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Prevalence of Four Common Bee RNA Viruses in Eastern Bee Populations in Yunnan Province, China

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Abstract

Deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV) are the most common RNA viruses in bee population worldwide. To determine the prevalence of these viruses in *Apis cerana* in Yunnan Province, China, 270 adult Eastern worker bee (*A. cerana*) samples have been collected from nine major bee breeding regions in Yunnan province for screening. Our data showed that BQCV, DWV, KBV and SBV are widely prevalent in Yunnan Province. The total prevalence rates of KBV, DWV, SBV and BQCV in Yunnan province were 3%, 14.8%, 24.1% and 36.3%, respectively. BQCV was detected in all selected regions except Jinghong City. The prevalence of KBV in Tengchong County is the first evidence, to our knowledge, showing that Eastern bees could be infected by KBV. The highest infection rate of SBV was found in Kunming city (56.7%). Sequence analysis demonstrated high nucleotide homology between the isolated BQCV, DWV, KBV and SBV strains. Phylogenetic analysis showed that except SBV (YN07), Yunnan isolates can be likely clustered into independent branches, which indicates the possibility of geographic origins.

Keywords: Black queen cell virus; Deformed wing virus; Kashmir bee virus; Sacbrood virus; Apis cerana; Prevalence; Yunnan

Introduction

The Eastern bee (*Apis cerana*) has unique biological and behavioral characteristics, playing unique and important roles during the pollination of plants [1]. Located in the far southwest in China, bordering Myanmar, Laos, and Vietnam, Yunnan is the most biologically and culturally diverse province. This province contains snow-capped mountains and true tropical environment, thus supporting an unusually full spectrum of species and vegetation types. Among the multitudinous plant species, 148 are honey plants, and 75 of them are used for producing commercial honey [2]. As a result, bee products from Yunnan province rank as the third largest production in China.

Bee colonies have been continually declining worldwide over the past 50 years, the reasons for which have often been linked to the spread of the parasitic mite *Varroa* destructor and its interaction with certain bee viruses [3]. Both the European bees and the Asian bees are susceptible to a variety of viruses and some other pathogens. At least 18 viruses have been globally identified in different stages and castes of bees including eggs, larvae, pupae, adult workers, drones, and queens [4]. Six of these, i.e., deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV), are the most common ribonucleic acid (RNA) viruses [5,6]. Like most insect-infecting RNA viruses, bee viruses usually persist in unapparent, asymptomatic infections, capable of replicating rapidly under certain conditions, which results in observable symptoms often leading to colony losses. Infection or co-infection with these viruses has been reported in the United States, Britain, France, Italy, Denmark, Japan and Thailand [5].

In the beginning of the 20th century, due to the lack of strict quarantine regulations, foulbrood in bees from Europe was transmitted to China which accompanied the introduction of the Western bee (*Apis mellifera*). Eastern bees were consequently infected and spread across China, causing huge losses to the bee keeping industry. In recent years, more and more Western bees have been introduced. The rising contact frequency between Western and Eastern bees has deteriorated the risk of the transmission of bee diseases. However, the prevalence state of the six most common RNA viruses in Eastern bees from Yunnan province is still unknown. In this study, we aim to investigate the prevalence of these six viruses in Eastern bees from nine major bee breeding regions of Yunnan province in China.

Material and Methods

Sample collection

Available beekeepers within the nine main beekeeping regions (Kunming City, Luoping County of Qujing prefecture, Mengzi City and Hekou County of Honghe autonomy prefecture, Jinghong City of Xishuangbanna autonomy prefecture, Wuding County and Dayao

County of Chuxiong autonomy prefecture, Tengchong County of Baoshan prefecture, and Deqing County of Diqing autonomy prefecture) in Yunnan province were selected for sampling, according to the previously described method [7]. From each region, thirty single adult worker bees without displaying any observable pathological symptoms were randomly collected from a single colony in the hive in March 2010. All the two hundred and seventy bee samples were preserved in individual microcentrifuge tubes (one bee in one tube) in liquid nitrogen, brought back to the laboratory and stored in −80°C for further analysis.

Primers

Based on sequence alignment, primers for DWV, SBV, CBPV, and ABPV were designed using Primier 5.0 (Premier Biosoft International, CA, USA). Primers for BQCV and KBV were selected from the literature [8]. All primers were synthesized by Sangon Biotech Co, Ltd (Shanghai, China) and listed in Table 1.

