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First identification of sac brood virus (SBV) in the not native species *Megachile sculpturalis*

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Abstract

The spillover of *Apis mellifera* L. pathogens represents one driver implicated in the decline of pollinators. The sac brood virus (SBV) is a widespread pathogen of honey bees, which mainly hits the brood, but it can remain active in pollen and asymptomatic adult bees, continuing the infection in the colony. This investigation aimed to evaluate the SBV infection in an Italian population of the non native giant resin bee *Megachile sculpturalis* (Smith). Among twenty-three *M. sculpturalis* sampled in the Italian Apennines, fifteen individuals (65.20%) scored positive for SBV. In all positive samples, the SBV was found replicative highlighting the active infection in the host. These results support the susceptibility of *M. sculpturalis* to honey bee pathogens, and this requires further investigation to better understand the ecological consequences of spillover.

Key words: spillover, invasive bee, *Apis mellifera*, honey bee virus, giant resin bee.

Introduction

Megachile sculpturalis (Smith), the giant resin bee, is a species native to East Asia (Iwata, 1933), that reached accidentally Europe (Ruzzier *et al*., 2020; Bila Dubaić and Lanner, 2021). This is the first non-native bee species introduced in Europe (Ribas-Marquès *et al*., 2021). Its arrival in Europe probably occurred with the introduction of a nest through timber or ornamental garden plant trading or other commercial routes (Lanner *et al*., 2020), even if the introduction of an adult fertilised female cannot be completely ruled out (Ribas-Marquès *et al*., 2021). Since its first European finding in Marseille (France) in 2008 (Vereecken and Barbier, 2009), this species has spread throughout the continent rather quickly. Other findings were also reported in 2009 in Italy (Quaranta *et al*., 2014), in 2015 in Hungary (Kovács, 2015) and Germany (Westrich *et al*., 2015), in 2016 in Slovenia (Gogala and Zadravec, 2018), in 2018 in Spain (Aguado *et al*., 2018), and 2019 in Liechtenstein (Lanner *et al*., 2020), Croatia (Ribas-Marquès *et al*., 2021), and Crimea (Ivanov and Fateryga, 2019). Like other Megachilidae species, the giant resin bee nests in pre-existing cavities, usually made by other insects, or in empty reeds, where the female lays her eggs lined. Each brood cell is built using mud and resin and the female supplies it with pollen and nectar, before laying the egg (Aguado *et al*., 2018; Lanner *et al*., 2020; Ribas-Marquès *et al*., 2021).

The sac brood virus (SBV), is an ssRNA(+) virus (Bailey *et al*., 1964; Li *et al*., 2019) belonging to the genus Iflavirus within the family Iflaviridae, according to the International Committee on Taxonomy of Viruses (ICTV). The virus is one of the most widespread pathogens of the honey bee *Apis mellifera* L., and, since its first isolation, SBV has been found in every part of the world where beekeeping practices are present (Chen and Siede, 2007; Boncristiani *et al*., 2020). The virus mainly affects the brood, giving it the characteristic sac-like appearance of infected larvae, since they are unable to get rid of their leathery endocuticle, under which a large amount of ecdysial fluid containing the viral particles accumulates between the larva body and its unshed cuticle (Chen and Siede, 2007). After the death of larvae, SBV remains active for up to one month in the carcasses, honey or pollen, spreading the infection at the colony level (Rana *et al*., 2011). Besides, asymptomatic infections are common in healthy bees, where the virus accumulates in the hypopharyngeal glands, contributing to the spread of the virus by feeding larvae with contaminated food (Anderson and Gibbs, 1988; Grabensteiner *et al*., 2001; Shen *et al*., 2005). The disease usually occurs from early spring, as the colony population increases, until early summer (Tentcheva *et al*., 2004; Berényi *et al*., 2006; Chen and Siede, 2007).

Among the recent interest in the honey bee pathogens spillover, this investigation aims to evaluate the presence of SBV in an Italian population of the non native species *M. sculpturalis.*

Materials and methods

Twenty-three free-ranging individuals of *M. sculpturalis* foraging on lavender (*Lavandula angustifolia*) were randomly collected on 31 July 2021 in Palazzuolo sul Senio (Florence, Italy). All the samples were individually stored in a tube at −20 °C, for one day, until analysis.

