



## Interim Report

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**One-sentence Summary:**

Ancient mitochondrial DNA shows that local hunter-gatherers were not the ancestors of Central and North-Eastern Europe's first farmers.

**Abstract:**

Following the domestication of animals and crops in the Near East some 11,000 years ago, farming reached much of Central Europe by 7,500 before present. The extent to which these early European farmers were immigrants, or descendants of resident hunter-gatherers who had learnt farming, has been widely debated. We compare new mitochondrial DNA (mtDNA) sequences from late European hunter-gatherer skeletons with those from early farmers, and from modern Europeans. We find large genetic differences between all three groups that cannot be explained by population continuity alone. Most (82 %) of the ancient hunter-gatherers share mtDNA types that are relatively rare in Central Europeans today. Together, these analyses provide persuasive evidence that the first farmers were not the descendants of local hunter-gatherers but immigrated into Central Europe at the onset of the Neolithic.

**Text:**

Europe has witnessed several changes in archaeological cultures since anatomically modern humans displaced the Neanderthal population 30-40,000 years ago (1, 2). Palaeolithic hunter-gatherers survived the Last Glacial Maximum (LGM) about 25,000 years ago in Southern and Eastern refugia (3), and resettled Central Europe following the retreat of the ice sheets. With the end of the Ice Age ~9,600 BC their Mesolithic descendants or successors had recolonized large parts of the deglaciated northern latitudes (4, 5). From around 6,400 BC the hunter-gatherer way of life gave way to farming cultures in a transition known as the “Neolithic Revolution” (6). The extent to which this important cultural transition was mediated by the arrival of new peoples, and the degree of European Mesolithic and early Neolithic ancestry in Europeans today, has been debated for more than a century (7-10).

To address these questions directly, we have obtained mitochondrial DNA types from 22 Central and Northern European post-LGM hunter-gatherer skeletal remains (Figure 1), and compared 20 of these – those for which full sequence information was available – to homologous mtDNA sequences from 25 early farmers (11, 12) and 484 modern Europeans, from the same geographic region. Our ancient sample spans a period from *ca.* 13,400 BC to 2,300 BC and includes bones from Hohle Fels in the

Ach valley (Late Upper Paleolithic) and Hohlenstein-Stadel in the Lone valley (Mesolithic). Extensive precautions were taken to ensure sequence authenticity (13), including extracting independent samples from different skeletal locations of the same individuals and examining remains only from high latitudes or cave sites with good biomolecular preservation.

An analysis of the molecular variance (14) showed that our early farmer and hunter-gatherers were from two well-differentiated populations ( $F_{ST} = 0.163$ ;  $P < 10^{-6}$ ). To compare with, two random modern day European samples would show  $F_{ST}$  values around # MT ###. We also found that our modern European sample was significantly different from the early farmer ( $F_{ST} = 0.0580$ ;  $P = 10^{-5}$ ) and the hunter-gatherer ( $F_{ST} = 0.0858$ ;  $P < 10^{-6}$ ) samples. To test if these genetic differences can be explained under the null hypothesis of population continuity alone, we performed coalescent simulations across a wide range of ancestral population size combinations. We conservatively assumed a modern female effective population size of  $N_0 = 12,000,000$  (one-tenth of the current female population size of Central and Northern Europe) and two periods of exponential growth; the first following an initial colonization of Europe 45,000 years ago of female effective population size  $N_{UP}$ , sampled from an ancestral African population of constant female effective size  $N_A = 5,000$ , and the second following the Neolithic transition in Central Europe 7,500 years ago of effective population size  $N_N$  (13). We sampled sequences from each simulation according to the numbers (hunter-gatherer  $N = 20$ ; early farmer  $N = 25$ , Modern  $N = 484$ ) and dates (see Table 1) of the data presented here and found the proportion of simulated  $F_{ST}$  values that were greater than those observed ( $P_{S>O}$ ). By exploring all combinations of 100 values for  $N_{UP}$  (ranging from 10 to 5,000) and 100 values for  $N_N$  (ranging from 1,000 to 100,000), we found the maximum  $P_{S>O}$  value between hunter-gatherers and early farmers was 0.022 (for  $N_{UP} = 4960$  and  $N_N = 1000$ ), and the maximum  $P_{S>O}$  value between hunter-gatherers and modern Central Europeans was 0.028 (for  $N_{UP} = 3560$  and  $N_N = 1000$ ). Most  $P_{S>O}$  values were considerably lower (see Figure 2). These results allow us to reject direct continuity between hunter-gatherers and early farmers, and between hunter-gatherers and modern Europeans.

