The evolution, diversity and host associations of rhabdoviruses

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Abstract

Metagenomic studies are leading to the discovery of a hidden diversity of RNA viruses. These new viruses are poorly characterised and new approaches are needed predict the host species these viruses pose a risk to. The rhabdoviruses are a diverse family of RNA viruses that includes important pathogens of humans, animals and plants. We have discovered 32 new rhabdoviruses through a combination of our own RNA sequencing of insects and searching public sequence databases. Combining these with previously known sequences we reconstructed the phylogeny of 195 rhabdovirus sequences, and produced the most in depth analysis of the family to date. In most cases we know nothing about the biology of the viruses beyond the host they were identified from, but our dataset provides a powerful phylogenetic approach to predict which are vector-borne viruses and which are specific to vertebrates or arthropods. By reconstructing ancestral and present host states we found that switches between major groups of hosts have occurred rarely during rhabdovirus evolution. This allowed us to propose 76 new likely vector-borne vertebrate viruses among viruses identified from vertebrates or biting insects. Based on currently available data, our analysis suggests it is likely there was a single origin of the known plant viruses and arthropod-borne vertebrate viruses, while vertebrate-specific and arthropod-specific viruses arose at least twice. There are also few transitions between aquatic and terrestrial ecosystems. Viruses also cluster together at a finer scale, with closely related viruses tending to be found in closely related hosts. Our data therefore suggest that throughout their evolution, rhabdoviruses have occasionally jumped between distantly related host species before spreading through related hosts in the same environment. This approach offers a way to predict the most probable biology and key traits of newly discovered viruses.

Keywords

Virus, Host shift, Arthropod, Insect, Rhabdoviridae, Mononegavirales

63 **Introduction**
64
65 RNA viruses are an abundant and diverse group of pathogens. In the past, viruses were
66 typically isolated from hosts displaying symptoms of infection, before being
67 characterized morphologically and then sequenced following PCR [1, 2]. PCR-based
68 detection of novel RNA viruses is problematic as there is no single conserved region of
69 the genome shared by all viruses from a single family, let alone across all RNA viruses.
70 High throughput next generation sequencing technology has revolutionized virus
71 discovery, allowing rapid detection and sequencing of divergent virus sequences simply
72 by sequencing total RNA from infected individuals [1, 2]
73
74 One particularly diverse family of RNA viruses is the *Rhabdoviridae*. Rhabdoviruses are
75 negative-sense single-stranded RNA viruses in the order *Mononegavirales* [3]. They
76 infect an extremely broad range of hosts and have been discovered in plants, fish,
77 mammals, reptiles and a broad range of insects and other arthropods [4]. The family
78 includes important pathogens of humans and livestock. Perhaps the most well-known is
79 rabies virus, which can infect a diverse array of mammals and causes a fatal infection
80 killing 59,000 people per year with an estimated economic cost of $8.6 billion (US) [5].
81 Other rhabdoviruses, such as vesicular stomatitis virus and bovine ephemeral fever
82 virus, are important pathogens of domesticated animals, while others are pathogens of
83 crops [3].
84
85 Arthropods play a key role in the transmission of many rhabdoviruses. Many viruses
86 found in vertebrates have also been detected in arthropods, including sandflies,
87 mosquitoes, ticks and midges [6]. The rhabdoviruses that infect plants are also often
88 transmitted by arthropods [7] and some that infect fish can potentially be vectored by
89 ectoparasitic copepod sea-lice [8, 9]. Moreover, insects are biological vectors;
90 rhabdoviruses replicate upon infection of insect vectors [7]. Other rhabdoviruses are
91 insect-specific. In particular, the sigma viruses are a clade of vertically transmitted
92 viruses that infect dipterans and are well-studied in *Drosophila* [10-12]. Recently, a
93 number of rhabdoviruses have been found to be associated with a wide array of insect
94 and other arthropod species, suggesting they may be common arthropod viruses [13,
95 14]. Furthermore, a number of arthropod genomes contain integrated endogenous viral
96 elements (EVEs) with similarity to rhabdoviruses, suggesting that these species have
97 been infected with rhabdoviruses at some point in their history [15-18].
98
99 Here we explore the diversity of the rhabdoviruses, and examine how they have
100 switched between different host taxa during their evolutionary history. Insects infected
101 with rhabdoviruses commonly become paralysed on exposure to CO_2 [19-21]. We
102 exploited this fact to screen field collections of flies from several continents for novel
103 rhabdoviruses that were then sequenced using metagenomic RNA-sequencing (RNA-
104 seq). Additionally we searched for rhabdovirus-like sequences in publicly available
105 RNA-seq data. We identified 32 novel rhabdovirus-like sequences from a wide array of
106 invertebrates and plants, and combined them with recently discovered viruses to
107 produce the most comprehensive phylogeny of the rhabdoviruses to date. For many of
108 the viruses we do not know their true host range, so we used the phylogeny to identify a

109 large number of new likely vector-borne viruses and to reconstruct the evolutionary
110 history of this diverse group of viruses.
111
112
113 **Methods**
114
115 *Discovery of new rhabdoviruses by RNA sequencing*
116
117 Diptera (flies, mostly Drosophilidae) were collected in the field from Spain, USA, Kenya,
118 France, Ghana and the UK (Data S1: http://dx.doi.org/10.6084/m9.figshare.1425432).
119 Infection with rhabdoviruses can cause *Drosophila* and other insects to become
120 paralysed after exposure to CO_2 [19-21], so we enriched our sample for infected
121 individuals by exposing them to CO_2 at 12°C for 15 mins, only retaining individuals that
122 showed symptoms of paralysis 30mins later. We extracted RNA from 79 individual
123 insects (details in Data S1 http://dx.doi.org/10.6084/m9.figshare.1425432) using
124 Trizol reagent (Invitrogen) and combined the extracts into two pools (retaining non-
125 pooled individual RNA samples). RNA was then rRNA depleted with the Ribo-Zero Gold
126 kit (epicenter, USA) and used to construct Truseq total RNA libraries (Illumina).
127 Libraries were constructed and sequenced by BGI (Hong Kong) on an Illumina Hi-Seq
128 2500 (one lane, 100bp paired end reads, generating ~175 million reads). Sequences
129 were quality trimmed with Trimmomatic (v3); Illumina adapters were clipped, bases
130 were removed from the beginning and end of reads if quality dropped below a
131 threshold, sequences were trimmed if the average quality within a window fell below a
132 threshold and reads less than 20 base pairs in length were removed. We *de novo*
133 assembled the RNA-seq reads with Trinity (release 2013-02-25) using default settings
134 and jaccard clip option for high gene density. The assembly was then searched using
135 tblastn to identify rhabdovirus-like sequences, with known rhabdovirus coding
136 sequences as the query. Any contigs with high sequence similarity to rhabdoviruses
137 were then reciprocally compared to Genbank cDNA and RefSeq nucleotide databases
138 using tblastn and only retained if they most closely matched a virus-like sequence. Raw
139 read data were deposited in the NCBI Sequence Read Archive (SRP057824). Putative
140 viral sequences have been submitted to Genbank (accession numbers in Tables S1 and
141 S2).
142
143 As the RNA-seq was performed on pooled samples, we assigned rhabdovirus sequences
144 to individual insects by PCR on RNA from individual samples. cDNA was produced using
145 Promega GoScript Reverse Transcriptase and random-hexamer primers, and PCR
146 performed using primers designed from the rhabdovirus sequences. Infected host
147 species were identified by sequencing the mitochondrial gene *COI*. We were unable to
148 identify the host species of the virus from a *Drosophila affinis* sub-group species
149 (sequences appear similar to both *Drosophila affinis* and the closely related Drosophila
150 *athabasca*), despite the addition of further mitochondrial and nuclear sequences to
151 increase confidence. In all cases we confirmed that viruses were only present in cDNA
152 and not in non reverse-transcription (RT) controls (i.e. DNA) by PCR, and so they cannot
153 be integrated into the insect genome (i.e. endogenous virus elements or EVEs [17]). *COI*
154 primers were used as a positive control for the presence of DNA in the non RT template.
155

