Genetic and phylogenetic analysis of Chinese sacbrood virus isolates from infected *Apis mellifera* (#36235)

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Genetic and phylogenetic analysis of Chinese sacbrood virus isolates from infected *Apis mellifera*

Ming Li [Equal first author, 1], **Li Sun** [Equal first author, 1], **Dongliang Fei** [1], **Mingxiao Ma** [Corresp. 1]

[1] College Animal Husbandry and Veterinary, Jinzhou Medical University, Jinzhou, Liaoning, China

Corresponding Author: Mingxiao Ma
Email address: lnjzmmx@163.com

Background. Sacbrood virus (SBV) is one of the most pathogenic honey bee viruses that exhibits host specificity and regional variations. The SBV strains that infect the Chinese honey bee *Apis cerana* are called Chinese SBVs (CSBVs).

Methods. In this study, a CSBV strain named AmCSBV-SDLY (GenBank accession no. MG733283) that infects *A. mellifera* was identified using electron microscopy, polyacrylamide gel electrophoresis, and agar gel immunodiffusion assays. To investigate phylogenetic relationships of the CSBV isolates, a phylogenetic tree of the complete ORF, of the CSBV sequences was constructed using MEGA 5.0, then the similarity and recombinant events of the CSBV isolated strains were analyzed using SimPlot and RDP4 software, respectively.

Results. Sequencing results revealed the complete 8,794-nucleotide long complete genomic RNA of the strain, with a single large open reading frame (189-8,717) encoding 2,843 amino acids. Comparison of the deduced amino acid sequence with the SBV/CSBV reference sequences deposited in the GenBank database identified helicase, protease, and RdRp domains; the structural genes were located at the 5′ end, whereas the non-structural genes were found at the 3′ end. Multiple sequence alignment showed that AmCSBV-SDLY had a 17 amino acid (aa) and a single aa deletion at positions 711-729 and 2,128, respectively, as compared with CSBV-GD-AF469603.1 and a 16 aa deletion (positions 711-713 and 715-728) as compared with AmSBV-UK-AF092924.1. However, AmCSBV-SDLY was similar to the CSBV-JL2014-KU574661.1 strain, which infects *A. cerana*. AmCSBV-SDLY ORF shared 92.4%-97.1% identity with the genomes of other CSBV strains (94.5%-97.7% identity for deduced amino acids). AmCSBV-SDLY was least similar (89.5%-90.4% identity) to other SBVs but showed maximum similarity with the previously reported CSBV-FZ-KM495267.1 strain. The phylogenetic tree constructed from AmCSBV-SDLY and 43 previously reported SBV/CSBV sequences indicated that SBV/CSBV strains were clustered according to the host species and country of origin; AmCSBV-SDLY clustered with other previously reported Chinese and Asian strains (named as the AC genotype SBV, as these strains originated from *A. cerana*) but was separate from the SBV genomes originating from Europe (named as the AM genotype SBV, originating from *A. mellifera*). SimPlot graph of SBV genomes confirmed the high variability, especially between the AC genotype SBV and AM genotype SBV. This genomic diversity may reflect the adaptation of SBV to specific hosts, ability of CSBV to cross the species barrier, and the spatial distances that separate CSBVs from other SBVs.

Title page

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Authors: Ming Li[#], Li Sun[#], Dongliang Fei, Mingxiao Ma[*]

Corresponding Author: Dr. Mingxiao Ma. E-mail: lnjzmmx@163.com

[#] **Co-author:** Li Sun

Author's Institution: College Animal Husbandry and Veterinary, Jinzhou Medical University, Jinzhou, China

First Author: Ming Li

Full address: No. 40, section 3 Songpo Road Jinzhou Liaoning Province 121001, China

Tel.: +86 18904965696

Fax: +86 04164673528

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Ming Li[#],Li Sun[#], Dongliang Fei, Mingxiao Ma[*].
College Animal Husbandry and Veterinary, Jinzhou Medical University, Jinzhou, China
*These authors contributed equally to this study
lnjzmmx@163.com(MMX)

ABSTRACT

Background. Sacbrood virus (SBV) is one of the most pathogenic honey bee viruses that exhibits host specificity and regional variations. The SBV strains that infect the Chinese honey bee *Apis cerana* are called Chinese SBVs (CSBVs).

Methods. In this study, a CSBV strain named AmCSBV-SDLY (GenBank accession no. MG733283) that infects *A. mellifera* was identified using electron microscopy, polyacrylamide gel electrophoresis, and agar gel immunodiffusion assays. To investigate phylogenetic relationships of the CSBV isolates, a phylogenetic tree of the complete ORF, of the CSBV sequences was constructed using MEGA 5.0, then the similarity and recombinant events of the CSBV isolated strains were analyzed using SimPlot and RDP4 software, respectively.

Results. Sequencing results revealed the complete 8,794-nucleotide long complete genomic RNA of the strain, with a single large open reading frame (189-8,717) encoding 2,843 amino acids. Comparison of the deduced amino acid sequence with the SBV/CSBV reference sequences deposited in the GenBank database identified helicase, protease, and RdRp domains; the structural genes

were located at the 5′ end, whereas the non-structural genes were found at the 3′ end. Multiple sequence alignment showed that AmCSBV-SDLY had a 17 amino acid (aa) and a single aa deletion at positions 711-729 and 2,128, respectively, as compared with CSBV-GD-AF469603.1 and a 16 aa deletion (positions 711-713 and 715-728) as compared with AmSBV-UK-AF092924.1. However, AmCSBV-SDLY was similar to the CSBV-JL2014-KU574661.1 strain, which infects *A. cerana*. AmCSBV-SDLY ORF shared 92.4%-97.1% identity with the genomes of other CSBV strains (94.5%-97.7% identity for deduced amino acids). AmCSBV-SDLY was least similar (89.5%-90.4% identity) to other SBVs but showed maximum similarity with the previously reported CSBV-FZ-KM495267.1 strain. The phylogenetic tree constructed from AmCSBV-SDLY and 43 previously reported SBV/CSBV sequences indicated that SBV/CSBV strains were clustered according to the host species and country of origin; AmCSBV-SDLY clustered with other previously reported Chinese and Asian strains (named as the AC genotype SBV, as these strains originated from *A. cerana*) but was separate from the SBV genomes originating from Europe (named as the AM genotype SBV, originating from *A. mellifera*). SimPlot graph of SBV genomes confirmed the high variability, especially between the AC genotype SBV and AM genotype SBV. This genomic diversity may reflect the adaptation of SBV to specific hosts, ability of CSBV to cross the species barrier, and the spatial distances that separate CSBVs from other SBVs.

69

70 Introduction

71 Sacbrood virus (SBV) is one of the most pathogenic honey bee viruses that
72 infects larvae and causes larval death. SBV is also known to infect adult bees and
73 reduce the production capacity of honey. At present, SBV has been globally found
74 (Allen et al.,1996; Berenyi et al.,2006; Ellis et al.,2005). SBV is a single positive-
75 stranded picornavirus of genus *Iflavirus* that encodes one large open reading frame
76 (ORF), with structural genes located at the 5′ end and nonstructural genes at the 3′
77 end (Chen et al.,2006). The complete genomic sequence of AmSBV-UK-
78 AF092924.1 (in this study, we refer to the previously reported SBV/CSBV strains
79 as "strains-GenBank accession numbers") was first determined by Ghosh et al.(
80 Ghosh et al., 1999) .

81 The SBV strains that infect the ~~Chinese~~ honey bee *Apis cerana* are called
82 CSBVs. There is no distinct difference between the nonstructural proteins of SBV
83 and CSBV; however, structural protein VP1 has the highest variation in the amino
84 acid sequence among the proteins from different SBV strains (Cheng et al.,2011) .
85 SBV has been divided into two major groups (AC genotype SBV originating from
86 *A. cerana* and AM genotype SBV from *A. mellifera*) based on the SBV VP1
87 sequence (Mingxiao et al,2013). Furthermore, the AC genotype may be divided
88 into subgroups. The differences between the AC and AM genotypes may result
89 from the adaptation of the virus to different hosts and the existence of different

subgroups of the AC genotype based on regional variations(Choe et al.,2012;Grabensteiner et al.,2001;Mingxiao et al,2013). The AC genotype SBV strains were mainly isolated from Asian countries, and their hosts include *A. cerana* (Dali et al.,2012; Guanghua et al.,1979; Kondreddy et al.,2017; Mingxiao et al.,2011; Nguyen et al.,2016; Reddy et al.,2016; Se E. et al.,2012; Xia et al.,2015; Ying et al.,2016; Yu et al.,2013; Zhang et al.2001;). But AM genotype SBV persists in the bee colony and may cause infection of *A. mellifera* (Allen et al.,1996; Berenyi et al.,2006; Ellis et al.,2005; Ian et al.,2016; Khaliunaa et al.,2016; Suresh et al.,2016). The AC genotype isolated from China, infecting the Chinese honey bee *A. cerana* was named as CSBV (Dali et al.,2012; Guanghua al.,1979; Hanrong et al.,2000;Mingxiao et al.,2011,2013; Ying et al.,2016;Zhang et al.,2001),ecent studies have shown that CSBV has no association with natural infections of *A. mellifera.*