Primer	Primer Sequence (5'-3')	Position	Reference sequence	Size of the amplicon (bp)	Corresponding gene
DWV-F	TCCATCAGGTTCTCCRATAAC	9256-9276	AJ489744	327	polyprotein
DWV-R	GCCACAGGTCTAGTTGGATG	9563-9582			
SBV-F	TCGGATCCACCAAGTTGGAGG	291-311	AF092924	349	polyprotein
SBV-R	ACCTCATCACTCTGGGTCCTT	619-639			
BQCV-F	TGGTCAGCTCCCACTACCTTAAAC	7850-7873	AF183905	700	Structural polyprotein
BQCV-R	GCAACAAGAAGAAACGTAAACCAC	8527-8550			
KBV-F	GATGAACGTCGACCTATTGA	5406-5424	AY275710	415	RdRp
KBV-R	TGTGGGTTGGCTATGAGTCA	5801-5819			
ABPV-F	TTATGTGTCCAGAGACTGTATCCA	8460-8483	AF150629	901	polyprotein
ABPV-R	GCTCCTATTGCTCGGTTTTTCGGT	9337-9360			
CBPV-F	CTTGGATCCGATTTCGCTC	55-73	AF375659	384	RdRp
CBPV-R	GAGGTTGTACTCGACCTGAT	419-438			

Table 1: Primers used in this study. F: forward, R: reverse.

RNA extraction

Individual adult worker bee sample was minced in liquid nitrogen and homogenized in Trizol (Invitrogen, California, USA). The total RNA for each sample was extracted following the manufacturer's protocol and dissolved in diethyl pyrocarbonate-treated water in the presence of an RNase inhibitor (Invitrogen). The concentration of total RNA was determined by measuring the absorption at 260 nm, and the purity of RNA was estimated by the absorbance ratio of 260 nm/280 nm using a spectrophotometer. The RNA samples were stored at −80°C prior to molecular detection of viruses.

RT-PCR detection

Using a one-step PCR kit (Takara Biotechnology Co Ltd, Dalian, China), PCR amplification conditions were optimized and finalized. The 25 μl reaction contains template 1 μl, $MgCl_2$ (25 mM) 5 μl, 10 × One Step RNA PCR Buffer 2.5 μl, dNTP mixture (each 10 mM) 2.5 μl, RNase Inhibitor (40 U/μl) 0.5 μl, AMV RTase XL (5 U/μl) 0.5 μl, AMV-Optimized Taq (5 U/μl) 0.5ul, RNase free dH2O 10.5 μl, forward primer and reverse primer 1 μl for each. The conditions are as follow: reverse transcription at 50°C for 30 minutes, denaturation at 94°C for 2 minutes, then 35 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds to corresponding optimal temperature (55°C for SBV, 54°C for BQCV, 53°C for DWV, KBV, and ABPV, and 56 °C for CPBV) and 1 minute elongation at 72°C.

Sequencing and phylogenetic analysis

The PCR amplification products were excised from the agarose gel and extracted using a PCR product purification kit (BioTeke Corporation, Beijing, China) and ligated into pMD18-T vectors (Takara Biotechnology Co Ltd). The plasmids were then used to transform *Escherichia Coli* DH5α cells (Takara Biotechnology Co Ltd). The plasmids were extracted and the inserts were sequenced by Sangon Biotech Co, Ltd. The nucleotide sequence similarities between isolated sequences for each virus were calculated using Lasergene sequence analysis tools MegAlign (DNASTAR Inc, Madison, USA). Phylogenetic and molecular evolutionary genetics analyses were conducted using the neighbor-joining method with MEGA 4 (Temple, AZ, USA) [9]. Numbers at nodes correspond to bootstrap percentages with values of greater than 50 percent being regarded as phylogenetic grouping. Forty-four related reference strains were used as references in the analysis (Table 2).

