Erratum to

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Ancient Hepatitis B viruses from the Bronze Age to the Medieval

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Abstract

Hepatitis B virus (HBV) is a major cause of human hepatitis. Approximately 257 million people are chronically infected and around 887,000 died in 2015 due to associated complications. We searched for HBV among 115 billion ancient DNA sequences in shotgun sequencing libraries obtained from 304 Central and Western Eurasian human skeletons, dated from the early Bronze Age to Medieval times. We recovered 12 full or partial ancient HBV genomes aged between 822 and 4488 years old. The ancient sequences group either within or in sister relationship to extant human or other ape HBV clades. With a few exceptions, the genome properties follow those of modern HBV. Different evolutionary models project the root of the heterochronous HBV tree to between 8.6 and 20.9 thousand years ago (kya), and estimate a substitution rate between $8.04 \times 10^{-6}$ and $1.51 \times 10^{-5}$ nucleotide substitutions per site per year (s/s/y). In several cases, the geographic locations of the ancient genotypes do not match present day distribution. Genotypes that today are typical of Africa and Asia, and a subgenotype from India, are here shown to have an early Eurasian presence. The geographic and temporal patterns we observe in ancient and modern HBV genotypes are compatible with well-documented human migrations during the Bronze and Iron Ages. We show evidence for the creation of genotype A via recombination and a long-term association of modern HBV genotypes with humans, including the discovery of a human genotype that is now extinct.

HBV is transmitted perinatally or horizontally via blood or genital fluids. The estimated global prevalence is 3.6%, ranging from 0.01% (UK) to 22.38% (South Sudan). In high endemicity areas, where prevalence is > 8%, 70-90% of the adult population show evidence of past or present infection. The virus persists in the liver of 10-90% of infected
individuals. The young and the immunocompromised are most likely to develop chronic HBV infection, which can result in high viremia over years to decades. Approximately 25% of chronically infected individuals die from HBV-induced liver cirrhosis or hepatocellular carcinoma.

Despite the prevalence and public health impact of HBV, its origin and evolution remain unclear. Inference of HBV nucleotide substitution rates is complicated by the fact that the virus genome consists of four overlapping open reading frames, and mutation rates differ between phases of chronic infection. Studies based on modern heterochronous sequences, sampled over a relatively short time period, find higher substitution rates, whereas rates estimated using external calibrations tend to be lower, leading to a wide range of estimated substitution rates (7.72x10^-4 to 3.7x10^-6) for HBV. Human HBV is classified into at least nine genotypes, A-I, roughly corresponding to sequence similarity of at least 92.5% within genotypes. Genotypes have a heterogeneous global distribution (Fig. 1a). Human and non-human primate (NHP) HBV are interspersed in the phylogenetic tree, for yet unknown reasons. Different hypotheses have been proposed to explain the origin of HBV and its diversification in humans and NHP, however none are conclusive. A New World origin with diversification and global spread after European contact in the last 400 years was proposed in an early study. This was later shown to be unlikely when the discovery of HBV in Old World NHP revealed a mixed phylogeny with humans, implying a very rapid dispersal and diversification of the virus. The subsequent finding of HBV in a 340 (±70) year old Korean mummy argued further against such a recent human origin. MacDonald et al. (2000) discussed co-speciation of a virus present in a human / NHP ancestor species, with cross-species transmission into and possibly out of humans in the last 35-10 million years. However, as they noted and as had been noted by
Simmonds (2001), the phylogeny of NHP HBV does not correspond to the host phylogeny as would be expected in a co-speciation scenario. In fact, some NHP HBVs group geographically, rather than by host species. For example, HBVs found in Orangutans and Gibbons that share habitats on the island of Borneo. Finally, it has been considered whether HBV co-evolved with ancient modern humans after leaving Africa 60-100 kya. However, the basal position of genotypes F and H and absence of viruses related or identical to those genotypes in North East Asians, who contributed considerably to the Native American gene pool, is incongruent with this hypothesis. Furthermore, this hypothesis does not explain the intermixing of the Old World NHP HBV with the human HBVs. The co-speciation and Out-of-Africa hypotheses are not necessarily exclusive. Current evolutionary hypotheses are vulnerable to sampling biases caused by our ignorance of viral lineages that infected humans during most of human history.

Recent advances in the sequencing of ancient DNA (aDNA) have yielded important insights into human evolution, past population dynamics, and diseases. However, ancient sequences have been recovered for only a handful of exogenous human viruses, including influenza (~100 years), variola (~350 years), and HBV (~340 years). The knowledge gained from these few cases emphasizes the general importance of ancient sequences for the direct study of long-term viral evolution. HBV has several characteristics that make it a good candidate for detection in an aDNA virus study: its extended high viremia during chronicity, the relative stability of its virion, and its small, circular, and partially double-stranded DNA genome.

Shotgun sequence data were generated from 167 Bronze Age and 137 predominantly Iron Age (Damgaard et al. 2017, in review) individuals from Central to Western Eurasia with a
sample age range of ~7.1-0.2 kya. We identified reads that matched the HBV genome in 25
samples (Table 1, Extended Data Table 1a, SI Table 3), spanning a period of almost 4000
years, from several different cultures and a broad geographical range (Fig. 1b, Table 1,
Extended Data Table 1a, SI Table 3). Using TaqMan PCR, we tested two samples with high
genome coverage (DA195, DA222) and two samples with low coverage (DA85, DA89) for
the presence of HBV. The high-coverage samples tested positive, whereas the low-coverage
samples tested negative (Extended Data Table 1b). This is consistent with shotgun
sequencing being more effective than targeted PCR for analysing highly degraded DNA.
Based on availability of sample material, libraries from 14 samples were selected for targeted
enrichment (capture) of HBV DNA fragments. This resulted in increased genome coverage
and an average of a 2.4-fold increase in number of HBV positive reads (Extended Data Table
1a, SI Table 3). In total, we obtained 17.9 to 100% HBV genome coverage from the sequence
data, with genomic depth ranging from 0.4x to 89.2x (Table 1, Extended Data Table 1a). We
selected 12 samples for phylogenetic analyses. Criteria for inclusion were at least 50%
genome coverage and clear aDNA damage patterns after capture (Extended Data Fig. 1, SI
Figure 1c).

For an initial phylogenetic grouping, we estimated a Maximum Likelihood (ML) tree using
the ancient HBV genomes together with modern human, NHP, rodent, and bat HBV genomes
(Dataset 1, see Methods). All ancient viruses fell within the diversity of Old World primate
HBV genotypes, which includes all human and other great ape genotypes, except human
genotypes F and H (Extended Data Fig. 2).

