AHDB Horticulture RESEARCH REPORT

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

New viruses discovered in Drosophila pest: the first steps on the road to a novel biopesticide.

Background and expected deliverables

Drosophila suzukii (Matsummura), also known as the spotted wing drosophila (SWD) is an invasive pest of soft and stone fruit crops. Its recent invasion of the fruit growing regions of North America and Europe and the damage it is causing there is driving interest in finding new control solutions. Conventional crop protection methods have many drawbacks and are difficult to implement within integrated pest management (IPM) programmes; consequently, the development of an alternative, IPM compatible biopesticide would be beneficial for growers, consumers, and pest management professionals alike.

The viruses of SWD offer good potential candidates for the development of a microbe-based bioinsecticide yet, to-date, the viruses of SWD remain almost completely unstudied. This study describes the viral diversity of SWD and aims to identify a pathogen suitable for the control of this pest in UK fruit crops. A combination of approaches, both innovative genetic techniques and traditional lab based investigation, will be used to identify the viruses infecting SWD from large samples of wild flies.

Summary of the project and main conclusions

This study characterises the viral diversity of SWD with the aim of identifying a pathogen suitable for the control of this pest in UK fruit crops. To do this we first used a metatransciptomic approach to identify viral genetic sequences from wild SWD. This was achieved by collecting large numbers of flies over three years in the UK, with additional samples from France (2014) and Japan (2016), extracting all genetic material, sequencing those samples and then reconstructing virus genomes from these datasets. The existence of viruses in the original samples was then confirmed by lab based methods. Through this process we have identified 18 new viruses from SWD alone. We describe members of the Picornavirales, Mononegavirales, Bunyavirales, Chuviruses, Nodaviridae, Tombusviridae, Reoviridae, and Nidovirales, and discuss their evolutionary relationships with previously known viruses. The new reovirus, ‘Eccles virus’ belongs to a family of viruses previously advocated for biological control in China. Eccles virus may represent the most promising candidate for further investigation of insecticidal activity. Our results were submitted for publication in the journal ‘Applied and Environmental Microbiology’ and are currently available online at https://www.biorxiv.org/content/early/2017/09/26/190322. We then assessed the mortality of viral biocontrol candidates by injection of viral extracts from wild flies or isolated cultures of our best

candidates from other Drosophila species. Unfortunately, the process of experimental infection did not yield a lethal viral extract and further work will be necessary to fully explore the potential of other viruses discovered. In light of this disappointing lack of mortality we also assessed the susceptibility of SWD to viral infection by exploring its immune response to two different viruses. To do this we conducted a comparative analysis of immune system gene expression between SWD and the closely related *Drosophila melanogaster*. Results are still under analysis but we have identified a number of genes that change expression significantly upon infection with virus. Finally, we are investigating the patterns of virus infection in several species of wild British Drosophila. To do this we have surveyed a large number of Drosophila from five different species giving us a picture of virus ecology and host specificity.

Financial benefits

The impact of this pest on the European horticultural industry has already been substantial, with SWD damage resulting in losses of over €8 million in fruit crops in Northern Italy in 2010 and 2011 and more than €1.5 million for French strawberries in 2011 (FERA, 2015). The European and Mediterranean Plant Protection Organisation (EPPO) in a recent 'Pest Risk Analysis' deemed this organism to be a potential threat to crops in its region. Potential damage is described as "massive" and the regions ability to control it as "with much difficulty" (EPPO, 2010). In the Pacific fruit growing regions of the USA, the estimated damage due to SWD has been calculated at over €400 million/year (Bolda et al., 2010). In Californian raspberries specifically, the damage caused by SWD between 2009 and 2014 has been calculated at $US 39.8 million in revenue losses, equivalent to 2.19% of realized revenues (Farnsworth et al., 2017). With damage estimates for the UK slow to emerge, it is hard to quantify the exact financial damage that this pest has done since its establishment here.

A key consideration for the damage caused by this pest is the effect of disrupting already established IPM programmes. Changes in management techniques, necessitated by the presence of this pest, often include the use of products incompatible with IPM programmes. Without IPM compatible products, damage is not limited to that done by the pest itself but also extends to secondary pest damage caused by the use of broader spectrum or longer persistence products.

Action points for growers

Because of the exploratory nature of this project, there are no action points for growers to date.

SCIENCE SECTION

Introduction

Drosophila suzukii

Belonging to the paraphyletic subgenus Sophophora and the *melanogaster* species group, *D. suzukii* is phylogenetically close to the famous lab model *Drosophila melanogaster* (Lewis et al., 2005, Kopp, 2006). Some striking morphological characters do, however, allow *D. suzukii* to be distinguished from its well-studied relative. Amongst these the presence of dark wing spots in the male (giving rise to the common species name 'Spotted Wing *Drosophila*') and a heavily sclerotized ovipositor bearing tooth-like bristles in the female are most prominent. It is this well-developed ovipositor that is considered to be the evolutionary innovation that allows *D. suzukii* to oviposit under the skin of ripening fruit still on the tree: a feature shared by few other *Drosophila* species (Atallah et al., 2014). Once laid, the eggs of *D. suzukii* develop through three larval instars inside the fruit, feeding on the mesocarp. Complete development, from egg to adult, takes approximately 8 to 10 days at 25 °C, and from 21 to 25 days at 15 °C according to early life history studies (Kanzawa, 1935, Kanzawa, 1939). Further information on oviposition behaviour (Mitsui et al., 2006), host range and overwintering (Walsh et al., 2011) being provided by more recent studies.

Plate 1: D. suzukii females on strawberry fruit. ©Sinclair Stammers

The monitoring scheme in the UK has reported the number of *D. suzukii* adults, caught in bait traps, to peak at some point between September and November depending on weather conditions. As British records of *D. suzukii* only date back three growing seasons, data on the phenology of the organism is still limited. A very broad range of host plants makes *D. suzukii* an especially difficult pest to control. *D. suzukii* is known to oviposit in a wide variety of commercial and wild soft skinned fruit (Walsh et al., 2011, Cini et al., 2012, Mitsui et al., 2010). This allows populations to reside in wild

refuges and may facilitate the reinvasion of crops after periods of intense spraying, fruit unavailability or cold weather. Further work on the small to medium scale population dynamics and ecology of this species are desperately needed in order to aid control.

Pattern of invasion

First described in Japan in 1916 (Matsumura, 1931), *D. suzukii* was reported to be widely distributed in Japan shortly after (Kanzawa, 1939). Although not certainly originating in Japan, this species was subsequently recorded across Asia during the last century: China (Peng, 1937), North and South Korea (Kang and Moon, 1968, Nagayama and Okamoto, 1940), India (Parshad and Duggal, 1965), Thailand (Okada, 1976), Burma (Toda, 1991), Eastern Russia (Sidorenko, 1992) and Pakistan (Amin ud Din et al., 2005). Recent studies, examining the genetic diversity within and between populations of *D. suzukii* populations from around the world, found Japanese populations had the largest number of unique haplotypes, supporting the theory that Japan falls within this species native range (Adrion et al., 2014, Carvajal and Markow, 2010). The first records of this pest from outside Asia came from Hawaii in the 1980's (Kaneshiro, 1983). Several, more recent, records of *D. suzukii* in Hawaii have been published (Beardsley et al., 1999, O'Grady et al., 2002), however, no crop damage is reported from these islands, despite the well-developed fruit growing industry there. Since its detection in Spain during 2008 (Calabria et al., 2012), *D. suzukii* has spread northwards through continental Europe (Vogt et al., 2012, Seljak, 2011, Baroffio and Fischer, 2011) and was reported for the first time in the UK in 2012 (Harris and Shaw, 2014). Further records of detection continue to be published from across Northern and Central Europe (Lavrinienko et al., 2017, Manduric, 2017, Kiss et al., 2016, Piotrowski et al., 2016), with the northerly most detection being in Scandia, Sweden (Manduric, 2017). A recent study found *D. suzukii* to be one of the four most abundant drosophilid species in the growing regions Apulia, Italy ((Antonacci et al., 2017).

Parallel to its spread across the Western Palearctic region, *D. suzukii* has simultaneously invaded the Neartic and Neo-tropical ecozones. The first detection of this species was logged in California (Bolda, 2008), with records soon following from across the western USA (Bolda et al., 2010, Goodhue et al., 2011). The pest was also detected on the eastern seaboard shortly after (Price et al., 2009) and is confirmed to now be breeding in wild fruit in the North Eastern states (Maier, 2012). The patterns of genetic diversity across the USA suggest a scenario in which colonisation has been passively mediated (anthropogenically or by wind) rather than through active dispersal by the species (Adrion et al., 2014). The pest has now been recorded from Canada, from British Columbia in the west (Bolda et al., 2010) to Dunham, Quebec in the east. Spreading southwards, *D. suzukii* has been recorded across Brazil (Deprá et al., 2014) with current records ranging as far south as south Argentina (Lue et al., 2017).

Control

Drosophila suzukii has the potential to cause severe damage to commercial soft fruit crops. During oviposition the female fly punctures the skin (exocarp) of the ripening fruit with her oviscapt. Even if no subsequent larval feeding takes place this wound allows fungi to begin degrading the fruit, rendering it unsalable. In cases where larval feeding occurs in the flesh (mesocarp), the fruit often collapses entirely also leaving that fruit unmarketable.

In light of the rapid spread of *D. suzukii* and potentially serious economic damage it can cause, a huge imperative lays on finding an effective control programme for this pest. The challenge for crop protection scientist is intensified by the biology of this particular organism: a short generation time, wide host range and cryptic feeding stages in close-to-harvest fruit combine to hinder conventional control. Furthermore, control techniques are often sought in crops with existing management programmes designed to control a range of pests whilst limiting chemical input, known as integrated pest management or IPM.

Integrated pest management programmes attempt to introduce alternatives to chemical pesticides: reducing the environmental impacts of pest control, managing resistance to pesticides, improving grower safety and reducing chemical residues in produce. This is achieved through the use of non-chemical control methods. These include: biological control; the introduction or augmentation of the pest's predators, parasitoids or pathogens, cultural control; preventative techniques such as plant variety selection or crop hygiene that pre-emptively reduce the susceptibility of a crop to pest attack and mechanical control; techniques that involve barriers, i.e. netting, or the physical removal of pests. Alongside non-chemical methods, the responsible use of synthetic pesticides, often those with a low environmental impact or high target specificity, also forms a part of most IPM programmes.

Many current control strategies for *D. suzukii* include an element of high volume, short persistence, pesticide sprays. High volume pesticide applications are undesirable for most parties involved in fruit production, firstly because of potential pesticide residue issues: As *D. suzukii* oviposits close to the time of harvest, targeted applications may cause unwanted residue on fruit at point of sale. Most fruit buyers, including supermarkets, and regulators have extremely low tolerances for pesticide residue, due largely to customer demand for pesticide free produce (Collins et al., 1993). A reduction in residues has been key driver in the development of IPM programmes in soft fruit (Cross and Berrie, 2006). Secondly the application of broad spectrum insecticides, as currently advocated for control of *D. suzukii* (Bruck et al., 2011), can have local environmental consequences that, not only effect wild ecosystems but also harm potentially useful biodiversity with the cropping system (reviewed in: Desneux et al., 2007, Biondi et al., 2012, Fountain and Medd, 2015, Crowder and Jabbour, 2014). Thirdly, high volume spray programmes run the risk of driving the rapid development of insecticide resistance in target and non-target pests. This has been the case for a number of invasive crop pests

where pesticide resistance has developed within non-native populations: The tomato pinworm, *Tuta absoluta* (Campos et al., 2014); the Colorado potato beetle, Leptinotarsa decemlineata (Sukhoruchenko and Dolzhenko, 2008, Sharif et al., 2007, Zamojska et al., 2011, Pourmirza, 2005, Stanković et al., 2004); the Asian citrus psyllid, *Diaphorina citri* (Tiwari et al., 2011); and the Q type tobacco whitefly, *Bemisia tabci* (Luo et al., 2010), to name just a few notable examples. Insecticide resistance is a well-studied area of evolutionary biology and consequently a good understanding of the genetic mechanisms behind resistance has been achieved, especially in *Drosophila*, which serves as a useful lab model for the study of insecticide resistance (Morton, 1993).

IPM compatible solutions for *D. suzukii* infestation are, however, emerging. Cultural control, in the form of crop hygiene, currently plays a large part in the control of *D. suzukii*. Collecting, neutralising and disposing of fruit waste correctly, although time consuming, has proven effective and is an important part of control recommendations disseminated to growers (ADHB, 2015). Increasing the overall number of harvests per week, shortening the amount of time that ripe fruit spends, vulnerable to attack, on the crop, is also proving a simple but effective measure to control populations of this pest (Cross, *pers comms*). Trapping has also formed a key component of many *D. suzukii* control programs to date. With various trap types and baits commercially available and a range of placement strategies proven to be effective (Lee et al., 2012, Grassi et al., 2014, Lee et al., 2013, Cha et al., 2013, Cha et al., 2015). Trapping is generally environmentally benign and compatible with existing IPM programmes. Placement of traps does, however, pose a large investment in labour time and expense for growers (Mazzi et al., 2017, Del Fava et al., 2017). Netting, another common cultural control method, including in native Japan (Madoka Nakai, *pers comms*; Plate 2), has proven to be potentially effective in raspberries (Leach et al., 2016) and blueberries (Cormier et al., 2015).

