Journal of Invertebrate Pathology 146 (2017) 14–23

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Replication of honey bee-associated RNA viruses across multiple bee species in apple orchards of Georgia, Germany and Kyrgyzstan

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ARTICLE INFO

Keywords:
ABPV
Apis mellifera
Bombus
CBPV
DWV-A
DWV-B
Pathogen
SBPV
SBV
Wild bee

ABSTRACT

The essential ecosystem service of pollination is provided largely by insects, which are considered threatened by diverse biotic and abiotic global change pressures. RNA viruses are one such pressure, and have risen in prominence as a major threat for honey bees (*Apis mellifera*) and global apiculture, as well as a risk factor for other bee species through pathogen spill-over between managed honey bees and sympatric wild pollinator communities. Yet despite their potential role in global bee decline, the prevalence of honey bee-associated RNA viruses in wild bees is poorly known from both geographic and taxonomic perspectives. We screened members of pollinator communities (honey bees, bumble bees and other wild bees belonging to four families) collected from apple orchards in Georgia, Germany and Kyrgyzstan for six common honey bee-associated RNA virus complexes encompassing nine virus targets. The Deformed wing virus complex (DWV genotypes A and B) had the highest prevalence across all localities and host species and was the only virus complex found in wild bee species belonging to all four studied families. Based on amplification of negative-strand viral RNA, we found evidence for viral replication in wild bee species of DWV-A/DWV-B (hosts: *Andrena haemorrhoa* and several *Bombus* spp.) and Black queen cell virus (hosts: *Anthophora plumipes,* several *Bombus* spp., *Osmia bicornis* and *Xylocopa* spp.). Viral amplicon sequences revealed that DWV-A and DWV-B are regionally distinct but identical in two or more bee species at any one site, suggesting virus is shared amongst sympatric bee taxa. This study demonstrates that honey bee associated RNA viruses are geographically and taxonomically widespread, likely infective in wild bee species, and shared across bee taxa.

1. Introduction

Animal-mediated pollination of many wild and crop plants is a key ecosystem service (Klein et al., 2007; Ollerton et al., 2011). The expansion of intensive agriculture increases demand for pollination delivered by insects and simultaneously generates growing pressures on pollinators (Bommarco et al., 2013; Graystock et al., 2015). Both wild and managed bees have declined in recent decades in the Northern Hemisphere (van Engelsdorp et al., 2011; Fitzpatrick et al., 2007; Potts et al., 2010) and multiple interacting factors are thought to have driven this process (Vanbergen et al., 2013). For instance, wild bee decline is thought to be primarily caused by loss and fragmentation of (semi-) natural, resource-rich habitats, which are converted into agricultural land and/or impervious surfaces (Brown and Paxton, 2009). In combination with additional stressors (e.g. pesticide exposure), this can lead to reduced population sizes and, subsequently, diminished genetic diversity of wild bee species, augmenting their susceptibility to infectious diseases (Whitehorn et al., 2011) such as honey bee-associated RNA viruses.

Although first described from honey bees in the early 1960s (Bailey et al., 1963), RNA viruses have recently emerged as a threat to apiculture since the widespread infestation of Western honey bee (*Apis mellifera* L. 1758) colonies with the exotic and invasive ectoparasitic mite, *Varroa destructor* Anderson and Trueman, 2000, which represents

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http://dx.doi.org/10.1016/j.jip.2017.04.002
Received 21 December 2016; Received in revised form 16 March 2017; Accepted 4 April 2017
Available online 06 April 2017
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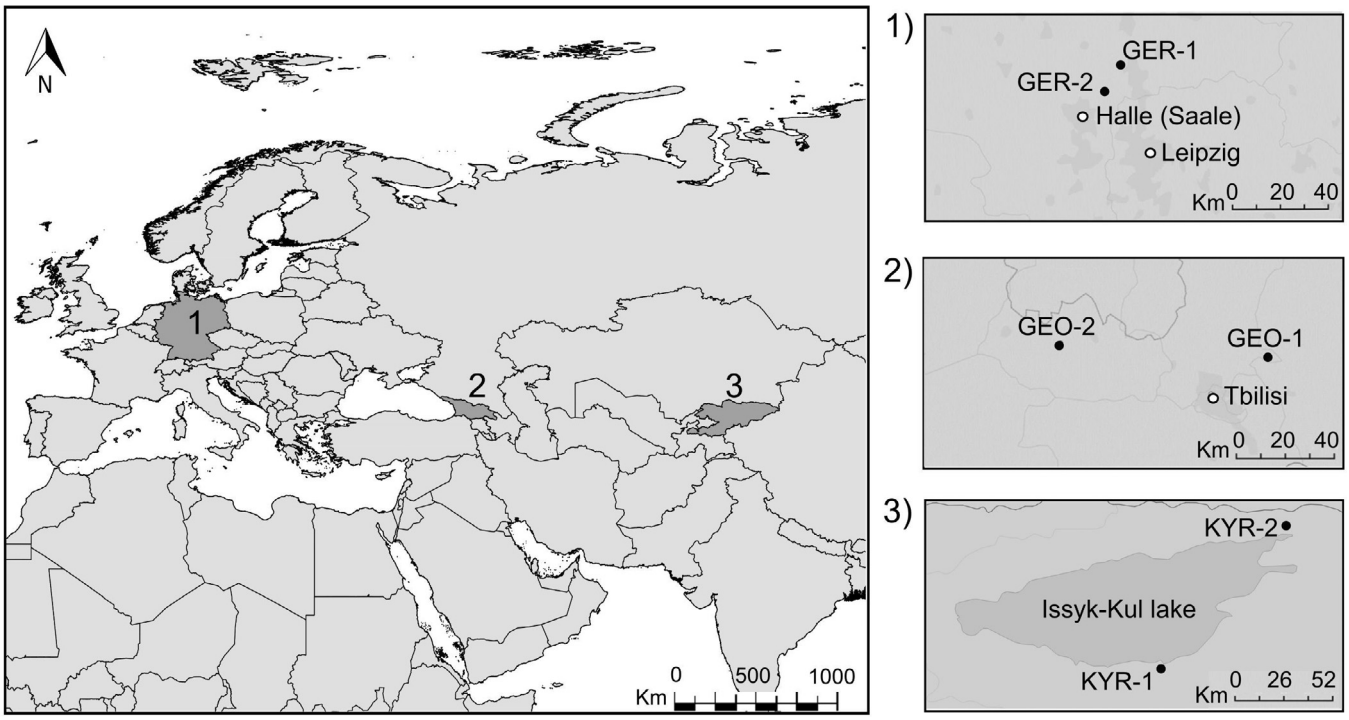

Fig. 1. Sample sites of bees in Germany (1, GER-1 and GER-2), Georgia (2, GEO-1 and GEO-2) and Kyrgyzstan (3, KYR-1 and KYR-2).

