Palaeogenomic and Biostatistical Analysis of Ancient DNA Data from Mesolithic and Neolithic Skeletal Remains

Dissertation

submitted in fulfilment of the requirements for the degree

Doktor der Naturwissenschaften

at Faculty of Biology
Johannes Gutenberg University Mainz

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Mainz, 2016

Abstract

Palaeogenomic data have illuminated several important periods of human past with surprising implications for our understanding of human evolution. One of the major changes in human prehistory was Neolithisation, the introduction of the farming lifestyle to human societies. Farming originated in the Fertile Crescent approximately 10,000 years BC and in Europe it was associated with a major population turnover. Ancient DNA from Anatolia, the presumed source area of the demic spread to Europe, and the Balkans, one of the first known contact zones between local hunter-gatherers and incoming farmers, was obtained from roughly contemporaneous human remains dated to $\sim 6^{\text{th}}$ millennium BC. This new unprecedented dataset comprised of 86 full mitogenomes, five whole genomes (7.1–3.7x coverage) and 20 high coverage (7.6–93.8x) genomic samples. The Aegean Neolithic population, relatively homogeneous on both sides of the Aegean Sea, was positively proven to be a core zone for demic spread of farmers to Europe. The farmers were shown to migrate through the central Balkans and while the local sedentary hunter-gathers of Vlasac in the Danube Gorges seemed to be isolated from the farmers coming from the south, the individuals of the Aegean origin infiltrated the nearby hunter-gatherer community of Lepenski Vir. The intensity of infiltration increased over time and even though there was an impact of the Danubian hunter-gatherers on genetic variation of Neolithic central Europe, the Aegean ancestry dominated during the introduction of farming to the continent.

Acknowledgements

First of all, I would like to thank my supervisor, Prof. Joachim Burger for giving me the opportunity to be his PhD student for this challenging project and for his guidance and wisdom during my time in Mainz. I also could not stress enough how important was for me the environment created by the members of the Palaeogenetic Group in Mainz. In the beginning, they were my colleagues and I enjoyed fruitful collaborations, inspiring discussions and active encouragement but they soon became my close friends and I will always be grateful for the privilege of getting to know them.

I am also grateful to the BEAN and PAT teams whose knowledge and scientific excellence inspired me and pushed me further. Special thanks to those who on the short notice invested their time, effort and knowledge into our joint articles. Especially, I would like to thank Krishna Veeramah for his comments and assistance with our joint SI chapter and Mathias Currat whose example of patient scientific reasoning during my stay at his lab helped me more than he probably realises. My work would be also hard without dedication of my students, Florian Gumboldt, Hannah Dehoust, Laura Winkelbach and Ilektra Schulz, who taught me a lot through our interactions.

My deepest gratitude goes to my loving family. To my parents who always supported me in whatever direction I fancied to go next, to my brother who could always put things into perspective and to my beloved husband who not only encouraged me in my dreams but made my dreams his.
Mám vás moc ráda!

Contents

1 Introduction

> *The great events of world history are, at bottom, profoundly unimportant. In the last analysis, the essential thing is the life of individual. This alone makes history, here alone do the great transformations take place, and the whole future, the whole history of the world, ultimately springs as a gigantic summation from these hidden source in individuals.*
>
> CG Jung, The Meaning of Psychology for Modern Man, 1934

By comparing DNA of different people, it is possible to observe patterns in genetic variation, and the main objectives are usually differences between populations that can be interpreted in relation to the past. For instance, similarities between two populations can be indicative of connections between them (e.g., *1*) or even of large prehistoric population movements (e.g., *2*). Population sizes can be derived from genetic diversity (e.g., *3*) and cryptic cultural borders can be discovered in seemingly homogeneous populations (e.g., *4*).

If we metaphorically consider DNA as a compendium of stories about our ancestors that has been combined and retold in every generation, we can effortlessly comprehend that the older the stories are, the harder it is to untangle them from each other. An opportunity to go back in time and obtain the story directly from one of the ancestors would be priceless. That is what ancient DNA is to genetics: a chance to get a glimpse of the genetic variation as it was, without the effects of following generations.

Human remains from archaeological sites are screened for signs of DNA survival and considerable effort and resources are spent on obtaining such genetic information ever since 1985 when DNA was extracted from 2,400 years old Egyptian mummy, the first attempt at DNA extraction from ancient human remains (*5*). Laboratory and analytical methods are being improved technologically (e.g., *6*) and conceptually (e.g., *7*) and all the data gained are repeatedly re-analysed to create a new, often surprising, perspective on genetic variation in the past and present (recently see *8*, *9*). Ancient DNA also brings large prospects for widening our knowledge of genetic processes, such as selection (*10*, *11*, *12*), the evolution of human beings (e.g., *13*, *14*), and health and disease in the past (e.g., *15*).

However, ever so often we should consider ancient DNA not only as an endless resource of new information. Our goal is to understand population history, to write a scientific record of the human past in the place of the non-written narratives. Moreover, because a population is a community of individuals exchanging genes, so its history is a combination of individual stories. Even though we are often able to see only the generalised population history, sometimes we gain insight to an individual's entire palaeogenome and to the story of this individual life told from the past.

1.1 Palaeogenomics as a tool for studying past populations

Obtaining complete palaeogenomes became possible only in recent years with the advances of next generation sequencing (NGS) techniques. Before that, only short stretches of mitochondrial DNA (mtDNA) could be gained and even that has happened relatively recently, increasingly so with the advent of PCR (polymerase chain reaction) (*16*, *17*).

While some anticipate that parts of history will be re-written by inferences based on palaeogenomic data obtained with NGS technologies (*18*), important ancient DNA (aDNA) inferences have already changed concepts about whole periods, even though sample sizes in the pre-NGS era were very small and studies relied on one locus (mtDNA, rarely Y chromosome) (*19*). Most notably, mitochondrial sequences from *Homo neanderthalensis* have resulted in a major leap in our understanding of human evolution since it was shown that inbreeding with this human species was low or non-existent (*20*, *21*), as this was interpreted to strongly support the recent African origin model of the transition of archaic to anatomically modern humans (*22*).

Ancient mtDNA studies also contributed to discussions about the first appearance of humans in North America (*23*, *24*), population history of the Andamanese people (*25*), relationships of pre-Columbian populations of Peru (*26*), the selective advantage of lactase persistence (*10*, *27*, *28*) or prehistoric and historic population movements in Europe and central Asia (e.g., *29*, *30*, *31*, *32*).

One of the most prominent question tackled (but not solved) before the advent of NGS was related to the beginning of farming in Europe, when Haak *et al.* (*33*) and Bramanti *et al.* (*34*) showed that direct continuity between first farmers in central Europe and hunter-gatherers living in Europe prior to the introduction of farming was excluded and a similar pattern was detected by aDNA analysis of early farmers from Iberia and Scandinavia (*35*, *36*).

The recent advances, however, all rely on NGS technologies applied in the field, most frequently on Illumina® massive parallel sequencing platform (*6*). This technology generates a large number of sequences, which is possible via library construction protocols that are used to attach adapter sequences to the fragmented DNA (*6*), in case of aDNA fragmented naturally during DNA degradation process (*37*). The massive output of Illumina sequencing allows to estimate error rates of individual sequencing runs (e.g., *38*, *39*), detect aDNA damage on the sequencing level (e.g., *39*, *40*) and sequence whole genomes of highly advanced age (e.g., *13*, *41*, *42*, *43*), even a 700,000-year old genome of prehistoric horse (*44*).

Not all the progress has been made solely thanks to the technological advancements though. Some of early aDNA studies resulted in incorrect ancient DNA, usually due tu contamination by human DNA of recent origin (*45*, *46*). Once the contamination issue was recognised, anti-contamination procedures in the dedicated laboratories and critical approaches to the results were implemented (*47*, *48*). While contamination is still an issue, its recognition and estimation makes it possible to verify the data (*40*, *49*).

Interestingly, even a relatively small improvement can change the field rapidly. The switch from teeth to petrous bones as the skeletal element used for DNA extraction instigated by Pinhasi *et al.* (*7*) has resulted in an increase of ancient DNA studies from regions that did not previously yield ancient DNA (*9*, *50*, *51*, *52*, *53*).

1.2 Main prehistoric demographic events in a view of palaeogenomics

The first ancient genomes came from very cold areas, where the preservation of ancient DNA was the highest (*54*). The first palaeogenome ever obtained was that of the mammoth (*54*, *55*) and the first ancient human genome was from a sample of Palaeo-Eskimo of the Saqqaq culture (*56*). The palaeogenome that got the most attention and led to major advancements in palaeogenomics was, however, the Neanderthal genome (*13*).

The full Neanderthal genome proved, contrary to the first interpretations of the Neanderthal mtDNA (*20*), that modern populations living outside of Africa shared low levels of ancestry with Neanderthals that were not present in modern African populations (via methods also used in this study, see section 3.13.2). That suggested an admixture event with Neanderthals that happened after humans spread from Africa (*13*). A high-coverage Neanderthal genome from the Denisova cave in the Altai Mountains (abbreviated often as "Altai") have further refined the estimate of a common ancestor of anatomically modern humans and Neanderthals to 383,000-257,000 years ago and the level of post-divergence admixture between them to approximately 1.5-2.1% (*57*).

At the same time the Neanderthal genomic analysis showed that notions obtained from mitochondrial DNA (*20*) do not have to be replicated when a large number of loci in the whole genome are analysed (*13*) and that aDNA, and especially mtDNA, analysis should be performed in a spatial and chronological framework to prevent over-interpretation of heterochronous data because spatially explicit modelling reanalysis of the Neanderthal mtDNA had reached a similar conclusion (a possible low amount of admixture) as whole genome analysis (*21*).

Another major shift in anthropology was caused by the DNA extracted from a finger bone of a juvenile hominin, also from the Denisova cave in Siberia. The mitochondrial DNA obtained from this individual was very different to that found in both modern humans and Neanderthals (based on the mtDNA, the estimated divergence from both was about one million years ago) (*58*). This often caused Denisovians to be assigned to a completely new species based solely on palaeogenomics (e.g., *59*). However, while the Denisova genome was highly differentiated from both Neanderthals and modern humans, the genomic data have showed that the Denisovians were a sister group of Neanderthals that diverged 239,000-190,000 years ago (i.e., after the split from anatomically modern humans) (*14*, *57*). One of the reasons for the previously noticed differences in mtDNA was probably low ancestral population sizes of both Neanderthals and Denisovians (*57*).

Differences in results from mitochondrial and genomic data were detected in another fossil hominin *Homo heidelbergensis*, the oldest known hominin genome yet. This approximately 430,000-year-old Sima delo Huesos sample was identified via mtDNA as being close to Denisovian (*43*), while deriving most of its genomic ancestry from a population related to the Neanderthals (*42*). The discrepancy was explained as an incomplete lineage sorting after the divergence of Neanderthals and Denisovians (*42*).

In this section, the focus will be placed on demographic events that involved Europe, notwithstanding the fact that ancient DNA is of great significance to questions involving other areas as well. A case in point is the Siberian Upper Palaeolithic genome Mal'ta, which proved to by partly related to a source population for Native Americans (other part of Native American ancestry seems to originate in a differentiated population related to modern East Asians) (*60*). Additionally, ancient Native American genome from Clovis culture site Anzick showed that the split between South and North American Native populations (while both having the same root) happened very shortly after the arrival of people to Americas, at least before approx. 10,500 cal. BC when the Anzick-1 individual lived (*61*, *62*).

1.2.1 Last Glacial Maximum

Our knowledge of genetic variation in the Upper Palaeolithic era is rather limited, with relatively few samples sequenced (*41*, *53*, *63*). Only very recently, nuclear target enrichment on a set of samples of this age was performed and total of 51 samples from this period were analysed (*41*). The authors of this study suggest that at the end of the Ice Age, when the climate became more suitable, two source populations repopulated Europe, one related to Upper Palaeolithic Iberia, the other (spreading later) linked to the Near East. In a related publication, mitochondrial sequences from these individuals were retrieved and modelled to support the hypothesis of a major population change 14,500 years ago (the Late Glacial period) and a significant decrease of population size during the Last Glacial Maximum (LGM; ∼23,050-17,550 cal BC) (*64*). While these findings will be certainly investigated further, the LGM bottleneck seems to be in contradiction to a recent study based on modern data and spatial modelling that did not observe the bottleneck but rather long-distance dispersals affecting current patterns of human diversity (*65*).

1.2.2 Neolithic Transition

The Neolithic way of life, usually described as sedentary farming with domesticated animals, first appeared in the Fertile Crescent (*66*). While there is no single generally accepted definition of Neolithisation (transition from foraging to farming), food production, a settled village life, a revolution of symbols, polished stone tools and pottery are usually present during Neolithic spread of economic domesticates (both domesticated plants and animals) and related subsistence strategies

(*67*). Özdoğan (*68*) postulates on the basis of archaeological data available that western part of the Anatolian peninsula became a (secondary) core area for the Neolithic spread to Europe after the initial expansion from the Fertile Crescent, the primary Neolithic core zone (see Figure 1 for dates of the appearance of the Neolithic throughout the region).

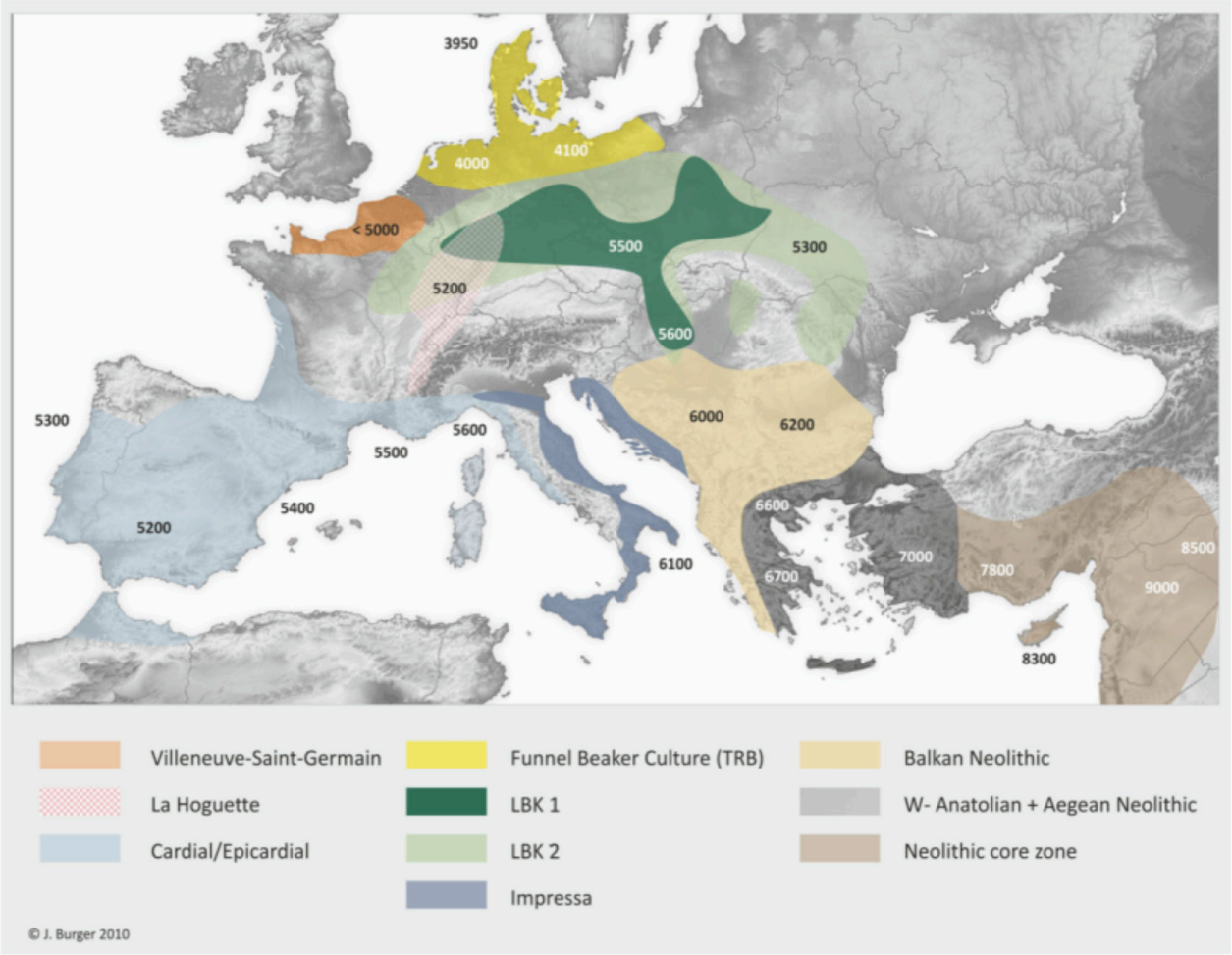


Figure 1: *The earliest dates of appearance of the Neolithic (in years cal BC) overlaying the geographical distribution of the Early Neolithic cultures. The figure was adopted from Burger & Thomas (69) with added novel dates for southern Greece (70) and central Macedonia (71).*

There were lengthy discussions how the spread of Neolithic innovations occurred. Two main theories were debated: cultural diffusion (spread of ideas) and demic diffusion (spread of people) (*72*). Additionally, a series of intermediate scenarios were suggested, leading to various manners of contact between Neolithic farmers and local hunter-gatherers, who occupied Europe during the preceding Mesolithic period (e.g., *73*) (in Europe the Mesolithic period is set between the beginning of Holocene cca 11,500 years ago and the introduction of Neolithic innovations in the region (*74*)). While archaeologically Neolithic innovations (e.g., pottery, polished stone tools, domesticates) could be consistent with the migration of people, the continuity of lithic technologies and sources indicates some level of contact (though it can also reflect similarity in the subsistence between early farmers and hunter-gatherers) (*75*).

Ancient mitochondrial DNA from the last hunter-gatherers and first farmers in Europe has shown that continuity between the two strata is not supported and this was interpreted as a clear sign of a newly arriving farming population (*33*, *34*). While the methods and absence of geographical and paternal information was criticised (*76*, *77*), palaeogenomic research (*40*, *78*, *79*) confirmed the mtDNA-based inferences as genomes from Mesolithic hunter-gatherers from Loschbour (contemporary Luxembourg) and La Braña (NW Iberia) (*78*, *79*) and an Early Neolithic farmer from Linearbandkeramik (LBK) culture site Viesenhäuser Hof in Stuttgart Mühlhausen (from here on called "Stuttgart") (*78*) proved to be from differentiated populations. The same conclusions were maintained when a larger set of data was obtained via nuclear target enrichment (*8*).

Later, however, it was shown that the hunter-gatherer ancestry did not entirely disappear. In Bollongino *et al.* (*80*) we demonstrated that, at least in certain contexts, hunter-gatherers and farmers lived in parallel even 2,000 years after the first presumed contact. Corresponding results showed that in the later stages of Neolithic period, the genetic ancestry of hunter-gatherers resurfaced in Neolithic populations (*8*). Interestingly, an individual discovered in Early Neolithic context in Hungary (at Tiszaszőlős-Domaháza) showed a genetic signature clearly similar to Loschbour and La Braña (a population defined as Western hunter-gatherers, WHG), suggesting contacts between the farmer and hunter-gatherer populations from the early stages (*52*).

However, the genetic variation of the presumed secondary Neolithic core zone for demic spread to Europe was not known until recently. Neolithic data from NW Anatolia (*12*, *51*) which was partly obtained for this study, has shown that the suggestions that western Anatolia was the core zone for European Neolithic were likely to be correct. Further works have suggested that Neolithic individuals in the western part of the Fertile Crescent were related closely to the NW Anatolian Neolithic population of the late 7^{th} millennium BC (*50*), whereas Neolithic individuals from the eastern part were extremely differentiated from them and possibly spread to the east of the area (*9*).

While the Neolithic Transition was seemingly illuminated by genetic data, details of the spread and of the contacts with the local hunter-gatherer population are still unknown. Also, spatially explicit simulations were rarely included in the studies and Mesolithic populations from the Balkans, the area of the first contact between farmers and hunter-gatherers, have not been analysed.

1.2.3 Population influx from the Pontic Steppe

A relative surprise evoked by palaeogenomics was the assertion of a large migratory event in Europe approximately 4,500 years ago. Relative changes between prehistoric periods in frequencies of haplogroups in Europe had been noticed previously (*81*), but it was the landmark study by Haak *et al.* (*8*) that brought convincing evidence of population turnover in the third millennium BC based on genomic data (∼390k captured SNPs to be exact). The study presented a large dataset dated from the Early Neolithic to Iron Age from central and western Europe and to the Mesolithic (i.e., Eastern hunter gatherers, EHG) and Late Copper Age (archaeologically assigned to Yamnaya culture) from

the Pontic Steppe. While the differences between the populations in Pontic Steppe and the rest of the prehistoric European samples prior to the Late Neolithic were found to be large, samples from the Late Neolithic Corded Ware culture from Germany traced ∼75% of their ancestry to the Yamnaya (*8*). The Pontic Steppe Yamnaya samples were therefore interpreted as related to the source of the differences between the Late Neolithic and previous periods, i.e., as the population migrating ∼4,500 years ago to central and western Europe, whose ancestry can still be detected ∼3,000 years ago and is present in European modern populations (*8*). Though it is not possible to pinpoint the migratory population without more data from the region (Asia is undersampled even for ancient DNA standards), the inference of Pontic Steppe migration in this era was also demonstrated by 100 low coverage genomes (*82*).

1.2.4 Challenges in palaeogenomic applications

After death of an individual, DNA undergoes degradation over time. One of the major obstacles in the field is to overcome the damage to the molecules in order to extract DNA and to produce reliable sequences without erroneous changes caused by degradation (*17*). The damage on the sequence level dominantly manifests as deamination of DNA bases from cytosine (C) to uracil (U) that is then replaced by thymine (T) in PCR and therefore results in generation of C ->T miscoding errors (*83*, *84*). The consequences of damage can be partly overcome by uracil DNA glycosylase (UDG) treatment via base-excision of U (*85*) or by selecting only transversions for the analysis (*86*). Another option is to select only the most frequent allele for the position and analyse this pseudo-haploid data (*87*). However, UDG treatment masks the damage patterns and it is therefore advisable to keep at least part of the sample non-UDG treated to confirm the advanced age of the sample (*88*). Other mentioned approaches cause a considerable loss of data. The alternative route for solving the problem of damage to ancient DNA is the likelihood-based variant calling approach with the incorporated post-mortem damage model (*51*, *89*).

Contamination is one of the main challenges in all human ancient DNA studies and it should be stressed that no matter the methodology (PCR or NGS), it is always possible that the DNA extracted from the sample did not belong to the ancient individual but to any of the human beings who were in (primary or secondary) contact with the sample. Since NGS methods allow for the sequencing of a large number of molecules from the sample (*90*), the actual ancient endogenous DNA would have to be largely absent in order for the sample to appear homogeneous and pass quality controls despite contamination. Still, especially for low coverage data, contamination cannot be excluded because it is possible that only a subset of positions in a sample would be affected, resulting in an artificial individual with a combination of endogenous and contaminating genetic patterns (*40*).

The damaged ends of the sequenced ancient molecules with a signal of elevated C ->T changes at 5' end due to deamination are highly indicative of the age of the sample and therefore the absence of this signal could signify contamination (*17*, *88*). However, the same damage patterns can be obtained

via the use of decontamination procedures (e.g., UV irradiation) (*91*). Therefore, conclusions based on aDNA should be scrutinised for unexpected results and confirmed on more than one sample, because complete or partial contamination can be present in datasets (*36*).

Many other challenges in palaeogenetic analysis stem from the fact that it is largely impossible to choose how many individuals from what populations can be selected for the analysis. The geographical distribution of archaeological material available for aDNA analysis is limited and uneven, and one cannot be sure that it completely reflects the distribution of human settlements in the past (*92*). For example, specific burial practices can cause some areas to be completely devoid of human remains at certain periods (*93*). Together with low DNA survival and limited resources, the scarcity of discovered human burials also leads to low numbers of analysed individuals. Additionally, there is no way to ascertain that the samples are random draws from past populations. For instance, some subsistence strategies and cultural practices might result in limited accessibility of the human remains and the genetic contribution of such subpopulations can be therefore underestimated.

Usual approaches to establishing the random selection of local individuals in contemporary population genetic studies (e.g., *2*) cannot be applied to palaeogenetics due to the limited amount of information obtainable from archaeological excavations. Individuals that are non-local to the sites are rarely recognised (*94*), and the way of life of past societies and their impact on genetic variation (e.g., high endogamy or exogamy) are poorly understood (*95*). Adding to the complexity, cultural and even chronological assessment of the samples can easily be called into question (*96*, *97*), while some analyses (especially mtDNA) require informed decisions on the grouping of the samples and others (such as *ADMIXTURE*) suffer from the inclusion of samples from too chronologically distant populations (*98*, *99*). These issues can be incorporated into statistical models as variables (e.g., as geography in *100*), but the more confounding variables and complex models there are, the higher the number of samples necessary for the analysis (*101*).

2 The skeletal material and its archaeological background

Nuanced and careful interpretations of palaeogenetic data with adequate regard to archaeology and anthropology are still not entirely common practice in palaeogenetics. This is the case, despite the fact that disregard for archaeological information has led to serious misinterpretations of palaeogenetic results and, unfortunately, also to certain level of distrust of the archaeological community towards results emanating from the palaeogenetic field in general (*97*).

On the other hand, while palaeogeneticists use terms originally defined in archaeology, the meaning in palaeogenetic literature has often already transferred. That is especially so in cases in which a sample or samples are referred to by their cultural label. The cultural context of studied human remains provides a label for a population whose relationships to both other ancient and modern populations are further studied. Unfortunately, this can often lead to expressions (e.g., "Yamnaya ancestry in modern Germans") that are reminiscent of *cultural-historical archaeology*, an archaeological paradigm that has been misused by nationalistic and racist movements. Labels are usually explained before usage, or they can be sourced in the original literature for the samples, yet in the condensed main text, abstract or oral presentation the meaning is sometimes unclear.

Due to the sensitivity of the subject, these forms might eventually be eradicated. At present, however, there are in use and probably will be for some time. Ancient populations studied in palaeogenetics need labels and often there is no other known option until more samples from the area are analysed and populations are defined genetically. Also, to have more statistical power, samples are often pooled together on an archaeological basis for genetic analysis. For individuals hereby studied, we attempted to use site names when possible (when a single sample/site was discussed) or geo-chronological names ("Neolithic Aegeans", often abbreviated to "Aegeans", and "Meso/Neolithic Danubians", often abbreviated as "Danubians"). We use "Steppe" for Yamnaya and Yamnaya-like samples (*12*, *78*). However, we use "LBK" for previously published samples from Transdanubia, pooled due to their affiliation with LBK culture.

It also should be noted that while archaeology is the ultimate source of research topics and testable hypotheses for palaeogenetics, sometimes the literature provides conflicting information about the samples. This often leads palaeogeneticists to disregard some publications because they believe they found what they were seeking (usually group assignment) in another publication. Even though the assumption of grouping individuals has to be made for some analyses (fortunately this is not necessary for many tests involving nuclear regions), the uncertainty of the group assignment has to be taken into account during interpretation.

2.1 Anthropological and archaeological background

The samples studied in this work were obtained from various geographical regions spanning the area of first contacts between farmers and hunter-gatherers (the Balkans) and a possible core area for the spread of farmers to Europe (western Anatolia, specifically northwestern Anatolia as Epipalaeolithic and Neolithic human remains are absent or rare in the region). Archaeological studies related to these areas have often differed in approaches, paradigms and research history. Fortunately, in recent years substantive efforts have been made to produce new comparable research and new archaeological sites have been discovered (*102*, *103*, *104*). This has paved the way for multidisciplinary studies and collaborations, such as the one this study is fortunately part of, namely BEAN (Bridging the European and Anatolian Neolithic) Marie Curie Initial Training Network, which involves 14 academic, research and commercial institutions from nine countries in the fields of anthropology, genomics, simulations and modelling, biostatistics, demography, and prehistoric archaeology.

Most of the human remains sourced for this study and further information related to them were kindly provided by Prof. Sofija Stefanović (samples from the Danube Gorges and other regions in Serbia), Prof. Necmi Karul (Aktopraklık samples), Prof. Arkadiusz Marciniak (Catalhöyük samples) and Assoc. Prof. Dr. Rana Özbal and Dr. Fokke Gerritsen (Barcın Höyük samples). The location of the sites can be seen in Figure 2 and ^{14}C dating is discussed and presented in section 2.4.

Figure 2: *The location of the sites that were sampled for this study (the map was generated from ESRI® map data using qGIS (105)).*

2.2 The Balkans in Meso-Neolithic Transition

As one of the first contact zones between farmers and hunter-gatherers (*104*, *106*) and at the same time the location of numerous human burial finds from the Mesolithic period, the Balkans have been extensively studied in terms of Meso-Neolithic Transition on the local scale (*66*). Recently, the region has been discussed more broadly as one of the corridors for the spread of farming from the Aegean to Europe (*75*). The first European appereance of farming is attributted Greece with the earliest phases dated to ∼6,700-6,500 cal BC (*70*, *107*).

The first Early Neolithic culture in the central Balkans was Starčevo, in Hungary it was named Körös and in Romania Criş. These cultures are often referred to jointly as the Starčevo-Körös-Criş complex (*108*). The Starčevo culture first appeared ∼6,200/6,000 cal BC (*109*) and while regionally it was succeeded after Early Neolithic by the Vinča culture in the late seventh millennium cal BC (*110*), the Starčevo culture also spread to the Danube Valley and established the first Linearbandkeramik (LBK) settlements in west Hungary ∼5,600/5,500-5,350 cal BC (*75*). The end of the Vinča culture (∼4,500 cal BC) approximately corresponds to the end of the Neolithic period and the beginning of Early Copper Age in the Balkans (*111*).

Together with the Marmara region in Northwest Anatolia, the main area studied for the purposes of this study was the Danube Gorges (see Figure 3), a region with frequent (∼500) human burials from both the Mesolithic and Early Neolithic (*112*). This area constitutes one of a few examples with continuous use of the sites from the Mesolithic to Neolithic (*113*), although the stratigraphy is still under discussion (*114*). Furthermore, DNA was successfully obtained from samples from several sites in other parts of the Balkans.

2.2.1 Lepenski Vir

Lepenski Vir is the most famous site in the Danube Gorges and one of the most known archaeological sites in general. This large settlement is a type-site of the Lepenski Vir culture (sometimes called Lepenski Vir-Schela Cladovei culture). The site with its abundance of burials has been under discussion ever since its excavation (started 1965 by Dragoslav Srejenović) and the chronology of the area was only relatively recently revised due to a large amount of new ^{14}C dating results (*116*). Here, we utilised the chronological schema used by Borić & Price (*94*) and further simplified it to form four groups of the individuals to be studied from the Danube Gorges (with the assistance of Prof. Sofija Stefanović): Early to Middle Mesolithic (∼9,500-7,400 cal BC; 3 Lepenski Vir individuals), Late Mesolithic (∼7,400-6,200 cal BC; burials from Lepenski Vir are missing from this period), Transition (∼6,200-6,000/5,950 cal BC; 17 Lepenski Vir individuals) and Neolithic (∼6,000/5,950-5,500 cal BC; 12 Lepenski Vir individuals) (see Table 1).

It is important to take into account the fact that only 15 of the samples were dated (see section 2.4) and it is possible that some samples were, on the basis of the archaeological context, assigned to

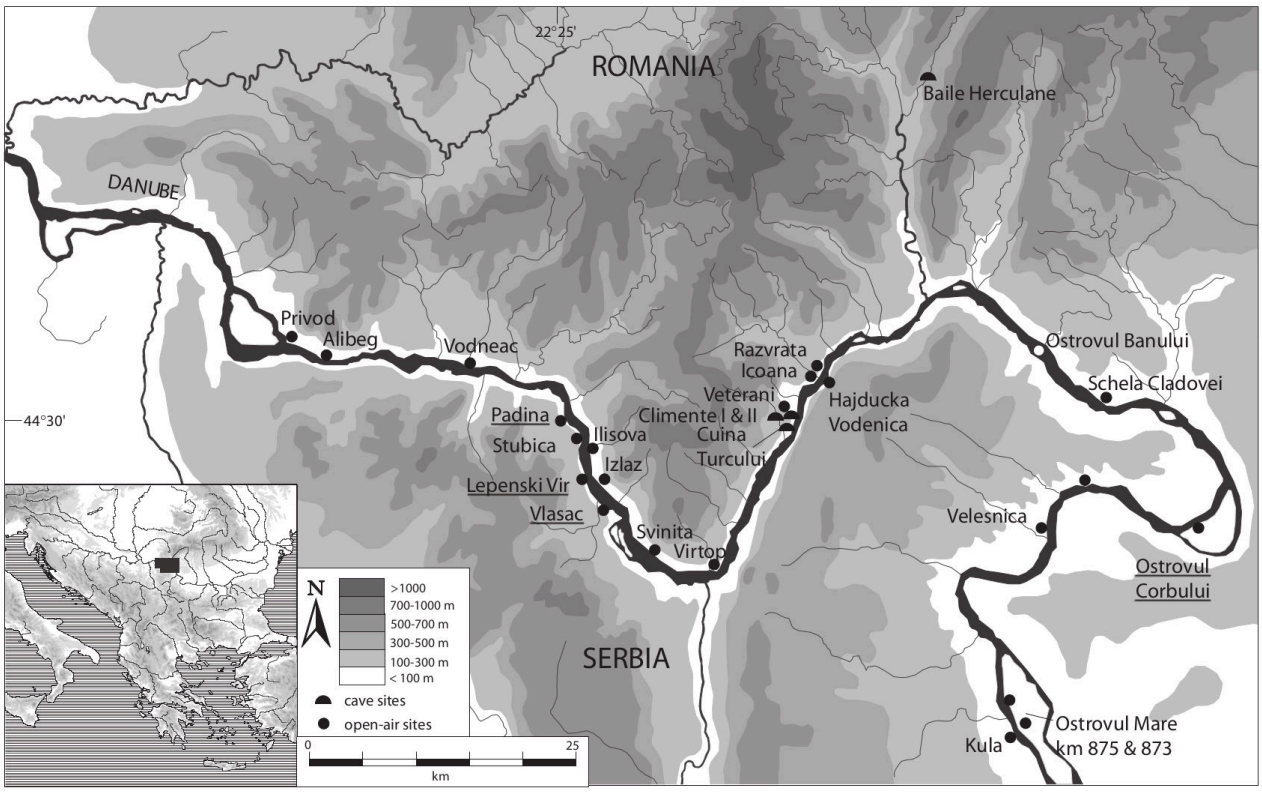


Figure 3: *Mesolithic and Early Neolithic sites in the Danube Gorges. The sites analysed in this study are underlined (adopted from 115).*

the groups incorrectly. However, the archaeological information and abundance of direct dates from the site from other than burial contexts (e.g., *94*) allow for a relatively high degree of certainty. It should be also noted that, except for the relationship of Mesolithic hunter-gatherers and Neolithic farmers, one of the questions related to this site is whether Mesolithic individuals from the early phases (∼9,500-7,400 cal BC) were the same as (at least partially hunter-gatherer) individuals in the Transition period (∼6,200-6,000/5,950 cal BC), because several authors have argued for a chronological gap between these two phases in Lepenski Vir during Late Mesolithic (∼7,400-6,200 cal BC) (e.g., *94*, *117*). One burial (Lepe2, grave 2; see Table 1) was assigned to the Eneolithic Sălcuţa culture (a sample dated to 4,451-3,980 cal BP) (*118*).

Table 1: *Lepenski Vir samples successfully analysed in this study. The anthropological sex and age assignment was provided by Stefanović (119) and the isotopic grouping based on $^{87}Sr/^{86}Sr$ ratio (see section 2.4) by Borić & Price (94). Question marks denote uncertainty of the assignment due to the fragmentary state of the human remains.*

Sample name	**Site**	**Individual**	**Sex**	**Age**	**$^{87}Sr/^{86}Sr$**	**Period**
Lepe49	Lepenski Vir	126	f?	35?	local range	Mesolithic
Lepe51	Lepenski Vir	68	f?	∼40	-	Mesolithic
Lepe47	Lepenski Vir	105	m?	35-40	-	Mesolithic
Lepe13	Lepenski Vir	21	m	30-35	local range	Transition
Lepe15	Lepenski Vir	26	m	20-25	local range	Transition
Lepe17	Lepenski Vir	27/b	f	35-45	local range	Transition
Lepe23	Lepenski Vir	43	-	∼12	local range	Transition
Lepe28	Lepenski Vir	54d	f	40-50	local range	Transition
Lepe42	Lepenski Vir	87/1	-	∼7	local range	Transition
Lepe27	Lepenski Vir	54e	f	∼20	non-local range	Transition
Lepe48	Lepenski Vir	122	f?	15-18	non-local range	Transition
Lepe7	Lepenski Vir	11	-	∼12	-	Transition
Lepe53	Lepenski Vir	27	-	-	-	Transition
Lepe18	Lepenski Vir	27/d	m	∼35	-	Transition
Lepe37	Lepenski Vir	79/b	m	>30	-	Transition
Lepe38	Lepenski Vir	79/c	m?	∼50	-	Transition
Lepe39	Lepenski Vir	82	m?	∼30	-	Transition
Lepe44	Lepenski Vir	89/a	f?	>30?	-	Transition
Lepe45	Lepenski Vir	91	f?	30-40	-	Transition
Lepe46	Lepenski Vir	93	f	30?	-	Transition
Lepe22	Lepenski Vir	39	f?	∼40	local range	Neolithic
Lepe34	Lepenski Vir	74	m?	>40	local range	Neolithic
Lepe52	Lepenski Vir	73	m?	30-40	local range	Neolithic
Lepe12	Lepenski Vir	20	f	∼40	non-local range	Neolithic
Lepe20	Lepenski Vir	32a	f	50-60	non-local range	Neolithic
Lepe32	Lepenski Vir	66	f?	∼25	non-local range	Neolithic
Lepe6	Lepenski Vir	8	f	30-40	non-local range	Neolithic
Lepe1	Lepenski Vir	35	-	10	-	Neolithic
Lepe11	Lepenski Vir	19	f	∼20	-	Neolithic
Lepe41	Lepenski Vir	86	f?	∼30	-	Neolithic
Lepe29	Lepenski Vir	57	-	∼15	-	Neolithic
Lepe3	Lepenski Vir	6	-	∼15	-	Neolithic
Lepe2	Lepenski Vir	2	-	adult	local range	Sălcuţa culture

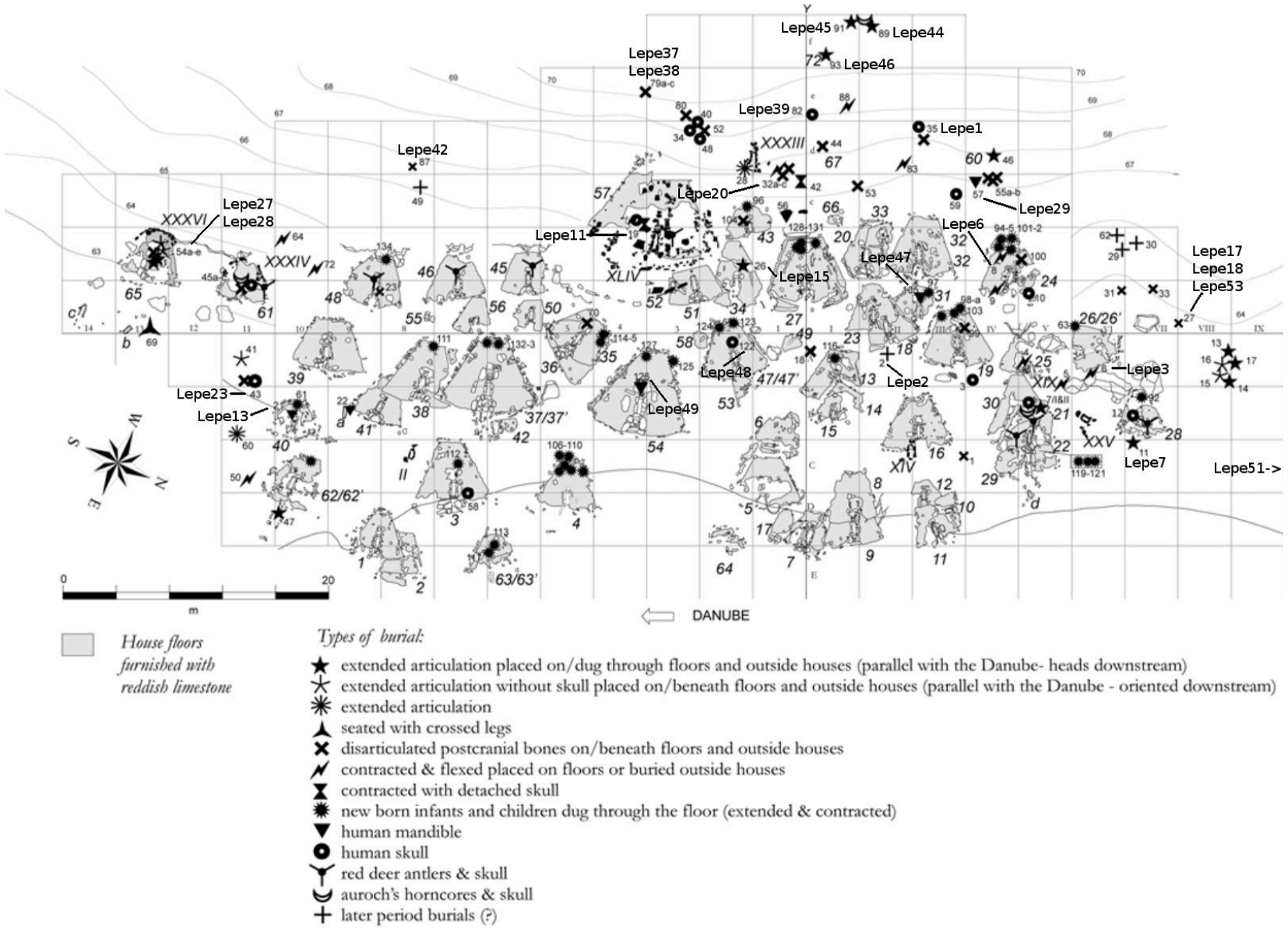


Figure 4: *The location of graves of studied individuals at Lepenski Vir site plan by Stefanović & Borić (120) (the plan is for the Mesolithic and Transition period of the site with several Neolithic burials included).*

2.2.2 Vlasac

The Vlasac site is geographically very close to Lepenski Vir (cca 3 km downstream) and was first excavated in the same period as Lepenski Vir (*121*). The site was assigned to the Lepenski Vir culture and is mostly dated to Late Mesolithic (∼7,400-6,200 cal BC), while there are dates as old as 9,800 cal BC known from the site (*112*, *117*, *122*). Additionally, new excavations (during seasons 2006–2009) showed that there was also occupation parallel to the Transition phase of Lepenski Vir with appearance of features influenced by Neolithic (Early Starčevo ceramics, *Spondylus* shells and discoid beads)(*113*). Most of the settlement was abandoned ∼6,300/6,200 cal BC, but the site might have served during the last centuries of 7^{th} millennium BC as an "ancestral" place and a cemetery for burial rites of Mesolithic tradition (*113*).

Given the likely gap of the occupation of Lepenski Vir in Late Mesolithic and the presence of Neolithic features in the later periods (*123*, *124*), Borić *et al.* (*113*) even suggested that in terms of the intensity of human activity and creative expression, the Lepenski Vir culture in the Danube Gorges should be renamed to "Vlasac-Schela Cladovei" culture.

All the 16 successful samples from Vlasac (see Table 2) were assigned to Late Mesolithic (∼7,400-6,200 cal BC; (*94*, *119*)) but only three were directly dated (see section 2.4).

Table 2: *Vlasac samples successfully analysed in this study. The anthropological sex and age assignment was provided by Stefanović (119) and the isotopic grouping based on $^{87}Sr/^{86}Sr$ ratio (see section 2.4) by Borić & Price (94). Question marks denote uncertainty of the assignment due to the fragmentary state of the human remains.*

Sample name	Site	Individual	Sex	Age	$^{87}Sr/^{86}Sr$	Period
Vlasa1	Vlasac	2	-	old adult	local range	Late Mesolithic
Vlasa2	Vlasac	9	m	old adult	local range	Late Mesolithic
Vlasa30	Vlasac	13	-	old adult	local range	Late Mesolithic
Vlasa32	Vlasac	16	m?	old adult	local range	Late Mesolithic
Vlasa4	Vlasac	18a	m	old adult	local range	Late Mesolithic
Vlasa41	Vlasac	30	m?	old adult	local range	Late Mesolithic
Vlasa7	Vlasac	31	m	adult	local range	Late Mesolithic
Vlasa44	Vlasac	47	f	adult	local range	Late Mesolithic
Vlasa20	Vlasac	69a	m	adult	local range	Late Mesolithic
Vlasa54	Vlasac	74	f	old adult	local range	Late Mesolithic
Vlasa56	Vlasac	78a	m?	old adult	local range	Late Mesolithic
Vlasa61	Vlasac	U64. x 11	-	-	-	Late Mesolithic
Vlasa37	Vlasac	24	f	old adult	-	Late Mesolithic
Vlasa10	Vlasac	41	m?	old adult	-	Late Mesolithic
Vlasa47	Vlasac	49(1)	-	-	-	Late Mesolithic
Vlasa48	Vlasac	52	-	-	-	Late Mesolithic

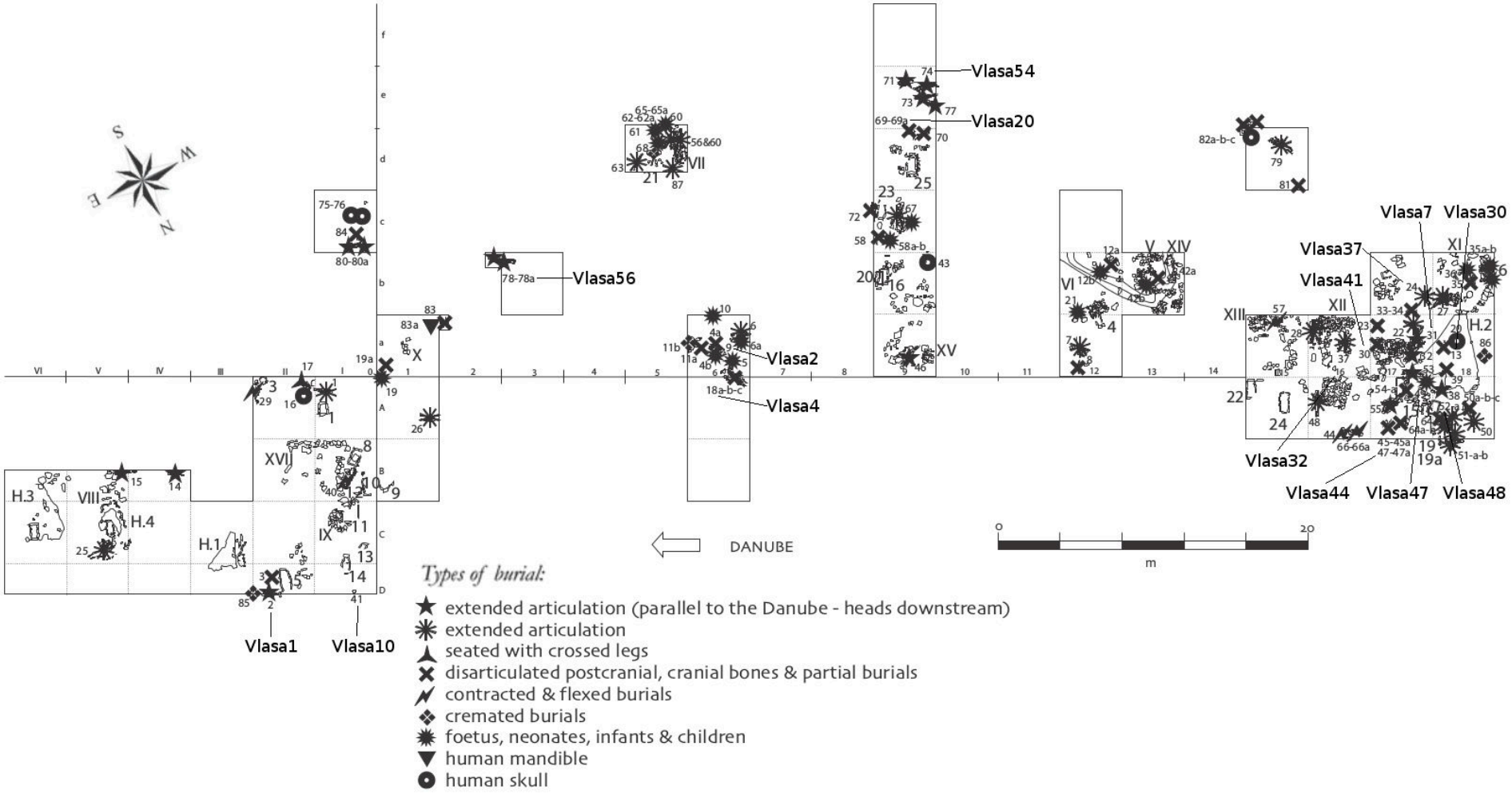


Figure 5: *The location of graves of studied individuals at Vlasac site plan by Borić & Stefanović (112) (except for Vlasa61, marked as U64. x 11 from the new excavation seasons 2006–2009) (125)).*

2.2.3 Padina

Padina is another Danube Gorges site with a superposition of Mesolithic and Early Neolithic structures. It is also associated with the Lepenski Vir culture, with additional later layers of Late Neolithic cultures present at the site (*115*). The assignment of the only ancient individual successfully analysed from this site (Pad 11, burial 30) to Early/Middle Mesolithic (∼9,500-7,400 cal BC) was provided by Borić & Price (*94*).

2.2.4 Ostrovul Corbului

Ostrovul Corbului is located on the Romanian side of the Danube Gorges, downstream from Lepenski Vir. It was originally assigned to the Schela Cladovei culture, which was later connected to the Lepenski Vir culture (*121*). The four samples analysed (see Table 3) were assigned to the phases Ostrovul Corbului II (6,782-6,360 cal BC) and Ostrovul Corbului I (with a date of 7,062 cal BC) (*126*) and they are therefore considered Mesolithic (∼9,500-6,200 cal BC; following the grouping described above for Lepenski Vir). However, it should be noted that there is some discussion regarding the Mesolithic provenance of the Ostrovul Corbului burials, because the context is often unclear and some burials were also discussed as Early Neolithic (*127*, *128*).

Table 3: *Ostrovul Corbului samples successfully analysed in this study. The anthropological sex and age assignment can be sourced in Boroneanț (127).*

Sample name	Site phase	Individual	Sex	Age	Period
Osco2	Ostrovul Corbului I	M2	m	adult	Mesolithic
Osco5	Ostrovul Corbului II	M47a	-	-	Mesolithic
Osco7	Ostrovul Corbului II	M57	-	-	Mesolithic
Osco6	Ostrovul Corbului II	M58	-	-	Mesolithic

2.2.5 Vinča

Vinča-Belo Brdo is a typical site of the eponymous Vinča culture with key Neolithic developments as the formation of large settlements and tells, the intensification of farming subsistence and the expansion of material networks (*110*). The culture occupied a large region of Serbia and several bordering countries between the late 6^{th} millennium BC and middle 5^{th} millennium BC (*129*). The only burial at the site is a collective burial at the lowermost level (*130*) and it is still under discussion whether this Starčevo level represents continuous occupation to later phases or an earlier abandoned settlement (*131*). The samples studied from this site can be seen in Table 4. Individual 2 from the collective burial (sample Vc1) was dated to 5,476-5,304 cal BC at a 95.4% confidence range, with correction for the fresh reservoir effect (see section 2.4) applied (*131*). Logically, the same date can be extended to other individuals from the collective burial.

Given the archaeological hypothesis that the Starčevo culture played a key role in the formation of LBK (Linearbandkeramik), the first Neolithic culture in Central Europe and Transdanubia (*75*), it is of interest to study whether these Vinča individuals genetically related to farmers from the presumed core zone (western Anatolia) and European early farmers to the north of the site (the Starčevo and LBK farmers from Central Europe and Transdanubia) or if they were related to the local hunter-gatherers from the Danube Gorges.

Table 4: *Vinča samples successfully analysed in this study.*

Sample name	Site	Individual	Period
Vc1	Vinča	2	Neolithic
Vc3	Vinča	5	Neolithic
Vc2	Vinča	4	Neolithic
Vc4	Vinča	6	Neolithic
Vc5	Vinča	7	Neolithic
Vc6	Vinča	8	Neolithic
Vc8	Vinča	10	Neolithic
Vc10	Vinča	12	Neolithic

2.2.6 Grivac

This well-stratified Neolithic site in central Serbia contains both proto-Starčevo and Vinča layers (*132*, *133*) and, for the purposes of this study, we opted to group the one Grivac sample successful in DNA extraction (burial 1 coded in this study as Gri1) with the Vinča-Belo Brdo samples from the Starčevo level.

2.2.7 Sultana Malu Roşu

The most recent individuals included in this study were from Sultana Malu Roşu (two samples from grave 1 and 2 coded as Smr1 and Smr2, respectively). This Eneolithic site is located on the bank of the Mostiştea River, about 15 km from the Danube (approximately 500 km from the Danube Gorges) (*134*). The community living at this site used the same cemetery as the one at Sultana Gheţărie belonging to the Boian culture (5,000-4,500 BC) (*135*), while Sultana Malu Roşu settlement itself is considered one of the most important sites of the Gumelniţa culture (4,500-3,700 BC) (*135*), which was known to have occupied a vast region that spanned from central Bulgaria and along the Black Sea coast to Thrace (*134*).

2.3 Neolithic in NW Anatolia

In Northwest Anatolia, the first known Neolithic sites appeared in the Marmara region, the oldest date being known from Barcın site (*136*). The distinct local Neolithic characteristics in the Marmara region are encapsulated by the term "Fikiterpe culture", coined by Kurt Bittel, one of the excavators of the eponymous site (*137*).

With respect to the arrival of the Neolithic way of life, Brami (*138*) has identified a 2,000-year long chronological gap in Neolithic occupation between central and western Anatolia, and suggested that central Anatolia formed a western border of the primary zone of Neolithic formation, while Neolithic innovations were introduced to western Anatolia later, already in a well established form (*67*). This confirms the previous notion that western Anatolia became a contact zone during the first stages of Neolithisation and later became a core zone for the further spread of the Neolithic lifestyle to Europe, while the contact barrier moved to Southeast Europe (*68*).

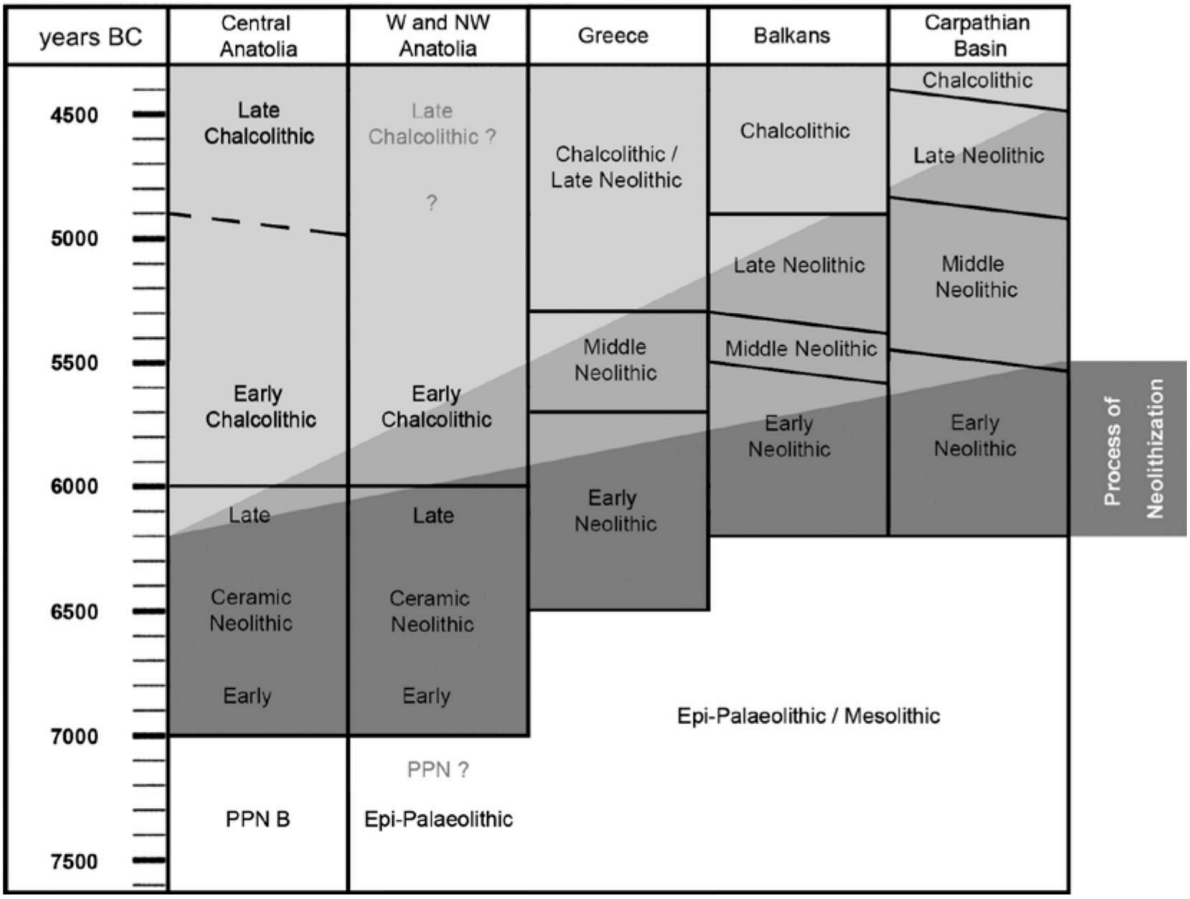


Figure 6: *The chronological chart for the areas relevant for this study (adopted from 139)*

The chronological schema of the Anatolian Plateau was applied as described by Baird (*140*): the periods of Neolithic (∼7,000-6,000 cal BC), Early Chalcolithic (∼6,000-5,400 cal BC), Middle Chalcolithic (∼5,400-4,500 cal BC) and Late Chalcolithic (∼4,500-3,200/3,000 cal BC) were discussed further in this study. It should be noted that in NW Anatolia some sites/phases dated to Early Chal-

colithic are referred to as Late Neolithic (*140*). The relationship between chrological periodisations of different areas studied is visualised in Figure 6 (though it should be noted that new excavations place the earliest Neolithic occupation in southern Greece to ∼6,700 cal BC (*70*)).

In contrast to other regions in western Anatolia (the Lake District and Aegean coast of Anatolia), burials are known primarily from the Marmara region (*102*). The reasons for which there are almost no burials and cemeteries elsewhere are not known. While it is possible that they will be found eventually, burial practices leading to the absence of discovered burials might be one of the reasons (*67*, p. 227). At present, NW Anatolia is the only possible source of population genetic information for Neolithic in western Anatolia.

2.3.1 Aktopraklık

Aktopraklık is a flat inland Fikiterpe site about 25 km from the city of Bursa (Marmara region) and its excavation showed uninterrupted occupation from the middle of the 7th millennium BC to the middle of the 6th millennium BC (*141*). Site Aktopraklık C served as a settlement (with associated human remains) during the earlier phases and as a cemetery during the later phases (the settlement moved to Aktopraklık B in Chalcolithic) (*141*). Therefore given the context of the site, one cannot formally discount that some graves assigned to early phases of Aktopraklık C were intrusive and only direct dating of the bones can confirm their belonging to the early cultural horizon (*67*). For this reason, and because we obtained reliable ancient DNA from only a small number of samples from the uninterrupted occupation of Aktopraklık (see Table 5), we have analysed the Aktopraklık samples as one group when grouping of samples was necessary (for calculation of mtDNA distances, section 3.11).

It should, however, be noted that only the Akt6 sample (17H 50.1) was directly assigned to the later phase of Early Chalcolithic (*142*). Two Neolithic samples from the same grave (Akt16 and Akt18, 89D 14.1 and 89D 17.1 respectively) were included in the analysis (*143*).

Table 5: *Aktopraklık samples successfully analysed in this study. The anthropological sex and age assignment can be sourced in Alpaslan-Roodenberg (143). Question marks denote uncertainty of the assignment due to the fragmentary state of the human remains.*

Sample name	Site	Individual	Sex	Age	Period
Akt16	Aktopraklık	89 D 14.1	m	-	Neolithic
Akt17	Aktopraklık	89 D 15.1	f?	25-35	Neolithic
Akt18	Aktopraklık	89 D 17.1	m	Mid-old age	Neolithic
Akt20	Aktopraklık	89 E 9.3	-	∼3	Neolithic
Akt26	Aktopraklık	90 D 4.4	f	35-45	Neolithic
Akt6	Aktopraklık	17H 50.1	m	35-45	Chalcolithic

2.3.2 Barcın Höyük

The Barcın site was occupied without interruptions between 6,600-6,000 cal BC (*136*) and shows continutity from pre-Fikiterpe to Fikiterpe horizons of the Neolithic period (Gerritsen in *51*). While the site shares common elements with other Fikiterpe sites in the Marmara region, there were differences in architecture and dietary habits noted between flat sites (e.g., Aktopraklık) and tell sites (e.g., Barcın) (*141*). This site is the oldest known Neolithic occupation in NW Anatolia (*136*) and from the start, the food economy was fully agrarian with absence of an earlier transitional phase from foraging to farming (Gerritsen, Galick in *51*, *144*, respectively). The dead were buried at several locations within the settlement: neonates and infants were buried within the houses, generally next to the walls, whereas juveniles and adults were buried in the central courtyard. The individuals were placed in primary single burials (*145*). Individuals that were successfully analysed in this study can be seen Table 6.