Among the AC genotype SBV strains, the first genome sequence was obtained for CSBV-GD-AF469603.1 (Zhang et al.,2001), and subsequently other CSBV genomes, including CSBV-FZ-KM495267.1, CSBV-JL2014-KU574661.1, CSBV-LN2009-HM237361.1, CSBV-SXYL-KU574662.1, and CSBV-BJ2012-KF960044.1, were sequenced. CSBV is similar to AmSBV-UK-AF092924.1 in terms of its physiological and biochemical features; however, the viruses differ in their antigenicity and do not show cross infection (Cheng et al.,2011; Choe et al.,2012; Guanghua et al.,1979;Mingxiao et al.,2013;). Sequence analysis indicated

that CSBV was different but highly homologous to AmSBV-UK-AF092924.1. The genetic characterization and phylogenetic relationship of SBV-infected honey bees collected from different hosts and various geographic regions have recently attracted attention. Previous studies have focused on the alignment, basic structure and composition of SBV/CSBV genomes, and host-specificity and geographic differences of SBV/CSBV (Choe et al.,2012; Grabensteiner et al.,2001; Kondreddy et al.,2017; Mingxiao et al.,2011,2013; Nguyen et al.,2016; Reddy et al.,2016;Se E et al.,2012; Xia et al.,2015; Ying et al.,2016; Yu et al.,2013; Zhang et al.,2001); however, whether there is recombination between SBV and CSBV and if CSBV breaks through the species barrier and develops cross infection and causes disease (kill larvae) in *A. mellifera* is unclear. In artificial-infection experiments, Hong-Ri Gong et al. have demonstrated that CSBV is able to infect *A. mellifera*, but no obvious signs of the disease were observed with low pathogenicity (Hong-Ri et al.,2016).

In this study, we have characterized a CSBV strain in *A. mellifera* from Shandong, China (henceforth referred to as AmCSBV-SDLY [GenBank accession no. MG733283]), and reported the molecular and biological characteristics of AmCSBV-SDLY. Furthermore, we have analyzed the virus recombination.

METHODS

Virus purification

AmCSBV-SDLY was obtained from a natural outbreak Linyi bee farm in Shandong, China. The infected *A. mellifera* larvae by AmCSBV-SDLY were allowed to collect by the owner of the Linyi bee farm. Fifty infected *A. mellifera* larvae were collected, weighed, and completely homogenized in sterile water (1.5× by weight) using a mortar and pestle. AmCSBV-SDLY purification was performed by cesium chloride gradient centrifugation, according to the method of Ma et al. (Mingxiao et al.,2011). The supernatant was then successively passed through 0.45-μm and 0.22-μm cell filters. Healthy larvae treated by the same method were used as the negative control. CSBV was subsequently identified by reverse-transcription polymerase chain reaction (RT-PCR) to exclude black queen cell virus (BQCV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), and Israeli acute paralysis virus (IAPV), following the method of Yu et al. (Yu et al.,2013). CSBV virus samples free of other viruses were stored at −80 °C until further use.

Electron microscopy and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for virus identification

As previously described (Mingxiao et al.,2011) , 100 μL of the purified viral suspension was directly pelleted onto carbon-coated Formvar copper grids by ultracentrifugation (15 min at 82,000 ×*g*) using a Beckman airfare. The grids were negatively stained with 2% sodium phosphotungstate at pH 6.8 for 90 s and observed using a Philips CM10 transmission electron microscope.

153 Structural proteins were separated by SDS-PAGE with 5% stacking and 12%
154 separating gels using standard protocols.

155 **Agar gel immunodiffusion (AGID) assay**

156 In brief, 1 g of agarose and 8 g of sodium chloride (NaCl) were added to
157 100mL phosphate buffer (0.01 M, pH 7.2), shaken well, and microwaved for 2 min
158 to prepare an agar solution. The solution was slightly cooled, poured into Petri
159 dishes (90 mm in diameter; 20-22 mL of agar per plate), and allowed to solidify.
160 Seven wells were made in the agar plates to identify AmCSBV-SDLY; the central
161 hole was loaded with antisera against CSBV-JL2014-KU574661.1 strain; the
162 surrounding wells 1 and 2 were loaded with purified AmCSBV-SDLY, wells 3 and
163 4 were loaded with a known positive control (purified CSBV-JL2014-
164 KU574661.1), and wells 5 and 6 were loaded with treated healthy larvae as the
165 negative control. The plates were then placed in a closed moisturized container and
166 incubated at 37 °C in a humidified chamber for 24 or 48 h. Precipitation was
167 visible after 24 h but became more distinct after 48 h of incubation.

168 **~~Amplification of the full-length~~ CSBV-SDLY genome**

169 The primers used in this study (Table 1) were designed based on the nucleotide
170 sequences of CSBV-JL2014-KU574661.1, AmSBV-UK- AF092924.1, and CSBV-
171 GD-AF469603.1. Full-length CSBV-SDLY genome was amplified by RT-PCR
172 and 3′ rapid amplification of cDNA ends (RACE) (Clontech-Takara, Mountain
173 View, CA), according to the method described by Ma et al. (Mingxiao et al.,2011).

The PCR amplification product was cloned in the pMD-18-T vector (Takara Biotechnology Co. Ltd., Dalian, China). The plasmids were then used to transform *Escherichia coli* DH5α cells (Takara Biotechnology Co. Ltd). The plasmids were extracted using a plasmid extraction kit (Axygen Biotechnology [Taizhou] Co. Ltd). Nucleotide sequencing was performed by Sangon Biotech Co. Ltd., and the nucleotide sequences from all the fragments were assembled to build a continuous complete sequence using the DNASTAR software.

Phylogenetic tree construction from AmCSBV-SDLY and SBV/CSBV genome sequence analysis

The amplified nucleotide fragments were assembled to generate the entire genome of AmCSBV-SDLY using the DNASTAR program. Multiple nucleotide and deduced amino acid sequence alignments were performed using ClustalW in the MegAlign program (DNAStar Inc., Madison, WI, USA) and the published SBV/CSBV sequences. A phylogenetic tree was constructed from the nucleotide sequences of the coding regions of 43 previously reported SBV/CSBV strains from various countries and the AmCSBV-SDLY isolated in this study. The phylogenetic tree constructed using the MEGA 4.1 package (Tamura et al.,2007) and neighbor-joining (NJ) method (Saitou et al.,1987) was computed using the Kimura 2 parameter method(Kimura 1980). A bootstrap value of 1,000 replicates was applied to yield a robust phylogeny.

Similarity analysis and virus recombination analysis

Based on the results of the phylogenetic analysis, the isolated CSBV/SBV strains were divided into nine groups as follows: A (CSBV-FZ-KM495267.1), B (AmCSBV-SDLY), C (CSBV-SXnor1-KJ000692.1), D (CSBV-SXYL-KU574662.1), E (CSBV-BJ2012-KF960044.1), F (CSBV-LN2009-HM237361.1), G (CSBV-JL2014-KU574661.1), H (CSBV-GD-AF469603.1), and I (AmSBV-UK-AF092924.1) for similarity analysis and virus recombination analysis. The RDP (Recombination Detection Program) values were computed using the RDP 4.0 software for the preliminary screening of the major parent and minor parent sequences. Similarity plots and BootScan were computed using Simplot software (Lole et al.,1999) with the following parameters: a window of 200 base pairs (bp; step: 20 bp), with gap-stripping and Kimura (two-parameter) correction, using AmCSBV-SDLY, CSBV-JL2014-KU574661.1, CSBV-GD-AF469603.1, and AmSBV-UK-AF092924.1 for FindSites, and AmCSBV-SDLY and CSBV-JL2014-KU574661.1 as the query sequences.

RESULTS

AmCSBV-SDLY was identified by electron microscopy and SDS-PAGE

Electron microscopy showed large amounts of typical CSBV particles in the preparations from virus-infected larvae; CSBV particles were icosahedrons and had an approximate diameter of 26 nm (Fig. 1). No virus particles were observed

215 in the control preparations from healthy larvae. The four main structural proteins of
216 CSBV were separated by SDS-PAGE (Fig. 2). The molecular weights of the four
217 proteins were about 44.2, 37.8, 31.5, and 30.5 kDa, respectively (Jianxu et al.,1998;
218 Mingxiao et al.,2011).
219
220 **AmCSBV-SDLY was identified by AGID assay**
221 AGID assay revealed the distinct CSBV-specific lines of precipitin observed
222 between the wells containing AmCSBV-SDLY and the antisera against CSBV-
223 JL2014-KU574661.1 as well as the positive control wells (Fig. 3). No precipitin
224 lines were observed in the negative control wells.
225 **Nucleotide sequence analysis**
226 The nucleotide sequence of the AmCSBV-SDLY genome was 8,794-bp long,
227 and the percentages of A, U, G, and C were 29.95%, 29.22%, 24.30%, and 16.52%,
228 respectively, similar to those reported for other CSBV/SBV strains encoding one
229 large OFR. Two AUG codons were located at positions 189 and 429 of the
230 AmCSBV-SDLY genome; however, AUG 189 may be the translation initiation
231 site, as unlike AUG 429, it was observed in a sequence (AUUAUGG) identical to
232 that of many invertebrate initiation codons (ANNAUGG). The size of the CSBV 3′
233 untranslated region (UTR) (77 nucleotides) was similar to that from other
234 picornaviruses (40-126 nucleotides). The 5′ UTR was generated from AmCSBV-
235 SDLY by RT-PCR with a primer designed using the CSBV-JL2014-KU574661.1

sequence, indicating that the CSBV 5′ UTR was similar to that of CSBV-JL2014-KU574661.1 (188 nucleotides). Multiple sequence comparisons showed that the sequence of AmCSBV-SDLY was similar to the sequences of the previously reported SBV/CSBV strains. Multiple sequence comparisons revealed a sequence homology of 92.4% to 97.1% among all CSBV isolates and a similarity of 94.5% to 97.7% in the deduced amino acid sequences. AmCSBV-SDLY was least similar (89.5% to 90.4% identity) to other SBVs but showed maximum similarity with CSBV-FZ-KM495267.1.

