Original Article

PATHOGEN DETECTION AND PHYLOGENETIC ANALYSIS OF *AETHINA TUMIDA* MURRAY IN SOUTH KOREA

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Abstract

The small hive beetle (SHB), *Aethina tumida* Murray, is a parasite of honey bee colonies and causes the fermentation of honey as well as colony collapse. Outbreaks have been confirmed in Africa, America, Europe as well as Asia, where an outbreak was reported in the Philippines and South Korea in 2014 and 2017, respectively. In South Korea, in September 2016, this honey bee parasite was first identified in apiaries in Miryang, Gyeongnam Province. However, the invasion pathway of SHB has not been identified, and honey bee pathogens harbored by SHB have not been well characterized. Therefore, phylogenetic analysis of SHB with the use of COI gene and detection of fourteen common honey bee pathogens were conducted in this study. The confirmation of the fourteen honey bee pathogens in SHB showed that this beetle carries black queen cell virus and deformed wing virus. Therefore, SHB could have a role in the spread of these viruses. The way of entry of the SHB to South Korea remains undetermined, but the phylogenetic analysis of the COI gene revealed that it was most similar to species found in the USA. There is an urgent need for national-level monitoring and quarantine measures for preventing the spread of SHB infestation in South Korea.

Keywords: *Aethina tumida,* black queen cell virus, deformed wing virus, phylogenetic analysis, small hive beetle

INTRODUCTION

The small hive beetle (SHB) *Aethina tumida* Murray 1867 is a serious parasite of honey bees belonging to the family Nitidulidae, order Coleoptera. The SHB feeds on honey, pollen and bee brood which results in colony mortality, honey fermentation and comb damage (Neumann & Elzen, 2004). The SHB is native to Sub-Saharan Africa (Lundie, 1940) where it is considered to have a minor influence on beekeeping (Evans et al., 2000) but can cause serious damage to apiculture in non-native countries (Lounsberry et al., 2010; Cordeiro et al., 2019; Namin et al., 2019). SHB infestations have been recorded globally, including the USA (Thomas, 1998), Australia (Gillespie et al., 2003), Italy (Mutinelli et al., 2014) and South America (Toufailia et al., 2017). In Asia, an outbreak of SHB was reported in the Philippines in 2014

(Cervancia et al., 2016) and in South Korea (Lee et. al., 2017). The SHB was detected in September 2016 in Miryang, Gyeongnam Province, South Korea (Lee et al., 2017) and then spread to surrounding regions threatening beekeeping in the country (Lee et al., 2017; Namin et al., 2019). However, the route of invasion into South Korea remains unknown, but its understanding is important to prevent the spread of SHB to other regions (Neumann et al., 2016).

Phylogenetic analysis with the use of mitochondrial DNA, due to its higher mutation rate compared with that of nuclear DNA, has been demonstrated to be useful for the identification of relationships among geographical strains and research on the origin of populations (Birky, 2001; Allio et al., 2017). Therefore, the mitochondrial cytochrome *c* oxidase I (COI) gene has been widely used as a tool for analyzing the relationships and origins of invasive species, including SHB, in different countries (Evans et al., 2000; Li et al., 2018; Cordeiro et al., 2019; Namin et al., 2019).

On the other hand, parasites of honey bee were also known to indirectly affect apiculture by carrying and spreading honeybee pathogens (Forfert et al., 2015; Posada-Florez et al., 2020). Some studies have reported deformed wing virus (DWV) (Eyer et al., 2009), microsporidian *Nosema ceranae* (Cilia et al., 2018), trypanosome honey bee parasites *Lotmaria passim* and *Crithidia bombi*, and *Apis mellifera* filamentous virus (de Landa et al., 2020) in SHB. In South Korea, the beekeeping industry is facing the challenge of various honey bee diseases caused by viral, bacterial and fungal pathogens (Kang et al., 2012; Jung & Lee, 2018). However, vectors of the diseases have not been well characterized, and the pathogens harboured by SHB still remain unknown. The identification of the honey bee pathogens carried by SHB could be important for further study on the propagation of related diseases and estimating the influence of SHB to apiculture.