156 We identified sigma virus sequences in RNA-seq data from *Drosophila montana* [22]. We
157 used RT-PCR on an infected fly line to amplify the virus sequence, and carried out
158 additional Sanger sequencing with primers designed using the RNA-seq assembly.
159 Additional virus sequences were identified from an RNA-seq analysis of pools of wild
160 caught *Drosophila*: DImmSV from *Drosophila immigrans* (collection and sequencing
161 described [23]), DTriSV from a pool of *Drosophila tristis* and SDefSV from
162 *Scaptodrosophila deflexa* (both Darren Obbard, unpublished data). Genbank accession
163 numbers for new virus sequences are (KR822817, KR822816, KR822823, KR822813,
164 KR822820, KR822821, KR822822, KR822815, KR822824, KR822812, KR822811,
165 KR822814 and KR822818). A full list of accessions can be found in tables S1 and S2.
166
167 *Discovery of rhabdoviruses in public sequence databases*
168
169 Rhabdovirus L gene sequences were used as queries to search (tblastn) expressed
170 sequence tag (EST) and transcriptome shotgun assembly (TSA) databases (NCBI). All
171 sequences were reciprocally BLAST searched against Genbank cDNA and RefSeq
172 databases and only retained if they matched a virus-like sequence. We used two
173 approaches to examine whether sequences were present as RNA but not DNA. First,
174 where assemblies of whole-genome shotgun sequences were available, we used BLAST
175 to test whether sequences were integrated into the host genome. Second, for the virus
176 sequences in the butterfly *Pararge aegeria* and the medfly *Ceratitis capitata* we were
177 able to obtain infected samples to confirm whether sequences are only present in RNA
178 by performing PCR on both genomic DNA and cDNA as described above (samples kindly
179 provided by Casper Breuker/Melanie Gibbs, and Philip Leftwich respectively)
180
181 *Phylogenetic analysis*
182
183 All available rhabdovirus-like sequences were downloaded from Genbank (accessions in
184 Data S2: http://dx.doi.org/10.6084/m9.figshare.1425419). Amino acid sequences for
185 the L gene (encoding the RNA Dependent RNA Polymerase or RDRP) were used to infer
186 the phylogeny (L gene sequences: http://dx.doi.org/10.6084/m9.figshare.1425067), as
187 they contain conserved domains that can be aligned across this diverse group of viruses.
188 Sequences were aligned with MAFFT [24] under default settings and then poorly aligned
189 and divergent sites were removed with either TrimAl (v1.3 strict settings, implemented
190 on Phylemon v2.0 server, alignment: http://dx.doi.org/10.6084/m9.figshare.1425069)
191 [25] or Gblocks (v0.91b selecting smaller final blocks, allowing gap positions and less
192 strict flanking positions to produce a less stringent selection, alignment:
193 http://dx.doi.org/10.6084/m9.figshare.1425068) [26]. These resulted in alignments of
194 1492 and 829 amino acids respectively.
195
196 Phylogenetic trees were inferred using Maximum Likelihood in PhyML (v3.0) [27] using
197 the LG substitution model [28] (preliminary analysis confirmed the results were robust
198 to the amino acid substitution model selected), with a gamma distribution of rate
199 variation with four categories and a sub-tree pruning and regrafting topology searching
200 algorithm. Branch support was estimated using Approximate Likelihood-Ratio Tests
201 (aLRT) that are reported to outperform bootstrap methods [29]. Figures were created
202 using FIGTREE (v. 1.4) [30].

203
204 *Analysis of phylogenetic structure between viruses taken from different hosts and ecologies*
205
206 We measured the degree of phylogenetic structure between virus sequences identified
207 in different categories of host (arthropods, vertebrates and plants) and ecosystems
208 (terrestrial and aquatic). Following Bhatia et al [32], we measured the degree of genetic
209 structure between virus sequences from different groups of hosts/ecosystems using
210 Hudson's F_{st} estimator [31] as in [32]. We calculated F_{st} as: 1- the mean number of
211 differences between sequences within or between populations, where a population is a
212 host category or ecosystem. The significance of this value was tested by comparison
213 with 1000 replicates with host categories randomly permuted over sequences. We also
214 measured the clustering of these categories over our phylogeny using the genealogical
215 sorting index (GSI), a measure of the degree of exclusive ancestry of a group on a rooted
216 genealogy [33], for each of our host association categories. The index was estimated
217 using the genealogicalSorting R package [34], with significance estimated by
218 permutation. The tree was pruned to remove strains that could not be assigned to one of
219 the host association categories under consideration. Finally, since arthropods are the
220 most sampled host, we tested for evidence of genetic structure within the arthropod-
221 associated viruses that would suggest co-divergence with their hosts or preferential
222 host-switching between closely related hosts. We calculated the Pearson correlation
223 coefficient of the evolutionary distances between viruses and the evolutionary distances
224 between their hosts and tested for significance by permutation (as in [35]). We used the
225 patristic distances of our ML tree for the virus data and a time-tree of arthropod genera,
226 using published estimates of divergence dates [36, 37].
227
228 *Reconstruction of host associations*
229
230 Viruses were categorised as having one of four types of host association: arthropod-
231 specific, vertebrate-specific, arthropod-vectored plant, or arthropod-vectored
232 vertebrate. However, the host association of some viruses are uncertain when they have
233 been isolated from vertebrates, biting-arthropods or plant-sap-feeding arthropods. Due
234 to limited sampling it was not clear whether viruses isolated from vertebrates were
235 vertebrate specific or arthropod-vectored vertebrate viruses; or whether viruses
236 isolated from biting-arthropods were arthropod specific viruses or arthropod-vectored
237 vertebrate viruses; or if viruses isolated from plant-sap-feeding arthropods were
238 arthropod-specific or arthropod-vectored plant viruses.
239
240 We classified a virus from a nematode as having its own host category. We classified
241 three of the fish infecting dimarhabdoviruses as vertebrate specific based on the fact
242 they can be transmitted via immersion in water containing virus during experimental
243 conditions [38-40], and the widely held belief amongst the fisheries community that
244 these viruses are not typically vectored [8]. However, there is some evidence these
245 viruses can be transmitted by arthropods (sea lice) in experiments [8, 9] and so we
246 would recommend this be interpreted with some caution. Additionally, although we
247 classified the viruses identified in sea-lice as having biting arthropod hosts, they may be
248 crustacean-specific. The two viruses from *Lepeophtheirus salmonis* do not seem to infect