Protein sequence analysis

The deduced amino acid sequences of AmCSBV-SDLY genomes and previously reported SBV/CSBV strains were aligned and compared. Results revealed that the structural and non-structural proteins were located at the 5′ and 3′ ends, respectively. Multiple sequence alignment showed that AmCSBV-SDLY had 17 and 1 amino acid deletions (positions 711-729 and 2,128, respectively) as compared with CSBV-GD-AF469603.1 and 3 and 13 amino acid deletions (positions 711-713 and 715-728, respectively) as compared to AmSBV-UK-AF092924.1; however, AmCSBV-SDLY was similar to the CSBV-JL2014-KU574661.1 strain that infects *A. cerana* (Fig. 4).

The amino acid sequence at the C-terminal region of the AmCSBV-SDLY polyprotein was similar to that for helicase, protease, and RNA-dependent RNA polymerase (RdRp) domains of the previously reported SBV/CSBV strains. The

257 highly conserved amino acids GPAGIGKS, QPVVVYDD, and
258 KKIRGNPLIVILLCNH corresponding to the helicase domains A, B, and C
259 (Supplementary 1), respectively, were located between the amino acid positions
260 1,353 and 1,473; however, the C domain containing KKIRGNPLIVILLCNH
261 appeared to be the most conserved, unlike mammalian picornaviruses. The
262 conserved cysteine protease motif ^{2249}GXCG^{2252} (GACG) and the putative
263 substrate-binding residue ^{2266}GxHxxG^{2271} (GMHFAG) were detected in the 3C
264 protease domains spanning amino acids 2,141 to 2,272 (Supplementary 2). In
265 addition, eight conserved domains identified in RdRp were also detected between
266 amino acid positions 2,444 and 2,813 in AmCSBV-SDLY (Supplementary 3).
267
268 **Phylogenetic analysis**
269 To assess the genetic relationship among the SBV/CSBV strains, a phylogenetic
270 tree based on the nucleotide sequence of the SBV/CSBV-coding region was
271 constructed by the NJ method for AmCSBV-SDLY and all SBV/CSBV reference
272 strains in GenBank. The tree (Fig. 5) revealed two clusters, one related to SBV
273 strains that originated from *A. cerana* (named as the AC genotype), and the other
274 related to the SBV strains that originated from *A. mellifera* (named as the AM
275 genotype). The AM cluster was distinctly separate from the AC lineages, as
276 supported by a clade credibility value of 100.0 (Fig. 5). The AC genotype was
277 further subdivided into several subtypes according to their countries of origin and

278 host species.

279 The phylogenetic tree also showed that AmCSBV-SDLY was classified into a
280 branch containing the CSBV and other Asian strains. These results indicate that the
281 AmCSBV-SDLY strain showed close genetic relationships with CSBV strains,
282 specifically the CSBV-FZ-KM495267.1 strain.

283

284 **Similarity analysis**

285 To identify differences in the full-length sequences and various genomic regions
286 from CSBV, AmSBV-UK-AF092924.1 and AmCSBV-SDLYA, the complete
287 coding regions were plotted using SimPlot, with AmCSBV-SDLY as the query
288 sequence (Fig. 6). AmCSBV-SDLY from *A. mellifera* was more similar to the
289 CSBV strains isolated than AmSBV-UK-AF092924.1. The CSBV strains isolated
290 formed a relatively independent separation group; there was an obvious deviation
291 compared to AmSBV-UK-AF092924.1, with maximum deviation (19.3%)
292 occurring at 2,181 and 2,221 bp. A high degree of consistency was observed
293 between the genomes of AmCSBV-SDLY and isolated CSBV strains, with more
294 than 85% similarity between the complete coding regions.

295 Among all the isolated CSBV strains, only recombinant CSBV-JL2014-
296 KU574661.1 was detected by RDP (Table 2) that exhibited three recombinant
297 fragments as follows: one obtained from AmCSBV-SDLY and two derived from
298 CSBV-FZ-KM495267.1. The minor parent was CSBV-LN2009-HM237361.1.

BootScan analysis showed that the cross recombination signals of AmCSBV-SDLY and CSBV-FZ-KM495267.1 were detected at position 1,957-2,162 and 6,635-7,110 using AmCSBV-SDLY and CSBV-JL2014-KU574661.1 as the query sequence, respectively (Fig. 7A, B).

Discussion

CSBV was first described in Guangdong in 1972 and later disseminated to the rest of China and Southeast Asian countries, causing a lethal disease in individual bees or the collapse of entire colonies, termed as the bee cancer by beekeepers (Mingxiao et al.,2010). More than 200,000 colonies (more than half of the total number of local colonies) were destroyed in Guangxi and Guangdong provinces from 1972 to 1994 (Hanrong et al.,2000), and re-emerged and caused the collapse of entire colonies in Liaoning China in 2008 (Dali et al.,2012; Mingxiao et al.,2010). Although CSBV and SBV are similar in structure, physiology, and biochemistry, these viruses differ in antigenicity and cross infection (Guanghua et al.,1979). SBV strains usually show host specificity in the structural polyprotein regions of their genomes (Cheng et al.,2011; Choe et al.,2012;Mingxiao et al.,2013). CSBV, which has been divided into different subtypes according to various geographic regions and host specificity (Mingxiao et al.,2013), contains three structural proteins, namely, VP1, VP2, VP3, and an unknown protein with a molecular weight of 44.2 kDa (Jianxu et al.,1998; Mingxiao et al.,2011). SBV

320 contains three structural proteins (VP1, VP2, and VP3). The proteins VP1, VP2,
321 and VP3 as well as the unknown 44.2 kDa protein of CSBV were detected by
322 SDS-PAGE in CSBV strains (termed as AmCSBV-SDLY) that infect *A. mellifera*,
323 and distinct CSBV-specific lines of precipitation were observed between
324 AmCSBV-SDLY and the antisera against CSBV-JL2014-KU574661.1 in the
325 AGID assay. Thus, AmCSBV-SDLY belongs to CSBV. As expected, the
326 phylogenetic and similarity analyses showed that AmCSBV-SDLY genome
327 isolated from *A. mellifera* was highly similar to the genomes of CSBV strains
328 derived from *A. cerana*. Similar to AmCSBV-SDLY, two Vietnamese SBVs
329 strains (AmSBV-Viet4-KM884993.1 and AmSBV-Viet6-KM884995.1) and two
330 Korean strain (AmSBV-Kor19-JQ390592.1 and AmSBV-Kor2-KP296801.1)
331 isolated from *A. mellifera* also belong to the AC type (Fig.5), consistent with the
332 previous reports (Kondreddy et al.,2017; Se E et al.,2012). Although no reports are
333 available to determine if this strain caused *A. mellifera* larval deaths, the results of
334 the present study show that the AC type SBV infecting and circulating in honey
335 bee populations for a long time may lead to exchange of viruses among the host
336 populations. Furthermore, phylogenetic and similarity analyses showed that the
337 virus strains from the same continent or country have higher levels of similarity
338 and genetic clustering.

339 The phylogenetic tree based on the nucleotide sequences of the SBV/CSBV-
340 coding region revealed the genetic relationship between different virus isolates

obtained from various geographic areas and hosts. Two distinct branches were formed according to bee species and higher level of similarity was observed between viral genomes from the same country, continent, or neighboring countries. Furthermore, CSBV genome sequences were closely related to those from Vietnam, Korea, and India, all of which formed a neighboring subcluster. The phylogenetic trees based on the SBV/CSBV-coding region and VP1 nucleotide sequences were identical (Mingxiao et al.,2013), indicating that SBV/CSBV strains may be divided into different groups and that VP1 may replace the complete genomic sequence of SBVs and CSBVs and be used as a specific target for studying genotyping, genetic evolution, and molecular epidemiology of SBV/CSBV strains in the future.