According to the present study, SHB infestation in apiaries was diagnosed and the phylogenetic relationship between South Korean SHB and those detected in other countries was investigated based on analysis of the COI gene, so that the route of invasion for this parasite in South Korea could be identified. In addition, fourteen honey bee pathogens in SHB were also detected to understand the types of honey bee pathogens that SHB are reservoirs for, in order to then determine potential honey bee diseases transmitted by SHB.

MATERIAL AND METHODS

Sample collection

SHB were diagnosed through the direct observation of whether eggs, larvae or adults were present in honey bee hives. Samples were collected according to the standard method of Neumann et al. (2013) from infested colonies between 2016 and 2018, from four apiaries in the city of Miryang where the SHB invasion was reported for the first time in South Korea and from one apiary in Damyang County, Jeollanam Province in 2021. One hive in each apiary with SHB was selected and transported to the Parasitic and Honey Bee Disease Laboratory, Animal and Plant Quarantine Agency, South Korea. Samples were used for species identification through morphological and molecular analysis. Morphological identification of *A. tumida* was carried out based on size, shape, colour and characteristics of the egg, larval, and adult stages of SHB (Lundie, 1940; Schmolke, 1974; Neumann et al., 2013). The specimens were observed under a stereomicroscope (M125; Leica, Wetzlar, Germany) for species identification. After species identification, the samples were stored at -20°C for further study.

Nucleic acid extraction

Total nucleic acid was extracted with the use of the Maxwell® RSC viral total nucleic acid purification kit (Promega, Madison, WI, USA). Two individual adult SHB or five larvae and 600 µL of PBS solution were added in a tissue-homogenizing tube with 2.381 mm diameter steel beads (SNC, Hanam, South Korea), and homogenized with the use of a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at four 15 s cycles at 5000 rpm.

Afterwards, 300 µL homogenate, 300 µL of lysis buffer, and 30 µL of proteinase K solution were added to each tube and incubated at 56°C for 10 min.

Nucleic acid was then purified automatically with the automated Maxwell® RSC instrument (Promega, Madison, WI) according to the manufacturer's instructions. Finally, 100 µl total nucleic acid acquired from each sample was used for the detection of honey bee pathogens. To extract DNA from SHB for the amplification of the COI gene, the FastDNA Spin Kit for Soil (Santa Ana, California, USA) was used with some modifications. Two individual adult SHB or five larvae were placed in a tissue-homogenizing tube with steel beads (SNC, Hanam, South Korea). After the addition of 978 µL sodium phosphate buffer and 122 µL MT buffer from the FastDNA Spin Kit for Soil (Santa Ana, California, USA), the samples were homogenized with the use of a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at four 15 s cycles at 5000 rpm. The following steps for DNA purification were done according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

The mitochondrial COI gene of four adult samples from four different apiaries was used for molecular identification of SHB and for phylogenetic analysis. A 1091-bp DNA fragment COI was amplified with a primer pair AT1904S (5′-GGTGGATCTTCAGTTGATTTAGC-3′) and AT2953A (5′-TCAGCTGGGGGATAAAATTG-3′) (Evans et al., 2000) and AccuPower PCR PreMix (Bioneer, Daejeon, Korea). The 20 µl reaction mix was composed of 5 µl of total nucleic acid, 1 µl (10 pmol) of each primer, and 13 µl of ddH_2O. PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 42°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 5 min. After confirming the expected band in electrophoresis gel, 1.5% agarose and 1× of RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, Inc., Gyeonggi-do, South Korea), the PCR products were sent to Macrogen (Seoul, South Korea) to be sequenced with the use of the Sanger sequencing method. The generated COI sequence was deposited on NCBI with accession number MZ234080. The COI sequence was compared to deposited sequences of *Aethina tumida* on NCBI with the use of the basic local alignment search tool (BLAST). Consensus sequences were aligned with the use of Clustal X2 (Larkin et al., 2007), and a neighbor-joining phylogenetic tree was constructed in MEGA7 (Kumar et al., 2016) with 1000 bootstrap iterations.