It should be noted that settlement on this twin mound resumed in the Late Chalcolithic period (∼4,500-3,200/3,000 cal BC), probably relatively shortly at the beginning of the fourth millennium BC (*146*). In the following eras, there were brief periods of occupation separated by long periods without any evidence for habitation or other activities (the Bronze Age, the Iron Age, the Roman/Hellenistic and Byzantine phases were additionally defined at the site) (*136*). A low saddle (of cca 300 m length) between the central points of the two parts of the twin mound served as a Byzantine graveyard during the later occupation (*147*). One sample from the Byzantine period was analysed together with the Neolithic samples (see Table 6).

Table 6: *Barcın samples successfully analysed in this study. The anthropological sex and age assignment can be sourced in Alpaslan-Roodenberg (145). Question marks denote uncertainty of the assignment due to the fragmentary state of the human remains.*

Sample name	Site	Individual	Sex	Age	Period
Bar3	Barcın	M10 / 166	-	6	Neolithic
Bar4	Barcın	L13/ 129	m	old adult	Neolithic
Bar6	Barcın	M10 / 173	f	old age	Neolithic
Bar7	Barcın	M 10 / 101	-	-	Neolithic
Bar8	Barcın	M 10 / 106	f?	25-35	Neolithic
Bar9	Barcın	M 10 / 115-b	f	Mid-old age	Neolithic
Bar11	Barcın	M11 / 93	m	35-45	Neolithic
Bar13	Barcın	M10 / 102	f?	Mid-old age	Neolithic
Bar15	Barcın	M10 / 115	f?	25-35	Neolithic
Bar16	Barcın	L10 / 187	-	-	Neolithic
Bar20	Barcın	M11S / 401	-	-	Neolithic
Bar31	Barcın	L11W / 546	-	-	Neolithic
Bar14	Barcın	L 12 / 49	-	-	Byzantine

2.3.3 Catalhöyük

The central Anatolian site of Catalhöyük became only the 11th site in Turkey to be inscribed in World Heritage List (UNESCO) in 2012, reflecting its uniqueness in terms of studying the Neolithic period in the region (*148*). We were working with samples from Catalhöyük East from the TP (Team Poznań) area, which can be dated to approximately the end of the seventh millennium BC (*149*) and they are therefore relatively contemporaneous with the samples we obtained from NW Anatolia.

The burial chamber (number 6000) in space 327 contained at least eight individuals and a number of disarticulated bones (*150*). These separate skeletal elements were labelled consecutively as 15839 and DNA from two of such individuals, B27 and B70(78) (Ch51 and Ch54, respectively) was successfully extracted.

2.4 Isotopic analysis

The samples from NW Anatolia and even more so those from the Danube Gorges have been subjected in the past to systematic isotopic analyses and radiocarbon dating (e.g., *94*, *142*). The ^{14}C dates for individuals that were analysed palaeogenetically (*94*, *123*, *131*, *133*) were assigned to the same chronological periods as described in the preceding sections (see Table 8 for dates obtained in previous studies and the respective periods).

Furthermore, samples that were chosen for costly genomic analysis and nuclear target enrichment procedures were dated separately for the purposes of this study. The successfully dated samples are reported in Figure 7 and Table 7. The obtained dates were calibrated with OxCal v4.2.4 using INTCAL13 to a 95.4% calibrated range.

In the Danube Gorges, the situation is complicated by a known reservoir effect that inflates the obtained dates in relation to a freshwater diet (*115*). The correction for fresh water reservoir effect (FRE) was made according to Method 2 of Cook *et al.* (*96*) utilising the percentage of nitrogen (^{15}N) to estimate a percentage of aquatic diet for the individuals. Nitrogen values for the corrections were obtained from the literature (*94*, *151*), except for one sample (Lepe45, grave 91), for which both the uncorrected date and a date with 100% FRE correction (i.e., 400 years closer to the present) are provided, see Table 7).

Mesolithic and Neolithic samples from the Danube Gorges were studied by Borić & Price (*94*) for their ^{87}Sr/^{86}Sr ratio in tooth enamel that forms in childhood (the strontium values do not change during life). By comparing results from local archaeological fauna and the geological background to the values obtained from human remains, Borić & Price (*94*) identified non-local individuals with values lower than 0.7085 and above 0.7100 (see Table 1 and 2). It should be noted that the local range of values was set experimentally and it could change with additional results. However,

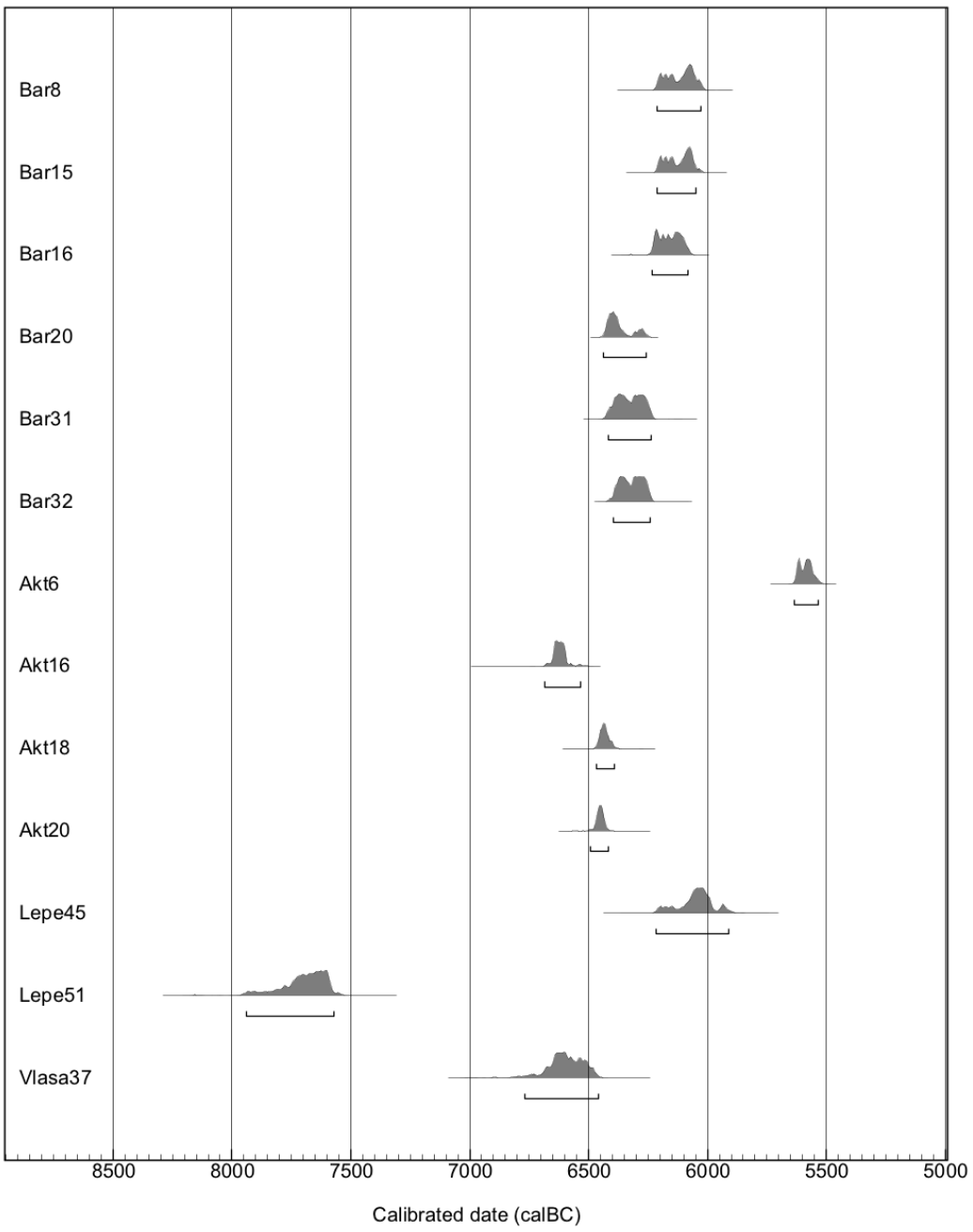


Figure 7: *Calibrated dates obtained for this study. Samples from the Danube Gorges were corrected for FRE (see details in Table 7).*

Table 7: *Calibrated dates obtained for this study. Samples Lepe51 and Vlasa37 were corrected with 100% offset from Method 2 of Cook et al. (96). For Lepe45, the correction was identically applied but recorded in grey (no ^{15}N values available). Akt18 sample is also recorded in grey as the collagen content (0.2) was too low to obtain reliable date (according to CEZ Archäometrie gGmbH, Mannheim).*

Sample	**Site**	**Individual**	**From cal BC**	**To cal. BC**	**^{14}C date uncal. BP**	±	**Sample ID**
Bar8	Barcın	M 10 / 106	-6212	-6030	7238	38	UBA-29837
Bar15	Barcın	M10 / 115	-6213	-6049	7245	29	MAMS25483
Bar16	Barcın	L10 / 187	-6233	-6084	7315	31	MAMS25484
Bar20	Barcın	M11S / 401	-6438	-6258	7499	30	MAMS25479
Bar31	Barcın	L11W / 546	-6419	-6238	7457	44	UBA-29838
Bar32	Barcın	L11 / 604	-6396	-6241	7447	30	MAMS25485
Akt6	Aktopraklık	17 H 50.1	-5633	-5535	6659	29	MAMS25474
Akt16	Aktopraklık	89 D 14.1	-6683	-6533	7792	28	MAMS25475
Akt18	Aktopraklık	89 D 17.1	-6469	-6393	7567	33	MAMS25476
Akt20	Aktopraklık	89 E 9.3	-6493	-6418	7602	30	MAMS25477
Lepe45	Lepenski Vir	91	-6215 -6588	-5911 -6395	7608	49	UBA-29841
Lepe51	Lepenski Vir	68	-7940	-7571	9092	59	UBA-29842
			^{15}N for correction from (*151*)				
Vlasa37	Vlasac	24	-6767	-6461	8210	48	UBA-29840
			^{15}N for correction from (*94*)				

the hypothesis that these individuals were migrants to the Danube Gorges region could be tested genetically.

Isotopic values were also important in studying the diet of the ancient individuals and they provided information about strong reliance of the Danube Gorges populations on fresh water fishing (*151*) and increasing differences between individuals in diet during the Final Mesolithic period (*151*, *152*). By contrast, the homogeneity of the isotope values and the high reliance on terrestrial resources was observed in Aktopraklık, even though freshwater and marine sources were in close proximity (*142*). These data from Northwest Anatolia also contrast interestingly with archaeologically detected reliance on maritime subsistence on the Aegean cost of Anatolia (*153*).

Table 8: *Calibrated dates for samples from this study obtained in previous studies.*

Sample name	**Site**	**Grave**	**Calibrated date (95.4% range)**	**Period**	**Source**	**Sample ID**
Vlasa7	Vlasac	31	6,823-6,436 cal BC	Late Mesolithic	(*123*)	AA-57777
Vlasa1	Vlasac	2	6,775-6,471 cal BC	Late Mesolithic	(*123*)	OxA-16541
Associated with Lepe37 & Lepe38	Lepenski Vir	79/a	6,372-6066 cal BC	Transition	(*94*)	OxA-11705
Lepe27	Lepenski Vir	54e	6,356-6064 cal BC	Transition	(*94*)	OxA-11697
Lepe28	Lepenski Vir	54d	6,000-5840 cal BC	Transition/ Neolithic	(*94*)	AA-57783
Lepe44	Lepenski Vir	89/a	6,210-5898 cal BC	Transition	(*133*)	OxA-11702
Lepe48	Lepenski Vir	122	6,208-5987 cal BC	Transition	(*94*)	OxA-16005
Lepe15	Lepenski Vir	26	6,078-5880 cal BC	Transition	(*94*)	AA-57782
Lepe20	Lepenski Vir	32a	6,076-5731 cal BC	Neolithic	(*94*)	OxA-5828
Lepe6	Lepenski Vir	8	6,016-5841 cal BC	Neolithic	(*94*)	AA-58319
Lepe52	Lepenski Vir	73	6,005-5845 cal BC	Neolithic	(*94*)	BA-10652
Lepe11	Lepenski Vir	19	5,983-5756 cal BC	Neolithic	(*133*)	OxA-16008
Lepe1	Lepenski Vir	35	5,748-5475 cal BC	Neolithic	(*133*)	OxA-5829
Vc1	Vinča	2	5,476-5,304 cal BC	Neolithic	(*131*)	OxA-15996
Lepe2	Lepenski Vir	2	4,361-4070 cal BC	Sălcuţa culture	(*94*)	OxA-11719

3 Methods

Three different strategies were applied to gain data from ancient human remains. The samples of the highest quality were chosen for the whole genome sequencing. The aim was to obtain at least two genomes of differing ages from each geographical region. A total of five samples were selected for this approach, from NW Anatolia Bar8 (6,212-6,030 cal BC) and Barc31 (6,419-6,238 cal BC), and from the Danube Gorges Vlasa37 (6,767-6,461 cal BC), Lepe51 (7,940-7,571 cal BC) and Lepe45 (6,588-6,395 cal BC, with the presumed FRE: 6,215-5,911 cal BC).

The second strategy was nuclear target enrichment, which results in high coverage nuclear data for the selected genomic regions. A total of 20 high quality samples, five from each major site (Aktoprakık, Barcın, Lepenski Vir and Vlasac, see Table 10), were carefully selected for this novel approach.

Population genetic data in the form of high coverage mitogenomes were obtained from samples with relatively low DNA quality by means of mitochondrial target enrichment (see Table S1). Additionally, the same data were gained as by-products of the previous two strategies. This resulted, after various discarding procedures, in 86 analysed samples.

3.1 Sample Preparation

The samples (Tables 9, 10 and S1) were analysed in the ancient DNA facilities of the Palaeogenetics Group, Institute of Anthropology, Mainz, following and further improving guidelines for good practices in ancient DNA analysis (*47*).

Details of the decontamination procedures and sample preparation of bones and teeth were described previously by Kreutzer *et al.* (Supplementary Section SI2 in *51*) and Scheu *et al.* (*154*). Prior to analysis, the bone samples were decontaminated (UV irradiation on both sides for 30–45 min) and photographed in detail, because laboratory procedures are by nature destructive and potential information from the skeletal remains can be lost. Generally, two sources of bone material were used – tooth roots and inner cores of petrous bones (Tables 9, 10 and S1).

To extract the inner section of the petrous bone, which was expected to contain a high amount of endogenous DNA (*7*), the petrous bone was separated from the temporal bone at the base of the petrous part (at the point where it fuses with the squamous and mastoid part). The outer surface of the petrous bone was removed with a saw (Electer Emax IH-300, MAFRA) in order to identify the densest parts of this bone fragment. All parallel canals, fossa, sinuses and canalliculi were cleaned by sandblasting (P-G 400, Harnisch & Rieth, Winterbach, Germany). The densest inner parts of the petrous bone were sawn into small cubes and UV-irridiated for 30 minutes per side before being milled into fine powder (MM200, Retsch).

The tooth root was cut away with a saw (Electer Emax IH-300, MAFRA) from the tooth crown at the cemento-enamel junction after dirt was removed from the tooth via sandblasting (P-G 400, Harnisch & Rieth, Winterbach, Germany). To avoid any further use of a heat-inducing saw, the complete tooth root was then UV-irradiated for 30 minutes and directly pulverised into powder (MM200, Retsch) to obtain material from the dentine and also from the DNA-rich cementum (*155*).

Milling controls as described in Scheu *et al.* (*154*) were processed in parallel to control for the decontamination procedure of the devices used. These controls were treated as samples for all subsequent steps, including extraction, library preparation and quantification.

3.2 Extraction

DNA extraction was performed similarly to Scheu *et al.* and Hofmanová *et al.* (*51*, *154*). During the lysis step, EDTA (5ml-15ml, 0.5M, pH8; Ambion/Applied Biosystems, Life technologies, Darmstadt, Germany), N-laurylsarcosine (250μl, 0.5%; Merck Millipore, Darmstadt, Germany) and proteinase K (30μl, 18Uμl; Roche, Mannheim, Germany) were mixed with the powdered sample. The lysis solution was incubated on rocking shakers at 37°C until the powder was dissolved and the DNA was released from the bone matter. The EDTA volume and incubation time was adjusted to the amount and density of the bone/tooth powder (200-810 mg). The DNA was isolated via phenol/chloroform/isoamyl alcohol (25:24:1, Roth, Karlsruhe, Germany) extraction, then desalted by means of stepwise washes with HPLC-water (5-20 ml; for one extract of Vlasa2, Vlasa4, Vlasa7 and Vlasa20, the wash volume was increased due to remaining dirt particles and suspected inhibition up to 45). Finally, it was concentrated to approximately 200μl using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore, Darmstadt, Germany).

Experimentally, a pre-lysis step was performed for 12 samples (1-5 from each site of Padina, Lepenski Vir, Vlasac, Barcın, Aktopraklık and Vinča) in order to increase the percentage of endogenous yields. Prior to lysis, the milled powder was incubated for 1 hour in 1-4 ml of EDTA (0.5M, pH8) and afterwards the supernatant was analysed separately, while the remaining pallet underwent normal lysis (see above). However, this pre-lysis procedure did not result in a remarkably increased number of endogenous molecules (see *156*, *157*) and in some cases (Pad11, Vc10, Lepe2, Lepe20) the pre-lysis supernatant contained enough DNA to be used for further analysis (mitochondrial target enrichment). Since target enrichment of both nuclear and mitochondrial DNA was the main goal for most of the samples, the pre-lysis was not applied to other samples as it would have been an additional step that could potentially result in contamination or loss of endogenous molecules that could be further captured.

To detect contamination and increase the DNA yields, several extractions were performed for each sample whenever possible (see Tables 9, 10 and S1). Blank controls were processed with every extraction and incorporated into all further analysis steps.

3.3 Library preparation

Libraries were prepared according to the protocol of Kircher *et al.* (*158*) with slight modifications as in Hofmanová *et al.* (*51*). Prior to the library preparation itself, USER^{TM} (NEB) treatment of the DNA extract was performed for ∼200 libraries (i.e., UDG treatment, see Tables 9, 10 and S1): 40 μl of DNA extract and 10 μl USER^{TM} was incubated for three hours. Inactivation of the enzyme was unnecessary, because that was achieved in the first step of library preparation (blunt-end repair). The rest of the libraries were untreated and full damage patterns were used to authenticate samples.

Blunt-end repair should eliminate 5' and 3' overhangs of DNA molecules and thus prepare the naturally fragmented molecules for ligation. Hybridised adapters P5 and P7 (IDT, Leuven, Belgium) were ligated to the ends of blunt-ended molecules in the ligation step (the adapter mix concentration of 2.5 μM was lowered to 1.25 μM and eventually to 0.75 μM to decrease adapter dimers appearing in samples with a low molecule count). After the adapter fill-in step (adapters have overhang ends to minimise the loss of molecules by allowing their ligation only at the blunt-ended side), amplifications of all libraries were performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystems) in 3-6 PCR parallels and primer concentration was lowered from 200 nM to 100 nM to avoid primer dimers. The number of cycles varied highly (8-27) because they were adjusted to the known or presumed quality of the sample and to the application (the lowest possible cycle number was chosen for genomic analysis, while a higher molecule count was preferable for target enrichment). Prior to target enrichment, additional reamplification was performed with Herculase II Fusion® DNA Polymerase to obtain enough DNA for hybridisation. Indices on both sides of the library molecule were added simultaneously during the initial amplification before the sample left the pre-PCR lab. Double indexing was performed according to Kircher *et al.* (*158*) and additional index sequences from the Nextera^{XT} index Kit v2 (Illumina) were added to increase the number of available combinations. Purification during library preparation was conducted using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and amplified libraries were purified with MSB ® Spin PCRapace (Invitek, Stratec Molecular, Berlin, Germany). Library concentrations were measured by Qubit® Fluorometric quantitation (dsDNA HS assay, Invitrogen) and fragment length distributions of libraries were estimated on the Agilent 2100 Bioanalyzer System (HS, Agilent Technologies) following the manufacturer's protocols. Occasional primer dimers of <100 bp length were removed prior to sequencing by additional purification with Agencourt® AMPure® XP beads (Beckmann Coulter).

Blank controls were processed with every library step and each PCR. In order to verify and relatively quantify the success of the library preparation, nonsense hybrids of known concentration were also processed alongside each reaction.

3.4 Quality assessment of samples

To divide samples for the differing approaches, we investigated the DNA quality of the extracts, mainly the endogenous DNA content. DNA extraction does not discriminate between the DNA of the individual whose skeletal remains are being investigated and DNA molecules that come from exogenous sources (e.g., bacterial DNA from the soil). Since human DNA does not chemically differ from the DNA of contaminants, the level of its presence was ascertained by means of shallow DNA screen sequencing.

These screening runs were performed on Illumina Miseq (50bp read length, single end) to evaluate each extract. Reads were processed as described in section 3.10. The endogenous percentage was calculated as the ratio of unique reads aligning to GRCh37/hg19 of the total number of reads passing the initial quality filters (see section 3.10). The duplication rate (difference between unique reads and all reads aligned to GRCh37/hg19) was also considered in the sample assessment, because a high duplication rate leads to an underestimation of the endogenous content in the calculation. More importantly, a high duplication rate suggests low complexity, and therefore low quality of the sample, and it is by itself very informative. However, even if the ratio of unique human sequences in the total of DNA extracted from a sample was high, the endogenous DNA content could still have been relatively low compared to other samples if the total number of molecules present in the extract prior to amplification was low.

3.5 Sex determination

The Miseq screen reads were used to determine the sex of the individuals by comparing a number of reads mapping to Y chromosome and a total of reads aligning to both allosomes. The applied approach of Skoglund *et al.* (*159*) allowed not only for sex determination in most cases but also for the calculation of confidence intervals (CI). If the CI falls above 0.075 or below 0.016, the sex is confidently assigned as male or female, respectively. It should be noted that the border values were obtained experimentally (*159*). The results were plotted in R.

3.6 Quantitative real-time PCR

The number of molecules in a library prior to amplification was estimated by quantitative real time PCR (qPCR) as described previously (*37*, *51*, *157*). qPCR was performed on aliquots of library fill-in products with KAPA Sybr Fast Universal Mastermix (PeqLab, VWR International) on a Step One Plus^{TM} Real-Time PCR system (Applied Bisosystems, Thermo Fisher Scientific) with primer pair IS7/IS8 (*37*) as set up by Kreutzer *et al.* (Supplementary Section SI2 in *51*). An artificial library molecule of 89bp was used as a synthetic standard (*160*). Bionalyzer (HS, Agilent Technologies) measurement was used to determine the fragment lengths of each library to correct

Table 9: *Details on sample preparation for genome analysis.*

Sample name	**Site**	**Skeletal element**	**N. of extractions**	**N. of libraries**	**N. of UDG libraries**
Bar8	Barcın	petrous bone	4	23	12
Bar31	Barcın	petrous bone	2	8	2
Lepe45	Lepenski Vir	petrous bone, tooth	3	17	14
Lepe51	Lepenski Vir	petrous bone	2	13	11
Vlasac37	Vlasac	petrous bone, tooth	6	31	15

resulting molecule numbers. The molecule numbers helped to determine how many independent libraries per sample should be pooled for subsequent experiments in order to obtain enough unique reads for sequencing (sequencing of duplicates does not provide any additional information and it is very costly).

3.7 Whole-genome sequencing

The complete DNA information obtainable from any sample is a whole genome sequenced to high coverage. This is a challenge for ancient DNA samples and in order to produce medium coverage genomes, we prepared additional libraries for the selected high quality samples (Bar8, Bar31, Lepe45, Lepe51, Vlasa37). For this purpose, decreased adapter (0.75 μM) and primer (100 nM) concentrations were used, the cycle number during PCR amplification was lowered (as low as possible for the library to be still detectable at Bionalyzer measurements) and the number of PCR parallels was increased from three to six.

Portions of the extracts were treated with the USER^{TM} enzyme before library preparation (i.e., UDG treatment; see Table 9). The selected samples were sequenced on Illumina Hiseq2500 (either a 100bp paired-end or single-end run) and Illumina NextSeq (75bp paired-end). Bar8 was sequenced on six lanes (one lane was paired-end), Bar31 on three lanes (all single-end), Vlasa37 on five lanes (one paired-end) and Lepe45 and Lepe51 on two lanes each (all single-end). For additional details regarding library preparation and sequencing, see Winkelbach and Hofmanová *et al.* (*51*, *157*).

3.8 Nuclear capture

Nuclear target enrichment (i.e., nuclear capture) aimed at obtaining accurate continuous stretches of DNA (as opposed to high amounts of low-coverage isolated SNPs (*8*)) was attempted on the ancient DNA samples of this age for the first time. Therefore, only the best samples from each site were selected and high complexity was ascertained by producing 6-10 libraries for each sample prior to the capture (see Table 10). Adjustments to the library preparation of nuclear capture samples were made similarly as for whole genome samples (see section 3.7).

Table 10: *Details on sample preparation for nuclear target enrichment.*

Sample name	**Site**	**Skeletal element**	**N. of extractions**	**N. of libraries**	**N. of UDG libraries**
Akt6	Aktopraklık	petrous bone	2	10	8
Akt16	Aktopraklık	petrous bone	2	10	8
Akt18	Aktopraklık	petrous bone	2	10	8
Akt20	Aktopraklık	petrous bone	2	10	8
Akt26	Aktopraklık	petrous bone	2	10	8
Bar11	Barcın	petrous bone	2	9	7
Bar15	Barcın	petrous bone	2	9	7
Bar16	Barcın	petrous bone	2	9	7
Bar20	Barcın	petrous bone	2	9	7
Bar32	Barcın	petrous bone	2	9	7
Lepe18	Lepenski Vir	petrous bone	2	7	5
Lepe39	Lepenski Vir	petrous bone, tooth	4	8	4
Lepe46	Lepenski Vir	petrous bone	2	7	4
Lepe52	Lepenski Vir	petrous bone	2	7	5
Lepe53	Lepenski Vir	petrous bone	2	8	5
Vlasa4	Vlasac	petrous bone	3	7	4
Vlasa10	Vlasac	tooth	3	8	5
Vlasa32	Vlasac	petrous bone	2	6	4
Vlasa41	Vlasac	petrous bone	2	7	5
Vlasa44	Vlasac	petrous bone	2	7	4

Majority of nuclear regions for target enrichment were chosen by Krishna Veeramah (*161*) from 37,574 regions previously identified as neutral (*162*) and overlapping with NRE, Neutral Region Explorer tool (*163*), with additional potentially problematic regions removed (e.g., regions with a background selection below 0.85, a recombination rate of <0.01cM and >10cM, extreme diversity values, closeness of <0.01cM from genes). This resulted in 4,653 neutral regions of 1,000 bp length. Approximately 529 neutral regions of 500 bp length and 496 phenotypic markers were added and the fivefold RNA baits tiling design was prepared by Sell (*164*) and Strobel (*165*). In this work, only SNPs overlapping with the reference dataset by Haak *et al.* (*8*) were further analysed because all the information contained will be further utilised for other projects when comparable data from other regions are available.

In-solution target enrichment (*166*) of these regions was conducted with a custom design MYbaits® Sequence Enrichment kit according to instruction manual v.3, with several modifications established by Melanie Strobel: adapter blockers were designed around index sequences for the reverse strand only, PCR in three PCR parallels was performed after capture on-bead with Herculase II Fusion® DNA Polymerase and purification was performed after PCR with MSB® Spin PCRapace (Invitek, Stratec Molecular, Berlin, Germany).

To obtain adequate coverage for the captured regions, a reaction was repeated on the already enriched sample for the second time ("double capture"). Only the cycle number (adjusted to the samples) differed between the first and second capture reaction (it was higher after the first capture to produce enough molecules for the second capture and to limit the duplication rate prior to sequencing). During the washing steps, DNA that was not hybridised to the beads was not discarded as stated in the manual, but this supernatant was kept and processed for mitochondrial capture. For additional details regarding nuclear capture of these samples, see Winkelbach (*157*).

3.9 Mitochondrial capture

As in Kreutzer *et al.* (Supplementary Section SI2 in *51*), we performed whole mitochondrial genome enrichment of the selected samples with Agilent's SureSelect^{XT} in-solution target enrichment kit (custom design) (*166*). The number of libraries pooled for a capture depended on the quality of the samples (see Table S1) and availability of further extracts. Supernatants from nuclear captures were purified with QIAquick PCR Purification Kit (QIAGEN®) and processed as a normal library pool.

120bp RNA baits covering the whole mitochondrial genome were built with eightfold tiling from the main mitochondrial haplogroups given by Phylotree 8 (mtDNA tree built14; 5. April 2012, *167*) by Kreutzer *et al.* (Supplementary Section SI2 in *51*). To ensure good coverage in the control region, additional baits (tenfold tiling) for this region (15900-16569 & 1-600) were designed for 18 different haplogroups and additional baits were introduced manually to close the circular structure of the mitochondrial genome. To correct for GC bias, extra baits for GC-low regions of the target sequence were introduced (*51*).

Capture reaction itself was performed according to a modified version of the manufacturer's protocol. The modifications were the same as for nuclear capture (see section 3.8). Additionally, the washing temperature was set to 57°C (*51*, *168*) and RNA-baits were diluted to allow for more reactions (the ancient DNA samples do not contain enough DNA to saturate the baits and the baits can therefore be used in lower concentrations). Additional hybridisation and washing buffers were prepared in-house (*51*, *169*). Double capture was also performed on the samples in order to increase the number of reads on target. For additional details regarding mitochondrial capture of the subset of these samples, see Schulz and Dehoust (*156*, *170*).

3.10 Read processing

Sequencing and read processing were performed almost exactly as in Sell *et al.* (Supplementary Section S3 in *51*), mostly thanks to scripts provided by Christian Sell. Illumina HiSeq 2000 runs were carried out at the sequencing facilities of the University of Mainz (Institute of Molecular Genetics, Mainz, Germany). One Illumina NextSeq run and all Illumina MiSeq runs were performed

by StarSEQ GmbH (Mainz, Germany). FASTQ files were generated and demultiplexed by the sequencing facility.

Adapter sequences were trimmed at the 3' end of each read according to Kircher (*171*), with criteria of at least 90% identity between adapter and read sequence, and a minimum adapter length of 1 bp. Reads with a base quality score ≤ 15 in more than 5% of the bases of a sequencing read were removed from the dataset (*171*). For runs with paired-end reads, a custom python script was used to order reads in pairs by their names and the ea-utils package (*172*) was applied to merge overlapping read pairs (the default parameters of ≥ 6 bp overlap and 92% sequence identity in the overlapping region).

Reads were aligned to the human reference build GRCh37/hg19 for whole-genome and nuclear capture data or to the revised Cambridge Reference Sequence (rCRS, the accession number NC_012920) for mitochondrial capture data with the default parameters by BWA aln (*173*). Duplicate reads were removed by the *MarkDuplicates* tool from the Picard tools package (picardtools, `http://broadinstitute.github.io/picard`). The Samtools package (*174*) was applied to sort and index the alignments and to merge different libraries and different runs of the same sample. Due to frequent discrepancies of short read alignments, all sequences with a length of <30 bp were removed from the alignment with NGSUtils (*175*). Local realignment was performed using *IndelRealigner* from GATK v. 3.3.0 (*176*) following the recommendations of the GATK development team. For all the libraries, damage patterns (5'- and 3'-deaminations) were obtained in MapDamage 2.0 (*177*).

Average coverage was determined in Samtools (*174*) as an average number of reads covering each base (mean depth). X-fold coverage was calculated as average coverage over the targeted region in the experiment (whole genome, nuclear regions or mitochondrial sequence) (*157*, *178*). The coverage variables were, together with results of the quality assessment (see section 3.4; endogenous content and the molecule copy number averaged over all measurements for a sample), compared in R (*179*). Standard t-test was used in case a normality was not rejected by the Shapiro-Wilk test (*180*). If the variable did not fit the normal distribution (i.e., normality was rejected), the Mann-Whitney-Wilcoxon test (*181*) was applied instead. Analogously, Spearman's rank correlation (*182*) was applied when normality was rejected to test correlations between studied variables.

3.11 Analysis of mitochondrial dataset

After the read processing (see section 3.10), mitochondrial data were further processed in GATK v. 3.3.0 (*176*). Base recalibration was performed according to the recommendations of the GATK development team using the *BaseRecalibrator* and the final variant calling was performed by the GATK *UnifiedGenotyper* (*176*) with ploidy set to 1 (*51*). Variants were then filtered for >5x coverage and >50 Phred quality. Scripts used for the data preparation described above were partially prepared by Christian Sell and Ilektra Schulz.

The consensus sequence of the complete mitochondrial genome was generated and transformed to FASTA via *samtools mpileup/bcftools/vcfutils*(*174*) with a minimum depth of 3 for a consensus call. Missing sites were replaced with "N". Haplogroups were estimated using Haplogrep 2.0 (*183*) from the final vcf files.

Consensus mitochondrial sequences were aligned with rCRS by *Clustal W* (*184*) with default parameters. The software *jailview* (*185*) was used to visually inspect the obtained sequences and cut them to the length of mitochondrial hypervariable region I (HVRI, positions 16013-16409). The HVRI sequences were realigned by *Clustal W* with default parameters to the reference panel of modern and ancient mitochondrial sequences of the same length (see Table S2, data prepared partly by Kreutzer (*186*)).

The mitochondrial population genetic analysis for both full mitogenomes and HVRI region was performed in *Arlequin 3.5* (*187*). Pairwise F_{ST} (*188*) was calculated as Slatkin's linearised F_{ST} (*189*) but Reynolds distance (*190*) was used (because it is bound between 0 and 1) for non-metric multidimensional scaling applied as *metaMDS* in R (*191*).

Mitochondrial regions were also extracted from whole-genome alignments to GRCh37/hg19 by *samtools* (*174*) and realigned against the rCRS. All further steps were identical to the data obtained from mitochondrial target enrichment. One sample (Bar32) from the nuclear target enrichment dataset was not successful in the mitochondrial capture of the supernatant (from the washing step of the nuclear capture). The mitochondrial reads for this sample were extracted from nuclear capture alignments to GRCh37/hg19 and processed with mitochondrial reads extracted from the whole genomes.

3.11.1 Contamination rate

Haploid mitochondrial data from both capture and whole-genome experiments can be used to assess the number of contaminating sequences present in a sample. We applied *contammix v. 1.0-10* (*192*) for this purpose because this likelihood-based method estimates percentages of contamination as differences in the affinity of all the mitochondrial reads from a sample to its consensus sequence and to mitochondrial genomes from diverse populations. As an additional measure, we manually investigated heterozygous (and therefore potentially contaminated) positions in the mitochondrial sequence, determined as heterozygous by GATK HaplotypeCaller (*176*). The contamination assessments were performed for each sample and library separately in order to determine if samples or libraries could be contaminated. The main criterion for exclusion was the level of authentic reads below 80% as determined by *contammix v. 1.0-10*, however for libraries or samples with low average coverage (below 5x), other contamination measures were considered with increased importance.

3.12 Y chromosomal variation

Similarly to Martiniano *et al.* (Supplementary Section SI4 in *51*), Y chromosomal lineages were investigated in nuclear data for the ancient samples that had proven to be male (see section 4.2). The software clean_tree (*193*) was applied to call alleles with *samtools mpileup* (*174*). We determined alleles at the SNP positions that were supplied with the clean_tree software (539 SNPs). The SNP positions and subsequent haplogroup determination was based on ISOGG 2013 (International Society of Genetic Genealogy; http://www.isogg.org/). We considered only the reads that had a quality above 30 and a base majority percentage of at least 80.

3.13 Nuclear data analysis

In order to make our results comparable to other recently published palaeogenomic studies, whole-genome analyses were limited to the ∼300K SNP positions utilised in Haak *et al.* (*8*). Danubian genome calls were merged with the dataset of Hofmanová *et al.* (*51*) already containing Anatolian genomic samples Bar8 and Bar31 (processed to obtain pseudo-haploid calls with a minimum quality of Q30 with a new calling method (*51*, *89*)). The ancient Danubian genomes were analysed using pseudo-haploid calls obtained via a custom script selecting a major allele for a position from reads above Q30 (courtesy of Jens Blöcher).

The capture data were processed similarly to the whole genomes from the Danube Gorges but the analysis was performed separately because far fewer SNPs were expected for joint analysis with the reference dataset. It should be noted that the high coverage capture data will be analysed in future projects to fully take advantage of the unique dataset, once comparable data from other geographical regions are produced.

3.13.1 f3-statistics

An f3 statistic is commonly notated as $F_3(C : A, B)$, where population C is examined as a *target* population for evidence of admixture with populations A and B, where a negative value indicates that C possesses ancestry from both populations. Since pseudo-haploid data were analysed, the *inbred* option was turned on when f3-statistics was applied to investigate admixture.

However, if C is chosen to be an *outgroup* population that has not experienced any post-divergence gene flow with either A or B, then the value of the f3 statistic will be a positive value proportional to the length of the shared drift path of populations A and B with C. Therefore, by fixing population A (or B) but substituting different populations for B (or A), it is possible to infer which populations (tested as B) are genetically more similar to population A based on the relative magnitudes of the f3 statistics. Under a simple three-population tree model with no post-divergence admixture, the relative f3 statistics for different B populations will be proportional to the relative population

divergence time of A and B and will be robust to differences in genetic drift that occurred after these populations diverged. However, if this simple tree model is violated, the f3 statistics will reflect a more complex demographic history of different timing of population divergence, the proportion of admixture and the population-specific drift (for example changes in N_e) that occurred during the period between divergence and the time of admixture.

The ≠Khomani San were selected as an *outgroup* because they are considered to be the most diverged extant human population, having diverged from all other modern humans at least 100kya, and are highly unlikely to have experienced substantial Eurasian admixture (*162*, *194*). However, we also tested Yoruba in the place of the ≠Khomani San and we note that our results were robust to the choice of the sub-Saharan African population that was utilised. The results are also consistent with all the levels of filtering tested and for the dataset without transitions.

3.13.2 D statistics

D statistics are commonly notated as $D(A, B : C, D)$ and provide a more model-based framework with which to investigate population similarity than *outgroup* f3-statistics, although their interpretation is also based on the extent to which populations share drift paths. If population D is chosen to be an *outgroup*, it provides a three-way test of population genetic similarity through the sum of genealogical topologies across loci. A D value of zero can be obtained in two ways. In the first, genealogical topologies are always consistent, with population A being closer to population B (i.e., population A and B form a bifurcating clade). In the second, there are balanced numbers of topologies, where A is closer to C and B is closer to C. However, positive and negative D values can be obtained only if the most common topologies are (A,C)(B,D) and (B,C)(A,D) respectively. Thus, the D statistic with an *outgroup* can be used to test whether population C is more similar to population A or B. An additional advantage of the D statistic is that it can be applied on a per sample basis (i.e., there is no need to pool multiple samples to represent a population to obtain allele frequencies). Results of f3-statistics and D statistics obtained with admixtools (*195*) for different *outgroups* or SNP positions were compared in R (*179*) with Spearman's rank correlation (*182*).

3.13.3 Admixture proportions

The nuclear (both whole genome and capture) datasets were further analysed with *ADMIXTURE* (*196*), a model-based maximum likelihood method of estimating the ancestries of individual samples. Known relatives of individuals from the reference dataset were excluded from the analysis, as in Hofmanová *et al.* (*51*) (relatives were marked in Haak *et al.* (*8*)). Similarly, SNPs that showed evidence of linkage disequilibrium were removed using PLINK (*197*). The maximum r^2 value was set to 0.5 and SNPs were analysed in sliding windows (window size of 200 SNPs, sliding 50 SNPs

per step). As *ADMIXTURE* is designed primarily for diploid genotype data, the program was run by treating each haploid allele presence call as a homozygote.

Genomic *ADMIXTURE* analysis was initially performed unsupervised and was limited to Neolithic and hunter-gatherer samples (without Danubians). The number of clusters to be estimated varied from K=2 to K=8 and the analysis for each K consisted of 100 independent runs with differing seeds. The cross-validation error (fivefold) was calculated to determine the optimal K. Results for each K were matched in *CLUMPP* (*198*) and plotted in *DISTRUCT* (*199*). Supervised *ADMIXTURE* analysis was also performed for K=2, where the allele frequencies were assumed to be known for two populations, with samples from Anatolia (Bar8 and Bar31) and Motala serving as proxies for the ancestral farmer and hunter-gatherer populations, respectively. Similarly, the analysis for K=2 to K=8 was performed for the dataset, including Caucasus hunter-gatherer (CHG) individuals (samples KK1 and SATP) from Jones *et al.* (*53*) and supervised runs were performed for K=3, with the CHG set as an additional known cluster. Additionally, the analysis for K=2 to K=8 and the supervised analysis for K=3 (supervised for CHG, Motala and Anatolian) was repeated while including Yamnaya individuals, a group that is considered to be associated with a major migration from the east during the Late Neolithic/Bronze Age period (*8*).

The capture dataset was processed identically to the genomic one but genomic samples from Danube Gorges and from Neolithic Iran (*9*) were also included. For the analysis, only hunter-gatherer (prior to the introduction of farming) and Early Neolithic samples from Spain, Central Europe, Greece, Iran and NW Anatolia were selected. Except for the 100 independent runs for each K=2 to K=8 as described above, supervised analysis was performed for K=2 (trained on Neolithic NW Anatolians and Mesolithic Vlasac hunter-gatherers as respective clusters). While the power of the *ADMIXTURE* analysis of capture dataset was depleted by the relatively low number of SNPs included, the overall results were aimed to complement the results of the D statistics.

It should be noted that, for some runs, the supervised version of *ADMIXTURE* did not output 100% assignment to a single cluster for some fixed source individuals (although the values were very close, 99%), which we believe might be due to a potential bug resulting from a rounding error. Consistent with the assumed model, in cases in which this occurred the individuals were restored to having 100% ancestry from a single cluster when plotted.

4 Ancient DNA analysis

Most of the studies of this extent follow the useful structure of strictly dividing the results and the discussion to separate parts. However, major inferences for this study were obtained via formally testing various hypotheses with D statistics, often on the basis of conclusions from the preceding tests. Full results for this method unbiased by any narrative would involve all combinations of samples tested (in the case of this reference dataset approx. 3.7×10^{10} values). The results of D statistics and, for consistency, the results of other methods in this study are therefore presented with explanations of particular conclusions similarly as customary in journals publishing studies from the field (e.g., *8*, *41*, *50*, *51*) and the discussion (section 5) is reserved for synthesis of the conclusions from various methods and datasets, placing the conclusions in the larger context and evaluating the impact of the inferences.

4.1 Evaluation of Anatolian and Danubian extraction success rate and ancient DNA quality and quantity

In this study, only successful samples are discussed in detail but it is important to state that 86 successful samples were selected from a total of 286 samples that were screened (*157*). The screening for all the samples was performed in the same way as described in section 3.1 but for diverse reasons not all the samples were selected for further analysis. The main criterion was the success of DNA extraction evaluated as successful library amplification, i.e., DNA detected after library preparation by Bioanalyzer and Qubit (at least after reamplification, see section 3.3). Samples were completely removed from further analysis only after they failed two library preparations from each of two independent extracts (when enough material was available). Additional criteria were endogenous contents, duplication rates and qPCR results, and further attempts were made to obtain DNA from sites with low numbers of analysed individuals, preferably to obtain large enough sample sizes for valid population genetic analysis. The last criterion (importance of a sample for population genetics) complicates formal evaluations of success rates between sites (as do differences in received skeletal elements available for analysis), yet it should be noted that the majority of unsuccessful samples were from Aktopraklık, followed by Barcın, whereas the Danube Gorges region provided more DNA-rich samples. That was consistent with expectations for DNA degradation based on climate (*17*) (the Anatolian climate is drier and warmer).

Irrespective of the source region, the successful sample yields from petrous bones (crudely measured here as average endogenous percentages of de-duplicated reads mapping to human genome in approximately similar ng of DNA sequenced, see Table S1) resulted in significantly higher yields than from teeth (Mann-Whitney-Wilcoxon test: W = 5450, p-value <2.2e-16), long bones (W = 320, p-value = 2.22e-04) or other bone elements (W = 512, p-value = 4.692e-06). The Mann-Whitney-Wilcoxon tests were applied because the neutrality of the variable (endogenous percentage) was rejected for each combination (e.g., Shapiro-Wilk test for endogenous percentages in petrous bones: W = 0.8845,

p-value = 2.193e-05). Thus, the success rate and endogenous content was higher from petrous bones than from other skeletal elements, including tooth roots (the various success rates of the samples are discussed further in the related works based on the same samples (*156*, *157*)).

While whole genome sequencing and nuclear target enrichment was successful for all the samples selected, some samples were discarded after mitochondrial target enrichment. The main criterion was the level of authentic reads below 80%, which was also associated with low coverages, ambiguous haplogroup assignments, low Haplogrep scores, elevated heterozygous calls and different haplogroup or sex assignments for different libraries. Again, most of the discarded samples came from Anatolia (Barcın, Aktopraklık and Catalhöyük; all the available samples from Catalhöyük were enriched for mitochondrial DNA, even if the libraries had low success rates and the discarding rate after mitochondrial enrichment was therefore higher). For two samples (Pad11 and Lepe6), it was not necessary to discard the whole sample, but when studying individual libraries only one library for each sample with problematic contamination values was identified. After this library was excluded, the samples were shown to be less contaminated (the haplogroup assignments and consensus call did not change showing that our methods were robust, even if a problematic library was present).

The contamination estimates on mitochondrial data (presented with mitochondrial results in section 4.3) indicated that the level of contamination for all the samples was low. These estimates can serve as a proxy for contamination levels for whole genome and capture data from the identical samples and libraries. Damage patterns obtained by Mapdamage2.0 (*177*) were typical of ancient DNA as C to T transitions occurred at the first base of the 5' end with a relatively high frequency; further details on the DNA quality assessment of the samples can be found in the related works (*51*, *156*, *157*). The blank controls with any measurable amounts of DNA and randomly selected blank controls from all stages of experiments were screened via shallow sequencing (see section 3.4) but no analysable data were produced, which confirmed the success of the decontamination procedures applied.

4.2 Sex determination

Sex assignments based on the ratios of Y chromosome reads in the screening results (see section 3.5) were successfully determined for 61 of the studied individuals (see Figure 8 and Table S3). It was of interest to see how they would compare with sex assignments from the skeletal remains that were available for 42 of the genetically assigned samples (*94*, *119*, *142*, *143*, *145*). 27 sex assignments based on skeletal remains were identical to the genetic results (64.29%), while 15 individuals were assigned to a different genetic sex. It should be noted that human remains from the prehistoric periods can be very fragmentary and the skeletal sex assignment was often assumed from scarce markers (the uncertainty was stated in the original articles and denoted as "considered" in Figure 8 and "?" in Table S3). The uncertain skeletal sex assignments account for eight out of 15 differing results and the concordance between the methods increases to 70.83% when the uncertain sex assignments were not considered (22 individuals were assigned sex via skeletal methods with certainty).

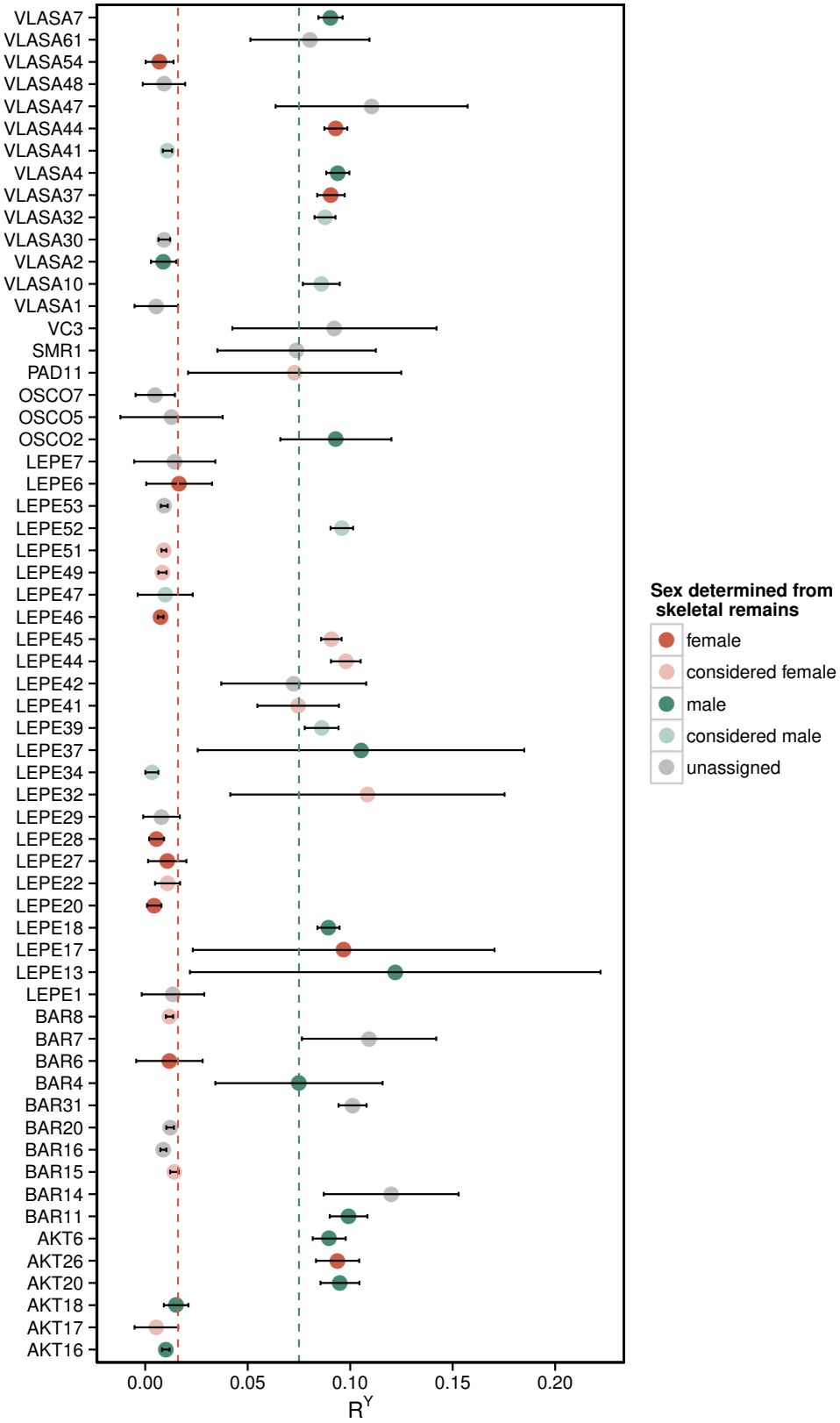


Figure 8: *Sex assignment of the studied individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown. Colours denote skeletal sex assignments (94, 119, 142, 143, 145). The full results are shown in Table S3.*

The sex of the ancient individuals, even when uncertain, is often used for interpretations. One example is the presumed difference between the frequency of females and males in the non-local category determined by different ratio of $^{87}Sr/^{86}Sr$ isotopes in human remains from Lepenski Vir site (see section 2.4, *94*). The abundance of presumably non-local females was interpreted as a possible influx of female farmers to Lepenski Vir community. However, the genetic sex of four individuals from this category was determined genetically and, while originally none was assigned as male, we were able to determine that one individual had been assigned incorrectly as female (Lepe32, grave 66). The ratio of women to men in the non-local category for our samples was still shifted towards females (3:1) but the low numbers do not allow for statistical testing. Still, given the different results of the genetic sex assignments, we advise caution when using morphological sex assignments to form such conclusions. Considering the importance of sex assignment results for the related fields, we were able to establish sex assignments also for some of individuals that were screened but not included in this study (98 additional individuals). These sex assignments are presented in Table S4 and their credibility is based on the assumption that the samples were not contaminated.

4.3 Maternal insight into population makeup of Meso-Neolithic Balkans and NW Anatolia

Before any mitochondrial haplogroup assignments are shown, it is important to state that we were acutely aware of the dangers of presenting any mitochondrial haplogroup as direct evidence of the genetic ancestry of any individual or of constructing narratives based on haplogroup assignments, a practice that has been justly criticised (*200*). Yet it has been shown that carefully considering haplogroups in certain cases can lead to hypotheses that can be further formally tested. One example is our recent work with mitochondrial genomes from the Late Neolithic cave Blatterhöhle (*80*).

4.3.1 Mitochondrial haplogroups identified for Mesolithic individuals from the Danube Gorges (∼9,500-6,200 cal BC)

U haplogroups, especially U5, were found to be very common among the pre-Neolithic individuals we examined in this study (see Table 11): ten out of 16 Vlasac Mesolithic and one out of three Lepenski Vir Mesolithic individuals were assigned to a haplogroup from U5 clade, consistently with previous studies that describe these haplogroups as characteristic for hunter-gatherers (e.g., *201*).

Still, all four samples from Ostrovul Corbului, the only sample from Padina and two out of three Mesolithic Lepenski individuals were not U5 and there were even several less expected haplogroup assignments detected for the Mesolithic individuals. K1f, a haplogroup previously identified only in Tyrolean "Iceman" Ötzi (*202*), was assigned to hunter-gatherers Vlasa10 and Vlasa37 (the latter of which was sequenced to obtain a whole genome and was ^{14}C dated to 6,767-6,461 cal BC, see Table

Table 11: *Haplogroups of ancient Balkan individuals assigned to the Mesolithic period (~9,500-6,200 cal BC). Local and non-local group assignment was based on interpretation of $^{87}Sr/^{86}Sr$ isotope ratio in Borić & Price (94).*

Sample name	**Site Date (cal BC)**	**Grave**	**$^{87}Sr/^{86}Sr$ group**	**Haplogroup**	**Score**	**Average coverage per base**	**min coverage**	**% authentic**
Vlasa1	Vlasac 6,775-6,471	2	local range	X2+225	99.4	46.13	0	98.66 - 93.76
Vlasa2	Vlasac	9	local range	U5a1c2a	96.7	99.04	4	99.98 - 99.04
Vlasa30	Vlasac	13	local range	U5b2c	97.3	122.15	14	99.17 - 97.99
Vlasa32	Vlasac	16	local range	U5a2+16294	94.7	296.85	25	99.02 - 98.19
Vlasa41	Vlasac	30	local range	U5b2b	97.8	407.51	25	98.46 - 97.58
Vlasa44	Vlasac	47	local range	U5b2b	97.8	368.57	32	98.89 - 98.01
Vlasa54	Vlasac	74	local range	U5a2+16362	97.8	123.41	6	99.61 - 98.63
Vlasa4	Vlasac	18a	local range	U5a1c2a	100	293.88	22	98.95 - 98.12
Vlasa20	Vlasac	69a	local range	U5a1c	91.2	11.10	0	99.55 - 91.74
Vlasa56	Vlasac	78a	local range	U4b1b1	98.1	42.41	0	99.8 - 98.51
Vlasa7	Vlasac 6,823-6,436	31	local range	U5a2a	95.2	172.97	12	99.59 - 98.46
Vlasa10	Vlasac	41	unknown	K1f	98.5	279.93	26	99.29 - 98.7
Vlasa48	Vlasac	52	unknown	U8b1b	97.8	115.35	2	99.71 - 98.33
Vlasa47	Vlasac	49(1)	unknown	U5a1c2a	98.4	171.96	8	99.64 - 98.32
Vlasa61	Vlasac	U64. x 11	unknown	U4b1b1	96.9	165.82	9	99.15 - 98.28
Vlasa37	Vlasac 6,767-6,461	24	unknown	K1f	93.9	57.96	0	99.98 - 99.46
Lepe49	Lepenski Vir	126	local range	H13	81.7	258.00	17	99.45 - 98.77
Lepe51	Lepenski Vir 7,940-7,571	68	unknown	U4a2	81.9	57.96	0	100 - 99.58
Lepe47	Lepenski Vir	105	unknown	U5a1c	94.5	232.16	20	99.77 - 98.81
Osco2	Ostrovul Corbului I	M2	unknown	H13	83.9	93.68	6	99.92 - 99.06
Osco5	Ostrovul Corbului II	M47a	unknown	H	100	194.32	8	99.99-99.45
Osco7	Ostrovul Corbului II	M57	unknown	H4a2	100	142.00	5	99.99-99.3
Osco6	Ostrovul Corbului II	M58	unknown	U3b	96.4	244.41	11	99.99-99.49
Pad11	Padina	30	unknown	I3a	100	52.16	3	93.35-88.02

7). The only other known example of a K1 clade in the Mesolithic context was K1c haplogroup detected in Mesolithic Greek samples Theo1 and Theo5 (7,288-6,771 cal BC and 7,605-7,529 cal BC, respectively; *51*). While similar haplogroups do not by any means suggest closeness between populations, the exceptional presence of a K1 clade could suggest that Danubians from Late Mesolithic could have some connection to a not-yet studied Mesolithic Aegean population.

H and H13 haplogroups were also identified in the Mesolithic dataset (one for Lepenski Vir, three for Ostrovul Corbului; see Table 11), but it should be noted that some of the individuals could have been misidentified in the chronological assignment (not all were dated and there are discussions about the Mesolithic/Neolithic provenance of Ostrovul Corbului samples, see section 2.2.4). Yet the diverse Mesolithic haplogroups (additionally to the previously described, the Ia3 haplogroup in Padina and

X2 haplogroup in Vlasac) could mean that the Mesolithic population on the border between central Europe and the Aegean might have been of a more complex ancestry than populations of similar age in northern and central Europe (*64*) and that the pooling of the Mesolithic samples for the analysis of genetic distances should be considered carefully.

4.3.2 Mitochondrial haplogroups identified in the Transition period (~6,200-6,000/5,950 cal BC) of Lepenski Vir

We obtained enough data to assign haplogroups to a total of 32 individuals from Lepenski Vir, 17 directly from a period of Meso-Neolithic Transition (abbreviated as "Transition", see Table 12). The haplogroups showed a mixture of both hunter-gatherer-like (seven U5) and farmer-like (seven of K1a, K1b, T2e, N1a and J2b) haplogroups (*33*, *34*, *201*). Interestingly, the isotopically identified non-local individuals Lepe27 (6,356-6,064 cal BC; *94*) and Lepe48 (6,208-5,987 cal BC; *94*) possessed the rather farmer-like K1a1 and J2b1d haplogroups, respectively. The six individuals identified as local in the Transition period did not show hints of belonging to only Mesolithic hunter-gatherer U haplogroups (also farmer haplogroups were present). This would be logical if the non-local individuals had descendants born in the Danube Gorges and therefore with the local isotopic signature.

Table 12: *Haplogroups of ancient Balkan individuals assigned to the Transition period (~6,200-6,000/5,950 cal BC). Local and non-local group assignments were based on interpretation of $^{87}Sr/^{86}Sr$ isotope ratio in Borić & Price (94).*

Sample name	Site Date (cal BC)	Grave	$^{87}Sr/^{86}Sr$ group	Haplogroup	Score	Average coverage per base	min coverage	% authentic
Lepe13	Lepenski Vir	21	local range	X2b	95.7	178.67	9	99.99 - 99.51
Lepe15	Lepenski Vir 6,078-5,880	26	local range	H5	100	242.52	14	100 - 99.58
Lepe17	Lepenski Vir	27/b	local range	N1a1a1b	92.6	93.28	4	99.41 - 98.15
Lepe23	Lepenski Vir	43	local range	K1b1b1	100	171.06	8	100 - 99.7
Lepe28	Lepenski Vir 6,000-5,840	54d	local range	K1b2	97.4	184.47	6	99.63 - 98.9
Lepe42	Lepenski Vir	87/1	local range	U5a2d	98.6	170.02	5	99.81 - 98.57
Lepe27	Lepenski Vir 6,356-6,064	54e	non-local range	J2b1d	97.1	224.21	11	99.23 - 98.21
Lepe48	Lepenski Vir 6,208-5,987	122	non-local range	K1a1	100	173.55	8	99.63 - 98.7
Lepe7	Lepenski Vir	11	unknown	K1a3a3	100	104.06	6	99.6 - 98.66
Lepe53	Lepenski Vir	27	unknown	U5b2c1	100	471.89	27	99.15 - 98.61
Lepe18	Lepenski Vir	27/d	unknown	U5a2	93.7	300.73	26	98.9 - 98.05
Lepe37	Lepenski Vir 6,372-6,066	79/b	unknown	U5a2+16362	95.6	79.63	1	98.95 - 96.16
Lepe38	Lepenski Vir	79/c	unknown	U5a2+16362	95.6	208.60	8	99.99 - 99.53
Lepe39	Lepenski Vir	82	unknown	T2e	98.7	292.43	20	99.53 - 98.85
Lepe44	Lepenski Vir 6,210-5,898	89/a	unknown	U5a2d	96.4	52.91	1	96.09 - 91.85
Lepe45	Lepenski Vir	91	unknown	U5a2d	86.3	75.87	0	99.86 - 98.83
Lepe46	Lepenski Vir	93	unknown	H	100	394.67	24	99.25 - 98.43

In the sample from the Transition period, two individuals from the same grave 27, Lepe17 (identified anthropologically as female but not consistent with XX genotype genetically, see Figure 8) and Lepe18 (both anthropologically and genetically male), were found not to be maternally related and differed in their haplogroups (Lepe17 with farmer-like N1a1a1b (*33*) and Lepe18 with hunter-gatherer-like U5a2 (*34*)). Different haplogroups (but both farmer-like) for individuals in the same burial were also detected for grave 54 (Lepe28 and Lepe27, both genetically and anthropologically female, see Figure 8). In a similar context, namely grave 79, two individuals, Lepe37 (g. 79b, anthropologically and genetically male, see Figure 8) and Lepe38 (g. 79c, anthropologically probably male), might have been maternally related to each other because they shared the same U5a2 (+16362 mutation) haplogroup; at least we cannot genetically exclude the maternal relationship.