Before extraction, all samples were washed with 95% ethanol to remove external microbial contaminations. Each individual was analysed individually, placing each sample in a 2mL microtube with 500 µL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and crushed with a TissueLyser II (Qiagen, Hilden, Germany) for 3 minutes at 30 Hz, as previously reported (Cilia *et al*., 2019; Nanetti *et al*., 2021b). Total RNA was extracted using Quick-RNA Microprep Plus Kit (Zymo Research) following the modified manufacturer's instructions for solid tissue processing (Mazzei *et al*., 2019). The obtained RNAs were eluted in 100 µL of DNAase-RNase-free water and the extracts were

stored at −80 °C until the qPCR assays.

To quantify the SBV abundance in the samples, all RNA extracts were analysed through Real-Time PCR, using Power SYBR™ Green Cells-to-CT™ Kit (ThermoFisher Scientific, Waltham, MA, USA), as previously reported (Cilia *et al*., 2021), using specific primer Fw SBV 311F 79 (5'-AAGTTGGAGGCGCGyAATTG-3') and Rev SBV 380R (5'- CAAATGTCTTCTTACdAGAGGyAAGGATTG-3') (Chantawannakul *et al*., 2006).

The Real-Time PCR assay was performed on QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific). SBV RNA previously extracted from positive honey bees was used as the positive control. All the analyses were conducted in duplicates.

For the quantification, a standard curve was generated by amplifying the serially diluted recombinant plasmids containing the pathogen-specific RNA fragment from 1×10^1 to 1×10^9 copies as previously reported (Mazzei *et al*., 2019; Cilia *et al*., 2021), following the amplification and quantification protocols (Chantawannakul *et al*., 2006).

The active replication of SBV was evaluated using strand-specific RT-PCR, as previously described (Mazzei *et al*., 2018; Nanetti *et al*., 2021b). Reactions were performed for all RNAs extracted using QuantiTect Reverse Transcription Kit (Qiagen), using the SBV primers. The obtained cDNAs were amplified by PCR for the related viral targets, and the amplicons were visualized on a 2% agarose gel. As positive control was used SBV previously isolated from honey bees, while as a negative control double distilled RNase-DNase-free water. Subsequently, the amplicons were sequenced (BMR Genomics, Padua, Italy) and analysed using BLAST (Altschul *et al*., 1990).

A phylogenetical analysis was performed on SBV polyprotein sequences deposited in GenBank using the Neighbor-Joining method (Saitou and Nei, 1987) associating taxa clustered together in the bootstrap test (500 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1112 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Results

Fifteen individuals (65.20%) scored positive for SBV (table 1). In the positive samples, the mean copy abundance was $2.52 \times 10^3 \pm 3.00 \times 10^3$ per bee.

The strand-specific RT-PCR demonstrated active viral replication of SBV in all positive PCR-positive samples (figure 1).

The SBV sequence was the same in all positive samples. The BLAST analysis performed on the obtained amplicons confirmed the same sequence for all positive individuals and highlighted the specificity of the sequences, with similarity (88.66% of identity, 1e-141 of E-value, 98% of Query Cover) to specific SBV genomes deposited in GenBank. The phylogenetic analysis and pairwise distance analysis indicated a similarity to SBV strains isolated from *A. mellifera* in Austria (figure 2).

Table 1. Summary of the *M. sculpturalis* (Ms) individuals analysed (* replicative).

Samples	SBV	Mean copy number per bee
Ms1	Positive*	2.25×10^2
Ms2	Negative	
Ms3	Positive*	1.34×10^2
Ms4	Negative	
Ms5	Negative	
Ms6	Positive*	5.39×10^3
Ms7	Positive*	6.51×10^2
Ms8	Positive*	2.68×10^3
Ms9	Negative	
Ms10	Positive*	4.44×10^1
Ms11	Positive*	9.47×10^3
Ms12	Positive*	8.29×10^2
Ms13	Negative	
Ms14	Positive*	6.07×10^2
Ms15	Positive*	5.91×10^3
Ms16	Positive*	1.30×10^2
Ms17	Positive*	5.80×10^2
Ms18	Negative	
Ms19	Positive*	4.75×10^2
Ms20	Positive*	6.07×10^3
Ms21	Negative	
Ms22	Positive*	4.67×10^3
Ms23	Negative	

The SBV sequences were deposited in GenBank with the Accession Number OM638044.