Detection of fourteen honey bee pathogens

Fourteen prevalent honey bee pathogens were detected in the total nucleic acid extracted from four different samples of collected adult SHB to confirm whether they were carrying these honey bee pathogens. The pathogens include the following viral pathogens: DWV type A, acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV), three fungal pathogens (*Nosema* spp., *Aspergillus flavus*, and *Ascosphaera apis*), two bacterial pathogens (*Paenibacillus larvae*, which causes American foulbrood, AFB; and *Melissococcus plutonius*, which causes European foulbrood, EFB), tracheal mite (*Acarapis woodi*), and phorid fly (*Apocephalus borealis*). Specific primers for detection of each pathogen are shown in Tab. 1. Detection of viral pathogens with an RNA genome was done through the use of conventional reverse transcription PCR (RT-PCR) and the AccuPower® RT-PCR premix (Bioneer, Daejeon, Korea). For the detection of other pathogens with a DNA genome, conventional PCR and the AccuPower® ProFi Taq PCR PreMix (Bioneer, Daejeon, Korea) were used. The 20 µl reaction mix was composed of 5 µl total nucleic acid, 1 µl (10 pmol) of each primer, and 13 µl of ddH_2O. Reverse transcription was done at 42°C for 60 min prior to PCR. PCR was performed at 94°C (5 min) for pre-denaturation, followed by 35 cycles of 94°C (30 sec) for denaturation, 30 sec for annealing, 72°C (1 min) for extension, and 72°C (10 min) for final extension. The annealing temperature of each primer pair is shown in Tab. 1.

Table 1.

Specific primers used for detection of honey bee pathogens

No.	Pathogen		Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp (°C)	Reference
1	Virus	SBV	F: ACCAACCGATTCCTCAGTAG R: CCTTGGAACTCTGCTGTGTA	487	57	Grabensteiner et al., 2001
2		ABPV	F: TTATGTGTCCAGAGACTGTATCCA R: GCTCCTATTGCTCGGTTTTTCGGT	901	55	Benjeddou et al., 2001
3		CBPV	F: AGTTGTCATGGTTAACAGGATACGAG R: TCTAATCTTAGCACGAAAGCCGAG	455	55	Ribiere et al., 2002
4		DWV	F: TCATCTTCAACTCGGCTTTCTACG R: CGAATCATTTTCACGGGACG	479	62	Lee et al., 2005a
5		BQCV	F: TGGTCAGCTCCCACTACCTTAAAC R: GCAACAAGAAGAAACGTAAACCAC	701	55	Benjeddou et al., 2001
6		KBV	F: GATGAACGTCGACCTATTGA R: TGTGGGTGGCTATGAGTCA	415	50	Stoltz et al., 1995
7		IAPV	F: GATTTGAGAGATGTATTTCCTTCT-GCGG R: ACACTTGCGTTGGTCCTGAATGT-TAATGG	725	52	This study
8	Bacteria	*Paenibacillus larvae*	F: GTGTTTCCTTCGGGAGACG R: CTCTAGGTCGGCTACGCATC	232	55	Lee et al., 2004
9		*Melissococcus plutonius*	F: AAGAGTAACTGTTTTCCTCG R: AAACCTTATCTCTAAGGCGT	583	52	Ha et al., 2005
10	Fungus	*Ascosphaera apis*	F: GGCTGTAGGGGGGAACCAGGA R: CGGGTGGTCGTTTCCAGCCTC	995	62	Lee et al., 2005b
11		*Aspergillus flavus*	F: ATCGGGCGGTGTTTCTATG R: ACCGGGCTATTTAAGGGCCG	311	55	Lee et al., 2004
12	Parasite	*Nosema* sp.	F: CTGCCTGACGTAGACGCTAT R: CTTCGATCCTCTAGCTTACG	592	50	Yoo et al., 2008
13		*Acarapis woodi*	F: CAGTAGGGCTAGATATCGATACC-CGAGCTT R: TGAGCTACAACATAATATCTGTCAT-GAAGA	247	55	This study
14		*Apocephalus borealis*	F: GTACACCTATACATTGGGTTCGTACAT-TAC R: GAGRGCCATAAAAGTAGCTACACC	500	57	This study

Abbreviation: SBV- sacbrood virus; ABPV- acute bee paralysis virus; CBPV- chronic bee paralysis virus; DWV- deformed wing virus; BQCV- black queen cell virus; KBV- Kashmir bee virus; IAPV- Israeli acute bee paralysis virus.

