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

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S **(SBV) is one of the most destructive viruses in the Asian honeybee *A*** **but is much less destructive in *A*** **. In previous studies, SBV isolates infecting *A.*** **(AcSBV) and SBV isolates infecting *A.*** **(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.*** **colonies and 64 *A.*** **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.*** **colonies and 37 *A.*** **colonies were positive for SBV infection. Phylogenetic analysis based on RNA-dependent RNA polymerase (RdRp) gene sequences indicated that *A.*** **isolates and most *A.*** **isolates formed two distinct clades but two strains isolated from *A.*** **were clustered with the *A.*** **isolates. In the artificial-infection experiments, AcSBV negative-strand RNA could be detected in both adult bees and larvae of *A.*** **, although there were no obvious signs of the disease, demonstrating the replication of AcSBV in *A.*** **. Our results suggest that AcSBV is able to infect *A.*** **colonies with low prevalence (0.63% in this study) and pathogenicity. This work will help explain the different susceptibilities of *A.*** **and *A.*** **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

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 μl was prepared using 2× 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′-AAT GGTGCGGTGGACTATGG-3′; SB15r, 5′-TGATACAGAGCGGCTCGA CA-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 H_2O 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 ± 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 μl 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/μl.

(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 μl artificial diet (40) and then were placed in an incubator set at 34 ± 0.5°C, with 70% ± 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 μl artificial diet, as described above. Fifteen microliters of AcSBV solution was added to each well for the experimental groups, and 15 μl 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 μl 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 ± 0.5°C and 70% ± 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 μl were carried out as described above. The cDNA was generated from 1 μg 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′-agcctgcgcaccgtggGGAGA TGTTAGAAATACCAACCGATTCC-3′ [lowercase letters indicate the Tag primer]); the reaction volume was 20 μl, with 4 μg RNA. The RT product was purified with the MinElute reaction cleanup kit (Qiagen,

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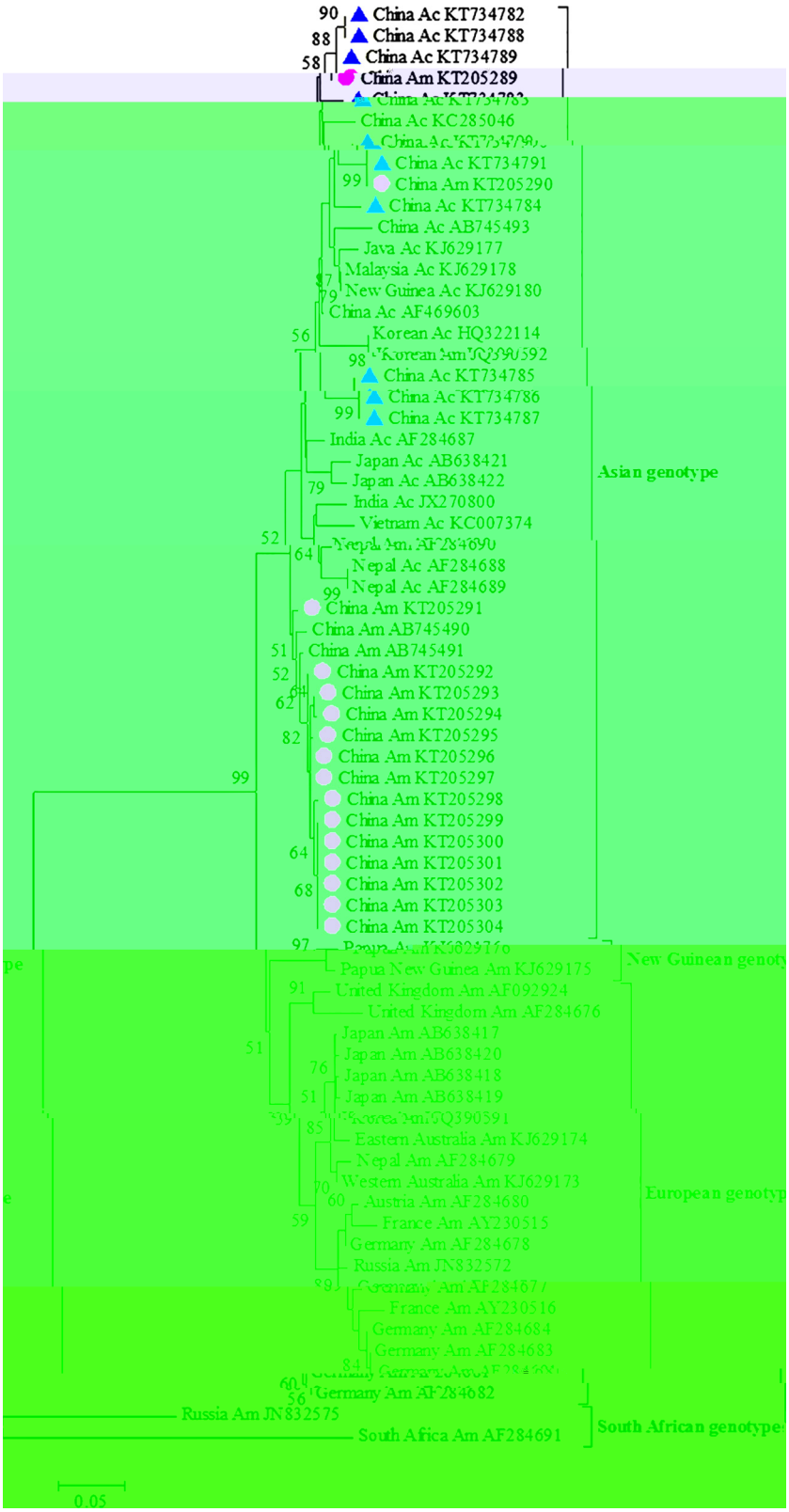

FIG 1 Maximum likelihood consensus tree of SBV isolates. A 386-bp region of the RNA-dependent RNA polymerase gene was used. The tree was constructed with a Tamura 3-parameter distance model. The statistical significance of a particular tree topology is evaluated by bootstrap resampling of the sequences 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*.

