



NOTE

Virology

Prevalence of honeybee viruses in *Apis mellifera* in Gifu prefecture of Japan

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ABSTRACT. Viral infection damages honeybee colony health. Viruses can be carried by queen bees and apicultural production materials when imported from foreign countries. We investigated seven honeybee viruses in worker bees (*Apis mellifera*) from 26 healthy apiaries in Gifu, Japan between 2018 and 2019. Black queen cell virus (BQCV) was detected in 23 (88.5%) of the apiaries, followed by Israeli acute paralysis virus (42.3%), deformed wing virus (DWV) (38.5%), and sacbrood virus (3.8%). In phylogenetic analysis, BQCV and DWV in Gifu were related to those in China and South Korea. Additionally, a high prevalence of BQCV was observed among worker bees in BQCV-positive colonies. Therefore, BQCV horizontal transmission among worker bees may contribute to the high prevalence of BQCV in Gifu.

KEY WORDS: *Apis mellifera*, Gifu, honeybee virus, transmission

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Honeybees have contributed to several fields such as medicine and agriculture because they produce not only honey but also propolis, royal jelly, and bee venom, and act as a pollinator in horticulture. European honeybees (*Apis mellifera*) have provided honey and pollination services since 1877 in Japan. Bee virus infection affects the colony health and life span of honeybees, resulting in decrease in honeybee population [6, 9, 20]. In addition, viral infection of honeybees is considered as one of the causes of colony collapse disorder, which has been reported in the United States, Europe, and Asia [9, 20]. Currently, decreases in honeybee populations have been reported in various regions of Japan, but the reasons are unknown.

International trade of honeybees and apicultural production materials can disseminate honeybee viruses to new countries [3]. The presence of black queen cell virus (BQCV), deformed wing virus (DWV), sacbrood virus (SBV), Israeli acute paralysis virus (IAPV), and chronic bee paralysis virus (CBPV) has been found in honeybees in Japan [11, 12, 14, 15]. Every year, honeybee queens (queen bees) are imported into Japan from Slovenia and Australia, where BQCV, DWV, SBV, IAPV, acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and CBPV are found in honeybees [17, 19]. In addition, apicultural production materials, such as pollen foods, are also imported from China and South Korea, where BQCV, DWV, SBV, IAPV, ABPV, KBV, and CBPV prevail [1, 4, 5, 16, 22]. Therefore, Japanese apiculture is exposed to the risks of several viral intrusions from foreign countries.

The present study elucidated the current situation and epidemiology of seven bee viruses (BQCV, DWV, SBV, IAPV, ABPV, KBV, and CBPV) in central Japan. In this study, we examined worker bees collected from several bee colonies in Gifu prefecture for selected honeybee viruses and assessed the genetic relatedness of BQCV, DWV, and IAPV between Japan and foreign countries.

Samples of worker bees were collected from 26 apiaries without clinical symptoms between May and July in 2018 and 2019 in the southwest area of Gifu prefecture. Bee samples in each apiary were collected in a plastic bag and immediately treated with carbon dioxide, and then brought back to the laboratory on ice. Samples from the same apiary were stored together in a container at -20°C until further study. Homogenates of five pooled worker bees in 2 ml distilled water were centrifuged at 4,000 rpm for 1 min at room temperature.

Total RNA was extracted from supernatants using NucleoSpin[®] RNA (Macherey-Nagel, Düren, Germany) or QIAamp Viral RNA mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Subsequently, 5 μl of total RNA was examined for the presence of seven honeybee viruses (Supplementary Table 1) by virus-specific reverse transcription-polymerase chain reaction (PCR) using Prime Script[™] RT-PCR Kit (TaKaRa Bio, Kusatsu, Japan) according to the manufacturer's protocol.

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The PCR cycling conditions were set as follows: 94°C for 1 min, 30 cycles (94°C for 30 sec, 50–60°C for 30 sec, 72°C for 1 min), and finally at 72°C for 5 min (see Supplementary Table 1 for the annealing temperature). The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced at the Life Sciences Research Center of Gifu University. Homologous sequences were searched in the nucleotide Basic Local Alignment Search Tool (BLASTn) program. For the phylogenetic analyses of BQCV, DWV, and IAPV, the maximum likelihood method was performed using Molecular Evolutionary Genetics Analysis (MEGA)-X [13]. The datasets generated during and/or analyzed during the current study are available in the GenBank repository with the following accession numbers: LC556264–LC556268, LC556271, LC556273, LC556275, LC556276–LC556279, LC556280, LC556281, LC556284, LC556285, LC556290, LC556297, LC601608, LC601610–LC601617, LC601623–LC601632, LC601634, LC601635, LC601637, LC601638, and LC635481.

