PALEOGENOMICS

Ten millennia of hepatitis B virus evolution

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Hepatitis B virus (HBV) has been infecting humans for millennia and remains a global health problem, but its past diversity and dispersal routes are largely unknown. We generated HBV genomic data from 137 Eurasians and Native Americans dated between ~10,500 and ~400 years ago. We date the most recent common ancestor of all HBV lineages to between ~20,000 and 12,000 years ago, with the virus present in European and South American hunter-gatherers during the early Holocene. After the European Neolithic transition, Mesolithic HBV strains were replaced by a lineage likely disseminated by early farmers that prevailed throughout western Eurasia for ~4000 years, declining around the end of the 2nd millennium BCE. The only remnant of this prehistoric HBV diversity is the rare genotype G, which appears to have reemerged during the HIV pandemic.

The World Health Organization (WHO) estimates that in 2015, 257 million people were living with chronic hepatitis B virus (HBV) infection, which causes close to 1 million deaths each year (*1*). HBV is transmitted through contact with bodily fluids, mainly in sexual and perinatal contexts (*2*), and has no known environmental or animal reservoir. Its spread is therefore tightly linked to the dispersal of humans, whose past population dynamics and migrations have likely shaped the genetic diversity of this partially double-stranded DNA virus, which is currently classified into nine genotypes associated with characteristic ethno-geographic ranges (Fig. 1) (*3*, *4*). However, the temporal and geographic context of HBV origins in humans, as well as its major routes of dissemination in the past, remain widely debated (*5*–*10*). Recent studies have retrieved HBV DNA from archaeological human remains (*11*–*16*), providing new avenues to address questions about HBV evolution and phylogeographic history. In particular, these studies revealed the presence of HBV in Europe as early as the Neolithic and ancient HBV lineages that are now seemingly extinct. Ancient DNA data permits molecular clock calibration, and the time to the most recent common ancestor (tMRCA) of all known HBV lineages has been dated to between ~21 thousand years ago (ka) and ~9 ka (*14*). However, the extent of the past diversity of this virus remains generally unknown because only 19 ancient HBV genomes with a limited temporal and geographic distribution have been reconstructed to date.

The MRCA of all known HBV lineages

Here, we report genomic evidence of HBV in the skeletal remains of 137 individuals from Eurasia and the Americas dated to between ~10,500 and ~400 years ago (Fig. 1, fig. S1 and data S1). Despite advances in molecular virology and numerous sequences from present-day HBV genomes, assessing the phylogenetic relationships among HBV genotypes has proven challenging (*7*, *17*–*20*), and doubts have been cast about its evolutionary rate and molecular clock–like behavior (*9*, *16*, *21*). Nevertheless, most HBV phylogenetic reconstructions have recovered a topology in which HBV genotypes typically found in Native Americans (genotypes F and H) represent a sister clade to the rest of worldwide HBV diversity (which we refer to as the Eurasian branch) (*18*). This topology was supported by a study incorporating 12 ancient HBV genomes (*14*) and was also reconstructed in this work (Fig. 2 and figs. S2 and S3). In particular, the monophyly of the American HBV branch, comprising all ancient genomes from the Americas dating back to as early as ~9 ka from the Cuncaicha rock shelter in the Andean highlands (CUN002), was highly supported. However, deep nodes within the Eurasian branch were not well resolved, pointing to plausible alternative topologies in which some of the earliest Eurasian lineages would have diverged before the American branch (figs. S4 and S5) (*22*). Our results confirm that HBV genomic data do exhibit a clear temporal structure when incorporating samples spanning several thousand years (fig. S3). Using the best-fitting uncorrelated relaxed clock model, we estimate the tMRCA of HBV, corresponding to the divergence of American and Eurasian HBV branches, to be between ~16 and ~12 ka [95%

highest posterior density (HPD)] (table S1), which is within the range of previous findings (*14*). This suggests that contacts between ancestral Eurasians and First Americans occurred until at least shortly before the Bølling-Allerød interstadial (~15 to 13 ka), a period of warming corresponding to widespread human expansion in North America (*23*, *24*). However, studies of ancient human genomes indicate that the ancestors of the First Americans likely began diverging from their closest Eurasian relatives between ~25 and 18 ka, possibly reflecting an extended isolation in a Beringian refugium during the Last Glacial Maximum, before dispersing into and across the Americas (*25*–*27*). The use of a time-dependent rate (TDR) model yielded an estimate of ~20 to 17 ka for the HBV tMRCA (95% HPD), which was more consistent in this regard. This suggests that not accounting for the time dependency of the evolutionary rate may have led to an underestimation of deep divergence times. However, model selection favored the use of a relaxed clock over a TDR model (log BF: 405) (*22*). Taken together, these results point to a scenario in which the MRCA of all HBV strains examined to date existed around the end of the Pleistocene and gave rise to one or several lineages that spread across Eurasia and eventually reached Africa and Oceania, and to another lineage that spread into the Americas with early settlers of this continent.

