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Genomic characterization and phylogenetic analysis of Chinese sacbrood virus isolated from Loess Plateau, China

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ABSTRACT. The complete genomic RNA of the Chinese sacbrood virus (CSBV) strain, which infects the honeybees in the Loess plateau, was sequenced and analyzed. The CSBV-SX strain contains 8705 nucleotides, which includes a single large open reading frame (99-8681 nucleotides) encoding 2860 amino acids. A novel efficient identification method was used to investigate the samples infected by CSBV. The putative amino acid sequence alignment analysis showed that, except for some normal well characterized domains such as RNA helicase, RNA protease, and RNA-dependent RNA polymerase domains, a calicivirus coat protein domain was identified at amino acids 493-564. Phylogenetic analysis indicated that CSBV-SX was closely related to CSBV-BJ, and this result was supported by nucleotide multiple sequence alignment and protein multiple

sequence alignment analysis results. These differences in the CSBV-SX strain may be related to virus adaptation to the xerothermic, low relative humidity, and strong ultraviolet radiation conditions in the Loess Plateau.

Key words: *Apis cerana*; Chinese sacbrood virus; Genome sequence; Honeybee

INTRODUCTION

Honeybees (*Apis cerana cerana* Fabricius) are important pollinators of agricultural and landscape plants cultivated worldwide, and are irreplaceable for their contribution to our lives (Winston, 1987; Martin, 2001). However, the health and vitality of honeybees is threatened by various pathogenic microorganisms, including fungi, bacteria, nematodes, parasitic mites, protozoa, and viruses (Bailey and Ball, 1991; Ellis and Munn, 2005). Among these disease agents, viruses can lead to a major economic consideration disease in apiculture.

Sacbrood virus (SBV) is one of the most common and widespread of the 19 viruses known to infect honeybees (Chen and Siede, 2007; Neumann and Carreck, 2010; VanEngelsdorp et al., 2010). SBV infects both larvae and adult bees, and there are no disease symptoms in SBV-infected adult bees (Berényi et al., 2007). The color of the SBV infected larvae change from pearly white to pale yellow, and fail to pupate, with ecdysial fluid accumulating beneath their unshed skin, forming a sac (Bailey, 1975). Sacbrood virus was first described in 1913, but it was not characterized until 1964 (Bailey et al., 1964). SBV is generally referred to as picornavirus for its biophysical properties and the presence of an RNA genome, but it has been reclassified into the genus *Iflavirus*, which contains linear positive single-stranded RNA viruses (Moore et al., 1985; Mayo, 2002; Baker and Schroeder, 2008). This RNA genome is monopartite monocistronic, with structural genes arranged at the 5' end and non-structural genes at the 3' end. The SBV particles are 28 nm in diameter and are non-enveloped, round, and featureless in appearance (Brcak and Kralik, 1965; Bailey, 1968).

The complete genomic sequence of SBV-UK was first determined by Ghosh et al. (1999). The Chinese SBV (CSBV) sequence was first determined in 2001, and 4 CSBV sequences have been reported, including CSBV-GZ, CSBV-LN, CSBV-BJ, and CSBV-FZ. CSBV is similar to SBV-UK in its physiological and biochemical features, but differs in antigenicity and do not show cross-infection. Sequence analysis indicated that CSBV has some differences, but is highly homologous to SBV-UK (Zhang et al., 2001). The CSBV genome is composed of a positive single-stranded RNA, which encodes for 4 structural proteins.

China includes a large amount of land, crossing nearly 63° in longitude and 50° in latitude; diverse landforms can be found in this range. There are large differences between the north and south in terms of climate as well as different landforms in China. These geographic variances or climate changes lead to differences between the different CSBV isolates reported in China. The CSBV reported in this study was isolated from the Loess Plateau, which has xerothermic climate, low relative humidity, and strong ultraviolet radiation. Therefore, studies of CSBV-SX provide information regarding the genetic background of SBV.

