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Original Article

MULTIPLE VIRUS INFECTIONS AND THE CHARACTERISTICS OF CHRONIC BEE PARALYSIS VIRUS IN DISEASED HONEY BEES (*APIS MELLIFERA* L.) IN CHINA

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Abstract

China has the largest number of managed honey bee colonies globally, but there is currently no data on viral infection in diseased *A. mellifera* L. colonies in China. In particular, there is a lack of data on chronic bee paralysis virus (CBPV) in Chinese honey bee colonies. Consequently, the present study investigated the occurrence and frequency of several widespread honey bee viruses in diseased Chinese apiaries, and we used the reverse transcription-polymerase chain reaction (RT-PCR) assay. Described was the relationship between the presence of CBPV and diseased colonies (with at least one of the following symptoms: depopulation, paralysis, dark body colorings and hairless, or a mass of dead bees on the ground surrounding the beehives). Phylogenetic analyses of CBPV were employed. The prevalence of multiple infections of honey bee viruses in diseased Chinese apiaries was 100%, and the prevalence of infections with even five and six viruses were higher than expected. The incidence of CBPV in diseased colonies was significantly higher than that in apparently healthy colonies in Chinese *A. mellifera* aparies, and CBPV isolates from China can be separated into Chinese-Japanese clade 1 and 2. The results indicate that beekeeping in China may be threatened by colony decline due to the high prevalence of multiple viruses with CBPV.

Keywords: *Apis mellifera* L, China, chronic bee paralysis virus (CBPV), diseased honey bees, multiple viruses, phylogeny.

INTRODUCTION

Honey bees (*Apis mellifera* L.) are one of the most important and economically valuable pollinators of crops worldwide and are the most commonly managed pollinator species. Colonies of *A. mellifera* serve as biological indicators and provide invaluable data on the environmental and ecological impacts of particular factors on nature in general (Celli and Maccagnani, 2003). However, the abundance of honey bees is currently declining (vanEngelsdorp et al., 2011). The possible reasons for this decline are pathogens and pesticides (Vejsnæs et al., 2010). To date, at least 18 viruses have been reported in honey bees colonies (de Miranda et al., 2013) with the following viruses being the most common: deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and chronic bee paralysis virus (CBPV). Multiple viral infections in honey bees have been reported in several countries (Berényi et al., 2006; Todd et al., 2007; Chen et al., 2011; Morimoto et al., 2012). Multiple viral infections are considered to

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be the cause of severe disease in honey bees particularly when accompanied with other stressors, such as *Varroa destructor* and *Nosema* infection, intoxication, poor nutrition, or cold weather (Allen and Ball, 1996; Chantawannakul et al., 2006). Thus, monitoring the occurrence and frequency of these viruses is vital for maintaining successful apiaries.

It has been reported that the following viruses can be present in apparently healthy honey bee colonies: DWV, BQCV, IAPV, SBV, ABPV, and KBV (Berényi et al., 2006; Ai et al., 2012; Morimoto et al., 2012; Yang et al., 2013; Jia et al., 2014). The majority of DWV infected bees were asymptomatic, while bees with high titres of the virus usually show overt infection, especially in *Varroa* infested colonies (Kojima et al., 2011; de Miranda and Genersch, 2010; de Miranda et al., 2013). Sacbrood virus, which can propagate in apparently healthy adult honey bees, primarily results in larval death (Berényi et al., 2006; Ai et al., 2012). Although BQCV clinically affects the pupae of the queen, it is commonly detected in apparently healthy adult bees, rather than in larvae or pupae (Kajobe et al., 2010). The description for ABPV, KBV, and IAPV is that of a complex of closely-related viruses in honey bees, with a predominantly sub-clinical etiology of honey bees (de Miranda and Genersch, 2010).

Infection with CBPV has been suggested as a significant factor that impacts on honey bee health (Bailey et al., 1963; Bailey and Woods, 1977; Ball, 1996; Ribière et al., 2007). A previous study in our laboratory reported that healthy honey bees injected with the purified preparation of CBPV from *A. mellifera* colonies (which were naturally affected with this viral type) showed paralytic symptoms after 4 days and death after 5 days. The mean mortality rate for the tested groups was nearly 100% at 10 days after injection (7.5% for the control group) (Feng et al., 1986). These results are partially consistent with results from other studies (Bailey et al., 1963; Bailey and Woods, 1977; Ball, 1996). Relative to the six aforementioned viruses, CBPV has been shown to have a lower prevalence in many countries (Kajobe et al., 2010; Kojima et al., 2011; Yang et al., 2013). The symptoms of CBPV infected bees include a trembling motion of the wings and bodies of adult bees, and diseased bees often crawl on the ground. Some bees are greasy, have dark body colorings, and a hairless appearance. They become flightless and die in a few days (Morimoto et al., 2012). However, the relationship between the presence of CBPV and overt infection remains controversial. Ribière et al. (2002) reported no relationship between CBPV and overt infection in France. In contrast, several studies have reported a higher incidence of CBPV in diseased honey bee colonies compared to apparently healthy colonies (Berényi et al., 2006; Cox-Foster et al., 2007; Todd et al., 2007; Morimoto et al., 2012; Yang et al., 2013). The samples infected with CBPV in these reports (fewer than 10 samples in each report), though, were too small to reach a final conclusion about the high incidence of CBPV in diseased honey bees.