RESULTS

Infestation of small hive beetle in apiaries

SHB infestation was detected through the observation of eggs, larvae or adults deposited in the combs of honey bee hives (Fig. 1). Morphological identification of SHB was carried out by the naked eye in the apiary and then by microscopy in the laboratory. Eggs of *A. tumida* were observed in egg clutches; they were around 1.4 × 0.26 mm (length × width), and two thirds the size of honey bee eggs and a pearly-white colour (Fig. 2E and 2F). The larvae were identified as being 10 mm in length and were characterized by three pairs of legs located close to the head, two rows of dorsal spines, and two larger paired spines on the posterior segment of the dorsum (Fig. 2B-2D). Adult



Fig. 1. Infestation of small hive beetle in honeybee hives. *Aethina tumida* in apiaries were diagnosed through the observation of the presence of adults (A), and larvae in supplied pollen (B) and honeycomb of infested hive (C).

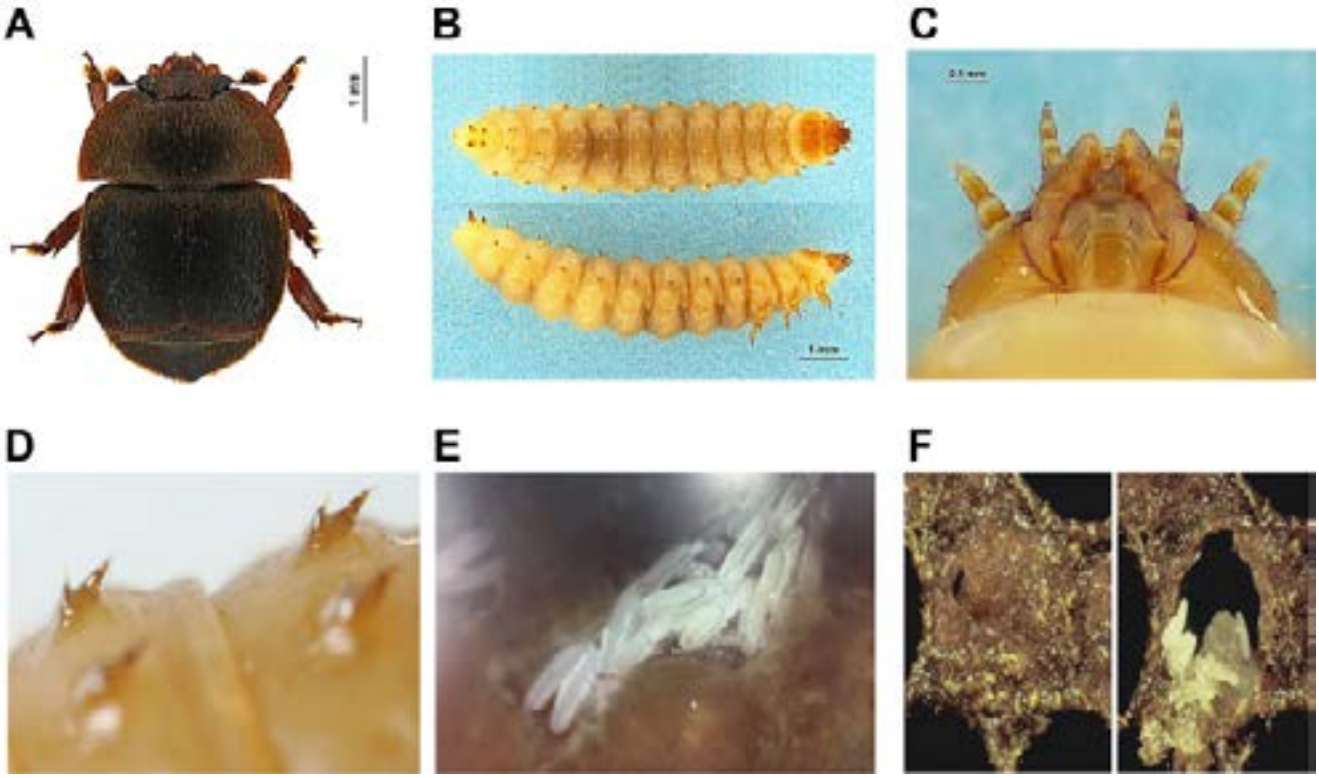


Fig. 2. Identification of small hive beetle based on morphological characteristics. Appearance of adults observed with 5~7 mm × 3~5 mm (length × width) of body size, dark brown colour, one pair of clubbed antennae on the head, and body shape of oval and dorsoventrally flattened (A). The larvae were characterized by three pairs of legs located close to the head, two rows of dorsal spines, two larger paired spines on the posterior segment of the dorsum, and body size was around 10 mm in length (B-D). Eggs of SHB were seen in capped bee brood with pearly-white colour and size of around 1.4 mm × 0.26 mm (length × width) (E and F).

A. tumida were identified as dark brown to black colour, having three parts of body (head, thorax, and abdomen), three pairs of legs and body size of 5~7 mm × 3~5 mm (length × width); they were oval and dorsoventrally flattened and had one pair of clubbed antennae on the head. The

pronotum was identified by sharp latero-posterior tips, and the abdomen was not entirely covered elytra (Fig. 2A).

Phylogenetic analysis of SHB

The COI gene of SHB collected from different apiaries in Miryang and Damyang, South Korea were 100% identitical to one another. These generated sequences also were 100% identical to reported COI sequences of SHB originating from the USA (NCBI accession no.: KC966652) and South Korea (MN023006). Phylogenetic analysis was conducted to identify the relationships among South Korean haplotypes and reported SHB haplotypes. The results showed a close relationship of the detected SHB strains to those originating from the USA, Canada and Costa Rica (Fig. 3).