Our findings challenge the view that current HBV diversity reflects early human dispersals out of Africa. This model is supported in particular by the exclusive association of HBV

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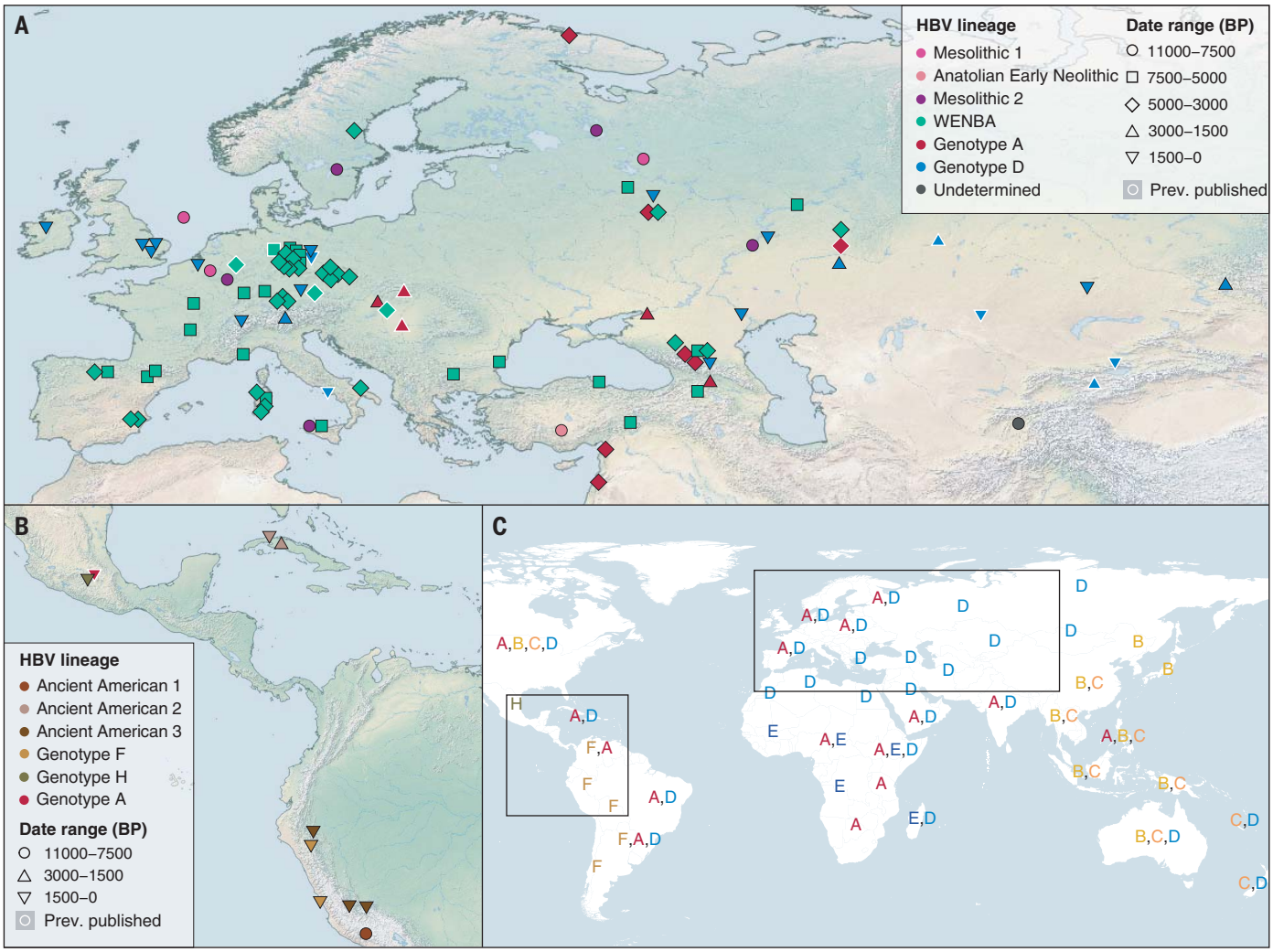

Fig. 1. Geographic location, time period, and lineage of ancient HBV genomes. (**A** and **B**) Lineages from (A) Eurasia and (B) the Americas. (**C**) Main distribution of present-day HBV genotypes [adapted from (*4*, *14*)].