China has the largest number of bee colonies (approximately 8.5 million) (Li, 2014) worldwide with the dominant honey bee species being *A. mellifera* ligustica. Epidemiological information on honey bee viruses is essential for both China and nearby countries affiliated with Chinese beekeeping activities.

With the exception of CBPV, the isolates of other common honey bee viruses (including DWV, BQCV, IAPV, SBV, ABPV, and KBV) from China, were found to belong to separate Chinese clades and showed the phylogenetic characteristic consistent with geographic separation (Yang et al., 2013; Li, 2014). There are currently only six CBPV isolates from Chinese *A. mellifera* colonies (Yang et al., 2013). These isolates could not be grouped into separate clusters. This small number of CBPV isolates is not sufficient to determine a clear relationship between the CBPV isolates in China and those in other countries. Furthermore, the CBPV tends to be isolated from diseased honey bee colonies (Allen and Ball, 1996; Berényi et al., 2006; Cox-Foster et al., 2007; Todd et al., 2007; Morimoto et al., 2012; Yang et al., 2013). It is necessary to investigate honey bee viruses in a broader number of diseased *A. mellifera* colonies in China.

Honey bee viral diseases are usually associated with *Varroa* mites and *Nosema* spp. infection. In North China, the general parasitic rate of *Varroa* mites on bees from July-August is the highest, and that from January-February is the lowest. *Nosema* spp. infection of this region from April-May is the highest, and that from January-February is the lowest. In South China, the parasitic rate of *Varroa* mites on bees in May and June is the highest, and that in December and January is the lowest. *Nosema* spp. infection of this region in March and April is the highest, and that in December and January is the lowest.

In the present study, the occurrence and frequency of seven common honey bee viruses (DWV, BQCV, IAPV, SBV, ABPV, KBV, and CBPV) were surveyed by RT-PCR. The difference in the incidence of a single virus in *A. mellifera* colonies was determined by comparing apparently healthy and diseased colonies

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in China. In addition, the phylogenetic relationships between RNA-dependent RNA polymerase (RdRp) proteins encoded by CBPV were analysed.

MATERIAL AND METHODS

Honey bee samples

Throughout China, beekeepers have reported serious colony declines in their apiaries. Beekeepers have even experienced losses of 10 colonies or more. Newly dead honey bees from one randomly selected diseased colony (with at least one of the symptoms, including depopulation, paralysis, dark body colorings and hairless bodies, and/or mass dead bees on the ground surrounding the beehives) per apiary were collected. A sample from an apparently healthy colony (collected in the apiaries in which there was no colony with significant losses) from another apiary in the same city and the same month was also randomly selected. The worker bees from the apparently healthy colonies were alive and collected from brood nests inside the hives. Each colony was sampled only once. Six diseased colonies and six apparently healthy colonies were randomly selected from each Chinese province, respectively.

A questionnaire was used to collect information about the tested colonies when the samples were selected. All samples were collected, visually examined for *Varroa destructor* mites and *Tropilaelaps* mites, and sent by post to the Key Laboratory of Pollinating Insect Biology (Beijing) by the beekeepers. Information on the level of these two kinds of mite infestations was also given by beekeepers. Each sample contained 30 to 50 honey bees. Fifteen bees from each sample were used for *Nosema* spp. detection which was based on the procedure described by Yang et al. (2013).

RNA extraction

Fifteen adult worker bees from each sample were homogenised in TRIzol® reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted following the manufacturer's standard protocol (EastepTM Universal RNA Extraction Kit, Promega, Madison, WI, USA). The RNA samples were stored at –80°C.

Table 1.

List of primers used for the detection of honey bee viruses

Target	Sequence of primer (5'-3')	Length of product (bp)	Reference
IAPV	GGT GCC TAT TTA GGG TGA GGA GGG AGT ATT GCT TTC TTG TTG TG	158	Sguazza et al., 2013
DWV	TGG TCA ATT ACA AGC TAC TTG G TAG TTG GAC CAG TAG CAC TCA T	269	Sguazza et al., 2013
SBV	CGT AAT TGC GGA GTG GAA AGA TT AGA TTC CTT CGA GGG TAC CTC ATC	342	Sguazza et al., 2013
ABPV	GGT GCC CTA TTT AGG GTG AGG A ACT ACA GAA GGC AAT GTC CAA GA	460	Sguazza et al., 2013
BQCV	CTT TAT CGA GGA GGA GTT CGA GT GCA ATA GAT AAA GTG AGC CCT CC	536	Sguazza et al., 2013
CBPV	AAC CTG CCT CAA CAC AGG AAC ACA TCT CTT CTT CGG TGT CAG CC	774	Sguazza et al., 2013
KBV	ATGACGATGATGAGTTCAAG AATTGCAAGACCTGCATC	290	Shen et al., 2005

