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RNA viruses of *Crithidia bombi*, a parasite of bumblebees

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ABSTRACT

Leishbuviridae (*Bunyavirales*) are a diverse monophyletic group of negative-sense single-stranded RNA virus infecting parasitic flagellates of the family Trypanosomatidae. The presence of RNA viruses in trypanosomatids can influence the virulence of the latter. Here, we performed a screening for viruses in *Crithidia bombi* – a common parasite of important pollinators *Bombus* spp. (bumblebees) that negatively affects its host in stressful conditions. The majority (8/10) of *C. bombi* isolates collected in Europe and North America were positive for a virus that we named *Crithidia bombi leishbuvirus 1* with high conservation of amino acid sequences between isolates. The results of our comparative phylogenetic analyses of the trypanosomatids and their viruses suggest that the high mobility of bumblebees and frequent coinfections by different strains of *C. bombi* determine an extensive viral exchange between the latter.

1. Introduction

Crithidia bombi is a widespread parasitic flagellate of the family Trypanosomatidae that inhabits the rectum of *Bombus* spp. (bumblebees) (Gallot-Lavallée et al., 2016; Lipa and Triggiani, 1988; Schmid-Hempel et al., 2014; Shykoff and Schmid-Hempel, 1991). Bumblebees are important pollinators playing crucial ecological roles and contributing substantial economic benefits to the human food supply (Corbet et al., 2015; Nogué et al., 2016). These insects are eusocial: every year, a female (a queen) founds a colony with a few dozen to a few hundred workers (Goulson, 2003). Such a habit facilitates spreading of the parasites and determines the high prevalence of *C. bombi* (Schmid-Hempel, 2021). The transmission of these flagellates is alimentary, i.e. occurs by feeding on a substrate contaminated with feces. Colony members can be infected either *via* their mother queen carrying parasites from the previous season or by foraging workers, which can bring the flagellates from flowers contaminated with feces (Durrer and Schmid-Hempel, 1994; Graystock et al., 2015; Piot et al., 2020). The infection develops quickly and the first infective cells are shed with feces after just a few days (Schmid-Hempel and Schmid-Hempel, 1993). The presence of *C. bombi* exerts various negative effects on its hosts. Under starvation, infected insects demonstrate significantly higher mortality rates (Brown et al., 2000). Infected queens are considerably less successful in colony founding, produce fewer progeny, and lose more body mass after long hibernation (Brown et al., 2003).

Many trypanosomatids, including members of the speciose subfamily Leishmaniinae, to which *C. bombi* belongs, bear RNA viruses (Grybchuk et al., 2018a). In general, the diversity of these viruses and their influence on the biology of trypanosomatids are understudied (Grybchuk et al., 2018b). Nevertheless, *Leishmaniavirus* (a double-stranded RNA virus of the family *Totiviridae*) has been implicated in increased virulence of human-infecting flagellates *Leishmania* spp. of the New World (Hartley et al., 2014; Ives et al., 2011). In addition to *Totiviridae*, recent surveys revealed the presence of other viral groups in trypanosomatids: *Narnaviridae*, *Leishbuviridae*, *Qinviridae,* tombus-like viruses, and *Ostravirus* (Grybchuk et al., 2018a; Grybchuk et al., 2018c; Klocek et al.,

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2023; Macedo et al., 2023). Of these, the family *Leishbuviridae* of the order *Bunyavirales* requires special attention. It is a speciose group of negative sense single-stranded RNA viruses documented in numerous lineages of trypanosomatids (Grybchuk et al., 2018a; Grybchuk et al., 2018c; Grybchuk et al., 2020). It appears to be exclusively associated with trypanosomatids, suggesting that the transition of the viruses from insects to these flagellates occurred only once. The subsequent evolution of *Leishbuviridae* was predominantly shaped by numerous horizontal viral transfers on the background of co-speciation with trypanosomatid hosts (Grybchuk et al., 2018a; Grybchuk et al., 2018c).

Typical bunyaviruses have tripartite genomes with large (L), medium (M), and small (S) segments, encoding an RNA-dependent RNA polymerase (RDRP), a surface glycoprotein precursor, and a nucleocapsid protein, respectively (Wichgers Schreur et al., 2018). They form enveloped virions (90–100 nm) that include each genomic segment in a complex with multiple nucleocapsid molecules and a single RDRP protein. Viral glycoproteins are incorporated into the membrane envelope (Elliott, 1990; Walter and Barr, 2011). Most bunyaviruses cause arthropod-borne diseases in vertebrates and plants (Junglen, 2016; Käfer et al., 2019).

