1 **RESEARCH ARTICLE**

2 **Do bed bugs transmit human viruses, or do humans transmit bed bug viruses? A worldwide**

3 **survey of the bed bug RNA virosphere**

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15 **ABSTRACT:**

16 Bed bugs (Hemiptera: *Cimicidae*) are a globally distributed hematophagous pest that

17 routinely feed on humans. Unlike many blood-sucking arthropods, they have never been linked

18 to disease transmission in a natural setting, and despite interest in their role as disease vectors,

19 little is known about the viruses that bed bugs naturally harbor. Here, we present a global-scale

20 survey of the bed bug RNA virosphere. We sequenced the metatranscriptomes of 22 individual

21 bed bugs (*Cimex lectularius* and *Cimex hemipterus*) from 8 locations around the world. We

22 detected sequences from two known bed bug viruses (Shuangao bedbug virus 1 and Shuangao

bedbug virus 2) which extends their geographical range and the host range of Shuangao bedbug virus 1 to *Cimex lectularius*. We identified three novel bed bug virus sequences from a tenui-like virus (*Bunyavirales*), a toti-like virus (*Ghabrivirales*), and a luteo-like virus (*Tolivirales*). Interestingly, some of the bed bug viruses branch near to insect-transmitted plant-infecting viruses, opening questions regarding the evolution of plant virus infection. When we analyzed the putative viral sequences by their host's collection location, we found unexpected patterns of geographical diversity that may reflect humans' role in bed bug dispersal. Additionally, we investigated the effect that *Wolbachia,* the primary bed bug endosymbiont, may have on viral abundance and found that *Wolbachia* infection neither promotes nor inhibits viral infection. Finally, our results provide no evidence that bed bugs transmit any known human pathogenic viruses.

34 **1. INTRODUCTION:**

35 Bed bugs (Hemiptera: Cimicidae) are globally distributed obligately hematophagous
36 ectoparasites (Doggett et al., 2012). Some species routinely feed on humans, but unlike many
37 other blood sucking insects, there is no evidence that bed bugs are human disease vectors
38 (Davies et al., 2012). Several studies have been conducted to assess if bed bugs could transmit
39 human pathogens, but transmission in the wild has not been documented (Blakely et al., 2018;
40 El Hamzaoui et al., 2019; Goddard & deShazo, 2009; Leulmi et al., 2015; Pietri, 2020).
41 Considering the consistent resurgence of bed bug populations leading to outbreaks in the last
42 20-30 years, it is important to understand what microbes they harbor along with their potential
43 as vectors (Davies et al., 2012; Doggett & Lee, 2023; Lewis et al., 2023).

44 Large scale metatranscriptomic studies have enhanced our knowledge of viral diversity
45 in invertebrates and have made detecting arthropod-associated viruses increasingly more
46 feasible (Käfer et al., 2019; Li et al., 2015; Shi et al., 2016). Bed bugs have been included in some
47 arthropod viromics-based studies, and four putative bed bug virus sequences have been
48 detected: Serbia reo-like virus 2 (NCBI:txid2771464), Serbia picorna-like virus 2
49 (NCBI:txid2771462), Shuangao bedbug virus 1 (NCBI:txid1608071) and Shuangao bedbug virus
50 2 (NCBI:txid1608072) (Li et al., 2015; Zhang et al., 2020). No further studies have been
51 conducted to examine the pathogenic properties of these putative viruses, but they do provide
52 evidence that bed bugs encounter viral infection. Furthermore, Ling *et al.* (2020) took a
53 metatranscriptomic approach to survey for viruses in bed bugs and found that some reads in a
54 sample of recently blood fed individuals aligned to hepatitis C virus. Although there was no
55 evidence that the virus was replicating (i.e., low number of reads mapped and an incomplete

56 genome), their study highlights the role that bioinformatic surveillance could play in detecting
57 human pathogen transmission in bed bugs. However, bed bug specific viruses were either not
58 detected or not reported in their study (Ling et al., 2020).

59 In this study, we conducted a worldwide survey of the bed bug RNA virosphere. Our
60 aims were to search for known human viral pathogens and novel bed bug viruses that could be
61 of interest to human health or biocontrol. We collected bed bugs (*Cimex lectularius* and *Cimex*
62 *hemipterus*) from 8 distinct locations around the world and sequenced RNA libraries from 22
63 individuals. We assembled bed bug metatranscriptomes, and conducted phylogenetic analyses
64 on the virus sequences that we detected. We also assessed viral diversity between bed bug
65 species and geographic location. Additionally, we investigated whether there is a correlation
66 between *Wolbachia* (a bed bug endosymbiont known to have an antiviral effect when infecting
67 other insect taxa) reads and bed bug virus reads in each sample (Cogni et al., 2021; Hussain et
68 al., 2023; Lindsey et al., 2018; Teixeira et al., 2008; Terradas & McGraw, 2017).

69 **2. MATERIALS AND METHODS:**

70 **2.1 Collection and extraction**:

71 The samples used in this study were obtained as part of a large international collection
72 of bed bugs provided by numerous pest control companies and researches. We included *Cimex*
73 *lectularius* samples from Czechia (n=3), France (n=1), the UK (n=3), Rome-Italy (n=3), Assisi-Italy
74 (n=3), Ohio-USA (n=3), the Harlan lab strain of *Cimex lectularius* (initially collected in Fort Dix,
75 New Jersey, USA and maintained at the King lab at Mississippi State University) (n=3), and
76 *Cimex hemipterus* samples from Madagascar (n=3). Each bug was washed in 95% ethanol and
77 total RNA was isolated using a standard TRIzol protocol (Invitrogen Waltham, MA) and further
78 purified using NEB's Monarch RNA cleanup kit was used (New England Biolabs Ipswich, MA).

79 **2.2 Library Prep and Sequencing:**

80 We checked total RNA quality using Nanodrop quantitation and agarose gel
81 electrophoresis, and we assessed RNA integrity with an Agilent 2100 bioanalyzer. Libraries were
82 prepared using a strand specific library prep with ribosomal RNA depletion. Sequencing was
83 conducted on the Illumina NovaSeq 6000 instrument for 150 base pair paired-end reads,
84 resulting in approximately 100-135 million reads per sample. The quality of the reads was
85 inspected using FastQC v0.11.5 (Andrews, 2010) and reads were quality trimmed using
86 trimmomatic v. 0.39 (Bolger et al., 2014).

87 **2.3 Virus Discovery:**

88 To enrich the data for viral reads, we filtered out *Cimex*, *Wolbachia*, and human reads
89 by mapping to the *Cimex lectularius* (GCF_000648675.2), *Cimex hemipterus* (GCA_001663875.1,
90 partial), *Wolbachia* endosymbiont of *Cimex lectularius* (GCF_000829315.1), and *Homo sapiens*

91 (GCF_000001405.40) genomes using the bbsplit.sh tool of the bbmap suite (version 38.46)
92 (sourceforge.net/projects/bbmap/) and retained all unmapped reads. We assembled the
93 unmapped reads using Trinity v.2.11.0 (Grabherr et al., 2011) both individually and by collection
94 location. We clustered the individual sample assemblies with cd-hit-est (W. Li & Godzik, 2006)
95 with a sequence similarity threshold of 90%, a minimum sequence length of 500 nt, and a word
96 size of 8. The assemblies were annotated using diamond blastx in the –very-sensitive mode
97 with an e-value cutoff of $1e^{-5}$ (Buchfink et al., 2015, 2021) and the annotated transcripts were
98 filtered for viral hits which were further inspected for false positives using NCBI's BLASTx
99 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nr protein database. To detect known viral
100 conserved domains, we also inspected each viral hit in NCBI's conserved domain database
101 (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and InterProScan
102 (https://www.ebi.ac.uk/interpro/search/sequence/) (Quevillon et al., 2005).
103 When putative multipartite virus sequences were present, we used a viral co-occurrence
104 detection method we previously described to identify the other genomic segments (Walt et al.,
105 2023). Briefly, this method calculates two metrics based off sample co-occurrence. The first
106 metric, V_{co}, determines the frequency at which a transcript occurs in a sample with a given viral
107 conserved sequence (e.g., transcripts with confirmed RdRp domains detected in BLAST
108 analysis). The second metric, T_{co}, determines if the transcripts found together with a viral
109 conserved sequence occur in other samples without the viral conserved sequence. We used the
110 thresholds V_{co}=0.75 and T_{co}=0.5 to determine candidate viral genomic segments. After running
111 the co-occurrence analysis, we only kept candidate sequences with an ORF size > 500 nt. We

112 further inspect these transcripts for conserved protein families and domains using NCBI's CDD
113 and InterProScan.