Results

RT-PCR detection

The prevalence of these six common bee RNA viruses in Eastern bee populations in Yunnan Province was summarized in Figure 1. For BQCV, we identified 20 positive samples in Kunming City (66.7%), 24

in Luoping County (80.0%), 16 in Mengzi City (53.3%), 15 in Wuding County (50.0%), 12 in Dayao County (40.0%), 2 in Tengchong County (6.7%), 8 in Deqin County (26.7%) and 1 in Hekou County (3.3%), with the total prevalence rate 36.3% (98/270). For DWV, 17, 12, 3, 2, and 2 positive bee samples were found in Kunming city (76.7%), Luoping County (40.0%), Wuding County (10.0%), Deqin County (6.7%) and Dayao County (6.7%), respectively. The overall prevalence rate of DWV was 14.8% (40/270). For KBV, 8 of 30 samples (26.7%) collected in Tengchong County were positive, with the other eight regions negative, and the overall total rate in Yunnan province was 3.0% (8/270). For SBV, 17 positive samples in Kunming City (56.7%), 9 in Luoping County (30.0%), 7 in Mengzi City (23.3%), 12 in Jinghong city (40.0%), 9 in Wuding County (30.0%) and 11 in Dayao County (36.7%) were detected, with a total prevalence rate of 24.1% (65/270). Primers for ABPV and CBPV did not reveal any positive samples in this study.

Viruses	Strain name	Accession No.	Isolation source	Country	Isolation/ submitted date
DWV	unknown	AJ489744	Apis mellifera	Italy	June 2002
	unknown	AY224602	Apis mellifera	France	Jan 2003
	PA	AY292384	Apis mellifera	USA	May 2003
	AUSTRIA-1	DQ224278	Apis mellifera	Austria	Jan 2007
	Warwick-2009	GU109335	Apis mellifera	UK	Oct 2009
	VDV-1-DWV-No-5	HM067437	Apis mellifera	UK	May 2009
	VDV-1-DWV-No-9	HM067438	Apis mellifera	UK	May 2009
	Warwick	HM162356	Apis mellifera	UK	May 2009
	Zriffin	**JF440526**	Zriffin Apiary	Israel	Oct 2008
	Chilensis A1	JQ413340	Apis mellifera	Chile	Oct 2011
SBV	Austria	AF284617	Apis mellifera	Austria	July 2000
	Germany 1	AF284618	Apis mellifera	Austria	July 2000
	Rothamstead	AF092924	Apis mellifera	UK	Sep 1998
	Riez2002	AY152711	Apis mellifera	France	Sep 2002
	Sophia1 R80	AY152712	Apis mellifera	France	Sep 2002
	AFSSA P12 2002	AY230517	Apis mellifera	France	Feb 2003
	CSBV-LN	HM237361	Apis cerana	China	Jan 2009
	Korea	HQ322114	Apis cerana	South Korea	Aug 2010
	AmSBV-Kor21	JQ390591	Apis mellifera	South Korea	2011
	CQ1	JQ796779	Apis cerana	China	March 2012
	Pennsylvania	AY626247	Apis mellifera	USA	May 2004
	T73/05A	EF570887	Apis mellifera	Denmark	Apr 2007
	South African	AF183905	Apis mellifera	South African	Sep 1999
	Pennsylvania	AY626246	Apis mellifera	USA	May 2004
BQCV	1	DQ364629	Apis mellifera	Uruguay	Jan 2006
	POLAND-4	EF517519	Apis mellifera	Poland	Mar 2007
	D6	EU292211	Apis mellifera	Brazil	Nov 2007
	CJ3	EU375537	unknown	South Korea	Nov 2007
	B1	FJ603458	unknown	South Korea	Dec 2008
	R4LY 51	GU903461	Apis mellifera	UK	Feb 2010

	20W1-PA-USA-2006	HQ655459	Apis mellifera	USA	Nov 2006
	Kor1-BQCV2	JN542438	Apis cerana	South Korea	Aug 2011
	YY1	JX149518	Apis mellifera	South Korea	Jun 2012
KBV	Canadian	AF034542	Apis mellifera	USA	Nov 1999
	unknown	AF035359	Apis mellifera	USA	Nov 2000
	unknown	AF052567	Varroa jacobsoni	USA	Nov 2001
	941227B-1	AF085478	Varroa jacobsoni	USA	Nov 1999
	mfb6	AF135861	Apis mellifera	USA	Mar 1999
	990806B-13	AF197906	unknown	Russia	Nov 1999
	BALL-BRL9apv	AF233366	Apis mellifera	USA	Feb 2000
	unknown	AY787143	Apis mellifera	Germany	Oct 2004
	Varroa	AY821562	unknown	Spain	Nov 2004
	JAK3	FJ225118	Apis cerana	Jordan	2007
	KBV_3_TW	GU108224	Apis cerana	China (Taiwan)	2008

Table 2: Reference sequences used in the phylogenetic analysis.

