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Genetic and phylogenetic analysis of the honey bee sacbrood virus from jiangxi isolates

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ABSTRACT

The high prevalence of honeybee viral diseases poses a severe threat to the health of honeybees and causes substantial economic losses worldwide. Sacbrood virus (SBV) is a single-strand RNA virus that infects honeybees at all life stages. The infection can shorten the lifespan of adult bees and is lethal to larvae. SBV is the major cause of honeybee losses in Asia. Genetic and phylogenetic analyses of SBV isolates from different areas have been previously conducted. However, the impact of *Apis mellifera* Linnaeus and *Apis cerana* Fabricius coexistence on the infection and phylogeny of SBV remains unknown. In this study, we collected *A. cerana* and *A. mellifera* samples from commercial apiaries, only *A. cerana* in mountainous region. SBV prevalence was evaluated in three commercial apiaries of Jinxi, Tonggu and Nanchang and two mountainous regions of Zixi and Yifeng. In our sampling location, we found a higher SBV prevalence in the mountainous regions than in commercial apiaries. Partial structural polyprotein coding sequences were sequenced and compared with other GenBank SBV isolates. Phylogenetic tree topologies showed that SBV isolates form two major groups based on their host specificity, and isolates from same country tend to cluster together in subclades, indicating that the host and geographic region has significant effects on SBV strain specificity.

Introduction

Honeybee is the most important insect pollinator in nature and is of great significance in agriculture. Notably, insect-pollinated crops directly contribute to 35% of the world's food (Klein et al., 2007). The global economic value of insect pollination, particularly by bees, is 153 billion per year, accounting for 9.5% of the world's gross agricultural food production (Gallai et al., 2009). In recent years, international studies have reported declining trends in honeybee populations (Yildirim et al., 2020; Kalayci et al., 2019). This decline has further triggered a pollination crisis, resulting in yield reduction of agricultural products that depend on insects for pollination (Klein et al., 2007), and subsequently, substantial economic losses worldwide.

The honeybee *Apis cerana* Fabricius is an important crop pollinator widely distributed in China (Shi et al., 2013), especially in mountainous regions. Recently, however, the number of colonies is declining due to the prevalence of bee viruses and other threatening factors (Hassanyar et al., 2019; Huang et al., 2017; Shan et al., 2017). Sacbrood virus (SBV) is common worldwide and is lethal to the Asia honeybee, *A. cerana* (Ai et al., 2012). The SBV-infected larvae gradually change from white to pale yellow and die before pupating (Bailey, 1975). SBV can also infect adult bees with no apparent clinical symptoms. Furthermore, SBV shortens the lifespan of honeybees (Bailey, 1969; Anderson and Gibbs, 1989). According to Bailey (1976), SBV is more prevalent during summer and spring, when the colony numbers increase the fastest, and there are more susceptible larvae and young adults. The Chinese SBV (CSBV) that infects the Asia honeybee *A. cerana* was first discovered in 1972 in Guangdong, China. The CBSV reappeared in 2008 in Liaoning, China, causing fatal pathema in individual bees and whole colony breakdown (Ma et al., 2010). SBV is a single strand RNA virus and belongs to the family Iflaviridae (Ghosh et al., 1999). Its 8832 bp genome consists of a single large open reading frame (179-8752). SBV contains three structural proteins, VP1, VP2, and VP3 (Ghosh et al., 1999), while CSBV has four structural proteins VP1, VP2, VP3, and VP4 (Ma et al., 2013).

; SBV, Sacbrood virus.

