VIRUS EVOLUTION

Virus Evolution, 2025, **11(1)**, veaf025

DOI: https://doi.org/10.1093/ve/veaf025
Advance Access Publication 5 April 2025
Research Article

Ancient origins and global spread of domestic cat hepatitis B virus

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Abstract

Mammalian hepadnaviruses have likely been evolving alongside their hosts for millions of years. Domestic cat HBV (DCHBV) has been detected in cats from several countries, but its genealogy, epidemiology, and host range remain unclear. Besides DCHBV, the only hepadnavirus identified among carnivores is the ringtail HBV (RtHBV). Because there is a gap in the felid fossil record of approximately 5–7 million years between the late Oligocene and the early Miocene, carnivore-derived viruses might help to shed light on Felidae evolution. Here, we screened 2260 sera and 154 paraffin-embedded liver samples from cats and 2123 sera from dogs sampled in Europe and South and Central America between 2018 and 2020 by PCR for DCHBV. We identified DCHBV genotype A (GtA) in 0.6% (7/1,195; 95% CI, 0.2–1.2) of cats sampled in Germany, France, Croatia, and Bulgaria and a genetically divergent DCHBV genotype B (GtB; 10.8% genomic sequence distance) in 0.2% of cats (2/1,065; 95% CI, 0.0–0.7) from Brazil. The detection rates of the two genotypes did not differ significantly (Fisher, $P = .19$). Viral loads ranged from 4×10^1–6×10^6 for DCHBV GtA to 5–7×10^3 for DCHBV GtB DNA copies per milliliter of serum. None of the cat livers or dog sera tested positive by PCR. Immunoglobulin G against the DCHBV core antigen (anti-DCHBc) was detected in 8/504 cat sera (1.6%; 95% CI, 0.7–3.1), without significant variation between countries (χ^2, $P = .17$), and in none of 180 dog sera by indirect immunofluorescence assay (IFA). Neither IFA (Fisher, $P = .11$; $n = 311$) nor PCR (Fisher, $P = .63$; $n = 699$) positivity was significantly associated with increased liver enzymes in cats, respectively. Coevolutionary reconciliations of virus and host phylogenies and Bayesian hypothesis testing suggested evolutionary origins of DCHBV during the Miocene, ∼8–17 million years ago (mya) from ancestral carnivores, consistent with long-term evolution. The long-term association of DCHBV with felines aids in elucidating orthohepadnaviral infection patterns and felid genealogy.

Keywords: hepatitis b; viral evolution; neotropics; carnivores; animal models

Introduction

More than 250 million people live with chronic hepatitis B globally, causing over 1.1 million deaths annually (WHO 2021). The causative agent, hepatitis B virus (HBV, genus *Orthohepadnavirus*), has been circulating among humans since at least the Neolithic (Krause-Kyora et al. 2018). Nonhuman HBV homologs have been found in different mammalian orders, including Primates and Rodentia (both belonging to the clade Euarchontoglires), as well as Eulipotyphla, Chiroptera, and Carnivora (all belonging to the clade Laurasiatheria) (Rasche et al. 2019b). The hepadnaviruses infecting avian, reptile, and amphibian hosts have evolved among vertebrates for >300 million years (Glebe et al. 2021), and cumulative evidence suggests that orthohepadnaviruses have been coevolving with their hosts for ∼80 million years, potentially originating in Laurasiatherian hosts via unclear macroevolutionary patterns, including both host shifts and coevolutionary processes

(Krause-Kyora et al. 2018, Simmonds et al. 2019, Rasche et al. 2019a, 2019b). However, the macroevolutionary patterns of orthohepadnaviruses are less well understood because those viruses do not integrate into the germline (de Carvalho Dominguez Souza et al. 2018, Rasche et al. 2019b).

The recently identified domestic cat hepadnavirus (DCHBV) and ringtail HBV (RtHBV) are the only hepadnaviruses identified among carnivores thus far (Aghazadeh et al. 2018, Jo et al. 2021). While the presence of a hepadnavirus in dogs was suggested years ago by serological studies that demonstrated antibody reactivity in dog sera against human HBV (Hoofnagle et al. 1983, Vieira et al. 2019, Al-Jumaa et al. 2020), no bona fide dog hepadnavirus has been found yet. DCHBV, on the other hand, has been reported sporadically in dogs from Italy (Diakoudi et al. 2022, Fruci et al. 2023) and Hong Kong (Choi et al. 2022).

DCHBV was initially detected in cats in Australia (Aghazadeh et al. 2018), and by mid-2024, it was detected in 12 countries globally (Fig. 1A) at highly different rates for unknown reasons (Lanave et al. 2019, Anpuanandam et al. 2021). A potential modifier causing relatively more frequent detection of DCHBV is its potential association with immunosuppression in cats, including those chronically infected and coinfected with immunosuppressive feline-specific retroviruses, feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) (Aghazadeh et al. 2018, Lanave et al. 2019). DCHBV infection has been associated with elevated serum alanine transferase (ALT) (Anpuanandam et al. 2021) and long-term infection for up to one year (Capozza et al. 2021, Takahashi et al. 2022) in cats with hepatic disease and hepatocarcinoma (Pesavento et al. 2019), but again, DCHBV pathogenicity remains unclear since the virus was also found in asymptomatic cats.

Finally, the limited genetic variability, narrow geographic range of DCHBV, and detection of genetically related viruses in ungulates challenge assessments of potential long-term evolutionary associations between DCHBV and felids (Gogarten et al. 2019, Rasche et al. 2021). Evolutionary analyses of DCHBV may be relevant not only for understanding the hepadnaviral, but also its host genealogy, as there is a gap in felid fossil record of ∼5–7 million years between the late Oligocene and the early Miocene ∼25–18 million years ago (mya), commonly termed the cat gap (Macdonald and Loveridge 2010). If DCHBV was associated with the felid lineage for a prolonged period of time, the detection of genetically related orthohepadnaviruses in diverse felids may aid in the elucidation of evolutionary events during the cat gap.

Here, we investigate the epidemiology and evolution of DCHBV in domestic cats and dogs on three continents and provide evidence for non-recent evolutionary origins of DCHBV.