When we considered continuity between early farmers and modern Europeans we did identify ancestral population size combinations where  $P_{S>O} > 0.05$  (black shaded area on Figure 2). Thus, there are demographic conditions under which the observed

genetic differences between early European farmers and modern Europeans can be explained by assuming population continuity. Those conditions include assuming  $N_N < 3000$ , an effective female population size that may be considered implausibly low and is certainly lower than the current archaeological census estimates of 124,000 (15). However, we note that (i) ancestral population sizes are notoriously difficult to estimate from archaeological data, and (ii) the relationship between effective and census population size is dependent on unknown factors including mating systems and population sub-structure.

Most modern European mitochondrial DNA lineages can be assigned to one of following clades or haplogroups: H, V, U (including K), J, T, all deriving from clade R; and I, W, X, the descendants of clade N. While some subclades, such as U5, are fairly specific to Europe, most are shared with adjacent areas of Asia and North Africa and are of uncertain antiquity in Europe. We are therefore cautious of treating specific clades as markers of particular past population groups or demographic episodes (16). Nonetheless, it is intriguing to note that 82% of our 22 hunter-gatherer individuals carried clade U (fourteen U5, two U4, and two unspecified U-types; table 1). A high incidence of U types (particularly those belonging to the U5 subclade) in Stone Age Europeans has been inferred from modern mtDNA (e.g. 7), but the frequencies found here are surprisingly high. Europeans today have moderate frequencies of U5 types, ranging from about 1-5% along the Mediterranean coastline to 5-7% in most core European areas, and rising to 10-20% in northeastern European Uralic-speakers, with a maximum of over 40% in the Scandinavian Saami. U4 types show frequencies between 1 % and 5 % in most parts of Europe, with western Europe at the lower end of this range, and northeastern Europe and central Asia showing percentages in excess of 7%.

The diversity among the hunter-gatherer U types presented here, together with their continued presence over 11 millennia, and the fact that U5 is rare outside Europe, raise the possibility that U types were common by the time of the post-LGM repopulation of Central Europe, which started around 23,000 years ago (3). In a previous study, we showed that the early farmers of Central Europe carried mainly N1a and H, but also J, K, T, V, and U3 types (11, 12). We found no U5 or U4 types in that early farmer sample. Conversely, no N1a- or H-types were observed in our hunter-gatherer sample, confirming the genetic distinctiveness of these two ancient

population samples. This is particularly surprising as there is clear evidence for some continuity in the material culture between Central European Mesolithic and the earliest LBK settlements (17). Thus, it seems that despite exchange of stone artifacts, genetic exchange between both groups, at least on the female side, was initially limited.

Taken together, our results indicate that the transition to farming in Central Europe was accompanied by a substantial influx of people from outside the region who, at least initially, did not mix significantly with the resident female hunter-gatherers. In this respect Ostorf is important among our sample sites, because culturally it was a mesolithic island surrounded by neolithic early farmers, and it is the only hunter-gatherer site where we found some non-U mtDNA types alongside the ubiquitous hunter-gatherer U types (Table 1). Further sampling from such local contexts may well shed further light on mesolithic-neolithic genetic contacts. For now, extent to which modern Europeans are descended from incoming farmers, their hunter-gatherer forerunners, or later incoming groups, remains unresolved. The predominant mtDNA types found in the ancient samples considered in this study are found in modern Europeans, but at considerably lower frequencies, suggesting that the diversity observed today cannot be explained by admixture between hunter-gatherers and early farmers alone. If this is the case, then subsequent dilution through migration and admixture, after the arrival of the first farmers, would need to be invoked, implying multiple episodes of population turnover which are not necessarily observable in the archaeological record. This, in turn, would mean that the classic model of European ancestry components (contrasting hunter gatherers versus early Neolithic farming pioneers) requires revision.