114 **2.4 Phylogenetic Analysis of Viruses**

115 We only used transcripts with confirmed RdRp domains for phylogenetic analyses. First,
116 we extracted and translated ORFs encoding for RdRp proteins using NCBI's ORFfinder
117 (https://www.ncbi.nlm.nih.gov/orffinder/) and we downloaded diverse representative
118 sequences from families within relevant viral orders from NCBI. We aligned RdRp amino acid
119 sequences using MAFFT v7.490 (using the E-INS-I, G-INS-i, and L-INS-i, algorithms) and MUSCLE
120 v3.8.1551 (Edgar, 2004; Katoh & Standley, 2013). We compared alignment qualities using
121 MUMSA (Lassmann & Sonnhammer, 2006) scores, and the highest scoring alignment was used
122 for tree inference. Phylogenetic analyses were conducted with IQ-TREE2 v.2.0.7 using
123 ModelFinder (Kalyaanamoorthy et al., 2017) to find the best fitting substitution model. We
124 assessed branch support using ultrafast bootstrap with 1000 replicates, Shimodaira-Hasegawa-
125 like approximate likelihood ratio test (SH-aLRT) with 1000 replicates, and the aBayes test
126 (Anisimova et al., 2011; Minh et al., 2013; Nguyen et al., 2015). All phylogenetic trees were
127 midpoint rooted (unless otherwise noted) and visualized using the Interactive Tree of Life (iTOL)
128 webserver (Letunic & Bork, 2021).

129 **2.5 Phylogeographic Analysis**

130 We retrieved all transcripts of putative viral origin from each individual bed bug
131 assembly using Diamond BLASTx and CD-hit output. We predicted coding sequences using
132 EMBOSS's getorf tool using the -find 2 option. We only used coding sequences with complete
133 RdRp domains for phylogenetic analysis and duplicate sequences within samples were

134 discarded. We conducted phylogenetic analysis in the same way as section 2.4, except that
135 nucleotide sequences were used instead of amino acid. We selected closely related taxa from
136 the analysis in 2.4 as outgroups for phylogeographic analysis. We calculated evolutionary
137 distance as p-distance using MEGA v.11.0.13 (Tamura et al., 2021). We did not conduct
138 phylogeographic analysis on Shaungao Bedbug virus 2 because it was only detected in one
139 location in our study.

140 **2.5 Correlation of Wolbachia and Viral Read Abundance**

141 We mapped the trimmed read datasets to all the viral genomes detected in this study
142 and the *Wolbachia* endosymbiont of *Cimex lectularius* genome (GCF_000829315.1) using
143 HISAT2 v.2.2.1 (Kim et al., 2019). We used the summary file output to obtain the percent of
144 reads that mapped to the virus genomes or the *Wolbachia* genome. To correlate the Wolbachia
145 and virus read abundances, we conducted a simple linear regression analysis in R v.4.2.2. (R
146 Core Team, 2020), using the percentage of reads that mapped to the viral genomes versus the
147 percentage of reads mapped to the *Wolbachia* genome.

148 **3. RESULTS**

149 **3.1 Read Mapping, Assembly, and Transcript Annotation**

150 To survey the bed bug virome, we sequenced the total RNA (ribosomal and small RNA
151 depleted) from 22 individual bed bugs collected from the United Kingdom, France, Chechia,
152 Italy, Madagascar, and the USA. To enrich for virus sequences in our bed bug samples, we
153 filtered out reads that mapped to genomes of organisms that could be represented in our
154 dataset. We classified an average of 85% of reads from each sample as bed bug (65.2% *C.*
155 *lectularius*, 19.8% *C. hemipterus*), *Wolbachia* (2.08%), or human reads (0.07%). Those reads
156 were not used for metatranscriptome assembly (**Figure 2**). We assembled the remaining reads
157 and aligned the assemblies to NCBI's nr protein database using diamond BLASTx, which
158 returned multiple significant hits to putative bed bug virus sequences. We detected sequences
159 from two known negative-sense single stranded RNA (-ssRNA) viruses associated with *C.*
160 *hemipterus*: Shuangao bed bug virus 1 (Sbbv1) and Shuangao bed bug virus 2 (Sbbv2), but we
161 did not detect the other two known bed bug viruses in our assemblies (C. X. Li et al., 2015;
162 Zhang et al., 2020). In addition, we detected 3 novel viral sequences. These sequences all
163 belong to the realm *Riboviria* and encode for 1.) a -ssRNA genome, 2.) a positive-sense single
164 stranded RNA (+ssRNA) genome, and 3.) a double stranded RNA (dsRNA) genome. Strikingly, all
165 putative viral sequences detected in this study are present intercontinentally, but none were
166 detected across all samples.

167 **3.2 Shuangao Bedbug Virus 1**

168 Sbbv1 is described as an unclassified rhabdovirus (-ssRNA) (NCBI:txid1608071) in the
169 NCBI database. Our phylogenetic analysis groups Sbbv1 within the *Bunyavirales* order

170 branching sister to the *Hantaviridae,* which is consistent with previous studies (C. X. Li et al.,
171 2015) (**Figure 3**). We detected Sbbv1 sequences in *C. hemipterus* samples from Madagascar,
172 and *C. lectularius* samples from France and Czechia. Our results extend Sbbv1's geographical
173 range from China (where it was first detected) to Madagascar, Czechia, and France, and its host
174 range to *C. lectularius*. We detected both known segments of Sbbv1: a ~7000 nt segment (L)
175 containing the RdRp domain, and a ~3000 nt (M) segment encoding for the glycoprotein
176 precursor. The complete M segment was detected in Czechia sample 3, France, and all three
177 Madagascar samples, but it was only partially complete in Czechia Sample 2.

178 **3.3 Shuangao Bedbug Virus 2**

179 Sbbv2 is described as an unclassified -ssRNA virus (NCBI:txid1608072) with a 10,925 bp
180 monopartite genome. Our analyses show that it groups with the *Rhabdoviridae* family (**Figure**
181 **4**), consistent with the findings of Li *et al.* (2015). We detected Sbbv2 sequences in *C.*
182 *hemipterus* samples from Madagascar samples 1 and 3. The genomes we assembled of this
183 virus were around 3 kb longer than those from Li et al. (2015), ranging from 12,802-13,477 bp.
184 Our results complete the genome of Sbbv2, as the extra 3 kb contains ORFs at the beginning of
185 the genome: one encoding for a rhabdovirus nucleoprotein (CDD E-value = $1.77e^{-06}$), and the
186 other encoding for what we suspect is the rhabdovirus phosphoprotein (**Supplementary Figure**
187 **1**). Although the latter ORF did not have any significant BLAST hits or conserved domains, we
188 assume that it is the phosphoprotein based off synteny to other rhabdovirus genomes which
189 generally encode for five proteins in the following order: nucleoprotein, phosphoprotein,
190 matrix protein, glycoprotein, and large protein (Walker et al., 2022). Our study also extends the

191 geographic range of this virus, as it was previously only detected in bed bugs from China (Li et
192 al. 2015).

193 **3.4 *Tenuiviridae***

194 We detected sequences that had significant BLAST hits to viruses in the family
195 *Phenuiviridae* (-ssRNA) in *C. lectularius* samples from Czechia, France, Rome-Italy, and Assisi-
196 Italy, and *C. hemipterus* samples in Madagascar. These viruses are typically multisegmented,
197 and from our BLASTx-based analysis, we detected a ~9000 bp putative L segment. We found
198 complete L segments in Czechia sample 3, Madagascar sample 2, Rome-Italy sample 1, and
199 Assisi-Italy samples 1, 2, and 3. We found partial L segments in Czechia sample 2, France, and
200 Rome-Italy sample 3. Our phylogenetic analysis groups these sequences in the family
201 *Phenuiviridae,* sister to a clade including the tenuiviruses, which are a genus of hemipteran-
202 transmitted plant infecting viruses (**Figure 3**). We refer to this putative virus as *Cimex* tenui-like
203 virus 1.

204 Because bunyaviruses are typically multisegmented, we used an approach previously
205 described in Walt et al. (2023) to identify candidate genomic segments based off sample co-
206 occurrence. We found a single transcript that met our requirements with a V_{co}= 0.86 and a T_{co} =
207 0.75. The transcript is 2,851 nt long and encodes for a 902 amino acid protein. Based on
208 transcript length in comparison to *Cimex* tenui-like virus 1's closest BLAST hit (*Solenopsis invicta*
209 virus 14: M segment length=2,705 bp), we propose this may be the M segment, which encodes
210 a glycoprotein. InterProScan predicted two transmembrane domains in the amino acid
211 sequence of this transcript, which is similar to the glycoprotein of *Solenopsis invicta* virus 14 as
212 it also has two transmembrane domains. These results need further confirmation, as this

213 transcript had no significant BLAST alignments to other known sequences, no predicted
214 conserved domains in the translated protein, and no similar transcripts were found in the Italy
215 samples, which also had *Cimex* tenui-like virus 1 sequences in them.