MATERIAL AND METHODS

Sample collection

Infected honeybees (*A. cerana*) used in this study originated from Yulin in northwest Shaanxi Province, China. The larvae were collected from sacbrood outbreaks in May 2012 and stored frozen at -80°C until use. The CSBV-SX sequence described in this study was submitted to GenBank under accession No. KJ000692.

Identification of CSBV with gradient band polymerase chain reaction (PCR)

Viral RNA was extracted from infected honeybees using RNAiso plus (TaKaRa, Shiga, Japan) according to the manufacturer instructions and digested with RNase-free DNase set (TakaRa). Synthesis of first-strand DNA complementary to the total RNA was carried out using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with oligo(dT) primers and random primers according to the manufacturer instructions.

All samples were first tested by nested-PCR with 4 pairs of outer primers and followed by 4 pairs of inner primers to confirm CSBV infection (Table 1). Another 5 pairs of primers (Table 1) located at 5 different regions of the CSBV genome were designed to produce increasing sizes of PCR products from 250 to 1500 bp. These primers would result in a gradient band version for fast identification of CSBV. rTaq DNA polymerase (TaKaRa) was used to amplify the products according to the manuscript in a S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). All products were sequenced and blasted to verify the accuracy of identification.

PCR amplification of viral RNA

To amplify the internal region of the CSBV-SX genome, 8 primer pairs (Table 1) were designed based on the sequences of CSBV-GZ and SBV-UK. cDNAs were synthesized as described above. Next, 1 μL each cDNA was PCR-amplified using HS Taq (TaKaRa) according to instructions. The amplified products were purified using a TIANgel Midi Purification Kit (Tiangen, Beijing, China) and ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) by T-A ligation. For each part, 5 clones were selected for sequencing.

The 5'RACE and 3'RACE procedure was performed using the 5'-Full RACE Kit and 3'-Full RACE Core Set Kit (TaKaRa) with 1 mg purified total RNA isolated from SBV-infected honeybee larvae. The amplified products were purified and ligated into the pGEM-T easy vector. Ten clones were selected for sequencing.

Nucleotide sequencing and analysis

The nucleotide sequence of each fragment was assembled to build a continuous complete sequence using the SeqMan program. Multiple nucleotide and deduced amino acid sequence alignments were performed using published SBV sequences. A phylogenetic tree was constructed using the MEGA 5.05 package and the neighbor-joining method (Saitou and Nei, 1987) with Kimura's 2-parameter model (Kimura, 1980), and bootstrap values were based on 500 replicates.

Table 1. Primers used to identify the virus and obtain the complete nucleotide sequence of CSBV-SX.