249 the fish they parasitise and are present in all developmental stages of the lice, suggesting
250 they may be transmitted vertically [41].
251
252 We simultaneously estimated both the current and ancestral host associations, and the
253 phylogeny of the viruses, using a Bayesian analysis, implemented in BEAST v1.8 [42, 43].
254 Since meaningful branch lengths are essential for this analysis (uncertainty about
255 branch lengths will feed into uncertainty about the estimates), we used a subset of the
256 sites and strains used in the Maximum Likelihood (ML) analysis. We retained 189 taxa;
257 all rhabdoviruses excluding the divergent fish-infecting novirhabdovirus clade and the
258 virus from *Hydra*, as well as the viruses from *Lolium perenne and Conwentzia*
259 *psociformis*, which had a large number of missing sites. Sequences were trimmed to a
260 conserved region of 414 amino acids where data was recorded for most of these viruses
261 (the Gblocks alignment trimmed further by eye:
262 http://dx.doi.org/10.6084/m9.figshare.1425431).
263
264 We used the host-association categories described above, which included ambiguous
265 states. To describe amino acid evolution we used an LG substitution model with gamma
266 distributed rate variation across sites [28] and an uncorrelated lognormal relaxed clock
267 model of rate variation among lineages [44]. To describe the evolution of the host
268 associations we used a strict clock model and a discrete asymmetric transition rate
269 matrix (allowing transitions to and from a host association to take place at different
270 rates), as previously used to model migrations between discrete geographic locations
271 [45] and host switches [43, 46]. We also examined how often these viruses jumped
272 between different classes of hosts using reconstructed counts of biologically feasible
273 changes of host association and their HPD confidence intervals (CI) using Markov Jumps
274 [47]. These included switches between arthropod-specific and both arthropod-vectored
275 vertebrate and arthropod-vectored plant states, and between vertebrate specific and
276 arthropod-vectored vertebrate states. We used a constant population size coalescent
277 prior for the relative node ages (using a birth-death prior gave equivalent results) and
278 the BEAUti v1.8 default priors for all other parameters [42] (BEAUti xml
279 http://dx.doi.org/10.6084/m9.figshare.1431922). In Figure 2 we have transferred the
280 ancestral state reconstruction from the BEAST tree to the maximum likelihood tree.
281
282 Convergence was assessed using Tracer v1.6 [48], and a burn-in of 30% was removed
283 prior to the construction of a consensus tree, which included a description of ancestral
284 host associations in the output file. High effective sample sizes were achieved for all
285 parameters (>200). Previous simulations, in the context of biogeographical inference,
286 have shown that the approach is robust to sampling bias [45]. However, to confirm this,
287 following [49], we tested whether sample size predicts rate to or from a host
288 association.
289
290 **Results**
291
292 *Novel rhabdoviruses from RNA-seq*
293
294 To search for new rhabdoviruses we collected a variety of different species of flies,
295 screened them for CO_2 sensitivity, which is a common symptom of infection, and

296 sequenced total RNA of these flies by RNA-seq. We identified rhabdovirus-like
297 sequences from a *de-novo* assembly by BLAST, and used PCR to identify which samples
298 these sequences came from.
299
300 This approach resulted in eleven rhabdovirus-like sequences from nine (possibly ten)
301 species of fly. Seven of these viruses were previously unknown and four had been
302 reported previously from shorter sequences (Tables S1 and S2
303 http://dx.doi.org/10.6084/m9.figshare.1502665). The novel viruses were highly
304 divergent from known viruses. Sigma viruses known from other species of *Drosophila*
305 typically have genomes of ~12.5Kb [12, 50], and six of our sequences were
306 approximately this size, suggesting they are near-complete genomes. None of the
307 viruses discovered in our RNA-seq data were integrated into the host genome (see
308 Methods for details).
309
310 To investigate the putative gene content of the viruses, we predicted genes based on
311 open reading frames (ORFs). For the viruses with apparently complete genomes (Figure
312 1), we found that those from *Drosophila ananassae, Drosophila affinis, Drosophila*
313 *immigrans* and *Drosophila sturtvanti* contained ORFs corresponding to the five core
314 genes found across all rhabdoviruses, with an additional ORF between the P and M
315 genes. This is the location of the X gene found in sigma viruses, and in three of the four
316 novel viruses it showed BLAST sequence similarity to the X gene of sigma viruses. The
317 virus from *Drosophila busckii* did not contain an additional ORF between the P and M
318 genes, but instead contained an ORF between the G and L gene.
319
320 Using the phylogeny described below, we have classified our newly discovered viruses
321 as either sigma viruses, rhabdoviruses or other viruses, and named them after the host
322 species they were identified from (Figure 1) [51]. We also found one other novel
323 mononegavirales-like sequence from *Drosophila unispina* that groups with a recently
324 discovered clade of arthropod associated viruses (Nyamivirus clade [13], see Table S5
325 and the full phylogeny: http://dx.doi.org/10.6084/m9.figshare.1425083), as well as five
326 other RNA viruses from various families (data not shown), confirming our approach can
327 detect a wide range of divergent viruses.

