

Evidence of *Apis cerana* Sacbrood virus Infection in *Apis mellifera*

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Sacbrood virus (SBV) is one of the most destructive viruses in the Asian honeybee *Apis cerana* but is much less destructive in *Apis mellifera*. In previous studies, SBV isolates infecting *A. cerana* (AcSBV) and SBV isolates infecting *A. mellifera* (AmSBV) were identified as different serotypes, suggesting a species barrier in SBV infection. In order to investigate this species isolation, we examined the presence of SBV infection in 318 *A. mellifera* colonies and 64 *A. cerana* colonies, and we identified the genotypes of SBV isolates. We also performed artificial infection experiments under both laboratory and field conditions. The results showed that 38 *A. mellifera* colonies and 37 *A. cerana* colonies were positive for SBV infection. Phylogenetic analysis based on RNA-dependent RNA polymerase (RdRp) gene sequences indicated that *A. cerana* isolates and most *A. mellifera* isolates formed two distinct clades but two strains isolated from *A. mellifera* were clustered with the *A. cerana* isolates. In the artificial-infection experiments, AcSBV negative-strand RNA could be detected in both adult bees and larvae of *A. mellifera*, although there were no obvious signs of the disease, demonstrating the replication of AcSBV in *A. mellifera*. Our results suggest that AcSBV is able to infect *A. mellifera* colonies with low prevalence (0.63% in this study) and pathogenicity. This work will help explain the different susceptibilities of *A. cerana* and *A. mellifera* to sacbrood disease and is potentially useful for guiding beekeeping practices.

Honeybees play vital roles in agriculture by providing pollination services to food crops and producing honey and other hive products (1). European honeybees (*Apis mellifera*) and Asian honeybees (*Apis cerana*) are two truly domesticated bee species in global apiculture (2, 3), but they are commonly threatened by a myriad of parasites and pathogens (4). **Sacbrood virus (SBV)**, the first virus identified in honeybees (5), is a single-stranded, positive-sense RNA virus belonging to the genus *Iflavirus* in the family *Iflaviridae* (6). The virus primarily affects the brood stage of honeybees, causing failure of pupation and larval death (7), but also affects adult bees without obvious signs of the disease (8–10). Sacbrood is characterized by the diseased-infected larva being encased in a tough, fluid-filled sac formed from the larval cuticle. The transmission of SBV can occur horizontally, vertically, or both (11). The prevalence of this virus has been investigated in various countries (4, 12–20). SBV infection appears to be less detrimental to *A. mellifera* colonies than to *A. cerana* colonies (21). In addition, it is unusual for SBV infection in *A. mellifera* colonies to cause colony death (22). However, SBV is deadly to Asian honeybees, and serious mortality rates for *A. cerana* due to SBV infection have been reported in Vietnam, Thailand, India, China, and South Korea (7, 23–26). Large-scale *A. cerana* colony losses caused by SBV infection were first reported in Guangdong Province, China, in 1972 (27). Infection also resulted in serious *A. cerana* colony losses in South Korea (26, 28).

Previous studies reported that SBV isolates infecting *A. cerana* (AcSBV) and SBV isolates infecting *A. mellifera* (AmSBV) represent two different serotypes (29) and are chemically distinct (30), suggesting that AcSBV and AmSBV confer unique pathogenicities to their respective hosts. To understand the susceptibilities of *A. cerana* and *A. mellifera* bees to SBV isolated from different honeybee species, studies of cross-species infections with AcSBV and AmSBV were performed. In previous cross-infection studies conducted using AcSBV and AmSBV to challenge *A. mellifera* larvae and *A. cerana* larvae, AmSBV and AcSBV were pathogenic only in *A. mellifera* larvae and *A. cerana* larvae, respectively (31–33).

An *A. mellifera* isolate, AmSBV-Kor19 (GenBank accession

number JQ390592), which is similar to *A. cerana* isolates across most of its genome, was reported recently (26). Roberts and Anderson (29) pointed out that cross-species SBV infection of *A. cerana* and *A. mellifera* may occur, because phylogenetic analysis of SBV revealed that AmSBV-Kor19 is grouped with other *A. cerana* isolates. Therefore, there have been nonidentical views on cross-species infection. The objectives of this study were (i) to survey for SBV infections in apparently healthy or weak *A. mellifera* and *A. cerana* colonies, (ii) to identify genotypes of SBV isolates from *A. mellifera* and *A. cerana* colonies, and (iii) to evaluate the infectivity of AcSBV in *A. mellifera*, both larvae and adults, under laboratory and field conditions.

