Time-calibrated tree reconciliations reveal frequent losses, intrahost speciations, and host switches in the evolution of herpesviruses

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SIGNIFICANCE

We reveal the dynamics by which herpesviruses (HVs) have evolved in association with their hosts, over 400 million years of viral losses, intrahost speciations, host switches, and cospeciations. Using time-calibrated tree reconciliations, we identify important disagreements between viral and host divergence times and tree topologies. By reconciling their trees, we find that: (i) HVs evolved mainly by intrahost speciations, while cospeciations were indeed rare events; (ii) viral losses were common, either indicating that undiscovered HVs may still exist in nature, or suggesting a high frequency of viral extinctions, and; (iii) host switches were observed principally among alphaherpesviruses. We uncover previously inaccessible aspects of HV evolution, combined with biogeographical and evolutionary information about their hosts.

Keywords: tree reconciliation; herpesvirus; phylogenetics; host transfers; virus-host evolution

ABSTRACT

Cospeciation has been suggested to be the main force driving the evolution of herpesviruses, with viral species co-diverging with their hosts along more than 400 million years of evolutionay history. Recent studies, however, have been challenging this assumption, showing that other co-phylogenetic events, such as intrahost speciations and host switches play a central role on their evolution. Most of these studies, however, were performed with undated phylogenies, which may mis-estimate the frequency of certain events. In this study we performed co-phylogenetic analyses using time-calibrated trees of herpesviruses and hosts. This approach allowed us to (i) perform better estimations of co-phylogenetic events over time, and (ii) integrate crucial information about continental drift and historical biogeography to better understand virus-host evolution. We observed that cospeciations were in fact relatively rare events, which took place mostly after the Late Cretaceous (~100 Millions of years ago). Host switches were particularly common among alphaherpesviruses, where at least 10 transfers were detected. Among beta- and gammaherpesviruses, transfers were less frequent, with intrahost speciation followed by loss playing a more prominent role, especially from the Early Jurassic to the Early Cretaceous, when these viral lineages underwent several intrahost speciations. Our study reinforces the evidence that cospeciations are indeed uncommon events in herpesvirus evolution. More than topological incongruences, mismatches in divergence times were the main disagreements between host and viral phylogenies. In most cases, host switches could not explain such disparities, highlighting the important role of losses and intrahost speciations in the evolution of herpesviruses.

INTRODUCTION

Herpesviridae is a diverse family of large double-stranded DNA viruses subdivided in three subfamilies – *Alpha*-, *Beta*-, and *Gammaherpesvirinae* –, which infect different groups of vertebrates, including birds, mammals and reptiles (1). The evolutionary history of HVs dates from the Early Devonian, approximately 400 Millions of years ago (Mya) (2). Up to recent years, HVs were considered to be species-specific, and to have evolved alongside their hosts mainly by cospeciation (2-5), an event that implies concurrent and interdependent splits of host and viral lineages over time (6). As more information about the divergence timing of host and viral species is available, the predominance of cospeciation as the main mechanism of HV evolution started to be challenged, especially due to mismatches between the divergence times of hosts and viruses, eliciting alternative hypothesis, such as evolution by host switches (transfers) (5, 7).

Transfers take place when viruses succeed in infecting a new host still unexplored by their ancestors (6). Recent studies have been suggesting that host switches are probably more frequent than previously thought (7, 8), but detecting host switches can be difficult, as extinctions of viruses transmitted to new hosts may occur frequently (8). Duplication of viral lineages (intrahost speciation) is another evolutionary process playing an important role on host-parasite evolution (6). By means of intrahost speciations, multiple species of viruses can explore a single host species, especially when new lineages occupy distinct biological niches (tissues) within the hosts (5). Finally, another event playing a key role in host-virus evolution is the loss of viral lineages, events that in a co-phylogenetic context can mean: symbiotic extinction (9); sorting events (10); or even rare/undiscovered species resulting in low sampling (11).

The aforementioned mechanisms can be inferred using co-phylogenetic analysis, such as tree

reconciliations, which help us understand the relationship between hosts and parasites over time (11). To do that, such methods identify differences and similarities between the topologies of host and parasite trees, where congruence may indicate points of co-divergence (cospeciation), while incongruences my imply host switches or intrahost speciations followed by losses (6). Topological congruence, however, not always is caused by cospeciation events: similar topologies of host and parasite phylogenies can happen by chance, as a result of repeated host switches (6). To better understand the intricate evolutionary processes of HVs, co-phylogenetic analyses can be applied to elucidate the pathways taken by viruses as their hosts and the environment evolve. By integrating time-calibrated phylogenies and historical biogeographical information, in this study we present detailed scenarios unravelling the evolution of herpesviruses with their hosts, providing answers to the following questions: (*i*) What are the main co-phylogenetic events underlying HV evolution?; (*ii*) How have herpesviruses achieved their broad host range?; (*iii*) Have the three HV subfamilies evolved following similar strategies?

METHODS

Host/virus species and phylogenetic analysis

A total of 72 herpesviruses (subfamilies *Alpha-, Beta-, and Gammaherpesvirinae*) and their 37 host species (mammals, birds and reptiles) were included in this study (supporting information (SI), Table S1). Only viral species with whole genomes available on NCBI Viral Genomes Resource (12) were considered in the analysis. To infer both host and viral species phylogeny, sequences of host nuclear genes and conserved viral proteins were retrieved from NCBI. Sequence of genes encoding BDNF, CNR1, EDG1, RAG1, and RHO were used for inferring the host tree, and the viral tree was generated using sequences of UL15, UL27 and UL30. For host genes, when species-specific sequences were not available, sequences from related species from the same taxonomic group were used. Each gene set was aligned using MAFFT (13), and models of nucleotide and amino acid substitution were determined using jModelTest (14) and ProtTest (15), respectively. The multiple sequence alignments were used as partitions in *BEAST to infer maximum clade credibility (MCC) species trees using the Markov Chain Monte Carlo (MCMC) Bayesian approach implemented in BEAST v2.4.5 (16).

Node ages were time-calibrated (in Millions of years, Myr) using as priors host divergence dates obtained from (17-19), and viral ones from (20, 21). Constraints of monophyly were applied for viral and host clades based on (22) and (17, 18), respectively. Three independent MCMC runs were performed using relaxed (uncorrelated lognormal) molecular clock with the Yule model as a coalescent prior. The host tree was run for 500 million generations, and the viral one for 35 million generations.

Tree reconciliation

Tree reconciliations were performed using the program Jane 4 (23). Such analyses were performed to find the least cost association between host and viral trees without changing their topologies. Taking advantage of their node height highest posterior density (HPD) intervals, the trees were converted into a Jane timed tree format using a Python script, which divided the trees in time zones of 5 Myr. This step was required to allow the assignment of their internal nodes to specific time frames, making sure that only events occurring in the same time zone could be associated for inferring potential host switches and cospeciation events, thus avoiding chronological inconsistencies. The algorithm implemented in Jane allows the nodes to be assigned to more the one time zone, and requires that all zones should be populated with at least one host node. To meet such requirement, an outgroup clade containing artificial taxa was added to the original host tree, ensuring that their internal nodes could span time zones not originally covered by the original host nodes. A similar outgroup was added to the viral tree, allowing the pairing of artificial virus-host pairs.

Since estimating the relative costs of events such as cospeciations, host switches and losses is difficult, we tested four cost regimes (Table 1) that penalized each event differently. Only regimes yielding single isomorphic solutions were considered, and the regime with lowest overall cost (cost regime 4) was chosen.

RESULTS

Phylogenetic analysis

Herpesvirus evolutionary history is older than that of their extant hosts (2). The MRCA (Most Recent Common Ancestors) of all hosts date back between 352 and 307 Mya, while the MRCA of all HV subfamilies existed prior to that period, between 416 and 373 Mya. As evidenced by the tanglegram in Figure 1, despite the apparent congruence of some clades in both phylogenies, topological disagreements are common for most virus-host pairs, as highlighted by the overcrossing connections between the trees.