Materials and methods

Sampling

In Brazil, dogs and cats were sampled between 2018 and 2020. Sampling and shipment were performed as approved by national authorities; thus, the analysis and activities within this study were approved by the Federal University of Bahia's animal ethics committee under authorization no. 74/2019. European samples of dogs and cats were collected from excess material of a fraction of samples, that were initially send in 2022 for routine diagnostics to a private laboratory in Germany, which would otherwise be discarded. According to the decision (RUF-55.2.2-2532-1-86-5) of the local competent authority (Regierung von Unterfranken) left-over material of samples, which were initially not taken for research purposes, can be repurposed for scientific investigations without further ethics application. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were obtained using an automatic chemical analyzer (Cobas 8000; Roche), which was provided (when available) together with the samples by the same private laboratory in Germany. Domestic cats were sampled for various reasons, such as checkups, blood transfusions, presurgical preparation, routine blood counts, and suspected infectious causes. The detailed list is available in Supplementary Table 2. In Costa Rica, liver biopsies were collected from the diagnostic service of the veterinary pathology department at the National University of Costa Rica between 2020 and 2023. The analysis and activities were performed as approved by national authorities. Sampling and shipment were approved by the Universidad Nacional de Costa Rica under permit nos. 0622-1 and 0069-23. All biopsies were subjected to histopathological analysis due to suspected liver disease by a referral veterinarian.

Viral detection and sequencing

Viral nucleic acids were extracted from animal sera using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Life Sciences). Viral nucleic acids were extracted from paraffin-embedded tissues using the MagNA Pure FFPET Buffer Set (Roche Life Sciences). A total of 4383 serum samples and 154 liver samples (Table 1) were initially screened for hepadnaviruses via a broadly reactive seminested PCR assay as previously described (Drexler et al. 2013). Viral loads in sera were determined via the following DCHBV-specific real-time PCR: forward primer (AGTACCAAACCTCCAAGCAC), reverse primer (AGCGAATATTCACAGCCTGAG) and probe (FAM-CGCAGACACATCCAAGGAAAGCCA-BHQ1) labeled with a dark quencher at the 3′-end and fluorescein amidite (FAM) at the 5′-end. Quantification was performed photometrically via previously quantified 0.5 kb synthetic DNA standards of the target (based on the polymerase of the Australian DCHBV, accession number: NC_040719). Then, 25 µl real-time PCR reactions were set up using Platinum Taq Kit (Invitrogen) with 1 µl of DNA, 400 nM each primer and probe, 1 µg of BSA, 0.2 mM each dNTP, and 2.5 mM MgCl2 and were carried out under the following thermocycling conditions: 94°C for 3 min, 45 cycles of 15 s at 94°C and 30 s at 58°C, followed by cooling for 30 s at 37°C. The DCHBV-specific real-time was able to detect up to 5 viral DNA copies/µl of serum. The complete genomic sequences (GenBank accession numbers: PQ468307- PQ468315) were obtained from positive sera via a PCR-based approach with primers designed to target available DCHBV sequences and RtHBV (Supplementary Table 1) or deep sequencing via Illumina MiSeq as previously described (Rasche et al. 2021b). Detailed information on the sequencing approach used for each obtained DCHBV sequence is available in Supplementary Table 2.

Evolutionary analysis

Host and hepadnavirus sequences were aligned using the MAFFT algorithm implemented in Geneious version 9.1.8 (www.geneious.com) (Katoh et al. 2017). Sites that were missing or ambiguous were excluded from the alignment. Percentage sequence distances and maximum likelihood trees of each translated open reading frame were calculated using MEGA X (Kumar et al. 2018). The host trees used for co-evolutionary analyses were constructed using cytochrome b sequences obtained from GenBank. To ensure phylogenetic accuracy, the host tree topologies were refined using monophyly priors based on previously established mammalian phylogeny (Foley, et al., 2016). Viral recombination was inferred using the Recombination Detection Program (RDP4) (Martin et al. 2015), which employs nine recombination detection methods (RPD, GENECONV, Bootscan, Maxchi, Chimaera, SISscan, PhylPro,

