The Genomic History of Southeastern Europe

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Abstract

Farming was first introduced to southeastern Europe in the mid-7th millennium BCE–brought by migrants from Anatolia who settled in the region before spreading throughout Europe. However, the dynamics of the interaction between the first farmers and the indigenous hunter-gatherers remain poorly understood because of the near absence of ancient DNA from the region. We report new genome-wide ancient DNA data from 204 individuals–65 Paleolithic and Mesolithic, 93 Neolithic, and 46 Copper, Bronze and Iron Age–who lived in southeastern Europe and surrounding regions between about 12,000 and 500 BCE. We document that the hunter-gatherer populations of southeastern Europe, the Baltic, and the North Pontic Steppe were distinctive from those of western Europe, with a West-East cline of ancestry. We show that the people who brought farming to Europe were not part of a single population, as early farmers from southern Greece are not descended from the Neolithic population of northwestern Anatolia that was ancestral to all other European farmers. The ancestors of the first farmers of northern and western Europe passed through southeastern Europe with limited admixture with local hunter-gatherers, but we show that some groups that remained in the region mixed extensively with local hunter-gatherers, with relatively sex-balanced admixture compared to the male-biased hunter-gatherer admixture that we show prevailed later in the North and West. After the spread of farming, southeastern Europe continued to be a nexus between East and West, with intermittent steppe ancestry, including in individuals from the Varna I cemetery and associated with the Cucuteni-Trypillian archaeological complex, up to 2,000 years before the Steppe migration that replaced much of northern Europe's population.

Introduction

The southeastern quadrant of Europe was the beachhead in the spread of agriculture into Europe from its source in the Fertile Crescent of southwestern Asia. After the first appearance of agriculture in the 7th millennium BCE[1,2], southeastern Europe incubated a succession of Early Neolithic cultures prior to the spread of farming westward via a Mediterranean route and northwestward via a Danubian route, reaching both Iberia and Central Europe by 5600 BCE.[3,4] Ancient DNA studies have shown that the spread of farming across Europe was accompanied by a massive movement of people[5-8] closely related to the farmers of northwestern Anatolia[9-11] but nearly all the evidence about the ancestry of the first farmers in Europe is from central and far western Europe, with only three individuals reported from northern Greece[9]. In the millennia following the establishment of agriculture in the Balkan

147 Peninsula, a series of complex societies formed, culminating in large tell settlements and sites
148 like the mid 5th millennium BCE necropolis at Varna, which has some of the earliest evidence
149 of extreme inequality in wealth, with one individual (ANI152/grave 43) from whom we
150 extracted DNA buried with more gold than is known from any site prior to that time. By the
151 end of the 6th millennium BCE, agriculture had reached eastern Europe, in the form of the
152 Cucuteni-Trypillian complex in the area of present-day Moldova, Romania and Ukraine, with
153 densely settled "mega-sites" in Ukraine housing hundreds, and perhaps thousands, of
154 people.[12] After around 4000 BCE, these societies further transformed, the tell settlements were
155 largely abandoned, and there is archaeological evidence of contact with nomadic pastoralist
156 populations from the Eurasian steppe. However, the population movements that accompanied
157 these events are not immediately evident from the archaeological record and remain largely
158 unknown.
159

160 ## Results

161 We generated new genome-wide data from 204 ancient humans (196 reported for the first
162 time), from the Balkan Peninsula, the Carpathian Basin, the North Pontic Steppe, and
163 neighboring regions mostly dated to between 12,000 and 1000 BCE (Figure 1A,
164 Supplementary Data Table 1, Supplementary Information, section 1). To obtain genome-wide
165 data in an efficient way, we enriched the DNA libraries in solution for sequences overlapping
166 1.24 million single nucleotide polymorphisms (SNPs) before sequencing.[7,10,13] We filtered out
167 individuals with low coverage (less than 15,000 SNPs covered by at least one sequence) or
168 that had unexpected ancestry for their archaeological context and were not directly dated. We
169 also report data for, but exclude from analysis, nine individuals that were first-degree relatives
170 of others in the dataset, resulting in an analysis dataset of 195 individuals.
171

172 We applied principal component analysis (PCA; Figure 1B, Extended Data Figure 1), and
173 supervised and unsupervised ADMIXTURE (Figure 1D, Extended Data Figures 2 and 3)[14] to
174 obtain an overview of this dataset in terms of population structure. We combined this genetic
175 assessment of structure with archaeological and chronological information to cluster the
176 individuals into populations. We used *D*-statistics to evaluate whether pairs of populations
177 were consistent with being clades[15] and the programs *qpAdm* and *qpGraph* to fit mixture
178 proportions and admixture graphs to the data[15]. To investigate how these individuals fit into
179 the wider context of European prehistory and present-day genetic diversity, we co-analyzed
180 them with data from 265 previously reported ancient individuals[9-11,16-28] as well as 799
181 present-day individuals genotyped on the Illumina "Human Origins" array[24], and 300 high
182 coverage genomes from the Simons Genome Diversity Project[29] (SGDP).

183 **Hunter-Gatherer substructure and transitions**

184 We report new genome-wide data for 101 individuals from Paleolithic, Mesolithic and eastern
185 European Neolithic contexts. In eastern Europe, the term "Neolithic" often refers to the
186 presence of pottery[30-32] (and may not include a transition to agricultural subsistence which is
187 the key criterion for the use of these terms in western Europe). Because of the differences in
188 meaning across our region of study, we avoid the use of "Neolithic" as a general term and use
189 terms corresponding to economic subsistence strategy (either "hunter-gatherer" or "farmer"),
190 or genetic ancestry (we use "hunter-gatherer ancestry" to refer to genetic ancestry derived
191 from a population closely related to Mesolithic Europeans, and "farmer ancestry" to refer to
192 ancestry derived from a population like northwestern Anatolian farmers).
193
194 Hunter-gatherers from central Europe have both western and eastern European hunter-
195 gatherer ancestry (WHG and EHG) – a cline that is clearly visible in PCA (Figure 1B). This
196 motivated us to investigate whether genetic population structure of Mesolithic and Early
197 Neolithic hunter-gatherers in Europe was determined purely by physical distance. We fit
198 admixture proportions with *qpAdm* and also a model estimating a spatially continuous
199 migration surface under an isolation-by-distance model,[33] and inferred a migration barrier
200 separating populations that are predominantly WHG from EHG, but with some diffusion of
201 ancestry across this boundary (Figure 2A). However, we also show that this frontier was not
202 static, with dramatic local shifts in ancestry over time (Figure 2B) and substructure in
203 phenotypically important variants (Supplementary Information, section 2).
204
205 From present-day Ukraine, our study reports new genome-wide data from 31 hunter-
206 gatherers: five Mesolithic individuals dating from 9500-6000 BCE, and 26 Neolithic and
207 Copper Age ("Eneolithic" or "Chalcolithic") individuals dating from ~6000-3500 BCE. On the
208 cline from western (WHG) to eastern hunter-gatherer ancestry (EHG, represented by
209 individuals from Karelia and Samara), the Ukrainian Mesolithic individuals fall towards the
210 eastern end, intermediate between EHG and hunter-gatherers from Sweden[7]. The Ukrainian
211 Neolithic population has significant differences in ancestry compared to the Ukrainian
212 Mesolithic population. A previous study of two individuals (for one of which we generated
213 new data) from the Mesolithic Vasil'evka 3 and Neolithic Vovnigi 2 sites[34] suggested that
214 between the Ukrainian Mesolithic and Neolithic there was an increase in Ancient North
215 Eurasian (ANE) ancestry related to the 24,000-year old Siberian Mal'ta 1.[26] However, our
216 larger sample shows the opposite – specifically that ANE ancestry decreases and WHG
217 ancestry increases – as shown by the statistics D(Mbuti, X, Ukraine_Mesolithic,
218 Ukraine_Neolithic), which is Z=-4.9 when X is the Mal'ta 1 individual and Z=9.1 for
219 X=WHG (Supplementary Data Table 2).

