



Was the Fishing Village of Lepenski Vir Built by Europe's First Farmers?

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Accepted: 12 August 2022 / Published online: 11 October 2022
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Abstract

It is now widely accepted that agriculture and settled village life arrived in Europe as a cultural package, carried by people migrating from Anatolia and the Aegean Basin. The putative fisher-forager site of Lepenski Vir in Serbia has long been acknowledged as an exception to this model. Here, the Mesolithic–Neolithic transition—possibly inspired by interaction with the new arrivals—was thought to have taken place autochthonously on site. Our reinterpretation, based on ancient genomes, as well as archaeological and isotopic evidence, indicates that here, too, house construction, early village society and agriculture were primarily associated with Europe's first farmers, thus challenging the long-held view of Lepenski Vir as a Mesolithic community that adopted Neolithic practices. Although aspects of the site's occupation, such as the trapezoidal houses, were inspired by local Mesolithic traditions, it is far from certain that the village was founded by Iron Gates foragers. A detailed timeline of population changes at the site suggests that Aegean incomers did not simply integrate into an established Mesolithic society, but rather founded new lineages and households. Iron Gates foragers and their admixed descendants largely appear to have been buried separately, on the fringes of the settlement. The diet of those buried outside in pits shows no major shift from aquatic to terrestrial food resources.

Keywords Farmer–forager interactions · Neolithic · Ancient DNA · Stable isotopes · Southeast Europe · Iron Gates

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Introduction

Whether the spread of agriculture into Europe was mediated by migration of new farming groups into the region or adoption by indigenous hunter-gatherer communities is one of the most important and long-standing questions of prehistoric archaeology. Traditionally cast as a complex and multi-faceted issue, the Neolithic or agricultural transition in Continental Europe is now thought to have primarily involved significant human migration from Anatolia and the Aegean Basin, accompanied in places by low levels of admixture with European Mesolithic populations (Bramanti et al., 2009; Hofmanová et al., 2016; Mathieson et al., 2015), as first postulated by Albert Ammerman and Luca Cavalli-Sforza in their ‘wave of advance’ model (Ammerman & Cavalli-Sforza, 1984). The farmers who migrated to Southeast and Central Europe in the 7th–6th millennia BC brought with them, in addition to plant and animal domesticates, new modes of production and symbolic activities centred on domestic houses and small village communities (Borić, 2008; Cauvin, 2000; Hodder, 1990; Özdoğan, 2011).

While the latest ancient DNA (hereafter aDNA) studies unequivocally show a small Mesolithic contribution to the Danubian Neolithic gene pool, of the order of 2–9% (Hofmanová et al., 2016; Lipson et al., 2017; Marchi et al., 2022; Mathieson et al., 2018; Nikitin et al., 2019), it is not clear how, practically, early farmers and foragers interacted at regional and site levels, nor if these interactions led to Mesolithic communities adopting agriculture and settled village life, as presumed by traditional frontier expansion models in archaeology (Dennell, 1983; Zvelebil & Rowley-Conwy, 1984; Voytek & Tringham, 1990; Zvelebil & Lillie, 2000). An equally plausible scenario, in view of the genomic data, is that European Mesolithic populations were gradually absorbed into Early Neolithic farming communities without an autochthonous Mesolithic–Neolithic transition (Shennan, 2018). Near the Blätterhöhle Cave of northwestern Germany, for example, foragers and early farmers were found to have lived side by side for 2000 years without a significant shift in subsistence strategies, despite clear signals of asymmetric admixture (Bollongino et al., 2013; Lipson et al., 2017).

Long viewed as a missing link between classical Mesolithic and Early Neolithic cultural groups of the Danubian region (Srejski, 1969), Lepenski Vir in today’s Serbia is represented in most archaeological textbooks as an atypical Mesolithic village that successfully exploited its rich and varied ecological niche to provide an alternative path to Neolithic complexity. Stable isotopic analyses, conducted since the mid 1990s on skeletal remains from the site, indicate that two distinct dietary patterns were present: one based on the exploitation of aquatic resources, the other on a mixture of aquatic and terrestrial resources, possibly reflecting changes in foraging practices and/or the actual introduction of domesticates (Fig. S1) (Bonsall et al., 1997, 2004). Although it is widely accepted that changes in subsistence strategies came about through interaction with farming communities outside the gorge, the idea of a local transformation from a predominantly Mesolithic to a predominantly Neolithic culture at Lepenski Vir has remained broadly unchallenged (Borić, 2019, p. 43). The site is thus ideally suited

to a re-examination of the processes behind the Neolithic transition in Europe and an exploration of patterns of Mesolithic–Neolithic interactions at a micro-scale, along one of the main corridors of early agricultural dispersal.

Set in the spectacular landscape of the Iron Gates, on the right bank of the Danube, c. 160 km downstream from Belgrade (Fig. 1), Lepenski Vir is thought to have been reached mainly by river, and is connected by a mountain path to the 2-km-long Boljetinska valley, which provides areas of pasture (Srejšović, 1969, p. 26). It is one of a series of sites that controlled access through the Iron Gates gorge, potentially acting as an important gateway for early farming expansion in Europe. Lepenski Vir was first used as a burial location during the eponymous ‘Proto-Lepenski Vir’ phase (~9500–7300 BC). Open-air stone rectangular hearths, as well as scatters of bone and lithics, were found underneath later building floors, suggesting periodic occupation by Iron Gates Mesolithic populations (Borić, 2003, p. 53). The site was resettled after a hiatus of at least a thousand years ~6200 BC, when the first houses with trapezoidal limestone floors were constructed (Table 1) (Bonsall et al., 2002; Borić, 2007, 2016). These semi-subterranean structures (‘pit-houses’), often described as ‘huts’ in the literature, range from 2 to 54 m² in size (median = 14 m²), and display

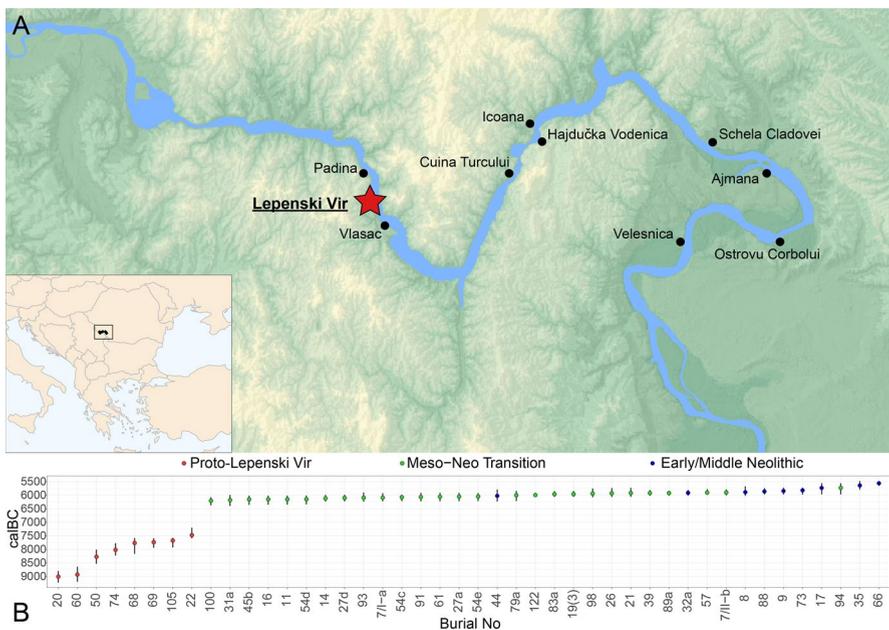


Fig. 1 **A** Map of the Iron Gates with the location of key Mesolithic and Neolithic sites including Lepenski Vir; topographic map © EuroGeographics; **B** temporal distribution of ¹⁴C dated burials of Lepenski Vir (full references in Electronic Supplementary Material 1). Dates were corrected for freshwater reservoir effect (Bonsall et al., 2015) and uniformly re-calibrated in OxCal 4.4.2 (Ramsey, 2009) using the IntCal20 calibration curve (Reimer et al., 2020). While Proto-Lepenski Vir and Mesolithic–Neolithic Transition burials are separated by a hiatus, Early/Middle Neolithic ones overlap chronologically with those of the preceding phase