4.3.3 Mitochondrial haplogroups identified for Neolithic and post-Neolithic individuals from NW Anatolian and the Balkans

Similarly to the Transition period, the haplogroups of samples from the Neolithic period in Lepenski Vir (see Table 13) showed a mixture of various haplogroups not completely typical for the early stages of the Neolithic period elsewhere – together with six farmer-like haplogroups (N1a, T1a, K1b, J2a, N1a, J1c) found previously in other Early Neolithic contexts there were also three hunter-gatherer-like U5 haplogroups identified (*33*, *34*, *81*). However, eight Neolithic individulas from Vinča, two from Sultana Malu Rošu and the only sample analysed from Grivac showed a previously observed farmer-like selection of haplogroups (*81*, *203*, *204*).

Table 13: *Haplogroups of ancient Balkan individuals assigned to the Neolithic period (∼6,000/5,950-5,500 cal BC). Local and non-local group assignments were based on interpretations of $^{87}Sr/^{86}Sr$ isotope ratio in Borić & Price (94).*

Sample name	Site Date (cal BC)	Grave	$^{87}Sr/^{86}Sr$ group	Haplogroup	Score	Average coverage per base	min coverage	% authentic
Lepe22	Lepenski Vir	39	local range	J2a1a1	100	41.34	2	99.19 - 97.01
Lepe52	Lepenski Vir 6,005-5,845	73	local range	H3v+16093	100	361.31	27	99.61 - 98.99
Lepe34	Lepenski Vir	74	local range	U5a1c	97.8	120.21	10	99.49 - 98.42
Lepe6	Lepenski Vir 6,016-5,841	8	non-local range	T1a4	100	44.77	1	88.29 - 81.89
Lepe12	Lepenski Vir	20	non-local range	U5a1	93.7	170.85	5	99.93 - 98.94
Lepe20	Lepenski Vir	32a	non-local range	H+16311	100	122.75	12	99.6 - 98.05
Lepe32	Lepenski Vir	66	non-local range	N1a1a1a3	94.8	142.79	7	99.94 - 99.11
Lepe3	Lepenski Vir	6	unknown	N1a1a1a2	94.1	237.73	13	99.97 - 99.37
Lepe11	Lepenski Vir	19	unknown	K1b1a	98.9	161.40	8	100 - 99.68
Lepe1	Lepenski Vir	35	unknown	H3v+16093	100	26.04	0	99.79 - 95.15
Lepe29	Lepenski Vir	57	unknown	U5b2b	97.4	148.15	12	99.79 - 98.58
Lepe41	Lepenski Vir	86	unknown	J1c5	100	178.69	12	100 - 99.79

Both of the haplogroup types (farmer-like and hunter-gatherer-like) were seen during Neolithic in Lepenski Vir (∼6,000/5,950-5,500 cal BC), even when we studied only the locals (one farmer-like and

one hunter-gatherer-like haplogroup) or only non-locals (two farmer-like and one hunter-gatherer-like haplogroup). While any notions from a few or even a single mitogenome are problematic, the non-local Lepe12 with haplogroup U5a1 could indicate that the non-local isotopic signature does not necessarily signify a migrant with fully Neolithic ancestry (if defined by no U5 haplogroups). This individual could be also a hunter-gatherer migrant from a site with a different isotopic signature or it can be a descendant of admixture with hunter-gatherers from elsewhere. However, it should be noted that the strontium ratio value for this sample (0.710007) was only very closely outside the local range (0.7085-0.7100) (*94*) and it is thus possible that the individual was actually local isotopically.

Table 14: *Haplogroups of ancient Anatolian individuals assigned to the Neolithic and Chalcolithic period (∼6,600-5,500 cal BC). Local and non-local group assignments were based on interpretations of $^{87}Sr/^{86}Sr$ isotope ratio in Borić & Price (94).*

Sample name	**Site Date (cal BC)**	**Grave**	**Haplogroup**	**Score**	**Average coverage per base**	**min coverage**	**% authentic**
Akt16	Aktopraklık 6683-6533	89 D 14.1	K1a3	96.6	586.51	32	95.69 - 94.29
Akt20	Aktopraklık 6493-6418	89 E 9.3	J2b1	100	239.31	16	97.15 - 95.89
Akt17	Aktopraklık	89 D 15.1	K1a	96.6	56.18	1	93.57 - 88.42
Akt18	Aktopraklık	89 D 17.1	H	100	233.38	17	98.99 - 97.82
Akt26	Aktopraklık	90 D 4.4	J2b1	97.0	407.11	25	98.53 - 97.82
Akt6	Aktopraklık 5633-5535	17H 50.1	T2b	96.1	384.03	36	90.44 - 90.44
Bar20	Barcın 6438-6258	M11S / 401	W5	100	315.08	14	99.01 - 98.27
Bar32	Barcın 6396-6241	L11 / 604	K1a2	100	4.84	0	99.78 - 93.23
Bar16	Barcın 6233-6084	L10 / 187	K1a1	100	326.84	17	98.15 - 97.34
Bar15	Barcın 6213-6049	M10 / 115	K1a2	97.9	389.70	18	98.29 - 97.53
Bar31	Barcın	L11W / 546	X2m2	91.0	50.85	0	99.98 - 98.28
Bar4	Barcın	L13/ 129	U3b	92.1	113.60	6	99.96 - 98.94
Bar7	Barcın	M 10 / 101	U8b1a2b	95.2	31.77	0	94.07 - 85.99
Bar8	Barcın	M 10 / 106	K1a2	92.5	80.20	0	99.69 - 98.56
Bar13	Barcın	M10 / 102	U3a	92.1	4.85	0	99.6 - 34.83
Bar3	Barcın	M10 / 166	H+16311	96.3	107.88	3	99.94 - 98.19
Bar6	Barcın	M10 / 173	K1a2	96.7	63.13	5	99.92 - 98.59
Bar11	Barcın	M11 / 93	X2e2c	99.7	407.97	20	83.99 - 81.06
Ch51	Catalhöyük	Space 327 15839 B27	K1b1c	98.0	86.68	2	95.05 - 91.49
Ch54	Catalhöyük	Space 327 15839 B70(78)	K2a11	90.3	21.95	1	93.5 - 84.44

The Anatolian Neolithic samples we tested included haplogroups previously identified in the Neolithic period in Europe (*205*). However, three samples from Barcın carried U haplogroups (not U5 though), again suggesting that dividing human genetic variation by branching of a single locus (mitogenome) could be misleading (see Table 14).

Haplogroups of individuals from later Neolithic stages (Vinča, Grivac and later Sultana Malu Roşu) showed a relatively similar composition to those of Anatolia (see Table 15) and previously published Neolithic samples (e.g., *33*, *205*).

Table 15: *Haplogroups of ancient Neolithic individuals (∼6,000/5,950-4,500 cal BC) outside of Danube Gorges.*

Sample name	**Site Date (cal BC)**	**Grave**	**Haplogroup**	**Score**	**Average coverage per base**	**min coverage**	**% authentic**
Vc1	Vinča 5,476-5,304	2	T2c1+146	95.4	138.92	4	99.57 - 98.44
Vc2	Vinča	4	H+16311	93.1	134.49	9	98.82 - 96.67
Vc3	Vinča	5	K1a1	100	211.75	11	99.99 - 99.55
Vc4	Vinča	6	T2b	95.9	130.43	8	99.95 - 99.06
Vc5	Vinča	7	H3h2	100	2 05.79	9	99.98 - 99.25
Vc6	Vinča	8	HV0a	93.1	194.10	10	99.99 - 98.99
Vc8	Vinča	10	K1a1	100	159.06	7	99.81 - 98.99
Vc10	Vinča	12	T2c1+146	93.8	68.42	1	93.15 - 88.51
Gri1	Grivac	1	H7	100	141.49	9	99.99 - 99.06
Smr1	Sultana Malu Roşu	1	K1a2	98.0	204.72545	15	99.99 - 99.5
Smr2	Sultana Malu Roşu	2	T2b3+151	95.0	66.63148	3	99.74 - 98.39

Incidentally, two samples that were later shown not to belong to the Neolithic and Mesolithic period were sampled (see Table 16). One Barcın individual was Byzantine (*206*) and one Lepenski Vir sample was found to belong to the Eneolithic Sălcuţa culture (*119*).

While these periods remain severely underexplored in terms of ancient DNA, a single mitogenome is not enough to reach any conclusions for the whole period. However, these sequences can be utilised for future studies. It should be noted that both of the haplogroups obtained from these samples would not be unique in the earlier populations, which highlights the fact that the archaeological context and preferably also ^{14}C dating is crucial for aDNA studies.

Table 16: *Haplogroups from ancient individuals assigned to periods other than Neolithic and Mesolithic (after 4,500 cal BC).*

Sample name	**Site Date (cal BC)**	**Grave**	**Culture**	**Haplogroup**	**Score**	**Average coverage per base**	**min coverage**	**% authentic**
Lepe2	Lepenski Vir 4,361-4070	2	Sălcuţa culture	K1a2	98.7	71.20	1	99.98 - 99.16
Bar14	Barcın	L 12 / 49	Byzantine	J1b9	92.3	167.76	6	97.24 - 95.55

4.3.4 Diversities indices comparison for farmers and hunter-gatherers

When investigating the mitochondrial variation of studied populations with distance measures, the first step was to group individuals as correctly as possible. We therefore proceeded stepwise and we first formed small groups that would reflect both periods and sites (see Table 17). While not all the samples were dated, we relied on archaeological information to form these groups (see section 2.1)

Table 17: *Pairwise linearised Slatkin's F_{ST} for samples grouped by site and, in the case of Lepenski Vir (LV), also by period. Sites with one sample per site or per period were disregarded (Grivac, Padina, Byzantine Barcın individual and Lepenski Vir Sălcuţa individual). Numbers in brackets reflect the number of individuals in the group (sample names related to the sites can be found at Tables 11, 12, 13. 14, 15). P-values are in the upper triangle. Grey-out F_{ST} values in the lower triangle are significant, light grey-out values were below 0.05 significance threshold before rounding.*

	Aktopraklık (6)	Barcın (12)	Catalhöyük (2)	Meso LV (3)	Neo LV (12)	Trans LV (17)	Ostrovul Corbului (4)	Sultana Malu Roşu (2)	Vinča (8)	Vlasac (16)
Aktopraklık	*	0.11	0.28	0.26	0.49	0.21	0.19	0.79	0.54	0.03
Barcın	0.08	*	0.20	0.12	0.01	0.05	0.04	0.38	0.04	0
Catalhöyük	0.17	0.12	*	0.11	0.07	0.12	0.05	0.33	0.14	0.02
Meso LV	0.05	0.14	0.55	*	0.74	0.63	0.57	0.39	0.22	0.46
Neo LV	0	0.10*	0.22	0	*	0.34	0.30	0.45	0.14	0.01
Trans LV	0.04	0.06	0.15	0	0	*	0.08	0.49	0.05	0.21
Ostrovul Corbului	0.12	0.22*	1.61	0	0.01	0.08	*	0.13	0.15	0.01
Sultana Malu Roşu	0	0.024	0.14	0.01	0	0	0.31	*	0.76	0.08
Vinča	0	0.13*	0.27	0.09	0.05	0.09	0.11	0	*	0
Vlasac	0.23*	0.19*	0.42*	0	0.10*	0.01	0.26*	0.22	0.28*	*

The analysis and especially the number of statistically significant values were strongly influenced by the number of individuals in each group (see Table 17), although we excluded sites and periods for which only one mitogenome had been obtained (Padina, Grivac, Lepenski Vir sample from Sălcuţa culture and Byzantine Barcın individual). The low number of groups and significant values lowered the informative value of the MDS visualisation (see Figure S3). Nevertheless, we obtained several statistically significant F_{ST} values (see Table 17), mainly for the Vlasac and Barcın datasets with a high number of samples (in any case, significant values for groups with very low sample sizes should be considered with a very high degree of caution).

As expected, we found highly significant values between the Mesolithic and Neolithic groups. The Vlasac group was differentiated from all the Anatolian groups (the highest value was for two Catalhöyük individuals) and the Neolithic individuals from Vinča and Lepenski Vir. Neolithic Barcın mitogenomes were also significantly differentiated from Mesolithic Ostrovul Corbului.

Interestingly, differences were also found among both the Neolithic and Mesolithic groups. There was a relatively high genetic distance between the Vlasac and Ostrovul Corbului groups, both considered Mesolithic. Neolithic Barcın mitogenomes were significantly differentiated from the Neolithic

individuals from Vinča and Lepenski Vir, but it should be noted that the differences were smaller than with the Mesolithic individuals.

The result of the Mesolithic comparison has to be interpreted with caution because we obtained only four full mitochondrial genomes from the Ostrovul Corbului and the samples were not dated. Yet their difference to both the Neolithic (Barcın and on the significance threshold to Catalhöyük) and Mesolithic (Vlasac) groups could suggest that there is some merit to suggestions that the Lepenski Vir culture was different from the Schela Cladovei culture of Ostrovul Corbului that are usually considered identical (*207*). On the other hand, while the differences between Barcın individuals and Neolithic individuals from Serbia are of interest, they are relatively small and could have easily arisen from a founder effect.

For further analysis, we formed bigger groups with our samples and for comparison with published data (grouped as in Table S2) the sequences were cut to the length of published sequences. We kept the samples from the Danube Gorges divided by period (Mesolithic, Transition and Neolithic, as in Tables 11, 12 and 13) and the Vlasac group separate. The two sequences from Sultana Malu Roşu were added to the published sequences from this site (*204*) and Grivac to Vinča group.

The different groupings did not affect the relationships between the samples studied (see Table 18). The Vlasac group was different from all except for Lepenski Vir individuals from the Transition period ("Trans Dan" as Transition Danubians) and it was still significantly different from a mixed group with samples from Mesolithic Ostrovul Corbului (4), Lepenski Vir (3) and Padina (1) ("Meso Dan" standing for Mesolithic Danubians). Neolithic Anatolian individuals from all three sites ("Neo Anat") were statistically different from all the other groups except for the Vinča group (which itself showed statistically significant values for other pairwise comparisons except for Neolithic and Transition Lepenski Vir samples "Neo Dan" and "Trans Dan", respectively).

The results (Table 18) were also visualised in Figure 9. Vlasac individuals were close to previously published sequences from Mesolithic and pre-Neolithic western Europe (this group was similar to Holocene and Late Glacial groups from Posth *et al.* (*64*)), while samples from the Transition phase of Lepenski Vir and even more the Neolithic phase of Lepenski Vir were closer to other groups from Neolithic Europe. This would suggest an increasing influx of individuals to the Danube Gorges over the periods of Meso-Neolithic Transition.

Table 18: *Pairwise linearised Slatkin's F_{ST} for reference samples grouped as in Table S2 and for samples obtained in this study grouped as described in section 4.3.4. P-values are in upper triangle. Grey-out F_{ST} values in lower triangle are significant, light grey-out values were below 0.05 significance threshold before rounding.*

	preLGM (15)	postLGM (9)	Late Glacial (5)	Holocene (17)	Starčevo (48)	LBK (39)	Early Neo CE (119)	Mid Neo CE (49)	Neo Anat (20)	Neo Dan (12)	Trans Dan (17)	Meso Dan (8)	Vinča (9)	Vlasac (16)	SuMalu Roşu (12)	SuValea Orb (12)	Vărăşti (14)
preLGM	*	0.32	0.05	0.00	0.00	0.00	0.00	0.02	0.00	0.17	0.12	0.33	0.00	0.03	0.00	0.00	0.00
postLGM	0.01	*	0.00	0.00	0.12	0.10	0.10	0.30	0.01	0.24	0.06	0.10	0.01	0.01	0.00	0.00	0.00
LateGlacial	0.23	0.85*	*	0.32	0.00	0.00	0.01	0.03	0.00	0.07	0.13	0.00	0.00	0.30	0.01	0.00	0.00
Holocene	0.24*	0.52*	0.00	*	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.19	0.00	0.00	0.00
Starčevo	0.09*	0.04	0.25*	0.35*	*	0.32	0.48	0.91	0.10	0.23	0.00	0.05	0.67	0.00	0.01	0.00	0.00
LBK	0.09*	0.04	0.24*	0.33*	0.01	*	0.60	0.60	0.00	0.22	0.01	0.08	0.39	0.00	0.00	0.00	0.00
EarlyNeo CE	0.08*	0.04	0.22*	0.31*	0.00	0.00	*	0.66	0.01	0.23	0.00	0.09	0.54	0.00	0.00	0.00	0.00
MidNeo CE	0.05*	0.01	0.20*	0.30*	0.00	0.00	0.00	*	0.04	0.22	0.02	0.14	0.59	0.00	0.00	0.00	0.00
Neo Anat	0.25*	0.23*	0.61*	0.68*	0.03	0.11*	0.05*	0.04*	*	0.01	0.01	0.01	0.22	0.00	0.00	0.00	0.00
Neo Dan	0.02	0.01	0.09	0.21*	0.02	0.02	0.01	0.01	0.10*	*	0.29	0.39	0.14	0.03	0.00	0.00	0.00
Trans Dan	0.04	0.09	0.06	0.08*	0.08*	0.09*	0.08*	0.06*	0.12*	0.01	*	0.10	0.05	0.33	0.00	0.00	0.00
Meso Dan	0.01	0.01	0.47*	0.41*	0.05	0.04	0.05	0.03	0.21*	0.01	0.06	*	0.04	0.01	0.00	0.00	0.00
Vinča	0.26*	0.24*	0.52*	0.61*	0.00	0.00	0.00	0.00	0.03	0.05	0.10	0.18*	*	0.00	0.01	0.00	0.00
Vlasac	0.12*	0.21*	0.03	0.02	0.22*	0.21*	0.20*	0.18*	0.34*	0.08*	0.00	0.13*	0.27*	*	0.00	0.00	0.00
SuMalu Roşu	0.29*	0.28*	0.55*	0.56*	0.11*	0.13*	0.13*	0.13*	0.34*	0.12*	0.19*	0.22*	0.24*	0.33*	*	0.16	0.71
SuValea Orb	0.45*	0.62*	0.84*	0.72*	0.21*	0.26*	0.22*	0.23*	0.53*	0.18*	0.27*	0.45*	0.50*	0.44*	0.02	*	0.21
Vărăşti	0.30*	0.29*	0.49*	0.58*	0.17*	0.20*	0.19*	0.19*	0.41*	0.15*	0.26*	0.28*	0.30*	0.41*	0.00	0.01	*

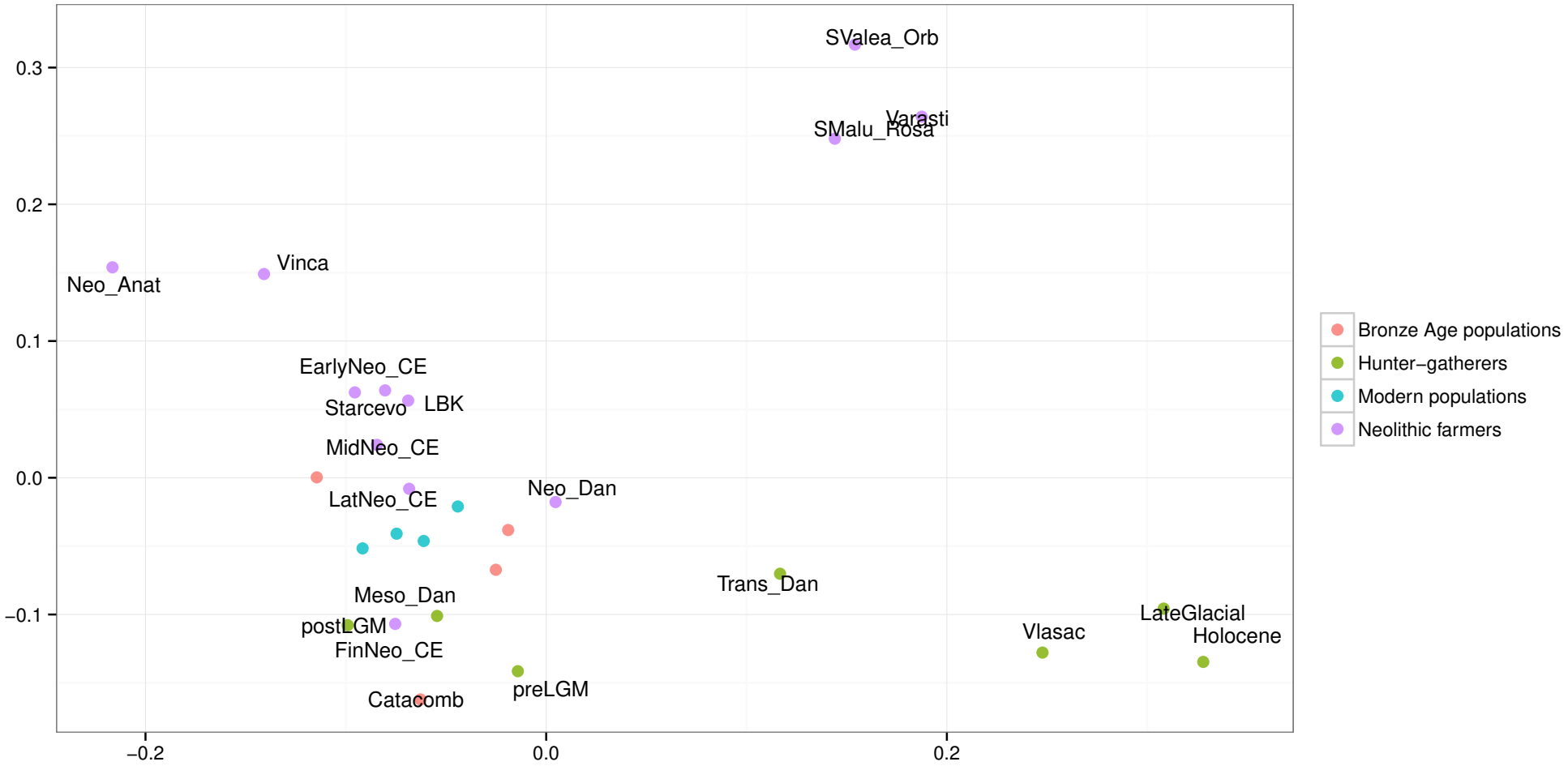


Figure 9: *Multidimensional scaling of Reynolds' distances. The stress value for MDS was 0.00127. Most of values were significant, see Table 18.*

Interestingly, Mesolithic Danubians other than Vlasac individuals were relatively close in Figure 9 to samples from Palaeolithic samples prior to and post LGM (*41*, *64*, *208*), but samples from central Europe Final Neolithic and Catacomb culture (*11*) were also relatively close. This could signify either that the mixed nature of Mesolithic Danubians produced a similar population genetic make-up to the one that was in Europe prior to the Late Glacial or in Ukraine in the Early Bronze Age, or that the Mesolithic samples from the Danube Gorges could be a relict of the hunter-gatherer population that survived the Last Glacial that is different to the Holocene populations of western Europe and Vlasac individuals. In the latter case, the geographical closeness of these two groups in the Danube Gorges would necessitate an explanation (e.g., cultural border or strong matrilocality).

The Vinča samples were the closest (see Figure 9 and Table 18) to the samples from Neolithic Anatolia and to those of Transdanubia LBK (denoted as LBK) and Starčevo (*203*), and from the Early Neolithic period from Germany (denoted as Early Neo CE, culturally also LBK) (*33*, *81*, *205*, *209*, *210*). This would be consistent with the presumed direction of Neolithic demic movement from Anatolia through the Balkans to central Europe.

It should be mentioned that samples from three sites of Middle/Late Neolithic in Romania (in a relative proximity to the Danube Gorges) were expected to be closer to the Neolithic samples from central Europe and the Danube Gorges. In the source publication from Hervella *et al.* (*204*), the sites of Sultana Malu Roşu, Sultana Valea Orbului and Vărăşti were not analysed separately, but were grouped with other sites from a wider region. We however opted to select only those three sites on the Danube because they were geographically relatively close to the Danube Gorges (although still around 500 km away) and they contained multiple individuals (more than 10 for each site) in order to avoid grouping. Also, our analysis differed in terms of what other populations were analysed and plotted on MDS. The results indicated that the closest group to these three sites were Hungarian Starčevo samples and Neolithic Danubians, which is consistent with published results (*204*), but that otherwise these groups were differentiated from the known genetic variation of the Neolithic and Bronze Age.

The same analysis was repeated with Neolithic and Transition Lepenski Vir individuals, which were grouped according to results of the $^{87}Sr/^{86}Sr$ isotope analysis (*94*). The three groups were formed from the samples of pooled Neolithic and Transition individuals from Lepenski Vir: locals (abbreviated as LocNT LV), non-locals (NonNT LV) and individuals for which no isotope values were published (UnkNT LV). The samples sizes were too low (as low as 6) to produce significant F_{ST} results (see Supplementary File S4) but when visualised in Figure 10, non-local individuals (presumed migrants in the area) were closer to the cluster of Neolithic groups than to individuals from the same area and period.

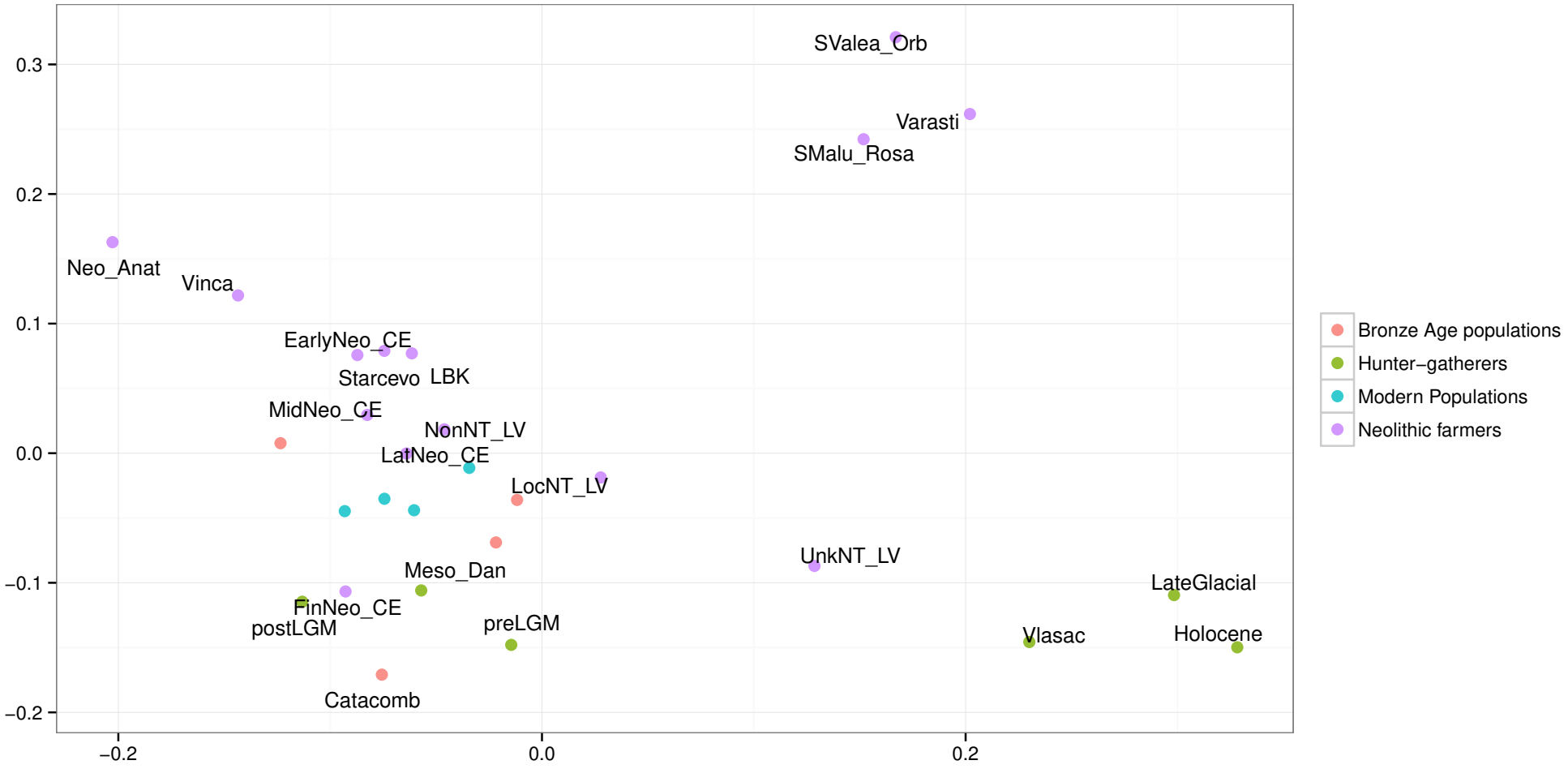


Figure 10: *Multidimensional scaling of Reynolds' distances. The stress value for MDS was 0.00132. Most of values were significant, see Table S2.*

Table 19: *Pairwise linearised Slatkin's F_{ST} with modern data, grouped as in Table S2. P-values are in upper triangle. Grey-out F_{ST} values in lower triangle are significant.*

	Modern Turk (102)	Modern Bulg (30)	Modern Cre (180)	Modern Hun (211)	Neo Anat (20)	Neo Dan (12)	Trans Dan (17)	Meso Dan (8)	Vinča (9)	Vlasac (16)
Modern Turk	*	0.26	0.10	0.01	0.00	0.14	0.00	0.60	0.04	0.00
Modern Bulg	0.00	*	0.40	0.40	0.00	0.10	0.01	0.91	0.08	0.00
Modern Cre	0.00	0.00	*	0.07	0.00	0.06	0.00	0.65	0.04	0.00
Modern Hun	0.01*	0.00	0.00	*	0.00	0.04	0.00	0.46	0.02	0.00
Neo Anat	0.06*	0.07*	0.08*	0.10*	*	0.01	0.01	0.01	0.22	0.00
Neo Dan	0.01	0.02	0.03	0.04*	0.10*	*	0.29	0.39	0.14	0.03
Trans Dan	0.06*	0.06*	0.07*	0.08*	0.12*	0.01	*	0.10	0.05	0.33
Meso Dan	0.00	0.00	0.00	0.00	0.21*	0.01	0.06	*	0.04	0.01
Vinča	0.03*	0.04	0.04*	0.04*	0.03	0.05	0.10	0.18*	*	0.00
Vlasac	0.15*	0.15*	0.16*	0.17*	0.34*	0.08*	0.00	0.13*	0.27*	*

While it seems that isotopic analysis allowed, on some level, a differentiation of individuals more related to early farmers, this differentiation does not seem to be complete (the non-locals were close to populations that had already experienced admixture in the Middle and Late Neolithic periods). The local group is also not close (yet relatively closer when compared with non-locals) to the Mesolithic cluster. Genetically, this was expected because the local descendants of incoming individuals would bear their genetic ancestry (in the case of mtDNA maternal) and would therefore not differ too much from the first generation migrants (non-locals).

The differences of studied groups to the selected modern populations were investigated with the same groupings and almost all the ancient groups studied were statistically differentiated from the modern mitochondrial sequences (see Table 19). Probably due to power (a low sample size), comparisons to Mesolithic and Neolithic Danubians (and in one case to Vinča) were often insignificant. However, these populations were also the closest to modern populations on MDS visualisation (see Figure 9). Nevertheless, without proper continuity analysis (preferably coalescent simulations), no claims on genetic continuity in the region should be even hinted at. Still, the difference between the hunter-gatherers from Vlasac and the modern populations was by far the largest, complementing previous results of population turnover in Neolithic (*33*, *52*, *203*).

4.4 Quality of nuclear dataset obtained

The sequencing of the whole genomes was successful for all the five samples (Bar8, Bar31, Lepe45, Lepe51 and Vlasa37) and the target x-fold coverage range (3x-7x) was attained. Most of nucleotides of the sequenced genomes were covered at least once (82.3-86.8%). It should be noted that Lepe51 and Lepe45 were sequenced only on two lanes (see section 3.7) and the results were still comparable to other samples. That is probably due to the higher quality of these two samples (measured both by endogenous content and copy number, see Table 20). However, the Barcın genome samples were also of high quality (e.g., endogenous content >48.5%, see Table 20) because Bar31 was sequenced

Table 20: *Summary of the whole genome dataset.*

Sample name	**Site Period Date (cal BC)**	**Individual**	**Coverage over genome (x-fold)**	**Genome covered (%)**	**Average endogenous content (%)**	**Average molecule count (μl)**	**Sex**	**SNPs within reference dataset (*8*)**
Bar31	Barcın Neolithic 6,419-6,238	L11W / 546	3.66	82.32	53.46	8.53e+07	XY	304,624
Bar8	Barcın Neolithic 6,212-6,030	M 10 / 106	7.13	86.65	48.55	2.56e+07	XX	342,398
Lepe51	Lepenski Vir Early Mesolithic 7,940-7,571	68	3.8	83.37	69.39	1.92e+09	XX	339,618
Vlasa37	Vlasac Late Mesolithic 6,767-6,461	24	5.1	86.82	27.86	9.1e+07	XY	266,254
Lepe45	Lepenski Vir Transition 6,588-6,395 FRE: 6,215-5,911	91	4.08	85.03	69.24	2.55e+08	XY	342,734

only on three lanes with eight libraries and 3.66x coverage was obtained. Vlasa37, which was chosen at the beginning of the project (and therefore had the lowest endogenous content), was sequenced on five lanes, with 31 libraries to balance the low endogenous content and reach 5.1x coverage.

The Danubian samples selected for capture were also of very high quality (see Tables 21 and 22) and it is therefore not surprising that the x-fold coverage obtained over the targeted regions (totalling 4,915,534 bp) was very high, between 25-94x. Anatolian samples had x-fold coverages between 28.4x and 7.6x (Akt18 was the lowest). The number of the libraries pooled for the capture was adjusted to the overall quality (10 for Aktopraklık, 9 for Barcın and 6-8 for Danubian samples), yet the endogenous content was still positively correlated with the resulting x-fold coverage (Spearman's rank correlation: $\rho = 0.7651$, p-value = 8.466e-05). No correlation was found between copy number and x-fold coverage (ρ=0.1249, p-value = 0.6088). While detailed investigations on relationships between sample quality and capture success was carried out on the samples used in this study separately (*156*, *157*), these general correlations would suggest that endogenous content (not copy number) is the main measurement to be evaluated prior to capture and that, even though increasing the libraries can lead to reasonable coverage for medium quality samples, it is possible that there is a limit to the level of improvement achievable via the library increase. A low copy number (or high duplication rate for that matter) should still lead to exclusion of a sample from further analysis.

Table 21: *Summary of the nuclear capture dataset. Direct dates for some of the samples are in Tables 7 and 8.*

Sample name	**Site Period Date (cal BC)**	**Individual**	**Coverage over target regions (x-fold)**	**Target bases covered (%)**	**Average endogenous content (%)**	**Average molecule count (μl)**	**Sex**	**SNPs within reference dataset (*8*)**
Akt16	Aktopraklık Neolithic 6,683-6,533	89D 14.1	39.66	97	39.39	4.25e+08	XX	38,942
Akt18	Aktopraklık Neolithic ∼6,500-6,000	89D 17.1	7.61	86.75	22.37	1.56e+08	XX	14,888
Akt20	Aktopraklık Neolithic 6,493-6,418	89E 9.3	23.3	98.64	17.62	1.63e+08	XY	28,307
Akt26	Aktopraklık Neolithic ∼6,500-6,000	90D 4.4	21.08	95.1	13.38	4.95e+08	XX	17,007
Akt6	Aktopraklık Chalcolithic 5,633-5,535	17H 50.1	35.78	98.29	25.01	4.10e+08	XY	32,671
Bar11	Barcın Neolithic ∼6,600-6,000	M11 / 93	26.92	98.27	34.07	2.09e+08	XY	21,117
Bar15	Barcın Neolithic 6,213-6,049	M10 / 115	28.44	97.75	43.96	1.60e+08	XX	40,006
Bar16	Barcın Neolithic 6,233-6,084	L10 / 187	25.05	95.14	41.5	5.65e+08	XX	28,916
Bar20	Barcın Neolithic 6,438-6,258	M11S / 401	15	98.05	45.73	8.63e+07	XX	33,810
Bar32	Barcın Neolithic 6,396-6,241	L11 / 604	14.69	98.01	45.19	6.87e+07	XX	45,259

Table 22: *Summary of the nuclear capture dataset. Direct dates for some of the samples are in Tables 7 and 8.*

Sample name	**Site Period Date (cal BC) $^{87}Sr/^{86}Sr$ group**	**Individual**	**Coverage over target regions (x-fold)**	**Target bases covered (%)**	**Average endogenous content (%)**	**Average molecule count (μl)**	**Sex**	**SNPs within reference dataset (*8*)**
Lepe18	Lepenski Vir Transition ∼6,200-6,000/5,950	27/d	86.33	99.23	67.45	2.54e+08	XY	74,952
Lepe39	Lepenski Vir Transition ∼6,200-6,000/5,950	82	25.87	99.17	19.69	3.39e+08	XY	30,547
Lepe46	Lepenski Vir Transition ∼6,200-6,000/5,950	93	57.95	99.45	59.61	1.40e+08	XX	33,611
Lepe52	Lepenski Vir Neolithic 6,005-5,845 local	73	67.28	99.38	64.38	1.69e+08	XY	69,483
Lepe53	Lepenski Vir Transition ∼6,200-6,000/5,950	27	40.05	99.48	53.54	5.56e+07	XX	68,787
Vlasa10	Vlasac Late Mesolithic ∼7,400-6,200	41	41.97	99.61	34.27	1.61e+08	XY	72,202
Vlasa32	Vlasac Late Mesolithic ∼7,400-6,200 local	16	47.51	99.28	63.2	1.25e+08	XY	96,419
Vlasa4	Vlasac Late Mesolithic ∼7,400-6,200 local	18/a	93.76	99.6	63.99	2.12e+08	XY	90,346
Vlasa41	Vlasac Late Mesolithic ∼7,400-6,200 local	30	61.89	99.36	57.85	1.87e+08	XX	61,819
Vlasa44	Vlasac Late Mesolithic ∼7,400-6,200 local	47	71.31	99.4	60.23	2.09e+08	XY	69,994

4.5 Using f-statistics to infer shared drift in Anatolians and Danubians

4.5.1 Mesolithic Danubian genomes compared to modern and ancient reference populations

Three Danubian whole genome samples from Lepenski Vir, Lepe51 (7,940-7,571 cal BC) and Lepe45 (6,588-6,395 cal BC, with the presumed FRE: 6,215-5,911 cal BC), and Vlasac, Vlasa37 (6,767-6,461 cal BC), were relatively compared to ascertain their similarity to modern and ancient reference populations by *outgroup* f3-statistics (*60*). For further details on this method, see section 3.13.1 and for the summary of the whole genome dataset see Table 20.

This test was performed in a form *f3(≠Khomani; TEST, Danubian)* and *f3(Yoruba; TEST, Danubian)* and modern populations that served as a *TEST* were ordered according to the f3 value produced. The ordering was the same for both the Yoruba and ≠Khomani sub-Saharan groups (see Figure 11 and Figure 12, respectively).

The grouping of individuals to these modern and ancient reference populations was kept as defined in Haak *et al.* (*8*) for comparability (the detailed results of these tests are in Supplementary File S2) with occasional shortening of the group names, whereas samples from additional studies were tested individually (see Hofmanová *et al.* (*51*) for samples added to the Haak *et al.* (*8*) reference dataset).

The affinities to modern populations were similar for all three Danubian samples, which differed in age by almost 3,000 years (see section 2.2), and the highest f3 value for all the samples was estimated for the Lithuanian population, closely followed by other Baltic (Estonian), Scandinavian (Norwegian, Finnish) and Eastern European (Belarusian, Ukrainian) populations. Among the populations from the north and east of Europe, the Basque population differed geographically. However, it had been previously found that this population might represent relicts of the hunter-gatherer population (*211*, *212*), even though this was recently disputed via findings of similarity between the Basques and Early Neolithic Atapuerca individuals (*213*).

The similarities to Scandinavian and Baltic populations were expected because these populations were also previously shown to have a higher ancestry related to pre-Neolithic population than the rest of the Europe (*86*).

When the same tests were performed for the ancient reference populations as *TEST* (see Figure 13 and Figure 14 for the ≠Khomani and Yoruba as *outgroup* populations, respectively), the results have shown the three Danubian samples to be again highly comparable. Danubians were genetically the most similar to the samples described as western hunter-gatherers (WHG), namely Loschbour (*78*), La Braña (*79*) and Bichon (*53*). A highly similar sample was also KO1 (*52*), which was discovered in Early Neolithic context at Tiszaszőlős-Domaháza, Hungary (in a relative geographical proximity to the Danube Gorges: ∼500 km). Despite the Neolithic context, this sample has been previously

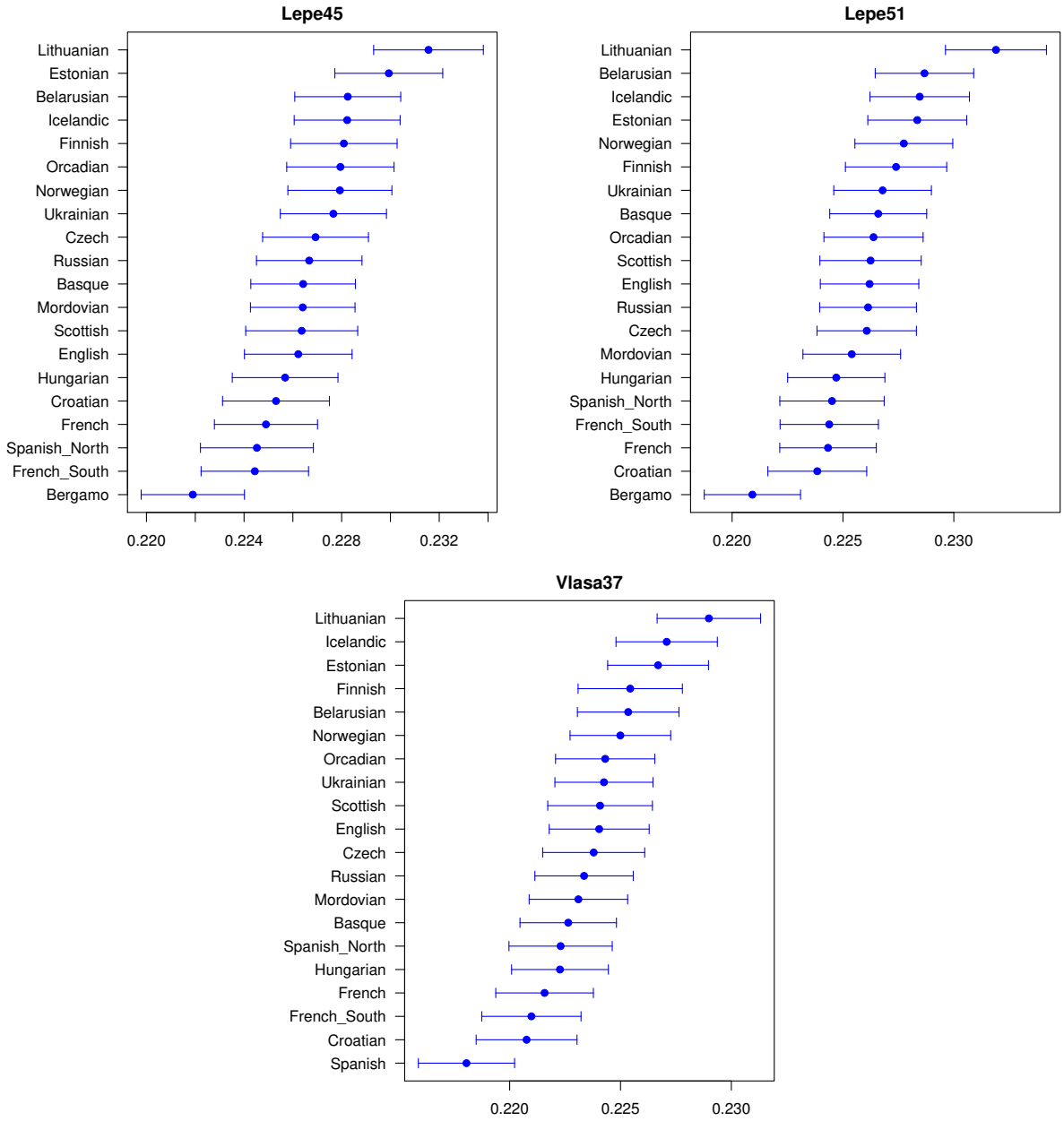


Figure 11: *f3(≠Khomani; Modern_population, Danubian). The highest 20 values shown.*

assigned to hunter-gatherers, given the similarity to WHG (*52*). KO1 and Loschbour were the only samples closer (yet within standard error) to Danubians than Danubians to each other.

After WHG, Motala (*78*) and SHG (here used as abbreviations for Swedish Neolithic and Mesolithic HG, *214*), followed by Eastern HG (EHG, Karelia from NW Russia and Samara from SE European Russia, *8*), showed high values in the *outgroup* f3 tests. Among the 20 highest values, there were also other samples from Hungarian Neolithic (NE3 and NE4) and Bronze Age (BR1 and BR2) contexts (*52*), as well as other post-Neolithic samples from central and northern Europe (*8*, *214*).

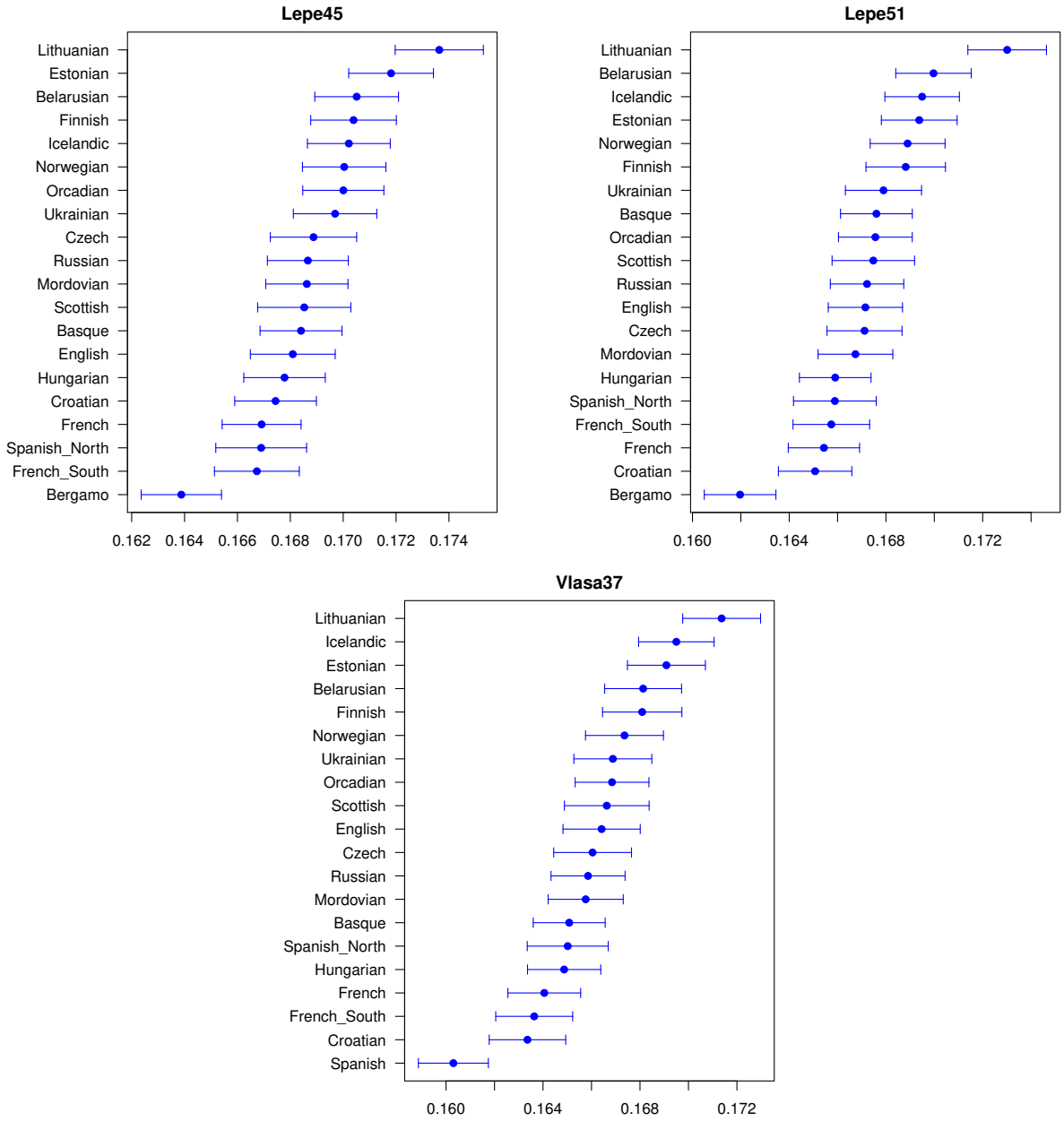


Figure 12: *f3(Yoruba; Modern_population, Danubian). The highest 20 values shown.*

4.5.2 Differences among Danubian samples

Outgroup f3 results indicated high concordance among the genetic affinities of Danubians. We performed a series of D statistics to investigate whether indeed Danubians (here represented by the three genomic samples analysed) did not differ in their genetic similarities to other ancient populations. These and other D tests were performed with both the Yoruba and ≠Khomani as *outgroup* populations, but since the results were again highly correlated (Spearman's rank correlation: $\rho =$ 0.91 and p-value <2.2e-16 for D values and $\rho = 0.87$ and p-value <2.2e-16 for Z-scores) and the main results were identical, we will further discuss only the results with ≠Khomani as an *outgroup*, unless otherwise stated.

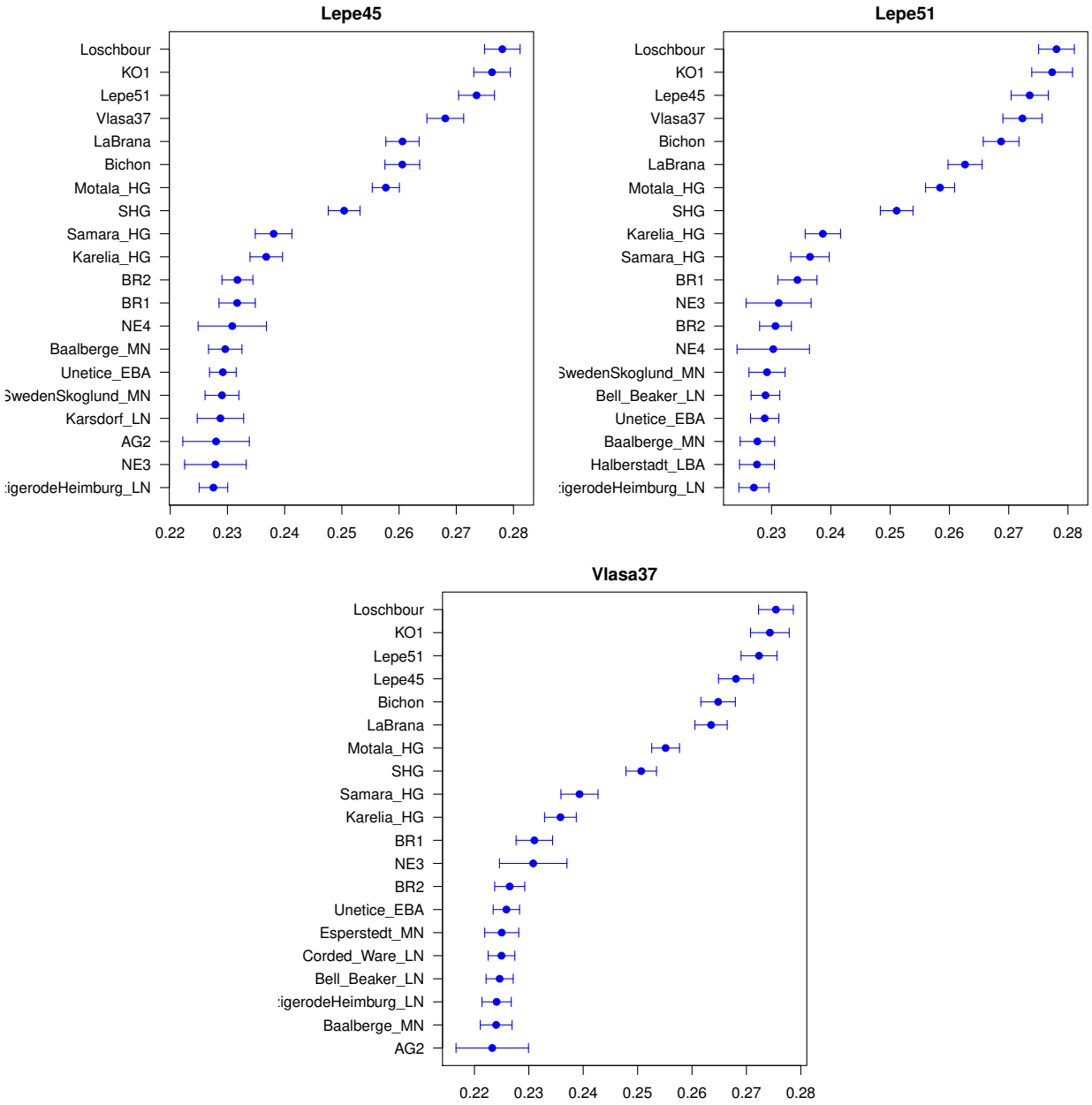


Figure 13: *f3(≠Khomani; Ancient_population, Danubian). The highest 20 values shown.*

The sustainability of a null hypothesis for Danubians branching together in respect to other populations can be tested as D test in a form *D(Danubian, Danubian, TEST, ≠Khomani)* with *TEST* being every modern or ancient reference population in the dataset. There were only a few significant values for *TEST* as a modern (see Table 23) and as an ancient (see Table 24) population. Interestingly, the significant values violating the null hypothesis for these tests show that compared to other Danubians Vlasa37 shares less drift with some modern populations from the Near East (the same test with Yoruba as an *outgroup* leads to more populations from the same region to pass the significance threshold). Also, and more importantly, Vlasa37 shares less drift with a few ancient populations, namely a rather low coverage Chalcolithic Anatolian Kumtepe6 individual (*53*) and Swedish Middle Neolithic samples (*214*), but also Early Neolithic LBK samples (*8*). The LBK result is present in the comparison of both the Lepenski Vir samples (Lepe45 and Lepe51) to Vlasac sample (Vlasa37)

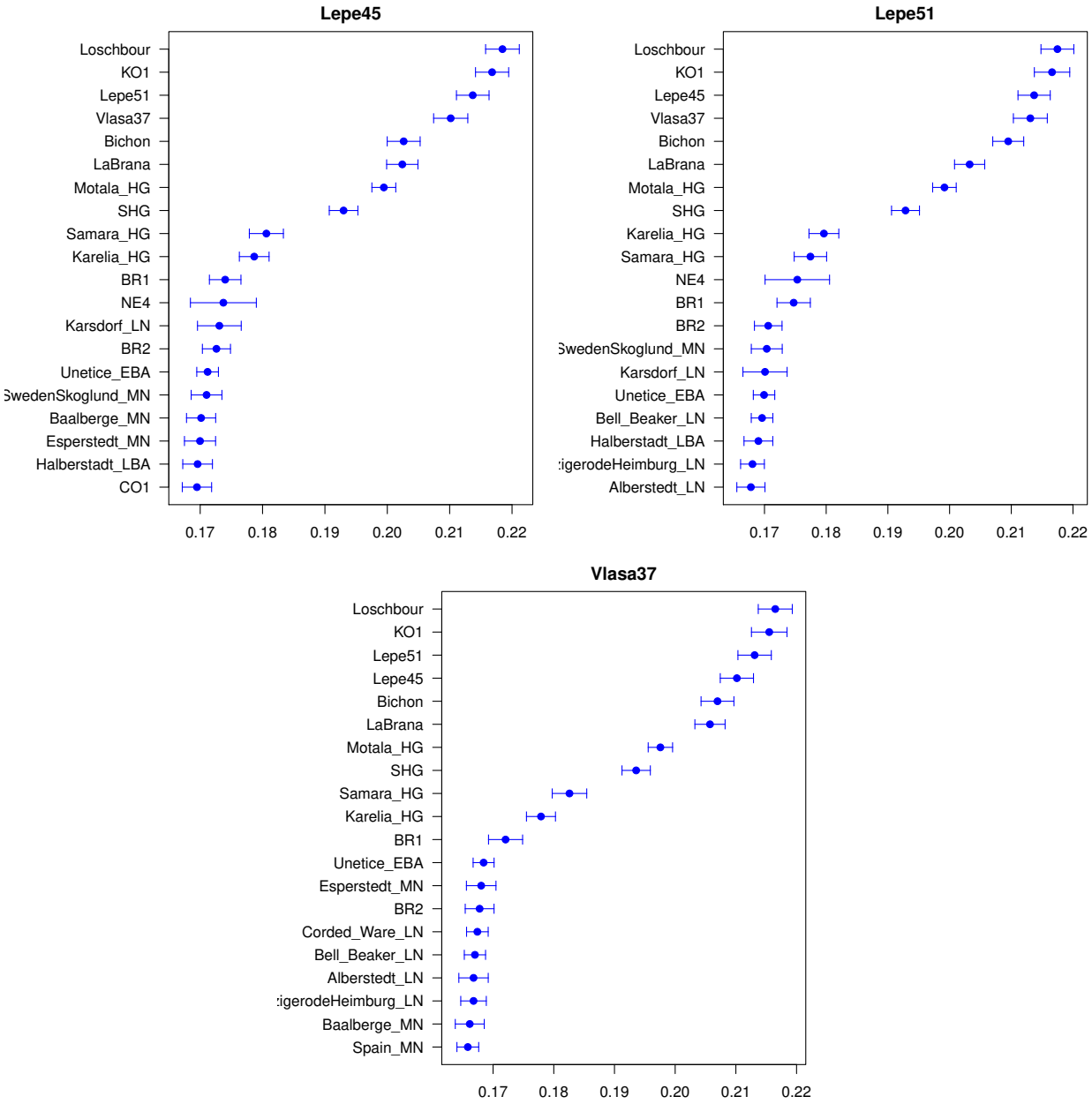


Figure 14: *f3(Yoruba; Ancient_population, Danubian). The highest 20 values shown.*

(and it is also confirmed for Yoruba as an *outgroup*). The higher sharing between Lepenski Vir and LBK when compared to Vlasac might suggest differences among Danubians in their relationship to LBK. However, a vast majority of D values for these tests is not significant and the null hypothesis that Danubians share the same amount of drift with various different populations is therefore overwhelmingly not violated. As such, this confirms the f3-statistics notion that Danubians were very similar to each other (for full results see Supplementary File S2).

So as not to rely only on the absence of evidence for similarity between Danubians, we tested an alternative scenario, namely that any Danubian sample would branch together with *TEST* population in respect to any other Danubian sample in a D form *D(Danubian, TEST, Danubian, ≠Khomani)*. For all modern populations as *TEST*, all D values were highly significantly positive (|Z|>15) show-

Table 23: *D(Danubian, Danubian, Modern population, ≠Khomani), values for |Z|>3 shown, identical values for different order of Danubians and all values can be seen in Supplementary File S2.*

Danubian	Danubian	Modern population	≠Khomani	D value	Z-score
Vlasa37	Lepe45	Georgian	Khomani	-0.0155	-3.285
Vlasa37	Lepe45	Saudi	Khomani	-0.0146	-3.142

Table 24: *D(Danubian, Danubian. Ancient population, ≠Khomani) , values for |Z|>3 shown, identical values for different order of Danubians and all values can be seen in Supplementary File S2.*

Danubian	Danubian	Ancient population	≠Khomani	D value	Z-score
Vlasa37	Lepe45	LBK_EN	Khomani	-0.017	-3.372
Vlasa37	Lepe51	LBK_EN	Khomani	-0.0154	-3.216
Vlasa37	Lepe45	Kumtepe6	Khomani	-0.0732	-3.101
Vlasa37	Lepe51	SwedenSkoglund_MN	Khomani	-0.0254	-3.072

ing that there was indeed a violation of the tested hypothesis and it was due to high amount of shared drift between Danubians. The results for ancient populations as *TEST* were also significantly positive, with the exception of a very low coverage sample Kumtepe4 (0.01x coverage) and WHG samples. Consistently with f3-statistics, only Loschbour and KO1 showed negative values (i.e., more shared drift between them and Danubian than between Danubians). However, it was significant only in a form *D(Vlasa37, Loschbour, Lepe45, ≠Khomani)* = -0.0264 with Z=-3.38 (for the complete results see Supplementary File S2). This means that, except for Loschbour, the similarity between Danubians is higher than any affinities to other populations.

4.5.3 Placing Danubians in the context of other hunter-gatherer genomes

The Danubian affinities to WHG are obvious from the *outgroup* f3 values for Danubians and also from the comparison of Danubians by D statistics (see sections 4.5.1 and 4.5.2). This was further tested by directly comparing the amount of drift Danubians shared with WHG with the amount of drift they shared with other HG. The form of this test was *D(Other HG, WHG, Danubian, ≠Khomani)* and, as expected, all the results were significantly negative, showing that the amount of ancestry shared between Danubians and WHG is higher than between Danubians and other HG (with three insignificant exceptions for HG from Motala (*78*), see Supplementary File S2). To further confirm this, we tested an alternative scenario that Danubians branched together with other HG in respect to WHG in a form *D(Danubian, Other HG, WHG, ≠Khomani).* The results were statistically positive for all other HG (see Supplementary File S2), suggesting that WHG shared more drift with Danubians than they did with other HG and that Danubians did not branch clearly together with any other HG.