216 **3.5 *Luteoviridae***

217 We detected a ~2800 bp luteo-like virus 1 sequence (+ssRNA) in the Harlan lab strain,
218 Czechia, and France samples, all of which were *C. lectularius*. We found complete transcripts in
219 Czechia samples 1,2, and 3, France, and Harlan Strain-USA samples 1 and 2, and found a partial
220 transcript in Harlan Strain-USA sample 3. Our phylogenetic analysis groups these viruses within
221 a clade of unclassified *Luteoviridae*, sharing a common ancestor with 4 viruses detected in
222 mosquitoes, and 1 virus detected in an anal swab from a bird (**Figure 5**). This clade branches
223 sister to a group of three viruses, *Miscanthus* yellow fleck virus, Rabbit luteovirus, and
224 *Arracacha* latent virus E. Rabbit luteovirus was discovered in a rabbit but was assumed to have
225 come from its diet (Tsoleridis et al., 2019), and the other two viruses are plant viruses that are
226 likely transmitted by aphids, another hemipteran insect (Bolus et al., 2020). We refer to this
227 putative virus as *Cimex* luteo-like virus 1.

228 **3.6 *Totiviridae***

229 We detected a toti-like virus (dsRNA) sequence in *C. lectularius* samples from five
230 locations: the Harlan lab strain-USA, Czechia, France, Assisi-Italy, and the UK. The complete
231 genome is around 7.8 kb and encodes for two large ORFs. One ORF (~3880 nt) has significant
232 BLASTx hits to other toti-like virus RdRps, and the other (~1860 nt) has significant BLAST hits to
233 other toti-like virus proline-alanine rich proteins (Spear et al., 2010). We recovered whole or
234 nearly complete genomes for the UK (all samples), Czechia (complete for samples 1 and 3,

235 partial for sample 2), France, and all three Assisi-Italy samples. Phylogenetically, this virus
236 shares a most recent common ancestor with an unclassified totivirus isolated from a flea. The
237 virus forms a clade with other Hemiptera and Thysanoptera associated toti-like viruses along
238 with two viruses isolated from plants (**Figure 6**). We hereon refer to this putative virus as *Cimex*
239 toti-like virus 1.

240 **3.7 Phylogeography of Bed Bug Viruses**

241 All the virus sequences that we detected were distributed across geographically distant
242 locations, so we investigated viral diversity between localities. We hypothesized that viral
243 evolutionary relationships would reflect the differences in host species, and that within a host
244 species, phylogenetic relationships would be reflective of geographic distance (Ballinger et al.,
245 2022; Longdon et al., 2014). Specifically, we would expect *C. lectularius* and *C. hemipterus* to
246 harbor closely related but distinct viral populations, and within *C. lectularius*, we would expect
247 samples from Europe to be distinct from those rom North America. We only used sequences
248 with complete RdRp domains and only included the unique sequences within individuals for our
249 phylogeographic analysis.

250 **3.7.1 *Cimex* tenui-like virus 1**

251 We found *Cimex* tenui-like virus 1 sequences in both *C. lectularius*, and *C. hemipterus*. In
252 the case of *C. lectularius*, sequences from this virus were found in one individual from Rome-
253 Italy, all three individuals from Assisi-Italy, one individual from Madagascar, the individual from
254 France, and one individual from Czechia. Interestingly, the Italian samples form a distinct clade
255 from the rest of the world (**Figure 7A**). When we grouped the samples by clade: Italy, world,
256 and outgroup and assessed evolutionary distance between the groups, the mean p-distance

257 between the Italian clade and the rest of the world clade was 22.4%, while the "within group"
258 mean p-distance was 3.0% and 1.1% for the Italian clade and the rest of the world, respectively
259 (**Table 1**). Interestingly, the "within group" distance is three times higher in the samples that
260 come from the same country (Italy) than those collected in different countries (France, Chechia,
261 and Madagascar).

262 Because the samples collected in Italy had a higher "within group" mean than the
263 samples from the rest of the world, we computed pairwise comparisons of p-distance for the
264 samples used in the phylogeny in **Figure 7A** (**Table 2**)**.** We found that one sequence of *Cimex*
265 tenui-like virus 1 (Rome-Italy 1) is the driver of the viral genetic diversity within the group of
266 samples collected in Italy, since the Assisi-Italy samples are identical, but the Rome-Italy 1
267 sample is 7.5% different from all the Assisi-Italy samples at the nucleotide level (**Table 2**).
268 Furthermore, the France and Czechia 3 *Cimex* tenui-like virus 1 sequences are nearly identical,
269 while the Madagascar 2 *Cimex* tenui-like virus 1 sequence is only 1.7% different from both the
270 France and the Czechia samples (**Table 2**), even though the Madagascar samples are *C.*
271 *hemipterus*, while the rest of the samples are *C. lectularius*.

272 **3.7.2 *Cimex* toti-like virus 1**

273 We only found *Cimex* toti-like virus 1 sequences in *C. lectularius* samples. We found
274 them in two of the three Czechia individuals, the France individual, all Assisi-Italy individuals,
275 and all UK individuals. Once again, the samples collected from Italy form a distinct clade from
276 the rest of the world (**Figure 7B**). We grouped the samples by the Italy clade, the rest of the
277 world clade and the outgroup and found that there was little difference between the Italy
278 viruses ("within group" mean p-distance = 0.3%), while rest of the world clade were more

279 genetically distant ("within group" mean p-distance = 10%) (**Table 1**). The "between group"
280 mean distance between the virus sequences detected in Italy and the virus sequences detected
281 from the rest of the world is 16.4%. Within the rest of the world clade, the viruses from Czechia
282 form one group and the France and UK viruses form another group, following expected
283 geographic patterns.

284 **3.7.3 *Cimex* luteo-like virus 1**

285 We only found *Cimex* luteo-like virus 1 sequences in *C. lectularius*, and we detected
286 them in all three Czechia samples, two of the Harlan Strain-USA samples, and the France
287 individual. The tree has low resolution other than one branch containing the viral sequences
288 detected in the Harlan Strain-USA bugs (**Figure 7C**). All the *Cimex* luteo-like virus 1 RdRp-
289 encoding transcripts have a mean p-distance of 4%.

290 **3.7.4 Shuangao Bedbug Virus 1**

291 We detected Sbbv1 sequences in all three *C. hemipterus* individuals collected from
292 Madagascar, and one *C. lectularius* individual from Czechia. The Madagascar samples and the
293 previously described Sbbv1 sample (detected in China) group together with 100% support
294 (**Figure 7D**). This reflects host taxonomy, as the Sbbv1 sample that was first detected in China
295 was also detected in *C. hemipterus*. Because the bed bugs collected in Czechia were *C.*
296 *lectularius,* our study expands the host range of Sbbv1 to *C. lectularius*. Overall, these viruses
297 are very similar to each other with an average p-distance of 2% in the RdRp-encoding
298 transcripts.

299 **3.8 Influence of *Wolbachia* on Viral Abundance**

300 Many hypotheses have been proposed to understand why bed bugs have never been
301 linked to pathogen transmission, including one involving their *Wolbachia* endosymbiont as a
302 potential factor (Pietri, 2020). *Wolbachia* has formed a nutritional symbiosis with bed bugs, as it
303 provides them with B-vitamins (Hosokawa et al., 2010). Other studies have shown that
304 Wolbachia colonization can confer resistance to viral infection in other insects such as
305 *Drosophila*, mosquitoes, and even hemipteran insects (Cogni et al., 2021; Gong et al., 2020;
306 Lindsey et al., 2018; Teixeira et al., 2008). A study investigating the influence of bed bug
307 *Wolbachia* on feline calicivirus titer has been conducted, but there was no evidence that the
308 virus ever replicated inside of the bed bugs (Fisher et al., 2019). To investigate the effects that
309 *Wolbachia* may have on viral abundance, we mapped all reads to the viral genomes detected in
310 this study and the *Wolbachia* endosymbiont of *Cimex lectularius* genome. **Table 3** shows the
311 percentage of reads that mapped to the virus and *Wolbachia* genomes from each sample. We
312 used these values to investigate the influence of Wolbachia reads on viral reads and we found
313 no correlation ($y = 0.41 - 0.042x$, $R = -0.14$, $p = 0.53$) between percent *Wolbachia* reads and
314 percent virus reads in a sample (**Figure 8**).