Studies into the biological control of *D. suzukii* using invertebrate natural enemies have given mixed results. Several studies have shown resistance in *D .suzukii* to attack by European parasitoid wasps (Chabert et al., 2012, Kacsoh and Schlenke, 2012, Poyet et al., 2013), whilst others report the spontaneous parasitism of *D. suzukii* in the field (Gabarra et al., 2014, Stacconi et al., 2013, Miller et al., 2015) and successful parasitism in controlled laboratory settings (Rossi Stacconi et al., 2015). Kacsoh and Schlenke (2012) and Poyet et al. (2013) report an association between resistance in *D. suzukii* to parasitoid attack and high haemocyte load in infected individuals. This correlation between increased haemocyte load and resistance to parasitoids has been noted for a number of other species in the melanogaster species group (Eslin and Prévost, 1998), however, total haemocyte load does not appear to be correlated with ability to encapsulate parasitoids in *D. melanogaster* itself despite a high natural variation in encapsulation ability across different European field collected lines (Gerritsma et al., 2013). *D. suzukii* also appears to increase its resistance to parasitoid attack through 'self-medication', i.e. preferentially laying eggs on substrates containing high levels of atropine, an

entomotoxic alkaloid, in the presence of parasitoids (Poyet et al., 2017). A similar behavioural immune response is also seen in *D. melanogaster* (Kacsoh et al., 2013).

Several studies have identified potential predators of *D. suzukii* in the predatory hymenopteran genus *Orius*. These small predatory bugs, or pirate bugs, are currently widely used as inundative biological control agents in covered horticulture. *Orius leavegatus* has been recovered from the field in *D. suzukii* vulnerable crops (Gabarra et al., 2014, Arnó et al., 2012) and proved an efficacious predator of *D. suzukii* eggs in lab condition strawberry fruits (Gabarra et al., 2014). *O. leavegatus* has also been shown to feed on *D. suzukii* adults under lab conditions (Cuthbertson et al., 2014b), however, neither *O. maiusculus*, *O. insidiosus* nor *O. leavegatus* proved particularly voracious in other lab conditions (Malagnini et al., 2014, Woltz et al., 2015) and their role in population suppression in the field remains questionable. Other generalist predators, earwigs for example may have a marginal role in supressing *D. suzukii* by consuming exposed larvae or pupae (Gabarra et al., 2014) but again these cannot be relied upon in isolation.

Another key branch of many IPM programmes is the use of microbial biopesticides. The susceptibility of *D. suzukii* to a number of microbial biological control agents has been tested. Several species of entomopathogenic fungi significantly reduce *D. suzukii* survival in laboratory assays: *Metarhizium anisopliae* (Woltz et al., 2015), *M. brunneum* (Cossentine et al., 2016, Fernández-Bravo, 2014), *Beauveria bassiana* (Cossentine et al., 2016, Cuthbertson et al., 2014a, Gargani et al., 2013, Cuthbertson and Audsley, 2016), *Lecanicillium muscarium* (Cuthbertson et al., 2014a), *Lecanicillium lecanii* (Cossentine et al., 2016) and *Isaria fumosorosea* (Cuthbertson and Audsley, 2016, Cossentine et al., 2016, Naranjo-Lázaro et al., 2014). Primary bioassays are, obviously a key first step to implementing any control measure, however, there is a need for more field scale data on the effectiveness of currently available microbial pesticides. Delivery methods, critical for success in ensuring the necessary spore-to-cuticle contact, along with a whole host of other variables must be tested before solid advice can be given to growers.

Characteristics of viral biological control agents

The viruses of *D. suzukii* offer an interesting potential source for a microbial biological control agent. Similarly to other microbial biological control agents: viruses potentially represent an environmentally benign control agent with high host specificity and low environmental persistence (Hunter-Fujita et al., 1998), making them eminently suitable for inclusion into existing IPM programs. Although some hurdles exist in the commercialisation of insect viruses as control agents (Carter, 1984), the improvement of culturing technologies and the rationalisation of restrictive regulations may, in time, alleviate some of the current difficulties (Sun and Peng, 2007).

Entomopathogenic viruses are represented in many of the known virus families with some families of virus are known to occur solely in arthropods (Hunter-Fujita et al., 1998). Commercial success as a

plant protection products has, however, been achieved only by a small selection of viruses. The two most notable both belonging to the family Baculoviridae. The family Baculoviridae consists of 600 described species in two genera: the Nuclear polyhedrosis viruses (NPV's) and the Granulosis viruses (GV's)(van Regenmortel et al., 2000). Only known to naturally infect arthropods, these viruses have been studied not only for their suitability as control agents but for their application in molecular biology as expression vectors (Smith et al., 1983, Luckow and Summers, 1988). Different species of baculovirus have been isolated from many different insect orders (Hunter-Fujita et al., 1998) but their deployment as biopesticides has mainly been against Lepidopteran pests (for review see Moscardi (1999)). Baculoviruses are enveloped and have a double stranded DNA genome of 80 to 200kb in length. Extracellular virions can be found in two forms: budded virions (BV's) which are formed during cell-to-cell transmission, or packaged in an occlusion body (OB) during host-to-host transmission (Granados, 1980). A feature almost unique to insect viruses, an OB is a proteinaceous, mainly polyhedrin, lattice that protects virions from the environment. Occlusion bodies vary in size from between 0.5 to >20µm in diameter and are often visible under a light microscope. Two other virus families contain occulated insect viruses: the dsRNA Reoviridae subfamily Spinareovirinae (Cytoplasmic polyhedrosis viruses, CPV) and the Poxviridae, specifically the subfamily Entomopoxvirinae.

Other viruses endorsed and tested for the control of insect pests belong to two other virus families: the Nudiviridae and the Parvoviridae. Oryctes nudivirus is a non-occluded dsDNA virus that was first described as *Rhabdionvirus oryctes* (Huger 1966). It was later defined as Oryctes virus and placed in a subgroup of the Baculoviridae by the International Committee on Taxonomy of Viruses (ICTV) before being incorporated into the Nudiviridae and designated as Oryctes rhinoceros nudivirus (OrNV) (Wang et al. 2007). This virus was introduced into Samoa in 1963, and later to other Pacific Ocean islands, to control the Coleopteran pest of cultivated Palms: *Orytes rhinoceros.* The virus is lethal to larvae and causes feeding cessation in adults and led to huge declines in pest population over the course of 1-3 years. Reapplication in areas of pest resurgence has proved effective, however, after 40 years a breakdown in control in certain locations is being reported by researchers (Jackson, 2009, Huger, 2005). The virus has been studied extensively in India where successful control of O. rhinoceros has also been achieved (Mohan and Pillai, 1993, Gopal et al., 2001). Closely related nudiviruses have recently been discovered in *Drosophila* (Unckless, 2011, Webster et al., 2015).

Drosophila virus diversity

Viruses are a ubiquitous threat to all living organisms. No organism is free from viruses, yet viruses are known from a comparatively few species of medical, economic or conservation importance. This is beginning to change. Modern metatransciptomic techniques have allowed a surge in the numbers of insect viruses described (Shi et al., 2016) and the genus *Drosophila* is no exception. Studies by

Webster *et al.* (2016, 2015) reported over 50 new viruses from the genus. Prior to these survey efforts only 11 viruses were known in *D. melanogaster* (Brun and Plus, 1980) with only five of these isolated, sequenced and available for experimental study: *Drosophila* melanogaster sigma virus (DmelSV), *Drosophila* C virus (DCV), *Drosophila* A virus (DAV), *Drosophila* Nora Virus and *Drosophila* X virus (DXV).

Sigma virus (DmelSV) was the first virus to be discovered in *Drosophila* (l'Héritier and Teissier, 1937). It was discovered by chance due to an unusual symptom of CO_2 sensitivity in infected flies and was later found to be transmitted vertically through eggs and sperm but also to be transmissible through injection, identifying a virus as the causal agent (L'Heritier, 1948). Further examination of the virus lead to its classification into the family rhabdoviridae (Teninges, 1968, Berkalof et al., 1965, Teninges et al., 1993). DmelSV is not the only sigma virus to infect *Drosophila*: *D. affinis*, *D. obscura*, *D. tristis*, *D. immigrans* and *D. ananassae* were all found to be infected with sigma viruses by screening for CO_2 sensitivity (Longdon et al., 2009, Longdon et al., 2011).

Drosophila C virus (DCV) was first isolated in a French strain of *D. melanogaster* (Jousset et al., 1972) and has since become one of the most well studied viruses of *Drosophila* (Huszar and Imler, 2008, Jousset et al., 1977). Closely related to another well studied insect virus, the Cricket Paralysis Virus (CpV), DCV belongs to the family Dicistroviridae. DCV is lethal to *D. melanogaster*, infecting the muscles around the fly's crop, foregut, casing acute cytopathology and intestinal obstruction in adult flies (Chtarbanova et al., 2014). DCV also infects *D. suzukii*, replicating successfully and causing increased mortality in lab reared flies (Lee and Vilcinskas, 2017).

Two less well studied viruses of *D. melanogaster* that afford mention are DAV and Nora virus. DAV is an unusual RNA virus described initially as a picorna-like virus (Brun and Plus, 1980, Plus et al., 1976) but with a diverse range of biological attributes that make it difficult to place systematically (Ambrose et al., 2009). It exhibits low pathogenicity in its host (Brun and Plus, 1980) despite interacting with antiviral RNAi pathways and has a global prevalence of between 5 and 10% (Webster et al., 2015). Also described as a picorna-like virus, *Drosophila* Nora virus is a small non-enveloped RNA virus infecting *D. melanogaster* and the closely related *D. simulans* (Habayeb et al., 2006). This virus is transmitted horizontally and has little effect on the longevity or fecundity of infected flies (Habayeb et al., 2009).

Drosophila X virus (DXV) is a non-enveloped dsRNA virus belonging to the family Birnaviridae. It was first discovered as a contaminant in a study on DSV in cell lines (Dobos et al., 1979). Little is known about the replication cycle of DXV and it has never been found as a natural pathogen of wild *Drosophila*. It has, however, been detected in *Culicoides* sp. (Adams and Bonami, 1991). The exact origin of the original contamination is not known.

Few studies have focused on the diversity of viruses in wild *Drosophila* populations. Recently, however, the development of metagenomic techniques has facilitated a new approach to viral discovery and has expanded our knowledge of insect virus diversity immensely (Liu et al., 2011). Webster et al. (2015) used next generation sequencing technology to identify more than 20 previously undescribed RNA and DNA viruses associated with *D. melanogaster*. Their survey of over 2000 individual wild flies showed 30% of flies to carry at least one virus and 6% of flies to carry multiple viruses. This study also involved the analysis of publically available RNA-seq datasets to estimate viral prevalence in laboratory stocks.

Less is known about the viruses infecting other species of *Drosophila* in the wild, *D. melanogaster* being by far the best studied. 25 new viruses, discovered through metatranscriptomic surveys were, however, described by Webster et al. (2016) in a number of British *Drosophila* species. Between one and five new viruses were described from pooled samples of the species: *D. tristis*, *D. subsilvestris*, *ScaptoDrosophila deflexa*, *D. obscura*, *D. subobscura* and *D. immigrans.*

A study by Unckless (2011) identified a DNA nudivirus infecting wild *Drosophila innubila*. This virus is closely related to the OrNV discussed above for its use as a biological control agent of coleopteran palm pests. Also closely related to OrNV, a nudivirus of *D. melanogaster* was discovered by Webster et al. (2015). Named Kallithea virus (KV), this virus was found to be relatively common in wild *D. melanogaster* (4.6% prevalence globally). KV infection is costly to adult *D. melanogaster*, causing substantial mortality, reduced fecundity and increased morbidity in the form of reduced movement (Palmer et al., *unpublished*). Kalithea represents a good candidate for the control of *D. suzukii* and we aim to assess the pathogenicity of Kalithea virus during this study.

Antiviral immunity in *Drosophila*

Insects rely almost entirely on an innate immune response, as opposed to the familiar, adaptive, immune response found solely in vertebrates. Several of the pathways involved in innate antiviral immune response were first identified in *Drosophila* and have since been proven to be highly conserved amongst the invertebrates and vertebrates alike. All start with pathogen recognition. Pattern recognition receptors (PRRs) recognise conserved components of different pathogens by what are known as pathogen-associated molecular patterns (PAMPs). There are several distinct classes of PRRs, acting as either membrane bound sensors (Toll-like receptors or C-type lectin receptors) or cytoplasmic sensors (Retenoic acid-inducable gene-like receptors or NOD-like receptors) (Akira et al., 2006). Binding of PAMPs activates signalling pathways resulting in the production of effector molecules that suppress pathogen replication. In *Drosophila* a range of different pathways are thought to be involved in the innate antiviral response: the Toll pathway, IMD pathway, JAK/Stat pathway, Toll-7 autophagy pathway, transcriptional pausing pathway and the RNA interference pathway (reviewed in (Sabin et al., 2010)).

The most important pathway in antiviral response is thought to be that of RNA interference (RNAi). Three RNAi pathways have been identified in *Drosophila*: the small-interfering (si)RNA pathway, the micro (mi)RNA pathway and the PIWI interacting (pi)RNA pathway (reviewed by Kim et al. (2009)). The siRNA pathway is most often associated with the antiviral response in insects. On infection by a virus, 'Dicer' proteins in the cytoplasm recognise and bind to viral dsRNA, cleaving it into siRNA fragments and initiating the pathway. These siRNAs are then loaded in to the RNA induced silencing complex (RISC) which guides the slicing enzyme Argonaut to complementary viral RNA sequences which are in turn cleaved preventing viral replication.