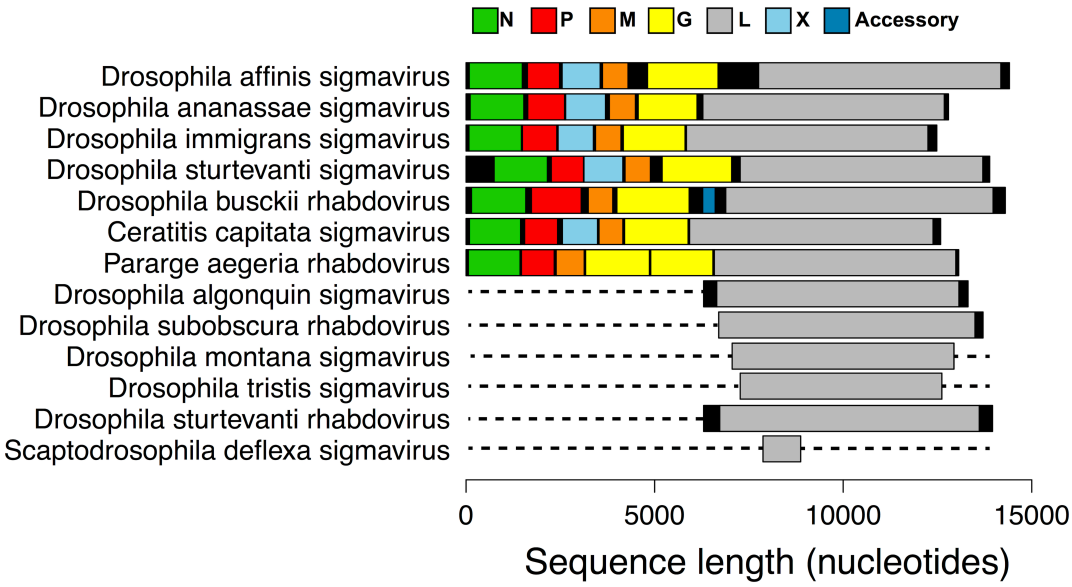


Figure 1. Genome organization of newly discovered viruses from metagenomic RNA sequencing of CO_2 sensitive flies.
Putative genes are shown in colour, non-coding regions are shown in black. ORFs were designated as the first start codon following the transcription termination sequence (7 U's) of the previous ORF to the first stop codon. Dotted lines represent parts of the genome not sequenced. These viruses were either from our own RNA-seq data, or were first found in in public databases and key features verified by PCR and Sanger sequencing. Rhabdovirus genomes are typically ~11-13kb long and contain five core genes 3'-N-P-M-G-L-5' [3]. However, a number of groups of rhabdoviruses contain additional accessory genes and can be up to ~16kb long [14, 52].

New rhabdoviruses from public databases

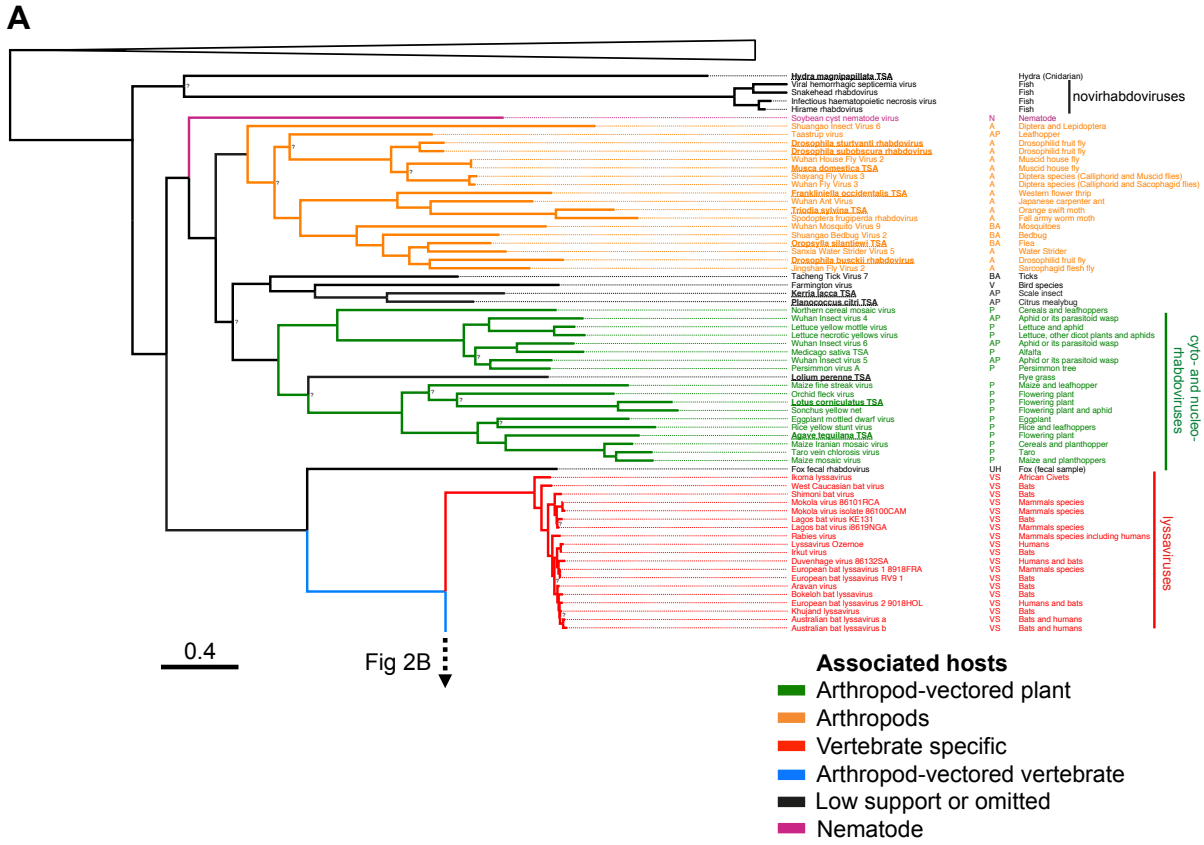
We identified a further 26 novel rhabdovirus-like sequences by searching public databases of assembled RNA-seq data with BLAST. These included 19 viruses from arthropods (Fleas, Crustacea, Lepidoptera, Diptera), one from a Cnidarian (*Hydra*) and 5 from plants (Table S3). Of these viruses, 19 had sufficient amounts of coding sequence (>1000bp) to include in the phylogenetic analysis (Table S3), whilst the remainder were too short (Table S4).

Four viruses from databases had near-complete genomes based on their size. These were from the moth *Triodia sylvina*, the house fly *Musca domestica* (99% nucleotide identity to Wuhan house fly virus 2 [13]), the butterfly *Pararge aegeria* and the medfly *Ceratitis capitata*, all of which contain ORFs corresponding to the five core rhabdovirus genes. The sequence from *C. capitata* had an additional ORF between the P and M genes with BLAST sequence similarity to the X gene in sigma viruses. There were several unusual sequences. Firstly, in the virus from *P. aegeria* there appear to be two full-length glycoprotein ORFs between the M and L genes (we confirmed by Sanger sequencing that both exist and the stop codon between the two genes was not an error). Secondly, the *Agave tequilana* transcriptome contained a L gene ORF on a contig that was the length of a typical rhabdovirus genome but did not appear to contain typical gene content, suggesting it has very atypical genome organization, or has been misassembled, or is integrated into its host plant genome [53]. Finally, the virus from