subgenotype C4 with the Aboriginal people of Australia, suggesting that this subgenotype may have been carried by the first settlers of Australia at least ~50 ka (*5*, *20*). Instead, in accordance with previous findings (*14*), our results indicate that all known modern and ancient HBV strains descend from a lineage that began to diversify at a more recent stage of human history and that subgenotype C4 was introduced in the Australian continent after ~4.5 ka (Fig. 2). Nevertheless, the age of the observed MRCA only represents a lower limit for the earliest presence of HBV in humans. Whether the latter has been preceded by long coevolution, a recent spillover from another animal species, or any intermediate scenario remains an open question. Other viruses from the Hepadnaviridae family have been recovered from a wide range of vertebrates, but none of them appear to represent an ancestral zoonotic source for the human HBV (*8*).

HBV circulated widely in western Eurasia as early as 10 ka

The retrieval of HBV genomes from around 10 ka in different parts of Europe and Anatolia indicates that the virus was widespread in western Eurasia at that time (Fig. 1 and fig. S1). The oldest HBV strains recovered in Europe form two distinct clades (Fig. 2, fig. S2, and table S2): one that was found in three hunter-gatherers (HGs) from northwestern Russia, Belgium, and Doggerland (Mesolithic 1) and another that was found in an HG from western Russia (Mesolithic 2). These two lineages are placed within the Eurasian branch as sister groups to the modern strains found in nonhuman primates (NHPs) from Southeast Asia and Africa, respectively. The position of NHP HBV lineages within human HBV diversity has been observed in most previous phylogenetic reconstructions and is thought to reflect spillover events from humans to NHPs (*7*, *22*, *28*). The HBV genome reconstructed from an early Anatolian farmer forms a separate lineage recovered at a phylogenetic position intermediate to the two European Mesolithic clades. Between ~9 and 7.5 ka, HBV strains found in HGs from Karelia (northwestern Russia), Sweden, Luxembourg, and Sicily all belonged to the Mesolithic 2 clade. Thus, although our data do not allow detailed phylogeographic inference, they suggest that during the early Holocene, HBV strains could spread over large parts of western Eurasia within a few thousand years. This is consistent with evidence of genetic connections between Europe and the Near East that predate the Neolithic transition (*29*, *30*) and with the observed genetic cline from Western to Eastern HGs (*31*). Our results further highlight that Mesolithic populations likely formed a network through which pathogens could spread.