Here, we screened various *C. bombi* isolates from Europe and North America for the presence of viral dsRNA, identified the discovered viruses, analyzed the genome of one of the viral strains, and compared their phylogeny with that of the respective trypanosomatid hosts.

2. Methods

2.1. Cultivation of C. bombi strains and their screening for the presence of dsRNA

Ten isolates of *Crithidia bombi* were analyzed in this work (Table 1). Cells were cultivated at 28 °C and 3% CO_2 in Brain Heart Infusion medium (VWR, Radnor, USA) supplemented with 20% heat-inactivated fetal bovine serum (BioSera Europe, Nuaillé, France), 2 μg/ml hemin (Jena Bioscience, Jena, Germany), 100U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific, Carlsbad, USA). Total RNA from 10^9 parasites per isolate was treated with DNase I/S1 nuclease (Isorce and Fasel, 2020) and purified using the Zymoclean Gel RNA Recovery kit (Zymo Research, Irvine, USA). The samples were run on a 0.8% agarose gel to check for dsRNA. One dsRNA-positive sample (isolate 5A) was sequenced on Illumina HiSeq 2500 at the Institute of Applied Biotechnologies (Olomouc, Czechia) as described before (Grybchuk et al., 2020).

Table 1
Origin of *Crithidia bombi* isolates.

Isolate	Collection date	Region, Site	Host	Caste
5A	15.05.2020	Czechia, Prague	*Bombus terrestris*	Queen
AK08-047	14.06.2008	Alaska, Willow Creek	*B. insularis*	Queen
AK08-052	14.06.2008	Alaska, Willow Creek	*B. insularis*	Queen
08–091	31.03.2008	Switzerland, Neunforn	*B. terrestris*	Queen
08–161	31.03.2008	Switzerland, Neunforn	*B. lapidarius*	Queen
08–261	02.04.2008	Switzerland, Neunforn	*B. terrestris*	Queen
12–450	23.03.2012	Switzerland, Aesch BL	*B. terrestris*	Queen
14–255	12.04.2014	Switzerland, Aesch BL	*B. terrestris*	Queen
*C*2-Q12	10.02.2012	France, Corsica	*B. terrestris*	Queen
S3-1	16.02.2012	Italy, Sardinia	*B. terrestris*	Worker

2.2. Next-generation sequencing and assembly of the viral genome

Read trimming and *de novo* assembly was accomplished in Trimmomatic v. 0.40 (Bolger et al., 2014) and Trinity v. 2.13.2 (Haas et al., 2013), respectively. Read mapping and coverage estimation were performed using Bowtie 2 v. 2.4.4 (Langmead and Salzberg, 2012), SAMtools v. 1.13 (Ramirez-Gonzalez et al., 2012), and BEDTools v. 2.30.0 (Quinlan, 2014). The L segment of the *C. bombi* LBV containing RDRP gene was identified by BLASTX (DIAMOND v. 2.0.2 (Buchfink et al., 2021)) search of *C. bombi* dsRNA contigs against the UniClust50 database (Mirdita et al., 2017). The M and S genomic segments were identified *via* TBLASTN searches of the corresponding sequences of a closely related Duke bunyavirus against the dsRNA contigs from *C. bombi*. The complementary terminal sequences were analyzed using iPknot v. 2.2.1 web server (Sato et al., 2011).

2.3. PCR

The 634-bp long fragment of the nucleocapsid-containing segment was amplified using the primers NC_F 5′-ccctcattccgtgttctgtt-3′ and NC_R 5′-catccacttcctctcggaat-3′. For partial amplification of the RDRP gene, the primer LeiBunyaF 5′-ttykcvacnttcaagaaragcac-3′ was used in combination with either LeiBunyaR 5′-ccagartcatcwgadgadaccat-3′ (989 bp, for most samples) or LeiBunyaR2-F4c 5′-ggcatrcaraaytgytgrcacca-3′ (494 bp, for the divergent strain AK08-047, from which the longer fragment could not be amplified). The obtained fragments were sequenced directly using the amplification primers.