Reverse transcription-polymerase chain reaction (RT-PCR) amplification

The occurrence and frequency of seven bee viruses were detected by RT-PCR. The virus specific primers were based on the previous reports for DWV, BQCV, IAPV, SBV, ABPV, and CBPV (Sguazza et al., 2013), and KBV (Shen et al., 2005) (Tab. 1). The reaction mixture and thermal cycling profiles of cDNA synthesis (Reverse Transcription System, Promega, Madison, WI) and the amplification, were performed as previously described (Sguazza et al., 2013). A negative and a positive DNA control were performed for each PCR reaction. The amplified bands were compared with the positive PCR products by electrophoresis on 2.0% agarose. Positive identification was confirmed by sequencing PCR products.

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Sequence of amplification fragments of each virus was aligned with the reported sequences of DWV, BQCV, IAPV, SBV, ABPV, KBV and CBPV in the GenBank database.

Phylogenetic analyses of CBPV

A phylogenetic tree of CBPV isolates from *A. mellifera* in China, was constructed. A total of 392 bp sequences (bases 958 – 1349) encoding a putative RNA-dependent RNA polymerase (RdRP) domain in the open reading frame 3 (ORF 3) of RNA segment 1 of CBPV were sequenced. The primer sets (F: TAYGAGYGATTTYTTGRGATCGAYTTCGCT and R: TGTAYTCGRCCTGATTRACGACRTTAGC) were based on previous reports (Olivier et al., 2008; Yang et al. 2013). All sequences were aligned using the MUSCLE program (Edgar, 2004), and the Kimura 2-parameter method with a gamma distribution selected as the best-fit substitution model. The total lengths of these alignments was 325 bp. The equivalent sequences of the Hepatitis C virus (AB207801) was used as an outgroup. Phylogenetic analysis was conducted using the Neighbour-Joining (NL) method and a bootstrap value of 1000 replicates in MEGA5.

Statistical analysis

The relationships between the incidence of each honey bee virus (DWV, BQCV, IAPV, SBV, ABPV, KBV, and CBPV) and the diseased colonies, were evaluated with the Chi-square test. P-values below 0.05 were considered significant. All statistical analyses were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL).

RESULTS

The occurrence of the various seven viruses in diseased honey bees

A total of 240 samples, from 20 Chinese provinces (South China: the provinces of Hunan, Hubei, Anhui, Sichuan, Guangzhou, Guizhou, Jiangsu, Jiangxi, Zhejiang, and Fujian; North China: the provinces of Henan, Hebei, Heilongjiang, Jilin, Liaoning, Gansu, Shaanxi, Shanxi, Shandong, and Beijing city), were submitted by beekeepers for analysis between January 2013 and December 2013. All samples were infected with multiple viruses. Six common honey bee viruses were detected, but ABPV was not detected. In diseased colonies, the most prevalent viruses were IAPV (99.17%), DWV (98.33%), SBV (98.33%), and CBPV (96.67%), followed by BQCV (63.33%), and KBV (13.33%). In apparently healthy colonies, the data was similar to that in the diseased colonies except for CBPV (5.83%). The most prevalent viruses were IAPV (94.17%), DWV (92.50%), SBV (92.50%), BQCV (58.33%), and KBV (10.83%).

There were 120 samples collected from diseased colonies. The following is a list of the months with the amount of collected samples noted after each month: January 9, February 10, March 10, April 12, May 10, June 11, July 10, August 10, September 10, October 9, November 10, and December 9. In China, there are four seasons (spring: March – May; summer: June – August; autumn: September – November; winter: December – February). The amount of samples in each season was 32, 31, 29, and 28 from spring to winter, respectively. No significant seasonal variance was identified in the detection frequency of the investigated viruses during the course of the year (Chi^2, DWV $p = 0.389$; BQCV $p = 0.987$; IAPV $p = 0.435$; SBV $p = 0.410$; KBV $p = 0.281$). Generally, spring, summer, and autumn are active beekeeping seasons, and winter is the non-active season in China. There were 92 infected samples in active beekeeping seasons and 28 in the non-active season. The incidence of each virus (DWV, BQCV, IAPV, SBV, KBV, or CBPV) did not significantly differ in active and non-active beekeeping seasons (Chi^2, DWV $p = 0.955$; BQCV $p = 1.000$; IAPV $p = 1.000$; SBV $p = 0.950$; KBV $p = 0.156$; CBPV $p = 1.000$).