Table 1 The main phases of occupation at Lepenski Vir and associated features

Early–Middle Neolithic (E–MN) c. 5900–5500 BC	LV IIIb LV IIIa	Quadrangular mud houses with domed ovens First domesticates (cattle/sheep, goats, pigs), pit dwellings, impressed, incised and painted pottery, flexed burials in oval pits, ‘cemetery’
Transformational/Early Neolithic (TEN) c. 6200–5900 BC	LV II LV Ie LV Id LV Ic LV Ib	Trapezoidal buildings with stone foundations, stone statues Subfloor burials (neonates) Vertically superimposed trapezoidal houses, both intramural and extramural burials in extended supine position
Late Mesolithic	LV Ia hiatus	First houses with trapezoidal limestone floors, organized settlement layout with radial axes, few intramural burials, head-like stone statues, monochrome pottery
Early/Middle Mesolithic c. 9000–7400 BC	Proto-Lepenski Vir	Rectangular stone-lined hearths; burial in extended supine and seated positions

Phasing after Borčić (2016), with modifications

remarkable geometric sophistication. The trapeziform shape was achieved by levelling a platform in the shape of the sector of a circle with the rear end cut into the hillslope (Srejšović, 1974, p. 364; Bonsall, 2008, p. 256; Perić & Nikolić, 2011, p. 54). There is still considerable uncertainty regarding the origin and function of the trapezoidal buildings at Lepenski Vir, which recall simpler structures observed in the region, but also display conspicuous Neolithic features, such as intramural burials, elaborate lime plaster floors with thin red or white polished surfaces, and a strict internal layout, segmented into zones for cooking and burial activities (Bonsall et al., 2008).

The rapid construction of the village as a planned settlement (confirmed by Bayesian modelling of published radiocarbon dates associated with transitional phase contexts: see Porčić & Nikolić, 2016), with rows of trapezoidal houses overlooking the river, coincides with the advent of agriculture in the Central Balkans (Fig. S2) (Krauß et al., 2018; Porčić et al., 2016; Živaljević et al., 2021). Yet with the exception of the dog, domesticates (sheep, goat, cattle and pig) are not found at the site until the latest phase of occupation, ~5900 BC, in the context of disused trapezoidal houses and layers with pit dwellings (Bökönyi, 1970; Borić & Dimitrijević, 2007). Given its location on the steep lower slope of the Koršo mountain, with no adjacent hinterland for cereal agriculture, it is unlikely that Lepenski Vir was ever a food-producing or Neolithic site in a traditional sense. Still, about 20% of the animal bones from Level III belong to domesticated species—older assemblages being too small to categorically rule out animal husbandry (Bonsall et al., 1997, p. 57). Small-scale agriculture, a few kilometres away through the gorge or in the hillsides, has not been proven but remains theoretically possible (Nandris, 1968, p. 67)—no systematic attempt at recovery of plant macro-remains has been made at the now submerged site (Bonsall et al., 1997, p. 57).

For this study, we revisited the interpretation of Lepenski Vir as a permanent settlement of hunters and gatherers in light of recent aDNA evidence for interactions between fisherfolk communities of the Iron Gates and incoming Aegean farmers ~6200 BC (González-Fortes et al., 2017; Hofmanová, 2016; Hofmanová et al., 2022; Marchi et al., 2022; Mathieson et al., 2018). Previously reported strontium isotope results seem consistent with a model in which ‘non-local’ Neolithic women, or at least a clear predomination of women, intermarried into the native Mesolithic population (Fig. S3) (Borić & Price, 2013). Such a scenario would result in sex-biased admixture, that is, predominantly male foragers mating with female farmers, and a predominance of females among Aegean incomers. The forager culture might also be expected to be ‘dominant’ at the site if migrants simply integrated into existing family structures (Borić, 2019; Borić et al., 2018). This is at odds with recent genetic evidence for both male and female incomers at Lepenski Vir (de Beccdelièvre et al., 2020; Hofmanová, 2016) and remains to be tested, taking into consideration a broader sample of burials, as well as the age and archaeological context of the burials.

To understand who was buried where at Lepenski Vir and how burials relate to the built environment of the site, we re-examined the context of deposition of 34 individuals that have produced autosomal or mitochondrial DNA data (Table 2). We compared our results with those obtained at other Iron Gates sites, giving a total of

Table 2 List of previously-reported Lepenski Vir genetic data used for this study and summary archaeological, anthropological, chronological and genetic information (for full details, see Electronic Supplementary Material 1; for molecular sexing results, see Electronic Supplementary Material 2)

Burial No.	Genetic ID	Sex	Age	Body position	cal BC (OxCal μ)	Ancestry	mtDNA	Y	References
<i>Mesolithic (c. 9500–7400 BC)</i>									
20	Lepe12	f	Adult	FL	9006	.	U5	–	(1), (2)
68	Lepe51	XX	Adult	ES	7764	Iron Gates HG	U4	–	(1), (2)
74	Lepe34	XX	Adult	FL	8017	.	U5	–	(1), (2)
105	Lepe47	XX	Adult	D	7674	.	U5	–	(1), (2)
126	15407/ Lepe49	XX	Adult	D	.	Iron Gates HG	H	–	(1), (2), (3)
<i>Mesolithic–Neolithic Transition (c. 6200–5900 BC)</i>									
11	Lepe7	XX	Subadult	ES	6153	.	K	–	(1), (2)
21	Lepe13	XY	Adult	D	5921	.	X	.	(1), (2)
26	Lepe15	m	Adult	ES	5930	.	H	.	(1), (2)
27a	Lepe53	XX	Adult	ES	6051	Admixed	U5	–	(1), (2)
27b	Lepe17	XY	Adult	ES	.	.	N1a	.	(1), (2)
27d	Lepe18	XY	Adult	ES	6099	Admixed	U5	I2	(1), (2)
39	Lepe22	XX	Adult	D	5921	.	J	–	(1), (2)
43	Lepe23	.	Subadult	D	.	.	K	.	(1), (2)
54d	Lepe28	XX	Adult	ES	6151	.	K	–	(1), (2)
54e	14665/ Lepe27	XX	Adult	ES	6047	Aegean	J	–	(1), (2), (3)
57	Lepe29	XX	Subadult	D	5904	.	U5	–	(1), (2)
61	14666	XY	Child	ES	6056	Aegean	H	R1b	(3)
79b	Lepe37	XY	Adult	D	.	.	U5	.	(1), (2)
79c	Lepe38	m?	Adult	D	.	.	U5	.	(1), (2)
82	Lepe39	XY	Adult	D	.	Aegean	T2	C2	(1), (2)
87(1)	Lepe42	XY	Child	D	.	.	U5	.	(1), (2)
89a	Lepe44	XY	Adult	ES	5920	.	U5	.	(1), (2)
91	Lepe45	XY	Adult	ES	6065	Iron Gates HG	U5	I2	(1), (2)
93	Lepe46	XX	Adult	ES	6091	Admixed	H	–	(1), (2)
122	Lepe48	XY	Subadult	D	5995	Aegean	K	C1	(1), (2), (4)
<i>Early/Middle Neolithic (c. 5900–5500 BC)</i>									
6	Lepe3	.	Subadult	FL	.	.	N1a	.	(1), (2)
8	Lepe6	XX	Adult	FL	5888	.	T1	–	(1), (2)
17	15405	XX	Subadult	FL	5731	Aegean	HV	–	(3)
19	Lepe11	f	Adult	FL	.	.	K	–	(1), (2)
32a	Lepe20	XX	Adult	FL	5912	.	HV	–	(1), (2)
35	Lepe1	XX	Adult	D	5640	.	H	–	(1), (2)
66	Lepe32	XY	Adult	FL	5559	.	N1a	.	(1), (2)
73	Lepe52	XY	Adult	FL	5819	Aegean	H	G	(1), (2), (4)
86	Lepe41	XY	.	D	.	.	J	.	(1), (2)