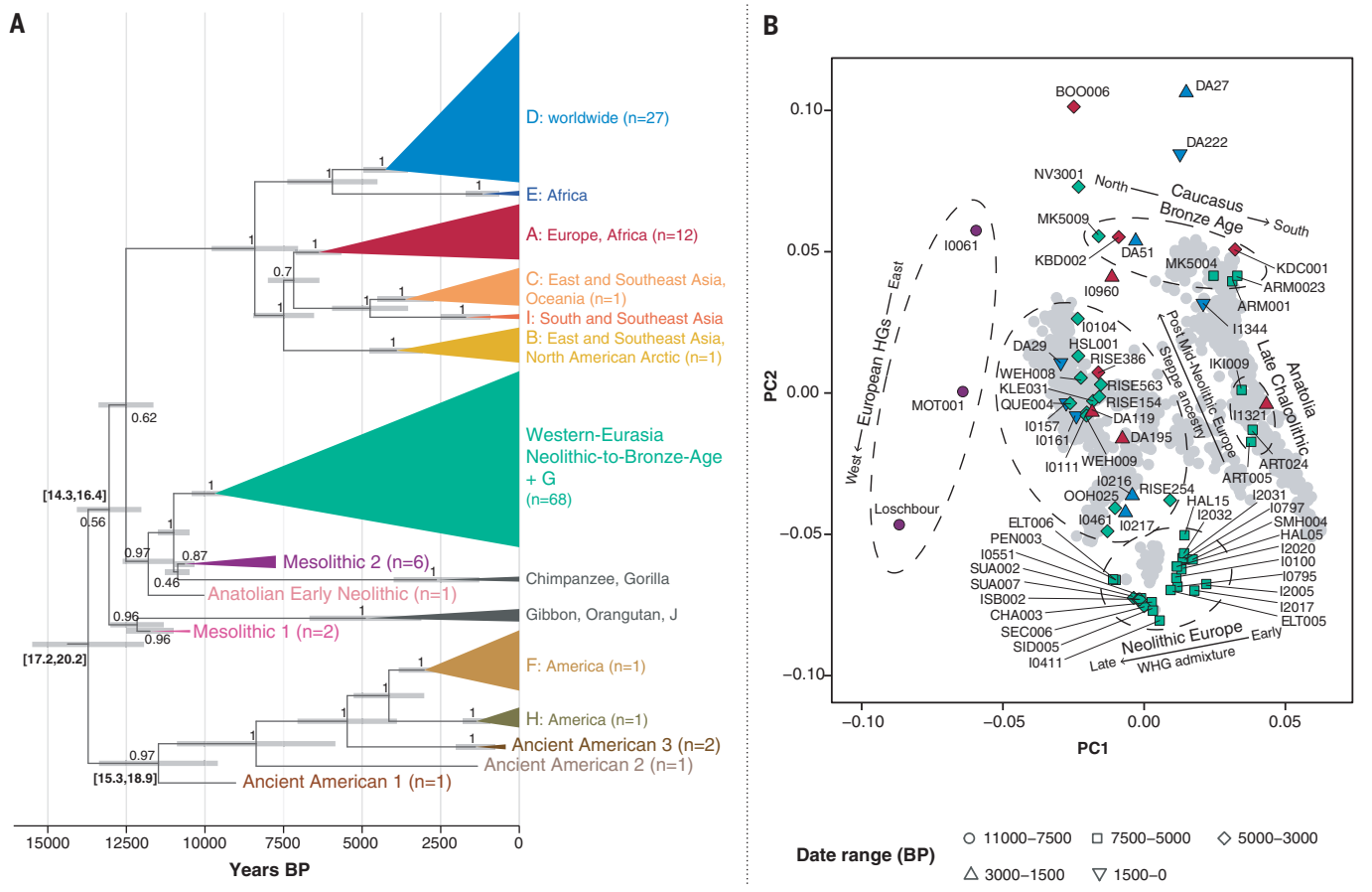

Fig. 2. HBV phylogeny and genetic profile of infected individuals. (**A**) Time-calibrated phylogenetic tree of HBV obtained by using a skyline coalescent tree prior and a lognormal relaxed clock. Main clades were collapsed and annotated with their typical present-day geographic location and the number of ancient genomes they contain. Posterior node supports and date estimates (gray bars, indicating 95% HPD) are reported. Additional time intervals written on deep nodes are 95% HPD estimates obtained with a time-dependent rate model. (**B**) Principal components analysis plot of modern and ancient western Eurasians summarizing the genetic variation of a subset of individuals for which human genetic data was available. Individuals are colored according to the lineage of the HBV strain they carried, as in the tree.

It has been suggested that most human-adapted pathogens emerged after the Neolithic transition in association with sedentary lifestyles, increased contact with domesticated animals, and higher population densities, a phenomenon sometimes referred to as the "first epidemiological transition" (*32*–*34*). Our finding of widespread HBV in HG populations indicates that HBV was present before the advent of agriculture and animal husbandry in different parts of the world. Today, HBV rarely causes lethal fulminant hepatitis but rather asymptomatic infections that may evolve into chronic forms, sometimes developing into liver complications and possible liver failure after decades of infection (*1*, *2*). Although it is difficult to extrapolate from present-day medical studies what the clinical impact of a pathogen would have been in the past—given different diets, disease burdens, and life expectancies—the virus has likely exhibited similar pathophysiological features. Consequently, our findings are consistent with the view that although small HG communities could not sustain highly epidemic "crowd" diseases, they could maintain chronic infectious agents (*35*, *36*).

A replacement of HBV diversity occurred with the Neolithic transition in Europe

Our data show that HBV remained widespread in Europe after the Neolithic transition (8 to 7 ka), with numerous strains recovered from early European farmers (EEF) across the continent (Fig. 3, fig. S1, and data S1). All of these strains belong to a single HBV lineage that does not descend from previously observed Mesolithic strains (Figs. 2 and 3 and fig. S2). We refer to this HBV lineage as the Western-Eurasian Neolithic–to–Bronze Age (WENBA) lineage. This transition is also observable at a microscale in Grotta dell'Uzzo (Sicily), where HBV strains recovered from Neolithic individuals are unrelated to a Late Mesolithic strain identified at the same site (figs. S1 and S2). This suggests that the HBV strains observed in EEFs were not acquired from local HGs in different areas but were rather disseminated by EEFs themselves. Although EEFs ultimately derived from early agricultural populations in the Near East (*37*, *38*), the strain we retrieved from an Anatolian farmer dated to ~10 ka was not ancestral to the WENBA lineage (Fig. 2). Therefore, even if EEFs were indeed key in disseminating WENBA strains, whether this lineage originated in Near Eastern centers of early agriculture or in another location along EEF's expansion routes remains to be determined. Furthermore, given the current sample availability for this period, a scenario in which the WENBA lineage would have originated and disseminated among European HGs shortly before the Neolithic transition cannot be completely excluded.