Direct sequencing of virus genome amplicons and comparison with known nucleotide sequences or deduced protein sequences of virus isolates obtained from various geographic areas and hosts may reveal the genome organization and molecular basis of pathogenicity of the newly isolated strains. The analysis of the deduced amino acid sequences showed that AmCSBV-SDLY, like CSBV, comprised conserved motifs within the helicase, protease, and RdRp domains. Furthermore, AmCSBV-SDLY from CSBVs was genetically similar to the previously reported strains CSBV-FZ-KM495267.1 and CSBV-JL2014-KU574661.1. We observed nucleotide transitions and insertions in the entire genome sequences, thereby reflecting the divergence of these genomes. In comparison to the CSBV strains that were isolated prior to 2014 (CSBV-GD-

AF469603.1, CSBV-LN2009-HM237361.1, CSBV-FZ-KM495267.1, CSBV-SXnor1-KJ000692.1, and CSBV-BJ2012-KF960044.1), AmCSBV-SDLY, CSBV-JL2014-KU574661.1, CSBV-SXYL-KU574662.1, and AmSBV-UK-AF092924.1 strains carried a deletion mutation at the amino acid residue 2,128, and AmCSBV-SDLY and CSBV-SXYL-KU574662.1 differed at positions 711-729. We speculated that AmCSBV-SDLY could cross the species barrier and cause death in *A. mellifera* larvae, probably owing to the deletion mutation at the amino acid residue 2,128. Furthermore, CSBV-JL2014-KU574661.1 could probably cross the species barrier and kill *A. mellifera* larvae because of the similarity at positions 711-729 and 2,128. In addition, we have demonstrated that CSBV-JL2014-KU574661.1 was a recombinant virus, and the sequence of its parent strain was derived from AmCSBV-SDLY, CSBV-FZ-KM495267.1, and CSBV-LN2009-HM237361.1 by RDP and BootScan. However, AmCSBV-SDLY was isolated after CSBV-JL2014-KU574661.1, and the recombinant signals obtained from CSBV-FZ-KM495267.1, CSBV-JL2014-KU574661.1, and CSBV-GD-AF469603.1 were detected by BootScan (Fig. 7); hence, we speculate that AmCSBV-SDLY may be a recombinant virus strain and its parent strain was derived from CSBV-FZ-KM495267.1, CSBV-JL2014-KU574661.1, and CSBV-GD-AF469603.1.

CONCLUSIONS

In summary, the strain AmCSBV-SDLY of CSBV was genetically similar to

383 the previously reported strains of CSBV; however, AmCSBV-SDLY may cross the
384 species barrier and cause death in *A. mellifera* larvae. This observation shows that
385 under natural selection and immune pressure, CSBV/SBV strains may be
386 exchanged between host ~~populations,~~ indicative of the independent evolution of
387 these viruses. Future studies will be directed to evaluate genetic variations and
388 population structures of CSBV/SBV strains infecting *A. mellifera* and *A. cerana* in
389 China and other countries.
390

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394 20180550289).

395 **Competing interests**

396 The authors declare no competing interests.

397 **Ethics Statement**

398 The use of the experimental animals involved in the article is in compliance with
399 the relevant provisions of the Animal Welfare and Ethics of Experimental Animals
400 of the Experimental Animal Center of Jinzhou Medical University, China. The
401 field studies did not involve endangered or protected species, and the owner of the
402 Linyi bee farm gave permission to conduct the study on this site.

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508

509 **Figure captions**

510 **Fig. 1 CSBV particles were observed with electron microscopy.**

511 Virus particles of approximately 26-nm diameter were observed in virus
512 preparations from the infected larvae by electron microscopy.

513 **Fig. 2 The four proteins of CSBV were detected by SDS-PAGE.**

514 The proteins were resolved on 12% SDS-polyacrylamide gels according to
515 standard protocols. A. representative protein markers. B. virus-free control. C.
516 AmCSBV-SDLY.

517 **Fig. 3 Identification of AmCSBV-SDLY by AGID assay.**

518 Antisera CSBV (center well), AmCSBV-SDLY (wells 1 and 2), known positive
519 control CSBV-JL2014-KU574661.1 (wells 3 and 4), and negative control (wells
520 5 and 6).

521 **Fig. 4 Protein domain alignments.** AmCSBV-SDLY had 17 and 1 amino acid
522 deletions (positions 711-729 and 2,128, respectively). However, AmCSBV-
523 SDLY had 3 and 13 amino acid deletions (positions 711-713 and 715-728,
524 respectively) as compared with AmSBV-UK-AF092924.1.

525 **Fig. 5 Phylogenetic analysis of all nucleotide sequences obtained from various**
526 **countries, including China, Korea, Vietnam, India, Australia, and the**
527 **United Kingdom.**

528 Numbers on the nodes indicate clade credibility values. Previously reported

529 sequence names are presented in the following format: strains-GenBank
530 accession numbers. The tree reveals two clusters, the AC and AM genotypes.
531 * The bar represents a genetic distance of 0.01. CSBV is marked with a red box,
532 the blue square represents that the AC genotype SBVs strains mainly were
533 isolated from *A. mellifera*.

534 **Fig. 6 SimPlot graphs comparing AmCSBV-SDLY with other CSBVs and**
535 **AmSBV-UK-AF092924.1.**

536 Each colored line indicates a group of SBV/CSBV strains (for interpreting the
537 color key in this figure legend, please refer to an online version of the article).

538 **Fig. 7 BootScan analysis of the recombinant sequence based on the**
539 **comparison between the complete coding regions of AmCSBV-SDLY and**
540 **other CSBVs and AmSBV-UK-AF092924.1**.

541 A. BootScan was conducted with the strain AmCSBV-SDLY as the query
542 sequence. B. BootScan was conducted with the strain CSBV-JL2014-
543 KU574661.1 as the query sequence. Note: The cross recombination position is
544 labeled by red lines.

545

546

Table 1(on next page)

Synthetic oligonucleotides for amplification of the CSBV genome

1 **Table 1. Synthetic oligonucleotides for amplification of the CSBV genome.**

Primers[a]	Sequence (5′ to 3′)	Nucleotide position[s]
S1 F	GAAATAAGAATACGAATCGT	1-20
S1 R	TAAACAAATCGGTATAAGAGTCC	379-401
S2 F	GACCCGTTTTCTTGTGAGTTTTAG	41-64
S2 R	GTGTAGCGTCCCCCTGAATAGAT	611-633
S3 F	TATTCAGGGGGACGCTACAC	614-633
S3 R	TATTCCATCGGGGTTATTTG	1713-1732
S4 F	GGAGACGCGCATGGTAAAGA	1644-1663
S4 R	GCGCGGTAAATAAACACTCG	2365-2384
S5 F	ATGGGGGTAAGGGACAATCTG	2290-2310
S5 R	TGCTCTAACCTCGCATCAAC	3423-3442
S6 F	TTACGGGAGCAGCACAACA	3391-3409
S6 R	ATTTCCGATTTACCGATACC	4287-4306
S7 F	CGGTGCGTTATGAACCTTTT	4243-4262
S7 R	AATGCGTAGATTGAGGTGCC	5333-5352
S8 F	GCGCAACTGGCACCTCAAT	5325-5343
S8 R	TTCCAAATATACTTCCCACTGC	6249-6270
S9 F	GTGACGGCAGTGGGAAGTAT	6262-6243
S9 R	GCAGCCTCCTCAGGTGTTAGT	7454-7474
S10 F	TTTGGTAGCGGGGTGTAAG	7322-7340
S10 R	CATTGCGTGGTATCATT	8501-8517

2

Table 2(on next page)

Recombinants detected with RDP

1

2 **Table 2. Recombinants detected with RDP.**

Recombinant	Major parent	Minor parent	Breakpoint	Av. P-Val
CSBV-JL 2014-KU 574661.1	CSBV-FZ-KM495267.1	CSBV-LN2009-HM237361.1	520-1277	1.048×10^{-18}
	AmCSBV-SDLY	CSBV-LN2009-HM237361.1	2212-3247	8.240×10^{-18}
	CSBV-FZ-KM495267.1	CSBV-LN2009-HM237361.1	7202-8336	7.771×10^{-17}

Figure 1(on next page)

CSBV particles were observed with electron microscopy.