315 **4. Discussion:**

316 **4.1 Detection and Phylogenetics of Bed Bug viruses**

317 Bed bugs are a worldwide urban pest, that have undergone population resurgence for
318 the last 20-30 years. Although their capacity to transmit human disease remains unknown,
319 interest in their vector competence is high because of the increasing frequency in outbreaks
320 (Doggett & Lee, 2023). We did not detect any known human viruses, but our study supports
321 that metatranscriptomic surveillance is a useful technique to detect what known or emerging
322 pathogens bed bugs could potentially transmit. We detected two previously known virus
323 sequences associated with *C. hemipterus*, and 3 novel putative bed bug viruses.

324 The two previously detected bed bug viruses, Sbbv1 and Sbbv2 had been found in China
325 associated with the tropical bed bug, *C. hemipterus* (C. X. Li et al., 2015). Our phylogeny groups
326 Sbbv1 with an insect virus sister to the *Hantaviridae* (**Figure 3**). This agrees with the findings of
327 Käfer et al. (2019) and supports the hypothesis that the *Hantaviridae* may have originated from
328 arthropod viruses, and subsequently shifted to infecting vertebrate hosts (Marklewitz et al.,
329 2015). Although Sbbv1 shares a common ancestor with the hantaviruses, it is unknown whether
330 it is of concern to humans. We extend Sbbv1's geographical range from China to Czechia,
331 Madagascar, and France, and extend its host range to *C. lectularius*, as it had previously only
332 been detected in the tropical bed bug, *C. hemipterus* (C. X. Li et al., 2015).

333 Sbbv2 provides an interesting insight to plant-infecting rhabdovirus evolution, as many
334 economically important plant rhabdoviruses are transmitted by hemipteran insects (Whitfield
335 et al., 2018). Our phylogeny agrees with Longdon et al. (2015), as Sbbv2 groups with insect-
336 specific clade of rhabdoviruses that shares a common ancestor with the cytorhabdoviruses and

337 the nucleorhabdoviruses (**Figure 4**). This supports the hypothesis that these viruses infected
338 hemipteran insects before they infected plants (Longdon et al., 2015). It is interesting to note
339 that Sbbv2 has persisted in the bed bug lineage despite shifts in feeding strategy from plants to
340 other insects, to obligate blood feeders (Johnson et al., 2018). Our bunyavirus phylogeny
341 (**Figure 3**) also suggests that the tenuiviruses, which are important insect-transmitted plant
342 viruses, infected their insect hosts before evolving the ability to infect plants. This is indicated
343 by *Cimex* tenui-like virus 1 branching sister to the tenuiviruses, and another insect virus, Wuhan
344 horsefly virus, branching with this group (**Figure 3**).

345 Totiviruses are dsRNA viruses that typically infect fungi, but there is a growing number
346 of toti-like viruses detected in arthropod and vertebrate metatranscriptomic studies (Tighe et
347 al., 2022). According to our phylogenetic analysis, *Cimex* toti-like virus 1 falls within a clade of
348 arthropod and plant infecting viruses, along with a toti-like virus 1 detected in an anal swab
349 from a bird (GenBank: QKN88741.1) (**Figure 6**). Interestingly *Cimex* toti-like virus 1 shares a
350 most recent common ancestor with a virus detected in fleas, which are also obligate blood
351 feeders (Harvey et al., 2019). This supports the hypothesis that similarities in ecological niche
352 could be more correlative of viral similarity than taxonomic relatedness (C. X. Li et al., 2015).
353 Most other viruses in this clade are Hemiptera or Thysanoptera-associated (a sister group to
354 the Hemiptera) viruses.

355 **4.2 Phylogeography of bed bug viruses**

356 Although our study design limited an extensive phylogeographic analysis, we found
357 unprecedented patterns of viral diversity. First, we found that bedbug viruses detected in this
358 study are not geographically restricted and can infect more than one host species. We detected

359 sequences from four of the five viruses in this study intercontinentally and sequences from two
360 out of five viruses were found in both *C. lectularius* and *C. hemipterus*. (**Figure 7**). Second, if we
361 found viruses that were present in both bed bug species, we expected these viruses to form
362 distinct clades reflecting bed bug taxonomy, as differences in host receptors between species
363 would add selective pressures to viral infection (Longdon et al., 2014). Our results did not
364 match that expectation, as the *Cimex* tenui-like virus 1 detected in the *C. hemipterus*
365 (Madagascar) samples grouped with the *Cimex* tenui-like virus 1 sequences from *C. lectularius*
366 (France and Czechia), while other *Cimex* tenui-like virus 1 sequences detected in *C. lectularius*
367 formed their own distinct clade (Rome-Italy and Assisi-Italy) (**Figure 7A**). Third, we expected
368 that viruses from similar geographic location would form distinct phylogenetic groups. This
369 trend was generally followed, but strikingly, in every case where viral sequences were detected
370 in samples from Italy, the sequences form their own distinct clades separated from the rest of
371 Europe (**Figure 7A&B**). Furthermore, even though the *Cimex* tenui-like virus 1 sequences from
372 Italy group together phylogenetically, there is higher mean evolutionary distance within these
373 samples than within the *Cimex* tenui-like virus 1 sequences from the rest of the world, which
374 include samples from France, Czechia and Madagascar, and include two different host species
375 (**Tables 1 & 2**). Previous studies of bed bug phylogeography have found low genetic diversity
376 within bed bug infestation sites, but high genetic diversity between infestation sites even of
377 relatively close proximity, which could be due to their dependence on humans for dispersal
378 (Fountain et al., 2014; Saenz et al., 2012). Bed bug dispersal by humans could also explain the
379 unexpected patterns of bed bug virus phylogeography, as the distinct groups of bed bug viruses
380 could be explained by Italy's popularity as a travel destination, with Rome being a hotspot for

381 tourism, and Assisi being a frequent site of pilgrimage. Along with this, the similarity between
382 viruses detected in Madagascar and Europe could reflect travel between these two places.

383 **4.3 *Wolbachia* influence on viral abundance**

384 As an additional exploration of our dataset, we investigated if the amount of *Wolbachia*
385 reads was correlated with viral abundance. It has been hypothesized that *Wolbachia* could have
386 a protective effect against viral infection in bed bugs, similar to what has been seen in
387 mosquitoes and *Drosophila* (Cogni et al., 2021; Fisher et al., 2019; Hussain et al., 2023; Lindsey
388 et al., 2018; Teixeira et al., 2008; Terradas & McGraw, 2017). We used percentage of reads
389 mapped to *Wolbachia* and percent mapped to the viral genomes detected in this study as a
390 proxy of abundance. We found that there was no correlation between *Wolbachia* and virus
391 abundance when a potential outlier sample was present (**Figure 8**). These results indicate that
392 unlike Diptera-associated *Wolbachia,* bed bug *Wolbachia* may not confer viral resistance.
393 Although our experimental design was not ideal to test *Wolbachia's* influence on bed bug virus
394 fitness, these results provide a preliminary look into how *Wolbachia* may affect viruses that
395 replicate inside of bed bugs.

396 **5. Conclusions**

397 Our study opens interesting questions about the bed bug virosphere but does not
398 provide evidence that bed bugs transmit human viruses. On the contrary, humans may drive
399 bed bug virus diversity by facilitating dispersal and local extinction of host populations
400 (Fountain et al., 2014). Future studies should assess the pathogenicity and transmission routes
401 of these viruses to have a more comprehensive understanding of their potential in biocontrol or

402 as emerging diseases. Along with this, a more comprehensive sampling strategy and

403 phylogeographic analysis could shed light on the interesting patterns of bed bug virus dispersal.

