Evolutionarily diverse origins of deformed wing viruses in western honey bees

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Novel transmission routes can allow infectious diseases to spread, often with devastating consequences. Ectoparasitic varroa mites vector a diversity of RNA viruses, having switched hosts from the eastern to western honey bees (*Apis cerana* to *Apis mellifera*). They provide an opportunity to explore how novel transmission routes shape disease epidemiology. As the principal driver of the spread of deformed wing viruses (mainly DWV-A and DWV-B), varroa infestation has also driven global honey bee health declines. The more virulent DWV-B strain has been replacing the original DWV-A strain in many regions over the past two decades. Yet, how these viruses originated and spread remains poorly understood. Here, we use a phylogeographic analysis based on whole-genome data to reconstruct the origins and demography of DWV spread. We found that, rather than reemerging in western honey bees after varroa switched hosts, as suggested by previous work, DWV-A most likely originated in East Asia and spread in the mid-20th century. It also showed a massive population size expansion following the varroa host switch. By contrast, DWV-B was most likely acquired more recently from a source outside East Asia and appears absent from the original varroa host. These results highlight the dynamic nature of viral adaptation, whereby a vector's host switch can give rise to competing and increasingly virulent disease pandemics. The evolutionary novelty and rapid global spread of these host–virus interactions, together with observed spillover into other species, illustrate how increasing globalization poses urgent threats to biodiversity and food security.

varroa | single-stranded RNA viruses | apis | phylogeography

Increased globalization, trade, and habitat fragmentation bring previously isolated species into contact, which risks increased transmission of diseases and parasites. These new connections caused global pandemics affecting populations of humans, animals, and plants. Many of these outbreaks have been caused by RNA viruses, and studies have highlighted them as primary etiological agents of human disease (1–4). The high adaptive potential of RNA viruses is linked to their high mutation rates, which are often an order of magnitude higher than those of DNA viruses (5) and their host range is frequently limited by their capacity to reach novel host populations (5, 6). Yet, our ability to predict specific viral host shifts and their consequences remains poor.

Many viruses solve the challenges of transmissibility by using vectors to connect potential hosts. As a result, changes in vector ecology, such as range expansions or host shifts, can drive viral pandemics. One model of viral spread involves anthropogenic changes in vector range, allowing viruses to spread to new geographical areas. For example, climate change has led to the poleward expansion of mosquito ranges bringing dengue to new human populations (7). Alternatively, viruses can acquire a novel host, driving their spread. This has happened in the case of the Chikungunya virus, originally found in arboreal mosquitoes feeding on primates in the sub-Saharan Africa, but has switched to urban-dwelling mosquitoes and spread through the human population to other continents (8). While human viruses have been studied extensively, these ecological and evolutionary dynamics are not limited to our species. Globalization has also caused pandemics among nonhuman animals (e.g., rabies) and plants (many diseases of crops) (9, 10). Yet, our ability to follow the dynamics of these pandemics is typically limited by a poor understanding of viral ecology in the original host, if the host is known at all.

Exceptionally, given their importance in world agriculture, there are excellent historical records on the spread of honey bee (genus *Apis*) parasites and viruses (11, 12). With their rapid generation times and large population sizes, arthropods provide an excellent opportunity to examine how vector host switches affect viral evolution. The eastern honey bee (*Apis cerana*) and the western honey bee (*Apis mellifera*) are sister species that diverged ~7 Mya (13). Eastern

Significance

RNA viruses adapt rapidly, sometimes switching hosts when given the opportunity. Many rely on arthropod vectors for transmission. Therefore, vector host switches expand evolutionary pathways for viruses, e.g., via spillover from the ancestral host, or via amplification of communities encountered posthost switch. We show that two closely related honey bee deformed wing virus (DWV) strains, transmitted by ectoparasitic varroa mites, show different patterns of spread. DWV-A was present in varroa's ancestral host in East Asia and spread worldwide postswitch. By contrast, DWV-B was most likely absent in the original host and was acquired later, replacing the original strain in many regions. DWV illustrates the dynamic nature of viral adaptation and competition, as well as the risks posed by globalization.