361 *Hydra magnipapillata* contained six predicted genes, but the L gene (RDRP) ORF was
362 unusually long. Some of the viruses we detected may be EVEs inserted into the host
363 genome and subsequently expressed [18]. For example, this is likely the case for the
364 sequence from the silkworm *Bombyx mori* that we also found in the silkworm genome,
365 and the L gene sequence from *Spodoptera exigua* that contains stop codons. Under the
366 assumption that viruses integrated into host genomes once infected those hosts, this
367 does not affect our conclusions below about the host range of these viruses [15-17]. We
368 also found nine other novel mononegavirale-like sequences that group with recently
369 discovered clades of insect viruses [13] (see Table S5 and
370 http://dx.doi.org/10.6084/m9.figshare.1425083).
371
372 *Rhabdovirus Phylogeny*
373
374 To reconstruct the evolution of the *Rhabdoviridae* we have produced the most complete
375 phylogeny of the group to date (Figure 2). We aligned the relatively conserved L gene
376 (RNA Dependant RNA Polymerase) from our newly discovered viruses with sequences
377 of known rhabdoviruses to give an alignment of 195 rhabdoviruses (and 26 other
378 mononegavirales as an outgroup). We reconstructed the phylogeny using different
379 sequence alignments and methodologies, and these all gave qualitatively similar results
380 with the same major clades being reconstructed (Gblocks:
381 http://dx.doi.org/10.6084/m9.figshare.1425083, TrimAl:
382 http://dx.doi.org/10.6084/m9.figshare.1425082 and BEAST:
383 http://dx.doi.org/10.6084/m9.figshare.1425436). The ML and Bayesian relaxed clock
384 phylogenies were very similar: 149/188 nodes are found in both reconstructions and
385 only 2 nodes present in the Bayesian relaxed clock tree with strong support are absent
386 from the ML tree with strong support. These are found in a single basal clade of
387 divergent but uniformly arthropod-specific strains, where the difference in topology will
388 have no consequence for inference of host association. This suggests that our analysis is
389 robust to the assumptions of a relaxed molecular clock. The branching order between
390 the clades in the dimarhabdovirus supergroup was generally poorly supported and
391 differed between the methods and alignments. Eight sequences that we discovered were
392 not included in this analysis as they were considered too short, but their closest BLAST
393 hits are listed in Table S4 (http://dx.doi.org/10.6084/m9.figshare.1502665).
394
395 We recovered all of the major clades described previously (Figure 2), and found that the
396 majority of known rhabdoviruses belong to the dimarhabdovirus clade (Figure 2b). The
397 RNA-seq viruses from *Drosophila* fall into either the sigma virus clade (Figure 2b) or the
398 arthropod clade sister to the cyto- and nucleo- rhabdoviruses (Figure 2a). The viruses
399 from sequence databases are diverse, coming from almost all of the major clades with
400 the exception of the lyssaviruses.
401
402 *Predicted host associations of viruses*
403
404 With a few exceptions, rhabdoviruses are either arthropod-vectored viruses of plants or
405 vertebrates, or are vertebrate- or arthropod- specific. In many cases the only
406 information about a virus is the host from which it was isolated. Therefore, *a priori*, it is
407 not clear whether viruses isolated from vertebrates are vertebrate-specific or

arthropod-vectored, or whether viruses isolated from biting arthropods (e.g. mosquitoes, sandflies, ticks, midges and sea lice) are arthropod specific or also infect vertebrates. Likewise, it is not clear whether viruses isolated from sap-sucking insects (all Hemiptera: aphids, leafhoppers, scale insect and mealybugs) are arthropod-specific or arthropod-vectored plant viruses. By combining data on the ambiguous and known host associations with phylogenetic information, we were able to predict both the ancestral and present host associations of these viruses (http://dx.doi.org/10.6084/m9.figshare.1425436). To do this we used a Bayesian phylogenetic analysis that simultaneously estimated the phylogeny and host association of our data. In the analysis we defined our host associations either as vertebrate-specific, arthropod-specific, arthropod-vectored vertebrate, arthropod-vectored plant, nematode, or as ambiguous between two (and in one case all five) of these states (see Methods).

This approach identified a large number of viruses that are likely to be new arthropod-vectored vertebrate viruses (Figure 2b). Of 80 viruses with ambiguous 89 host associations were assigned a host association with strong posterior support (>0.95). Of the 52 viruses found in biting arthropods, 45 were predicted to be arthropod-vectored vertebrate viruses, and 6 to be arthropod-specific. Of the 30 viruses found in vertebrates, 22 were predicted to be arthropod-vectored vertebrate viruses, and 2 were predicted to be vertebrate-specific (both fish viruses). Of the 7 viruses found in plant-sap-feeding arthropods (Figure 2a), 3 were predicted to be plant-associated and 2 arthropod-associated.



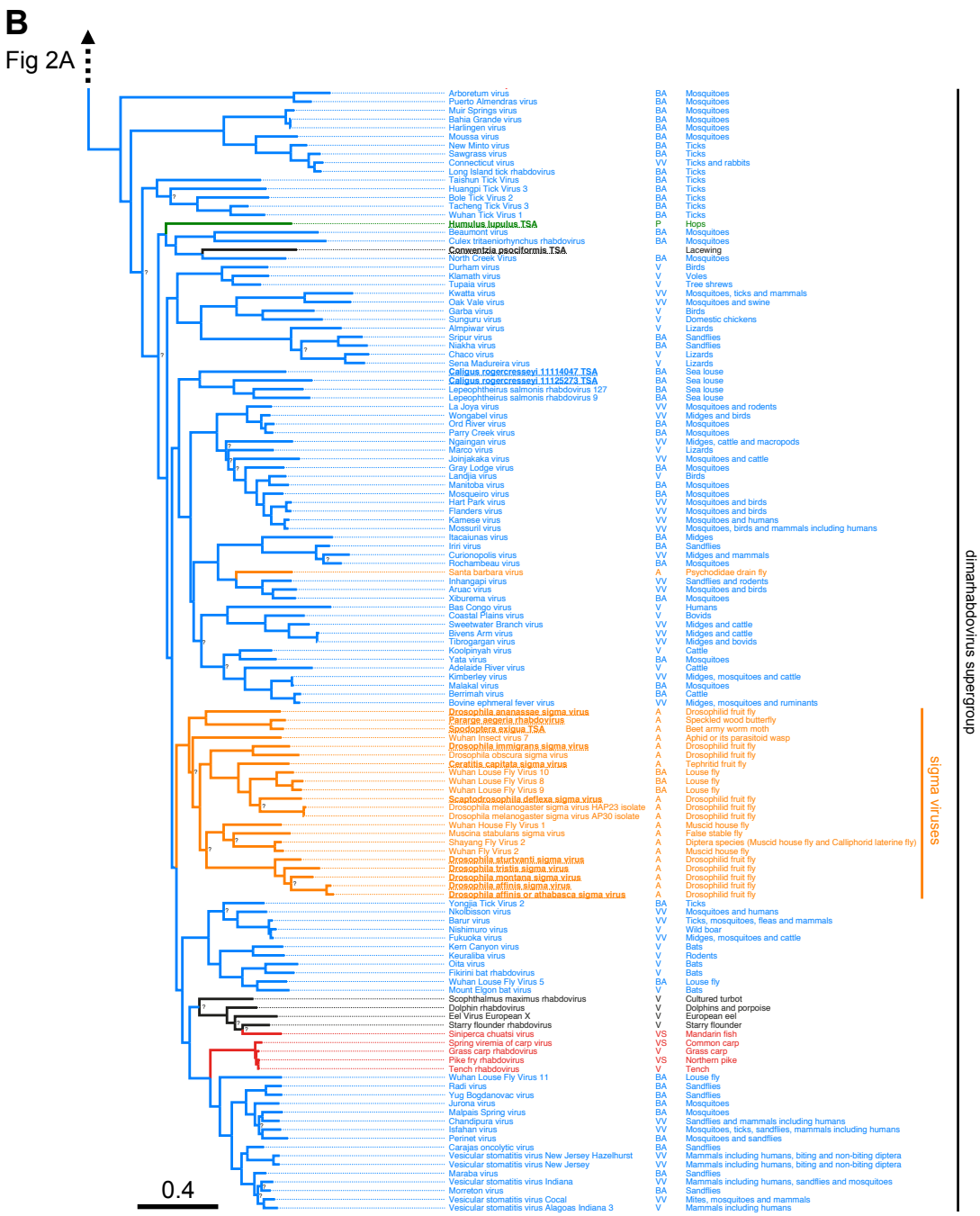


Figure 2. Maximum likelihood phylogeny of the *Rhabdoviridae*.
(A) shows the basal fish-infecting novirhabdoviruses, an unassigned group of arthropod associated viruses, the plant infecting cyto- and nucleo- rhabdoviruses, as well as the vertebrate specific lyssaviruses. **(B)** shows the dimarhabdovirus supergroup, which is predominantly composed of arthropod-vectored vertebrate viruses, along with the arthropod-specific sigma virus clade. Branches are coloured based on the Bayesian host association reconstruction analysis. Black represents taxa omitted from host-state reconstruction or associations with <0.95 support. The tree was inferred from L gene sequences using the Gblocks alignment. The columns of text are the virus name, the host category used for reconstructions, and known hosts (from left to right). Codes for the host categories are: VS= vertebrate-specific, VV = arthropod-vectored vertebrate, A= arthropod specific, BS = biting-arthropod (ambiguous state), V = vertebrate (ambiguous state) AP =plant-sap-feeding-arthropod (ambiguous state), UH = uncertain-host (ambiguous across all states) and N = nematode. Names in bold and underlined are viruses discovered in this study. The tree is rooted with the Chuvirus clade (root collapsed) as identified

as an outgroup in [13] but we note this gives the same result as midpoint and the molecular clock rooting. Nodes labelled with question marks (?) represent nodes with aLRT (approximate likelihood ratio test) statistical support values less than 0.75. Scale bar shows number of amino-acid substitutions per site. Bayesian MCC tree used to infer ancestral traits: http://dx.doi.org/10.6084/m9.figshare.1425436.

To test the accuracy of our predictions of current host associations we randomly selected a set of viruses with known associations, re-assigned their host association as ambiguous between all possible states (a greater level of uncertainty than we generally attributed to viruses in our data), and re-ran our analysis. We repeated this 10 times for 9 sets of 10 viruses and one set of 9 viruses (randomly sampling without replacement from the 99 viruses in our data with known host associations). These analyses correctly returned the true host association for 95/99 viruses with strong posterior support (>0.9) and 1 with weak support (mean support = 0.99, range = 0.73-1.00; Data S3 http://dx.doi.org/10.6084/m9.figshare.1538584). All three cases in which the reconstruction returned a false host association involved anomalous sequences (e.g., a change in host association on a terminal branch). Note, there would be no failure in cases where there was no phylogenetic clustering of host associations. In such cases the method would – correctly – report high levels of uncertainty in all reconstructed states.

We checked for evidence of sampling bias in our data by testing whether sample size predicts rate to or from a host association [49]. We found there is a high level of uncertainty around all rate estimates, but that there is no pattern of increased rate to or from states that are more frequently sampled.

Ancestral host associations and host-switches

Viral sequences from arthropods, vertebrates and plants form distinct clusters in the phylogeny (Figure 2). To quantify this genetic structure we calculated the F_{st} statistic between the sequences of viruses from different groups of hosts. There is strong evidence of genetic differentiation between the sequences from arthropods, plants and vertebrates (P<0.001, Figure S1 http://dx.doi.org/10.6084/m9.figshare.1495351). Similarly, viruses isolated from the same host group tend to cluster together on the tree (GSI analysis permutation tests: arthropod hosts GSI = 0.43, P<0.001, plant hosts GSI = 0.46, P<0.001, vertebrate hosts GSI = 0.46, P < 0.001).

Our Bayesian analysis allowed us to infer the ancestral host association of 176 of 188 of the internal nodes on the phylogenetic tree (support >0.95), however we could not infer the host association of the root of the phylogeny, or some of the more basal nodes. A striking pattern that emerged is that switches between major groups of hosts have occurred rarely during the evolution of the rhabdoviruses (Figure 2). There are a few rare transitions on terminal branches (Santa Barbara virus and the virus identified from the plant *Humulus lupulus*) but these could represent errors in the host assignment (e.g. cross-species contamination) as well as recent host shifts. Our analysis allows us to estimate the number of times the viruses have switched between major host groups across the phylogeny, while accounting for uncertainty about ancestral states, the tree topology and root. We found strong evidence of only two types of host-switch across our phylogeny: two transitions from being an arthropod-vectored vertebrate virus to being

arthropod specific (modal estimate = 2, median = 3.1, CI's= 1.9–5.4) and three transitions from being an arthropod-vectored vertebrate virus to a vertebrate-specific virus (modal estimate = 3, median = 3.1, CI's= 2.9–5.2). We could not determine the direction of the host shifts into the other host groups.

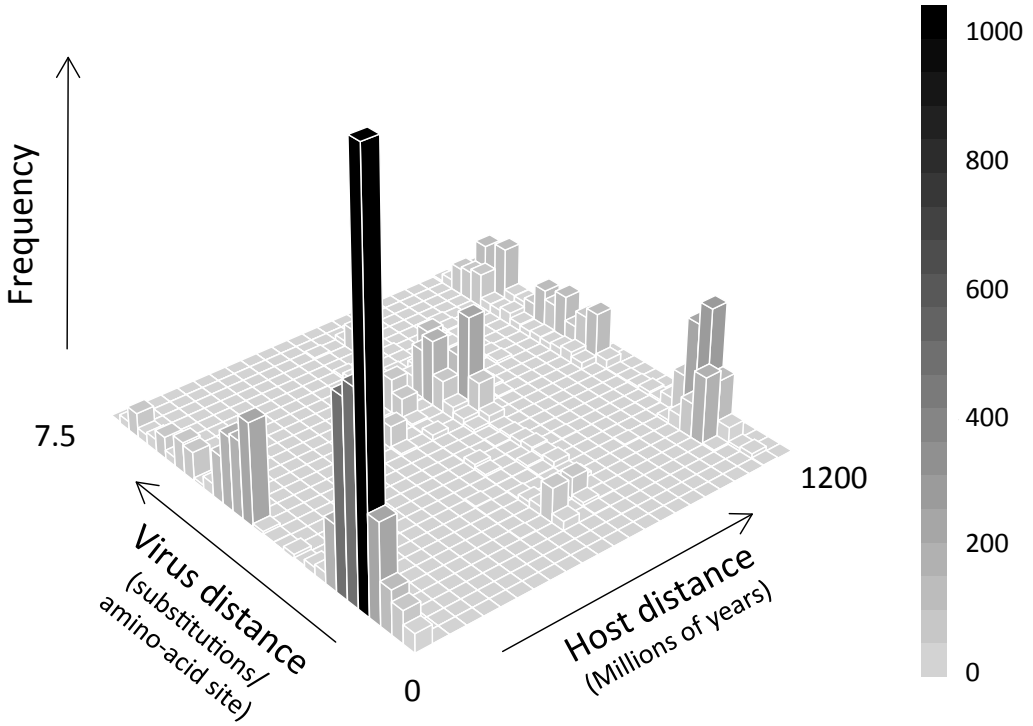
Vertebrate–specific viruses have arisen once in the lyssaviruses clade [3], as well as at least once in fish dimarhabdoviruses (in one of the fish-infecting clades it is unclear if it is vertebrate-specific or vector-borne from our reconstructions). There has also likely been a single transition to being arthropod-vectored vertebrate viruses in the dimarhabodovirus clade.

Insect-vectored plant viruses in our dataset have arisen once in the cyto- and nucleo-rhabdoviruses, although the ancestral state of these viruses is uncertain. A single virus identified from the hop plant *Humulus lupulus* appears to fall within the dimarhabdovirus clade. However, this may be because the plant was contaminated with insect matter, as the same RNA-seq dataset contains *COI* sequences with high similarity to thrips.

There are two large clades of arthropod-specific viruses. The first is a sister group to the large clade of plant viruses. This novel group of predominantly insect-associated viruses are associated with a broad range of insects, including flies, butterflies, moths, ants, thrips, bedbugs, fleas, mosquitoes, water striders and leafhoppers. The mode of transmission and biology of these viruses is yet to be examined. The second clade of insect-associated viruses is the sigma virus clade [11, 12, 19, 50]. These are derived from vector-borne dimarhabdoviruses that have lost their vertebrate host and become vertically transmitted viruses of insects [10]. They are common in Drosophilidae, and our results suggest that they may be widespread throughout the Diptera, with occurrences in the Tephritid fruit fly *Ceratitis capitata*, the stable fly *Muscina stabulans,* several divergent viruses in the housefly *Musca domestica* and louse flies removed from the skin of bats. For the first time we have found sigma-like viruses outside of the Diptera, with two Lepidoptera associated viruses and a virus from an aphid/parasitoid wasp. All of the sigma viruses characterised to date have been vertically transmitted [10], but some of the recently described viruses may be transmitted horizontally – it has been speculated that the viruses from louse flies may infect bats [54] and Shayang Fly Virus 2 has been reported in two fly species [13] (although contamination could also explain this result). Drosophila sigma virus genomes are characterised by an additional X gene between the P and M genes [50]. Interestingly the two louse fly viruses with complete genomes, Wuhan insect virus 7 from an aphid/parasitoid and *Pararge aegeria* rhabdovirus do not have a putative X gene. The first sigma virus was discovered in *Drosophila melanogaster* in 1937 [55]. In the last few years related sigma viruses have been found in other *Drosophila* species and a Muscid fly [10-12, 50] and here we have found sigma-like viruses in a diverse array of Diptera species, as well as other insect orders. Overall, our results suggest sigma-like viruses may be associated with a wide array of insect species.

Within the arthropod-associated viruses (the most sampled host group) it is common to find closely related viruses in closely related hosts (Figure 1). Viruses isolated from the

542 same arthropod orders tended to cluster together on the tree (GSI analysis permutation
543 tests: Diptera GSI = 0. 57, P<0.001, Hemiptera GSI = 0.34, P<0.001, Ixodida GSI = 0.38,
544 P<0.001, Lepidoptera GSI = 0.15, P=0.089). This is also reflected in a positive correlation
545 between the evolutionary distance between the viruses and the evolutionary distance
546 between their arthropod hosts (Pearson's correlation=0.36, 95% CI's=0.34-0.38,
547 P<0.001 based on permutation, Figure 3 and Figure S2
548 http://dx.doi.org/10.6084/m9.figshare.1495351). Since the virus phylogeny is
549 incongruent with that of the respective hosts, this suggests rhabdoviruses preferentially
550 host shift between closely related species [56, 57].



551
552 **Figure 3. The relationship between the evolutionary distance between viruses and the**
553 **evolutionary distance between their arthropod hosts (categorised by genus).** Closely
554 related viruses tend to be found in closely related hosts. Permutation tests find a significant
555 positive correlation (correlation=0.36, 95% CI's=0.34-0.38, P<0.001) between host and virus
556 evolutionary distance (see Figure S2).
557
558 We also find viruses clustering on the phylogeny based on the ecosystem of their hosts;
559 there is strong evidence of genetic differentiation between viruses from terrestrial and
560 aquatic hosts (F_{st} permutation test P=0.007, Figure S3
561 http://dx.doi.org/10.6084/m9.figshare.1495351; GSI analysis permutation tests:
562 terrestrial hosts= 0.52, aquatic hosts= 0.29, P<0.001 for both). There has been one shift
563 from terrestrial to aquatic hosts during the evolution of the basal novirhabdoviruses,
564 which have a wide host range in fish. There have been other terrestrial to aquatic shifts
565 in the dimarhabdoviruses: in the clades of fish and cetacean viruses and the clade of
566 viruses from sea-lice.
567
568
569 **Discussion**
570

571 Viruses are ubiquitous in nature and recent developments in high-throughput
572 sequencing technology have led to the discovery and sequencing of a large number of
573 novel viruses in arthropods [13, 14, 64]. Here we have identified 43 novel virus-like
574 sequences from our own RNA-seq data and public sequence repositories. Of these, 32
575 were rhabdoviruses, and 26 were from arthropods. Using these sequences we have
576 produced the most extensive phylogeny of the *Rhabdoviridae* to date, including a total of
577 195 virus sequences.
578
579 In most cases we know nothing about the biology of the viruses beyond the host they
580 were isolated from, but our analysis provides a powerful way to predict which are
581 vector-borne viruses and which are specific to vertebrates or arthropods. We have
582 identified a large number of new likely vector-borne viruses – of 85 rhabdoviruses
583 identified from vertebrates or biting insects we predict that 76 are arthropod-borne
584 viruses of vertebrates (arboviruses). The majority of known rhabdoviruses are
585 arboviruses, and all of these fall in a single clade known as the dimarhabdoviruses. In
586 addition to the arboviruses, we also identified two clades of likely insect-specific viruses
587 associated with a wide range of species.
588
589 We found that shifts between distantly related hosts are rare in the rhabdoviruses,
590 which is consistent with previous observations that both rhabdoviruses of vertebrates
591 (rabies virus in bats) and invertebrates (sigma viruses in Drosophilidae) show a
592 declining ability to infect hosts more distantly related to their natural host [46, 57, 58].
593 It is thought that sigma viruses may sometimes jump into distantly related but highly
594 susceptible species [56, 57, 59], but our results suggest that this rarely happens between
595 major groups such as vertebrates and arthropods. It is nonetheless surprising that
596 arthropod-specific viruses have arisen rarely, as one might naively assume that there
597 would be fewer constraints on vector-borne viruses losing one of their hosts. However,
598 this would involve evolving a new transmission route among insects, and this may be an
599 important constraint. Within the major clades, closely related viruses often infect closely
600 related hosts (Figure 2). For example, within the dimarhabdoviruses viruses identified
601 from mosquitoes, ticks, *Drosophila*, Muscid flies, Lepidoptera and sea-lice all tend to
602 cluster together (Figure 2B). However, it is also clear that the virus phylogeny does not
603 mirror the host phylogeny, and our data on the clustering of hosts across the virus
604 phylogeny therefore suggests that viruses preferentially shift between more closely
605 related species (Figures 3, S1 and S2) in the same environment (Figure S3).
606
607 There has been a near four-fold increase in the number of recorded rhabdovirus
608 sequences in the last five years. In part this may be due to the falling cost of sequencing
609 transcriptomes [60], and initiatives to sequence large numbers of insect and other
610 arthropods [37]. The use of high-throughput sequencing technologies should reduce the
611 likelihood of sampling biases associated with PCR, where people look for similar viruses
612 in related hosts. Therefore, the pattern of viruses forming clades based on the host taxa
613 they infect is likely to be robust. However, sampling is biased towards arthropods, and it
614 is possible that there may be a great undiscovered diversity of rhabdoviruses in other
615 organisms [61].
616