Individuals associated with the Bronze Age Yamnaya Complex from Ukraine, like previously reported Yamnaya individuals from Samara[7] and Kalmykia[16], have little evidence of WHG ancestry, but do have a third source of ancestry related to hunter-gatherers from the Caucasus [20] (CHG) and early Iranian farmers[23,35] (Supplementary Data Table 3). Two Yamnaya individuals – one from Ozera in Ukraine and one from Bulgaria (I1917 and Bul4, both dated to ~3000 BCE) – in addition have evidence of early European farmer related admixture, which is the first evidence of such ancestry in Yamnaya individuals (Figure 1B,D, Supplementary Data Table 2). Similarly, one Copper Age individual (I4110) dated to ~3600-3400 BCE from Dereivka in Ukraine has both CHG and farmer ancestry (Figure 1D, Supplementary Data Table 2). This is by far the earliest appearance of farmer ancestry this far East in Eurasia, which was previously not known on the Steppe until the Srubnaya Complex after ~1800 BCE.

At Zvejnieki in Latvia (18 newly reported individuals, added to 4 first reported in Ref. 34 for which we report additional data here) there is a transition in hunter-gatherer ancestry that is the opposite of that seen in Ukraine. Consistent with similar data from the Baltic States [34,36,37], we find that Mesolithic and Early Neolithic individuals associated with the Kunda and Narva cultures had ancestry that was genetically intermediate between western and eastern hunter-gatherers (we estimate 70% WHG and 30% EHG, but perhaps with some differential relatedness to ANE, relative to EHG; Supplementary Data Table 3). However, there is a dramatic shift between the Early Neolithic and the Middle Neolithic Comb Wear Complex, who are almost entirely EHG in ancestry (we estimate 73% EHG, but two out of four individuals appear almost 100% EHG in PCA space). The most recent individual, associated with the Final Neolithic Corded Ware Complex, provides evidence of yet another transition, clustering closely with Yamnaya from Samara[7], Kalmykia[16] and Ukraine.

We report new data from hunter-gatherers from France, Sicily and Croatia, as well as higher coverage data from three previously published hunter-gatherers from France and Germany.[18] The Sicilian and Croatian individuals dating to 12,000 and 6100 BCE cluster closely with western hunter-gatherers, including individuals from Loschbour[24] (Luxembourg, 6100 BCE), Bichon[20] (Switzerland, 11,700 BCE), and Villabruna[18] (Italy 12,000 BCE). These results demonstrate that the "western hunter-gatherer" population[24] was widely distributed from the Atlantic seaboard of Europe in the West, to Sicily in the South, to the Balkan Peninsula in the Southeast, for at least six thousand years, strengthening the evidence that the western hunter-gatherers represent a population that expanded from a southeastern European refugium following the last Ice Age around 15,000 years ago–in the process displacing or admixing with the existing population of western Europe.[18 38]

A particularly important hunter-gatherer population that we newly report in this study is from the Iron Gates region that straddles the border of present-day Romania and Serbia. This region is close to the route taken by farmer migrants on their way from the Balkans to central Europe, and the population is represented in our study by 35 individuals from the Iron Gates sites of Hajdučka Vodenica, Ostrovul Corbului, Padina, Schela Cladovei and Vlasac, one individual (I2534) with similar (but more EHG) hunter-gatherer ancestry from the Early Neolithic site of Măgura Buduiasca 250km east of the Iron Gates, and two individuals with farmer ancestry from the Mesolithic-Neolithic site of Lepenski Vir. Modeling Iron Gates hunter-gatherers as a mixture of WHG and EHG (Supplementary Table 3) shows that they are – as expected given their geographic location and the hunter-gatherer ancestry cline – intermediate between WHG (87%) and EHG (13%). However, this *qpAdm* model does not fit well (p=0.0003, Supplementary table 3) and we note that the Iron Gates hunter-gatherers carry mitochondrial haplogroups K1 (8/36) as well as other subclades of haplogroup U (27/36) and haplogroup H (1/36). This contrasts with WHG, EHG and Scandinavian hunter-gatherers who almost all carry haplogroup U5 or U2. Therefore the Iron Gates hunter-gatherers have ancestry that is not present in WHG or EHG. This suggests either genetic contact between the ancestors of the Iron Gates population and hunter-gatherers from Anatolia, or that the Iron Gates population is related to the source population from which the WHG split off during a post-LGM re-expansion into Europe.

In contrast, the two individuals (I4665 and I4666, dated to 6205-5907 calBCE and 6222-5912 calBCE respectively) that we sampled from Iron Gates site of Lepenski Vir are genetically farmers rather than hunter-gatherers, despite having been buried in the local Mesolithic tradition. Strontium isotope data shows that many of the individuals buried after ~6100 BCE at Lepenski Vir–including one of the two that we sampled (I4665, burial 54E)–were not originally from the Danube Gorges.[39] These observations, combined with one individual from Padina (I5232), dated to 6061-5841 calBCE that has both farmer and hunter-gatherer ancestry, demonstrates that the Iron Gates region was one where farmer and hunter-gatherer groups interacted both genetically and culturally, and provides a window into the first few generations of interactions between these disparate groups.

Population structure and transformation of farmers in southeast Europe

Neolithic populations from present-day Bulgaria, Croatia, Macedonia, Serbia and Romania cluster closely with the Neolithic populations of northwestern Anatolia (Figure 1). Modeling Balkan Neolithic populations as a mixture of Anatolia Neolithic, western hunter-gatherer and Ukraine Mesolithic, we estimate that Balkan Neolithic populations derive 98% (95% confidence interval [CI]; 97-100%) of their ancestry from populations related to those of the

294 northwestern Anatolian Neolithic. A striking exception to the pattern of limited hunter-
295 gatherer admixture in Balkan Neolithic populations is evident in 8 out of 9 individuals from
296 Malak Preslavets, a site in present-day Bulgaria close to the Danube river.[40] These individuals
297 likely lived in the mid-6th millennium BCE and have significantly more hunter-gatherer
298 ancestry than other Balkan Neolithic individuals as shown by PCA and ADMIXTURE as well
299 as *D*-statistics and *qpAdm* modeling (Figure 1B,D, Extended Data Figures 1-3,
300 Supplementary Data Tables 2-4). We find that a model of 82% (CI: 77-86%) Anatolian
301 Neolithic, 15% (CI: 12-17%) WHG, and 4% (CI: 0-9%) EHG ancestry is a good fit to the
302 data. This hunter-gatherer ancestry with a ~4:1 WHG:EHG ratio plausibly represents a
303 contribution from local Balkan hunter-gatherers like those that we sampled from the Iron
304 Gates. By the Late Mesolithic, hunter-gatherers populations in the Balkans were likely
305 concentrated in sites along the coast and major rivers such as the Danube,[41] which directly
306 connects the Iron Gates with Malak Preslavets. This suggests a heterogeneous landscape of
307 farmer populations with different proportions of hunter-gatherer ancestry during the early
308 Neolithic, and that farmer groups with the most hunter-gatherer ancestry, like those at Malak
309 Preslavets and, possibly, Lepenski Vir were those that lived close to the highest densities of
310 hunter-gatherers.
311
312 In the Balkans, Copper Age populations have significantly more hunter-gatherer ancestry than
313 Neolithic populations as shown, for example, by the statistic D(Mbuti, WHG,
314 Balkans_Neolithic, Balkans_Chalcolithic); Z=5.18 (Supplementary Data Table 2). This is
315 consistent with changes in funeral rites[42] and roughly contemporary with the "resurgence" of
316 hunter-gatherer ancestry previously reported in central Europe and Iberia[7,10,43].
317
318 We also report the first data from the Late Neolithic Globular Amphora Complex. Globular
319 Amphora individuals from two sites in Poland and Ukraine form a tight genetic cluster,
320 showing genetic homogeneity over a large distance (Figure 1B,D). We find that this
321 population had more hunter-gatherer ancestry than Middle Neolithic groups from Central
322 Europe[7] (we estimate 25% [CI: 22-27%] WHG ancestry, similar to Chalcolithic Iberia). This
323 finding further extends our knowledge of the variable landscape of hunter-gatherer and farmer
324 admixture proportions in Europe (Supplementary Data Table 3). In east-central Europe, the
325 Globular Amphora Complex immediately precedes the Corded Ware Complex that marks the
326 first appearance of steppe ancestry in the region.[7,16] The Globular Amphora abutted
327 populations with steppe-influenced material cultures for hundreds of years and yet the
328 individuals in our study have no evidence of steppe ancestry, suggesting that this persistent
329 culture frontier corresponded to a genetic barrier.
330