The geographic origin of the demographic processes that brought the early farmer mtDNA types to Central Europe now becomes a major question. On the one hand, all of the early farmer remains analyzed here are associated with the LBK culture of Central Europe. Based on ceramic typology, the LBK culture is thought to have originated in present day western Hungary and southwestern Slovakia, with a possible predecessor in the southeast European Starčevo-Kris culture (18, 19). These cultural source locations may provide the most plausible origins or routes for the geographic spread of the early farmers, considering the LBK was the first major farming culture in Central and Northern Europe, is archaeologically attested to have disseminated

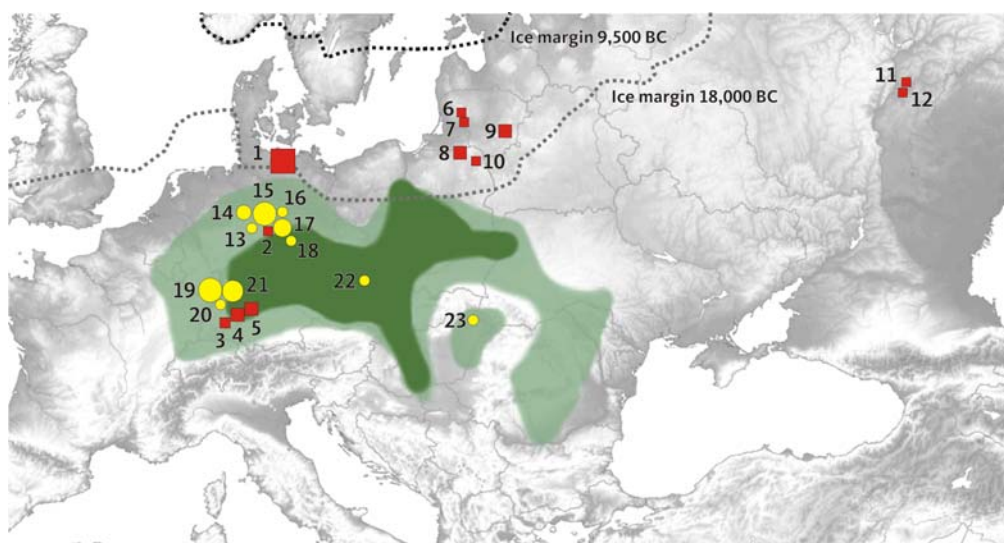
over five centuries, and covered nearly a million square kilometers. Alternatively, the farmers' mtDNA types may have an origin closer to the Neolithic core zone in southwestern Asia. Further ancient DNA analysis of early farmer samples from southeastern Europe and Anatolia will be required to resolve this question.