Primer	Sequence (5'-3')	Location	PCR products (bp)
Nested PCR primers			
CSBV1O-F	CAAGGTGGCTTCAGAAATAGCT	520-541	351
CSBV1O-R	GCGTGAGTTGACAGAAAATC	852-871	
CSBV1I-F	AGTATATTGGTGCAATTTGT	549-568	297
CSBV1I-R	GATGTAATATTGTGCTAAAA	826-845	
CSBV2O-F	CCTAGTGTGAATCCGAGTA	2871-2889	430
CSBV2O-R	TTGCACGCTCAGGAGTTACT	3281-3300	
CSBV2I-F	GTACCATTTGATACTGAAAA	2901-2920	369
CSBV2I-R	CATATTACACAACGTACTAA	3250-3269	
CSBV3O-F	GATTGTTACTTTTCTGA	5961-5977	410
CSBV3O-R	AATGTTCTAGTTGTTCGCCT	6351-6370	
CSBV3I-F	AACGGTATAAGGAGGAAAA	6004-6023	321
CSBV3I-R	TAGGAACGAACTCAACACGC	6305-6324	
CSBV4O-F	GCCCATTACCGTGGTGATA	8141-8159	400
CSBV4O-R	TAAATCAGCACATTCCCATA	8481-8500	
CSBV4I-F	AGTAAAGAAAGAGGGCAAAC	8214-8233	253
CSBV4I-R	GTGGATACACTTCGTGGG	8449-8466	
Gradient bands PCR primers			
CSBV250-F	AGTAAAGAAAGAGGGCAAAC	8068-8087	250
CSBV250-R	GTGGATACACTTCGTGGG	8300-8317	
CSBV500-F	GCGTGTTGAGTTCGTTCCTAGTGG	7520-7543	500
CSBV500-R	TTGGGGCCAACAATAAGGACCCT	7997-8019	
CSBV750-F	GTACGGAGAGGCCCCACTTT	1025-1044	750
CSBV750-R	CTTCTAAACTGGGTCCTTCCCAA	1752-1774	
CSBV1000-F	CCAGGTGTATTACTATCCGCC	4120-4140	1000
CSBV1000-R	TCTAGATAAGATTGGAGAGGCAC	5097-5119	
CSBV1500-F	CCCAACGGTATAAGGAGGAA	6000-6019	1500
CSBV1500-R	AATACTGTATATCAGGAAAGCCA	7478-7500	
RACE analysis primers			
5RACEouter	TTCCTTAGAACTTTGCTGTGTAGCG	626-650	
5RACEUinner	TTCCTTCTGCGGTCTTAATTGACACTGCACGTCTAA	448-483	
3RACEDouter	TGTACCAACAGAAGTGTGGGTC	8107-8128	
3RACEDinner	AGAATTGTTCTGCTACGGTGATGATTTGATAATGTC	8258-8293	
Genome partial sequence primers			
CSBVP1-F	TCGAGATTTACCTTGACGG	119	1500
CSBVP1-R	CACTCTTACCTTTCCCATG	1482-1500	
CSBVP2-F	TTATCAAGCTGATAATATAA	1001-1020	1500
CSBVP2-R	GATACTTGGGTGTCCAAAGA	2481-2500	
CSBVP3-F	AAGGCAGATAATGTTAGTAA	2001-2020	1500
CSBVP3-R	AGCCTCCTTAACCGCTGTA	3482-3500	
CSBVP4-F	TGGTTGTAACACCTGTA	3001-3017	1500
CSBVP4-R	GCTGGTATAATTCCGAAA	4483-4500	
CSBVP5-F	AACATCGGCTACGATACA	4001-4081	1500
CSBVP5-R	CCCATAATTAATGCACGCC	5482-5500	
CSBVP6-F	GATGTTACGACTGGGTT	5001-5017	1500
CSBVP6-R	CACACGGCCCGCGGGTTTAT	6481-6500	
CSBVP7-F	CCCAACGGTATAAGGAGGA	6001-6019	1500
CSBVP7-R	AATACTGTATATCAGGA	7484-7500	
CSBVP8-F	GGGTCGAGGTGAGGGAT	7001-7017	1740
CSBVP8-R	ATATGAGACCTTAAAAAACA	8721-8740	

RESULTS

Gradient band reverse transcription-PCR analysis

All samples were first tested by nested-PCR with 4 pairs of outer primers, followed by another 4 pairs of inner primers (Table 1). Four clear bands of expected size were observed with the first round of PCR amplification using the outer primers (Figure 1A). In the second

round of PCR amplification using the 4 pairs of inner primers, another 4 bands of predicted size were observed (data not shown). All PCR products were sequenced and blasted, revealing high identity with CSBV-BJ, indicating that our samples were CSBV-infected and the bands were from the CSBV genome.