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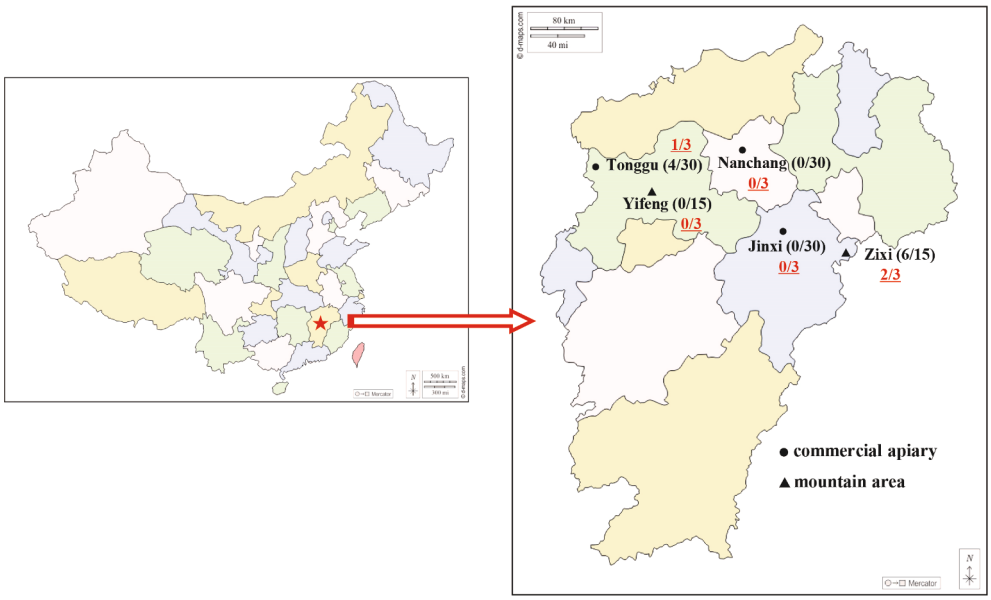

Fig. 1. The red pentagram indicates the location of Jiangxi Province in the map of China, and the arrow points to the sampling locations in Jiangxi province. Numbers in the brackets show positive samples and the total number of tested samples. Underlined numerals indicate the numbers of positive and tested apiaries. The 30 tested samples in commercial apiary included 15 *A. cerana* samples and 15 *A. mellifera* samples, all 15 tested samples in mountain region were *A. cerana* samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Recently, the focus has shifted to the genetic characterization and phylogenetic relationship of SBV isolates (Choi et al., 2008; Li et al., 2016). SBV showed host sepcificity, where virus isolating from *A. cerana* and *A. mellifera* were segregated into two clusters in sympatric population (Ma et al., 2013). By including allopatric population, additional clusters were identified. The authors suggested that the SBV strains diversity separated among geographical regions (Blanchard et al., 2014; Yildirim et al., 2020; Kalayci et al., 2019). Further study confirmed that both geographical distribution and host affiliation shaped the diversity SBV isolates (Yang et al., 2013).

Jiangxi Province is mainly a mountainous region. Only *A. cerana* is found in the mountainous region, and it has very little interaction with the outside world. However, *A. mellifera* and *A. cerana* frequently coexist in many commercial apiaries. Studies have reported that the coexistence of *A. mellifera* and *A. cerana* reduced the incidence of SBV in *A. cerana* colony (Vung et al., 2018). However, it is unknown whether the coexistence of *A. mellifera* and *A. cerana* has an effect on SBV infection and genetic diversity, therefore we sought to investigate SBV prevalence and understand the genetic relationship among distinct virus isolates. In addition, the SB1-2 fragment of SBV was used for genetic characterization and phylogenetic analysis to elucidate their evolutionary relationship (Grabensteiner et al., 2001).

Materials and methods

Sample collection

A total of 120 samples (45 *A. mellifera*, 75 *A. cerana*) from 15 apiaries in three commercial apiaries (Jinxi: 116.77°E, 27.92°N; Tonggu: 114.37°E, 28.53°N; Nanchang: 115.85°E, 28.68°N) and two mountainous regions (Zixi: 117.07°E, 27.7°N; Yifeng: 114.78°E, 28.38°N) in Jiangxi province of China were obtained during the summer of 2019. There are only *A. cerana* in the mountainous regions, however, *A. mellifera* and *A. cerana* frequently coexist in commercial apiaries. The distribution of the 15 apiaries from which the samples were collected is shown in Fig. 1. The bees were brought to the laboratory and stored at −80 °C for subsequent molecular analysis.

RNA extraction and cDNA synthesis

The TransZol reagent (Transgen Biotech, www.transgen.com.cn) was used to extract total RNA following the manufacturer's instructions. A spectrophotometer (GeneQuant, Pharmacia) was used to determine the RNA concentration of each RNA sample. The purity of the total RNA was determined at 260 nm/280 nm ratio with expected values between 1.8 and 2.0, then stored at −80 °C until use. The Primer-Script RT reagent Kit (TaKaRa, www.takara-bio.com) was used to synthesize cDNA from the 2 μg RNA following the manufacturer's instructions and stored at −20 °C.