a vector for several RNA viruses (Genersch and Aubert, 2010). Particularly for the Deformed wing virus complex (DWV mutant clouds A and B, hereafter DWV-A and DWV-B, respectively), the vector *V. destructor* has led to an increase in viral prevalence (Martin et al., 2012; Mondet et al., 2014) and potentially in viral virulence (McMahon et al., 2016) in honey bees. However, as RNA viruses can also be transmitted horizontally via shared, contaminated flowers and pollen (McArt et al., 2014; Singh et al., 2010), they may potentially endanger a wide range of wild pollinator species, too. Although virus-carrying honey bees are mainly asymptomatic (Genersch and Aubert, 2010), severe infections are lethal or lead to impaired development of pupae, morphological deformities and behaviour aberrations of the adult bees, for instance, paralysis, shivering and changes in brood care and foraging behaviour (reviewed in Aubert, 2008; Bailey and Ball, 1991). Fürst et al. (2014) have showed experimentally that infection with DWV reduces the survival of workers in the buff-tailed bumble bee, *Bombus terrestris* (L., 1758). Furthermore, wild bumble bees exhibiting deformed wings, similar to those of clinically DWV-infected honey bees, were reported under natural conditions in Germany (Genersch et al., 2006); wing deformities were associated with presence of DWV in the abdomen and thorax of the symptomatic bumble bees, suggesting potential detrimental effects of this virus on other host bee species in addition to the Western honey bee.

Commercial transport of (asymptomatically) infected honey bee colonies for crop pollination and their subsequent co-occurrence with diverse wild bee species within the same pollinator community facilitates interspecific pathogen spill over (Graystock et al., 2015). Indeed, a Great Britain-wide study (Fürst et al., 2014; McMahon et al., 2015) revealed associations between sympatric honey bee and bumble bee populations in prevalence and infection levels of the most prevalent honey bee-associated RNA viruses, the DWV complex and Black queen cell virus (BQCV). Furthermore, shared haplotypes of the DWV complex were detected between co-occurring honey bees and bumble bees, suggesting on-going pathogen spill over *in situ* (Fürst et al., 2014). However, our knowledge of pathogen presence and infectivity in the vast majority of wild bee species and in other geographic regions is far more limited despite its relevance for the threatened ecosystem service of pollination (Potts et al., 2016).

To date, reviews of the most common RNA viruses in wild pollinator species (Manley et al., 2015; Tehel et al., 2016) suggest that they have a broad host range and thus are potential candidates for cross-species transmission. Previous studies have shown that the most widespread honey bee-associated RNA viruses, the DWV complex and BQCV, infect bumble bees (Fürst et al., 2014; Peng et al., 2011), but very little is known as to whether other wild bee species can also serve as host for these viruses (Dolezal et al., 2016; Manley et al., 2015; Tehel et al., 2016). Earlier studies have been limited by small sample sizes, limited taxonomic breadth, and limited geographic range, with the focus on a few Western European countries and the United States (e.g. Evison et al., 2012; Levitt et al., 2013; Ravoet et al., 2014; Singh et al., 2010; Dolezal et al., 2016).

Because there is a gap in our knowledge of the geographic and taxonomic breadth of the distribution of honey bee-associated viruses in wild bee species, we tested for viral prevalence in six wild bee communities by screening a broad taxonomic range of bee visitors to apple flowers in Georgia, Germany and Kyrgyzstan of the six most common RNA viral complexes known to infect *A. mellifera*. We additionally tested for the presence of the negative strand viral RNA of two positive-sense single-stranded RNA (+ss RNA) viruses as evidence of viral replication in wild bees. We also sequenced viral amplicons to confirm viral identity and support viral sharing across bee species at a sampling site.

2. Materials and methods

2.1. Field sites and sample collection

Sampling was carried out in six apple orchards in spring 2014 during apple bloom: two sites in Georgia, two in Germany and two in Kyrgyzstan (Fig 1, Supplementary Table A1). Apple is a mass blooming spring crop that attracts managed and diverse wild bee pollinators (Blitzer et al., 2016). It thus can potentially facilitate pathogen spillover due to increased horizontal virus transmission via shared flowers (Singh et al., 2010). Orchards under study were a subset of a wider sampling scheme designed to explore associations between pollinator diversity and pollination service provision to apple and to assess the

current state of pollinator diversity and health in understudied regions. All sites were > 15 km apart, beyond the flight range of bees (Greenleaf et al., 2007), and therefore can be considered independent. The density of managed honey bee hives per orchard area varied from 0 to 8 hives/ha (Supplementary Table A1). Colonies of commercial bumble bees or mason bees (*Osmia* spp.) were not introduced for pollination in or near the orchards under study.

Each bee was individually sampled from apple flowers during the peak of apple bloom, either directly into a 1.5 ml microfuge tube or with a sweep net and then placed into its own 1.5 ml microfuge tube. All individuals from one site were collected on the same day. We assigned bees collected from apple flowers into three main groups or taxa: the Western honey bee (*A. mellifera*), bumble bees (*Bombus* spp.) and other wild bees (all remaining wild bee species); the latter were also determined visually to genus at collection. We aimed to collect 20 individuals from each taxon per site, but final sample sizes varied due to unequal occurrence across sites (Supplementary Table A2). To prevent RNA degradation, bees were either frozen on dry ice immediately upon collection and stored at −80 °C until further processing (German samples) or preserved in RNAlater (Sigma-Aldrich, St. Louis, Missouri, USA) and subsequently stored at −24 °C (Georgian and Kyrgyz samples).

2.2. Identification of bee species

Honey bees could be unambiguously identified visually. For bumble bees and other wild bees, we used DNA barcoding (Hebert et al., 2004) to determine species identity. One mid-leg of each specimen was dissected immediately before RNA extraction (see 2.3. below) and used to extract genomic DNA using a Chelex™-based protocol (Walsh et al., 1991). A partial region of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified with universal primers LCO-1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO-2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994) or a combination of a modified forward primer (5′-GCT TTC CCA CGA ATA AAA TAA TA-3′) (Schmidt et al., 2015) and HCO-2198.

For both primer pairs, PCR reactions (10 μL final volume) consisted of 1 × GoTaq PCR buffer (Promega, Madison, WI, USA), 200 μM of each dNTP, 0.3 μM of each primer, 2 mM Mg^{2+}, 1.5 U *Taq*-Polymerase (Promega) and 2 μL of template DNA (ca. 25–50 ng). PCRs were performed in a Biometra TProfessional basic gradient thermocycler (Biometra, Göttingen, Germany) under the following thermal regime: 2 min at 95 °C for initial denaturation, followed by 35 cycles of 30 s at 94 °C, 45 s at 51 °C and 1 min at 72 °C, with a final elongation step at 72 °C for 5 min. PCR products were stained with Diamond Nucleic Acid Dye (Promega, Madison, WI, USA) and resolved on 2% agarose gels under UV light to ensure a single PCR product of the correct size (ca. 650 bp or ca. 400 bp for LCO-1490/HCO-2198 and modified forward primer/HCO-2198 primer pairs, respectively) had been amplified. Amplicons were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced commercially on an automated DNA sequencer (GATC Biotech GmbH) with the forward PCR primer.

The Barcode of Life (BOLD; Ratnasingham and Hebert, 2007) database was used to determine species identity of specimens based on their COI barcodes. In total, 188 sequences were submitted to the NCBI GenBank database and are available under Accession numbers KX957806 - KX957928, KY234214 - KY234230 (this study) and KY121827 - KY121869 (Kirkitadze et al., unpublished data).