Table 2 (continued)

Phasing of burials follows Borić (2016), except where new ^{14}C dates change interpretation—i.e. Burials 20, 74 (Jovanović et al., 2021). Genetic sex reported as XX/XY (where available). Anthropological sex reported as m/f. Key: D: disarticulated; ES: extended supine; FL: flexed. References: (1) Hofmanová (2016); (2) Hofmanová et al. (2022); (3) Mathieson et al. (2018); (4) Marchi et al. (2022)

96 individuals with full, partial and/or mitochondrial genome data on which to base our analyses (González-Fortes et al., 2017; Hofmanová, 2016; Hofmanová et al., 2022; Marchi et al., 2022; Mathieson et al., 2018).

Results

Limited Gene Flow Between Aegean and Iron Gates Populations in Pre-Neolithic Times

In line with previous whole-genome and genome-wide studies (Marchi et al., 2022; Mathieson et al., 2018), we find that Iron Gates Mesolithic populations cluster near so-called Western European Hunter-Gatherers (WHG) on a principal component analysis plot, represented for example by Loschbour man in present-day Luxembourg (Fig. 2). However, a recent study by Marchi et al. has shown that southeastern European hunter-gatherers were separated from the central and western European hunter-gatherers since ~20 thousand years ago (Marchi et al., 2022). The ancestry of native Mesolithic populations in the Iron Gates is therefore being described throughout this paper as ‘Iron Gates HG’. Aegean and Central Anatolian foragers and early farmers (hereafter ‘Aegeans’), from e.g. Pinarbaşı, Boncuklu and Barcın, represent a distinct ancestry cluster on the PCA (Feldman et al., 2019; Hofmanová et al., 2016; Kılınç et al., 2016; Mathieson et al., 2018). Consequently, in a two-population admixture scenario, individuals from the Mesolithic or Proto-Lepenski Vir phase (~9500–7400 BC) at the eponymous site of Lepenski Vir are well modelled as 100% Iron Gates HGs, without Aegean ancestry, which arrives only after 6200 BC (Figs. 2; S4A, S4B, S5A, S5B). Even though no obvious geographic barrier to gene flow separates the Danube region and the Aegean—which are connected by, for example, the Vardar–Morava river systems and the Black Sea shoreline (Childe, 1929; Nandris, 1970)—our analyses confirm that Iron Gates populations were not part of the same mating networks as Aegean and Anatolian populations before the advent of agriculture in the Central Balkans (Mathieson et al., 2018).

Several lines of evidence point nonetheless to a small but non-negligible genetic contribution from Aegean to Iron Gates populations in pre-Neolithic times, as previously reported (Mathieson et al., 2018, Table S2): (1) minimal admixture in five HG individuals from Schela Cladovei and Hajdučka Vodenica (Figs. 2; S4A), and (2) unusual mitochondrial lineages traditionally associated with Southwest Asian prehistoric populations, namely K1 and H, already present in the Iron Gates respectively since the 10th and 8th millennia BC (Fig. S6). The significance of these maternal lineages in Mesolithic context depends on the time of admixture between

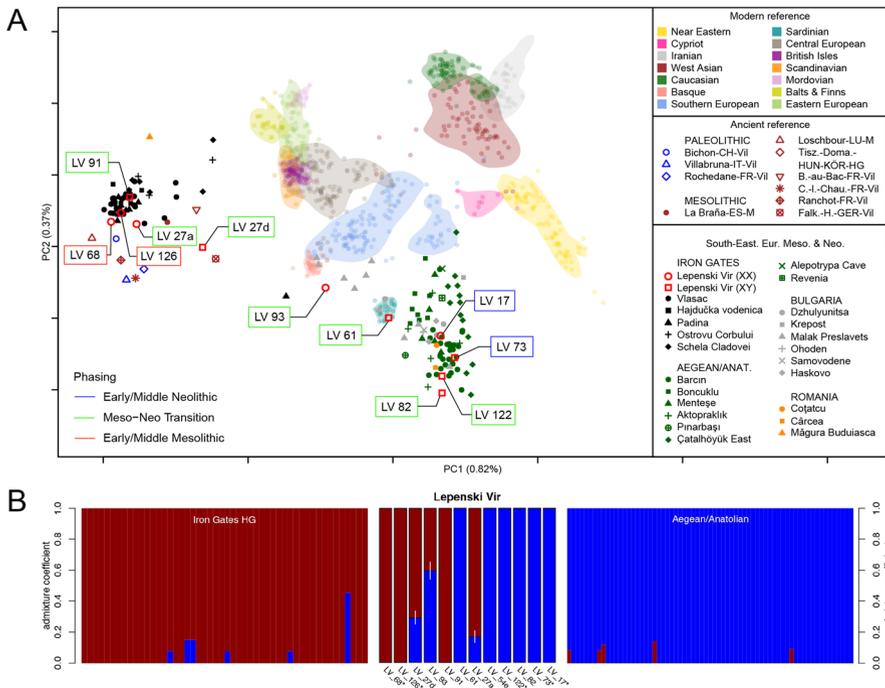


Fig. 2 **A** PCA of modern and projected ancient West Eurasians and Southwest Asians highlighting the Lepenski Vir (LV) individuals of the different phases; **B** ancient individuals shown in (A) modelled as mixtures of Iron Gates HG and Aegean–Anatolian ancestry components with qpADM. Each panel ordered chronologically from oldest to youngest. Lepenski Vir burial No. abbreviated as LV

European and Southwest Asian populations, indicating either recent but limited gene flow from the Aegean across the Aegean–Danubian frontier in Mesolithic times, or traces of a more distant admixture process that gave rise to the Aegean–Anatolian farming ancestry, as found by Marchi et al. (2022). The latter is the more likely cause for remnants of European Mesolithic-like ancestry inferred in five genomes from Epipalaeolithic Pınarbaşı and Neolithic Boncuklu, Çatalhöyük East and Barcın (Fig. S4B). In any case, traces of Southwest Asian ancestry in Iron Gates HG populations are too small to account for the abrupt change in ancestry observed ~6200 BC at Lepenski Vir, leaving migration as the only plausible explanation.