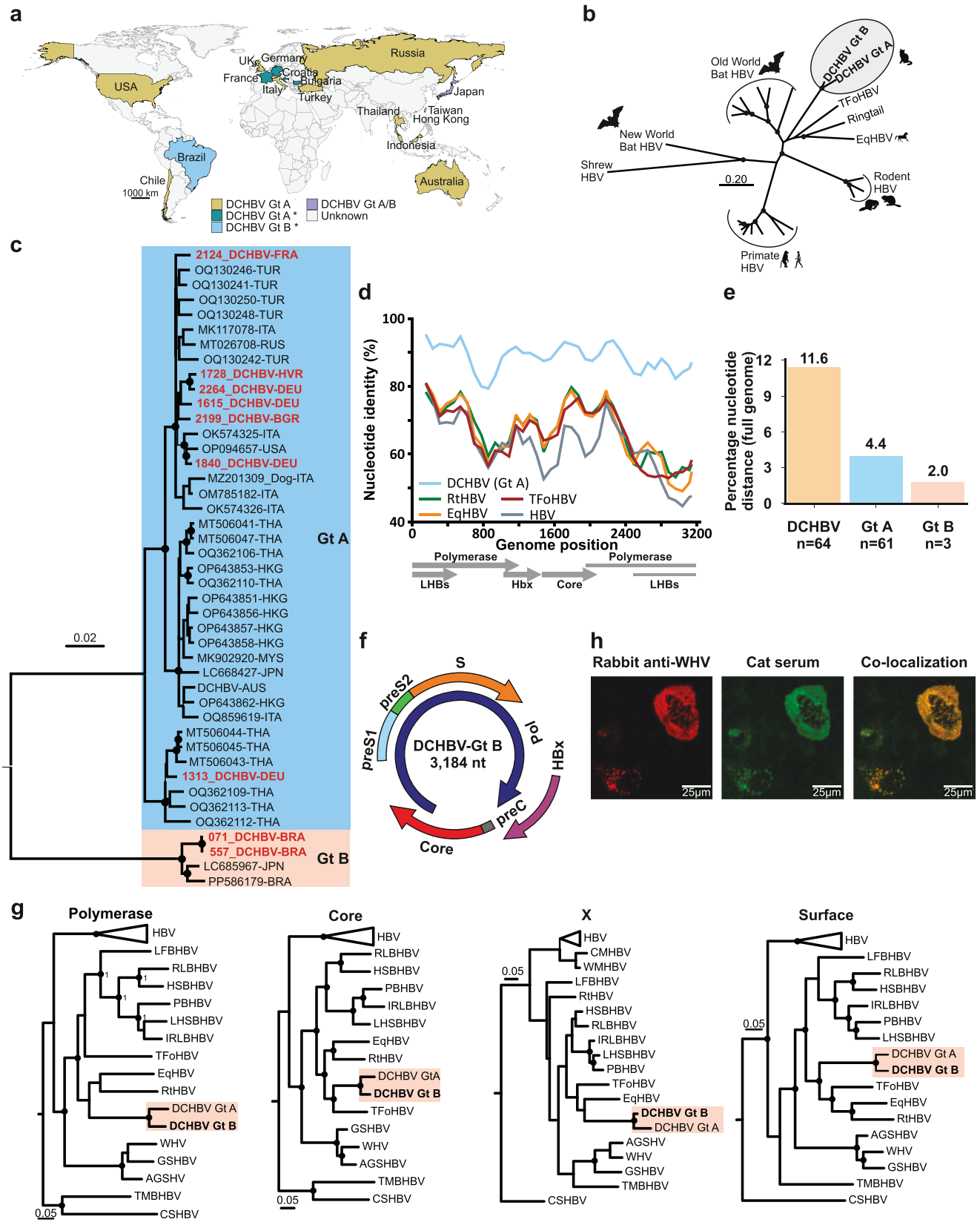

Figure 1. Molecular epidemiology of DCHBV. (a) Worldwide distribution of DCHBV genotypes. (b) Maximum-likelihood phylogenetic tree based on the complete genome of mammalian hepadnaviruses. (c) Maximum-likelihood phylogenetic tree of DCHBV without recombinant sequences using the white sucker hepatitis B virus as an outgroup (see Appendix for detailed information). Sequences identified in this study are highlighted in bold(d) Pairwise comparison of DCHBV and related hepadnaviruses. (e) Nucleotide-level intra- and inter-genotypic pairwise comparison of DCHBV sequences . (f) Genome characteristics of DCHBV genotype B. (g) Translated open reading frame phylogenies of DCHBV genotypes and other hepadnaviruses. (h) Immunofluorescence assay co-staining of the DCHBV core transfected into Vero E6 cells using rabbit anti-woodchuck hepatitis virus and a seropositive domestic cat serum.

Table 1. Sample characteristics.

Material	Species	Country	No.	PCR positivity (%)	95% CI	IFA			Year sampled
						No.	(%)	95% CI	
Sera	Cats	Brazil	1065	0.2	0.0–0.7	194	2.6	0.8–5.9	2018–2020
		Germany	976	0.4	0.1–1.1	310	1.0	0.2–2.8	2022
		Czech Republic	26	0.0	-	-	-	-	2022
		Austria	25	0.0	-	-	-	-	2022
		Estonia	19	0.0	-	-	-	-	2022
		Sweden	18	0.0	-	-	-	-	2022
		Luxembourg	15	0.0	-	-	-	-	2022
		Romania	15	0.0	-	-	-	-	2022
		Italy	13	0.0	-	-	-	-	2022
		Switzerland	11	0.0	-	-	-	-	2022
		France	9	11.1	0.3–48.3	-	-	-	2022
		Croatia	8	12.5	0.3–52.7	-	-	-	2022
		Other[a]	60	1.7	0.0–8.9	-	-	-	2022
		Total cat (sera)	2260	0.4	0.2–0.8	504	1.6	-	2018–2022
	Dogs	Brazil	923	0.0	-	90	0.0	-	2018–2020
		Germany	1200	0.0	-	90	0.0	-	2022
		Total dog (sera)	2123	0.0	-	180	0.0	-	2018–2022
FFPE	Cats	Costa Rica	154	0.0	-	-	-	-	2020–2023

CI, confidence interval; [a] Detailed information including sampling sites (countries) with fewer than eight samples is available in Supplementary Table 2.

LARD, and 3Seq). Predicted recombinant events that were confirmed by more than three different methods were considered and the recombining regions were manually removed from the alignment. Our analysis detected multiple sites of non-recent recombination events, mostly in the polymerase, and after removing all recombinant sites from the alignment, a total of 1911 remaining sites were used for further evolutionary inferences. Ancestral state reconstructions (ASRs) and hypothesis testing were performed in BEAST v. 1.10.4 (Suchard et al. 2018). Hypothesis testing was performed as previously described (de Carvalho Dominguez Souza et al. 2018, Rasche et al. 2021), with an alignment including all defined species of mammalian hepadnaviruses. Initially, we performed Nested Sampling in Beast 2 (Bouckaert et al. 2019) to determine the best-fitting clock model. In brief, we compared strict, relaxed exponential, relaxed log-normal, optimized relaxed and random local clock models, selecting the one with the highest likelihood estimation. We employed a mean substitution rate of 1.0×10^{-5} nucleotide substitutions per year, according to previous calibrations for hepadnaviruses (Muhlemann et al. 2018, Simmonds et al. 2019). To further calibrate the molecular clock and for the hypothesis testing, we constrained the most recent common ancestor (MRCA) of the root at 80.5 million years ago (mya) (normally distributed; mean, 80.5; standard deviation, 0.5 [quantiles: 2.5%, 79.02; 97.5%, 80.98]), corresponding to the estimated origin of Laurasiatheria (Foley et al. 2016). Additionally, we calibrated the node of Carnivores-ungulate-derived viruses at 56 mya (normally distributed; mean, 56.0; standard deviation, 0.5 [quantiles: 2.5%, 55.02; 97.5%, 56.98]), corresponding to the estimated origin of carnivores (Foley et al. 2016).

Antibody detection

There is no commercial DCHBV-specific serological test available for DCHBV. Therefore, we developed an IFA based on the DCHBV GtB core protein (DCHBc), as described previously for other hepadnaviruses (Jo et al. 2021, Rasche et al. 2021). Cross-reactive rabbit polyclonal serum raised against the woodchuck hepatitis virus(WHV) core protein was used as positive control. In brief, Vero E6 cells (C1008, ATCC CRL-1587) were transfected with a pcDNA 3.1+ expression plasmid containing the core protein of DCHBV-GtB as described previously for other hepadnaviruses (Drexler et al. 2013). Fixed and permeabilized cells were incubated for 2 h at 37°C with serum samples diluted 1:40 in 0.1% FCS (Fetal Calf Serum (Thermo Fischer, Cat. no. A5256701) in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fischer, Cat. no. 12 491 015). After the cells were carefully washed three times with Phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Thermo Fischer, Cat. no. 10 700 941) (PBS-T), the cells were incubated with a 1:500 dilution of the following secondary antibodies: anti-cat (goat anti-cat-FITC-IgG, Jackson Immunoresearch, West Grove, USA; Cat no. 102-095-003), anti-dog (rabbit anti-dog-FITC, Jackson ImmunoResearch, West Grove, USA; Cat no. 304-095-003) and anti-rabbit (goat-anti-rabbit-Cy3, Jackson ImmunoResearch, West Grove, USA; Cat no. 111-165-144). After 1 h of incubation at 37°C, the cells were washed three times with PBS-T and the slides were rinsed once in water. Dried cells were mounted with DAPI ProLong Mounting Medium (Thermo Fisher).