Sequence and phylogenetic analysis

After alignment, the viruses sharing the same sequences were considered as the same strains. In total, 12 BQCV, 8 DWV, 8 KBV, and 8 SBV strains were identified from the two hundred and seventy bee samples, with the sequences confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank, with the accession numbers and geographic origins listed in Table 3.

Viruses	Nomination	Accession No.	Origin
KBV	kbv-YN01	JX232598	Tengchong County
	kbv-YN02	JX232599	Tengchong County
	kbv-YN03	JX232600	Tengchong County
	kbv-YN04	JX232601	Tengchong County
	kbv-YN05	JX232602	Tengchong County
	kbv-YN06	JX232603	Tengchong County
	kbv-YN07	JX232604	Tengchong County
	kbv-YN08	JX232605	Tengchong County
DWV	dwv-YN01	JX679473	Kunming City
	dwv-YN02	JX679474	Kunming City
	dwv-YN03	JX679475	Kunming City
	dwv-YN04	JX679476	Luoping County
	dwv-YN05	JX679477	Luoping County
	dwv-YN06	JX679478	Wuding County
	dwv-YN07	JX679479	Dayao County
	dwv-YN08	JX679480	Deqing County
SBV	sbv-YN01	JX679481	Kunming City
	sbv-YN02	JX679482	Kunming City
	sbv-YN03	JX679483	Dayao County
	sbv-YN04	JX679484	Mengzi City
	sbv-YN05	JX679485	Jinghong City
	sbv-YN06	JX679486	Luoping County
	sbv-YN07	JX679487	Luoping County
	sbv-YN08	JX679488	Wuding County
BQCV	bqcv-YN01	JN185928	Luoping County
	bqcv-YN02	JN185929	Luoping County
	bqcv-YN03	JN185930	Luoping County
	bqcv-YN04	JN185931	Kunming City
	bqcv-YN05	JN185926	Wuding County
	KM1	JN185927	Kunming City
	bqcv-YN06	JN185932	Dayao County
	bqcv-YN07	JN379018	Deqin County
	bqcv-YN08	JX679489	Kunming City
	bqcv-YN09	JX679490	Kunming City
	bqcv-YN10	JX679491	Mengzi City
	bqcv-YN11	JX679492	Tengchong County

	bqcv-YN12	JX679493	Hekou County

Table 3: KBV, DWV, SBV and BQCV strains detected in Eastern bees from Yunnan Province.