The migrations from the Pontic-Caspian steppe associated with the Yamnaya Cultural Complex in the 3rd millennium BCE made a profound contribution to the genetic ancestry of central Europe, contributing about 75% of the ancestry of individuals associated with the Corded Ware Complex and about 50% of the ancestry of succeeding material cultures such as the Bell Beaker Complex.[7,16] In a few individuals from southeastern Europe, we find evidence of steppe-related ancestry far earlier (defined here as a mixture of EHG and CHG similar to the genetic signature of individuals of the later Yamnaya; Figure 1B,D). One individual (ANI163) from the Varna I cemetery dates to 4711-4550 BCE, one (I2181) from nearby Smyadovo dates to 4550-4450 BCE, and a third individual (I1927) from Verteba cave, associated with the Cucuteni-Trypillian complex, dates to 3619-2936 BCE. These findings push back by almost 2000 years the first evidence of steppe ancestry this far West in Europe, demonstrating the resumption of genetic contact between southeastern Europe and the Steppe that also occurred in the Mesolithic. Other Copper Age (~5000-4000 BCE) individuals from the Balkans have little evidence of steppe ancestry, but Bronze Age (~3400-1100 BCE) individuals do (we estimate 30%; CI: 26-35%). The four latest Balkan Bronze Age individuals in our data (later than ~1700 BCE) all have more steppe ancestry than earlier Bronze Age individuals (3200-2500 BCE; Figure 1D), showing that the contribution of the Steppe to southeast European populations increased further during the Bronze Age.

New evidence about the spread of farming into, and throughout, Europe

This study resolves two open questions about the initial spread of farming into Europe. The first is the question of whether the first farmers of the Danubian Route that brought agriculture to northern Europe along the Danube River valley, and those that spread along the Mediterranean coast to Iberia and other southern European locations, were derived from a single ancestral population or instead represent separate migrations from different Anatolian sources. A challenge in studying the relationship among Early Neolithic populations is the different proportions of hunter-gatherer ancestry they carry, which obscures the more subtle differences between their farmer ancestries. One approach to this problem is to explicitly model both sources of ancestry using an admixture graph (Supplementary Information, section 3). We confirm that Mediterranean populations, represented in our study by individuals of the Impressa complex from Croatia and the Epicardial Early Neolithic from Spain[7], are closely related to the Danubian population represented by the *Linearbandkeramik* (LBK) from central Europe[7,44] and show that both groups are closely related to the Balkan Neolithic population. These three populations form a clade with Northwest Anatolians as an outgroup, consistent with a single migration from a population closely related to the northwestern Anatolian Neolithic farming population into the Balkan peninsula, which then split into two populations that followed the Danubian and Mediterranean routes.

368

369 A related question about the spread of farming into Europe concerns whether its initial arrival
370 in present-day Greece and subsequent expansion was mediated by a single population
371 migrating from Anatolia – as has been consistent with genetic data up until now[9] – or whether
372 there were multiple initial groups, as suggested by the archaeological record.[45,46] We find that
373 four southern Greek (Peloponnese) Neolithic individuals – three from Diros Cave and one
374 from Franchthi Cave, plus one previously published individual from Diros[27] – are not
375 consistent with descending from the same source population as other European farmers. In
376 PCA these individuals are outliers; shifted away from northwestern Anatolian and European
377 Early Neolithic individuals, in a direction opposite from WHG. *D*-statistics (Supplementary
378 Data Table 2) show that in fact, these "Peloponnese Neolithic" individuals have less WHG-
379 related ancestry than Anatolia Neolithic ones, and that they form an outgroup relative to
380 Anatolian and Balkans Neolithic populations, suggesting an independent migration into
381 Europe from a population that split off from the ancestors of the northwest Anatolian
382 individuals from which we have data. Admixture graph modeling (Supplementary
383 Information, section 3) supports this interpretation, confirming that their Near Eastern
384 ancestry is derived from a lineage that is close, or basal, to the non-WHG component of
385 Anatolian Neolithic ancestry. One possibility is that this independent migration is related to
386 an earlier Aceramic Neolithic in Greece that was derived from the pre-pottery Neolithic
387 (PPNB) of Cyprus and the Levant[46]. Under this model, the earliest Neolithic populations in
388 Greece migrated from the Levant, perhaps via the southwestern Anatolian coast as early as
389 7000 BCE,[45,46] but the majority of Neolithic ancestry arrived around 500 years later via a route
390 that passed through northwestern Anatolia. The predictions of this hypothesis could be further
391 tested with genome-wide data of Early Neolithic individuals from Cyprus, Crete and
392 southwest Anatolia. Populations related to the Peloponnese Neolithic potentially made a small
393 contribution to the ancestry of other Mediterranean Neolithic populations like Early Neolithic
394 Iberia and Neolithic farmers from northern Greece[9] but we do not strongly reject models
395 without such a contribution (Supplementary Information, section 3).
396

397 **Sex-biased admixture between hunter-gatherers and farmers**

398 We provide the first evidence for sex-biased admixture between hunter-gatherers and farmers
399 in Europe, showing that the Middle Neolithic "resurgence" of hunter-gatherer ancestry[7,43] in
400 central Europe and Iberia was driven more by male than by female hunter-gatherers (Figure
401 3B&C, Supplementary Data Table 5). One way of detecting this is to compare ancestry
402 proportions on the autosomes and the X chromosome. Since males always inherit their X
403 chromosome from their mothers, differences between ancestry on the autosomes and
404 chromosome X imply sex-biased mixture. In the Balkan Neolithic there is no evidence of sex

405 bias using ancestry estimates obtained from *qpAdm* (Z=-0.65 where a positive Z-score implies
406 male hunter-gatherer bias), nor in the LBK and Iberian_Early Neolithic (Z=-0.24 and 1.04).
407 However, in the Middle Neolithic and later populations, this effect reverses. In the Balkan
408 Copper Age there is weak evidence of bias (Z=1.77) but in Iberia and central Europe Middle
409 Neolithic there is clear bias is in favor of male hunter-gatherer ancestry (Z=2.98, and 2.77 in
410 Iberia Copper Age and central European Middle Neolithic). This result is independently
411 supported by uniparental markers (Figure 3C). Proportions of typically hunter-gatherer
412 mitochondrial haplogroups (haplogroup U)[47] are low in all populations and within the
413 intervals of genome-wide ancestry proportions. On the other hand, hunter-gatherer Y
414 chromosomes (haplogroups I2, R1 and C1)[18] are much more common: 6/7 in the Iberian
415 Neolithic/Copper Age and 7/8 in Middle-Late Neolithic central Europe (Central_MN and
416 Globular_Amphora). Under a single pulse model of admixture, the autosomal/X chromosome
417 ancestry proportions imply that in the central European Middle Neolithic population that
418 shows the strongest evidence of sex bias, 35-50% of the male ancestors were hunter-
419 gatherers, compared to 0-5% of the female ancestors (Extended Data Figure 4).
420
421 The merging of hunter-gatherer and farmer populations was a dynamic process that unfolded
422 over thousands of years, and proceeded in a profoundly different way in different parts of
423 Europe. Our analysis shows that in some places – for example at Malak Preslavets in Bulgaria
424 – there was extensive mixing between hunter-gatherers and farmers, likely driven by the high
425 local hunter-gatherer population density. In other places–in particular in western, central and
426 northern Europe–hunter-gatherers and farmers lived in close proximity for long periods of
427 time with minimal mixture[6,43,48]. When they did finally mix, we find evidence that the hunter-
428 gather admixture was male-biased, implying a different dynamic. Farming was initially
429 unable to expand widely in central and northern Europe because early farming techniques
430 were only suitable for specific regions within the loess belt of the northern European plain.
431 Thus, northern and central European hunter-gatherers were protected from the demographic
432 impact of farming migrations, resulting in persistent frontiers between farmers and hunter-
433 gatherers.[49,50] This may have given hunter-gatherers and farmers time to learn from each other
434 and interact in a different way than during the more rapid expansion of the first farmers in the
435 South.
436
437 **No evidence of Copper Age Balkans-to-Anatolia migration**
438 One version of the Steppe Hypothesis of Indo-European language origins suggests that Proto-
439 Indo European languages developed in the steppe north of the Black and Caspian seas, and
440 that the earliest known diverging branch – Anatolian – was spread into Asia Minor by
441 movements of steppe peoples through the Balkan peninsula during the Copper Age around

4000 BCE, as part of the same incursions from the steppe that coincided with the decline of the tell settlements.[51] If this were correct, then one way to detect evidence of it would be the appearance of large amounts of characteristic steppe ancestry first in the Balkan Peninsula, and then in Anatolia. However, our genetic data do not support this scenario. While we find steppe ancestry in Balkan Copper Age and Bronze Age individuals, this ancestry is sporadic across individuals in the Copper Age, and at low levels in the Bronze Age. Moreover, while Bronze Age Anatolian individuals[27] have CHG / Iran Neolithic related ancestry, they have neither the EHG ancestry characteristic of all steppe populations sampled to date[20], nor the WHG ancestry that is ubiquitous in southeastern Europe in the Neolithic (Figure 1A, Supplementary Data Table 2, Supplementary Information section 1). This pattern is consistent with that seen in northwestern Anatolia[11] and later in Copper Age Anatolia[23], suggesting continuing migration into Anatolia from the East rather than from Europe.

