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Short communication

Replicative DWV type A in *Bombus terrestris* in Pantelleria island (Sicily, Italy)

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ABSTRACT

The deformed wing virus (DWV) is one of the most common bee pathogens, deemed responsible for both honey bee colony losses and general pollinator decline. That virus may infect both managed and wild bumblebees. In this study, the DWV infection was investigated in 52 free-flying *Bombus terrestris* (L., 1758) individuals from Pantelleria. This is a volcanic island in the Sicilian Channel. Of the collected individuals, 59.62% scored positive for DWV, with a mean abundance of $2.97 \times 10^5 \pm 1.46 \times 10^6$ copies per bee. Active replication of the virus could be demonstrated in all positive samples. All the sequences belonged to DWV type A. However both phylogenetic and pairwise distance analysis indicated a low similarity to Italian and Tunisian strains. Further studies are needed to elucidate the epidemiology of DWV in *B. terrestris* and the drivers of possible genetic modifications of the virus on Pantelleria island.

Introduction

Islands and areas at the boundaries of different bioregions are highly interesting for their species richness levels, which depend on their specific geographical position (Minelli, 2012).

Pantelleria is a volcanic island, that emerged approximately 324,000 years ago. Since its appearance, it has never had direct contact with the emerged mainland (Agnesi and Federico, 1995). The most relevant event in the determination of the present faunistic composition was the eruption that occurred approximately 45 thousand years ago, resulting in the coverage of the entire island with a stone layer approximately 5 m thick ("green ignimbrite"). Considering the Mediterranean area, Pantelleria stands out for its isolation, being located 70 and 120 km off the Tunisian and Sicilian coasts, respectively (Agnesi and Federico, 1995). Due to its position in the Sicilian Channel, Pantelleria hosts unique faunistic assemblages with affinities with both the European and African communities and includes endemic taxa also. For example, Pantelleria thrives as residents typically North African bird species (Corso et al., 2012), like the North African blue tit (*Cyanistes teneriffae* (Lesson 1831)), as well as at least 20 endemic invertebrate species (Muscarella and Baragona, 2017). Also, the island hosts several wild bee species, which naturally live in the Mediterranean area including Italy and Tunisia (Pagliano, 2003). Unfortunately, these species are not well studied, and more surveys is needed.

Honey bees (*Apis mellifera*) are kept on the island in form of two different subspecies: *A. m. siciliana* (Dalla Torre, 1896), which is naturally distributed in all Sicilian areas, and *A. m. ligustica* (Spinola, 1806), that was introduced by professional beekeepers not living in the island. Besides, Pantelleria hosts several wild and unmanaged honey bee colonies belonging to the African lineage, which live in natural cavities of the volcanic stone.

Despite beekeeping was not established recently on Pantelleria, little is known about the epidemiology of pathogens in honey bees and wild pollinators living on the island. To the best of the authors' knowledge, deformed wing virus DWV and black queen cell virus (BQCV) were the only two pathogens present in wild and managed honey bee colonies, while sacbrood virus (SBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV) were not detected (Freda et al., 2022). Indeed, the small islands represent very important case studies for bee research, especially for the pathogens transmission investigation; i.e the *Varroa destructor*-free honey bee colonies in Gorgona islands (Tuscany, Italy), or the epidemiology of nosemosis in the Tuscanian Archipelago (Italy) and Azores (Portugal), or the spillover transmission in Hawaii (U.S.A.) (Cilia et al., 2019; Giusti et al., 2016; Lopes et al., 2022; Santamaria et al., 2018).