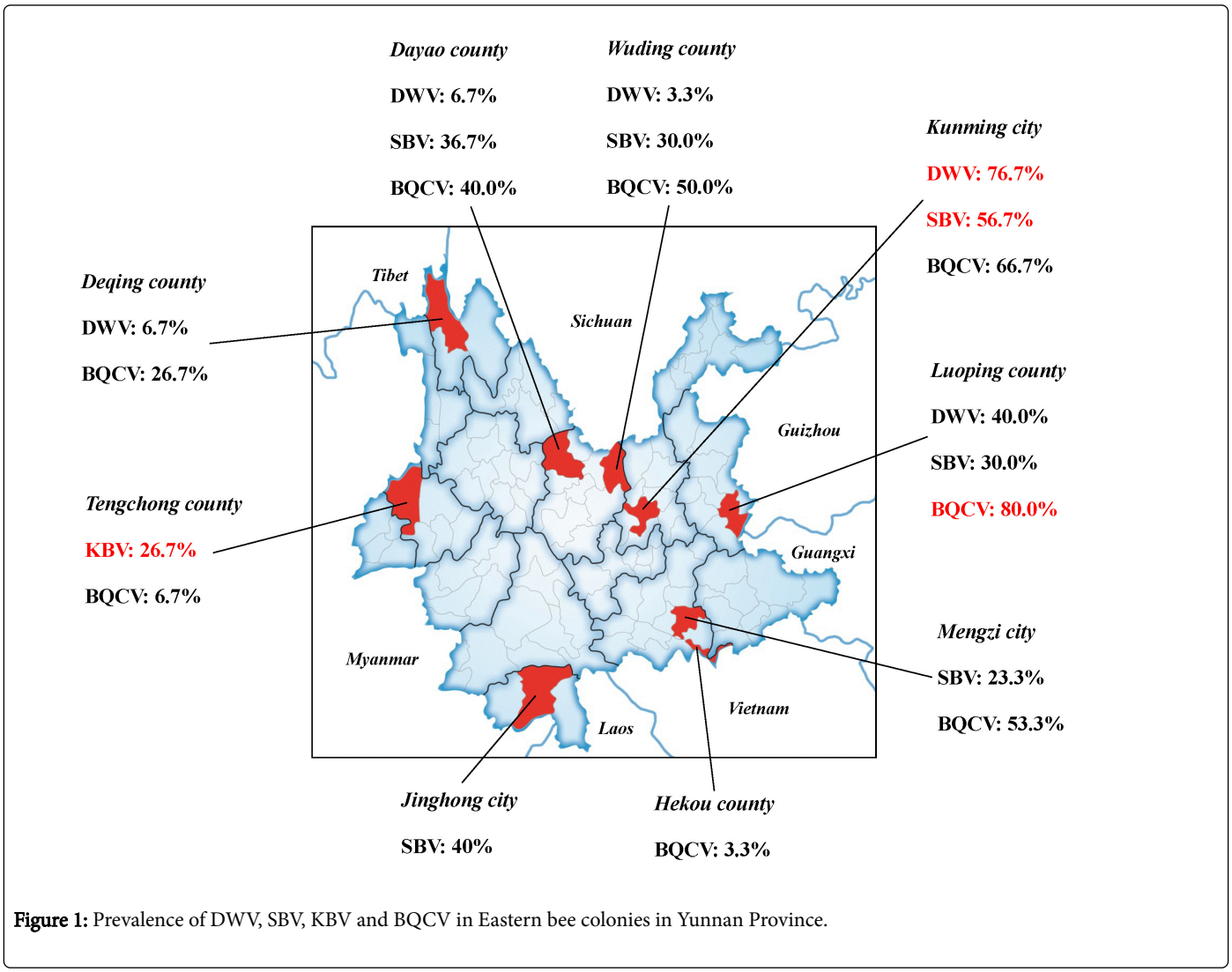

Figure 1: Prevalence of DWV, SBV, KBV and BQCV in Eastern bee colonies in Yunnan Province.

Phylogenetic trees were constructed and bootstrap analysis was performed (Figure 2). Overview, all the virus isolates of BQCV, DWV, KBV and SBV from Yunnan province were grouped into independent cluster, respectively, except SBV YN07 fell into the reference sequence group. The BQCV samples in this study came from *A. cerana* in Yunnan province while the BQCV reference strains came from *A. mellifera* in Korea, USA, South Africa and Brazil. The nucleotide acid sequences of the isolated BQCV were 98.1%–100% identical between the 12 Yunnan isolates, and 94.3–99.6% to the 11 selected reference strains. Eight Chinese BQCV sequences in this study were aligned and compared to the sequences of the reference strains. The phylogenetic tree revealed that the eight BQCV isolated strains separated into three sub-groups: JH1 strain (JN379018) isolated in Xishuangbanna was more closely related to two references strains (EU375535, EU639830) that are known to be originally present in *A. mellifera* but were far from other isolates, while the Kunming isolated strains were located on an independent branch. Our results revealed that the virus has widely spread in *A. cerana* with prevalence rates as high as 80%. The nucleotide acid sequences of the amplified products of the DWV isolated strains were 95.4%–99.7% identical between the eight Yunnan isolates, and 92.7%–96.6% to the 10 reference sequences selected from GenBank. Further sequence analysis revealed that the eight isolated KBV strains shared 99.0%–100% nucleotide homology between the Yunnan isolates and 76.1%–95.4% nucleotide homologies with the 11 selected reported strains. The nucleotide acid sequences of the amplified fragments of the eight isolated SBV strains share 94.3%–100% nucleotide homology between the isolates and 94.0%–98.7% with the 12 selected references strains.