Cost regimes

The co-phylogenetic analysis of 72 herpesviral species and their respective 37 hosts has provided different possible scenarios to explain their evolution, based on distinct event costs. We tested four different cost regimes (CR), which favoured or penalized events differently. CR1 corresponds to the default parameters, and favoured host switches, while reducing intrahost speciations (duplications) and losses along the evolution (Figure 2A). CR2 penalized cospeciations, and raised the number of transfers, producing an expensive scenario. CR3, on the other hand, penalized host switches decreasing its overall occurrence, while inflating duplications and losses. Finally, by favouring cospeciations, CR4 has shown the lowest overall costs in

all reconciliations, and was selected to explain the topological disagreements between viral and host trees, while the remaining CRs were not considered in further analysis. For the sake of clarity, reconciliations between HVs from different subfamilies were treated individually, by splitting their family tree into subtrees for Alpha-, Beta-, and GammaHVs. It allowed us to examine the predominance of each co-phylogenetic event across different HV genera (Figure 2B), as shown in the following sections.

Early co-phylogenetic events explaining herpesviral dispersal

Intrahost speciations, losses and host transfers were key phenomena defining the current distribution of HVs across placentals, marsupials, birds and reptiles (Figure 3). Early in the evolution of these viruses, two intrahost speciations occurred along the Devonian and the Carboniferous (~416-330 Mya), period when the three known herpesviral subfamilies originated and diverged while infecting ancestors of amniotes. At the split of sauropsids (bird/reptile ancestors) and synapsids (mammal ancestors), losses of HVs may have occurred, defining the apparent absence of some HV subfamilies infecting extant host groups (e.g. Beta- or GammaHVs infecting birds). Finally, after the Jurassic, two independent events of host switch established AlphaHVs in placentals, and later in Marsupials. The next sections provide detailed explanations for these and other events.

Cospeciations along the last 100 millions of years

Cospeciations were only found after the Late Cretaceous (~100 Mya), and have shown to be a rare event among HVs, being the least common event for alphaherpesviruses, and the second least common among Beta- and Gammaherpesviruses (Figure 2). For alphaherpesviruses, seven cospeciation events were reconstructed, the oldest one assigned to the Late Cretaceous (~92-81 Mya), involving ancestors of *Varicellovirus* infecting *Laurasiatheria* mammals (see Figure 4). This event was followed by at least other two cospeciations, one at the split of ancestors of canines, felines and equines (*Zooamata*); and another at the split of *Carnivora* ancestors. Among mardiviruses, ancestors of GaHV1 and PsHV1 (genus *Iltovirus*) co-diverged with bird ancestors (*Neognathae*) around 78-94 Mya, similar to what occurred for viruses infecting ancestors of chickens and turkeys (*Phasianinae*) around 36-26 Mya. Finally, among simplexviruses infecting *Catarrhini* (Old World Monkeys and Apes), two cospeciations were found, one taking place most likely at the Oligocene (~36-26 Mya); and the second at the Miocene (~9-5 Mya), involving viruses infecting ancestors of Humans and Chimpanzees (*Hominini*).

In Betaherpesviruses, only two cospeciations were reported, both along the lineage of cytomegaloviruses infecting ancestors of *Cercopithecine* (macaques and baboons) (Figure 5): the first one at the Paleogene-Neogene boundary (~24-21 Mya); and the second one around 0.8 Mya, the most recent cospeciation found in this study, involving ancestors of CyCMV and McHV3, at the split between *Macaca fascicularis* (Mfa) and *M. mulatta* (Mma).

Among Gammaherpesviruses, three cospeciations were found in different periods (Figure 6). The earliest event involved *Macavirus* ancestors, and took place at the split between *Laurasiatheria* and *Euarchontoglires* (Early Cretaceous, ~99 Mya). Following this event, HVs infecting ancestors of bats, equines and felines (*Pegasoferae*) co-diverged with their hosts at the Late Cretaceous (~88-79 Mya); and finally, at the Paleogene-Neogene boundary (~25-16 Mya), *Rhadinovirus* ancestors co-diverged with New World Monkeys (*Platyrrhini*).

Intrahost speciations: duplications of viral lineages

Intrahost speciations occur when a parasite diverges and both lineages remain within the same host species, and represent alternative modes of evolution that explain mismatches between divergence times of viruses and hosts (6). Intrahost speciations were the second most frequent co-phylogenetic event in the evolution of HVs. Along the evolution of AlphaHV, such events were particularly common after the Early Cretaceous. During the Permian and Triassic periods, when AlphaHVs infected ancestors of birds and reptiles (*Archosauromorpha*), two important intrahost speciations gave rise to ancestors of the main genera of *Alphaherpesvirinae* (Figure 4). Later in their evolution, along the Paleogene and Neogene, these viruses underwent a process of diversification that gave rise to multiple species infecting common hosts, as observed for Gallid (GaHV2, 3); Equid (EHV1, 3, 4, 8, 9); and Bovine alphaherpesviruses (BHV1, 5). Differently from these recent duplications, older duplication events were always followed by losses, which may explain, for example, the apparent lack of scutaviruses infecting birds, or mardiviruses and iltoviruses infecting reptiles. The evolutionary histories of BetaHVs and GammaHVs are also largely marked by intrahost speciations (Figure 2B). As a consequence of early intrahost speciations, ancestors of BetaHV and GammaHV co-existed infecting early placental mammals from the Late Triassic to the Early Cretaceous, period when such viral lineages experienced extensive diversification (Figure 5 and Figure 6).

Losses: extinctions, sorting events and undiscovered herpesviruses

Among the four co-phylogenetic events, losses were the most common, with a frequency of 52.9% in AlphaHVs, 72.7% in BetaHVs, and 70.4% in GammaHVs (Figure 2). Remarkably, considering all HV subfamilies, more than half of the losses (nearly 54%) were assigned to the Cretaceous period (~145-66 Mya). As previously mentioned, losses are usually preceded by intrahost speciations, and highlight host clades that lack viruses from certain lineages. At this point it is essential to

emphasize that in the context of host-parasite tree reconciliations, losses can be interpreted in at least three distinct ways: (1) as lineage sorting events ('missing the boat'), when a parasite fails to disperse to one of the new host species after their speciation (10); (2) as undiscovered or rare parasites (low sampling) (11); or (3) as genuine events of parasite extinctions (9). In the latter scenario, if extinctions explain the absence of viruses infecting certain host clades, it is important to consider that points of losses in reconciliations do not reflect the exact time of the extinctions, but rather highlight a point from which such events could have happened at any subsequent time.

Throughout the evolution of herpesviruses, in Figure 4, Figure 5, and Figure 6, viral losses are depicted as dashed lines pointing towards the opposite direction of the loss. In the evolution of AlphaHVs, for example, the oldest loss dates from the Carboniferous period, before the split between sauropsids (bird/reptile ancestors) and synapsids (mammal ancestors) (Figure 4). This loss was inferred due to the potential lack of a basal group of AlphaHVs infecting synapsids in that time. Most losses of AlphaHVs were assigned to periods after the Early Cretaceous, along the earliest events of diversification of modern birds and mammals. Except for *Scutavirus*, a genus here represented by a single species (TeHV3), all genera of *Alphaherpesvirinae* show extensive losses between 110 and 70 Mya. Proportionally, losses were the most common events in the evolution of the genus *Iltovirus* (Figure 2B), an old clade of HVs infecting distantly related avian families: *Psittacidae* and *Phasianidae*. Another context where losses played a central role was during the evolution of varicello- and simplexviruses infecting *Euarchontoglires* (a group that includes rabbits and primates). The evolution of these viruses alongside their hosts could only be explained by means of multiple losses, especially from ~90 Mya onwards, where at least 12 losses were observed.