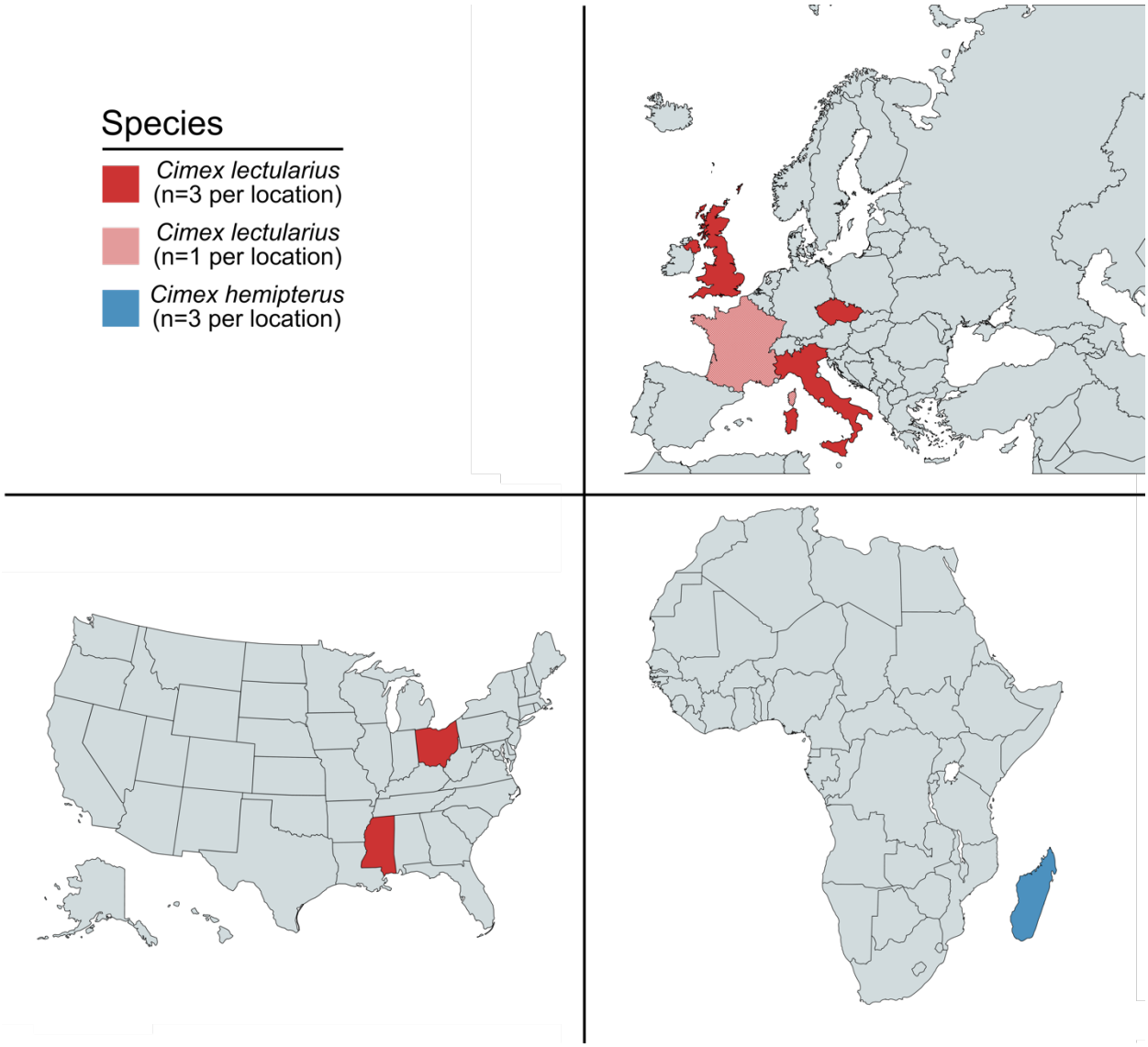


Figure 1: Locations of sample collection sites. Samples were collected at nine distinct sites around the world: France (*Cimex lectularius,* n=1), the UK (*Cimex lectularius,* n=3), Czechia (CR) (*Cimex lectularius,* n=3), Rome-Italy (*Cimex lectularius,* n=3), Assisi-Italy (*Cimex lectularius,* n=3), Madagascar (*Cimex hemipterus,* n=3), Ohio-USA (*Cimex lectularius*, n=3), and the King lab at Mississippi State University-USA (Harlan Strain-USA *Cimex lectularius,* n=3).

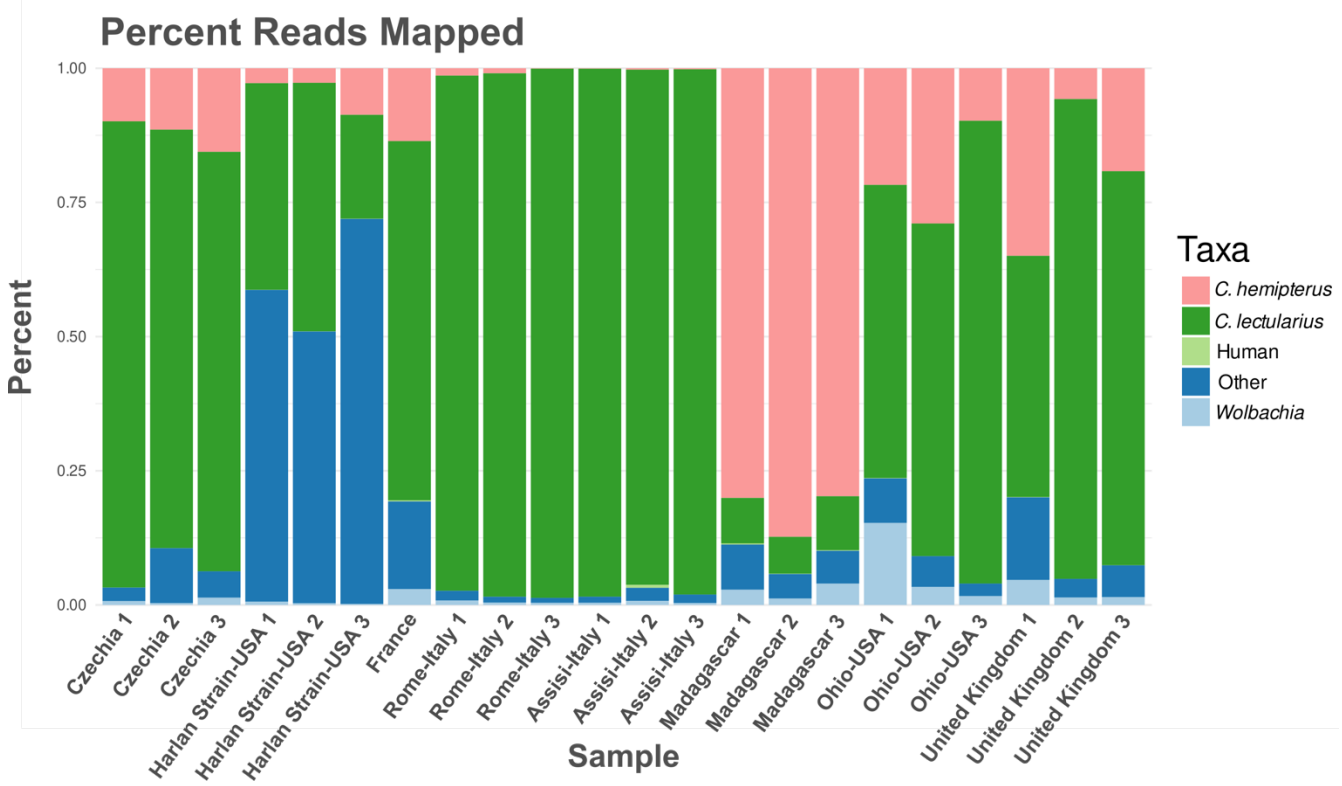


Figure 2: Percentage of reads mapped to the bed bug genomes, human genome, and *Wolbachia* genome per sample.

