

ORIGINAL RESEARCH ARTICLE

Potential for virus transfer between the honey bees *Apis mellifera* and *A. cerana*

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Summary

Viruses seem to play a key role for European honey bee, *Apis mellifera*, health, and have a much broader host spectrum than previously thought. Few studies have investigated interspecific virus transfer within the genus *Apis*. The introduction of *A. mellifera* into Asia exposed endemic *Apis* species to the risk of obtaining new viruses or viral strains and vice versa. To investigate the potential for host shifts, virus prevalence and sequences were monitored over three years in single and mixed-species apiaries hosting introduced *A. mellifera* and / or endemic *A. cerana* alone or in combination. DWV, IAPV, BQCV and SBV were found, but not KBV, VDV-I, ABPV or CBPV. Virus infections and prevalence was generally lower in *A. cerana* compared to *A. mellifera*, and varied over the years. The sequence data provided evidence for interspecific transfer of IAPV, BQCV and DWV, but SBV strains seem to be species specific. Prevalence and sequence results taken together indicate that interspecific transfers of viruses are rare, even if honey bees are kept in close proximity. We discuss the pattern observed in the context host specificity and resistance. Our understanding of the extent of these exchanges is limited by a lack of knowledge on the mechanisms of adaptation of viruses to different hosts.

Keywords: *Apis cerana*, *Apis mellifera*, honey bee, interspecific transmission, host specificity, virus

Short title: Interspecific virus transfer in honey bees

Introduction

Elevated global losses of managed western honey bee, *Apis mellifera* L. colonies have worried beekeepers, the general public and scientists in recent years. Despite increased research efforts on the causes of these losses, no single culprit has been identified, but beekeeping practices and honey bee pathologies seem to be the main driver of mortalities (Genersch *et al.*, 2010; vanEngelsdorp *et al.*, 2010). Among the prime suspects for pathogens involved in colony mortalities are the ectoparasitic mite *Varroa destructor* (e.g. Dietemann *et al.*, 2012; Guzman-Novoa *et al.*, 2010; Le Conte *et al.*, 2010; Neumann and Carreck, 2010) and honey bee viruses (Berthoud *et al.*, 2010; Carreck *et al.*, 2010; Martin *et al.*, 2010). The absence of consensus on the causes for the losses results from regional variations in the suspected agents, and considerable gaps in our knowledge of the epidemiology of honey bee pathogens.

For example, our understanding of pathogen transmission between species is poor, despite its fundamental importance given the potential for increased virulence after host shifts (Woolhouse *et al.*, 2002). Honey bee viruses can adapt to different hosts, including other hymenoptera (e.g. Fürst *et al.*, 2014; Genersch *et al.*, 2006; Singh *et al.*, 2010; Yañez *et al.*, 2012b) and to parasites of their own hosts (e.g. *V. destructor*, Neumann *et al.*, 2012). As an illustration of such interactions, evidence has recently been gathered for the selection of particular strains of the deformed wing virus (DWV) by *V. destructor* (Martin *et al.*, 2012; Ryabov *et al.*, 2014). Although interspecific transmission of pathogens has been hypothesized for a diversity of taxa, few studies have investigated such occurrences between species within the genus *Apis*.

In contrast to some parts of Asia, where many *Apis* species naturally occur in sympatry, the western honey bee *A. mellifera* had been isolated for a long evolutionary time span and coevolved with a range of endemic pathogens and parasites. However, in the times of global trading, geographical obstacles to pathogen dispersal between species have been overcome. This has especially been the case for the western honey bee, which was introduced in many parts of the world for beekeepers to benefit from its high honey yields. It was thus brought into Asia, where all other *Apis* species are endemic. The physical proximity (e.g. workers visiting the same flowers (Singh *et al.*, 2010) and interspecific robbing (Adlakha and Sharma, 1974)) provides ample opportunities for pathogens to switch to a new host. A typical example of such host switch resulting from human-mediated sympatry is the spread of the mite *V. destructor* from the eastern honey bee *A. cerana* to *A. mellifera*, allowing this mite to spread to most of the world, with dramatic economic consequences (Cook *et al.*, 2007).

In a similar way, viruses might have been able to switch to a new host when *A. mellifera* was brought into the natural distribution range of *A. cerana*.

There is little knowledge on the host specificity of honey bee viruses and on their species of origin. Viruses can only be detected with methods that postdate the human mediated sympatry of honey bees and the potential exchange of viruses. In general, presence and prevalence of viruses in Asian honey bees are poorly characterized (Allen and Ball, 1996; Fries, 2011; Yañez *et al.*, 2012b), but Apis iridescent virus (AIV), black queen cell virus (BQCV), DWV, Kashmir bee virus (KBV), sacbrood virus (SBV) and Israeli acute paralysis virus (IAPV) have been reported in a few localities. Recently, several larger-scale surveys in China, Japan and Korea have confirmed and extended these characterizations (Ai *et al.*, 2012; Choe *et al.*, 2012; Kojima *et al.*, 2011; Li *et al.*, 2012; Yang *et al.*, 2013). Whether these viruses represent strains specific to the eastern bees or are the same strains as in *A. mellifera* has been investigated by Kojima *et al.* (2011) and Yang *et al.* (2013) at a nationwide scale. Despite all these recent efforts to document viral infections in eastern honey bees, the potential of transfer of honey bee viruses between *Apis* species has not been systematically tested (Li *et al.*, 2012). Here, we investigated the potential for the exchange of viruses between sympatric honey bee species in China over three years.

To improve our understanding of population dynamics of honey bee viruses, we focused on the transmission of viruses between neighboring colonies at the apiary and regional scale, where robbing, drifting (Moritz and Neumann, 1996) and usage of the same foraging range constitute potential pathways for interspecific pathogen transmission. This differs from the previous studies of virus prevalence in *A. cerana* (e.g. Ai *et al.*, 2012; Kojima *et al.*, 2011; Yang *et al.*, 2013), which did not consider mixed apiaries or regional scale. Although we could detect transfers that occurred any time after introduction of *A. mellifera*, mixed apiaries are potential hot spots for interspecies virus transfer due to the close spatial proximity of colonies of both species, thus maximizing the probability to find evidence for such transfers if they occur. We investigated the occurrence of eight of the most common honey bee viruses (DWV, IAPV, SBV, KBV, BQCV, Varroa destructor virus (VDV-I), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV)), and compare their prevalence in mixed-species apiaries in which *A. mellifera* and *A. cerana* are kept in close proximity to that of apiaries hosting these species separately. We consider the latter as the natural situation with species hosting their natural pathogens and the former as apiaries where potential host shifts are more likely to occur. We built virus phylogenies based on the

sequences of the viruses detected and compare these across species and apiary types as well as with GenBank entries to determine whether we find evidence for host change. The presence of the same virus strain in both species in the mixed-species apiaries would suggest the recent occurrence of viral transmission. The presence of similar strains in more distant colonies of both species would suggest transfers in the past. Such evidence was found during our survey, but the relatively low virus prevalence measured in *A. cerana* over three years suggests limited occurrence of such transfers or higher resistance to the viruses. We discuss the patterns observed in the context of interspecific exchange of honey bee viruses and host specificity.