According to the reports of the beekeepers, all of the investigated colonies (240 colonies) were infested with *Varroa destructor*. Of the apiaries surveyed, 72.5% were host to *Tropilaelaps* mites. The infestation level with the two kinds of mites was low, because these colonies were all regularly treated with acaricide and sublimed sulphur. There was no significant correlation between the level of *Varroa destructor* mite infestation and the pattern of sampling in this study. Typical symptoms of chronic bee paralysis were observed in some of the collected, newly-dead bees (see supplementary data).

Nosema ceranae was indentified in 67.5% of the samples from diseased colonies and in 62.5% from apparently healthy colonies. *Nosema apis* was not detected in all samples. There was no significant difference in the incidence of the investigated honey bee virus between *N. ceranae* infected samples and non-infected samples from diseased colonies collected from South China (Ch^2, DWV $p = 0.874$; BQCV $p = 0.943$; IAPV $p = 0.653$; SBV $p = 0.995$; KBV $p = 0.227$; CBPV $p = 1.000$) or North China (Ch^2, DWV $p = 0.971$; BQCV $p = 0.932$; IAPV $p = 0.771$; SBV $p = 0.870$; KBV $p = 0.439$; CBPV $p = 0.916$).

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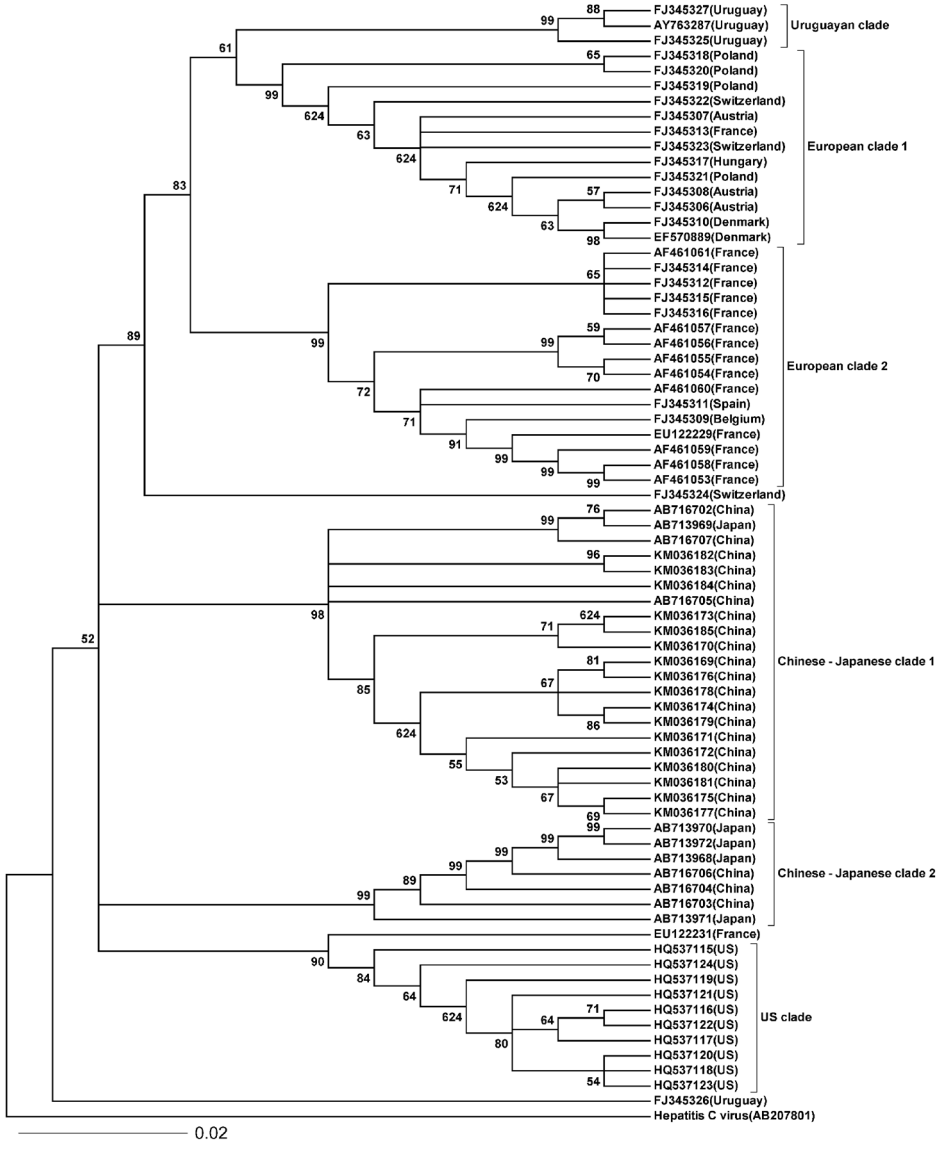

Fig. 1. Phylogenetic relationship of chronic bee paralysis virus (CBPV) isolates from China and other countries. The condensed phylogenetic tree based on alignment of the putative RdRP gene sequences of Chinese, Japanese, mainland US, European, and Uruguayan. The Neighbour-Joining (NJ) method was used. Hepatitis C virus (AB207801) was used as an outgroup to root the tree. The number of each node represents the bootstrap value resulting from 1000 replicates, and the nodes supported by bootstrap values>50 are shown. Each isolate is indicated by its accession number, followed by the collected locations.