MATERIALS AND METHODS

Sample collection for SBV detection. To survey SBV infections in field colonies, we sampled 279 *A. mellifera* colonies from 20 apiaries and 38 *A. cerana* colonies from 7 apiaries in Zhejiang Province. These colonies were apparently healthy and strong, and 100 adult worker bees were randomly sampled from each of them. To increase the chances of positive detection of SBV, 39 weak *A. mellifera* colonies and 26 *A. cerana* colonies showing symptoms of honeybee diseases were sampled. The symptoms of honeybee diseases were defined as dead broods being observed in cells and/or significant numbers of dead adult worker bees being observed in the hives or near the entrances. From each of these colonies, 10 larvae were collected. Samples were stored at –80°C until used.

RNA extraction, RT-PCR, and sequencing. RNA extraction and cDNA synthesis (except the cDNA synthesis for the detection of negative-strand RNA) were performed using TRIzol reagent (Adilab, Beijing,

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China) and ReverTra Ace qPCR RT (quantitative PCR-reverse transcription) [Note that I restored qPCR RT since that is likely how the ReverTra Ace product is worded.] master mix (catalog number FSQ201; Toyobo, Shanghai, China), respectively, by following the manufacturers' instructions. The PCR volume of 50 μ l was prepared using 2 \times Taq PCR StarMix (GenStar, Beijing, China), according to the manufacturer's protocol, and the PCR amplification was conducted with an initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and finally 5 min at 72°C, with the primers SB14f and SB15r (SB14f, 5'-AATGGTGC GGCTGGACTATGG-3'; SB15r, 5'-TGATACAGAGCGGCTCGACA-3') (14), amplifying 579 bp of the RNA-dependent RNA polymerase (RdRp) region. Each PCR amplification included a negative control, in which the same volume of H₂O was used instead of template cDNA. The PCR products were analyzed by 2.5% agarose gel electrophoresis, and the positive products were directly sequenced using an ABI 3730XL automatic sequencing system (Ruidi, Shanghai, China). Sequence specificity analysis was performed using BLAST (NCBI).

Phylogenetic analysis. MEGA5 was used to perform the phylogenetic analysis (34). The phylogenetic analysis was conducted with the RdRp gene sequences of SBV-positive isolates obtained in this study and other representative SBV homologous sequences, which were retrieved from the GenBank database. Phylogenetic trees were constructed with the maximum likelihood (ML) method. The bootstrap values were the results of 1,000 replicates, and the values below 50% were omitted.

Inoculation experiments. (i) AcSBV preparation and quantitation. Larvae with overt symptoms of SBV infection were sampled from an *A. cerana* colony; the colony had been confirmed to be infected with SBV but not with the other six common honeybee viruses, namely, *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Deformed wing virus* (DWV), *Israeli acute paralysis virus* (IAPV), and *Kashmir bee virus* (KBV), using RT-PCR with the method described previously (35). The SBV isolate was identified as typical SBV infecting *A. cerana*, namely, AcSBV, based on the alignment of 579-bp RdRp gene sequences. The procedure to obtain concentrated AcSBV was conducted as described by Bailey (9) but with dilution with phosphate-buffered saline (PBS) (pH 7.4 \pm 0.2). Ten larvae were homogenized in 10 ml PBS with a mortar and pestle and then centrifuged for 40 min at 10,000 rpm in a centrifuge (Hitachi, Japan). The supernatant was collected and used for inoculation; 180 μ l of the solution was used for RNA extraction, and RT-PCR was performed again, to confirm the presence of SBV and the absence of the other common viruses described above. Sequencing of the PCR products was conducted to confirm the identity of the virus. The SBV concentration was determined by an absolute quantification assay according to the method described previously (36), with the following modifications. The primers were used for qPCR assays as reported previously (37), and the primers were used to construct recombinant plasmids as reported previously (38). The qRT-PCRs were performed in triplicate in an Eppendorf Mastercycler ep realplex system using SYBR green (Thunderbird SYBR qPCR mix; Toyobo, Shanghai, China). The AcSBV concentration for the subsequent experiments was approximately 4.0×10^5 copies/ μ l.

(ii) Screening for viruses to identify healthy colonies. One hundred adult worker bees collected from *A. mellifera* or *A. cerana* colonies were checked, as a pooled sample, for the presence of SBV and the other six common honeybee viruses mentioned above. The colonies with no detectable viruses were used as the source of honeybee workers and larvae for inoculation.