Virus particles of approximately 26-nm diameter were observed in virus preparations from the infected larvae by electron microscopy

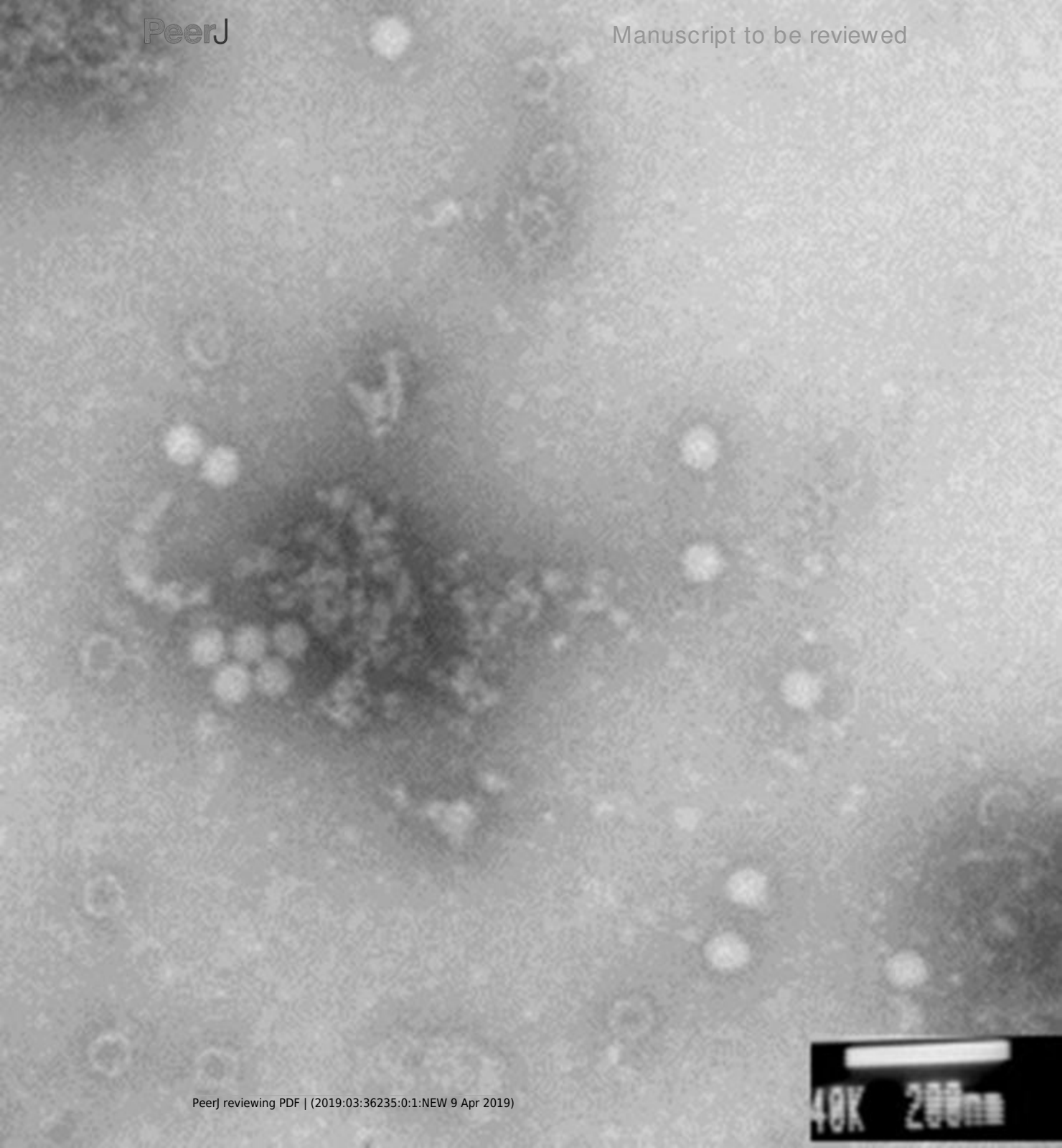
40K 200nm

Figure 2(on next page)

The four proteins of CSBV were detected by SDS-PAGE.

The proteins were resolved on 12% SDS-polyacrylamide gels according to standard protocols. A. representative protein markers. B. virus-free control. C. AmCSBV-SDLY

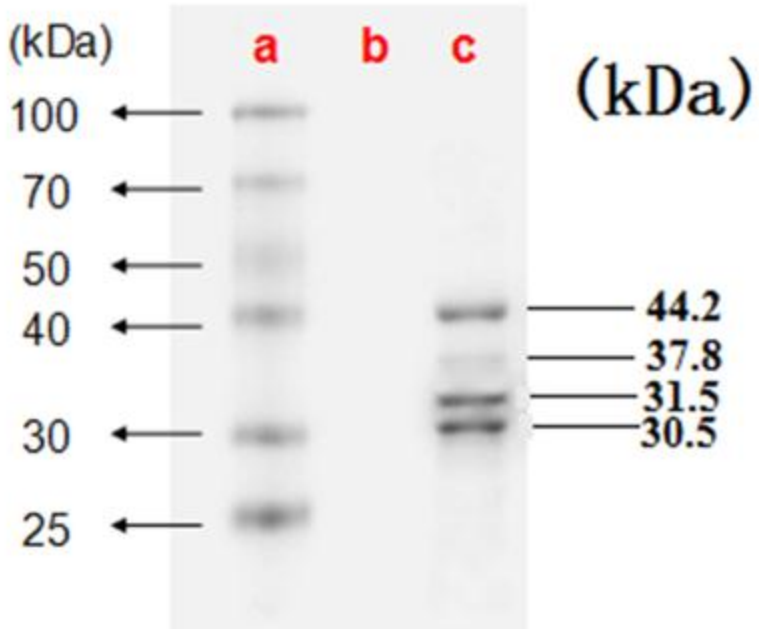
(kDa)
a
b
c
100
70
50
40
30
25
(kDa)
44.2
37.8
31.5
30.5

Figure 3(on next page)

Identification ofAmCSBV-SDLY by AGID assay

Antisera CSBV (center well), AmCSBV-SDLY (wells 1 and 2), known positive control CSBV-JL2014-KU574661.1 (wells 3 and 4), and negative control (wells 5 and 6)

