E394.

Watson E., Bauer K., Aman R., Weiss G., von Haeseler A., and Pääbo S. (1996) mtDNA sequence diversity in Africa. American Journal of Human Genetics, 59: 437–444.

Wilde S., Timpson A., Kirsanow K., Kaiser E., Kayser M., Unterländer M., Hollfelder N., Potekhina I.D., Schier W., Thomas M.G., and Burger J. (2014) Direct evidence for positive selection of skin, hair, and eye pigmentation in Europeans during the last 5,000 y. Proceedings of the National Academy of Sciences of the USA, 111: 4832–4837.

Yang D.Y., Eng B., Waye J.S., Dudar J.C., and Saunders S.R. (1998) Technical note: improved DNA extraction from ancient bones using silica-based spin columns. American Journal of Physical Anthropology, 105: 539–543.

Zvelebil M. and Dolukhanov P. (1991) The transition to farming in Eastern and Northern Europe. Journal of World Prehistory, 5: 233–278.