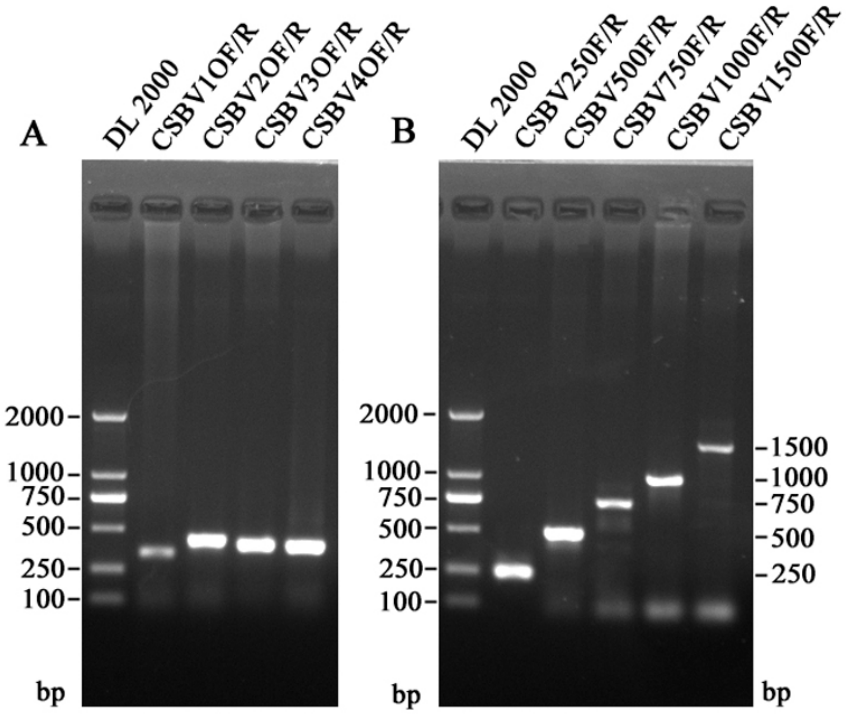

Figure 1. SBV-infected sample identified by reverse transcription-PCR. **A.** PCR products with outer primers of nested PCR electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized under UV light; **B.** representative PCR products separated by 1% agarose gel electrophoresis as a stair-step graph by gradient band PCR. *Lane*: DL 2000, DNA ladder 2000 (TaKaRa).

To investigate the CSBV infection in a more efficient manner, an additional 5 pairs of primers were used to amplify 5 parts located in 5 different regions of the SBV genome were used (Table 1). With the amplification of these gradient band primers, 250-, 500-, 750-, 1000-, and 1500-bp PCR products were presented in each lane as gradient up-steps, which were in similar to the predicted sizes with each pair of primers, respectively (Figure 1B). This gradient band phenomenon is strong evidence for SBV-positive samples. Sequencing was conducted to confirm these results.

Nucleotide sequence analysis

The complete genome of CSBV-SX was composed of 8705 bp, excluding the poly A tail. The base composition of CSBV-SX was A (29.73%), C (16.32%), G (24.53%), and U (29.39%). The CSBV-SX genome contained a single large open reading frame encoding

2860 amino acids, starting at nucleotide 99 and ending with a stop codon TAG at nucleotide 8681. Multiple sequence comparison showed that CSBV-SX was genetically closely related to CSBV-GZ (93.2%) and was closest to CSBV-BJ (97.5%) (Table 2).

Table 2. Homology (%) analysis between CSBV-SX and other referenced virus strains.

	SBV-UK	CSBV-BJ	CSBV-GZ	CSBV-LN	SBV-Kor19	SBV-Kor21	SBV-Korean
Nucleotide sequence	88.9%	97.5%	93.2%	90.9%	90.5%	88.3%	90.8%
Putative amino acid sequence	95.5%	98.8%	96.3%	96.0%	95.1%	94.6%	95.4%

Multiple sequence alignment showed that the 5' sequence of CSBV-SX was similar to that of the SBV-UK strain. Alignment of the region showing the largest number of differences, from 3160 to 3205 nucleotides, showed that the CSBV-SX strain contained a sequence that was similar to those in the CSBV-BJ, CSBV-GZ, or SBV-Kor21 strains. Compared with SBV-Korean or SBV-Kor19, CSBV-SX contained a region that was 34-nucleotides longer. An additional 30-nucleotide addition was observed compared with CSBV-LN, SBV-K5B, and SBV-K1A. In this region showing variability in sequence length, CSBV-SX was 3 nucleotides longer than SBV-UK (Figure 2).