Discussion

Pathogens and parasites are deemed drivers of pollinator decline, together with other factors including pesticides and global warming. The honey bee pathogens spillover seems to represent one cause of this decline with severe ecological aspects (Meeus *et al*., 2011; Pritchard *et al*., 2021). The present study showed for the first time the presence of SBV in free-flying *M. sculpturalis* collected in Italy. The viral replication highlighted the active SBV infection, demonstrating the adaptation in the new host. This is not the first time that SBV is detected in wild bees; the virus was previously detected in several *Andrena*, *Bombus*, *Halictus* and *Lasioglossum* species, as well as in *Anthophora plumipes* (Pallas), *Ceratina dentiventris* Gerstaecker, *Eucera nigrescens* Perez, *Nomiapis diversipes* (Latreille), *Pseudoanthidium scapulare* (Latreille), *Xylocopa violacea* (L.) (Nanetti *et al*., 2021a; Cilia *et al*., 2022). Focusing on Megachilidae family, SBV infection was also reported in *Megachile melanopyga* Costa, *Megachile albisecta* (Klug), *Megachile brevis* Say and *Megachile rotundata* (F.) in Italy, France, USA and Canada, respectively (Dolezal *et al*., 2016; Melathopoulos *et al*., 2017; Dalmon *et al*., 2021; Cilia *et al*., 2022) and in