The similarity between WHG and Danubians is clear, but because human populations are rarely tree-like, it was important to investigate whether any other HG shared different amounts of genetic

ancestry with Danubians than with other WHG. First, we tested geographically relatively close Eastern hunter-gatherers (EHG), samples from Karelia and Samara (*8*) in a form *D(Danubian, WHG, EHG, ≠Khomani)*. Whereas the results for KO1 and Loschbour did not show any significant differences, we observed an increase of EHG ancestry in Danubians when compared to Bichon and La Braña (see Table 25). This is consistent with the age and geographical location of the samples (Bichon is relatively older than other WHG and La Braña was located a considerable distance from EHG).

Table 25: *D(Danubian, WHG, EHG, ≠Khomani), values for |Z|>3 shown, all values can be seen in Supplementary File S2.*

Danubian	WHG	EHG	≠Khomani	D value	Z-score
Lepe51	La Braña	Karelia_HG	Khomani	0.0242	3.226
Vlasa37	Bichon	Karelia_HG	Khomani	0.0252	3.426
Lepe45	Bichon	Karelia_HG	Khomani	0.0302	3.719
Lepe51	Bichon	Karelia_HG	Khomani	0.0357	4.483
Vlasa37	Bichon	Samara_HG	Khomani	0.0308	3.397
Vlasa37	La Braña	Samara_HG	Khomani	0.0323	3.731

Similar patterns were seen for SHG and Motala (Motala is often analysed as a part of SHG; here it is divided purely because Motala samples coming from one site can be considered a separate population) in a D form *D(Danubian, WHG, SHG/Motala, ≠Khomani)*. From WHG, only Bichon and La Braña showed a significantly different (and lower) amount of ancestry shared with SHG/Motala than with Danubians (see Table 26).

Table 26: *D(Danubian, WHG. SHG/Motala, ≠Khomani), values for |Z|>3 shown, all values can be seen in Supplementary File S2.*

Danubian	WHG	SHG/Motala	≠Khomani	D value	Z-score
Lepe45	La Braña	SHG	Khomani	0.0275	3.678
Vlasa37	La Braña	SHG	Khomani	0.0283	3.955
Lepe51	La Braña	SHG	Khomani	0.0309	4.25
Lepe45	Bichon	SHG	Khomani	0.0335	4.662
Vlasa37	Bichon	SHG	Khomani	0.0341	4.665
Lepe51	Bichon	SHG	Khomani	0.0371	5.357
Lepe51	Bichon	Motala_HG	Khomani	0.0193	3.35
Vlasa37	La Braña	Motala_HG	Khomani	0.0247	4.418
Lepe51	La Braña	Motala_HG	Khomani	0.0326	5.927

Another important HG group, CHG (Caucasus hunter-gatherers), was tested for its differential genetic similarity to WHG and Danubians in a D form *D(Danubian, WHG. CHG, ≠Khomani)*. These samples (KK1 and SATP) indicated (contrary to SHG/Motala and EHG) significantly more drift shared with Bichon than with Danubians (see Table 27). For other WHG samples, the results were also mostly negative but not significant (see Supplementary File S2 for all results). This confirms the finding of Jones *et al.* (*53*) and Broushaki *et al.* (*9*) that after very ancient split of

CHG and WHG there might have been gene flow between the populations. It is of interest to note that, compared to Danubians, only Bichon, the oldest WHG sample, was shown to have significant levels of unique drift shared with CHG compared to drift shared with Danubians. This would suggest gene sharing between CHG and a population close to Bichon but still differentiated from Danubians, even though other WHG (KO1) and Danubians were geographically closer to CHG.

Table 27: *D(Danubian, WHG. CHG, ≠Khomani), values for |Z|>3 shown, all values can be seen in Supplementary File S2.*

Danubian	WHG	CHG	≠Khomani	D value	Z-score
Lepe51	Bichon	SATP	Khomani	-0.1126	-12.806
Vlasa37	Bichon	SATP	Khomani	-0.1169	-11.942
Lepe45	Bichon	SATP	Khomani	-0.0995	-11.162
Vlasa37	Bichon	KK1	Khomani	-0.0794	-10.298
Lepe45	Bichon	KK1	Khomani	-0.0684	-9.068
Lepe51	Bichon	KK1	Khomani	-0.0623	-7.909

Except for CHG, there was only one other combination that showed significantly less drift shared with Danubians than with other WHG. According to the results of *D(Danubian, WHG, Kostenki, ≠Khomani)*, there is significantly more unique drift (except for Lepe51 but still with Z<-2.4) between Kostenki (*63*) and Loschbour than between Danubians and Kostenki (see Table 28). For other WHG, the D values were also often negative (see Supplementary File S2). Still, it is especially surprising that a very old Kostenki sample (∼37, 000 years old) showed differences in affinities to rather homogeneous WHG and Danubians, and it was a relatively recent Loschbour sample (dated to approx. 6000 BC) that was shown to be related to this very old sample.

Table 28: *D(Danubian, WHG, Kostenki, ≠Khomani), values for |Z|>2 shown, all values can be seen in Supplementary File S2.*

Danubian	WHG	Kostenki	≠Khomani	D value	Z-score
Vlasa37	Loschbour	Kostenki	Khomani	-0.0257	-3.208
Lepe45	Loschbour	Kostenki	Khomani	-0.0225	-3.057
Lepe51	Loschbour	Kostenki	Khomani	-0.0194	-2.437

4.5.4 Genetic structure of Western hunter-gatherers

Western hunter-gatherers have been described first for Mesolithic Loschbour (*78*) and La Braña (*79*) samples dated to 6^{th}–7^{th} millennium BC. The WHG genomic similarity was extended to a Bichon sample from Switzerland (*53*) that is more than 13,000 years old and to KO1 sample from a Hungarian Neolithic context (*52*). Since Danubians are dated approximately between Loschbour/La Braña and Bichon samples and are geographically the closest to KO1, it begs the question of what their comparative relationship to other WHG samples was. We tested all combinations with D statistics in a form *D(WHG, WHG, Danubian, ≠Khomani)* in order to compare the WHG samples

by their genetic similarity to Danubians. Negative (and mostly statistically significant) results for all WHG except for La Braña in comparison to Bichon in *D(Bichon, WHG, Danubian, ≠Khomani)* show that there was a shared drift between central (Loschbour) and south-eastern (KO1 and Danubians) Europe, which happened chronologically between Loschbour/KO1 and Bichon (see Table 29). That would be logical, given the geographical proximity and the amount of time in which gene flow could have happened. Interestingly, this shared ancestry was not observed for NW Iberian La Braña sample and when directly tested as *D(La Braña, WHG, Danubian, ≠Khomani)*, we found statistically significant negative values for all Danubians when compared to KO1 and Loschbour (see Table 30). This suggests that even though there was relative genetic similarity over the region, some level of differentiation between Iberia and the rest of Mesolithic Europe was present. A direct comparison of La Braña and Bichon was not statistically significant (even though the comparison to the oldest sample Lepe51 was, with Z=2.406 in the direction of more shared ancestry with Bichon, see Table 29). The comparison between two younger samples, Loschbour and KO1, in their genetic similarity to Danubians did not show any significant differences (see Supplementary File S2).

Table 29: *D(Bichon, Other WHG, Danubian, ≠Khomani), all values shown.*

Bichon	**Other WHG**	**Danubian**	**≠Khomani**	**D value**	**Z-score**
Bichon	Loschbour	Lepe45	Khomani	-0.056	-6.967
Bichon	KO1	Lepe45	Khomani	-0.0425	-4.936
Bichon	Loschbour	Vlasa37	Khomani	-0.0347	-3.815
Bichon	Loschbour	Lepe51	Khomani	-0.0295	-3.573
Bichon	KO1	Vlasa37	Khomani	-0.0253	-2.706
Bichon	KO1	Lepe51	Khomani	-0.0245	-2.661
Bichon	La Braña	Lepe45	Khomani	0.001	0.123
Bichon	La Braña	Vlasa37	Khomani	0.0035	0.399
Bichon	La Braña	Lepe51	Khomani	0.0202	2.406

Table 30: *D(La Braña, Other WHG. Danubian, ≠Khomani), all values shown.*

La Braña	**Other WHG**	**Danubian**	**≠Khomani**	**D value**	**Z-score**
La Braña	Loschbour	Lepe45	Khomani	-0.0525	-6.844
La Braña	Loschbour	Lepe51	Khomani	-0.0475	-5.84
La Braña	KO1	Lepe45	Khomani	-0.0435	-5.279
La Braña	KO1	Lepe51	Khomani	-0.0448	-5.076
La Braña	Loschbour	Vlasa37	Khomani	-0.0354	-4.159
La Braña	KO1	Vlasa37	Khomani	-0.0308	-3.386

To compare the shared drift of Danubians and WHG to a drift the other WHG shared with each other, we tested all combinations of *D(Danubian, WHG, WHG, ≠Khomani)*. A different ancestral relationship to La Braña was confirmed as *D(Danubian, La Braña, KO1, ≠Khomani)* values were the only positive and significant ones for Danubians (see Table 31). Apart from that the values are either insignificant (especially for other comparisons involving KO1, see Supplementary File S2), or significantly negative (see Table 32). It should be especially noted that there is a violation of tests

having a null hypothesis of branching of Loschbour with Danubians with respect to Bichon and La Braña *D(Danubian, Loschbour, La Braña/Bichon, ≠Khomani)*, which is significantly negative for all comparisons. Together with the previous results comparing the shared drift with Danubians, we concur that WHG ancestry has isolation by distance pattern from Iberia, over central Europe to Hungary and the Danube Gorges. Except for Bichon, the age of the samples does not seem to play a role (at least not on a level observable by f-statistics).

Table 31: *D(Danubian, WHG, WHG, ≠Khomani) , values for Z >3 shown, all values can be seen at Supplementary File S2, significantly negative values in Table 32.*

Danubian	**WHG**	**WHG**	**≠Khomani**	**D value**	**Z-score**
Vlasa37	La Braña	KO1	Khomani	0.0476	5.182
Lepe45	La Braña	KO1	Khomani	0.0487	5.754
Lepe51	La Braña	KO1	Khomani	0.0542	6.204

Table 32: *D(Danubian, WHG. WHG, ≠Khomani) , values for Z <-3 shown, all values can be seen at Supplementary File S2, significantly positive values in Table 31.*

Danubian	**WHG**	**WHG**	**≠Khomani**	**D value**	**Z-score**
Lepe45	Loschbour	Bichon	Khomani	-0.0987	-11.349
Lepe51	Loschbour	Bichon	Khomani	-0.0723	-8.227
Vlasa37	Loschbour	Bichon	Khomani	-0.0835	-8.417
Lepe45	Loschbour	La Braña	Khomani	-0.0545	-6.73
Lepe51	Loschbour	La Braña	Khomani	-0.0488	-6.271
Vlasa37	Loschbour	La Braña	Khomani	-0.0408	-5.118
Lepe45	Bichon	Loschbour	Khomani	-0.0429	-4.669
Lepe51	Bichon	Loschbour	Khomani	-0.0428	-4.876
Vlasa37	Bichon	Loschbour	Khomani	-0.049	-5.238
Lepe45	Bichon	La Braña	Khomani	-0.0295	-3.496
Lepe45	La Braña	Bichon	Khomani	-0.0285	-3.252

4.5.5 Relationship of Greek and Anatolian genome samples to other ancient and modern populations

As a part of Hofmanová *et al.* (*51*), we have performed genomic analysis jointly for genomes from Neolithic NW Anatolia (abbreviated in the following sections as "Anatolian", see Table 20 for the summary of the whole genome dataset) and Neolithic Greece (Rev5 from Early Neolithic and Klei10 and Pal7 from Final Neolithic, *51*) and when referred to jointly, a term "Aegean" was used. The relative genetic similarity of Greek and Anatolian Neolithic samples to other ancient populations was estimated using an *outgroup* f3-statistic as for the Danubians (see 3.13.1). Our form of the *outgroup* f3 statistic was *f3(≠Khomani; TEST, Greek/Anatolian)* where *TEST* was one of the ancient populations from the reference datasets (see Figure 15).

The greatest amount of genetic similarity as reflected by the largest f3 statistics is generally found between the Greek (*51*) and NW Anatolian Neolithic genomes generated in this study and the Chalcolithic Anatolian sample, Kumtepe6 (see Figure 15). Other populations demonstrating high f3 values with the Greek/Anatolian population (considered either separately or together) are other European Early and Middle Neolithic populations. Especially high amounts of shared drift can be seen with Spanish Neolithic farmers, LBK and Starčevo. This suggests a common ancestry component for Neolithic populations found throughout Europe during this era. Interestingly Pal7, and Klei10 demonstrate relatively high levels of drift with the KK and SATP genomes from Jones *et al.* (*53*) compared to the other Aegean samples, potentially indicating some admixture between late Neolithic Greeks and a population similar to Caucasus hunter-gatherers (CHG) not present in the region previously.

We also used the *outgroup* f3-statistic to examine the level of genetic similarity between our Greek/ Anatolian Neolithic samples and contemporary humans populations (see Figure 16 and detailed results in Supplementary File S1). The geographical distributions of the values can be seen in Figure 17. The modern populations with the highest f3 statistics are those located in the Mediterranean area (Italians, Sardinians, Greeks etc.) as well as Basques. All the highest values were obtained for modern Sardinians, a population previously noted for its genetic similarity to early farmers (*78*), possibly because of their relative geographic isolation from mainland Europe. However, we did not observe particularly high genetic similarity between the ancient samples excavated in Anatolia and the geographically closest modern Turkish populations. This pattern is also supported by PCA, mixture model and simulation-based continuity analysis in Hofmanová *et a.* (*51*).

4.5.6 Genetic Structure of Aegean Neolithic populations

Outgroup f3 statistics (see Figure 15) indicate that the Greek and Anatolian samples are highly similar compared to all other populations. However, the D statistic allowed us to more explicitly

Table 33: *D(Anatolian, Greek, Early farmer, ≠Khomani), values for |Z|>2 shown.*

Anatolian	Greek	Early farmer	≠Khomani	D value	Z-score
Bar31	Klei10	Spain_EN	≠Khomani	-0.0129	-2,017
Bar8	Pal7	LBK_EN	≠Khomani	-0.0103	-2,033
Bar31	Pal7	Spain_EN	≠Khomani	-0.0112	-2,130
Bar31	Pal7	LBKT_EN	≠Khomani	-0.0440	-2,163
Bar8	Pal7	Stuttgart	≠Khomani	-0.0199	-2,169
Bar8	Pal7	Stuttgart	≠Khomani	-0.0199	-2,169
Bar31	Pal7	Alberstedt_LN	≠Khomani	-0.0173	-2,193
Bar8	Pal7	Spain_MN	≠Khomani	-0.0154	-2,198
Bar8	Rev5	LBK_EN	≠Khomani	-0.0101	-2,223
Bar8	Klei10	LBK_EN	≠Khomani	-0.0133	-2,227
Bar8	Pal7	Alberstedt_LN	≠Khomani	-0.0204	-2,621
Bar8	Pal7	Spain_EN	≠Khomani	-0.0178	-2,820
Bar8	Klei10	Spain_MN	≠Khomani	-0.0230	-3,322
Bar8	Klei10	Spain_EN	≠Khomani	-0.0242	-3,518
Bar8	Klei10	Esperstedt_MN	≠Khomani	-0.0319	-3,757

examine the level of population genetic structure amongst these samples with regard to geography and chronology.

We first examined the D statistics of the form *D(Anatolian, Greek, Early_farmer, ≠Khomani)* (see Table 33 and Supplementary File S1) in order to examine whether there are differences in the level of non-Aegean early farmer ancestry in Greek versus Anatolian samples. While not all pairwise comparisons were significant, there was a slight general trend of negative D values indicating that Greeks were more genetically similar to other early farming populations from Spain and central Europe (perhaps indicative of a movement of Neolithic farmers across the Aegean sea from Anatolia into the rest of Europe, though this also may simply be an isolation by distance pattern). However, we note that no significant comparisons were observed when using only transition mutations (i.e., removing potential post-mortem damage but lowering the number of positions analyzed). If population structure did exist between the Anatolian and Greek Neolithic farmers it was likely relatively subtle.

Given that there is a substantial time gap of ∼2,000 years between Early Neolithic (Rev5, Bar8, Bar31) and Middle Neolithic (Klei10, Pal7) samples and a gap of ∼200 years between Bar8 and Bar31 (see section 2.4), we calculated D statistics additionally in the forms *D(Greek1, Greek2, Early_farmer, Khomani)* and *D(Bar8, Bar31, Early_farmer, ≠Khomani).* We found no significant pairwise comparisons using these chronological groupings (see Supplementary File S1). Assuming the Aegean as the source of European Neolithic ancestry, this would indicate that once early European farmers diverged from this source, the Aegean populations remained relatively isolated from later European farmers (i.e., there were no major episodes of gene flow back into Aegean farming populations from the west).

Table 34: *D(Early farmer, Iceman, Aegean, ≠Khomani), values for |Z|>3 shown.*

Early farmer	**Iceman**	**Aegean**	**≠Khomani**	**D value**	**Z-score**
Hungary_CA	Iceman	Pal7	≠Khomani	-0.0373	-5,409
SwedenSkoglund_MN	Iceman	Bar31	≠Khomani	-0.0358	-4,062
Hungary_EN	Iceman	Pal7	≠Khomani	-0.0207	-3,801
SwedenSkoglund_MN	Iceman	Pal7	≠Khomani	-0.0305	-3,542
Hungary_EN	Iceman	Bar31	≠Khomani	-0.0193	-3,084
Hungary_EN	Iceman	Rev5	≠Khomani	-0.0228	-3,021

When Western and Eastern hunter-gatherers were included in our analysis using D statistics of the form *D(Aegean, Aegean, HG, ≠Khomani)* (see Supplementary File S1), we obtained no significantly positive values for *D(Greek, Anatolian, HG, ≠Khomani)* comparisons, suggesting that Aegean populations also formed a clade with respect to HG and we did not observe significant shared drift violating this tree.

4.5.7 The Aegean as the source for early farming populations in Europe

We next estimated D values using the form *D(Early_farmer, post-Neolithic, Aegean, ≠Khomani)* in order to formally examine whether the Greek and Anatolian Neolithic samples were genetically closer to other Early and Middle Neolithic European farmers compared to other post Neolithic European and Middle Eastern populations. As expected, almost all D values were significantly positive (with |Z|>>3, see Supplementary File S1) (for the only exception, see Iceman below), consistent with the Aegean Neolithic populations being more genetically similar, as defined by greater levels of shared drift paths, to other early European farmers than with any other tested populations from more recent eras. Similarly, D tests of the form *D(Early_farmer, HG, Neolithic Greek/Anatolian, ≠Khomani)* (all values significantly positive, see Supplementary File S1) and *D(Neolithic Greek/Anatolian, HG, Early_farmer, ≠Khomani)* (all values positive, >90% significant with the insignificant combinations involving only populations represented by a single individual of low coverage, see Supplementary File S1) also clearly demonstrated that the Neolithic Aegeans were genetically more related to early farmers than any hunter-gatherer populations.

Interestingly, comparisons of our Neolithic Greek/Anatolian samples with the Late Neolithic/Early Bronze Age Iceman (*202*) resulted in significantly negative values when compared to Neolithic farmers (see Table 34 and Supplementary File S1). If our Aegean populations are assumed to be the source of Neolithic genetic ancestry, it is thus possible that Ötzi and his ancestors did not admix with local populations after an initial spread from the Aegean to the same extent as other Middle and Late Neolithic farmer populations. This unique drift shared between Iceman and Aegeans might also suggest that the ancestors of this individual either shared substantial exchange with the Aegean farmer core area after the original spread, or they migrated from the core area later.

Based on the genetic similarity between the Early and Middle Neolithic populations and the archaeological context of the samples, it is reasonable to assume that this genetic ancestry arose in and around Anatolia and spread out to the rest of Europe. Given the presence of Early Neolithic farmers stretching from Anatolia all the way to Spain, might this spread have arisen via a serial range expansion moving westwards? If this was the case, then some non-Aegean early farmers further along this route might be expected to share unique drift compared to the original source Aegean farmers. However, D statistics of the form *D(Early_farmer2, Aegean, Early_farmer1, ≠Khomani)* using all pairwise combinations of non-Aegean Early Neolithic farmers from different geographic locations (Spain, Hungary/central Europe) demonstrated negative or non-significant values rather than positive ones with the exception of *D(LBK_EN, Bar8, SPAIN_EN, ≠Khomani)* (see Table 35).

These results suggest that either the initial Neolithic expansion from the Aegean region to the rest of Europe involved multiple independent migrating groups from the same source or was very rapid such that there was insufficient time for genetic differentiation. Interestingly, positive D values were obtained when comparing pairs of non-Aegean early farmers from the same region but different time periods, suggesting some geographic-specific drift once these populations were established and diverged from Aegeans. Some positive values were also obtained when comparing Middle Neolithic Spanish to Middle Neolithic Esperstedt, which may be the result of the proposed resurgence of hunter-gatherer ancestry during this era and the Late Neolithic. It is of interest to note that the positive significant values were observed almost exclusively with Anatolian samples (see in Table 36), which is consistent with the previous observation from the *D(Anatolian, Greek, Early_farmer, ≠Khomani)* test that the Greek Aegean samples are genetically closer to Early Neolithic farmers.

Under a scenario of a rapid migration from central Europe and then to Spain, we would assume that non-Aegean farmers would form a clade to the exclusion of Aegeans. However, when performing a test of the form *D(Early_farmer1, Early_farmer2, Aegean, ≠Khomani)*, we observed unique drift between Aegeans and Spanish farmers (see Table 37). This points to a gene flow event through the Mediterranean between Greece and Spain that did not include central Europe. Given the previously discussed result (see Table 33) and the archaeological record, the most likely scenario would be an independent migration of Aegean farmers to Iberia distinct from an initial migration to central Europe (though migration from Spain back to the Aegean would also fit the data). Due to the observation of unique drift between LBK and Spanish early farmers after their split from Bar8 (one significant positive value for *D(LBK_EN, Bar8, SPAIN_EN, ≠Khomani)*, see Table 35), we can speculate that it happened chronologically after this individual lived (6,212-6,030 cal BC).

4.5.8 The relationship between Neolithic Aegeans and Chalcolithic Anatolians

Given their geographic proximity, the Aegean population characterized by the genomes sequenced in this study could potentially be the source population for both the Anatolian Kumtepe (*215*) that is dated to Chalcolithic as well as European Neolithic farmers. Interestingly, D tests of the

Table 35: *D(Early_farmer2, Aegean, Early_farmer1, ≠Khomani), values for Z>3 shown.*

Early_farmer2	**Aegean**	**Early_farmer1**	**≠Khomani**	**D value**	**Z-score**
Esperstedt_MN	Bar8	Baalberge_MN	≠Khomani	0.0248	3,158
LBK_EN	Bar8	Stuttgart	≠Khomani	0.0153	3,290
LBK_EN	Bar8	Stuttgart	≠Khomani	0.0153	3,290
Starcevo_EN	Rev5	Hungary_EN	≠Khomani	0.0277	3,316
Spain_EN	**Bar8**	**LBK_EN**	**≠Khomani**	**0.0130**	**3,352**
Starcevo_EN	Bar8	Hungary_EN	≠Khomani	0.0203	3,360
Spain_EN	Bar31	Spain_MN	≠Khomani	0.0161	3,505
Starcevo_EN	Bar8	Esperstedt_MN	≠Khomani	0.0438	3,532
Spain_MN	Rev5	Esperstedt_MN	≠Khomani	0.0221	3,603
Spain_EN	Bar31	Esperstedt_MN	≠Khomani	0.0249	3,614
Baalberge_MN	Bar8	Esperstedt_MN	≠Khomani	0.0304	3,732
Spain_MN	Bar31	Esperstedt_MN	≠Khomani	0.0289	3,816
Stuttgart	Bar8	Esperstedt_MN	≠Khomani	0.0218	4,004
Stuttgart	Bar8	Esperstedt_MN	≠Khomani	0.0218	4,004
Starcevo_EN	Bar8	LBK_EN	≠Khomani	0.0269	4,057
Esperstedt_MN	Bar31	LBK_EN	≠Khomani	0.0188	4,075
Esperstedt_MN	Bar31	Spain_MN	≠Khomani	0.0220	4,379
Esperstedt_MN	Bar8	Spain_EN	≠Khomani	0.0245	4,647
Spain_MN	Bar8	Spain_EN	≠Khomani	0.0234	4,696
LBK_EN	Bar31	Esperstedt_MN	≠Khomani	0.0294	4,755
Esperstedt_MN	Bar8	Spain_MN	≠Khomani	0.0329	5,514
Spain_EN	Bar8	Spain_MN	≠Khomani	0.0276	5,667
Esperstedt_MN	Bar8	LBK_EN	≠Khomani	0.0299	6,215
Spain_EN	Bar8	Esperstedt_MN	≠Khomani	0.0371	6,441
LBK_EN	Bar8	Esperstedt_MN	≠Khomani	0.0394	7,725
Spain_MN	Bar8	Esperstedt_MN	≠Khomani	0.0417	8,020

Table 36: *D(Early_farmer2, Aegean, Early_farmer1, ≠Khomani), values for Z<-3 shown.*

Early_farmer2	**Aegean**	**Early_farmer1**	**≠Khomani**	**D value**	**Z-score**
Hungary_EN	Pal7	Spain_MN	≠Khomani	-0.0314	-7,625
Hungary_EN	Klei10	Spain_MN	≠Khomani	-0.0322	-7,047
Hungary_EN	Pal7	Spain_EN	≠Khomani	-0.0282	-6,825
Stuttgart	Pal7	Spain_MN	≠Khomani	-0.0277	-5,806
Stuttgart	Pal7	Spain_MN	≠Khomani	-0.0277	-5,806
Hungary_EN	Klei10	Spain_EN	≠Khomani	-0.0279	-5,332
Stuttgart	Klei10	Spain_MN	≠Khomani	-0.0288	-5,055
Stuttgart	Klei10	Spain_MN	≠Khomani	-0.0288	-5,055
Stuttgart	Bar31	Spain_MN	≠Khomani	-0.0206	-4,642
Stuttgart	Bar31	Spain_MN	≠Khomani	-0.0206	-4,642
LBK_EN	Pal7	Spain_MN	≠Khomani	-0.0155	-4,292
Hungary_EN	Bar31	Spain_MN	≠Khomani	-0.0194	-4,268
LBK_EN	Pal7	Spain_EN	≠Khomani	-0.0139	-4,119
Spain_MN	Pal7	Stuttgart	≠Khomani	-0.0295	-3,950
Spain_MN	Pal7	Stuttgart	≠Khomani	-0.0295	-3,950
Stuttgart	Pal7	Spain_EN	≠Khomani	-0.0244	-3,875
Stuttgart	Pal7	Spain_EN	≠Khomani	-0.0244	-3,875
Hungary_EN	Rev5	Spain_MN	≠Khomani	-0.0206	-3,711
Hungary_EN	Rev5	Spain_EN	≠Khomani	-0.0179	-3,610
Hungary_EN	Pal7	LBK_EN	≠Khomani	-0.0124	-3,560
Hungary_EN	Bar31	Spain_EN	≠Khomani	-0.0141	-3,460
Stuttgart	Klei10	Spain_EN	≠Khomani	-0.0237	-3,447
Stuttgart	Klei10	Spain_EN	≠Khomani	-0.0237	-3,447
Spain_MN	Bar31	Stuttgart	≠Khomani	-0.0187	-3,193
Spain_MN	Bar31	Stuttgart	≠Khomani	-0.0187	-3,193
Spain_MN	Pal7	Hungary_EN	≠Khomani	-0.0143	-3,180
Stuttgart	Rev5	Spain_MN	≠Khomani	-0.0232	-3,113
Stuttgart	Rev5	Spain_MN	≠Khomani	-0.0232	-3,113
SwedenSkoglund_MN	Klei10	LBK_EN	≠Khomani	-0.0165	-3,046

Table 37: *D(Early farmer1, Early farmer2, Aegean, ≠Khomani), values for Z<-2 shown.*

Early farmer1	**Early farmer2**	**Aegean**	**≠Khomani**	**D value**	**Z-score**
Hungary_EN	Spain_EN	Pal7	≠Khomani	-0.0236	-5.379
Hungary_EN	Spain_EN	Klei10	≠Khomani	-0.0255	-5.201
Hungary_EN	LBK_EN	Rev5	≠Khomani	-0.0142	-4.352
Hungary_EN	Spain_MN	Klei10	≠Khomani	-0.0194	-4.26
Hungary_EN	Spain_EN	Rev5	≠Khomani	-0.0172	-3.859
Stuttgart	Starcevo_EN	Klei10	≠Khomani	-0.0377	-3.387
Stuttgart	Starcevo_EN	Klei10	≠Khomani	-0.0377	-3.387
Hungary_EN	Starcevo_EN	Klei10	≠Khomani	-0.029	-3.369
Hungary_EN	LBK_EN	Bar31	≠Khomani	-0.0092	-3.334
Hungary_EN	LBK_EN	Klei10	≠Khomani	-0.0125	-3.264
Hungary_EN	Spain_MN	Pal7	≠Khomani	-0.0171	-3.245
Stuttgart	Spain_EN	Klei10	≠Khomani	-0.0201	-3.133
Stuttgart	Spain_EN	Klei10	≠Khomani	-0.0201	-3.133
Hungary_EN	LBK_EN	Pal7	≠Khomani	-0.0118	-3.107
Stuttgart	Starcevo_EN	Bar8	≠Khomani	-0.0264	-3.095
Stuttgart	Starcevo_EN	Bar8	≠Khomani	-0.0264	-3.095
LBK_EN	Starcevo_EN	Bar8	≠Khomani	-0.0194	-3.031
Hungary_EN	Starcevo_EN	Rev5	≠Khomani	-0.0269	-2.996
LBK_EN	Spain_EN	Klei10	≠Khomani	-0.013	-2.871
Hungary_EN	Spain_EN	Bar31	≠Khomani	-0.0128	-2.811
LBK_EN	Spain_EN	Pal7	≠Khomani	-0.0121	-2.8
Stuttgart	Spain_MN	Klei10	≠Khomani	-0.0154	-2.548
Stuttgart	Spain_MN	Klei10	≠Khomani	-0.0154	-2.548
Hungary_EN	Starcevo_EN	Bar8	≠Khomani	-0.0175	-2.453
Hungary_EN	Stuttgart	Pal7	≠Khomani	-0.018	-2.25
Hungary_EN	Stuttgart	Pal7	≠Khomani	-0.018	-2.25
Hungary_EN	Spain_MN	Rev5	≠Khomani	-0.0111	-2.19
LBK_EN	Starcevo_EN	Klei10	≠Khomani	-0.0185	-2.107
Hungary_EN	Starcevo_EN	Pal7	≠Khomani	-0.0203	-2.10

form *D(Aegean, Kumtepe, Early_farmer, ≠Khomani)* were often significantly positive (see Table 38 and Supplementary File S1), suggesting that Aegeans share ancestry with Neolithic European farmers (especially with LBK, Starčevo and other Early Hungarian Neolithic farmers) not present in Kumtepe samples. Thus, the Kumtepe likely descended from an Aegean population that was somewhat differentiated from the one that expanded from Anatolia into the rest of Europe.

We also examined whether Kumtepe shared more unique drift with Anatolian samples from Barcın or later Greek samples by performing D tests of the form *D(Greek, Kumtepe, Neo_Anatolian, ≠Khomani)* and *D(Neo_Anatolian, Kumtepe, Greek, ≠Khomani)* (Table 39). Kumtepe6 demonstrated unique drift with Neolithic Greeks, especially Late Neolithic ones (Klei10, Pal7), which could be explained by gene flow that was maintained over the Aegean throughout the Neolithic. Results for Kumtepe4 showed indications of shared ancestry in the opposite direction (i.e., greater affinity with Barcın), but this result was barely significant, perhaps as a consequence of the much lower coverage of this genome.

Finally, D statistics of the form *D(Aegean, Kumtepe, CHG, ≠Khomani)* showed that CHG populations shared unique drift with Kumtepe6 when compared to both Greek and Anatolian Aegeans (Table 41). Though little is known about hunter-gatherers in Anatolia, this suggests that towards the end of, or directly following, the Neolithic expansion there was gene flow from the Caucasus and neighboring regions to Anatolia. If there was continued gene flow across the Aegean at this time between Greece and Anatolia, this would also be compatible with the f3 outgroup results which show the later Greek samples to be closer to CHG than the Rev5 and two early Neolithic Anatolian samples.

Table 38: *D(Aegean, Kumtepe, Early farmer, ≠Khomani), values for |Z3|>3 shown.*

Aegean	**Kumtepe**	**Early farmer**	**≠Khomani**	**D value**	**Z-score**
Bar8	Kumtepe6	Hungary_EN	≠Khomani	0.0706	4.526
Bar31	Kumtepe6	LBK_EN	≠Khomani	0.0571	4.321
Klei10	Kumtepe6	Hungary_BA	≠Khomani	0.083	4.101
Bar8	Kumtepe6	LBK_EN	≠Khomani	0.0514	4.002
Klei10	Kumtepe6	Hungary_EN	≠Khomani	0.0616	3.71
Klei10	Kumtepe6	Stuttgart	≠Khomani	0.0851	3.63
Klei10	Kumtepe6	Stuttgart	≠Khomani	0.0851	3.63
Bar31	Kumtepe6	Hungary_BA	≠Khomani	0.0682	3.588
Pal7	Kumtepe4	Esperstedt_MN	≠Khomani	0.2761	3.502
Bar8	Kumtepe6	Corded_Ware_LN	≠Khomani	0.0604	3.476
Klei10	Kumtepe6	Bell_Beaker_LN	≠Khomani	0.0633	3.439
Bar8	Kumtepe6	Hungary_BA	≠Khomani	0.0662	3.436
Bar31	Kumtepe6	Bell_Beaker_LN	≠Khomani	0.0493	3.404
Bar31	Kumtepe6	Hungary_EN	≠Khomani	0.0519	3.331
Klei10	Kumtepe6	LBK_EN	≠Khomani	0.0576	3.33
Klei10	Kumtepe4	Esperstedt_MN	≠Khomani	0.2394	3.268
Rev5	Kumtepe6	Hungary_EN	≠Khomani	0.0649	3.188
Pal7	Kumtepe4	Spain_MN	≠Khomani	0.1862	3.164
Bar31	Kumtepe6	Hungary_CA	≠Khomani	0.0833	3.058
Klei10	Kumtepe6	Starcevo_EN	≠Khomani	0.1247	3.028
Pal7	Kumtepe6	Hungary_CA	≠Khomani	0.1179	3.018
Klei10	Kumtepe6	Esperstedt_MN	≠Khomani	0.0863	3.013

Table 39: *D(Greek, Kumtepe, Neo Anatolian, ≠Khomani) and D(Neo_Anatolian, Kumtepe, Greek, ≠Khomani), all values shown. Kumtepe6 shows negative, Kumtepe4 positive values.*

Greek	**Kumtepe**	**Neo Anatolian**	**≠Khomani**	**D value**	**Z-score**
Bar8	Kumtepe6	Pal7	≠Khomani	-0.1438	-6.821
Bar31	Kumtepe6	Pal7	≠Khomani	-0.1183	-4.571
Bar8	Kumtepe6	Klei10	≠Khomani	-0.0672	-3.097
Bar8	Kumtepe6	Rev5	≠Khomani	-0.0652	-2.739
Pal7	Kumtepe6	Bar31	≠Khomani	-0.0465	-1.935
Rev5	Kumtepe6	Bar8	≠Khomani	-0.0395	-1.557
Rev5	Kumtepe6	Bar31	≠Khomani	-0.0547	-1.544
Bar31	Kumtepe6	Klei10	≠Khomani	-0.0296	-1.018
Pal7	Kumtepe6	Bar8	≠Khomani	-0.0255	-1.004
Bar31	Kumtepe6	Rev5	≠Khomani	-0.0114	-0.415
Klei10	Kumtepe6	Bar8	≠Khomani	-0.0072	-0.291
Klei10	Kumtepe6	Bar31	≠Khomani	-0.0017	-0.055
Bar31	Kumtepe4	Klei10	≠Khomani	0.0473	0.599
Bar8	Kumtepe4	Rev5	≠Khomani	0.1316	1.47
Klei10	Kumtepe4	Bar8	≠Khomani	0.1158	1.493
Klei10	Kumtepe4	Bar31	≠Khomani	0.1029	1.513
Pal7	Kumtepe4	Bar31	≠Khomani	0.1203	1.725
Bar8	Kumtepe4	Klei10	≠Khomani	0.1132	1.787
Bar31	Kumtepe4	Rev5	≠Khomani	0.1647	1.857
Rev5	Kumtepe4	Bar8	≠Khomani	0.1453	2.026
Bar31	Kumtepe4	Pal7	≠Khomani	0.1825	2.243
Bar8	Kumtepe4	Pal7	≠Khomani	0.1655	2.283
Pal7	Kumtepe4	Bar8	≠Khomani	0.1892	2.327
Rev5	Kumtepe4	Bar31	≠Khomani	0.2766	3.529

Table 40: *D(Aegeans, Kumtepe, WHG, ≠Khomani), all values shown.*

Aegeans	**Kumtepe**	**WHG**	**≠Khomani**	**D value**	**Z-score**
Pal7	Kumtepe4	La Braña1	≠Khomani	0.0846	1.143
Rev5	Kumtepe4	La Braña1	≠Khomani	0.0805	1.082
Klei10	Kumtepe4	La Braña1	≠Khomani	0.0731	1.015
Pal7	Kumtepe6	La Braña1	≠Khomani	0.0026	0.088
Klei10	Kumtepe6	La Braña1	≠Khomani	0.0007	0.025
Rev5	Kumtepe6	La Braña1	≠Khomani	-0.0003	-0.008
Bar31	Kumtepe6	La Braña1	≠Khomani	-0.0006	-0.025
Bar8	Kumtepe6	La Braña1	≠Khomani	-0.002	-0.086
Bar31	Kumtepe4	La Braña1	≠Khomani	-0.0538	-0.725
Bar8	Kumtepe4	La Braña1	≠Khomani	-0.0756	-1.203
Rev5	Kumtepe6	Loschbour	≠Khomani	0.0771	2.791
Bar8	Kumtepe6	Loschbour	≠Khomani	0.0521	2.24
Klei10	Kumtepe6	Loschbour	≠Khomani	0.0609	2.12
Bar31	Kumtepe6	Loschbour	≠Khomani	0.0418	1.438
Rev5	Kumtepe4	Loschbour	≠Khomani	0.0933	1.21
Pal7	Kumtepe6	Loschbour	≠Khomani	0.0298	0.92
Pal7	Kumtepe4	Loschbour	≠Khomani	0.0091	0.119
Klei10	Kumtepe4	Loschbour	≠Khomani	-0.0139	-0.211
Bar8	Kumtepe4	Loschbour	≠Khomani	-0.0167	-0.248
Bar31	Kumtepe4	Loschbour	≠Khomani	-0.1371	-1.797

Table 41: *D(Aegean, Kumtepe, CHG, ≠Khomani), all values shown.*

Aegeans	**Kumtepe**	**CHG**	**≠Khomani**	**D value**	**Z-score**
Bar8	Kumtepe6	SATP	≠Khomani	-0.1584	-6.017
Bar8	Kumtepe6	KK1	≠Khomani	-0.0855	-4.505
Rev5	Kumtepe6	KK1	≠Khomani	-0.099	-4.116
Bar31	Kumtepe6	KK1	≠Khomani	-0.0836	-3.639
Rev5	Kumtepe6	SATP	≠Khomani	-0.1119	-3.218
Bar31	Kumtepe6	SATP	≠Khomani	-0.0917	-2.954
Klei10	Kumtepe6	SATP	≠Khomani	-0.1049	-2.93
Pal7	Kumtepe6	SATP	≠Khomani	-0.0975	-2.82
Pal7	Kumtepe6	KK1	≠Khomani	-0.0728	-2.81
Klei10	Kumtepe6	KK1	≠Khomani	-0.0684	-2.793
Bar8	Kumtepe4	SATP	≠Khomani	0.0699	0.813
Bar31	Kumtepe4	KK1	≠Khomani	0.0733	0.945
Bar31	Kumtepe4	SATP	≠Khomani	0.111	1.248
Bar8	Kumtepe4	KK1	≠Khomani	0.0913	1.609
Pal7	Kumtepe4	KK1	≠Khomani	0.1294	1.858
Klei10	Kumtepe4	SATP	≠Khomani	0.21	2.156
Pal7	Kumtepe4	SATP	≠Khomani	0.2387	2.876
Rev5	Kumtepe4	SATP	≠Khomani	0.2859	2.877
Klei10	Kumtepe4	KK1	≠Khomani	0.2282	3.118
Rev5	Kumtepe4	KK1	≠Khomani	0.2555	3.833

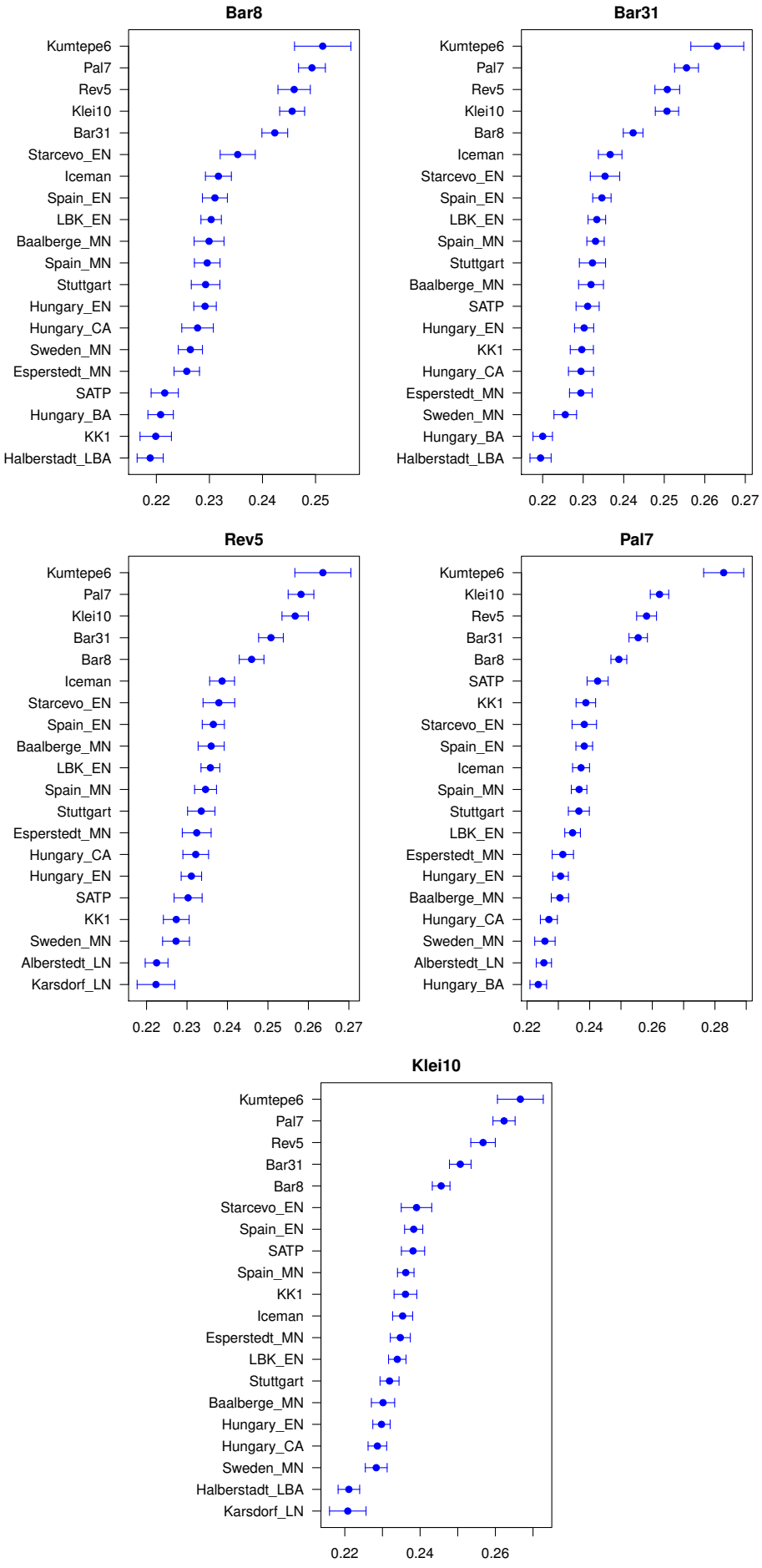


Figure 15: *f3(≠Khomani; Ancient_population, Greek/Anatolian). The highest 20 values shown.*

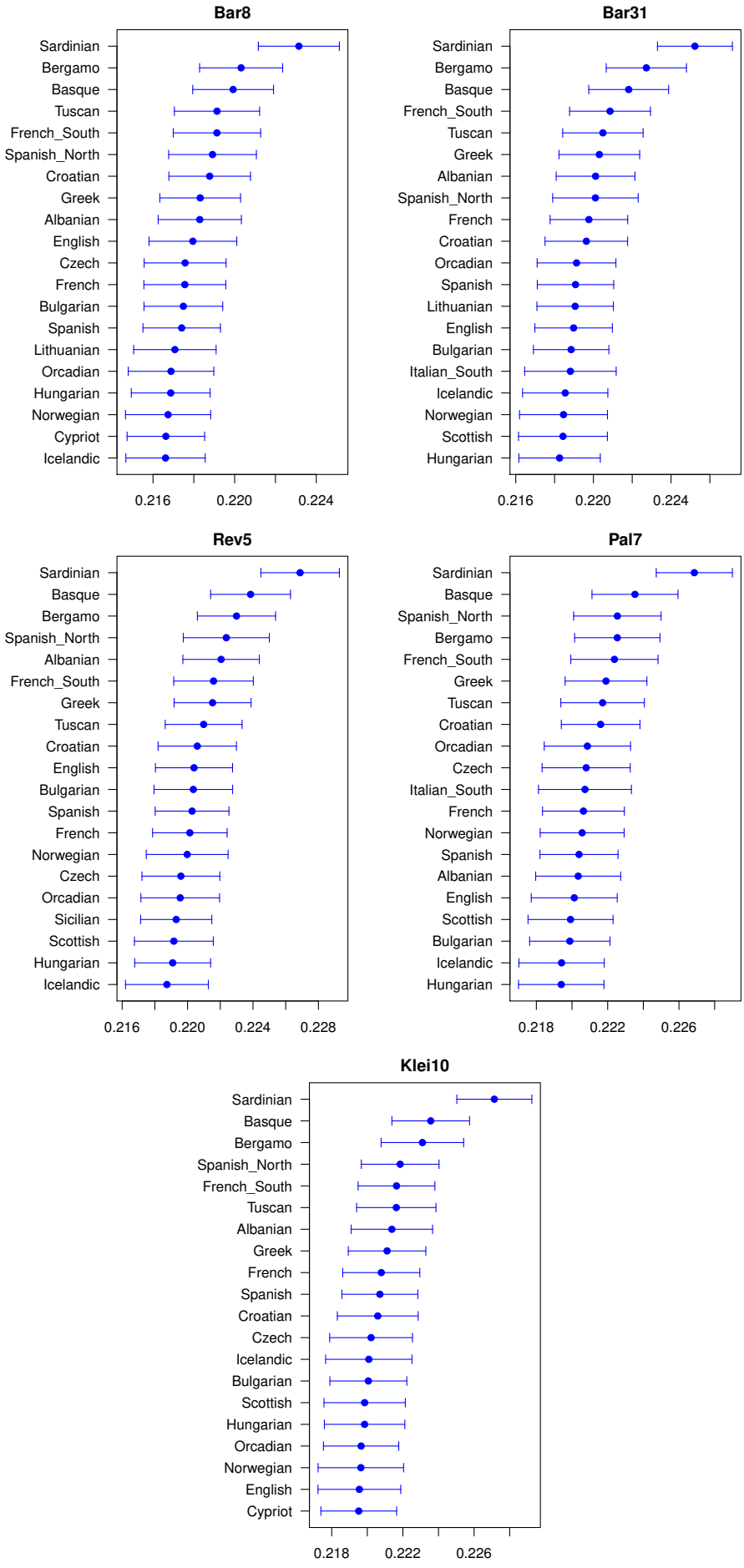
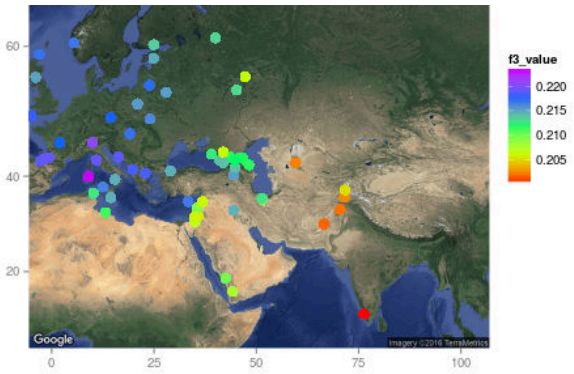
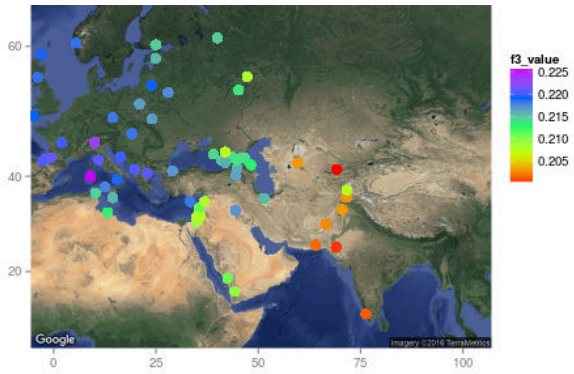


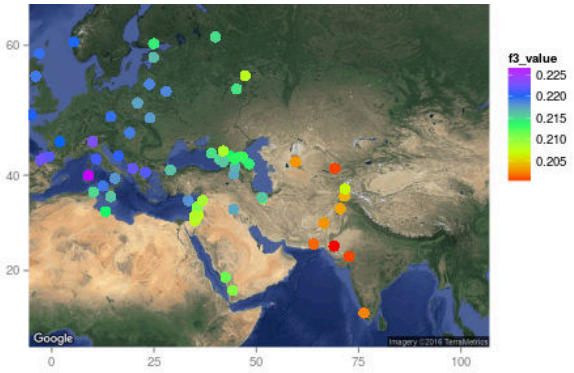
Figure 16: *f3(≠Khomani; Modern_population, Greek/Anatolian). The highest 20 values shown.*



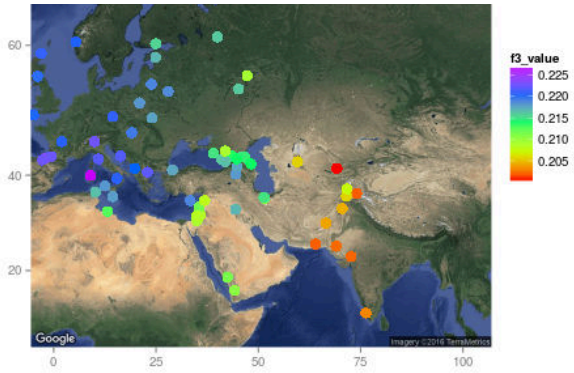
(a) Bar8



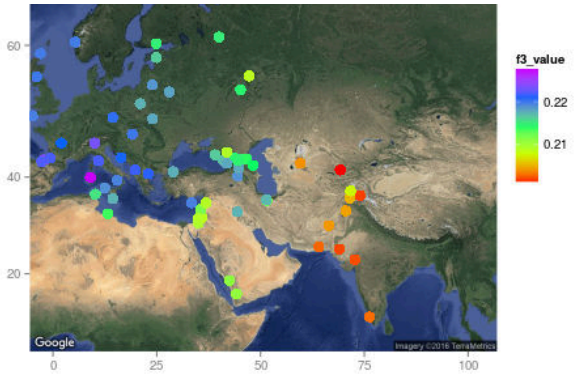
(b) Bar31



(c) Rev5



(d) Pal7



(e) Klei10

Figure 17: *f3(≠Khomani; Modern_population, Greek/Anatolian). Values above 0.2 in the relative geographical proximity shown.*

4.5.9 Hunter-gatherer contributions to farming societies

Recent studies have shown that European Neolithic populations likely experienced some level of western hunter-gatherer (WHG) admixture. In particular, Haak *et al.* (*8*) have suggested there was a resurgence of hunter-gatherer ancestry in Middle and Later Neolithic European farmers. Our NW Anatolian farmers probably possessed genetic ancestry that is most representative of the ancestral Neolithic component, thus presenting an opportunity for us to refine our understanding of the degree of Neolithic vs. WHG admixture in Europe.

Again we used the D statistic, this time of the form *D(Neolithic_farmer, Anatolian, HG, ≠Khomani)*. The observation of a positive value under this test would indicate admixture between the Neolithic farmer and hunter-gatherer populations. The results shown (see Table 42) utilise Loschbour to represent *HG* (other HG in Supplementary File S1).

Amongst Early Neolithic populations, only Neolithic Iberian and Hungarian early farmers show significant positive values ($|Z|>3$; see Table 42) and therefore evidence of hunter-gatherer gene flow. As previously noted by Haak *et al.* (*8*), there is, however, evidence of a resurgence of the hunter-gatherer admixture component in Middle and Late Neolithic samples from Iberia, Hungary and central Europe (see Supplementary File S1).

Regarding the differential affinities of Kumtepe and Neolithic Aegeans to hunter-gatherers, we did not observe significant drift with WHG for any of Neolithic or Chalcolithic Aegeans studied. If the Final and Chalcolithic Aegeans samples are representative of their respective populations, we can then conclude that the WHG resurgence did not happen in the Aegean. However, it should be noted that for *D(Aegeans, Kumtepe6, Loschbour, ≠Khomani)*, the values are positive and some Z-scores are above 2 for Bar8 and Rev5 (see Table 40), showing rather an opposite trend (the decrease of WHG-like ancestry over time in the Aegean).

When we examined a D statistic of the form *D(Neolithic_farmer, Aegeans, CHG, ≠Khomani)*, we obtained almost exclusively negative results, consistent with CHG admixture with the Aegean (see Supplementary File S1). Again, consistent with the results described the f3 tests, a D test of the form of *D(Aegean, Aegean, CHG, ≠Khomani)* (Table 43) demonstrated greater shared drift between CHG and Late Neolithic Greeks.

4.5.10 Differences between Danubian and WHG heritage in farming societies

Danubians are the geographically closest WHG samples to the Aegean population and are directly placed between the Aegean and central Europe. It is therefore important to compare the results regarding the detection of HG admixture obtained previously (see Table 42) with Loschbour to Danubians. We therefore performed a test *D(Neolithic_farmer, Anatolian, Danubian, ≠Khomani)* (see Table 44). The results indicated that, while the analysis with Loschbour revealed the admix-

Table 42: *D(Early farmer, Greek/Anatolian, HG, ≠Khomani), all values shown.*

Early farmer	**Greek/Anatolian**	**HG**	**≠Khomani**	**D value**	**Z-score**
Hungary_EN	Bar8	Loschbour	≠Khomani	0.0205	4,189
Hungary_EN	Bar31	Loschbour	≠Khomani	0.0271	3,786
Hungary_EN	Klei10	Loschbour	≠Khomani	0.0182	2,551
Hungary_EN	Rev5	Loschbour	≠Khomani	0.0155	2,397
Hungary_EN	Pal7	Loschbour	≠Khomani	0.0140	2,205
LBK_EN	Pal7	Loschbour	≠Khomani	0.0045	0,807
LBK_EN	Bar31	Loschbour	≠Khomani	0.0201	2,977
LBK_EN	Bar8	Loschbour	≠Khomani	0.0133	2,738
LBK_EN	Klei10	Loschbour	≠Khomani	0.0132	1,984
LBK_EN	Rev5	Loschbour	≠Khomani	0.0061	1,066
LBKT_EN	Pal7	Loschbour	≠Khomani	0.0089	0.433
LBKT_EN	Rev5	Loschbour	≠Khomani	0.0348	1,822
LBKT_EN	Bar8	Loschbour	≠Khomani	0.0216	1,386
LBKT_EN	Bar31	Loschbour	≠Khomani	0.0215	1,248
LBKT_EN	Klei10	Loschbour	≠Khomani	-0.0186	-1,077
Spain_EN	Bar8	Loschbour	≠Khomani	0.0258	5,239
Spain_EN	Bar31	Loschbour	≠Khomani	0.0307	4,215
Spain_EN	Klei10	Loschbour	≠Khomani	0.0267	3,868
Spain_EN	Rev5	Loschbour	≠Khomani	0.0175	2,771
Spain_EN	Pal7	Loschbour	≠Khomani	0.0168	2,625
Stuttgart	Bar31	Loschbour	≠Khomani	0.0226	2,809
Stuttgart	Bar8	Loschbour	≠Khomani	0.0155	2,190
Stuttgart	Klei10	Loschbour	≠Khomani	0.0172	2,018
Stuttgart	Pal7	Loschbour	≠Khomani	0.0112	1,305
Stuttgart	Rev5	Loschbour	≠Khomani	0.0097	1,105

Table 43: *D(Aegean, Aegean, CHG, ≠Khomani), all values shown.*

Aegean	**Aegean**	**CHG**	**≠Khomani**	**D value**	**Z-score**
Bar8	Bar31	SATP	≠Khomani	-0.0298	-3.877
Bar8	Bar31	KK1	≠Khomani	-0.0279	-3.487
Rev5	Bar31	SATP	≠Khomani	-0.018	-1.659
Rev5	Bar31	KK1	≠Khomani	-0.0126	-1.291
Klei10	Pal7	SATP	≠Khomani	-0.0166	-1.249
Klei10	Pal7	KK1	≠Khomani	-0.0091	-0.934
Rev5	Bar8	KK1	≠Khomani	0.0078	1.021
Rev5	Bar8	SATP	≠Khomani	0.0121	1.188
Pal7	Bar31	KK1	≠Khomani	0.0219	2.056
Klei10	Bar31	SATP	≠Khomani	0.0214	2.11
Klei10	Bar31	KK1	≠Khomani	0.0228	2.369
Pal7	Bar31	SATP	≠Khomani	0.0334	2.69
Klei10	Rev5	SATP	≠Khomani	0.0312	2.746
Pal7	Rev5	KK1	≠Khomani	0.0349	3.298
Pal7	Rev5	SATP	≠Khomani	0.0476	3.318
Klei10	Rev5	KK1	≠Khomani	0.0321	3.322
Pal7	Bar8	SATP	≠Khomani	0.0671	5.69
Pal7	Bar8	KK1	≠Khomani	0.0528	5.752
Klei10	Bar8	KK1	≠Khomani	0.0491	6.07
Klei10	Bar8	SATP	≠Khomani	0.0535	6.398

Table 44: *D(Early farmer, Greek/Anatolian, Danubian, ≠Khomani), all values shown.*

Early farmer	Greek/Anatolian	Danubian	≠Khomani	D value	Z-score
Starcevo_EN	Bar31	Lepe51	Khomani	0.0296	3.19
Stuttgart	Bar31	Lepe51	Khomani	0.0228	3.151
LBK_EN	Bar8	Lepe45	Khomani	0.0179	3.07
LBK_EN	Bar31	Lepe51	Khomani	0.02	3.461
NE1	Bar8	Lepe45	Khomani	0.0336	4.759
NE1	Bar31	Lepe45	Khomani	0.0267	3.559
NE1	Bar8	Lepe51	Khomani	0.0255	3.459
NE1	Bar31	Lepe51	Khomani	0.0362	4.636
NE1	Bar8	Vlasa37	Khomani	0.0243	3.073
NE1	Bar31	Vlasa37	Khomani	0.0284	3.347
NE3	Bar8	Vlasa37	Khomani	0.062	3.455
NE5	Bar8	Vlasa37	Khomani	0.0292	3.597
NE5	Bar31	Vlasa37	Khomani	0.0282	3.471
NE7	Bar8	Lepe45	Khomani	0.0294	3.837
NE7	Bar31	Lepe45	Khomani	0.0262	3.032
NE7	Bar31	Lepe51	Khomani	0.034	3.889
NE7	Bar31	Vlasa37	Khomani	0.0271	3.073
Spain_EN	Bar8	Lepe45	Khomani	0.0204	3.473
Spain_EN	Bar31	Lepe51	Khomani	0.0256	3.848
Spain_EN	Bar8	Vlasa37	Khomani	0.0207	3.158
Spain_EN	Bar31	Vlasa37	Khomani	0.0243	3.799

ture only for Iberian and Hungarian Early Neolithic farmers, with Danubians as HG we can show unique ancestry between Danubians and all European Early Neolithic populations (Starčevo, LBK, LBK from Transdanubia, Early Neolithic Iberians and almost all Neolithic samples from Neolithic Hungary).

We have already described that there was a significant difference between Danubians in terms of the amount of the shared drift with LBK (see Table 24), because Vlasa37 shared less drift with the LBK (*8*) as *TEST* than Lepenski Vir samples in the D form *D(Danubian, Danubian, TEST, ≠Khomani)*. It is possible that high genetic drift in Vlasac population could be a cause for such results.