Recombination is known to occur in HBV. Using RDP4, we found strong evidence that an
ancient sequence, HBV-DA51, and an unknown parent recombined to form the ancient
genotype A sequences. Although that cannot literally be the case due to sample ages, the
logical interpretation is that an ancestor of HBV-DA51 was involved in the recombination.
The same recombination is also suggested for the two modern genotype A sequences that
were included in the analysis. The ancient genotype B (HBV-DA45), a modern genotype B,
and two modern genotype C sequences were not similarly flagged, suggesting that the
possible recombination occurred after genotypes A, B, and C had diverged. The predicted
recombination break points (Extended Data Table 2, Extended Data Fig. 3) correspond
closely to the polymerase gene. Thus, it is possible that the polymerase from an unknown
parent and the remainder of the genome from an HBV-DA51 ancestor recombined to form
genotype A (see Methods).

For detailed phylogenetic analyses, we used a set of 112 reference human and NHP HBV
sequences (Dataset 2, see Methods). An ML phylogenetic tree based on these reference
sequences and all ancient sequences was constructed (Extended Data Fig. 4). Regression of
root-to-tip genetic distances against sampling dates, as well as date randomisation tests,
showed a clear temporal signal in the data (Fig. 2a, SI section 8), suggesting that molecular
clock models can be applied. A dated coalescent phylogeny was constructed using BEAST2
(Fig. 2b). The molecular clock was calibrated using tip dates. Strict and relaxed lognormal
molecular clocks were tested with constant, exponential, and Bayesian skyline population
priors (Extended Data Table 3a). Model comparisons favoured a relaxed molecular clock
model with lognormally distributed rate variation and an exponential population prior
(Extended Data Table 3a). The root of the resulting tree dates to 11.6 kya (95% Highest
Posterior Density (HPD) interval: 8.6 to 15.3 kya) and the clock rate is estimated to be
1.18x10^-5 s/s/y (95% HPD interval: 9.21x10^-6 to 1.45x10^-5 s/s/y). Under a strict molecular
clock, a Bayesian skyline population prior was favoured, in which case the root age is 15.6
kya (95% HPD interval: 13.7 to 17.8 kya) and the substitution rate \(9.48 \times 10^{-6} \text{s/s/y} (95\% \text{ HPD interval: } 8.3 \times 10^{-6} \text{ to } 1.07 \times 10^{-5} \text{s/s/y})\) (Extended Data Tables 3b and 3c).

Under all model parameterisations used here, the substitution rate we find is lower than rates estimated from phylogenies built using either modern heterochronous sequences\(^7\) or sequences from mother-to-child transmissions\(^16\), but higher than rates inferred using external calibrations based on human migrations\(^8\). A lower rate is consistent with Tedder et al. (2013)\(^{35}\), who found that although short-term mutation rates may be high, mutations within an individual often revert back to the genotype consensus, and thus rarely lead to long-term sequence change. It is also consistent with the so-called time-dependent rate phenomenon, observed for many viruses, which shows that short term evolutionary rates are higher than long term rates\(^{36}\).

The knowledge of ancient HBV genomes enables us to formally evaluate hypotheses concerning HBV origins using path sampling of calibrated phylogenies based on appropriate external divergence date assumptions. We tested several calibration points implied by a co-expansion of HBV with humans after leaving Africa\(^{20,21}\) for support of congruence between migrations and geographical locations of HBV clades. We find weak evidence for a split of the F/H clade between 13.4 and 25.0 kya under a strict, but not a relaxed clock model. We do not find support for the divergence of subgenotype C3 strains between 5.1-12.0 kya, leading to a distribution in different regions of Polynesia, or for divergence of Haitian A3 strains from other genotype A strains between 0.2-0.5 kya under either strict or relaxed clock models (Extended Data Table 3d, Methods).
In the dated coalescent phylogeny, four ancient sequences (from youngest to oldest: HBV-DA119, -DA195, -RISE386, and -RISE387) group with genotype A. The first three fall well within the 7.5% nucleotide divergence criterion used to delimit membership in HBV genotypes. HBV-RISE387 is right on this limit (Extended Data Table 4a)\(^1\). The three oldest samples lack a six nucleotide insertion at the carboxyl end of the Core gene that is present in all modern genotype A viruses (Table 2). HBV-RISE387 encodes a stop codon in its pre-Core peptide that would have ablated the expression of the immune modulator HBe antigen (HBeAg), a phenomenon known in modern HBV infections (Table 2). This characteristic viral mutant is usually found in chronic HBV carriers who seroconverted from HBeAg to anti-HBe. Interestingly, RISE386 and RISE387 are archaeologically dated only ~100 years apart and both come from the Bulanovo site in Russia, but their viruses show only 93.37% sequence identity (Extended Data Table 4b), indicating the existence of significant localized HBV diversity ~4.2 kya.

The ancient sequence HBV-DA45 phylogenetically groups with genotype B and has 97.6% sequence identity with modern genotype B (Extended Data Table 4a).

Sequences HBV-DA51, -DA27, -DA222, and -DA29 phylogenetically group with the modern genotype D. They have high sequence identity (96.9 to 98.7%) with modern genotype D sequences (Extended Data Table 4a), and have the typical 33 nucleotide deletion in the PreS1 sequence of the S-gene, encoding the three HBV surface proteins\(^5\) (Table 2).

Sequences HBV-RISE154, -RISE254, and -RISE563 are in sister relationship to the Chimpanzee/Gorilla HBV clade (Fig. 2b). All three have the same 33 nucleotide deletion in the PreS1 sequence that is shared with NHP HBV and human genotype D. HBV-RISE563
does not encode a functional pre-Core peptide (Table 2). Based on sequence similarity across
the whole genome, HBV-RISE563 and -RISE254 together might be classified as a new
human genotype that is extinct today, and HBV-RISE154 as possibly another (Extended Data
Tables 4a and 4b). However, HBV-RISE154 has low genome coverage, which precludes an
exact calculation. The sister relationship of these three sequences with modern Chimpanzee
and Gorilla HBV could be interpreted as a consequence of relatively recent transmission(s) of
HBV from humans to NHPs. However, other scenarios and confounding factors are possible,
as these lineages are deeply separated in the tree. Incomplete lineage sorting combined with
viral extinction (possibly boosted by massive recent reductions in great ape populations)
should be considered. Much more data on current and, if possible, ancient NHP-associated
HBV will be necessary to reach definitive conclusions.

The geographic locations of some of the ancient virus genotypes do not match the present-
day genotype distribution, and also do not match dates and/or locations inferred in previous
studies of HBV. While it is important to keep in mind that the data presented here are limited,
they provide important spatiotemporal reference points in the evolutionary history of HBV.
Their synopsis suggests a more complicated ancestry of present-day genotypes than
previously assumed, especially in light of recent insights into the history of human migration.
Based on all currently available information we can outline a tentative evolutionary history of
our ancient samples and HBV genotypes A and D. However, it must be emphasized that these
speculations are based on limited data, and may change as more information becomes
available.