In Beta- and Gammaherpesviruses losses were predominant. Among the BetaHV genera, *Roseolovirus* has shown the highest level of losses (Figure 2B). Ancestors of these viruses originated most likely in the Early Cretaceous (~146-119 Mya), and across their evolution they were either lost in most host groups or may still exist as rare/undiscovered viruses. In GammaHVs, the genus *Macavirus* showed the second highest relative frequency of losses. Viruses in this taxonomic group are known to infect *Cetartiodactyla* hosts, mostly ruminants and swine (24). Since the MRCAs of these viruses and their *Laurasiatheria* hosts existed around 87 Mya (Late Cretaceous), in case they were not extinct, macaviruses infecting *Pegasoferae* (felines, canines, equines and bats) may still exist in nature

Host switches

Host switches (transfers) take place when parasites succeed on infecting new hosts not yet explored by their ancestors (6). In all scenarios (cost regimes) investigated, host switches were invoked to explain herpesvirus-host evolution. Based on the CR4, at least 10 host switches were reported for AlphaHVs. The oldest host switch took place when HVs from bird ancestors (sauropsids) got transferred to placental mammals in the Early Cretaceous (~144 Mya), before the split between *Mardivirus* and *Varicellovirus*. Later (~130 Mya), a second host switch took place, giving rise to ancestors of all varicelloviruses, which currently are found only in a wide range of mammalian hosts (25) (Figure 4). Interestingly, other than these two early transfers, all the remaining host switches of AlphaHVs were assigned to periods after the Cretaceous-Paleogene boundary. In the *Mardivirus* lineage, viruses now infecting falconids and columbids (*Neoaves*) have likely originated after two host transfers: one in the Paleocene (~67 Mya) involving viruses from ancestors of modern chicken and turkeys (*Phasianinae*); and a second one in more recent times, when viruses infecting pigeons got transferred to falcons in the upper Pleistocene (~150 thousands of years ago), the most recent transfer identified in this study.

Most notably, host switches were particularly common among simplexviruses, where more than 2/3 of the transfers in AlphaHVs were observed (Figure 2B). In this genus, two early transfers from primates gave rise to viruses now infecting Rabbits, Bats and Marsupials: ancestors of *Leporid alphaherpesvirus* (LHV4) switched from primates to rabbits around 76-52 Mya; and later primate HVs switched to bats in the Eocene (~54-41 Mya). Descendants of these viruses were later transferred to marsupials in the Oligocene (~33 Mya), giving rise to the present-day *Macropodid herpesvirus* (MaHV1). The remaining transfers involved the interchange of simpleviruses among Old World Monkeys and Apes (*Catarrhini*) in the Pliocene. Ancestors of HVs now infecting monkeys of the genus *Macaca* (Rhesus, infected by CeHV1), *Papio* (Baboons, infected by CeHV16), and *Cercopithecus* (Guenons, infected by CeHV2) were likely transferred among these monkey species between 3.3 and 2.1 Mya. The exact origin of their ancestors, and the polarity of their host switches were not possible to be determined only by phylogenetic analysis, as multiple possible scenarios have shown the same overall costs. Finally, an important *Simplexvirus* transfer occurred between 3.6 and 0.1 Mya, when viruses infecting Chimpanzees (genus *Pan*) were transferred to humans, giving rise to HHV2, as already reported in a previous study (21).

In Beta- and GammaHVs, host switches were less prominent, with only one event being assigned to each of these groups, both involving primate hosts. Among BetaHVs, the species here named CeHV5-B (originally known as isolate OCOM4-52 (26)) originated in the Miocene (~12 Mya) from viruses transferred from Guenon ancestors (genus *Cercopithecus*). Finally, for GammaHVs our results provide a possible explanation for the origins of HHV4 (Epstein-Barr Virus). Among lymphocryptoviruses, McHV4, a *Macacine gammaherpesvirus* infecting Rhesus macaques (*M. mulatta*), is the non-human HV more closely related to HHV4. Ancestors of these viruses infected either Apes (early hominids) or Old World Monkeys (early cercopithecids) around 13.3 - 2.1 Mya, when these HVs switched hosts. For this particular event, the directionality of the host transfer (hominids ↔

cercopithecids) was not possible to be determined because given tree topologies and node heights, both directions of transfer had the same overall cost. Despite that, other than host switch, no other co-phylogenetic event can explain the existence of these viruses in macaques and humans.

DISCUSSION

To understand the current distribution of herpesviruses in distinct host groups, co-phylogenetic analysis with time-calibrated trees can provide consistent explanation for the temporal dynamics of HVs evolution alongside their hosts. Furthermore, by adding chronological references into tree reconciliations, we could also incorporate information about geological and biogeographic events that shaped life on Earth.

A previous study placed the origin of herpesviruses in the Devonian (~400 Mya) (20), a time reference used as one of the priors to calibrate the herpesviral phylogeny, and estimate the timing of all events underlying their evolution from that period to the present. Our reconciliation analysis revealed that ancestors of viruses belonging to all known subfamilies of Herpesviridae (*Alpha*-; *Beta*; and *Gammaherpesvirinae*) co-infected populations of fish-like animals before their split into the two major amniote groups (synapsids and sauropsids), which later on evolved into the existing mammals, birds and reptiles (27).

For a long time, cospeciation was considered to be the main evolutionary mechanism driving the evolution of herpesviruses and their hosts (2-5). However, most previous studies performed reconciliations using trees with few taxa, and no time calibration, thus disregarding the divergence times of host and viral ancestors. Beyond topological congruence, proportional branch lengths and correspondent divergence times are essential for cospeciations to happen (6). In this way, the absence of temporal references calibrating internal nodes may overestimate the occurrence of certain events due to chronological inconsistency. In the present study we performed such an analysis using time-calibrated trees with divergence times as priors to date internal nodes of both viral and host trees. Due to temporal incompatibilities, when such trees were reconciled we observed that cospeciation was in fact extremely rare in herpesviruses, with other events playing more predominant roles.

After extensive phylogenetic characterization of herpesviruses, topological disagreements between host and viral trees became evident, and since cospeciations alone could no longer explain the evolution of HVs, host transfers were initially presumed to account for such incongruences (7, 24). In contrast to this assumption, our results revealed that while transfers play a key role on *Alphaherpesvirinae* evolution, they are uncommon in *Beta*- and *Gammaherpesvirinae*. For these two subfamilies, intrahost speciations followed by losses were abundant (Figure 2B), and may provide better explanations for the topological disagreements between their phylogenies and those of their hosts.

Viral losses do not necessarily mean extinctions, as they can be a result of low sampling or undiscovered viruses. Since the highest levels of losses were observed for herpesviral genera with few sampled taxa (Figure 2B), a hypothesis of undiscovered viruses appears to be plausible, as the relative frequency of losses were to some extent influenced by the number of sampled taxa. If extinctions are invoked to explain these losses, some events linked to the evolution of the hosts can be appointed as potential causes of the elimination of viral clades, one of them being host extinction. Although each mass extinction could have wiped out 76-96% of the ancient species, most extinctions along the Phanerozoic Eon were the result of minor events taking place in between mass extinctions (28). Since the mean duration of a species is four Myr, while a genus lasts for around 28 Myr (28), symbiont extinction may explain some of the losses inferred in the present study.

Apart from host populations being wiped out causing viral elimination, cataclysmic events can also cause drastic decreases in host populations (29), which may promote bottleneck effects directly affecting viral adaptation to hosts, and leading to viral extinctions. It occurs especially because bottleneck effects can increase the likelihood of fixation of some host alleles, including those granting host resistance towards parasites (29). Among bird species, for example, a strong bottleneck effect impacted avian populations after the 5th mass extinction (30), decreasing by nearly half the net diversification rates of birds along the Paleocene and Eocene (~66-45 Mya) (18). Such a drop in host diversity may possibly explain some of the losses assigned to avian HVs, especially those of *Iltovirus* (Figure 2B and Figure 4).

As direct or indirect contact are required for viral spread into new hosts, the geological movement of landmasses can split or merge host populations, in this way allowing or preventing certain host switches (9). For a better understanding of ancestral host transfers it is important to consider the biogeography (distribution) of ancestral hosts and the geological history of the Earth. As shown in Figure 4, Figure 5 and Figure 6, alongside the evolution of hosts and their associated viruses, the planet underwent drastic changes. All hosts included in this study are tetrapods, a group of organisms that diverged from aquatic vertebrates between 385 and 375 Mya (27). Given that the MRCA of herpesviruses dates from 416 and 373 Mya, ancestors of such viruses probably infected marine organisms, and along their terrestrialization, their viruses diverged into ancestors of Alpha-, Beta-, and GammaHVs (Figure 3). Being the first to diverge, AlphaHVs were probably lost on synapsids (proto-mammals) in the Carboniferous, reappearing as mammalian HVs much later, when avian AlphaHVs got transferred to mammals in at least two independent events. Such transfers, which gave rise to *Simplexvirus* and *Varicellovirus*, were already suggested in previous studies to explain the dispersal of HVs in mammalian hosts (2, 20). Although parasites are more likely to jump between closely related hosts, as they share similar ecological, physiological and chemical characteristics (6, 8), transfers can also occur over great phylogenetic distances, as hosts from closely related

clades can independently acquire or lose immunogenetic traits, affecting their levels of susceptibility to pathogens (31). As host populations could have been physically and genetically closer in the Early Cretaceous than they are now, the origin of mammalian HVs by means of host transfers from avian ancestors is a plausible explanation.