An alternative hypothesis is that the ultimate homeland of Proto-Indo European languages was in the Caucasus or in Iran. In this scenario, westward movement contributed to the dispersal of Anatolian languages, and northward movement and mixture with EHG was responsible for the formation of the population associated with the Yamnaya complex. These steppe pastoralists plausibly spoke a "Late Proto-Indo European" language that is ancestral to many of the non-Anatolian branches of the Indo-European language family.[52] On the other hand, our data could still be consistent with the Steppe-Balkans-Anatolia route hypothesis model, albeit with constraints. It remains possible that populations dating to around 1600 BCE in the regions where the Indo-European Luwian, Hittite and Palaic languages were spoken did have European hunter-gatherer ancestry. However, our results would require that such ancestry was not ubiquitous in Bronze Age Anatolia, and was perhaps tightly linked to Indo-European speaking groups. We predict that additional insight about the genetic origins of the potential speakers of early Indo-European languages will be obtained when ancient DNA data become available from additional sites in this key period in Anatolia and the Caucasus.

Discussion

Our study shows that southeastern Europe consistently served as a genetic contact zone between different populations. This role likely contributed to the extraordinary series of cultural innovations that characterize the region, from the elegant figurines of the Neolithic to the ornaments and precious metalwork of Varna. Before the arrival of farming, this region saw constant interaction between highly diverged groups of hunter-gatherers, and this interaction continued, perhaps accelerating, after the arrival of farming. We find evidence that some early farmers from Greece derived ancestry from a different source compared to the one

that contributed the majority of ancestry of all other farmers in Europe. In eastern Europe we document the appearance of CHG/Iranian Neolithic ancestry north of the Black Sea, and its eventual extension as far north as the Baltic. In some ways, this expansion parallels the expansion of Anatolian farmer ancestry into western Europe although it is less dramatic, and several thousand years later. These expansions set up the two, largely separate, populations in western and eastern Europe that would come together in the Final Neolithic and Early Bronze Age to form the ancestry of present-day Europe.

This study describes key ancestral components that contributed to present-day West Eurasian genetic diversity. However, the more recent processes that created present-day southeastern European populations are unknown, and understanding this will require dense sampling of Bronze Age, Iron Age, Roman, and Medieval groups and comparison to present-day populations. At the most ancient end of our time series, while information about hunter-gatherer population structure in northern and western Europe now extends back throughout the Upper Paleolithic,[18] we have little data about how these populations fit into a wider Eurasian context, and more data from hunter-gatherer populations in Anatolia, the Near East and East Asia will be needed to resolve that question. Finally, many questions about the nature of the interactions between populations remain unresolved. For example, we report evidence for sex-bias in one particular set of interactions between hunter-gatherers and farmers, and other interactions may have had similar dynamics[37,53]. However, many more examples of such interactions need to be collected before it will become possible to make generalizable claims about the patterns of sex-biased interactions among human populations as they came into contact and mixed during prehistory.

501 ## Methods

502

503 **Ancient DNA Analysis**

504 We extracted DNA and prepared next-generation sequencing libraries in four different
505 dedicated ancient DNA laboratories (Adelaide, Boston, Budapest, and Tuebingen). Sample
506 powder was also generated in a fifth laboratory (Dublin) and sent to Boston for DNA
507 extraction and library preparation (Supplementary Table 1).

508

509 Two samples were processed at the Australian Centre for Ancient DNA, Adelaide, Australia,
510 according to previously published methods[7] and sent to Boston for subsequent screening,
511 1240k capture and sequencing.

512

513 Seven samples were processed as previously described[28] at the Institute of Archaeology RCH
514 HAS, Budapest, Hungary, and amplified libraries were sent to Boston for screening, 1240k
515 capture and sequencing.

516

517 Seventeen samples were processed at the Institute for Archaeological Sciences of the
518 University of Tuebingen and at the Max Planck Institute for the Science of Human History in
519 Jena, Germany. Extraction[54] and library preparation[55,56] followed established protocols. We
520 performed in-solution capture as described below ("1240k capture") and sequenced on an
521 Illumina HiSeq 4000 or NextSeq 500 for 76bp either single or paired-end.

522

523 The remaining 195 samples were processed at Harvard Medical School, Boston, USA. From
524 about 75mg of sample powder from each sample (extracted in Boston or University College
525 Dublin, Dublin, Ireland), we extracted DNA following established methods[54] replacing the
526 column assembly with the column extenders from a Roche kit.[57] We prepared double
527 barcoded libraries with truncated adapters from between a ninth to a third of the DNA extract.
528 Most libraries included in the nuclear genome analysis (90%) were subjected to partial
529 ("half") Uracil-DNA-glycosylase (UDG) treatment before blunt end repair. This treatment
530 reduces by an order of magnitude the characteristic cytosine-to-thymine errors of ancient
531 DNA data[58], but works inefficiently at 5' ends,[56] and thereby leaves a signal of characteristic
532 damage at the terminal ends of ancient sequences. Some libraries were not UDG treated
533 ("minus"). For some samples we increased coverage by preparing additional libraries from
534 the existing DNA extract using the partial UDG library preparation, but replacing the
535 MinElute column cleanups in between enzymatic reactions with magnetic bead cleanups, and
536 the final PCR cleanup with SPRI bead cleanup.[59,60]

537

538 We screened all libraries from Adelaide, Boston and Budapest by enriching for the
539 mitochondrial genome plus about 3000 (50 in an earlier, unpublished, version) nuclear SNPs
540 using a bead-capture[61] but with the probes replaced by amplified oligonucleotides synthesized
541 by CustomArray Inc. After the capture, the adapter sites were completed by PCR, and thereby
542 dual index combinations[62] were attached to each enriched library. We sequenced the products
543 of between 100 and 200 libraries together with the non-enriched libraries (shotgun) on an
544 Illumina NextSeq500 using v2 150 cycle kits for 2x76 cycles and 2x7 cycles.
545

546 In Boston, We performed two rounds of in-solution enrichment ("1240k capture") for a
547 targeted set of 1,237,207 SNPs using previously reported protocols.[7,13,24] When we enriched
548 additional libraries to increase coverage, multiple libraries from the same sample were pooled
549 in equimolar ratios before the capture. All sequencing was performed on an Illumina
550 NextSeq500 using v2 150 cycle kits for 2x76 cycles and 2x7 cycles. We attempted to
551 sequence each enriched library up to the point where we estimated that it was economically
552 inefficient to sequence further. Specifically, we iteratively sequenced more and more from
553 each individual and only stopped when we estimated that the expected increase in the number
554 of targeted SNPs hit at least once would be less than about one for every 100 new read pairs
555 generated. After sequencing, we removed individuals with evidence of contamination based
556 on mitochondrial DNA polymorphism[63] or difference in PCA space between damaged and
557 undamaged reads[64], a high rate of heterozygosity on chromosome X despite being male[64,65], or
558 an atypical ratio of X to Y sequences. We report, but do not analyze, data from 17 individuals
559 that had low coverage (less than 15,000 SNPs hit on the autosomes), were first-degree
560 relatives of others in the dataset, or were undated and had unusual ancestry for their
561 archaeological context.
562