If the colonies were positive for the pooled samples, the remaining five worker bees were crushed in 1 ml distilled water individually, and individual samples were subjected to PCR detection.

This study was approved by the Ethics Committee for Animal Research and Welfare of Gifu University (approval number H30-009).

BQCV was the most frequently observed virus in the colonies (23 colonies, 88.5%), followed by IAPV (11 colonies, 42.3%), DWV (10 colonies, 38.5%), and SBV (1 colony, 3.8%) ($P < 0.05$, Fisher's exact test with Bonferroni adjustment). In contrast, in a previous study, DWV was the most prevalent (84.0% of 65 apiaries), followed by BQCV (77.0%), IAPV (66.0%), and SBV (39.0%) in 36 prefectures of Japan between 2009 and 2010 [12]. Although bee samples were collected throughout the year in the previous study [12], they were collected between May and July in the present study. Seasonal variations in viral prevalence have been observed in China [4], Germany [7], and France [18]. For example, in a study conducted in China, the highest prevalence of BQCV was observed in April compared with other seasons, whereas DWV showed a high prevalence in October [4]. In Germany [7] and France [18], BQCV was more frequently detected between May and August and between June and August, respectively, whereas DWV was more abundant between November and February and between August and November, respectively. Although differences in viral prevalence rates between the present study and the previous study conducted in Japan might be due to variations in sampling period and area, sampling season may also partially contribute to the prevalence of the aforementioned four viruses. Moreover, previous studies have suggested that BQCV, IAPV, and DWV may be transmitted among colonies and within a colony [10] and besides honeybees, *Varroa destructor* mite [3, 8, 21] or *Nosema apis* [2] may be responsible for transmission. In the present study, the examination of 5 individual bees revealed the presence of IAPV and DWV in 3 or more bees in 2 of 7 colonies tested (28.6%) and 2 of 6 colonies tested (33.3%), respectively, and BQCV was detected in 3 or more bees in 7 of the 10 colonies tested (70.0%) (Table 1). Therefore, horizontal transmission of BQCV inside a colony may also partially contribute to the high prevalence of BQCV in Gifu.

DWV was divided into D-I and D-II clades (Fig. 1). DWV in Gifu were grouped into the D-I clade, which also included sequences from China and South Korea, whereas those from European countries were grouped into the D-II clade. DWV in the D-I clade had a sequence similarity of 95.0–100.0% with