(iii) Laboratory inoculation of larvae. To determine the effects of AcSBV on the mortality and infection rates of third-instar larvae, which represent the most sensitive stage for SBV infection (39), second-instar larvae of *A. cerana* and *A. mellifera* were carefully transferred to 48-well culture plates containing 150 μ l artificial diet (40) and then were placed in an incubator set at 34 \pm 0.5°C, with 70% \pm 5% relative humidity (Stik, Shanghai, China). The second-instar larvae were obtained as described previously (33). After 24 h, the third-instar larvae of *A. cerana* and *A.*

mellifera were transferred individually to other 48-well culture plates containing 130 μ l artificial diet, as described above. Fifteen microliters of AcSBV solution was added to each well for the experimental groups, and 15 μ l PBS was added for the control groups. After another 24 h, the bee larvae were washed 3 times with ultrapure water and kept at -80°C for later use. Every treatment was tested with 3 replicates, and each replicate contained 20 larvae. The deaths of larvae were confirmed by identifying the color of their bodies changing from white to yellow or even dark gray and then noting no response when the larvae were touched with a soft tip. RT-PCR was performed to confirm whether the larvae were infected with AcSBV (15). In total, 15 positive samples of *A. mellifera* were randomly chosen to verify the identity of strains by sequencing. RNA from another 5 positive samples from each replicate was pooled and used to detect the negative-strand AcSBV RNA.

(iv) Laboratory inoculation of adult bees. Newly emerged bees (*A. mellifera* and *A. cerana*) were individually inoculated with 3 μ l AcSBV preparation (experimental groups) or PBS containing 10% honey (control groups), using a Hamilton syringe to feed the bees. Fifty honeybees per cage were kept at 34 \pm 0.5°C and 70% \pm 5% relative humidity in an incubator (Stik, Shanghai, China) and were fed pollen that had been treated with UV light for 24 h and 50% (vol/vol) sucrose solution. Four treatments, each with 3 replicates, were established. Dead bees were recorded and removed daily until day 10 after inoculation. Four bees were collected from each cage at days 0, 2, 6, and 10 and were used to quantify the AcSBV. RNA from another 6 positive adult workers from days 2, 6, and 10 was pooled and used to detect the negative-strand AcSBV RNA.

(v) Quantification of AcSBV in adult bees by real-time qPCR. The titers of AcSBV in adult bees (*A. mellifera* and *A. cerana*) were quantified by a two-step SYBR green real-time qPCR assay. RNA extraction, cDNA synthesis, and a qPCR assay with a final total volume of 20 μ l were carried out as described above. The cDNA was generated from 1 μ g of total RNA and diluted 1:5. The primer pairs for AcSBV (22) and the reference gene (β -actin) (41) were reported previously. The PCR program was as follows: 1 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melt curve analysis. PCR amplification for each sample was performed in triplicate, to address the variability of the analysis process. The relative titers of AcSBV were calculated according to the $2^{-\Delta\Delta CT}$ method (42).

(vi) Colony inoculation. In order to investigate the ability of AcSBV to infect *A. mellifera* in the field, three *A. mellifera* colonies that were free of SBV infection, as confirmed by RT-PCR, were selected for the inoculation experiment. One liter of sugar syrup mixed with 2 ml AcSBV solution was fed to each colony. At day 5 after feeding, the clinical symptoms of SBV were observed in the colonies, and 20 larvae that were ready to pupate and 20 adult worker bees were randomly collected from each experimental colony. Each larva or bee was checked by RT-PCR for the presence of AcSBV, according to the procedure introduced above (15). PCR products of 5 positive samples were randomly selected to confirm the identity of SBV by sequencing. RNA of 10 positive adult workers and 10 positive larvae from each colony was pooled and used to detect the negative-strand AcSBV RNA.

Tagged RT-PCR for detection of negative-strand AcSBV RNA. Negative-strand (replicative intermediate) RNA has been regarded as an indicator of ongoing replication of positive-strand RNA viruses (43). Therefore, detection of negative-strand RNA is indicative of viral replication. Tagged RT-PCR has been developed to improve the specific detection of negative-strand RNA (44) and has already been used to confirm the replication of honeybee viruses (45–47). The reverse transcription (RT) reaction was performed using the ReverTra Ace RT-qPCR kit (catalog number FSQ101; Toyobo, Shanghai, China), by following the standard protocol with modifications. The reaction was performed at 55°C for 30 min in the presence of the primer Tag-SB7f (5'-agcctgcgcaccgtggGAGATGTTAGAAATACCAACCGATTCC-3' [lowercase letters indicate the Tag primer]); the reaction volume was 20 μ l, with 4 μ g RNA. The RT product was purified with the MinElute reaction cleanup kit (Qiagen,