RT-PCR amplification

The SB1-2 specific primer pairs (F: 5′-CCAACCGATTCCTCAGTAG-3′, R: 5′-CCTTGGAACTCTGCTGTGTA-3′, size: 469 bp, nucleotide position: 221-689) from Grabensteiner et al. (2001) were used to amplify SBV fragments for phylogenetic analyses. The SB1-2 fragment are highly conserved region of the structural polyprotein of SBV (Choe et al., 2012a). Phylogenetic treewere then constructed using the SB1-2 fragments. The primers mentioned above were obtained from Sangon Biotech (Sangon Biotech Shanghai, China Co., Ltd). A 20 μL PCR reaction diagnosed each virus isolate. The reaction contained: 10 μL SinBio 2× master Mix (GenStar BioSolutions), 1 μL SB1-2 forward primer, 1 μL SB1-2 reverse primer, 6 μL RNase Free water, and 2 μL cDNA template. The PCR thermocycling conditions were: 95 °C for 5 min, then 40 cycles at 95 °C for 20 s, 50 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. PCR products were analyzed using 1.5% agarose gel electrophoresis. The amplified products were visualized under ultraviolet light against a standard DNA marker (1000 bp).

Nucleotide sequence and phylogenetic analysis

SBV was detected in 10 samples, i.e., six samples from Zixi and four from Tonggu. The ten amplification products were then directly sequenced. Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) was used to identify the SBV nucleotide sequence (Altschul, 2012). BLAST was also used for multiple sequence alignments. The phylogenetic tree was constructed using the neighbor-joining (NJ) method through the Mega 5 software (Tamura et al., 2011; Saitou, 1987) and computed with the Kimura 2-parameter method (Kimura, 1980). A bootstrap value of 1000 replicates was adopted to maintain the phylogeny stability. The DNA Sequence polymorphism software was used for synonymous and non-synonymous substitution rate analyses of SB1-2 fragment. Clustal X 2.0 was used for multiple alignments of nucleotide sequence (Larkin et al.,

Table 1
SB1-2 fragment nucleotide sequence homology in 47 reference strains with Zixi and Tonggu isolates.

Isolates	Accession number	Tonggu (%)	Zixi (%)
Ac-CQ/China	JQ796779	98.57	97.99
Ac-CQ/China	JQ796780	97.99	97.99
Ac-CQ/China	JQ796781	97.99	97.99
Ac-CQ/China	JQ796782	97.71	97.71
Am-Vietnam	KM884993	97.42	97.42
Ac-Vietnam	KM884991	97.42	97.42
Ac-Vietnam	KM884992	97.42	97.42
Ac-Vietnam	KM884990	97.42	97.42
Ac-Vietnam	KM884994	96.85	96.85
Ac-YN/China	JX679485	97.11	97.69
Ac-YN/China	JX679484	97.11	97.69
Ac-YN/China	JX679486	96.82	97.40
Ac-YN/China	JX679483	96.82	97.40
Am-Germany	AF284624	95.42	96.85
Am-Germany	AF284618	95.42	96.85
Am-Germany	AF284620	95.42	96.85
Am-Germany	AF284621	95.42	96.85
Am-Germany	AF284622	95.42	96.85
Am-Vietnam	KM884995	95.42	97.13
Ac-LN/China	HM237361	95.42	97.13
Am-Germany	AF284619	95.13	96.56
Am-France	AY230517	95.13	96.56
Am-Russia	KC513760	95.13	96.56
Am-France	KC513753	94.84	96.28
Am-Austria	KC513758	94.84	96.28
Am-Russia	KF274667	94.56	96.56
Am-France	KC513754	94.56	95.99
Am-France	KC513752	94.56	95.99
Am-Russia	KC020674	94.56	96.56
Am-Austria	AF284617	94.56	95.99
Am-Denmark	KC513757	94.56	95.99
Am-Korea	JQ390591	94.56	95.42
Ac-India	AF284626	94.56	96.85
Ac-India	JX270796	94.27	96.56
Am-Denmark	EF570887	93.98	95.70
Am-Russia	KF274668	94.72	96.19
Am-Korea	JQ390592	93.98	94.84
Ac-India	JX270795	93.98	96.28
Ac-India	JX270797	93.70	95.99
Ac-Korea	HQ916829	93.70	95.99
Ac-Korea	HQ916828	93.70	95.99
Ac-Korea	HQ916830	93.70	95.99
Ac-Korea	HQ916831	93.70	95.99
Ac-Korea	HQ916832	93.70	95.99
Am-UK	AF092924	93.12	94.56
Am-UK	AF284616	92.26	93.70
Ac-Korea	HQ916827	92.28	95.13

Note: CQ/China means Chongqing city, China. YN/China means Yunnan province, China. LN/China means Liaoning province, China. Ac and Am indicated that the host of the isolates was Apis. cerana and Apis. cerana. The same below.