Figure 2. Alignment of nucleotide sequences of the reported SBV strains at the region located between nucleotides 3160 and 3205, which was the most different region in the genome alignment.

Amino acid sequence analysis

The BLAST result of the predicted amino acid sequence for CSBV-SX showed that an RNA-dependent RNA polymerase domain (located at amino acids 2504-2795), 3 picornavirus capsid protein domain (located at amino acids 213-388, 494-681, and 791-983), and an RNA helicase motif (located at amino acids 1377-1486) were found in this deduced amino acid sequence. Interestingly, a calicivirus coat protein motif was found at amino acids 439-564, which was the first time this motif has been identified in the SBV encoded protein. These results indicate that structural proteins are located in the N-terminal region, while nonstructural proteins are located in the C-terminal region.

The predicted amino acid sequences for CSBV-SX and other strains were then aligned and compared as described above. Multiple sequence comparison showed that the deduced CSBV-SX amino acid sequence was closely related to CSBV-GZ (96.3%) and most closely related to CSBV-BJ (98.8%) (Table 2). This result was supported by our nucleotide sequence comparison results. Analysis of the structural protein regions indicated that the length of the amino acid sequence of CSBV-SX was similar to those of CSBV-BJ, CSBV-GZ, and SBV-Kor21 from 693 to 762 amino acids, which was 1 amino acid longer than that of SBV-UK in this region and 13 amino acids longer than CSBV-LN (Figure 3).

Figure 3. Alignment of the putative amino acid sequences regions between amino acids 693 and 762 of the SBV strains.

Next, we determined the homology of the deduced amino acid sequences for alignment analysis focused on the conserved domains. As a representative of the *Iflaviridae* family, deformed wing virus and *Varroa destructor* virus were selected with SBV strains for comparison, and acute bee paralysis virus, Kashmir bee virus, *Drosophila* C virus, black queen cell virus, *Plautia stali* intestine virus, and *Phopalosiphum padi* virus were used to represent the *Dicistorviridae* family. In the alignment, conserved RNA-dependent RNA polymerase domains were identified in amino acid sequences between amino acids 2496 and 2674 in the *Iflaviridae* family and from amino acid position 1300 to 1748 in the *Dicistroviridae* family (Figure 4). The RNA helicase domains, which contained the highly conserved amino acids GxxGxGKS and Qx5DD (Koonin and Dolja, 1993), were located between amino acids 1348 and 1574 in the *Iflaviridae* family (Figure 5). These positions were found between amino acids 431 to 690 in the *Dicistorviridae* family (Figure 5). The GxCG and GxHxxG domains were identified within the protease domain in the deuced amino acid sequences of the viruses. These motifs were found between amino acids 2219 and 2342 in the *Iflaviridae* family, and from amino acids 1050 to 1430 in the *Dicistroviridae* family (Figure 6).

Phylogenetic analysis

Phylogenetic analysis was conducted using complete CSBV-SX genome sequences to determine the probable genetic relationships among the virus strains (Figure 7). The viruses were segregated into 2 groups in the phylogenetic tree according to their taxonomic

classifications (*Iflavirus* and *Dicistroviridae*). The phylogenetic tree showed that CSBV-SX was classified onto the same branch as the CSBV-BJ strain, and next to the branch containing the CSBV-LN strain, which was slightly farther away from the branch containing the SBV-UK and SBV-Kor21 strains.