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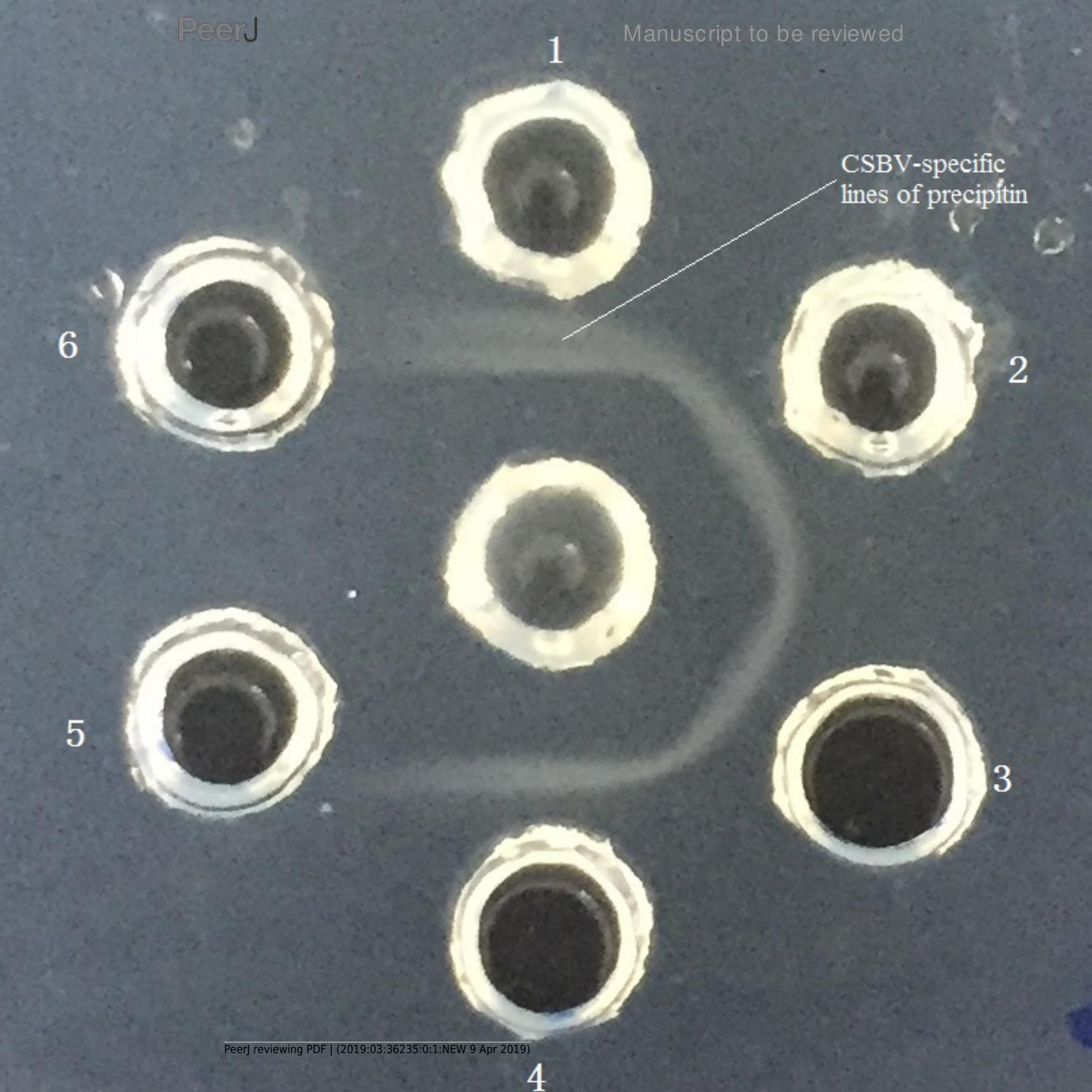
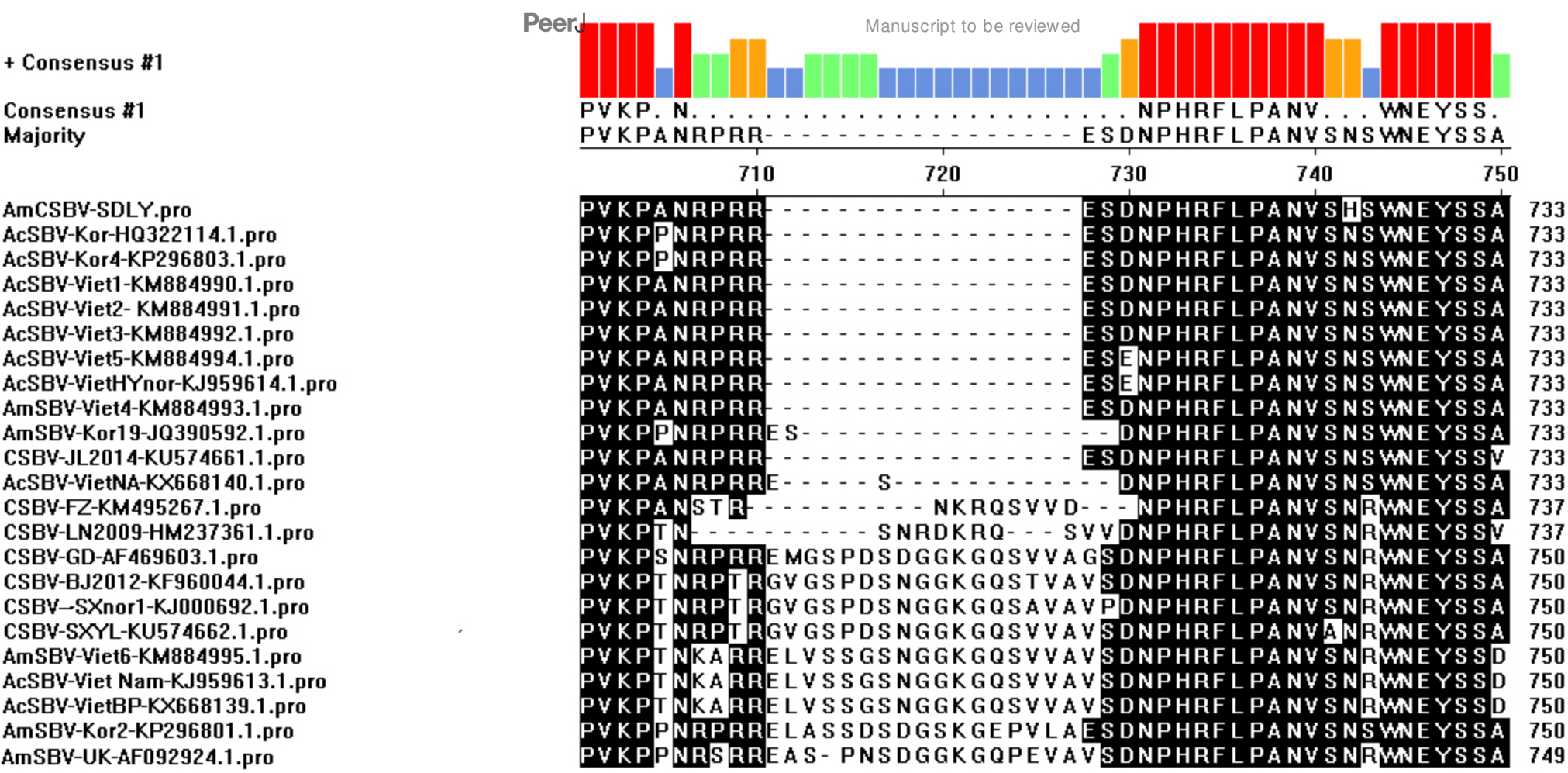


Figure 4(on next page)

Protein domain alignments

AmCSBV-SDLY had 17 and 1 amino acid deletions (positions 711-729 and 2,128, respectively). However, AmCSBV-SDLY had 3 and 13 amino acid deletions (positions 711-713 and 715-728, respectively) as compared with AmSBV-UK-AF092924.1


+ Consensus #1
Consensus #1
Majority
710
720
730
740
750
AmCSBV-SDLY.pro
AcSBV-Kor-HQ322114.1.pro
AcSBV-Kor4-KP296803.1.pro
AcSBV-Viet1-KM884990.1.pro
AcSBV-Viet2- KM884991.1.pro
AcSBV-Viet3-KM884992.1.pro
AcSBV-Viet5-KM884994.1.pro
AcSBV-VietHYnor-KJ959614.1.pro
AmSBV-Viet4-KM884993.1.pro
AmSBV-Kor19-JQ390592.1.pro
CSBV-JL2014-KU574661.1.pro
AcSBV-VietNA-KX668140.1.pro
CSBV-FZ-KM495267.1.pro
CSBV-LN2009-HM237361.1.pro
CSBV-GD-AF469603.1.pro
CSBV-BJ2012-KF960044.1.pro
CSBV--SXnor1-KJ000692.1.pro
CSBV-SXYL-KU574662.1.pro
AmSBV-Viet6-KM884995.1.pro
AcSBV-Viet Nam-KJ959613.1.pro
AcSBV-VietBP-KX668139.1.pro
AmSBV-Kor2-KP296801.1.pro
AmSBV-UK-AF092924.1.pro
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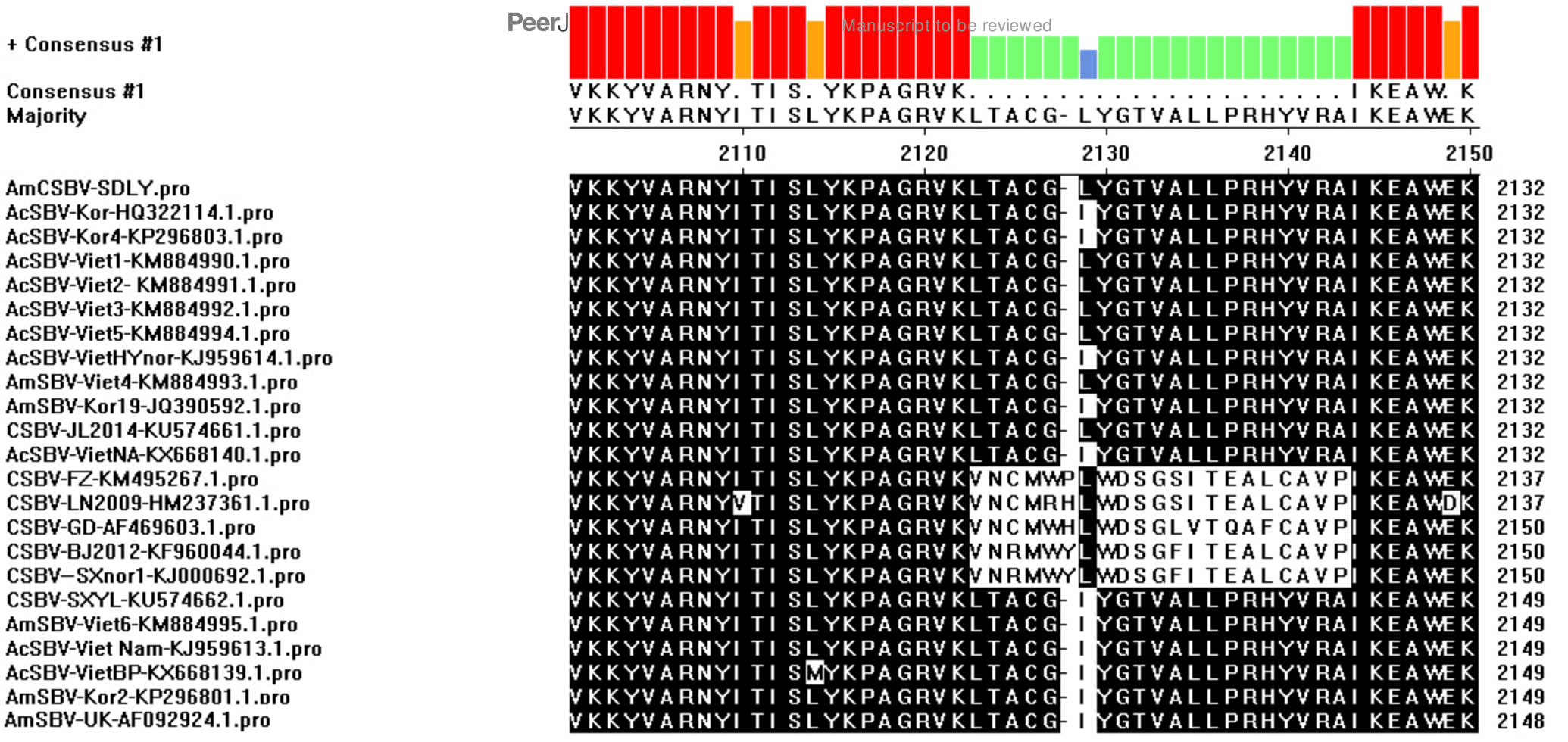

Figure 5(on next page)

Phylogenetic analysis of all nucleotide sequencesobtained from various countries, including China, Korea, Vietnam, India,Australia, and the United Kingdom

Numbers on the nodes indicate clade credibility values. Previously reported sequence names are presented in the following format: strains-GenBank accession numbers. The tree reveals two clusters, the AC and AM genotypes.