Incoming Aegean Farmers Admixed with Local Hunter-Gatherers but Eventually Supplanted or Fully Assimilated Them

Patterns of admixture observed among individuals buried at Lepenski Vir (and the neighbouring site of Padina) are consistent with the arrival of communities that are closely related to early farmers of the Aegean Basin during the transitional phase (Figs. 2; S4A; S4B). Of the six individuals modelled as deriving ~100% of their ancestry from an Aegean-like source, three are adult or adolescent males

(Burials 73, 82, 122) and one is a child of about eight, genetically male (Burial 61) (Table 2), suggesting that residence practices and mating patterns were not restricted to Neolithic females moving into Mesolithic communities of the Iron Gates, as proposed on the basis of the strontium evidence (Borić & Price, 2013). Even though sample size is small, the fact that residents of both sexes and all ages display virtually ‘unadmixed’ Aegean genetic signatures suggests that entire families, as opposed to individual females, relocated to Lepenski Vir after 6200 BC.

Alternative explanations have been considered to account for the abrupt change in genetic ancestry observed at Lepenski Vir, including the possibility that not all human remains found at the site belonged to people who lived there. A prime contender would be the skull of adolescent male 122 (15–18 years old), which is genetically Aegean-like and was found deposited without a mandible in the packing layer between the floors of trapezoidal houses 47 and 47', at the centre of the settlement. Much of the iconography at Lepenski Vir, including the fish statues, relates to human heads (Srejović, 1972), suggesting they formed a ritual or funerary focus for the community. Burial 122 may relate to the ritual or funerary deposition of a community member; however, it is also possible that the skull was brought to the site as a trophy or a gift.

At least two of the individuals that have produced genomic information consistent with an Aegean origin (Burials 54e and 61) are primary burials stratigraphically associated with residential floors of trapezoidal houses. Fully articulated skeletons may be expected to belong to people who died at the site or were transported there as corpses shortly after death. Burial 54e lay over stone slabs at the floor level of house 65 and was placed within a stone construction or cairn that was erected upon the trapezoidal house (Bonsall et al., 2008, p. 190; Borić, 2016, p. 510). The burial relates to either the end of the house's use life, or a later re-use. Burial 61, on the other hand, appears to have been inserted through the plaster floor of trapezoidal house 40 (Borić, 2016, p. 512); the fact that the burial was sealed by an anthropomorphic sandstone sculpture at the level of the building floor may suggest that the building was still in use when the burial took place. Burials 54e and 61 were both in extended supine position, confirming their attribution to the Mesolithic–Neolithic transition phase.

We observe both the appearance of new mitochondrial lineages (incl. J, X, T, N1a) and the continuation of existing ones (U5, H) during the Mesolithic–Neolithic Transition phase (Fig. S6). Two of the individuals that have to be considered as clearly admixed based on the genomic analysis carry U5 maternal lineages (Burials 27a, 27d). Since U5 lineages are rare among Aegean early farmers, it is likely that these individuals had female Mesolithic ancestors, suggesting that local women were part of the mating network of the incoming group that settled at Lepenski Vir ~6200 BC. Conversely, the presence of the R1b Y haplogroup in the transitional phase and its low frequency in Aegean individuals (none in 32 [95% CI: 0–0.058], Electronic Supplementary Material 1) hints at an HG male contribution to the post-6200 BC gene pool. Consistent with the latter, we observe that Iron Gates HG males were occasionally buried at Lepenski Vir (Burial 91).

Ancestry proportions estimates for one of the admixed males (Burial 27d) are consistent with 75% Iron Gates HG ancestry on the autosomes and 100% HG ancestry on the X chromosome (Fig. S7), as expected on average if this individual had one Aegean and three Iron Gates HG grandparents. Based on this and the X chromosome of individual 27d being of Iron Gates HG descent, a scenario in which a local HG woman was mating with an admixed male is twice as likely as one in which an admixed female was mating with an HG male. The likelihood of the latter scenario producing the observed data is still 0.5. Assuming the U5 mitochondrial lineage in 27d traces back to an HG woman, admixture must have happened at most two generations in the past (in agreement with the scenario presented in Hofmanová et al. (2022) for this individual, there labelled as LEPE18).

The picture changes during the latest phase of occupation at Lepenski Vir ~5900 BC with the disappearance of the once-prevalent U5 maternal clade and the corresponding rise of pre-existing lineages, such as T, N1a and H, suggesting a disproportionate increase of maternal lineages of ultimate Aegean origin (Fig. S6). Considering all available ancient mitochondrial genome data from the Iron Gates, haplogroup diversities (H) are lowest in the Mesolithic phase ($H=0.38$; 95% CI: 0.36–0.41), rising considerably in the transitional phase ($H=0.73$; 95% CI: 0.70–0.77), to settle at a high level in Neolithic-period genomes ($H=0.81$, 95% CI: 0.70–0.92) (Electronic Supplementary Material 1).

In a randomly mating population with no survival or fertility differences leading to differential population growth, we expect two base populations with distinguishable ancestry to evolve towards stable ancestry proportions at the individual level over time. Yet individuals of mixed genomic ancestry are only found in the transitional phase dated to ~6200–5900 BC, whereas later individuals, including adult male 73, are modelled as 100% Aegean. While survival or fertility differences cannot be ruled out, these processes are slow, so that continuous influx of Aegean farmers to the site leading to dilution of Iron Gates HG ancestry over time, or even a single replacement event, are more likely (Fig. 3). Interestingly, individual 73—previously described as ‘local’ (Borić & Price, 2013), albeit with a borderline strontium isotope signature and almost entirely of Aegean ancestry—has a long Run of Homozygosity (~46 cM; Fig. S8; see also Marchi et al., 2022), indicative of recent inbreeding between close relatives (Ringbauer et al., 2021). Although anecdotal, this might be interpreted as a signal of regulated mating patterns during the last phase of occupation at Lepenski Vir (Level III) despite ‘continuous influx’ of Aegean farmers.

Evidence for Structure in the Spatial Distribution of the Burials at Lepenski Vir

We observe structure in the spatial distribution of the burials during the Mesolithic–Neolithic Transition phase, with Aegean-related individuals primarily buried within the habitation space, either inside or between houses, while Iron Gates HG individuals appear to be preferentially buried in the upslope area at the rear of the village, in large multi-inhumation pits (Fig. 3). These pits are unusual features ~3–4 m in diameter, encompassing multiple inhumations, deposited one on top of the other in disarticulated or extended supine position parallel to the Danube, as