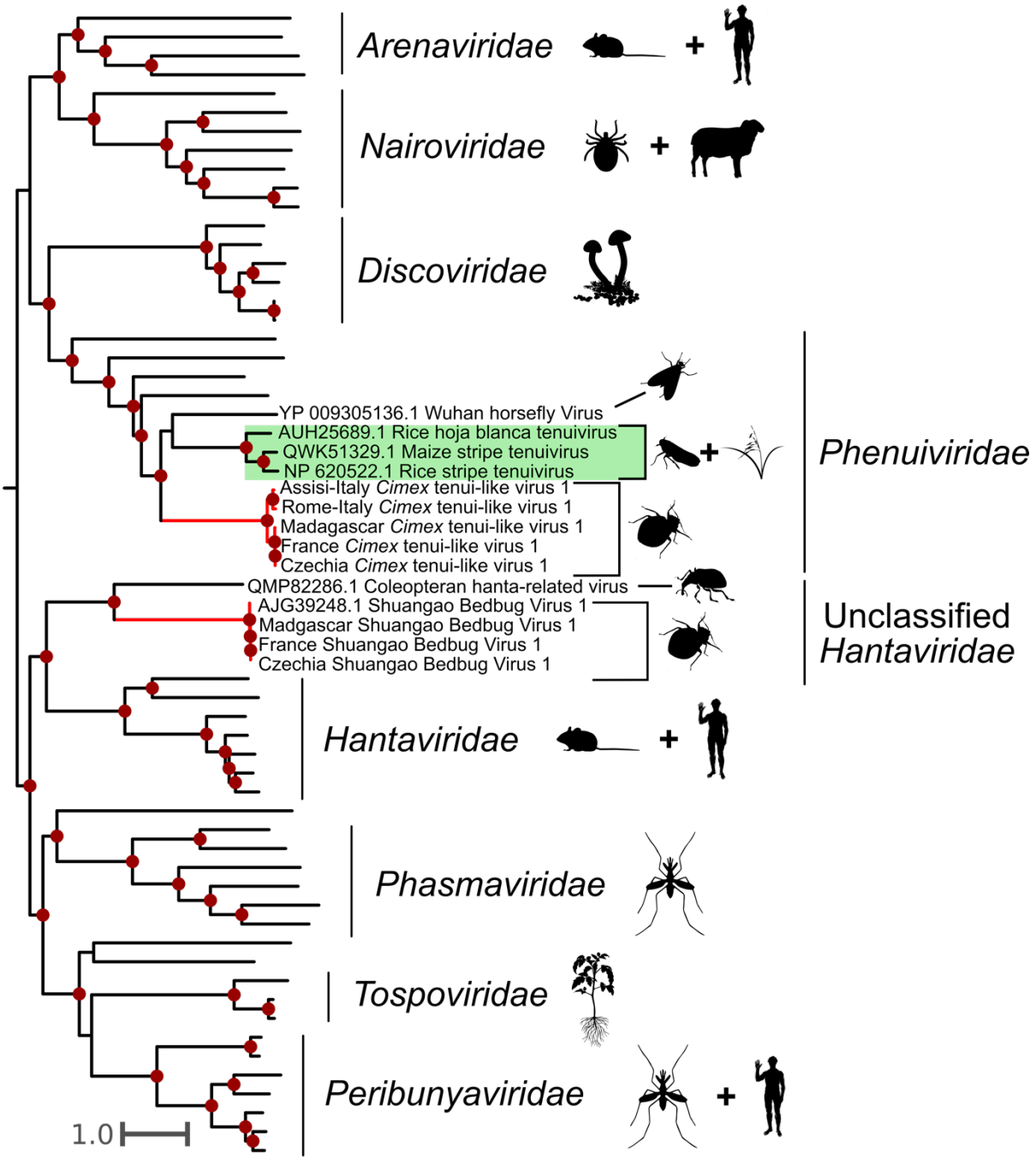


Figure 3: Phylogeny of the *Bunyavirales* including viruses detected in this study. Viruses found in this study are labeled by their location, and their branches are colored red. Dark red dots indicate bootstrap support greater than 75. Silhouette images represent the general host taxa of a virus or virus clade. The tree supports that we detected Shuangao bedbug virus 1 in

421 multiple samples, and that it branches sister to the *Hantaviridae* (a clade of viruses that infects
422 vertebrates) along with another insect-associated virus. *Cimex* tenui-like virus 1 groups within
423 the family *Phenuivirudae*. Notably, *Cimex* tenui-like virus 1 branches sister to a clade including
424 the *Tenuiviridae* genus (highlighted in green), which is comprised of important hemipteran-
425 transmitted plant viruses.

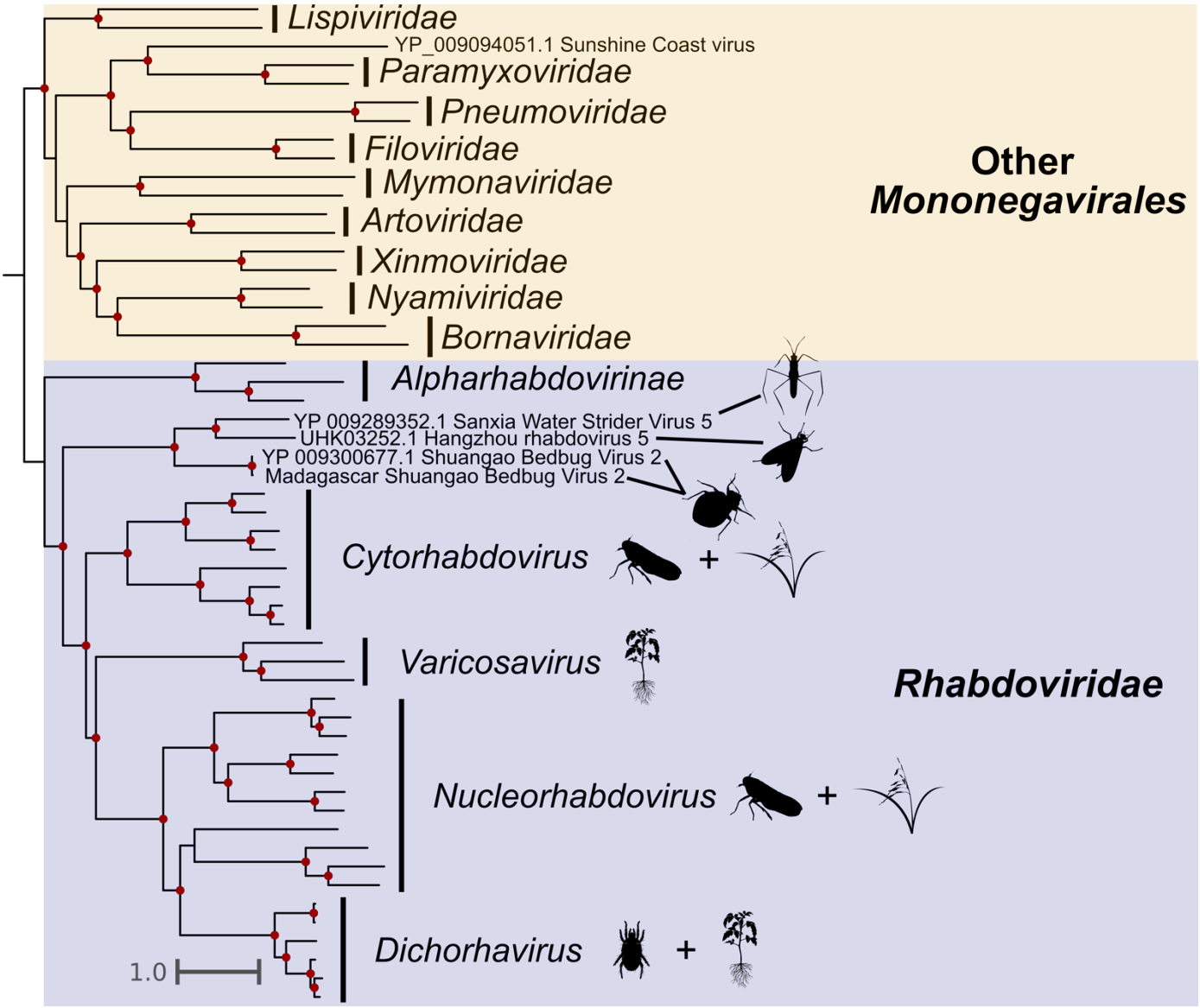


Figure 4: Phylogeny of the Mononegavirales with expansive *Rhabdoviridae* representation. Red dots indicate bootstrap support greater than 75. Sbbv2 is shown by a red branch. Non-rhabdovirus mononegaviruses are shown in yellow, while rhabdoviruses are shown in blue. Silhouette images represent the general host taxa of a virus or virus clade. Our phylogeny supports that Sbbv2 branches with other insect-associated viruses before the cytorhabdoviruses, nucleorhabdoviruses, and other plant infecting *Rhabdoviridae*.