Results

Rare detection of DCHBV in cats from South America and Europe

To investigate the presence of DCHBV in domestic carnivores, we tested sera from 1065 cats and 923 dogs from Brazil, as well as from 1195 cats from several European countries, 1200 dogs from Germany and 154 liver samples from Costa Rica (Table 1). Serum and liver samples were sent for routine diagnostics to four different laboratories between 2018 and 2023 (Fig. 2A). Using broadly reactive and highly sensitive seminested PCR (Drexler et al. 2013), we detected DCHBV in two cats (2/1,065, 0.2%; 95% CI, 0.0–0.7) in one location in northeastern Brazil (Table 1). In Europe, we detected DCHBV in seven cats (7/1195, 0.6%; 95% CI, 0.2–1.2), including animals sampled in Germany, France, Croatia, and Bulgaria, with detection rates per country ranging from 0.0 to 12.5 (1/8; 95% CI, 0.3–52.7) (Table 1). All 2123 dog sera tested from Brazil and Germany were negative. Our data revealed a relatively lower overall frequency of DCHBV infection in cats than that reported in previous PCR-based studies, at 6.5–17.8% in Italy (Lanave et al. 2019, Capozza et al. 2021) or up to 12.3% in Malaysia (Anpuanandam et al. 2021), but our findings are in agreement with a previous

DCHBV detection rate of 0.8% reported in Japan (Takahashi et al. 2022). One possible reason for such a large discrepancy in DCHBV detection rates in cats is likely related to different study setups (e.g. randomized studies, sampling only symptomatic animals, or coinfection with other pathogens) or the age of the animals sampled. Nevertheless, our detection rate was comparable to those reported for other animal species using identical laboratory methodology, without significant difference compared with the 0.3% reported for bats (χ^2, $P = .656$) and 1.0% reported for equids (χ^2, $P = .013$), whereas our rate was lower than the 2.5% reported for shrews (χ^2, $P < .001$), corroborating that detection rates vary among different study samples (Drexler et al. 2013, Rasche et al. 2019a, 2021).

Detection of a divergent DCHBV genotype in South America

Phylogenetic analysis based on the full viral genomes generated by our combined PCR- and Illumina high-throughput sequencing-based workflows as well as publicly available DCHBV sequences indicated that while sequences from Bulgaria, Croatia, France, and Germany clustered together with other European, Asian, and North American sequences [henceforth termed genotype A (GtA)], the sequences obtained from Brazilian cats clustered together with DCHBV sequences from Japan (available in GenBank, accession number: LC685967) and from southern Brazil (Tessmann et al. 2024) in a separate clade (Fig. 1C). Genotyping of orthohepadnaviruses is defined on the basis of a divergence >7.5% at the genomic nucleotide level (Okamoto et al. 1988, Norder et al. 2004, Schaefer 2007, Kramvis 2014). The genome of our Brazilian DCHBV strains was 10.2% divergent compared to that of the other DCHBV complete genomic sequences (Table 1, Fig. 1D, Fig. 1E), suggesting that the Brazilian and Japanese DCHBV sequences represent a respective DCHBV genotype B (GtB). DCHBV GtB exhibited a typical hepadnavirus genome composed of 3184 nucleotides (Fig. 1F), and its genome organization included four predicted overlapping open-reading frames encoding predicted core (HBc), HBe, polymerase, surface (HBs), and X (HBx) proteins (Fig. 1F, Fig. 1G). Viral loads, determined using a DCHBV-specific real-time PCR assay capable of detecting and quantifying both genotypes, ranged from 4.0×10^1 to 6.0×10^6 DNA copies/ml for DCHBV GtA and from 5.0×10^3 to 7.0×10^3 DNA copies/ml for DCHBV GtB (Supplementary Table 2).

Low DCHBV seroprevalence in cats and lack of antibody detection in dogs

We tested a total of 504 cat and 180 dog sera available in sufficient volumes by a DCHBV core (DCHBc) IFA, which we developed by expressing the complete open reading frame of GtB DCHBc in Vero E6 cells. For comparison, the genetic variability of HBc at the amino acid level among human HBV genotypes is ~11.4%, yet single-genotype HBc-based serological tests are used to detect HBc-specific antibodies in patients irrespective of the infecting genotypes (Hussain et al. 2003, Kramvis et al. 2008). Therefore, considering that the DCHBc amino acid distance between GtA and GtB is 0.9% (Table 1), our test should reliably detect antibodies against both DCHBV genotypes. Anti-DCHBc IgG antibodies were detected in 1.6% of cats tested (8/504; 95% CI, 0.7–3.1), ranging from 1.0% (3/310; 95% CI, 0.2–2.8) in Germany to 2.6% (5/194; 95% CI, 0.9–5.4) in Brazil, without a statistically significant difference (χ^2, $P = .167$). End-point IFA titers ranged from 1:640 to 1:10,240 serum dilution (Supplementary Table 3). The serological reactivity pattern of domestic cat sera was more concentrated in the cytoplasm and slightly scattered in the nucleus, resembling the reactivity of positive control sera raised in rabbits against woodchuck hepadnavirus (WHV) (Fig. 1H, Supplementary Fig. 1). None of the 180 dog sera tested positive by IFA.