Germany), according to the manufacturer's instructions, which can avoid false-positive results (48), and then was used to conduct PCR assays using the primer pair Tag (forward primer, 5'-AGCCTGCGCACCGTGG-3') (45) and SB8r (reverse primer, 5'-CCATTAAACAAATCGGTATAAGAGTCCACT-3'). The reaction mixtures were first denatured at 94°C for 30 s and then subjected to 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 5 min. The RT reaction without the primer Tag-SB7f served as the negative control. At the end of the PCR, 5 µl product per reaction was used for analysis on a 2.5% agarose gel.

Data analysis. The normality of data sets was tested using the Shapiro-Wilk test. Data sets with normal distribution were compared with the parametric Student's *t* test. Data sets with nonnormal distribution were analyzed using the lower-power nonparametric Mann-Whitney U test. A chi-square test was performed to compare SBV infection rates in *A. mellifera* and *A. cerana* colonies. Kaplan-Meier survival curves were used to plot and to interpret the survival data. Survival curves were compared using log rank tests. Data are shown as averages ± standard deviations (SDs). *P* values of <0.05 were considered statistically significant.

Nucleotide sequence accession numbers. The genomic sequence of SBV reported in this paper has been submitted to the GenBank database under accession numbers KT205289 to KT205304 and KT734782 to KT734791.

RESULTS

Rates of SBV infection in *A. mellifera* and *A. cerana* colonies. Of the 279 *A. mellifera* colonies and 38 *A. cerana* colonies examined with adult bees, SBV was identified in 29 (10.39%) and 11 (28.95%), respectively, showing a significant difference between species ($\chi^2 = 8.83$; *P* = 0.003). In the *A. mellifera* colonies showing disease symptoms (see "Sample collection for SBV detection" above for the definition), the SBV infection rate was 23.08%; in the diseased *A. cerana* colonies, the infection rate was found to be 100% ($\chi^2 = 37.14$; *P* < 0.001).

Phylogenetic analysis. The evolutionary tree of SBV isolates is presented in Fig. 1, with representative homologous sequences retrieved from the NCBI database and obtained in the survey. As reported by Roberts and Anderson (29) and Grabensteiner et al. (14), the phylogenetic tree of SBV based on RdRp gene sequences had four clades, corresponding to four genotypes of SBV, namely, Asian genotype, New Guinean genotype, European genotype, and South African genotype. *A. mellifera* SBV isolates were clustered with the two previously reported SBV isolates isolated from *A. mellifera* in China (49), forming a separate clade. *A. cerana* SBV strains isolated in this study were clustered with other SBV strains isolated from *A. cerana* in Asian countries. However, it must be noted that two *A. mellifera* SBV isolates were clustered with *A. cerana* SBV isolates and two previously reported *A. mellifera* isolates (GenBank accession numbers JQ390592 and AF284690), but in this case the occurrence rate was only 0.63%.

Mortality and infection rates of larvae after laboratory AcSBV challenge. Infection was identified in 100% of *A. mellifera* and *A. cerana* larvae challenged with AcSBV. No clinical symptoms were observed in *A. mellifera* larvae infected with AcSBV. No significant difference in mortality rates for *A. mellifera* larvae was found between the AcSBV-inoculated larvae (8.3%) and the control group (5.0%) (*U* = 2.500; *P* = 0.346) (Fig. 2A). However, the mortality rate of *A. cerana* larvae (51.7%) challenged with AcSBV was significantly higher than that of *A. cerana* larvae (13.3%) without the AcSBV challenge (*U* = 0; *P* = 0.043) (Fig. 2A).

Survival of inoculated adult bees. Log rank tests indicated that there was no significant difference in survival between *A. mellifera* fed AcSBV and PBS ($\chi^2 = 0.912$; *P* = 0.340) (Fig. 2B). In addition,

AcSBV treatment did not significantly affect the survival of *A. cerana*, compared with the control group treated with PBS ($\chi^2 = 1.210$; *P* = 0.271) (Fig. 2B).