We also found WENBA HBV strains in two HGs from transitional Neolithic contexts in western Russia dated to ~7.2 and ~6.4 ka (JAZ001 and MUR007), as well as on both

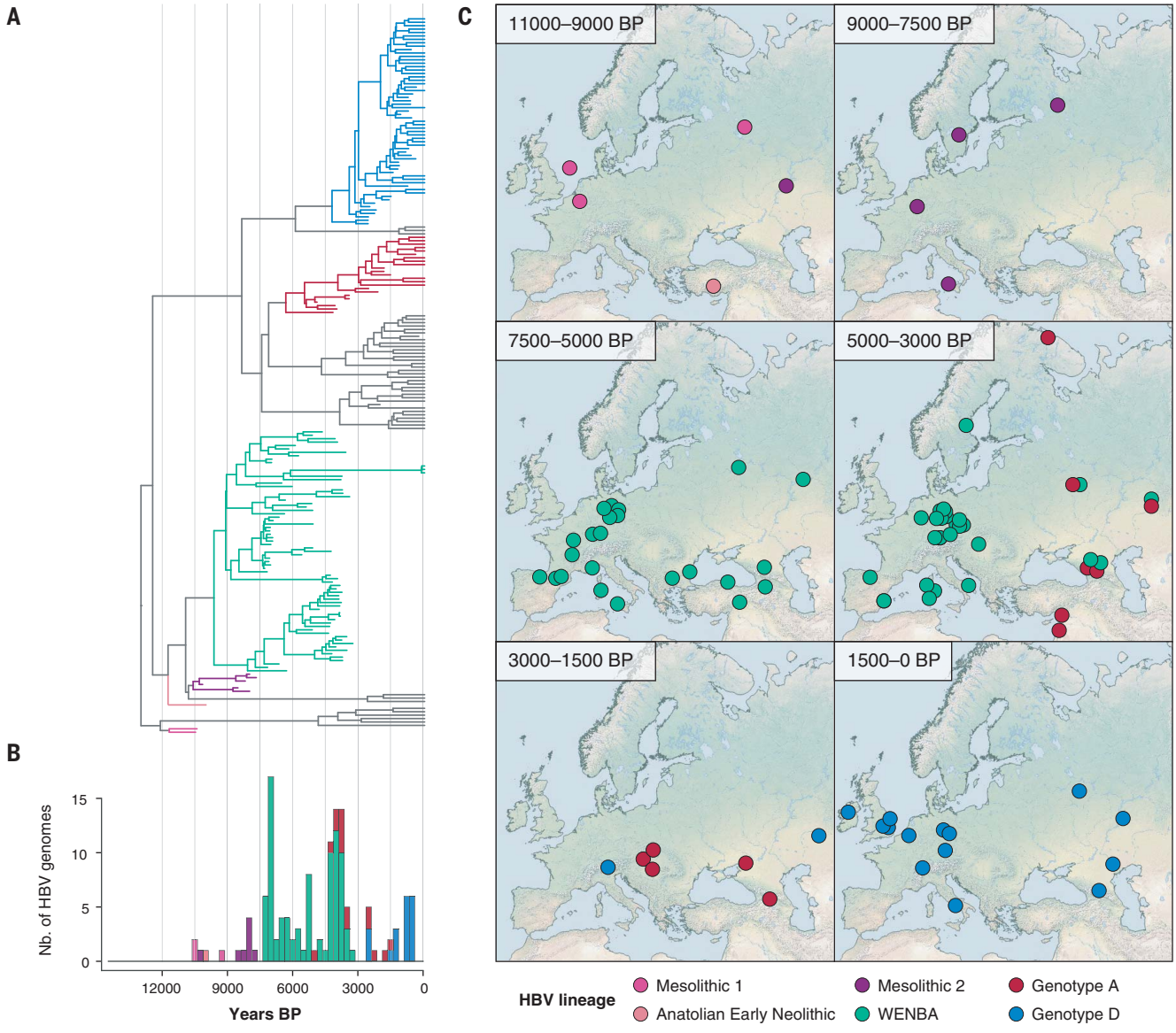

Fig. 3. Spatiotemporal distribution of ancient western Eurasian HBV strains. (**A**) Time-calibrated phylogenetic tree (Eurasian branch). Lineages containing ancient HBV genomes are colored. (**B**) Histogram showing the number of recovered ancient HBV genomes belonging to each lineage through time. (**C**) Geographic distribution of ancient HBV genomes within different time-periods, colored by lineage.