The recombination analysis suggests that genotype A was formed from the recombination of
a genotype D ancestor and an unknown genotype, approximately 6-7 kya. Given the
geographic location of the modern genotypes closest to genotype A (B and C), we could
guess that the recombination occurred in Eastern Asia. We find genotype A in South-Western
Russia by 4.3 kya (RISE386, RISE387), in individuals belonging to the Sintashta culture.
Further, we find genotype A in a sample (DA195) that is ~2500 years old, from the Scythian
culture. The western Scythians are related to the Bronze Age cultures of the Western Steppe
populations (Damgaard et al., 2017, in review, 37) and their shared ancestry suggests that the
modern genotype A may descend from this ancient Eurasian diversity and not, as previously
hypothesized, from African ancestors38-40. This is also consistent with phylogeny (Figure 2b),
as well as the fact that the three oldest ancient genotype A sequences (HBV-DA195, -
RISE386, and -RISE387) lack the six nucleotide insertion found in the youngest (HBV-
DA119), as well as all modern A sequences. The ancestors of subgenotypes A1 and A3 could
have been carried into Africa subsequently, via migration from western Eurasia. This
possibility is suggested by human genetic studies showing admixture of west Eurasians in
east and southern Africans 2.7-3.3 kya41. Subsequent dispersal of subgenotypes A1 and A3
from Africa into South Asia and the New World, potentially via the East Indian and trans-
Atlantic slave trade routes, might explain the present distribution pattern of these clades and
the structure of the phylogenetic tree42,43. Note that the structure of the phylogenetic tree
offers evidence against an introduction of genotype A into Southern Asia during well-
described Bronze Age migrations.

The ancient HBV genotype D sequences presented in this paper were all found in Central
Asia. Phylogenetically, they group with clades currently found around the world. The most
ancient genotype D sequence (HBV-DA51, dated to 2.3 kya) was found in Kyrgyzstan and
falls basal to all modern genotype D sequences. Furthermore, HBV-DA27, found in
Kazakhstan and dated to 1.6 kya, falls basal to the modern subgenotype D5 sequences that
today are found in the Paharia tribe from eastern India. DA27 and the Paharia people in India are linked by their Tibeto-Burman ancestry (Damgaard et al., 2017, in review). The central Steppe populations, including the four individuals carrying genotype D, were heavily admixed with East Asian sources during the Iron Age (Damgaard et al., 2017, in review), possibly suggesting a westward expansion of genotype D from Eastern into Central Asia. The absence of genotype A in our Central Asian samples may suggest a replacement of genotype A with genotype D during the Iron Age.

Meanwhile, at least one previously unknown genotype (HBV-RISE154, -RISE254, -RISE563) had a presence in Europe by ~5 kya. These genotypes became extinct, possibly following the arrival from the east of either genotype A or, later, genotype D.

Although the ancient HBV sequences presented here can conclusively rule out some possibilities, they cannot provide the true evolutionary history of HBV. In fact, these sequences illustrate the fragility of inferences regarding the geographic and temporal origins of HBV genotypes based on modern heterochronous data and external calibrations. Although incorporating 12 temporally and spatially distinct ancient sequences increases our specific knowledge of HBV evolution, conclusions based on such limited data are likely to be refined or corrected by future discoveries and should be regarded as provisional.

To our knowledge, we report the oldest exogenous viral sequences recovered from DNA samples of ancient humans, or any vertebrate. We show that humans throughout Eurasia were widely infected with HBV for thousands of years. Despite the age of the samples and the imperfect diagnostic test, our dataset contained a surprisingly high proportion of HBV-positive individuals. The actual ancient prevalence during the Bronze Age and thereafter
might have been higher, reaching or exceeding the prevalence typically found in contemporary indigenous populations\(^4\). The ancient data reveal aspects of complexity in HBV evolution that are not apparent when only modern sequences are considered. They show the existence of ancient HBV genotypes in locations incongruent with their present-day distribution, contradicting previously suggested geographic or temporal origins of genotypes or sub-genotypes; evidence for the creation of genotype A via recombination; at least one now-extinct human genotype; and ancient genotype-level localized diversity. These suggest that the difficulty in formulating a coherent theory for the origin and spread of HBV may be due to genetic evidence of an earlier evolutionary scenario being overwritten by relatively recent alterations, as also suggested by Simmonds et al., in the context of recombination\(^{32}\). The lack of ancient sequences limits our understanding of the evolution of HBV and, very likely, of other viruses. Discovery of additional ancient viral sequences may provide a clearer picture of the true origin and early diversification of HBV, enable us to address questions of paleo-epidemiology, and broaden our understanding of the contributions of natural and cultural changes (including migrations and medical practices) to human disease burden and mortality.
Figure 1: Geographic distribution of analysed samples and modern genotypes

A. Distribution of modern human HBV genotypes. Genotypes relevant to the manuscript are shown in colour. Coloured shapes indicate the locations of the HBV-positive samples included for further analysis, as in panel B.

B. Locations of analysed Bronze Age samples taken from Allentoft et al., 2015 are shown as circles, Iron Age and later samples, from Damgaard et al., 2017, in review, as triangles. Coloured markers indicate HBV-positive samples. Ancient genotype A samples are found in regions where genotype D predominates today, and DA27 is of sub-genotype D5 which today is found almost exclusively in India.