AcSBV proliferation in inoculated adult bees. The genotypes of strains were verified to be AcSBV, and the samples of *A. mellifera* and *A. cerana* used for qRT-PCR analysis were all positive. The proliferation of AcSBV in *A. mellifera* and *A. cerana* is presented in Fig. 3A and B, respectively. In terms of AcSBV in *A. mellifera*, the AcSBV titers on days 2, 6, and 10 showed 6.42 ± 0.73-fold, 259.18 ± 124.34-fold, and 359.39 ± 161.76-fold changes, respectively, compared to the day 0 titer. In addition, there were significant differences in AcSBV titers for day 0 versus day 2 (*U* = 0; *P* < 0.001) and day 2 versus day 6 (*U* = 14.000; *P* = 0.004), but no significant difference was observed for day 6 versus day 10 (*U* = 52.000; *P* = 0.833). Compared with day 0, the titers of AcSBV in *A. cerana* on days 2, 6, and 10 showed 33.63 ± 10.54-fold, 353.25 ± 63.18-fold, and 255.96 ± 74.56-fold changes, respectively. Furthermore, significant differences were found for day 0 versus day 2 (*U* = 12.000; *P* < 0.001) and day 2 versus day 6 (*U* = 2.000; *P* < 0.001), but there was no significant difference between day 6 and day 10 (*t* = 0.952; *P* = 0.353). In addition, it should be noted that the increases in AcSBV titers in *A. mellifera* were less than those in *A. cerana* during the first 6 days after inoculation. Surprisingly, however, the increases in AcSBV titers within 10 days were more substantial in *A. mellifera*.

Infection in *A. mellifera* colonies fed AcSBV. At day 5 after feeding, no clinical symptoms of SBV were observed in the colonies. However, the infection rates for larvae and adult bees in the colonies fed AcSBV solution were found to be 90.5% (54/60 larvae) and 62.2% (37/60 adult bees), respectively (*U* = 2.000; *P* = 0.268).

Presence of AcSBV negative-strand RNA. Figure 4 shows the results of detection of AcSBV negative-strand RNA in *A. mellifera* adult bees and larvae inoculated under laboratory and field conditions. AcSBV negative-strand RNA could be detected in all *A. mellifera* samples randomly collected in the experiments. When the Tag-SB7f primers were omitted from the cDNA synthesis, no false-positive PCR signals occurred. Compared with the samples inoculated under laboratory conditions, the nucleic acid bands (Fig. 4C and D) of samples inoculated under field conditions were much weaker.

DISCUSSION

Most previous studies (31–33) concluded that there was no cross-species infection between AcSBV and AmSBV, based on the pathogenicity in larvae during the course of the experiments. However, an *A. mellifera* isolate, AmSBV-Kor19 (GenBank accession number JQ390592), was found to be distinct from other *A. mellifera* isolates in the genome (26) and clustered together with *A. cerana* isolates in the phylogenetic tree (29). One explanation is that this special *A. mellifera* isolate was the SBV that typically infects *A. cerana* or the SBV was recently transferred from *A. cerana*.

In the present study, most *A. mellifera* SBV isolates were clustered with the two previously reported SBV strains isolated from *A. mellifera* in China (49), forming a separate clade. This suggests a consequence of the climatic conditions of China and the resulting changes in host biology. However, two special AmSBV isolates were found among 38 *A. mellifera* isolates, which were recovered from 318 *A. mellifera* colonies. Similar to AmSBV-Kor19, these two AmSBV isolates were grouped together with *A. cerana* isolates