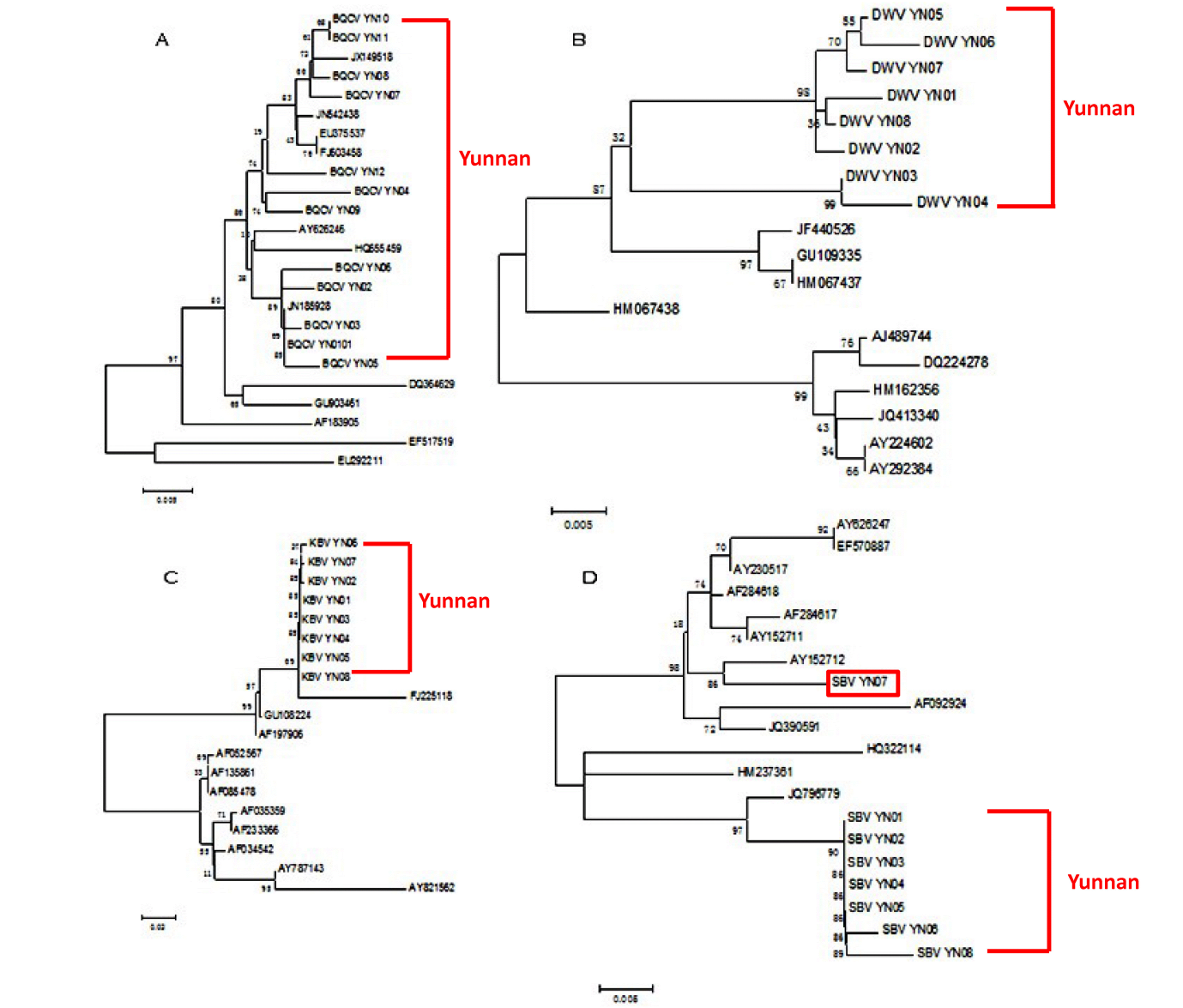

Figure 2: Phylogenetic tree constructed by aligning the BQCV, DWV, KBV and SBV detected in Yunnan Province.

Determination of co-infection

The present study found multiple virus infections in 20.0% (54/270) of the bee samples. Among the samples with multiple infections, the dual-infection (SBV/DWV, BQCV/SBV, BQCV/DWV or BQCV/KBV) rate was 83.3% (45/54) and the triple-infection (BQCV/SBV/DWV) rate was 16.7% (9/54). Co-infection with SBV and BQCV occurred at the highest frequency (35.2%; 19/54). The most common regions for co-infection were Kunming City and Luoping County (Table 4).

Co-infection type	Sampling site						
	Kunming	Luoping	Mengzi	Wuding	Dayao	Tengchong	Total
SBV/DWV	5	1	0	0	0	0	6
BQCV/SBV	6	4	2	4	3	0	19
BQCV/DWV	10	5	0	0	1	0	16

BQCV/KBV	0	0	0	0	0	4	4
BQCV/SBV/DWV	4	4	0	1	0	0	9
Total	25	14	2	5	4	4	54

Table 4: Frequency of dual and triple co-infections. The numbers shows the combinations of multiple virus infections, co-infection was found in 6 of 9 study regions.