Table 1: Overview of samples used for phylogenetic analyses

<table>
<thead>
<tr>
<th>Sample</th>
<th>¹⁴C age (standard)</th>
<th>Median cal BP age, or Approx. sample age</th>
<th>Site</th>
<th>Culture or period</th>
<th>Sex</th>
<th>Reads included in consensus</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample ID</td>
<td>Estimate (in years)</td>
<td>Deviation</td>
<td>Consensus</td>
<td>Depth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISE563</td>
<td>3955 (35)</td>
<td>4421</td>
<td>4488</td>
<td>100%</td>
<td>79.3x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA222</td>
<td>N/D</td>
<td>1200-1000</td>
<td>1167</td>
<td>100%</td>
<td>89.2x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA195</td>
<td>2479 (35)</td>
<td>2578</td>
<td>2645</td>
<td>99.9%</td>
<td>29.2x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA51</td>
<td>2220 (37)</td>
<td>2230</td>
<td>2297</td>
<td>99.2%</td>
<td>14.5x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISE254</td>
<td>3631 (29)</td>
<td>3942</td>
<td>4009</td>
<td>99.0%</td>
<td>36.6x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA119</td>
<td>N/D</td>
<td>1500</td>
<td>1567</td>
<td>98.8%</td>
<td>53.1x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISE386</td>
<td>3758 (34)</td>
<td>4121</td>
<td>4188</td>
<td>97.8%</td>
<td>7.0x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA27</td>
<td>1641 (33)</td>
<td>1543</td>
<td>1610</td>
<td>90.0%</td>
<td>14.3x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA29</td>
<td>849 (25)</td>
<td>755</td>
<td>822</td>
<td>87.5%</td>
<td>4.8x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA45</td>
<td>2083 (27)</td>
<td>2053</td>
<td>2120</td>
<td>87.2%</td>
<td>4.3x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISE387</td>
<td>3822 (33)</td>
<td>4215</td>
<td>4282</td>
<td>86.6%</td>
<td>6.2x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISE154</td>
<td>3522 (24)</td>
<td>3784</td>
<td>3851</td>
<td>57.2%</td>
<td>2.0x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Samples included in phylogenetic analysis, by decreasing genome coverage. Criteria for inclusion were at least 50% genome coverage and sufficient aDNA damage patterns after capture. The read count indicates the number of reads used to make consensus sequences. N/D (not determined) indicates samples where dating was not performed or where osteological sex was undetermined. See Methods for information on sequence matching, consensus making, and sample dating.
Figure 2: Phylogenetic analyses of HBV sequences

a, Root-to-tip regression. Branch lengths were inferred using neighbour joining (NJ), ML, and Bayesian methods. Root to tip distances were extracted in TempEst⁴⁶, and the regression analyses were performed with Scipy⁴⁷. The slopes are 1.01E-05, 1.20E-05 and 4.21E-06 and Correlation coefficients are 0.45 ($R^2=0.2$), 0.36 ($R^2=0.13$), and 0.51 ($R^2=0.26$) for ML, Bayesian, and NJ trees, respectively. b, Dated maximum clade credibility tree calculated using BEAST2⁴⁴. A lognormal relaxed clock and coalescent exponential population prior were used. The X axis shows years into the past. Grey horizontal bars indicate the 95% HPD interval of the age of the relevant node. Larger numbers on the nodes indicate the age and 95% HPD interval of the age under a strict clock and Bayesian skyline tree prior. Taxon names indicate: genotype / subgenotype, accession number, sample age, country abbreviation of sequence origin, region of sequence origin, host species, and optional additional remarks.
Table 2: Genome properties of ancient sequences included in phylogenetic analyses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype of closest sequence</th>
<th>% sequence identity to closest sequence</th>
<th>Genome length</th>
<th>Serotype</th>
<th>Insertions / deletions</th>
<th>Predicted HBeAg status</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA119</td>
<td>A3</td>
<td>97.8%</td>
<td>3221</td>
<td>adw2</td>
<td>6nt insert at the carboxyl end of core region</td>
<td>Positive</td>
</tr>
<tr>
<td>DA195</td>
<td>A3</td>
<td>96.2%</td>
<td>3215</td>
<td>adw2</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>RISE386</td>
<td>A</td>
<td>95.2%</td>
<td>3215</td>
<td>adw2</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>RISE387</td>
<td>A</td>
<td>92.5%</td>
<td>3215</td>
<td>adw2</td>
<td>None</td>
<td>Negative, Pre-core stop codon</td>
</tr>
<tr>
<td>DA45</td>
<td>B1</td>
<td>96.6%</td>
<td>3215</td>
<td>ayw1</td>
<td>None</td>
<td>Positive</td>
</tr>
<tr>
<td>DA29</td>
<td>D3</td>
<td>98.5%</td>
<td>3182</td>
<td>ayw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Positive</td>
</tr>
<tr>
<td>DA222</td>
<td>D3</td>
<td>98.7%</td>
<td>3182</td>
<td>ayw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Positive</td>
</tr>
<tr>
<td>DA27</td>
<td>D1</td>
<td>97.2%</td>
<td>3182</td>
<td>ayw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Positive</td>
</tr>
<tr>
<td>DA51</td>
<td>D1</td>
<td>96.7%</td>
<td>3182</td>
<td>ayw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Positive</td>
</tr>
<tr>
<td>RISE154</td>
<td>Chimp.</td>
<td>92.5%</td>
<td>Ambiguous</td>
<td>adw2</td>
<td>Ambiguous</td>
<td>Positive</td>
</tr>
<tr>
<td>RISE254</td>
<td>Chimp.</td>
<td>95.2%</td>
<td>Ambiguous</td>
<td>adw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Positive</td>
</tr>
<tr>
<td>RISE563</td>
<td>Gorilla</td>
<td>92.7%</td>
<td>3182</td>
<td>adw2</td>
<td>33nt deletion at the amino terminus of the preS1 region</td>
<td>Negative, Pre-core stop codon</td>
</tr>
</tbody>
</table>

Genotype groups are sorted by increasing sample age. Serotypes are inferred from sequence data, as in Table 1 in Kay and Zoulim, 2007. *Serotype could not be determined unambiguously, due to lack of coverage.
Methods

HBV datasets

The following HBV datasets were used in the present study. Full listings of accession numbers are given in the Supporting Information.

**Dataset 1**: 26 HBV genomes, covering all species in the Orthohepadnaviruses. This includes one sequence each from the human HBV genotypes (A-J), Orangutan, Chimpanzee, Gorilla, Gibbon, Woolly monkey, Woodchuck, Ground squirrel, Arctic ground squirrel, Horseshoe bat, and 4 sequences from Roundleaf bats and 3 from Tent-making bats, largely following Drexler et al.\(^49\)

**Dataset 2**: 124 HBV genomes, from humans and NHP. This set contains 92 sequences from Paraskevis et al.\(^8\) (excluding their incomplete sequences), 7 additional genotype D sequences, the Korean mummy genotype C sequence\(^14\), the 12 ancient sequences from the present study, and 12 full genomes selected from a set of 9066 full HBV genomes downloaded from NCBI\(^50\) on 2017-08-24 (Entrez query: hepatitis b virus[organism] not rna[title] not clone[title] not clonal[title] not patent[title] not recombinant[title] not recombination[title] and 3000:4000[sequence length]) corresponding to the closest, non-artificial match for each of the ancient sequences. Dates for these sequences were acquired by looking for a date of sample collection in the NCBI entry, or the paper where the sequence was first published. If a range of dates was mentioned, the mean was used. If no date of sample collection was found in this way, either the year of the publication of the paper, or the year of addition of the sequence to GenBank was used, whichever was earlier.