To investigate whether other WHG differed from Danubians in terms of the affinities to farmers (since we saw differences in abundance of significant values), we tested a D form *D(Danubian, WHG, Neolithic/Post-Neolithic, ≠Khomani)*. Importantly, more drift shared between Danubians and early farmers (both Neolithic and post-Neolithic) than between WHG and the early farmers was observed only when WHG tested were Bichon and La Braña. This confirms that the genetic split between Bichon/La Braña and Danubians was also maintained in the ancestries of upcoming European farmer populations.

Table 45: *D(Danubian, WHG, Early farmer, ≠Khomani), values for Z>3 shown.*

Danubian	WHG	Early farmer	≠Khomani	D value	Z-score
Lepe45	Bichon	LBK_EN	Khomani	0.027	5.333
Lepe51	Bichon	LBK_EN	Khomani	0.0228	4.428
Lepe45	La Braña	LBK_EN	Khomani	0.0179	3.919
Lepe45	Bichon	NE1	Khomani	0.0457	6.408
Lepe51	Bichon	NE1	Khomani	0.0392	5.298
Vlasa37	Bichon	NE1	Khomani	0.0245	3.223
Lepe45	La Braña	NE1	Khomani	0.0245	3.345
Lepe51	Bichon	NE4	Khomani	0.0603	3.269
Lepe45	Bichon	NE4	Khomani	0.059	3.109
Lepe45	Bichon	NE6	Khomani	0.0356	4.165
Lepe51	Bichon	NE6	Khomani	0.0285	3.722
Lepe45	La Braña	NE6	Khomani	0.029	3.557
Lepe51	La Braña	NE6	Khomani	0.0256	3.301
Lepe45	Bichon	NE7	Khomani	0.0354	4.234
Lepe51	Bichon	NE7	Khomani	0.0322	3.861
Lepe51	Bichon	Stuttgart	Khomani	0.0261	3.662
Lepe45	Bichon	Stuttgart	Khomani	0.023	3.255
Lepe51	La Braña	Stuttgart	Khomani	0.0224	3.114

This increased genetic affinity of Neolithic farmers to Danubians was observed for Neolithic Hungarians (*52*), LBK from central Europe (*8*) and LBK Stuttgart sample (*78*) (see Table 45). Some post-Neolithic samples also proved to share more drift with Danubians (see Table 46), again samples from Hungary (Bronze Age and Copper Age samples (*52*) and also Yamnaya and samples with elevated Yamnaya ancestry (Early Bronze Age samples from Únětice, Bell Beaker samples, Late Neolithic Karlsdorf sample and Corded Ware samples) (*8*). It should be noted that the increased shared drift with Vlasa37 samples was present only three times for both comparisons and previously mentioned significant tests in vast majority involve the Lepenski Vir genomic samples (see Table 46 and 45). Therefore, the ancestry of Lepenski Vir individuals was more prevalent in the later populations than that of other WHG, namely Bichon and La Braña.

The opposite, decreased levels of affinities to Danubians when compared by *D(Danubian, WHG, Neolithic farmer, ≠Khomani)* to early farmers (i.e., statistically significant negative values) were observed again mostly for Bichon sample (see Table 47). Almost all combinations of *D(Danubian, Bichon, Aegean/Kumtepe6, ≠Khomani)* and *D(Danubian, Bichon, Neo/IA_Iranians, ≠Khomani)* were significantly negative. These results agree with the affinities of CHG and Bichon compared to Danubians shown previously (see Table 27), because these samples are comparably close to CHG (*9*, *51*). Additionally, the negative significant values in comparison with Loschbour were present for Middle Neolithic Iberian samples from La Mina (*8*) and with comparison to LaBraña for Chalcolithic Anatolian sample Kumtepe6 (see Table 47). This could suggest that Iberian Middle Neolithic individuals had ancestry that involved HG related rather to Loschbour than to Danubians.

Table 46: *D(Danubian, WHG, Post-neolithic farmer, ≠Khomani), values for Z>3 shown.*

Danubian	**WHG**	**Post-neolithic farmer**	**≠Khomani**	**D value**	**Z-score**
Lepe45	Bichon	Unetice_EBA	Khomani	0.0263	5.109
Lepe51	Bichon	Unetice_EBA	Khomani	0.0251	4.889
Lepe51	La Braña	Unetice_EBA	Khomani	0.024	4.826
Lepe45	La Braña	Unetice_EBA	Khomani	0.0241	4.809
Lepe45	Bichon	Yamnaya	Khomani	0.0238	4.736
Lepe51	Bichon	Yamnaya	Khomani	0.0239	4.729
Vlasa37	Bichon	Yamnaya	Khomani	0.0194	3.751
Lepe51	La Braña	Yamnaya	Khomani	0.0173	3.623
Lepe45	La Braña	Yamnaya	Khomani	0.0154	3.241
Lepe45	Bichon	Corded_Ware_LN	Khomani	0.02	3.595
Lepe51	Bichon	Corded_Ware_LN	Khomani	0.0199	3.357
Lepe45	La Braña	Karsdorf_LN	Khomani	0.038	3.114
Lepe51	Bichon	Bell_Beaker_LN	Khomani	0.0226	4.088
Lepe45	Bichon	Bell_Beaker_LN	Khomani	0.0161	3.041
Lepe51	La Braña	Bell_Beaker_LN	Khomani	0.0229	4.395
Lepe45	La Braña	Bell_Beaker_LN	Khomani	0.0153	3.036
Lepe51	Bichon	BR1	Khomani	0.0346	3.754
Lepe45	Bichon	BR2	Khomani	0.0395	5.326
Lepe51	Bichon	BR2	Khomani	0.036	5.019
Vlasa37	Bichon	BR2	Khomani	0.0246	3.255
Lepe45	La Braña	BR2	Khomani	0.029	4.175
Lepe51	La Braña	BR2	Khomani	0.0248	3.585
Lepe45	Bichon	CO1	Khomani	0.0371	4.57
Lepe51	Bichon	CO1	Khomani	0.0335	4.01
Lepe45	La Braña	CO1	Khomani	0.0259	3.311
Lepe51	La Braña	CO1	Khomani	0.0255	3.241

Table 47: *D(Danubian, WHG, Neolithic farmer, ≠Khomani), values for Z<-3 shown.*

Danubian	WHG	Neolithic/Post-neolithic farmer	≠Khomani	D value	Z-score
Lepe51	Bichon	Bar31	Khomani	-0.0341	-4.305
Vlasa37	Bichon	Bar31	Khomani	-0.043	-5.225
Vlasa37	Bichon	Bar8	Khomani	-0.0279	-3.649
Lepe45	Bichon	IA_Iranian	Khomani	-0.0542	-6.712
Lepe51	Bichon	IA_Iranian	Khomani	-0.0577	-6.824
Vlasa37	Bichon	IA_Iranian	Khomani	-0.0622	-7.185
Lepe45	Bichon	Klei10	Khomani	-0.0496	-6.288
Lepe51	Bichon	Klei10	Khomani	-0.055	-6.601
Vlasa37	Bichon	Klei10	Khomani	-0.0675	-7.951
Lepe45	Bichon	Kumtepe6	Khomani	-0.132	-6.385
Vlasa37	Bichon	Kumtepe6	Khomani	-0.2169	-9.261
Lepe51	Bichon	Kumtepe6	Khomani	-0.2009	-9.574
Lepe45	Bichon	Neo_Iranian	Khomani	-0.0684	-11.422
Lepe51	Bichon	Neo_Iranian	Khomani	-0.0775	-12.973
Vlasa37	Bichon	Neo_Iranian	Khomani	-0.0859	-13.274
Lepe45	Bichon	Pal7	Khomani	-0.0651	-7.446
Vlasa37	Bichon	Pal7	Khomani	-0.0845	-8.71
Lepe51	Bichon	Pal7	Khomani	-0.0777	-8.84
Lepe45	Bichon	Rev5	Khomani	-0.032	-3.471
Lepe51	Bichon	Rev5	Khomani	-0.031	-3.516
Vlasa37	Bichon	Rev5	Khomani	-0.0416	-4.411
Vlasa37	Bichon	Spain_MN	Khomani	-0.0205	-3.555
Vlasa37	La Braña	Kumtepe6	Khomani	-0.0818	-3.339
Lepe51	La Braña	Kumtepe6	Khomani	-0.0851	-3.819
Lepe51	Loschbour	Spain_MN	Khomani	-0.0183	-3.257
Lepe45	Loschbour	Spain_MN	Khomani	-0.0194	-3.384
Vlasa37	Loschbour	Spain_MN	Khomani	-0.027	-4.56

4.5.11 Patterns of allele correlations among Anatolian and Danubian capture samples and reference populations

As in the previous sections (4.5.1 and 4.5.5), exploratory *outgroup* f3-statistics were performed for each of the samples studied with target enrichment of nuclear regions (see Table 21 and 22 for the summary of the nuclear capture dataset). The results for the Anatolian capture samples from both Barcın and Aktopraklık, dated to the Neolithic period (6,600-6,000 cal BC, see section 2.3) with the exception of Chalcolithic Akt6 individual (5,633-5,535 cal BC, see section 2.4), largely agreed with the genomic comparisons of Bar8 and Bar31 samples to modern populations (see Figure 16). Mainly Mediterranean populations, especially western Mediterranean, analysed as *TEST* in *f3(Khomani; TEST, Anatolian)* produced the highest values and were therefore the closest to the Neolithic and Chalcolithic northwestern "Anatolians" (abbreviated as Anatolians in the following text) (see Figures 20 and 21).

Vlasac capture samples from the Danube Gorges dated to Late Mesolithic (∼7,400-6,200 cal BC, see section 2.2.2) show similar patterns in affinities to modern populations as genomic samples from the Danube Gorges (see Figure 11). Populations from northern and eastern Europe, especially Lithuanians, Estonians, Orcadians, Finns and Belarusians, showed the highest values as *TEST* in *f3(Khomani; TEST, Danubian)* (see Figure 25).

Lepenski Vir capture samples from the same area produced much more diverse results. Samples Lepe18, Lepe46 and Lepe53 from the Transition period (∼6,200-6,000/5,950 cal BC, see section 2.2.1) yielded similar patterns to Vlasac capture samples and all the genomic samples (Lepe51, Lepe45 and Vlasa37, see Figure 11), whereas Lepe39 also from Transition period (∼6,200-6,000/5,950 cal BC, see section 2.2.1) and Neolithic (6,005-5845 cal BC, see Table 8) Lepe52 produced a pattern more similar to Aegean (Anatolian and Greek) ancient samples (see Figure 15, 20 and 21) with affinities to Mediterranean rather than to northern European populations (see Figure 23).

Corresponding results were produced by a test of *f3(≠Khomani; TEST, Anatolian)*, where *TEST* was any ancient sample from the reference dataset. Analogously to Bar8 and Bar31 (see Figure 15), captured Anatolian samples (both from Aktopraklık and Barcın) showed affinities to other Anatolian individuals and to Neolithic samples from Greece (Rev5, Klei10 nad Pal7), Hungary and Germany (from Gamba *et al.* (*52*) and Haak *et al.* (*8*)) and notably also to Lepenski Vir samples Lepe52 and Lepe39 (precisely those Lepenski Vir individuals that demonstrated patterns equivalent to Anatolians in *outgroup* f3-statistics with modern populations).

There were no evident differences in terms of affinities of different Aktopraklık and Barcın individuals, with the exception of Bar15 and Bar8, whose relationship was noticeably closer than that of Bar15 to any other sample. Given the close similarity of age (see Figure 7) and presence of both individuals in the same cluster of graves (*145*), it is highly likely that these two women, abbreviated as Bar8 and Bar15, were related (the extent of the relatedness would have to be evaluated in future studies on heterozygous calls, preferably on genotype likelihoods).

In the form of *f3(≠Khomani; TEST, Vlasac)* with *TEST* as an ancient population, all Vlasac samples showed close affinities with other Vlasac, WHG and Lepenski Vir samples without strong Aegean-like signals (these are capture samples Lepe18, Lepe46 and Lepe53 and genomic samples Lepe51 and Lepe45) (see Figure 24).

The Lepenski Vir individuals in *f3(Khomani; TEST, Lepenski Vir)* (*TEST* as an ancient population) once again proved to be a complex group (see Figure 22). Lepe39 and Lepe52 samples had clear affinities with Aegean individuals and other Early Neolithic samples from Europe, especially to an Early Neolithic sample of the Starčevo culture from Hungary ("Starcevo_EN", Alsónyék-Bátaszék, Mérnöki Telep site from Haak *et al.*(*8*)) and other Neolithic Hungarian samples from the reference dataset (*52*).

While only samples Lepe39 and Lepe52 showed these almost Anatolian patterns in their affinities with other samples, some other Lepenski Vir samples (Lepe18 and Lepe46) as *TEST* still produced relatively high values for *f3(≠Khomani; TEST, Lepe39/Lepe52)*. Likewise, even though Lepe18 and Lepe46 generally had Vlasac-like affinities to Danubian and WHG samples serving as *TEST* in *f3(≠Khomani; TEST, Lepe18/46*, there were still relatively high values for a few Anatolian samples for this test. Both of these notions are stronger for Lepe46 rather than Lepe18 and are missing for Lepe53 (which is more similar to the genomic samples Lepe45, Lepe51 and Vlasa37). These results could indicate that Lepe18 and Lepe46, while deriving most of their ancestry from WHG-like Danubians, are in part descendants of Aegean-like migrants to the region.

4.5.12 Intrapopulation genetic structure in Anatolia

Relationships among the samples at the different sites were further studied via D statistics. When we studied the relationships among Neolithic Anatolians *D(Anatolian, Anatolian, Anatolian, ≠Khomani)*, we again identified a strong link between Bar8 and Bar15, because many combinations of *D(Bar8/Bar15, Anatolian, Bar8/Bar15, ≠Khomani)* were significantly positive (see Supplementary File S3). The only significant combinations with other samples of inter-Anatolian D statistics are presented in Table 48.

It should be noted that Akt20 and Bar31 as *Anatolian* are significant for a form *D(Bar8/Bar15, Bar8/Bar15, Anatolian ≠Khomani)*. This could mean that even though Bar8 and Bar15 do seem to be related to each other, there are other samples relatively close to them. We did not observe systematic significant differences between Aktopraklık and Barcın samples and we do not consider, based on the nuclear capture dataset, that these sites represent different populations.

When we studied differences between Anatolian samples with respect to their relationship with ancient hunter-gatherers, we identified only a few spurious significant results for *D(Anatolian, Anatolian, HG/Danubian, ≠Khomani)* in Table 49 and no significantly negative value for *D(Anatolian,*

Table 48: *f4(Anatolian, Anatolian. Anatolian, ≠Khomani), values for |Z|>3 shown except for combinations proving a close relationship between Bar8 and Bar15. Identical analysis for a different order of Anatolians and all values can be seen at Supplementary File S3.*

Anatolian	**Anatolian**	**Anatolian**	**≠Khomani**	**D value**	**Z-score**
Bar15	Bar8	Akt20	Khomani	0.1323	3.107
Bar8	Bar15	Bar31	Khomani	0.0713	4.378
Akt18	Bar8	Bar11	Khomani	0.1748	3.193
Akt26	Bar11	Bar16	Khomani	0.1901	3.197
Bar8	Bar32	Bar31	Khomani	0.0604	3.539
Bar8	Akt6	Bar31	Khomani	0.0522	3.053

Table 49: *D(Anatolian, Anatolian. HG/Danubian, ≠Khomani), values for Z >3 shown. All values can be seen at Supplementary File S3.*

Anatolian	**Anatolian**	**HG/Danubian**	**≠Khomani**	**D value**	**Z-score**
Akt16	Akt18	AG2	Khomani	0.413	3.198
Bar11	Bar32	Karelia_HG	Khomani	0.1015	3.143
Akt26	Akt18	Kostenki	Khomani	0.2101	3.825
Akt26	Bar20	MA1	Khomani	0.1508	3.05
Akt18	Bar15	MA1	Khomani	0.148	3.01
Akt26	Akt20	Samara_HG	Khomani	0.191	3.352
Akt20	Bar11	Ust_Ishim	Khomani	0.1265	3.144
Akt16	Bar31	Vlasa37	Khomani	0.0581	3.756
Akt26	Akt20	Vlasa37	Khomani	0.1702	3.589
Bar11	Bar31	Lepe52	Khomani	0.121	3.547

HG/Danubian, Anatolian ≠Khomani) (except for one value of *D(Bar8, Lepe52, Bar11, ≠Khomani)* discussed bellow, see Supplementary File S3).

Table 50: *D(Anatolian, Anatolian, Neolithic/Post-Neolithic, ≠Khomani), values for Z >3 shown except for those discussed in section 4.5.13. All values can be seen at Supplementary File S3.*

Anatolian	Anatolian	Neolithic/Post-Neolithic	≠Khomani	D value	Z-score
Bar11	Akt16	Iceman	Khomani	0.1187	3.23
Bar31	Bar32	Kumtepe6	Khomani	0.2029	3.67
Bar31	Bar15	Kumtepe6	Khomani	0.1792	3.172
Bar8	Akt16	Kumtepe6	Khomani	0.2186	3.858
Bar8	Akt6	Kumtepe6	Khomani	0.1701	3.13
Bar8	Bar15	Kumtepe6	Khomani	0.2076	3.064
Akt6	Bar20	NE3	Khomani	0.3894	3.394
Bar11	Bar32	NE5	Khomani	0.1382	3.07
Akt18	Bar11	NE7	Khomani	0.1746	3.182
Bar16	Bar11	NE7	Khomani	0.1656	3.362
Bar31	Bar11	NE7	Khomani	0.0916	3.504
Bar31	Bar11	Spain_EN	Khomani	0.0468	3.128
Bar8	Akt16	Spain_EN	Khomani	0.0316	3.166
Bar31	Bar15	Spain_MN	Khomani	0.0369	3.151
Bar31	Bar20	Spain_MN	Khomani	0.0453	3.075
Bar8	Bar20	Spain_MN	Khomani	0.0426	3.667

By contrast, Anatolians differed in relationship to Neolithic and post-Neolithic samples in *D(Anatolian, Anatolian, Neolithic_farmer, ≠Khomani).* The signals were mostly detected in the relationship of Anatolian genomic samples (Bar8 and Bar31) to reference samples, logically given the large number of SNPs obtained for the genomic samples. The increased affinity of these samples in respect to other Anatolians was detected especially to Neolithic Greeks (Klei10, Pal7 and Rev5) and, to a lesser extent, to Neolithic and Iron Age Iranians (see Supplementary File S3). It should be noted that these samples were all analysed with a different variant calling method and without further studies we cannot exclude the possibility that it contributed to the result (*9*, *51*).

There were also some significant (yet unsystematic) differences between Anatolian samples in terms of their relatedness to Kumtepe6, Neolithic Hungarian and Iberian samples (see Table 50), again with slightly inflated results in respect to the whole genome samples of higher quality. To a lesser

Table 51: *D(Anatolian, Neolithic/Post-Neolithic, Anatolian, ≠Khomani), values for Z <-3 shown except for those discussed in section 4.5.13. All values can be seen at Supplementary File S3.*

Anatolian	Neolithic/Post-Neolithic	Anatolian	≠Khomani	D value	Z-score
Bar31	KO2	Bar15	Khomani	-0.1322	-3.106
Bar16	Kumtepe6	Bar31	Khomani	-0.2054	-3.486
Bar16	NE3	Akt6	Khomani	-0.4086	-4.035
Akt6	NE3	Bar16	Khomani	-0.4402	-3.782

Table 52: *D(Danubian, Danubian, Lepe39/Lepe52, ≠Khomani) , values for Z >3. All values can be seen at Supplementary File S3.*

Danubian	Danubian	Lepe39/Lepe52	≠Khomani	D value	Z-score
Lepe18	Vlasa37	Lepe39	Khomani	0.095	3.241
Lepe46	Vlasa37	Lepe39	Khomani	0.139	3.942
Lepe46	Lepe51	Lepe52	Khomani	0.0773	3.082
Lepe46	Vlasa37	Lepe52	Khomani	0.0908	3.454

extent, we also detected a shared drift between Neolithic Greeks as *Neolithic_farmer* and Anatolians via *D(Anatolian, Neolithic_farmer, Anatolian, ≠Khomani)* but this weak signal was again detected mostly only for the genomic samples Bar8 and Bar31. A few additional significantly negative values for this test are reported in Table 51.

4.5.13 Intrapopulation genetic structure in the Danube Gorges

Tests of genetic structure among Danubians in a form *D(Danubian, Danubian, Danubian, ≠Khomani)* revealed that even though Lepe39 and Lepe52 both showed Aegean-like signals in *outgroup* f3 statistics, they did not show the same closeness as Bar8 and Bar15 – none of the *D(Lepe39/Lepe52, Danubian, Lepe39/Lepe52, ≠Khomani)* was significant (see Supplementary File S3).

This is understandable because those two samples, in contrast to Bar8 and Bar15, had been archaeologically assigned to different periods (Lepe52 to Neolithic period, Lepe39 to Transition period). It is thus less likely that Lepe52 was a descendant of an earlier Lepe39 sample without any admixture with local WHG-like individuals. However, many other samples did show significant unique drift with each other when contrasted to Lepe39 and Lepe52 (a large number of significant values in Supplementary File S3) in tests *D(Danubian, Lepe39/Lepe52, Danubian, ≠Khomani)*, suggesting that other Danubians shared unique ancestry not present in Lepe52 and Lepe39.

Vlasac individuals did not show any hints of the same pattern of exclusion as Lepe52/Lepe39 but Lepe46 and, to lesser extent, Lepe18 did. Thus, the notion from *outgroup* f3-statistiscs that Lepe18 and (more so) Lepe46 show certain hints of common ancestry with the Aegean-like samples seemed to be confirmed by often significant values of *D(Danubian, Lepe18/Lepe46, Danubian, ≠Khomani)* (see Supplementary File S3).

Also, the only test for which Lepe52 or Lepe39 shared unique drift with a Danubian compared to another Danubian in *D(Danubian, Danubian, Lepe39/Lepe52, ≠Khomani)* was with Lepe18/Lepe46 (see Table 52). This would suggest that Lepe18 and Lepe46 could be descendants of individuals that migrated to this region from the Aegean (while most of their ancestry was still local). Other intra-Danubian comparisons did not form a consistent pattern and can be seen in Table 53.

Table 53: *D(Danubian, Danubian, Danubian, ≠Khomani), values for |Z|>3 shown except for those discussed in section 4.5.13. All values can be seen at Supplementary File S3.*

Danubian	Danubian	Danubian	≠Khomani	D value	Z-score
Vlasa41	Lepe45	Lepe51	Khomani	0.0359	3.038
Lepe51	Lepe45	Vlasa10	Khomani	0.0424	3.073
Vlasa41	Lepe45	Vlasa10	Khomani	0.0618	3.096
Lepe51	Lepe45	Vlasa32	Khomani	0.0415	3.452
Vlasa4	Lepe45	Vlasa32	Khomani	0.051	3.015
Lepe51	Lepe45	Vlasa4	Khomani	0.0325	3.35
Lepe51	Lepe45	Vlasa44	Khomani	0.0492	3.767
Vlasa4	Vlasa37	Lepe51	Khomani	0.0451	3.65
Vlasa44	Vlasa37	Lepe51	Khomani	0.0447	3.101
Lepe51	Vlasa37	Vlasa32	Khomani	0.0436	3.412
Vlasa4	Vlasa37	Vlasa32	Khomani	0.0642	3.929
Lepe51	Vlasa37	Vlasa4	Khomani	0.0373	3.299
Lepe51	Vlasa37	Vlasa41	Khomani	0.0594	4.661
Vlasa44	Vlasa37	Vlasa41	Khomani	0.1146	4.878
Lepe51	Vlasa37	Vlasa44	Khomani	0.0487	3.243
Lepe51	Lepe53	Vlasa10	Khomani	0.0748	3.264

Table 54: *D(Lepe46, Lepe39/Lepe52, HG, ≠Khomani), values for |Z|>3 shown. All values can be seen at Supplementary File S3.*

Lepe46	Lepe39/Lepe52	HG	≠Khomani	D value	Z-score
Lepe46	Lepe52	Bichon	Khomani	0.1092	3.551
Lepe46	Lepe52	KO1	Khomani	0.1183	3.509

The patterns observed in the previous tests were confirmed when we compared Danubians in terms of their relationship to other samples. The test in a form of *D(Danubian, Danubian, HG, ≠Khomani)* was shown to be significantly positive for a large number of combinations of *D(Danubian, Lepe39/Lepe46/Lepe52, HG, ≠Khomani)* and, similarly, the results of *D(Lepe39/Lepe46/Lepe52, HG, Danubian, ≠Khomani)* were often significantly negative (see Supplementary File S3). Therefore, not only other WHG but also SHG, EHG and in some cases also Kostenki, Mal'ta and AG2 shared unique ancestry with Danubians that was not present in Lepe39, Lepe52 and Lepe46. It should be noted that Lepe46 had slightly less of these significant values and this sample also shared unique ancestry with other HG (Bichon, KO1) when compared with Lepe52 (see Table 54), again confirming that female Lepe46 could be an admixed individual tracing her ancestry to both the Aegean and the Danube Gorges regions. A few additional significant results for Lepe18 (another possible admixed individual) and other Danubians are presented in Table 55 and 56. These values can be also explained as a potential (but weak) substructure among WHG-like Danubians.

Table 55: *D(Danubian, Danubian, HG, ≠Khomani), values for |Z|>3 shown except for those discussed in section 4.5.12. All values can be seen at Supplementary File S3.*

Danubian	**Danubian**	**HG**	**≠Khomani**	**D value**	**Z-score**
Vlasa44	Lepe18	KO1	Khomani	0.0828	3.288
Lepe45	Lepe18	Loschbour	Khomani	0.0447	3.238
Lepe51	Vlasa32	Loschbour	Khomani	0.0368	3.143
Vlasa37	Vlasa32	Loschbour	Khomani	0.0351	3.026
Vlasa4	Vlasa37	Ust_Ishim	Khomani	0.0375	3.445
Vlasa44	Vlasa41	Motala_HG	Khomani	0.062	3.37

Table 56: *D(Danubian, HG, Danubian, ≠Khomani), values for Z <3 shown except for those discussed in section 4.5.12. All values can be seen at Supplementary File S3.*

Danubian	**HG**	**Danubian**	**≠Khomani**	**D value**	**Z-score**
Lepe18	KO1	Lepe45	Khomani	-0.0474	-3.427
Lepe18	KO1	Vlasa37	Khomani	-0.0434	-3.043
Lepe18	Loschbour	Lepe45	Khomani	-0.066	-4.666
Lepe18	Loschbour	Lepe51	Khomani	-0.0385	-3.508
Lepe53	KO1	Lepe45	Khomani	-0.0413	-3.102
Lepe53	Loschbour	Lepe45	Khomani	-0.0421	-3.318
Vlasa32	Loschbour	Lepe45	Khomani	-0.0313	-3.041
Vlasa37	Loschbour	Lepe45	Khomani	-0.027	-3.633
Vlasa37	Loschbour	Vlasa41	Khomani	-0.0394	-3.046
Vlasa41	Loschbour	Vlasa37	Khomani	-0.0521	-3.134

4.5.14 Relationships between Danubians and Anatolians

Lepe52 and Lepe39, consistently with the results of *outgroup* f3 statistics, proved to have a very close relationship to Neolithic Anatolians (see Table 57), Neolithic Greeks and early Neolithic samples from Iberia and Hungary (see Tables 58 and 59) via significantly positive values of *D(Lepe39/Lepe52, Danubian, Neolithic_farmer/Anatolian, ≠Khomani)*. The detailed results presented confirm the important notion that Neolithic farmers shared more ancestry with these two samples than with other Danubians.

In tests of *D(Danubian, Neolithic_farmer, Danubian, ≠Khomani)* and *D(Danubian, Anatolian, Danubian, ≠Khomani)* (see Supplementary File S3 and Table 61 respectively), this relationship was also confirmed in the opposite direction by the abundance of significantly negative values between the same Early Neolithic populations and Lepe52/Lepe39. That proves that the two samples shared more ancestry with the Neolithic samples than with samples from the Danube Gorges. Lepe52 even produced a significantly negative value for *D(Bar8, Lepe52, Bar11, ≠Khomani)*, which highlights that this individual was so closely related to the Anatolians that there were even Anatolian samples (Bar11 in this case) that shared more ancestry with him than with other Anatolian (Bar8). The clear results of these tests strongly suggest that the two Lepenski Vir males Lepe39 and Lepe52 were connected to the Aegean region. Even though Lepe52 was identified as local by the strontium isotope study of Borić & Price (*94*), on the basis of these D statistics results it is arguable that Lepe52 might have been non-local (though it could have been also an unadmixed local descendent of incoming individuals). The non-local origin of this sample would be additionally supported by the fact that $^{87}Sr/^{86}Sr$ ratio of Lepe52 was the lowest among the individuals assigned to the local range (and only by 0.0003 from the non-local range).

Interestingly, Lepe46, a sample that has often showed a weak Aegean-like signal, had a large number of positive yet mostly insignificant values for *D(Lepe46, Danubian, Neolithic_farmer, ≠Khomani)* and only one positive value over the significance threshold (Z=3) for *D(Lepe46, Danubian, Anatolian, ≠Khomani)* (see Supplementary File S3 and Table 57, respectively). Nevertheless, a lack of significance does not constitute a lack of the Aegean-like signal and a test of another potentially admixed sample, Lepe18, in the form *D(Lepe18, Danubian, Neolithic_farmer/Anatolian, ≠Khomani)* resulted in several significant comparisons (see Table 57 and 58). Thus, it is still not inconceivable that these two Lepenski Vir samples are descendants of individuals who migrated to this region from the Aegean (or from a population related to Aegeans).

We further investigated how the individuals from the Danube Gorges with their complex ancestral history compare to other Neolithic samples from Europe and Anatolia. We performed a series of tests *D(Danubian, Neolithic_farmer, Anatolian, ≠Khomani)*, *D(Anatolian, Danubian, Neolithic_farmer, ≠Khomani)* and *D(Neolithic_farmer, Anatolian, Danubian, ≠Khomani)*, each of which formally tested a slightly different aspect of their relatedness. In general, we detected significant values that were in concordance with our expectations informed by the genomic analysis: NW Anatolian and

Table 57: *D(Danubian, Danubian, Anatolian, ≠Khomani), values for Z >3 shown. All values can be seen at Supplementary File S3.*

Danubian	**Danubian**	**Anatolian**	**≠Khomani**	**D value**	**Z-score**
Lepe18	Lepe51	Bar20	Khomani	0.0814	3.318
Lepe18	Lepe45	Akt26	Khomani	0.0993	3.011
Lepe46	Lepe51	Bar32	Khomani	0.1124	3.227
Lepe39	Lepe51	Bar31	Khomani	0.0676	4.332
Lepe39	Vlasa44	Bar16	Khomani	0.2034	3.948
Lepe39	Vlasa37	Bar31	Khomani	0.0647	3.893
Lepe39	Lepe51	Bar20	Khomani	0.1173	3.841
Lepe39	Vlasa4	Bar8	Khomani	0.1035	3.516
Lepe39	Lepe51	Akt16	Khomani	0.1262	3.305
Lepe39	Lepe45	Bar31	Khomani	0.0523	3.143
Lepe39	Lepe45	Akt16	Khomani	0.1121	3.08
Lepe39	Vlasa32	Bar15	Khomani	0.1372	3.077
Lepe39	Lepe51	Bar16	Khomani	0.1115	3.015
Lepe52	Vlasa37	Bar31	Khomani	0.0684	5.507
Lepe52	Lepe45	Bar32	Khomani	0.1026	4.714
Lepe52	Lepe51	Bar31	Khomani	0.0506	4.678
Lepe52	Lepe45	Bar8	Khomani	0.0418	4.464
Lepe52	Lepe51	Bar8	Khomani	0.0449	4.426
Lepe52	Lepe53	Bar8	Khomani	0.0788	4.199
Lepe52	Vlasa32	Akt20	Khomani	0.145	4.07
Lepe52	Lepe45	Bar11	Khomani	0.1197	3.949
Lepe52	Lepe53	Akt20	Khomani	0.1357	3.689
Lepe52	Lepe53	Bar31	Khomani	0.0857	3.606
Lepe52	Vlasa32	Bar11	Khomani	0.171	3.587
Lepe52	Vlasa37	Bar8	Khomani	0.0421	3.558
Lepe52	Lepe51	Akt20	Khomani	0.1097	3.551
Lepe52	Lepe45	Bar31	Khomani	0.0419	3.459
Lepe52	Lepe51	Bar20	Khomani	0.0916	3.286
Lepe52	Vlasa41	Bar8	Khomani	0.0633	3.277
Lepe52	Lepe45	Bar15	Khomani	0.0889	3.2
Lepe52	Lepe18	Bar8	Khomani	0.0672	3.195
Lepe52	Vlasa41	Akt18	Khomani	0.1612	3.191
Lepe52	Lepe51	Bar11	Khomani	0.1145	3.127
Lepe52	Lepe53	Akt18	Khomani	0.1577	3.056
Lepe52	Lepe45	Akt6	Khomani	0.0874	3.024
Lepe52	Vlasa44	Bar11	Khomani	0.1749	3.001

Table 58: *D(Danubian1, Danubian, Neolithic_farmer, ≠Khomani), values for Z >3 shown for Danubian1 as Danubians except for Lepe52 (see Table 59). All values can be seen at Supplementary File S3.*

Danubian1	**Danubian**	**Neolithic_farmer**	**≠Khomani**	**D value**	**Z-score**
Lepe39	Lepe45	Klei10	Khomani	0.0758	3.631
Lepe39	Vlasa41	Klei10	Khomani	0.103	3.065
Lepe39	Vlasa37	Klei10	Khomani	0.0645	3.059
Lepe39	Vlasa37	LBK_EN	Khomani	0.0541	5.074
Lepe39	Lepe45	LBK_EN	Khomani	0.0375	4.106
Lepe39	Vlasa44	LBK_EN	Khomani	0.0723	4.07
Lepe39	Vlasa32	LBK_EN	Khomani	0.0557	4.054
Lepe39	Vlasa4	LBK_EN	Khomani	0.0519	3.788
Lepe39	Lepe51	LBK_EN	Khomani	0.0409	3.69
Lepe39	Lepe51	NE5	Khomani	0.0607	3.146
Lepe39	Vlasa37	NE6	Khomani	0.0802	3.925
Lepe39	Lepe51	Pal7	Khomani	0.0604	3.218
Lepe39	Vlasa37	Rev5	Khomani	0.0931	4.432
Lepe39	Lepe51	Rev5	Khomani	0.0935	4.173
Lepe39	Vlasa32	Rev5	Khomani	0.1128	3.845
Lepe39	Lepe45	Rev5	Khomani	0.0925	3.754
Lepe39	Lepe51	Starcevo_EN	Khomani	0.1172	4.111
Lepe39	Lepe45	Starcevo_EN	Khomani	0.0934	3.516
Lepe39	Lepe51	Stuttgart	Khomani	0.0575	3.828
Lepe39	Vlasa41	Stuttgart	Khomani	0.0794	3.531
Lepe39	Vlasa44	Stuttgart	Khomani	0.0959	3.16
Lepe18	Vlasa37	LBK_EN	Khomani	0.0289	3.852
Lepe18	Vlasa37	NE7	Khomani	0.0642	4.627
Lepe18	Vlasa37	Spain_EN	Khomani	0.0253	3.281

Table 59: *D(Lepe52, Danubian, Neolithic_farmer, ≠Khomani), values for Z >3 shown. All values can be seen at Supplementary File S3.*

Lepe52	**Danubian**	**Neolithic_farmer**	**≠Khomani**	**D value**	**Z-score**
Lepe52	Vlasa37	Klei10	Khomani	0.0746	5.626
Lepe52	Lepe51	Klei10	Khomani	0.0639	5.083
Lepe52	Lepe45	Klei10	Khomani	0.068	4.73
Lepe52	Vlasa32	Klei10	Khomani	0.0919	4.311
Lepe52	Vlasa10	Klei10	Khomani	0.0819	3.445
Lepe52	Lepe53	Klei10	Khomani	0.0801	3.17
Lepe52	Vlasa41	Klei10	Khomani	0.0889	3.15
Lepe52	Vlasa37	LBK_EN	Khomani	0.0429	6.256
Lepe52	Lepe45	LBK_EN	Khomani	0.0395	5.929
Lepe52	Lepe51	LBK_EN	Khomani	0.0412	5.214
Lepe52	Vlasa10	LBK_EN	Khomani	0.0688	5.194
Lepe52	Vlasa32	LBK_EN	Khomani	0.0519	5.01
Lepe52	Vlasa44	LBK_EN	Khomani	0.0593	4.295
Lepe52	Lepe18	LBK_EN	Khomani	0.0474	3.877
Lepe52	Lepe53	LBK_EN	Khomani	0.0518	3.834
Lepe52	Vlasa41	LBK_EN	Khomani	0.0484	3.597
Lepe52	Vlasa10	NE1	Khomani	0.0562	3.014
Lepe52	Vlasa32	NE5	Khomani	0.0921	4.617
Lepe52	Lepe45	NE5	Khomani	0.0428	3.766
Lepe52	Lepe51	NE5	Khomani	0.0332	3.145
Lepe52	Vlasa37	NE6	Khomani	0.0452	3.327
Lepe52	Lepe18	NE6	Khomani	0.0704	3.128
Lepe52	Vlasa37	Pal7	Khomani	0.0492	3.598
Lepe52	Lepe45	Pal7	Khomani	0.0506	3.592
Lepe52	Lepe53	Pal7	Khomani	0.0959	3.425
Lepe52	Lepe18	Rev5	Khomani	0.1	5.059
Lepe52	Lepe51	Rev5	Khomani	0.0608	4.255
Lepe52	Vlasa32	Rev5	Khomani	0.0775	3.835
Lepe52	Vlasa37	Rev5	Khomani	0.0549	3.81
Lepe52	Lepe45	Rev5	Khomani	0.0537	3.475
Lepe52	Vlasa10	Rev5	Khomani	0.1059	3.451
Lepe52	Lepe45	Spain_EN	Khomani	0.0357	4.94
Lepe52	Lepe51	Spain_EN	Khomani	0.035	4.587
Lepe52	Vlasa37	Spain_EN	Khomani	0.031	3.929
Lepe52	Lepe18	Spain_EN	Khomani	0.0541	3.844
Lepe52	Vlasa32	Spain_EN	Khomani	0.0498	3.709
Lepe52	Lepe53	Spain_EN	Khomani	0.0488	3.402
Lepe52	Vlasa10	Spain_EN	Khomani	0.0468	3.041
Lepe52	Vlasa10	Stuttgart	Khomani	0.0849	4.203
Lepe52	Lepe45	Stuttgart	Khomani	0.0495	4.018
Lepe52	Vlasa32	Stuttgart	Khomani	0.0548	3.276
Lepe52	Vlasa37	Stuttgart	Khomani	0.0391	3.239

European Neolithic farmers shared unique drift, not present in Danubians (both from the Late Mesolithic and Transition period, see Table 22 and Supplementary File S3). There were, however, exceptions because Neolithic northwestern Anatolians were often closer to Lepe52 and Lepe39 (from the Neolithic and Transition period, respectively) than to other European Neolithic samples (e.g., Table 62).

Since we already established that Lepe39 and Lepe52 are Aegean-like, these results are not surprising, but it was also important to establish whether we could consider such incoming individuals as Lepe39 and Lepe52 as ancestors of early farmers in other parts of Europe. For this, we tested whether other European Neolithic farmers shared the same amount of ancestry with Anatolians as with the Aegean-like Danubians or if they were closer to either of the groups in a test of *D(Anatolian, Danubian, Neolithic_farmer, ≠Khomani)*. It was likely that the difference would not be large (it is usually considered that the migration of farmers to Europe was relatively fast and there was no time to accumulate genetic variation leading to differences along the path (see *51*). Yet we detected two significant values for Lepe52 (for Rev5, early Neolithic Rev5 from Greece and middle Neolithic NE5 from Hungary, see Table 63).

One of the main conclusions observable for genomic Danubians (one from each of the Early Mesolithic, Late Mesolithic and Transition periods) is that their genetic signal contributed to the ancestry of Early Neolithic populations in Europe (see Table 44 for the results on genomic samples). The results with capture data, consistently with results for genomic samples, confirmed that there was some level of admixture with local HG present along the way to central Europe and Iberia (see Table 60).

We formally investigated what WHG population was the source of this HG admixture in Early Neolithic individuals by comparing the HG that lived in the Danube Gorges with other WHG in terms of their relatedness to Early Neolithic samples in *D(WHG-Danubian, WHG, Neolithic_farmer, ≠Khomani)* but the results were rarely significant and not conclusive (see Supplementary File S3). We therefore repeated the test by pooling the Danubians without the Aegean-like signal into one group "WHG-Danubian" (i.e., Danubians without Lepe18, Lepe39, Lepe46 and Lepe52 because with them the test would have been biased through the shared Aegean-like ancestry). Signals in either direction were still not very strong, but we demonstrated that Danubians were closer to the Neolithic farmers in Europe than Bichon, Upper Paleolithic sample from today's Switzerland (see Supplementary File S3) and closer to central European farmers than La Braña and not close to Iberian farmers from the Middle Neolithic when compared to Loschbour and KO1 (see Table 64).

Table 60: *D(Early farmer, Anatolian, Danubian, ≠Khomani), values Z >3 shown. All values can be seen at Supplementary File S3.*

Early farmer	Anatolian	Danubian	≠Khomani	D value	Z-score
Klei10	Bar20	Lepe51	Khomani	0.048	3.108
KO2	Bar15	Lepe51	Khomani	0.1384	3.201
Starcevo_EN	Bar16	Lepe53	Khomani	0.1513	3.098
Starcevo_EN	Bar20	Vlasa37	Khomani	0.0804	3.362
Stuttgart	Bar31	Vlasa10	Khomani	0.0408	3.616
LBK_EN	Bar15	Lepe51	Khomani	0.0348	3.075
LBK_EN	Bar20	Lepe51	Khomani	0.0346	3.434
NE1	Bar15	Lepe51	Khomani	0.0504	3.082
NE1	Bar8	Lepe53	Khomani	0.0404	3.388
NE1	Bar31	Lepe53	Khomani	0.0411	3.445
NE1	Bar31	Vlasa10	Khomani	0.0378	3
NE1	Bar31	Vlasa32	Khomani	0.036	3.53
NE2	Bar11	Vlasa32	Khomani	0.2229	3.57
NE2	Bar31	Vlasa41	Khomani	0.0964	3.143
NE4	Akt26	Lepe45	Khomani	0.2133	3.523
NE5	Bar31	Vlasa44	Khomani	0.0466	3.118
NE6	Bar31	Vlasa44	Khomani	0.0348	3.007
NE7	Bar20	Lepe51	Khomani	0.0585	3.059
NE7	Bar31	Vlasa32	Khomani	0.0451	3.324
Spain_EN	Bar11	Vlasa32	Khomani	0.0744	3.071
Spain_EN	Bar31	Vlasa10	Khomani	0.0326	3.075
Spain_EN	Bar8	Vlasa41	Khomani	0.0308	3.26

Table 61: *D(Danubian, Anatolian, Danubian, ≠Khomani), values Z <-3 shown. All values can be seen at Supplementary File S3.*

Danubian	Anatolian	Danubian	≠Khomani	D value	Z-score
Lepe51	Akt16	Lepe39	Khomani	-0.1303	-3.064
Vlasa37	Bar31	Lepe39	Khomani	-0.0763	-4.386
Lepe51	Bar31	Lepe39	Khomani	-0.0683	-3.919
Lepe45	Bar31	Lepe39	Khomani	-0.0596	-3.256
Lepe45	Akt18	Lepe52	Khomani	-0.1181	-3.241
Lepe45	Akt26	Lepe52	Khomani	-0.1208	-3.508
Lepe45	Bar11	Lepe52	Khomani	-0.1845	-5.586
Vlasa32	Bar11	Lepe52	Khomani	-0.1869	-4.582
Lepe51	Bar11	Lepe52	Khomani	-0.1385	-3.924
Lepe53	Bar11	Lepe52	Khomani	-0.1911	-3.673
Vlasa37	Bar11	Lepe52	Khomani	-0.128	-3.452
Vlasa10	Bar11	Lepe52	Khomani	-0.2007	-3.426
Vlasa41	Bar11	Lepe52	Khomani	-0.1619	-3.133
Lepe45	Bar15	Lepe52	Khomani	-0.1089	-3.87
Lepe51	Bar15	Lepe52	Khomani	-0.1031	-3.599
Vlasa37	Bar15	Lepe52	Khomani	-0.0942	-3.482
Vlasa37	Bar31	Lepe52	Khomani	-0.0518	-3.848
Lepe45	Bar31	Lepe52	Khomani	-0.0452	-3.529
Lepe51	Bar31	Lepe52	Khomani	-0.0416	-3.22
Vlasa37	Bar8	Lepe52	Khomani	-0.0377	-3.336

Table 62: *D(Danubian, Neolithic_farmer, Anatolian, ≠Khomani), values Z >3 shown. All values can be seen at Supplementary File S3.*

Danubian	Neolithic_farmer	Anatolian	≠Khomani	D value	Z-score
Lepe52	Spain_EN	Bar11	Khomani	0.0964	4.105
Lepe39	Klei10	Akt16	Khomani	0.1432	3.789
Lepe39	LBK_EN	Akt16	Khomani	0.1023	3.492
Lepe39	NE1	Akt16	Khomani	0.0994	3.104
Lepe39	NE1	Bar16	Khomani	0.1276	3.055
Lepe39	NE2	Bar16	Khomani	0.3496	3.417
Lepe39	NE7	Bar15	Khomani	0.1286	3.704
Lepe39	NE7	Akt6	Khomani	0.1334	3.108
Lepe46	NE7	Bar11	Khomani	0.1393	3.36
Lepe52	NE7	Bar11	Khomani	0.1807	4.445
Lepe52	Stuttgart	Bar11	Khomani	0.0913	3.045

Table 63: *D(Anatolian, Danubian, Neolithic_farmer, ≠Khomani), values Z <-3 shown. All values can be seen at Supplementary File S3*

Anatolian	**Danubian**	**Neolithic_farmer**	**≠Khomani**	**D value**	**Z-score**
Bar20	Lepe39	NE5	Khomani	-0.1167	-3.168
Akt16	Lepe52	Rev5	Khomani	-0.1016	-3.083

Table 64: *D(WHG-Danubian, WHG, Neolithic_farmer, ≠Khomani), values for |Z|>3. WHG-Danubians were samples assigned to Mesolithic period: Vlasac samples and Lepe51. All values can be seen at Supplementary File S3.*

WHG-Danubian	**WHG**	**Neolithic_farmer**	**≠Khomani**	**D value**	**Z-score**
WHG-Danubian	Bichon	LBK_EN	Khomani	0.0147	3.256
WHG-Danubian	Bichon	NE1	Khomani	0.0304	6.105
WHG-Danubian	Bichon	NE5	Khomani	0.019	3.127
WHG-Danubian	Bichon	NE6	Khomani	0.0256	4.127
WHG-Danubian	Bichon	NE7	Khomani	0.0195	3.071
WHG-Danubian	Bichon	Stuttgart	Khomani	0.0225	3.694
WHG-Danubian	La Braña	NE6	Khomani	0.0196	3.724
WHG-Danubian	La Braña	Stuttgart	Khomani	0.0167	3.273
WHG-Danubian	Loschbour	Spain_MN	Khomani	-0.0263	-5.243
WHG-Danubian	KO1	Spain_MN	Khomani	-0.0142	-3.313

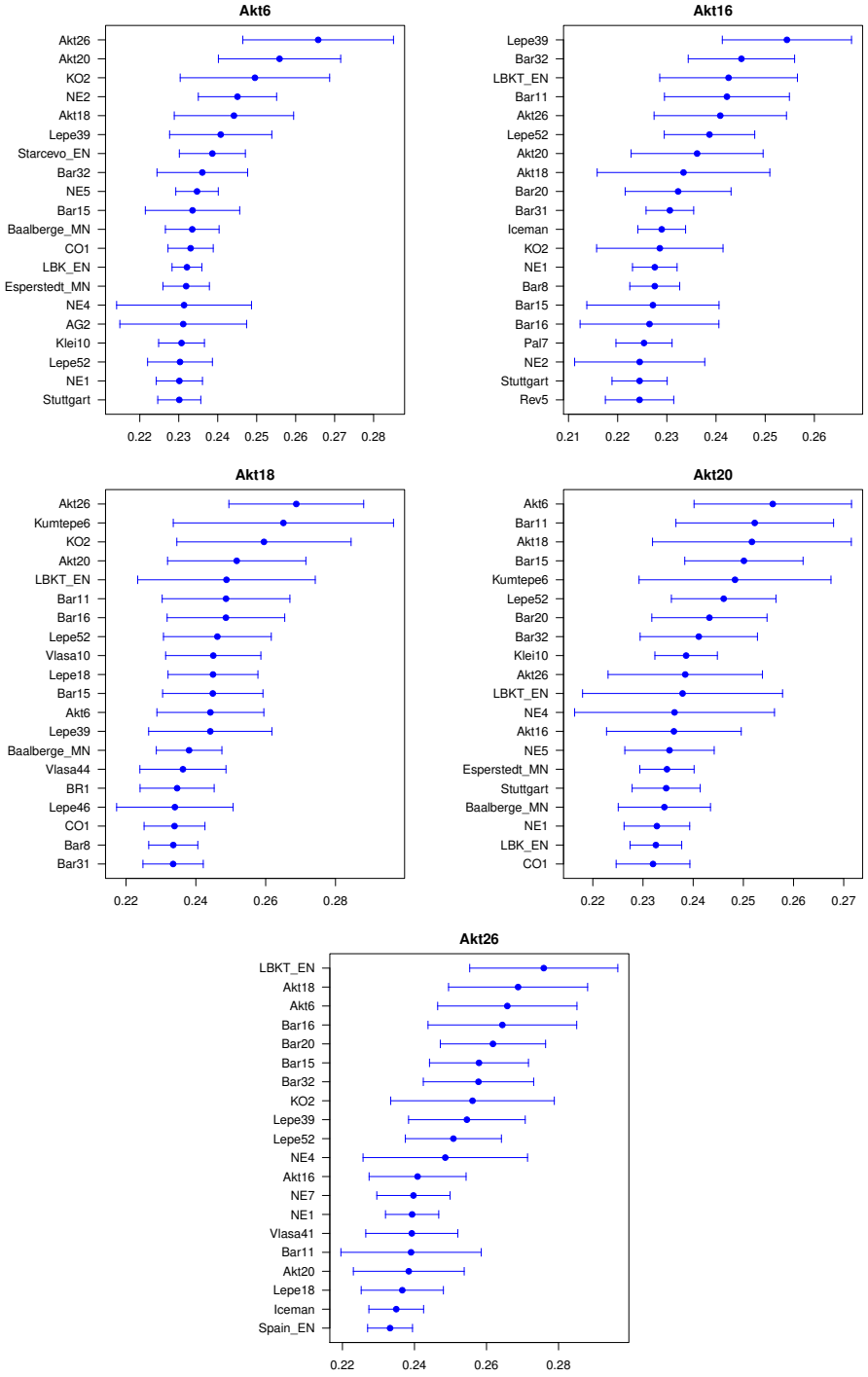


Figure 18: *f3(≠Khomani; Ancient_population, Aktopraklık captured sample). The highest 20 values shown.*

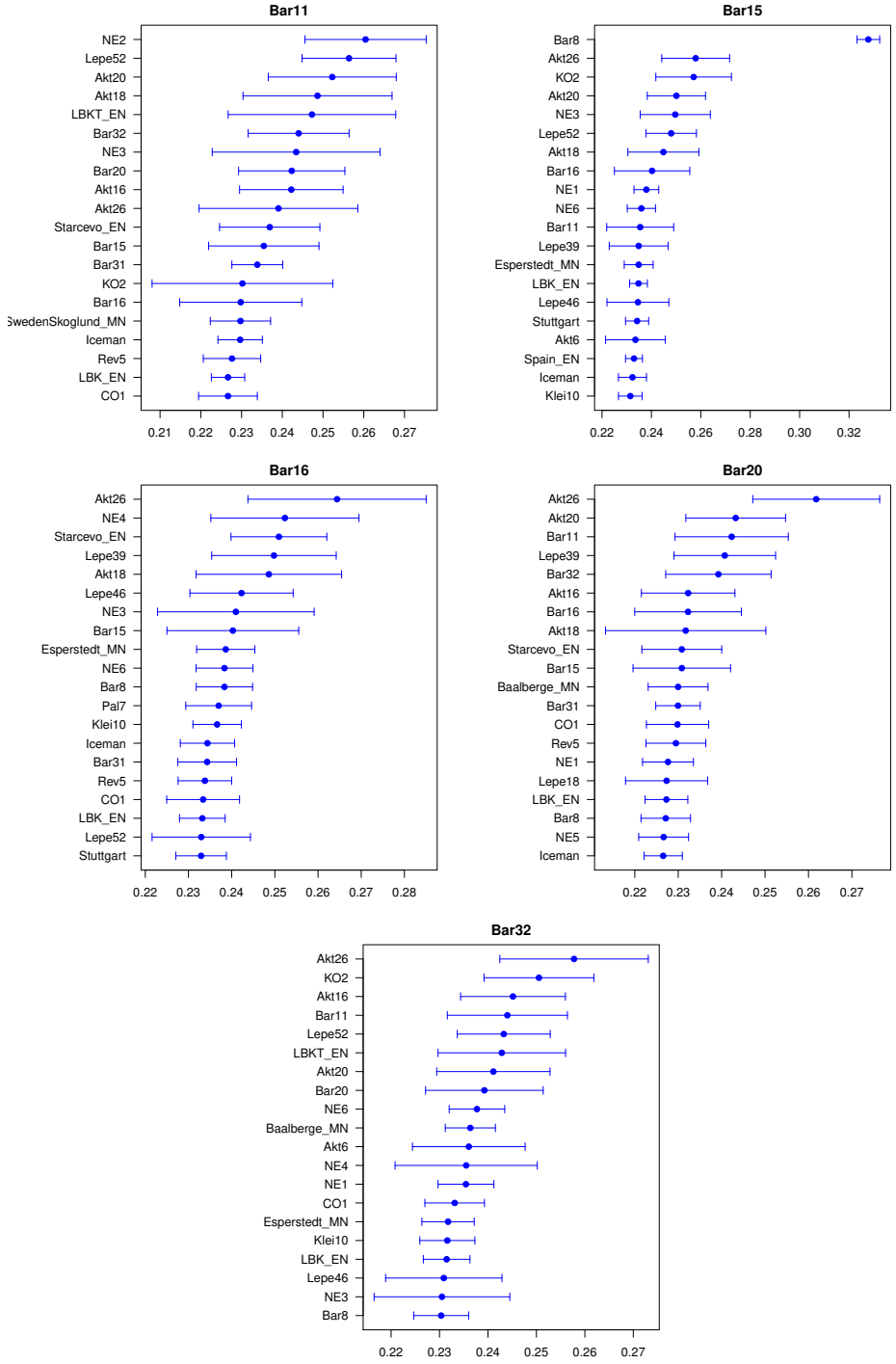


Figure 19: *f3(≠Khomani; Ancient_population, Barcin captured sample). The highest 20 values shown.*

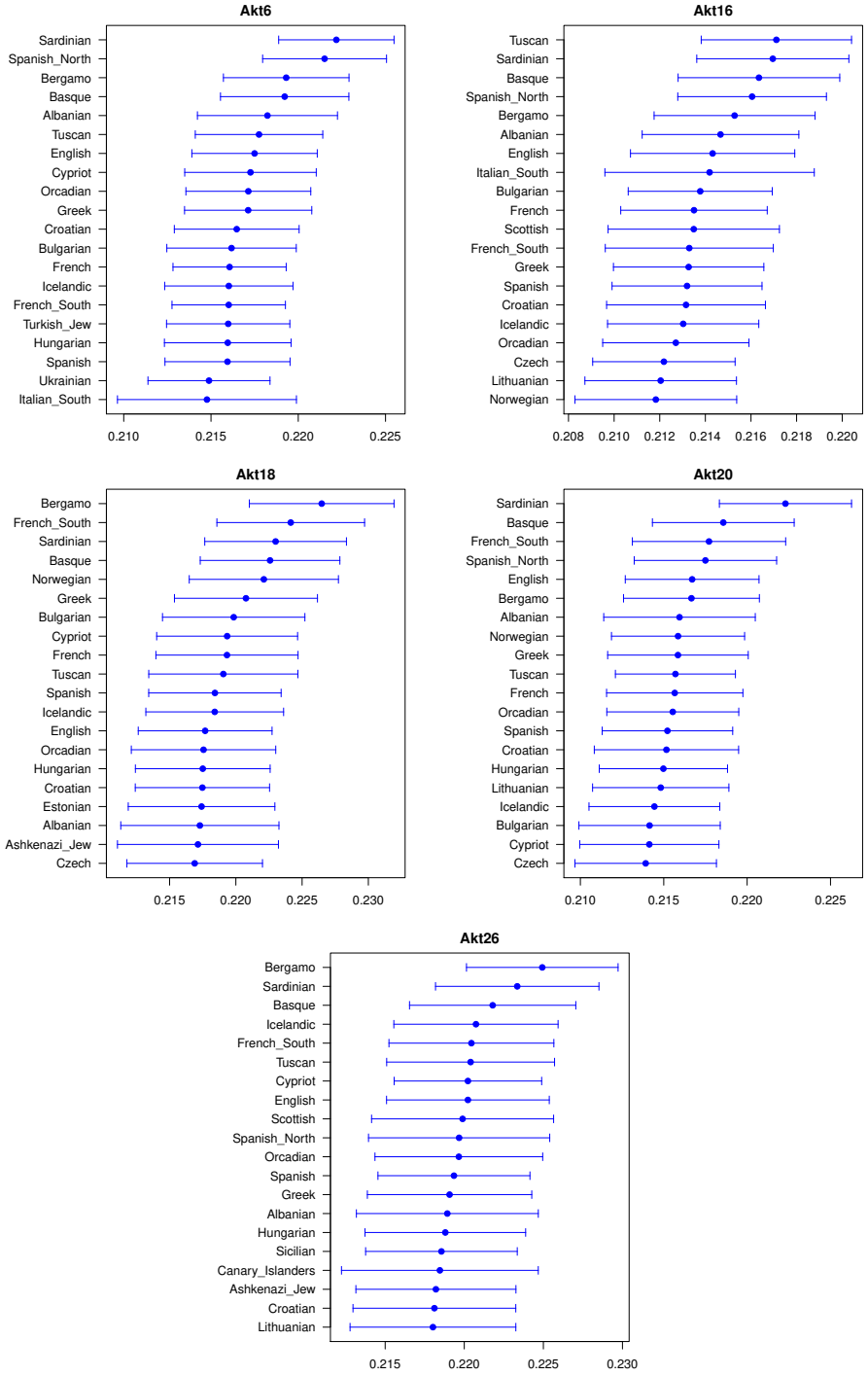


Figure 20: *f3(≠Khomani; Modern_population, Aktopraklık captured sample). The highest 20 values shown.*

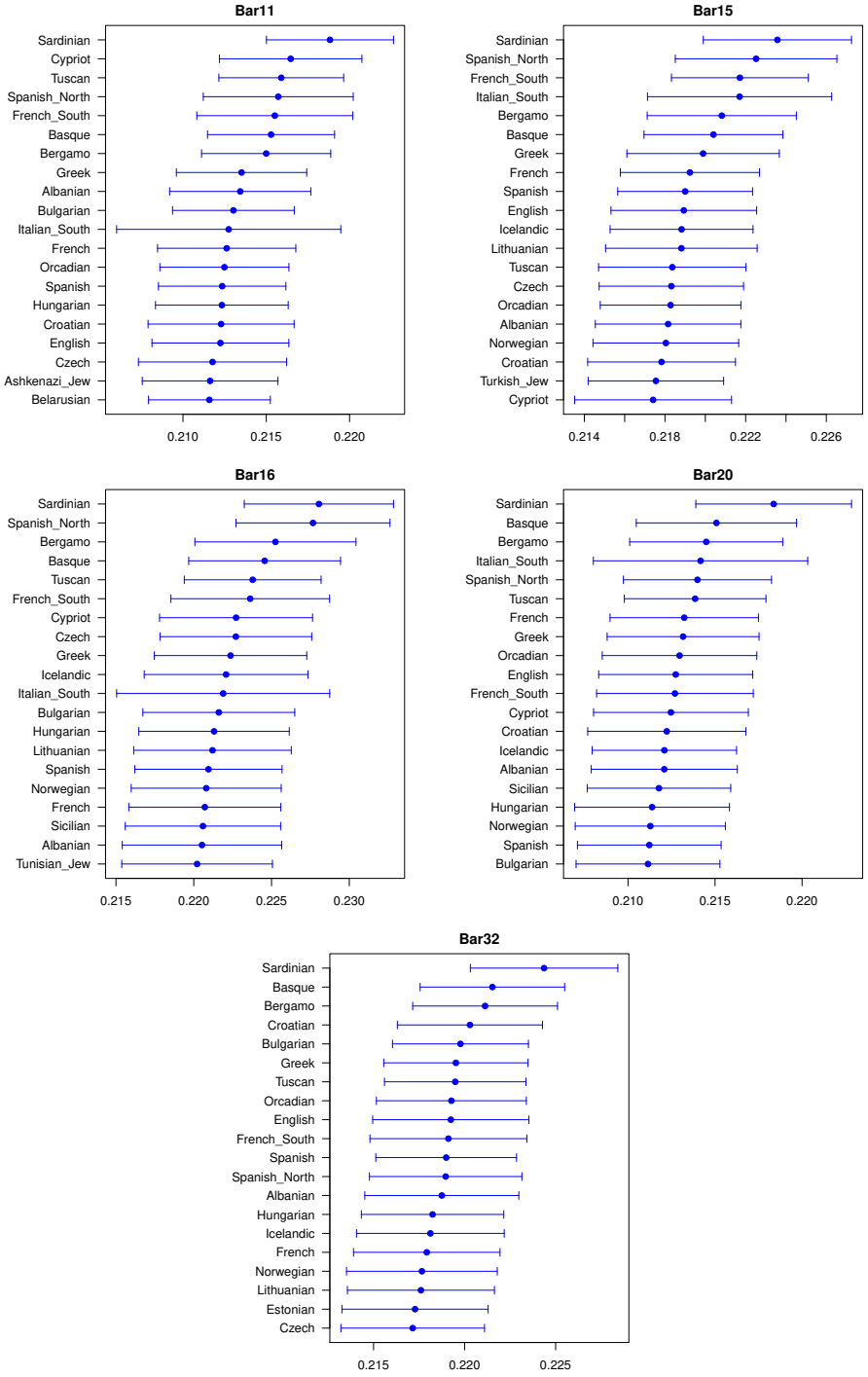


Figure 21: *f3(≠Khomani; Modern_population, Barcin captured sample). The highest 20 values shown.*