2.3. RNA extraction and virus detection

As previous studies have found RNA viruses across various tissue and body parts of honey bees and bumble bees (Peng et al., 2011; Yue and Genersch, 2005), we extracted total RNA from the whole insect with an RNeasy Mini kit (Qiagen, Hilden, Germany) in a Qiacube extraction robot (Qiagen) following the manufacturer's recommendations. We employed reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA, henceforth: MLPA; de Miranda et al., 2013; de Smet et al., 2012) using the SALSA RT-MLPA kit (MRC-Holland, Amsterdam, Netherlands) to screen RNA extracts for the following five composite +ss RNA viral complexes widely considered to be 'honey bee viruses': (i) the Deformed wing virus complex ((DWV-A/DWV-B); DWV-A encompasses Deformed wing virus and Kakugo virus, and DWV-B is otherwise known as Varroa destructor virus-1); (ii) Black queen cell virus (BQCV); (iii) the Acute bee paralysis virus, Israeli acute paralysis virus and Kashmir bee virus complex (ABPV/IAPV/KBV); (iv) Slow bee paralysis virus (SBPV); (v) Sacbrood virus (SBV); and (vi) Chronic bee paralysis virus (CBPV). Products of RT-MLPA were resolved on a QIAxcel automated capillary electrophoresis system (Qiagen). We scored samples as positive if the intensity of the amplified probe specific for a particular viral complex exceeded a threshold of 0.1 relative fluorescent units (RFU).

The *β-actin* gene was simultaneously screened in MLPAs as an internal sample quality control of successful RNA extraction for honey bees and wild bee species belonging to the family Apidae (genus *Bombus* (except subgenus *Psithyrus*), *Anthophora*, *Eucera*, *Melecta* and *Xylocopa*). We again applied a minimum threshold of 0.1 RFU to *β-actin* and accepted samples above this threshold for MLPA analysis of viral targets.

However, we found that available MLPA primers and probes for *β-actin*, which were developed for the honey bee (de Smet et al., 2012), do not function in phylogenetically more distantly related wild bee species such as *Andrena* spp. (Andrenidae), *Osmia* spp. (Megachilidae) or *Lasioglossum* spp. (Halictidae). We tested RNA quality of these samples separately via reverse transcription and subsequent PCR of the highly conserved nuclear long-wavelength rhodopsin gene, using Opsin-For3 (mod) (5′-TTC GAY AGA TAC AAC GTR ATC GTN AAR GG-3′) and LWRhRev (5′-ATA TGG AGT CCA NGC CAT RAA CCA-3′) primers (Almeida and Danforth, 2009). To do so, we converted 500 ng of sample RNA to total cDNA using M-MLV Revertase (Promega, Mannheim, Germany) following the manufacturer's recommendations. We used the same PCR reaction mix as for partial COI amplification, but changed the primers and replaced template DNA with 1 μL of cDNA diluted in RNAse-free water (1:5, v/v). PCR conditions were as follows: 2 min at 95 °C; 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C; and a final elongation step of 10 min at 72 °C. PCR products were stained with Diamond Nucleic Acid Dye (Promega), resolved on 1% agarose gels and visualized by UV light for an amplification product of the correct length (~700 bp). Only samples passing these quality control standards for RNA extraction were analysed for viral targets by MLPA.

In total, 142 honey bees, 110 bumble bees and 109 other wild bees passed our RNA quality control thresholds (Table A2); we excluded five honey bees (one from a German site, 4 from Kyrgyz sites) and two other wild bees (both from a Kyrgyz site) due to low RNA quality. The 361 remaining individuals were used in subsequent statistical analysis.

2.4. Amplification of negative-strand viral RNA

The negative-strand RNA intermediate of a +ss RNA virus is present only during active viral replication. Hence, detection of negative-strand viral RNA in a host may be considered indicative of viral replication (Yue and Genersch, 2005), although we caution that it does not imply host pathology (Tehel et al., 2016). We screened our bumble bees and other wild bee samples which were DWV-A/DWV-B- or BQCV-positive by MLPA for the presence of the negative-strand viral RNA using negative-strand-specific RT-PCR with tagged primers, as described in Yue and Genersch (2005) for DWV-A/DWV-B and Peng et al. (2011) for BQCV. We did not screen honey bees for the negative-strand RNA intermediate of these viruses because they are well known to be infective in *A. mellifera* (Reddy et al., 2013; Yue and Genersch, 2005).

All RT-PCRs were carried out with a Biometra TProfessional basic

gradient thermocycler (Biometra). A negative control containing RNAse-free water (Qiagen) was included into each reaction array. As a positive control, we used RNA extracts from honey bees which were experimentally successfully infected with BQCV (Doublet et al., 2015), DWV-A or DWV-B (McMahon et al., 2016). We stained PCR products (8 µl per reaction) with Nucleic Acid Dye (Promega) and resolved them on 2% agarose gels under UV light for an amplification product of the correct length (DWV-A/DWV-B: 451 bp; BQCV: 420 bp).

2.5. Sequencing

We cloned and sequenced positive-strand partial amplicons of the most common viruses in our samples, DWV-A/DWV-B and BQCV, from one bee individual per taxon per site in order to confirm virus identity and variant of the viral major master sequence. We synthesized total cDNA as described above and amplified a ca. 296 bp long partial sequence of BQCV capsid protein gene with primers BQCV-qF7893 (5′-AGT GGC GGA GAT GTA TGC-3′) and BQCV-qB8150 (5′-GGA GGT GAA GTG GCT ATA TC-3′) (Locke et al., 2012) or a ca. 452 bp long partial sequence of DWV RNA-dependent RNA polymerase (*RdRp*) gene using the primers F15 (5′-TCC ATC AGG TTC TCC AAT AAC GGA-3′) and B23 (5′-CCA CCC AAA TGC TAA CTC TAA GCG-3′) (Yue and Genersch, 2005).

Although primers F15/B23 apparently amplify both DWV-A and DWV-B, we found that amplification of DWV-B in our positive controls with these primers was poor. Furthermore, they did not generate a product for several German and Georgian samples positive for the DWV-A/DWV-B complex by MLPA. Therefore, we employed the DWV-B-specific primers VDV-F1409 (5′-GCC CTG TTC AAG AAC ATG-3′) (Locke et al., 2012) and DWV-B1806 (5′-CTT TTC TAA TTC AAC TTC ACC-3′) (Yanez et al., 2012) to additionally screen all (DWV-A/DWV-B)-MLPA-positive samples for the presence of DWV-B via PCR amplification of a ca. 415 bp partial sequence of the DWV-B l-protein. None of the samples amplified with both F15/B23 and these DWV-B-specific primers, indicating the presence of either DWV-A or DWV-B within an individual sample.