563 After removing a small number of sites that failed to capture, we were left with a total of
564 1,233,013 sites of which 32,670 were on chromosome X and 49,704 were on chromosome Y,
565 with a median coverage at targeted SNPs on the 138 new individuals of 0.73 (range 0.017-
566 9.2; Supplementary Table 1). We generated "pseudo-haploid" calls by selecting a single read
567 randomly for each individual at each SNP. Thus, there is only a single allele from each
568 individual at each site, but adjacent alleles might come from either of the two haplotypes of
569 the individual. We merged the newly reported data with previously reported data from 266
570 other ancient individuals[9-11,16-28], making pseudo haploid calls in the same way at the 1240k
571 sites for individuals that were shotgun sequenced rather than captured.
572

573 Using the captured mitochondrial sequence from the screening process, we called
574 mitochondrial haplotypes. Using the captured SNPs on the Y chromosome, we called Y
575 chromosome haplogroups for males by restricting to sequences with mapping quality ≥30 and
576 bases with base quality ≥30. We determined the most derived mutation for each individual,
577 using the nomenclature of the International Society of Genetic Genealogy
578 (http://www.isogg.org) version 11.110 (21 April 2016).
579
580 **Population genetic analysis**
581 To analyze these ancient individuals in the context of present day genetic diversity, we
582 merged them with the following two datasets:
583
584 1. 300 high coverage genomes from a diverse worldwide set of 142 populations
585 sequenced as part of the Simons Genome Diversity Project[29] (SGDP merge).
586
587 2. 799 West Eurasian individuals genotyped on the Human Origins array[24], with
588 597,573 sites in the merged dataset (HO merge).
589
590 We computed principal components of the present-day individuals in the HO merge and
591 projected the ancient individuals onto the first two components using the "*lsqproject: YES*"
592 option in *smartpca (v15100)*[66] (https://www.hsph.harvard.edu/alkes-price/software/).
593
594 We ran *ADMIXTURE (v1.3.0)* in both supervised and unsupervised mode. In supervised mode
595 we used only the ancient individuals, on the full set of SNPs, and the following population
596 labels fixed (so, for k=4, we used labels 1-4 in the list below, for example):
597 *1. Anatolia_Neolithic*
598 *2. WHG*
599 *3. EHG*
600 *4. Yamnaya*
601 *5. Ukraine_Mesolithic*
602 *6. SHG*
603 For unsupervised mode we used the HO merge, including 799 present-day individuals. We
604 flagged individuals that were genetic outliers based on PCA and ADMIXTURE, relative to
605 other individuals from the same time period and archaeological culture.
606
607 We computed *D*-statistics using *qpDstat (v710)*, and fitted admixture proportions with *qpAdm*
608 *(v610* and admixture graphs with *qpGraph (v6021)*[15] (https://github.com/DReichLab/
609 AdmixTools), in each case using the SGDP merge. We computed standard errors for Z scores
610 with the default block jackknife parameters. For *qpAdm* we used the following set of seven
611 populations as outgroups or "right populations":

- *Mbuti.DG*
- *Ust_Ishim_HG_published.DG*
- *Mota.SG*
- *MA1_HG.SG*
- *Villabruna*
- *Papuan.DG*
- *Onge.DG*
- *Han.DG*

For some analyses (Extended Data Table 4) we used an extended set of 14 right populations, including additional Upper Paleolithic European individuals[18]:

- *ElMiron*
- *Mota.SG*
- *Mbuti.DG*
- *Ust_Ishim_HG_published.DG*
- *MA1_HG.SG*
- *AfontovaGora3*
- *GoyetQ116-1_published*
- *Villabruna*
- *Kostenki14*
- *Vestonice16*
- *Karitiana.DG*
- *Papuan.DG*
- *Onge.DG*
- *Han.DG*

For 40 Mesolithic individuals we estimated an effective migration surface using the software *EEMS* (https://github.com/dipetkov/eems)[33]. We computed pairwise differences between individuals using the *bed2diffs2* program provided with *EEMS*. We set the number of demes to 400 and defined the outer boundary of the region by the polygon (in latitude-longitude co-ordinates) [(66,60), (60,10), (45,-15), (35,-10), (35,60)]. We ran the MCMC ten times with different random seeds, each time with one million burn-in and four million regular iterations, thinned to one in ten thousand.

To analyze potential sex bias in admixture, we used *qpAdm* to estimate admixture proportions on the autosomes (default option) and on the X chromosome (option "*chrom: 23*"). We computed Z scores for the difference between the autosomes and the X chromosome as $Z = \frac{p_A - p_X}{\sqrt{\sigma_A^2 + \sigma_X^2}}$ where p_A and p_X are the hunter-gatherer admixture proportions on the autosomes and the X chromosome, and σ_A and σ_X are the corresponding jackknife standard deviations. Thus, a positive Z-score means that there is more hunter-gatherer admixture on the autosomes than on the X chromosome and thus the hunter-gatherer admixture was male-biased. Because X chromosome standard errors are high and *qpAdm* results can be sensitive to which

654 population is first in the list of outgroup populations, we checked that the patterns we observe
655 were robust to cyclic permutation of the outgroups. To compare frequencies of hunter-
656 gatherer uniparental markers we counted the individuals with mitochondrial haplogroup U
657 and Y chromosome haplogroups C2, I2 and R1 which are all common in Mesolithic hunter-
658 gatherers but rare or absent in the Anatolian Neolithic. The Iron Gates hunter-gatherers also
659 carry H and K1 mitochondrial haplogroups so the proportion of haplogroup U represents the
660 minimum maternal hunter-gatherer contribution. We computed binomial confidence intervals
661 for the proportion of markers using the Agresti-Coull method[67,68] implemented in the *binom*
662 package in *R*.
663
664 Given autosomal and X chromosome admixture proportions, we estimated the proportion of
665 male and female hunter-gatherer ancestors by assuming a single pulse model of admixture. If
666 the proportions of male and female ancestors that are hunter-gatherer are given by *m* and *f*,
667 respectively, then the proportions of hunter-gatherer ancestry on the autosomes and the X
668 chromosome are given by $\frac{m+f}{2}$ and $\frac{m+2f}{3}$. We approximated the sampling error in the
669 observed admixture proportions by the estimated jackknife sampling error, computed the
670 likelihood surface for *(m,f)* over a grid ranging from (0,0) to (1,1).
671
672 **Direct AMS ^{14}C Bone Dates**
673 We report new direct AMS ^{14}C bone dates in this study from multiple AMS radiocarbon
674 laboratories. In general, bone samples were manually cleaned and demineralized in weak HCl
675 and, in most cases (PSU, UCIAMS, OxA), soaked in an alkali bath (NaOH) at room
676 temperature to remove contaminating soil humates. Samples were then rinsed to neutrality in
677 Nanopure H_2O and gelatinized in HCL.[69] The resulting gelatin was lyophilized and weighed
678 to determine percent yield as a measure of collagen preservation (% crude gelatin yield).
679 Collagen was then directly AMS ^{14}C dated (Beta, AA) or further purified using ultrafiltration
680 (PSU/UCIAMS, OxA, Poz, Wk, MAMS).[70] It is standard in some laboratories
681 (PSU/UCIAMS, OxA, Wk) to use stable carbon and nitrogen isotopes as an additional quality
682 control measure. For these samples, the %C, %N and C:N ratios were evaluated before AMS
683 ^{14}C dating. C/N ratios for well-preserved samples fall between 2.9 and 3.6, indicating good
684 collagen preservation.[71]
685
686 All ^{14}C ages were δ^{13}C-corrected for mass dependent fractionation with measured ^{13}C/^{12}C
687 values[72] and calibrated with OxCal version 4.2.3 [73] using the IntCal13 northern hemisphere
688 calibration curve.[73]
689