**Dataset 3**: 124 HBV genomes, from humans, NHP, and a variety of other Orthohepadnavirus host species, including Woolly monkey, Roundleaf and Tent-making bat, Ground and Arctic ground squirrel, Woodchuck, and Snow goose. This set contains 113 sequences that are the
union of a selection of 91 sequences from Paraskevis et al.\textsuperscript{38} and 29 from Drexler et al.\textsuperscript{49}, and
11 additional sequences.

**Dataset 4:** 3505 HBV genomes. 3384 are from Bell et al., (2016)\textsuperscript{51}, divided into ten human genotypes. To these we added 17 Chimpanzee, 56 Gorilla, 12 Gibbon and 36 Orangutan full HBV genome sequences downloaded from NCBI on 2017-01-18, resulting in 14 genome categories.

**Dating of ancient samples**

Sample ages were determined by direct \(^{14}\)C-dating. These ages were calibrated using OxCal\textsuperscript{52} (version 4.3) using the IntCal13 curve\textsuperscript{53}. Table 1 shows the \(^{14}\)C age and standard deviation for each sample. This is followed by the median probability calibrated age before present (cal BP), where “present” is defined as 1950. RISE386 was \(^{14}\)C dated twice, with ages (standard deviation) of 3740 (33) and 3775 (34), so a rounded mean of 3758 (34) was used for its calibration. DA29 was dated at 822 years using \(^{14}\)C and also at ~700 years using multi-proxy methods, the former was used for consistency. The dates for DA119, DA222, RISE548, RISE556, RISE568, and RISE597 are best estimates, based on sample context.

**Data and data processing**

We analysed 101 Bronze Age samples published in Allentoft, et al.\textsuperscript{29}, 137 predominantly Iron Age samples published in Damgaard et al., (2017, in review), and 66 additional samples from the Bronze Age. A total of 114.58x10\(^9\) Illumina HiSeq 2500 sequencing reads were processed.

AdapterRemoval\textsuperscript{54} (version 2.1.7) was used with its default settings to remove adaptors from all sequences, to trim N bases from the ends of reads, and to trim bases with quality ≤ 2.
Reads were aligned against a human genome (GRCh38\textsuperscript{55}) using BWA\textsuperscript{56} (version 0.7.15-r1140, mem algorithm). Reads that did not match the human genome were then mapped against the NCBI viral protein reference database containing 274,038 viral protein sequences (downloaded on 2016-08-31) using DIAMOND\textsuperscript{57} (version 0.8.25). Protein matches were grouped into their corresponding viruses. Reads matching HBV were found in 25 samples.

The non-human reads from the HBV-positive samples that had more than three reads matching HBV using DIAMOND were selected for a subsequent BLAST\textsuperscript{58} (version 2.4.0) analysis. A BLAST database was made from Dataset 3, and samples were matched using blastn (with arguments -task blastn -evalue 0.01). Matching reads with bit scores greater than 50 for all samples (except DA222 (70) and DA45 (55)) were selected for subsequent processing. The number of reads selected from the BLAST matches, per sample, is shown in Table 1, with additional detail in Extended Data Table 1. Across all samples 11,149 reads matched against HBV sequences.

**PCR confirmation**

Real-time PCR was established using primers and TaqMan probes as described by Drosten et al.,\textsuperscript{59} which amplifies a 91 base pair amplicon of the HBV genome. Primers and probe were added to QuantiTect PCR mix (Qiagen #204343) in a final concentration of 400 nM or 200 nM, respectively, in a total reaction volume of 25 ul, including 5 ul template. Using the Roche LC480 or Agilent Mx3006p instruments, PCRs were incubated for 15 min. at 95°C followed by 45 cycles of 15 seconds at 94°C and 60 seconds at 60°C, measuring fluorescence from the 6-carboxy-fluorescein/BHQ1-labelled probe and the passive dye (ROX) at the end of each cycle.

Careful precautions were taken to prevent PCR contamination. PCR mastermixes were prepared in dedicated ancient DNA clean lab facilities, in which no prior targeted work has
been carried out on HBV. Ancient DNA extracts and non-template controls (NTC) were
added into PCR reactions in this location too, which were not subsequently opened. Positive
control material was handled in labs in a physically separated building. Here, standard
material, diluted to 5-50 copies/reaction, was added to duplicate PCR reactions along with
additional NTCs.

**Virus capture**

14 samples with sufficient sample material were selected for virus capture (DA27, DA29,
DA45, DA51, DA85, DA89, DA119, DA195, DA222, RISE254, RISE386, RISE416,
RISE568, RISE556). The viral reference genomes for probes were selected as follows. The
International Committee for Taxonomy of Viruses (ICTV) 2012 listed 2618 viral species. As
many had no associated reference genomes or merely partial sequence information, we
selected 2599 sequences of full-length viral genomes, available from GenBank (June 2014),
representing viral species found in vertebrates excluding fish. Sequences <1000 nt were
discarded. Sequences with identical length and organism ID were regarded as duplicates and
thus reduced to 1. For a number of specific viral taxa for which a large number of similar
reference sequences are available, we manually selected representative genomes or genome
segments (SI Tables 1 and 2). For example, among 72 available Hepatitis C virus genome
sequences, we selected one genome per subtype (1a-c, g; 2a-c, i, k; 3a, b, i, k; 4a-d, f, g, k-r,
t; 5a; 6a-u; 7a). Likewise, 12 HIV-1 genomes were selected representing groups M (subtypes
A-D, F1, F2, H, J, K, N, O, and P). For influenza A virus, we included only sequences from
segment 7 and segment 5 encoding the conserved matrix proteins M1/M2 and the
nucleocapsid protein NP, respectively. We selected 82 M1/M2 segments and 115 NP
segments among the available segments sequences. All available segments were included
from genomes belonging to * Arenaviridae, Bunyaviridae, and Reoviridae*. For members of
*Poxvirinae* for which full genomes were unavailable (Skunk-, Racoon-, and Volepox virus)
sequences representing the conserved gene encoding the DNA-dependent RNA polymerase were included (n=22). In addition, 2 partial genomes of Squirrelpox virus were included. By mistake 2 and 9 partial sequences were included from *Iridoviridae* (1.5-2.5 kb) and *Coronaviridae* (1.3-14.5 kb), respectively, already represented by full genomes. Likewise, sequences representing Merkel cell polyomavirus and KI polyomavirus were not included among the reference genomes used for probe design. SeqCap EZ hybridization probes were designed and synthesized by Roche NimbleGen (Madison, USA) based on the resulting reference sequences. Capture was performed on double-indexed libraries prepared from ancient DNA, following the manufacturer’s protocol (version 4.3) with the following modifications. Briefly, 1.8 to 2.2 µg of pooled libraries were hybridized at 47°C for 65-70 hours with low complexity C\textsubscript{0}T-1 DNA, specific P5/P7 adaptor-blocking oligonucleotides each containing a hexamer motif of inosine nucleotides to match individually indexed adapters, hybridization buffer containing 10% formamide, and the capture probes. Dynabeads M-270 (Invitrogen) were used to recover the hybridized library fragments. After washing and eluting the libraries, the post-capture PCR amplification was performed with KAPA Uracil+ polymerase (Kapa Biosystems). PCR cycling conditions were as follows: 1 cycle of 3 min at 95°C, followed by 14 cycles of: 20 sec denaturation at 98°C, 15 sec annealing at 65°C and 30 sec elongation at 72 °C, ending with 5 min at 72°C. The amplified captured libraries were purified using AMPureXP beads (Agencourt). Shotgun sequencing data was generated as described in Allentoft et al. (2015). Sequencing of target-enriched libraries was performed on Illumina Hiseq2500 SR80bp, V4 chemistry. The resulting reads were compared to Dataset 2 using BLASTn (with arguments -task blastn -evalue 0.01). Matching reads with bit scores greater than 50 for all samples (except DA222
(70) and DA45 (55)) were selected for subsequent processing. In total, 6757 reads matched HBV after capture.