Material and methods

Sampling, RNA extraction and PCR assays

Single and mixed-species apiaries were selected in Zhejiang Province, China (Figure 1), in which only introduced *A. mellifera ligustica* (n=5), only endemic *A. cerana cerana* (n=5) or both species (n=5) were kept. Apiaries were separated by 2 to 140 km. While the *A. cerana* colonies were not chemically treated against *V. destructor*, the *A. mellifera* colonies were treated against parasitic mites with fluvalinate. Samples of 30-50 workers of unknown age were taken in October of 2010, April of 2011 and April of 2012 from three to six colonies per apiary. Autumn and spring correspond to the main honey bee seasons in this province. Seasonal prevalence of viruses in Asia is poorly documented, but viruses have been detected during these seasons in other regions of the world (e.g. Tentcheva *et al.*, 2004) and thus pathogen transfer is likely to occur. Samples were collected in 95 % EtOH and stored at -20 °C.

Pooled honey bee workers (n=30-50) from each colony were suspended in TN buffer (Tris-Cl 10 mM, NaCl 10 mM) and homogenized using MACS M-tubes and the gentleMACS™ Dissociator machine (Miltenyi Biotec GmbH; Bergisch Gladbach, Germany). Total RNA from pooled honey bees per colony was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel; Dueren, Germany) following the manufacturer recommendations including the step for DNA digestion. Extracted RNA was eluted in 50 µl of RNase-free water. Reverse transcription was performed using M-MLV RT Thermoscript® RT-PCR (Invitrogen; CA, US) using approximately 1 µg of extracted RNA in 20 µl final volume following the manufacturer recommendations. Before PCR amplifications the resulting cDNAs were diluted 10-fold. Amplifications for the detection of viruses were performed

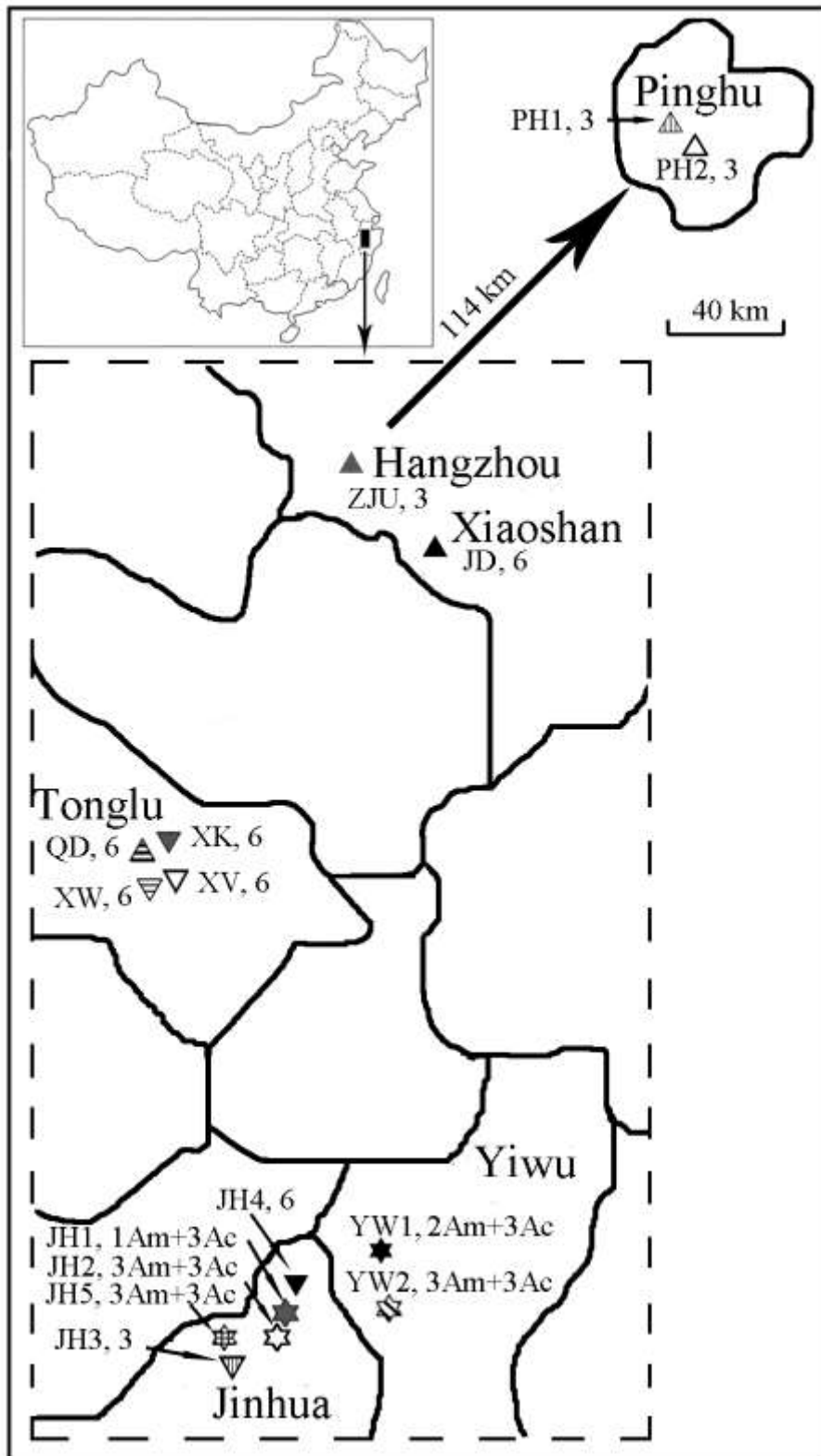


Figure 1. Map indicating the sampling locations in Zhejiang Province, China. Symbols represent the different types of apiaries (triangle pointing up = *A. mellifera* single-species apiary, pointing down = *A. cerana* single-species apiary, star = mixed-species apiary). Fill patterns differentiate apieries within the same type. For each location, the apiary code is given, followed by the number of colonies (for mixed apiaries, the number of colonies per species is mentioned: Am = *A. mellifera*, Ac = *A. cerana*).

using AmpliTaq® Gold (Applied Biosystems; CA, US) reagents in 50 µl volumes containing 5 µl template cDNA, 5 µl of 10X Reaction Buffer, 1 µl of dNTPs Mix (10 mM), 2 µl of each forward and reverse primers (10 µM), 3 µl of MgCl₂ (25 mM) and 0.25 µl of *Taq* polymerase (1.25 U). The PCR assays consisted of 2 min incubation at 95 °C and 35 cycles of 30 sec at 95 °C for denaturation, 30 sec at 57 °C for annealing and 30 sec at 72 °C extension and then a final step of 7 min at 72 °C. The primers used for the PCR amplifications are shown in Table I. Bands were visualized under UV light after electrophoresis running in 1.2 % agarose. Single-band PCR products at the expected size were purified using the Kit NucleoSpin Extract II (Macherey-Nagel; Dueren, Germany) and were sequenced by a commercial company (Fasteris; Geneva, Switzerland). GenBank accession numbers are given in Figures 3 to 6.