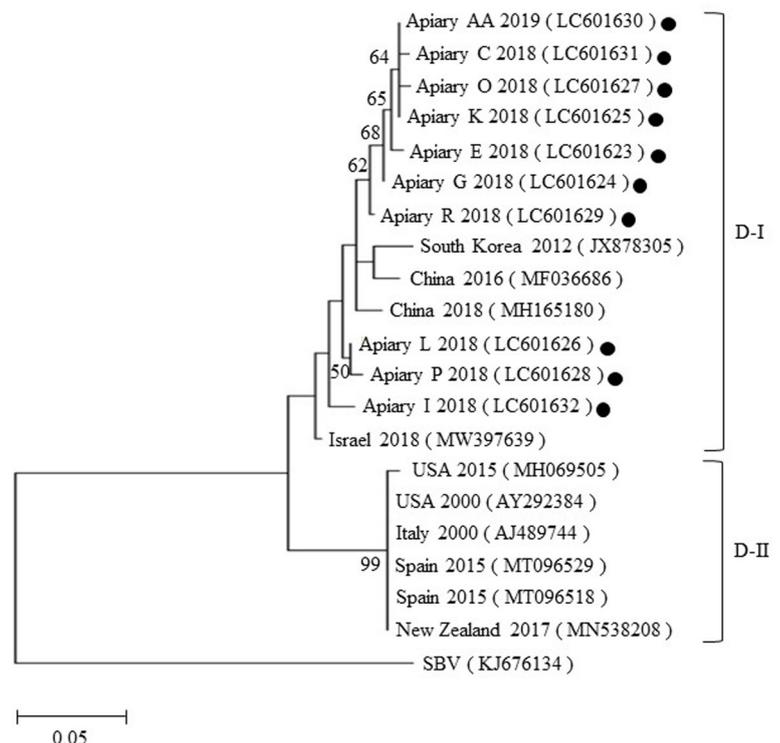


Fig. 1. Phylogenetic tree of deformed wing virus based on partial nucleotide sequence of RNA-dependent RNA polymerase (*RdRp*) gene (159 bp), constructed using the maximum likelihood method under the Kimura 2-parameter (K2). Bootstrap confidence limits were determined from 1,000 replications and nodes supported by bootstrap values ≥ 50 are shown. The current sequences are shown with apiary name, isolation year, and solid circles. The outgroup is the sequence of the sacbrood virus (KJ676134). The sequences used as references are as follows: China (MF036686 and MH165180), Israel (MW397639), Italy (AJ489744), New Zealand (MN538208), South Korea (JX878305), Spain (MT096518 and MT096529), and United States (AY292384 and MH069505).

Table 1. Viral prevalence of five worker bees in colonies positive for black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), and/or deformed wing virus (DWV)

Positive numbers of five worker bees	Colonies positive for BQCV (n=10)	Colonies positive for IAPV (n=7)	Colonies positive for DWV (n=6)
5	1	2	1
4	4	0	1
3	2	0	0
2	2	0	1
1	1	1	0
0	0	4	3

each other and 90.6–94.3% with those in the D-II clade. DWV was found in 94.0% and 8.1% of worker bees in China [1] and South Korea [5], respectively. In a previous study, although the relationship between DWV in Japan and South Korea was not mentioned, DWV detected in Japan before 2018 were related to Chinese samples according to the analyses of the capsid protein [22]. Therefore, these results suggest two possibilities: DWV has been imported into Japan from China or DWV has been maintained in Japanese apiaries. DWV prevailed in 70.0% of worker bees in Slovenia [19]; however, DWV lineages distributed in other European countries were not found in Gifu. Therefore, queen bees from Slovenia may not have contributed to the distribution of DWV in Gifu.

BQCV was clustered into three clades (B-I, B-II, and B-III) (Fig. 2). BQCV detected in Gifu were grouped into the B-I clade that included sequences from Japan and other Asian countries, whereas those from South Africa were grouped into the B-II clade,

and those from Australia and Slovenia were grouped into the B-III clade. BQCV in the B-I clade had a sequence similarity of 93.5–100.0% with each other, 93.2–97.5% with those in the B-II clade, and 90.4–97.1% with those in the B-III clade. Accordingly, the nucleotide sequence of the capsid protein region showed low divergence and there was no clear differentiation in BQCV nucleotide sequences among the B-I, B-II, and B-III clades. Although this region may not be suitable for the analysis of phylogenetic relatedness, we targeted this region because previous studies [12, 14, 22] have reported the nucleotide sequences of this gene region detected in Japan. The phylogenetic analysis of BQCV in the present study suggests not only intrusion from China and South Korea but also circulation in domestic honeybee populations, along with DWV.

Phylogenetic analysis revealed that IAPV did not split into clades (Supplementary Fig. 1). IAPV showed sequence similarity of 94.6–99.8% among Gifu, South Korea, US, Australia, and Spain.

Reddy [16] reported that the 5'-untranslated region (UTR) had high similarity among IAPV detected in countries such as South Korea, China, Israel, US, and Australia. Since the targeted regions in the present study were the 5'-UTR and the helicase gene, similarities may be observed between IAPV in Gifu and that found in other countries. In the present study, IAPV sequences in Gifu differed among apiaries (Supplementary Fig. 1). Our previous study [11] using this gene region showed that IAPV from honeybees were very closely related to honeybees and wild arthropods in the same apiary, but not to these species in other apiaries. Therefore, these results suggest that IAPV may not be frequently transmitted among apiaries in Gifu.