2007). DnaSP version 5.10 was used to determine the genetic characteristics, such as synonymous and nonsynonymous substitution rates of SBV isolates (Rozas, 2009).

Results

Nucleotide sequence analysis

SBV was found only in *A. cerana* samples, so we only discuss *A. cerana* in the following. RT-PCR showed that 20% (6/15) of tested apiaries were infected with SBV, and 13.3% (10/75) of tested *A. cerana* samples were infected with SBV. The amplified fragment had the expected size. Nucleotide SBV sequences were uploaded to the GenBank database under the accession numbers MW892515-MW892524. Multiple sequence alignment revealed high similarity (>97.14%) between SBV isolates from Zixi and Tonggu. Chinese isolates (JQ796779) had the highest similarity with Zixi (97.99%) and Tonggu isolates (98.57%), while the UK isolates (AF284616) had the lowest similarity with Zixi (93.70%) and Tonggu (92.26%) isolates (Table 1).

Phylogenetic analysis

The phylogenetic tree constructed from the partial structural polyprotein coding sequence amplified through the SB1-2 primer pair is presented in Fig. 2. Bootstrap analysis showed suitable confidence values, indicating a statistically validated clustering. The phylogenetic tree had two main branches according to their host, Am and Ac. The isolates from the *A. mellifera* in European and Korea tended to clustered together formed one main branch. In this group, at least three subclusters occur, in which two UK isolates are closely related and formed one sub-group and two korea isolates from *A. mellifera* formed second sub-group, the other European isolates cluster together to form the three sub-group. The isolates in this study with other isolates from the *A. cerana* in Asia are closely related, and cluster together formed another main branch. In this group, the isolates from same geographic region each formed their own subclade. Interestingly, the Vietnam Am