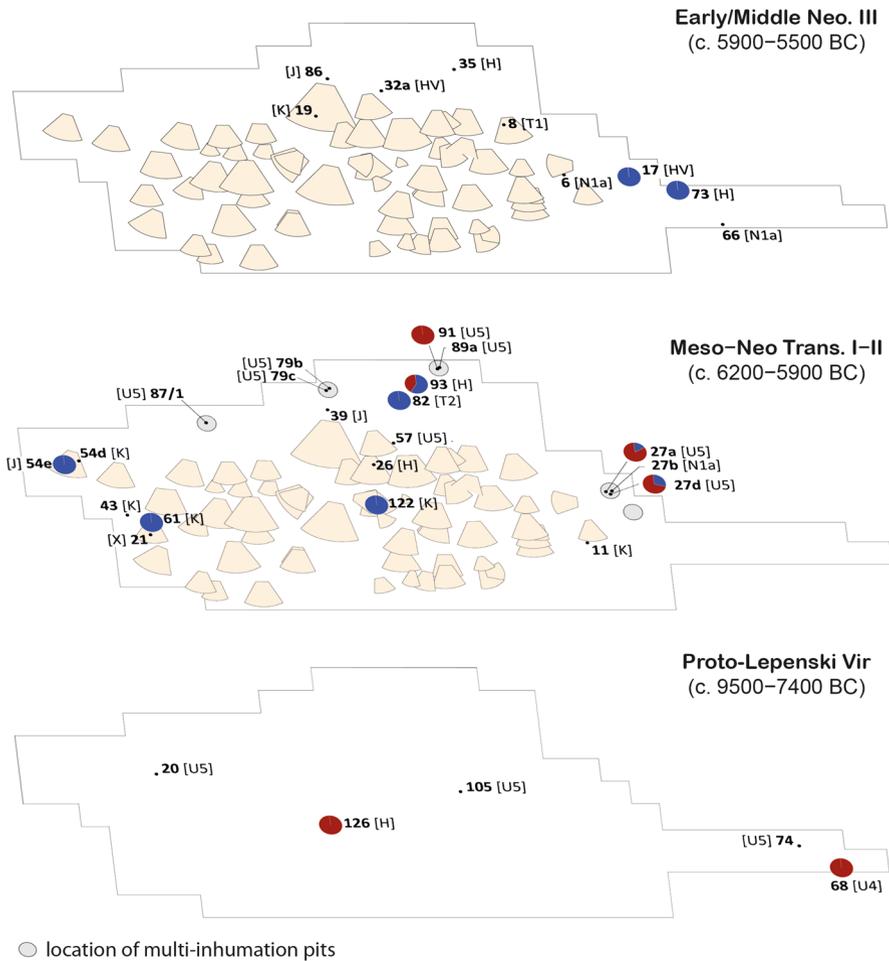


Fig. 3 Distribution of ancestry proportions (see Fig. 2B; pie charts: red: Iron Gates HG; blue: Aegean–Anatolian) and mitochondrial haplogroups (square brackets) for selected individuals during the three main phases of occupation at Lepenski Vir. While trapezoidal houses were no longer in use during the Early/Middle Neolithic phase, their footprints were still visible and served for burial activities

among Late Mesolithic communities of the Iron Gates (Borić, 2016; Radovanović, 2000). Five such collective burial pits, encompassing Burials 13–16, 27a–f, 89–91, 79(a–c) and 87(1–3), have been identified at Lepenski Vir, one of which (Burial 87) may have had cremations associated with it (Borić, 2016, p. 189).

With mitochondrial haplogroups inferred for as many as ~20% of known Lepenski Vir individuals, we detect a statistically significant association between U lineages and stacked burials outside in pits (Fisher’s exact test, $p < 0.0007$). To confirm that this association holds at nuclear genomic level, we performed a two-sample permutation test comparing the inferred ancestry proportions between individuals buried inside and outside in pits. The three whole-genomes from the external pits

(Burials 27a, 27d and 91) have more Iron Gates HG ancestry on average than other individuals from the transitional phase (permutation test, one-sided $p < 0.0119$).

It is difficult to confirm that areas inside and outside the village were in use at precisely the same time. This is due to the complex stratigraphy of Lepenski Vir, characterised by parallel house sequences that rarely intersect, and the uncertainty of ^{14}C dates on human bones, which must be corrected for freshwater reservoir effect in addition to the calibration process (Cook et al., 2001). Summed probability distributions of calibrated radiocarbon dates for burials inside, and outside in pits indicate broad chronological simultaneity, but do not rule out more subtle differences in dating (Figs. S9–S10). A sequence of overlapping burials with cremations, resembling the multi-inhumation pits encountered at the periphery of Lepenski Vir, has been documented at the nearby site of Vlasac, dating to the 7th–6th millennia BC, suggesting continuation of a Late Mesolithic practice in the Neolithic period (Borić et al., 2014).

Based on previously-published stable isotope evidence, it is likely that those individuals buried in pits still consumed high amounts of aquatic proteins, while individuals from the village show a wider range of isotopic signatures, consistent with both a terrestrial diet (plant and/or animal-based) and fishing activities (Fig. 4). To some extent, body position in the grave reflects the same intra-site variation: Aegean incomers were occasionally buried like Iron Gates HGs in extended supine position, but none of the Iron Gates HGs or U5-carrying individuals identified to date were buried in a flexed position during the transitional phase—a practice observed among Early Neolithic communities of Southeast Europe and among later burials of Lepenski Vir (Table 2).

Taken together, our results suggest that Iron Gates HGs and their descendants were primarily buried on the fringes of the village, maintaining economic and burial practices that were more attuned to a Mesolithic lifestyle. We caution against interpreting our results as deliberate exclusion of Iron Gates HGs from the space of habitation, given limited sample size and the fact that burial location may not reflect where people stayed during their lifetime. Sample bias (Booth, 2019) may be of concern if, for

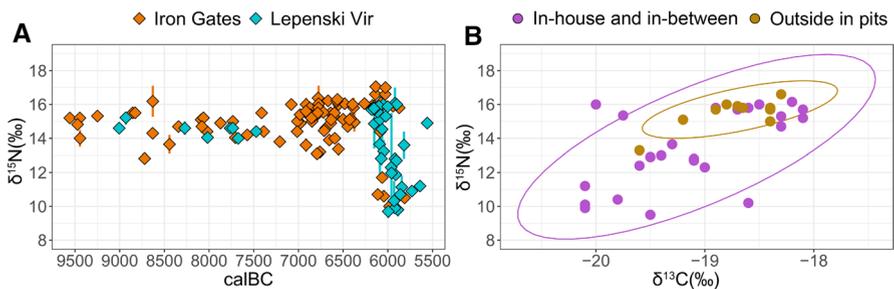


Fig. 4 **A** Shift in $\delta^{15}\text{N}$ values observed in directly-dated humans from the Iron Gates (Oxcal μ). Any $\delta^{15}\text{N}$ value above 10‰ is likely to indicate some intake of aquatic proteins; **B** paired median $\delta^{13}\text{C}/\delta^{15}\text{N}$ signatures for individuals of the Mesolithic–Neolithic transition phase at Lepenski Vir, coloured according to burial location. Ellipses generated with `stat-ellipse` (assuming a multivariate t-distribution) from the `ggplot2` R library. Based on previously reported ^{14}C dates and stable isotope values (full references in Electronic Supplementary Material 1)

example, descendants of Aegean farmers were more likely to bury their dead than Iron Gates HGs, who sometimes practised cremation (Borić et al., 2014; Borić, 2016, p. 260). There could have been fugitive architecture elsewhere with two styles of community present that the original excavation might not have revealed. Local foragers may have decided to live side by side with incoming farmers but maintain separate identities with regard to rituals of death and burial. Conversely, Lepenski Vir may have attracted burials beyond the immediate community. Differential access to resources such as domesticated food plants across multiple generations of inhabitants might highlight conservative dietary traditions, food taboos or even differences in status based on ancestry.

No close genetic relationships between individuals buried at Lepenski Vir could be found that would allow for the reconstruction of familial ties in a biological sense, a fact that may be explained by the lack of systematic sampling of DNA (Fig. S11; for the interpretation of archaeological kinship results see Ensor, 2021). This includes the HGs buried at the periphery of the site in multi-inhumation pits. To explore relatedness beyond immediate kin in a spatial context, we tested for correlation between physical distance among contemporaneous burials at the site and genetic distance measured by outgroup f_3 statistics through a Mantel test, but found no correlation (Mantel test, $p > 0.4143$). While these observations remain preliminary, they can be contrasted with those reported for Vlasac, where two neonate brothers (U62 and U69) were buried close together (Mathieson et al., 2018). Beside this and another previously reported first degree relationship between a father and his daughter (Burials 12 and 24) at Padina, we detect one additional first or second degree relationship between two adult males (Burial 45 and Burial 47) at Mesolithic Vlasac and document a number of additional second to third degree relationships among Iron Gates HGs (Fig. S11).