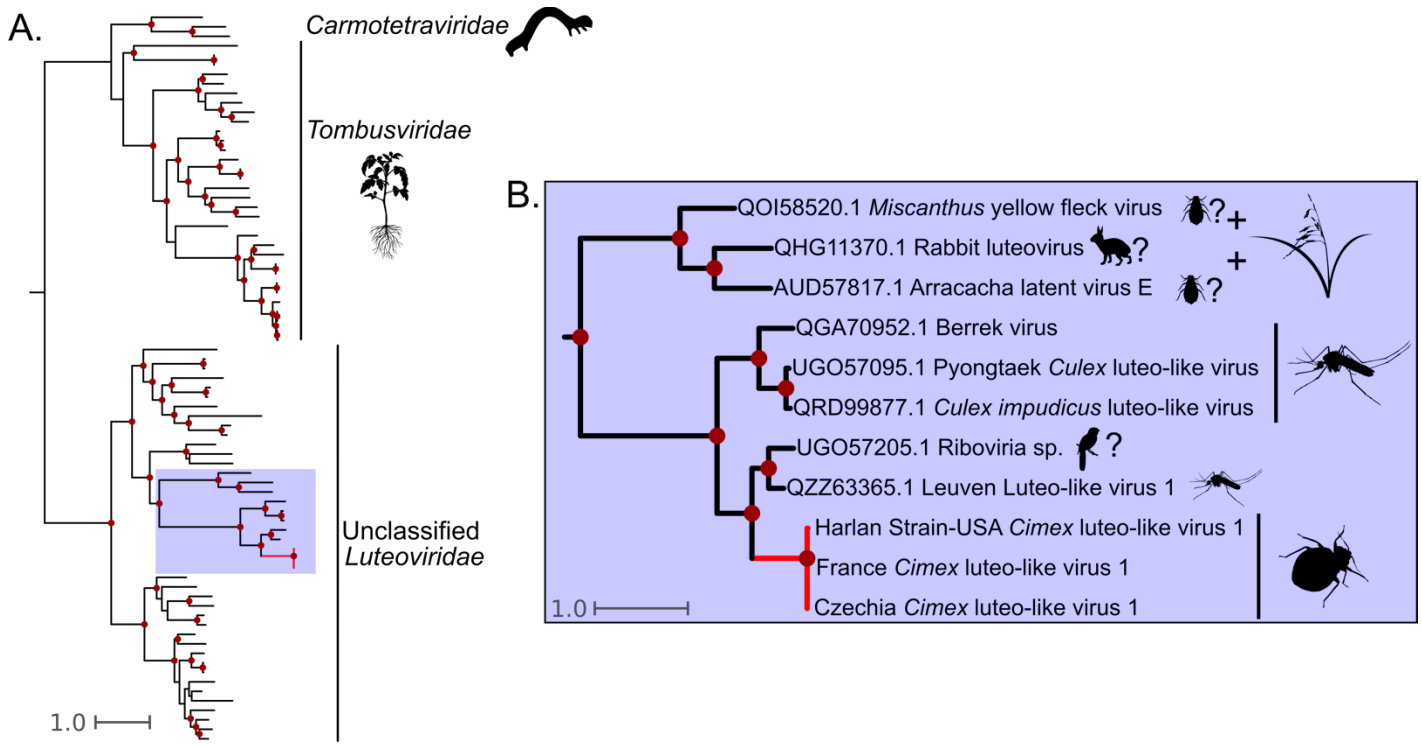


Figure 5: Phylogeny of the *Tolivirales*, including the ssRNA(+) virus discovered in this study. **A.)** Full phylogeny of the *Tolivirales*. The red branch indicates the *Cimex* luteo-like virus 1. Red dots along the tree show bootstrap support greater than or equal to 75. **B.)** Zoomed in view of the clade that *Cimex* luteo-like virus 1 groups in. Each virus is labeled by the respective location in which it was found. Dark red dots indicate bootstrap support greater than 75. Silhouette images represent the general host taxa of a virus or virus clade. *Cimex* luteo-like virus 1 groups with a clade of mosquito-associated viruses, sister to a clade of plant viruses that are putatively transmitted by aphids.

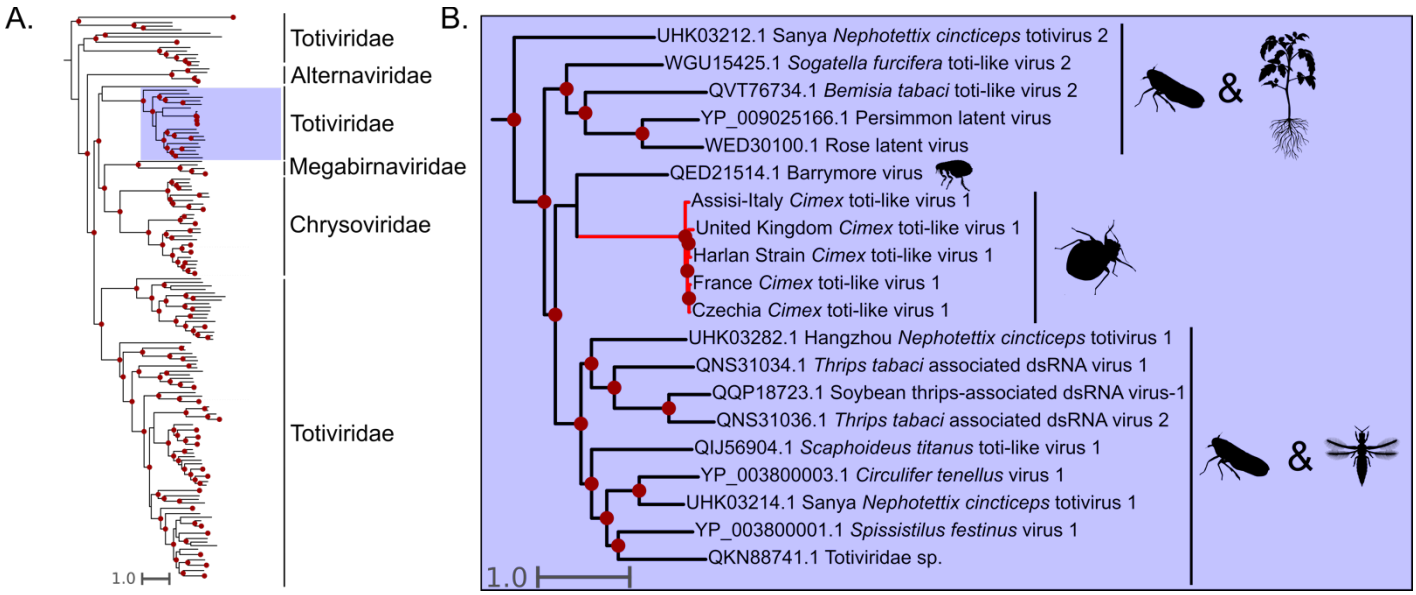


Figure 6: Phylogeny of the *Ghrabrivirales*, including the dsRNA virus discovered in this study. **A.)** Phylogeny of the *Ghrabrivirales* including diverse taxa from all families. *Cimex* toti-like virus 1 is shown on red branches and the clade it groups with is highlighted in red. Dots indicate a bootstrap value of 75 or above. **B.)** Zoomed-in view of the clade that *Cimex* toti-like virus 1 groups in. Silhouette images represent the general host taxa of a virus or virus clade.