We could not amplify DWV-A or DWV-B in two (DWV-A/DWV-B)-MLPA-positive samples (a wild bee from Georgian site GEO-2 and a bumble bee from German site GER-1) using either primers F15/B23 or VDV-F1409/DWV-B1806. For these samples, a shorter, ca. 120 bp partial sequence of the *RdRp* gene was successfully obtained using another pair of DWV-B-specific primers VDV-F2 (5′-TATCTTC ATTAAAACCGCCAGGCT-3′)/ VDV-R2a (5′-CTTCCTCATTAACTGAG TTGTTGTC-3′) (McMahon et al., 2015).

All PCR reactions were carried out using the same PCR mix as for DNA barcoding (see Section 2.2 above) under the following PCR thermal regime: 95 °C for 2 min; 36 cycles of 95 °C for 30 s, 60 °C (for BQCV) or 55 °C (for DWV-A and DWV-B) for 45 s and 72 °C for 1 min; 72 °C for 8 min. A virus-positive honey bee sample and a negative control containing RNA-free water (Qiagen) were included in each reaction.

PCR products were stained with Nucleic Acid Dye (Promega), resolved on 2% agarose gels and screened under UV light for the presence of non-specific bands. When we amplified a single band of the expected length, we purified the PCR product using the Qiaquick PCR Purification Kit (Qiagen) and cloned it using the pGEM-T Easy Vector system (Promega, Mannheim, Germany) following the manufacturer's instructions. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Up to five clones per sample were commercially sequenced and aligned by eye to reference genomes of BQCV (NC_003784), DWV-A (NC_004830) and DWV-B (NC_006494). Our partial sequences of BQCV, DWV-A and DWV-B were submitted to NCBI GenBank and are available under the accession numbers KX911740 - KX911791, KX911810 - KX911833 and KX911792 - KX911809, respectively.

2.6. Statistical analyses

Statistical analyses were conducted in R v.3.3.1 (R Core Team, 2013). True prevalence of the viruses under study and their 95% confidence intervals were estimated using the R package *epir* v.09-54 (Stevenson et al., 2015). True prevalence accounts for bias in observed virus prevalence estimates due to sensitivity and specificity of the detection method (MLPA) being less than 100%. Sensitivity and specificity were both set at 95% based on previous studies (McMahon et al., 2015). Differences in the prevalence of DWV-A/DWV-B and BQCV and SBPV between host taxa (*A. mellifera* vs. *Bombus* spp. vs. other wild bees) across sites were tested using a generalized linear mixed-effects model (GLMM) with binomial error distribution using the R package *lme4* v.1.0-6 (Bates et al., 2015) followed by Tukey HSD post hoc comparisons using the R package *multcomp* (Hothorn et al., 2008). Sites nested within country were included as random effect factor to account for the hierarchical structure of our sampling design. SBV, CBPV and ABPV/IAPV/KBV were not modelled due to an insufficient number of positive samples ($n = 23$, $n = 8$ and $n = 4$ positive samples across all bee taxa, respectively).

2.6.1. Sequence analysis

As well as confirming the identity of viruses detected by PCR, we also used sequences of viral amplicons to determine if the same viral variant is shared across bee taxa within a site rather than within a bee taxon across multiple sites (e.g. see Fürst et al., 2014; McMahon et al., 2015). To do so, we constructed Median Joining haplotype networks (Bandelt et al., 1999) for BQCV, DWV-A and DWV-B in PopART v.1.7 (Leigh and Bryant, 2015). The parameter ε was set to 10. Haplotype networks were colour-coded according to geographical sampling location and virus host morphogroup. Amplicons of DWV-B, amplified with primer pair VDV-F2/VDV-R2a, were not included into haplotype network analysis due to their short length and non-overlapping sequence with the main amplicon of DWV-B, amplified with primer pair VDV-F1409/DWV-B1806.

3. Results

Of 110 bumble bee and 109 other wild bee specimens collected across our six sites and that passed RNA quality control, we successfully amplified and sequenced partial COI sequences of 108 and 86 individuals, respectively. Ten bumble bee species (including two species of cuckoo bumble bees, *Bombus rupestris* (Fabricius, 1793) and *Bombus vestalis* Geoffroy, 1785) and 17 other wild bee species were unambiguously identified based on DNA barcodes. Other wild bees in our data set belonged to four families: Andrenidae (1 genus, 8 species), Apidae (4 genera, 4 species), Halictidae (1 genus, 2 species) and Megachilidae (1 genus, 3 species) (Table A2). These included six bumble bee species and fifteen wild bee species that we have tested for the presence of the most common honey bee viruses for the first time (Table 1).

3.1. Prevalence of RNA viruses across bee taxa

All six virus complexes were geographically widespread, being found in all countries, though not necessarily all sites (Fig. 2). Across bee taxa and sites, the DWV-A/DWV-B complex was the most prevalent (22% of individuals), followed by BQCV (17%), SBPV (16%), and then SBV (6%). The other two virus complexes were far less prevalent; the ABPV/IAPV/KBV complex was found in only 4 individuals (1%) whilst CBPV was found in only 8 individuals (2%; Fig. 2).

These overall patterns of viral prevalence belie differences in virus composition among taxa. All six viral complexes were detected in honey bees (Table 1). Across bumble bee taxa, SBPV was detected in 73% of species, BQCV in 45% of species and the DWV-A/DWV-B complex in 36% of species (Table 1). The DWV-A/DWV-B complex nevertheless exhibited the widest taxonomic distribution of potential hosts, encom-

Table 1
Summary of viruses detected per bee species by MLPA. +, detection by MLPA; §, detection of negative-strand of the two most prevalent viruses (DWV-A/DWV-B and BQCV) in negative-strand specific RT-PCR of bumble bees and other wild bees; *, species tested for the presence of RNA viruses for the first time.

	Virus or virus complex					
Species (n of individuals analysed)	DWV-A/ DWV-B	BQCV	ABPV/ IAPV/KBV	SBPV	SBV	CBPV
(a) **Honey bee**						
Apis mellifera (142)	+	+	+	+	+	+
(b) **Bumble bees**						
Bombus spp. (2)	+(§)	−	−	−	−	−
*Bombus cryptarum** (2)	−	−	−	+	−	−
*Bombus laesus** (14)	−	+(§)	−	+	−	−
Bombus lapidarius (14)	−	−	−	+	−	−
Bombus lucorum (6)	+	−	−	+	−	−
Bombus pascuorum (2)	−	−	−	+	−	−
*Bombus rupestris** (4)	−	+	−	−	−	−
*Bombus soroeensis** (2)	−	+(§)	−	+	−	−
*Bombus sylvarum** (6)	+(§)	−	−	+	−	−
Bombus terrestris (53)	+(§)	+(§)	−	+	+	+
*Bombus vestalis** (5)	−	+(§)	−	−	+	−
(c) **Other wild bees**						
Andrenidae						
*Andrena bicolor** (8)	+	−	−	−	−	−
*Andrena falsifica** (1)	−	−	−	−	−	−
*Andrena gravida** (5)	−	−	−	−	−	−
*Andrena haemorrhoa** (11)	+(§)	−	−	−	−	−
*Andrena helvola** (3)	+	−	−	−	−	−
*Andrena thoracica** (1)	+	−	−	−	−	−
*Andrena trimmerana** (3)	+	−	−	−	−	−
*Andrena wilkella** (1)	−	−	−	−	−	−
Andrena spp. (7)	−	−	−	−	−	−
Apidae						
*Anthophora plumipes**(23)	+	+(§)	+	+	+	−
Anthophora spp. (11)	+	+	+	+	+	−
*Eucera nigrescens** (1)	−	−	−	−	−	−
*Melecta albifrons** (4)	−	−	−	+	−	−
*Xylocopa dissimilis** (1)	−	−	−	−	−	+
Xylocopa spp. (4)	−	+(§)	−	−	+	−
Halictidae						
*Lasioglossum calceatum** (3)	−	−	−	−	−	−
*Lasioglossum subfasciatum** (1)	−	−	−	−	−	−
Lasioglossum spp. (3)	+	−	−	−	−	+
Megachilidae						
Osmia bicornis (18)	+	+(§)	+	+	+	+
*Osmia brevicornis** (1)	−	−	−	−	−	−
Osmia cornuta (1)	+	−	−	−	−	−