sides of the Greater Caucasus Mountain range and in Anatolia as early as ~5.6 ka (fig. S1). In general, phylogenetic relationships among HBV sublineages within the WENBA clade do not exhibit a strong geographical structure (fig. S2), nor do they seem to reflect the material culture or genetic profile of the individuals in which they were found (fig. S6). Furthermore, our phylodynamic reconstruction indicates that after an initial growth phase, the transmission of WENBA HBV reached an equilibrium from ~7.5 to ~3.5 ka (fig. S7). Overall, this suggests that HBV strains disseminated by EEFs quickly spread throughout much of western Eurasia beyond the limits of the European agricultural expansion, where they became endemic and continued to circulate widely across different populations, for several thousand years. In particular, we do not observe substantial changes in the HBV genetic landscape associated with the expansion of steppe-related ancestry that dramatically altered the genetic profile of Europeans from ~5 ka onward (Fig. 2, fig. S2, and data S1) (*37*). Sexual and perinatal transmission have likely always been the major mechanisms of HBV infection in humans, but cultural practices involving contact with blood [such as tattooing (*39*)] or nonsexual violent interactions (*40*) could also have played a role in the spread of the virus in the past. In general, our findings attest to a degree of interconnectivity among prehistoric populations of different origins, subsistence modes, and cultures that allowed for the dissemination of directly transmitted pathogens.

The collapse of WENBA HBV during the 2nd millennium BCE

After the Early Neolithic (8 to 7 ka), the WENBA HBV lineage prevailed in most parts of western Eurasia for more than 4000 years (Fig. 3). However, the latest occurrence of a WENBA strain in our dataset is dated to ~3.3 ka, after which this lineage is no longer observed (figs.

S1 and S2). By contrast, genotype A, which we first observed at the eastern edge of Europe and in the Near East between ~5 and ~3.5 ka, still appears after ~2.5 ka, by which time it had reached the Carpathian Basin in central Europe. Around the same date, we first observed genotype D in two individuals from the Italian Alps, as well as in various locations in the western steppe, before prevailing in large parts of Europe during the Medieval period. Thus, it seems that as most WENBA HBV lineages disappeared by the end of the 2nd millennium BCE, genotypes A and D subsequently spread from eastern reservoirs to eventually reach western regions that had previously only harbored WENBA strains (*22*).

The second half of the 2nd millennium BCE bears witness to major cultural shifts in the archaeological record in western Eurasia, including the sudden disappearance of tell settlements in the Carpathian Basin (*41*), the expansion of the Urnfield culture and the increase of military conflicts in large parts of Europe (*42*–*45*), the breakdown of the Terramare culture in northern Italy (*46*), and the so-called Late Bronze Age collapse of most state societies in the eastern Mediterranean region and Near East (*47*, *48*). Some of these societal transformations could have been triggered by underlying phenomena such as climatic events (*49*) or the spread of epidemic diseases (*50*) and were likely associated with substantial shifts in population densities, transregional networks, and modes and scales of human mobility. The observed decline of WENBA HBV diversity, as well as our phylodynamic reconstruction (fig. S7), further point to major changes in epidemiological dynamics over large parts of western Eurasia during this period. However, although our data suggests that new lineages disseminated across Europe only later on, the lack of observations around 3 ka (Fig. 3) could reflect sampling biases related to the widespread adoption of cremation practices around that time (*42*–*44*) rather than a decrease of HBV prevalence. Searching for the virus in a large number of systematically dated samples across this period could help to better characterize the process that ultimately led to the renewal of western Eurasian HBV diversity after the end of the 2nd millennium BCE.

Recent reemergence of the WENBA HBV lineage

The majority of HBV strains circulating in western Eurasia today belong to genotypes A and D (*3*, *4*), thus only reflecting a relatively recent part of the phylogeographic history of this virus. However, our results show that despite the seemingly complete disappearance of WENBA HBV strains around the end of the 2nd millennium BCE, one lineage descending from this clade has persisted to the present. The latter gave rise to a group of modern strains classified as genotype G (Fig. 2 and fig. S2), a rare, recently described genotype for which the biology is poorly understood (*51*). First discovered in patients from France and the United States, genotype G was later found in other parts of Europe, the Americas, and in Asia, making its geographic origin unclear (*52*). Despite its wide distribution, genotype G exhibits remarkably low genetic diversity (*53*), suggesting a recent reemergence after thousands of years of low-level persistence. Furthermore, genotype G has mostly been found in HIV-positive patients, and phylodynamic patterns have pointed to a sharp increase of its dissemination co-occurring with the HIV pandemic, possibly associated with highly sexually active groups and injection-drug users (*52*).