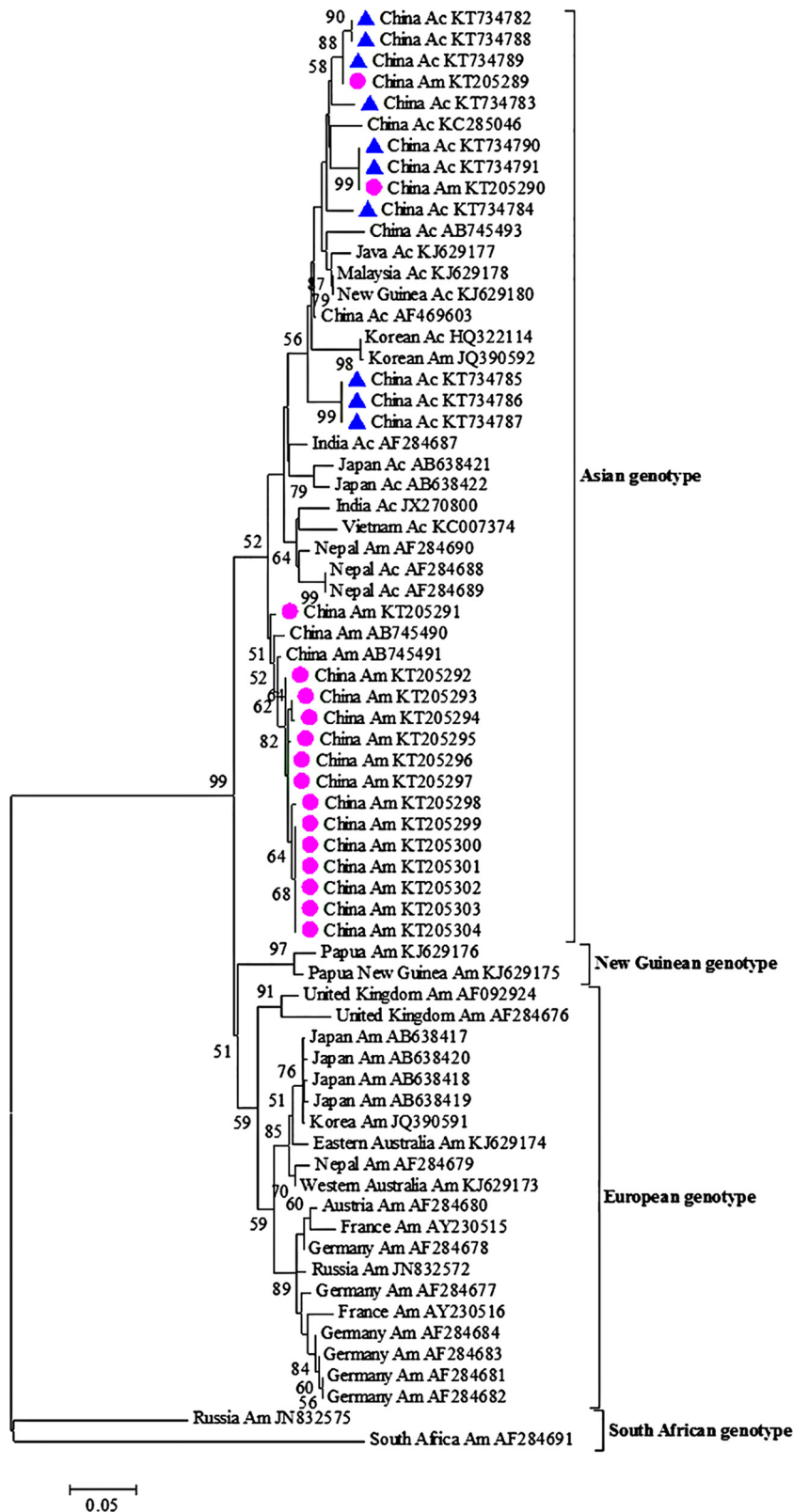


FIG 1 Maximum likelihood consensus tree of SBV isolates. A 386-bp region of the RNA-dependent RNA polymerase gene was used. The tree was constructed with a Tamura 3-parameter distance model. The statistical significance of a particular tree topology is evaluated by bootstrap resampling of the sequences 1,000 times, and bootstrap values of <50% are omitted. Numbers on the nodes indicate clade credibility values. New isolates of *A. mellifera* (circles) and *A. cerana* (triangles) described in this study are indicated. Strains are annotated with respect to country of isolation, virus host, and GenBank accession number. Am, *A. mellifera*; Ac, *A. cerana*.