* The bar represents a genetic distance of 0.01. CSBV is marked with a red box, the blue square represents that the AC genotype SBVs strains mainly were isolated from *A. mellifera*.

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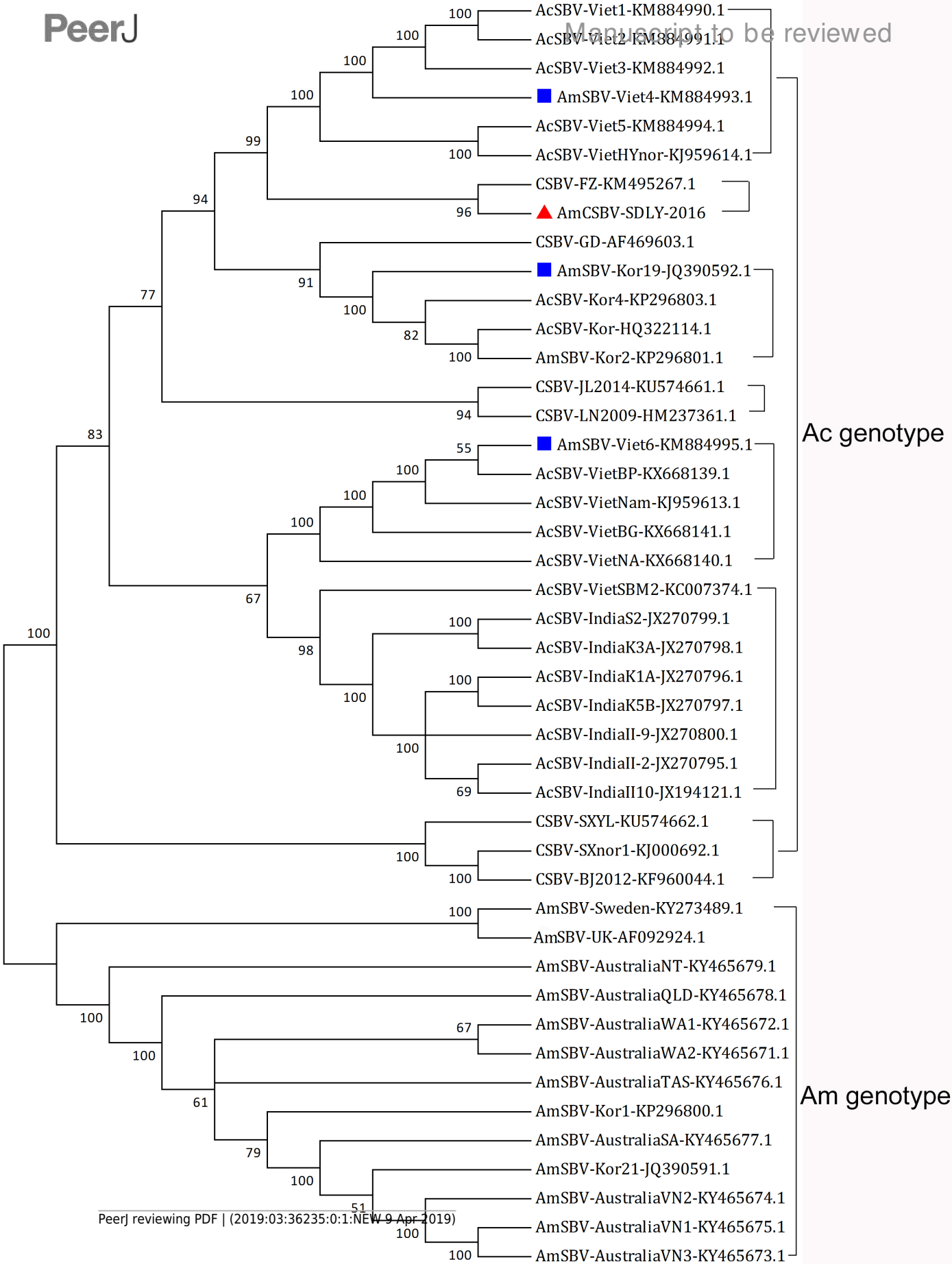


Figure 6(on next page)

SimPlot graphs comparing AmCSBV-SDLY with other CSBVsand AmSBV-UK-AF092924.1

Each colored line indicates a group of SBV/CSBV strains (for interpreting the color key in this figure legend, please refer to an online version of the article)

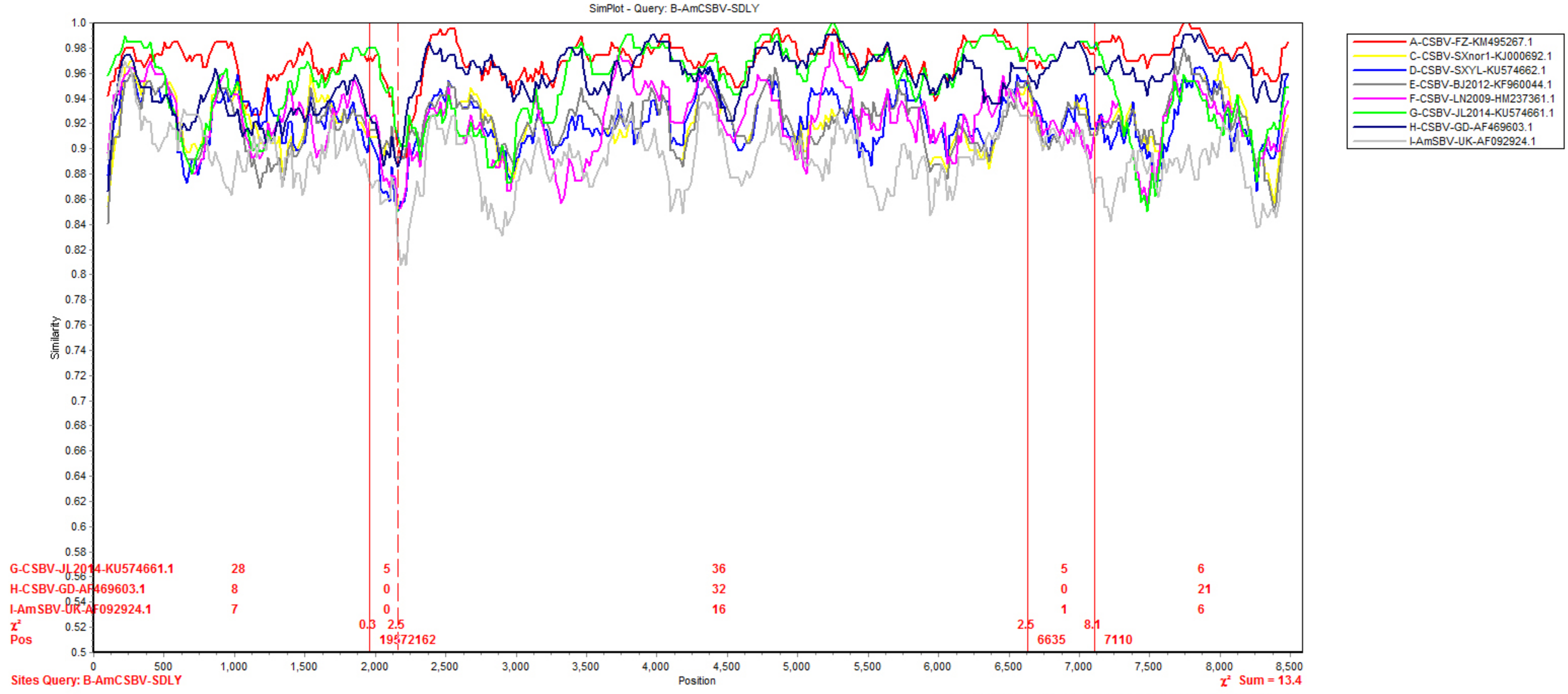
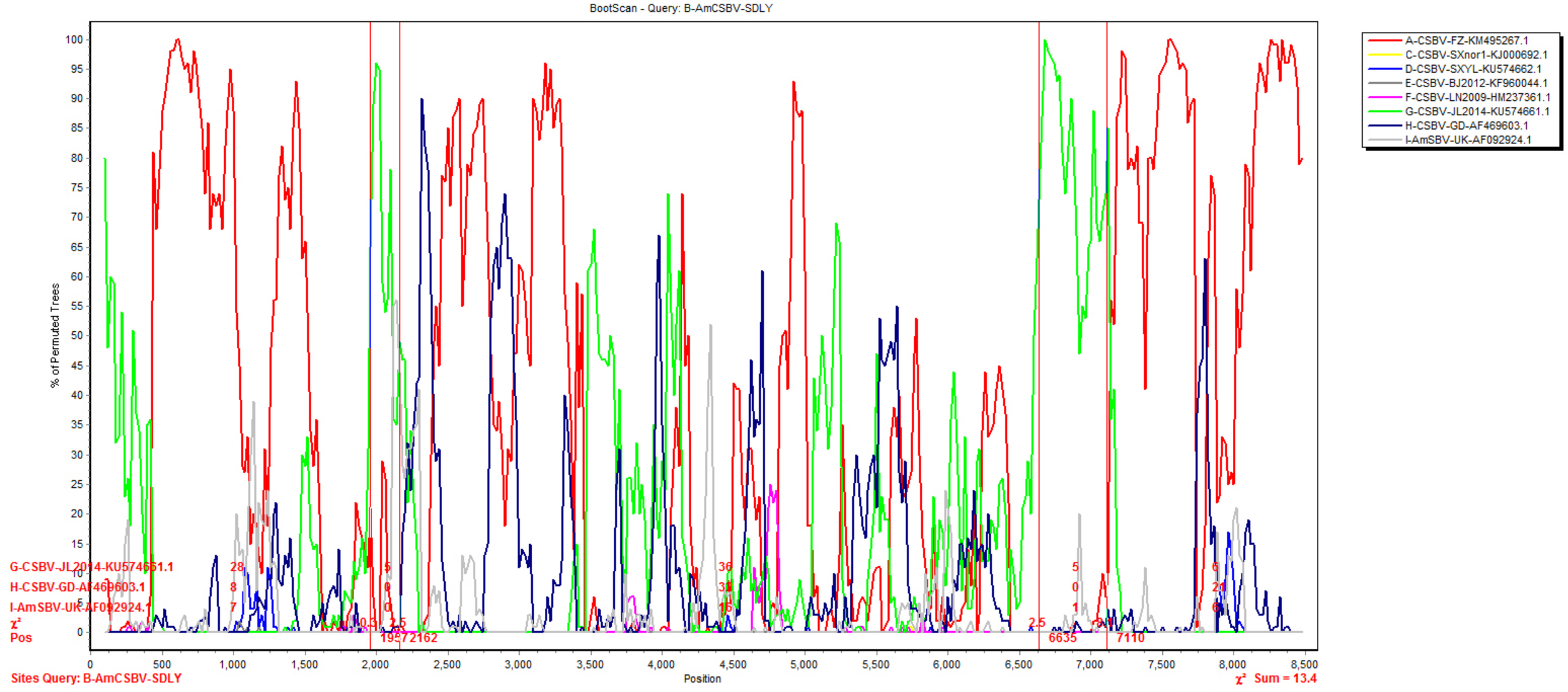