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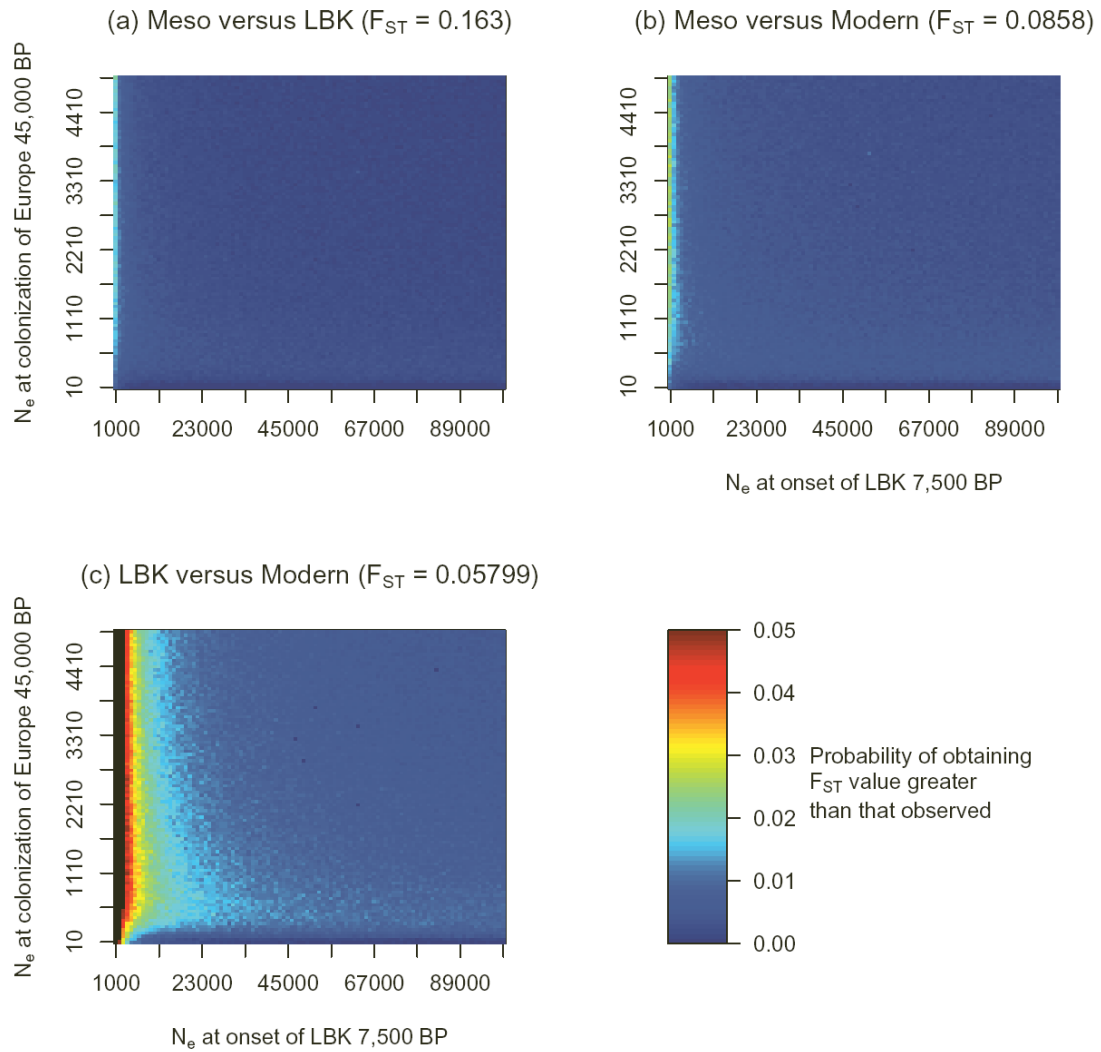
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**Fig. 1: mtDNA types from prehistoric samples of hunter-gatherers and farmers.** The green shading represents the first farming areas (Neolithic LBK culture, 5.500-5.000BC) in Central Europe, based on archaeological finds, while squares represent successfully analysed Late Palaeolithic, Mesolithic and Ceramist hunter-gatherers dating from 13,400BC- 2300BC. The term ‘Neolithic’ is sometimes applied to the eastern European Ceramist culture because of their use of pottery, but this does not imply a farming economy (20). Previously analysed (11, 12) LBK farming sites are marked with circles for comparison. The area of each square or circle is proportional to the number of individuals successfully investigated. In red are labelled archaeological sites with one or more U4/U5 individuals; in yellow, sites with other mitochondrial DNA types, highlighting the specificity of U types in the prehistoric hunter-gatherers. The sites are as follows: 1. Ostorf; 2. Bad Dürrenberg; 3. Falkensteiner Höhle; 4. Hohler Fels; 5. Hohlenstein-Stadel; 6. Donkarnis; 7. Spiginas; 8. Dudka; 9. Kretuonas; 10. Drestwo; 11. Chekalino; 12. Lebyazhinka; 13. Unseburg; 14. Unterwiederstedt; 15. Derenburg/Meerenstieg; 16. Eilsleben; 17. Halberstadt; 18. Seehausen; 19. Flomborn; 20. Vaihingen an der Enz; 21. Schwetzingen; 22. Asparn/Schletz; 23. Ecsegfalva.



**Fig. 2. Probabilities of obtaining observed genetic differences**, as measured by  $F_{ST}$ , between (a) Mesolithic hunter-gatherers and LBK early farmers, (b) Mesolithic hunter-gatherers and modern Europeans, and (c) LBK early farmers and modern Europeans, across a range of assumed ancestral population size combinations. Two phases of exponential growth were considered, the first following the initial colonization of Europe 45,000 years ago, of assumed effective female population size  $N_{UP}$  (y-axis) and ending when farming began in Central Europe 7,500 years ago when the assumed effective female population size was  $N_N$  (x-axis), and the second leading up to the present, when the assumed effective female population size is 12 million. The initial colonizers of Europe were sampled from a constant ancestral African population of 5000 effective females. The  $F_{ST}$  values are those observed from the data presented in this study. The black shaded area indicates probabilities  $> 0.05$ .

**Table 1. Stone Age individuals and their mtDNA results. Notes:** A = DNA of the archaeologists available for comparison, D = diagenetical analysis, M = multiple extractions and number of these, C = clones of HVS-I and number of these, N = positive amplification of nuclear DNA; Rf = RFLP analysis; SNP = SNPs from the coding region of mtDNA obtained by means of multiplex amplification. The mtDNA was sequenced from np 15997 to np 16409. mtDNA positions are numbered according to the rCRS (21), minus 16,000. Fourteen individuals did not yield results (tab. S1), whereas for two individuals the mtDNA sequences were not determined (n.d.), thus not considered in the AMOVA analysis. (\*) Radiocarbon dates with Laboratory-numbers refer to direct dates of the skeleton and were calibrated with the program CalPal (22) on the basis of Intcal04. Corrections of reservoir effects were applied where identified.