Pattern of multiple virus infection in diseased honey bees

Among the infected samples, 51.67% showed co-infection with five viruses (DWV/BQCV/IAPV/SBV/CBPV and DWV/IAPV/SBV/KBV/CBPV), 33.33% showed co-infection with four viruses (DWV/IAPV/SBV/CBPV and DWV/BQCV/IAPV/SBV), 10.83% showed co-infection with six viruses (DWV/BQCV/IAPV/SBV/KBV/CBPV), and the remaining colonies (4.17%) showed co-infection with three viruses (IAPV/SBV/CBPV, DWV/IAPV/CBPV, BQCV/IAPV/SBV, DWV/BQCV/IAPV, and DWV/SBV/CBPV). In addition, the most prevalent co-infection patterns were DWV/BQCV/IAPV/SBV/CBPV (49.17%) for five viruses, and DWV/IAPV/SBV/CBPV (31.67%) for four viruses.

The incidence of each virus in diseased and apparently healthy colonies

The incidence of CBPV in diseased colonies (116 infected samples out of 120 samples from diseased colonies, 96.67%) was significantly higher than that in apparently healthy colonies (5.83%) (Chi^2, $p = 0.000$). The incidence of other viruses

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(DWV 98.33%, BQCV 63.33%, IAPV 99.17%, SBV 98.33%, and KBV 13.33%) did not significantly differ in the diseased and apparently healthy colonies (DWV 92.50%, BQCV 58.33%, IAPV 94.17%, SBV 92.50%, and KBV 10.83%) (Chi^2, DWV $p = 0.064$; BQCV $p = 0.508$; IAPV $p = 0.072$; SBV $p = 0.064$; KBV $p = 0.552$).

Phylogenetic relationship of CBPV isolates from different countries

To analyse the phylogeny of Chinese CBPV isolates, a region of the putative RdRP coding sequences was analysed based on the availability of this sequence in most isolates from different countries. It was found that 17 isolates had sequence variations (KM036169 – KM036185, see Fig. 1). The phylogeny of Chinese, Japanese, Uruguayan, mainland US, and European CBPV isolates was constructed using the Neighbour-Joining (NJ) method and the putative RdRP coding sequences (Fig. 1). The European isolates were grouped into two major clusters (European clade 1 and 2), except for the two isolates, EU122231 and FJ345324. The mainland US isolates and Uruguayan isolates (except of FJ345326) formed independent clades, the US clade and the Uruguayan clade, respectively, as previously reported (Blanchard et al., 2009; Chen et al., 2011; Morimoto et al., 2012; Yang et al., 2013). All of the 17 CBPV isolates included in the present study, along with three additional Chinese isolates (AB716702, AB716705 and AB716707; Yang et al., (2013)), and one Japanese isolate (AB713969; Morimoto et al., 2012) formed a mixed clade; the Chinese–Japanese clade 1. In addition, four Japanese isolates (AB713968, AB713970, AB713971, and AB713972; Morimoto et al., (2012)) and three Chinese isolates (AB716703, AB716704, and AB716706; Yang et al., (2013)) formed the Chinese-Japanese clade 2. Finally, two new clades (Chinese-Japanese clade 1 and 2) formed the Asian clade. Although there were several exceptions (EU122231 and FJ345324), the Asian, European, mainland US, and Uruguayan clades showed the trend of geographic separation.

DISCUSSION

High prevalence of multiple virus infections in diseased honey bees in China

The pattern of multiple viral infections in diseased *A. mellifera* apiaries in China is unique to this region. When compared with data from other countries, the prevalence of multiple viral infections was extremely high, specifically for co-infection with five and six viruses. These patterns have seldom been reported in other countries, and rarely in apparently healthy colonies. Interestingly, bees infected with two viruses or one virus were not found in China.