Genotype G has sometimes been referred to as "aberrant" because of its distinctive genomic features: a 36-nucelotide insertion near the 5′ end of the core gene and two nonsense mutations in the precore region (*51*, *54*). These changes inhibit production of the immunotolerogen e antigen (HBeAg), which appears to be essential for the establishment of a persistent HBV infection, and alter the structure of the HBV core protein, which may impair packaging and replication of the viral genetic material (*54*, *55*). This likely explains why in the vast majority of cases genotype G occurs in co-infections with other HBV genotypes, which can provide the HBeAg and core protein production functions lacking in genotype G (*54*–*56*). We identified similar insertions and stop codons in 14 ancient HBV genomes ranging in age between ~7 and 3.5 ka, which form the WENBA subclade from which genotype G descends (fig. S8). Additionally, most of these ancient genomes were found in individuals showing signs of infections with several HBV variants (fig. S8 and data S2) (*22*). Cases of mixed infection were exclusively found in individuals carrying WENBA HBV strains, among which they were very frequent (22 of 83 individuals, likely underestimating the true frequency). In all cases, both major and minor strains appeared to belong to the WENBA lineage, and sequencing data were partially supporting a ~40–base pair insertion at the 5′ end of the core gene (table S3 and data S1).

Therefore, although genotype G is considered rare today, it seems that the cotransmission of its ancestral form together with another $HBeAg^+$ WENBA strain was a common epidemiological feature of HBV between ~7.5 and 3.5 ka. Notably, this functionally limited variant persisted until today, whereas the rest of the WENBA HBV diversity seemingly went extinct. Virologic studies indicate that genotype G tends to outcompete HBeAg-producing strains during late HBV infection stages after anti-HBeAg seroconversion (*56*–*58*). Although these short-term selection patterns parallel the survival of this lineage over thousands of years, the latter may rather be related to less deterministic factors. One of the closest Bronze Age ancestors of genotype G was recovered at the archaeological site of Shagara in the eastern European forest zone (SGR003) (figs. S1 and S2), a location where the present-day widespread genotype A was already circulating (SGR004). Genotype A is the most common genotype found in mixed infections with genotype G today (*55*, *57*). The discovery of ancestral forms of both genotypes at the same archaeological site, albeit from different individuals and time periods, may indicate that this viral association had already formed during prehistory in eastern Europe.

Conclusions

This study demonstrates the value of large-scale paleogenomic analyses for studying the phylogeographic history of HBV. DNA enrichment allowed us to reconstruct large proportions of more than 100 ancient HBV genomes from a variety of skeletal tissues, opening possibilities for future paleovirologic studies. We show that HBV was already widely present in humans during the early Holocene and that its phylogeographic history reflects several well-known human migrations and demographic events, including the expansion of First American populations in the Americas and the Neolithic transition in Europe, but not others, such as later Bronze Age steppe ancestry expansions. Furthermore, our results reveal patterns that were not expected on the basis of human genetic and archaeological data alone, such as the near complete renewal of western Eurasian HBV diversity around the end of the 2nd millennium BCE. These findings highlight that the reconstruction of ancient viral diversity has great potential to contribute to our understanding of human history.