Other components of the *Drosophila* antiviral immune response include two signalling pathways that largely mediate the expression of antimicrobial peptides (AMPs), the Toll and Immune deficiency (Imd) pathways. Despite their primary association with antifungal and antibacterial defence, studies in *Drosophila* and Anopheles mosquitos have shown a role for these pathways in the antiviral response (Avadhanula et al., 2009, Costa et al., 2009, Zambon et al., 2005). The Jak-STAT pathway, which is responsible for the expression of several other immune related proteins and the promotion of cellular immune responses (Sorrentino et al., 2004), is also required for antiviral defence in *D. melanogaster* (Dostert et al., 2005, Huang et al., 2013). Interestingly, a ligand activating the Jak-STAT pathway, vago, is dependent on Dicer-2 for expression, providing a possible interaction between the RNAi and Jak-STAT pathways (Deddouche et al., 2008, Paradkar et al., 2012). A mechanism independent of these pathways, Toll-7 activated autophagy, has also proposed as a constituent part of the antiviral response in *Drosophila* (Nakamoto et al., 2012, Shelly et al., 2009).

Ecoimmunology of *D. suzukii*

As an invasive species, *D. suzukii* caught in the UK today are potentially is experiencing a different immunological environment to their recent ancestors. Rapid introduction into a new ecosystem can bring with it a reduction in the diversity of natural enemies adapted to prey on or infect the invasive organism, a concept known as the enemy realise hypothesis or ERH, (Keane and Crawley, 2002). Indeed, a reduction in the number of compatible enemies, or their effect on the introduced species, has been demonstrated for numerous different invasive organisms in their naturalised ranges (Callaway et al., 2004, Torchin et al., 2001, Wolfe, 2002, Beckstead and Parker, 2003), especially on the leading edge of an invasion where parasites have been found to lag behind their hosts (Phillips et al., 2010). This reduction could in turn impart an ecological advantage to the invasive species, aiding range expansion and establishment, not only by a reduction in extrinsic population control but by providing an evolutionary opportunity to reallocate resources away from costly defences (Blossey and Notzold, 1995). Although seemingly intuitive, evidence for the ERH is incomplete and the true reasons for increased abundance or impact of introduced species may be far more complex (Colautti et al., 2004). It has been argued that invasive species may not free up defence resources evenly but

shift immune defences against well adapted native specialists to defence against more general threats (Joshi and Vrieling, 2005). Another potential adaptation to invasion might be to increase pathogen tolerance. Tolerant individuals alleviated of the fitness consequences of infection could increase pro-invasive behaviours such as dispersal and reproduction. This could have potentially negative impacts on related native fauna through 'pathogen spillback' (Kelly et al., 2009).

All adaptations in immune function made by the invasive host species are constrained by the amount of genetic diversity within the invading population. As invaders often experience population bottlenecks during the introduction process, diversity may be reduced, and vulnerability to infectious disease increased (O'Brien and Evermann, 1988), a concept often associated with agricultural crops (Zhu et al., 2000, Duvick, 1984, Staskawicz et al., 1995). A reduction in haplotype diversity has been observed in *D. suzukii*, with European populations being the least diverse compared to flies of the native range (Adrion et al., 2014)

Aims of this Study

- To describe the diversity of viruses infecting *D. suzukii* in its native and naturalised ranges.
- To examine the antiviral immune function of *D. suzukii* in relation to other *Drosophila* species
- To test the pathology of known and novel *Drosophila* viruses in *D. suzukii*
- To examine the patterns of virus occurrence across a range of Drosophilid hosts

Materials and methods

Sample collection

In total, approximately 4450 individual *D. suzukii* were collected across a three-year period between September 2013 and September 2016, including 230 larvae in 2016. Flies were collected near Montpellier, France (43.59 N, 3.78 E) in 2013, in Kent, UK (51.284 N, 0.465 E) during the late summer of 2014, 2015 and 2016, and in three locations across Honshu, Japan, during May 2016: Tokyo University of Agriculture and Technology, Fuchu (plate 2) (35.683 N, 139.481 E); Naganuma Park, Tokyo (35.637 N, 139.375 E); Shimaminami Shima, Yamagata Prefecture (38.351 N, 140.276 E); Agriculture Total Center Kaju Research Institute, Fukushima (37.813 N, 140.443 E); and Fuefukigawa Fruit Park, Yamanashi (35.700 N, 138.666 E). A combination of commercial bait traps were used with cotton soaked in a proprietary liquid attractant (DROSO TRAP® and DROS'ATTRACT®, Biobest, Belgium, NV), and a standard sweep net to catch adult flies. Traps, hung at field margin and woodland sites, were collected at intervals of two to three days. All individuals were sorted into vials by trap and species within three hours of collection. We aimed to morphologically identify all species of *Drosophila* caught (Bächli et al., 2004), however, we also subsequently examined RNA pools for potential contamination due to misidentification. Other species of *Drosophila* were caught in these traps and

we collected them together with *D. suzukii*, but they were not analysed further. Wild-collected flies were maintained on solid agar/sugar medium, before being macerated in sterile Ringer's solution (to allow for future experimental virus culture and isolation). Larvae were dissected from infested fruit collected in 2016 from UK and Japan with sterile forceps.

RNA was immediately extracted from a subsample of the fly (or larva) homogenate using TRIzol® (Invitrogen), before storage at -80°C. Pooled RNA samples were treated for possible DNA contamination using DNAse (Turbo DNA-free, Ambion) prior to library preparation. For flies collected in the UK and Japan, library preparation and sequencing were performed by Edinburgh Genomics (Edinburgh, UK) using the Illumina Hi-Seq platform with 120 or 150nt paired end reads. To increase representation of viral and host protein coding RNAs, all libraries underwent depletion of rRNA using Ribo-Zero rRNA Removal Kit (Illumina). Flies collected in France during 2013 were sequenced separately at Beijing Genomics Institute (BGI tech solutions, Hong Kong) using paired-end 90nt reads. These samples underwent Duplex-Specific Thermostable Nuclease (DSN) normalisation and poly-A selection. This process, although enriching for viruses by rRNA depletion, biases virus discovery towards poly-adenylated genomic products only produced by certain viral taxa (e.g. Picornavirales). All raw reads have been submitted to the NCBI sequence read archive under project accession PRJNA402011 (Japan SRR6019484; France SRR6019487; Kent: SRR6019485, SRR6019486, and SRR6019488).

Plate 2: Netted Blueberry plots, TUAT, Tokyo.

Virus identification and Phylogenetic Analysis

To remove those reads derived from *Drosophila*, raw reads were mapped against the *D. suzukii* genome and transcriptome using Bowtie2 (Langmead and Salzberg, 2012) with the '--very-fast' command-line option. Trimmomatic (Bolger et al., 2014) was used to quality trim and remove adapter sequences from the remaining unmapped raw reads (as pairs) using default parameters, before *de*

novo assembly using Trinity version 2.2.0 (Grabherr et al., 2011), retaining a minimum contig length of 500nt. All translations of all open reading frames (ORFs) in each resulting contig were concatenated, and only those with an open reading frame of 150 codons or greater were retained. These concatenated protein sequences were used to search against a custom database using Diamond (Buchfink et al., 2015) with an e-value threshold of 0.01, retaining a single top hit. This database comprised all of the viral proteins from the Genbank non-redundant protein database, 'nr' (Clark et al., 2016), and all of the prokaryote, protist, fungal, nematode, hymenopteran, and dipteran sequences from NCBI refseq protein. Contigs for which the top hit was a virus were imported into Geneious®8.0.2 sequence analysis software (Kearse et al., 2012) for manual analysis. Putative virus fragments were grouped taxonomically according to their initial best blast hit, assembled (Geneious) and manually curated them with reference to closest relatives in Genbank, to give the longest viral sequences consistent with the predicted protein content and structure of that virus taxon.

To infer phylogenetic relationships, RNA-dependent RNA polymerase (RdRp) coding sequences were used unless otherwise stated. The RdRp is generally the most conserved protein across RNA viruses, making it suitable for phylogenetic analysis of this diverse set of virus taxa (Koonin et al., 1993, Shi et al., 2016). RdRp gene sequences were translated and aligned with homologous sequences from their close relatives, as identified by BLAST (Altschul et al., 1990). ClustalW (Thompson et al., 2002) with BLOSSOM cost matrix (Henikoff and Henikoff, 1992) were used to align multiple protein sequences. Regions of poor alignment at the 5' and 3' ends of the alignment were manually identified and removed before further analysis. Maximum-likelihood phylogenetic trees were inferred using PhyML 2.2.3 (Guindon and Gascuel, 2003) with the LG substitution model (Le and Gascuel, 2008). Branch support was calculated using the Shimodaira-Hasegawa-like nonparametric version of an approximate likelihood ratio test implemented in PhyML, aLRT (Anisimova et al., 2011). Trees in Figures are clusters from within of larger trees, realigned and reconstructed using the same methods.

Detection by RT-PCR

To confirm the presence of the newly discovered viruses in original RNA pools, and to estimate prevalence of known viruses in pools, Reverse Transcription PCR (RT- PCR) was used to screen for short amplicons of each virus' longest ORF, where possible spanning part of the RdRp gene. Primers were designed using the Primer3 (Rozen and Skaletsky, 1999) plugin for Geneious (Kearse et al., 2012). RNA virus sequences identified by metagenomic methods may derive from viral elements endogenised into genomic DNA, if they are expressed (Katzourakis and Gifford, 2010). To test for endogenised viral elements (EVEs) PCRs (without a reverse transcription step) were conducted on RNA samples that contained genomic DNA from the original phenol-chloroform extraction. As RNA viruses do not produce a DNA intermediate, any viruses detected in this way are likely to be EVEs.

Virus Genome Annotation

For viruses with complete, or near complete genomes, genome structure was inferred and protein functional domains identified by first identifying ORFs and then comparing these to the Conserved Domain Database with an expected value threshold of 5×10^{-3}, and searching the NCBI 'nr' protein database using BLASTp. Only ORFs of 100 amino acids or longer were annotated, unless notable similarity to closely related viruses was evident. ORFs of less than 200 amino acids that were nested completely with larger ORFs were disregarded, unless they displayed high similarity to known proteins.

Distribution of RNA sequence reads across samples

To estimate the number of virus reads in each pooled sample, and to detect any cross-species contamination in fly collections, trimmed forward reads were mapped to all new and previously published *Drosophila* virus genomes (including multiple divergent isolates where they were available); a selection of *Drosophila* ribosomal sequences, and a short region of cytochrome oxidase 1 (COI) that has discriminatory power between *Drosophila* species. Sequences were mapped with Bowtie2 (Langmead and Salzberg, 2012) using the '--very-sensitive' option. These are reported after normalisation by the number of non-ribosomal reads and the length of each target sequence. An arbitrary lowest level detection threshold was applied for each putative species of 0.5 total reads per Kb per million non-rRNA reads to reduce spurious signals caused by low level species contamination, library barcode switching, and cross-mapping to close relatives.

Pathology Testing

To test if any virus within the pools of wild flies contained a virus pathogenic to *D. suzukii,* lab stocks were injected with viral extracts of wild flies. To do this, homogenate of wild flies was macerated in sterile Ringers buffer solution and filtered through 0.22µm Millex® Sterile syringe filter units, filtering out all microorganisms aside from viruses by size. To exclude the possibility that occluded viruses larger than 0.22 µm in diameter were filtered out, some homogenate was also treated by centrifugation at 4°C and 4000 x g for 15 minutes to remove large debris (mainly fly), before extracting the supernatant and centrifuging at 15000 x g for 5 minutes to remove bacterial and fungal contaminants. 69.0nl of this solution was then injected into anesthetised flies via micro-injection (Drummond™, NanoJect®). Control groups were injected with sterilised ringers solution, and either passaged in the same manner as treatment groups (Control, C) or discarded and new fly groups injected with buffer every week (Weekly control, WC). Flies were then monitored at regular intervals for a period of 7-21 days to assess mortality. To allow for replication of viruses present at very low titres in the original solution, extracts of infected flies were serially passaged through fresh groups of flies every seven days for a total of four passages. Mortality was monitored throughout.

The to test the pathogenicity of Kalithea virus to *D. suzukii,* 50 nL of 10^5 ID50 KV was injected. Virus was isolated from wild *D. melanogaster* by gradient centrifugation as described by Palmer et al. (*unpublished*). Flies were monitored for mortality over 21 days. Flies were also inoculated orally with KV to mimic the natural route of infection after application of the virus as a control product. A liquid solution of 2000 X ID50 was made up using sterile 10mM Tris buffer and administered to vials of standard lewis food medium. The solution was allowed to dry for 1hr before 10 adult flies, separated by sex, were transferred to vials. Mortality was monitored over 21 days to assess mortality. Further to assessment of adult mortality, the effect of kalithea treatment on larval development was also tested. To do this, 5 mated females were allowed to oviposit into standard food vials for 48hrs then applied 200µl of 2000 X ID50 KV solution to the surface of the media. Vials were monitored for adult emergence daily and the total number of emergent adult flies counted after 21 days.