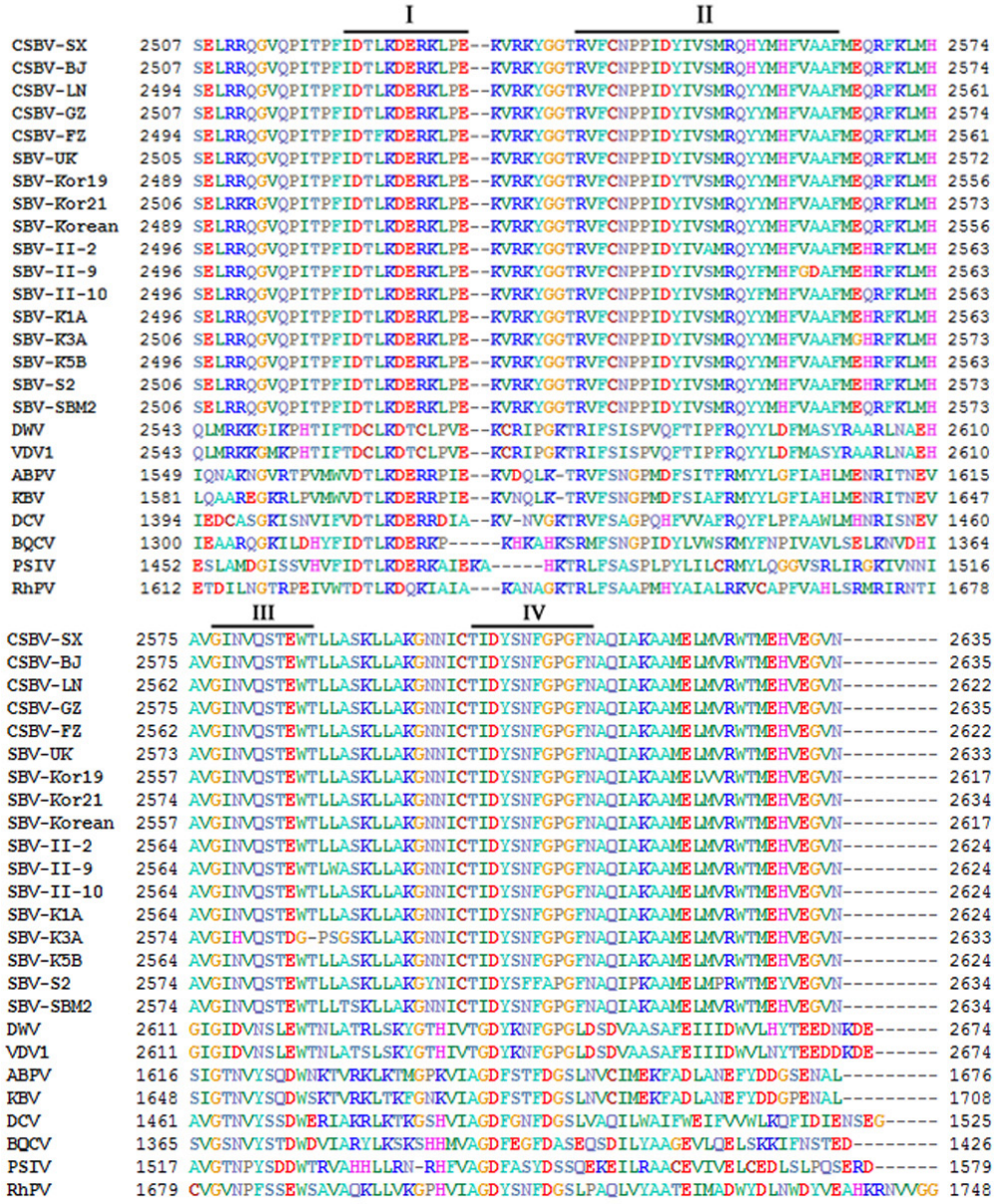

Figure 4. Alignment of the putative RNA-dependent RNA polymerase domains (RdRp) from all selected virus strains. The motifs identified as RdRp are lined with I-IV.