2.4. Phylogenetic analyses

2.4.1. Phylogenetic position of the new virus

The LBV phylogeny was inferred based on the RDRP gene. Related *Leishbuviridae* and *Phenuiviridae* sequences were aligned in MAFFT v. 7.511 (Katoh and Standley, 2013) with G-INS-i algorithm and trimmed in TrimAl v. 1.4 with the "automated1" option (Capella-Gutiérrez et al., 2009). The resulting matrix (1,863 positions) was used for maximum likelihood (ML) analysis in IQ-Tree v. 2.2.0 (Minh et al., 2020) with the automatically selected best-fit classical model LG + I + F + G4 and 1,000 standard bootstrap replicates. Bayesian inference was performed in MrBayes v. 3.2.7. (Ronquist et al., 2012) with default settings (two runs, four chains each, 100,000 generations with sampling every 100 generations) and the same substitution model as above.

2.4.2. Relationships between Crithidia bombi isolates

Phylogenetic relationships between *C. bombi* isolates were inferred using one of the most variable parts of the available genomic data, the maxicircle kDNA (Gerasimov et al., 2020; Kaufer et al., 2019). The maxicircle sequence-containing contig MDUH01002070 was identified in the assembly of *C. bombi* IL132 by BLAST against the corresponding sequence of *Leishmania tarentolae* (GenBank NC_000894). A reliable alignment could be obtained for the major (~16.2 kb) part of these two sequences, including all rRNA and protein-coding genes as annotated for *L. tarentolae*. This segment was extracted and used as a reference for the assembly of maxicircle sequences of the studied isolates. The assembly was performed in Geneious Prime v. 2023.0.4 (Dotmatics, Boston, USA) by mapping Illumina reads onto the reference using the Geneious mapper with the following parameters: reads trimming with 0.95 probability limit, trimming of paired read overhangs, maximum 5% gaps per read, word length of 18, 10% maximum mismatches *per* read, and other parameters as in the medium–low sensitivity preset. For the isolate 5A, the NGS data generated here were used; for the others, the SRAs ERR3418932, ERR3418937, ERR3418938, ERR3418945, ERR3418946, ERR3418948, ERR3418953, ERR3418956, and ERR3418960 were downloaded from the GenBank. The assembled sequences were trimmed according to the reference (at the borders of *12S* rRNA and *ND5* genes), and an alignment containing 16,195 positions was obtained in MAFFT

with the automatic algorithm. The ML and Bayesian analyses were performed as above, but the best-fit model was HKY + F and the run in MrBayes lasted 300,000 generations.

2.4.3. Diversity of the viruses

The sequences of the nucleocapsid and RDRP-containing segments assembled from the NGS data for the isolate 5A, obtained by PCR for other isolates studied here, and those available in the GenBank for Duke bunyavirus (KY094605 and KY094607) were aligned (segment-wise) by MAFFT and manually trimmed to fit the length of the amplified fragments. The two resulting alignments were concatenated in BioEdit (Hall, 1999) and used for phylogenetic inference following the same scheme as above, but with substitution models TIM2 + F and GTR + F, for IQ-TREE and MrBayes, respectively.

3. Results and discussion

Out of ten *C. bombi* isolates screened for the presence of dsRNA, eight (one out of two from Alaska and seven out of eight from Europe) were positive. They displayed the same band pattern (Fig. 1A), suggesting the presence of a single species of the family *Leishbuviridae* (LBV): while S (0.8 kb) and L (6.2 kb) segments were clearly visible on a gel, the M segment was not perceptible, as frequently happens with these viruses (Grybchuk et al., 2020). However, the missing segment (along with the other two) could be detected in next-generation sequencing data and its length was 1.1 kb. Therefore, this virus has a typical leishbunyaviral organization, which is schematically shown in Fig. 1B.

Each of the three segments harbors a single ORF, the nucleotide sequences of which are highly similar to those of Duke bunyavirus (KY094605–KY094607) previously discovered in a bee metatranscriptome from the USA (RDRP – 97.7%, glycoprotein – 93.2%, nucleocapsid – 92.3%). Due to the high sequence similarity and proximity of these two viruses on a phylogenetic tree (Fig. 2A), we consider them a single species – *Crithidia bombi leishbuvirus 1* (*Cbom*LBV1). Its terminal complementary sequences (ACACAAAGA…UCUUUGUCU (Fig. 3)) are typical for most *Leishbuviridae* and *Phenuiviridae* (Elliott, 1990; Grybchuk et al., 2018a). Since the finding of Duke bunyavirus was not described in a publication, and nothing but the sequences of its three segments is available, it remains to be established whether it was hosted by *C. bombi*. The corresponding GenBank records report the honeybee (i.e., *Apis mellifera*) as the host. Although *C. bombi* cannot develop in honeybees, it has been experimentally shown that the latter can act as paratenic hosts (carriers) for this trypanosomatid (Ruiz-González and Brown, 2006). Indeed, screening of honeybees in nature revealed occasional occurrence of *C. bombi* in their gut (Bartolomé et al., 2020; Bartolomé et al., 2018; Graystock et al., 2015; Lim et al., 2023). In addition, this parasite has been documented to infect two species of North American solitary bees, *Osmia lignaria* and *Megachile rotundata* of the family Megachilidae (Figueroa et al., 2021), phylogenetically distant from both *A. mellifera* and *Bombus* spp., which belong to Apidae. Since these two species of bees are superficially similar to honeybees, the insect harboring Duke bunyavirus might have been misidentified. Of note, in a whole-insect metatranscriptome of *A. mellifera* from South Africa, *Apis bunyavirus 1*, which is a member of *Leishbuviridae* distantly related to *Cbom*LBV1 (Fig. 2A), has been found along with *Lotmaria passim*, a common trypanosomatid parasite (Remnant et al., 2017).