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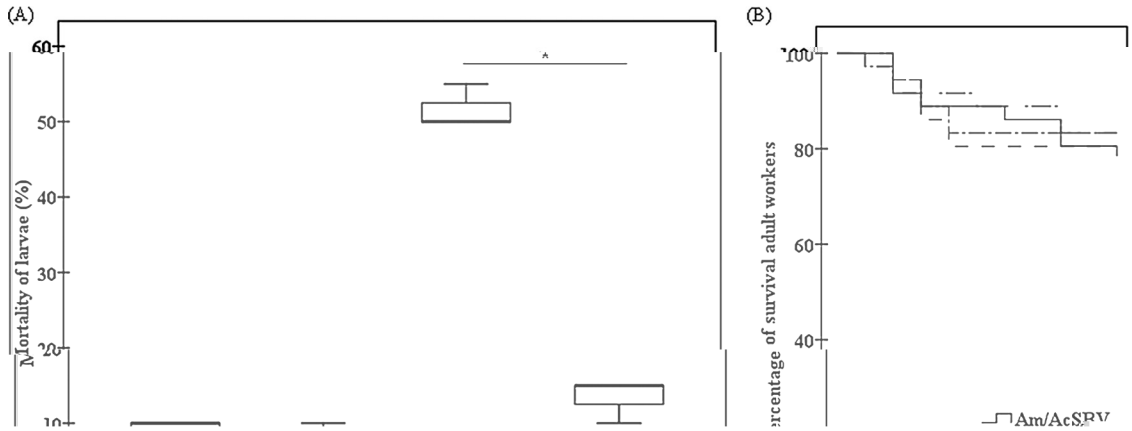

FIG 2 Larval mortality rates at 24 h after inoculation (A) and survival rates of adult workers (B) of *A. cerana* (Ac) and *A. mellifera* (Am) inoculated with AcSBV under laboratory conditions. In panel A, the boxes represent first quartiles and third quartiles, the horizontal lines indicate the median, 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

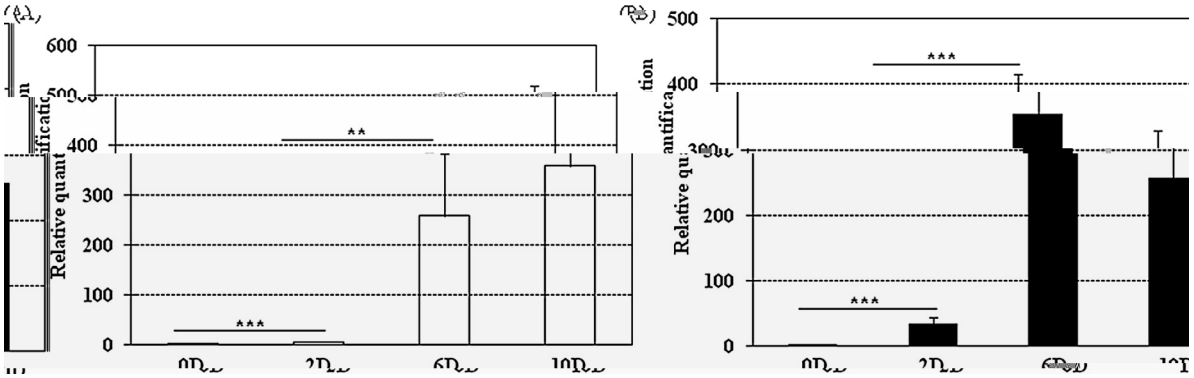

FIG 3 Relative quantification of AcSBV in *A. mellifera* (A) and *A. cerana* (B) adult workers inoculated under laboratory conditions. Twelve bees were collected on days 0, 2, 6, and 10 and used for quantification. D, day. **, $P < 0.01$; ***, $P < 0.001$.

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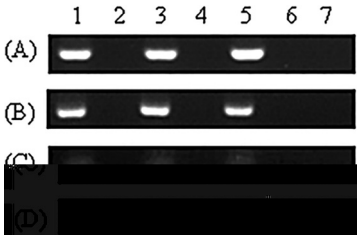

FIG 4 Detection of negative-strand RNA of AcSBV in *A. mellifera* larvae and adult workers. Lanes 1, 3, and 5, samples obtained on days 2, 6, and 10, respectively; lanes 2, 4, and 6, negative controls without Tag-SB7f primers; lane 7, negative control without the DNA template. Lanes 1, 3, and 5 in panel 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.

ACKNOWLEDGMENTS

We thank the three anonymous reviewers for their constructive comments in improving the manuscript.

FUNDING INFORMATION

This work was supported by earmarked funds from the Modern Agroindustry Technology Research System (grant CARS-45) and the Science and Technology Department of Zhejiang Province, China (grant 2012C12906-19).

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