honey bees are found throughout Asia, while western honey bees naturally range from Europe to Africa but are nowadays distributed all over the world except Antarctica. Western honey bees have larger colony sizes and produce more honey, making them the mainstay of the beekeeping industry worldwide. Western honey bees existed in allopatry from other congenerics, isolating them from many honey bee diseases and parasites. However, that changed in the 19th century when beekeepers took western honey bees to Asia where they encountered eastern honey bees and Varroa mites, which are obligate parasites of honey bee brood. By the mid-20th century, *Varroa destructor* switched to its novel honey bee host (*A. mellifera*), followed by *Varroa jacobsoni* in the early 21st century (11, 14, 15). *V. destructor* then spread worldwide, transmitting a wide variety of bee viruses and causing global population collapses of western honey bees (12).

The well-documented history of the varroa host switches makes this an excellent system to examine how novel transmission routes affect viral disease emergence, as well as providing insights into the evolution of viral diseases (16). Here, we focused on understanding (1) how host bee species affect the virome of varroa and (2) reconstructing the geographic spread of the two most common and important viruses vectored by varroa—deformed wing virus (DWV) strains A and B that diverged about 200 y ago (17). A previous phylogeographic investigation of DWV-A found that the virus most likely originated in Europe and has increased in frequency after the introduction of varroa mites (18), though the inference was based on less than 10% of the viral genome. The origins of DWV-B remain poorly understood to date, though its spread in honey bees appears to have happened in recent decades (19). While these two strains are similar in sequence and biology, they differ significantly in how they interact with both bees and mites (20, 21). For example, DWV-B, but not DWV-A, replicates in Varroa (22, 23), and is also more virulent to honey bees (24). This study aimed at using full-genome sequences to examine whether these viruses had similar or different phylogeographic histories, evolutionary origins, and population dynamics.

Materials and Methods

Data analysis pipelines and R scripts are available at: https://github.com/nonnohasegawa/varroa-transcriptomics.

Mite Collection, Sequencing, and Bioinformatic Analysis. Varroa mites were collected from 1989 to 2019 from 43 countries and islands spanning six continents (*SI Appendix*, Tables S1 and S2, Dataset S1, and Fig. 1*A*). Mite RNA was extracted from single adult female mites using TRIzol and prepared for sequencing using the previously described methodology (25). Sequencing was performed on an Illumina NovaSeq S2 at 150 cycles with paired-end reads. Raw sequence data were first mapped to *V. destructor* reference genome Vdes_3.0 [GCF_002443255.1] (26) using Bowtie2 (27). The unmapped reads from the previous step were mapped to a selection of viruses that are known to be associated with varroa mites (21). Using VariantBam (28), duplicates were removed and downsampled to a maximum coverage to 200. A pileup format was created using SAMtools (29), then variants were called using VarScan (30). Indels, which are phylogenetically uninformative, were removed using vcftools (31). Then, viral sequences of each sample were extracted using BCFtools (29). In addition, we quantified viral abundances using Kallisto (32) on raw reads mapped to reference viral genomes using the set of viral genomes from ref. 21.