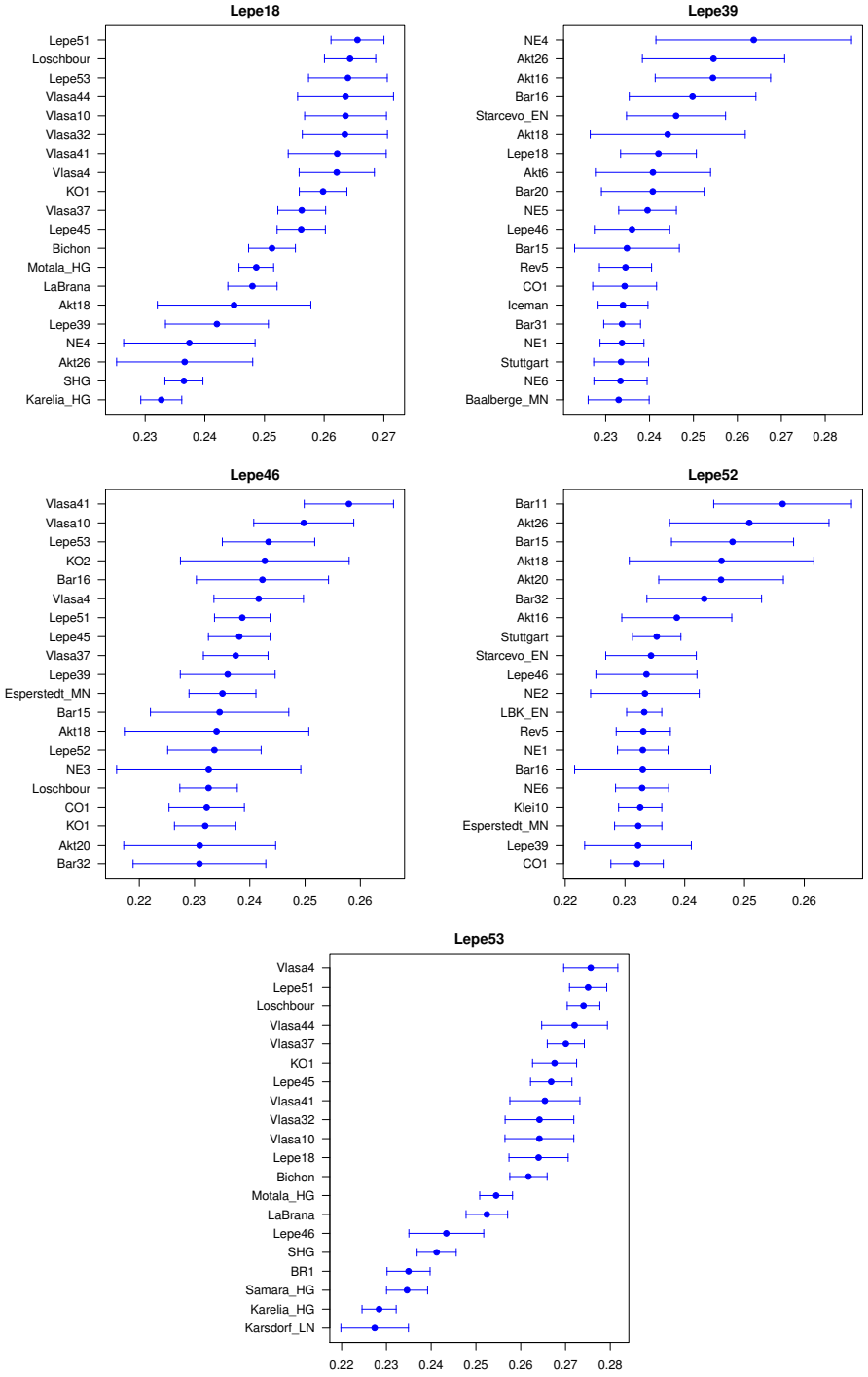


Figure 22: *f3(≠Khomani; Ancient_population, Aktopraklık captured sample). The highest 20 values shown.*

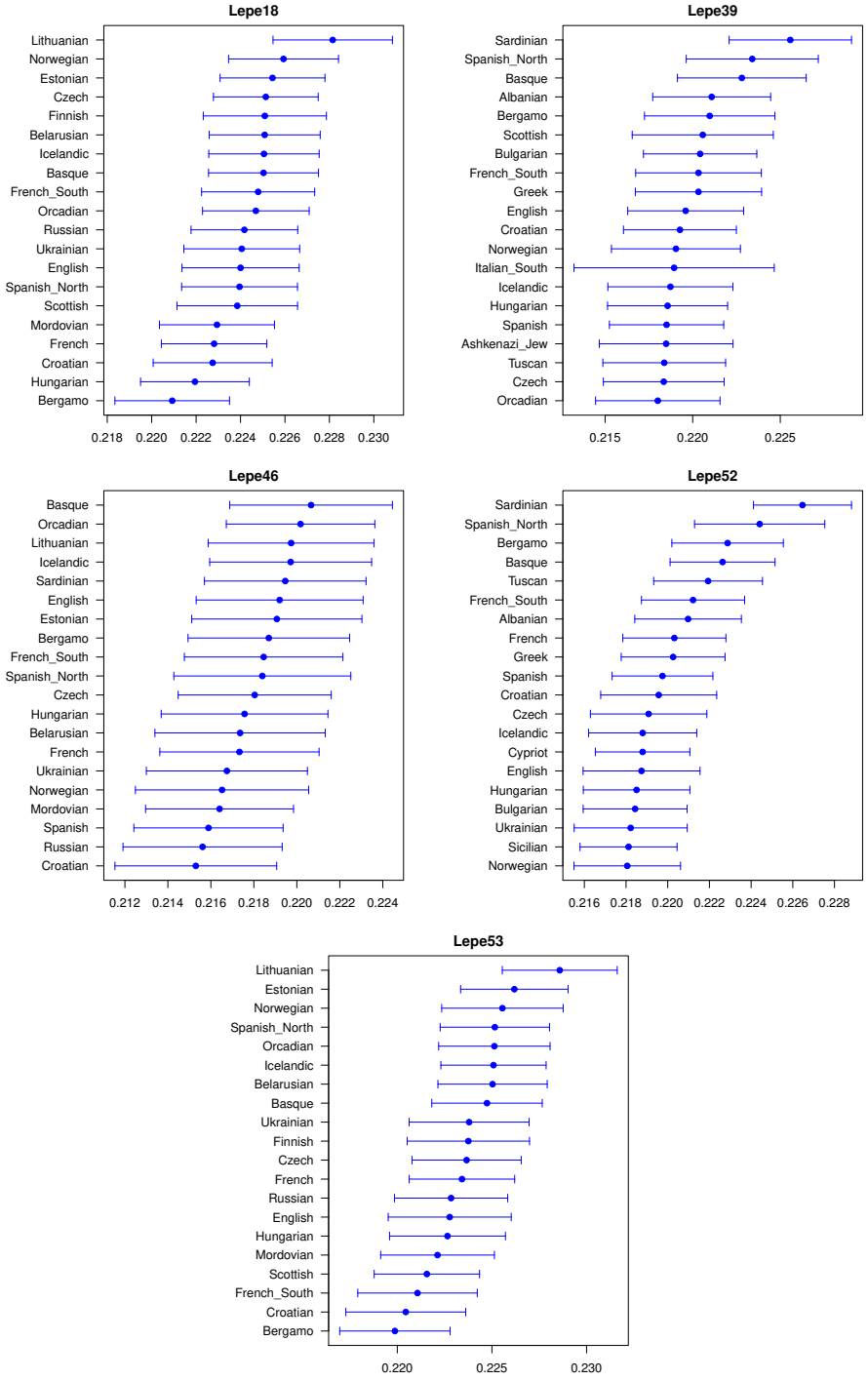


Figure 23: *f3(≠Khomani; Modern_population, Lepenski Vir captured sample). The highest 20 values shown.*

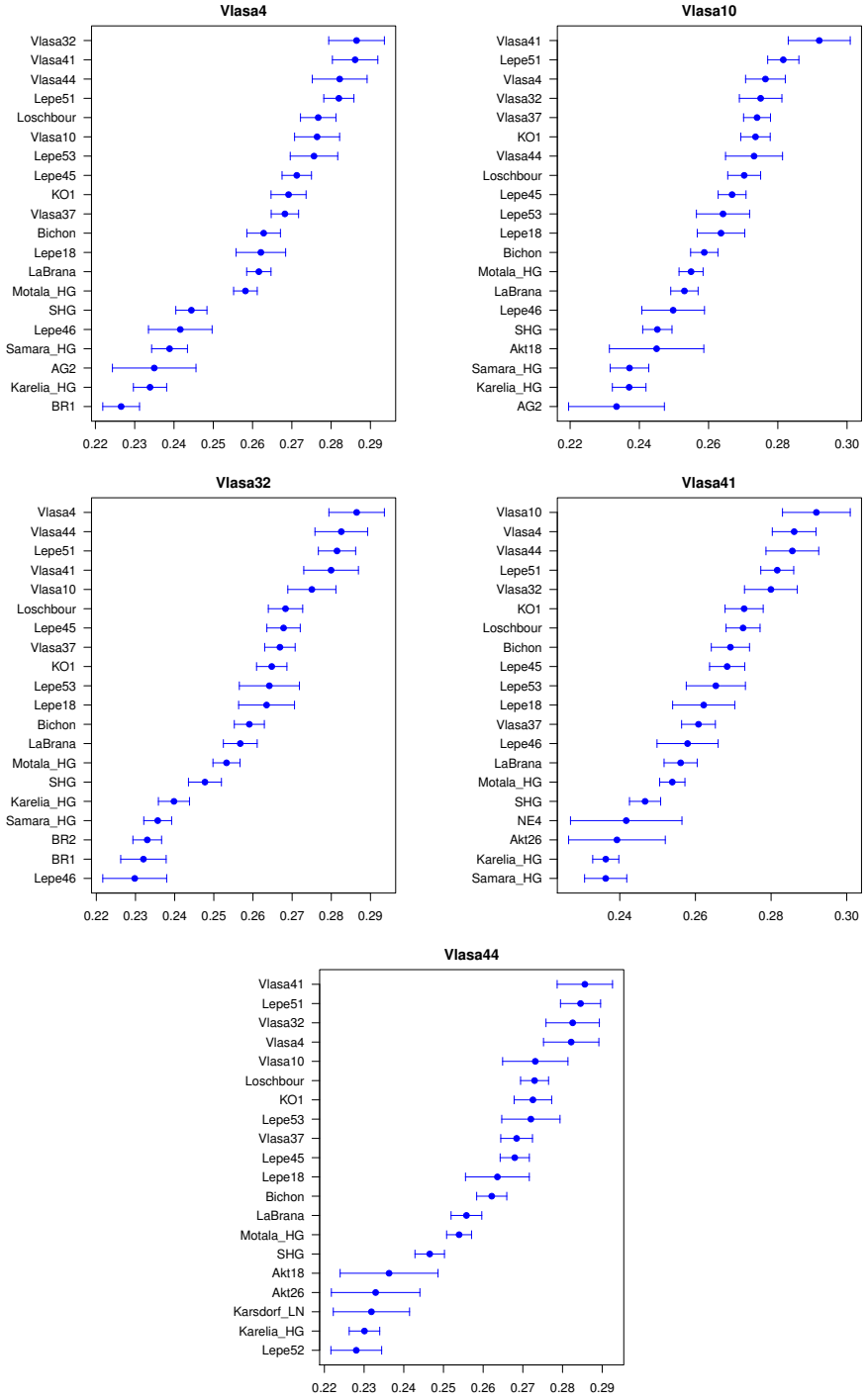


Figure 24: *f3(≠Khomani; Ancient_population, Vlasac captured sample). The highest 20 values shown.*

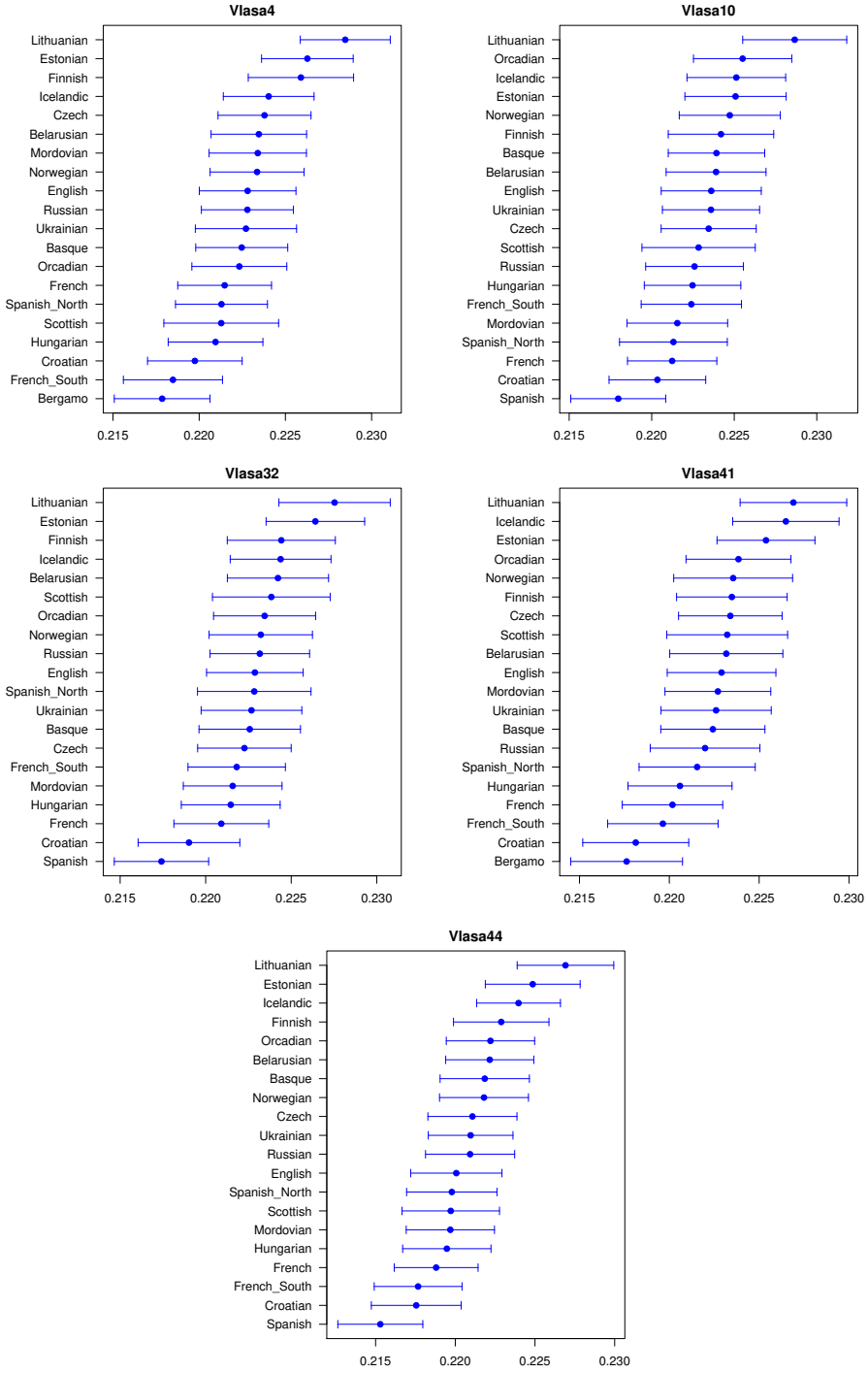


Figure 25: *f3(≠Khomani; Modern_population, Vlasac captured sample). The highest 20 values shown.*

4.5.15 Confirming D statistics results on the capture dataset

Even though the capture dataset does not provide as much power as the genomic one, we were able to confirm the main conclusions of sections 4.5.1–4.5.10. The notions from the samples from Lepenski Vir were less clear, because Lepe52 and Lepe39 can be considered Aegean in their affinities and other samples (Lepe18 and Lepe46) yielded admixed signals (as argued in the previous section). Except for the lack of increased Neolithic Iranian ancestry in the Anatolian capture samples (related to the discussed differences in relation to CHG), we did not observe notable differences in the signals between the genomic samples and capture samples (see Supplementary File S3).

4.6 Ancestral components of studied populations

4.6.1 NW Anatolian samples form an ancestral cluster to Neolithic farmers in Europe

The results of unsupervised *ADMIXTURE* analysis for Neolithic Anatolian genomic samples are shown in Figure S1a. The cross-validation error was lowest at K=2 (Figure 26). The sample clustering is highly similar between the supervised and unsupervised run (see Figure 30 and Figure S1a, respectively). This suggests that the Anatolian samples could be considered good proxies for the ancestral farmer component, though we note that most other early Neolithic farmers also show the same ancestry component with no evidence of admixture with hunter-gatherers. The only exceptions are NE1, NE3 and NE4 (data from Gamba *et al.* (*52*)). This result agrees with the D statistics analysis (see section 4.5, Table 42), where the Hungary_EN group containing these samples also demonstrated an apparent signal of admixture with hunter-gatherers. Interestingly, an older Neolithic sample from the same region (KO2 from Gamba *et al.* (*52*)) demonstrates no evidence of hunter-gatherer admixture, while another sample of the same age (KO1 from Gamba *et al.* (*52*)) is genetically most similar to hunter-gatherers. While hunter-gatherer ancestry is largely absent in Early Neolithic farmers according to ADMIXTURE results, it is increasingly apparent transitioning into the Middle and Late Neolithic. It should be noted that Kumtepe4 is also showing apparent admixture with the non-farmer cluster, however under higher K, it is obvious that there is no high affinity of Kumtepe4 to Western hunter-gatherers (this result is probably due to low quality of the sample).

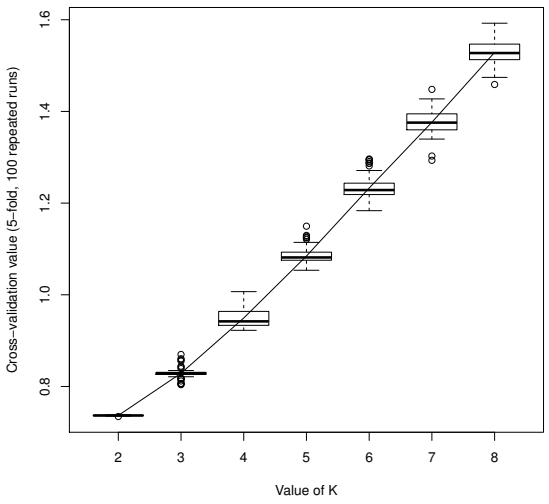


Figure 26: *Cross-validation error (5-fold) of 100 iterations of unsupervised ADMIXTURE run with Neolithic samples.*

4.6.2 CHG affinities to farmers

Results for unsupervised runs of K=2 to K=8 when including CHG samples are shown in Figure S1b. The cross-validation error pattern did not change with the addition of the CHG samples (the lowest was for K=2, see Figure 27). For the most likely clustering of K=2, the main conclusion of all Early Neolithic samples clustering with Aegeans was maintained. For K=3, all Neolithic samples demonstrated mixed ancestry with at least some CHG-defined component in addition to the WHG-defined component described in section 4.6.1 (see the supervised run in Figure 31 and unsupervised in Figure S1b). Interestingly, the CHG cluster was found at a higher proportion in Aegeans than other Early Neolithic samples, especially for Kumtepe4. The difference between Kumtepe4 and earlier Aegeans in terms of higher CHG influence was also observed using D statistics (see section 4.5).

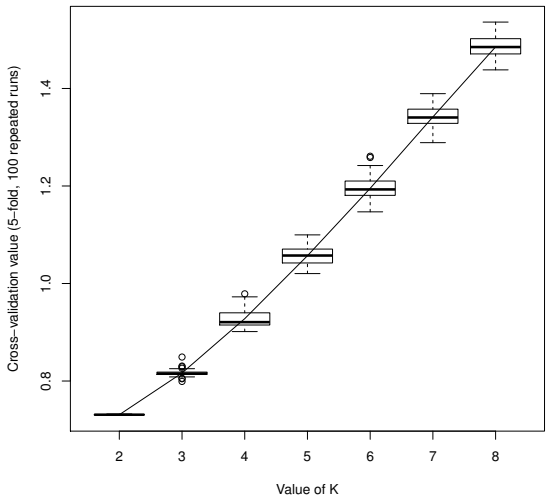


Figure 27: *Cross-validation error (5-fold) of 100 iterations of unsupervised ADMIXTURE run with CHG samples.*

4.6.3 Yamnaya signal in Late Neolithic

The results of our *ADMIXTURE* analysis for the dataset including also Yamnaya samples are shown in Figure S1c. The cross-validation error was the lowest for K=2 (Figure 28). Supervised (Figure 32) and unsupervised analyses for K=3 are again highly concordant (see Figure S1c). Early Neolithic farmers again demonstrate almost no evidence of hunter-gatherer admixture, while it is observable in the Middle Neolithic farmers. However, much of the Late Neolithic hunter-gatherer ancestry from the previous analysis is replaced by Yamnaya ancestry. These results are consistent with the results of Haak *et al.* (*8*) who demonstrated a resurgence of hunter-gatherer ancestry followed by the establishment of Eastern hunter-gatherer ancestry.

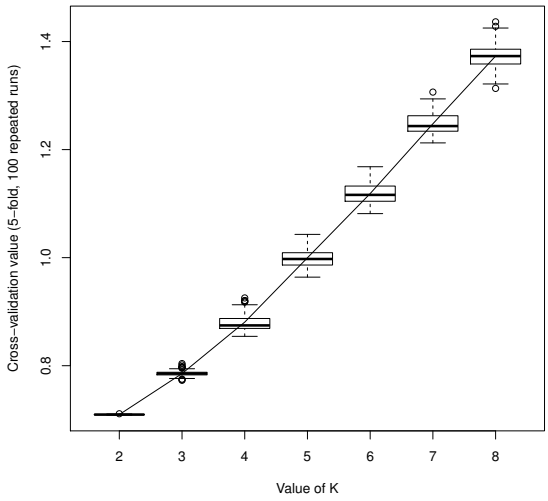


Figure 28: *Cross-validation error (5-fold) of 100 iterations of unsupervised ADMIXTURE run with Yamnaya samples.*

4.6.4 Danubians during Neolithic Transition

The capture dataset, including Neolithic and Mesolithic genomic samples from Anatolia, the Danube Gorges and Iran (*9*), was analysed with *ADMIXTURE* to investigate how the Danubian population compared to previously known hunter-gatherers and additional samples from Neolithic Anatolia. The cross-validation error supported K=2 result (Figure 29). The results from the Anatolian data for both supervised and unsupervised runs (see Figure S2 and Figure 33, respectively) for K=2 largely supported our previous conclusions from D statistics that Neolithic NW Anatolians were very similar to each other, to the Neolithic Aegeans and to early Neolithic samples from Europe. Also corresponding to the previous results, Danubians from Vlasac belonged to the same cluster as other WHG samples included in the analysis (Loschbour, KO1 and La Braña) and the same was true for most of the samples from Lepenski Vir. The presumed incomers, Lepe52 and Lepe39, were shown to have the same ancestral cluster as Neolithic NW Anatolians and Neolithic farmers from Europe. While Lepe46 was identified as an admixed individual (the cluster assignment was almost perfectly divided between the Anatolian-like and WHG-like cluster) similarly as in D statistics, Lepe18 did not show any level of Aegean-like ancestry. The absence of Aegean-like signal for Lepe18 weakly detected by D statistics (see section 4.5.11) can be explained by a real absence of this signal or a lack of power with low number of SNPs.

Additionally, we noticed a small level of WHG-like cluster for Neolithic sample Akt16 (see Figure 33). However, the number of SNPs in the capture dataset does not allow for a conclusive analysis on the individual basis (capture samples were not intended for population genetic analysis with reference datasets targeting different nuclear regions) and this result is also not confirmed by D statistics. Still, it cannot be excluded that there was some influence on Neolithic and post-Neolithic

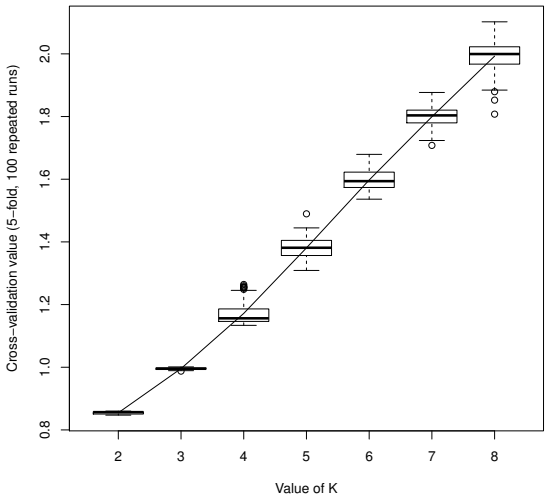


Figure 29: *Cross-validation error (5-fold) of 100 iterations of unsupervised ADMIXTURE run with joined dataset of genomic and capture samples.*

Aegean genetic variation from a WHG-like population (or rather a population with some levels of admixture with WHG, for example from the Balkans).

It should be noted that for the higher clusters (Figure S1d), we have observed higher similarity between Neolithic genomic samples from the Aegean and Neolithic Iranians (and CHG) than between Neolithic Iranians (and CHG) and the additional Neolithic NW Anatolians from the capture dataset. While this can be due to low coverages of capture samples targeted for specific regions rather than whole genomes, the same was found in D statistics (see section 4.5.11) and therefore it could be a relict of the different calling method applied (genomic samples from Anatolia were called by a likelihood-based method in Hofmanová *et al.* (*51*), while here we used major calls for the capture samples). However, we observed a similar pattern when we experimentally examined the complete dataset with majority calls and the Iranian/CHG-like cluster was also present in Iberian Middle Neolithic samples (see Figure S1d) that were not called with the method from Hofmanová *et al.* (*51*). Therefore, the difference could be due to a real genetic structure in Neolithic NW Anatolian population, especially since a similar genetic structure was observed during the Neolithic period in central Anatolia (*216*).

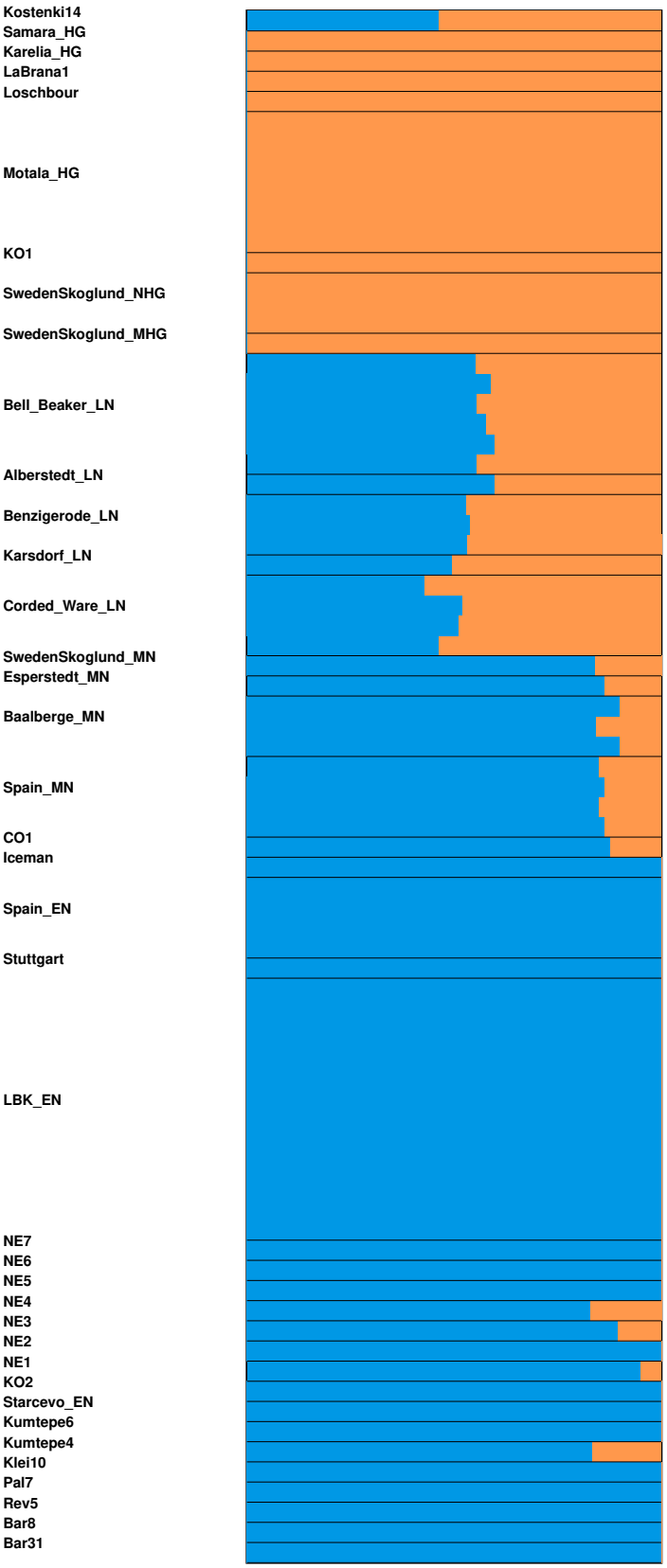


Figure 30: *Supervised run of ADMIXTURE. The clusters to supervise were chosen to best fit the presumed ancestral populations (for HG Motala and for farmers Bar8 and Bar31).*

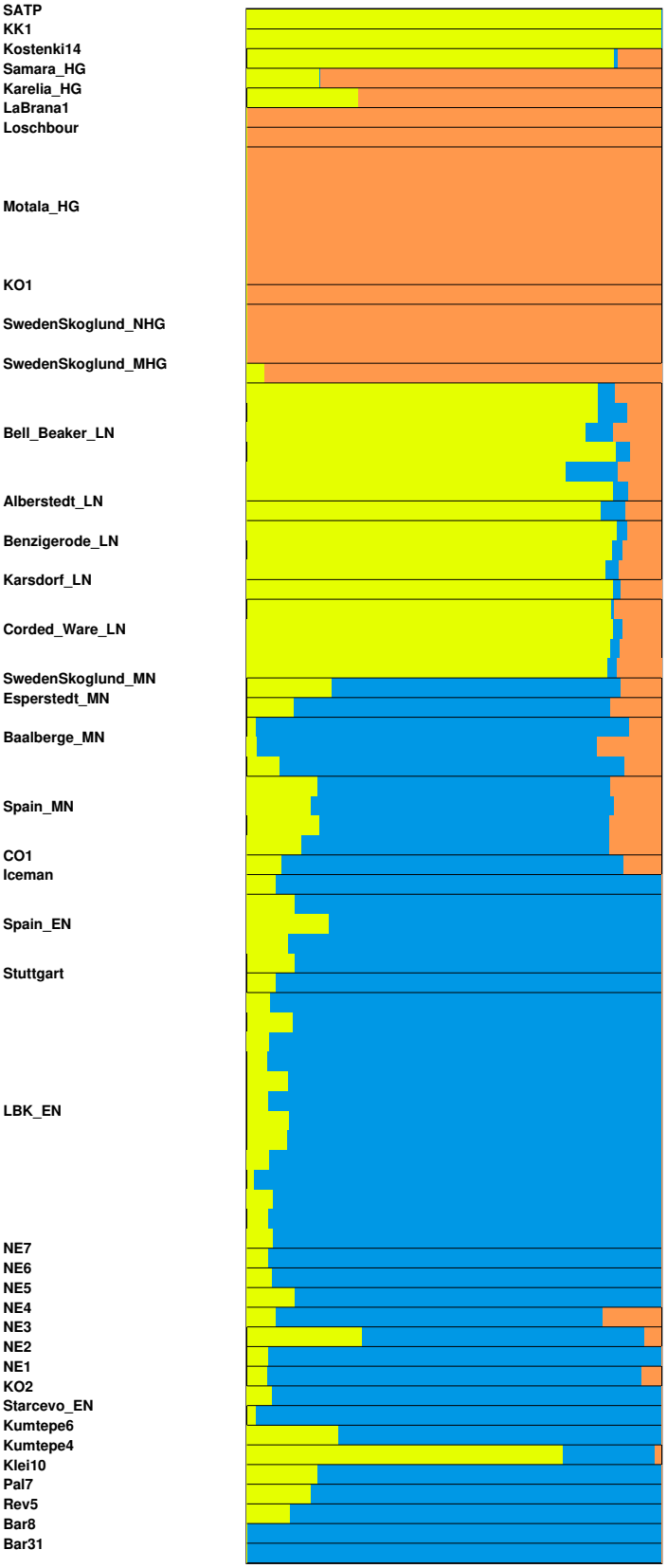


Figure 31: *Supervised run of ADMIXTURE. The clusters to be supervised were chosen to best fit the presumed ancestral populations (for WHG Motala, for CHG KK1 and SATP and for farmers Bar8 and Bar31).*

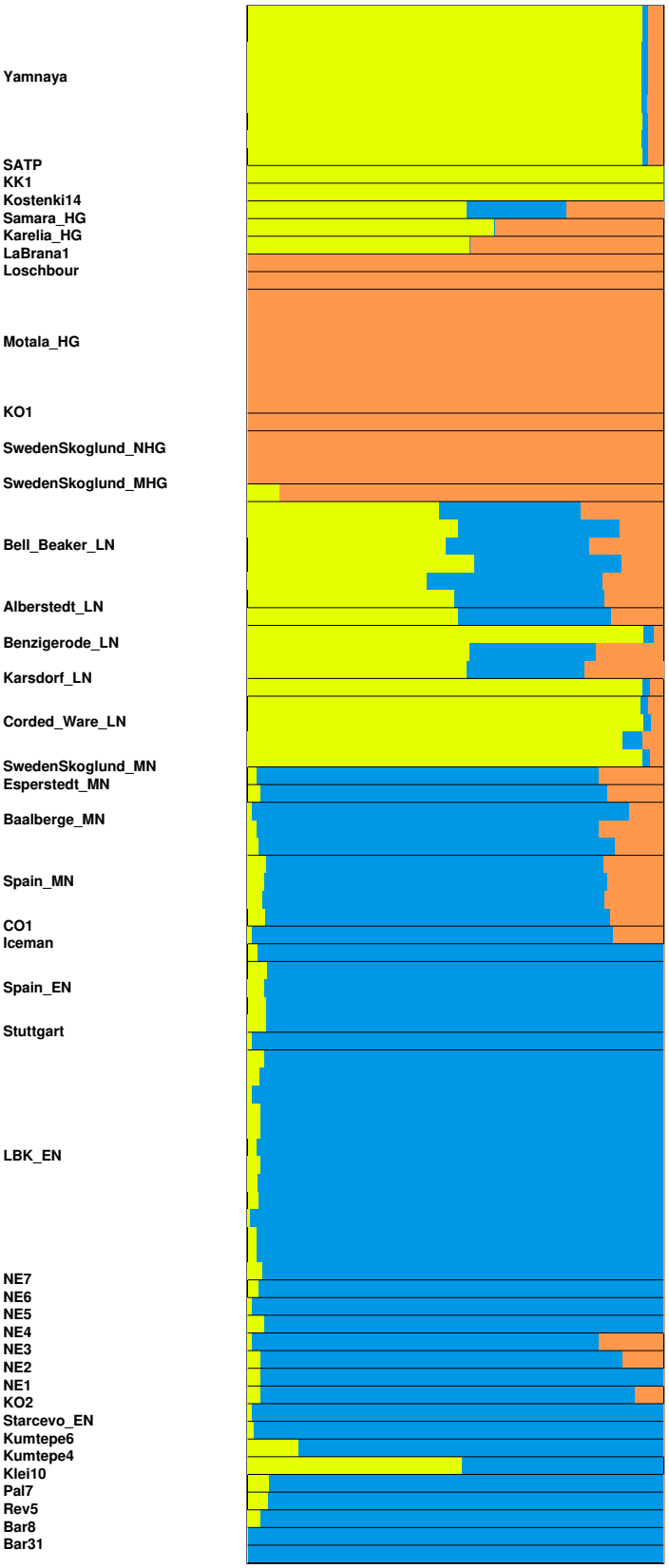


Figure 32: *Supervised run of ADMIXTURE. The clusters to be supervised were chosen to best fit the presumed ancestral populations (for HG Motala and for farmers Bar8 and Bar31 and for later Eastern migration Yamnaya).*

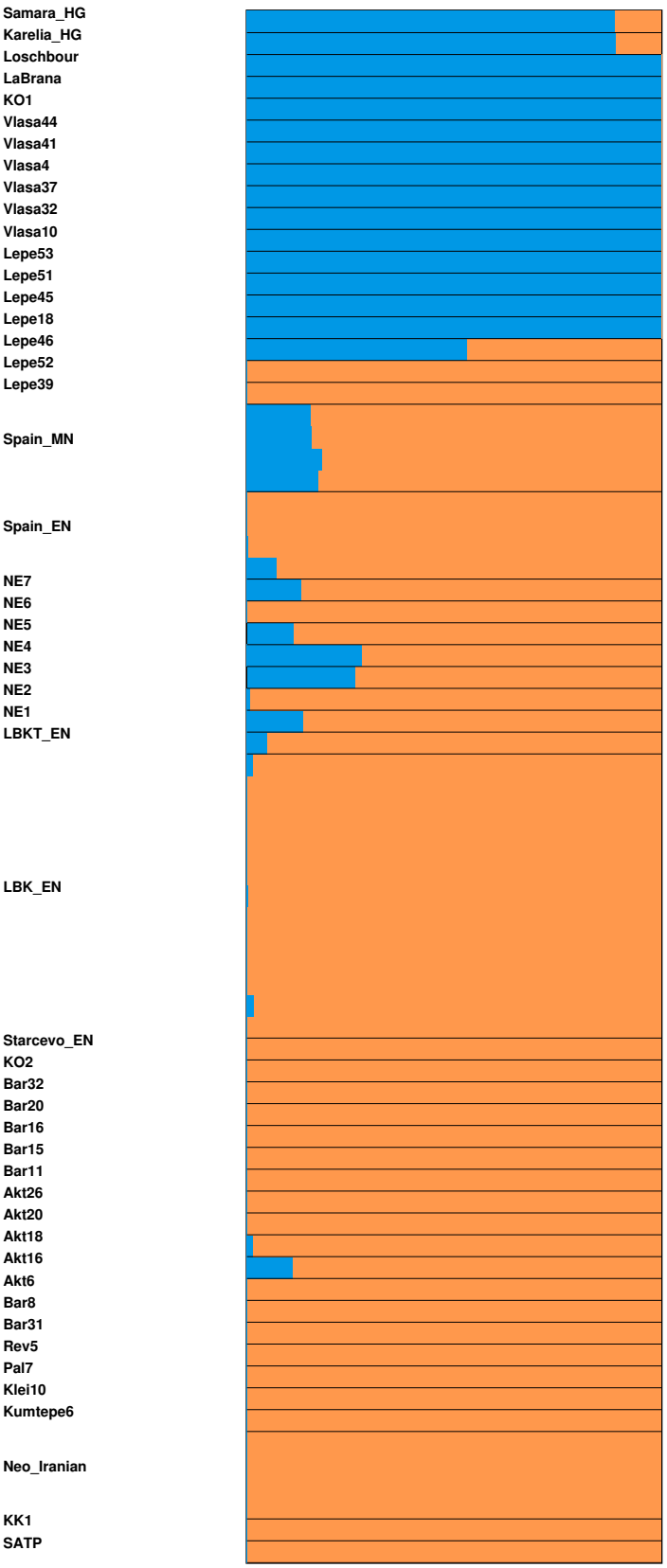


Figure 33: *Unsupervised run of ADMIXTURE for joined dataset of genomic and capture samples for K=2. The less likely K shown in Figure S1d.*

Table 65: *Informative derived alleles from samples from Anatolia, samples without identifying derived SNPs positions were excluded. Note that the haplogroup was confirmed by higher number of independent derived markers only for Bar31.*

Sample name	Position	SNP	Haplogroup	Coverage	Mutation
Akt20	16638804	M438	I2	19	A->G
Bar11	21741703	M35.1	E1b1b1	4	G->C
Bar31	17174741	L156	G2	2	A->T
	23244026	P15	G2a	4	C->T
	14692227	L32	G2a2b	6	T->C

4.7 Remarks on Y chromosomal variation

The Y chromosome is often used in population genetic studies to investigate paternal ancestral histories, especially in comparison to maternally determined mtDNA. In this study, Y chromosome sequencing was not the objective, but it was possible to obtain general (and by no means conclusive) information on Y chromosome haplogroups from several of the studied samples. For genomic samples the haplogroup assignment was supported by more than one derived mutation. From the male samples enriched for nuclear DNA, but not enriched specifically for many Y chromosome positions, a few derived informative positions were identified and can serve as supporting information for the assignment of genomic samples.

As previously analysed in more detail by Martiniano (Supplementary Section SI4 in *51*), the genomic sample Bar31 was identified as G2a2b (see Table 65), a part of G2a lineage that had been previously identified in Neolithic farmers from Germany, the western Mediterranean (*8*, *35*, *205*, *217*) and also the Near East (*9*, *50*). The same haplogroup was observed for Lepe52, a sample identified in this study as of Aegean-like origin. Other Y haplogroups from Anatolia (I2 for Akt20, E1b1b1 for Bar11, see Table 65) were both previously observed in ancient Near East samples (*12*, *50*). Yet the I2 haplogroup was also identified for Lepe45, Lepe18, Vlasa4 and Vlasa44 (see Table 66 and 67), which is understandable since this haplogroup is very frequent among the known WHG from Bichon, Loschbour and Motala (*12*, *53*, *78*). Another haplogroup that was assigned to several of the Vlasac samples is R1, in Vlasa37 it was possible to identify R1b1c. While this haplogroup has usually been connected to changes in genetic variation in the Bronze Age (*8*, *81*), it was already observed in an EHG individual from Samara region and in an early Neolithic individual from El Troc, Spain (*8*).

The Y chromosome group assignments then generally fall into previously observed genetic variation for this marker. The only surprise was a derived allele for P53.1 for Lepe39 sample, assigning it to the C2c haplogroup typical of Asia (*218*). However, it should be noted that this position is very likely placed in an unstable part of the Y chromosome because it appears in many sections of the Y chromosomal tree (International Society of Genetic Genealogy; http://www.isogg.org/) and the haplogroup assignment for Lepe39 can be thus considered highly uncertain.

Table 66: *Informative derived alleles from samples from Lepenski Vir, samples without identifying derived SNPs positions were excluded. Note that the haplogroup was confirmed by higher number of independent derived markers only for Lepe45.*

Sample name	Position	SNP	Haplogroup	Coverage	Mutation
Lepe45	16638804	M438	I2	4	A->G
	18700150	L68	I2	2	C->T
	13992338	P216	I2a2	3	C->G
	7628484	P217	I2a2	2	C->T
	7716262	L34	I2a2a	4	A->C
Lepe18	16638804	M438	I2	109	A->G
Lepe39	14491649	P53.1	C2c	7	T->C
Lepe52	23244026	P15	G2a	51	C->T

Table 67: *Informative derived alleles from samples from Vlasac, samples without identifying derived SNPs positions were excluded. Note that the haplogroup was confirmed by higher number of independent derived markers only for Vlasa37.*

Sample name	Position	SNP	Haplogroup	Coverage	Mutation
Vlasa37	6868118	S9	R	2	T->C
	8050994	P229	R	4	G->C
	7570822	P294	R1	3	G->C
	18914441	L278	R1b1	3	C->T
	4862861	PF6279	R1b1c	2	C->T
Vlasa4	7173143	L16	IJK	2	G->A
	16638804	M438	I2	116	G->A
Vlasa10	7570822	P294	R1	14	G->C
Vlasa32	7570822	P294	R1	12	G->C
Vlasa44	8590752	P127	IJ	2	C->T
	16638804	M438	I2	64	G->A

5 Discussion

During the course of this study, we obtained a relatively large dataset of 86 samples, which was diverse methodologically (5 genomes, 20 samples with nuclear regions and 86 mitogenomes), geographically (from both southestern Europe and Anatolia) and chronologically (the difference between obtained samples was in extreme cases up to 8,000 years). Traditional challenges to ancient DNA work were emphasised by the relatively advanced age of the samples (up to approx. 9,500 years old, see section 2.4), among the oldest in the majority of even recently published ancient DNA studies (*9*, *41*, *50*). However, the main framework of Neolithic Transition has enabled us to connect the inferences and compare their relative methodological advantages for answering the questions at hand and at the same time to consider the studied populations in a previously not attempted complexity.

5.1 Population demographics during the spread of farming

5.1.1 Western Anatolia as a core zone for Neolithic spread to Europe

The Aegean was inferred by our dataset as a source area for the demic spread of farmers to Europe mainly due to the shared ancestry and similarity of Early Neolithic farmers from central and western Europe and newly obtained Aegean Neolithic samples at both uniparental (the same haplogroups and low F_{ST} values, see section 4.3) and nuclear levels (see f3-statistics in Figures 15, 18 and 19 and D statistics described in section 4.5.7). These genetic affinities are especially important in contrast to the differences of both the European and Aegean early farmers to hunter-gatherers previously inhabiting Europe (mtDNA: contrasting haplogroups and high F_{ST} values, see section 4.3; nucDNA: less shared drift compared to other populations, measured via D statistics, see section 4.5.7). In the past, similar statements were made (*33*) but only now are there direct observations of genetic variation in Neolithic western Anatolia (this study, *12*, *51*) and not through proxies such as southern European modern populations (*86*).

Another important supporting argument for the spread of farmers from Anatolia is that Early Neolithic farmers probably did not migrate to Iberia through central Europe but rather from the Aegean region directly (see section 4.5.7). The Mediterranean route for the spread of the Neolithic populations from the Aegean to Iberia has been expected archaeologically (*219*) but has not yet been demonstrated by palaeogenetic means. It should be added that recently sequenced Iberian Neolithic farmers showed an admixture with hunter-gatherers, assumed to occur either on the Iberian Peninsula or along the Mediterranean coast (*213*).

Of course, there is always a possibility that the farmers migrating to Europe were not originally Neolithic W Anatolians but rather a population related to them. In this respect, the Fertile Crescent, a region where agriculture first appeared (*66*), should be mentioned as a potential direct source of farmers coming to Europe, for example through archaeologically supported maritime prehistoric

connections (*153*). Recently nuclear data from the Fertile Crescent were obtained and while Neolithic individuals from the eastern part of the Fertile Crescent (samples from the Zagros Mountains, Iran) were related to the Neolithic population of Europe and the Aegean only very distantly (and therefore did not contribute to the ancestry of the first European farmers) (*9*), pre-Neolithic (Natufians from Raqefet Cave, Israel) and continuing Neolithic populations from the western part of the Fertile Crescent (Levant individuals from Ain Ghazal, Jordan and Motza, Israel) were closely related to the early farmers that spread to western Anatolia and then to Europe) (*50*).

However, unique ancestry between western Anatolian and European Neolithic samples not present in the Neolithic samples from the western Fertile Crescent (Levant) was detected (*50*) and therefore, the newly appearing farming population in Europe could not have come directly from the Fertile Crescent and must have shared genes with Neolithic W Anatolians or another population related to Neolithic W Anatolians (e.g., other Aegeans) along the way. This unique Neolithic W-Anatolian-European shared ancestry of farmers could have been a result of a serial founder effect due to migration from the Fertile Crescent to western Anatolia (which would accelerate genetic drift and accumulate genetic variation not present in the Neolithic Levant). That would be consistent with low diversity observed in central Neolithic Anatolians from Boncuklu (∼8,300 and 7,500 cal BC), sampled along the potential terrestrial migratory route from the Fertile Crescent to western Anatolia (*216*). However, autochthonous Mesolithic hunter-gatherer populations in the Aegean (so-far genetically uncharacterised) could have also played a role in creating the ancestral connection.

In Hofmanová *et al.* (*51*), we argued that, due to the K1c mitochondrial haplogroups of two Theopetra Cave samples (dated to 7,605-6,771 cal BC; Thessaly, Greece), we cannot exclude the possibility that Mesolithic Aegean hunter-gatherers played some role in the makeup of Neolithic Aegean and consequently that of European farmers. Without nuclear data from Mesolithic Aegean individuals, this hypothesis still cannot be excluded. However, because of the K1f haplogroups of two individuals from Mesolithic Vlasac (see Table 11), which were both determined to be on the nuclear basis related to hunter-gatherers (WHG) and distinct from the expanding farmer population (see section 4.5), the importance of the haplogroup assignment of Theopetra samples for the interpretation of the Mesolithic Aegean diminishes. Futhermore, influences of Mesolithic Danubians (∼9,500-6,600 cal BC) on the ancestry of Neolithic Aegeans are inconsistent with the genomic differentiation between these groups (see especially sections 4.5.5 and 4.5.14) and with the similarity of Neolithic farmers from the Aegean with Neolithic populations in central Anatolia (*216*) and Levant (Israel and Jordan) (*50*).

5.1.2 Neolithic NW Anatolians in a context of known regional genetic variation

No strong differences in mitochondrial and nuclear variation were observable among the Northwest Anatolian Neolithic samples and sites Aktopraklık and Barcın (see sections 4.3.4 and 4.5.12), even though distinctions were noticed archaeologically between these Fikiterpe sites (*136*, *141*). While we

obtained only two mitochondrial genomes for central Anatolian site Catalhöyük, their similarity to Northwest Anatolian samples corresponds to the recent analysis that suggests that central Anatolian whole genome samples from Boncuklu (∼8,300 and 7,500 cal BC) and Tepecik-Çiftlik (∼7,500 and 5,800 cal BC) were similar to Neolithic farmers from central Europe and NW Anatolia (Barcın) (*216*).

The successful extraction of DNA from Catalhöyük indicates that DNA extraction on a genomic level from this important Neolithic centre is not beyond current technological limitations and more samples should be screened and analysed (preferably using petrous bones). Such analysis could confirm the genetic similarity of Neolithic farmers from central and Northwest Anatolia, which is also supported archaeologically (e.g., *67*). In particular, the effect of chronological gap detected archaeologically between the areas (*138*) could be further investigated with increased sample sizes and genomic data from central Anatolia.

Interestingly, Greek whole genome samples from the Early and Final Neolithic periods analysed alongside the Anatolian whole genome samples have proven to be, on the basis of genomic analysis (f3 and D statistics and *ADMIXTURE*; see especially sections 4.5.6 and 4.6.1, *51*), similar to the Neolithic Anatolians to such an extent that they could be considered as originated from the same Neolithic Aegean population. Archaeologically detected strong maritime connections and the reliance on deep sea fishing in the Neolithic Aegean, argued especially for Ulucak Höyük and Çukuriçi Höyük (*153*, *220*), support this conclusion.

The "eastern" influence of a population related to Caucacus hunter-gatherers (CHG) on genetic variation in Chalcolithic Anatolia was first observed via the comparable analysis of Kumtepe6 (∼4,850 cal BC) (*215*) and our Neolithic Aegean genomes (see section 4.5.8, *51*), and it was further confirmed by Lazaridis *et al.* (*50*) with a Chalcolithic sample from Barcın (3,943-3,708 cal BC). While our Neolithic Aegean (especially Final Neolithic Greek) whole genome samples (see section 4.5.8, *51*) already show small levels of this CHG-like ancestry, our Neolithic Anatolian capture dataset (of less SNPs, see Figure 33) is more consistent with data from Mathieson *et al.* (*12*), i.e., without noticeable levels of CHG affinity (*50*). Also central Anatolian individuals from Tepecik-Çiftlik (∼7,500 and 5,800 cal BC) have shown diversity in the relationship to CHG, with only one individual (out of five analysed) showing CHG-like admixture (*216*). Still, it should be noted that the differences in the affinities to CHG between whole genome and capture samples from Barcın (also differing in the amount of SNPs in the analysed panel) can be further confirmed only when all the samples are analysed with the same calling methods (for Aegean genomic samples the advanced likelihood-based calling method was applied, whereas capture data were analysed with majority calling strategy, see section 3.13).

5.1.3 Mesolithic settlements in the Danube Gorges

Lepenski Vir and Vlasac are the sites that were sampled most extensively in this study and therefore they contributed most of the DNA-rich samples to the analysis of both mitochondrial and nuclear data. When we investigated the origin of the Danubian Mesolithic hunter-gatherer population from both sites genomically (especially attested by the whole genome of Lepe51, cca 9,700-year-old sample), we observed strong affinities with other analysed Holocene hunter-gatherers of western origin (WHG, see section 4.5.1). The structure of WHG (containing Danubians) was investigated and genetic links between Danubians and Mesolithic Loschbour individual and between Danubians and Eastern hunter-gatherers (EHG) in respect to the rest of WHG (Mesolithic individuals from La Braña and Bichon) were additionally observed (see section 4.5.3). Given especially these results, an isolation by distance pattern between Iberia (La Braña), central Europe (Loschbour) and Danubians can be assumed. However, further genomic analyses of the origin of Danubians should be performed, especially in relation to recently obtained data from Pleistocene samples (e.g., Dolní Věstonice) (*41*).

Our results from mitochondrial data from Mesolithic layers of Ostrovul Corbului were quite differentiated from Late Mesolithic individuals from Vlasac. Even when pooled with other Early/Middle Mesolithic samples from the area (Padina and Lepenski Vir including Lepe51 sample, see section 4.3.4), strong differentiation was observed between them and Holocene samples from Vlasac and other areas to the north and west of the Danube Gorges (Holocene and post-LGM groups mainly obtained and grouped by Posth *et al.* (*64*), see Table S2). There could be several reasons for the discrepancy between these mitochondrial results and strong affinities of Danubian genomes to each other and to WHG; the main ones being the absence of genomic analysis with Pleistocene samples, the absence of genomic data from Ostrovul Corbului and the fact that Ostrovul Corbului samples are not directly dated and could be younger (see sections 2.2.4).

Nevertheless, until proven otherwise, we cannot completely discard the hypothesis that hunter-gatherers of the early strata in the Danube Gorges could have been differentiated from the Late Mesolithic Vlasac individuals, especially since a gap in the occupation of Lepenski Vir was observed chronologically (*122*). Moreover, given the absence of differences between the mitochondrial data of Early/Middle Mesolithic Danubians and Plesitocene samples before Last Glacial period, this population could have been a relict of the genetic variation present in Europe before cca 14,500 years when a genetic turnover in Europe probably occurred (*41*, *64*). Yet we conclude that the strongest evidence was observed in the genomic analysis that showed no difference between the Lepenski Vir individual from the Early Mesolithic period (Lepe51, grave 68; 7,940-7,571 cal BC) and the WHG-like individuals from the Transition period (three individuals from ∼6,200-6,000/5,950 cal BC; see sections 4.5.2 and 4.5.13).

5.1.4 The Balkans in a time of change

Lepenski Vir individuals from the Transition (∼6,200-6,000/5,950 cal BC) and Neolithic (∼6,000/5,950-5,500 cal BC) periods have shown, both on the nuclear and mitochondrial levels, a mixed ancestral signal interpreted as admixture of populations of diverse origin. That is consistent with archaeological interpretations that appeared since new radiocarbon dating projects of old finds showed that the Danube Gorges layers from the Lepenski Vir culture were parallel to appearance of Neolithic in the region (*109*), which would have logically led to contacts between the incoming farmers (with Aegean-like ancestry) and local hunter-gatherers (with WHG-like ancestry).

No Aegean-like individuals were detected in the nuclear dataset of Late Mesolithic Vlasac, which seemed to be restricted to individuals related to WHG only and on mtDNA basis differentiated from all other groups except for Holocene and Last Glacial European hunter-gatherer populations. On the other hand, Lepenski Vir individuals from the Transition period were related almost equally to Mesolithic hunter-gatherers and Early Neolithic farmers on the mitochondrial level (see section 4.3.4) and in the nuclear dataset (e.g., section 4.5.11) there were individuals with both the full Aegean-like (Lepe39, grave 82) and WHG-like affinity (Lepe18, Lepe45 and Lepe53, graves 27/d, 91 and 27, respectively). Moreover, the contact between the groups at Lepenski Vir site also resulted in a directly admixed individual from the Transition period, Lepe45 (from grave 93), a descendant of individuals with both ancestries.

While the nuclear dataset is too small to compare the Lepenski Vir Neolithic and Transition groups (only one individual was from the Neolithic period), mitochondrial data strongly suggest that Aegean-like ancestry already present in Lepenski Vir in the Transition period increased during the Neolithic stage (see section 4.3.4). This increase could have been caused by an continuing influx of incoming individuals with this genetic affinity to the community or by a difference in the number of children Aegean-like farmers might have had (*221*). However, the incoming individuals would have to frequently select mates with the same ancestry (assortative mating) and they would have to bring and maintain a different subsistence or behaviour towards children compared to the local population (e.g., weaning of children is expected to have an effect of increasing the number of children per woman (*222*)). At the same time, many authors point out that the population density increase (Neolithic Demographic Transition) could have been rather connected to the advent of sedentarism, probably common for the whole community in the Danube Gorges (*133*, *223*).

There are reasonable reservations for the usage of strontium isotopes to estimate a level of migration by detecting non-local individuals from a site (*224*). These strontium isotope inferences in the Danube Gorges by Borić & Price (*94*) are likely not valid for every individual tested, as one presumably non-local individual was identified with U5 haplogroup (see section 4.3). Still, the increase of Aegean-like ancestry from the Transition to Neolithic period corresponds to the increase of non-locals in Lepenski Vir identified in the isotopic study of Borić & Price (*94*). Furthermore,

non-locals seem to be closer to other Early Neolithic populations than a local group genetically (see Figure 10).

Taken together, the genetic and isotopic data suggest that there was continuous gene flow in the Transition and Neolithic periods to the Lepenski Vir settlement rather than one single migration event. However, given changes in the sex assignment of several individuals due to genetic data (see section 4.2) and genetic affinities of isotopically border-line individuals (see sections 4.3.4 and 4.5.11), the suggestion of Borić & Price (*94*) that non-locals were mostly women should be further investigated with both genetic and isotopic data (only 4 non-local individuals were assigned sex genetically). More importantly, we also do not observe any genetic hints that Aegean-like ancestry was carried to the Danube Gorges mostly by women. While the methods applied in this study did not allow to examine how sex differences contributed to population ancestry, both individuals that had Aegean-like ancestry in our nuclear capture dataset were male and one (Lepe52) had a Y chromosome haplogroup identical to the ones associated with Neolithic in Europe (*35*) and Anatolia (*12*, *51*). Of course, the contribution of migrating women must have been very significant, as otherwise the Aegean-like signal in maternally inhereted mitochondrial DNA would not be present (see section 4.3). Still, without deducing the sex ratio of the migrating population from our dataset, we suggest that the situation was very likely more complex than an exchange of females.

Established Neolithic societies of Vinča-Belo Brdo and Sultana Malu Roşu did not provide high quality samples for nuclear enrichment and only a limited number of samples was analysed for mtDNA. Still, it was obvious (see Figure 9) that the samples from Vinča site were very similar to Neolithic Anatolians (even when compared to Early Neolithic farmers from central Europe – individuals assigned to Starčevo or LBK from Transdanubia and LBK from Germany). On the other hand, Sultana-Malu Roşu, together with other Late Neolithic/Eneolithic sites reanalysed in this study with the newly obtained mitochondrial data (see Table S2), showed to be quite genetically differentiated from Early, Middle, Late Neolithic and Bronze Age samples from central Europe and, surprisingly, also from Neolithic Anatolians. Hervella *et al.* (*204*) suggest that the differentiation of these groups is due to additional migration from Anatolia. Given our data, it is obvious that if the hypothesis is true, then the genetic variation of Anatolia must have remarkably changed from what was observed in the Neolithic period. That could be consistent with the presumed population change during Chalcolithic in Anatolia (see section 4.5.8, *50*, *51*).

5.2 Implication for understanding of Neolithisation

Archaeological links between Mesolithic hunter-gatherers and Early Neolithic farmers in central Europe, especially in the continuity of the Mesolithic lithic tradition (*75*), were to the certain extent in contrast with the observed absence of genetic contribution of the previously sampled local Mesolithic hunter-gatherers to European Early Neolithic populations (*34*, *78*). It should, however, be emphasised that the introduction of the Neolithic innovations (e.g., farming subsistence, pottery, polished stone tools) (*67*) is consistent with the genetic inferences of the demic spread.