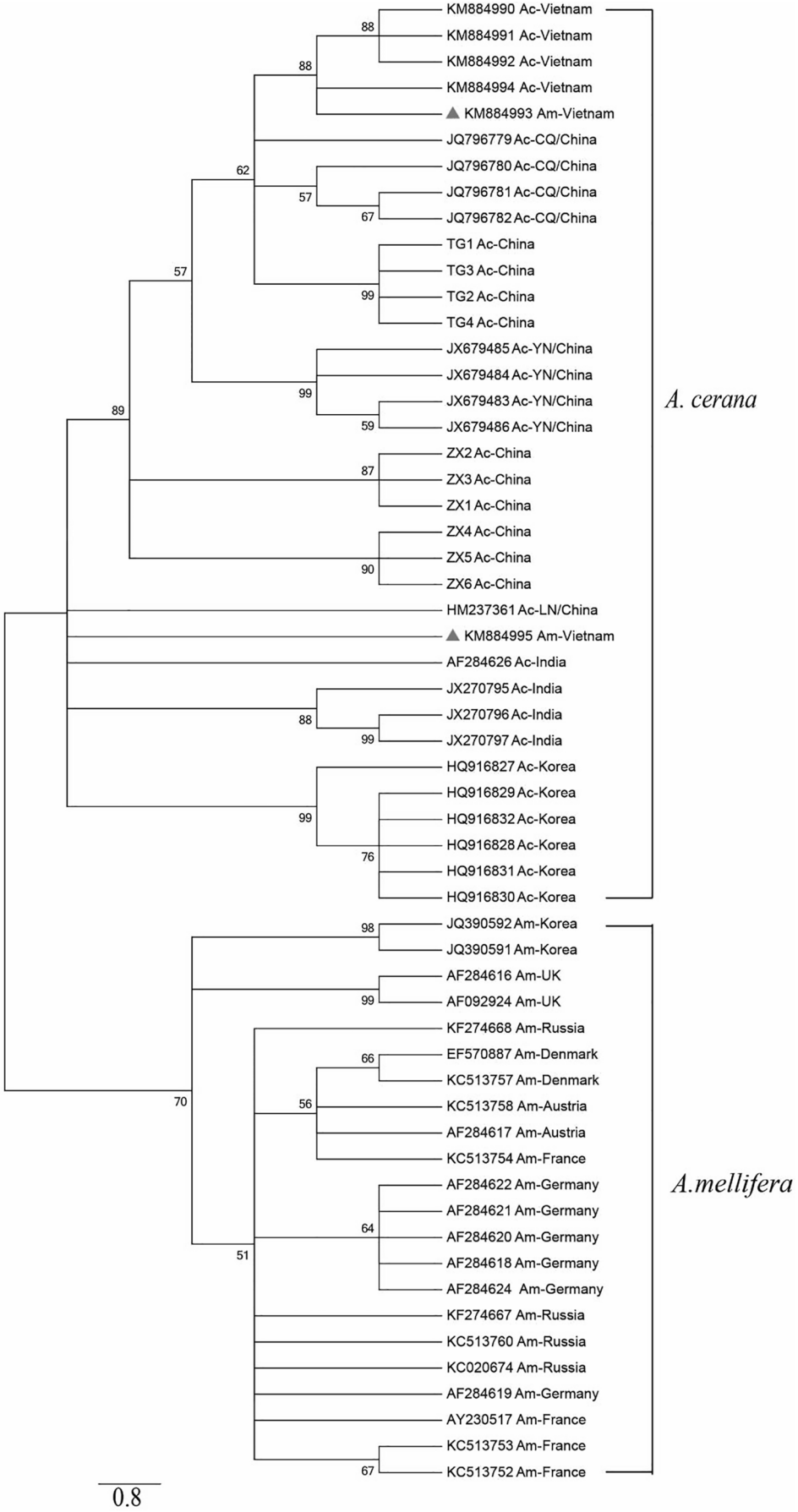

Fig. 2. Phylogenetic tree of SBV isolates constructed using the structural protein-coding sequence amplified via SB1–2 primers. Overall, the SBV diverged into two main branches, which is consistent with their host, suggesting a host specificity of virus. However, the viruses cross-over event might be present, as two Vietnam samples (highlighted with triangle) tended to clustered together with Asian honeybees. Numerals represent bootstrap values (%) from 1000 replicates. ZX1-ZX6 and TG1-TG4 are the isolates in this study, other SBV isolates from Genbank. "*A. cerana*" and "*A. mellifera*" represent the host of the isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Nucleotide substitution rate of SB1-2 fragment from Tonggu SBV isolates and other SBV isolates.

Tonggu				
Isolates	Accession number	Ks	Ka	Ka/Ks
Ac-Korea	HQ916832	0.0775	0.0576	0.7432
Ac-Korea	HQ916831	0.0775	0.0576	0.7432
Ac-Korea	HQ916830	0.0775	0.0576	0.7432
Ac-Korea	HQ916828	0.0775	0.0576	0.7432
Ac-Korea	HQ916827	0.0930	0.0701	0.7538
Ac-Korea	HQ916829	0.0775	0.0576	0.7432
Ac-LN/China	HM237361	0.0769	0.0414	0.5384
Ac-YN/China	JX679483	0.0415	0.0272	0.6554
Ac-YN/China	JX679484	0.0416	0.0233	0.5601
Ac-YN/China	JX679485	0.0416	0.0233	0.5601
Ac-YN/China	JX679486	0.0413	0.0273	0.6610
Ac-CQ/China	JQ796781	0.0273	0.0194	0.7106
Ac-CQ/China	JQ796780	0.0274	0.0194	0.7080
Ac-CQ/China	JQ796782	0.0273	0.0233	0.8535
Ac-CQ/China	JQ796779	0.0274	0.0115	0.4197
Ac-India	AF284626	0.0994	0.0434	0.4366
Ac-India	JX270796	0.0857	0.0514	0.5998
Ac-India	JX270795	0.0707	0.0596	0.8430
Ac-India	JX270797	0.0857	0.0596	0.6954
Ac-Vietnam	KM884990	0.0135	0.0313	2.3185
Ac-Vietnam	KM884994	0.0137	0.0391	2.8540
Ac-Vietnam	KM884992	0.0135	0.0313	2.3185
Ac-Vietnam	KM884991	0.0135	0.0313	2.3185
Am-Vietnam	KM884993	0.0272	0.0273	1.0037
Am-Vietnam	KM884995	0.0695	0.0394	0.5669
Am-Korea	JQ390592	0.0836	0.0558	0.6675
Am-Korea	JQ390591	0.0689	0.0517	0.7504
Am-Denmark	EF570887	0.0991	0.0434	0.4379
Am-Denmark	KC513757	0.0991	0.0434	0.4379
Am-Austria	KC513758	0.0841	0.0434	0.5161
Am-Austria	AF284617	0.0844	0.0475	0.5628
Am-Russia	KF274668	0.0835	0.0476	0.5701
Am-Russia	KC020674	0.0834	0.0435	0.5216
Am-Russia	KC513760	0.0838	0.0394	0.4702
Am-Russia	KF274667	0.0838	0.0394	0.4702
Am-France	AY230517	0.0838	0.0394	0.4702
Am-France	KC513753	0.0841	0.0434	0.5161
Am-France	KC513752	0.0991	0.0434	0.4379
Am-Austria	KC513754	0.0841	0.0475	0.5648
Tonggu				
Isolates	Accession number	Ks	Ka	Ka/Ks
Am-UK	AF284616	0.1297	0.0683	0.5266
Am-UK	AF092924	0.0984	0.0642	0.6524
Am-Germany	AF284622	0.0692	0.0394	0.5694
Am-Germany	AF284621	0.0692	0.0394	0.5694
Am-Germany	AF284620	0.0692	0.0394	0.5694
Am-Germany	AF284619	0.0838	0.0394	0.4702
Am-Germany	AF284618	0.0692	0.0394	0.5694
Am-Germany	AF284624	0.0692	0.0394	0.5694