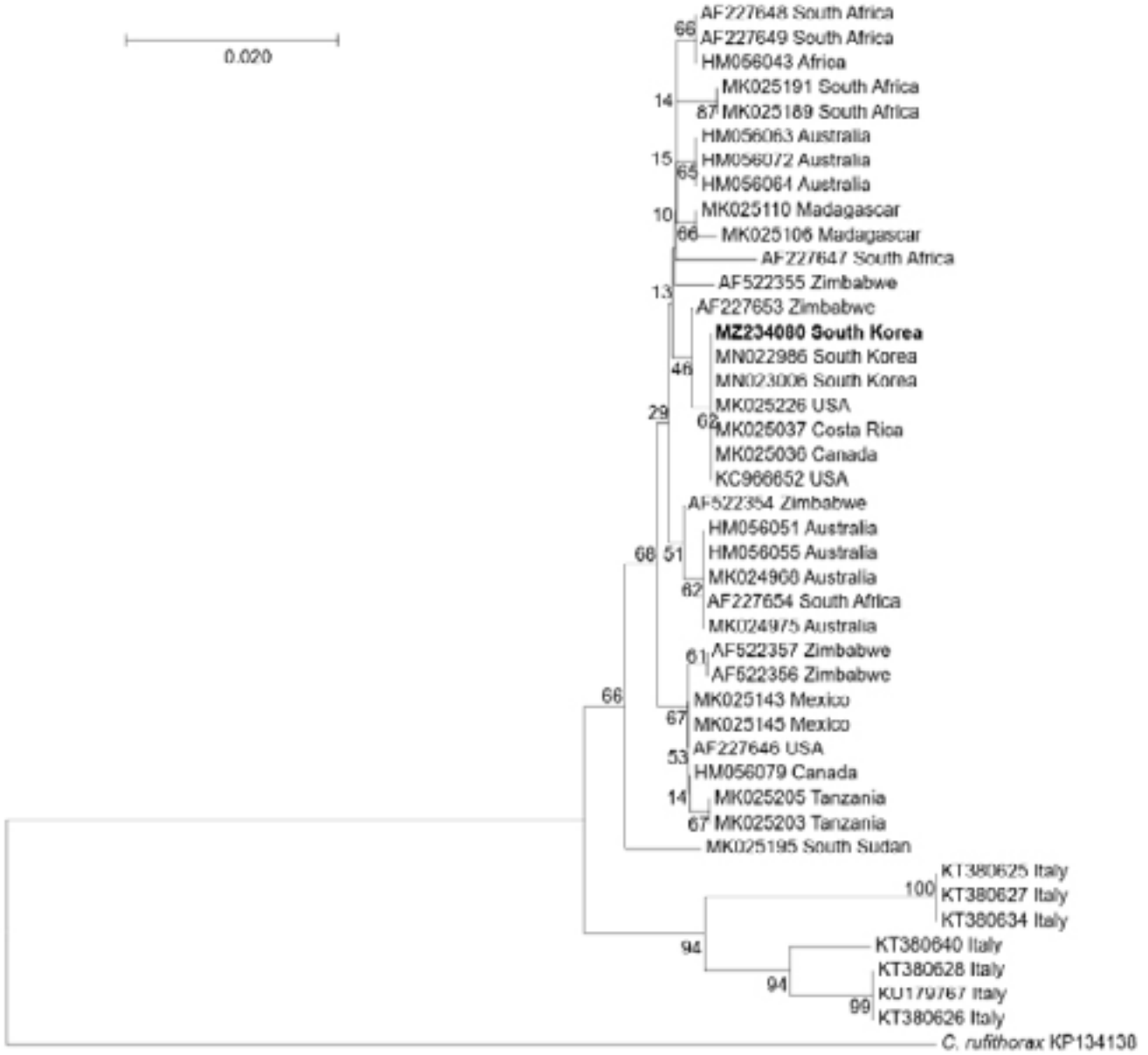


Fig. 3. Phylogenetic tree of mitochondrial cytochrome *c* oxidase I (COI) gene sequences from small hive beetle (SHB). Neighbor-joining phylogenetic tree was created based on mitochondrial COI gene of SHB with 1000 bootstrap iterations in MEGA7. *Aethina tumida* detected in this study with NCBI accession number MZ234080 was written in bold. Other reference strains of *A. tumida* with NCBI accession numbers and country names are shown. *Cratonura rufithorax* was used as an outgroup. Number above line represents bootstrap percentages.