Table I. Set of primers used for the diagnostic testing for honey bee viruses.

	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Size (bp)	References
DWV	CGTCGGCCTATCAAAG	CTTTTCTAATTCAACTTCACC	417	(Yañez et al., 2012a)
IAPV	CCATGCCTGGCGATTAC	CTGAATAATACTGTGCGTAT C	203	(Gauthier et al., 2011)
SBV	GGATGAAAGGAAATTACCAG	CCACTAGGTGATCCACACT	426	(Tentcheva et al., 2004)
KBV	GATGAACGTCGACCTATTGA	TGTGGGTTGGCTATGAGTCA	414	(Tentcheva et al., 2004)
BQCV	GTGGCGGAGATGTATGCGCTTT ATC	CTGACTCTACACACGGTTTCG ATTAG	511	(Yang et al., 2013)
VDV-I	GCCCTGTTCAAGAACATG	CTTTTCTAATTCAACTTCACC	430	(Gauthier et al., 2011)
ABPV	CTCAAGTTATACGTAAAATAGC TGGAATT	AACCAACCTTGCTTCCCTTT A	646	(Tentcheva et al., 2004)
CBPV	AGTTGTCATGGTTAACAGGATA CGAG	TCTAATCTTAGCACGAAAGC CGAG	455	(Tentcheva et al., 2004)

Virus prevalence between species was compared statistically with the Chi-square test (Systat 12) when sample size allowed for such analyses.

Phylogenetic trees

The sequences of DWV, IAPV, BQCV and SBV obtained from the samples were aligned using the MUSCLE program and compared using the maximum likelihood method with the MEGA5.1 program under the Tamura 3-parameter. The tree topology was evaluated by bootstrap resampling (1,000 times). Regarding DWV, sequences corresponded to the DWV-Lp gene, the most variable part of the DWV genome (Lanzi et al., 2006), raising the

probability of finding genetic variability between samples. DWV-Lp sequences from previous isolates from Italy, Switzerland and Japan were included as references. VDV-I virus was included in the tree as reference. IAPV sequences from the capsid protein gene (VP2) were compared with previous isolations from the USA, Poland, Israel, Japan and China. Previous isolates of the BQCV capsid protein gene from the USA, South Africa, Brazil, Uruguay, Spain, Japan and China were used as references for the BQCV phylogenetic tree. SBV sequences from the RNA-dependent RNA polymerase (RdRp) gene were compared with previous *A. mellifera* isolates from the UK, Germany, South Africa, China and *A. cerana* isolates from Korea, Vietnam and China. GenBank accession numbers from the isolates are provided in the figures.

Results

The prevalence of the detected viruses (DWV, IAPV, SBV and BQCV), in single-species and mixed-species apiaries is shown in Figure 2.

Single-species apiaries

SBV was neither found in *A. mellifera* nor *A. cerana* single-species apiaries. In the single-species *A. mellifera* apiaries, DWV occurrence ranged from apiaries with all sampled colonies positive to apiaries where all colonies sampled were negative. DWV prevalence was significantly different between years (fall 2010: 100 % of three colonies; spring 2011: 67 % prevalence of six colonies; spring 2012: 8 % of 12 colonies; Pearson Chi Square test, Chi Square=11.5, df=2, p=0.003). IAPV occurrence also ranged from apiaries with all sampled colonies positive to apiaries where none of colonies harbored this virus. The prevalence decreased over time, but differences were not significant between years (fall 2010: 100 % of three colonies; spring 2011: 33.3 % of six colonies; spring 2012: 25.0 % of 12 colonies; Chi Square=5.8, df=2, p=0.06). BQCV was not detected in the years 2010 and 2011. In contrast, all 12 colonies sampled from 2 apiaries in 2012 were positive for BQCV, which constituted a significant increase (Chi Square=31.0, df=2, p<0.001).

In the single-species *A. cerana* apiaries, DWV and IAPV were rare. Each was detected in one colony in a single year only, with a prevalence of 11.1 % of nine colonies for 2010 and 5.6 % of 18 colonies in 2012, respectively (Figure 2). BQCV was detected only in 2012 with a prevalence of 67 % in 18 colonies, resulting in significant differences between years (Pearson Chi Square test, Chi Square=10.8, df=1, p=0.001).

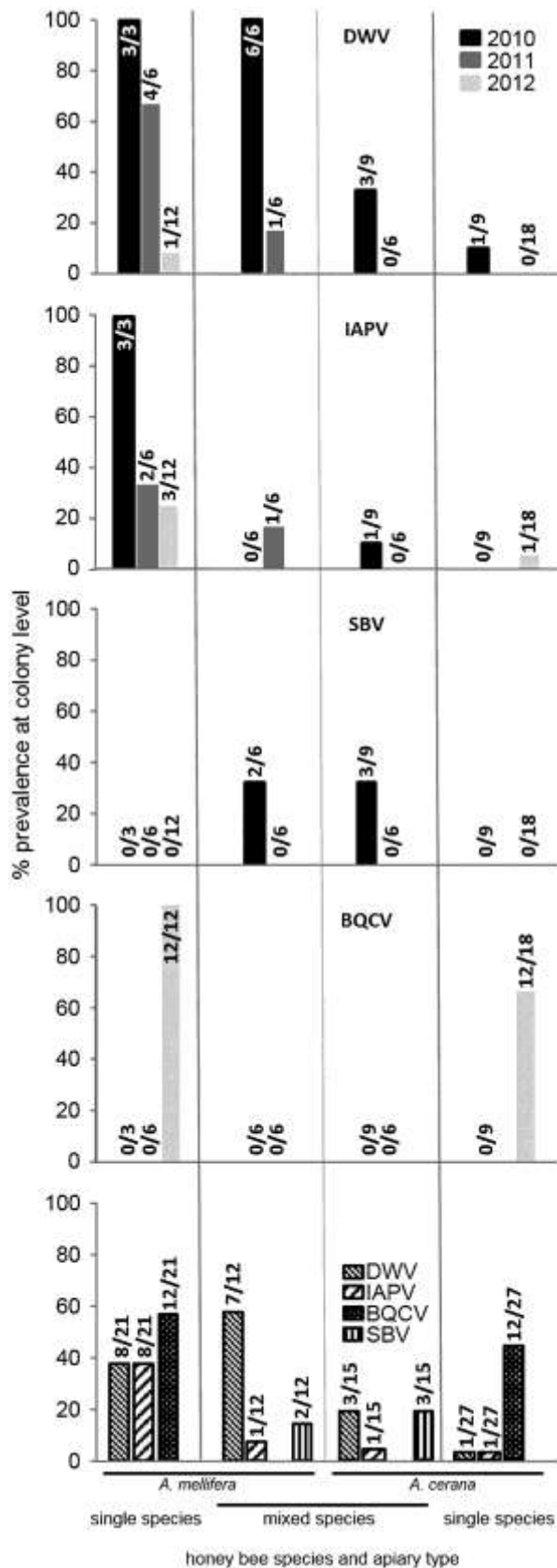


Figure 2. Prevalence of DWV, IAPV, SBV and BQCV, in single-species and mixed-species apiaries of *A. mellifera* and *A. cerana*. Data are presented for each virus and sampling year (top four graphs) and pooled over the three years of the survey (bottom graph). Figures in or above the bars represent the number of positive colonies / the total number of colonies screened.