Immune Expression Analysis

In order to characterise the transcriptomal immune response of D*. suzukii* in relation to *D. melanogaster*, 6 vials of 10 male and 10 female flies of both species were injected with either isolates of DCV, KV or a control consisting of sterile isolation medium. At point of infection flies were mated and 5-7 days old. Three days post infection flies were homogenised in Trizol® solution and extracted total RNA. These samples were enriched for mRNA through treatment with DNAse and poly-A selected before preparation of strand-specific paired-end libraries using the NEB Next Ultra Directional RNA Library Prep Kit. Libraries were then pooled and sequenced by Edinburgh Genomics over three lanes of an Illumina HiSeq 4000 platform with strand-specific 75 nucleotide paired end reads.

Known sequence contaminants (primer and adapter sequences) were removed from the paired end reads with cutadapt (V1.8.1; Martin, 2011) and remaining reads were mapped to the D. suzukii genome (NCBI: PRJNA325161) and all known Drosophila virus genomes using STAR (V2.5.3a; Dobin et al, 2013), with a maximum intron size of 100 KB, but otherwise default settings. The number of reads mapping to each gene were counted using the 'featurecounts' command in the 'subread' package (V1.5.2; Liao et al, 2013) and these raw count data used as input to DESeq2 (V1.16.0; Love et al, 2014) for differential expression analysis. DESeq2 fits a generalised linear model for each gene, where read counts (K) are modelled as a negative binomially distributed variable (Anders and Huber, 2010; Love et al, 2014). The design matrix included sex, virus infection status, and the interaction between the two, allowing tests for expression changes following either virus infection and how these changes differed between the sexes. Log2 fold changes in DESeq2 were calculated and tested for significance using Wald tests. The 'plotPCA' function implemented in DESeq2 was used to perform principal component analysis of the rlog-transformed read count data.

Results

In total, approximately 280 million read pairs were generated, ranging from 33 million pairs (Kent 2016) to 105 million pairs (France 2013) per library. Assemblies comprised between 18,431 (Japan 2016) and 56,384 (Kent 2015) putative transcript contigs. Among these, 18 new RNA viruses associated with *D. suzukii* were identified (Table 1.). These viruses represent a variety of RNA virus taxa with positive sense single stranded (+ssRNA), negative sense single stranded (-ssRNA), and double stranded RNA (dsRNA) genomes, and include representatives of the Picornavirales, Mononegavirales, Bunyavirales, Chuviruses, Nodaviridae, Tombusviridae, Reoviridae and Nidovirales. No DNA viruses were identified. Any viruses detected in *D. suzukii* that are identical, or near identical (>95% amino acid similarity in the polymerase), to previously published viruses are not reported as new. Those previously described viruses that were detected in *D. suzukii* are detailed in supplementary material (S1_Table.) and relative read counts in each pool are shown in Figure 6.

These viruses are provisionally named according to the location from which the hosts were sampled. It was decided not to include taxonomic or host information in the provisional name of the virus, as these are subject to change as phylogenetic relationships are revised and alternative or additional hosts discovered. The one exception to this rule is *D. suzukii* Nora Virus. This virus is sufficiently closely related to the *D. melanogaster* Nora virus and *D. immigrans* Nora virus that a name outside of this local scheme may cause confusion for future studies. During Phylogenetic analysis, a number of virus-like sequences were identified by BLAST in the public Transcriptome Shotgun Database (TSA). These have been included in analyses to improve accuracy of phylogenetic inference, but are not further discussed.

From the sample pools, reads mapping to a number of known *Drosophila* viruses were detected. Because of the presence of closely related viruses in *D. suzukii* and other *Drosophila* species there is a possibility that reads of true *D. suzukii* viruses may cross map to their close relatives in other fly species. In addition, COI read mapping suggests a low level of species contamination (notably *D. melanogaster* and *D. immigrans*) in some pools.

Viruses with single-stranded positive sense RNA genomes.

Ten of the viruses described here encode their genomes in +ssRNA. Of these, Teise virus was found at the highest titre. Teise virus is a sobemo-like virus closely related to Prestney Burn Virus of *D. subobscura* (Webster et al., 2016) and Motts Mill virus of *D. melanogaster* (Webster et al., 2015), with 90.9% and 88.6% RdRp amino acid similarity respectively (Figure 1).

Table 1. Novel viruses detected in *D. suzukii*. [a]PCR reactions performed on cDNA. [b]PCR reactions performed on extractions containing nuclear DNA.

Provisional Name	Accession	Host	Taxon	Genome	Longest contig (kb)	Sample(s)	Detected RT-PCR[a]	Detected PCR[b]
Beult virus	MF893261, MF893262	Dsuz	Negevirus	+ssRNA	12	France2013, UK2014, UK2015, UK2016, Japan	+	-
Saiwaicho virus	MF893256	Dsuz	Negevirus	+ssRNA	10	Japan2016	+	-
Luckshil l virus	MF893250	Dsuz	Virgavirus	+ssRNA	3.5	UK2016	+	-
Tiese virus	MF893259	Dsuz	Luteoviridae	+ssRNA	4.5	France2013, UK2014, UK2015, UK2016, Japan2016	+	-
Tama virus	MF893258	Dsuz	Sobemovirus	+ssRNA	3.5	Japan2016	+	-
Medway virus	MF893251	Dsuz	Sobemovirus	+ssRNA	2.7	UK2014	+	-
Dsuz Nora virus	MF893254	Dsuz	Picornaviridae	+ssRNA	12	Japan2016	+	-
Naganuma virus	MF893253	Dsuz	Nodaviridae	+ssRNA	1.6	Japan2016	+	-
Fuefuki virus	MF893247	Dsuz	Nidoviridae	+ssRNA	16	Japan2016	+	-
Cyril virus	MF893263	Dsuz	Virgavirus	+ssRNA	1.5	UK2016	+	-
Eccles Virus	MF893265-MF893270	Dsuz	Reoviridae	dsRNA	4.2	UK2014	+	-
Larkfield virus	MF893249	Dsuz	Totiviridae	dsRNA	6	UK2015	+	-
Snodland virus	MF893257	Dsuz	Totiviridae	dsRNA	1.6	UK2015	+	-
Mogami virus	MF893252	Dsuz	Chuvirus	-ssRNA	10.5	Japan2016	+	-
Ditton virus	MF893264	Dsuz	Phasmaviridae	-ssRNA	7.3	UK2015	+	-
Barming virus	MF893260	Dsuz	Phleboviridae	-ssRNA	6.5	UK2016	+	-
Notori virus	MF893255	Dsuz	Phasmaviridae	-ssRNA	7	Japan2016	+	-
Kiln Barn virus	MF893248	Dsuz	Chuvirus	-ssRNA	3.7	UK2016	+	-

The single-stranded positive sense genome of these viruses comprises two unjoined fragments, which may represent subgenomic products (Webster et al., 2015, Shi et al., 2016, Tokarz et al., 2014). However, *de novo* assembly of putative fragments of Teise virus produced contigs bridging the two

fragments together, and PCR primers were designed that resulted in an amplicon bridging these fragment. The genome of this virus is therefore presented as one contiguous fragment of 4.5 kb, while noting conserved domains (Fig. 2). Teise virus is the most geographically widespread virus of *D. suzukii*, with reads appearing in high numbers in both native and naturalised ranges (Fig. 6).

Medway virus (Fig. 1, G) shares close relationship to Braid Burn virus, previously described from *Drosophila* subsilvestris in the UK (Webster et al., 2016). These viruses belong to a clade of insect viruses distantly related to the Sobemo and Polero viruses of plants. Medway virus appears at low copy-number in our samples with a small number of reads being detected in UK samples from 2014 and 2015. As for other viruses in this section of the Luteo-Sobemo group, the Medway virus genome probably consists of two genomic RNA segments. However, the second RNA segment was not detected here and the virus is described only from an RNA fragment that contains two ORFs, including the RdRp (Fig. 2). Tama virus, a third virus in the Luteo-Sobemo clade, was only detectable by PCR in Japanese samples. In the *D. suzukii* collections three separate Nora viruses were detected: *D. melanogaster* Nora Virus (Habayeb et al., 2006), *D. immigrans* Nora Virus (van Mierlo et al., 2014) and the new Nora virus, most closely related to that of *D. immigrans*, but sufficiently divergent from both (37.1% and 30.4% amino-acid divergence at the RdRp locus, respectively) to merit description (Fig. 1, J). This clade of viruses also evidently infects other families of 'fruit fly', as they are detectably in the transcriptomes of two species of tephritids (*Bactrocera latifrons* and *Ceratitis capitata*), and can also be found in the transcriptomes of their parasitoid, *Fopius arisanus* (Fig. 1, J).

Beult virus was the most geographically widespread virus identified: Beult virus was detected across sampling locations and years, with reads being especially abundant in samples from the UK in 2014 and Japan in 2016. Belonging to a clade of Virga-like viruses, it is very closely related to Bofa virus and Buckhurst virus of *D. melanogaster* and *D. obscura*, respectively (Webster et al., 2016). Two different haplotypes of this virus were identified which share a 98.9% nucleotide similarity: one from the UK, and a second divergent lineage from Japan. Saiwaicho virus also belongs to the Hepe-Virga clade of viruses and is closely related to the Negevirus genus. For the Hepe-Virga clade viruses it was possible to identify domains for transferases, helicases, and polymerases (Fig. 2).

A single Nidovirus was detected in our samples from the UK and Japan. This has been provisionally named the Fuefuki virus, and it has the longest contig recovered for any of our putative viruses, at over 16.5 kb. Within this near-complete genome, five ORFs were identified but only one conserved domain: the RdRP (Fig. 2). Fuefuki virus is very closely related to Wuhan nido-like virus 1 (Shi et al., 2016) at 94.8% amino acid similarity in the polymerase. Along with Hubei *Tetragnatha maxillosa* virus 7 and Wuhan insect virus 19 (Shi et al., 2016) these four viruses form a distinct cluster near to the Coronaviridae, a family containing some notable vertebrate pathogens, including the SARS virus (Fig. 1, I).

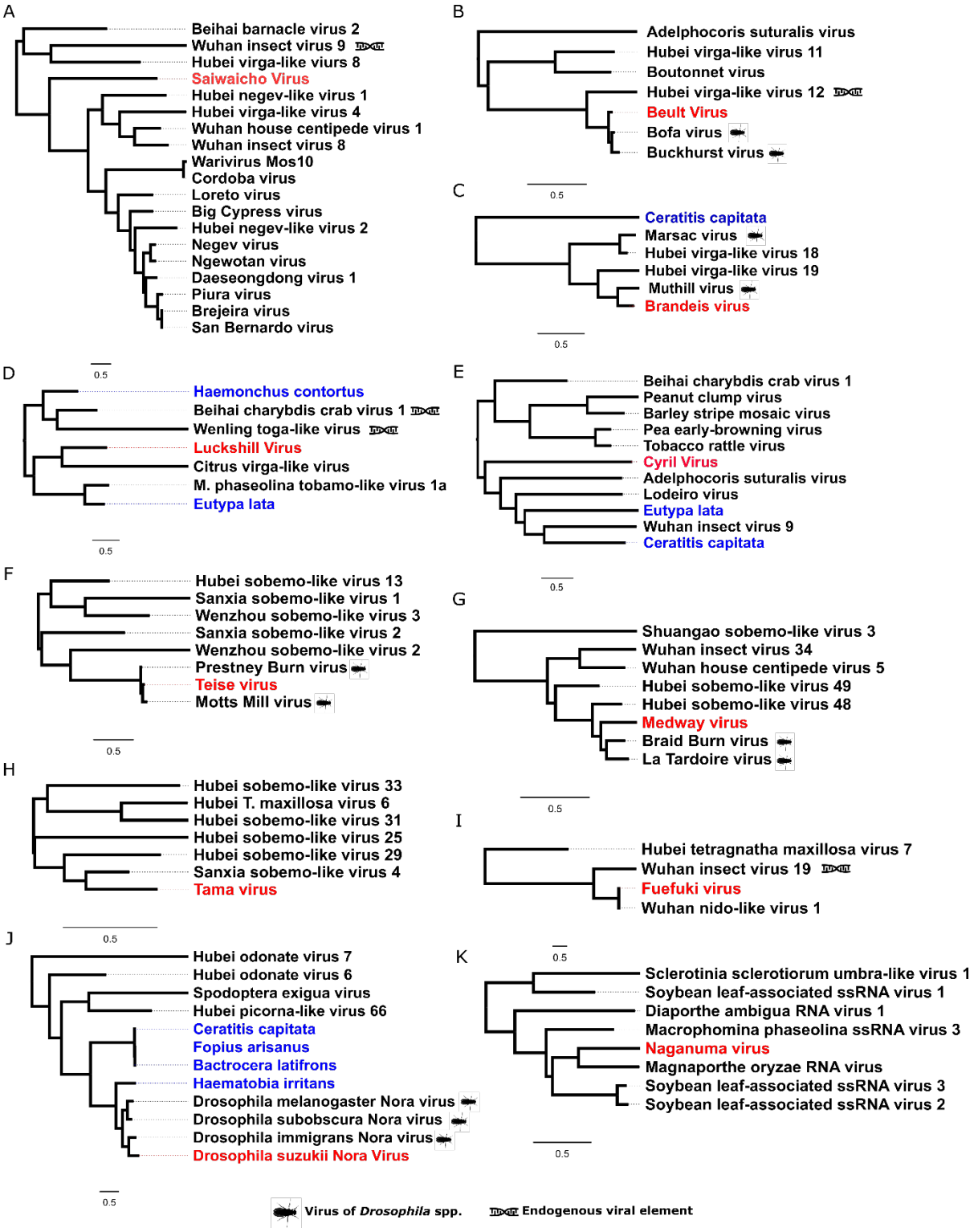

Figure 1. Positive sense single stranded RNA viruses. Midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase sequences. Scale bar represents 0.5 substitutions per site. Putative viruses newly described in association with *D. suzukii* (red) are highlighted alongside virus-like sequences identified in public transcriptome datasets (blue). Viruses previously described as endogenous viral elements are also marked. Tree **A:** Negeviruses and nearby clusters from the Hepe-Virga clade; **B,C:** Virga-like viruses belonging to the Hepe-Virga like clade; **D:** A small cluster of toga-like viruses neighbouring the Alphaviruses, Togaviridae; **E:** A small cluster of virga-like viruses neighbouring the Cileviruses, Hepe-virga clade; **F,G,H:** Sobemo-like viruses belonging to clusters within the Luteo-Sobemo clade; **I:** a cluster in the Nidoviruses close to the Coronaviridae; **J:** Noraviruses and related cluster of the Picora-Calici clade. **K:** Cluster of Nodaviruses within the Tombus-Noda clade;