Among the bee pathogens, the DWV is deemed responsible for the

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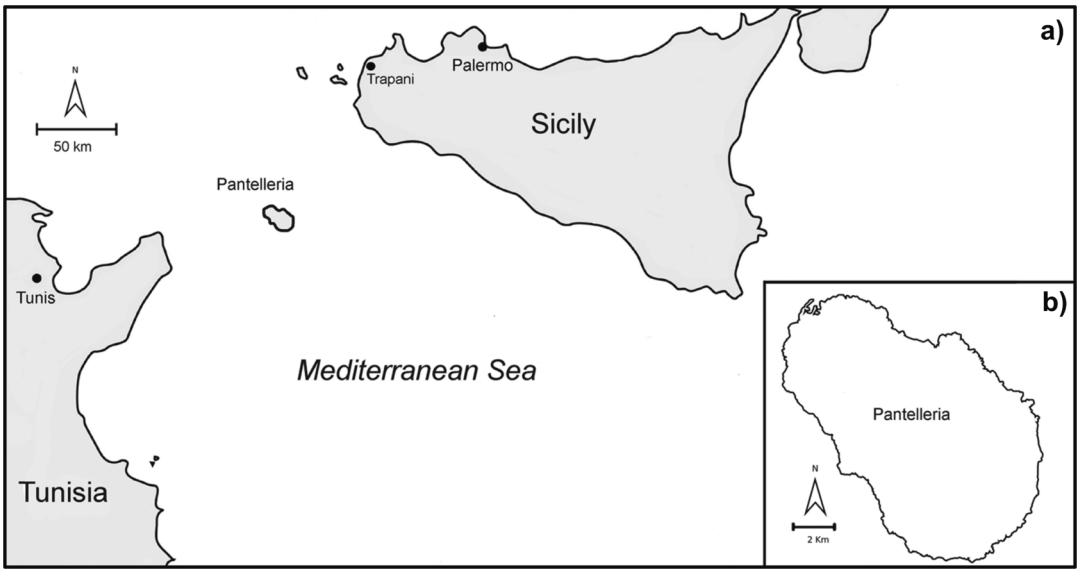

Fig. 1. Map showing the position of Pantelleria in the Sicily Channel of the Mediterranean Sea (a) Detailed map of Pantelleria (b).

honey bee colony losses and, as such, it significantly impacts the ecosystem (McMenamin and Genersch, 2015; Steinhauer et al., 2018). The DWV belongs to the Picornaviridae family, within the Iflavirus genus, with a positive-sense ssRNA (de Miranda and Genersch, 2010; Genersch and Aubert, 2010). The virus is spread globally (Buendía et al., 2018; de Miranda and Genersch, 2010; Genersch and Aubert, 2010; Martin et al., 2012) and the infections are generally detected due to the presence of symptomatic honey bees, characterized by deformed or missing wings and shortened abdomens (de Miranda and Genersch, 2010). In most cases, both in honey bees and other pollinators, the infections show asymptomatic and become chronic, latent, seasonal or apparent under stressful conditions (Alonso-Prados et al., 2021; Cilia et al., 2022b; Dalmon et al., 2019; Tentcheva et al., 2004). The DWV is characterized by three genetic variants that are acknowledged and named type A, B, and C (McMahon et al., 2016; Mordecai et al., 2016), with type A being the most prevalent (McMahon et al., 2016), although type B is apparently to be replacing the other variant (Natsopoulou et al., 2017; Paxton et al., 2022).

In honey bees, DWV infection is often associated with *Varroa destructor* (Anderson & Trueman, 2000) infestations, although the virus has been detected in *Varroa*-free individuals (Ryabov et al., 2014). The trophic activity of the mite is responsible for the intra-colony transmission to larvae, pupae and adults (Yue et al., 2007), but the infection may also spread horizontally by trophallaxis, direct contacts (Ball and Allen, 1988; Gisder et al., 2009; Lanzi et al., 2006; Nordström, 2003; Shen et al., 2005), and ingestion of contaminated food (Chen et al., 2006; Mazzei et al., 2014; Mockel et al., 2011). However, due to the multiple possible transmission routes, DWV may generate spillover to other sympatric hymenopterans (Cilia et al., 2022a; Martin and Brettell, 2019; Nanetti et al., 2021a; Tehel et al., 2022), including bumblebees and other species used for commercial pollination (Gisder and Genersch, 2017; Gusachenko et al., 2020; Ravoet et al., 2014; Singh et al., 2010; Tehel et al., 2016).