In our analysis, except for the two transfers discussed above, all host switches were found in more recent times, especially after the Cretaceous/Paleogene boundary (~66 Mya). This pattern was already expected, as shown in a previous study that observed recent host switches as a predominant pattern across the evolution of most DNA and RNA viruses (8). Our results revealed that simplexviruses are particularly prone to switch hosts. A striking example is that involving FBaHV1 and MaHV1, which are closely related to primate HVs, but infect distantly related hosts (bats of the genus *Pteropus* and marsupials, respectively). As *Pteropus sp.* ancestors (Megabats) inhabited Eurasia alongside primate ancestors in the Paleocene and Eocene (~59-38 Mya) (32, 33), transfers of HVs from primates to bats were probably common in that period. The subsequent host switch from megabats to macropods (Kangaroo ancestors) can be mainly explained by their current and ancestral distributions in Australia, and by the dispersal capabilities of bats using powered flight (32).

Interestingly, host switches involving primate HVs were observed in all subfamilies, most of them taking place from ~10 Mya, along the Miocene, Pliocene and Quaternary. Wertheim and colleagues (21) performed an in depth analysis of the evolution of Human simplexviruses (HHV1 and HHV2) and suggested that HHV2 could have originated from host switches of HVs from Chimpanzees around 1.6 Mya. Our findings have confirmed this transfer, positioning it slightly earlier in time, around 2 Mya (95% HPD = 3.6 - 0.1 Mya), after the divergence of ChHV1 and HHV2. The same study has already pointed out another possible host switch involving ancestors of CeHV2. Our results revealed that simplexviruses infecting Old World Monkeys, such as CeHV1, CeHV2, and CeHV16 (Figure 1), underwent host switches after 4.3 Mya. Strikingly, all species of the genera *Cercopithecus* and *Papio* (except *P. hamadryas*) are found only in Africa, while those of *Macaca* are mostly found in Asia (except *M. sylvanus*) (33). Ancestors of *Macaca* dispersed from Africa to Asia in the Pliocene, between 5.4 and 2.3 Mya (33), a period that matches the tMRCA of simplexviruses infecting Old World Monkeys. Thereby, our findings allow us to suggest that transfers of HVs from *Cercopithecus* to *Macaca* took place before the migration of the latter to Asia, which explains the presence of CeHV1 infecting *M. Mulatta*. Finally, between 3.5 and 0.07 Mya, transfers from *Cercopithecus* to ancestors of *Papio* took place in Africa, the place of origin and current area of distribution of most species from both genera (33).

It is known that herpesviral host switches occurred frequently in the past, especially among closely related organisms (34, 35). As mutation rate and effective population size (*Ne*) affect the likelihood of adaptation of a pathogen in a new host (31), the extinction of viruses transmitted to new host species may occur frequently (8). As a result, most host switches cannot be easily detected, and the number of transfers inferred in our co-phylogenetic analysis is probably underestimated.

To conclude, to our knowledge this study is the first one to apply time-calibrated virus-host phylogenies to perform tree reconciliations. This detailed approach was able to not just detect topological disagreements between viral and host trees, but more importantly, revealed major chronological mismatches on divergence times of animal species and their symbionts, showing the relevance and fundamental contribution of chronological references for reconciling phylogenies. Our dated reconciliations highlighted the central roles of intrahost speciations in the evolution of herpesviruses, events that in most cases were followed by losses, which were predominant along the Cretaceous period. It was not possible to clarify what such losses represent in the evolution of herpesviruses, as they can represent lineage sorting events, undiscovered viruses, or even episodes of viral extinctions. As more viral samples are incorporated in tree reconciliations, the real nature of such losses will be revealed. Host switches were particularly frequent among alphaherpesviruses, especially those of the genus *Simplexvirus*, which deserve further studies as to what genetic traits favoured the colonization of new hosts. Finally, contrasting what has been hypothesized, cospeciations between herpesviruses and their hosts are in fact rare events, restricted to a few virus-host co-divergences, most of them involving alphaherpesviruses.