Note: Ks: Synonymous substitution rate, Ka: Nonsynonymous substitution rate.

Table 3
Nucleotide substitution rate of SB1-2 fragment from Zixi SBV isolates and other SBV isolates.

Zixi				
Isolates	Accession number	Ks	Ka	Ka/Ks
Ac-Korea	HQ916832	0.0276	0.0432	1.5652
Ac-Korea	HQ916831	0.0276	0.0432	1.5652
Ac-Korea	HQ916830	0.0276	0.0432	1.5652
Ac-Korea	HQ916828	0.0276	0.0432	1.5652
Ac-Korea	HQ916827	0.0420	0.0472	1.1238
Ac-Korea	HQ916829	0.0276	0.0432	1.5652
Ac-LN/China	HM237361	0.0344	0.0253	0.7355
Ac-YN/China	JX679483	0.0278	0.0193	0.6942
Ac-YN/China	JX679484	0.0278	0.0154	0.5540
Ac-YN/China	JX679485	0.0278	0.0154	0.5540
Ac-YN/China	JX679486	0.0277	0.0193	0.6968
Ac-CQ/China	JQ796781	0.0419	0.0115	0.2745
Ac-CQ/China	JQ796780	0.0420	0.0115	0.2738
Ac-CQ/China	JQ796782	0.0419	0.0154	0.3675
Ac-CQ/China	JQ796779	0.0421	0.0115	0.2732
Ac-India	AF284626	0.0559	0.0272	0.4866
Ac-India	JX270796	0.0422	0.0350	0.8294
Ac-India	JX270795	0.0278	0.0431	1.5504
Ac-India	JX270797	0.0422	0.0431	1.0213
Ac-Vietnam	KM884990	0.0276	0.0232	0.8406
Ac-Vietnam	KM884994	0.0279	0.0310	1.1111
Ac-Vietnam	KM884992	0.0276	0.0232	0.8406
Ac-Vietnam	KM884991	0.0276	0.0232	0.8406
Am-Vietnam	KM884993	0.0418	0.0193	0.4617
Am-Vietnam	KM884995	0.0558	0.0233	0.4176
Am-Korea	JQ390592	0.0699	0.0434	0.6209
Am-Korea	JQ390591	0.0553	0.0393	0.7107
Am-Denmark	EF570887	0.0557	0.0312	0.5601
Am-Denmark	KC513757	0.0557	0.0312	0.5601
Am-Austria	KC513758	0.0414	0.0312	0.7536
Am-Austria	AF284617	0.0415	0.0352	0.8482
Am-Russia	KF274668	0.0411	0.0353	0.8589
Am-Russia	KC020674	0.0411	0.0313	0.7616
Am-Russia	KC513760	0.0412	0.0273	0.6626
Am-Russia	KF274667	0.0412	0.0273	0.6626
Am-France	AY230517	0.0412	0.0273	0.6626
Am-France	KC513753	0.0414	0.0312	0.7536
Am-France	KC513752	0.0557	0.0312	0.5601
Am-Austria	KC513754	0.0414	0.0352	0.8502
Zixi				
Isolates	Accession number	Ks	Ka	Ka/Ks
Am-UK	AF284616	0.0850	0.0556	0.6541
Am-UK	AF092924	0.0553	0.0515	0.9313
Am-Germany	AF284622	0.0272	0.0273	1.0037
Am-Germany	AF284621	0.0272	0.0273	1.0037
Am-Germany	AF284620	0.0272	0.0273	1.0037
Am-Germany	AF284619	0.0412	0.0273	0.6626
Am-Germany	AF284618	0.0272	0.0273	1.0037
Am-Germany	AF284624	0.0272	0.0273	1.0037