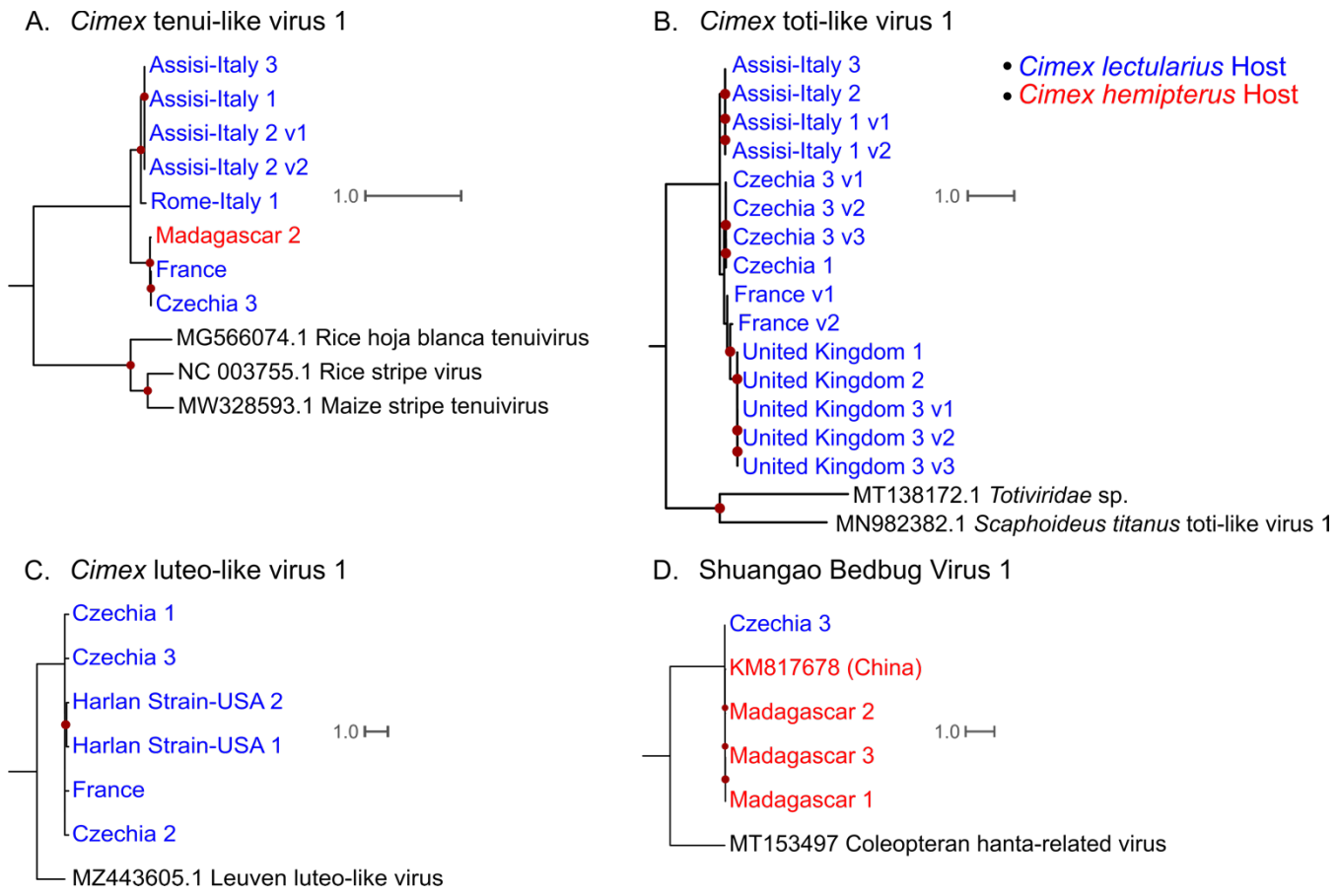
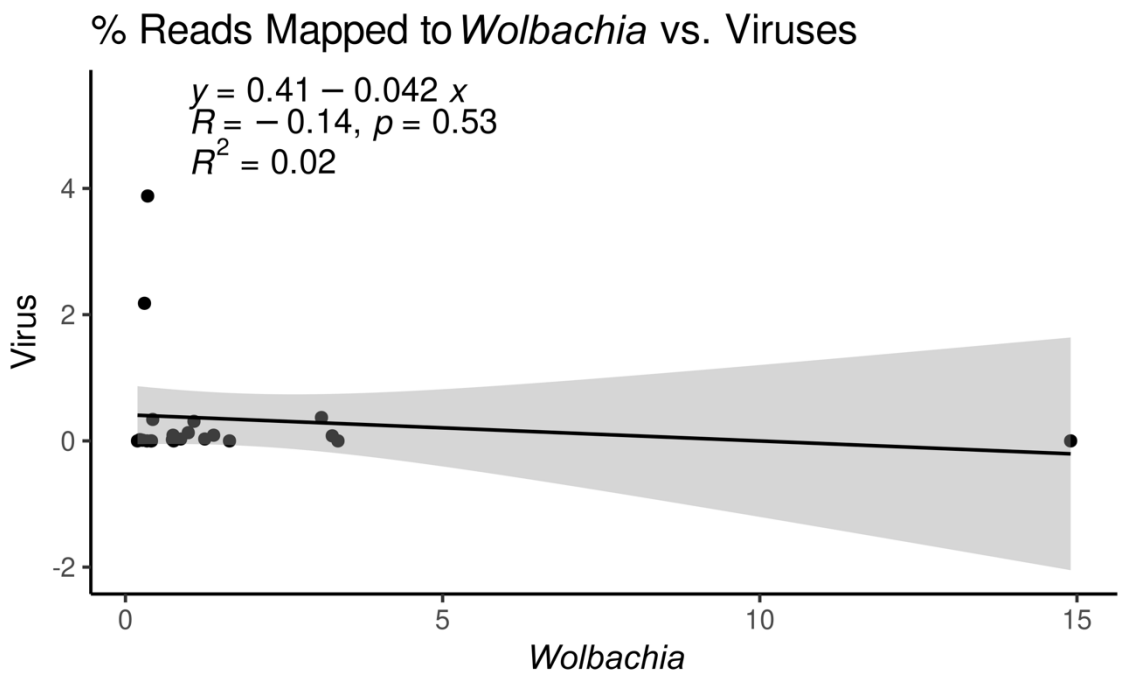


Figure 7: Phlyogeographic analysis of the putative bed bug viruses detected in this study. Only the coding sequences where an RdRp domain was detected were used. All trees were rooted using the closest phylogenetic neighbors from phylogenetic analyses in **Figures 4-7.** Dark red dots indicate bootstrap support greater than 75. Samples from *C. lectularius* hosts are indicated in blue text, and samples from *C. hemipterus* hosts are indicated in red text. **A.)** Phlyogeographic analysis of *Cimex* tenui-like virus 1. **B.)** Phylogeographic analysis of *Cimex* toti-like virus 1. **C.)** Phylogeographic analysis of *Cimex* luteo-like virus 1. **D.)** Phylogeographic analysis of Sbbv1.



458

459 **Figure 8: Correlation of the percentage of reads mapped to *Wolbachia* endosymbiont of**

460 ***Cimex lectularius* and number of reads mapped to the viruses detected in this study.** There is

461 no correlation between percent of Wolbachia reads present in a sample and percent of RNA

462 virus reads present in a sample.

463 **Table 1: Mean evolutionary distance between distinct clades of viruses from phylogeographic**

464 **analysis.** Only *Cimex* tenui-like virus 1 and *Cimex* toti-like virus 1 are shown as they had highly

465 supported trees containing many viral genomes. Distinct phylogenetic clades were formed from

466 the Italy samples, the rest of the world, and the outgroups.

467

Cimex tenui-like virus 1	P-distance		
	Italy	Rest of the World	Outgroup
Italy	3.0%		
Rest of the World	22.4%	1.1%	
Outgroup	55.8%	55.9%	36.5%
Cimex toti-like virus 1			
Italy	0.3%		
Rest of the World	16.4%	10.0%	
Outgroup	51.5%	51.8%	50.8%

468

469 **Table 2: Pairwise p-distances of *Cimex* tenui-like virus 1 RDRP coding sequences.** The matrix includes the outgroup sequences used

470 in Figure 7A**.** RHBTV = Rice hoja blanca tenuivirus, RSV = Rice Stripe Virus, MSTV = Maize Stripe Tenuivirus.

471

	Rome-Italy 1	Assisi-Italy 1	Assisi-Italy 2 (1)	Assisi-Italy 2 (2)	Assisi-Italy 3	Czechia 3	France	Madagascar 2	RHBTV	RSV	MSTV
Rome-Italy 1	-										
Assisi-Italy 1	7.5%	-									
Assisi-Italy 2 (1)	7.5%	0.0%	-								
Assisi-Italy 2 (2)	7.5%	0.0%	0.0%	-							
Assisi-Italy 3	7.5%	0.0%	0.0%	0.0%	-						
Czechia 3	23.1%	20.6%	22.6%	21.3%	22.6%	-					
France	23.1%	22.6%	22.6%	20.6%	22.6%	0.0%	-				
Madagascar 2	23.0%	22.6%	22.6%	21.3%	22.6%	1.7%	1.7%	-			
RHBTV	56.3%	56.3%	56.3%	54.9%	56.2%	56.2%	55.3%	56.0%	-		
RSV	56.7%	56.5%	56.5%	54.9%	56.5%	56.7%	56.1%	56.8%	39.5%	-	
MSTV	55.1%	55.4%	55.4%	54.4%	55.4%	55.4%	54.8%	55.4%	39.3%	30.7%	-

472

473 **Table 3: Percent reads mapped to the viruses detected in this study, or *Wolbachia***

474 **endosymbiont of *Cimex lectularius*.**

Sample	% Reads Mapped to Viruses	% Reads mapped to *Wolbachia*
Czechia 1	0.02	0.74
Czechia 2	3.88	0.35
Czechia 3	0.09	1.39
Harlan Strain-USA 1	0.00	0.40
Harlan Strain-USA 2	2.18	0.30
Harlan Strain-USA 3	0.00	0.19
France	0.34	0.43
Rome-Italy 1	0.01	0.76
Rome-Italy 2	0.00	0.41
Rome-Italy 3	0.00	0.34
Assisi-Italy 1	0.01	0.27
Assisi-Italy 2	0.09	0.75
Assisi-Italy 3	0.02	0.23
Madagascar 1	0.03	1.25
Madagascar 2	0.03	0.87
Madagascar 3	0.08	3.26
Ohio-USA 1	0.00	14.90
Ohio-USA 2	0.00	3.35
Ohio-USA 3	0.00	1.64
United Kingdom 1	0.37	3.09
United Kingdom 2	0.31	1.08
United Kingdom 3	0.13	0.99

475

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DATA AVAILABILITY:

All newly reported virus genome sequences used in this study will be made available in GenBank, and all reads generated in this study will be deposited to NCBI's Sequence Read Archive (SRA) database.

SUPPLEMENTARY DATA:

Supplementary data will be available online.

CONFLICT OF INTEREST:

The authors declare no conflict of interest

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