passing all four wild bee families surveyed (Table 1); it was the only virus detected in andrenid bees. All six virus complexes were found in *Osmia bicornis* (L., 1758) and all but CBPV in *Anthophora plumipes* (Pallas, 1772) and *Anthophora* spp. This is also the first report of DWV-A/DWV-B and CBPV in halictid bees (*Lasioglossum* spp.) in Eurasia (Table 1).

The viral targets with the highest true prevalence per taxon across sites were the DWV-A/DWV-B complex in honey bees (28%, 95% CI: 19–36%) and BQCV in honey bees (27%, 95% CI: 18–35%) (Table A3, Fig. 2). The true prevalence of SBPV and SBV was lower whilst the ABPV/IAPV/KBV complex and CBPV exhibited the lowest prevalence across all host taxa (Fig. 2). BQCV was the only virus consistently detected in honey bees across all six sites in our study, but this pattern was not observed in other bee taxa. DWV-A/DWV-B was not found in any host taxon at one German site and SBV was absent in all samples from Georgia.

The prevalence of DWV-A/DWV-B was significantly higher in *A. mellifera* (29%) than in *Bombus* spp. (18%; GLMM, $z = -2.36$; Tukey's HSD, $p = 0.05$) or in wild bees (16%; GLMM, $z = -3.49$; Tukey's HSD, $p < 0.001$). The difference between *Bombus* spp. and wild bees was not significant (Tukey's HSD, $p = 0.62$) (Fig. 3a). The same pattern was found for BQCV: it was at a significantly higher prevalence in *A. mellifera* (27%) than in *Bombus* spp. (7%; GLMM, $z = -3.70$; Tukey's HSD, $p < 0.001$) and in wild bees (8%; GLMM, $z = -3.45$; Tukey's HSD, $p < 0.001$), but its prevalence did not differ between bumble bees and wild bees (Tukey's HSD, $p = 0.91$) (Fig. 3b). The prevalence of SBPV did not vary across bee taxa (honey bees, 17%; bumble bees, 17%; other wild bees, 10%; GLMM, $p > 0.05$).

The DWV-A/DWV-B complex was only found in bumble bees and other wild bees if also present in honey bees (Fig. 2a). Honey bees at all sites harbored BQCV (Fig. 2b) and so little can be drawn from patters of prevalence across species for this virus. For SBPV and SBV, in contrast, both viruses were found in bumble bees and other wild bees at sites at which these viruses were not detected in honey bees (Fig. 2c and d respectively).

3.2. Viral replication in wild bees

In total, 20 DWV-A/DWV-B-positive bumble bee samples and 18 samples of other wild bee species were screened for the presence of DWV-A/DWV-B-negative-strand RNA. We found contrasting patterns in the two bee taxa: the replicative intermediate of DWV-A/DWV-B was detected in 75% of screened bumble bee individuals (belonging to *Bombus sylvarum* (L., 1761), *B. terrestris* and *Bombus* spp.) but only in a single sample of other wild bees (*Andrena haemorrhoa* (Fabricius, 1778) from site GER-1). To our knowledge, this is the first report of DWV-A/DWV-B replication in andrenid bees and in *B. sylvarum*. All but one bumble bee sample positive for the negative strand of DWV-A/DWV-B were collected in Georgia; 74% of these samples (n = 10) came from site GEO-2, which also had the highest true prevalence of DWV-A/DWV-B in honey bees (Fig. 2a). The DWV-A/DWV-B-negative-strand was not detected in wild bee samples from Kyrgyzstan.

In contrast to DWV-A/DWV-B, the prevalence of the replicative intermediate of BQCV was similar in bumble bees and other wild bees. Negative-strand RNA of BQCV was detected in 67% (6 of 9 samples) of bumble bees (4 species, Table 1) and in 56% (5 of 9 samples) of other wild bees identified as BQCV-positive in MLPA. We found negative strand BQCV more frequently in bumble bees and other wild bees in Kyrgyzstan (n = 10) than in Georgian (n = 2) or German samples (n = 5), a pattern opposite to the one observed for DWV-A/DWV-B. We found the replicative intermediate of BQCV in *B. terrestris* (3 samples), *Bombus* (*Psithyrus*) *vestalis* Geoffroy, 1785 (1 sample), *Bombus laesus* Morawitz, 1875 (1 sample) and *Bombus soroeensis* (Fabricius, 1777) (1 sample). In other wild bees, it was detected in *Osmia bicornis* (3 samples), *Anthophora plumipes* (1 sample) and *Xylocopa* sp. (1 sample) (Table 1). Our results show the replication of BQCV in all aforementioned species for the first time.

3.3. Viral sharing across bee taxa from sequence analysis

We successfully cloned and sequenced 52 amplicons of BQCV (from 13 individuals), 24 of DWV-A (from 5 individuals) and 22 of DWV-B (18 amplicons from 5 individuals amplified with primer pair VDV-F1409/DWV-B1806 and 4 amplicons from 1 individual amplified with primer pair VDV-F2/VDV-R2a, see Section 2.5 Sequencing). All clones mapped to the sequences of a corresponding virus in GenBank with > 98% sequence identity. DWV-A occurred only in Georgia and Kyrgyzstan, but was not found in German samples (Fig. 4a). Rather, DWV-B was found in German honey bees and wild bees, including the single sample of *Andrena haemorrhoa* from site GER-1, in which we also detected the negative stand of this virus, and in Georgian honey bees and bumble bees (Fig. 4c and d). To our knowledge, this is the first report of DWV-A