Host, Mite Species, and Geographic Effects on the Virome. For the analysis of virome composition, we first filtered out 8 failed libraries. Read counts are compositional data, which means that an increase in one virus abundance in a sequenced sample must lead to a proportional decrease in the abundances of other viruses. To address this issue, we performed an additive log-ratio transform on the count data (33), using the number of reads mapped to the varroa genome as a common denominator. This procedure makes the viral titers independent of one another, so that increasing the titer of one virus does not lead to a corresponding decrease in the others, given a fixed number of reads, i.e., the resulting ratios should be independent of each other. We then used the ratios to perform a principal component analysis and to quantitatively assess the effects of honey bee host species, mite species, and geographic region using analysis of matrix distances analysis using Euclidean distances (34). Statistical analyses were conducted on a total of 166 libraries using the R programming environment using the vegan package (35).
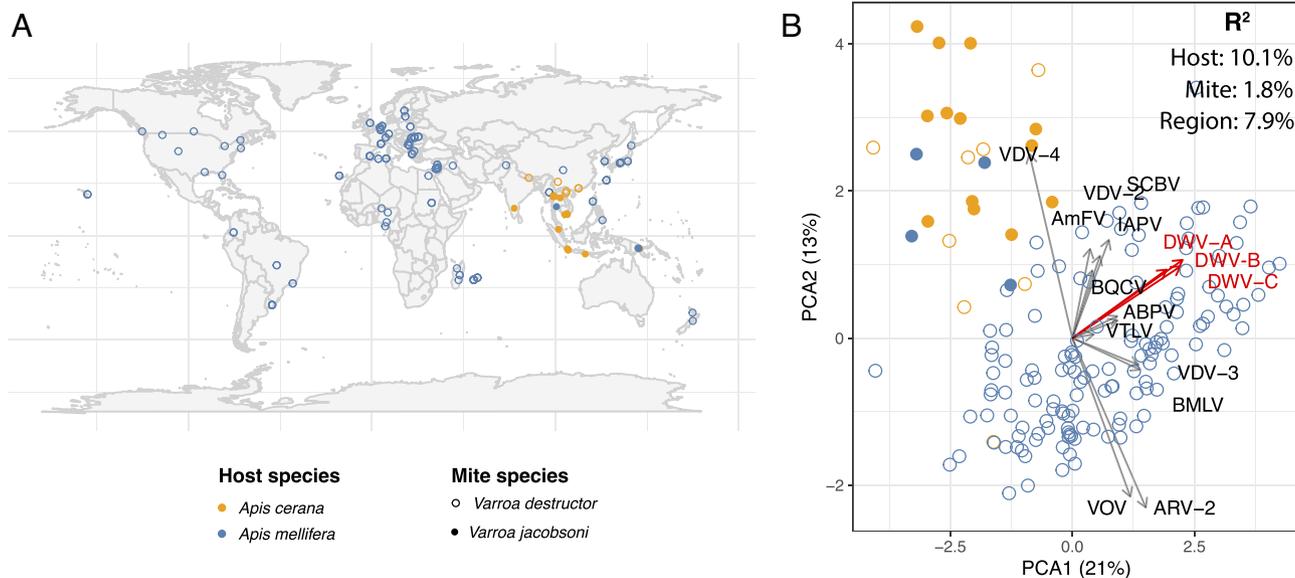
Fig. 1. Varroa viromes are most strongly affected by the host bee species. (*A*) Map of 166 samples used in the virome analysis. (*B*) The Principal Component Analysis (PCA) plot of varroa virome composition. Arrows represent loadings of each virus onto the principal component axis (i.e., how correlated its titer is with each of the two PCA axes). Loadings show that mites from eastern honeybee have generally lower titers of deformed wing viruses (DWV-A, DWV-B, and DWV-C), as well as most other viruses, except VDV-4, which was more abundant. Note that short-read data will not distinguish between master and recombinant viral types, making loadings of closely related recombining strains, such as the DWV variants, more similar to each other than they are in reality. These community-level data show that the viral landscape inside the mite is most likely shaped by the host bee, either by direct uptake of viruses by mites from bees, or via tripartite coevolutionary relationships. This relationship is quantified by R^2 values from an ANOVA of viral abundance distance matrices, suggesting that much of the mite virome were acquired from the host, relative to the effects of the mite itself. However, all effects were significant ($P < 0.001$), indicating that the virome is shaped by all the three factors. Abbreviations: AmFV–*Apis mellifera* filamentous virus; ABPV–acute bee paralysis virus; ARV–Apis rhabdovirus; BMLV–*Bombyx mori* latent virus; BQCV–black queen cell virus; IAPV–Israeli acute paralysis virus; SCBV–sacbrood virus; VTLV–Varroa Tymo-like virus; VOV–Varroa orthomyxovirus.

Phylogeographic Analysis. Consensus sequences extracted from varroa mite transcriptomes were aligned. We excluded genomes with insufficient sequence coverage, less than 90% for DWV-A and 80% for DWV-B. To this dataset, we added complete viral genomes of DWV-A and DWV-B from NCBI and consensus sequences from a published dataset (36), excluding those identified as recombinants by other studies (Dataset S2). While recombinant viruses play increasingly important roles in honey bee pathology (37, 38), their sequences do not follow the tree-like topology and excluding them is generally desirable for clock-based and phylgeographic analyses. To improve alignment quality, all genomes were trimmed to remove the untranslated regions and the polyprotein was codon aligned. All viral genomes were screened for possible recombination events using RDP4 (39). Sequences were considered likely recombinants if most detection methods implemented in RDP4 showed significant evidence for recombination. Overall, we identified one DWV-A sequence (out of 56) and two DWV-B sequences (out of 32) that were likely to be recombinants and excluded them from further analysis that relied upon a tree-like sequence topology.

The phylogenetic history of DWV isolates was reconstructed jointly with their geographic movement using a discrete-trait continuous-time Markov chain phylogeographic model in BEAST v. 2.6 (40, 41). Viral sequences were assumed to evolve under a general time reversible substitution model with gamma-distributed rate heterogeneity across sites. To obtain a time-calibrated tree, we assumed a strict molecular clock with a LogNormal (mean = 0.001; SD = 2.5) prior to the clock rate. For the phylogeographic reconstruction, sampling locations were binned into seven discrete locations (Africa, Asia, Europe, the Mediterranean, North America, Oceania, and South America). Bayesian Stochastic Search Variable Selection was used to identify movement rates between locations significantly higher than zero (41). The geographic origin of each DWV strain was then reconstructed based on the (posterior) ancestral state/location probabilities computed at the node representing the MRCA of each strain. Inferred origins, therefore, take into account uncertainty both in the phylogenetic (tree) and reconstructed ancestral states. We provide the input BEAST XML files for further details on the phylogenetic analysis (Dataset S1).

We also reconstructed the demographic history of each DWV strain using a Bayesian Skyline analysis in BEAST 2 (42). To account for phylogenetic uncertainty, phylogenetic trees were jointly reconstructed along with demographic parameters using the same substitution and clock models as above. Historical changes in effective population sizes (N_e) were reconstructed under a Skyline coalescent tree prior 10 coalescent time intervals, each with a Jeffrey's prior on N_e.

Results

Each library generated an average of 9,216,119 reads (SD 3,057,629), of which an average of 57.7% (SD 12.8%) mapped to viruses (ranging between 21.7 to 93.3%). We extracted 83 viral genome sequences, including 54 from our study, adding a further 29 reference sequences for phylogenetic analysis (Datasets S1 and S2).

Host Effects on the Virome. Our dataset consisted of RNA-seq data that captured viruses from two species of mites collected from either original or novel bee hosts (*A. cerana* and *A. mellifera*). We used these data to determine the relative importance of host and vector species for the composition of the varroa virome, which showed that the host bee species played a greater role than the species of the mite (Fig. 1). Viromes of *V. destructor* mites which were collected from *A. mellifera* host had a higher viral load, notably of DWVs (Fig. 1*B*). On the contrary, viromes of varroa mites collected from *A. cerana* hosts exhibited higher association with VDV-4 and not to DWVs. We had far fewer *V. jacobsoni* samples, but they appeared to show a qualitatively similar trend (Fig. 1*B*).

Origins of DWVs. We found that DWV-A and DWV-B diverged from each other 308 y ago (95% HPD 266 to 353 y), predating the varroa host switches. There are other DWV strains, the most common being DWV-C. While we did not encounter enough of this strain to conduct phylogeographic analysis, we could compute its divergence from the other two strains, which was

even more ancient, 979 y ago (HPD 836 to 1128). We found strong evidence that DWV-A originated in East Asia (Fig. 2*A*), and that its population expanded rapidly after the varroa host switch to western honey bees. The expansion occurred around 1970 according to our analysis, consistent with spillover onto a novel host (posterior probability = 0.97). By contrast, while the most ancestral DWV-B samples originate from the Middle East, this origin was less supported (posterior probability = 0.48), and no evidence of an increase in global post-varroa host switch population size could be detected. However, when focusing only on Europe and North America, there was a signal of increased population size around 1970 (Fig. 2*B*). An East Asian origin for DWV-B is not likely (posterior probability = 0.12).
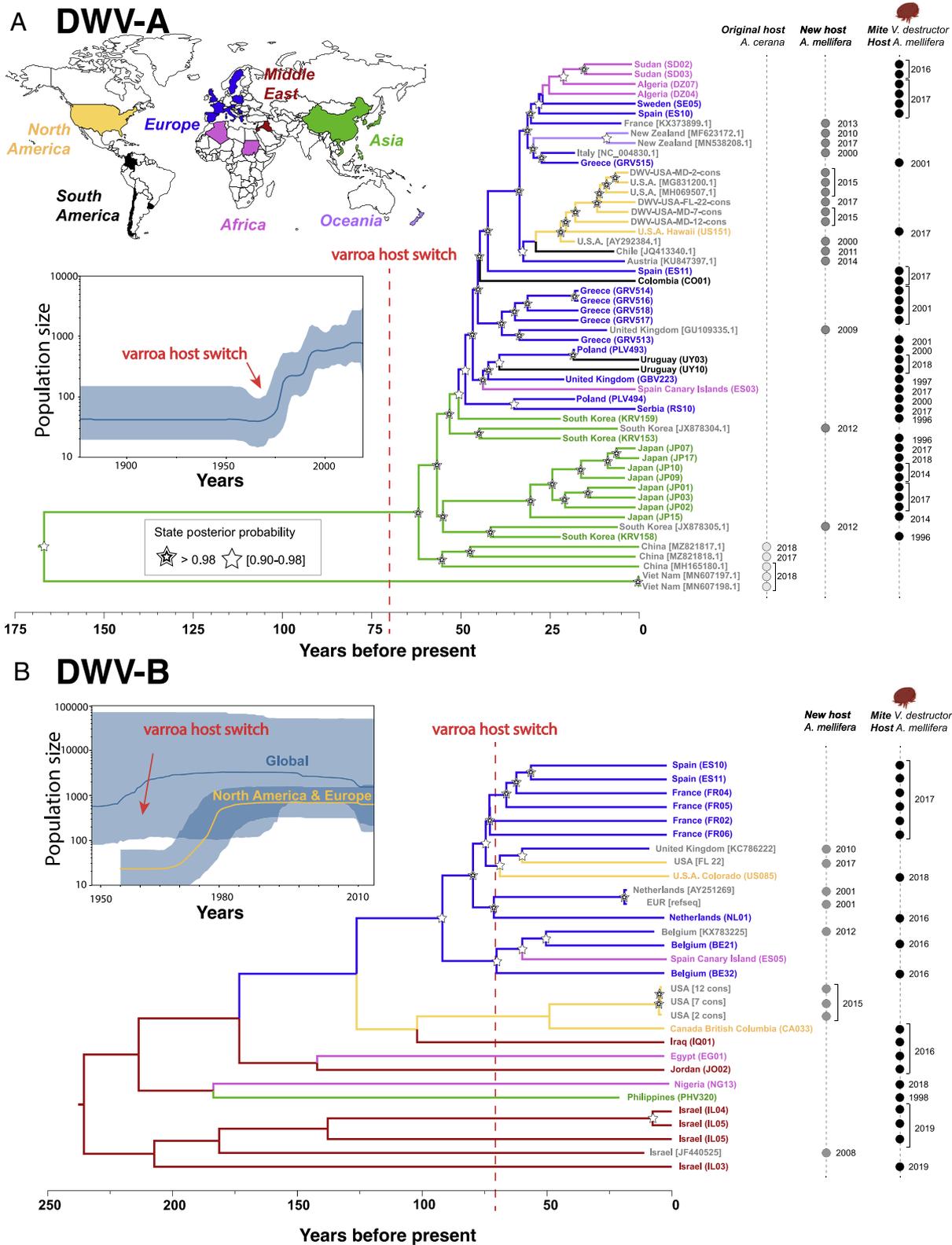
Discussion

Host availability limits the spread of many RNA viruses, and changes in vector ecology can lead to novel transmission pathways. For example, climate-driven expansion of mosquito ranges leads to larger endemic ranges of many mosquito-borne diseases (7). Alternatively, acquiring a new host and/or vectors can allow a virus to spread globally, as in the case of the Chikungunya virus (8). Mirroring these human vector–borne viruses, we found evidence for both mechanisms in DWV. DWV-A spilled over into western honey bees in association with the varroa host switch. While the exact geographic origin and host of DWV-B remain unclear, several lines of evidence suggest that it was acquired by varroa after its host switch. First, it appears to be absent in eastern honey bees, whereas DWV-A is detected consistently (43–45). Second, its split from DWV-A predates the varroa pandemic by hundreds of years (Fig. 2). Third, epidemiological data suggest a spread that started over the past two decades, long after the varroa host switch that started in the mid-20th century (19, 36, 46). These lines of evidence point to the dynamic interplay between vector and disease ecology, and the potential for continuing viral emergence after a vector host switch.

Virome Analysis. Taking advantage of viromes collected from different species of mites across different bee hosts, we could ask which factors play a greater role in shaping viral community composition. Most of the differentiation was associated with the bee host rather than the mite species (Fig. 1). These findings are interesting considering recent work suggesting that viruses interact with varroa gene expression in species-specific ways that affect viral titers (21). DWV-A titer was shown to be strongly correlated with changes in mite gene expression, whereas DWV-B titer was not. However, relatively few viruses replicate in varroa, and therefore must be taken up from the bee hosts. Thus, the biology of the host bee likely remains the primary driver of viral community composition.

An important caveat to the virome analysis is that short-read data cannot accurately distinguish recombinant genotypes which are increasing in frequency worldwide (37, 38). They were relatively rare in our phylogeographic dataset, much of which are historic, but are likely more prevalent in more modern collections. A larger database of recombinant sequences could allow for a more detailed study of how recombination affects DWV genomes, which is expected to play a major role in viral adaptation by generating sequence diversity and reducing genetic linkage (47). Additional insights could come from time series data tracking the frequency of recombinant lineages, which will provide additional insights into molecular mechanisms underlying viral competition.

Phylogeographic Analysis. Phylogeographic analysis suggests that DWV-A was already present in eastern honey bees and spilled over to western honey bees at the same time as the varroa host switch

Fig. 2. Divergence and phylogeography of deformed wing viruses based on sequences from mites and bees. Locations where samples were collected are color coded. Sample identifiers refer to samples sequenced in our study (Dataset S1) or via their NCBI identifiers (Dataset S2). (*A*) Divergences between the three main DWV strains predate the host switch by *V. destructor* by hundreds of years. Phylogeographic reconstruction indicates that DWV-A originated in Asia (posterior probability 0.97). (*B*) By contrast, the provenance of DWV-B remains unclear with the strongest evidence for a Middle Eastern origin (posterior probability 0.48). Bayesian skyline reconstruction shows a sharp increase in DWV-A populations in the mid-20th century concurrent with varroa's host switch and subsequent global spread (95% posterior probability shown in blue shading). This suggests that the host switch was mechanistically linked to the demographic expansion of the virus. A similar pattern is seen in the clade of North American and European DWV-B. In contrast, the globally DWV-B population size is more stable, suggesting that transmission dynamics may vary regionally. Also, note the different time scales in the two panels, with most divergences in the DWV-B tree being much deeper than those in DWV-A. These data show that the two closely related viruses most likely have distinctly different geographic and evolutionary origins as well as dynamics of spread, while predominantly relying on the same host and vector.

(Fig. 2). These findings differ significantly from earlier work that excluded an Asian origin and suggested that the virus was endemic to western honey bee populations before the arrival of varroa (18). The reason for these discrepancies is not clear, though our study used nearly complete viral genomes instead of fragments, and could pick up stronger signals. One of the signals detected in this study is the explosive growth of DWV-A associated with the origins of varroosis, which supports the link between mite host switch and viral spread (Fig. 2*A*). It is possible that other DWV strains existed in Europe prior to the arrival of the current East Asian DWV-A strain.

Our findings that varroa drove the spread of DWV-A need to be reconciled with other work that found DWV-A in regions where varroa has not yet been detected. For instance, in Australia (18), where subsequent work has shown DWV to be absent (Roberts et al. 48), or in advance of the invasion front in Hawaii (49). There are several possibilities for reconciling the absence of Varroa with the presence of DWV-A. It is possible that once DWV-A became established in western honey bees post-varroa host switch, it gained access to additional transmission pathways that allowed spread without varroa. Another possibility is that some of these results were caused by contamination; e.g., a subsequent in-depth study of Australian viruses, where libraries were prepared and sequenced in Australia, without the possibility of crosscontamination, failed to find DWV (48). To further illustrate this point, a cursory examination of sequenced negative controls in the NCBI SRA database (search terms negative control AND "*A. mellifera*"[orgn] in RNA data, 19 January 2023), yielded just two sequencing runs of library blanks (50), and both have DWV contamination present (accession numbers DRR333172 and DRR333191). We recommend that future studies aiming to ascertain the presence of DWV rule out contamination by sequencing negative controls. This is particularly important for studies aiming to detect viral spillover into non-*Apis* taxa, which are usually prepared and sequenced alongside honey bee samples.

In contrast to DWV-A, we found no evidence that DWV-B population history was affected by the Varroa host switch at a global level. It was first detected in the Netherlands in 2001 (51). The fact that it was detected decades after DWV-A suggests that it was either a virus novel to honey bees or was previously geographically restricted and not detected until later. Thus, it is possible that DWV-B was already present in some western honey bee populations before Varroa but the mites have offered it a new mode of transmission. Alternatively, it may have been acquired by honey bees from another host after the varroa host switch. While bearing in mind the caveat above regarding contamination, DWV-B is found in other taxa (52, 53), and some of them could be potential sources of infection. Yet, there are increases in DWV-B levels in honey bees, particularly in Europe and North America (19, 36, 54), and we see an increase in effective population size in that lineage of DWV-B that is contemporaneous with the spread of varroa in these regions (Fig. 2*B*). Future work should investigate the origins of DWV-B to ascertain how new viruses can enter the varroa–bee ecosystem. This may cause repeated viral emergence, particularly given the recent arrival of varroa in Australia, which is home to a diverse picornavirus community (48) that could be the source of future varroa-transmitted diseases.

One limitation of phylogeographic studies is that sampling biases can affect outcomes since reconstructions of ancestral states are sensitive to the number of samples from a particular geographic region (55). To minimize the potential impact of sampling bias we attempted to get as broad a range of specimens as possible. In the case of DWV-A, this approach yielded many sequences with good geographic coverage. The number of DWV-B sequences was considerably lower, making

it impossible to infer its geographic origins with certainty. However, the reconstructed location of the most ancestral lineages in our dataset appears in the Middle East, and an East Asian origin is unlikely. Additional sampling of DWV-B could resolve this uncertainty as well as potentially provide more details into temporal changes in effective population size.

Our study illustrates the dynamic interaction between virus, vector, and host. Although the original host switch of *V. destructor* took place in the mid-20th century, (co-)evolutionary dynamics continue in this system with the acquisition of novel viruses and novel vectors (14, 15). Viral fitness depends on successfully exploiting both vectors and hosts, and how viruses evolve to do that remains an open question. For example, DWV-A appears to affect varroa gene expression more than DWV-B (21), suggesting that even relatively closely related viruses may have significantly different ways of interacting with their vector. The dynamism of the bee–varroa–virus system can make it an excellent model for viral emergence and evolution, as well as broadly applicable strategies for viral control.

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