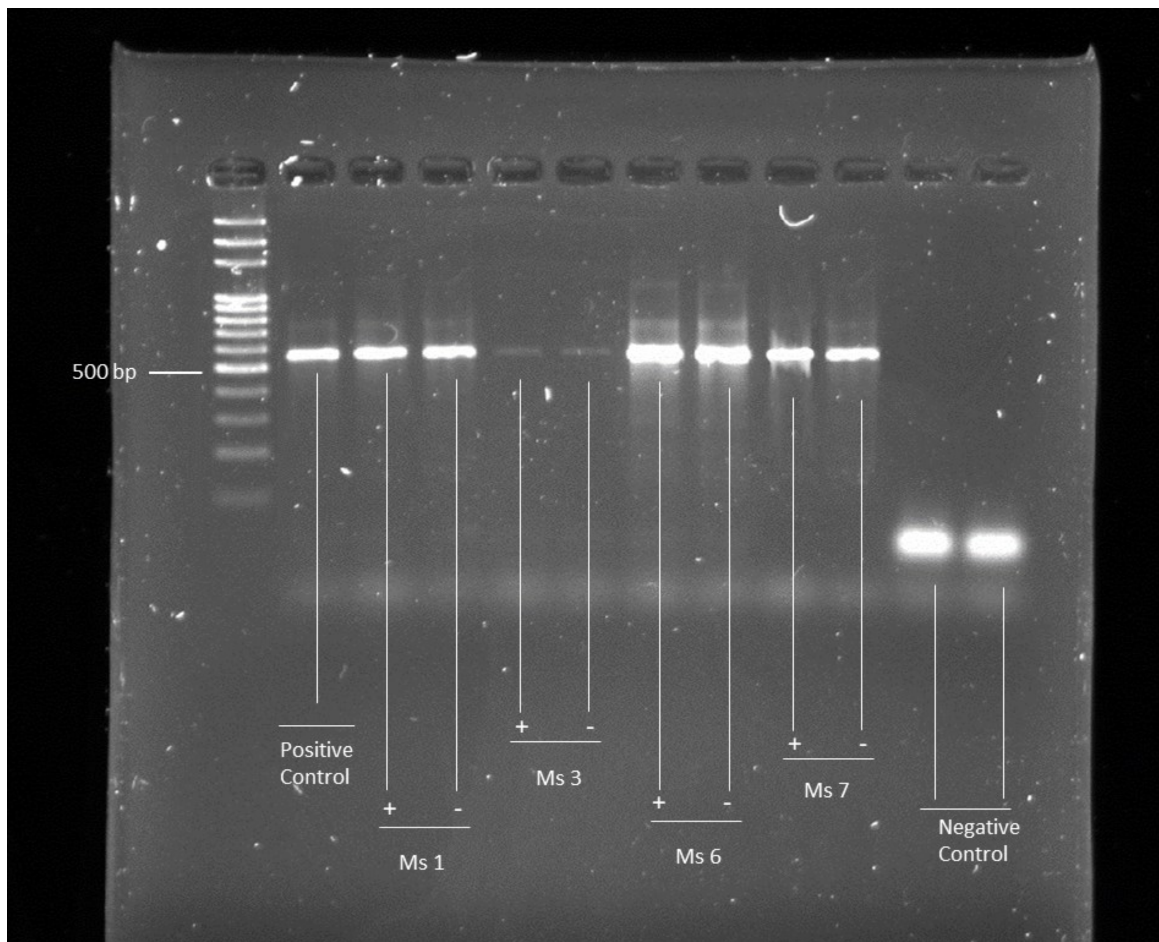

Figure 1. Evidence of genomic and replicative SBV in *M. sculpturalis.* Gel electrophoresis of strand specific RT-PCR performed on cDNA obtained from positive individuals. Genomic strand (+) and replicative strand (-). Replicative strand (-) and genomic strand (+). As positive control was used SBV previously isolated from *A. mellifera.* As a negative control double distilled RNase-DNase-free water. Ms = *Megachile sculpturalis*.

Osmia bicornis (L.) populations used for apple orchards pollination in Georgia, Germany and Kyrgyzstan (Radzevičiūtė *et al*., 2017). Excluding bees, the viral RNA was also detected in Vespidae and syrphids, as well as in *Aethina tumida* Murray, *Galleria melonella* (L.), *Blattella germanica* (L.) and *Polistes dominula* (Christ) (Nanetti *et al*., 2021a; Cilia et al., 2022). Although the number of investigated individuals is limited, the prevalence of SBV-positive detected in this study resulted higher (65.20%), than reported in *M. albisecta* (33.3%), *M. brevis* (17.0%), and *M. rotundata* (14.3%) (Dolezal *et al*., 2016; Melathopoulos *et al*., 2017; Dalmon *et al*., 2021).

The generalist SBV infection in several insect species (Nanetti *et al*., 2021a), could be an important key to evaluate the epidemiology of this virus. Usually, the SBV infection is associated with honey bee brood (Chen and Siede, 2007), but the detections and isolations of SBV variants in different insect species may suggest the possible spillover from the reservoir (*A. mellifera*) or it could mean that the virus has always been present in many different species and spillover and spillback events occurs, as recently verifies for other viruses in bumblebees (Martin *et al*., 2021; Pereira *et al*., 2021). However, the low viral abundance in each individual seems to indicate a chronic SBV infection, suggesting that a balanced host-pathogen interaction that would be occurred after a long time of co-existence. No SBV sequences isolated in Italy are deposited in GenBank, and the virus identified in this study showed similarity (88.66% identity) with an SBV sequence isolated from the honey bees in Austria (Grabensteiner *et al*., 2001).

The replicative viruses finding in *M. sculpturalis* highlighted the active infection of SBV, although the transmission route must be clarified. The foraging activity could also promote virus spillover (Grozinger and Flenniken, 2019). The SBV can be transmitted to the giant resin bee by the ingestion of contaminated pollen and nectar (Graystock *et al*., 2013; Burnham *et al*., 2021), or by direct contact with infected flower visitors (Singh *et al*., 2010; Mazzei *et al*., 2014). Besides, vertical transmission is not excluded, as reported for honey bees (Chen and Siede, 2007; Rana *et al*., 2011). Indeed, the high SBV prevalence detected in the investigated *M. sculpturalis* individuals could be associated with vertical transmission, opposing the idea of spillover, but justifying a possible adaptation of the pathogen in the investigated species.

To investigate the mode of transmission, it would be interesting to verify the presence and effects of SBV on *M. sculpturalis* larvae, by analysing the brood cells inside the nest.