Furthermore, in the present study, no samples were positive for ABPV, KBV, and CBPV. In a previous study by Kojima *et al.* [12], these three viruses were not detected either. ABPV was found in 6.0% of worker bees in China and 40.0% in Slovenia, whereas KBV was detected in 1.6% of worker bees in South Korea and 1.7% in Slovenia [1, 5, 19]. CBPV was also found in China, South Korea, and Slovenia at the rates of 6.0%, 0.4%, and 18.3%, respectively [1, 5, 19]. Therefore, although the low prevalence of ABPV, KBV, and CBPV in these countries may be one of the reasons for the lack of detection of these viruses in Japan, our results suggest that the impact of imports on viral distribution in Japan may be limited.

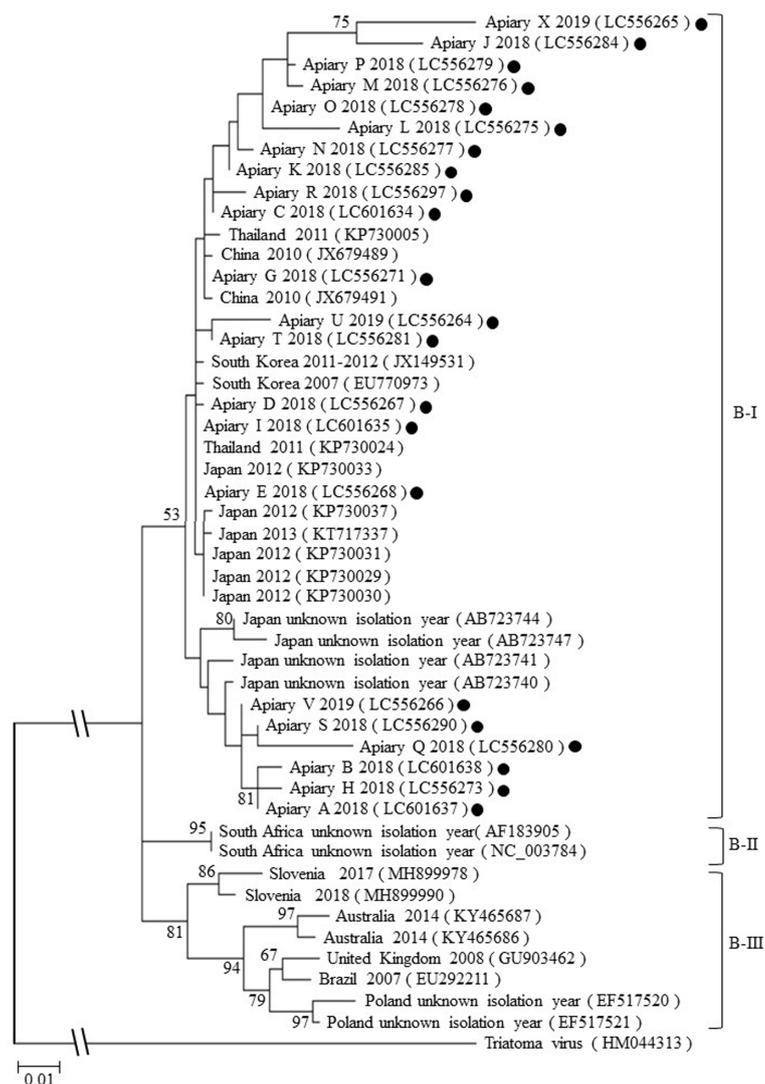


Fig. 2. Phylogenetic tree of black queen cell virus based on partial nucleotide sequence of capsid protein (511 bp), constructed using the maximum likelihood method under the Tamura 3-parameter with a discrete gamma distribution model (T92+G). Bootstrap confidence limits were determined from 1,000 replications and nodes supported by bootstrap values ≥ 50 are shown. The current sequences are shown with apiary name, isolation year, and solid circles. The outgroup is the sequence of *Triatoma virus* (HM044313). The sequences used as references are as follows: Australia (KY465686 and KY465687), Brazil (EU292211), China (JX679489 and JX679491), Japan (AB723740, AB723741, AB723744, AB723747, KT717337, KP730029–KP730031, KP730033, and KP730037), Poland (EF517520 and EF517521), Slovenia (MH899978 and MH899990), South Africa (AF183905 and NC_003784), South Korea (EU770973 and JX149531), Thailand (KP730005 and KP730024), and United Kingdom (GU903462).

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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