**Sequence authenticity and ruling out contamination**

The following evidence leads us to believe that the ancient HBV sequences are authentic and that the possibility of contamination can be excluded:

1. Standard precautions for working with ancient DNA were applied\(^{60}\).
2. Sequences were checked for typical ancient DNA damage patterns using mapDamage\(^{61}\) (version 2.0.6). Whenever sufficient amounts of data were available (>200 HBV reads), we found C>T mutations at the 5’ end, typical of ancient DNA\(^{31}\) (see Extended Data Fig. 1 and SI Fig. 1a).
3. Capture was performed on sample DA222 DNA extracts with and without pre-treatment by Uracil-Specific Excision Reagent (USER)\(^{62}\). After USER treatment (3h at 37°C) of the aDNA extract, the damage pattern is eliminated (SI Fig. 1b).
4. As the ancient viruses are from three different HBV genotypes (A, B, D) and a clade in sister relationship to NHP viruses, any argument that samples were contaminated would have to account for this diversity as well as the sequence novelty.
5. HBV sequences were identified in 25 of 305 analysed samples (Table 1), showing that the findings cannot be due to a ubiquitous laboratory contaminant.
6. Despite the low frequency of positive samples, we sequenced extraction blanks to provide additional evidence against the possibility that the HBV sequences stemmed from sporadic incorporation, amplification, and sequencing of background reagent contaminants into the ancient DNA libraries. The negative extraction controls were amplified for 40 PCR cycles, and BLAST was used to match the read sequences against Dataset 3, with the same parameters used for the
ancient samples. Because the ancient HBV positive reads used to assemble genomes all had bit scores of at least 50 (see Data and Data Processing, above), we filtered the negative extraction control BLAST output for reads with a bit score $\geq 45$. No reads (out of 23 million) matched any HBV genome at that level.

(6) HBV is a blood-borne virus that is mainly transmitted by exposure to infectious blood and that does not occur in the environment$^2$, making contamination during archaeological excavation extremely unlikely.

Consensus sequences

Reads from the original sequencing and from the capture were aligned to a reference genome (SI Table 3) in Geneious$^{63}$ (version 9) using Medium Sensitivity / Fast and Iterate up to 5 times. Because aDNA damage often clusters towards read termini$^{31}$, the resulting alignments were carefully curated by hand to remove non-matching termini of reads if the majority of the read showed a very good match with the reference sequence.

Genotyping

All reads used to construct the ancient HBV consensus sequences were matched against the full NCBI nucleotide (nt) database (downloaded December 28, 2016) using BLAST. 97.5% of the reads had HBV as their top match. All ancient consensus sequences were matched against the full HBV genomes of Dataset 4 with the Needleman-Wunsch algorithm$^{64}$, as implemented in EMBOSS$^{65}$ (version 6.6.0.0). For each ancient sequence, the percent sequence identity for each modern genotype and four NHP species is listed in Extended Data Table 3a. The Needleman-Wunsch algorithm was also used to calculate the pairwise sequence similarity between all ancient sequences (Extended Data Table 3b).

Recombination analysis
The Recombination Detection Program\textsuperscript{33}, version 4 (RDP4) was used to search for evidence of recombination within the 12 ancient sequences and a selection of 15 modern human and NHP sequences (see SI section 6). Recombination with HBV-RISE387 as the recombinant and HBV-DA51 as one parent, was suggested at positions 1567-2256, by seven recombination methods (RDP\textsuperscript{66}, GENECONV\textsuperscript{67}, BootScan\textsuperscript{68}, MaxChi\textsuperscript{69}, Chimaera\textsuperscript{70}, SiScan\textsuperscript{71}, and 3Seq\textsuperscript{72}) with p-values from 1.179x10^{-6} to 5.336x10^{-11} (Extended Data Table 2). The same recombination was suggested for all 4 ancient genotype A and two modern genotype A sequences. Graphical evidence of the recombination and the predicted break point distribution for sequences HBV-RISE386 and HBV-RISE387 from three methods (MaxChi, Bootscan, and RDP) is shown in Extended Data Fig. 3.

**Phylogenetic analysis**

**Initial maximum likelihood phylogenies**

An initial Maximum Likelihood (ML) tree was generated to ascertain that the ancient sequences fall within the primate HBV clades. Dataset 1 and the ancient sequences were aligned in MAFFT\textsuperscript{73} (version 7). The ML tree was constructed using PhyML\textsuperscript{74} (version 20160116), optimizing topology, branch lengths, and rates. We used a GTR substitution model, with base frequencies determined by ML, and an ML-estimated proportion of invariant sites and 100 bootstraps (Extended Data Fig. 2). Furthermore, an ML tree (Extended Data Fig. 4) was generated based on a MAFFT alignment of Dataset 2 and the ancient sequences, using the same parameters as outlined above. The final trees show nodes with support values less than 70 as polytomies.