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Figures

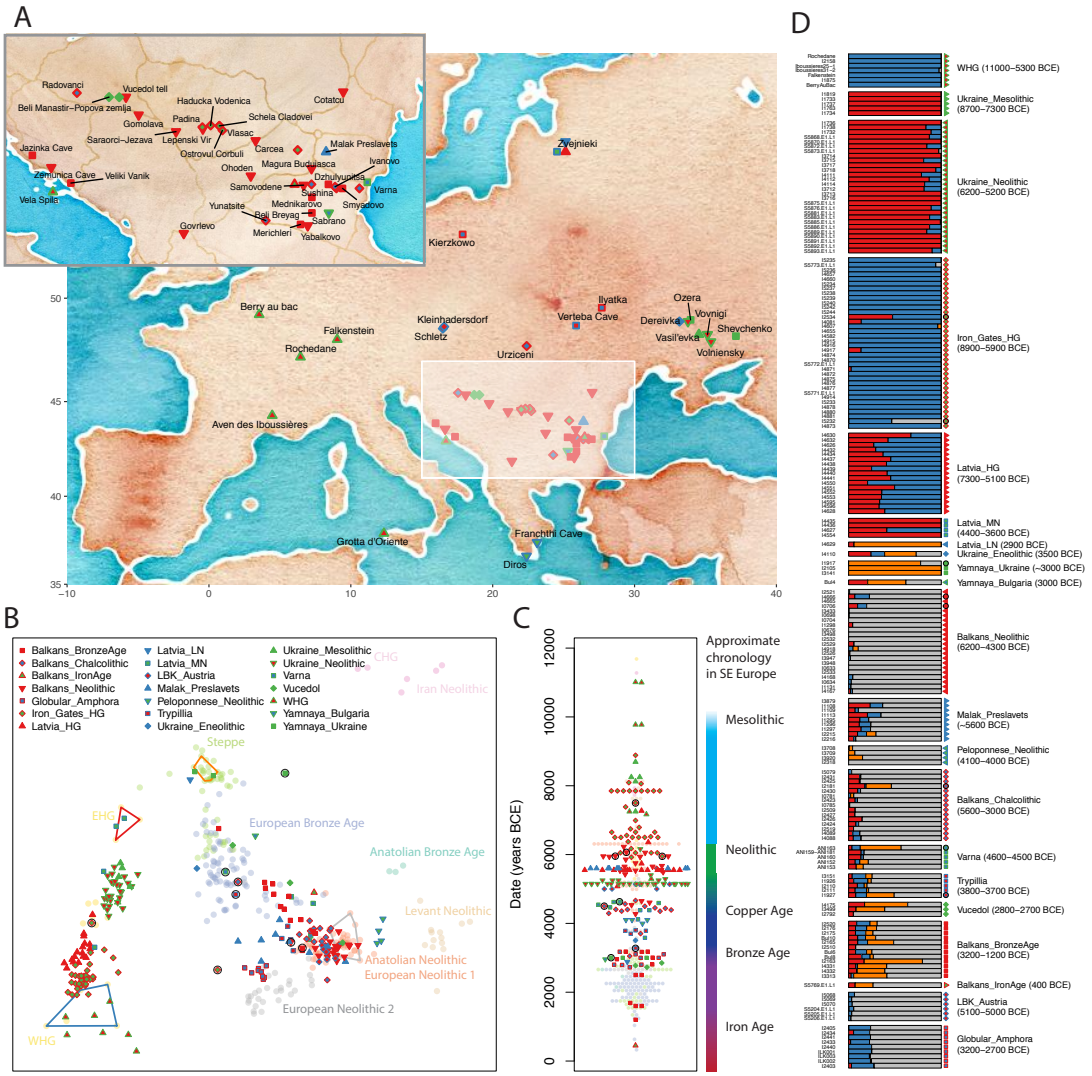


Figure 1: Geographic locations and genetic structure of newly reported individuals. **A**: Location and groupings of newly reported individuals. **B**: Individuals projected onto axes defined by the principal components of 799 present-day West Eurasians (not shown in this plot for clarity, but shown in Extended Data Figure 1). Projected points include selected published individuals (faded colored circles, labeled) and newly reported individuals (other symbols; outliers shown by additional black circles). Colored polygons indicate the individuals that had cluster memberships fixed at 100% for the supervised admixture analysis in D. **C**: Estimated age (direct or contextual) for each sample. Approximate chronology used in southeastern Europe shown to the right **D**: Supervised ADMIXTURE plot, modeling each ancient individual (one per row), as a mixture of populations represented by clusters containing Anatolian Neolithic (grey), Yamnaya from Samara (orange), EHG (red) and WHG (blue). Dates indicate approximate range of individuals in each population. Map tiles in **A** by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under CC BY SA.

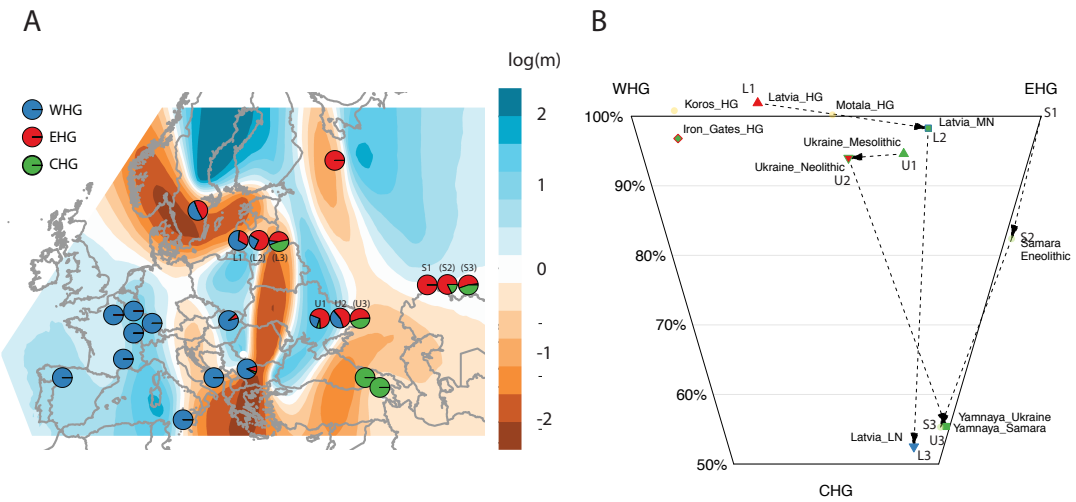


Figure 2: Structure and population change in European populations with mainly hunter-gatherer ancestry. **Background:** Effective migration surface[33] inferred using 109 Mesolithic and Early Neolithic genomes. Blue indicates regions of high migration and red regions of low migration (relative migration rate *m*). **Pies:** Each pie represents one or more individuals. Blue, red and green segments pies show, respectively, WHG, EHG and CHG ancestry – we chose a 2-population model if it fitted in *qpAdm* with p>0.01 (Supplementary Data Table 3). Pies with 100% ancestry are those that were fixed as source populations. In Latvia, Samara and Ukraine, sets of three pies show the transition in ancestries, approximately from Mesolithic-Neolithic-Yamnaya (Exact labels in panel B). Populations with labels in brackets were not used to fit the migration surface. **B**: Ancestry transitions for hunter-gatherer populations in the 3-population WHG+EHG+CHG model. Dashed lines show populations with geographic continuity.