It is worth pointing out that non-kinship factors, such as relative age, are likely to have played a role in choice of burial location and burial practice at Lepenski Vir (Fig. S12), trumping ancestry in the relevant context. Newborn infants ($N=41$), for instance, were almost without exception buried as foundation deposits beneath trapezoidal house floors during the Mesolithic–Neolithic Transition phase (Stefanović & Borić, 2008). Owing to the liminal status of this age group, it is likely that infants under two months old were afforded different burial rites compared to older children and adults, who were sometimes interred through the floors, that is, when the buildings were in use, or after their abandonment (Radovanović, 2000, p. 340). Consequently, we expect neonate burials to show different ancestry profiles compared to other community members buried within the houses, who may not have been co-residents. Further DNA analysis will clarify the position of the neonates within the settlement of Lepenski Vir, and their relationship to adults buried at the site.

Discussion

Newcomers were Men, Women, as Well as Children Born to Immigrant Parents

Recent biomolecular results are fundamentally changing our image of Lepenski Vir as a Mesolithic village in the process of transition to agriculture. Summing up the

evidence at our disposal, we observe that mating was not part of regular interactions between Iron Gates and Aegean populations before the advent of agriculture in the Central Balkans, ~6200 BC. While material objects and exotic resources travelled between the Aegean Basin and the Iron Gates in Mesolithic times, eventually facilitating the migration of early farmers to the Danube region (Chapman, 2020; Cristiani et al., 2016), farmers and forager populations remained well-differentiated genetically (and phenotypically: see Hofmanová et al., 2022). Following occasional visits by hunter-gatherers, Lepenski Vir was re-settled by a population consisting of first-generation Aegean migrants, Iron Gates HGs and their admixed descendants. By ~5900 BC, the incomers' ancestry became effectively dominant over that of the native Mesolithic population. Unless the site was refounded by another Neolithic community after a gap, this observation is not consistent with the idea of a stable Mesolithic population integrating some Neolithic people and adopting their culture. Only Lepenski Vir and Padina demonstrate significant levels of Aegean ancestry, whereas none of the individuals sequenced to date at Vlasac, including those radiocarbon-dated to the 6th millennium BC (Burials H53, U62, U21), show any sign of admixture with Aegean farmers, suggesting that communities existed side by side in the Iron Gates for hundreds of years without regular mating interactions (Fig. S4A).

Assuming that people buried at the site lived there and were actively involved in its construction, it is likely that Aegean women, men and children settled at Lepenski Vir during the transitional phase (6200–5900 BC). A scenario in which Neolithic women married into a Mesolithic group can be rejected, in light of (i) the almost equal representation of females and males among first generation migrants; (ii) admixture patterns that are not consistent with predominantly male foragers mating with female farmers; and (iii) potential evidence for immigrant children (Burial 61). More genomes and high coverage genomes respectively are needed to increase confidence in the latter two conclusions (see [Materials and Methods/F-Statistics](#)). At present, our results do not rule out the possibility that Aegean migrants moved to Lepenski Vir as, for example, marriage partners, slaves and/or war captives (Bonsall et al., 2008, p. 194). Should additional genomes indicate, in future, that neonates were born to immigrant parents and their descendants at the site, a case could be made for whole families, as opposed to individuals alone, relocating to Lepenski Vir.

The idea that Lepenski Vir was significantly inhabited during the transition phase by migrant farmers, who traced their ancestry back to the Aegean Basin, is consistent with previously published nitrogen isotope values, which show a shift to lower-trophic level food and presumably cereal-based diets ~6200 BC in some humans buried at the site (Fig. S1). Aspects of the Neolithic Demographic Transition, such as high mortality rates among children below weaning age, and population growth, are already visible during the Mesolithic–Neolithic transition phase (Porčić & Nikolić, 2016), suggesting that patterns of replacement observed at genomic level are best explained by continuous gene flow from Neolithic communities outside the Iron Gates and/or higher fertility among individuals with an Aegean genetic background. Interestingly, those individuals with a more terrestrial isotopic signature show the closest genetic proximity to first farmers of Anatolia and the Aegean Basin (Burials 82 and 122). Changes in human isotopic signatures precede the introduction

of domesticates at the site, suggesting that newcomers either acquired food from agricultural communities outside the gorge or came to the site already looking like farmers, isotopically. In all likelihood, they supplemented their diet by fishing in the Danube and raised their children as fishers, hence the wide range of isotopic signatures observed among Aegean farmers (de Beccdelièvre et al., 2020).

Trapezoidal Houses at Lepenski Vir Associated With Incoming Aegean Farmers and Their Descendants

Further questions should be raised about the identification of Lepenski Vir as an established Mesolithic community, in light of evidence for structure in the spatial distribution of the burials during the transitional phase. If Lepenski Vir was indeed a Mesolithic village that integrated a few Neolithic individuals, we might expect members of the community to be buried inside or in close proximity to trapezoidal houses (i.e. their homes). However, individuals assigned to the U mitochondrial clade, who presumably descended from Iron Gates HG populations (or at least had hunter-gatherer or admixed mothers), were all buried, with one exception, some distance away from trapezoidal houses in the upslope area at the rear of the village and show elevated $\delta^{15}\text{N}$ isotopic signatures consistent with a fish-based diet (Figs. 4, 5). As things stand, our results speak against a piecemeal adoption of agriculture by Iron Gates HGs and their admixed descendants, who appear to have maintained separate habitation, burial and dietary traditions at Lepenski Vir.

While the location was repeatedly visited by foragers, who used it as a burial place and seasonal campsite from at least the 10th millennium BC, the construction of the settlement with the distinctive trapezoidal houses was likely triggered by the arrival of Aegean farmers and their descendants, who were buried inside or in close proximity to houses. Among those individuals that show no signs of admixture with Iron Gates HGs in a two-population qpAdm model, at least three are directly associated with sequences of trapezoidal houses (Burials 54e, 61, 122) (Fig. 2B). Child 61,

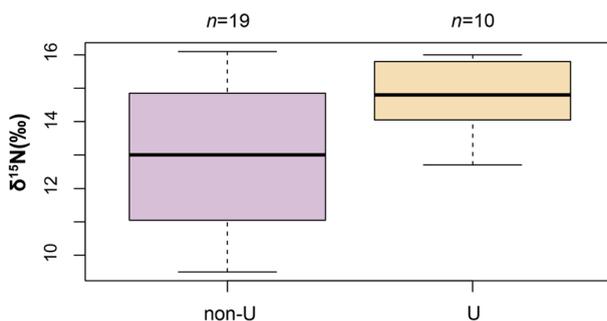


Fig. 5 Distribution of median $\delta^{15}\text{N}$ values for Lepenski Vir individuals assigned to U and non-U mitochondrial clades (full references in Electronic Supplementary Material 1). The U clade, including U5 and U4, is seen as a marker for European hunter-gatherer ancestry. For a fuller overview of the relationship between specific haplogroups and stable isotopic signatures see de Beccdelièvre et al. (2020, fig. 2.5)

who is unlikely to have had more than one hunter-gatherer grandparent if admixed at all (Mathieson et al., 2018), was discovered buried beneath the rear wall of trapezoidal building 40, stratigraphically one of the oldest houses at the site (Bonsall et al., 2002; Borić et al., 2018; Radovanović, 2000).