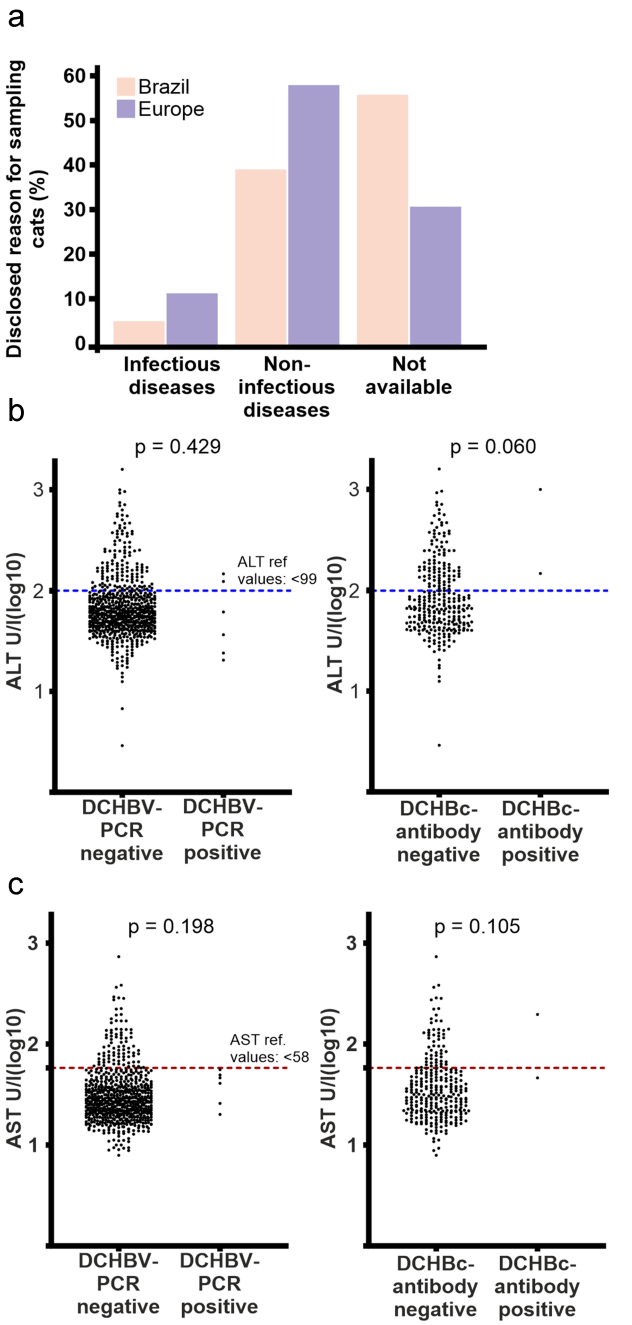

Figure 2. Clinical characteristics of domestic cats. (a) Reasons for collecting samples from cats in Brazil and Europe. The lack of more detailed information on the reasons for sampling was due to local data protection legislation. (b) Serum alanine transaminase (ALT) levels and the presence of DCHBV DNA or antibodies against DCHBV in domestic cats. (c) Serum aspartate aminotransferase (AST) levels and the presence of DCHBV DNA or antibodies against DCHBV in domestic cats.

Evidence for predominantly mild DCHBV infection

To assess the symptoms associated with DCHBV infection, we investigated the presence of DCHBV in paraffin-embedded liver

samples from cats with hepatic degeneration and liver neoplasia and compared the levels of aspartate aminotransferase (AST) (n = 696) and alanine aminotransferase (ALT) (n = 734) in the serum of cats that tested positive and negative for DCHBV DNA and anti-DCHBc. Liver enzymatic activity was altered in 20.8% of the sera for ALT (153/737, reference value: <99 U/l) and in 12.3% for AST (86/699; reference value: <58 U/l). None of the 154 liver samples tested positive by PCR. One of the PCR-positive cats from Brazil was an 8-year-old animal diagnosed with pancreatitis and liver dysfunction, whereas the other PCR-positive animals presented either no apparent symptoms or the reason why they were sampled was not disclosed (Fig. 2A). No significant associations were detected between increased ALT or AST levels and the presence of anti-DCHBc (Mann–Whitney; ALT, $P = .060$; AST, $P = .105$) or DCHBV DNA (Mann–Whitney; ALT, $P = .429$; AST, $P > .198$) (Fig. 2B and Fig. 2C).

Long-term evolutionary association of DCHBV and carnivore hosts

To infer macroevolutionary events shaping the genealogy of DCHBV, we inferred the level of congruence between the host and virus phylogenies in two separate analyses. In the first analysis, we investigated symmetries between genetic distances using the ParaFit program (Legendre et al. 2002). Coevolutionary reconstructions based on sequence distances supported cospeciation between DCHBV and domestic cats as well as among the other hepadnaviruses in the clade and their respective carnivore and ungulate hosts (Fig. 3A). In the second analysis, using the CoRe-PA program, we compared the topologies of the host and virus phylogenies to infer the nature and frequency of distinct evolutionary events (Merkle et al. 2010). Event-based reconciliation at the ancestral node of carnivores and ungulates revealed four sorting events, two duplication events, and three co-speciation events. Sorting events are common in viral macroevolutionary reconstructions and are consistent with a long-standing host–virus association. Here, the observed sorting events could hypothetically be due to unknown genetically related carnivore- and ungulate-derived viruses. On the other hand, statistical support for cospeciation ($P < .05$) was found only between DCHBV and RtHBV and their respective hosts, the domestic cat and the ringtail (Fig. 3B). Therefore, both evolutionary inferences suggested the occurrence of cospeciation during the evolutionary history of carnivore hepadnaviruses (DCHBV and RtHBV) (Fig. 3A, Fig. 3B, Fig. 3C), in contrast to the results found for ungulate viruses (EqHBV infecting donkeys globally and zebras (Rasche et al. 2021), as well as the Taï Forest HBV (TFoHBV) found in a Maxwell's duiker sampled in 2015 in Taï National Park in Côte d'Ivoire (Gogarten et al. 2019)), which share a recent common ancestor with carnivore-derived HBV. Bayesian ancestral state reconstruction (ASR) calibrated by mammalian host orders in which hepadnaviruses have been reported provided evidence that the DCHBV clade originated from carnivore-derived hepadnaviruses (posterior probability, 0.74) (Fig. 3C). Next, we performed hypothesis testing in a time-calibrated Bayesian framework by inferring whether the time to the most recent common ancestor (TMRCA) of DCHBV corresponded to essential events in Felidae evolution. Initially, the TMRCA of the root was calibrated to mya, corresponding to the origin of Laurasiatheria (Foley et al. 2016). Because previous analyses suggested coevolution, we investigated the likely origin of DCHBV by conducting a hypothesis test comparing two priors "Estimated age of the wild cat ancestor" (about 131 kya), the beginning of modern felid radiation (estimated around 10.8 mya ago) and the most recent occurrence of Proailurus, marking the estimated beginning of the cat gap period (Macdonald et al. 2010, Johnson et al. 2006, Driscoll et al. 2007). The high BF obtained in our analyses (BF = 870) provided decisive support (Kass and Raftery 1995) for the origin of DCHBV coinciding with the radiation of modern felids, rather than with the common ancestor of *Felis catus* (Fig. 4A). A comparable time frame was corroborated by further Bayesian analysis, which estimated a range of 8.4 and 16.6 mya (95% of highest posterior density [HDP]), with a geometric mean of 12.1 mya (Fig. 4B). On the other hand, the accuracy of this inference must be relativized considering the limited availability of other carnivore-derived-hepadnavirus sequences and the temporal overlap between the radiation of modern felids and the prehistoric ancestor Pseudaelurus. Nevertheless, all our analyses consistently support a long-term evolutionary origin extending into the Miocene period.