Country	Site, skeleton	Basis of dating(*)	Dating calBC(*)	Analyses	mtDNA Sequence	Mt-type
Lithuania	Spiginas 4	GIN-5571: 7470 ± 60 BP	ca. 6350 calBC	A, M3, C109, Q, Rf	<b>356c</b>	<b>U4</b>
	Donkalis 1	Cultural context	Mesolithic	A, D, M4, C79, N, Rf, SNP	<b>192t 270t</b>	<b>U5b2</b>
	Kretuonas 3	OxA-5926: 5580 ± 65 BP	ca. 4450 calBC	A, M4, C72, N, Rf, SNP	<b>192t 270t</b>	<b>U5b2</b>
	Kretuonas 1	OxA-5935: 5350 ± 130 BP	ca. 4200 calBC	A, M5, C56, N, Rf, SNP	<b>192t 270t</b>	<b>U5b2</b>
Poland	Dudka 2	<sup>14</sup> C-date on charcoal	ca. 3650 calBC	A, M3, C80, N, Rf	<b>189c 270t</b>	<b>U5b1</b>
	Dudka 3	Cultural context	4000-3000 calBC	A, M3, C127, Q, Rf	<b>189c 265g 270t</b>	<b>U5b1</b>
	Drestwo 2	Ua-13085: 3805 ± 70 BP	ca. 2250 calBC	D, M4, C102, N, Rf	<b>192t 256t 270t</b>	<b>U5a</b>
Russia	Chekalino IVa	<sup>14</sup> C-date shell	ca. 7800 calBC	A, D, M2, C83, Rf	<b>192t 256t 270t</b> <b>294t</b>	<b>U5a</b>
	Lebyazhinka IV	<sup>14</sup> C-date shell and cultural context	8000-7000 calBC	A, D, M2, C60, Rf	<b>192t 241a/c</b> <b>256t 270t 399g</b>	<b>U5a1</b>
Germany	Bad Dürrenberg 2	OxA-3136: 7930 ± 90 BP	ca. 6850 calBC	A, D, M2, C 119, Rf	<b>356c</b>	<b>U4</b>
	Hohlenstein-Stadel, 5830a	ETH-5732: 7835 ± 80 BP	ca. 6700 calBC	M1, SNP	<b>114a 192t 256t</b> <b>294t 311c</b>	<b>U5a1</b>
	Hohlenstein-Stadel, 5830b	ETH-5732: 7835 ± 80 BP	ca. 6700 calBC	M1, SNP	<b>192t 270t</b>	<b>U5b2</b>
	Hohler Fels, 49 Ib1 66	<sup>14</sup> C-dates bone (H 5312-4907: 12 770 ± 110 BP; H 5119-4601: 13 085 ± 95 BP) and cultural context	Magdalenian ca. 13,400 calBC	M2, SNP	<b>CRS</b>	<b>U</b>

Germany	Hohler Fels, 10 Ic 405	<sup>14</sup> C-dates bone (H 5312-4907: 12 770 ± 110 BP; H 5119-4601: 13 085 ± 95 BP) and cultural context	Magdalenian ca. 13,400 calBC	M2, SNP	<b>n.d.</b>	<b>U</b>
	Falkensteiner Höhle, FH	ETH-7615: 8185 ± 80 BP	ca. 7 200 calBC	M2, SNP	<b>n.d.</b>	<b>U5b2</b>
	Ostorf SK28a	<sup>14</sup> C-dates and context	ca. 3200 calBC	A, M2, C18	<b>224c 311c</b>	<b>K</b>
	Ostorf SK8d	<sup>14</sup> C-dates and context	ca. 3200 calBC	A, M2, C16	<b>270t</b>	<b>U5</b>
	Ostorf SK35	<sup>14</sup> C-dates and context	ca. 3100 calBC	A, M2	<b>270t</b>	<b>U5</b>
	Ostorf SK12a	<sup>14</sup> C-dates and context	ca. 3000 calBC	A, M2	<b>093y 126c 153a 294t</b>	<b>T5</b>
	Ostorf SK45a	<sup>14</sup> C-dates and context	ca. 3000 calBC	A, M2, C16	<b>069t 126c</b>	<b>J</b>
	Ostorf SK18	<sup>14</sup> C-dates and context	ca. 3000 calBC	A, M4	<b>093c 126c 153a 294t</b>	<b>T5</b>
	Ostorf SK19	<sup>14</sup> C-dates and context	ca. 2950 calBC	A, M3	<b>168t 192t 256t 270t 302g</b>	<b>U5a</b>