Given small sample size and lack of systematic sampling strategy within each sequence of trapezoidal houses, we cannot rule out that Aegean incomers were simply reusing a deserted village (Perić & Nikolić, 2016), while the actual inhabitants were those buried outside in pits. Such a scenario appears unlikely in view of the dating of some Aegean-related intramural burials (~6050 BC), their context of deposition (extended supine position), and the aforementioned stratigraphic relationships with trapezoidal floors and stone statues; however, it should not be discounted until more genomes are analysed. Intramural burials are unevenly distributed across the site and rarely exceed three per house, which speaks against a strict interpretation of in-house burials as family graves (Fig. S12). Trapezoidal buildings may not all have been used as houses. Some may have had specialised functions associated with birth or collective child-rearing, hence the high number of neonate burials associated with them (Stefanović, 2006).

The association detected in this article between trapezoidal houses and Aegean ancestry at Lepenski Vir adds a new twist to the long-standing debate regarding the origins of this house form in the Iron Gates (Bonsall et al., 2008). Initially described as an autochthonous Mesolithic phenomenon (Srejović, 1972), trapezoidal houses were subsequently regarded as a marker of hybridisation between Mesolithic and incoming Early Neolithic cultural groups, pottery having been found within some of the trapeziform houses at Padina (B. Jovanović, 1969, 1972). Starčevo-like Early Neolithic pottery, imported Balkan flint and ground-edge tools were later reported from trapezoidal house floors at Lepenski Vir, including a complete pot in the ‘ash-place’ of House 54 (Bonsall et al., 2008, p. 192; Garašanin & Radovanović, 2001).

Further research on the Mesolithic of the Iron Gates has indicated that small trapeziform pit structures, occasionally associated with rectangular stone-bordered hearths, were already being constructed at Vlasac, Schela Cladovei and other Late Mesolithic Iron Gates sites (Bonsall, 2008, p. 256; Borić et al., 2008, p. 278; 2014; Boroneanţ et al., 2014; Rusu, 2011, 2019). These structures fit within a local tradition of increased sedentism and open-air settlements on the Danube starting ~7600 BC (Bonsall, 2008, p. 240), their unusual shape being thought to reference the trapezoidal Treskavac mountain on the other side of the river at Lepenski Vir (Fig. S13; Chapman, 2020, p. 52). As larger trapezoidal structures with a broader end facing the river appear to be predominantly associated with layers that are Early Neolithic in date (Bonsall, 2008, p. 256), a fusion between a local building form and the habitation practices of incoming farmers seems to have taken place—Lepenski Vir being the most spectacular product of that syncretic process. Trapeziform pit structures with their back against the hillslope may have been ideally suited for the gorge environment, which was prone to mudslides, as indicated by hillwash deposits at Vlasac and other Iron Gates sites (Borić et al., 2008).

The arrival of Aegean farmers in the Iron Gates likely led to renewed elaboration and construction of systematically-planned villages such as Lepenski Vir, which displays typical Early Neolithic practices, including subfloor burials and vertical

superimposition of houses, leading to *tell*-like accumulation of deposits by the side of the mountain (Borić, 2008; Chapman, 2000). Burials occurred within a symbolic environment that was not unlike that observed at early agricultural sites, with distinct areas reserved for burial activities at the rear of the houses, away from the central fire installation (Hodder, 1990). The pyrotechnology needed for making elaborate red limestone floors has good parallels in Neolithic Anatolia and reflects what John Nandris has described as a ‘Neolithic mode of behaviour’ (Nandris, 1988, p. 14).

If Iron Gates HGs maintained separate traditions, as suggested here, how to explain the ubiquity of Mesolithic practices at Lepenski Vir? The hybrid character of the site is generally well accepted, with the forager culture seen as ‘dominant’ during the transitional phase (Borić, 2019, p. 43). Assuming that burial in extended supine position parallel to the Danube with the head pointing downstream was the norm among Late Mesolithic Iron Gates HGs (Bonsall & Boroneanț, 2018), then some of the incoming Aegeans can be considered to be buried in a similar fashion to local people during the transitional phase (e.g. Burials 61, 54e). Conversely, not a single Iron Gates HG was found buried in a typically Aegean Neolithic manner—the only U5-carrying individuals displaying a flexed position (Burials 20, 74) having been recently re-dated to the 10th and 9th millennia BC (J. Jovanović et al., 2021). While burial practices are intimately bound with identity, our results confirm that culture and genetics rarely match 1:1, even in cases, like this, of ‘folk migration’ (the so-called ‘pots-equal-people’ paradigm, widely discredited in archaeological scholarship; for a recent discussion of the ancient DNA evidence and its implications for Neolithic archaeology, see Furholt, 2021).

Cultural syncretism has been described in many parts of the world and usually implies competitive rather than harmonious interactions, as explained by Robert Hayden in his work on competitive sharing of religious sites in South Asia and the Balkans (Hayden, 2002). The fact that Aegean first farmers occasionally buried their dead in extended supine position should not be interpreted as a conversion to Mesolithic lifeways. What we are seeing is a brief ‘window’ in the development of farming, where farmers (already familiar with fishing, as evidenced by e.g. fishing hooks at typical farming villages such as Barcın in Anatolia—see Gerritsen et al., 2013) change their subsistence strategies and adopt forager practices when confronted with well-established local traditions and locally available resources. Importantly, this suggests that agriculture is not quite as robust a tipping point as usually assumed. Those individuals identified genetically as ‘Aegeans’ display a remarkable ability to adopt and transform local practices, confounding expectations of a unidirectional shift from Mesolithic to Neolithic societies.

Conclusion

A model of Mesolithic–Neolithic transition for inland Europe hinging on Lepenski Vir as a Mesolithic village that transitioned to agriculture is not sustainable. Even in a remote environment like the Iron Gates, the influence of Europe’s first farmers was pervasive. The arguments outlined in this article do not in any way challenge

the exceptional nature of Lepenski Vir, which remains (possibly alongside Padina) the only Early Neolithic-period site in Europe with evidence of recent admixture (two generations or less) between early farmers and foragers. Especially noteworthy is the readiness of Aegean farmers to adopt local Mesolithic burial and dietary practices, a fact which may be contrasted with the more conservative outlook of sampled Iron Gates HG individuals. Lepenski Vir clearly differs from traditional Early Neolithic communities of Southeast and Central Europe, which show little if any influence from the Mesolithic world. Why Aegean farmers chose to settle in a location that was not well suited for cereal agriculture is an intriguing question, which will no doubt come under sharp scrutiny in light of our re-interpretation of the site. At Lepenski Vir, the wild and the domestic were never very far apart.

Materials and Methods

Bioinformatics Pipeline

Variant Call

All variant calling was carried out following the approach described in Hofmanová et al. (2016), by using the ATLAS package (Link et al., 2017; commit=647daf7). Majority-allele calls (method=majorityBase) were produced for the SNPs overlapping the 1240K capture array (Mathieson et al., 2015), as well as for the mitochondrial and the Y-chromosome. Diploid Genotype calls were obtained with the maximum likelihood approach described in Hofmanová et al. (2016) (method=MLE) for sites found to be bi-allelic in the 1000 Genomes data-set (The 1000 Genomes Project Consortium et al., 2010).