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FIGURES

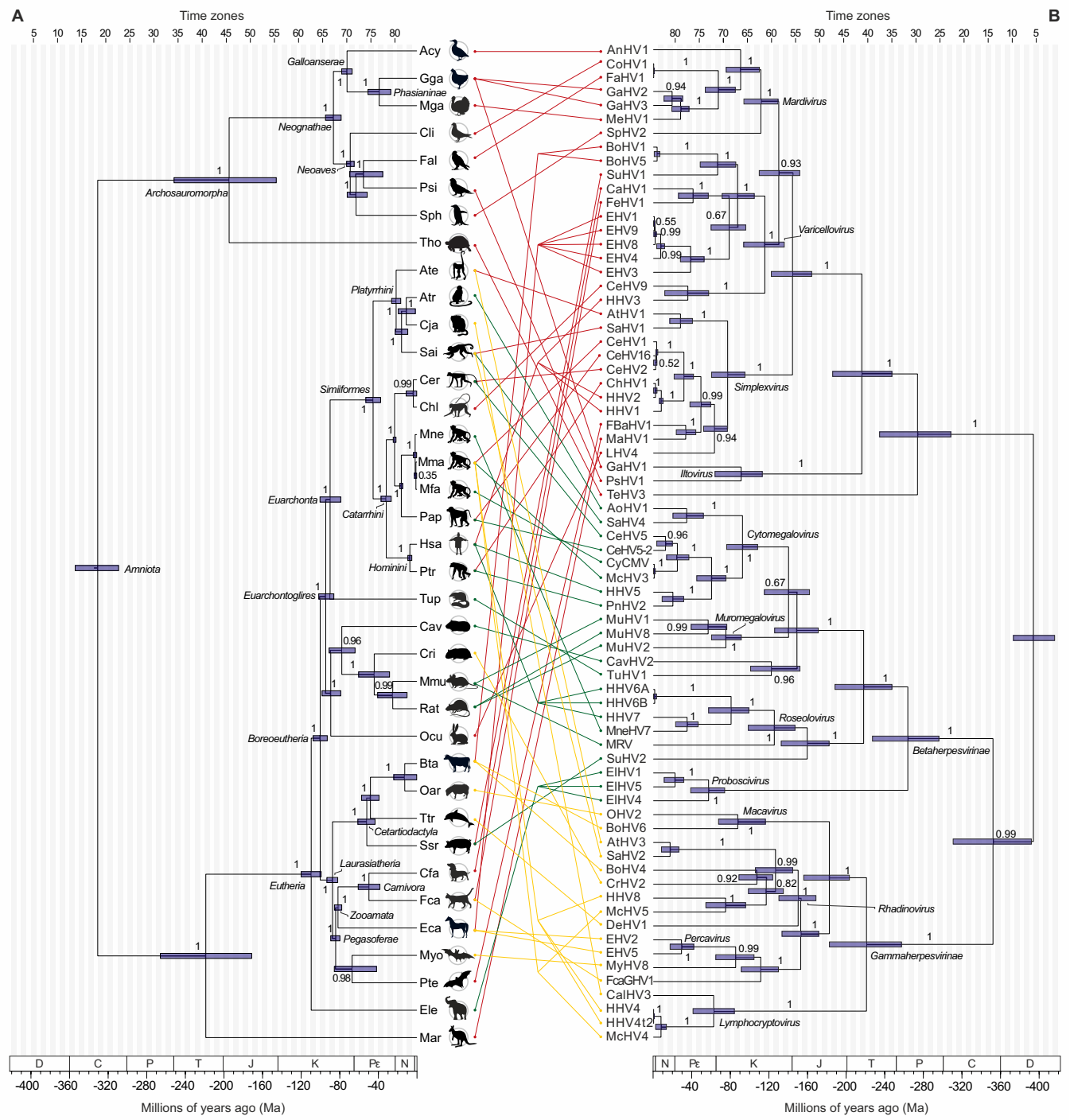

Figure 1. Host-virus tanglegram. Viruses (right) are connected to their hosts (left) with lines whose colours denote the three herpesviral subfamilies: *Alpha-* (red); *Beta-* (green) and *Gammaherpesvirinae* (yellow). Node height HPD intervals of hosts and viruses are shown as bars, labels are provided for some taxonomic groups, and all nodes are assigned with posterior probabilities. Both trees are divided in time zones of 5 Myr, as shown by the scale at the top, and some node HPD intervals span more than one time zone. The geologic time scale is set according to (36), where D = Devonian period; C = Carboniferous; P = Permian; T = Triassic; J = Jurassic; K = Cretaceous; Pε = Paleogene; N = Neogene; and * = Quaternary period. Black diamonds denote events of mass extinction, as described in (28). Host accronyms are defined as follows: Acy = *Anser cygnoides*; Ate = *Ateles sp.*; Atr = *Aotus trivirgatus*; Bta = *Bos taurus*; Cav = *Cavia porcellus*; Cer = *Cercopithecus aethiops*; Cfa = *Canis lupus familiaris*; Chl = *Erythrocebus patas*; Cja = *Callithrix jacchus*; Cli = *Columba livia*; Cri = *Cricetidae*; Eca = *Equus caballus*; Ele = *Elephas maximus*; Fal = *Falco mexicanus*; Fca = *Felis catus*; Gga = *Gallus gallus*; Hsa = *Homo sapiens*; Mar = *Macropodidae*; Mfa = *Macaca fascicularis*; Mga = *Meleagris gallopavo*; Mma = *Macaca mulatta*; Mmu = *Mus musculus*; Mne = *Macaca nemestrina*; Myo = *Myotis velifer*; Oar = *Ovis aries*; Ocu = *Oryctolagus cuniculus*; Pap = *Papio sp.*; Psi = *Amazona oratrix*; Pte = *Pteropus sp.*; Ptr = *Pan troglodytes*; Rat = *Rattus sp.*; Sai = *Saimiri sp.*; Sph = *Spheniscus sp.*; Ssr = *Sus scrofa*; Tho = *Testudo horsfieldii*; Ttr = *Tursiops truncatus*; and Tup = *Tupaiidae*. For more details on host and viral taxonomy, accession numbers, and TaxID can be found in the Supplementary Material.

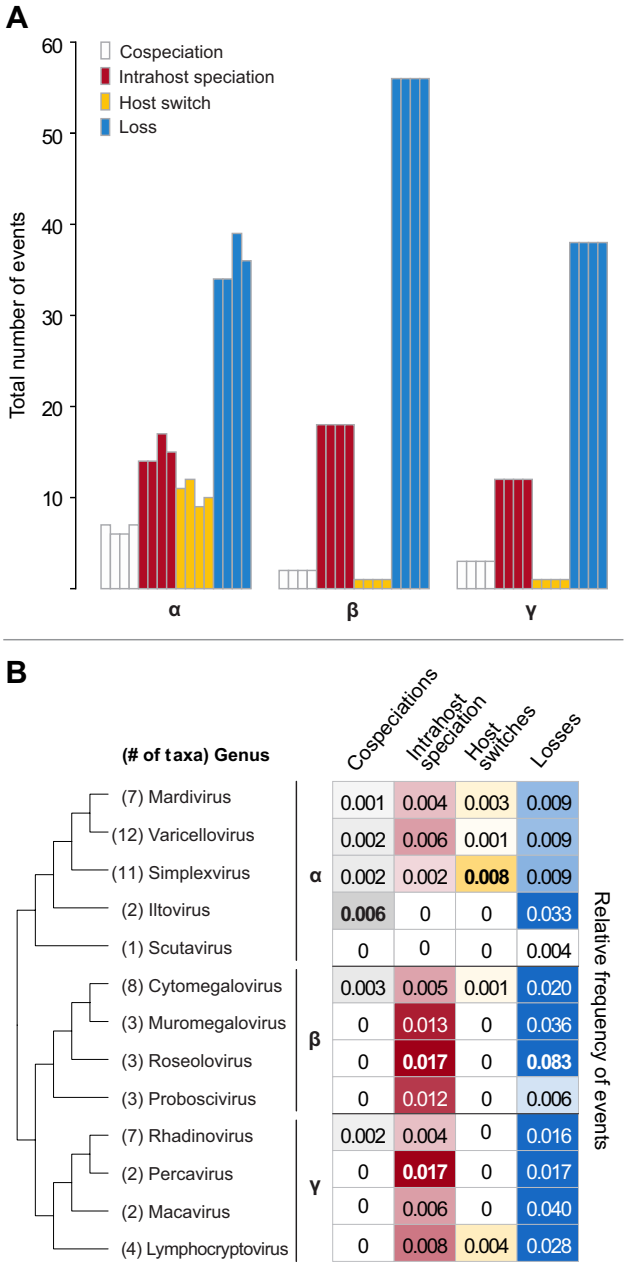

Figure 2. Co-phylogenetic events inferred in reconciliations of herpesvirus and host trees. A) Each bar shows the total number of Cospeciations (white); Intrahost speciations (red); Host switches (yellow); and losses (blue). The ordering of the bars at each group denotes the cost regimes they belong to: CR1 (bars at the first position), CR2 (second), CR3 (third) and CR4 (fourth position). B) Predominance of co-phylogenetic events per herpesviral genus, normalized by the total number of taxa in each genus, and their respective times to the MRCA. As highlighted, losses were the most frequent co-phylogenetic events, followed by intrahost speciations.

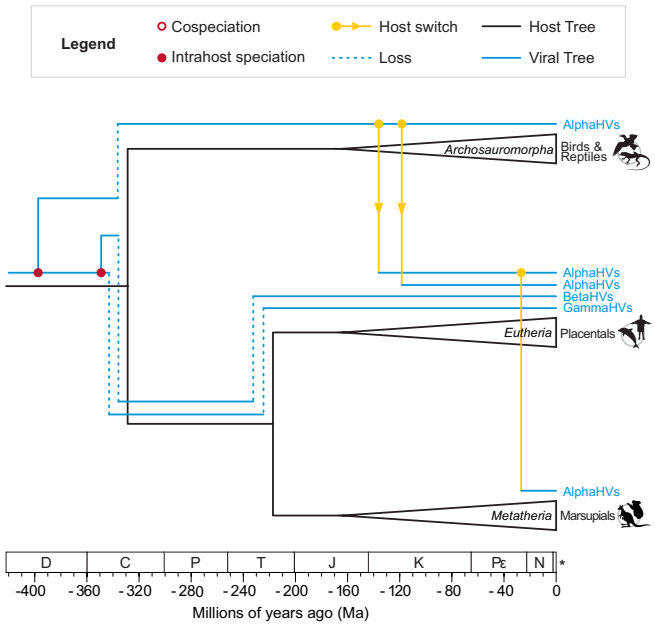

Figure 3. Main events explaining the dispersal of herpesviruses across different host groups. The time scale is shown as in Figure 1.