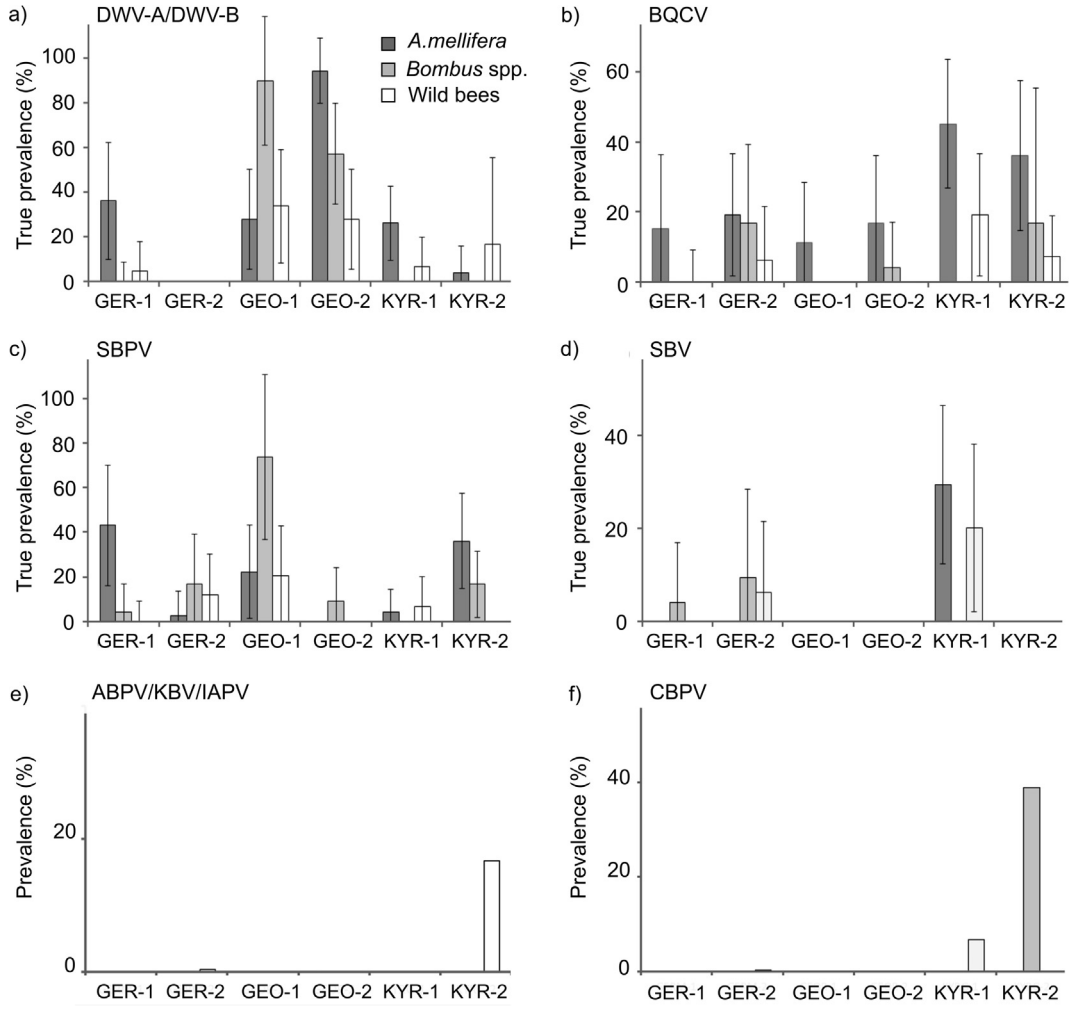

Fig. 2. True prevalence (in % ± 95% confidence intervals) of RNA viruses per site: (a) DWV-A/DWV-B, (b) BQCV, (c) SBPV, (d) SBV. Observed prevalence per site is presented for (e) ABPV/KBV/IAPV and (f) CBPV because of the low number of positive samples (n = 4, n = 8, respectively). Honey bees are in dark grey, bumble bees in light grey and other wild bees in white. Key to sampling site codes on abscissa: GER, Germany; GEO, Georgia; KYR, Kyrgyzstan. Note the different scale of the y-axis across the four viruses.

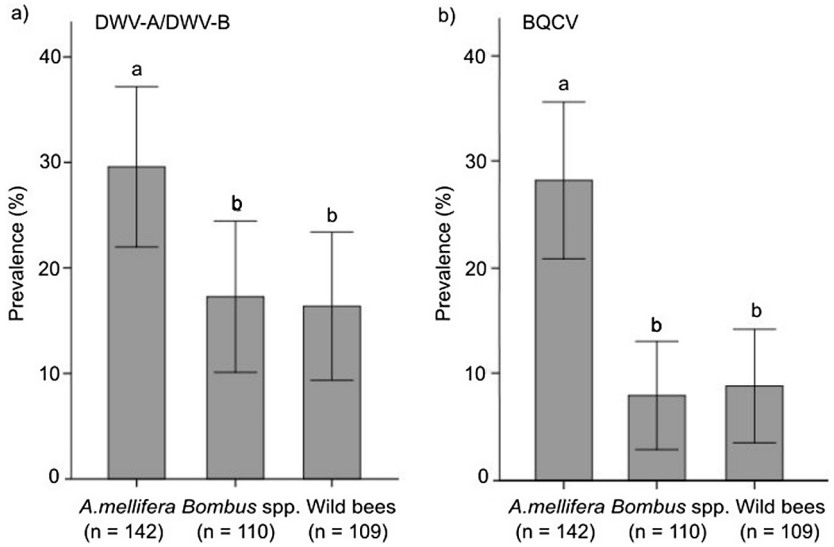

Fig. 3. Prevalence (in % ± 95% confidence intervals) of (a) DWV-A/DWV-B and (b) BQCV for each host taxon. Different letters above the bars indicate significant differences after Tukey's HSD ($p < 0.05$ for DWV-A/DWV-B and $p < 0.001$ for BQCV).