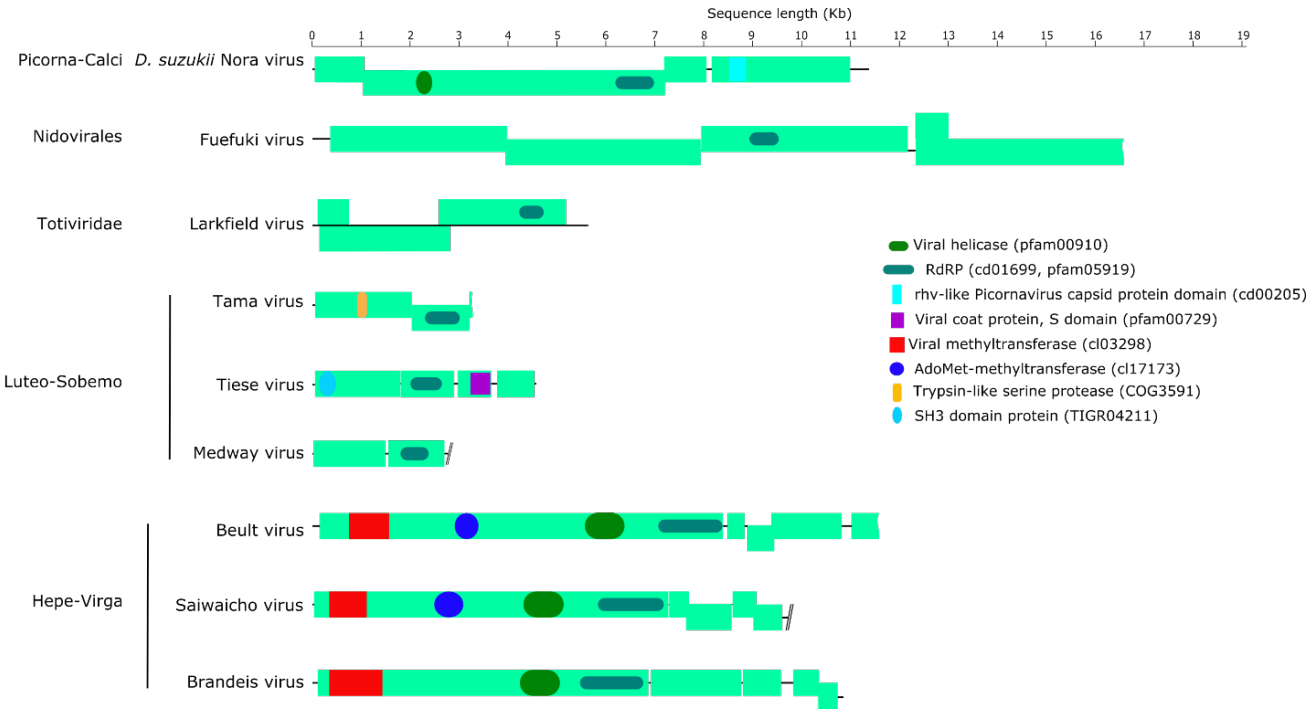

Figure 2. The structure of selected +ssRNA and dsRNA virus genomes for which we recover complete or near complete genome sequences. Relative positions of protein coding regions are calculated with reference to the NCBI conserved domain database.

Viruses with single-stranded negative sense RNA genomes

Five of the viruses we detected have -ssRNA genomes. Three of these belong to the Bunya-Arena clade of viruses: Notori virus, Ditton virus and Barming virus. Notori and Ditton viruses can be further classified as Phasmaviruses. These were detected in the samples as contigs of around 7kb in length that represent complete, or near-complete L- segments (Bishop and Shope, 1979) (Fig. 5). Barming virus, the third putative Bunya-Arena clade virus we identified, belongs to the Phlebo-like cluster of the clade. It too is known from a contig of just over 6kb, also representing the L-segment of the Bunyavirus genome, consisting of one ORF containing the viral RdRp (Fig. 4). The closest relative of Barming virus was a viral-like sequence identified in the TSA database from *Colletotrichum cereale*, a plant disease that has been found to cause crown rot anthracnose of turf grass (Crouch et al., 2006).

The remaining -ssRNA viruses we identified belong to the Mono-Chu clade of -ssRNA viruses. Kiln Barn virus was identified from fly samples collected in the UK in 2014 , represented by a 3.7 kb contig. This clusters phylogenetically with a group of viruses close to the Chuviruses *sensu stricto,* and we find its closest relatives to be Hubei rhabdo-like virus 4 (Shi et al., 2016) and a viral sequence identified in the transcriptome of the Shiitake mushroom fungus *Lentinula edodes* (AGH07920.1). The other virus identified from this clade, Mogami virus, is closely related to Shayang fly virus 1, a Chuvirus detected in Chinese diptera (Shi et al., 2016), and was represented by a 10.5kb contig in which from which both glycoprotein and polymerase ORFs were identified.

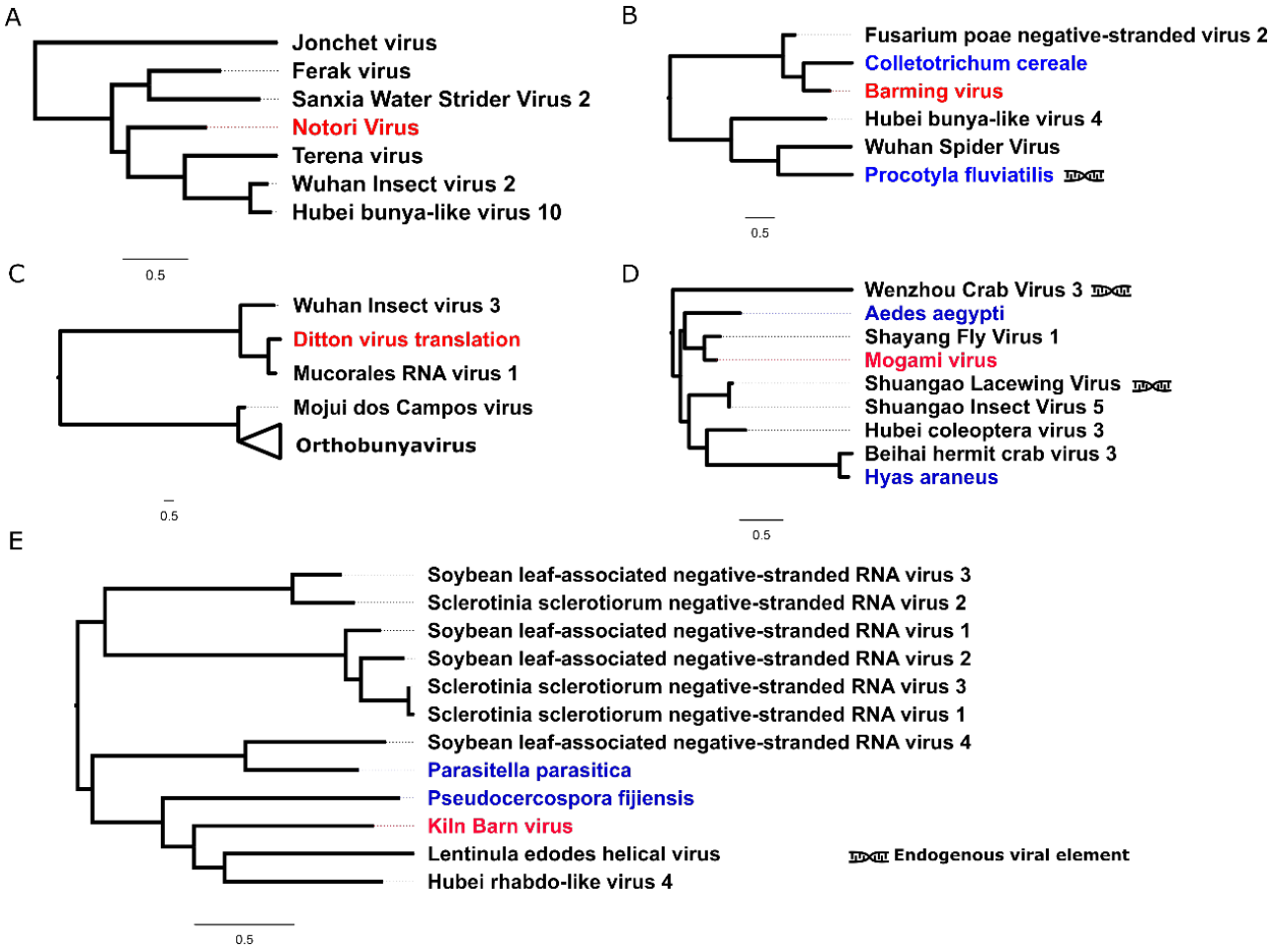

Figure 3. Negative sense single-stranded RNA viruses. Midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase sequences. Scale bar represents 0.5 substitutions per site. Viruses newly described in association with *D. suzukii* (red) are highlighted alongside viral-like sequences identified in public transcriptome datasets (blue). Viruses previously described by the original authors as endogenous viral elements are also marked. Tree **A:** Viruses close to Phasmaviruses in the Bunya-Arena group; **B:** Viruses belonging to the Phlebo-like cluster of the Bunya-Arena group; **C:** Orthobunyaviruses (collapsed) and small sister clade consisting of three viruses, including the newly described Ditton virus; **D:** Cluster of the Chuviruses; **E:** Cluster of viruses close to Chuviruses in the Mono-Chu clade.

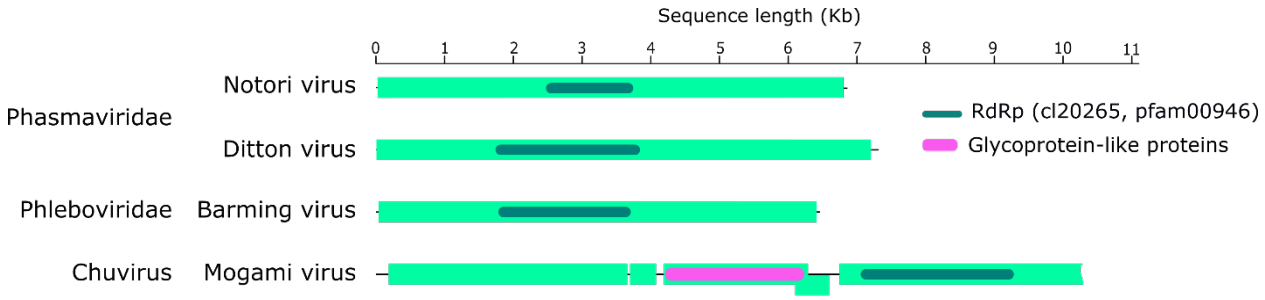

Figure 4. The structure of selected -ssRNA virus genomes for which we recover complete or near complete genome sequences. Relative positions of glycoprotein like proteins and RNA dependent RNA polymerase regions (RdRp) are calculated with reference to the NCBI conserved domain database.

Viruses with double-stranded RNA genomes.

We discovered three viruses predicted to possess double-stranded RNA genomes. These included two Totiviruses, Snodland virus and Larkfield virus, both reported from partial protein coding sequences. Both have closest relatives discovered in insect pool sequencing by Shi et al. (2016). Larkfield shares a cluster within the Totiviruses which includes a number of ant viruses: two discovered by Koyama et al. (2015) and Koyama et al. (2016) in genus *Camponotus*, and one found here as a virus like sequence in a published transcriptome of the black garden ant: *Lasius niger* (Fig. 5). Its closest relative, Hubei toti-like virus 14, is described as an endogenous viral element (Shi et al., 2016). Snodland virus clusters with a small group of other insect viruses, neighbouring a cluster of mycoviruses associated primarily with powdery mildews (Fig. 5).

The final dsRNA virus identified, Eccles virus, is our only representative of a virus family that has been previously advocated for the biological control of insect pests (Peng et al., 2000): the Reoviridae. Eccles virus is most closely related to Hubei diptera virus 21 (Shi et al., 2016) and a reovirus of the geometrid, *Operophtera brumata* (Graham et al., 2006). Homology predicts this virus has a multipartite genome consisting of 11 segments, although only 6 of those segments could be assembled from our samples.