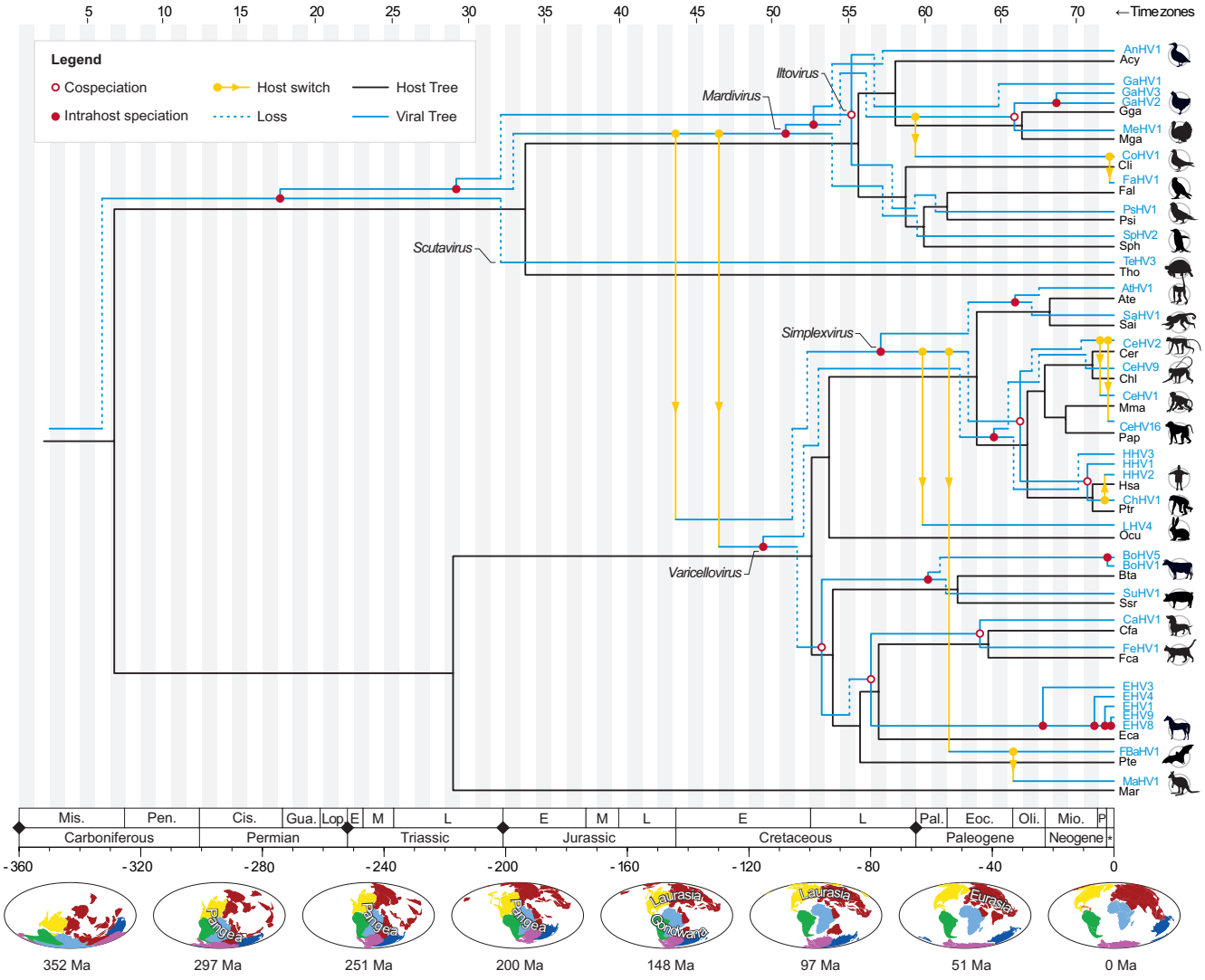

Figure 4. Tree reconciliation of Alphaherpesviruses and their hosts. In this representation, the host tree is shown in black, and the viral tree is vertically twisted, without changing its original topology and time zones (as shown in Figure 1). The maps below show changes of landmasses (continental drift) over time, and were retrieved from PBDB (37). Cis. = Cisuralian; E = Early; Eoc. = Eocene; Gua. = Guadalupian; L = Late; Lop. = Lopingian; M = Middle; Mio. = Miocene; Mis. = Mississippian; Oli. = Oligocene; P = Pliocene; Pal. = Paleocene; Pen. = Pennsylvanian; and * = Quaternary.

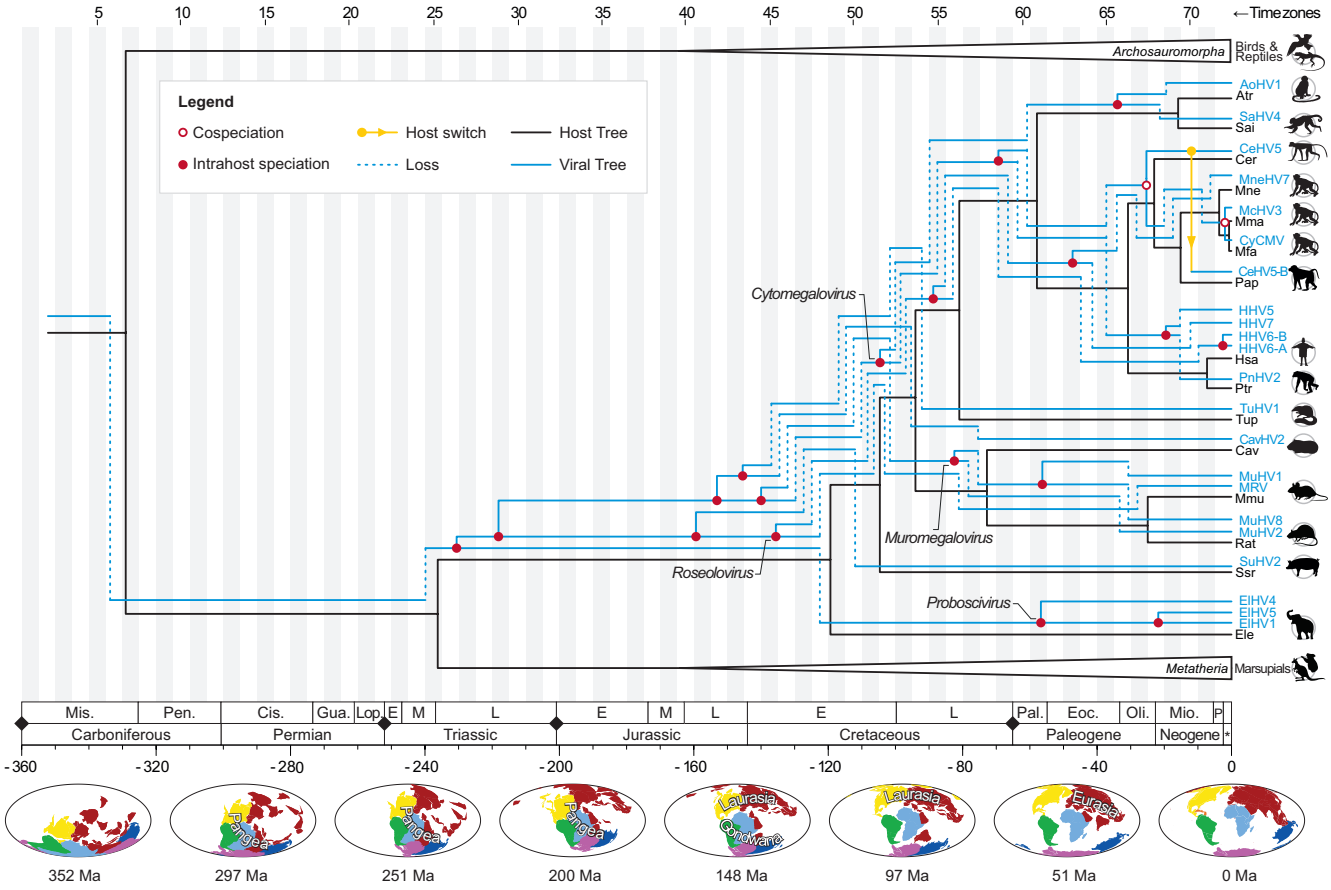

Figure 5. Tree reconciliation of Betaherpesviruses and their hosts.