BQCV was significantly more prevalent in *A. mellifera* (*A. mellifera* 100 % of 12 colonies, *A. cerana* 66.7 % of 18 colonies; Chi Square=5.0, df=1, p=0.03 in 2012). DWV was significantly more prevalent in *A. mellifera* in 2010, but not in 2012 (Chi Square₂₀₁₀=8.0, df=1, p₂₀₁₀=0.005; Chi Square₂₀₁₂=1.6, df=1, p₂₀₁₂=0.21). Similarly, this difference was significant for IAPV in 2010, but not in 2012 (Chi Square₂₀₁₀=14.9, df=1, p₂₀₁₀<0.001; Chi Square₂₀₁₂=1.2, df=1, p₂₀₁₂=0.27).

Mixed-species apiaries

DWV was found to occur with significant prevalence variation between years in *A. mellifera* (from 100 to 17 %; Pearson Chi Square test, Chi Square=8.6, df=1, p=0.003). In two apiaries, it was also found in *A. cerana* colonies (Figure 2) with large, but non-significant prevalence variation between years (from 33.3 to 0 %; Chi Square=2.5, df=1, p=0.11). DWV was not found in *A. cerana* without it being detected in the respective *A. mellifera* colonies of the same apiary. The prevalence in *A. mellifera* was significantly higher than in *A. cerana* in 2010, but not in 2011 (Chi Square₂₀₁₀=6.7, df=1, p₂₀₁₀=0.01; Chi Square₂₀₁₁=1.1, df=1, p₂₀₁₁=0.30). IAPV was detected in a single colony of both *A. cerana* and *A. mellifera*, but neither in the same apiary nor in the same year; this corresponded to a prevalence of 11.1 and 16.7 % for 2010 and 2011, respectively. SBV was found only in 2010 in a single apiary in two out of two colonies of *A. mellifera* and three out of three colonies of *A. cerana* sharing this apiary. Overall, prevalence of 33.3 % was not significantly different between the species in this year (Chi square=0.0; df=1, p=1.0).

Comparison between single and mixed apiaries

In *A. cerana*, DWV in 2010 was the only case where virus prevalence was high enough to allow for a statistical comparison between apiary types. On this year, prevalence at the colony level was not significantly different in mixed-species compared to single-species apiaries (Chi-square=1.3, df=1, p_{DWV}=0.26). In *A. mellifera*, all colonies of both types of apiaries were infected by DWV.

Comparison between species

DWV prevalence at the colony level was significantly higher in *A. mellifera* (n=9 colonies) compared to *A. cerana* (n=18 colonies) in 2010 (mixed and single-species apiaries included; Chi Square=14.5, df=1, p<0.001). BQCV prevalence was significantly higher in *A. mellifera*

than in *A. cerana* in 2012 at the colony level (only detected in single-species apiaries, see corresponding results above). SBV prevalence at the colony level is 33.3 % in both species in 2010. IAPV was detected in single-species apiaries of both species in 2010 and 2012 with significantly higher prevalence in *A. mellifera* in the first year, but not two years later (see above results for single-species apiaries).

Phylogenetic analyses

The DWV phylogenetic tree was inferred using 14 isolates from single- and mixed-species apiaries detected in 2010 and 2011. These Chinese DWV sequences are well separated from the clusters that represent the European, South and North American, and Japanese isolates (Figure 3). The phylogeny shows that some of the isolates cluster together according to their region of collection, such as isolates from the Zhejiang University apiary (marked by the grey triangles pointing up in Figure 3). Other isolates, especially from the Jinhua region (marked by the grey and white stars in Figure 3), are distributed in the tree irrespective of the apiary or the bee species of origin. The *A. cerana* isolates CerJH4c and CerJH1b and the *A. mellifera* isolate MelJH2a were found in different apiaries, but cluster together in the tree (Figure 3). Isolates from *A. mellifera* and *A. cerana* from the mixed-species apiaries JH1 and JH2 (grey and white stars in Figure 3, respectively) do not form clusters that represent the apiary.

The SBV phylogenetic tree was inferred from isolates detected in 2010 from *A. mellifera* and *A. cerana* colonies kept in a mixed-species apiary in the Yiwu region (YWI, Figures 1 and 4). These isolates cluster with other previous Asian isolates and are well separated from those found on other continents (Figure 4). The *A. mellifera* isolate (MelYWI) clusters with other previous SBV isolates from the same species and from China. Similarly, the *A. cerana* isolates (CerYWIa, b and c) show close relation to previous Chinese and Asian isolates from the same species (Figure 4).

The IAPV phylogenetic tree was inferred from *A. mellifera* and *A. cerana* isolates detected in 2010. These isolates are more closely related to previous *A. mellifera* isolates from China, Korea and Israel than isolates from Japan, Australia, Europe and North America. Our isolates were collected from different bee species and different regions (Jinhua and Hangzhou, approximately 140 km apart), but were more closely related to each other than to other previous isolates from China (Figure 5).