Our conclusions are likely to be robust to biases in the data or limitations in the analysis. By reconstructing host associations using the Bayesian methods in the BEAST software [42] we have avoided most of the simplifying assumptions of earlier methods (e.g. symmetric transition rate matrices, lack of uncertainty associated with estimates). Nonetheless all such methods depend on there being some of sort of "process homogeneity" over the phylogeny [62]. Such analyses are of course limited by sampling; for example, if a past host is now extinct, it will never be reconstructed as an ancestral state. Nevertheless, previous studies have shown that the method is relatively robust to uneven sampling across hosts [45]. Furthermore, when we have viruses from under-sampled groups like cnidarians, fungi, nematodes, they fall outside the main clades of viruses that we are analysing. The limitations of the approach are evident in our results: we were unable to reconstruct the host associations of the root or most basal nodes of the phylogeny. The reconstructions were, however, very successful within clades that were strongly associated with a single host or clades where the less common hosts tend to form distinct subclades. As a result of this high level of phylogenetic structure, our approach was able to reconstruct the current host associations of many viruses for which we had incomplete knowledge of their host range. To check that this approach is reliable, we repeated the analysis on datasets where we deleted the information about which hosts well-characterised viruses infect. Our analysis was found to be robust, with 97% of reconstructions being accurate. The method only failed for strains with irregular host associations for their location in the phylogeny (i.e. recent changes in host on terminal branches)– a limitation that would be expected for such an analysis.

Rhabdoviruses infect a diverse range of host species, including a large number of arthropods. Our search has unearthed a large number of novel rhabdovirus genomes, suggesting that we are only just beginning to uncover the diversity of these viruses. The host associations of these viruses have been highly conserved across their evolutionary history, which provides a powerful tool to identify previously unknown arboviruses. The large number of viruses being discovered through metagenomic studies [13, 63, 64] means that in the future we will be faced by an increasingly large number of viral sequences with little knowledge of the biology of the virus. Our phylogenetic approach could be extended to predict key biological traits in other groups of pathogens where our knowledge is incomplete. However, there are limitations to this method, and the rapid evolution of RNA viruses may mean that some traits change too quickly to accurately infer traits. Therefore, such an approach should complement, and not replace, examining the basic biology of novel viruses.

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Contributions

BL and FMJ conceived and designed the study. BL and JD carried out molecular work. BL, WJP, DJP and DJO carried out bioinformatic analysis. BL, GGRM and JJW carried out phylogenetic analysis. BL GGRM, JJW and FMJ wrote the manuscript with comments from all other authors. All authors gave final approval for publication.

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868
869 **Supplementary materials**
870
871 Tables S1-5. List of newly discovered viruses:
872
873 Figures S1-S3
874

875 Supplementary Figure 1.
876
877 Results of permutation tests of population differentiation between the sequences of
878 viruses taken from different categories of host: (a) between arthropods and vertebrates,
879 (b) between arthropods and plants and (c) between plants and vertebrates. Red lines
880 show the degree of differentiation, measured using Hudson's F_{st} estimator, and grey
881 histograms show the F_{st} values from 1000 unique random permutations of host
882 categories over viral sequences. We found significant differentiation between each pair
883 of categories (F_{st} values: a=0.07, b=0.35, c=0.48) with none of the 1000 permutations
884 resulting in as high an F_{st} value as the data (P<0.001 for all).
885
886 Supplementary Figure 2.
887
888 Correlation of the evolutionary distances between rhabdovirueses and the evolutionary
889 distances between their arthropod hosts. Figure shows the results of a permutation test
890 of this relationship (measured by Pearson's correlation coefficient). The red line shows
891 the true correlation coefficient (0.36), and the grey histogram shows the correlation
892 coefficients returned from 1000 unique random permutations of host genera over viral
893 sequences (see Methods). We found a significant association between viral and host
894 phylogenies with none of the 1000 permutations resulting in as strong a correlation as
895 the data (P<0.001).
896
897 Supplementary Figure 3.
898
899 Results of the permutation test of population differentiation between the sequences of
900 viruses taken from land and aquatic arthropod and vertebrate hosts. Only arthropod
901 and vertebrate hosts were included in the test, since aquatic hosts only included
902 arthropods and vertebrates. The red line shows the true degree of differentiation,
903 measured using Hudson's F_{st} estimator, and the grey histogram shows the F_{st} values
904 returned from 1000 unique random permutations of host categories over viral
905 sequences. Viruses from terrestrial and aquatic hosts were found to have significant
906 population differentiation (with only 7 of the 1000 permutations resulted in as high an
907 F_{st} value as the data (F_{st} =0.07, P=0.007).
908
909
910 **All data has been made available in public repositories:**
911
912 NCBI Sequence Read Archive Data: SRP057824
913 Data S1, sample information: http://dx.doi.org/10.6084/m9.figshare.1425432
914 Data S2, virus ID, Genbank accession numbers and host information:
915 http://dx.doi.org/10.6084/m9.figshare.1425419
916 Data S3, results from testing ancestral trait reconstructions predictions:
917 http://dx.doi.org/10.6084/m9.figshare.1538584
918 L gene sequences fasta: http://dx.doi.org/10.6084/m9.figshare.1425067
919 TrimAl alignment fasta: http://dx.doi.org/10.6084/m9.figshare.1425069
920 Gblocks alignment fasta: http://dx.doi.org/10.6084/m9.figshare.1425068
921 Phylogenetic tree Gblocks alignment: http://dx.doi.org/10.6084/m9.figshare.1425083
922 Phylogenetic tree TrimAl alignment: http://dx.doi.org/10.6084/m9.figshare.1425082
923 BEAST alignment fasta: http://dx.doi.org/10.6084/m9.figshare.1425431
924 BEAUti xml file: http://dx.doi.org/10.6084/m9.figshare.1431922
925 Bayesian analysis tree: http://dx.doi.org/10.6084/m9.figshare.1425436
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