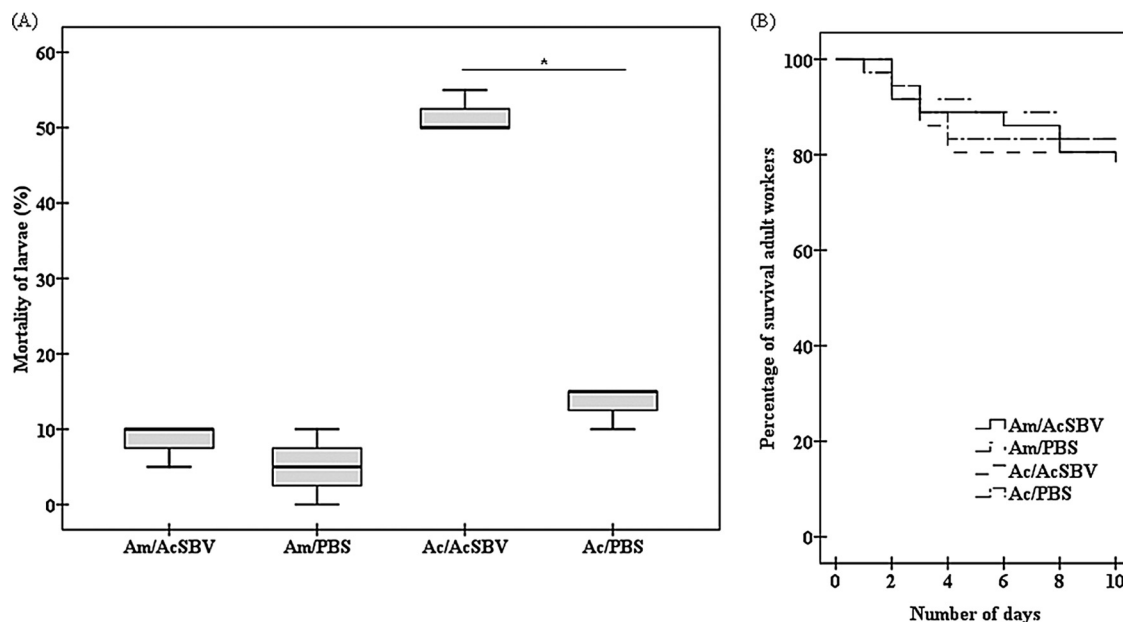


FIG 2 Larval mortality rates at 24 h after inoculation (A) and survival rates of adult workers (B) of *A. cerana* (Ac) and *A. mellifera* (Am) inoculated with AcSBV under laboratory conditions. In panel A, the boxes represent first quartiles and third quartiles, the horizontal lines indicate the median, and the whiskers represent the minimum and maximum for all observed values. Mortality rates of larvae and survival rates of adult workers were calculated from 20 larvae and 50 adult workers per treatment group, respectively. Am/PBS, *A. mellifera* larvae fed PBS; Am/AcSBV, *A. mellifera* larvae infected with AcSBV; Ac/PBS, *A. cerana* larvae fed PBS; Ac/AcSBV, *A. cerana* larvae infected with AcSBV. The data are averages from three replicates. *, $P < 0.05$.

in the phylogenetic tree. These results supported the hypothesis that cross-species infection of AcSBV may occur, but with low occurrence rates.

Our inoculation experiments with AcSBV under laboratory and field conditions confirmed the hypothesis. Unlike previous studies in which only death and pupation of larvae were observed (31–33), we employed another diagnostic method by examining the presence of AcSBV negative-strand RNA. This method allowed us to detect the replication of virus even when the pathogenicity was very low.

Although the *A. mellifera* larvae infected with AcSBV under either laboratory or field conditions had no obvious signs of the disease, as demonstrated in previously reported experiments (31–33), AcSBV negative-strand RNA could be detected in *A. mellifera* larvae (Fig. 4B and D), which suggests that the genome of AcSBV

is able to replicate in *A. mellifera* larvae. However, additional studies must be performed to determine whether a large dose induces clinical disease.

In agreement with previous results (8–10), SBV affects adult bees without obvious signs of the disease, although the virus titers increased significantly. The titers of AcSBV in *A. mellifera* and *A. cerana* adult workers inoculated under laboratory conditions had similar growth trends, increasing significantly in the first 6 days after inoculation and reaching a plateau (Fig. 3A and B). Although the increases in AcSBV titers in *A. mellifera* were less than those in *A. cerana* during the first 6 days after inoculation, the increases in AcSBV titers in *A. mellifera* within 10 days were more substantial. We suspect that, when infecting *A. mellifera*, AcSBV may take time to adjust to the new host during the initial infection phase and then replicates efficiently by an unknown mechanism. This result