The partial RDRP and nucleocapsid sequences obtained for the remaining virus-positive isolates showed 90.8–99.6% and 91.8–100% pairwise nucleotide identity, respectively, confirming that they belong to the same species as the viruses discussed above. The phylogenetic analyses revealed a well-defined split between European and North American *Cbom*LBVs, which was also apparent in the tree of the trypanosomatid strains (Fig. 2B). Beyond this, the poor resolution of European LBVs did not allow detection of any signs of potential co-evolution. The only recognizable LBV clade with moderate support included isolates 08–161 and S3-1, which was absent from the *C. bombi* tree (Fig. 2B). At the same time, the trypanosomatid isolates 08–261 and 14–255 displayed almost identical maxicircle sequences, but the former was virus-free. All these facts suggest that the viruses are transmitted between strains of *C. bombi*. This agrees with frequent coinfections of bumblebees by different strains of this flagellate (Schmid-Hempel et al., 2011; Tognazzo et al., 2012). The absence of apparent geographic subdivisions within the European clade of *C. bombi* (Fig. 2B) also suggests an exchange of flagellate strains between countries, which can be explained by the high mobility of bumblebees. For example, the isolate 5A from Czechia is very similar to 08–161 from Switzerland, and, notably, their viruses do not mirror this relationship. Indeed, bumblebees have been repeatedly reported to perform massive migrations in spring over distances of hundreds of kilometers, which can be explained by higher survival rates of queens upon shorter hibernation periods and/or by the scarcity of disused vole burrows serving as nesting sites (Fijen, 2021). Random migration of individual queens also cannot also be excluded. Although the impact of *C. bombi* infection on bumblebee mobility has not been studied, it is unlikely that the latter significantly decreases in the presence of the parasite. If bumblebees do not starve (queens migrating in the spring definitely can feed), the parasite affects only their reproductive function by reducing the size of ovaries (partial parasitic castration), which is even accompanied by an increase of fat content in the body (Brown et al., 2000; Brown et al., 2003).

The exchange of LBVs between *C. bombi* isolates residing in the gut of one insect individual may occur in a number of ways. Firstly, the enveloped virions of LBVs enable transmission between trypanosomatid cells *via* direct shedding and endocytosis. Secondly, extracellular vesicles can mediate sharing of viruses, as demonstrated in *Leishmaniavirus* (Atayde et al., 2019; Lafleur and Olivier, 2022). Lastly, *C. bombi* practices regular genetic exchanges with Mendelian genotype segregation