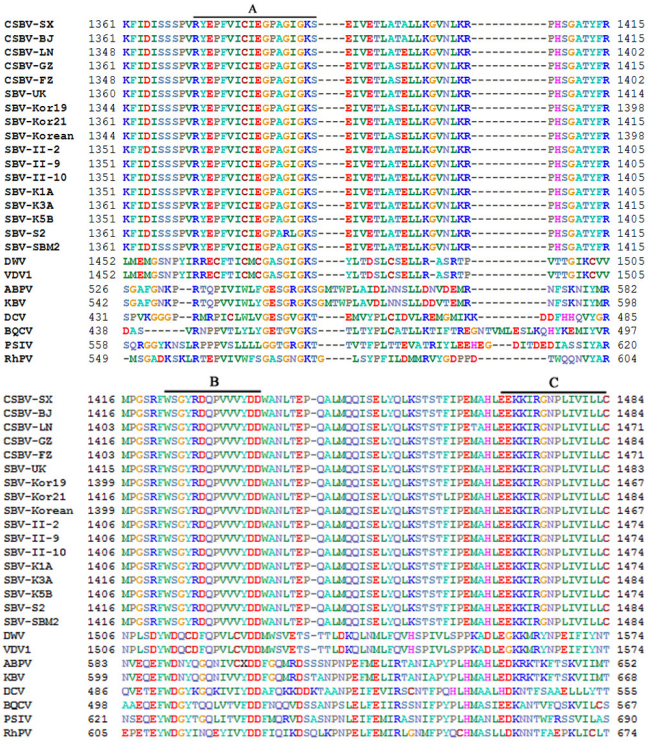

Figure 5. Alignment of the putative RNA helicase domain from all selected virus strains. The motifs identified as RNA helicase domain are lined with A, B, and C.

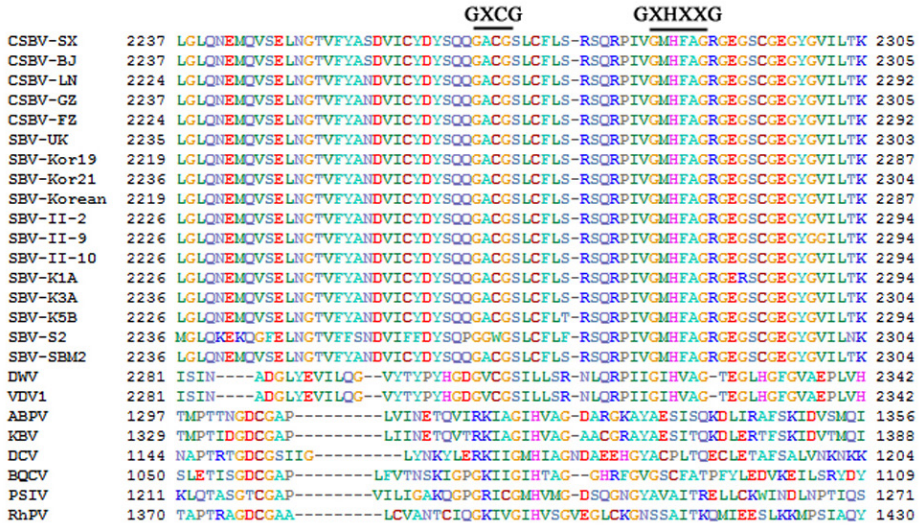

Figure 6. Alignment of the putative RNA protease domain from all selected virus strains. GxCG and GxHxxG motifs were lined in the predicted domain.