Note: Ks: Synonymous substitution rate, Ka: Nonsynonymous substitution rate.

isolates (KM884993, KM884995) were included in the Ac group in our phylogenetic tree.

Synonymous and nonsynonymous substitution rates analysis

The substitution rate of SB1-2 fragment sequences of Zixi and Tonggu isolates and other sequences downloaded from the NCBI were analyzed separately. The synonymous substitution level of Zixi isolates and Tonggu isolates was significantly higher than the nonsynonymous substitution level. The Ka/Ks values of most Zixi isolates and Tonggu isolates were less than 1 (Table 2, Table 3).

Discussion

Sacbrood virus (SBV) was first reported in honeybees in 1913 in America (White, 1913). SBV pose serious threats to honeybees in Asia, where it has since been reported in China (Ai et al., 2012), South Korea (Choi et al., 2008), Japan (Kojima et al., 2011), Thailand (Shah and Shah, 1988), and Vietnam (Forsgren et al., 2015). In this study, SBV was detected only in Zixi (mountainous region) and Tonggu (commercial apiary), and the SBV prevalence was higher in Zixi (40%) than Tonggu (26.7%), indicating SBV infection is common in mountainous region with superior geographical isolation conditions. In addition, SBV was not detected in any of the *A. mellifera* samples, therefore, we speculated that the coexistence of *A. mellifera* and *A. cerana* had little influence on SBV infection.

The nucleotide sequence and genetic diversity analyses of SBV from different countries is the current study hotspot (Choe et al., 2012a; Li et al., 2016). Phylogenetic tree analysis showed that the Zixi and Tonggu SBV isolates are most closely related to Chinese isolates, which may reflect their relatively close geographic proximity. Multiple sequence alignment also indicated that Zixi and Tonggu SBV isolates had higher

nucleotide similarity to Chinese isolates than the reference sequences downloaded from the NCBI.

The phylogenetic tree formed two main groups, Am and Ac, consistent with previous studies (Li et al., 2016; Yang et al., 2013). The SBV isolates form different clades depending on their host, *A. cerana* or *A. mellifera*, which have the characteristics of specific infection of *A. cerana* and *A. mellifera* (Kojima et al., 2011). SBV might have a species barrier for the infection of *A. mellifera* and *A. cerana* (Yang et al., 2013). In addition, the isolates from same geographic region closely related and each formed their own subclade, thus it can be seen that geographic region influence the phylogenetic relationship of SBV. This is in agreement with the results of the previous study conducted by Li et al. (2016). The Vietnam Am isolates were mixed into the Ac group, this was probably because the adaptation of SBV to different hosts and the coexistence of *A. cerana* and *A. mellifera* in Vietnam (Reddy et al., 2017). This hypothesis has also been addressed in several previous studies such as Grabensteiner et al. (2001), Choe et al. (2012b). Moreover, this result indicated that SBV can cross-infect between *A. cerana* and *A. mellifera* species. One way to evaluate the selective pressures on evolution is to compare the rate of Ks and Ka. Ks is the estimated number of synonymous changes per synonymous site and corresponds to the rate of amino acid-neutral evolution. Ka, on the other hand, is the number of nonsynonymous substitutions per nonsynonymous site. Ka/Ks <1 in this study, indicated negative (purifying) selection (Duret, 2000).

In conclusion, Zixi and Tonggu isolates in this study are genetically closely related to Chinese strains. Furthermore, the host specificity and geographic region significantly influence the phylogeny of SBV isolates. Our findings provide a basis for further control and prevention of SBV. It also sheds light on the transmission mechanism of pathogens among honeybee populations in different regions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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