Moreover, the multiple virus infection of DWV/IAPV/SBV/CBPV was surprisingly consistent in Chinese diseased samples (all these viruses were detected in 95.83% of diseased colonies). In other reports, the largest number of viruses in a multiple infection was 5 (1% of the samples) and the smallest number was 1 (8%) based on a report from Austria (Berényi et al., 2006), 5 (3.45%), and free of viruses (82.76%) based on a report from Japan (Morimoto et al., 2012), 4 (< 16.67%) and 1 (4.60%) based on a report from New Zealand (Todd et al., 2007), and 6 (25%) and 1 (25%) based on a report from the USA (Cox-Foster et al., 2007). The current study showed that *Varroa* and associated viruses were detected in most of the samples, and the *Tropilaelaps* spp. infestation was widespread (72.5%). Low levels of *Varroa* and *Tropilaelaps* spp. infestations were reported by beekeepers, but beekeepers' experiences might lack accuracy. Considering the impact of *Tropilaelaps* spp., *Varroa*, and associated viruses in honey bees, investigating colonies for the level of these two kinds of mite infestations should be conducted by researchers in the future. In addition, there is a similar incidence of *N. ceranae* in apparently healthy (62.5%) and diseased colonies (67.5%) here. The examination of only 15 bees might underestimate the true incidence of this microsporidium. The level of *Nosema* infection was not investigated in this study. *Nosema apis* appears to be associated with the pathogenesis of BQCV outbreaks (Allen and Ball, 1996). The relationship between *N. ceranae* and BQCV, and their role in colony losses also needs further study. It is noteworthy that the SBV observed in the current study appears unusual. This virus seems to be widespread in Chinese *A. mellifera* apiaries, but this virus causes covert infections, which is consistent with reports referring to Chinese areas (Ai et al., 2012; Yang et al., 2013; Jia et al., 2014). However, sacbrood virus can also be a cause of colony depopulation, and the symptoms in the colonies might have been ignored by beekeepers in this study. In addition, the relative viral load from diseased colonies was 10-126 folds higher than in bees from apparently healthy colonies (Bailey et al., 1963; Berényi et al., 2006). Thus, viral load may determine whether the infection is covert or overt. An investigation into the symptoms of SBV infection and viral load in diseased and apparently healthy colonies is still needed.

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Previous studies on apiaries outside of China have reported occasional co-infection with four, five, and six honey bee viruses. But the most prevalent pattern of multiple viral infection in diseased and apparently healthy colonies has been reported to be co-infection with two and three viruses (Berényi et al., 2006; Todd et al., 2007; Morimoto et al., 2012). The results of the present study show that the prevalence of multiple viral infections in diseased honey bees in China, is one of the highest in the world. The viral analysis of diseased colonies in this study provides a more informative picture of the prevalence of multiple viral infections in China. The results of the present study suggest that beekeeping in China may be threatened by colony decline due to the high prevalence of multiple viruses. Solutions must be found to avoid colony decline within Chinese *A. mellifera* colonies. Our study results also suggest that beekeepers in China or other counties with a close relationship with Chinese beekeeping activities, ought to be cautious and diligent about virological analysis before importing and purchasing bee colonies and products.

The characteristics of CBPV in diseased colonies in China

Our results demonstrate that five common honey bee viruses (DWV, BQCV, IAPV, SBV, and KBV), but not CBPV, resulted in a similar incidence, no matter whether in diseased or apparently healthy colonies, and that the 5 viruses induced covert infections in apparently healthy colonies in China. The phylogenetic characterisation of the five viruses' isolates (DWV, BQCV, IAPV, SBV, and KBV) has been widely studied due to the high occurrence and global prevalence of these viruses (Todd et al., 2007; Yang et al., 2013), while data on CBPV isolates from different countries remains limited, particularly in China. Thus, the current study also focused on the characteristics of CBPV in diseased colonies in China. The incidence of CBPV in diseased colonies was significantly higher than in what were apparently healthy colonies. Previously published data, in conjunction with the findings of the present study, provide further support for this hypothesis. Our previous work (Jia et al., 2014) showed that the pattern of multiple viral infections of DWV/BQCV/IAPV/SBV was consistent (100% of the samples) in apparently healthy *A. mellifera* colonies of randomly selected apiaries from four counties in Beijing while the diseased colonies showed different co-infection (DWV/BQCV/IAPV/SBV/CBPV). Such a pattern indicates that the difference between the two populations was most likely due to the presence of CBPV. In addition, Ai et al. (2012, 6%), and Yang et al. (2013, 0%) and the present study (3.33%), found a low level of CBPV infection in apparently healthy Chinese *A. mellifera* colonies, which supports the findings in other countries (Berényi et al., 2006; Sanpa and Chantawannakul, 2009; Kajobe et al., 2010). In contrast, CBPV was found in 6 out of 6 samples (100%) of diseased *A. mellifera* worker bees in Chinese apiaries in a previous study (Yang et al., 2013), and in 116 of 120 diseased samples (96.67%) in the present study.

In previous reports, Asian (Chinese and Japanese) isolates did not form mixed clades as there were only six Chinese isolates (Yang et al., 2013). The results of the current study show that 17 CBPV isolates clustered in the same clade (the Chinese–Japanese clade 1). This finding indicates that for the first time, Chinese isolates can be separated into two clades (the Chinese-Japanese clade 1 and 2). In addition, the results of the present study show that one CBPV isolate from Japan (AB713696), which is close to the mainland US and Japanese isolates (Morimoto et al., 2012), is clustered in the Chinese–Japanese clade 1.