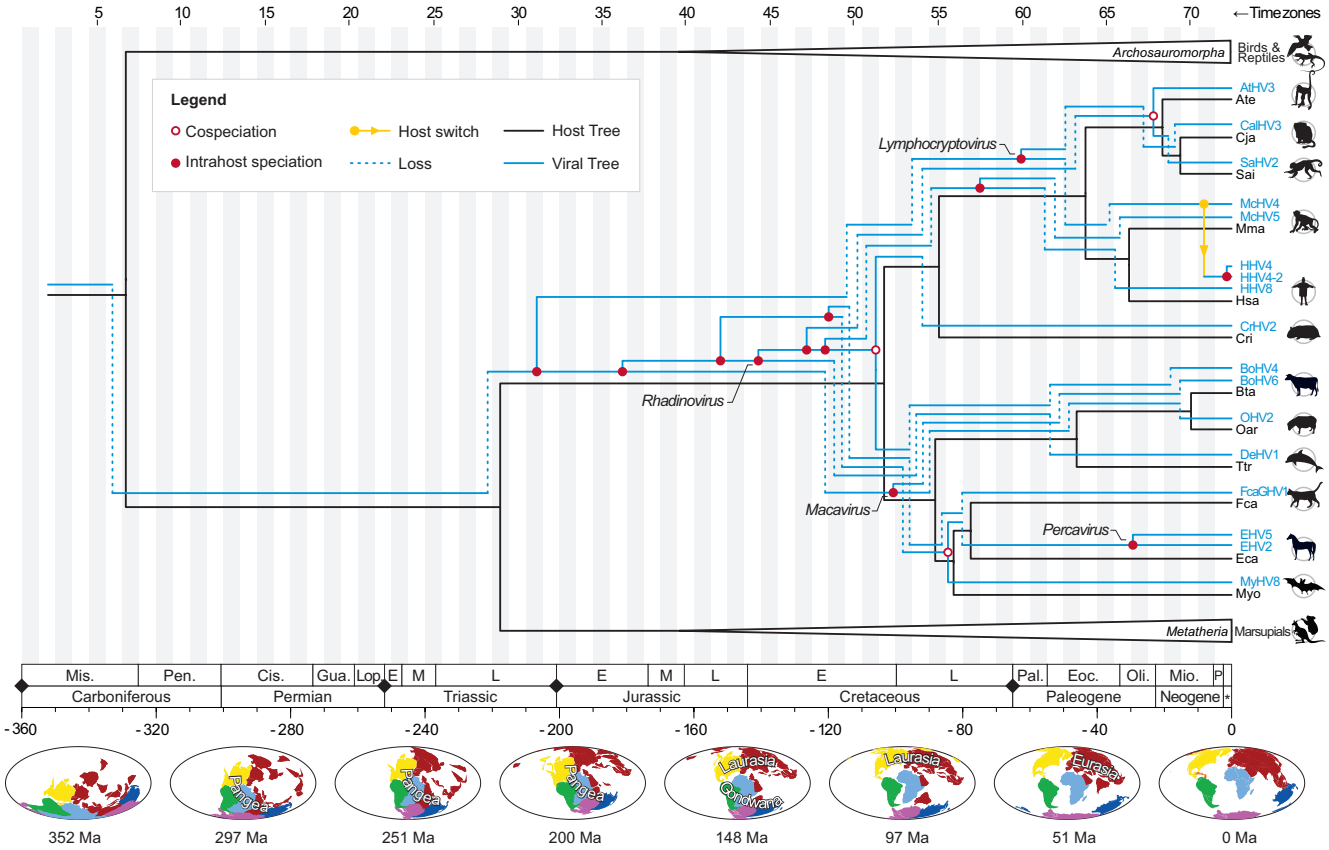

Figure 6. Tree reconciliation of Gammaherpesviruses and their hosts.

TABLE

Table 1. Cost regimes used for host-viral tree reconciliation analysis. CO = Cospeciation; IS = Intrahost speciation; HS = Host Switch; and LO = Loss. The numbers correspond to the cost assigned for each event, followed by the overall costs for each cost regime, per HV subfamily (α, β and γ).

Cost regime	CO	IS	HS	LO	Overall costs		
					α	β	γ
CR1	0	1	2	1	70	76	52
CR2	2	1	1	2	106	135	95
CR3	0	0	4	1	75	60	42
CR4	-1	1	2	0	28	18	11

SUPPLEMENTARY MATERIAL

Table S1. Herpesviruses and hosts included in this study. Subfamilies (S) are represented as: α = *Alphaherpesvirinae*; β = *Betaherpesvirinae*; and γ = *Gammaherpesvirinae*.

S	Genus	Viral TaxID	Genome AccNo	Viral name	Viral species	Host TaxID	Host species	Host name	Common name
α	*Iltovirus*	10386	NC_006623	GaHV1	*Gallid alphaherpesvirus 1*	9031	*Gallus gallus*	Gga	Chicken
α	*Iltovirus*	50294	NC_005264	PsHV1	*Psittacid alphaherpesvirus 1*	9224	*Amazona oratrix*	Psi	Yellow-headed parrot
α	*Mardivirus*	1890678	NC_033464	SpHV2	*Spheniscid herpesvirus 2*	9231	*Spheniscus sp.*	Sph	Banded penguin
α	*Mardivirus*	10390	NC_002229	GaHV2	*Gallid alphaherpesvirus 2*	9031	*Gallus gallus*	Gga	Chicken
α	*Mardivirus*	35250	NC_002577	GaHV3	*Gallid alphaherpesvirus 3*	9031	*Gallus gallus*	Gga	Chicken
α	*Mardivirus*	93386	NC_034266	CoHV1	*Columbid alphaherpesvirus 1*	8932	*Columba livia*	Cli	Pigeon
α	*Mardivirus*	1510155	NC_024450	FaHV1	*Falconid herpesvirus 1*	8952	*Falco mexicanus*	Fal	Prairie falcon
α	*Mardivirus*	104388	NC_013036	AnHV1	*Anatid alphaherpesvirus 1*	8840	*Anser cygnoides*	Acy	Swan goose
α	*Mardivirus*	37108	NC_002641	MeHV1	*Meleagrid alphaherpesvirus 1*	9103	*Meleagris gallopavo*	Mga	Turkey
α	*Scutavirus*	1561226	NC_027916	TeHV3	*Testudinid herpesvirus 3*	101699	*Testudo horsfieldii*	Tho	Russian tortoise
α	*Simplexvirus*	340907	NC_007653	CeHV16	*Papiine alphaherpesvirus 2*	9554	*Papio sp.*	Pap	Baboon
α	*Simplexvirus*	332937	NC_023677	ChHV1	*Chimpanzee herpesvirus strain 105640*	9598	*Pan troglodytes*	Ptr	Chimpanzee
α	*Simplexvirus*	1343901	NC_024306	FBaHV1	*Fruit bat alphaherpesvirus 1*	1562770	*Pteropus sp.*	Pte	Fruit bats
α	*Simplexvirus*	10317	NC_006560	CeHV2	*Cercopithecine alphaherpesvirus 2*	9533	*Cercopithecus aethiops*	Cer	Guenon
α	*Simplexvirus*	10298	NC_001806	HHV1	*Human alphaherpesvirus 1*	9606	*Homo sapiens*	Hsa	Human
α	*Simplexvirus*	10310	NC_001798	HHV2	*Human alphaherpesvirus 2*	9606	*Homo sapiens*	Hsa	Human
α	*Simplexvirus*	137443	NC_029132	MaHV1	*Macropodid alphaherpesvirus 1*	38609	*Macropodidae*	Mar	Macropod
α	*Simplexvirus*	481315	NC_029311	LHV4	*Leporid alphaherpesvirus 4*	9986	*Oryctolagus cuniculus*	Ocu	Rabbit
α	*Simplexvirus*	10325	NC_004812	CeHV1	*Macacine alphaherpesvirus 1*	9544	*Macaca mulatta*	Mma	Rhesus macaque
α	*Simplexvirus*	35243	NC_034446	AtHV1	*Ateline alphaherpesvirus 1*	9511	*Ateles sp.*	Ate	Spider monkey
α	*Simplexvirus*	10353	NC_014567	SaHV1	*Saimiriine alphaherpesvirus 1*	9520	*Saimiri sp.*	Sai	Squirrel monkey
α	*Varicellovirus*	10334	NC_013590	FeHV1	*Felid alphaherpesvirus 1*	9685	*Felis catus*	Fca	Cat
α	*Varicellovirus*	10320	NC_001847	BoHV1	*Bovine alphaherpesvirus 1*	9913	*Bos taurus*	Bta	Cattle
α	*Varicellovirus*	35244	NC_005261	BoHV5	*Bovine alphaherpesvirus 5*	9913	*Bos taurus*	Bta	Cattle
α	*Varicellovirus*	170325	NC_030117	CaHV1	*Canid alphaherpesvirus 1*	9615	*Canis lupus familiaris*	Cfa	Dog
α	*Varicellovirus*	10326	NC_001491	EHV1	*Equid alphaherpesvirus 1*	9796	*Equus caballus*	Eca	Horse