**Dated coalescent phylogenies**

In order to check for a temporal signal in the data, a root-to-tip regression and date randomisation tests were performed. For the root-to-tip regression, input trees were calculated using Dataset 2 with the addition of a Woolly Monkey sequence (GenBank
Accession Number: AF046996) as an outgroup. Three phylogenetic algorithms were used, Neighbour Joining, ML (PhyML), and Bayesian (MrBayes\textsuperscript{75} (version 3.2.5)) methods (SI Figures 2-4). Root-to-tip distances were extracted using TempEst\textsuperscript{46} (version 1.5). For ML and Bayesian, root distances for tip taxa (in substitutions per site) were extracted from optimized tree topologies (ML and Maximum Clade Credibility trees, respectively). For NJ, root-to-tip distances were averaged over 1000 bootstrap replicates. Regression analyses were performed with Scipy\textsuperscript{47} (version 0.16.0). For the date randomisation tests, we used three different approaches to randomise tip dates: First, tip dates were randomised between all sequences in the phylogeny. Second, tip dates were randomised only among the ancient sequences presented in this paper, as well as the Korean mummy sequence (accession number JN315779). The modern sequences retained their correct ages. Third, dates were randomised within a clade. For each of the three approaches, we did two independent randomisations. This resulted in a total of six analyses, which were run for 100,000,000 generations each, under the relaxed lognormal clock model and coalescent exponential tree prior. We also ran the same analysis under a strict clock and Bayesian skyline tree prior, which were run for 20,000,000 generations. We used a GTR substitution model with unequal base frequencies, four gamma rate categories, estimated gamma distribution of rate variation, and estimated proportion of invariant sites, as found by bModelTest\textsuperscript{76} (version 1.0.4). None of the analyses using the relaxed clock converged (Estimated Sample Size (ESS) < 200). This is most likely because the mis-specification of the dates leads to an incongruence between the sequence and time information. Under the strict clock model, all runs converged, and none of the 95% HPD intervals of the tree height overlapped between the randomised and the non-randomised runs, fulfilling the criteria for evidence of a temporal signal\textsuperscript{77} (SI section 8, SI Figure 6).

Dated phylogenies were estimated using BEAST\textsuperscript{2} (version 2.4.4, prerelease). We used a MAFFT alignment of Dataset 2. Using bModelTest\textsuperscript{76}, we selected a GTR substitution model.
with unequal base frequencies, four gamma rate categories, estimated gamma distribution of rate variation, and estimated proportion of invariant sites. Proper priors were used throughout. Path sampling, as implemented in BEAST2, was performed to select between strict or relaxed lognormal clock and a constant coalescent population, an exponential coalescent population, or a coalescent Bayesian skyline tree prior (Extended Data Table 2a). Likelihood values were compared using a Bayes factor test. According to Kass and Raftery\textsuperscript{78}, a Bayes factor in the range of 3-20 implies positive support, 20-150 strong support, and >150 overwhelming support. The relaxed lognormal clock model in combination with a coalescent exponential tree prior was favoured. For the final tree, a Markov chain Monte Carlo analysis was run until parameters reached an ESS > 200, sampling every 2000 generations. Convergence and mixing were assessed using Tracer\textsuperscript{79} (version 1.6). The final tree files were subsampled to contain 10,000 or 10710 (for the relaxed lognormal clock, coalescent exponential tree prior) trees, with the first 25% of samples discarded as burn-in. Maximum clade credibility trees were made using TreeAnnotator\textsuperscript{34} (version 2.4.4 prerelease).

In order to formally test the Out of Africa hypothesis, calibration points were tested using path sampling as implemented in BEAST2. Calibration points were constrained as follows. Split of genotypes F and H: The MRCA of all genotype F and H sequences was constrained using a uniform(13,400: 25,000) distribution, as this is the range of estimates for when the Americas were first colonized\textsuperscript{80,81}. Split of subgenotype A3 in Haiti: The MRCA of FJ692598 and FJ692611 was constrained using a uniform(200: 500) distribution, due to the timing of the slave trade to Haiti\textsuperscript{43}. Split of C3 in Polynesia: The MRCA of X75656 and X75665 was constrained using a uniform(5,100: 12,000) distribution, due to the range of estimates for the MRCA of Polynesian populations\textsuperscript{8,82}. Calibration points were tested under both a relaxed...
lognormal clock, coalescent exponential tree prior, and a strict clock, Bayesian skyline tree prior.

**Data availability**

All HBV DNA sequence reads and consensus sequences that support the findings of this study will be deposited in the Sequence Read Archive and GenBank (respectively) prior to publication.
References


Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions

BM: wrote the paper, computational analysis, consensus sequences, phenotype and mutation analysis, recombination analysis, genotyping, Bayesian trees, calibrations, and hypothesis testing, DNA damage analysis, regression analysis, created figures and tables, participated in interpretation of results.

TJ: wrote the paper, computational pipeline and analysis, recombination analysis, genotyping, similarity analysis, ML trees, negative extraction control analysis, created figures and tables, participated in interpretation of results.
PD: wrote the paper, conducted sampling and generation of the sequence data, participated in interpretation of results.

MA: wrote the paper, conducted sampling and generation of the sequence data, participated in interpretation of results.

IS, AL, EU: excavated and curated the analyzed samples.

IP: excavated, curated, and analyzed archaeological context of data used for the sampling.

BB, TB: conducted sampling, provided archaeological background, participated in interpretation of results.

KT, VM, NL: excavated and curated the analyzed samples.

VS: excavated and curated the analyzed samples and participated in interpretation of results from a paleopathological point of view.

DV, EK, AE, DP, MV, TDP, VM: excavated and curated the analyzed samples.

AH: designed virus capture probes.

LO: scientific discussions and editing paper.

SR: participated in interpretation of results.

MS: participated in interpretation of results.

LV: wrote the paper, conducted TaqMan PCR, designed virus capture probes and performed all target enrichment experiments.

AO: initiated and provided critical input on the development of NGS bioinformatics tools.

DS: wrote the paper, computational analysis, participated in interpretation of results.

DG: wrote the paper, genotyping, serotyping, phenotype and mutation analysis, participated in interpretation of results.

RF: wrote the paper, recombination analysis, computational analysis, participated in interpretation of results.
CD: wrote the paper, recombination analysis, Bayesian trees and hypothesis analysis, ML trees, regression analysis, PCR probe design, participated in interpretation of results.

KS, KK: conducted sampling and archaeological background.

EW: initiated the work, wrote the paper, led sampling and generation of the sequence data, participated in interpretation of results.

**Author Information**

Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints). The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to E. W. (ewillerslev@snm.ku.dk).
Extended Data table and figure titles and legends

Extended Data Table 1 | Extended overview of samples with reads matching HBV and PCR results

a, Extended overview of samples with reads matching HBV. Rows are sorted by decreasing consensus coverage. Explanation of column titles, from left to right starting from the second column: $^{14}$C age and standard deviation; Median cal BP age or estimate (in years); approximate sample age in years; site; culture or period; gender; number of sequencing reads that matched HBV using DIAMOND$^{57}$; number of HBV proteins matched by those reads; number of sequencing reads that matched HBV using a BLASTn$^{58}$ database built from Dataset 3 (see Methods); the number of reads from the capture that matched HBV using BLASTn (as above); the bit score cut-off above which matching reads were used to form consensus sequences; the percentage of the consensus genome covered by matching reads; average depth of coverage across the reference genome, as reported by Geneious$^{63}$. When reading sample information across a row, an empty cell will be encountered when processing on that sample was concluded, either (in column 6) due to too few matching reads or (penultimate column) consensus coverage less than 50%. b, TaqMan PCR results.