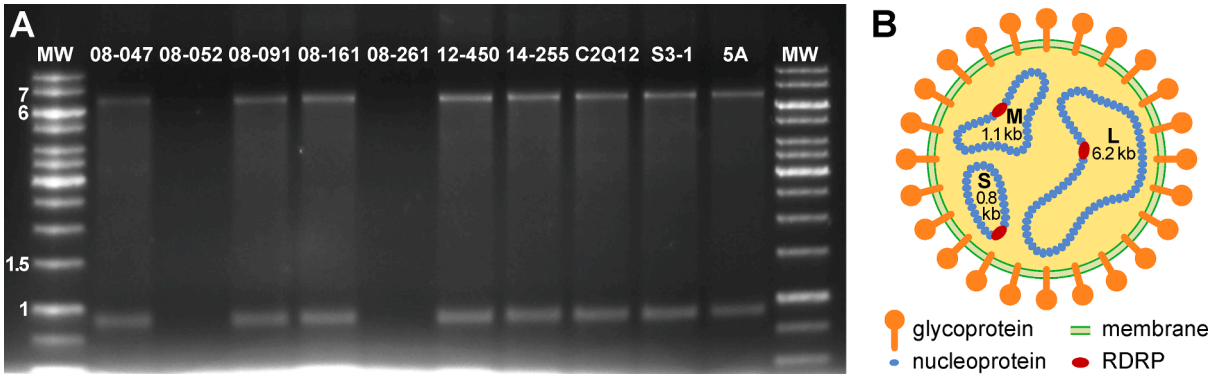

Fig. 1. *Crithidia bombi leishbuvirus 1*. (A) Screening of *Crithidia bombi* isolates for dsRNA presence. MW - GeneRuler 1 kb DNA ladder. Indicated sizes are in kilobases. (B) Scheme of the virus organization based on the obtained genomic data.