α	*Varicellovirus*	80341	NC_024771	EHV3	*Equid alphaherpesvirus 3*	9796	*Equus caballus*	Eca	Horse
α	*Varicellovirus*	10331	NC_001844	EHV4	*Equid alphaherpesvirus 4*	9796	*Equus caballus*	Eca	Horse
α	*Varicellovirus*	39637	NC_017826	EHV8	*Equid alphaherpesvirus 8*	9796	*Equus caballus*	Eca	Horse
α	*Varicellovirus*	55744	NC_011644	EHV9	*Equid alphaherpesvirus 9*	9796	*Equus caballus*	Eca	Horse
α	*Varicellovirus*	10335	NC_001348	HHV3	*Human alphaherpesvirus 3*	9606	*Homo sapiens*	Hsa	Human
α	*Varicellovirus*	35246	NC_002686	CeHV9	*Cercopithecine alphaherpesvirus 9*	392815	*Erythrocebus patas*	Chl	Patas monkey
α	*Varicellovirus*	10345	NC_006151	SuHV1	*Suid alphaherpesvirus 1*	9823	*Sus scrofa*	Ssr	Pig
β	*Cytomegalovirus*	1667587	NC_027016	CeHV5-B	*Papio ursinus cytomegalovirus*	9554	*Papio sp.*	Pap	Baboon
β	*Cytomegalovirus*	188763	NC_003521	PnHV2	*Panine betaherpesvirus 2*	9598	*Pan troglodytes*	Ptr	Chimpanzee
β	*Cytomegalovirus*	1919083	NC_033176	CyCMV	*Cynomolgus cytomegalovirus*	9541	*Macaca fascicularis*	Mfa	Crab-eating macaque
β	*Cytomegalovirus*	50292	NC_012783	CeHV5	*Cercopithecine betaherpesvirus 5*	9533	*Cercopithecus aethiops*	Cer	Guenon
β	*Cytomegalovirus*	10359	NC_006273	HHV5	*Human betaherpesvirus 5*	9606	*Homo sapiens*	Hsa	Human
β	*Cytomegalovirus*	50290	NC_016447	AoHV1	*Aotine betaherpesvirus 1*	9504	*Aotus trivirgatus*	Atr	Night monkey
β	*Cytomegalovirus*	47929	NC_006150	McHV3	*Macacine betaherpesvirus 3*	9544	*Macaca mulatta*	Mma	Rhesus macaque
β	*Cytomegalovirus*	1535247	NC_016448	SaHV4	*Saimiriine betaherpesvirus 4*	9520	*Saimiri sp.*	Sai	Squirrel monkey
β	*Muromegalovirus*	10366	NC_004065	MuHV1	*Murid betaherpesvirus 1*	10090	*Mus musculus*	Mmu	Mouse
β	*Muromegalovirus*	1261657	NC_019559	MuHV8	*Murid betaherpesvirus 8*	10114	*Rattus sp.*	Rat	Rat
β	*Muromegalovirus*	79700	NC_002512	MuHV2	*Rat cytomegalovirus Maastricht*	10114	*Rattus sp.*	Rat	Rat
β	*Proboscivirus*	548914	NC_028379	ElHV4	*Elephant endotheliotropic herpesvirus 4*	9780	*Elephas maximus*	Ele	Asian elephant
β	*Proboscivirus*	768738	NC_024696	ElHV5	*Elephant endotheliotropic herpesvirus 5*	9780	*Elephas maximus*	Ele	Asian elephant
β	*Proboscivirus*	146015	NC_020474	ElHV1	*Elephantid betaherpesvirus 1*	9780	*Elephas maximus*	Ele	Asian elephant
β	*Roseolovirus*	32603	NC_001664	HHV6-A	*Human betaherpesvirus 6A*	9606	*Homo sapiens*	Hsa	Human
β	*Roseolovirus*	32604	NC_000898	HHV6-B	*Human betaherpesvirus 6B*	9606	*Homo sapiens*	Hsa	Human
β	*Roseolovirus*	10372	NC_001716	HHV7	*Human betaherpesvirus 7*	9606	*Homo sapiens*	Hsa	Human
β	*Roseolovirus*	1940555	NC_033620	MRV	*Murine roseolovirus*	10090	*Mus musculus*	Mmu	Mouse
β	*Roseolovirus*	1846169	NC_030200	MneHV7	*Macaca nemestrina herpesvirus 7*	9545	*Macaca nemestrina*	Mne	Pig-tailed macaque
β	*Unassigned*	33706	NC_020231	CavHV2	*Caviid betaherpesvirus 2*	10140	*Cavia porcellus*	Cav	Guinea pigs
β	*Unassigned*	1608255	NC_022233	SuHV2	*Suid betaherpesvirus 2*	9823	*Sus scrofa*	Ssr	Pig
β	*Unassigned*	10397	NC_002794	TuHV1	*Tupaiid betaherpesvirus 1*	9393	*Tupaiidae*	Tup	Treeshrew
γ	*Lymphocryptovirus*	106331	NC_004367	CalHV3	*Callitrichine gammaherpesvirus 3*	9483	*Callithrix jacchus*	Cja	Common marmoset
γ	*Lymphocryptovirus*	10376	NC_007605	HHV4	*Human gammaherpesvirus 4*	9606	*Homo sapiens*	Hsa	Human
γ	*Lymphocryptovirus*	12509	NC_009334	HHV4-2	*Human herpesvirus 4 type 2*	9606	*Homo sapiens*	Hsa	Human
γ	*Lymphocryptovirus*	45455	NC_006146	McHV4	*Macacine gammaherpesvirus 4*	9544	*Macaca mulatta*	Mma	Rhesus macaque
γ	*Macavirus*	1504288	NC_024303	BoHV6	*Bovine gammaherpesvirus 6*	9913	*Bos taurus*	Bta	Cattle
γ	*Macavirus*	10398	NC_007646	OHV2	*Ovine gammaherpesvirus 2*	9940	*Ovis aries*	Oar	Sheep
γ	*Percavirus*	12657	NC_001650	EHV2	*Equid gammaherpesvirus 2*	9796	*Equus caballus*	Eca	Horse
γ	*Percavirus*	10371	NC_026421	EHV5	*Equid gammaherpesvirus 5*	9796	*Equus caballus*	Eca	Horse
γ	*Rhadinovirus*	2022783	NC_035117	DeHV1	*Common bottlenose dolphin gammaherpesvirus 1*	9739	*Tursiops truncatus*	Ttr	Bottlenose dolphin
γ	*Rhadinovirus*	10385	NC_002665	BoHV4	*Bovine gammaherpesvirus 4*	9913	*Bos taurus*	Bta	Cattle
γ	*Rhadinovirus*	37296	NC_009333	HHV8	*Human gammaherpesvirus 8*	9606	*Homo sapiens*	Hsa	Human

γ	*Rhadinovirus*	154334	NC_003401	McHV5	*Macacine gammaherpesvirus 5*	9544	*Macaca mulatta*	Mma	Rhesus macaque
γ	*Rhadinovirus*	1605972	NC_015049	CrHV2	*Cricetid gammaherpesvirus 2*	337677	*Cricetidae*	Cri	Rice rat
γ	*Rhadinovirus*	85618	NC_001987	AtHV3	*Ateline gammaherpesvirus 3*	9511	*Ateles sp.*	Ate	Spider monkey
γ	*Rhadinovirus*	10381	NC_001350	SaHV2	*Saimiriine gammaherpesvirus 2*	9520	*Saimiri sp.*	Sai	Squirrel monkey
γ	*Unassigned*	1452540	NC_028099	FcaGHV1	*Felis catus gammaherpesvirus 1*	9685	*Felis catus*	Fca	Cat
γ	*Unassigned*	1780507	NC_029255	MyHV8	*Myotis gammaherpesvirus 8*	9435	*Myotis velifer*	Myo	Vesper bats