In summary, CBPV was rarely detected in apparently healthy *A. mellifera* colonies, yet present in nearly all diseased Chinese *A. mellifera* colonies. Toward minimising the impact of viral pathogens, diseased or dead honey bees in apiaries should be collected on a regular basis (and either buried or burned). This may help to eliminate an important viral source that could result in further disease propagation. Routine strategies for detection of honey bee viruses, especially CBPV, should be considered by beekeepers before purchasing honey bees or bee bread. In this study, 15 bees from the brood nest were examined, which was based on previous studies (de Miranda et al., 2013; Yang et al., 2013), while the foragers were reported to have a higher level of CBPV infection than young bees from the inside of hives (Bacandritsos et al., 2010). Therefore, the prevalence of CBPV in apparently healthy colonies might actually be higher. In addition, visible symptoms of CBPV infection have also been reported by beekeepers because it was difficult and costly for researchers to reach each involved apiary in China. The early stage of CBPV infection, which was less obvious, was excluded from this study. Further researches are required to investigate the symptoms of CBPV infection by researchers and to detect the viral load in more forager bees from diseased and apparently healthy colonies in China.

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CONCLUSIONS

The prevalence of multiple virus infections is high in *A. mellifera* colonies in China, especially a prevalence of five and six viruses at a time in diseased colonies. Except CBPV, the incidence of DWV, BQCV, IAPV, SBV, ABPV, or KBV showed no significant difference between diseased (with at least one of the symptoms, including depopulation, paralysis, dark body colorings and hairless, or mass dead bees on the ground surrounding the beehives) and apparently healthy colonies; and CBPV, the isolates from China can be separated into Chinese-Japanese clade 1 and 2.

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Supplementary data:
The presence of IAPV, DWV, SBV, BQCV, CBPV, KBV and ABPV in diseased *A. mellifera* colonies by RT-PCR

Region		IAPV	DWV	SBV	BQCV	CBPV	KBV	ABPV	Symptom of chronic bee paralysis
	Spring								
Hebei	Mar.	1	1	1	0	1	0	0	1
Henan	Mar.	1	1	1	1	1	0	0	1
Shaanxi	Mar.	1	1	1	0	1	0	0	0
Shandong	Mar.	1	1	1	1	1	0	0	0
Anhui	Mar.	1	1	1	1	1	0	0	1
Guizhou	Mar.	1	1	1	0	1	0	0	0
Hunan	Mar.	1	1	1	1	1	0	0	0
Jiangxi	Mar.	1	1	1	1	1	0	0	0
Zhejiang	Mar.	1	1	1	0	1	0	0	0
Beijing	Apr.	1	1	1	1	1	0	0	1
Gansu	Apr.	1	1	1	1	0	0	0	0
Hebei	Apr.	1	1	1	1	1	1	0	1
Jilin	Apr.	1	1	1	0	1	0	0	1
Liaoning	Apr.	1	1	1	1	1	0	0	1
Shandong	Apr.	1	1	1	0	1	0	0	1
Shanxi	Apr.	1	1	1	1	1	0	0	1
Anhui	Apr.	1	1	1	0	1	0	0	0
Hubei	Apr.	1	1	1	1	1	0	0	1
Hunan	Apr.	1	1	1	0	1	0	0	0
Jiangsu	Apr.	1	1	1	1	1	1	0	0
Jiangxi	Apr.	1	1	1	0	1	0	0	0
Gansu	May	1	1	1	1	1	1	0	0
Jilin	May	1	1	1	0	1	1	0	1
Liaoning	May	1	1	1	1	1	0	0	0
Shaanxi	May	1	1	1	1	1	0	0	0
Anhui	May	1	1	1	0	1	0	0	0
Fujian	May	1	1	1	1	1	0	0	1
Hunan	May	1	1	1	0	1	0	0	0
Sichuan	May	1	1	1	1	1	0	0	1
Zhejiang	May	1	1	1	1	1	0	0	1
Heilongjiang	May	1	1	1	1	1	0	0	1
	No. of positive	31	31	31	19	30	4	0	0
	Summer								
Gansu	Jun.	1	1	1	1	1	1	0	1
Heilongjiang	Jun.	1	1	1	0	1	0	0	0
Henan	Jun.	0	1	1	0	1	0	0	0
Jilin	Jun.	1	1	1	1	1	0	0	0
Liaoning	Jun.	1	1	1	1	1	0	0	0