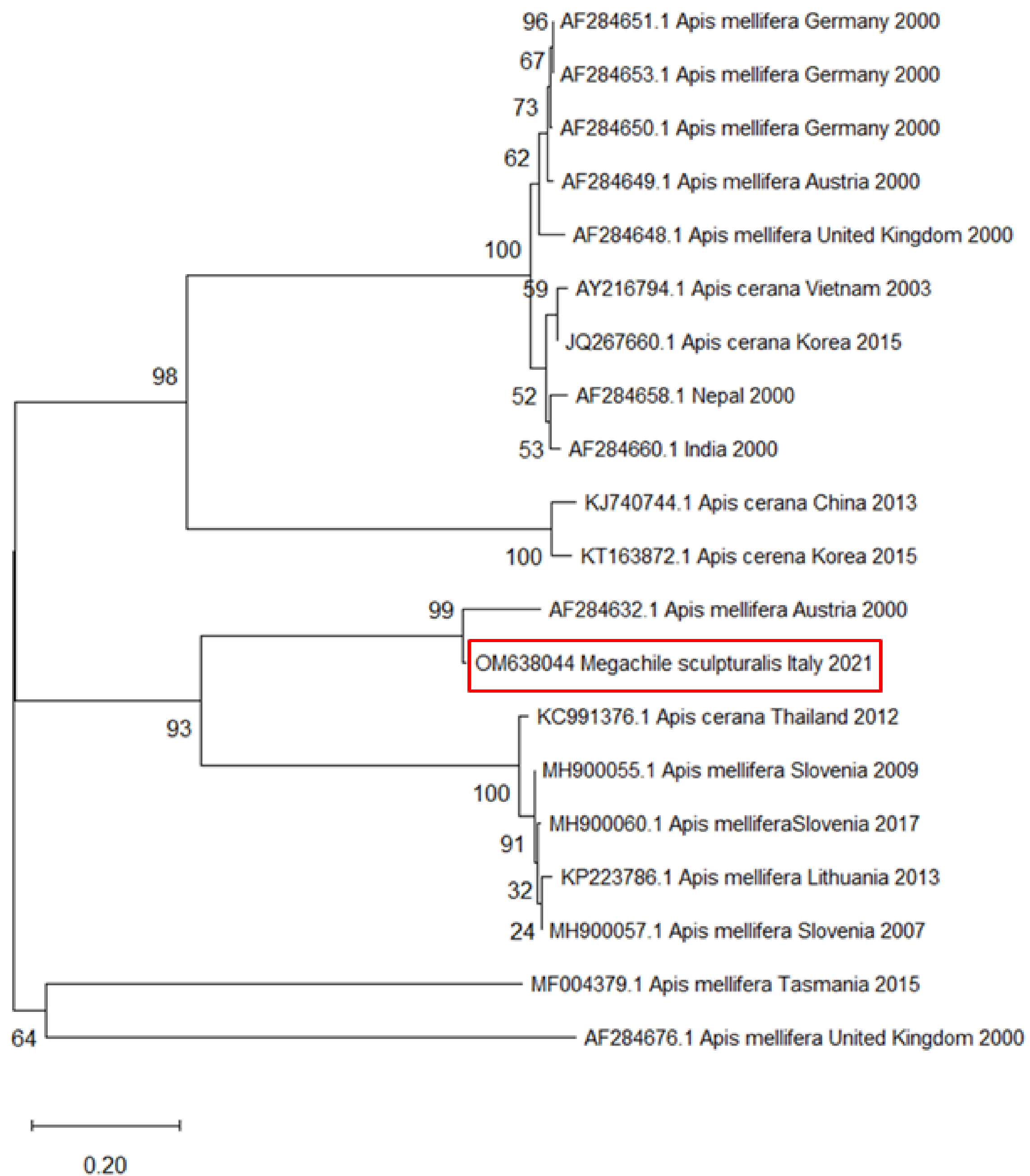

Figure 2. Molecular phylogenetic analysis for polyprotein gene of sac brood virus (SBV) using the Neighbor-Joining method. Accession number, host, state, and year of available GenBank SBV sequences are shown. SBV sequence accession numbers are reported and associated with year and site of origin and type. The SBV sequence obtained from the *M. sculpturalis* samples is in a red box.

In conclusion, the spillover of honey bee pathogens to wild bees may represent a threat to the health of pollinators with a high impact on their ecology (Graystock *et al*., 2013; Gisder and Genersch, 2017; Nanetti *et al*., 2021b). Further studies are needed to elucidate the mechanism of infection and the role of *M. sculpturalis* in the SBV spread and to evaluate the real effect of honey bee pathogens spillover to wild pollinators.

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