The BQCV phylogenetic tree was inferred using six isolates detected in 2012 from single-species apiaries of both bee species from the Tonglu region. BQCV was not detected

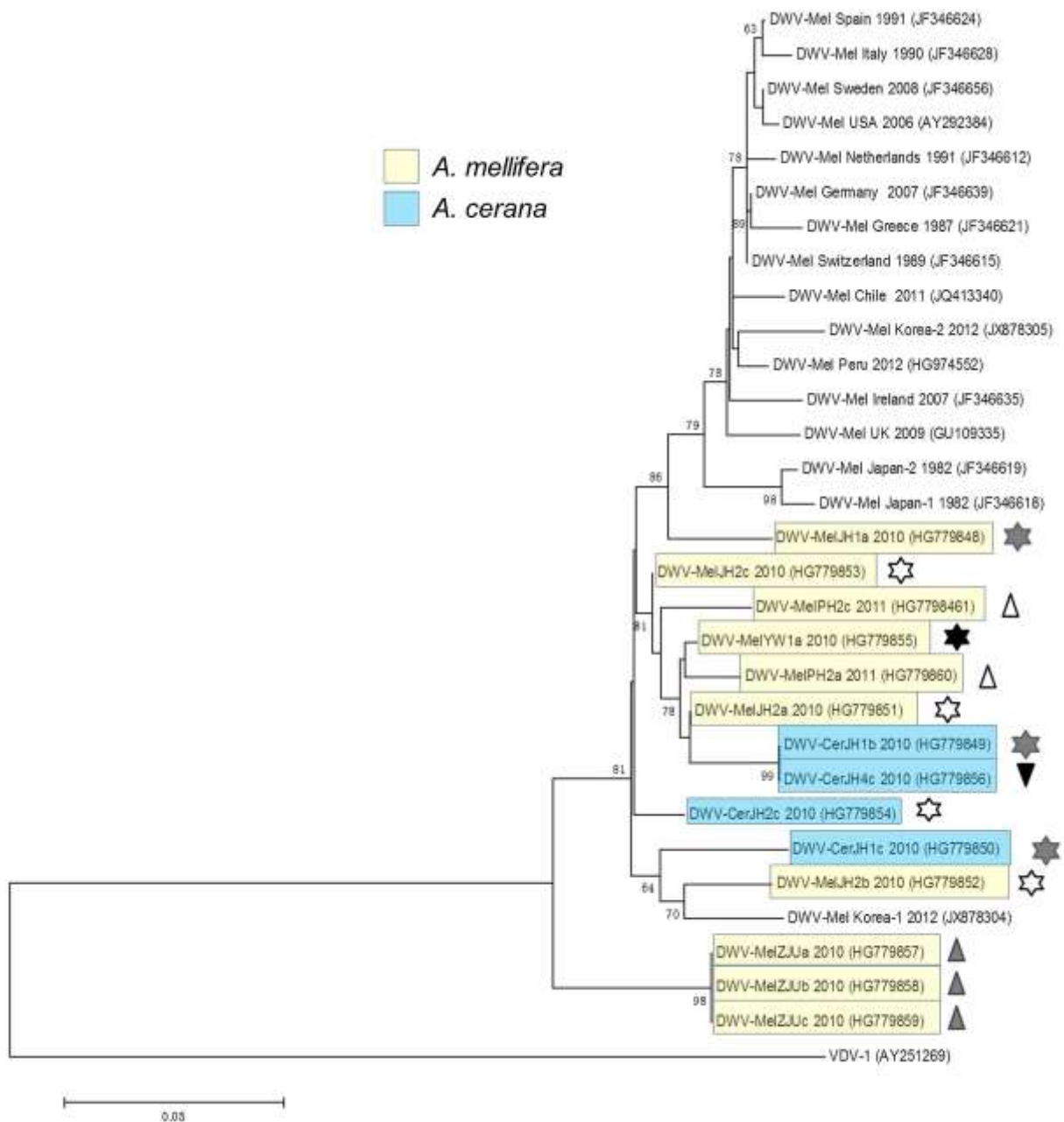


Figure 3. Phylogenetic tree of DWV isolates. Isolates from Zhejiang Province, China, are identified by their code, year of collection and accession number. Isolates originating from different continents with their GenBank accession numbers are included as references. VDV-1 is presented as outgroup sequence. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values >50 are shown in the corresponding nodes. Symbols and their shading correspond to those in Figure 1.

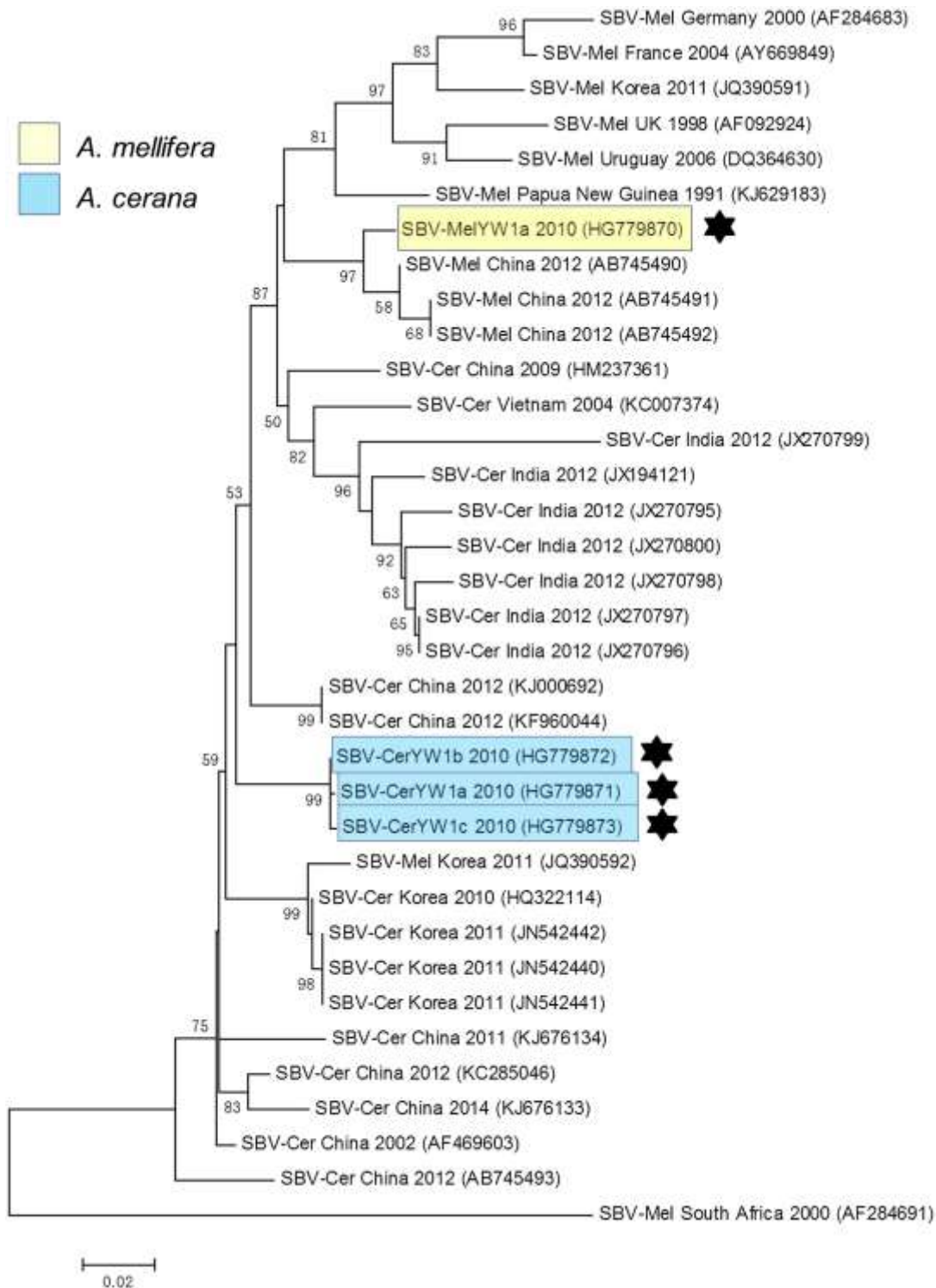


Figure 4. Phylogenetic tree of SBV isolates. Isolates from Zhejiang Province, China, are identified by their code, year of collection and accession number. SBV sequences found in an *A. mellifera* colony and three *A. cerana* colonies originating from the same mixed-species apiary (star symbol, see Figure 1) are compared with previously described SBV isolates. Their sequences were obtained from GenBank and are shown with their country of origin and accession numbers. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values >50 are shown in the corresponding nodes. Symbols and their shading correspond to those in Figure 1.