Due to the recent interest in the interspecific transmission of the pathogen within pollinators, the dimension of the small island and the geographical position between Italy and Tunisia, this investigation aims to evaluate the peculiar DWV infection in free-flying *Bombus terrestris* (L., 1758) individual sampled on Pantelleria islands.

Material and methods

Sample collection

From 16 to 20 May 2022, free-flying worker bumblebees (*Bombus terrestris)* were sampled on Pantelleria island (Fig. 1). Due to the entire island is a National Park, sampling was performed under the authorisation of the administration "Ente Parco Nazionale Isola di Pantelleria". The specimens were collected during their foraging activity using the sweep net technique (Cilia et al., 2022a). Each individual was introduced in a sterile 15 mL tube and placed in a cooler bag with freezer packs for transportation. Once in the laboratory, the samples were stored a −80 °C until the analysis.

Extraction of total RNA

Before the extraction of total RNA, all samples were washed by full immersion in 95% ethanol for 10 s to remove any external viral contamination(Cilia et al., 2021). Each sample was processed individually, placed in a 2 mL microtube with 300 μL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and crushed with a TissueLyser II (Qiagen, Hilden, Germany) for 3 min at 30 Hz, as previously reported (Cilia et al., 2022b). The extraction of the nucleic acids was performed with STAR BEADS Pathogen DNA/RNA Extraction kit (Cyanagen, Bologna, Italy) using Auto-Pure32A (Allsheng, Zhejiang, China). Extracts were stored at − 80 °C until the analysis.

Detection and quantification of the deforming wing virus (DWV)

The extracted RNAs were analysed by qRT-PCR to detect and quantify the presence of DWV in bumblebees. Primers amplified a 132-bp fragment within the highly conserved region coding for the RNA-dependent RNA polymerase (*Rd-Rp)* gene, commonly expressed in all virus variants, using the primer pair previously reported (Mazzei et al., 2014). The viral genomes were amplified using Power SYBR® Green RNA-to-Ct™ 1-Step Kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's instruction. The qPCR assay was performed on QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific). For the target gene, a total reaction volume of 20 μL was used following the protocols previously described (Mazzei et al., 2018, 2014).

Virus loads were quantified with absolute quantification of the number of DWV copies in each ng of RNA (copies/ng RNA). The

Table 1
Summary of *Bombus terrestris* individuals analyzed. The number of DWV copies is the average of two technical replicates.

Sample	DWV copies	Sample	DWV copies	Sample	DWV copies	Sample	DWV copies
ID 1	Nd	*ID 14*	4.12×10^2	*ID 27*	Nd	*ID 40*	7.14×10^3
ID 2	2.45×10^3	*ID 15*	6.15×10^4	*ID 28*	8.16×10^3	*ID 41*	8.13×10^6
ID 3	3.27×10^2	*ID 16*	4.31×10^3	*ID 29*	6.31×10^4	*ID 42*	7.11×10^5
ID 4	Nd	*ID 17*	6.25×10^2	*ID 30*	Nd	*ID 43*	8.01×10^2
ID 5	5.23×10^2	*ID 18*	Nd	*ID 31*	Nd	*ID 44*	7.10×10^2
ID 6	6.12×10^3	*ID 19*	Nd	*ID 32*	Nd	*ID 45*	Nd
ID 7	2.16×10^2	*ID 20*	Nd	*ID 33*	Nd	*ID 46*	6.93×10^3
ID 8	Nd	*ID 21*	Nd	*ID 34*	9.15×10^2	*ID 47*	Nd
ID 9	4.36×10^4	*ID 22*	9.15×10^3	*ID 35*	Nd	*ID 48*	8.11×10^1
ID 10	6.13×10^2	*ID 23*	6.23×10^4	*ID 36*	3.64×10^3	*ID 49*	9.03×10^2
ID 11	7.25×10^3	*ID 24*	Nd	*ID 37*	Nd	*ID 50*	6.51×10^3
ID 12	Nd	*ID 25*	Nd	*ID 38*	7.42×10^2	*ID 51*	3.42×10^2
ID 13	Nd	*ID 26*	7.16×10^2	*ID 39*	6.61×10^4	*ID 52*	Nd

Note. Nd: not detected.