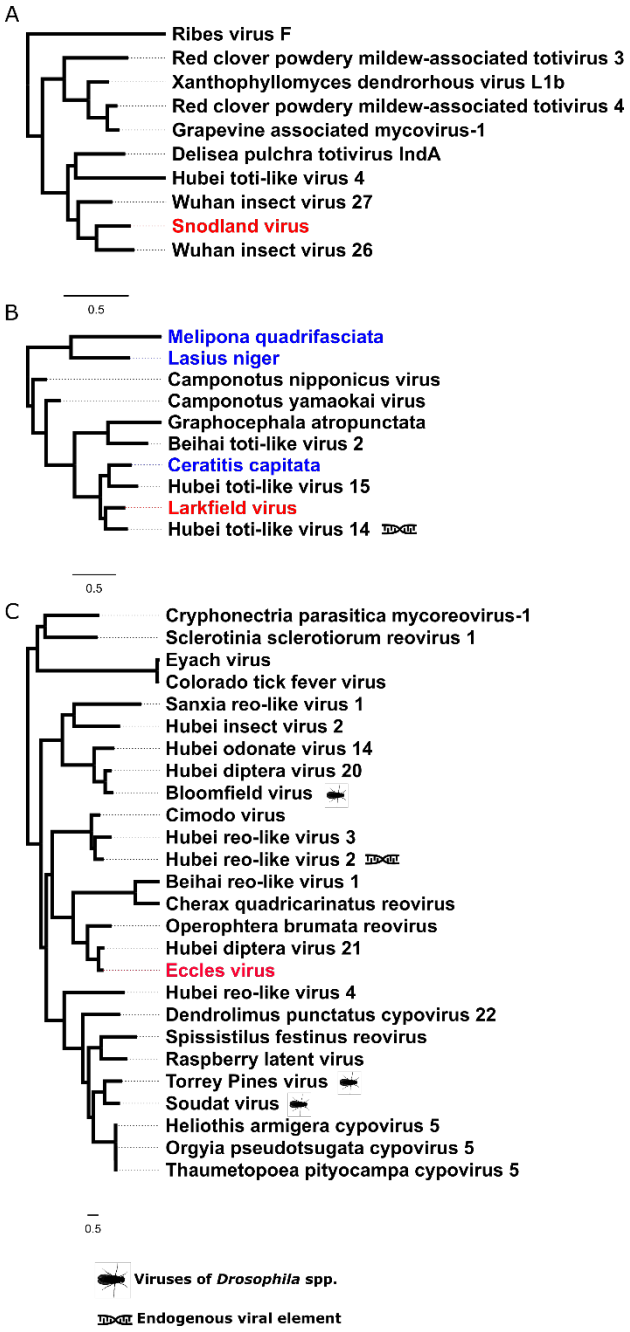

Figure 5. Double stranded RNA viruses. These midpoint-rooted, maximum-likelihood trees were inferred from viral polymerase sequences. Putative viruses newly described in association with *D. suzukii* (red) are highlighted alongside viral-like sequences identified in public transcriptome database (blue). Viruses previously described from a *Drosophila* spp. and viruses described by the original authors as endogenous viral elements are also marked. Tree **A:** Totiviruses, Totiviridae; **B:** Viruses belonging to a c;ade of the totiviridae, Toti-Chryso clade; **C:** Reoviruses, including Coltiviruses (Eyach virus and Colarado tick fever virus) and viruses close to Fijiviruses.

Known *Drosophila* Viruses

18 further viruses previously described from other species of *Drosophila* were also detected. Three known viruses were detected at very high levels (below), and are therefore highly likely to represent infections of *D. suzukii.* The first of these is Brandeis virus (MF953177) the genome of which is reported here for the first time. Although originally detected by Webster et al. (Webster et al., 2015) in public *D. melanogaster* transcriptome datasets (PRJNA159179; Rodriguez et. al., 2012) and provisionally named, it has not previously been detected in wild flies. It is detected here at high levels (26.8% of all remapped virus reads) in *D. suzukii* samples from France in 2013. Brandeis virus belongs to the Hepe-Virga clade of +ssRNA viruses and is closely related to Muthill virus, a virus of *Drosophila*: *D. immigrans* (Webster et al., 2016). A contig of 10.7 kb was assembled, which given homology to closely related virga-like viruses, is likely to represent a near-complete genome. Detection of Muthill virus itself is likely due to cross mapping of reads to this Brandeis virus genome. The other previously reported *Drosophila* viruses that we reidentified with confidence here are the iflaviruses Kinkell virus and La Jolla virus. Kinkell virus, first described by Webster et al. (Webster et al., 2016) was detectable in *D. suzukii* from the UK in 2016, and La Jolla in all samples from all locations. La Jolla virus reads were detected at high titre in all samples, comprising up to 30.7% of viral reads in British flies from 2014, and on average 15.0% of virus reads across all samples.

Four viruses of other *Drosophila* species also appear to be present in *D. suzukii* populations. For example, Corseley virus, a virus most associated with *D. subobscura* (Webster et al., 2016), which was detected at fairly high levels in British caught *D. suzukii* from 2016. It is uncommon in other *Drosophila* species (Webster et al., 2016) and is sufficiently divergent from any newly described *D. suzukii* viruses to minimise cross-mapping of reads. Galbut and Chaq viruses are both known infectious agents of *D. melanogaster*, but appear to be at high levels in 2015 *D. suzukii*. Cross-mapping to these viruses is unlikely due to their divergence from other *Drosophila* viruses, and host species contamination is unlikely to explain the high numbers of re-mapped reads observed. Dkikkawai virus (Webster et al., 2015) may represent true association for the same reasons. It was detected in Japanese flies only, although not at such high titre. Bloomfield virus, a reovirus of *D. melanogaster*, also likely represents true association with *D. suzukii* as we identified a divergent haplotype of one of the 10 genomic segments in *D. suzukii* that has not previously been seen in *D. melanogaster.* It is tempting to speculate that this reflects a history of host shifting and segment reassortment in this virus.

The remaining previously published viruses, were detected at much lower levels, and it is likely that some of them represent a low level of cross mapping from newly described but closely related viruses. For example, the low number of reads mapping to Buckhurst virus, a virus of *D. obscura* (Webster et al., 2016), may be mismapped Beult virus reads, the few reads mapping to Prestney Burn virus and Motts Mill (Webster et al., 2016, Webster et al., 2015) are most likely mismapped Teise virus reads.

Reads mapping to *D. immigrans* Nora virus and *D. melanogaster* Nora virus may actually belong to the *D. suzukii* Nora virus. In addition, a small number may result from sample contamination by misidentified flies and/or library cross-contamination, such as barcode-switching (Sinha et al., 2017, Kircher et al., 2011, Ballenghien et al., 2017). This includes viruses with no close relative associated with *D. suzukii*, such as Thika virus, Craigies Hill virus and Ashworth virus (*unpublished*), or viruses with biologically constrained host ranges, such as the Sigma viruses, along with *Drosophila* A virus (DAV), *Drosophila* C virus (DCV), and *D. melanogaster* Nora virus that were known to be present in *D. melanogaster* samples run alongside the 2016 *D. suzukii* samples.

Virus titre and composition varies among samples

To estimate the amount of virus in each sample all raw reads were mapped back to newly discovered and established putative *Drosophila* virus genomes (Fig. 6). The percentage of non-rRNA reads that mapped to any *Drosophila* virus varied from 0.09% in the poly-A selected French sample up to 5.14% in UK sample from 2016, with an average of 4.27% of reads being viral in Japanese and British pools. The virus composition varied markedly among samples from different times and locations. Despite applying a detection threshold for very low viral read numbers, it is not possible to formally analyse patterns of virus sharing among years or sampling locations from these data, for three reasons. First, the possibility of cross-mapping between some highly-conserved regions of the most closely related viruses means that the presence and absence of those viruses cannot accurately be inferred. Second, barcode switching (Sinha et al., 2017, Kircher et al., 2011, Ballenghien et al., 2017) and other sources of cross-contamination between libraries sequenced together on the Illumina platform may allow miss-assignment of reads between the Japanese and British samples from 2016, and also from other drosophilid libraries analysed at the same time. Finally, cytochrome oxidase (COI) read mapping suggests a small proportion of contaminating reads deriving from *D. melanogaster* and *D. immigrans* were present in some of the datasets. For example, in the Japanese sample of 2016 1.3% of COI reads mapped to *D. immigrans* (potentially misidentified larvae) and in the UK sample of 2015 0.74% of reads mapped to *D. melanogaster*. The *D. melanogaster* reads may represent misidentification or cross-mapping, as the species are quite closely related, but it is more likely that they are the result of

contamination across libraries through barcode switching as *D. suzukii* samples were sequenced in parallel with unrelated drosophilid libraries.

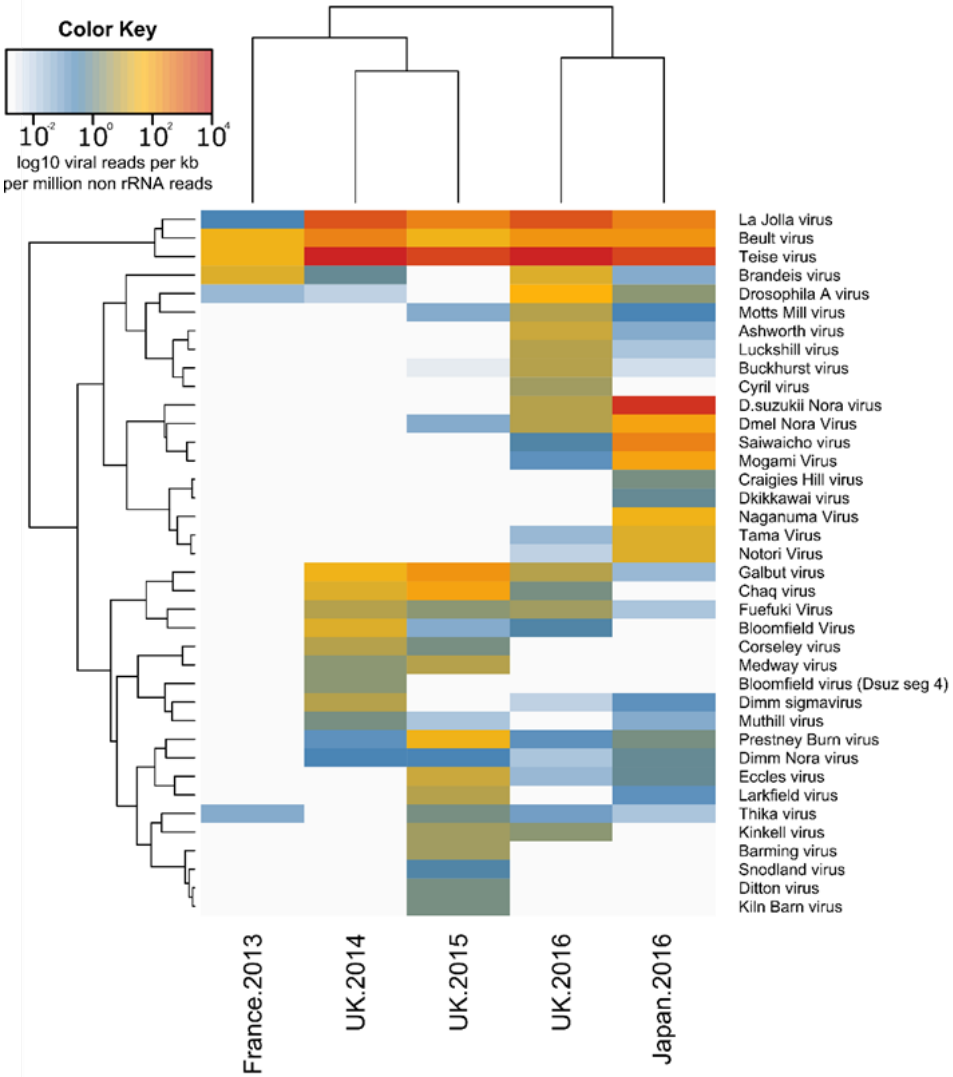

Figure 6. The heatmap shows the relative number of reads (log_{10} reads per kb per million non-ribosomal RNA reads) from each library mapping to each of the *Drosophila* viruses. Rows and columns are clustered by their similarity in read frequency on a log_{10} scale. A threshold for detection of 0.5 reads per kb per million non-rRNA reads was applied, however, a small amount of cross mapping is possible between closely related viruses and this may explain the detection of viruses with very low read counts. The low diversity of viruses in the France 2013 sample may be attributable to poly-A selection of RNA libraries. Some host species contamination and /or library cross-contamination (e.g. barcode switching) is possible in all samples, with a low proportion of COI reads mapping to *D. melanogaster* and *D. immigrans*. This could potentially explain the apparent presence of Sigma and Nora viruses from other drosophilids. Created using the 'heatmap2' function of the gplots package (Warnes et al., 2016) in R (R Core Team, 2017).

Virus Pathology

Pooled viral extracts from wild flies induces some mortality in lab reared *D. suzukii* (Figure 7). Flies injected with extract filtered through 0.22µm filter or centrifuged extract showed an increased mortality compared to flies injected with fresh ringers weekly (WC) but not compared to flies injected with passaged control (C). This effect grew in later passages with passaged controls causing significant mortality compared to the fresh controls in passage 4 (Figure 7). After four passages we tested by RT-PCR for infection by known viruses and all passaged treatments tested positive for DCV.

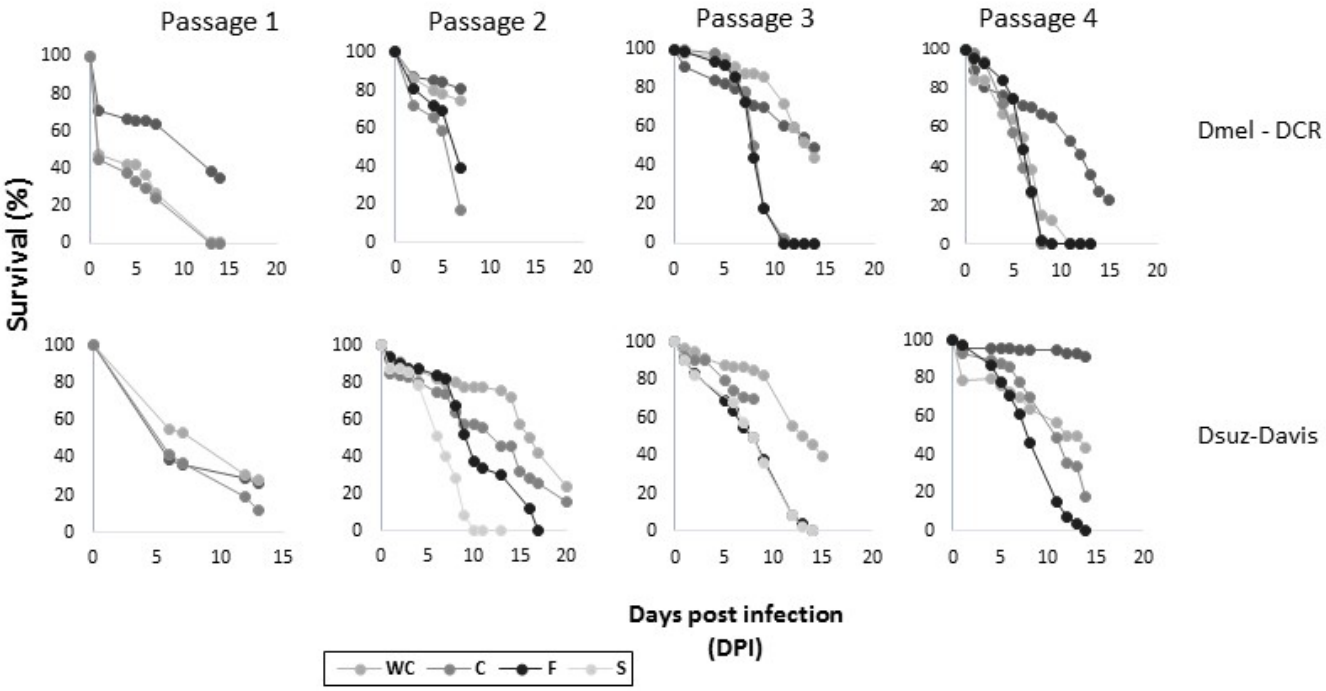

Figure 7. Survival of flies after injection with a pooled wild fly extract and serial passaged four times. WC= weekly or sham control with filtered ringers sol., C = passaged control, F= wild fly treatment passed through 0.22µm filter and S = wild fly treatment centrifuged to remove bacteria, not filtered

D. suzukii treated with concentrated solution of Kallithea virus did not show increased mortality compared to control flies (Fig. 8). Adult flies introduced to food vials heavily dosed with virus solution survived as well as control flies, showing no significant mortality over a three week period (Cox's mixed effects model; Z=-0.81, P= 0.42). Application of concentrated virus solution also showed no effect on larval development. Virus treatment did not effect the number of larvae developing to adulthood (Fig. 9A; Welch Two Sample t-test; t = 1.0658, p-value = 0.3011) nor did it effect the length of larval development (Fig. 9B; Welch Two Sample t-test; t = 0, p-value = 1).

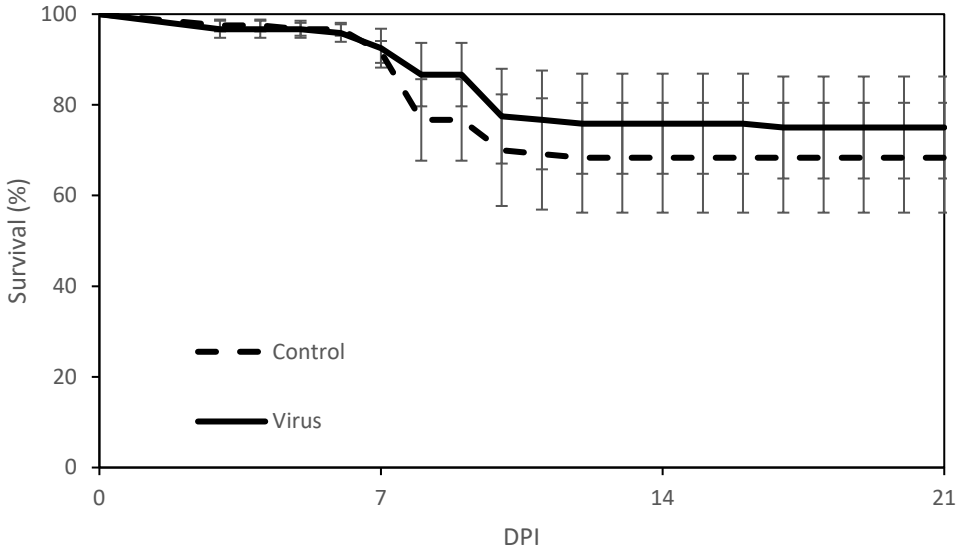

Figure 8. Survival of adult *D. suzukii* exposed to Kallithea virus oral infection. Virus infected flies (solid line) exposed to viral solution of 2000 X ID50 Kallithea virus preparation. Control flies (dotted line) were exposed to sterile Tris buffer.

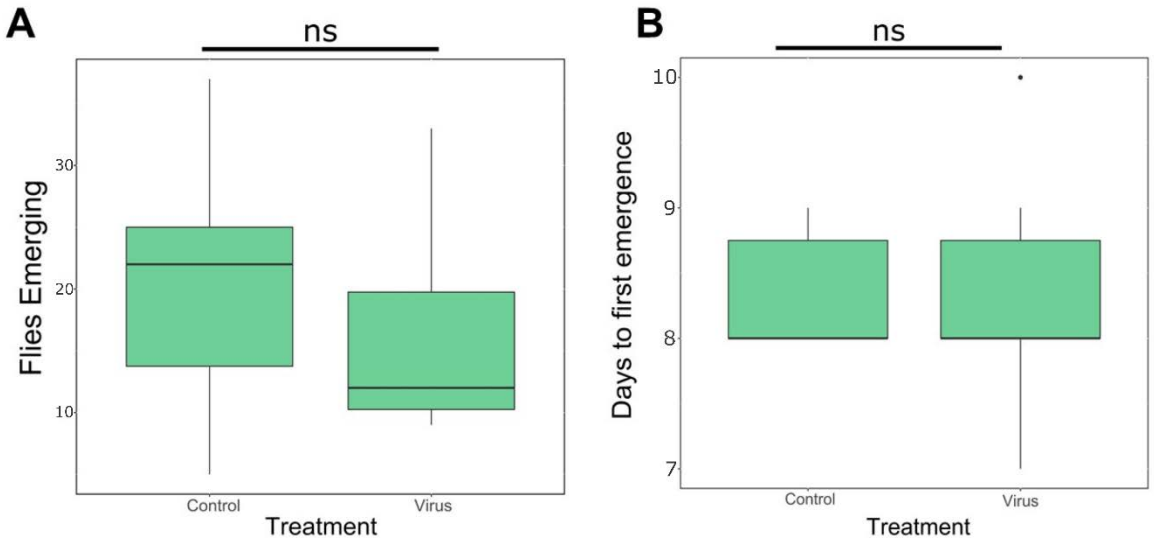

Figure 9. Survival and development of larval *D. suzukii* exposed to Kallithea virus solution. Groups of virus treated larvae were exposed to a solution of 2000 X ID50 Kallithea virus as first instar larvae. Control groups were exposed to sterile Tris buffer.

Antiviral Gene Expression

Gene expression associated to virus infection is being investigated for infection with two viruses known to be pathogenic in *D. melanogaster*. Early results show a selection of genes in *D. suzukii* that have a significant change in regulation under virus challenge. Kallithea virus seems to elicit a greater transcriptional response than DCV in *D. suzukii* (Fig. 10). Gene expression data is currently being analysed to test for differences between *D. suzukii* and *D. melanogaster*.

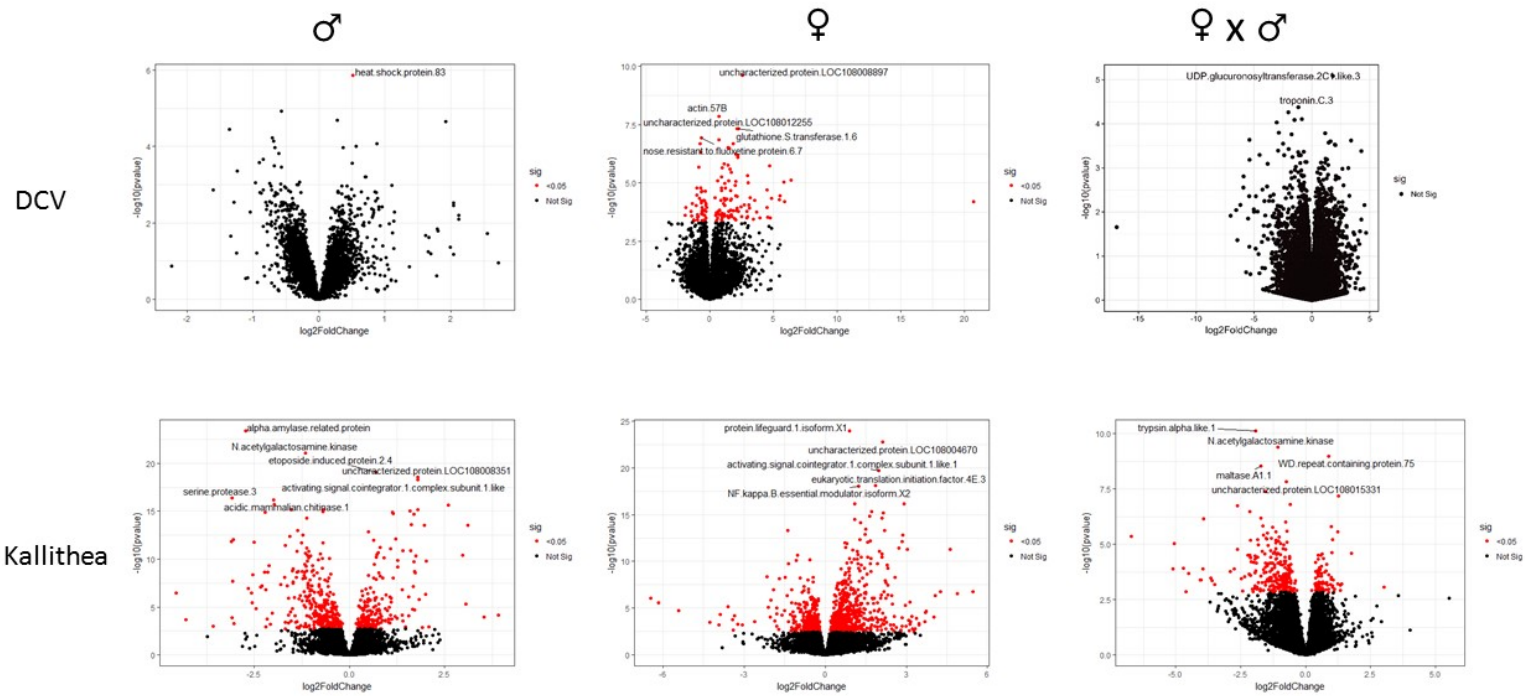

Figure 10. Volcano plots showing fold changes and p-values from Wald tests for differential expression of *D. suzukii* genes following infection with Drosophila C virus (DCV)(upper row) or Kallithea virus (lower row) for females (left), males (center), those different between the sexes (right). In each panel up to 7 genes with the smallest p-values are labelled. Many of the genes differentially regulated between the sexes are those highly significant in females.

Discussion

Here we make a first survey of the viruses associated with the invasive *Drosophila* pest *D. suzukii* in its native and invasive ranges. Alongside 18 new viruses, not previously described from any organism, we confidently identified a further seven viruses associated with this novel invasive host that had previously been described from other *Drosophila* species. Some novel viruses were detected solely from the native range of *D. suzukii* and others from the invasive range, but rarely from both habitats.

These viruses were identified from metagenomic sequencing of samples of wild *D. suzukii*, and although their presence as RNA but not DNA implies that they are not expressed endogenised viral elements (i.e. EVEs), it remains possible that some are not truly infections in this fly, but may be contaminants of the surface of the fly or infect a commensal, pathogenic, or food organism within the fly's gut lumen. However, we believe that this is unlikely to be the case for most sequences, as previous studies that additionally used the presence of virus-derived 21nt short interfering RNAs to demonstrate active replication (Webster et al., 2015) found that the majority of viruses identified in similar metatransciptomic sequencing of *D. melanogaster* constituted active infections. There is also a possibility of some cross-species contamination, barcode-switching and cross-mapping could result in spurious host allocation but this is not compatible with the read number or distribution of the majority of viruses (above).