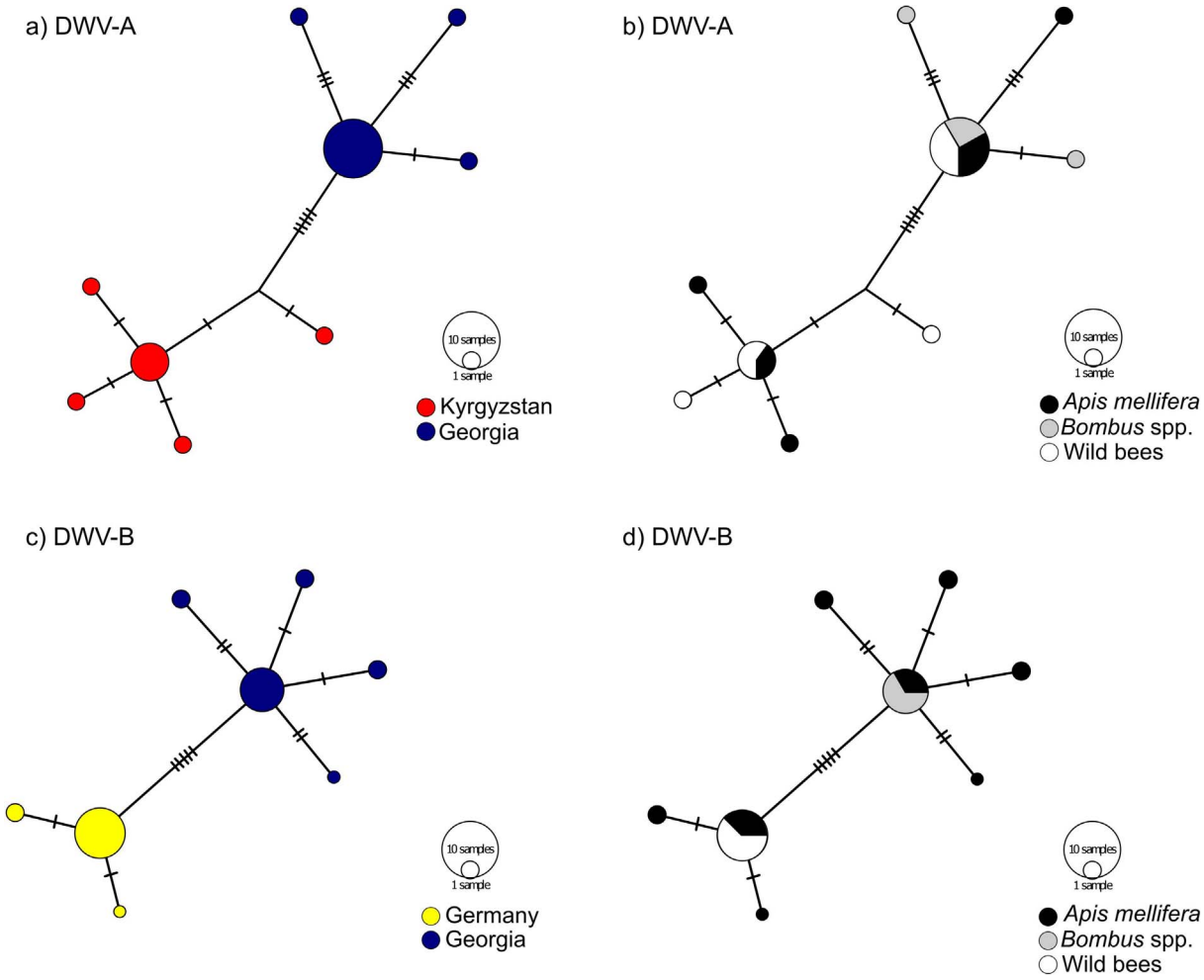

Fig. 4. Median Joining haplotype networks of cloned partial sequences of DWV-A RNA-dependent RNA polymerase (*RdRp*) gene (420 bp, $N_{sequences}$ = 24) and DWV-B L-protein gene (413 bp, $N_{sequences}$ = 18); (a) DWV-A haplotypes per sampling location (GEO-1 in Georgia and KYR-1 in Kyrgyzstan), (b) DWV-A haplotypes per host morphogroup, (c) DWV-B haplotypes per sampling location (GEO-2 in Georgia and GER-1 in Germany), (d) DWV-B haplotypes per host morphogroup. The size of the circle representing a haplotype is proportional to the haplotype's frequency. Hatch marks indicate mutational steps.

and DWV-B from the Transcaucasian region.

cDNA sequence analysis revealed clustering according to geographic origin in DWV-A and DWV-B (Fig. 4). All five DWV-A haplotypes obtained from Kyrgyz bees, which encompassed partial virus sequences from both honey bee and wild bee samples, formed a distinct cluster separated by at least five mutational steps from the Georgian DWV-A haplotypes (Fig. 4a and b). The most common of the latter was shared between all three host morphogroups (Fig. 4b). Similarly, the five detected DWV-B haplotypes from Georgia clustered separately from German DWV-B haplotypes, which differed from each other just by one mutation event (Fig. 4c). The most abundant DWV-B haplotype in Georgia was shared between honey bees and bumble bees and the most common German DWV-B haplotype was found both in honey bees and wild bees. These data support the idea that DWV is shared between taxa at the same site. In contrast, no well-resolved geographic pattern was found in the haplotype network of BQCV (Fig. 5), most likely due to lack of genetic variability in the highly conservative gene (structural capsid protein) used to confirm identity of this virus (Reddy et al., 2013).

4. Discussion

Honey bee-associated RNA viruses have been found in several species of wild bee (reviewed in Manley et al., 2015; Tehel et al., 2016), raising concern about pathogen spill-over between managed and wild pollinator species (Fürst et al., 2014). Here we show that sharing of several RNA viruses associated with honey bees is both geographically and taxonomically widespread and that these viruses may be infective not only for *A. mellifera* but also for many other bee species. Moreover, we provide evidence that DWV-A and DWV-B are shared across species at the same site.

We tested 33 bee species (including *A. mellifera*) and found DWV-A/DWV-B to have the highest true prevalence across taxa, in line with the findings of a UK-wide survey (McMahon et al., 2015). Across sites, 28% of honey bees, 15% of bumble bees and 13% of wild bees in our study harbored this virus complex whereas SBV, ABPV/IAPV/KBV and CBPV were rarely detected (Table A3). However, we are aware that our study could potentially underestimate the actual pervasiveness of these pathogens in pollinator communities because we collected only active foragers from apple blossoms and thus excluded severely infected bees incapable of flying.

We found variable composition of viruses among different bee taxa. Andrenid bees in our study harbored only DWV-A/DWV-B, although previous studies have found BQCV and IAPV in andrenid bees (Ravoet et al., 2014; Singh et al., 2010; Dolezal et al., 2016). *Osmia bicornis* (Megachilidae) was the only wild bee species carrying all six tested viruses/virus complexes. To our knowledge, commercially reared *O. bicornis* individuals were not introduced for apple pollination in our sampling sites, but we cannot exclude the possibility of their dispersal from surrounding orchards. In contrast, *Anthophora plumipes*, which