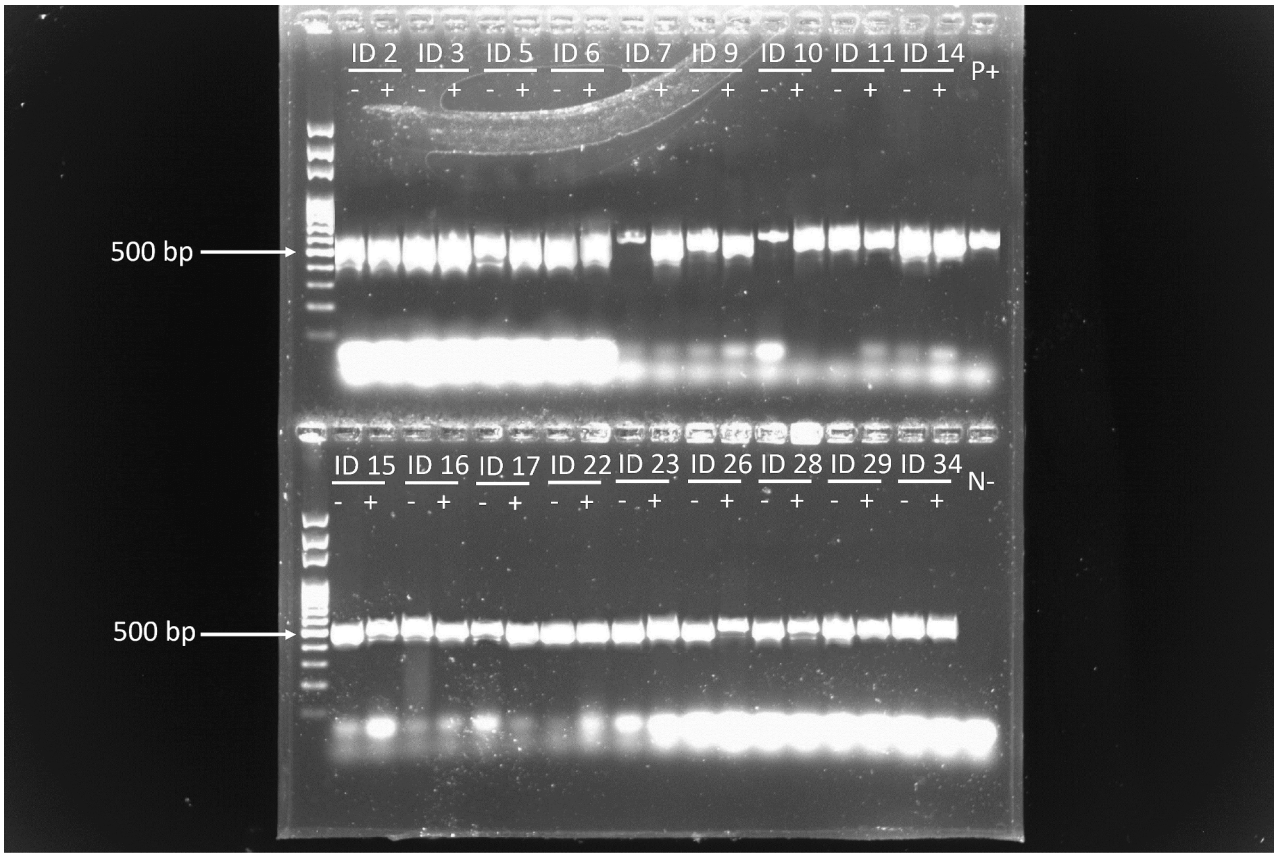

Fig. 2. Evidence of replicating and genomic DWV in *B. terrestris* in Pantelleria. Gel electrophoresis of strand specific RT-PCR performed on some cDNA obtained from positive individuals. Replicative strand (−) and genomic strand (+). As a posive control (P+) cDNA obtained from a DWV infected honey bee samples. As a negative control (N−) was used sterile water.

successful amplification of reference gene β-Actin, with the previously described primers (Chen et al., 2005), was used to confirm the sample integrity from the RNA extraction to the qPCR analysis.