Discussion

Here, we describe divergent DCHBV genotypes circulating in Europe and South America and corroborate a long-term evolutionary association between carnivores and DCHBV.

Estimating the cost and impact of DHCBV infection on the health of domestic cats is challenging. Despite the low prevalence and likely onset of mild disease or asymptomatic infection, the potential harm to animal health is certainly more considerable with the prospect of chronic infection. In particular, considering that cats can live between 11 and 13 years, with some reaching up to 17 years, and a large global population estimated at 0.6 to 1 billion animals (Montoya et al. 2023, Review 2024). Therefore, future studies should address the medium- and long-term effects of DCHBV infection, including coinfections with other pathogens (e.g. cat retroviruses) and other debilitating chronic conditions, such as neoplasia or degenerative diseases.

We did not find a significant association between altered liver function and the presence of DCHBc-specific antibodies or DCHBV DNA. The lack of altered liver function and hepatic disease associated with DCHBV infection contrasts with recent findings reported in cats (Pesavento et al. 2019, Takahashi et al. 2022). However, the association of viral infection with clinical disease or carcinogenesis and the analogy with the effect of HBV in humans must be considered carefully. First, the association of a virus with tumor formation is complex and multifactorial, and unlike in humans, where liver tumors are very common, liver tumors account for only 1–2% of feline primary neoplasia cases (Hammer and Sikkema 1995). Furthermore, the effects of HBV infection on liver disease and carcinogenesis in humans might be measurable only after years or decades of chronic infection (Xie 2017). For example, the positive animal with hepatic disease reported in our study was already in a declining age group, which increases the risk of developing both liver and pancreas complications (Ferreri et al. 2003, Hoskins 2005). Therefore, whether DCHBV infection is associated with disease or increases the odds of developing liver neoplasia in cats is still unclear and needs further investigation in long-term clinical studies.

The lower seroprevalence rates found in cats are compatible with the results of the molecular investigation, suggesting that DCHBV is scarcely distributed in Brazilian and German cat populations. The seroprevalence rates reported here differ substantially from those of another study reporting rates of up to 25% in cats from Italy using a DCHBV-specific ELISA (Fruci et al. 2022). However, our data are comparable to the seroprevalence rates ranging from 1.2% to 5.4% reported in equids, bats and shrews (Drexler et al. 2013, Rasche et al. 2019a, 2021). One possible explanation for this discrepancy could be differences in test sensitivity

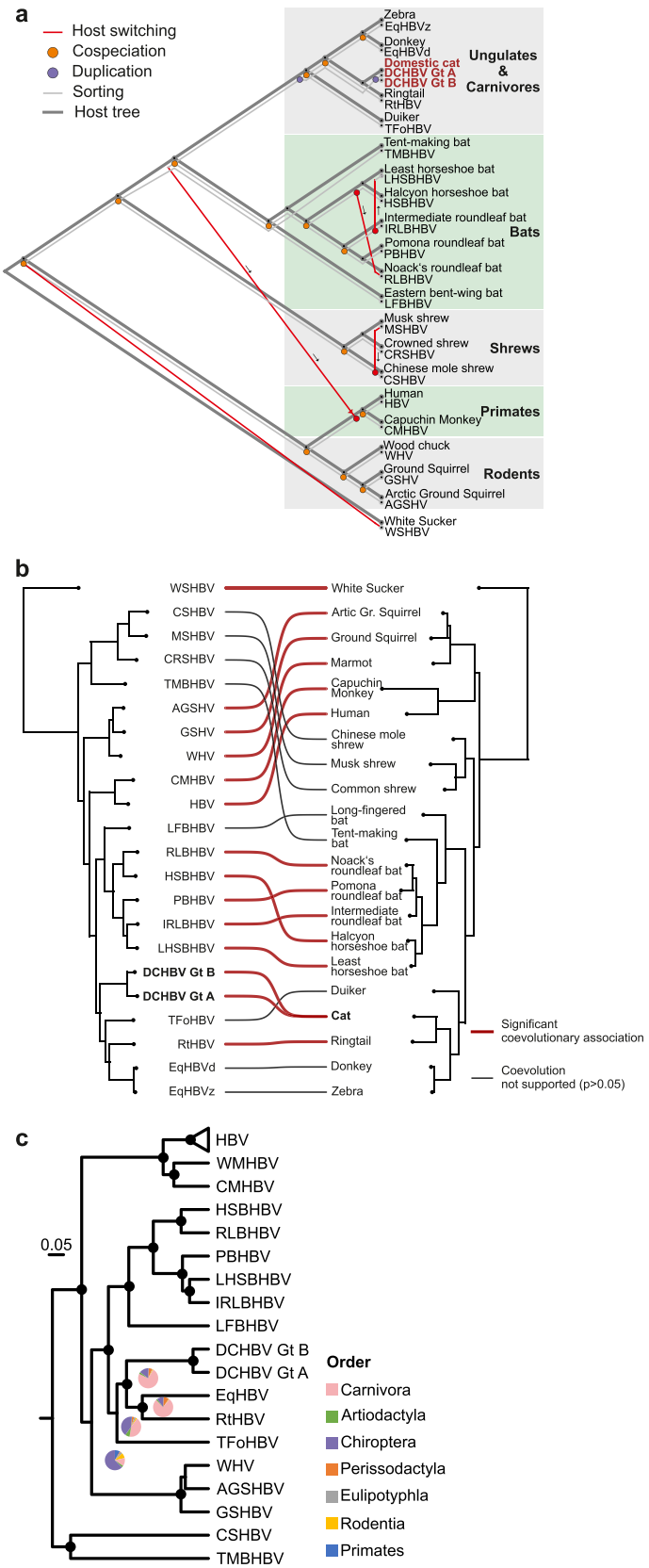

Figure 3. Evolutionary characteristics of DCHBV. (a) Representation of the most parsimonious reconciliation of host and hepadnavirus coevolutionary events. (b) Distance-based coevolutionary analysis. (c) Bayesian ASR. Pie charts indicate the posterior probabilities of ancestral traits for mammalian orders. Black dots indicate posterior probability support of grouping > 0.9. Trees (a-c) were built from nucleotide alignments of host and viral sequences.