Uniparental Markers and Molecular Sexing

The molecular sex of each sample was determined following the approaches described in Skoglund et al. (2013) and Mittnik et al. (2016) (Electronic Supplementary Material 2). Y-chromosomal haplogroups were assigned from BAM files using Y-leaf (Ralf et al., 2018). For mitochondrial haplogroup determination, majority-allele calls were uploaded to HaploGrep2 (Weissensteiner et al., 2016). Haplogroup diversity H was estimated as:

$$H = \frac{N}{N-1} \left(1 - \sum_i x_i^2 \right)$$

based on the haplotype frequency x_i in N samples. 95% confidence intervals were obtained via leave-one-out jackknife resampling.

Population Genetics

Principal Component Analysis

PCA was performed with LASER version 2.04 (Wang et al., 2014) following the approach described in Hofmanová et al. (2016). After generating a reference space of modern Eurasian individuals (data published as part of reference Lazaridis et al., 2016; see Supplementary Methods for list of populations), we projected BAM files (see Supplementary Methods for list of individuals) on to the reference space via Procrustes analysis implemented in LASER averaged over ten replicates, using pileup files generated with Samtools version 1.9 (Li et al., 2009), with the suggested filter criteria of minimum mapping quality 30 and minimum base quality 20.

f-Statistics

All *f*-statistics, that is, outgroup f_3 and f_4 admixture proportions, were computed with qp3Pop and qpAdm respectively from the ADMIXTOOLS (Patterson et al., 2012) package, with default parameters and on the sites defined by the *HOIII* set of SNPs (Lazaridis et al., 2016). All qp3Pop analyses are performed with Khomani as outgroup, all qpAdm runs used the set of outgroups Han, Karitiana, Mbuti, Onge, Papuan, Mota, Ust'-Ishim, MA1, El Miron, GoyetQ116-1. Pseudohaploid genomes were retrieved from David Reich's lab website (<https://reich.hms.harvard.edu/allen-ancient-dna-resource-aadr-downloadable-genotypes-present-day-and-ancient-dna-data>; downloaded January 20, 2021); in addition, we used our own majority allele calls for the individuals from Yaka et al. (2021) as described in section [Bioinformatics Pipeline/Variant Call](#). See Supplementary Methods for the list of individuals used to represent the Iron Gates hunter-gatherer and Aegean/Anatolian farmer ancestry components.

Note that due to different sets of source individuals our ancestry proportions may differ from previously published ones. For example, individual LV 61 (I4666) was originally described as a mixture of northwestern-Anatolian-Neolithic-related and hunter-gatherer-related ancestry (Mathieson et al., 2018). We find no statistical support for a two-component model over a simpler exclusively Neolithic one, although both fit well (mixture of 9% Iron Gates hunter-gatherer and 91% Aegean/Anatolian farmer: tail probability 0.85; pure Aegean/Anatolian farmer: tail probability 0.88, *p*-value for nested model 0.52). This may be explained by the relatively small number of 44,695 SNPs available for the analysis of LV 61.

X/Autosomal Ancestry

For the comparison of ancestry patterns on X and autosomes, individual ancestries were estimated based on the *HOIII* set of SNPs (Lazaridis et al., 2016). Following Oteo-García and Oteo (2021), we quantified hunter-gatherer ancestry by computing $f_3(\text{Aegean, Test, HG})/f_2(\text{Aegean, HG})$ separately for the X chromosome and autosomes. Here, 'Aegean' and 'HG' stand for the same sets of samples used in

the qpAdm analysis to represent Aegean/Anatolian farmer and Iron Gates hunter-gatherer ancestry respectively. 95% confidence intervals were obtained by re-sampling, leaving out SNPs in 5 cM windows.

Kinship Analyses

Biological relatedness of the Iron Gates samples was determined following the methodology outlined in Yaka et al. (2021), using READ (Monroy Kuhn et al., 2018); lcMLkin (Lipatov et al., 2015); and ngsRelate (Hanghøj et al., 2019; Korneliussen & Moltke, 2015). For the READ analysis, majority-allele calls overlapping the 1240K capture array (Mathieson et al., 2015) were used. For lcMLkin and ngsRelate, phred-scaled genotype likelihoods from the MLE-calls were used as input. All analyses were repeated three times on three different datasets: first for all samples jointly; second for all samples with predominantly hunter-gatherer ancestry, as well as admixed individuals; and third with all samples with predominantly farmer ancestry, as well as admixed individuals. Admixture proportions were taken from the qpAdm analysis described above. The merged MLE-calls were pruned, based on linkage disequilibrium with plink (`--indep-pairwise 200 25 0.4` [Purcell et al., 2007]), and only polymorphic SNPs with a frequency $\geq 5\%$ —which were present in at least 30% of the samples—were kept. For ngsRelate and lcMLkin, only results for sample pairs with at least 5000 overlapping SNPs were considered.

Radiocarbon Dating

For an overview of the radiocarbon dates considered for this project see Electronic Supplementary Material 1. All dates were uniformly re-calibrated in OxCal 4.4.2 (Ramsey, 2009) using the IntCal20 calibration curve (Reimer et al., 2020). Correction for freshwater reservoir effect follows Bonsall et al. (Bonsall et al., 2015): for human bones with $\delta^{15}N \geq 8.3\%$, uncalibrated dates of the form $\mu \pm \sigma$ were adjusted to $(\mu - 545c) \pm \sqrt{\sigma^2 + (70c)^2}$ where $c = \frac{\delta^{15}N - 8.3}{17 - 8.3}$.

Summed probability distributions of calibrated radiocarbon dates were calculated in OxCal using the Sum() function.

Statistical Analyses

Fisher's exact test was run in R with the command 'fisher.test', and the permutation test to compare ancestry proportions inside and outside in pits was performed with 'permTS' from the 'perm' R library. The Mantel test for correlation between out-group f_3 and spatial distance was performed with the command 'mantel.rtest' from the ade4 R library. Note that a Mantel-test assumes a smooth correlation, which may not be the case on the f_3 scale, especially given the admixture scenario. The two-sample Kolmogorov–Smirnov test comparing the summed probability distributions of calibrated radiocarbon dates ($n_1 = 8, n_2 = 7$) at significance level $\alpha = 0.05$ was implemented in R by testing if the maximal absolute difference between the

cumulative probability distributions exceeds $\sqrt{-z\left(\frac{\alpha}{2}\right)\frac{1+\frac{n_1}{n_2}}{2n_1}}$. The credible interval for the R1b Y-haplogroup frequency given no R1b in n observed male individuals was computed as the 95th quantile of $Beta(0.5, 0.5 + n)$.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10963-022-09169-9>.

Acknowledgements We thank the editors and three anonymous referees for their constructive comments and suggestions. Bogdana Milić offered helpful archaeological insights and Katie Meheux, David Shankland and Stephen Shennan kindly commented on a preliminary draft of this article. Errors and omissions remain our own. Finally, we are grateful to Prince Parise for the permission to reproduce the Discover illustration. Maxime Brami was supported by an Individual Fellowship from the H2020 Marie Skłodowska-Curie Actions (793893) and by the Institut National de Recherche Archéologique (INRA), Luxembourg.

Funding Open Access funding enabled and organized by Projekt DEAL. Parts of this research were conducted using the supercomputer Mogon and/or advisory services offered by Johannes Gutenberg University Mainz (hpc.uni-mainz.de), which is a member of the AHRP (Alliance for High Performance Computing in Rhineland Palatinate, www.ahrp.info) and the Gauss Alliance e.V.

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