For the first time by comparison with the actual presumed source population for migration (Neolithic Aegeans), this study showed that while most of the ancestry of central European early farmers was indeed Aegean-like (see sections 4.5.7 and 4.6), minor influences of WHG (more specifically Danubian) ancestry were present (see sections 4.5.9 and 4.5.15). At the same time, we confirm and demonstrate again that this was the case also for Early Neolithic Iberians (as in *213*).

The geographical location of the Danubian population along the route from the Aegean to central Europe and the importance of the large Lepenski Vir settlement for the Mesolithic Balkans (*133*) could suggest that the source of the hunter-gatherer ancestry in the early farmers could have been directly in the Danube Gorges hunter-gatherer community; at least the Danubians were more genetically related to the admixing hunter-gatherers than other tested samples (section 4.5.14). Still, while the levels of hunter-gatherer admixture might have been relatively high at the local sites in the central Balkans where admixture and incorporation was proven (i.e., Lepenski Vir), the conclusion on the continental scale remains that the impact on the genetic variation of early central European farmers was low (see section 4.5.7 and 4.6).

It should, however, be noted that signals of admixture are highly dependent on many factors, including prior genetic variation, the differentiation of both groups before admixture, population sizes, migration rates and the geographical distribution of settlements. A number of scenarios can thus easily result in identical patterns of genetic variation; for example, as famously demonstrated by Arenas *et al.* (*225*), clines of decreasing genetic similarity in one direction could have easily been caused by a migration from a completely different direction. These genetic mechanisms can be formally explored with spatial explicit modelling (*100*) and even though such models cannot fully accommodate all unknown variables and local conditions leading to minor or major variations in results, it is necessary to investigate simple demographic scenarios before invoking more complex ones (*226*). Therefore, without further studies exploring at least some of these uncertainties, we cannot conclude what process or processes created the observed pattern of low hunter-gatherer admixture in central Europe and if the instance of the farmers incorporation to the Danube Gorges hunter-gatherer community significantly contributed to the genetic ancestry of early farmers over the whole continent.

In this study, we instead concentrated on several different local cases of contact between farmers and hunter-gatherers in the corridor from the Aegean to central Europe and while it cannot be

extrapolated that the same processes as observed here were repeated elsewhere, at least it can provide a notion what processes in particular should be studied further.

5.2.1 Summary of differences between sites

With cases of Lepenski Vir (∼9,500-5,500 cal BC), Vlasac (∼7,400-6,200 cal BC) and Vinča (∼5,476-5,304 cal BC), we observed distinctly different reactions to Neolithic Transition. Whereas in Lepenski Vir we detected the incorporation of Aegean-like individuals (presumably farmers in origin) to the Danubian society of fishermen and hunter-gatherers, in Vlasac the isolation of the hunter-gatherer population seems to be more probable (while we cannot completely exclude the existence of non-sampled Aegean-like individuals in Vlasac, it is very unlikely given the mtDNA results). Except for Lepenski Vir, the only other known example of possible incorporation of farmers to hunter-gatherer context was discovered in Ostrof (*34*) around 3,000 years later.

Given the geographical proximity of Vlasac and Lepenski Vir (cca 3 km), the differentiation between the sites is surprising and it is reminiscent of the Late Neolithic Blatterhöhle cave, where individuals with fisher-hunter-gatherer subsistence and WHG-like ancestry lived in parallel yet were genetically separated from a population of local farmers (*80*). It should, however, be noted that Lepenski Vir and Vlasac sites might not have been contemporaneous for most of their existence (*94*).

Similarly, Aegean-like Early Neolithic Vinča individuals seemed to be on the basis of ancient DNA genetically isolated with absence of local hunter-gatherer mitochondrial sequences. These results interestingly contrast with a case detected by Gamba *et al.* (*52*), in which an individual (KO1) from Tiszaszőlős-Domaháza, Hungary (5,650-5,780 cal BC) with strong genetic affinities to WHG was buried in a clearly Early Neolithic Körös context.

5.2.2 Comparison to archaeological models

How do these results compare with archaeological theory? Zvelebil (*73*) has defined seven mechanisms that could lead to Neolithisation. The spread of agriculture only via *contact (mechanism 1)* and exchange of ideas with hunter-gatherers can be excluded in view of current palaeogenetic knowledge. Also, *frontier mobility (mechanism 2)* involving small-scale movements, the slow spread of genes and fast spread of ideas along the farmer/hunter-gatherer border is improbable, given the dates of appearance of the first Neolithic sites in central Europe, the Balkans and Anatolia (*227*) and expectations of a higher genetic heritage of hunter-gatherers among Early Neolithic farmers in central Europe than detected (*8*, *33*, *51*, *78*).

So-called *folk migration (mechanism 3)*, defined as a major population movement causing complete and sudden gene replacement, can be considered for central Europe (*34*) but this study (see also *51*) and a recent study involving farmers in Iberia (*213*) have shown that there was a genetic impact,

albeit small, of hunter-gatherers on LBK, Starčevo and Iberian Early Neolithic farmers, followed by resurgence of hunter-gatherer ancestry in the Middle and Late Neolithic period (*8*), while at least in one case (Blatterhöhle) the hunter-gatherer population kept its genetic heritage for 2,000 years after the first contact with farmers (*80*).

The other four mechanisms of Zvelebil are more difficult to distinguish genetically because they all involve some level of admixture of both populations and, as noted by Richards (*228*), they are also often not mutually exclusive. The most known is *demic diffusion (mechanism 4)*, a wave-of-advance model that suggests farmer enclaves that grow and serve as local core zones for further spread. This model, coined by Ammerman & Cavalli-Sforza (*227*), is often used and supported by genetic simulations (*76*, *229*). Early enclaves of this form could be Starčevo settlements such as Vinča-Belo Brdo, at which no mitochondrial hints of hunter-gatherer ancestry were detected. The Vinča group is genetically placed between the Anatolian source and Early Neolithic groups in Hungary and Germany (see Figure 9). Series of founder effects during *demic diffusion* could lead to this genetic pattern. However, the low sample size and especially the specificity of the context of Vinča-Belo Brdo collective burial should deter from attempting a strict interpretation of Vinča samples as a population completely representative of the local genetic variation at the time. Still, even relatively frequent incorporation of hunter-gatherers into Neolithic society, as in Tiszaszőlős-Domaháza (*52*), would be concordant with the model and could still lead to the observed low impact on the general genetic variation of central European Neolithic societies (*226*, *229*).

Infiltration (mechanism 5) is very likely for the Transition and Neolithic periods of Lepenski Vir. This case is in effect the first time Neolithic individuals of Aegean-like origin were genetically observed to incorporate into a hunter-gatherer settlement of WHG-like affinities. The community probably maintained, to some extent, its dependence on the fishing subsistence even after the incorporation, but the dietary differences of the population in different periods are still under discussion (*94*, *114*, *125*, *151*). The *infiltration* mechanism suggests that the incoming population fills a niche or brings other benefits to the locals (such as expertise in farming) but so far this has not been conclusively associated with the non-locals (determined as such by $^{87}Sr/^{86}Sr$ isotopes)(*94*). However, some cultural traits such as crouched/flexed burial positions seem to be more frequent among isotopically non-locals (*94*). It is possible that cases of infiltration were rare because there are not many Mesolithic sites similar to Lepenski Vir in terms of the number of inhabitants, complexity of cultural traits and benefits from a high-energy source of food (fish) (*133*).

Elite dominance (mechanism 6) is defined as incorporation of elites into hunter-gatherer societies with subsequent subsistence change and it could manifest genetically similarly to *infiltration* in Lepenski Vir. However, it would probably result in a higher genetic input of hunter-gatherers than observed if this social structure was more prominent in Europe. Although the hints of cultural differences between isotopically determined locals and non-locals (*94*) could be interpreted as a difference in social status, more studies comparing local and non-local groups need to be carried out before this mechanism could be argued. Such studies should take into account genetic information

because at least for one isotopically non-local individual we expect, on the basis of mtDNA, the local WHG origin (see section 4.3).

The most complicated mechanism to evaluate genetically is *leapfrog movement (mechanism 7)*, defined as a selective colonisation of an area occupied by local hunter-gatherers by a small group of farmers. As Zvelebil (*73*) states, such a mechanism would result in different admixture scenarios, such as the incorporation of hunter-gatherers into Early Neolithic settlement (as in case of KO1 from Tiszaszőlős-Domaháza (*52*)) and local islands of different genetic ancestry (e.g., Vlasac, Vinča). However, the infiltration in Lepenski Vir does not correspond with this mechanism being applied generally.

To conclude the comparison of the mechanisms, we observe several distinct scenarios consistent with the mechanisms defined by Zvelebil (*73*). When introducing the contemporaneous occupation of the Balkans by both farmer and hunter-gatherer societies, Whittle *et al.* (*109*) suggests that contacts between these societies could have involved a mosaic of differing processes and while we generally concur with this conclusion on the basis of ancient DNA, we would be cautious in any application of the same explanation to sites other than those studied here. Except for the specificity of the Danube Gorges, another reason is that the central Balkan Early Neolithic demographic study of Porcic *et al.* (*133*) seem to be in agreement with predictions of Neolithic Demographic Transition as determined in central Europe by Shennan *et al.* (*230*) and therefore the main mechanisms involved were likely similar between central and western Europe (*133*).

It is important to add that the local diversity in modes of Neolithisation in the Balkans might not have played such an important role as previously thought in creating the genetic variation of Neolithic central Europe. Shennan (*231*) argues that there is no basis for the assumption that the farmers would spread only when the carrying capacity of one area was saturated. He suggests that farming communities did not grow locally but instead new generations, and possibly also incoming inhabitants, occupied new patches of the land at a distance to the already occupied ones in order to secure the ideal land for farming. Under this so-called despotic distribution, the observed overall rapid migration from the Aegean would be explained (*231*). This could also explain why we see local admixture with hunter-gatherers (as at Lepenski Vir) without more pronounced impact on the genetic variation of central European Early Neolithic. A similar mechanism, long-distance dispersals, has been recently incorporated into spatial explicit models (*65*) but that hypothesis can be formally tested only once data adequately cover the Neolithic migration routes.

5.2.3 Hypothesis of the Neolithic spread through the Aegean and the Balkans

Given all the information available, we can deduce that the first farmers that reached Europe (probably Greece with the earliest phases dated to ∼6,700-6,500 cal BC; *70*, *107*) maintained connections to the contemporaneous western Anatolian Neolithic settlements (e.g., Aktoprakık and Barcın) first appearing at the same time (∼6,600 cal BC) (*136*). These early Aegean farmers might have relatively

soon come into contact with the large and geographically relatively close Danube Gorges communities; definitely during the Balkan Transition period (∼6,200-6,000/5,950 cal BC), but maybe even earlier during the Late Mesolithic occupation of Vlasac (∼7,400-6,200 cal BC). The contact with a different environment (the continental climate of the central Balkans) and a different population (WHG-like Danubian hunter-gatherers) might have mitigated a consolidation of farming populations in the Aegean for a few centuries and a cultural barrier might have prevented a gene flow between the farmers and hunter-gatherers during the earliest phases of contact. However, since the Danube Gorges sources of food (fish) and potentially also other knowledge of the local environment were beneficial to the newly arrived farmers, some farmers in the periphery of the Neolithic Aegean zone increasingly over time incorporated to the Lepenski Vir community during the Transition (∼6,200-6,000/5,950 cal BC) and Neolithic periods (∼6,000/5,950-5,500 cal BC), while bringing the Neolithic innovations connected to the food production. Additionally, independent Starčevo farming settlements (∼6,000/5,950-5,500 cal BC) also appeared with the continuously increasing northward migration of farmers from the Aegean. It is possible that the relatively dense Danubian occupation led farmers to migrate even faster to the north of the Danube Gorges and from there to central Europe, yet also the despotic distribution of the farming land could have the same effect (the earliest LBK appeared in western Hungary already in 5,600/5,500-5,350 cal BC (*75*)). Some of the individuals with the local hunter-gatherer ancestry, potentially even directly from the mixed Danubian communities, might have been incorporated to the expanding farming population (possibly the instance of Körös WHG-like individual from Tiszaszőlős-Domaháza, Hungary) with a relatively limited genetic impact on the overall population ancestry of central and western European early farmers.

Under this hypothesis, differing speeds of the Neolithic spread (fast over the Aegean, relatively slower infiltration of the central Balkans in the Danube Gorges and a fast spread over the rest of the Balkans and to the central Europe) would lead to more complex scenarios than previous demic models not considering heterogeneity of the Neolithic migration wave and its temporal fluctuations (*226*). Such complexity could explain the variability in Neolithisation processes observed between the sites in the Balkan region (Vlasac, Lepenski Vir, Vinča)-Belo Brdo). Genetically uncharacterised hunter-gatherers from the Aegean could fit in this hypothesis both in the case they were WHG-like (if their population size was low and/or they did not contribute to the Neolithic Aegean genetic variation) or Aegean-like (if a genetic barrier in the Mesolithic period between the Danube Gorges and the Aegean could be explained). The Mediterranean migration route was independent to the route through the central Balkans, but was similar in terms of intake of some level of WHG-like ancestry (though not from the Danube Gorges).

5.3 Methodological considerations

5.3.1 Processing of old and damaged samples

For years, the main approach to ancient DNA was to analyse mitochondrial DNA that is present in the samples in multiple copies and which is relatively informative for one locus (*201*). The analysis of mtDNA is still the most advantageous course of action for samples with very low content of endogenous DNA molecules. In this manner, we have obtained DNA from samples of young individuals (e.g., estimated ages of 6, 7 and 10 years for samples Bar3, Lepe42 and Lepe1, respectively (*119*, *145*)), solitary finds of skeletal elements in a collective burial (a case of Catalhöyük (*150*)) and skeletal elements that are usually not considered a good source of DNA (Vlasa7 metacarpal and Vlasa2 phalang bone). However, petrous bones were selected for analysis for 23 samples in this study and, consistently with previous findings (*7*), they were found to contain more DNA than other skeletal elements (see section 4.1). For that reason, petrous bone samples were selected for nuclear target enrichment and whole genome sequencing.

Nevertheless, 26 of the samples analysed were estimated to have endogenous content below 1% (the lowest was 0.14% for Bar13). Apart from the technological advancements in the field, the main contribution to the success of these samples was probably the increased number of extractions and independently indexed libraries for poor samples, which led to an increased number of unique molecules available for target enrichment (for more discussion see *156*, *157*). Additionally, this allowed for separate analysis of each extract and each library and an exclusion of contaminated ones (two libraries in total, see section 4.1).

This strenuous approach of numerous independently indexed libraries was also applied to samples analysed for their nuclear DNA and the number of libraries pooled for the purpose of this analysis was further increased because the target regions were much larger (∼5 Mb). In similar nuclear target enrichment approaches, up to 1,240k SNPs were captured with a median coverage of 0.67 (min 0.03, max 25.37) (*12*, *50*), whereas our approach led to a median coverage of 37.72 (min 7.61, max 93.76). While the approaches are not comparable (our approach targeted continuous stretches of DNA not SNPs), it demonstrates that this data can be further used for confident variant calling and therefore also for deep data analysis on this level (*232*). These high quality nuclear data were not analysed here to their fullest potential and new information will be gained from joint analysis with similar data obtained in future from other regions and periods.

Medium coverages of the genomes (3.7x-7.1x) allowed for two of them (Bar8 and Bar31) to be included in the study of Hofmanová *et al.* (*51*), where the data were used for improvements in variant calling methods and heterozygosity estimation (Supplementary Section SI9 by Kousathanas *et al.* in *51*, *89*), whole-genome population continuity testing (Supplementary Section SI9 by Díez del Molino *et al.* in *51*) and allele matching profiles (Supplementary Section SI10 by van Dorp,

Lopes *et al.* in *51*). The methods were applied and further developed in another landmark study of Broushaki *et al.* (*9*) that reinvented our understanding of past genetic variation.

5.3.2 Sex determination

We were able to determine the sex, not only of the individuals that were sequenced for nuclear data, but also from the screening sequencing runs in our pipeline (see section 4.2). The information on the sex of the individuals is being used only relatively marginally in population genetic studies that focus on alleles representing populations rather than individuals (it can be used, for example, to determine which individual has Y chromosome that can be studied further). However, sex assignment is of major importance for the understanding of past social and cultural traits and some population genetic studies use sex information to their advantage (*77*).

Morphological sex assignments in archaeology are usually based on the assumption that the skeletal differences between the sexes can be approximated for the ancient populations from the measurements on known contemporary populations (*233*). Unfortunately, inter-population tests have shown that this can lead to erroneous sex assignments (*234*). That is especially true for juvenile individuals (*235*) and the fragmentary state of many human remains discovered in archaeological contexts further complicates the issue (*236*). Still, it is surprising to find that seven individuals were genetically assigned to the incorrect sex (29.17%) and another eight individuals were genetically assigned to a different sex than expected (i.e., for these eight individuals the skeletal elements did not allow for conclusive sex assignment; see section 4.2). To our knowledge, a comparison of sex determination on genetic and skeletal data has not previously been performed on such a high number of individuals and the results presented here suggest that physical anthropology could benefit from the genetic information on this issue. A study that would compare different methods for sex determination from skeletal elements to genetic information on at least a subset of samples from the site could help calibrate the morphological methods. However, since shallow screening sequencing is a part of the NGS pipeline in many laboratories (*41*, *50*, *213*), if enough DNA is present the sex determination can be performed even without further analysis of the sample. If there is enough DNA present, there is also no age limit in sex determination. It was therefore even possible to determine sex (male) for Akt20, an individual approximately three years old (*143*), which is usually not possible from archaeological material (*235*).

The genetic sex, however, does not necessarily represent the gender role assigned to the individual in life and in some (very rare) cases it is possible that the individual's morphological traits or cultural traits associated with his or her burial could be more indicative of the gender role than the genetic sex assignment. For example, the sex of individual DV 15 from Dolní Věstonice recently genetically determined to be male (*41*) was under scrutiny for a long time due to inconclusive skeletal features and also because of the burial context (another male individual from this triple grave was reaching a pubic region of the studied individual) (*237*). While there is not enough data to suggest that

XY individual DV 15 was assigned a female gender role or was intersex, this possibility should not be discarded, especially since buried individuals of Upper Palaeolithic era often exhibit physical diversity associated with peculiar burial practices (*237*).

5.3.3 Comparison to the context

Mobility in Lepenski Vir was estimated via strontium isotopes (see section 2.4, *94*) and groups of local and non-local individuals were identified via comparison to the local range of $^{87}Sr/^{86}Sr$ isotope ratio values. To our knowledge, this is the first study in which two groups of individuals differentiated by strontium isotopes were directly compared genetically. While the comparison did not conclusively support differentiation between local and non-local groups in Lepenski Vir, distances to other farmer groups were smaller for the non-local group (see Figure 10). The reason for the absence of a significant F_{ST} value between local and non-local groups could be the low sample sizes and that the non-local individuals, while having been born locally, carry the ancestry of the non-local individuals that arrived in previous generations. However, the assignment of U5a1 haplogroup to Lepe12 individual with a non-local strontium isotope signature seems slightly problematic. This would suggest that this individual is non-local with some level of hunter-gatherer ancestry (or it could be the first U5 haplogroup detected among individuals of fully Aegean-like origin). It should, however, be considered that this individual was assigned to the non-local group on the basis of a borderline value (0.001 above the local range) and it is possible that the real local range of strontium values could be slightly higher than established experimentally in Borić & Price (*94*). Analogously, the local assignment of Lepe52 sample could be reconsidered due to a marginal strontium ratio value (only 0.003 above the non-local range) (*94*) and the nuclear data that strongly point to the Aegean origin of this individual. As a result, it is arguable that local and non-local assignments based on strontium values might profit from a probability-based framework with confidence intervals.

Another aspect of the context is the suspected relatedness of the samples. In section 4.3, potential relatives in collective burials (on the basis of mtDNA) are discussed and it should be stressed again that the same mtDNA sequences only signify *possible* maternal relatedness, whereas different mitogenomes exclude maternal (but not paternal) relatedness. On the basis of nuclear DNA (see e.g., Figure 19), we have identified Bar8 and Bar15 as more related than other samples from the same population but the applied analysis did not allow for confirmation of the relatedness, let alone for estimating the degree of familial relationship. Such analysis would have to be performed on genotypic data, preferably genotypic likelihoods. No sample was excluded from the nuclear population genetic analysis for the unconfirmed relatedness because we analysed the samples individually. It is, however, possible that it could have had a slight impact on *ADMIXTURE* analysis (*98*, *196*). Relatedness among individuals analysed for mtDNA could not be excluded or confirmed and consequently they were treated as independent samples from the population.

5.3.4 Different datasets could lead to different conclusions

It was quite insightful to consider how our conclusions would have changed if only one of the obtained datasets had been studied. The genomic dataset that usually relies on obtaining one or a few moderately covered genomes would put Danubians firmly into WHG genetic variation (because all three samples Lepe51, Lepe45 and Vlasa37 overwhelmingly show the WHG signal, see section 4.5.1). The situation could have then been easily interpreted as the cultural transmission of Neolithic during Transition period of Lepenski Vir. Yet with an increased number of samples in the nuclear capture dataset, we were able to detect that two of the seven studied Lepenski Vir individuals were actually of completely different Aegean-like ancestry, while another individual showed high admixture levels between both of the groups, quite likely as a very early descendent of the *in situ* process of mixing (see sections 4.5.11 and 4.6.4).

Similarly, if only mitochondrial data were studied, mitochondrial haplogroups could have caused misinterpretations. For example, the whole genome of Vlasa37 was very WHG-like but the sample's haplogroup was K1f, previously detected only for Tyrolean Iceman (*202*). The haplogroup of the same clade (K1c) for Theopetra samples (*51*) prevented the exclusion of Mesolithic Aegean ancestry in the Aegean farmer population. Following similar logic, Vlasa37 could have been without the whole genome interpreted similarly – as an individual with some level of Aegean-like origin or with ancestry that contributed to the Neolithic Aegean population. Even though it still cannot be excluded that there were some links between Mesolithic Aegean and both Tyrolean Iceman and Vlasa37, it seems unlikely given highly different genomic ancestries of Vlasa37 and Iceman.

U groups detected in Barcın individuals could have also led to a wrong conclusion of relatedness between these samples and known U haplogroup individuals with WHG ancestry. However, it should be noted the Barcın haplogroups were not U5, usually considered as "typically" farmer (*201*) (and already the same U haplogroups have been detected in Anatolia in parallel studies of Anatolian prehistoric genetic variation (*12*, *50*)). Contrary to some mitochondrial haplogroups, distances calculated on the basis of mtDNA data seemed to be consistent with the results from nuclear data.

Different conclusions could have been drawn also upon different grouping of the samples in the mitochondrial DNA analysis of genetic distances (section 4.3.4). The grouping procedure is the most challenging in this kind of approach, because when a group is formed the group assignment for each sample is one of the assumptions of the analysis (and wrong assignments could lead to wrong results). At the same time, faultlessly determining group affiliations of different burials from different sites is almost impossible, especially within complex archaeological contexts and with not enough samples from all periods and sites. Though grouping assumptions of mitochondrial tests were carefully considered during the analysis and all decisions to group samples were substantiated by archaeological literature (see section 2.1), it is always possible that other literature not presented in this study would have led to different group assignments or that the context was misidentified or simply not properly understood. While we obtained and utilised expert information as much

as possible (e.g., *119*, *206*), a direct collaboration with archaeologists and anthropologist would be advisable for the reanalysis of these samples.

For the nuclear analysis, grouping assumptions were avoided by individual analysis of the samples. Even though such an approach lowered the statistical power of the analysis, it means that the statistically significant conclusions obtained repeatedly on more samples are certain and individual differences in one site (as for Lepenski Vir individuals with Aegean-like ancestry) could be studied. While in other studies (*8*, *12*, *50*), individual differences between the samples within one group were detected via PCA and *ADMIXTURE* only, f-statistics were not performed individually. In our individualised approach it was, for example, possible to observe some levels of Aegean-like ancestry even for sample Lepe18 or to show that Aegean-like Lepe39 and Lepe52 individuals were likely not related and even to notice differences between Vlasa37 and other Lepenski Vir genomic samples. While these results were not repeated on *ADMIXTURE* analysis and they were therefore not discussed further, they could be important as a basis for a detailed study on a genotypic level. Also, no samples were excluded for "outlier" genetic ancestry differing from genetic ancestry observed in other samples from the same group as in other studies (*8*, *12*, *50*). Although it is understandable that PCA outliers are excluded from the analysis for GWAS studies (*238*), ancient DNA population genetic study should not exclude samples from a site based on a differing ancestry. We rather argue that at least for the first publication (if no follow-up publications are planned), the samples should be analysed as individually as possible with regard to context, especially to isotopic data and signs of different cultural affiliations. Otherwise, problematic groupings could easily propagate to other studies. Importantly, even though the general group-based conclusions are probably correct, individual ancestries relevant to interpretation of past societies might be lost.

6 Conclusion

The first farmers spreading throughout Europe from the 7th millenium onwards had been expected to come from western Anatolia and genetic data obtained in this study fully support this archaeological hypothesis. Rather unexpected genetic homogeneity across the Aegean Sea provides information about the genetic impact of maritime connections during the time of the Neolithic farmers in the area. Early Neolithic populations in central Europe and Iberia resulted from migrations via independent routes from this Aegean core zone and, to a limited extent, both populations experienced admixture with local hunter-gatherers along the way from the Aegean.

The migration corridor to central Europe led through the central Balkans, a region inhabited by sedentary hunter-gatherers alongside the Danube. Whereas Danubian hunter-gatherer individuals from Vlasac site (∼7,400-6,200 cal BC) showed absence of gene flow with the contemporaneous farmers inhabiting the Aegean, the so far genetically unobserved incorporation of early farmers into a hunter-gatherer context occurred in Lepenski Vir during the Transition (∼6,200-6,000/5,950 cal BC) and more so during the Neolithic period (∼6,000/5,950-5,500 cal BC). Individual ancestries at this large and culturally unique site are diverse, with individuals with a local (WHG-like) ancestry, individuals of a fully Aegean origin and individuals that could trace their ancestry partly locally and partly to the Aegean. Another Early Neolithic community in the central Balkans, Vinča-Belo Brdo was closely related to the Neolithic Aegeans and could have represented a settlement independent on the earlier occurrence of the Neolithic (and Aegean-like individuals) in the Danube Gorges.

The effects of the observed admixture in Lepenski Vir did not propagate with the same intensity to central Europe because hunter-gatherer ancestry was low among Early Neolithic farmers from central Europe; probably due to the effects of geographical distances, population size differences or varying migration speeds on the genetic patterns. Mitochondrial data from Romanian Eneolithic individuals (from Sultana Malu Roşu and additional published sites) suggest that during the later stages of the Neolithic era (∼5th millennium BC) another population turnover occurred in the Balkans, which could be even related to the observed genetic impact of "eastern" (CHG-like) populations on individuals in the Chalcolithic period in western Anatolia (6,000-3,200/3,000 cal BC).

Neolithic Transition in the Balkans proved to be genetically a diverse process (with differing genetic variation in Vlasac, Lepenski Vir and Vinča-Belo Brdo) and, in the light of the current data, simplistic models of Neolithic spread might need to be reconsidered, especially to add variable migration speeds, temporal fluctuations and relaxing the assumption of the saturated carrying capacity. The individual ancestries at the studied sites were also used to test archaeological hypotheses at the sites (e.g., to show the absence of genetic differences between Aktopraklık and Barcın in western Anatolia), to confirm signals from strontium isotopes (at Lepenski Vir and Vlasac) and to provide genetic sex assignments for a large collection of samples (160 individuals).

References

1. Auton A et al. (2015) A global reference for human genetic variation. *Nature* 526(7571):68–74.

2. Tishkoff SA et al. (2009) The genetic structure and history of Africans and African Americans. *Science* 324(5930):1035–1044.

3. Li H, Durbin R (2011) Inference of human population history from individual whole-genome sequences. *Nature* 475(7357):493–496.

4. Hellenthal G et al. (2014) A genetic atlas of human admixture history. *Science* 343(6172):747–751.

5. Pääbo S (1985) Molecular cloning of ancient Egyptian mummy DNA. *Nature* 314(6012):644–645.

6. Knapp M, Hofreiter M (2010) Next Generation Sequencing of Ancient DNA: Requirements, Strategies and Perspectives. *Genes* 1(2):227–243.

7. Pinhasi R et al. (2015) Optimal ancient DNA yields from the inner ear part of the human petrous bone. *PLoS ONE* 10(6):e0129102.

8. Haak W et al. (2015) Massive migration from the steppe was a source for Indo-European languages in Europe. *Nature* 522(7555):207–211.

9. Broushaki F et al. (2016) Early Neolithic genomes from the eastern Fertile Crescent. *Science* 353(6298):499–503.

10. Gerbault P et al. (2011) Evolution of lactase persistence: an example of human niche construction. *Philosophical Transactions of the Royal Society B: Biological Sciences* 366(1566):863–877.

11. Wilde S et al. (2014) Direct evidence for positive selection of skin, hair, and eye pigmentation in Europeans during the last 5,000 years. *Proceedings of the National Academy of Sciences* 111(13):4832–4837.

12. Mathieson I et al. (2015) Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* 528(7583):499–503.

13. Green RE et al. (2010) A draft sequence of the Neandertal genome. *Science (New York, N.Y.)* 328(5979):710–22.

14. Reich D et al. (2010) Genetic history of an archaic hominin group from Denisova Cave in Siberia. *Nature* 468(7327):1053–60.

15. Bos KI et al. (2014) Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. *Nature* 514(7523):494–497.

16. Higuchi R, Bowman B, Freiberger M, Ryder OA, Wilson AC (1984) DNA sequences from the quagga, an extinct member of the horse family. *Nature* 312(5991):282–284.

17. Pääbo S et al. (2004) Genetic analyses from ancient DNA. *Annual Review of Genetics* 38(1):645–679.

18. Der Sarkissian C et al. (2014) Ancient genomics. *Philosophical Transactions of the Royal Society B: Biological Sciences* 370(1660):20130387–20130387.

19. Kirsanow K, Burger J (2012) Ancient human DNA. *Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft* 194(1):121–32.

20. Krings M et al. (1997) Neandertal DNA sequences and the origin of modern humans. *Cell* 90(1):19–30.

21. Currat M, Excoffier L (2004) Modern humans did not admix with Neanderthals during their range expansion into Europe. *PLoS Biology* 2(12):2264–2274.

22. Veeramah KR, Hammer MF (2014) The impact of whole-genome sequencing on the reconstruction of human population history. *Nature Reviews Genetics* 15(3):149–162.

23. Gilbert MTP et al. (2008) DNA from pre-Clovis human coprolites in Oregon, North America. *Science* 320(5877):786–789.

24. Poinar H et al. (2009) Comment on "DNA from pre-Clovis human coprolites in Oregon, North America". *Science* 325(5937):148–148.

25. Endicott P et al. (2003) The genetic origins of the Andaman islanders. *The American Journal of Human Genetics* 72(1):178–184.

26. Fehren-Schmitz L, Reindel M, Cagigao ET, Hummel S, Herrmann B (2009) Pre-Columbian population dynamics in coastal southern Peru: A diachronic investigation of mtDNA patterns in the Palpa region by ancient DNA analysis. *American Journal of Physical Anthropology* pp. 208–221.

27. Burger J, Kirchner M, Bramanti B, Haak W, Thomas MG (2007) Absence of the lactase-persistence-associated allele in early Neolithic Europeans. *Proceedings of the National Academy of Sciences of the United States of America* 104(10):3736–41.

28. Malmström H et al. (2010) High frequency of lactose intolerance in a prehistoric hunter-gatherer population in northern Europe. *BMC evolutionary biology* 10(1):1.

29. Belle EMS, Ramakrishnan U, Mountain JL, Barbujani G (2006) Serial coalescent simulations suggest a weak genealogical relationship between Etruscans and modern Tuscans. *Proceedings of the National Academy of Sciences* 103(21):8012–8017.

30. Pilipenko AS, Romaschenko AG, Molodin VI, Parzinger H, Kobzev VF (2010) Mitochondrial DNA studies of the Pazyryk people (4th to 3rd centuries BC) from northwestern Mongolia. *Archaeol Anthropol Sci* 2(4):231–236.

31. Guimaraes S et al. (2009) Genealogical discontinuities among Etruscan, Medieval, and contemporary Tuscans. *Molecular Biology and Evolution* 26(9):2157–2166.

32. Sampietro ML et al. (2005) The genetics of the pre-Roman Iberian Peninsula: A mtDNA study of ancient Iberians. *Ann Human Genet* 69(5):535–548.

33. Haak W et al. (2005) Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. *Science (New York, N.Y.)* 310(5750):1016–8.

34. Bramanti B et al. (2009) Genetic discontinuity between local hunter-gatherers and central Europe's first farmers. *Science (New York, N.Y.)* 326(5949):137–40.

35. Lacan M et al. (2011) Ancient DNA suggests the leading role played by men in the Neolithic dissemination. *Proceedings of the National Academy of Sciences of the United States of America* 108(45):18255–9.

36. Malmström H, StoråJ, Dalén L, Holmlund G, Götherström A (2005) Extensive human DNA contamination in extracts from ancient dog bones and teeth. *Molecular biology and evolution* 22(10):2040–7.

37. Meyer M, Kircher M (2010) Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor protocols* 2010(6):pdb.prot5448.

38. Wang XV, Blades N, Ding J, Sultana R, Parmigiani G (2012) Estimation of sequencing error rates in short reads. *BMC Bioinformatics* 13(1):185.

39. Racimo F, Renaud G, Slatkin M (2016) Joint estimation of contamination, error and demography for nuclear DNA from ancient humans. *PLOS Genetics* 12(4):e1005972.

40. Skoglund P et al. (2014) Separating endogenous ancient DNA from modern day contamination in a Siberian Neandertal. *Proceedings of the National Academy of Sciences* 111(6):2229–34.

41. Fu Q et al. (2016) The genetic history of Ice Age Europe. *Nature*.

42. Meyer M et al. (2016) Nuclear DNA sequences from the Middle Pleistocene Sima de los Huesos hominins. *Nature* 531(7595):504–507.

43. Meyer M et al. (2013) A mitochondrial genome sequence of a hominin from Sima de los Huesos. *Nature* 505(7483):403–406.

44. Orlando L et al. (2013) Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature* 499(7456):74–78.

45. Willerslev E, Cooper A (2005) Review paper. Ancient DNA. *Proceedings of the Royal Society B: Biological Sciences* 272(1558):3–16.

46. Zischler H et al. (1995) Detecting dinosaur DNA. *Science* 268(5214):1192–1193.

47. Cooper A (2000) Ancient DNA: Do it right or not at all. *Science* 289(5482):1139b–1139.

48. Gilbert MTP, Bandelt HJ, Hofreiter M, Barnes I (2005) Assessing ancient DNA studies. *Trends in Ecology & Evolution* 20(10):541–544.

49. Krause J et al. (2010) A complete mtDNA genome of an early modern human from Kostenki, Russia. *Current biology : CB* 20(3):231–6.

50. Lazaridis I et al. (2016) Genomic insights into the origin of farming in the ancient Near East. *Nature*.

51. Hofmanová Z et al. (2016) Early farmers from across Europe directly descended from Neolithic Aegeans. *Proc Natl Acad Sci USA* 113(25):6886–6891.

52. Gamba C et al. (2014) Genome flux and stasis in a five millennium transect of European prehistory. *Nature Communications* 5:5257.

53. Jones ER et al. (2015) Upper Palaeolithic genomes reveal deep roots of modern Eurasians. *Nature communications* 6:8912.

54. Poinar HN et al. (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. *Science (New York, N.Y.)* 311(5759):392–4.

55. Miller W et al. (2008) Sequencing the nuclear genome of the extinct woolly mammoth. *Nature* 456(7220):387–90.

56. Rasmussen M et al. (2010) Ancient human genome sequence of an extinct Palaeo-Eskimo. *Nature* 463(7282):757–62.

57. Prüfer K et al. (2013) The complete genome sequence of a Neanderthal from the Altai Mountains. *Nature* 505(7481):43–49.

58. Krause J et al. (2010) The complete mitochondrial DNA genome of an unknown hominin from southern Siberia. *Nature* 464(7290):894–7.

59. Paixão Côrtes V, Viscardi L, Salzano F, Hünemeier T, Bortolini M (2012) Homo sapiens, Homo neanderthalensis and the Denisova specimen: New insights on their evolutionary histories using whole-genome comparisons. *Genetics and Molecular Biology* S4(35):904–911.

60. Raghavan M et al. (2014) Upper Palaeolithic Siberian genome reveals dual ancestry of Native Americans. *Nature* 505(7481):87–91.

61. Rasmussen M et al. (2014) The genome of a Late Pleistocene human from a Clovis burial site in western Montana. *Nature* 506(7487):225–229.

62. Skoglund P, Reich D (2016) A genomic view of the peopling of the Americas. *Current Opinion in Genetics & Development* 41:27–35.

63. Seguin-Orlando A et al. (2014) Genomic structure in Europeans dating back at least 36,200 years. *Science (New York, N.Y.)*.

64. Posth C et al. (2016) Pleistocene mitochondrial genomes suggest a single major dispersal of non-Africans and a Late Glacial population turnover in Europe. *Current Biology* 26(6):827–833.

65. Alves I et al. (2015) Long-distance dispersal shaped patterns of human genetic diversity in Eurasia. *Molecular Biology and Evolution* 33(4):946–958.

66. Price TD, Bar-Yosef O (2011) The origins of agriculture: New data, new ideas: An introduction to supplement 4. *Current Anthropology* 52(S4):S163–S174.

67. Brami M (2015) Ph.D. thesis (University of Liverpool).

68. Özdoğan M (2011) Archaeological evidence on the westward expansion of farming communities from eastern Anatolia to the Aegean and the Balkans. *Current Anthropology* 52(S4):S415–S430.

69. Burger J, Thomas MG (2011) The palaeopopulationgenetics of humans, cattle and dairying in Neolithic Europe. *Human Bioarchaeology of the Transition to Agriculture* pp. 369–384.

70. Perlès C, Quiles A, Valladas H (2013) Early seventh-millennium AMS dates from domestic seeds in the initial Neolithic at Franchthi Cave (Argolid, Greece). *Antiquity* 87(338):1001–1015.

71. Karamitrou-Mentessidi G (2014) About prehistoric sites in western Macedonia: prefectures of Kozani and Grevena in *A century of Research in Prehistoric Macedonia. International Conference Proceedings 22-24 November 2012*, eds. Stefani, Merousis, Dimoula. (Archaeological Museum of Thessaloniki, Thessaloniki), pp. 233–249.

72. Cavalli-Sforza LL (1997) Genes, peoples, and languages. *Proceedings of the National Academy of Sciences* 94(15):7719–7724.

73. Zvelebil M (2001) The agricultural transition and the origins of Neolithic society in Europe in *Documenta Praehistorica XXVIII, Neolithic studies*, ed. Budja M. (University of Ljubljana) Vol. 8, pp. 1–26.

74. Bailey G, Spikins P (2008) *Mesolithic Europe*. (Cambridge University Press).

75. Oross K, Bánffy E (2009) Three successive waves of Neolithisation: LBK development in Transdanubia. *Documenta Praehistorica* 36:175–189.

76. Currat M, Silva NM (2013) Investigating European genetic history through computer simulations. *Hum Hered* 76(3-4):142–153.

77. Rasteiro R, Chikhi L (2013) Female and male perspectives on the Neolithic transition in Europe: clues from ancient and modern genetic data. *PloS one* 8(4):e60944.

78. Lazaridis I et al. (2014) Ancient human genomes suggest three ancestral populations for present-day Europeans. *Nature* 513(7518):409–413.

79. Olalde I et al. (2014) Derived immune and ancestral pigmentation alleles in a 7,000-year-old Mesolithic European. *Nature* 507(7491):225–228.

80. Bollongino R et al. (2013) 2000 years of parallel societies in Stone Age Central Europe. *Science (New York, N.Y.)* 342(6157):479–81.

81. Brandt G et al. (2013) Ancient DNA reveals key stages in the formation of central European mitochondrial genetic diversity. *Science (New York, N.Y.)* 342(6155):257–61.

82. Allentoft ME et al. (2015) Population genomics of Bronze Age Eurasia. *Nature* 522(7555):167–172.

83. Gilbert MTP et al. (2003) Characterization of genetic miscoding lesions caused by postmortem damage. *The American Journal of Human Genetics* 72(1):48–61.

84. Gilbert MTP et al. (2006) Recharacterization of ancient DNA miscoding lesions: insights in the era of sequencing-by-synthesis. *Nucleic Acids Research* 35(1):1–10.

85. Der Sarkissian C et al. (2015) Evolutionary genomics and conservation of the endangered Przewalski's horse. *Current Biology* 25(19):2577–2583.

86. Skoglund P et al. (2012) Origins and genetic legacy of Neolithic farmers and hunter-gatherers in Europe. *Science (New York, N.Y.)* 336(6080):466–9.

87. Fu Q et al. (2015) An early modern human from Romania with a recent Neanderthal ancestor. *Nature* 524(7564):216–219.

88. Briggs AW et al. (2007) Patterns of damage in genomic DNA sequences from a Neandertal. *Proceedings of the National Academy of Sciences* 104(37):14616–14621.

89. Kousathanas A et al. (2016) Inferring heterozygosity from ancient and low coverage genomes. *bioRxiv*.

90. Kircher M, Heyn P, Kelso J (2011) Addressing challenges in the production and analysis of Illumina sequencing data. *BMC genomics* 12(1):382.

91. Mitchell D, Willerslev E, Hansen A (2005) Damage and repair of ancient DNA. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 571(1-2):265–276.

92. Neustupný E (2002) *Archeologie nenalézaného: sborník přátel, kolegů a žáků k životnímu jubileu Slavomila Vencla.* (Aleš Čeněk).

93. Çilingiroğlu A, Cevik O, Çilingiroğlu Ç (2012) Towards understanding the Neolithic of central-western Anatolia: The contribution of Ulucak in *The Neolithic in Turkey: New Excavations and New Research. Northwestern Turkey and Istanbul*, eds. Özdoğan M, Başgelen N, Kuniholm P. (Archaeology and Art Publications, Istanbul) Vol. 4, pp. 139–175.

94. Borić D, Price TD (2013) Strontium isotopes document greater human mobility at the start of the Balkan Neolithic. *Proceedings of the National Academy of Sciences of the United States of America* 110(9):3298–303.

95. Heyer E, Chaix R, Pavard S, Austerlitz F (2011) Sex-specific demographic behaviours that shape human genomic variation. *Molecular Ecology* 21(3):597–612.

96. Cook G et al. (2002) Problems of dating human bones from the Iron Gates. *Antiquity* 76(291):77–85.

97. Banffy E, Brandt G, Alt KW (2012) 'Early Neolithic' graves of the Carpathian Basin are in fact 6000 years younger - Appeal for real interdisciplinarity between archaeology and ancient DNA research. *Journal of Human Genetics* 57(7):467–469.

98. Pritchard J, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotypes data. *Genetics* 155:945–959.

99. Pickrell JK, Pritchard JK (2012) Inference of population splits and mixtures from genome-wide allele frequency data. *PLoS Genet* 8(11):e1002967.

100. Ray N, Currat M, Foll M, Excoffier L (2010) Splatche2: a spatially explicit simulation framework for complex demography, genetic admixture and recombination. *Bioinformatics* 26(23):2993–2994.

101. Jobling M, Hurles M, Tyler-Smith C (2004) *Human Evolutionary Genetics: Origins, Peoples & Disease.* (Garland Science).

102. Çilingiroğlu Ç, Çakırlar C (2013) Towards configuring the Neolithisation of Aegean Turkey. *Documenta Praehistorica* 40:21–29.

103. Weninger B et al. (2014) Neolithisation of the Aegean and Southeast Europe during the 6600–6000 cal BC period of Rapid Climate Change. *Documenta Praehistorica* 41:1–31.

104. Özdoğan E (2015) Current research and new evidence for the Neolithization process in western Turkey. *European Journal of Archaeology* 18(1):33–59.

105. QGIS Development Team (2009) *QGIS Geographic Information System* (Open Source Geospatial Foundation).

106. Reingruber A (2008) *Die Argissa-Magula. Das frühe und das beginnende mittlere Neolithikum im Lichte transägäischer Beziehungen. Die deutschen Ausgrabungen auf der Argissa-Magula in Thessalien II. Beiträge zur ur- und frühgeschichtlichen Archäologie des Mittelmeer-Kulturraumes.* (Dr. Rudolf Habelt GmbH, Bonn) Vol. 35.

107. Reingruber A, Thissen L (2005) ^{14}C database for the Aegean catchment (eastern Greece, southern Balkans and western Turkey) 10,000–5500 cal BC in *How did farming reach Europe?*, ed. Lichter C. (BYZAS 2), pp. 295–327.

108. Tringham R (2000) Southeastern Europe in the transition to agriculture in Europe: bridge, buffer, or mosaic in *Europe's First Farmers*, ed. Price TD. (Cambridge University Press), pp. 19–56. Cambridge Books Online.

109. Whittle A, Bartosiewicz L, Borić D, Pettitt P, Richards MP (2002) In the beginning: New radiocarbon dates for the Early Neolithic in northern Serbia and south-east Hungary. *Antaeus* 25:63–118.

110. Tasić N et al. (2015) The end of the affair: formal chronological modelling for the top of the Neolithic tell of Vinča-Belo Brdo. *Antiquity* 89(347):1064–1082.

111. Yerkes RW, Gyucha A, Parkinson W (2009) Multiscalar approach to modeling the end of the Neolithic on the Great Hungarian Plain using calibrated radiocarbon dates. *Radiocarbon* 51:1071–1109.

112. Borić D, Stefanović S (2004) Birth and death: infant burials from Vlasac and Lepenski Vir. *Antiquity* 78:526–546.

113. Borić D et al. (2014) Late Mesolithic lifeways and deathways at Vlasac (Serbia). *Journal of Field Archaeology* 39(1):4–31.

114. Bonsall C et al. (2015) Holocene climate change and prehistoric settlement in the lower Danube valley. *Quaternary International* 378:14–21.

115. Borić D, Miracle P (2004) Mesolithic and Neolithic (dis)continuities in the Danube Gorges: new AMS dates from Padina and Hajducka Vodenica (Serbia). *Oxford J Archeol* 23(4):341–371.

116. Borić D (2002) The Lepenski Vir conundrum: reinterpretation of the Mesolithic and Neolithic sequences in the Danube Gorges. *Antiquity* 76(294):1026–1039.

117. Bonsall C et al. (2000) Stable isotopes, radiocarbon and the Mesolithic-Neolithic transition in the Iron Gates. *Documenta Praehistorica* 27(7):119–132.

118. Pătroi CN (2013) About the Sălcuţa Eneolithic culture. *Annales d'Université Valahia Targoviste* 15:117–140.

119. Stefanović S (2016) Information on dating and sex assignment of individuals from Lepenski Vir (personal communication).

120. Stefanović S, Borić D (2008) New-born infant burials underneath house floors at Lepenski Vir: in pursuit of contextual meanings in *The Iron Gates in Prehistory: New Perspectives*, BAR international series, eds. Bonsall C, Boroneanţ V, Radovanović I. (Archaeopress), pp. 131–170.

121. Boroneanţ A (2011) A suggested chronology for the Iron Gates Mesolithic. *Buletinul Muzeului Judeţean Teleorman, Seria Arheologie* 3:21–39.

122. Borić D, French C, Dimitrijević V (2008) Vlasac revisited: formation processes, stratigraphy and dating. *Doc. praeh.* 35(0):261.

123. Borić D, Dimitrijević V (2007) When did the "Neolithic package" reach Lepenski Vir? Radiometric and faunal evidence. *Documenta Praehistorica* 34:53–72.

124. Bonsall C et al. (2008) Dating burial practices and architecture at Lepenski Vir. *British Archaeological Reports International Series* 1893.

125. Cristiani E, Radini A, Edinborough M, Borić D (2016) Dental calculus reveals Mesolithic foragers in the Balkans consumed domesticated plant foods. *Proceedings of the National Academy of Sciences* p. 201603477.

126. Roksandić M (1999) Ph.D. thesis (Simon Fraser University).

127. Boroneanţ A (2010) Ostrovul Corbului - o discuţie asupra mormintelor mezolitice şi neolitice timpurii. *Buletinul Muzeului Judeţean Teleorman, Seria Arheologie* 2:5–27.

128. Boroneanţ A, Bonsall C (2012) Burial practices in the Iron Gates Mesolithic in *Homines, Funera, Astra. Proceedings of the International Symposium on Funerary Anthropology (5-8 June 2011, Romania) British Archaeological Reports -International Series*, eds. Kogalniceanu R, Curca R, Gligor M, Stratton S. (Archaeopress) Vol. 2410, pp. 232–239.

129. Borić D (2015) The end of the Vinča world: Modelling the Neolithic to Copper Age transition and the notion of archaeological culture in *Neolithic and Copper Age Between the Carpathians and the Aegean Sea: Chronologies and Technologies from the 6th to the 4th Millennium BCE : International Workshop Budapest 2012*, eds. Hansen S, Anders A, Raczky P, Reingruber A. (Habelt-Verlag), pp. 157–217.

130. Dimitrijević V (2014) The provenance and use of fossil scaphopod shells at the Late Neolithic/Eneolithic site Vinča-Belo Brdo, Serbia. *British Archaeological Reports International Series* 2666.

131. Borić D (2009) Absolute dating of metallurgical innovations in the Vinča culture of the Balkan in *Metals and Societies. Studies in honour of Barbara S. Ottaway*, eds. Kienin T, Roberts B. (Habelt, Bonn), pp. 191–245.

132. Bogdanović M (2008) *Grivac: Settlements of Proto-Starčevo and Vinča Culture.* (Center for Scientific Research of Serbian Academy of Sciences and Arts).

133. Porčić M, Blagojević T, Stefanović S (2016) Demography of the Early Neolithic population in central Balkans: Population dynamics reconstruction using summed radiocarbon probability distributions. *PLoS ONE* 11(8):e0160832.

134. Lazăr C, Andreescu R, Ignat T, Florea M, Astaloş C (2008) The Eneolithic cemetery from Sultana Malu Roşu (Călăraşi county, Romania). *Studii de Preistorie.*

135. Lazăr C, Voicu M, Vasile G (2012) Traditions, rules and exceptions in the Eneolithic cemetery from Sultana-Malu Roşu (Southeast Romania) in *Homines, Funera, Astra. Proceedings of the International Symposium on Funerary Anthropology (5-8 June 2011, Romania) British Archaeological Reports -International Series*, eds. Kogalniceanu R, Curca R, Gligor M, Stratton S. (Archaeopress) Vol. 2410, pp. 107–118.

136. Gerritsen FA, Özbal R, Thissen L (2013) The earliest Neolithic levels at Barcın Höyük, Northwestern Turkey. *Anatolica* 39:53–92.

137. Özdoğan M (2000) The appearence of early Neolithic cultures in Northwestern Turkey. Some problems in *Karanovo. Beiträge zum Neolithikum in Südosteuropa*, eds. Hiller S, Nikolov V. (Phoibos, Wien) Vol. 3, pp. 165–170.

138. Brami MN (2014) A graphical simulation of the 2,000-year lag in Neolithic occupation between central Anatolia and the Aegean basin. *Archaeol Anthropol Sci* 7(3):319–327.

139. Krauß R (2011) Neolithization between Northwest Anatolia and the Carpathian Basin – an Introduction in *Beginnings - New Research in the Appearance of the Neolithic Between Northwest Anatolia and the Carpathian Basin: Papers of the International Workshop, 8th-9th April 2009, Istanbul, Organized by Dan Ciobotaru, Barbara Horejs and Raiko Krauß*, Forschungscluster 1, Von der Sesshaftigkeit zur komplexen Gesellschaft, ed. Krauß R. (VML, Verlag Marie Leidorf), pp. 1–7.

140. Baird D (2012) The Late Epipalaeolithic, Neolithic and Chalcolithic of the Anatolian Plateau, 13,000–4,000 BC in *A Companion to the Archaeology of the Ancient Near East*, A Companion to the Archaeology of the Ancient Near East, ed. Potts D. (Wiley) No. v. 1.

141. Karul N, Avcı MB (2013) Aktopraklık in *The Neolithic in Turkey: New Excavations and New Research. Northwestern Turkey and Istanbul*, eds. Özdoğan M, Başgelen N, Kuniholm P. (Archaeology and Art Publications, Istanbul) Vol. 5, pp. 45–68.

142. Budd C, Lillie M, Alpaslan-Roodenberg S, Karul N, Pinhasi R (2013) Stable isotope analysis of Neolithic and Chalcolithic populations from Aktopraklık, northern Anatolia. *Journal of Archaeological Science* 40(2):860–867.

143. Alpaslan Roodenberg S (2011) A preliminary study of the burials from Late Neolithic-Early Chalcolithic Aktopraklık. *Anatolica* 37:17–43.

144. Arbuckle BS et al. (2014) Data sharing reveals complexity in the westward spread of domestic animals across Neolithic Turkey. *PLoS ONE* 9(6).

145. Alpaslan Roodenberg S, Gerritsen F, Özbal R (2013) Neolithic burials from Barcın Höyük. *Anatolica* 39:93–111.

146. Gerritsen FA, Özbal R, Thissen L, Özbal H, Galik A (2010) The Late Chalcolithic settlement of Barcin Höyük. *Anatolica* 36:197–225.

147. Roodenberg J, Alpaslan Roodenberg S (2008) Barcın Hüyük in the plain of Yenişehir (2005–2006). A preliminary note on the fieldwork, pottery and human remains of the prehistoric levels. *Anatolica* 34:53–60.

148. Hodder I (2007) Çatalhöyük in the context of the Middle Eastern Neolithic. *Annual Review of Anthropology* 36(1):105–120.

149. Marciniak A, Czerniak L (2007) Social transformations in the Late Neolithic and the Early Chalcolithic periods in central Anatolia. *Anatolian Studies* 57:115–130.

150. Marciniak A, Czerniak L (2008) The excavations of the TP (Team Poznań) area in the 2008 season. Online; accessed 01-Aug-2016.

151. Nehlich O, Borić D, Stefanović S, Richards MP (2010) Sulphur isotope evidence for freshwater fish consumption: a case study from the Danube Gorges, SE Europe. *Journal of Archaeological Science* 37(5):1131–1139.

152. Bonsall C et al. (2004) Radiocarbon and stable isotope evidence of dietary change from the Mesolithic to the Middle Ages in the Iron Gates: New results from Lepenski Vir. *Radiocarbon* 46:293–300.

153. Horejs B et al. (2015) The Aegean in the early 7th millennium BC: Maritime networks and colonization. *Journal of World Prehistory* 28(4):289–330.

154. Scheu A et al. (2015) The genetic prehistory of domesticated cattle from their origin to the spread across Europe. *BMC Genetics* 16(1):54.

155. Adler C, Haak W, Donlon D, Cooper A (2011) Survival and recovery of DNA from ancient teeth and bones. *Journal of Archaeological Science* 38(5):956–964.

156. Schulz I (2016) Master's thesis (JGU Mainz).

157. Winkelbach L (2016) Master's thesis (JGU Mainz).

158. Kircher M, Sawyer S, Meyer M (2012) Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic acids research* 40(1):e3.

159. Skoglund P, StoråJ, Götherström A, Jakobsson M (2013) Accurate sex identification of ancient human remains using DNA shotgun sequencing. *Journal of Archaeological Science* 40(12):4477–4482.

160. Bunce M, Oskam CL, Allentoft ME (2012) Quantitative real-time PCR in aDNA research in *Ancient DNA*, eds. Shapiro B, Hofreiter M. (Springer), pp. 121–132.

161. Veeramah K (2016) Information on regions selected for nuclear capture (personal communication).

162. Gronau I, Hubisz MJ, Gulko B, Danko CG, Siepel A (2011) Bayesian inference of ancient human demography from individual genome sequences. *Nature Genetics* 43(10):1031–1034.

163. Arbiza L, Zhong E, Keinan A (2012) NRE: a tool for exploring neutral loci in the human genome. *BMC Bioinformatics* 13(1):301.

164. Sell C (2016) Information on nuclear capture design (personal communication).

165. Strobel M (2016) Information on nuclear capture design and selection of phenotypic positions (personal communication).

166. Gnirke A et al. (2009) Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nature biotechnology* 27(2):182–9.

167. van Oven M, Kayser M (2009) Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. *Hum. Mutat.* 30(2):E386–E394.

168. Dabney J et al. (2013) Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. *Proceedings of the National Academy of Sciences* 110(39):15758–15763.

169. Blumenstiel B et al. (2001) Targeted exon sequencing by in-solution hybrid selection. *Current Protocols in Human Genetics*.

170. Dehoust H (2015) Master's thesis (JGU Mainz).

171. Kircher M (2011) Analysis of high-throughput ancient DNA sequencing data. *Ancient DNA* pp. 197–228.

172. Aronesty E (2013) Comparison of sequencing utility programs. *TOBIOIJ* 7(1):1–8.

173. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25(14):1754–1760.

174. Li H et al. (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16):2078–2079.

175. Breese MR, Liu Y (2013) NGSUtils: a software suite for analyzing and manipulating next-generation sequencing datasets. *Bioinformatics* 29(4):494–496.

176. DePristo MA et al. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics* 43(5):491–498.

177. Jonsson H, Ginolhac A, Schubert M, Johnson PLF, Orlando L (2013) mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. *Bioinformatics* 29(13):1682–1684.

178. Bloöcher J (2014) Master's thesis (JGU Mainz).

179. R Core Team (2016) *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna, Austria).

180. Royston P (1995) Remark AS R94: A remark on algorithm AS 181: The W-test for normality. *Journal of the Royal Statistical Society. Series C (Applied Statistics)* 44(4):547–551.

181. Bauer DF (1972) Constructing confidence sets using rank statistics. *Journal of the American Statistical Association* 67(339):687–690.

182. Best DJ, Roberts DE (1975) Algorithm AS 89: The upper tail probabilities of Spearman's rho. *Applied Statistics* 24(3):377.

183. Weissensteiner H et al. (2016) HaploGrep 2: mitochondrial haplogroup classification in the era of high-throughput sequencing. *Nucleic Acids Res.* 44(W1):58–63.

184. Larkin M et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21):2947–2948.

185. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview version 2 – A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9):1189–1191.

186. Kreutzer S (2016) Information on the dataset presented in Table S2 (personal communication).

187. Excoffier L, Lisher HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10(3):564–567.

188. Weir BS, Cockerham CC (1984) Estimating f-statistics for the analysis of population structure. *Evolution* 38(6):1358–1370.

189. Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139(1):457–462.

190. Reynolds J, Weir BS, Cockerham CC (1983) Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics* 105(3):767–779.

191. Oksanen J et al. (2013) *vegan: Community Ecology Package.* R package version 2.0-10.

192. Fu Q et al. (2013) A revised timescale for human evolution based on ancient mitochondrial genomes. *Current Biology* 23(7):553–559.

193. Ralf A, Oven M, Zhong K, Kayser M (2015) Simultaneous analysis of hundreds of Y-chromosomal SNPs for high-resolution paternal lineage classification using targeted semiconductor sequencing. *Human mutation* 36(1):151–159.

194. Veeramah KR et al. (2011) An early divergence of KhoeSan ancestors from those of other modern humans is supported by an ABC-based analysis of autosomal resequencing data. *Molecular Biology and Evolution* 29(2):617–630.

195. Patterson N et al. (2012) Ancient admixture in human history. *Genetics* 192(3):1065–93.

196. Alexander DH, Novembre J, Lange K (2009) Fast model-based estimation of ancestry in unrelated individuals. *Genome Research* 19(9):1655–1664.

197. Chang CC et al. (2015) Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaSci* 4(1).

198. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23(14):1801–1806.

199. Rosenberg NA (2003) distruct: a program for the graphical display of population structure. *Molecular Ecology Notes* 4(1):137–138.

200. Gerbault P et al. (2014) Storytelling and story testing in domestication. *Proceedings of the National Academy of Sciences* 111(17):6159–6164.

201. Pinhasi R, Thomas MG, Hofreiter M, Currat M, Burger J (2012) The genetic history of Europeans. *Trends in genetics : TIG* 28(10):496–505.

202. Keller A et al. (2012) New insights into the Tyrolean Iceman's origin and phenotype as inferred by whole-genome sequencing. *Nature communications* 3:698.

203. Szécsényi-Nagy A et al. (2015) Tracing the genetic origin of Europe's first farmers reveals insights into their social organization. *Proceedings of the Royal Society of London B: Biological Sciences* 282(1805):20150339.

204. Hervella M et al. (2015) Ancient DNA from South-East Europe reveals different events during Early and Middle Neolithic influencing the European genetic heritage. *PLoS ONE* 10(6):e0128810.

205. Haak W et al. (2010) Ancient DNA from European early Neolithic farmers reveals their near Eastern affinities. *PLOS biology* 8(11):e1000536.

206. Gerritsen F (2016) Information on archaeological context of individuals from Barcın (personal communication).

207. Dinu A (2010) Mesolithic fish and fishermen of the Lower Danube (Iron Gates) in *Documenta Praehistorica XXXVII, Neolithic studies*, ed. Budja M. (University of Ljubljana) Vol. 17, pp. 299–310.

208. Schulz A (2015) Ph.D. thesis (JGU Mainz).

209. Brotherton P et al. (2013) Neolithic mitochondrial haplogroup H genomes and the genetic origins of Europeans. *Nature communications* 4:1764.

210. Bollongino R (2012) Sequences of Central European early Neolithic samples from Dillingen (7), Essenheim (1), Herxheim (9) and Otzingen (1). Unpublished data.