RNA previously extracted from DWV-infected honey bee samples was used as a positive control. Sterile water was used as a negative control in all analytical steps. All the analyses were conducted in duplicate.

For each target gene, a standard curve was generated by amplifying serially diluted recombinant plasmids containing the pathogen-specific DNA fragment from 1×10^1 to 1×10^9 copies/ng in a qPCR assay on QuantStudio™ 3 Real-Time PCR System (ThermoFisher Scientific), as previously reported (Mazzei et al., 2019, 2018), following the amplification and quantification protocols (Mazzei et al., 2014).

Strand-specific RT-PCR

The DWV replication was evaluated through a strand-specific RT-PCR using specific primers, which amplify a 504-bp fragment of the *Rd-Rp*, as previously described (Mazzei et al., 2014). All amplicons were visualized on a 1.5% agarose gel.

Phylogenetic analysis

The strand-specific RT-PCR-obtained amplicons were sequenced (BMR Genomics, Padua, Italy) and analysed using BLASTn to standard databases with default parameters for megablast (Altschul et al., 1990). To build the phylogenetic tree, the sequences with a high Max Score and

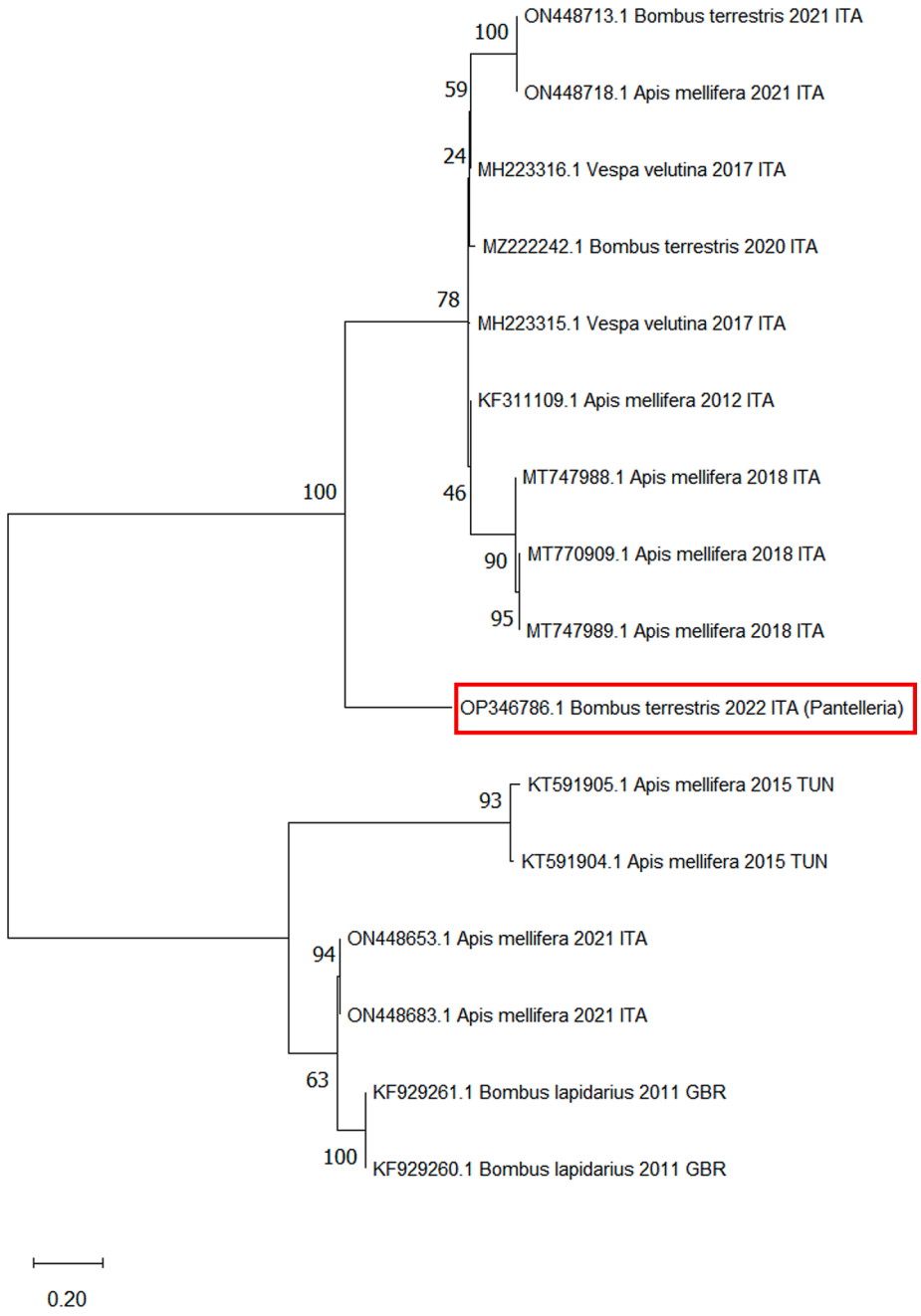

Fig. 3. Molecular phylogenetic analysis for polyprotein gene of deformed wing virus (DWV) using the Neighbor-Joining method. Accession number, host, state, and year of available GenBank DWV sequences are shown. The DWV sequence obtained from the *B. terrestris* samples collected in Pantelleria is in a red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a Query cover $\geq$ 30% in the BLAST analysis were selected. The phylogenetic analysis was performed by the maximum likelihood method based on the Tamura–Nei model with a bootstrap test using MEGA software (Kumar et al., 2018).

Results