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ACKNOWLEDGMENTS

We thank M. Harbeck, L. Bondioli, R. Risch, and H. Duday for their contribution to the archaeological interpretation; R. Heyne and H. Heyne for comments concerning clinical manifestation of hepatitis B infections; B. Mühleman and T. C. Jones for helpful discussion on genome assemblies and mixed infections; and M. O'Reilly for her help on the design of the figures. **Funding:** The research was funded by the Max Planck Society, the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (771234–PALEoRIDER, to W.H.; 805268–CoDisEASe to K. Bos; 834616–ARCHCAUCASUS to S.H.), the Slovak Academy of Sciences and the European Union's Seventh Framework Programme and Marie Curie Actions under the Programme SASPRO (1340/03/03 to P.C.R.), the ERA.NET RUS Plus–S&T programm of the European Union's Seventh Framework Programme (277–BIOARCCAUCASUS to S.Re. and S.H.), the Werner Siemens Stiftung ("Paleobiochemistry", to CW), the Award Praemium Academiae of the Czech Academy of Sciences (to M.E.), the Institute of Archaeology of the Czech Academy of Sciences (RVO 67985912, to M.Dobe.), the Russian Foundation for Basic Research (19-09-00354a, to M.K.K. and V.V.K.; 19-78-10053 to SSh), the German Research Foundation (DFG-HA-5407/4-1–INTERACT to W.H. and RE2688/2 to S.Re.), the French National Research Agency (ANR-17-FRAL-0010–INTERACT, to M.F.D., M.Ri., S.Ro., S.Sai., D.Bi., and P.Le.), the Wenner-Gren Dissertation Fieldwork Grant (9558 to S.Sab.), and the Ministry of Education and Science of the Republic of Kazakhstan (AP08856654 to L.B.D., L.M., and E.Kh. and AP08857177 to A.Z.B.). **Author contributions:** D.K. and J.K. initiated and supervised the study. S.F., V.Sc., V.K., M.K.K., M.S.C., V.V.K., A.A.Kh., A.A.C., D.A.S., A.F.K., C.T.R., Í.G.-M.d.L., H.A.M., R.G.P., J.I.R.G., J.N., S.Ro., S.K., S.Sai., E.Ka., A.B.B., P.V., P.Li., M.Ko., L.Lo., E.P., R.C., A.L., R.M., Y.C.d.A., S.T.H.G., D.I.H.Z., J.P., D.Bi., P.Le., A.R.K., V.E.M., L.La., M.Z., J.F.B., M.L., A.D., T.I., G.G.A., M.P.d.M.I., A.R., A.Sp., S.B., S.Sal., E.D.Z., D.V.V., K.v.H., R.L.B., L.C.S., L.A., M.N., E.R., C.A.F., V.Sl., A.A.Ka., B.C.A., E.B., M.A.C., M.S., R.K., J.J.E., M.Fr., S.Sh., P.d.K., E.A., K.V.d.V., L.F.S., T.A.T., S.L., M.Dobr., N.M., C.R., M.V.T., C.S., P.C.R., M.A., K.A.Y., E.C.B., F.C., V.Ma., P.U., K.R., D.Ba., P.S., L.M.M., M.Ro., H.S., D.C.S.G., N.S., Y.S.E., F.H., Y.B., K.Boy., M.Kü., D.S., P.O., R.Sk., M.R.G., A.B., L.B.D., A.Z.B., Z.S., K.Mas., M.Ma., V.Mo., K.Man., S.Re., S.H., E.P.K., M.Dobe., M.E., H.M., and K.W.A. conducted the archaeological/anthropological work or the curation of the archeological material. J.K., W.H., C.Po., K.Bos., P.W.S., S.Sc., C.W., M.F.D., O.B., E.Kh., E.F.D., B.L., A.Sa., E.B., E.K.G., L.M., and M.Ri. organized the sample collection for genetic analyses. R.B., L.P., M.A.S., F.A., R.St., A.W., A.M., V.V.M., G.U.N., M.Ri., M.S.v.d.L., K.Maj., R.I.T., L.M., A.G., S.P., S.Sab., M.Mi., J.G., E.A.N., T.F., K.N., C.Pa., M.Ke., E.K.G., M.Fe., S.E., E.S., K.G., G.A.G.R., D.I.H.Z., B.L., E.F.D., O.B., M.F.D., and W.H. participated in the laboratory work, sample management, and gathering of contextual information. A.K., F.M.K., M.A.S., and R.H. performed HBV screening of the sequencing data, which was supervised by A.H.; A.K. and K.P. performed the bioinformatic analyses. A.K. and D.K. performed the phylogenetic analyses. L.P. performed the human population genetics analyses. A.K. and A.B.R. performed the statistical analyses. All authors contributed to the interpretation of the data. A.K. wrote the first version of the manuscript, which was edited by D.K., J.K., W.H., A.H., C.Po., K.Bos., P.W.S., S.Sc., C.W., F.v.B., M.P., L.P., R.B., F.M.K., and M.A.S., and all authors contributed to its improvement. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The sequencing data generated in this study has been deposited at the European Nucleotide Archive (www.ebi.ac.uk/ena/data/view/PRJEB45699). Complete HBV sequence alignments are available in the supplementary materials.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.abi5658
Materials and Methods
Supplementary Text
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Data S1 to S4
MDAR Reproducibility Checklist

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18 March 2021; accepted 17 August 2021
10.1126/science.abi5658

Science

Ten millennia of hepatitis B virus evolution

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Science, 374 (6564),

Ancient DNA traces the history of hepatitis B

Hepatitis B virus (HBV) infections represent a worldwide human health concern. To study the history of this pathogen, Kocher *et al*. identified 137 human remains with detectable levels of virus dating between 400 and 10,000 years ago. Sequencing and analyses of these ancient viruses suggested a common ancestor between 12,000 and 20,000 years ago. There is no evidence indicating that HBV was present in the earliest humans as they spread out of Africa; however, HBV was likely present in human populations before farming. Furthermore, the virus was present in the Americas by about 9000 years ago, representing a lineage sister to the viral strains found in Eurasia that diverged about 20,000 years ago. —LMZ