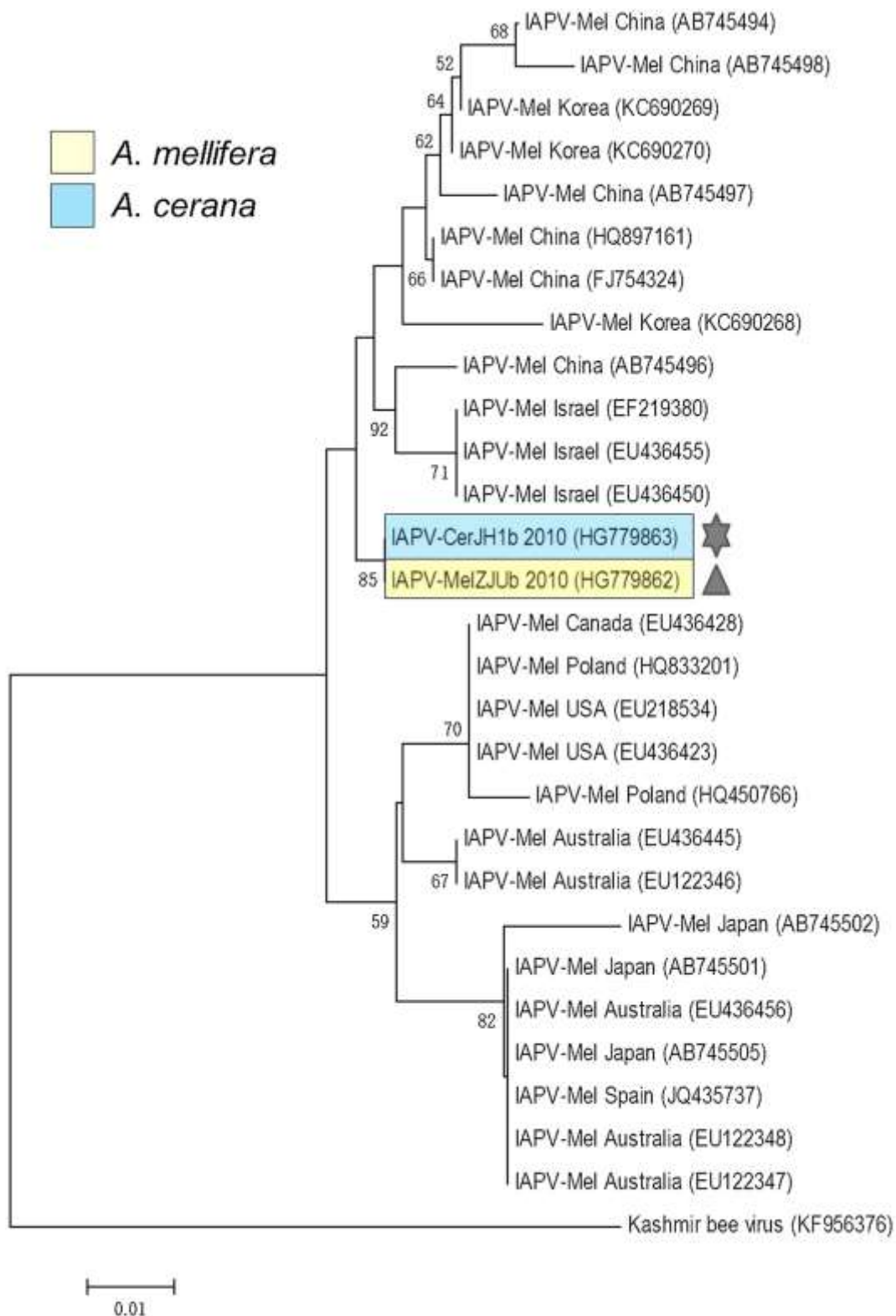


Figure 5. Phylogenetic tree of IAPV isolates. Isolates from Zhejiang Province, China, are identified by their code, year of collection and accession number. IAPV sequences from an *A. mellifera* colony and an *A. cerana* colony are compared with previously described IAPV isolates. Their sequences were obtained from GenBank and shown with their country of origin and accession numbers. Kashmir bee virus is presented as the outgroup sequence. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values >50 are shown in the corresponding nodes. Symbols and their shading correspond to those in Figure 1.