211. Cavalli-Sforza L, Menozzi P, Piazza A (1993) Demic expansions and human evolution. *Science* 259:639–646.

212. Behar D et al. (2012) The Basque paradigm: Genetic evidence of a maternal continuity in the Franco-Cantabrian region since pre-Neolithic times. *The American Journal of Human Genetics* 90(3):486–493.

213. Günther T et al. (2015) Ancient genomes link early farmers from Atapuerca in Spain to modern-day Basques. *Proc Natl Acad Sci USA* 112(38):11917–11922.

214. Skoglund P et al. (2014) Genomic diversity and admixture differs for Stone-Age Scandinavian foragers and farmers. *Science* 344(6185):747–750.

215. Omrak A et al. (2016) Genomic evidence establishes Anatolia as the source of the European Neolithic gene pool. *Current Biology* 26(2):270–275.

216. Kılınç G et al. (2016) The demographic development of the first farmers in Anatolia. *Current Biology*.

217. Lacan M et al. (2011) Ancient DNA reveals male diffusion through the Neolithic Mediterranean route. *Proceedings of the National Academy of Sciences U S A* 108(24):9788–9791.

218. Zhong H et al. (2010) Extended Y chromosome investigation suggests postglacial migrations of modern humans into East Asia via the northern route. *Molecular Biology and Evolution* 28(1):717–727.

219. Cortés Sánchez M et al. (2012) The Mesolithic-Neolithic transition in southern Iberia. *Quaternary Research* 77(2):221–234.

220. Çilingiroğlu Ç (2012) The Neolithic pottery of Ulucak in Aegean Turkey: Organization of production, interregional comparisons and relative chronology. *British Archaeological Reports International Series* 2426.

221. Goodale NB (2009) Ph.D. thesis (Washington State University).

222. De Becdelièvre C et al. (2015) Prehistoric motherhood: Diet from pregnancy to baby-led weaning in the Danube Gorges Mesolithic-Neolithic. *American Journal of Physical Anthropology* 156:65–334.

223. Bocquet-Appel J, Bar-Yosef O (2008) *The Neolithic Demographic Transition and its Consequences*. (Springer Netherlands).

224. Price TD, Burton JH, Bentley RA (2002) The characterization of biologically available strontium isotope ratios for the study of prehistoric migration. *Archaeometry* 44(1):117–135.

225. Arenas M, François O, Currat M, Ray N, Excoffier L (2013) Influence of admixture and paleolithic range contractions on current European diversity gradients. *Molecular biology and evolution* 30(1):57–61.

226. Currat M, Excoffier L (2005) The effect of the Neolithic expansion on European molecular diversity. *Proceedings of the Royal Society B: Biological Sciences* 272(1564):679–688.

227. Ammerman AJ, Cavalli Sforza L (1984) *The Neolithic Transition and the Genetics of Populations in Europe*. (Princeton University Press, San Francisco, USA).

228. Richards M (2003) The Neolithic transition in Europe: archaeological models and genetic evidence in *Documenta Praehistorica XXX, Neolithic studies*, ed. Budja M. (University of Ljubljana) Vol. 10, pp. 159–167.

229. Chikhi L, Nichols RA, Barbujani G, Beaumont MA (2002) Y genetic data support the Neolithic demic diffusion model. *Proceedings of the National Academy of Sciences* 99(17):11008–11013.

230. Shennan S et al. (2013) Regional population collapse followed initial agriculture booms in mid-Holocene Europe. *Nat Comms* 4.

231. Shennan S (2009) Evolutionary demography and the population history of the European Early Neolithic. *Human Biology* 81(2-3):339–355.

232. Koboldt DC, Ding L, Mardis ER, Wilson RK (2010) Challenges of sequencing human genomes. *Briefings in Bioinformatics* 11(5):484–498.

233. White T, Folkens P (2005) *The Human Bone Manual*. (Elsevier Science).

234. Sierp I, Henneberg M (2015) The difficulty of sexing skeletons from unknown populations. *Journal of Anthropology* 2015:1–13.

235. Wilson LA, Cardoso HF, Humphrey LT (2011) On the reliability of a geometric morphometric approach to sex determination: A blind test of six criteria of the juvenile ilium. *Forensic Science International* 206(1-3):35–42.

236. Nakahashi T, Nagai M (1986) Sex assessment of fragmentary skeletal remains. *Journal of the Anthropological Society of Nippon* 94(3):289–305.

237. Formicola V, Pontrandolfi A, Svoboda J (2001) The Upper Paleolithic triple burial of Dolní Věstonice: Pathology and funerary behavior. *American Journal of Physical Anthropology* 115(4):372–379.

238. Price AL et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. *Nature Genetics* 38(8):904–909.

239. Hughey JR et al. (2013) A European population in Minoan Bronze Age Crete. *Nature Communications* 4(May):1871–1877.

240. Calafell F, Underhill P, Tolun A, Angelicheva D, Kalaydjieva L (1996) From Asia to Europe: mitochondrial DNA sequence variability in Bulgarians and Turks. *Ann Human Genet* 60(1):35–49.

241. Martinez L, Mirabal S, Luis JR, Herrera RJ (2008) Middle Eastern and European mtDNA lineages characterize populations from eastern Crete. *American Journal of Physical Anthropology* 137(2):213–223.

242. Irwin J et al. (2006) Hungarian mtDNA population databases from Budapest and the Baranya county Roma. *International Journal of Legal Medicine* 121(5):377–383.

243. Schönberg A, Theunert C, Li M, Stoneking M, Nasidze I (2011) High-throughput sequencing of complete human mtDNA genomes from the Caucasus and West Asia: high diversity and demographic inferences. *European Journal of Human Genetics* 19(9):988–994.

244. Comas D, Calafell F, Mateu E, Pérez-Lezaun A, Bertranpetit J (1996) Geographic variation in human mitochondrial DNA control region sequence: the population history of Turkey and its relationship to the European populations. *Molecular Biology and Evolution* 13(8):1067–1077.

Supplementary material

Supplementary Figures and Tables

Figure S1 Graphs showing ancestry estimated amongst various hunter-gatherer and farmer palaeogenomes using *ADMIXTURE* for K=2 to K=8. Analysis was performed for the selected reference individuals and Neolithic Aegean genomes without CHG and Yamnaya (a), with CHG (b) and with both Yamnaya and CHG (c). Additionally, capture nuclear dataset with Danubian genomic samples with reference individuals selected for the implication for Neolithic Transition was analysed in the same manner (d).

Figure S1a: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with Neolithic samples for K=2 to K=5.*

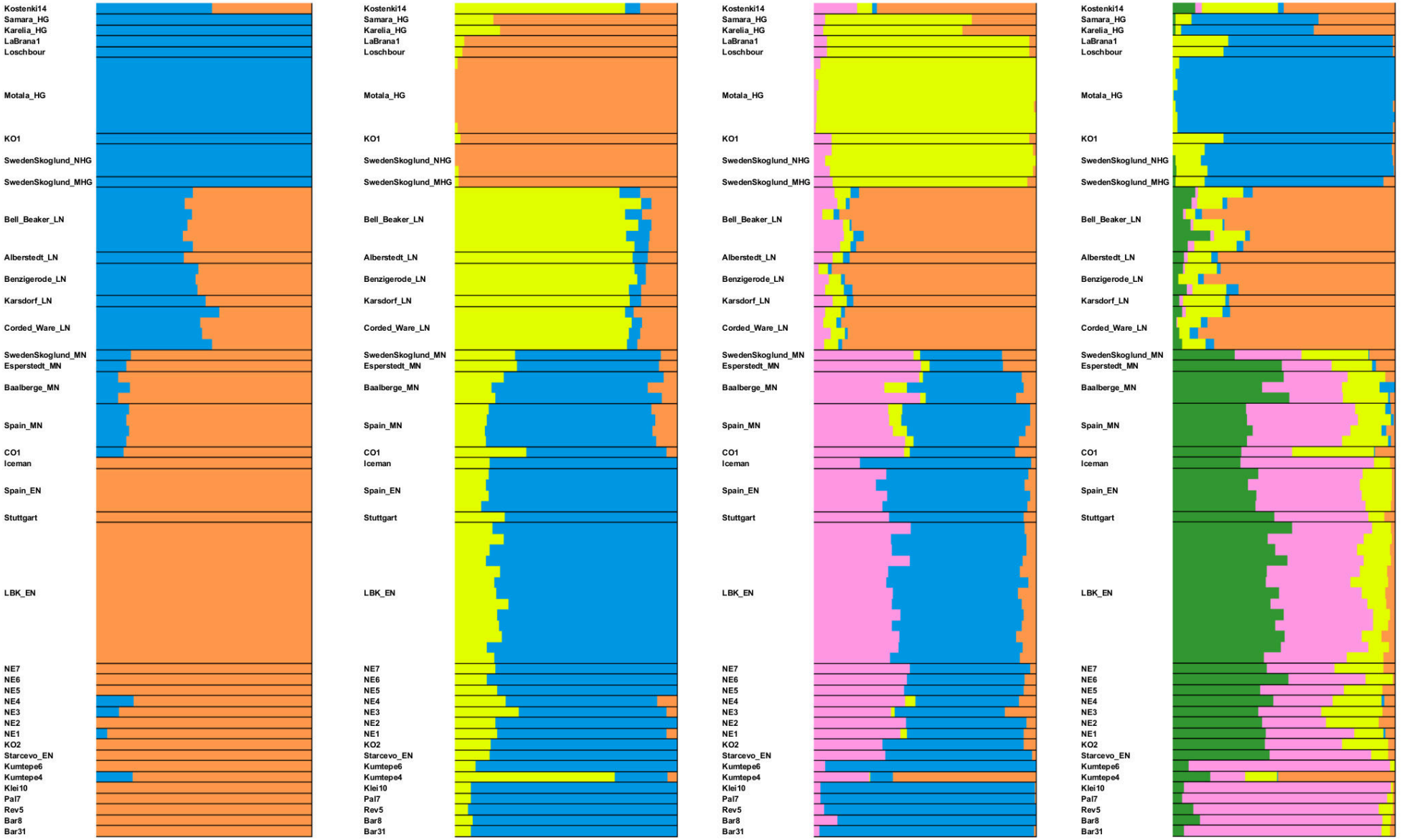


Figure S1a: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with Neolithic samples for K=6 to K=8.*

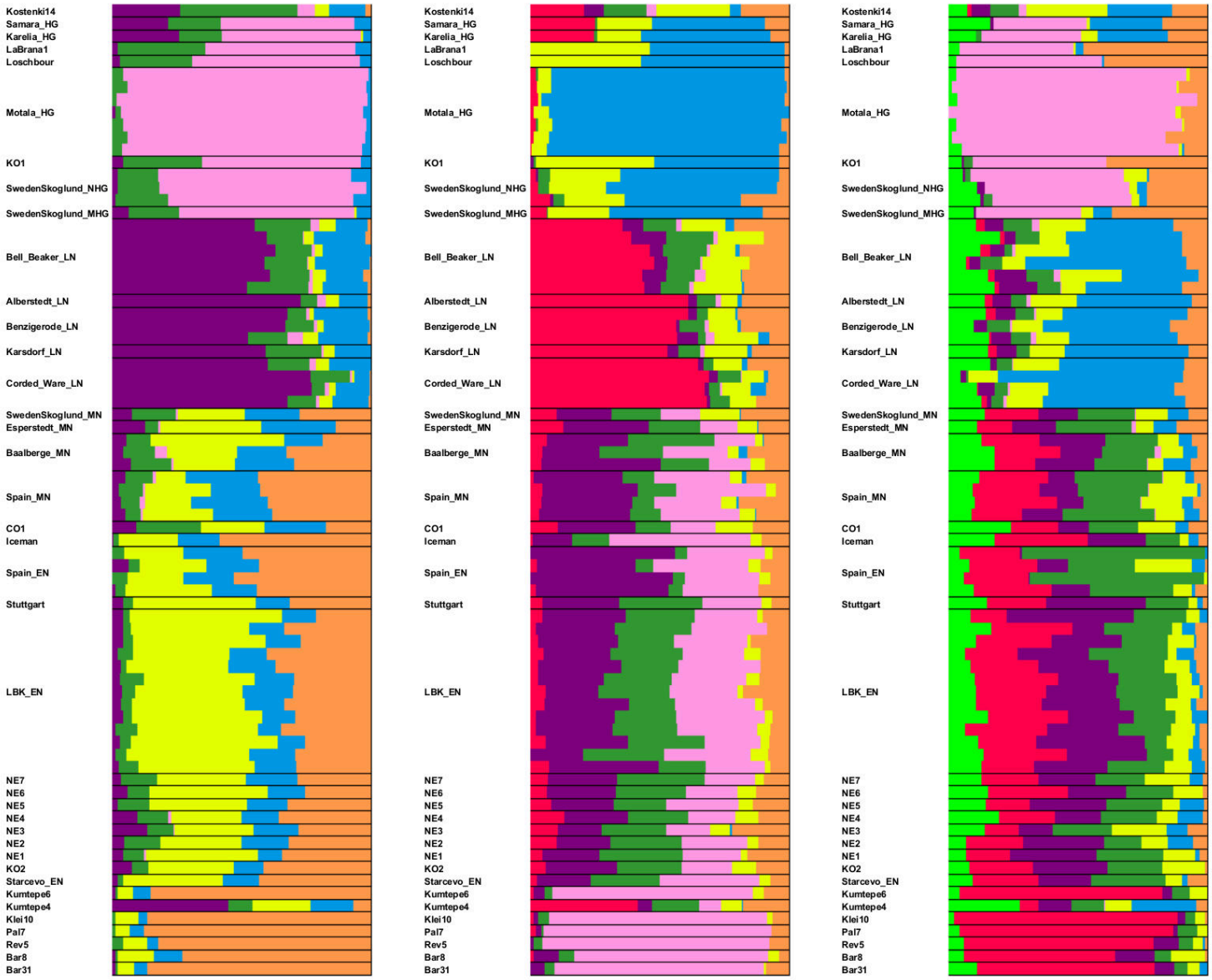


Figure S1b: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with CHG samples for K=2 to K=5.*

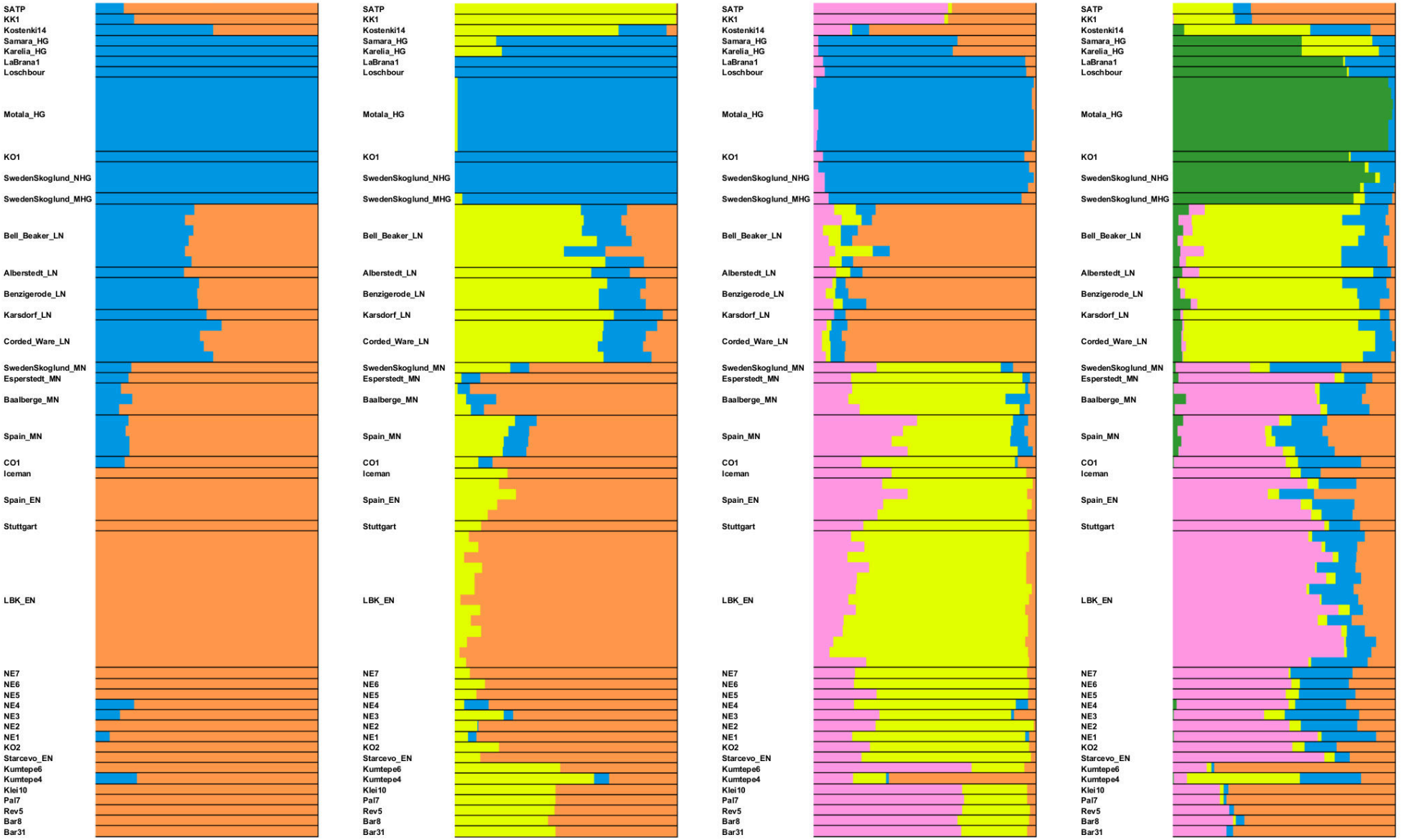


Figure S1b: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with CHG samples for K=6 to K=8.*

Figure S1c: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with Yamnaya samples for K=2 to K=5.*

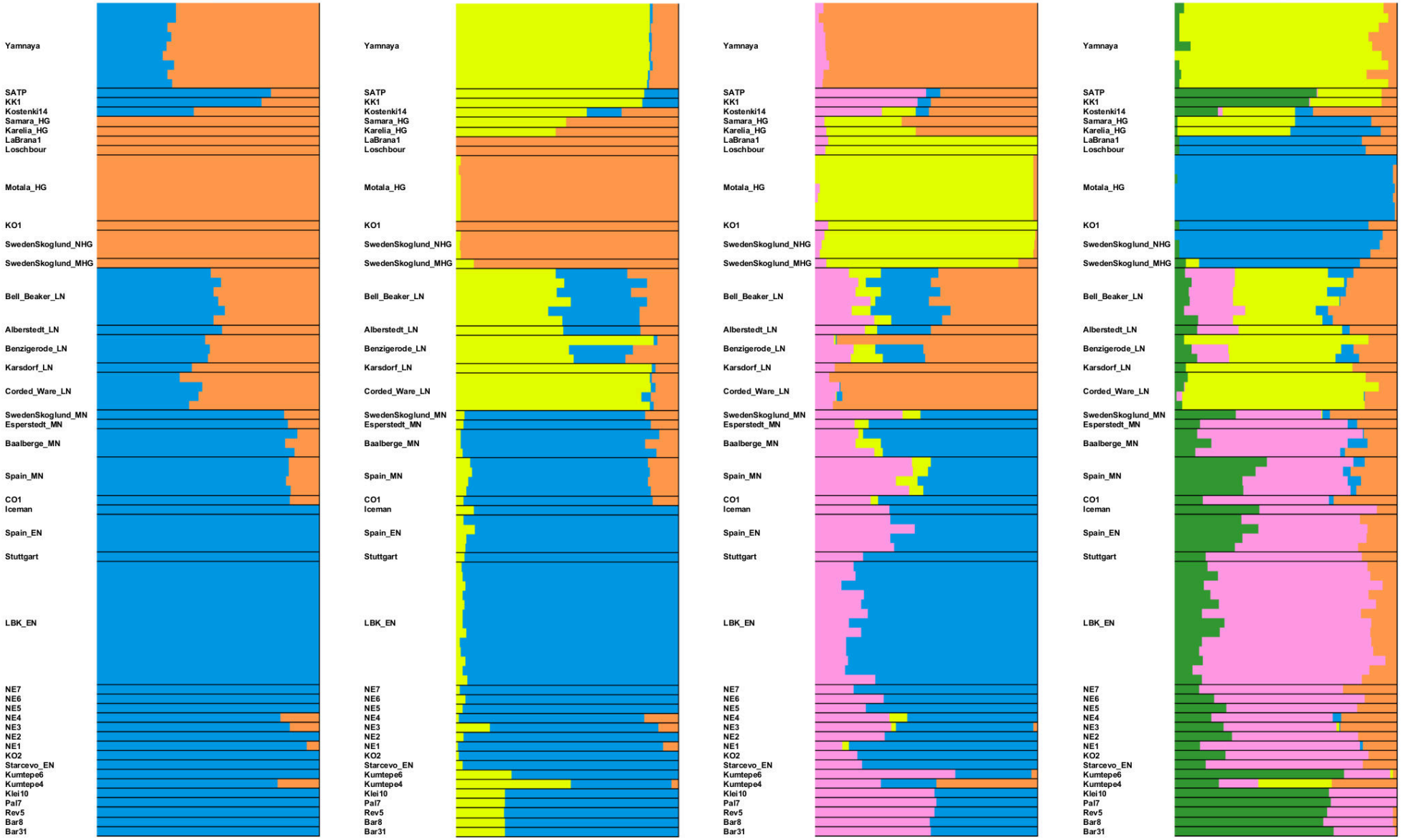
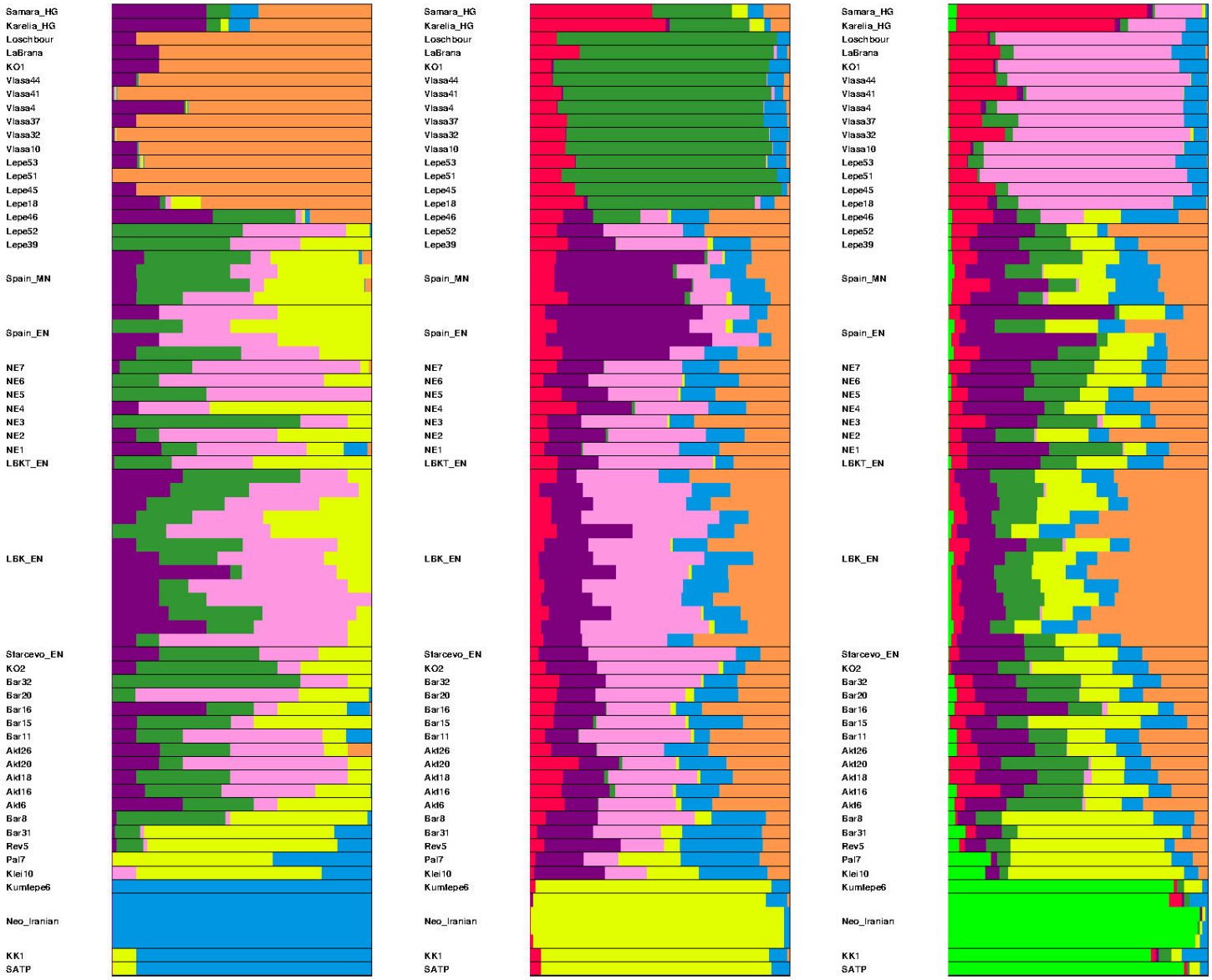


Figure S1c: *Unsupervised run of ADMIXTURE for the Anatolian genomic dataset with Yamnaya samples for K=6 to K=8.*

Figure S1d: *Unsupervised run of ADMIXTURE for joined dataset of genomic and capture samples for K=3 to K=5.*

Figure S1d: *Unsupervised run of ADMIXTURE for joined dataset of genomic and capture samples for K=6 to K=8.*



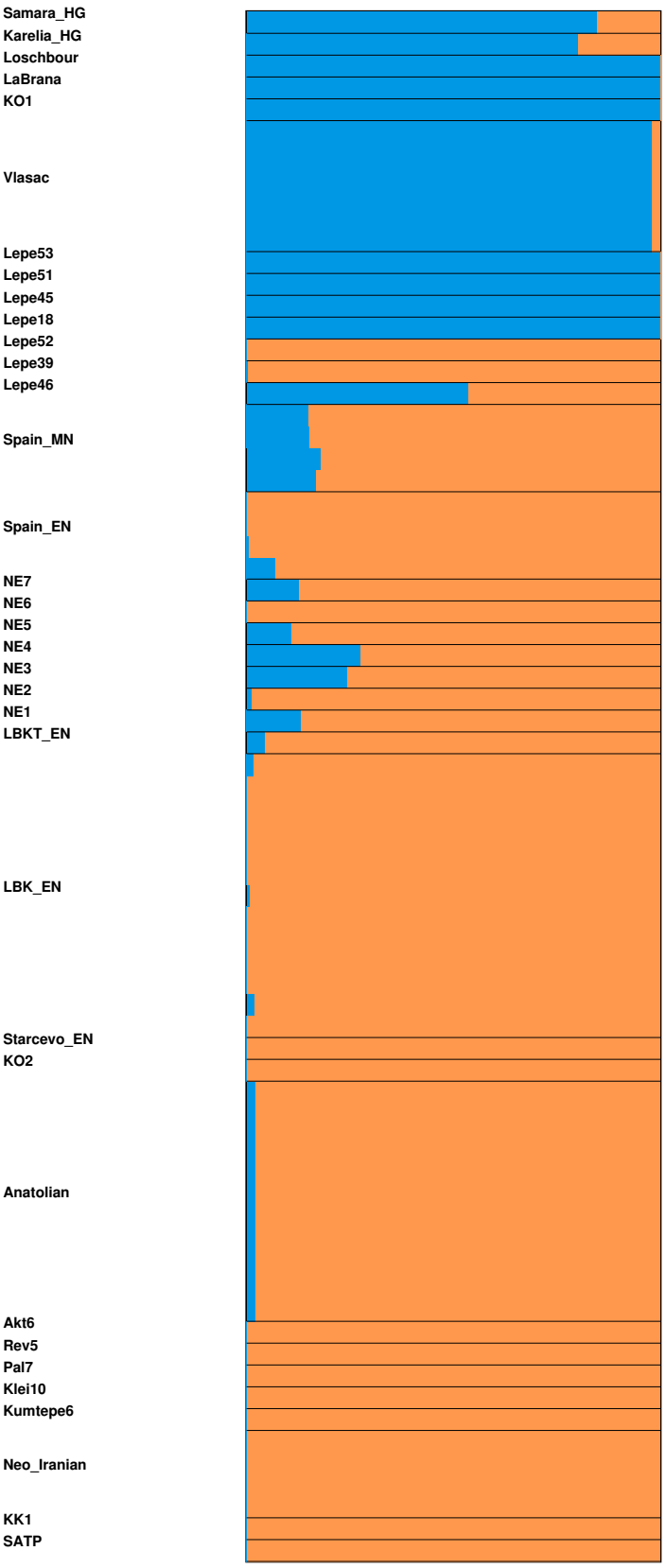


Figure S2: *Supervised run of ADMIXTURE for joined dataset of genomic and capture samples.*

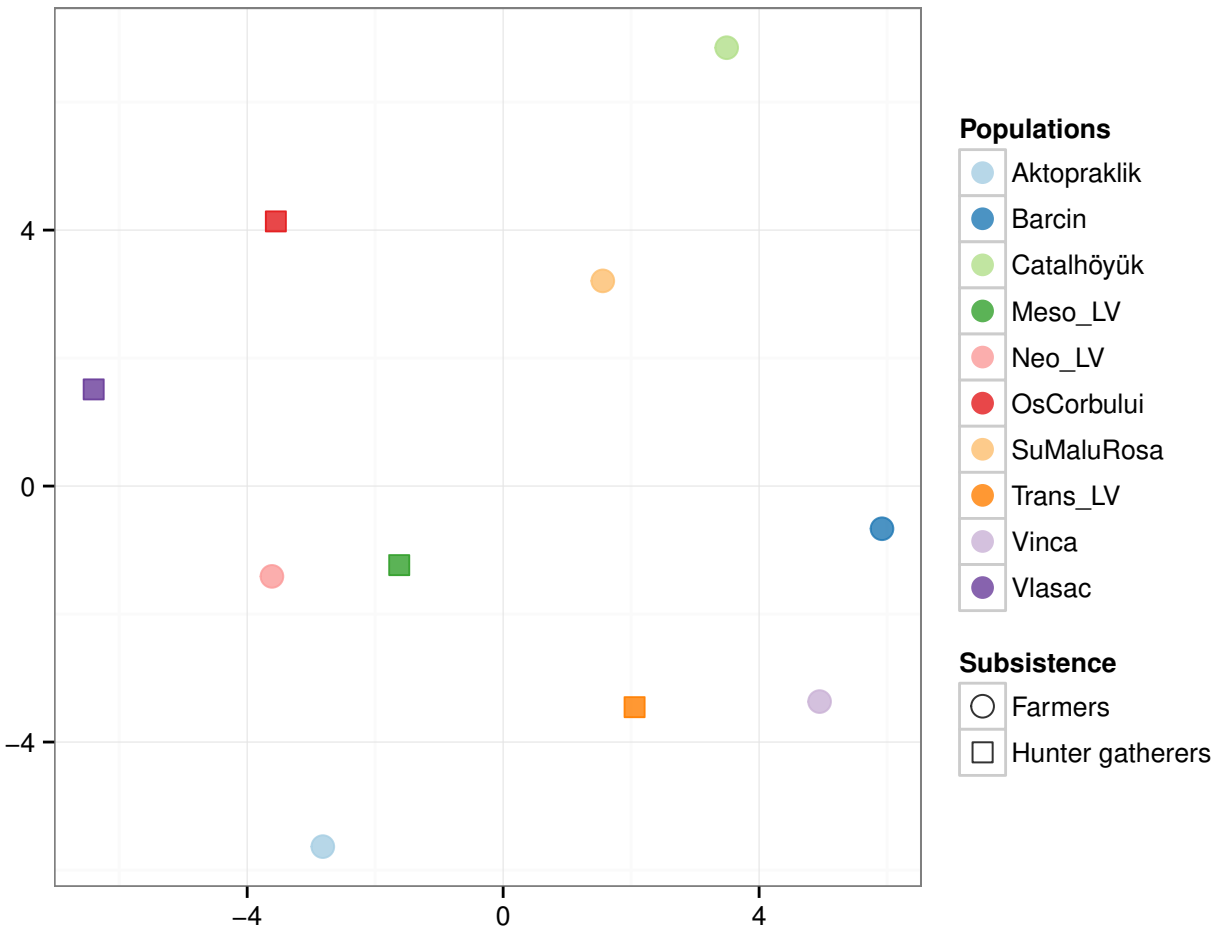


Figure S3: *Multidimensional scaling of Reynolds' distances for full mitogenomes. The stress value for MDS was 0.00167. Most of values were not significant, see Table 17.*

Table S1: *Information on samples selected for mitochondrial capture.*

Sample name	**Skeletal element**	**N. of extractions**	**N. of libraries**	**N. of UDG libraries**	**Average CN**	**Average endogenous percentage**
Akt16	petrous bone	2	10	8	424500000	39.39
Akt17	tooth	3	5	0	760000000	0.38
Akt18	petrous bone	2	10	8	156250000	22.37
Akt20	petrous bone	2	4	2	163300000	17.62
Akt26	petrous bone	2	10	8	495050000	13.38
Akt6	petrous bone	2	4	2	409999999	25.01
Bar11	petrous bone	2	5	3	209050000	34.07
Bar13	left femur	2	5	0	298000000	0.14
Bar14	left femur	2	5	0	102000000	3.39
Bar15	petrous bone	2	9	7	160000000	43.96
Bar16	petrous bone	2	9	7	564800000	41.5
Bar20	petrous bone	2	9	7	86299999	45.73
Bar3	tooth	2	5	0	59000000	0.55
Bar4	left femur	2	5	0	28000000	0.84
Bar6	left femur	2	5	0	85000000	0.86
Bar7	left femur	2	5	0	5200000	6.04
Bar8	tooth	2	5	0	254033333	9.87
Ch51	tooth	2	5	0	267000000	0.67
Ch54	tooth	2	5	0	n.d.	0.21
GRI1	tooth	2	4	0	288000000	0.95
Lepe1	skull fragment	3	3	0	56800000	0.95
Lepe11	tooth	2	3	0	129000000	0.52
Lepe12	tooth	2	4	0	53000000	1.18
Lepe13	tooth	2	4	2	342000000	0.55
Lepe15	tooth	4	4	1	1320000000	0.24
Lepe17	tooth	2	4	2	25099999	1.59
Lepe18	petrous bone	2	4	3	339000000	67.21
Lepe2	tooth	2	3	1	235000000	0.7
Lepe20	tooth	2	3	1	35450000	9.6
Lepe22	tooth	5	9	2	28336666	1.3
Lepe23	tooth	2	3	0	74600000	0.61
Lepe27	tooth	2	3	0	244000000	1.87
Lepe28	tooth	1	4	2	80600000	6.2
Lepe29	tooth	2	3	0	69100000	2.27
Lepe3	tooth	2	4	0	125000000	0.84
Lepe32	tooth	2	4	0	163000000	1
Lepe34	tooth	2	3	2	58200000	6.57
Lepe37	tooth	2	4	0	26200000	1.73

Table S1: *Information on samples selected for mitochondrial capture.*

Sample name	**Skeletal element**	**N. of extractions**	**N. of libraries**	**N. of UDG libraries**	**Average CN**	**Average endogenous percentage**
Lepe38	tooth	2	3	0	224000000	0.99
Lepe39	petrous bone, tooth	3	4	1	424333333	20.19
Lepe41	tooth	2	3	2	24600000	4.88
Lepe42	tooth	2	4	0	150000000	2.59
Lepe44	tooth	2	3	0	41900000	1.29
Lepe46	petrous bone	2	7	4	140400000	59.61
Lepe47	tooth	2	3	0	312000000	1.29
Lepe48	tooth	2	4	0	36800000	1.08
Lepe52	petrous bone	2	5	3	169000000	64.38
Lepe53	petrous bone	2	8	5	55599999	53.54
Lepe6	tooth	1	4	0	12700000	2.56
Lepe7	tooth	1	4	2	26700000	0.67
Lepe2	tooth	2	3	2	197250000	35.39
Osco2	tooth	2	3	1	28000000	4.25
Osco5	tooth	1	4	0	372000000	0.71
Osco6	tooth	1	4	0	1720000000	0.55
Osco7	tooth	1	3	0	104000000	1.53
Pad11	tooth	4	6	0	87125000	0.77
Smr1	tooth	2	2	0	657666666	1.78
Smr2	mandibula	1	1	0	1830000000	n.d.
Vc1	tooth	2	4	0	55100000	1.09
Vc10	tooth	2	4	0	18610000	1.31
Vc2	tooth	2	4	0	149000000	0.82
Vc3	tooth	2	4	0	381000000	1.47
Vc4	tooth	2	4	0	102000000	0.41
Vc5	tooth	2	4	0	271000000	0.41
Vc6	tooth	2	4	0	84000000	0.36
Vc8	tooth	2	4	0	213000000	0.46

Table S1: *Information on samples selected for mitochondrial capture.*

Sample name	**Skeletal element**	**N. of extractions**	**N. of libraries**	**N. of UDG libraries**	**Average CN**	**Average endogenous percentage**
Vlasa1	tooth	4	6	0	87125000	0.77
Vlasa10	tooth	1	3	2	160000000	48.96
Vlasa2	tooth, phalang	2	3	0	51400000	3.98
Vlasa20	tooth	6	6	0	3719999	2.68
Vlasa30	tooth	2	3	1	8430000	9.38
Vlasa32	petrous bone	2	4	2	124550000	63.2
Vlasa4	petrous bone	3	4	3	133000000	63.32
Vlasa41	petrous bone	2	7	5	187150000	57.85
Vlasa44	petrous bone	2	4	1	208500000	60.23
Vlasa47	tooth	2	4	0	160000000	1.51
Vlasa48	tooth	2	4	0	65400000	2.02
Vlasa54	tooth	1	4	2	14700000	5.44
Vlasa56	tooth	1	4	2	7510000	0.72
Vlasa61	tooth	2	3	2	36100000	7.06
Vlasa7	petrous bone, metacarpal tooth	4	5	0	59466666	21.38

Table S2: *Reference data from mitochondrial analysis, partly aggregated and grouped by Kreutzer (186).*

Site	**Country**	**Approximate age (cal. BC)**	**Group**	**N. of individuals**	**Source**
Goyet	Belgium	32845-24370	preLGM	7	(*64*)
Cioclovina	Romania	31262	preLGM	1	(*64*)
Paglicci	Italy	31050-26446	preLGM	2	(*64*)
Dolni Vestonice	Czech Republic	29205-28027	preLGM	4	(*41*, *64*)
La Rochette	France	25642	preLGM	1	(*64*)
Paglicci	Italy	16635	postLGM	2	(*64*)
HohleFels	Germany	13959-13520	postLGM	3	(*64*)
Rigney	France	13515	postLGM	1	(*64*)
Goyet	Belgium	13055	postLGM	1	(*64*)
Brillenhohle	Germany	12830	postLGM	1	(*64*)
Burkhardtshohle	Germany	12665	postLGM	1	(*64*)
Maszycka Höhle	Polen	16364	postLGM	1	(*208*)
Rochedane	France	11010	LateGlacial	1	(*64*)
Aven des Iboussières	France	9870	LateGlacial	3	(*64*)
Oberkassel	Deutschland	11507	LateGlacial	1	(*41*)
Ranchot	France	8134	Holocene	1	(*64*)
Les Closeaux	France	7955	Holocene	1	(*64*)
Mareuil Les Meaux	France	7340	Holocene	1	(*64*)
Falkenstein	Germany	7251	Holocene	1	(*64*)
Felsdach	Germany	6730	Holocene	1	(*64*)
Ofnet	Germany	6342	Holocene	1	(*64*)
Cuiry Les Chaudardes	France	6255	Holocene	1	(*64*)
Bockstein	Germany	6223	Holocene	1	(*64*)
Berry Au Bac	France	5294	Holocene	1	(*64*)
Bad Dürrenberg	Deutschland	6848	Holocene	1	(*34*)
Hohlenstein-Stadel	Deutschland	6743	Holocene	1	(*64*)
Blätterhöhle	Deutschland	9210-8638	Holocene	5	(*80*)
Loschbour	Luxenburg	6097	Holocene	1	(*78*)
Alsónyék-Bátaszék Mérnöki telep	Hungary	5800 - 5200	Starcevo	26	(*203*)
Lánycsók, Gata-Csotola	Hungary	6000-5500	Starcevo	4	(*203*)
Lánycsók, Csata-alja	Hungary	5680-5560	Starcevo	3	(*203*)
Vinkovci	Kroatien	6000-5500	Starcevo	8	(*203*)
Vukovar Gimnazija	Kroatien	6000-5500	Starcevo	3	(*203*)

Table S2: *Reference data from mitochondrial analysis, partly aggregated and grouped by Kreutzer (186).*

Site	**Country**	**Approximate age (cal. BC)**	**Group**	**N. of individuals**	**Source**
Balatonszemes Bagódomb	Hungary	5300-4900	LBK	4	(*203*)
Bölcske-Gyűrűsvölgy	Hungary	5300-4900	LBK	5	(*203*)
Balatonszárszó Kis-erdei-dűlő	Hungary	5210-4940	LBK	5	(*203*)
Budakeszi 4/8 Szőlőskert-Tangazdaság	Hungary	5220-5040	LBK	12	(*203*)
Harta-Gátőrház	Hungary	5300-4900	LBK	5	(*203*)
Kóny, Proletár-dűlő II	Hungary	5300-4900	LBK	4	(*203*)
Szemely-Hegyes	Hungary	5210-4940	LBK	2	(*203*)
Tolna-Mözs	Hungary	5310-5000	LBK	2	(*203*)
Derenburg	Deutschland	5500-4775	EarlyNeo_CE	22	(*33*, *205*, *209*)
Halberstadt-Sonntagsfeld	Deutschland	5500-4775	EarlyNeo_CE	31	(*33*, *81*)
Karsdorf	Deutschland	5500-4775	EarlyNeo_CE	23	(*81*, *209*)
Naumburg	Deutschland	5500-4775	EarlyNeo_CE	4	(*81*)
Oberwiederstedt 1 Unterwiederstedt	Deutschland	5500-4775	EarlyNeo_CE	8	(*33*, *81*)
Dillingen	Deutschland	5000	EarlyNeo_CE	7	(*210*)
Essenheim	Deutschland	5000	EarlyNeo_CE	1	(*210*)
Herxheim	Deutschland	5000	EarlyNeo_CE	9	(*210*)
Otzingen	Deutschland	5000	EarlyNeo_CE	1	(*210*)
Eilsleben	Deutschland	5000	EarlyNeo_CE	1	(*33*)
Schwetzingen	Deutschland	5500-4775	EarlyNeo_CE	4	(*33*)
Vaihingen	Deutschland	5500-4775	EarlyNeo_CE	1	(*33*)
Seehausen	Deutschland	5500-4775	EarlyNeo_CE	1	(*33*)
Flomborn	Deutschland	5500-4775	EarlyNeo_CE	6	(*33*)

Table S2: *Reference data from mitochondrial analysis, partly aggregated and grouped by Kreutzer (186).*

Site	Country	Approximate age (cal. BC)	Group	N. of individuals	Source
Sultana Malu Roşu	Romania	5500-4500	Smalu_Rosa	12	(*204*)
Vărăşti	Romania	5500-4500	Varasti	12	(*204*)
Sultana Valea Orbului	Romania	5500-4500	Svalea_Orb	12	(*204*)
Esperstedt	Deutschland	4632	MidNeo_CE	1	(*81*)
Halberstadt Sonntagsfeld	Deutschland	4500	MidNeo_CE	2	(*81*)
Oberwiederstedt 3 Schrammhoehe	Deutschland	4620-4500	MidNeo_CE	8	(*81*, *209*)
Oberwiederstedt 4 Arschkerbe Ost	Deutschland	4500	MidNeo_CE	1	(*81*)
Wittmar	Deutschland	4519-4415	MidNeo_CE	4	(*208*)
Rössen	Deutschland	4075	MidNeo_CE	2	(*208*)
Salzmünde-Schiebzig	Deutschland	4149-4013	MidNeo_CE	33	(*81*, *209*)
Esperstedt (Esp30)	Deutschland	3859	LatNeo_CE	1	(*209*)
Halle-Queis	Deutschland	3650	LatNeo_CE	1	(*81*, *209*)
Karsdorf (21+22)	Deutschland	3650	LatNeo_CE	2	(*81*)
Quedlinburg VII 2	Deutschland	3650	LatNeo_CE	8	(*81*)
Quedlinburg IX	Deutschland	3650-3504	LatNeo_CE	6	(*81*)
Salzmünde-Schiebzig (55)	Deutschland	3650	LatNeo_CE	1	(*81*)
Salzmünde-Schiebzig	Deutschland	3200	LatNeo_CE	27	(*81*, *209*)
Esperstedt (Esp24)	Deutschland	3200	LatNeo_CE	1	(*81*)
Benzingerode I	Deutschland	3326-2870	LatNeo_CE	17	(*81*)
Benzingerode-Heimburg	Deutschland	2270	FinNeo_CE	2	(*81*, *209*)
Esperstedt	Deutschland	2469-2387	FinNeo_CE	13	(*81*, *209*)
Eulau	Deutschland	2700-2352	FinNeo_CE	12	(*81*)
Karsdorf	Deutschland	2742-2420	FinNeo_CE	13	(*81*)
Oberwiederstedt 2	Deutschland	2420	FinNeo_CE	4	(*81*)
Quedlinburg VII 2	Deutschland	2420	FinNeo_CE	1	(*81*)
Quedlinburg XII	Deutschland	2343-2317	FinNeo_CE	2	(*81*, *209*)
Alberstedt	Deutschland	2437-2334	FinNeo_CE	2	(*81*, *209*)
Benzingerode-Heimburg	Deutschland	2132	FinNeo_CE	7	(*81*, *209*)
Eulau	Deutschland	2270	FinNeo_CE	3	(*81*)
Karsdorf	Deutschland	2317-2127	FinNeo_CE	3	(*81*)
Quedlinburg VII 2	Deutschland	2270	FinNeo_CE	9	(*81*, *209*)
Quedlinburg XII	Deutschland	2222	FinNeo_CE	5	(*81*, *209*)
Rothenschirmbach	Deutschland	2458-2270	FinNeo_CE	8	(*81*, *209*)

Table S2: *Reference data from mitochondrial analysis, partly aggregated and grouped by Kreutzer (186).*

Site	**Country**	**Approximate age (cal. BC)**	**Group**	**N. of individuals**	**Source**
Ayios Charalambos	Greece	1750	Minoan_BA	37	(*239*)
Yamnaya culture sites	Bulgaria	3050-2550	Yamnaya_BA	28	(*11*)
Catacomb culture sites	Ukraine	2750-2050	Catacomb_BA	23	(*11*)
Central European sites from Bronze Age	Germany	2200-1550	CE_BA	110	(*81*)
Modern populations	Bulgaria	present	Mod_Bulg	30	(*240*)
Modern populations	Greece	present	Mod_Cre	180	(*241*)
Modern populations	Hungary	present	Mod_Hun	211	(*242*)
Modern populations	Turkey	present	Mod_Turk	102	(*240*, *243*, *244*)

Table S3: *Sex assignment of the studied individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown. The visualisation of these results is presented in Figure 8.*

Sample	**Site**	**Individual**	**Anthropological sex**	**Ry (95% CI)**	**Genetic sex assignment**
Akt16	Aktopraklık	89 D 14.1	m	0.0082-0.0119	XX
Akt17	Aktopraklık	89 D 15.1	f?	0-0.0161	consistent with XX but not XY
Akt18	Aktopraklık	89 D 17.1	m	0.0091-0.0211	consistent with XX but not XY
Akt20	Aktopraklık	89 E 9.3	unknown	0.0855-0.1045	XY
Akt26	Aktopraklık	90 D 4.4	f	0.0833-0.1044	XY
Akt6	Aktopraklık	17 H 50.1	m	0.0817-0.0978	XY
Bar11	Barcın	M11 / 93	m	0.09-0.1084	XY
Bar14	Barcın	L 12 / 49	unknown	0.0871-0.1529	XY
Bar15	Barcın	M10 / 115	f?	0.0122-0.0163	consistent with XX but not XY
Bar16	Barcın	L10 / 187	unknown	0.0074-0.0103	XX
Bar20	Barcın	M11S / 401	unknown	0.0103-0.014	XX
Bar31	Barcın	L11W / 546	unknown	0.0943-0.108	XY
Bar4	Barcın	L13/ 129	m	0.0342-0.1158	consistent with XY but not XX
Bar6	Barcın	M10 / 173	f	0-0.028	consistent with XX but not XY
Bar7	Barcın	M 10 / 101	unknown	0.0764-0.142	XY
Bar8	Barcın	M 10 / 106	f?	0.0101-0.0136	XX

Table S3: *Sex assignment of the studied individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown. The visualisation of these results is presented in Figure 8.*

Sample	**Site**	**Individual**	**Anthropological sex**	**Ry (95% CI)**	**Genetic sex assignment**
Lepe1	Lepenski Vir	35	unknown	0-0.0288	consistent with XX but not XY
Lepe13	Lepenski Vir	21	m	0.0218-0.2221	consistent with XY but not XX
Lepe17	Lepenski Vir	27/b	f	0.0232-0.1704	consistent with XY but not XX
Lepe18	Lepenski Vir	27/d	m	0.084-0.0948	XY
Lepe20	Lepenski Vir	32a	f	0.0009-0.0079	XX
Lepe22	Lepenski Vir	39	f?	0.0048-0.017	consistent with XX but not XY
Lepe27	Lepenski Vir	54e	f	0.0014-0.0201	consistent with XX but not XY
Lepe28	Lepenski Vir	54d	f	0.0019-0.0092	XX
Lepe29	Lepenski Vir	57	unknown	0-0.0169	consistent with XX but not XY
Lepe32	Lepenski Vir	66	f?	0.0415-0.1753	consistent with XY but not XX
Lepe34	Lepenski Vir	74	m?	0.0001-0.0064	XX
Lepe37	Lepenski Vir	79/b	m	0.0256-0.1849	consistent with XY but not XX
Lepe39	Lepenski Vir	82	m?	0.0778-0.0943	XY
Lepe41	Lepenski Vir	86	f?	0.0547-0.0945	consistent with XY but not XX
Lepe42	Lepenski Vir	87/1	unknown	0.0371-0.1078	consistent with XY but not XX
Lepe44	Lepenski Vir	89/a	f?	0.0906-0.1051	XY
Lepe45	Lepenski Vir	91	f?	0.0858-0.0958	XY
Lepe46	Lepenski Vir	93	f	0.0062-0.0089	XX
Lepe47	Lepenski Vir	105	m?	0-0.0232	consistent with XX but not XY
Lepe49	Lepenski Vir	126	f?	0.0064-0.0104	XX
Lepe51	Lepenski Vir	68	f?	0.0079-0.0103	XX
Lepe52	Lepenski Vir	73	m?	0.0904-0.1014	XY
Lepe53	Lepenski Vir	27	unknown	0.0076-0.011	XX
Lepe6	Lepenski Vir	8	f	0.0005-0.0326	consistent with XX but not XY
Lepe7	Lepenski Vir	11	unknown	0-0.0342	consistent with XX but not XY

Table S3: *Sex assignment of the studied individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown. The visualisation of these results is presented in Figure 8.*

Sample	**Site**	**Individual**	**Anthropological sex**	**Ry (95% CI)**	**Genetic sex assignment**
Osco2	Ostrovul Corbului	M2	m	0.0659-0.1201	consistent with XY but not XX
Osco5	Ostrovul Corbului	M47a	unknown	0-0.0378	consistent with XX but not XY
Osco7	Ostrovul Corbulu	M57	unknown	0-0.0145	XX
Pad11	Padina	30	f?	0.0209-0.1249	consistent with XY but not XX
Smr1	Sultana Malu Roşu	2	unknown	0.0352-0.1125	consistent with XY but not XX
Vc3	Vinca	5	unknown	0.0425-0.1421	consistent with XY but not XX
Vlasa1	Vlasac	2	unknown	0-0.016	XX
Vlasa10	Vlasac	41	m?	0.0769-0.0949	XY
Vlasa2	Vlasac	9	m	0.0028-0.0151	XX
Vlasa30	Vlasac	13	unknown	0.0065-0.0121	XX
Vlasa32	Vlasac	16	m?	0.0826-0.0928	XY
Vlasa37	Vlasac	24	f	0.0839-0.0973	XY
Vlasa4	Vlasac	18a	m	0.0883-0.0996	XY
Vlasa41	Vlasac	30	m?	0.0086-0.0131	XX
Vlasa44	Vlasac	47	f	0.0874-0.0986	XY
Vlasa47	Vlasac	49(1)	unknown	0.0636-0.1573	consistent with XY but not XX
Vlasa48	Vlasac	52	unknown	0-0.0195	consistent with XX but not XY
Vlasa54	Vlasac	74	f	0.0002-0.0138	XX
Vlasa61	Vlasac	U64/X11	unknown	0.0513-0.1094	consistent with XY but not XX
Vlasa7	Vlasac	31	m	0.0845-0.0963	XY

Table S4: *Sex assignment of additional screened individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown.*

Sample name	**Site**	**Individual**	**R_y 95% Cl**	**Assignment**
Akt1	Aktopraklık	89 D 4.4	0.0376-0.208	consistent with XY but not XX
Akt10	Aktopraklık	88 E 12.1	0.0041-0.0195	consistent with XX but not XY
Akt22	Aktopraklık	89 E 11.1	0.01-0.0253	consistent with XX but not XY
Akt25	Aktopraklık	90 C 5.1	0-0.0475	consistent with XX but not XY
Akt27	Aktopraklık	90 D 11.1	0.0055-0.0075	XX
Akt29	Aktopraklık	89 E 17	0.0835-0.1103	XY
Akt4	Aktopraklık	16 H 25	0.0055-0.0075	XX
Akt48	Aktopraklık	14 G 4	0-0.0371	consistent with XX but not XY
Akt5	Aktopraklık	16 H 136.1	0.0299-0.2101	consistent with XY but not XX
Akt53	Aktopraklık	89 F 20.1	0.0055-0.0075	XX
Akt55	Aktopraklık	89 E 17	0.0055-0.0075	XX
Akt60	Aktopraklık	88 D 106.2	0.0055-0.0075	XX
Akt61	Aktopraklık	19 K 35.2	0.0614-0.107	consistent with XY but not XX
Akt62	Aktopraklık	19 K 35.1	0.0055-0.0075	XX
Bar17	Barcın	L10 / 221	0.0779-0.0958	XY
Bar18	Barcın	M11 / 271	0.0095-0.0166	consistent with XX but not XY
Bar2	Barcın	M10 / 185	0.0432-0.1037	consistent with XY but not XX
Bar21	Barcın	L12 / 401	0-0.0221	consistent with XX but not XY
Bar22	Barcın	L12 / 417	0.0606-0.1234	consistent with XY but not XX
Bar23	Barcın	M115 / 435	0-0.0446	consistent with XX but not XY
Bar24	Barcın	M10 / 445	0.0031-0.0105	XX
Bar25	Barcın	M10 / 455	0.0898-0.1065	XY
Bar26	Barcın	M10 / 462	0.0775-0.0984	XY
Bar28	Barcın	M11 / 499	0.0757-0.1078	XY
Bar29	Barcın	M11 / 508	0.0614-0.0896	consistent with XY but not XX
Bar30	Barcın	M11 / 516	0.0037-0.0155	XX
Ch50	Catalhöyük	15839 B114(320)	0-0.0181	consistent with XX but not XY

Table S4: *Sex assignment of additional screened individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown.*

Sample name	**Site**	**Individual**	**R_y 95% CI**	**Assignment**
Hd1	Hajdučka vodenica	20	0.0672-0.0925	consistent with XY but not XX
Hd2	Hajdučka vodenica	Profile 11-20	0.0071-0.013	XX
Lepe10	Lepenski Vir	16	0.0037-0.0093	XX
Lepe14	Lepenski Vir	22	0.0744-0.1008	consistent with XY but not XX
Lepe16	Lepenski Vir	27/a	0.0048-0.0082	XX
Lepe19	Lepenski Vir	28	0-0.0084	XX
Lepe21	Lepenski Vir	37	0.0767-0.1017	XY
Lepe24	Lepenski Vir	48	0.0722-0.0953	consistent with XY but not XX
Lepe25	Lepenski Vir	49	0.0055-0.0075	XX
Lepe26	Lepenski Vir	50	0.0333-0.1306	consistent with XY but not XX
Lepe30	Lepenski Vir	60	0.0546-0.081	consistent with XY but not XX
Lepe31	Lepenski Vir	64	0.0785-0.128	XY
Lepe33	Lepenski Vir	69	0.0391-0.1132	consistent with XY but not XX
Lepe35	Lepenski Vir	75	0.068-0.1532	consistent with XY but not XX
Lepe36	Lepenski Vir	79/a	0.0421-0.1774	consistent with XY but not XX
Lepe4	Lepenski Vir	7/I	0.0634-0.1016	consistent with XY but not XX
Lepe40	Lepenski Vir	83/a	0.0046-0.0127	XX
Lepe43	Lepenski Vir	88	0.0039-0.0138	XX
Lepe50	Lepenski Vir	32b	0-0.0138	XX
Lepe8	Lepenski Vir	13	0-0.0467	consistent with XX but not XY
Lepe9	Lepenski Vir	14	0.0034-0.0095	XX
Osco1	Ostrovul Corbului	M24	0.0792-0.1017	XY
Osco3	Ostrovul Corbului	M32	0.0022-0.0086	XX
Osco4	Ostrovul Corbului	M45	0.052-0.1379	consistent with XY but not XX
Pad1	Padina	4	0.0866-0.1057	XY
Pad10	Padina	26	0-0.0204	consistent with XX but not XY
Pad2	Padina	6	0.0044-0.0093	XX
Pad4	Padina	12	0.0616-0.1223	consistent with XY but not XX
Pad6	Padina	17	0.0021-0.0139	XX
Pad7	Padina	18/b	0.0047-0.01	XX
Pad8	Padina	22	0.0763-0.1092	XY
Pad9	Padina	24	0.0063-0.0211	consistent with XX but not XY
Per2	Perlez-Batka	C/trench II grave 1	0-0.0155	XX
Rud1	Rudnik Kosovski	1	0.0033-0.011	XX

Table S4: *Sex assignment of additional screened individuals according to Skoglund et al. (159, see section 3.5). Measurements with CI below 0.075 (inconsistent with XY) and above 0.016 (inconsistent with XX) shown.*

Sample name	Site	Individual	R_y 95% Cl	Assignment
Vlasa11	Vlasac	43	0.0807-0.1088	XY
Vlasa12	Vlasac	45	0.0055-0.0075	XX
Vlasa13	Vlasac	46	0.0003-0.0203	consistent with XX but not XY
Vlasa15	Vlasac	51a	0.0055-0.0115	XX
Vlasa16	Vlasac	53	0.0055-0.0075	XX
Vlasa17	Vlasac	56	0.0039-0.0085	XX
Vlasa18	Vlasac	60	0.0055-0.0075	XX
Vlasa19	Vlasac	67	0.0055-0.0075	XX
Vlasa22	Vlasac	77	0.0055-0.0117	XX
Vlasa24	Vlasac	82a	0.0729-0.1028	consistent with XY but not XX
Vlasa27	Vlasac	unit 232 T. 1/2006	0.0037-0.0095	XX
Vlasa28	Vlasac	4a	0.0584-0.0828	consistent with XY but not XX
Vlasa29	Vlasac	6	0.0773-0.1073	XY
Vlasa31	Vlasac	15	0.0063-0.0123	XX
Vlasa33	Vlasac	17	0.0244-0.0976	consistent with XY but not XX
Vlasa35	Vlasac	19a	0.0036-0.0168	consistent with XX but not XY
Vlasa36	Vlasac	23	0.0061-0.0118	XX
Vlasa39	Vlasac	27	0.0061-0.0095	XX
Vlasa40	Vlasac	28	0.0713-0.0961	consistent with XY but not XX
Vlasa42	Vlasac	32	0.0015-0.0101	XX
Vlasa45	Vlasac	48	0.004-0.0091	XX
Vlasa46	Vlasac	49	0.0001-0.0082	XX
Vlasa5	Vlasac	26	0.0774-0.1238	XY
Vlasa50	Vlasac	65	0.0612-0.1202	consistent with XY but not XX
Vlasa51	Vlasac	69	0.0067-0.0091	XX
Vlasa52	Vlasac	71	0-0.0577	consistent with XX but not XY
Vlasa53	Vlasac	73	0.0038-0.0092	XX
Vlasa55	Vlasac	78	0.0055-0.0075	XX
Vlasa59	Vlasac	84	0.0449-0.2884	consistent with XY but not XX
Vlasa6	Vlasac	29	0.0018-0.0116	XX
Vlasa60	Vlasac	U53	0-0.0158	XX
Vlasa62	Vlasac	44	0.0058-0.0084	XX
Vlasa63	Vlasac	37	0.0086-0.0207	consistent with XX but not XY
Vlasa64	Vlasac	8	0.0054-0.0093	XX
Vlasa8	Vlasac	38	0.0059-0.0094	XX
Vlasa9	Vlasac	40	0.0075-0.0225	consistent with XX but not XY

Additional files

Supplementary File S1 Tables containing results of f3-statistics and D-statistics for Anatolian genomes.

Supplementary File S2 Tables containing results of f3-statistics and D-statistics for Danubian genomes.

Supplementary File S3 Tables containing results of f3-statistics and D-statistics for capture dataset.

Supplementary File S4 Tables containing results of F_{ST} analysis for mitochondrial dataset.