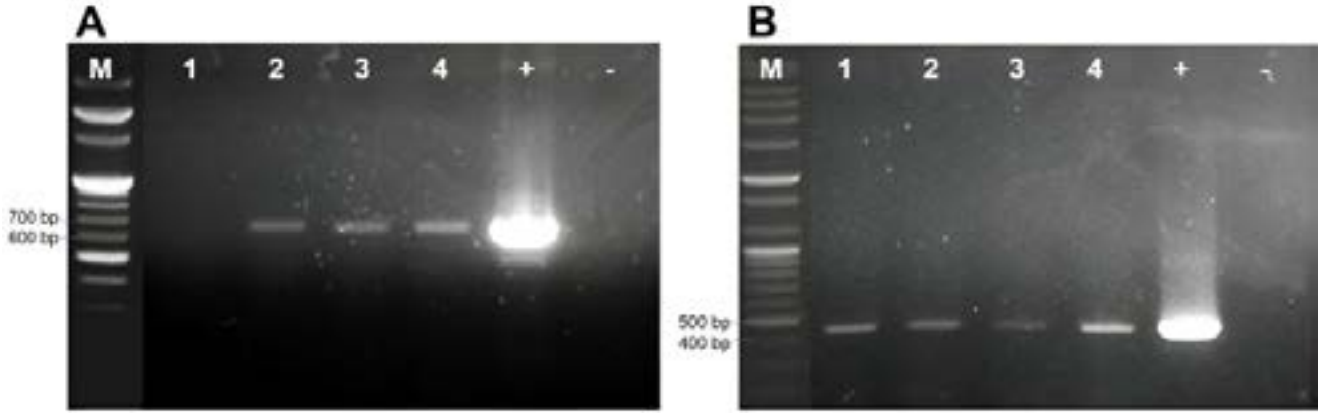


Fig. 4. Detection of honeybee pathogens in small hive beetle (SHB). Positive results of black queen cell virus (A) and deformed wing virus (B) detection were confirmed with expected band, 701 bp and 479 bp long, respectively, in electrophoresis. Lanes 1 to 4 were for four SHB samples. "M" is a 100-bp DNA marker. "+" and "-" are positive using recombinant DNA and negative control without DNA template, respectively.

Detection of honey bee pathogens in SHB

Four adult samples and one larval sample of SHB were used for the detection of fourteen prevalent honey bee pathogens by specific conventional PCR (Tab. 1). The results showed that two of the fourteen honey bee pathogens were detected in the adult SHB (DWV and BQCV), of which, DWV was detected in all four adult samples, and BQCV was detected in three adult samples. The result was confirmed through electrophoresis with the expected band of 479 bp and 701 bp long for DWV and BQCV detection, respectively (Fig. 4).

DISCUSSION

An SHB infestation was recorded in 2016 in Miryang, and this outbreak spread to the surrounding areas, including Changyeong in the same province (Lee et al., 2017; Namin et al., 2019). The invasion poses a threat to apiculture in South Korea, particularly regarding the possibility of spreading to Asian honey bee (*Apis cerana*) populations. Therefore, various studies on the control of this parasite have been conducted (Kim et al., 2017; Hong & Jung, 2018). Our results revealed that the collected SHB samples carried two honeybee pathogens, DWV and BQCV, and showed a close relationship between the South Korean SHB and the SHB that originated from the USA, Canada, and Costa Rica.

The positive detection of DWV and BQCV among the fourteen prevalent honey bee pathogens in SHB suggests that this pest has the potential to be a natural reservoir of these two viruses, and the spread of SHB could contribute to the transmission of these two honey bee pathogens in South Korean apiculture. BQCV was detected in SHB for the first time in this study. Increasing the scale of surveillance across the whole country for detection of SHB, honey bee pathogens carried by SHB, and the role of SHB in the spread of honey bee pathogens is important for controlling SHB and to mitigate the damage to the beekeeping industry.

Phylogenetic methods are useful for tracing the origin of SHB (Li et al., 2018). The high sequence homology of the COI gene of the South Korean SHB with that of the USA populations and phylogenetic analysis showed a close relationship between the Korean SHB and those originated from USA, Canada and Costa Rica. The analysis of the COI gene suggested that the USA population originated from the SHB population in South Africa (Evans et al., 2000), which was also the origin of the Australian population (Lounsberry et al., 2010; Namin et al., 2019). This suggests that the Korean and Canadian variant of SHB could have originated from the USA. Furthermore, the low variation of the COI gene and restricted area of the original invasion of SHB in South Korea suggest that this pest was introduced into South Korea from a single

source to a specific region. However, it could not be confirmed whether the South Korean SHB was introduced from USA, Canada, or Costa Rica. Further study on microsatellite analysis (Evans et al., 2008) of samples collected from these countries could be useful in identifying the origin of Korean SHB. Understanding the global movement of SHB is important to identify the routes of invasion and to establish a strategy to control the spread of SHB through the trade of honey bees and such bee products as beeswax (Idrissou et al., 2019).

Our results showed that the SHB detected in South Korea carried two honey bee viral pathogens. This information is useful for further research on the role of SHB in the transmission of honey bee pathogens. The present study was also conducted to analyse the phylogenetic relationships of South Korean SHB collected in Damyang and Miryang where the invasion of SHB into South Korea was first reported. The sequence analysis of the COI gene showed a close relationship between the South Korean SHB and those from USA, Canada and Costa Rica. However, the pathway of invasion remains unclear. Therefore, the strict examination of trade in honey bees or honey bee products could be important to control the spread of SHB.

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