In total, fifty-two individuals were collected, thirty-one of which (59.62%) scored positive for DWV (Table 1). In the positive samples, the mean abundance was $2.97 \times 10^5 \pm 1.46 \times 10^6$ (s.d.) copies per bee. On the other hand, the median value of abundance was 4.31×10^3 copies.

The strand-specific RT-PCR demonstrated active viral replication of DWV in all PCR-positive samples (Fig. 2).

The DWV sequence, deposited in GenBank with the Accession Number OP346786, 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 (73.60% of identity, 5e-07 of E-value, 36% of Query Cover) to specific DWV type A genomes deposited in GenBank. The phylogenetic analysis and pairwise distance analysis indicated a low similarity to DWV strains previously isolated (Fig. 3).

Discussion

Pathogens and parasites are deemed drivers of pollinator decline together with other factors including pesticides, land fragmentation and global warming (Meeus et al., 2011; Pritchard et al., 2021). Deformed

wing virus is the most widespread honey bee pathogen, as it may infect several flower-visiting insects other than bees (Cilia et al., 2022a; Dalmon et al., 2021; Genersch et al., 2006; Gisder and Genersch, 2017; Ravoet et al., 2014; Singh et al., 2010; Tehel et al., 2016), including hornets (Forzan et al., 2017; Mazzei et al., 2018), wasps (Brenton-Rule et al., 2018), ants (Sébastien et al., 2015), and beetles (Eyer et al., 2009; Huwiler et al., 2020; Nanetti et al., 2021b). The active DWV replication in different insect species highlights the high adaptation of the virus, which can infect managed and wild bees (Cilia et al., 2022a; Nanetti et al., 2021a).

The present study showed the presence of DWV in free-flying *B. terrestris* collected on the island of Pantelleria. The viral replication highlighted the active DWV infection, demonstrating the adaptation to the host.