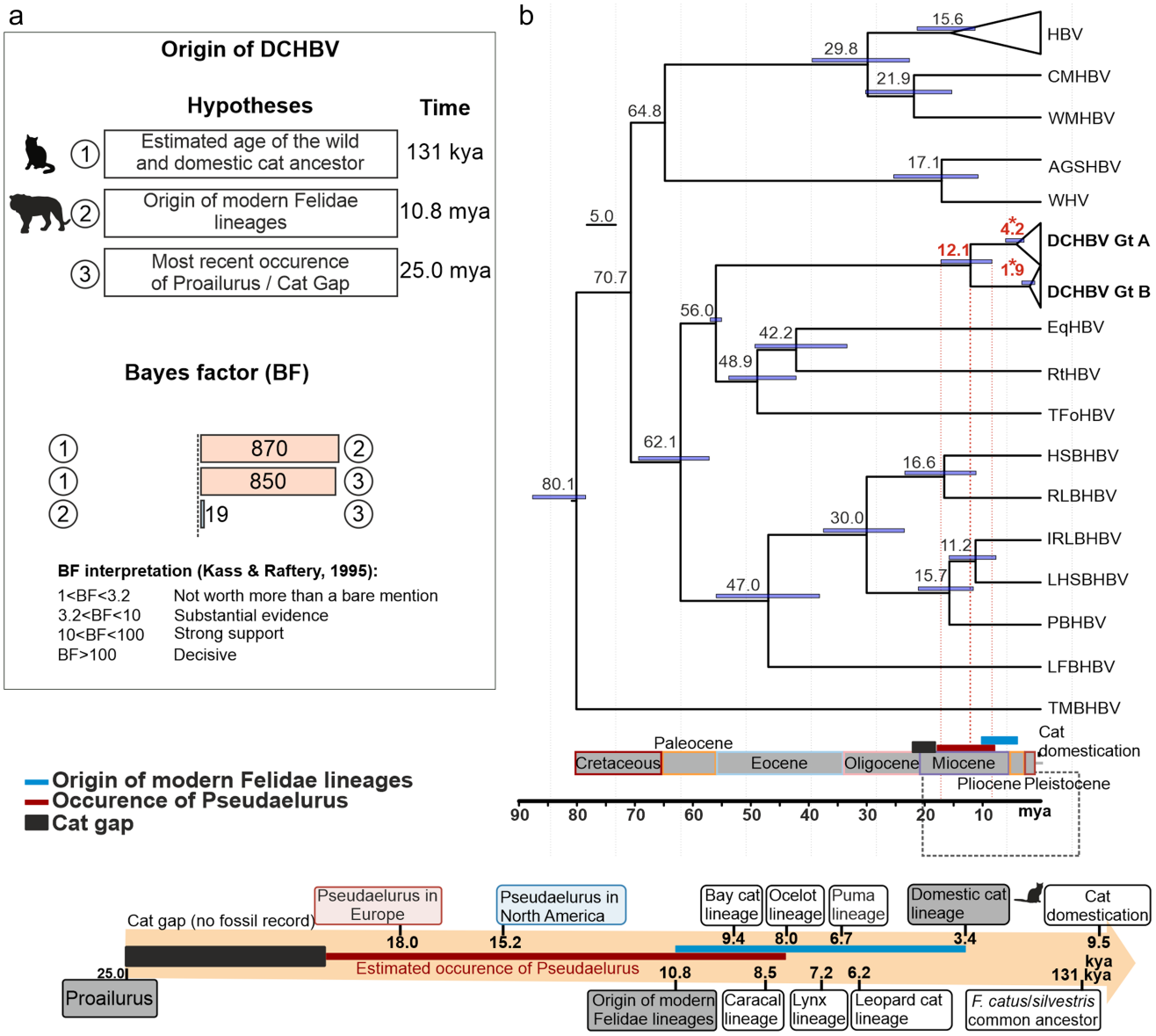

Figure 4. Evolutionary origins of DCHBV. (a) Hypothesis testing. The priors used for calibrating the hypothesis testing are labeled with numbers to improve visualization. Bayes factors (BF) allow the comparison of two priors. Abbreviations: kya, thousand years ago; mya, million years ago. (b) Bayesian phylogenetic tree and the timeline of selected events in domestic cat evolution. Asterisks indicate that the estimation of the DCHBV genotypes' TMRCA estimates should be carefully considered since there may be other DCHBV and related carnivore-derived sequences to be discovered.

and specificity, but further investigation is needed. We did not find evidence of DCHBV infection in domestic dogs from Brazil or Germany. The lack of DCHBV PCR-positive and seropositive dogs found here contrasts with previous PCR-based detection rates of DCHBV ranging from 0.4% to 6.3% (Choi et al. 2022, Diakoudi et al. 2022) and DCHBc seroprevalence rates of up to 30% in dogs (Fruci et al. 2023). The hepadnavirus found in dogs thus far is DCHBV, suggesting cat-to-dog viral transmission. Dog and cat interspecies pathogen circulation has been documented for other viruses (e.g. H3N2 influenza A and rabies virus), protozoans (*Babesia vogeli*, *Leishmania amazonensis*, *L. braziliensis*) and bacteria (e.g. *Bordetella bronchiseptica*) (Egberink et al. 2009, Song et al. 2011, Kim et al. 2013, Dantas-Torres and Otranto 2014, Dias et al. 2019). The interspecies transmission of DCHBV could also be supported by the genetic similarities observed in the viral receptor sodium/bile acid cotransporter (NTCP) of dogs and cats (Shofa et al. 2023). Therefore, our molecular, serological and evolutionary data suggest that DCHBV strains previously found in dogs so far are likely rare and geographically confined transmission events.