Four extracts from samples with HBV reads were selected for TaqMan PCR confirmation: two with a large proportion of HBV reads (DA222 and DA195), two with a small proportion of HBV reads (DA85 and DA89), and one with no HBV reads (DA351). HBV was detected in extracts from DA222 and DA195, whereas the three low- and zero-read samples were negative, as were all non-template controls.

Extended Data Table 2 | Genotype A predicted recombination break points and p-values

a, The p-values assigned to the predicted genotype A recombination by the seven methods used by RDP4$^{33}$, in the order given by RDP. The number of sequences in which the
recombination was predicted is always 6, corresponding to the 4 ancient and two modern genotype A sequences. b, The predicted begin and end break points for each of the 6 genotype A sequences. Sequences are ordered from oldest to youngest. The 99% confidence intervals for the begin and end points are also shown, and are identical for all sequences. Note that the predicted break points are close to the boundaries of the polymerase. For example, for the modern genotype A sequence LC074724, the polymerase is found in regions 1-1623 and 2307-3221 and the predicted break points are 1622 and 2256. If recombination formed an HBV-RISE387/6 ancestor, it is possible that the entire polymerase gene was contributed by one parent.

Extended Data Table 3 | Model testing and inferred age of genotypes
Models were compared using Path Sampling, as implemented in BEAST2. Likelihood values were compared using a Bayes factor test. A positive value for the Bayes factor implies support for model 1, a negative value support for model 2. According to Kass and Raftery, a Bayes factor in the range of 3-20 implies positive support, 20-150 strong support, and >150 overwhelming support. a, Results of testing different clock models and population assumptions to be used for dated phylogenies. Positive numbers indicate support for the columns model, negative number for the rows model. b, MRCA age of individual nodes under a strict clock and Bayesian skyline tree prior or under a relaxed lognormal clock and coalescent exponential tree prior. c, Root age and substitution rates under different clock models and tree priors. d, Results of testing different calibration point hypotheses under a strict clock and Bayesian skyline tree prior or under a relaxed lognormal clock and coalescent exponential tree prior.

Extended Data Table 4 | Consensus sequence identity
a, Best consensus sequence identity with 14 groups of HBV full genomes. The Needleman-Wunsch algorithm (as implemented in EMBOSS) was used to globally align each sample consensus sequence against each of the 3384 full HBV genomes of Dataset 4 (see
Methods). The table shows the best nucleotide (nt) similarity percentage for each sample consensus against 14 genome groups from the full set of HBV genomes. In cases where the consensus length is less than the genome length, the given figure is the percentage of identical nucleotides (nts) in the matching region, not counting any alignment gaps or ambiguous consensus nts. For each sample, the genome group with the highest identity is highlighted in bold. b, Inter-consensus sequence identity. The Needleman-Wunsch algorithm was used to globally align all sample consensus sequences against one another. The table shows the nt identity percentage for each alignment. In cases where the consensus lengths were unequal, the given figure is the percentage of identical nts in the matching region, not counting any alignment gaps or ambiguous consensus nts.

Extended Data Figure 1 | Ancient DNA damage patterns for the samples included for further analysis

The frequencies of the mismatches observed between the HBV reference sequences (Extended Data Table 1) and the reads are shown as a function of distance from the 5' end. C>T (5') and G>A (3') mutations are shown in red and blue, respectively. All other possible mismatches are reported in gray. Insertions are shown in purple, deletions in green, and clippings in orange. The count of reads matching HBV for each sample is shown in parentheses. a, RISE563. b, DA222. c, DA119. d, RISE254. e, DA195. f, DA27. g, DA51. h, RISE386. i, RISE387. j, DA29. k, DA45. l, RISE154.

Extended Data Figure 2 | Hepadnavirus Maximum Likelihood tree

Shows 26 sequences from the Orthohepadnavirus species (Dataset 1, see Methods) including the ancient HBV sequences. Ancient genotype A sequences are shown in red, ancient genotype B sequences in orange, ancient genotype D sequences in blue and novel genotype sequences in green. The tree was constructed in PhyML, optimizing for topology, branch lengths, and rates, with 100 bootstraps (see Methods). Internal nodes with <70% bootstrap support are shown as polytomies.
Extended Data Figure 3 | Genotype A recombination break point evidence

RDP4 was used to analyse the set of 12 ancient sequences plus a representative set of 15 modern human and NHP sequences (see Methods). The seven recombination programs used by RDP4 suggested that all genotype A sequences are recombinants, with the genotype D sequence HBV-DA51 as the minor parent and an unknown major parent. The obvious interpretation is that recombination formed an ancestor of the oldest sequences, evidence of which is still present in the less ancient and the modern representatives. The panel shows the graphical evidence and predicted recombination break point distribution for the two oldest genotype A sequences, HBV-RISE386 and HBV-RISE387, according to three of the RDP4 methods (MaxChi, Bootscan, and RDP). In all sub-plots the predicted location of the break points is shown by a dashed vertical line and the surrounding gray area shows the 99% confidence interval for the break point. Sub-plots on the same row share their Y axis and those in the same column share their X axis. a, HBV-RISE386 analysed by MaxChi. b, HBV-RISE386 analysed by Bootscan. c, HBV-RISE386 analysed by RDP. d, HBV-RISE387 analysed by MaxChi. e, HBV-RISE387 analysed by Bootscan. f, HBV-RISE387 analysed by RDP.

Extended Data Figure 4 | HBV Maximum likelihood tree

The sequences from Dataset 2 (see Methods) and the ancient sequences were aligned in MAFFT. The tree was constructed in PhyML, optimizing for topology, branch lengths, and rates, with 100 bootstraps (see Methods). Internal nodes with <70% bootstrap support are shown as polytomies. Ancient genotype A sequences are shown in red, ancient genotype B sequences in orange, ancient genotype D sequences in blue and novel genotype sequences in green. Letters on internal branches indicate the genotype. Taxon names indicate: genotype / subgenotype, GenBank accession number, age, country abbreviation of sequence origin, region of sequence origin, host species, and optional additional remarks. Note that the ML tree shows topological uncertainty (polytomies) in areas where the
BEAST2\textsuperscript{34} tree (Figure 2b) is well resolved. This is the case for two reasons. Firstly, BEAST2 always produces a fully-resolved binary topology without polytomies. Second, and more important, BEAST2 creates a time tree and uses tip dates to constrain the possible topologies under consideration. Thus BEAST2 can know that certain topologies are unlikely or impossible, whereas ML cannot and thus inherently has greater uncertainty regarding tree topology.