Previous surveys detected DWV in various bumblebee species worldwide, where it may be contributing to the decline of both wild and managed populations (McMahon et al., 2015; Meeus et al., 2011). DWV infections were reported in *B. terrestris* (Arismendi et al., 2021; Cilia et al., 2021; Dalmon et al., 2021; Evison et al., 2012; Fürst et al., 2014; Jabal-Uriel et al., 2017; Tehel et al., 2016), *B. pascuorum* (Scopoli, 1763) [25,41,52,58,60], *B. impatiens* Cresson, 1863 (Levitt et al., 2013; Li et al., 2011; Sachman-Ruiz et al., 2015; Singh et al., 2010; Tehel et al., 2016), *B. atratus* Franklin, 1913 (Gamboa et al., 2015; Reynaldi et al., 2013), *B. vagans* Smith, 1853 (Levitt et al., 2013; Singh et al., 2010), *B. huntii* Greene, 1860 (Li et al., 2011), *B. ruderatus* (Fabricius, 1775) (Arismendi et al., 2021), *B. ternarius* Say, 1837 (Singh et al., 2010), *B. lapidarius* (L., 1758), *B. lucorum* (L., 1761), and *B. monticola* Smith, 1848 (Fürst et al., 2014).

Although DWV was frequently detected in *B. terrestris* (Gisder and Genersch, 2017; Tehel et al., 2016), symptomatic adults were seldom described. Only a few studies found *B. terrestris* specimens and *B. pascuorum* workers both showing symptoms attributable to a DWV infection and reporting replicative viral RNA (Cilia et al., 2021; Genersch et al., 2006).

Although highly variable, the DWV load found in each sampled *B. terrestris* individual is comparable to values detected in asymptomatic honey bees (from 10^2 to 10^6 copies/bee) (Mazzei et al., 2014). Finding the replicative form of the virus in the investigated DWV-positive *B. terrestris* specimens highlights that a viral infection was active. Nevertheless, the transmission route in the specific conditions of that island is anything but clarified.

Indeed, on Pantelleria island, the presence of pathogens in managed and wild honey bees is poorly investigated. However, the DWV is present on Pantelleria, where in some colonies it exceeds the abundance of 10^8 copies per bee during the autumn (Freda et al., 2022), suggesting a wide viral circulation on the island. Within the honey bee colonies, *V. destructor* plays a pivotal role in DWV transmission (de Miranda and Genersch, 2010; Gisder et al., 2009; Gisder and Genersch, 2017; Martin et al., 2012; Mockel et al., 2011). Missing known vectors in bumblebees, the infections are likely to propagate horizontally or vertically (Yañez et al., 2020). The foraging activity promotes virus transmission (Grozinger and Flenniken, 2019). The DWV can be transmitted by the ingestion of contaminated pollen and nectar (Burnham et al., 2021; Graystock et al., 2013), or by direct contact with infected flower visitors (Mazzei et al., 2014; Singh et al., 2010). The flowers may become a DWV hotspot when visited by infected bees, thus representing sites for interspecific transmission to other pollinators, wild bumblebees included (Cilia et al., 2022a; Dalmon et al., 2021; Gajger et al., 2021; Mazzei et al., 2014; Mockel et al., 2011; Toplak et al., 2020). Besides, vertical transmission is not excluded, as reported for honey bees (Chen and Siede, 2007; Rana et al., 2011).

Finally, the phylogenetic analysis highlighted that the obtained DWV belonged to type A but with low similarity with Italian and Tunisian strains (Cilia et al., 2022a, 2021; Fürst et al., 2014; Haddad et al., 2017; Marzoli et al., 2020; Mazzei et al., 2018, 2014). The phylogenetic analysis on our samples showed high similarity to both Italian and Tunisian sequences. This finding may suggest the occurrence of recombination events between different DWV strains parasitizing the same host insect, whether it be a bumblebee or another pollinator. Further research is essential to elucidate the genetic aspects of the DWV variant found in Pantelleria.

Conclusions

The spread of pathogens among sympatric bees represents an important threat to the health of pollinator populations and a harbinger of high impact on their ecology (Gisder and Genersch, 2017; Graystock et al., 2013; Nanetti et al., 2021b), especially in a limited area such as an island. Further studies are needed to elucidate the details of DWV epidemiology and the mechanics leading to the genetic modifications detected in the Island of Pantelleria.

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CRediT authorship contribution statement

Simone Flaminio: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Antonio Nanetti:** Investigation, Writing – review & editing. **Laura Bortolotti:** Investigation, Writing – review & editing, Funding acquisition. **Giovanni Cilia:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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