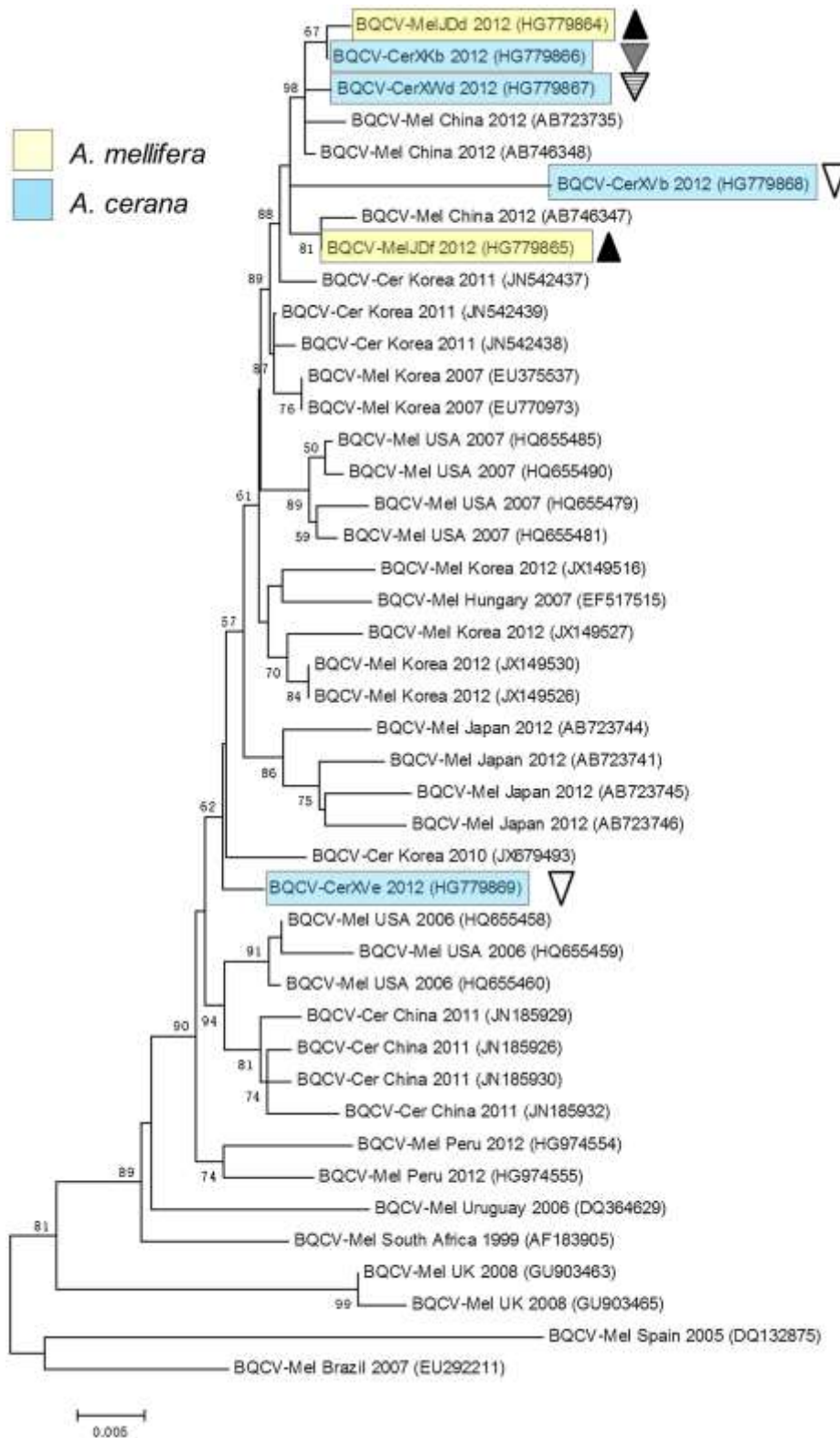


Figure 6. Phylogenetic tree of BQCV isolates. Isolates from Zhejiang Province, China are identified by their code, year of collection and accession number. BQCV sequences from an *A. mellifera* and three *A. cerana* apiaries are compared with previously described BQCV isolates. Their sequences obtained from GenBank are denoted with their country of origin and accession numbers. The bar indicates the genetic distance scale (number of nucleotide differences per site). Bootstrap values >50 are shown in the corresponding nodes. Symbols and their shading correspond to those in Figure 1.

in our mixed-species apiaries. The BQCV phylogenetic tree shows high variability of sequences in countries as USA, Korea and China, with isolates from the same country forming several clusters. The cluster for Korea includes sequences of both species. Similarly, some of our Chinese isolates are dispersed in the tree and some isolates from *A. mellifera* and *A. cerana* are clustering together (Figure 6).

Discussion

The prevalence of several viruses was measured in single and mixed-species apiaries of *A. mellifera* and *A. cerana* in order to investigate the likelihood of interspecific viral transmission between honey bee species kept separately or in close spatial proximity. The data confirm four (BQCV, DWV, IAPV and SBV) out of the six viruses, which have previously been reported for *A. cerana* (Ai *et al.*, 2012; Choe *et al.*, 2012; Kojima *et al.*, 2011; Li *et al.*, 2012). The present sampling scheme allowed for the detection of a virus when only 6-10 % of workers are infected with a probability of 0.96 (Pirk *et al.*, 2013). Therefore, our detection power was sufficient to identify even colonies with low infection rates.

In *A. mellifera*, the high DWV prevalence at the apiary level (80 %) confirms earlier studies (94 % Kojima *et al.*, 2011; 84 % Ai *et al.* 2012). While the IAPV prevalence was intermediate in our samples (40 %) compared to these studies (18 % and 66 %, respectively), BQCV was lower (20 % vs. 44 % and 77 %). The same was true for SBV (with 10 % compared to 21 % and 39 %, respectively). With a higher sampling size and survey area, Ai *et al.* (2012) and Kojima *et al.* (2011) detected ABPV and CBPV, which we did not find. In *A. cerana*, we detected DWV, IAPV, BQCV and SBV, while Li *et al.* (2012) only detected DWV and BQCV. Ai *et al.* (2012), Choe *et al.* (2012) and Kojima *et al.* (2011) found, in addition, CBPV and KBV. While IAPV prevalence in *A. cerana* was higher in our samples with 20 % compared to 7 % for both Kojima *et al.* (2011) and Ai *et al.* (2012), DWV and BQCV were intermediate compared to these studies with 30 % compared to 8-64 %, and 30 % compared to 7-75 % respectively. SBV prevalence was lower with 10 % compared to 30-86 % in the previous studies (Kojima *et al.*, 2011; Ai *et al.*, 2012). These differences suggest considerable regional and/or seasonal variation in virus infections and/or may reflect host specificity of some strains. Our current knowledge of virus infections in *A. cerana* is too limited to identify informative pattern, e.g. the possible impact of seasonality on virus

prevalence has not yet been characterized. For instance, in our data, the prevalence was different between two consecutive springs.

In general, we found that virus infections and prevalence in endemic eastern honey bees *A. cerana* were relatively low compared to imported western honey bees *A. mellifera*. This pattern confirms earlier studies (Kojima *et al.*, 2011; Ai *et al.*, 2012), with the exception of SBV, which was more prevalent in *A. cerana* (86 %) than in *A. mellifera* (21 %; Ai *et al.*, 2012). We could not find evidence for recent virus transfer in mixed-species apiaries based on prevalence trends because of the rarity of viruses in *A. cerana* colonies kept in mixed-species apiaries. In contrast, evidence for transfers was found in the phylogenetic patterns observed for DWV, IAPV and BQCV, but not for SBV.

SBV was not detected in single-species apiaries, but in both species kept in the same mixed-species apiary. Despite their close spatial proximity, the isolates found showed closer relationship with previous isolates originating from the same host species. The species-specific clustering of the sequences thus suggested that our isolates were not recently transferred between species. This is in line with previous studies in which different isolates were found in *A. cerana* and *A. mellifera* by Bailey *et al.* (1982), Verma *et al.* (1990), Kojima *et al.* (2011) and Yang *et al.* (2013). If only minor SBV quasi-species variants, which are underrepresented in the consensus sequence, are able to replicate in *A. cerana*, the separated clusters in the phylogeny could be an artifact and bias our perception of interspecies transmission. Further research is needed to clarify the dynamics of viral strain replication in different honey bee hosts.