Discussion

Before the introduction of superior varieties of Western bees into China, Eastern bees were the only breeding species. Eastern bees have unique biological and behavioral characteristics and play important roles as unique plant pollinators for commercially valuable crops, including oil crops, vegetables, and fruit trees, and as a wild nectar source [2]. Eastern bees are widely distributed in Yunnan province, from the low and hot valley of Hekou County (altitude 76.4 m, south of Yunnan) to the high and cold region of Zhongdian County (altitude 3600 m, northwest of Yunnan). Eastern bees can survive both in cold winter and in hot summer, providing various bee products (for example, honey and wax) with economic importance.

Bees in most parts of the world have undergone marked declines since the 1940s, one of the reason for this is that bees can be infected by at least 18 viruses and other pathogens [10]. Viral infection can occur during various growth and developmental stages of bees, but only a few of them result in serious disease. In earlier days, laboratory diagnosis of bee viruses was established based on electronic microscopic identification of the virus particles and antigens, and using techniques such as immunodiffusion test, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) [11]. The sensitivity and specificity of most of these tests is low or they suffer from non-specific amplification and it is difficult to distinguish different virus types using these traditional methods. The lack of cell culture and in vitro techniques further hampered the development of molecular diagnostic methods. This study established RT-PCR methods for the rapid detection of six of the most common bee RNA viruses with high sensitivity and analytic specificity, and identified the prevalence status of DWV, SBV, KBV and BQCV in Eastern Bee colonies in Yunnan province. Among the nine regions in which BQCV, SBV, and DWV were screened, Luoping County showed very high infection rates. This is of particular concern as thousands hectares of canola are planted in this area each year and, while canola plants are in bloom, large numbers of hives arrive to this relatively small geographical location from many different regions of the country. In addition, it is well known that bee breeding enhances virus transmission.

A survey of the prevalence of seven bee viruses in Western and Eastern bees in 18 provinces in China found that infection rates varied dramatically between the two bee species. DWV, BQCV, SBV, Israel acute paralysis virus (IAPV), ABPV and CBPV were prevalent in Western bees, with DWV being the most prevalent at 94%. IAPV, DWV, CBPV, BQCV and SBV were found in Eastern bees, with SBV being the most dominant and present in 86% of apiaries [7].

SBV primarily affects bee broods, with color change in infected larvae from pearly white to pale yellow, and shortly after death they dry out, forming a dark brown gondola-shaped scale [12]. In China, SBV was first described in Guangdong province in 1972, and reemerged in Liaoning province in 2008, which caused lethal diseases in individual bees and the collapse of entire colonies [13]. A novel strain of SBV in Chinese bees that shares 86.8% nucleotide sequence homology and 93.4% amino-acid identity with reference strains has been detected in Guangdong Province [14]. SBV infection has also been reported prevalent in our neighboring countries like Thailand [15]. The present study revealed the presence of SBV (24.1%) of the screened samples in Yunnan province, of which the highest infection rate was in Kunming (56.7%). Considering that SBV mainly infects larvae, further studies focusing on the prevalence of this virus in larvae are needed to acquire specific prevalence data.

DWV infection is the most common in bees which has established persistent infection in most apiaries in the world since the first isolation of DWV in the early 1980s. DWV Infection is found in different bee castes including queens, drones, and workers, as well as in different bee developmental stages including eggs, larvae, pupae and adults. This virus is transmitted both vertically and horizontally [16]. While DWV usually persists as an asymptomatic infection, the virus can be re-activated when triggered by various host stress, causing symptoms of illness in infected bees [17]. The manifestations of the disease caused by DWV infection include shrunken and crumpled wings, decreased body size, discoloration in adult bees and a reduction in life span. The severe symptoms of DWV infections appear to be associated with *Varroa* destructor infestation of the bee hive, and studies have shown that *Varroa* destructor harbors even greater levels of the virus than found in severely infected bees [18]. A recent study [19] on 1 140 *A. cerana* worker samples was performed in 19 provinces in China between November 2010 and January 2011. However, no data about DWV from Yunnan province was available in this study and only very limited spots were sampled. Our study revealed that 14.8% of screen samples were positive for DWV, the highest of which was present in Kunming (76.7%), and this virus was also prevalent in Luoping County, Wuding County, Dayao County and Deqing County.