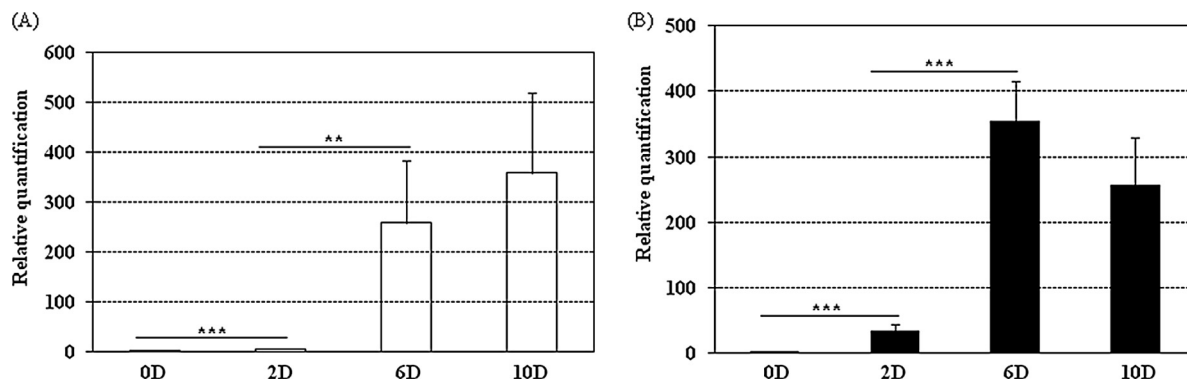


FIG 3 Relative quantification of AcSBV in *A. mellifera* (A) and *A. cerana* (B) adult workers inoculated under laboratory conditions. Twelve bees were collected on days 0, 2, 6, and 10 and used for quantification. D, day. **, $P < 0.01$; ***, $P < 0.001$.

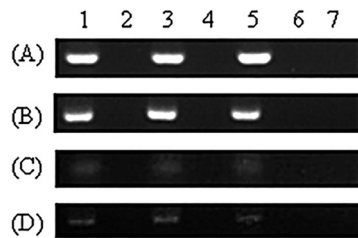


FIG 4 Detection of negative-strand RNA of AcSBV in *A. mellifera* larvae and adult workers. Lanes 1, 3, and 5, samples obtained on days 2, 6, and 10, respectively; lanes 2, 4, and 6, negative controls without Tag-SB7f primers; lane 7, negative control without the DNA template. Lanes 1, 3, and 5 in panel A represent pooled samples of 6 bees from each of 3 replicates on each day; in panels B, C, and D, results of one replicate are shown. (A) Adult worker bees challenged with AcSBV in the laboratory. (B) Larvae challenged with AcSBV in the laboratory. (C) Adult worker bees challenged with AcSBV in a colony. (D) Larvae challenged with AcSBV in a colony.

indicates that AcSBV successfully replicated in *A. mellifera* adult workers. In addition, the AcSBV negative-strand RNA could be detected in *A. mellifera* adult workers inoculated in the laboratory (Fig. 4A) and under field conditions (Fig. 4C). As with the larvae, compared with the samples prepared under laboratory conditions, the nucleic acid bands (Fig. 4C and D) of samples inoculated under field conditions were much weaker. This may be due to the different amounts of virus inoculated into the bees and/or different levels of resistance of the bees to the virus under different conditions. Nevertheless, these results suggest that AcSBV is able to replicate in *A. mellifera* adult worker bees.

Therefore, by quantifying the titers of AcSBV in *A. mellifera* adult worker bees and detecting AcSBV negative-strand RNA in adult worker bees and larvae in artificial inoculation experiments, we concluded that AcSBV is able to infect *A. mellifera*, which is different from previous reports. This is of significance not only for the study of the pathogenicity of SBV but also for the protection of *A. mellifera* and *A. cerana*. In agreement with a previous investigation performed in China by Ai et al. (35), the infection rate of *A. cerana* colonies was much higher than that of *A. mellifera* colonies. With the finding that AcSBV can infect *A. mellifera* colonies, as shown in this study, *A. cerana* colonies serve as a large reservoir of SBV infection for *A. mellifera* colonies. Although the AcSBV infection rate in *A. mellifera* colonies was very low and symptoms were not seen, this effect should not be neglected and efforts should still be made to monitor the dynamic changes of AcSBV in *A. mellifera* colonies. Due to beekeeping practices, the numbers of *A. mellifera* colonies are much larger than those of *A. cerana* colonies in most beekeeping areas in Asia. Therefore, although the AcSBV infection rate in *A. mellifera* colonies is very low (0.63% in this study), the fact that *A. mellifera* colonies serve as a reservoir for SBV infection for *A. cerana* colonies should not be neglected, especially when the two honeybee species are located in close proximity.

Although quantification at the nucleic acid level with the qRT-PCR approach is one of the most common methods used for the quantification of virus, it should be noted that the stringency of this technique may be doubted, since nucleic acids are not necessarily part of an infectious particle (50). Our data on SBV detection in field colonies, virus RNA genome quantification, and negative-strand RNA detection have strongly supported our conclusion, while it remains unclear whether new proteins were

produced and new virus particles were assembled. It would be worthwhile to quantify virus proteins or particles to determine whether new proteins are produced or new particles are assembled. In addition, because *A. mellifera* colonies very commonly are infected by a variety of bee viruses and rarely are infected by only SBV, purified AmSBV was not obtained for cross-species inoculation in our study. The ability of AmSBV to infect *A. cerana* should be studied in the future whenever possible.

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