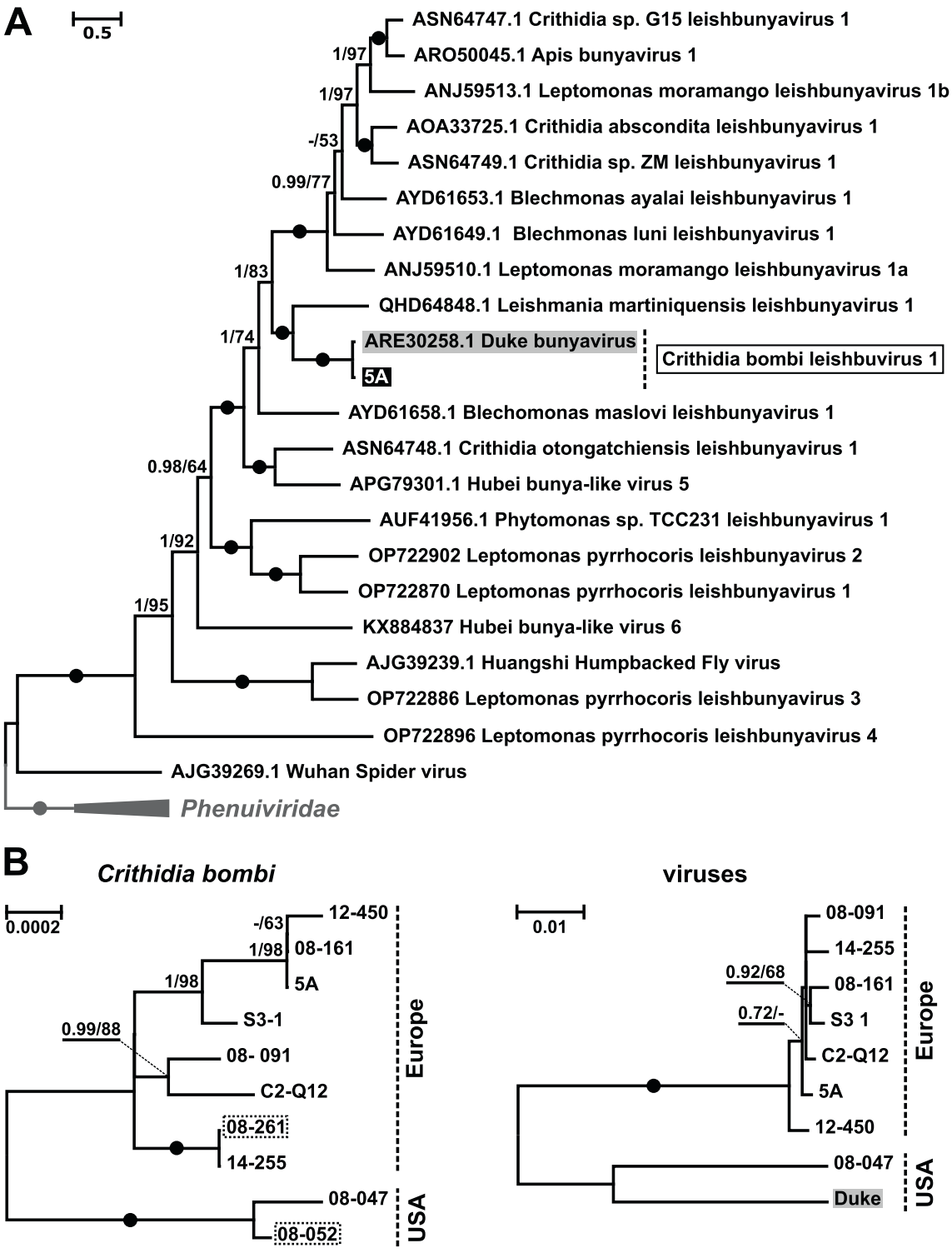

Fig. 2. Maximum likelihood phylogenetic reconstructions. (A) Position of *Crithidia bombi leishbuvirus 1* (boxed) and, in particular, the isolate 5A (highlighted in black) on an RDRP-based phylogenetic tree of *Leishbuviridae*, rooted using *Phenuiviridae* as outgroup. The Duke bunyavirus (not being part of the study) is highlighted in grey. (B) Midpoint-rooted trees for the studied *C. bombi* strains and their viruses based on kDNA and concatenated partial nucleocapsid + RDRP sequences, respectively. The virus-free strains have dotted outline. The Duke bunyavirus (not being part of the study) is highlighted in grey. Bayesian posterior probabilities and bootstrap supports are shown at branches, their respective values below 0.5 and 50 are omitted or replaced with dashes. Branches with absolute (1/100) supports are marked with black circles. Scale bar corresponds to the number of substitutions *per* site.

(Schmid-Hempel et al., 2011), implying a sexual process, which represents one more way of viral transmission. With our small sample size of *C. bombi* isolates, we did not detect viral co-infections. However, frequent mixed infections in bumblebees and (presumably) easy viral exchange should lead to such cases, which may result in reassortment or recombination. Such processes might be responsible for the poor resolution observed in the European clade of *Cbom*LBVs.

To sum up, *Cbom*LBV1 is a typical representative of the family *Leishbuviridae* being a common and, so far, the only known virus of *C. bombi*. We propose that the high mobility of bumblebees creates local

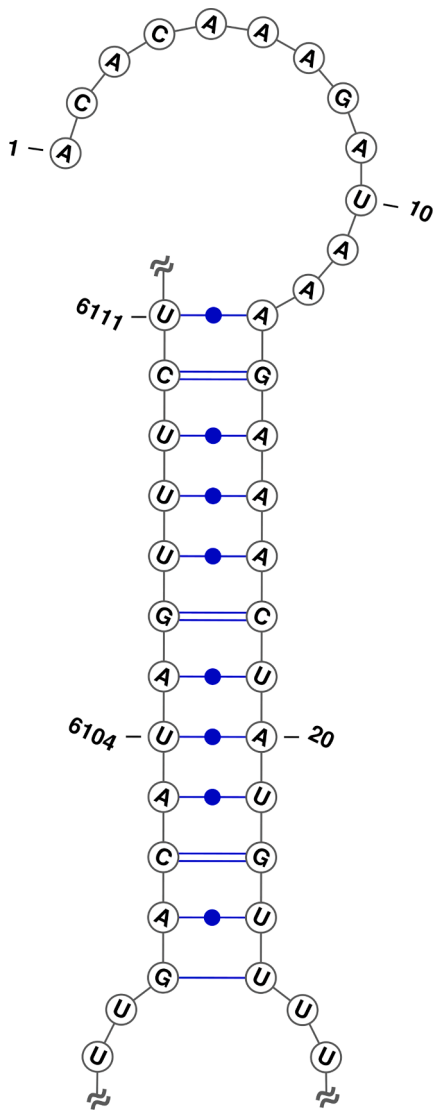

Fig. 3. Terminal secondary structure of the L segment of Crithidia bombi leishbuvirus 1. The 3′-end of the hairpin is incomplete.

heterogeneity of European populations of *C. bombi*, which, due to the high prevalence of the parasite, results in frequent mixed infections. In turn, this creates favorable conditions for regular inter-strain viral transfers.

4. Data accessibility

All sequence data obtained in this work were submitted to GenBank with the following accession numbers: OR146996, OR146997, and OR146998 for the L, M and S segments, respectively of *Cbom*LBV1 isolate 5A; OR208631 – OR208671 and OR208638 – OR208644 for partial sequences L (RDRP) and S (nucleocapsid) segments of viruses of other isolates; as well as OR211421 – OR211430 for trypanosomatid maxicircle sequences of all analyzed *C. bombi* isolates.

Authors' contributions

The study was designed by V.Y. and A.Y.K. Data collection and analyses were carried out by D.K., D.G., D.H.M., J.V., A.R., R.S.-H., P.S.-H., and A.Y.K. V.Y. and A.Y.K. drafted the manuscript. All authors contributed to the final draft and editing, giving their approval for publication, and agreeing to be held accountable for the work performed herein.

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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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