SimPlot - Query: B-AmCSBV-SDLY
A-CSBV-FZ-KM495267.1
C-CSBV-SXnor1-KJ000692.1
D-CSBV-SXYL-KU574662.1
E-CSBV-BJ2012-KF960044.1
F-CSBV-LN2009-HM237361.1
G-CSBV-JL2014-KU574661.1
H-CSBV-GD-AF469603.1
I-AmSBV-UK-AF092924.1
Similarity
Position
G-CSBV-JL2014-KU574661.1 28 5 36 5 6
H-CSBV-GD-AF469603.1 8 0 32 0 21
I-AmSBV-UK-AF092924.1 7 0 16 1 6
χ² 0.3 2.5 2.5 8.1
Pos 1972162 6635 7110
Sites Query: B-AmCSBV-SDLY
χ² Sum = 13.4
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2.0

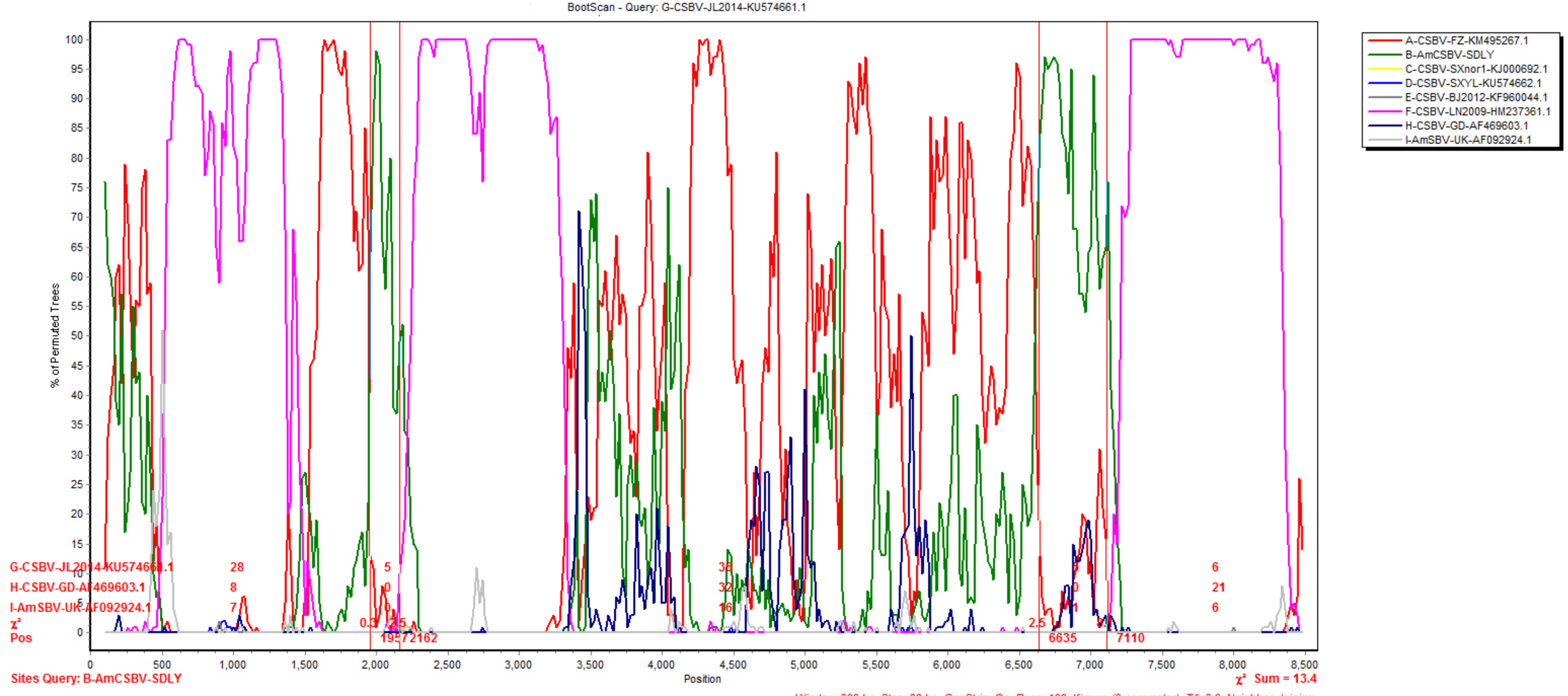
Figure 7(on next page)

BootScan analysis of the recombinant sequence based onthe comparison between the complete coding regions of AmCSBV-SDLY and otherCSBVs and AmSBV-UK-AF092924.1 .

A. BootScan was conducted with the strain AmCSBV-SDLY as the query sequence. B. BootScan was conducted with the strain CSBV-JL2014-KU574661.1 as the query sequence.

Note: The cross recombination position is labeled by red lines


BootScan - Query: B-AmCSBV-SDLY
A-CSBV-FZ-KM495267.1
C-CSBV-SXnor1-KJ000692.1
D-CSBV-SXYL-KU574662.1
E-CSBV-BJ2012-KF960044.1
F-CSBV-LN2009-HM237361.1
G-CSBV-JL2014-KU574661.1
H-CSBV-GD-AF469603.1
I-AmSBV-UK-AF092924.1
% of Permuted Trees
Position
G-CSBV-JL2014-KU574661.1 28 5 36 5 6
H-CSBV-GD-AF469603.1 8 0 34 0 21
I-AmSBV-UK-AF092924.1 7 0 15 1 6
χ² 0.5 2.5 2.5 3.4
Pos 1972162 6635 7110
Sites Query: B-AmCSBV-SDLY
χ² Sum = 13.4
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining


BootScan - Query: G-CSBV-JL2014-KU574661.1
A-CSBV-FZ-KM495267.1
B-AmCSBV-SDLY
C-CSBV-SXnor1-KJ000692.1
D-CSBV-SXYL-KU574662.1
E-CSBV-BJ2012-KF960044.1
F-CSBV-LN2009-HM237361.1
H-CSBV-GD-AF469603.1
I-AmSBV-UK-AF092924.1
% of Permuted Trees
Position
G-CSBV-JL2014-KU574661.1 28 5 36 6
H-CSBV-GD-AF469603.1 8 0 32 21
I-AmSBV-UK-AF092924.1 7 16 6
χ² 0.3 2.5 2.5
Pos 1957 2162 6635 7110
Sites Query: B-AmCSBV-SDLY
χ² Sum = 13.4
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining