Title: Twenty five new viruses associated with the Drosophilidae (Diptera)

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Abstract

Drosophila melanogaster is an important laboratory model for studies of antiviral immunity in invertebrates, and *Drosophila* species provide a valuable system to study virus host range and host switching. Here we use metagenomic RNA sequencing of *ca.* 1400 adult flies to discover 25 new RNA viruses associated with six different drosophilid hosts in the wild. We also provide a comprehensive listing of viruses previously reported from the Drosophilidae. The new viruses include Iflaviruses, Rhabdoviruses, Nodaviruses, and Reoviruses, and members of unclassified lineages distantly related to Negeviruses, Sobemoviruses and Poleroviruses, Flaviviridae, and Tombusviridae. Among these are close relatives of Drosophila X virus and Flock House Virus, which we find in association with wild *Drosophila immigrans*. These two viruses are widely used in experimental studies, but have not been reported to naturally infect *Drosophila*. Although we detect no new DNA viruses, in *D. immigrans* and *D. obscura* we identify sequences almost identical to *Armadillidium vulgare* Iridescent Virus (Invertebrate Iridescent Virus 31), bringing the total number of DNA viruses found in the Drosophilidae to three.

Key Words:

Drosophila, Virus, Metagenomics, Transcriptome, Drosophila X Virus, Flock House Virus,

Introduction

Drosophila melanogaster is an important model system for the study of antiviral immunity in invertebrates[1-4], and has been instrumental in defining all of the major insect antiviral immune mechanisms, including the RNAi, IMD, Toll, autophagy, and Jak-Stat pathways, and the role of *Wolbachia*[5-10]. However, from an evolutionary perspective, the value of *D. melanogaster* is not just in its experimental tractability, but also in its close relationship to many other experimentally tractable species[11]. For example, experimental viral infection studies of more than 50 species of Drosophilidae (representing around 50 million years of evolution) have shown how susceptibility to viral infection has a strong phylogenetic component, such that more closely-related host species display more similar viral replication rates and virulence[12], and closer relatives of the virus' natural host tend to support higher viral replication rates[13]. To understand how such phylogenetic patterns relate to host and virus biology in the wild, we need to know the natural host range and frequency of host switching of these viruses. Thus, to capitalise on the value of the Drosophilidae as a model clade, we require a broader perspective on *Drosophila* viruses than *D. melanogaster* alone.

Prior to the advent of modern molecular biology, a handful of *Drosophila* viruses had been described on the basis of traditional virological techniques[14]. Starting with Sigmavirus in *D. melanogaster* (DMelSV, Rhabdoviridae[15]), which was initially identified by the failure of infected flies to recover from CO_2 anaesthesia[16,17], these 'classical' *Drosophila* viruses also include Drosophila P Virus (DPV, Picornavirales[18]), Drosophila C Virus (DCV, Cripavirus[19]), Drosophila A Virus (DAV[20]), Drosophila F Virus (Reoviridae[21]), and Drosophila G Virus (Unclassified[21]) from adult flies, and Drosophila X Virus (DXV, Entomobirnavirus[22]), Drosophila K Virus (Reoviridae,[23]), and unnamed Reoviruses from cell culture (e.g. ref. 24, and see also ref 25). In broadly the same period, Iota Virus (Picornavirales[26]) was identified from *D. immigrans* and was shown to be serologically similar to DPV, RS Virus was identified in *D. annanassae* and members of the *D. montium* group[21] and shown to be morphologically similar to Chronic Bee Paralysis Virus, and Drosophila S Virus (Reoviridae[27]) was identified from *D. simulans*. Unfortunately, of these 'classical' viruses, only DAV, DCV, DXV, and DMelSV remained in culture into the era of routine sequencing, and the others have been lost—making their classification tentative and relationships to each other and subsequently discovered viruses uncertain.

As large-scale sequencing became more routine, it led to the serendipitous discovery of *Drosophila* viruses amongst host RNA sequenced for other purposes. Starting with the discovery of Nora virus (unclassified Picornavirales) in a *D. melanogaster* cDNA library[28], such discoveries have included six viruses from small RNAs of *D. melanogaster* cell culture and *D. melanogaster* laboratory stocks (American Nodavirus, *D. melanogaster* totivirus, *D. melanogaster* Birnavirus, and Drosophila tetravirus[29]; *Drosophila* uncharacterized virus and *Drosophila* reovirus[30]), a novel Cripavirus in *D. kikkawai*[31], and a new Sigmavirus in *D. montana*[32]. At the same time, PCR surveys of other *Drosophila* species using primers designed to *D. melanogaster* viruses were used to detect novel Nora viruses in *D. immigrans*, and *D. subobscura*[33], and new Sigmaviruses in CO_2-sensitive individuals of *D. affinis* and in *D. obscura*[34], and subsequently in *D. immigrans*, *D. tristis*, and *D. annanassae*[35].

With the widespread application of high-throughput sequencing technologies, the metagenomic (transcriptomic) sequencing of wild-collected flies is now starting to revolutionise our understanding of the drosophilid virome. The first explicitly metagenomic virus study in *Drosophila* discovered the first DNA virus of a drosophilid, *D. innubila* Nudivirus[36]. Subsequently, RNA and small-RNA sequencing of around 3000 *D. melanogaster* from the United Kingdom and 2000 individuals of several species from Kenya and the USA (primarily *D. melanogaster*, *D. ananassae*, *D. malerkotliana* and *Scaptodrosophila latifasciaeformis*) has been used to identify more than 20 new RNA virus genomes and genome fragments, and a single near-complete DNA virus (Kallithea virus, Nudivirus)[31]. Metagenomic sequencing targeted to CO_2 sensitive individuals has also recently been

used to identify novel Sigmaviruses and other Rhabdoviruses in *D. algonquin*, *D. sturtevanti*, *D. busckii*, *D. subobscura*, *D. unispina*, and *S. deflexa* [32].

In total, studies using classical virology, serendipitous transcriptomic discovery, and metagenomic sequencing have reported more than 60 viruses associated with the Drosophilidae and *Drosophila* cell culture (for a comprehensive list, see supporting information file 1). And, while the lost 'classical' viruses and incomplete metagenomic genomes make the exact number uncertain, around 50 distinct viruses are currently represented by sequence data in public databases. From these it is possible to draw some general observations about the virus community of the Drosophilidae. For example, it is clear that RNA viruses substantially outnumber DNA viruses: of the *ca.* 50 viruses with published sequence, only two are DNA viruses (the Nudiviruses of *D. innubila*[36] and *D. melanogaster*[31]). However, the extreme sampling bias introduced by targeted viral discovery, such as CO_2-sensitivity analysis for Sigmaviruses (Rhabdoviridae[32]), makes it difficult to draw robust conclusions about the taxonomic composition of the *Drosophila* viruses. For example, amongst the RNA viruses, positive sense single stranded (+ssRNA) viruses are generally more common than other groups, but in fact negative sense viruses (-ssRNA, including the Rhabdoviridae) constitute around 30% of classifiable *Drosophila* RNA viruses, and double-stranded (dsRNA) viruses nearly as high a proportion (supporting online file 1). To generalise such patterns, and to gain broader insight into the host-range of *Drosophila* viruses and their relationship to the viruses of other organisms will require further, unbiased, metagenomic sequencing.

Here we report the viruses discovered through metagenomic sequencing of RNA from around 1600 wild-collected flies of the species *D. immigrans*, *D. obscura*, *D. subobscura*, *D. subsilvestris*, *D. tristis* and *S. deflexa*, and the re-analysis of two putatively virus-like sequences previously identified in a large pool of mixed *Drosophila*[31]. In total we describe 25 new viruses, and place these within the phylogenetic diversity of known viruses and undescribed virus-like sequences from public transcriptomic datasets. Remarkably, in wild *D. immigrans* we identify new viruses that are extremely closely related to the laboratory models DXV (previously known only from *Drosophila* cell culture) and Flock House Virus (originally isolated from beetles), and we detect the presence of *Armadillidium vulgare* iridescent virus[37] in *D. immigrans* and *D. obscura*—only the third DNA virus to be reported in a drosophilid. We find that a few viruses, such as La Jolla virus[31], appear to be generalists, and that many viruses are shared between the closely-related members of *obscura* group of *Drosophila*, but that viruses are not often shared between more distantly-related species. We discuss our findings in the context of the Drosophilidae as a model clade for studying host-virus coevolution, and the diversity and host range of invertebrate viruses more generally.

Methods

Sample collections and sequencing

We collected around 1400 adult flies representing five species in the United Kingdom in summer 2011 (*D. immigrans*, *D. obscura*, *D. subobscura*, *D. subsilvestris*, and *D. tristis*) and 200 *S. deflexa* in France in summer 2012. Flies were netted or aspirated from banana/yeast bait in wooded and rural areas at intervals of 24 hours for up to a week at each location. They were sorted morphologically by species, and RNA was extracted using Trizol (Ambion) according to the manufacturer's instructions. Females of the obscura group (including *D. obscura*, *D. subobscura*, *D. subsilvestris*, and *D. tristis*) are hard to identify morphologically, and for these species only males were used for RNA extraction and sequencing.

In total, 498 *D. immigrans* were collected in three groups (63 flies in July 2011 Edinburgh 55.928N, 3.170W; 285 flies in July 2011 Edinburgh 55.921N, 3.193W; 150 flies July 2011 Sussex 51.100N,

0.164E). 502 *D. obscura* males were collected in four groups (280 flies collected in July 2011 Edinburgh 55.928N, 3.170W; 52 flies October 2011 Edinburgh 55.928N, 3.170W; 115 flies July 2011 Sussex 51.100N, 0.164E; 55 flies August 2011 Perthshire 56.316N, 3.790W). 338 *D. subobscura* males were collected in four groups locations (60 flies collected in July 2011 Edinburgh 55.928N, 3.170W; 60 flies in October 2011 Edinburgh 55.928N, 3.170W; 38 flies July 2011 Sussex 51.100N, 0.164E; 180 flies August 2011 Perthshire 56.316N, 3.790W). 64 *D. subsilvestris* were collected in three groups (44 flies collected in July 2011 Edinburgh 55.928N, 3.19W; 15 flies in October 2011 Edinburgh 55.928N, 3.19W; 5 flies in August 2011 Perthshire 56.316N, 3.790W). 29 *D. tristis* in two groups from a single location (21 flies collected July 2011 Edinburgh 55.928N, 3.190W; 8 flies October 2011 Edinburgh 55.928N, 3.190W), and approximately 200 *S. deflexa* in a single collection (August 2012 in Le Gorges du Chambon, France 45.66N, 0.556E). Sequence data subsequently showed that some of these collections may be contaminated with other species. In particular, around 2% of reads in the *D. subobscura* sample appear to derive from *D. tristis*, and around 5% of reads in the *D. subsilvestris* sample may derive from *D. bifasciata*.

RNA was treated with DNAse (Turbo DNA-free, Ambion) to reduce DNA contamination, and precipitated in RNAstable (Biomatrica) for shipping. All library preparation and sequencing was performed by the Beijing Genomics Institute (BGI tech solutions, Hong Kong) using the Illumina platform and either 91nt or 101nt paired-end reads. Raw data have been submitted to the sequencing read archive (SRA) under project accession SRP070549. Initially, two separate sequencing libraries were prepared for *D. immigrans*, the first used Ribo-Zero (Illumina) depletion of rRNA to increase the representation of viruses and host mRNAs (SRR3178477), and the second used duplex-specific nuclease normalisation (DSN) to increase the representation of rare transcripts (SRR3178468). Subsequently, for each of the other species a single library was prepared, again using DSN normalisation (*D. obscura* SRR3178507; *D. subobscura* SRR3180643; *D. subsilvestris* SRR3180644; *D. tristis* SRR3180646; *S. deflexa* SRR3180647). Unfortunately, due to a miscommunication with the sequencing supplier, these six libraries were subject to poly-A selection prior to normalisation. This process substantially increases the amount of viral sequence available for assembly and identification (by excluding rRNA), but will strongly bias viral discovery toward virus genomes and subgenomic products that are poly-adenylated (e.g. Picornavirales). Sequencing resulted in an average of 48 million read pairs per library, ranging between 47.3M read pairs for *D. subobscura* and 52.7M read pairs for the *D. immigrans* DSN library.

Virus genome assembly and identification

Raw reads were quality-trimmed using sickle (version 1.2[38]) only retaining reads longer than 40nt, and adapter sequences were removed using cutadapt (version 1.8.1[39]). Paired-end sequences were then *de novo* assembled using Trinity (version 2.0.6[40]) with default parameters, and the resulting raw unannotated assemblies are provided in supporting information file 2.

We took two approaches to identify candidate 'virus-like' sequences for further analysis. First, for each nominal gene assembled by Trinity, we identified and translated the longest open reading frame, and used these translations to query virus sequences present in the Genbank non-redundant protein database ('nr')[41] using blastp (blast version 2.2.28+)[42] with default parameters but an e-value threshold of 10^{-5} and retaining the single 'best' hit. Second, for each nominal gene, we used the transcript with the longest open reading frame to query virus sequences in 'nr' using blastx with default parameters, but again using an e-value threshold of 10^{-5} and retaining the single 'best hit'. These two candidate lists, comprising all the sequences for which the top hit was a virus, were then combined and used to query the whole of 'nr' using blastp, using an e-value threshold of 10^{-5} and retaining the top 20 hits. Sequences for which the top hit was still a virus, and sequences with a blastx hit to viruses but no other blastp hits in 'nr', were then treated as putatively viral in origin, and subject

to further analysis. In parallel with these analyses, raw data that we previously reported from *D. melanogaster*[31] were re-assembled and re-analysed in the same way.

For each putative virus fragment we identified other virus-like fragments in the same host that showed sequence similarity to the same virus taxonomic group, e.g. combining all Negevirus-like sequences in *D. immigrans*, or all Rhabdovirus-like sequences in *D. obscura*. We then manually ordered and orientated these fragments by reference to the closest relatives in Genbank to identify longer contigs that had not been assembled by Trinity. In some cases we were able to identify very long contigs (i.e. near-complete viral genomes) in the Genbank Transcriptome Shotgun Assembly database ('tsa_nt'), and used these to order and orientate virus fragments. Where helpful, we used the longer TSA sequences to query our *Drosophila* metagenomic data using tblastx and thereby identify further fragments to complete viral genomes. In cases of ambiguity (e.g. due to the presence of two closely related viruses in the same host), we took a conservative approach, and did not manually join contigs. Near-complete genome sequences from Nora viruses of *D. immigrans* and *D. subobscura*, and Sigmaviruses of *D. tristis* and *S. deflexa*, were reported previously and are not further analysed here[32,33]. The remaining virus contigs are reported here and have been submitted to Genbank under accession numbers KU754504-KU754539.

Phylogenetic analysis

We inferred the phylogenetic placement of each virus using a conserved region of coding sequence. Where possible, this was the RNA polymerase, as these tend to be highly conserved in RNA viruses. We used blastp to query the Genbank non-redundant protein database ('nr') and tblastn to query the Genbank Transcriptome Shotgun Assembly database ('tsa_nt') to identify potential relatives for inclusion in the phylogenetic analysis. For viruses that could be tentatively assigned to a well-studied group (e.g. Iflaviruses, Nodaviruses), we additionally selected key representative members of the clade from the NCBI Virus genomes reference database[43]. We aligned protein sequences using Mcoffee from the T-Coffee package[44], combining a consensus of alignments from ClustalW[45], T-coffee[44], POA[46], Muscle[47], Mafft[48], DIALIGN[49], PCMA[50] and Probcons[51]. Consensus alignments were examined by eye, and the most ambiguous regions of alignment at either end removed. Nevertheless, as expected for an analysis of distantly-related and rapidly-evolving RNA viruses, these alignments retain substantial ambiguity and deep relationships within the resulting phylogenetic trees should be treated with caution. Alignments are provided in supporting information file 3.

Alignments were used to infer maximum-likelihood trees using PhyML (version 20120412)[52] with the LG substitution model[53], empirical amino-acid frequencies, and a four-category gamma distribution of rates with an inferred shape parameter. Maximum parsimony trees were used to provide the starting tree for the topology search, and the preferred tree was the one with the highest likelihood identified after both nearest-neighbour interchange (NNI) and sub-tree prune and re-graft (SPR) searches. Support was assessed in two ways, first using the Shimodaira-Hasegawa-like nonparametric version of an approximate likelihood ratio test (see ref. 54), as implemented in PhyML, and second by examining 100 bootstrap replicates.

Origin of RNA sequence reads

To infer the proportion of reads mapping to each virus, and to detect potential cross-species contamination in the fly collections, quality-trimmed reads were mapped to all the new and previously published drosophilid virus genomes, and to a 343 nt region of Cytochrome Oxidase I that provides a high level of discrimination between drosophilid species. Mapping was performed using Bowtie 2 (version 2.2.5)[55] with default parameters and global mapping, and only the forward read in each read pair was mapped. To reduce the potential for cross-mapping between closely-related sequences, we excluded all trimmed reads with fewer than 80 contiguous non-N characters.

Results

In total we identified 25 new RNA viruses through metagenomic sequencing of wild caught Drosophilidae. Among those that could easily be classified were four members of the Picornavirales, three Rhabdoviruses, two Nodaviruses, two Reoviruses and an Entomobirnavirus (Fig 1). Among those lacking a current classification were five viruses distantly related to Negeviruses, four viruses distantly related to Sobemoviruses and Polerovirus, two distantly related to Flaviviruses, and two distantly related to Tombusviruses (Fig 2). It is striking that among this latter group there are many viruses that are closely related to unrecognised virus-like sequences in transcriptomic data. Indeed, of the 368 virus-like sequences we included in our phylogenetic analyses, more than half (63%) were derived from transcriptome data rather than from published viruses, illustrating the under-sampling of RNA viruses generally. All phylogenetic trees, including support values and Genbank accession numbers, are provided in supporting information file 4.

Following common practice, we have provisionally named the new *Drosophila* viruses after localities near to our collection sites. We have chosen this approach as it avoids associating the sequence with higher levels of either the host or virus taxonomy, when both may be uncertain. The new *Drosophila* viruses are each represented by between 1.8 kbp and 13.7 kbp of sequence (Tartou virus of *S. deflexa*, and Lye Green Virus of *D. obscura*, respectively), and six are likely to be near-complete genomes with more than 9 kbp of sequence each. We have not named, and do not report, virus sequences that were near-identical to previously published viruses (i.e. K_s<0.3, or falling within the published diversity of other viruses), but for read numbers of previously published viruses see Fig 3.

New viruses closely related to viruses of D. melanogaster

For around half of the newly-discovered viruses (11 of 25), the closest previously-reported relative was associated with *D. melanogaster*. Most striking of these is Eridge virus, a segmented dsRNA Entomobirnavirus closely related to the *D. melanogaster* laboratory model, Drosophila X virus[56] (Fig 1 D; 78% sequence identity and 83% amino-acid identity in Segment A). DXV has not yet been observed in wild flies, but has been reported from flies injected with fetal bovine serum, and has therefore been considered a cell culture contaminant[57]. We detect sequences >98% identical to Eridge virus in some *Drosophila* cell cultures in addition to DXV (e.g. ModEncode dataset SRR1197282 from S2-DRSC cells[58]), showing that fly cell cultures can harbor both viruses.

Other viruses that are also closely related to a published *Drosophila* viruses include Machany virus of *D. obscura* (unclassified Picornavirales close to Kilifi virus and Thika virus of *D. melanogaster*; Fig 1 A), Grange virus of *D. subobscura* (a Reovirus close to Bloomfield virus of *D. melanogaster*; Fig 1 F), Craigmillar Park virus of *D. subsilvestris* (an Alphanodavirus close to Craigie's Hill virus of *D. melanogaster*; Fig 1 C), Grom virus and Prestney Burn virus (of *D. obscura* and *D. subobscura* respectively, both close to Motts Mill virus of *D. melanogaster*; Fig 2 C), and Muthill virus and Marsac virus (of *D. immigrans* and *S. deflexa* respectively Fig 2 F; both close to Brandeis virus identified in publicly available *D. melanogaster* sequence data from laboratory stocks[31,59]).

New viruses very closely related to viruses of other species

We identified two new viruses that are extremely closely related to viruses reported from other taxa. Newington virus of *D. immigrans* is an Alphanodavirus extremely similar to Boolarra virus[60] (isolated from the lepidopteran *Oncopera intricoides*; 84% nucleic acid identity and 89% amino-acid identity in the polymerase), the widely-used laboratory model Flock House virus[60] (from the coleopteran *Costelytra zealandica*; 79% nucleic acid and 87% amino acid identity) and American Noda virus (ANV, identified from small RNAs of *D. melanogaster* cell culture[29]). This clade of closely-related nodaviruses also includes Bat Nodavirus (detected in the brain tissue of the insectivorous bat

Eptesicus serotinus[61]) and transcriptome sequences from the flies *Bactrocera cucurbitae*[62] and *Ceratitis capitate* [63].

We also identified a novel Cripavirus in *S. deflexa* that is very closely related to Goose Dicistrovirus (90% sequence identity, 92% amino-acid identity), recently identified in a faecal sample from geese[64]. However, given that the next closest relatives to this sequence are a transcriptome sequence from the stalk-eyed fly *Teleopsis dalmanni*[65], and a Cripavirus present in publicly available transcriptome data from *D. kikkawai* (supplementary information in ref. 31), we think it likely that these represent invertebrate viruses. To reflect this, and given the divergence between them, we have decided to consider the *S. deflexa*-associated sequence as a new virus, and have provisionally named it Empeyrat virus.

New viruses without close relatives

The remaining half of the new viruses (13 of 25) do not have published close relatives, although many are related to unrecognised viruses in transcriptome datasets. Most notable among these are Kinkell virus of *D. subsilvestris* and Corseley virus of *D. subobscura*. Kinkell virus, along with transcriptome sequences from the fly genera *Bactrocera*[62,66] and *Ceratitis*[63], the beetle *Colaphellus*[67], the thrip *Frankliniella*[68], and the spider *Latrodectus*[69], appears to define a major new clade that falls within or close to the Iflaviruses (Fig 2 D). Similarly, Corseley virus, which is almost identical to transcriptome sequences from *D. pseudoananassae*[70] and is related to transcriptome sequences from the bug genus *Lygus*[71] and the beetle genus *Anoplophora*[72], appears to define an entirely new group of viruses distantly related to Tombusviridae and the recently-described *Diaphorina citri* associated C virus[73] (which is itself closely related to the newly identified Tartou virus of *S. deflexa*; Fig 2 E).

Two other groups are also noteworthy. First, the clade that includes Takaungu virus—which we have identified through re-analyses of mixed Drosophilid sequences from Kenya[31]—and Hermitage virus of *D. immigrans*. These viruses are most closely related to a transcriptome sequence from the Neuropteran *Conwentzia psociformis*, and the enigmatic Gentian Kobu-sho-associated virus, which is reported to be an extremely large dsRNA relative of the Flaviviruses (Fig 1 B; ref. 74, see also ref. 75). Second, the clade that includes Blackford virus of *D. tristis*, Buckhurst virus of *D. obscura*, and Bofa virus (also derived from the Kenyan pool[31], incorporating three unnamed fragments KP757936, KP757935, and KP757975). These viruses, along with seven transcriptome sequences from various arthropods and Muthill, Marsac, and Bradeis virus (described above), appear to represent a major group of insect-infecting viruses that fall between the recently proposed Negeviruses[76] and the plant virus family Virgaviridae.

A DNA Iridescent virus in Drosophila

In *D. immigrans* and *D. obscura* we identified more than 900 read pairs almost identical to the DNA iridescent virus of *Armadillidium vulgare* (Invertebrate Iridovirus 31, ref. 37). Although read numbers were relatively small (around 700 high-quality read pairs in *D. obscura* and 250 read pairs in *D. immigrans*), they do not represent low-complexity sequence, they are widely distributed around the viral genome, and they suggest that viral genes were being expressed (i.e. present in RNA). The longest contiguous region of coverage in *D. obscura* corresponded to the virus major capsid protein, and displayed 98% sequence identity to the virus (K_S=0.08). These data suggest that this virus has a broad host range, and represent the third DNA virus to be identified naturally infecting a drosophilid.

The distribution of virus reads across host species

To explore the distribution of viruses across hosts, we mapped high-quality reads from all libraries to new and previously reported *Drosophila* virus sequences (Fig. 3). We included a UK sample of *D. melanogaster* and a mixed drosophilid pool from Kenya and the USA, that we presented previously[31]. Overall, approximately 1% of reads were viral in origin, ranging from 0.02% in the *D. tristis* pool to

6.96% in the mixed drosophilid pool. As expected, many published viruses were absent. These include all the Rhabdoviruses from host species not present in our collections (Rhabdoviruses from *D. affinis*, *D. busckii*, *D. montana*, *D. surtevanti*, *D. algonquin* and *D. unispina*[32]) and the Cripavirus identified in public RNA reads from *D. kikkawai*[31] (host also absent from our collections). Absent viruses also included the five that have only been previously identified in cell culture (Drosophila X Virus, American Nodavirus, *D. melanogaster* Birnavirus, *D. melanogaster* Totivirus[29], and the totivirus from public dataset SRR1197466 [31]), and also Berkeley virus (identified in reads from SRR070416[31]).

The number of viruses varied substantially among metagenomic pools. Normalising by sequence length and by the number of reads from host Cytochrome Oxidase 1 (to account for different total read numbers, rRNA contamination, and sequence lengths) we were able to detect between 4 viruses (*D.tristis*) and 27 viruses (*D. immigrans* DSN) per pool at 0.001% of COI expression. The number of detectable viruses was positively correlated with the number of flies in the single-species samples, and the strength of the relationship increased with the expression threshold for inclusion (Spearman rank correlations: at 0.001% of COI ρ=0.86, p=0.02; at 0.01% of COI ρ=0.96, p=0.0008; and at 0.1% of COI ρ=0.96, p=0.003). For *D. immigrans*, the DSN library detected more viruses than the rRNA depleted library, regardless of threshold. Note that the presence of some cross-mapping between related viruses means that estimates of the number of viruses will tend to be slightly inflated at low thresholds.

Although our sampling scheme precludes a rigorous formal analysis of host range, some viruses do appear to be generalists and others specialists. Using the 0.01% threshold, the majority of Rhabdoviruses (including Sigmaviruses) appeared to be restricted to a single host: assuming the apparant low level of DImmSV in *D. melanogaster* is due to cross-mapping, only Cherry Gardens Virus (related to Soybean cyst nematode associated northern cereal mosaic virus[77]) was present in two host species (*D. subobscura* and *D. subsilvestris*). In contrast, a few viruses appeared to have a broad host range: La Jolla virus (Iflavirus), Blackford virus (related to Negeviruses and the Virgaviridae), Corseley Virus (related to Tombusviruses), and Pow Burn virus (Picornavirales, related to Fisavirus 1) were each present in four species at >0.01% COI, and small numbers of La Jolla virus reads were detected in all pools except *S. deflexa*. Considering read frequencies across all viruses, members of the obscura group displayed the greatest similarity to each other (Fig.3; *D.obscura*, *D. subobscura*, *D. tristis* and *D. subsilvestris*), while *S. deflexa* was the most distinct, with 6 of its viruses not present in any other pool, and only two of the viruses from other pools present in *S. deflexa*.

Discussion

New viruses of Drosophila

The twenty-five new viruses we present here bring the total number of viruses reported from the Drosophilidae to approximately 85 (see supporting information file 1). However, as with the majority of metagenomic studies, it remains uncertain whether these viruses constitute active infections of *Drosophila*, or whether they are contaminants of the host surface or gut lumen, or infections of an unrecognised parasite. Small RNA sequencing can be used to demonstrate that viruses do replicate within arthropod hosts, and are targeted by their immune system[30,31]. As two of the viruses reported here (Takaungu virus and Bofa virus) were identified through re-analysis of data from Webster *et al* [31], we were able to test whether these viruses show the expected siRNA profile. As expected, we do detect 21nt siRNAs from both strands of these two viruses, consistent with their replication in *Drosophila*. Indeed, in the earlier analysis[31] we identified an unnamed but putatively viral sequence purely on the basis of 21nt siRNAs that can now be shown to be part of Bofa virus (Genbank accession KP757975; sufficient similarity to identify Bofa virus using blast is now provided by

Buckhurst virus). Although siRNA data are not available for the other 23 novel viruses, fifteen them are so closely related to known insect viruses or sequences from insect transcriptomes (Fig 1, Fig 2), and/or are present at such high levels (greater than 10% of host COI in the cases of Muthill Virus and Eridge Virus), that is seems highly likely that the associated drosophilid is indeed the host. For the remaining eight—namely Braid Burn virus, Cherry Gardens Virus, Blackford virus, La Tardoire virus, Hermitage virus, Pow Burn virus, Tartou virus, and Soudat virus—conclusive demonstration of a drosophilid host must await siRNA sequencing or experimental confirmation.

Three groups of newly discovered and currently unclassified viruses seem particularly prominent within the drosophilid samples presented here. First, near to the Sobemoviruses and Poleroviruses are a large clade of invertebrate-infecting viruses defined by Ixodes Tick Associated viruses 1 and 2 (ref 80), Humaita-Tubiacanga virus[30], the *Drosophila*-associated Grom virus, Prestney Burn virus, Motts Mill virus, Braid Burn Virus and La Tardoire virus, and transcriptome-derived sequences predominantly from Hymenoptera and Hemiptera. Second, branching basally to the Negeviruses (and potentially between Negeviruses and Virgaviridae) are two clades including the *Drosophila*-associated Blackford virus, Bofa virus, Buckhurst Virus, Brandeis virus, Muthill virus and Marsac virus, along with transcriptome-derived sequences dominated by Diptera and Hymenoptera. Third, near to the Tombusviridae are the clades defined by *Diaphorina citri* associated C virus[73], Tartou virus and Corseley virus from the Drosophilidae, and transcripts from various invertebrates. All three groups appear to represent common and widespread infections of invertebrates that warrant taxonomic recognition.

Virus diversity and host range

Rapid viral discovery, facilitated by large-scale metagenomic sequencing and the serendipitous discovery of viral genomes in transcriptomic data, is revolutionising our understanding of virus diversity. The Drosophilidae provide a clear example of this, with approximately ten viruses reported prior to the year 2000, eleven more between 2001 and 2014, and more than 60 since 2015. Particularly striking is the frequency with which completely new, and deeply divergent, lineages of RNA viruses are being identified. Recent examples include the enormous and unexpected diversity of basally-branching –ssRNA viruses[78], the diversity of basal Flaviviridae[75], the Negeviruses[76], and the Phasmoviruses (Bunyaviridae)[79].

How many invertebrate viruses are there, and when will the accelerating virus-discovery curve start to saturate? The *ad hoc* but intensive sampling of *Drosophila* suggests that such questions will require systematic estimates of the distribution of virus host ranges, the distribution of virus geographic ranges, and the distribution of virus prevalences. First, many *Drosophila* viruses are multi-host and widely-distributed. Around 10 of the 25 new viruses we report are detectable in multiple species, and we also detect previously published viruses of *D. melanogaster* in *D. immigrans* and members of the obscura group (Fig. 3). Similarly, our earlier PCR survey of *D. melanogaster* viruses[31] detected 12 of 16 viruses in more than a third of *D. melanogaster* populations, and 10 of them in at least one *D. simulans* population. Second, it seems likely that more closely-related hosts share more viruses. This is consistent with the apparently high overlap in virus community between *D. melanogaster* and *D. simulans*[31] and among members of the obscura group, and the divergent set of viruses associated with *S. deflexa* (Fig. 3). It is also consistent with the absence of *D. melanogaster* viruses from metagenomic surveys of other invertebrate taxa (although Goose dicistrovirus is closely related to Empeyrat virus of *S. deflexa*). Third, it is clear that viruses vary enormously in prevalence, such that few viruses are common and many are rare. Of the 16 viruses we previously surveyed by PCR, only three ever exceeded 50% prevalence, and most only exceeded 10% prevalence in two or three of the surveyed populations. This is consistent with the strong relationship we find between sample size and virus number, and suggests that many hundreds of *Drosophila* individuals are required to comprehensively survey a population.

Conclusions

The 25 new viruses we present here expand the catalogue of recorded drosophilid-associated viruses by nearly 50%, and identify several new clades of insect-associated viruses. These include a new clade of Iflaviruses (Kinkell virus), new clades related to the Tombusviridae (Corseley virus and Tartou virus), and new clades related to the Negeviruses and Virgaviridae (including 6 viruses detected in *Drosophila*). Nevertheless, the large numbers of undescribed viruses present in transcriptome datasets illustrates that, across the invertebrates as a whole, there are many more viruses and many more deeply divergent virus lineages to uncover.

We expect that the future isolation of these *Drosophila*-associated viruses will provide useful laboratory tools to better understand host-virus biology and host range. However, it is possible to capitalise on viral sequences to address these questions even in the absence of viable viral isolates, and new virus sequences *per se* are likely to prove valuable[33]. In addition, given the widespread experimental use of model viruses that are not known to infect *D. melanogaster* in the wild, such as Flock House Virus[81,82], Drosophila X Virus[83,84], and Invertebrate Iridovirus 6 (ref. 85), it is reassuring to know that these viruses have close relatives naturally associated with the Drosophilidae (Newington Virus, Eridge Virus, and *Armadillidium vulgare* iridescent virus, respectively, in *D. immigrans*).

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Author Contributions

Conceived and designed the experiments: DJO BL. Performed experiments: DJO BL SHL CLW. Analysed the data: DJO. Wrote the first draft of the manuscript: DJO. Contributed to the writing of the manuscript: DJO BL SHL CLW.

Figure 1: Viruses related to well-studied clades

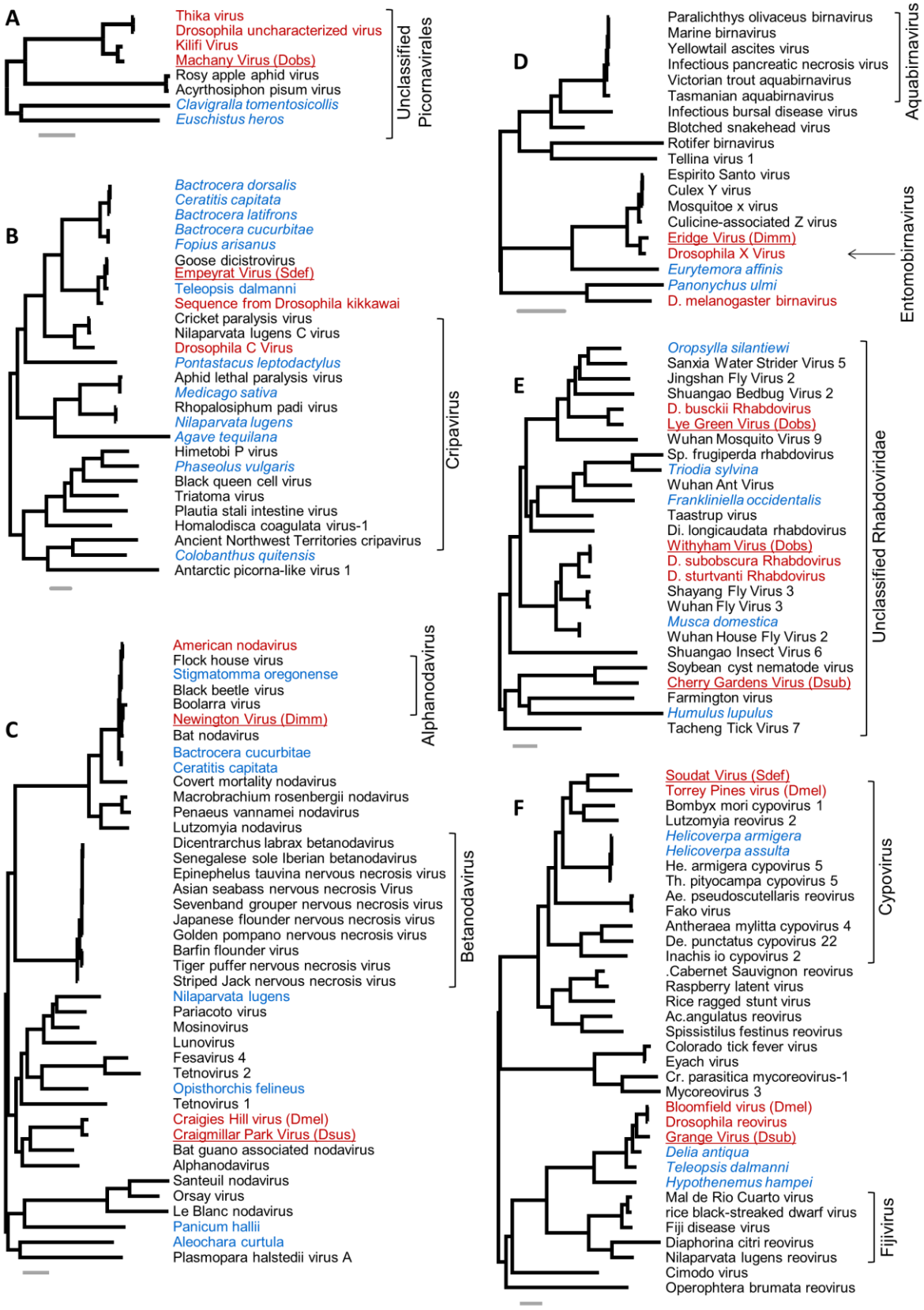

Figure 1: Viruses related to well-studied clades

Mid-point rooted maximum-likelihood phylogenetic trees for the viruses reported here, inferred using polymerase protein sequences. The grey scale bars represent 0.5 amino-acid substitutions per site. In each tree, viruses reported from Drosophilidae in labelled in red, published viruses from other taxa are labelled in black, and unannotated virus-like sequences from publicly-available transcriptome datasets labelled in blue. Viruses newly-reported here are underlined, and *Drosophila* species abbreviations are given for the reference sequence (Dimm – *D. immigrans*; Dobs – *D. obscura*; Dsub – *D. subobscura*; Dsus – *D. subsilvestris*; Dtri – *D. tristis*; Sdef – *Scaptodrosophila deflexa*). Tree A: Viruses near to the Dicistroviridae (Picornavirales). B: Putative Cripaviruses (Dicistroviridae, Picornavirales). C: Nodaviruses. D: Birnaviruses. E: Unclassified members of the Rhabdoviridae that form the sister clade to the Cytorhabdoviruses and the Nucleorhabdoviruses[32]. F: Reoviridae. Alignments are provided in online supporting file 3, and clade support values and sequence accession identifiers are provided in online supporting file 4.

Figure 2: Viruses not closely related to well-studied clades

See Fig. 1 for a key to the colours and abbreviations. Tree A: Unclassified Picornavirales. B: Unclassified clade of basally-branching Flavi-like viruses[75]. C: An unclassified clade that branches basally to Poleroviruses and Sobemoviruses[80]. C: Nodaviruses. D: Iflaviruses, including a new clade that falls within (or close to) the Iflaviruses. E: Two unclassified clades related to the Tombusviridae[73]. F: Two unclassified clades related to the Negeviruses and the Virgaviridae. Alignments are provided in online supporting file 3, and clade support values and sequence accession identifiers are provided in online supporting file 4.

Figure 2: Viruses not closely related to well-studied clades

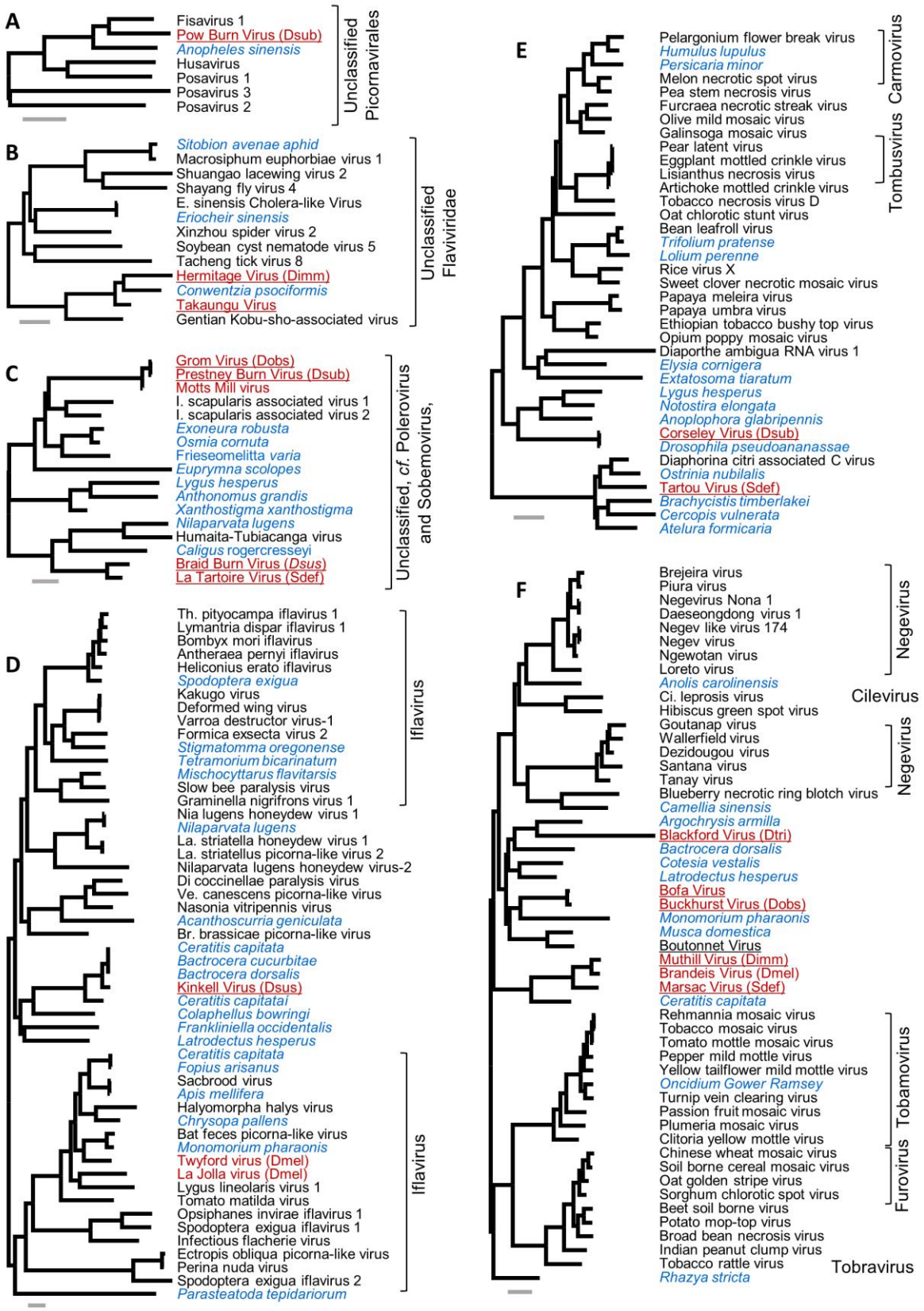

Figure 3: Virus read numbers (relative to host COI, normalised for length)

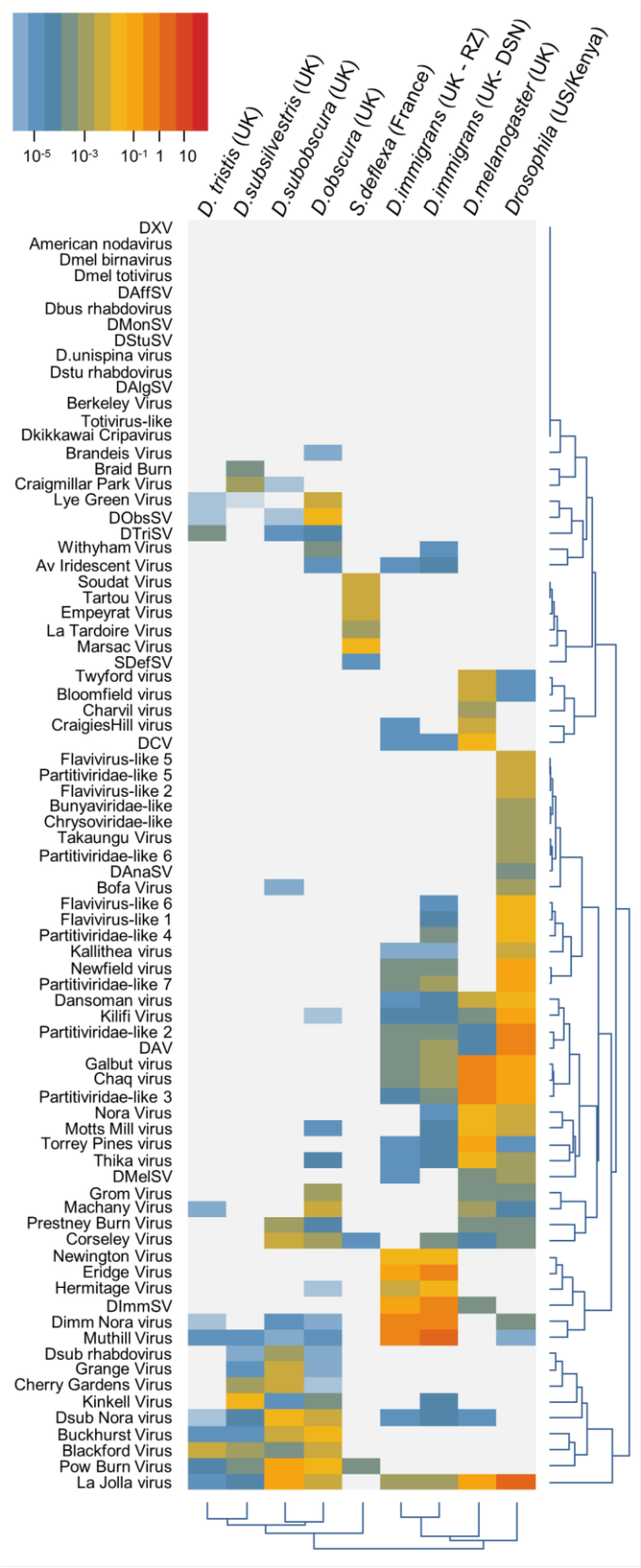

Figure 3: Virus read numbers

A heatmap showing the relative number of high quality (80 nt) forward reads from each library that map to each of the *Drosophila* viruses. Read numbers are normalised by target sequence length and by the number of reads mapping to a fragment of the host Cytochrome Oxidase 1 gene, so that a value of 1 implies equal read numbers per unit length of the virus and the host COI. Rows and columns are clustered by the similarity in read frequency on a log scale. Note that some viruses may be sufficiently similar for a small proportion of reads to cross-map.

Tables

Table 1: New viruses reported here.

Provisional Name	Host	Classification	Accession	Description
Blackford virus	Dtri	*cf.* Negevirus	KU754514	+ssRNA. Distantly related to Brandeis virus (detected in RNA-seq data from *D. melanogaster*), to virus-like transcripts from a range of invertebrates, and to Negeviruses [76]. [4.5 kbp fragment encoding a single ORF]
Bofa Virus	(Pool)	*cf.* Negevirus	KU754515	+ssRNA. Distantly related to Brandeis virus (detected in RNA-seq data from *D. melanogaster*, [31]), to virus-like transcripts from a range of invertebrates, and to Negeviruses. Derived from pools E and K of Webster *et al* [31]and replaces two Negevirus-like sequences (KP757936 KP757935) and a small-RNA rich sequence (KP757975) previously reported there. [10.7 kbp near-complete genome encoding a two ORFs]
Braid Burn virus	Dsus	*cf.* Polerovirus Sobemovirus	KU754508	+ssRNA. Related to Motts Mill Virus of *D. melanogaster*, to *Ixodes scapularis* associated viruses 1 and 2 (ref [80]), to Humaita-Tubiacanga virus [30] and to virus-like transcripts from a range of invertebrates. Distantly related to plant Poleroviruses and Sobemoviruses. [2.5 kbp fragment encoding two ORFs]
Buckhurst virus	Dobs	*cf.* Negevirus	KU754516	+ssRNA. Distantly related to Brandeis virus (detected in RNA-seq data from *D. melanogaster*, see supporting information in ref. [31]), to virus-like transcripts from a range of invertebrates, and to Negeviruses. [11.1 kbp near-complete genome encoding a two ORFs]
Cherry Gardens virus	Dsub	Rhabdoviridae	KU754524	-ssRNA. Related to Soybean cyst nematode associated northern cereal mosaic virus [77]. [5.7 kbp fragment encoding a partial polymerase]
Corseley virus	Dsub	Unclassified	KU754520	+ssRNA. Very closely related to virus-like transcripts from a range of invertebrates, including near-identical virus-like transcripts from *D. pseudoananassae*. Distantly related to the Tombusviridae and *Diaphorina citri* associated C virus [73]. [4 kbp fragment encoding three ORFs]

Craigmillar Park virus	Dsus	Alphanodavirus	KU754525 KU754526	+ssRNA Segmented. Closely related to Craigie's Hill virus of *D. melanogaster*, and related to Bat guano-associated Nodavirus [86]. [Near-complete genome of two segments: RNA1 is 2.8 kbp encoding a polymerase, RNA2 is 1.8kbp encoding a putative coat-protein precursor]
Empeyrat virus	Sdef	Cripavirus	KU754505	+ssRNA. Very closely related (90% AA identity) to 'Goose Dicistrovirus' from goose faeces [64], to a virus-like transcript from *Teleopsis dalmanni*, and to a Cripavirus present in raw RNAseq data from *D. kikkawai* supporting material of [31]. [9.2 kbp near-complete genome encoding two open reading frames]
Eridge virus	Dimm	Entomobirnavirus	KU754527 KU754528	dsRNA Segmented. Closely related to Drosophila X Virus (and similarly present in some *D. melanogaster* cell cultures. [Near-complete genome of two segments: Segment A is 3.4 kbp, Segment B is 3.2kbp and encodes a putative polymerase]
Grange virus	Dsub	Reoviridae	KU754536- KU754538	dsRNA Segmented. Related to Bloomfield virus of *D. melanogaster* (ref. [31], see also ref. [23,25,30]), to virus-like transcripts from a range of invertebrates, and to Fijiviruses. By similarity to Bloomfield virus, fragments of segments 1, 2, 6, and 7 are identifiable. [Segment 1 is a 1.7 kbp fragment encoding a partial polymerase, Segment 2 is a 1.9 kbp fragment encoding the partial major core protein, Segment 6 is a 1.1 kbp, fragment Segment 7 is a 1.3 kbp fragment]
Grom virus	Dobs	*cf.* Polerovirus Sobemovirus	KU754506	+ssRNA. Related to Motts Mill Virus of *D. melanogaster*, to *Ixodes scapularis* associated viruses 1 and 2 (ref. [80]), to Humaita-Tubiacanga virus [30] and to virus-like transcripts from a range of invertebrates. Distantly related to plant Poleroviruses and Sobemoviruses. [3 kbp fragment encoding an ORF]
Hermitage virus	Dimm	Unclassified	KU754511 KU754512	RNA. Related to Gentian Kobu-sho-associated virus (reported to be dsRNA, [74] and a virus-like transcript from *Conwentzia psociformis*. Distantly related to Soybean cyst nematode virus 5 and the Flavivirus-like Xinzhou spider virus 2. [Two un-joined contigs of 3.2 kbp and 3.5 kbp encoding a putative polyprotein]
Kinkell virus	Dsus	Iflavirus	KU754510	+ssRNA. Closely related to virus-like transcripts from *Ceratitis* and *Bactrocera*, equally distantly related to Deformed Wing virus and Sacbrood Virus. [6.7 kbp fragment encoding a putative incomplete polyprotein]
La Tardoire virus	Sdef	*cf.* Polerovirus	KU754509	+ssRNA. Related to Motts Mill Virus of *D. melanogaster*, to *Ixodes scapularis*

		Sobemovirus		associated viruses 1 and 2 (ref. [80]), to Humaita-Tubiacanga virus [30] and to virus-like transcripts from a range of invertebrates. Distantly related to plant Poleroviruses and Sobemoviruses. [2.3 kbp fragment encoding two ORFs]
Lye Green virus	Dobs	Rhabdoviridae	KU754522	-ssRNA. Related to *Drosophila busckii* Rhabdovirus [32]. [14.5 kbp near-complete genome encoding five ORFs]
Machany virus	Dobs	Picornavirales	KU754504	+ssRNA. Related to Kilifi virus and Thika virus of *D. melanogaster*, and to Rosy Apple Aphid virus and Acyrthosiphon pisum virus. [4.9 kbp fragment encoding a putative polyprotein]
Marsac virus	Sdef	*cf.* Negevirus	KU754518	+ssRNA. Related to Brandeis virus (detected in RNA-seq data from *D. melanogaster* [31]), to a virus-like transcript from *Ceratitis capitata*, and to Negeviruses. [11.1 kbp near-complete genome encoding a two ORFs]
Muthill virus	Dimm	*cf.* Negevirus	KU754517	+ssRNA. Closely related to Brandeis virus (detected in RNA-seq data from *D. melanogaster* [31]), to a virus-like transcript from *Ceratitis capitata*, and to Negeviruses. [10.6 kbp near-complete genome encoding a two ORFs]
Newington virus	Dimm	Alphanodavirus	KU754529 KU754530	+ssRNA Segmented. Very closely related to Boolarra virus and Bat nodavirus. [Near-complete genome of two segments: RNA1 is 3 kbp encoding a polymerase, RNA2 is 1.2kbp encoding a putative coat-protein precursor]
Pow Burn virus	Dsub	Picornavirales	KU754519	+ssRNA. Related to Fisavirus 1 and to a virus-like transcript from *Anopheles sinensis*. [9.3 kbp near-complete genome encoding a single polyprotein]
Prestney Burn virus	Dsub	*cf.* Polerovirus Sobemovirus	KU754507	+ssRNA. Related to Motts Mill Virus of *D. melanogaster*, to *Ixodes scapularis* associated viruses 1 and 2 (ref. [80]), to Humaita-Tubiacanga virus [30] and to virus-like transcripts from a range of invertebrates. Distantly related to plant Poleroviruses and Sobemoviruses. [3 kbp fragment encoding two ORFs]
Soudat virus	Sdef	Cypovirus	KU754531- KU754534	dsRNA Segmented. Related to Torrey Pines virus of *D. melanogaster* and to Bombyx mori Cypovirus 1 and Lutzomyia reovirus 2 [30]. By similarity to Torrey Pines virus, fragments of segments 1, 2, 3, and 5 are identifiable. [Segment 1 is near-complete 3.7 kbp encoding a polymerase, Segment 2 is a 0.6 kbp fragment, Segment 3 is near-complete 3.9 kbp encoding the major core protein, Segment 5

				is a 1.3 kbp fragment]
Takaungu virus	(Pool)	Unclassified	KU754513 KP757925	RNA. Related to Gentian Kobu-sho-associated virus (reported to be dsRNA; [74]) and a virus-like transcript from *Conwentzia psociformis*. Distantly related to Soybean cyst nematode virus 5 and the Flavivirus-like Xinzhou spider virus 2 (ref. [75]). Derived from pools E and K of Webster *et al* [31], this virus incorporates Flavivirus-like sequence KP757925 that was previously reported there. [Two un-joined contigs of 2.3 kbp and 3.9 kbp encoding a putative polyprotein]
Tartou virus	Sdef	Unclassified	KU754521	++ssRNA. Related to *Diaphorina citri* associated C virus [73] and virus-like transcripts from a range of invertebrates. Distantly related to the Tombusviridae. [1.8 kbp fragment encoding a single ORF]
Withyham virus	Dobs	Rhabdoviridae	KU754523	-ssRNA. Very closely related to *Drosophila subobscura* Rhabdovirus [32]. [6.9 kbp fragment encoding the polymerase]

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Supporting Online Data

Supporting online file 1: Viruses of the Drosophilidae

A comprehensive list of all *Drosophila* viruses reported to date (excluding retroelements) is provided as an xlsx file. Recorded data include: the virus name, its Baltimore classification, the drosophilid hosts in which it has been detected (excluding experimental infections), its year of discovery, its approximate classification, reference Genbank accession identifiers, and citation for its discovery.

Supporting online file 2: Raw metagenomic assemblies

Compressed fasta files containing the transcriptome assemblies generated for this study.

Supporting online file 3: Alignments used for phylogenetic inference

Compressed fasta-format protein alignments used to infer phylogenetic trees

Supporting online file 4: Phylogenetic trees

Mid-point rooted maximum likelihood trees are presented, with percentage support marked on nodes for which either method identified less than 100% support (marked as 'SH|bootstrap'), and NCBI accession identifiers for the sequences used to infer the phylogeny.