While absent in the first two years of the survey, BQCV was detected with a high prevalence in the single-species apiaries of *A. mellifera* and *A. cerana* only in the third year, in a pattern suggestive of an epidemic (Fries and Camazine, 2001; Ribière *et al.*, 2008). The phylogenetic tree indicates the presence of BQCV strains with heterogeneous origin in the countries screened. Some of these clusters may contain sequences isolated from both *Apis* species (e.g. Korea and in our Chinese samples, despite the low number of sequences available), a pattern supporting the occurrence of interspecific transmission of this virus. This is in line with the results of Yang *et al.* (2013) based on the same capsid coding protein region of BQCV, suggesting that interspecific exchange of this virus might be comparatively more likely compared to others.

In *A. mellifera* and in *A. cerana*, IAPV was found both in single and mixed-species apiaries. The prevalence of IAPV in *A. cerana* colonies was higher in mixed-species than in

single-species apiaries, although not significantly so. This trend may be the result of virus transfer from neighboring *A. mellifera* colonies, but should be confirmed by surveying a greater number of colonies in mixed and single-species apiaries. Potential transfer mechanisms are the frequent direct contacts between the honey bee species (Sakagami, 1959; Adlakha and Sharma, 1974) and spillover of the ectoparasitic mite *V. destructor*, which can transmit IAPV to its host (Di Prisco *et al.*, 2011). Since *V. destructor* builds up much larger populations in the susceptible *A. mellifera* than in their original host *A. cerana* (Rosenkranz *et al.*, 2010), these mites could transfer IAPV between species and thus contribute to an increase in virus prevalence in neighboring *A. cerana* colonies. Despite the low number of sequences available, we found support for the occurrence of virus transfer in the absence of variability of the IAPV target-sequence corresponding to the 3' end of the virus genome between the isolates from *A. mellifera* and *A. cerana*. The low variability of IAPV could also be attributed to a methodological bias resulting from the short size of the target sequence (203bp). We consider this alternative unlikely since the sequence was variable enough to find that our isolates differed from those from distant origin (e.g. USA, Poland and Japan), but also clearly segregated isolates from within the same country as it is the case for China and Korea (Figure 5; Palacios *et al.*, 2008).

DWV was the most prevalent virus detected in the apiaries of both species surveyed. DWV was found more frequently in *A. mellifera* in single- as well as in mixed-species apiaries compared to *A. cerana*. Its spread within *A. mellifera* therefore seems more frequent than to and within *A. cerana*, which could be related to the role of *V. destructor* as a virus vector (Bowen-Walker *et al.*, 1999; Neumann *et al.*, 2012). It is also possible that this virus has lower rates of infection and replication in the eastern honey bee. This would indicate partial host specificity, which is not expected given the finding and replication of DWV in bumble bees (Genersch *et al.*, 2006). Alternatively, the lower DWV infection levels in *A. cerana* may reflect levels of resistance of the eastern honey bee to this virus or to its vector, the mite *V. destructor* (Bowen-Walker *et al.*, 1999; Rosenkranz *et al.*, 2010). These arguments could also apply to IAPV, which showed a similar prevalence pattern. In addition, treatment against mites may interfere with virus prevalence in *A. mellifera* (Francis *et al.*, 2013; Locke *et al.*, 2012; Martin *et al.*, 2010). Different beekeeping management might also play a role in the observed patterns. For example, significantly higher virus prevalence (BQCV, DWV, SBV) and higher percentage of multiple viral infections in bees of migratory apiaries were found compared to sedentary apiaries of *A. mellifera* (Welch *et al.*, 2009). Similar results were found

by Kojima *et al.* (2011) when comparing managed and feral *A. cerana* colonies. Another factor with a potential effect on viral infections is the occurrence of endosymbionts (Aebi and Neumann, 2011).

Some DWV sequences detected in *A. mellifera* colonies belonging to the same apiary formed clusters. This relatedness is expected since the apiary could be considered as a level of geographic isolation, harboring its own strain. In contrast, we found samples with similar sequences (i.e. the same strain) separated geographically. This can be explained by the movements of colonies, queens or captured swarms between apiaries. In the Jinhua region, isolates from *A. mellifera* and *A. cerana* colonies from neighboring apiaries cluster together, suggesting that exchanges between species occurred in the past. This can also be concluded from the dispersion in the tree of isolates belonging to the same mixed-species apiaries. Transmission between both bee species could have occurred by direct or indirect contact via flowers, workers or hive material, or via a vector such as *V. destructor* (Chen *et al.*, 2006; Fries and Camazine, 2001). The transfer of these mites originating in infested *A. mellifera* colonies (e.g. via interspecific robbing, Adlakha and Sharma, 1974) could directly be responsible for the transfer of the virus.

Even though different genomic regions were used, our phylogenetic patterns are comparable to those of Kojima *et al.* (2011) and Yang *et al.* (2013), who sampled over a larger geographical scale. The data available to date thus show the occurrence of strains separated geographically that cluster according to their countries or continent of origin. Also obvious is a high diversity of strains, with closely related strains shared between Asian and European honey bees, in areas where both species cohabit. However, this and our data do not provide any information of the directionality of virus transfers, similar to that of Fürst *et al.* (2014) for the transfer of viruses between honey bees and neighboring bumble bees. The increasing number of studies on prevalence of DWV occurrence and adaptation to various species (i.e., the mite *Tropilaelaps mercedesae* (Dainat *et al.*, 2009; Forsgren *et al.*, 2009), the small hive beetle *Aethina tumida* (Eyer *et al.*, 2009) or bumble bees (Fürst *et al.*, 2014)) improves our understanding of the host specificity and epidemiology of this virus.

We have identified the presence of DWV, IAPV, BQCV and SBV in the Zhejiang Province of China. The phylogenetic trees suggest that our virus isolates from *A. mellifera* and *A. cerana* varies from apparently species-specific sequences (SBV) to similarity of strains (DWV, IAPV and BQCV) suggesting virus transfer. However, given the low prevalence of these viruses, we were not able to detect evidence of recent transfers within the mixed-

species apiaries screened. This suggests that such exchanges are infrequent and that the proximity of colonies does not necessarily imply interspecies virus transfer. Our results provide an insight into the differential transmission capabilities and host specificity of the honey bee viruses in colonies located in close proximity, at a scale making the direct transfer of viruses possible. More sample sequences should be acquired from mixed-species apiaries during frequent systematic surveys to confirm the trends observed. The apparent low frequency of transfers could also be explained by a higher degree of resistance against these viruses by *A. cerana*. To prove the function of these two honey bee species as vectors and hosts for these viruses and assess their degree of resistance, controlled infection and transmission experiments as well as evidence for replication are necessary. A better understanding of the molecular mechanism of host adaptation could help determine why patterns of transfers between honey bee species differed for the four viruses detected. Characterization of the host spectrum of viruses affecting honey bees is important for our understanding of their epidemiology and of the role played by these pathogens in colony losses and pollinator declines in times of global trading.

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