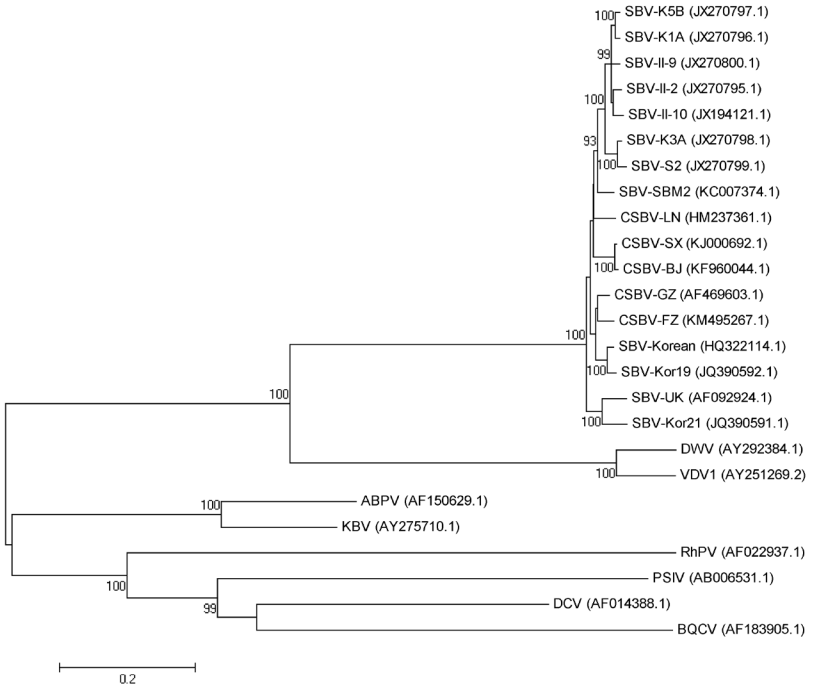

Figure 7. Complete genome sequences of CSBV-SX strain and other complete genome sequences from the GenBank database were used to construct a phylogenetic tree. The GenBank accession Nos. of each virus are shown.

DISCUSSION

In the present study, a novel gradient band reverse transcription-PCR method was used to identify CSBV infection in *A. cerana* larvae, and the nucleotide sequence of this CSBV was determined. The CSBV-SX genome were monopartite monocistronic and contained a single large open reading frame staring at nucleotide 99 and terminating with a stop codon at nucleotide 8681. Nucleotide alignment analysis and phylogenetic analysis indicated that the CSBV-strain was most closely related to the CSBV-BJ strain. The genomic organization of CSBV-SX was similar to that of the *Iflavirus* family with structural proteins at the 5' end and non-structural proteins at the 3' end.

Reverse transcription-PCR was successfully used to identify the RNA genomic virus infection, and nested or seminested PCR was used in these identification procedures (Grabensteiner et al., 2001; Choe et al., 2012; Reddy et al., 2013). For nested PCR or seminested PCR, at least 2 rounds of PCR amplification were needed to complete and verify the identification, which were followed by nucleotide sequencing. The gradient band PCR method used in this study was used to identify SBV infection in a rapid manner. The samples infected with SBV exhibited a stair-step graph, with the PCR products separated by agarose gel electrophoresis (Figure 1). Only one round of PCR amplification was required using this novel identification method. This method did not require the PCR products to be sequenced,

as it is nearly impossible for infection by another virus infection to lead to a similar stair-step graph.

CSBV-SX contained a base composition very similar to that of other SBV strains, including CSBV-BJ, CSBV-GZ, SBV-UK, and SBV-19 (Ghosh et al., 1999; Grabensteiner et al., 2001; Zhang et al., 2001; Chen et al., 2006; Mingxiao et al., 2011; Choe et al., 2012). Genomic alignment analysis showed that CSBV-SX shared high homology with other SBV strains (88.3-97.5%), and that it was classified into the same group as the CSBV-BJ strain in the complete genomic phylogenetic analysis.

Many well-characterized domains were identified in the CSBV-SX amino acid sequence, such as an RNA helicase domain, RNA protease domain, and RNA-dependent RNA polymerase domain (Koonin and Dolja, 1993; Choe et al., 2012). However, a newly calicivirus coat protein domain located at amino acids 493-564 was identified during BLAST analysis. Whether the protein structure was changed remains unknown. These results indicate that the putative amino acid sequence of CSBV-SX contains universal properties included in other SBV strains, which also includes some unique properties.

In summary, a new strain of SBV was identified and characterized in this study. Additionally, using the gradient band PCR method established in this study, we found that this method is efficient, and it can be applied to identify various types of RNA virus infection. The CSBV-SX strain showed high homology with other SBV strains, including a calicivirus coat protein domain-encoding sequence, which was first reported in the SBV genome. This may be because the virus adapted to the unique climate in the Loess plateau, which is xerothermic, has low relative humidity, and has strong ultraviolet radiation.

Conflicts of interest

The authors declare no conflict of interest.

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