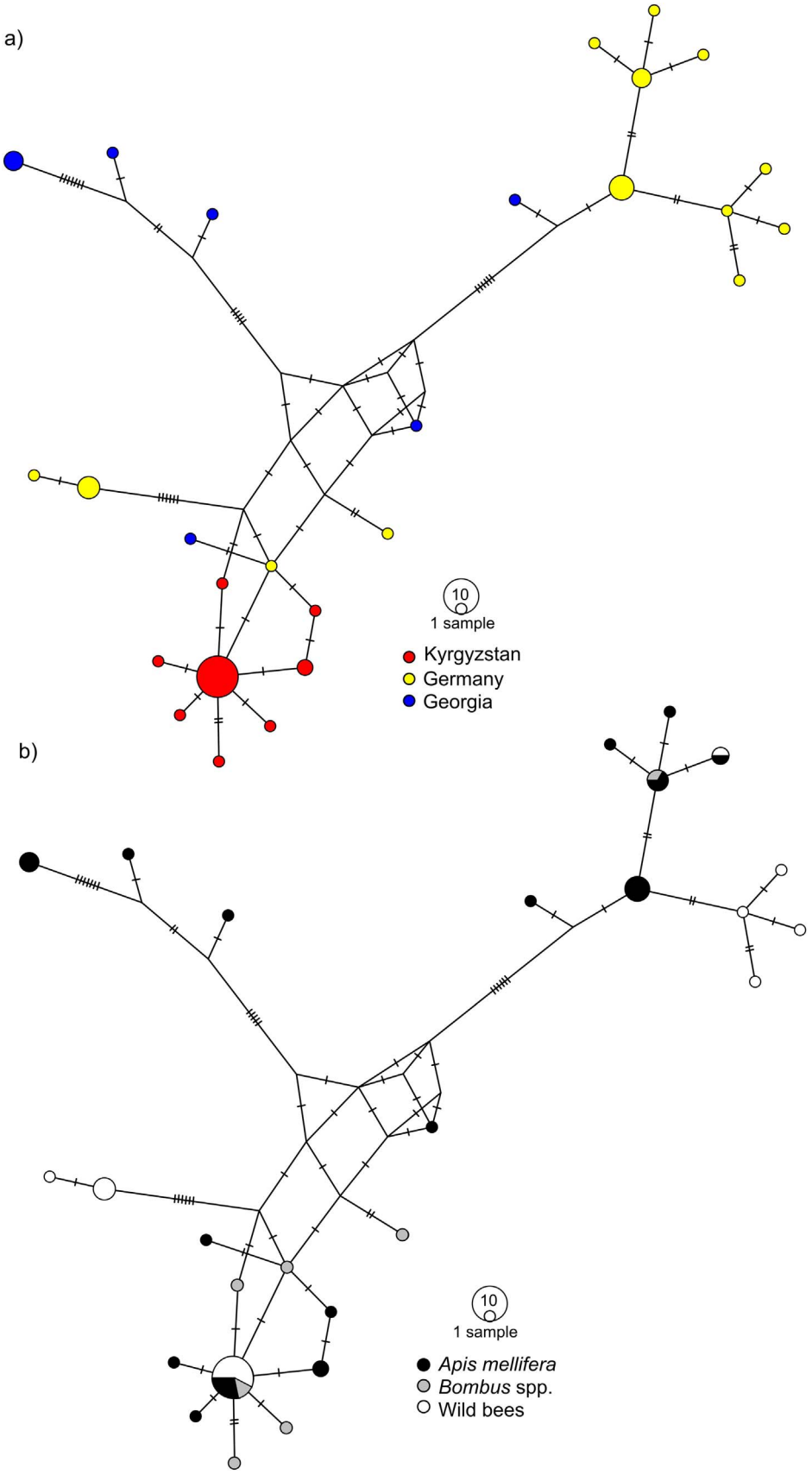

Fig. 5. Median Joining haplotype networks of cloned partial sequences of BQCV capsid protein gene, 296 bp, $N_{sequences} = 24$) per (a) sampling location (aggregated by country) and (b) host morphogroup. The size of the circle representing a haplotype is proportional to the haplotype's frequency. Hatch marks indicate mutational steps. For a version of (a) by individual sampling location within a country, see Supplementary Fig. A1.

carried all screened viruses except CBPV, is an unmanaged wild bee species, common in spring during apple bloom. The broad range of viruses detected in this bee species could be explained by its closer phylogenetic relatedness to honey bees and bumble bees (Michener, 2007) (and thus more similar molecular structure of viral cell receptors), its relatively high abundance and its high flower visitation rate, increasing the probability the uptake of virus particles with pollen or nectar from shared flowers (McArt et al., 2014; Singh et al., 2010).

DWV-A/DWV-B was the only viral complex we detected across the other wild bee species belonging to all four bee families under study (Table 1), suggesting this virus quasispecies to be a generalist bee pathogen. Among *Bombus* spp., we found four of seven species to harbour the viral complex and found its negative strand in three of these four, suggesting the virus is infective for many *Bombus* spp.; Fürst et al. (2014) also found negative strand of the DWV complex to be widespread in British bumble bees and experimentally demonstrated that it is pathogenic when fed to *B. terrestris*. Our screening of DWV-A/DWV-B-MLPA positive other wild bee samples for the presence of the negative-strand intermediate RNA revealed replication of DWV-A/DWV-B (and thus a potential infection) in just 6% of tested wild bee individuals, and only *Andrena haemorrhoa* out of ten other wild bee species tested. This is the first evidence that andrenid bees can also serve as a host species for DWV-A/DWV-B in natural pollinator communities. In contrast to DWV-A/DWV-B, BQCV had a lower prevalence across our samples yet was found to be replicating in 56% of tested wild bee individuals, including members of *O. bicornis*, *A. plumipes* and *Xylocopa* sp. and four *Bombus* species: *B. laesus*, *B. soroeensis*, *B. terrestris* and *B. vestalis*.

Our findings thus extend the known range of wild bee species harbouring DWV-A/DWV-B and BQCV as well as those in which viral replication occurs. Previous studies have found DWV-A/DWV-B to be able to replicate in *Osmia cornuta* (Latreille, 1805) (Mazzei et al., 2014) and a range of bumble bee species (Fürst et al., 2014), whereas BQCV has only been shown to replicate in *Bombus huntii* Greene, 1860 (Peng et al., 2011). However, though evidence of viral replication is suggestive of infection, it is not definitive evidence for pathology (Tehel et al., 2016); experimental infection is necessary to demonstrate that a virus causes disease, as shown for DWV fed to *B. terrestris* in reducing host lifespan (Fürst et al., 2014). Whether replication of DWV-A/DWV-B or BQCV, as we found, also causes pathology across a wide range of host species remains to be shown.

Our analysis of sequences of DWV-A/DWV-B amplicons indicated the presence of DWV-A in honey bees and wild bees in both Georgia and Kyrgyzstan whereas DWV-B was found in German honey bees and wild bees and in Georgian honey bees and bumble bees (Fig. 4). To our knowledge, this is the first report of DWV-A in Georgia and Kyrgyzstan and of DWV-B in Georgia. Our sequences of viral amplicons suggest viruses are shared across host species at the same site, at least for the DWV-A/DWV-B complex, as also seen in Great Britain for *A. mellifera* and *Bombus* spp. (Fürst et al., 2014). McMahon et al. (2016) have recently demonstrated experimentally that DWV-B is more virulent than traditional DWV-A in honey bees. That DWV-B is also widely shared across host species at the same site, as we find here, therefore represents a potentially higher threat to wild bee species than DWV-A. However, we still know surprisingly little about the pathology of honey bee-associated viruses in wild bees, despite their potential role in pollinator decline (Tehel et al., 2016). Large scale standardized surveys of prevalence and replication of honey bee-associated RNA viruses in sympatric pollinator communities coupled to experiments testing for viral virulence are thus crucial and urgently needed to determine the epidemiology of these pathogens, to understand their dynamics under field conditions, and to quantify their impact on wild bee populations.

Acknowledgments

This study was supported by the Volkswagen Foundation (AZ:86880), the Georgian National Science Foundation (DO /372/10-101/14), the University of Leipzig, the Helmholtz Research School for Ecosystem Services under Changing Land-use and Climate (ESCALATE) and the German Research Foundation (DFG) (Pa632/10). We thank Christina Jenkins for comments on the manuscript and Kerstin Göβel, Veronika Lutz, Anja Miertsch and Gitana Timofejavaitė for their assistance in the lab. We thank all landowners for their permission to collect bees from their apple orchards. Bees in Germany were collected under permit number RL-0387 from the Saxony-Anhalt State Bureau for Environmental Protection, for which we thank Per Schnitter; licences were not required for collection of bees in Georgia and Kyrgyzstan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2017.04.002.

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