Estimating the ancestral origin of hepadnaviruses can be challenging, as illustrated by the discrepancies in multiple orders of magnitude in different attempts to date its origin (Fares and Holmes 2002, Zhou and Holmes 2007, Rasche et al. 2016). Cats were domesticated roughly 9500 years ago in western Asia (Driscoll et al. 2007), and modern felids emerged from a series of speciation events that began ~11 mya from the common ancestor Pseudaelurus (Westbury et al. 2021). Our data supported long-term evolutionary associations and suggested that DCHBV originated 8–17 mya during the Miocene period, likely from the forebears of Pseudaelurus after the cat gap, overlapping with

the radiation of modern Felid lineages (Johnson et al. 2006). The estimated TMRCA found here should be interpreted cautiously. First, as evolutionary rates depend on their scale of measurement and tend to decay over time (Aiewsakun and Katzourakis 2015, Simmonds et al. 2019), the long-term evolutionary rate of DCHBV may differ substantially from the short-term rate. We applied a rate of 1.0×10^{-5}, whereas reported hepadnavirus rates vary widely, ranging from 2.1×10^{-4} to 6.8×10^{-8} (Simmonds et al. 2019). Secondly, hepadnavirus evolution is highly complex due to prolonged interactions with the host immune system, particularly in persistently infected individuals. Here mutations may cyclically disappear and reemerge, especially in key genomic regions such as the surface protein and polymerase. This dynamic process has been proposed as an example of the Red Queen's dilemma in HBV evolution (Tedder et al. 2013). However, whether the same mechanism applies to DCHBV remains unclear. Finally, the entire time calibration framework may shift as hepadnaviruses are likely to be discovered among carnivores, especially in the Procyonidae and Felidae families, in which those hepadnaviruses were reported. Nevertheless, further discovery of new hepadnaviruses in Felidae species would contribute in the resolution of evolutionary events during the "cat gap".

On the other hand, it is unusual that TFoHBV and EqHBV hosted by species that are usually prey are derived from carnivore viruses, which are considered predators (French and Holmes 2020). Hypothetically, viruses derived from other animals might have been involved in the spillover to ungulates, which could be related to the need for adjustments due to food shortages, as potentially occurred during the cat gap period (Retallack 2004), or that those reconstructions are affected by the scarcity of known carnivore-derived hepadnaviruses. Furthermore, the reconstruction of bat-derived virus ancestors by ASR in the DCHBV clade, albeit at relatively low statistical support (posterior probability, 0.15), and more robustly in the carnivore/ungulate-associated orthohepadnavirus clade (posterior probability, 0.63), could suggest the existence of unidentified bat-associated ancestors. This interpretation is compatible with a likely origin of orthohepadnaviruses in Laurasiatheria and early radiation of bats (order Chiroptera) within Laurasiatheria over 60 mya, as indicated previously (Foley et al. 2016, Rasche et al. 2016). Therefore, the possible ancient bat origins of carnivore-derived hepadnaviruses warrant further investigation. Finally, the possible impact of DCHBV or homologous hepadnaviruses infecting extinction-threatened wild carnivore species, such as jaguars and tigers, needs further investigation. This is especially pertinent for Brazil, considering the large wild carnivore fauna and the large domestic cat population with approximately 24 million animals (Dantas-Torres and Otranto 2014), including a large number of free-roaming cats that interact and eventually exchange pathogens with wildlife (Dantas-Torres and Otranto 2014).

Limitations of our study include the fact that our sampling was not representative of each country or region and was performed for different reasons at different points in time. Although we investigated more than 4000 samples from dogs and cats in Brazil, Germany and other European countries, it is not possible to exclude sampling bias. This may affect the apparent absence of DCHBV in dogs and influence our reported incidence and seroprevalence rates at the different study sites. Using opportunistic sampling from commercial laboratories and limited to legally available information prevented us from obtaining detailed clinical information (e.g. age, clinical history) and contacting owners directly for a second sampling, which is relevant for revealing the occurrence of chronic DCHBV infection. However, if chronic infection is common in cats, more PCR-positive animals would be expected. Moreover, a low antibody detection rate is a sign of limited transmission, severe disease, or death. Nevertheless, the large number of samples tested in our study and the consistency between the serological and molecular results corroborate the robustness of our data. Another limitation is the imperfect association of carnivore viruses with the MRCA of the clade containing hepadnaviruses derived from both ungulates and carnivores. However, because the rise of both modern ungulates and carnivores occurred between the late Miocene and early Eocene (Johnston and Anthony 2012, Orlando et al. 2013), it is not clear whether further calibration might substantially change tree topology and evolutionary reconstructions. Finally, the number of tissue samples in our study was relatively small, and only animals from Costa Rica were included. Therefore, conclusions regarding the association between DCHBV infection and liver disease are limited. In addition, in our study, we did not investigate possible differences in the clinical course of the two DCHBV genotypes, as observed among the HBV genotypes in humans (Schaefer 2005). This highlights the importance of conducting controlled infection studies without other pathogens that can cause liver damage, which might involve costly experimental studies with ethical restrictions. However, future follow-up epidemiological studies investigating the natural infection of domestic cats with DCHBV can be readily conducted via the tools developed in our study.

In summary, the natural occurrence of an evolutionarily likely well-adapted hepadnavirus in cats may enable the use of a unique animal model to investigate the viral persistence and immunopathogenesis of acute and potentially chronic hepatitis B, including viral coinfection. The reconstructed long-term evolutionary association between DCHBV and feline hosts provides a new tool for studying *Felidae* evolution and suggests that genetically related viruses are present in extant *Felidae* species.

Acknowledgements

We thank Victor Carvalho Urbieta and Michele Kolasa for technical assistance. We thank Dieter Glebe for providing the anti-WHV polyclonal antibodies.

Supplementary data

Supplementary data is available at *VEVOLU* online.

Conflict of interest: None declared.

Funding

This work was funded by the Global Centers for Health and Pandemic Prevention from the German Academic Exchange Service (GLACIER; 57592642) and by funding from the German Research Foundation (DFG, Grant DR 810/12-1; project no. 537500489).

Data availability

Raw datasets, including sequencing data, and sequence alignments, will be available in the public repository Zenodo (DOI.10.5281/zenodo.15280993).

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Virus Evolution, 2025, 11(1), veaf025 , DOI: https://doi.org/10.1093/ve/veaf025, Advance Access Publication 5 April 2025, Research Article