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Shanxi	Jun.	1	1	1	0	1	0	0	1
Guangdong	Jun.	1	1	1	0	1	0	0	1
Guizhou	Jun.	1	1	1	1	1	0	0	1
Hubei	Jun.	1	1	1	0	1	0	0	0
Sichuan	Jun.	1	1	1	1	1	1	0	0
Zhejiang	Jun.	1	1	1	0	1	0	0	0
Beijing	Jul.	1	1	1	1	1	0	0	1
Gansu	Jul.	1	1	0	0	1	0	0	0
Hebei	Jul.	1	1	1	1	1	0	0	1
Heilongjiang	Jul.	1	1	1	0	1	0	0	0
Liaoning	Jul.	1	1	1	1	1	0	0	1
Anhui	Jul.	1	1	1	1	1	0	0	1
Fujian	Jul.	1	1	1	1	0	0	0	0
Hubei	Jul.	1	1	1	1	1	0	0	0
Hunan	Jul.	1	1	1	1	1	1	0	1
Jiangxi	Jul.	1	1	1	1	1	1	0	0
Gansu	Aug.	1	1	1	1	1	1	0	1
Heilongjiang	Aug.	1	1	1	1	1	0	0	1
Jilin	Aug.	1	1	1	1	1	0	0	0
Liaoning	Aug.	1	1	1	0	1	0	0	0
Shanxi	Aug.	1	1	1	1	1	0	0	1
Anhui	Aug.	1	1	1	0	1	0	0	0
Hubei	Aug.	1	1	1	1	1	0	0	0
Jiangsu	Aug.	1	1	1	1	1	0	0	0
Jiangxi	Aug.	1	1	1	0	1	0	0	0
Zhejiang	Aug.	1	1	1	0	1	0	0	1
	No. of positive	30	31	30	19	30	5	0	0
	Autumn								
Beijing	Sep.	1	1	1	0	1	0	0	0
Heilongjiang	Sep.	1	1	1	1	1	0	0	1
Henan	Sep.	1	1	1	0	1	0	0	1
Jilin	Sep.	1	1	1	1	1	0	0	0
Liaoning	Sep.	1	1	1	1	1	0	0	1
Guizhou	Sep.	1	1	1	1	1	0	0	1
Hunan	Sep.	1	1	1	0	1	0	0	0
Jiangsu	Sep.	1	1	1	1	1	0	0	0
Jiangxi	Sep.	1	1	1	1	1	0	0	0
Sichuan	Sep.	1	1	1	0	1	0	0	1
Hebei	Oct.	1	1	1	1	1	0	0	1
Shaanxi	Oct.	1	1	1	1	1	0	0	0
Shandong	Oct.	1	1	1	1	1	0	0	0
Shanxi	Oct.	1	1	1	1	1	1	0	1
Anhui	Oct.	1	0	1	1	0	0	0	0
Fujian	Oct.	1	1	1	1	1	0	0	0
Guangdong	Oct.	1	1	1	0	1	0	0	1
Jiangsu	Oct.	1	1	1	1	1	1	0	0

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Zhejiang	Oct.	1	1	1	1	1	0	0	1
Beijing	Nov.	1	1	1	0	1	1	0	0
Gansu	Nov.	1	1	1	1	1	0	0	1
Heilongjiang	Nov.	1	1	1	1	1	0	0	1
Jilin	Nov.	1	1	1	1	1	0	0	1
Shanxi	Nov.	1	1	1	1	1	1	0	1
Guangdong	Nov.	1	1	1	0	1	0	0	0
Guizhou	Nov.	1	1	1	1	1	0	0	0
Hubei	Nov.	1	1	1	0	1	1	0	1
Hunan	Nov.	1	1	1	0	1	0	0	0
Jiangxi	Nov.	1	1	1	0	1	0	0	0
	No. of positive	29	28	29	19	28	5	0	1
	Winter								1
Hebei	Dec.	1	1	1	1	1	0	0	0
Henan	Dec.	1	1	1	0	1	0	0	0
Shaanxi	Dec.	1	1	1	1	1	0	0	1
Shandong	Dec.	1	1	1	0	1	0	0	0
Fujian	Dec.	1	1	1	1	1	0	0	0
Guangdong	Dec.	1	1	1	0	1	0	0	1
Guizhou	Dec.	1	1	1	1	1	0	0	0
Jiangsu	Dec.	1	1	1	1	1	0	0	1
Sichuan	Dec.	1	1	1	1	1	0	0	1
Beijing	Jan.	1	1	1	1	1	0	0	0
Henan	Jan.	1	1	1	0	1	0	0	1
Shaanxi	Jan.	1	1	1	1	1	0	0	0
Shandong	Jan.	1	1	1	0	1	0	0	1
Fujian	Jan.	1	1	1	1	1	1	0	1
Guangdong	Jan.	1	1	1	1	1	0	0	1
Guizhou	Jan.	1	1	1	1	1	0	0	0
Sichuan	Jan.	1	1	1	1	1	0	0	0
Zhejiang	Jan.	1	1	1	0	1	0	0	1
Hebei	Feb.	1	1	1	1	1	0	0	0
Henan	Feb.	1	1	1	0	1	0	0	0
Shaanxi	Feb.	1	1	1	1	1	0	0	0
Shandong	Feb.	1	0	1	0	1	0	0	0
Shanxi	Feb.	1	1	0	1	0	0	0	0
Fujian	Feb.	1	1	1	1	1	0	0	0
Guangdong	Feb.	1	1	1	0	1	0	0	0
Hubei	Feb.	1	1	1	1	1	0	0	1
Jiangsu	Feb.	1	1	1	0	1	0	0	0
Sichuan	Feb.	1	1	1	1	1	0	0	0
	No. of positive	28	27	27	18	27	1	0	

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