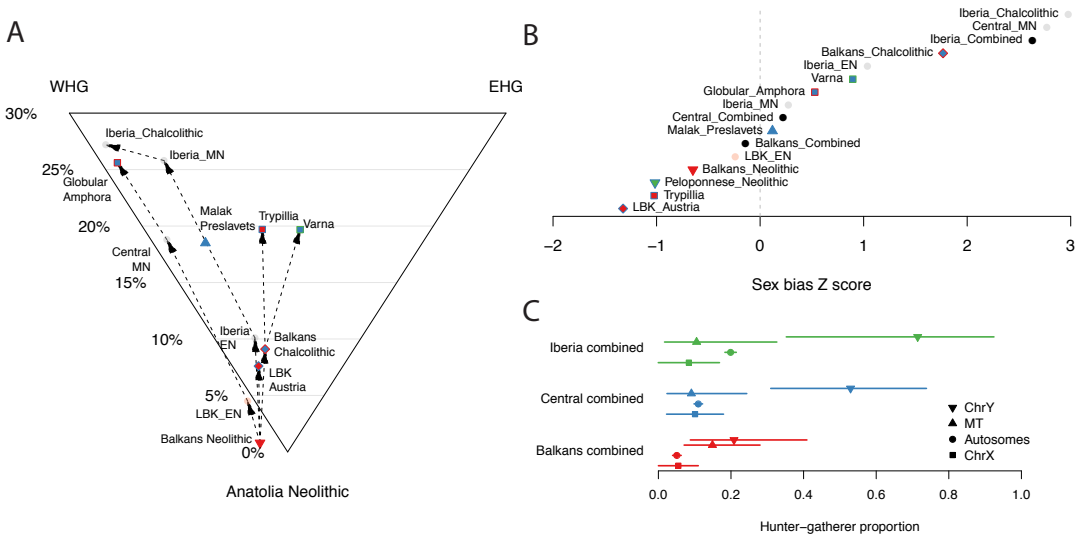
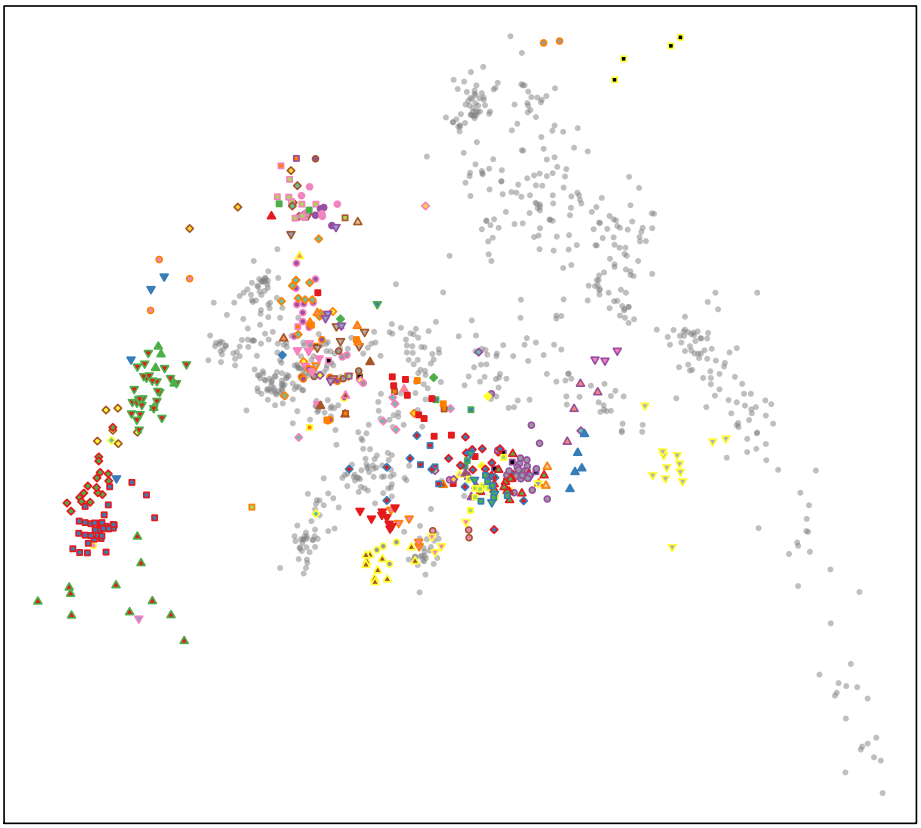


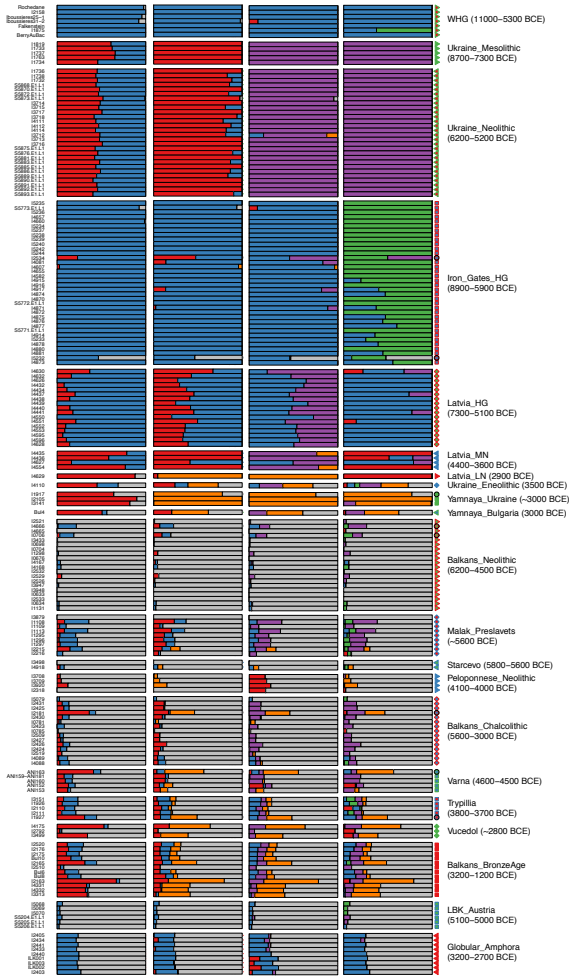
Figure 3: Structure and population change in European populations with farmer ancestry. **A**: each population is modeled as a mixture of Anatolia Neolithic, WHG, and EHG. Dashed lines show temporal relationships between populations from the same geographic areas with similar ancestries. **B**: Z-scores for the difference in hunter-gatherer ancestry on the autosomes compared to the X chromosome when populations are modeled as a mixture of Anatolia Neolithic and WHG. A positive score indicates that there is more hunter-gatherer ancestry on the autosomes and therefore the hunter-gatherer ancestry is male-biased. **C**: Hunter-gatherer ancestry proportions on the autosomes, X chromosome, mitochondria (i.e. mt haplogroup U), and Y chromosome (i.e. Y chromosome haplogroups I2, R1 and C2). Bars show approximate 95% confidence intervals.