KBV was first confirmed in *A. cerana* Fabricius and analysis of the genome sequence shows that KBV is a cricket paralysis-like virus (*Dicistroviridae cripavirus*) [20,21]. Like most bee viruses, KBV is thought to persist as an asymptomatic infection within the bee community, until stress or an alternative vector (such as *Varroa*) activates it to become epidemic and lethal [22]. There is a report indicating that when KBV carrier bees are pierced and suckled by *Varroa* destructor, KBV is activated which results in viremia, thus causing a large number of bee deaths. However, as of yet, no one has found a way to culture bee viruses in vitro, which has hampered the study of viral diseases in bees. As a result, the relationship between crawling disease and *Varroa* destructor infection and the prevalence of KBV in China has yet to be fully elucidated. We found the positive rate of KBV in Yunnan province is 26.7%, which is the first report of KBV infection in Eastern bees in China.

BQCV infection was more prevalent in adult bees than in pupae, but the infected worker bees normally do not exhibit significant disease symptoms and that the incidence of BQCV was higher in spring and

summer than in autumn [23]. BQCV is the most common cause of death of queen larvae in Australia and Poland [21]. More and more BQCV strains have been isolated and identified from *A. mellifera,* making it the second most prevalent virus infection in this species after DWV [24]. The variability of BQCV isolates collected from a variety of geographic regions can be further understood by molecular comparison and determination of genomic relationships between the strains.

DWV is present in all developmental stages and castes, with significant economic importance due to its close association with bee colony collapse induced by *Varroa* destructor. DWV is considered as a common global bee virus which is present in both laboratory-reared and wild populations of bumble bees (*Bombus huntii*) [1,25] DWV and BQCV are common multi-host and prevalent viruses in European bees. Queen, worker or drone broods can be infected by these two viruses without apparent morphologic alterations, although symptomatic BQCV infection of such broods has also been reported [26].

Multiple infections with different viruses are very common in both Western and Eastern bees [27]. This phenomenon has also been reported in many countries including France, Hungary, USA, Austria, Southwest England and South Korea [7]. The dynamics of these coexisting virus populations within these colonies is of great interest and it would be useful to determine how they fluctuate with respect to each other [28]. Therefore, the incidence of dual and triple infections confirmed in this study is unsurprising.

ABPV and CBPV were not detected in this study, which is coincident with a previous investigation in Japan [29], where the study of *Apis mellifera* and *Apis cerana* japonica colonies with RT-PCR has revealed CBPV infection in *A. mellifera* but not in A. c. japonica colonies in Japan. The possible reasons why we did not detect ABPV or CBPV from Yunnan could be as follows: 1). The *Apis cerana* populations in the areas under investigation in Yunnan province are free of infection of ABPV and CBPV; 2). The virus titer was so low, which is beyond the detection sensitivity of classical RT-PCR: 3). After propagation and adaption in the *Apis cerana*, ABPV and CBPV accumulated sequence variation within the primer regions where we started the amplifications.

Although Eastern honey bee population in China is often infected with *Varroa*, the samples we collected were free of *Varroa* infestation. However, *Varroa* infestation was observed in the beehives where we collected the samples, which could be one of the reasons of virus transmission. Since these viruses are often latent in honey bees and our study was based mainly on qualitative detection of the viruses, it is unclear, to what extent the results are relevant for pathology, which is worth of further investigation. Furthermore, due to the unavailability of cell culture models for honey bee viruses, quantitative analysis of the titers of the viruses is technically and practically difficult. Recently, new viruses were discovered in *Apis mellifera* like Lake Sinai Virus [30], it can also be worthy to investigate these viruses in *A. cerana* in the future study.

In conclusion, our study investigated the prevalence of the six most common bee RNA viruses that are known to cause diseases in bee colonies in Yunnan Province of China. We demonstrated that BQCV, DWV, KBV and SBV are already widely prevalent in Yunnan province and that Luoping County and Kunming city had the highest infection rates. For the first time we showed that KBV could infect *A. cerana* and is already prevalent in Tengchong County. Phylogenetic analysis revealed that most of the Yunnan isolates are likely clustered into independent branches. We demonstrated a geographic separation of Yunnan isolates from most of the reference isolates. The lack of major exchange of honey bees between Europe/mainland US and Yunnan for the recent decades may have resulted in the geographic separation of Yunnan isolates. Therefore, the results in our study strongly suggest that sequence analysis can play an important role in tracing the origins of honey bee virus strains and diagnosis of honey bee disease in Chinese populations during epizootics.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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