Extended Data Figures

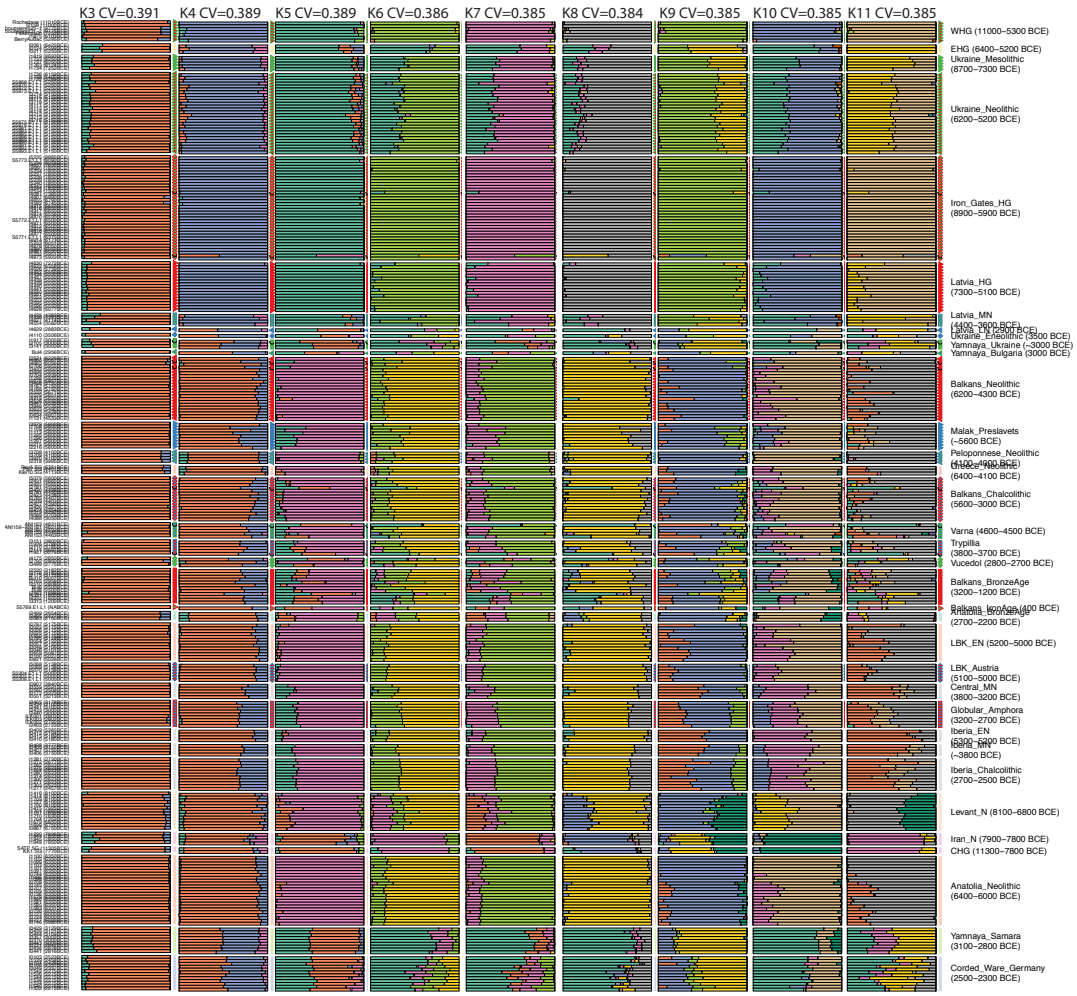


Afanasievo.SG
AfontovaGora3
Alberstedt_LN
ALPc_MN
Anatolia_BronzeAge
Anatolia_Neolithic
Anatolia_Neolithic_Boncuklu.SG
Anatolia_Neolithic_Kumtepe.SG
Anatolia_Neolithic_Tepecik_Ciftlik.SG
Andronovo.SG
Baden_LCA
Balkans_Chalcolithic_outlier
Balkans_Neolithic_outlier
BattleAxe_Sweden.SG
Bell_Beaker_Czech.SG
Bell_Beaker_Germany
Bell_Beaker_Germany.SG
BenzigerodeHeimburg_LN
Buekk_MN
Central_MN
CHG
Corded_Ware_Estonia.SG
Corded_Ware_Germany
Corded_Ware_Germany.SG
Corded_Ware_Proto_Unetice_Poland.SG
EHG
ElMiron
Germany_Bronze_Age.SG
Greece_Neolithic
Halberstadt_LBA
Hungary_LBA
Hungary_Mako_EBA
Hungary_MBA.SG
Iberia_Chalcolithic
Iberia_EN
Iberia_MN
Iran_N
Iron_Gates_HG_outlier
Karsdorf_LN
Koros_EN
Koros_HG
LBK_EN
LBKT_MN
Lengyel_LN
Levant_N
MA1_HG.SG
Maros.SG
Motala_HG
Nordic_BA.SG
Nordic_LBA.SG
Nordic_LN.SG
Nordic_MN_B.SG
Poltavka
Poltavka_outlier
Potapovka
Remedello_BA.SG
Russia_EBA.SG
Samara_Eneolithic
Scythian_IA
Sintashta_MBA_RISE.SG
Srubnaya
Srubnaya_Outlier
Starounetice_EBA.SG
Trypillia_outlier
Unetice_EBA
Unetice_EBA.SG
Varna_outlier
Vatya.SG
Vestonice16
Villabruna
Yamnaya_Kalmykia.SG
Yamnaya_Samara
Yamnaya_Ukraine_outlier
Balkans_BronzeAge
Balkans_Chalcolithic
Balkans_Neolithic
Globular_Amphora
Iron_Gates_HG
Latvia_HG
Latvia_LN
Latvia_MN
LBK_Austria
Malak_Preslavets
Peloponnese_Neolithic
Starcevo
Trypillia
Ukraine_Eneolithic
Ukraine_Mesolithic
Ukraine_Neolithic
Varna
Vucedol
WHG
Yamnaya_Bulgaria
Yamnaya_Ukraine

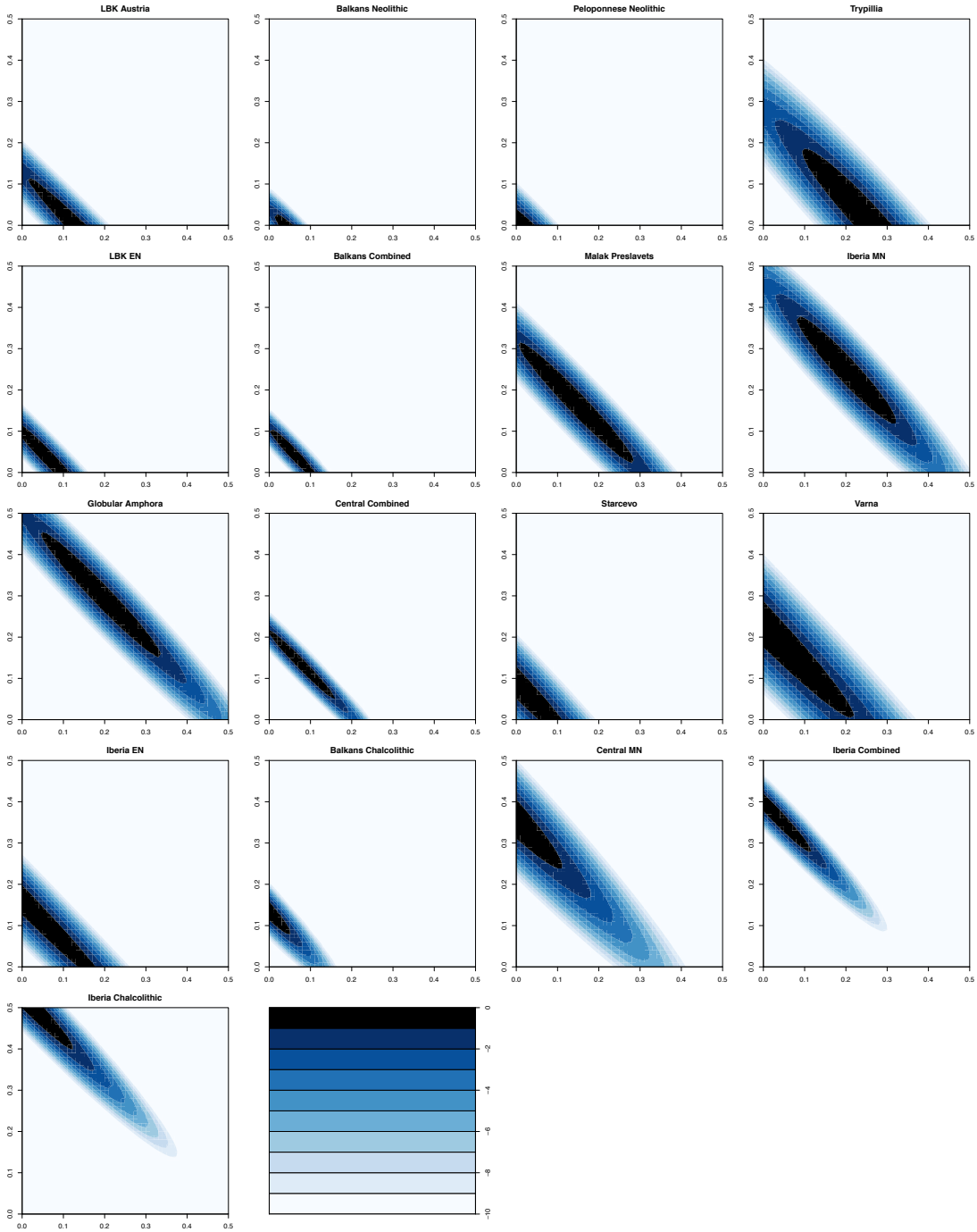
Extended Data Figure 1: PCA of all ancient individuals, projected onto principal components defined by 799 present-day West Eurasian individuals. (This differs from Figure 1B in that the plot is not cropped and the present-day individuals are shown.)



Extended Data Figure 2: Supervised admixture plot for K=3 to 6. We ran on 357 ancient individuals and show newly reported ancient individuals with some previously published ancient individuals for comparison. K=4 is shown in Figure 1D. Clusters constrained to include individuals from Anatolia_Neolithic (grey), WHG (blue), EHG (red), Yamnaya_Samara (orange), Ukraine_Mesolithic (purple), Motala_HG (green).



Extended Data Figure 3: Unsupervised ADMIXTURE plot from k=3 to 11, on a dataset consisting of 799 present-day individuals and 405 ancient individuals. We show newly reported ancient individuals and some previously published individuals for comparison.



Extended Data Figure **4**: log-likelihood surfaces for the proportion of female (x-axis) and male (y-axis) ancestors that are hunter-gatherers for the populations analysed in Figure 3. Log-likelihood scale ranges from 0 to -10, where 0 is the feasible point with the highest likelihood.

Supplementary Tables

Supplementary Table 1: Details of ancient individuals analyzed in this study.

Supplementary Table 2: Key *D*-statistics to support statements about population history.

Supplementary Table 3: *qpAdm* models with 7-population outgroup set.

Supplementary Table 4: *qpAdm* models with extended 14-population outgroup set.

Supplementary Table 5: *qpAdm* models for Neolithic populations for chromosome X.

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