In addition, more recent large-scale invertebrate virus discovery projects (Shi et al., 2016) give us a greatly increased confidence in the phylogenetic relationships of newly identified virus sequences. In particular, despite some virus taxa having a diverse host ranges, it seems reasonable to infer that *D. suzukii* is the true host for viruses with very close relatives confirmed to infect another insect. For example, Mogami virus (Chuvirus) is distantly related to any known *Drosophila* virus, but is closely related to Shayang Fly virus 1 (Shi et al., 2016) and clusters within a group of viruses that are only described from insect samples (see Fig. 4, D). Nevertheless, this pattern is not true for all viruses described here. Specifically, two of the 18 novel viruses in this study (Ditton virus and Barming virus), are more closely related to Mycoviruses than they are to any entomopathogenic viruses and one (Luckshill virus) is most closely related to a sequence found in a parasitic nematode of ruminants. And, while this pattern does not exclude the possibility of these being true viruses of *D. suzukii*—as many viral families contain a broad range of hosts including those of different phyla and patterns of host switching are still little understood—these are among the best candidates to be infections of *Drosophila* parasites or gut fauna, rather than *D. suzukii* itself.

The potential of these viruses to be used as biological control agents is currently unclear. Commercially successful viral biocontrol agents have in the past only come from the dsDNA virus family Baculoviridae, which was not represented in our collections, and most lineages represented here have not been investigated for their ability to be cultured and applied as control agents. Indeed, few viruses in the families here have been successfully isolated for experimentation, and many are known only from metagenomic sequencing. Kallithea virus of *D. melanogaster* is one virus for which we do have working isolates, however our work shows that the lethality of this virus to *D. suzukii* is far lower than that expected of a successful biological control agent. The only virus family we found in associated with *D. suzukii* that has any history as a control agent (Zeddam et al., 2003, Peng et al., 1998, Peng et al., 2000) is the reovirus 'Eccles virus'. Eccles virus was relatively rare in our samples, but this may speak to the potential pathogenicity of the virus, as flies harbouring a

particularly pathogenic virus, especially one that has a short latency period, may be less likely to visit baited traps (74). Further investigation of this virus, including isolation and pathogenicity assays, are needed before any further conclusions can be drawn about its utility as a control agent. Virus enriched extracts from wild *D. Suzukii* potentially harbour some of the viruses identified by the metatransciptomic survey and may be in-part responsible for increased mortality seen during experimental infections. Detected contamination does, however make determining the pathology of such viruses extremely difficult. Viruses potentially lethal to *D. suzukii* may also await discovery in other species of *Drosophila*. Indeed, pathogens have the potential to display increased virulence following a host shift event (Longdon et al., 2015) and the susceptibility of D. suzukii to viruses of D. melanogaster has been shown experimentally (Cattel et al., 2016, Lee and Vilcinskas, 2017). Here we show the potential association of viruses from D. melanogaster, D. immigrans and D. subobscura with D. suzukii in the wild. Further investigation of the viral community experienced by many different Drosophila in nature may, therefore, be of both academic and applied interest.

Given our focus on an invasive species, the potential for a shift in the virological environment associated with invasion is of particular interest. Theory predicts that organisms may experience a 'release' from natural enemies, including pathogens, in their invasive range due to low host densities and founder effects at the invasive edge (Keane and Crawley, 2002): However, this idea remains contentious, as supporting evidence is limited (Colautti et al., 2004). It has also been hypothesised that invasives, rather than experience a drop in overall number of enemies, undergo a shift in the type of enemy encountered, from co-evolved specialists in the native range to more generalist enemies, quickly able to adapt to a new host, in the naturalized range (Joshi and Vrieling, 2005). In this study, we do detect an apparently marked difference in the virus communities of flies from different areas within its expanding geographical range. Although a low level of species contamination in certain pools means that these findings should be treated with some caution, five of the new viruses described (Saiwaicho virus, Tama virus, Mogami virus, Naganuma virus and Notori virus) were only detected at high levels in Japanese (native) flies. These five viruses are not particularly closely related to any previously described *Drosophila* viruses (Fig. 2 and Fig. 4) and may represent a more specialized relationship with *D. suzukii*. In contrast, the three most ubiquitous viruses across all samples, La Jolla virus, Teise virus and Beult virus are either a known generalist (La Jolla) or very closely related to a virus in another related hosts (Fig.2, A and E). If confirmed, this pattern could reflect a shift in natural enemy type from native to invasive range of *D. suzukii*.

Conclusions

- 18 new viruses have been discovered infecting *Drosophila suzukii*
- A similar number of know *Drosophila* viruses also infect *D. suzukii* in the wild
- Kalithea virus, a nudivirus of *D. melanogaster* does not cause significant mortality in *D. suzukii*

- Assays confirming virus presence by RT-PCR have allowed an estimate of prevalence for newly discovered and previously discovered viruses.
- Growth of wild viruses in lab strains of *D. suzukii* is poor, limiting the ability to isolate viruses.

Knowledge and Technology Transfer

2017

- Talk: SWD steering group meeting, Belgravia, London
- Poster: Ecological Immunology meeting, Blossin, Germany

2016

- Talk: ICE2016 Florida, *D. suzukii* symposium
- Poster: Popgroup 49, Edinburgh
- Talk: Guest Seminar, Tokyo University of Agriculture and Technology.
- Poster: IEB student poster day, Winner.
- Poster and Talk: AHDB studentship conference 2016.
- Poster: RES insect infection and immunity special interest group.

2015

- Poster: HDC conference, Herefordshire

Glossary

Amplicon: the section of genetic material targeted and amplified by a particular PCR reaction.

Contig: Section of RNA or DNA sequence assembled from the smaller reads produced by our sequencing.

DNA: Deoxyribonucleic acid, the primary coding molecule for most living genomes.

DNA virus: A virus in which genomic sequence is made up of DNA.

ID_{50}: Infective Dose at which 50% of hosts or cells (if calculated from cell culture) are killed.

Metagenomics: A method for sequencing all genetic material present in an environmental or whole-organism sample. Results in the identification of all species present in that sample.

mRNA: 'messenger' Ribonucleic acid, the messenger molecule acting as an intermediate to translate the cell's DNA blueprint into proteins, the building blocks of an organism.

Nucleotide (nt): a single letter in the genetic code. One half of a 'base pair'(bp). A, C, G, T or U (in RNA).

Open reading frame (ORF): A stretch of sequence uninterrupted by a 'stop codon'. This can be loosely interpreted as a protein-coding region or gene.

PCR: Polymerase Chain Reaction. A molecular method used to amplify particular segments of DNA.

Primers: a short sequence of DNA used during a PCR reaction to amplify a particular piece of target DNA.

Read: A short fragment of RNA or DNA produced by sequencing.

RNA: Ribonucleic acid, includes messenger molecule acting as a translator between DNA and proteins, the genomic molecule of certain viruses and

RNA virus: A virus in which genomic sequence is made up of RNA.

RT-PCR: Reverse Transcriptase PCR (see above). During the RT reaction RNA is transcribed into a complimentary DNA which can be taken forward into a conventional PCR protocol.

References

ADAMS, J. R. & BONAMI, J. R. 1991. Atlas of invertebrate viruses. *Atlas of invertebrate viruses.*

ADHB 2015. Laboratory determination of the environmental conditions needed to eliminate *D. suzukii* and pest attractiveness from waste. http://horticulture.ahdb.org.uk/sites/default/files/research_papers/SF%20145_Report_Annual_2015_0.pdf: East Malling Research.

ADRION, J. R., KOUSATHANAS, A., PASCUAL, M., BURRACK, H. J., HADDAD, N. M., BERGLAND, A. O., MACHADO, H., SACKTON, T. B., SCHLENKE, T. A. & WATADA, M. 2014. Drosophila suzukii: The Genetic Footprint of a Recent, Worldwide Invasion. *Molecular biology and evolution,* 31**,** 3148-3163.

AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen recognition and innate immunity. *Cell,* 124**,** 783-801.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *J Mol Biol,* 215**,** 403-10.

AMBROSE, R. L., LANDER, G. C., MAATY, W. S., BOTHNER, B., JOHNSON, J. E. & JOHNSON, K. N. 2009. Drosophila A virus is an unusual RNA virus with a T= 3 icosahedral core and permuted RNA-dependent RNA polymerase. *Journal of general virology,* 90**,** 2191-2200.

AMIN UD DIN, M., MAZHAR, K., HAQUE, S. & AHMED, M. 2005. A preliminary report on Drosophila fauna of Islamabad (Capital, Pakistan). *Drosoph. Info. Serv.,* 88**,** 6-7.

ANISIMOVA, M., GIL, M., DUFAYARD, J. F., DESSIMOZ, C. & GASCUEL, O. 2011. Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst Biol,* 60**,** 685-99.

ANTONACCI, R., TRITTO, P., CAPPUCCI, U., FANTI, L., PIACENTINI, L. & BERLOCO, M. 2017. Drosophilidae monitoring in Apulia (Italy) reveals Drosophila suzukii as one of the four most abundant species. *Bulletin of Insectology,* 70**,** 139-146.

ARNÓ, J., RIUDAVETS, J. & GABARRA, R. 2012. Survey of host plants and natural enemies of Drosophila suzukii in an area of strawberry production in Catalonia (northeast Spain). *IOBC/WPRS Bulletin,* 80**,** 29-34.

ATALLAH, J., TEIXEIRA, L., SALAZAR, R., ZARAGOZA, G. & KOPP, A. 2014. *The making of a pest: the evolution of a fruit-penetrating ovipositor in Drosophila suzukii and related species*.

AVADHANULA, V., WEASNER, B. P., HARDY, G. G., KUMAR, J. P. & HARDY, R. W. 2009. A Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. *PLOS Pathogens,* 5**,** e1000582.

BÄCHLI, G., VILELA, C. R., ESCHER, S. A. & SAURA, A. 2004. *The Drosophilidae (Diptera) of Fennoscandia and Denmark*, Brill Academic Publishers.

BALLENGHIEN, M., FAIVRE, N. & GALTIER, N. 2017. Patterns of cross-contamination in a multispecies population genomic project: detection, quantification, impact, and solutions. *BMC biology,* 15**,** 25.

BAROFFIO, C. & FISCHER, S. 2011. Neue bedrohung für obstplantagen und beerenpflanzen: die kirschessigfliege. *UFA-Revue.(11)***,** 46-47.

BEARDSLEY, J., ARAKAKI, K., UCHIDA, G., KUMASHIRO, B. & PERREIRA, W. 1999. New records for Diptera in Hawai'i. *Bishop Museum Occasional Papers, Records of the Hawaii Biological Survey,* 58**,** 51-57.

BECKSTEAD, J. & PARKER, I. M. 2003. INVASIVENESS OF AMMOPHILA ARENARIA: RELEASE FROM SOIL-BORNE PATHOGENS? *Ecology,* 84**,** 2824-2831.

BERKALOF, A., BREGLIAN, J. C. & OHANESSI, A. 1965. Mise en evidence de virions dans des drosophiles infectees par le virus hereditaire sigma. *COMPTES RENDUS HEBDOMADAIRES DES SEANCES DE L ACADEMIE DES SCIENCES,* 260**,** 5956-&.

BIONDI, A., MOMMAERTS, V., SMAGGHE, G., VIÑUELA, E., ZAPPALÀ, L. & DESNEUX, N. 2012. The non-target impact of spinosyns on beneficial arthropods. *Pest management science,* 68**,** 1523-1536.

BISHOP, D. H. L. & SHOPE, R. E. 1979. Bunyaviridae. *In:* FRAENKEL-CONRAT, H. & WAGNER, R. R. (eds.) *Comprehensive Virology: Newly Characterized Vertebrate Viruses.* Boston, MA: Springer US.

BLOSSEY, B. & NOTZOLD, R. 1995. Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. *Journal of Ecology,* 83**,** 887-889.

BOLDA, M. 2008. *New fruit fly pest in strawberries and caneberries* [Online]. University of California, Division of Agriculture and Natural Resources. Available: http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=821 [Accessed 10/11/2014 2014].

BOLDA, M. P., GOODHUE, R. E. & ZALOM, F. G. 2010. Spotted wing drosophila: potential economic impact of a newly established pest. *Agricultural and Resource Economics Update,* 13**,** 5-8.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics,* 30**,** 2114-2120.

BRUCK, D. J., BOLDA, M., TANIGOSHI, L., KLICK, J., KLEIBER, J., DEFRANCESCO, J., GERDEMAN, B. & SPITLER, H. 2011. Laboratory and field comparisons of insecticides to reduce infestation of Drosophila suzukii in berry crops. *Pest management science,* 67**,** 1375-1385.

BRUN, G. & PLUS, N. 1980. The viruses of Drosophila. *The genetics and biology of Drosophila,* 2**,** 625-702.

BUCHFINK, B., XIE, C. & HUSON, D. H. 2015. Fast and sensitive protein alignment using DIAMOND. *Nat Meth,* 12**,** 59-60.

CALABRIA, G., MÁCA, J., BÄCHLI, G., SERRA, L. & PASCUAL, M. 2012. First records of the potential pest species Drosophila suzukii (Diptera: Drosophilidae) in Europe. *Journal of Applied entomology,* 136**,** 139-147.

CALLAWAY, R. M., THELEN, G. C., RODRIGUEZ, A. & HOLBEN, W. E. 2004. Soil biota and exotic plant invasion. *Nature,* 427**,** 731.

CAMPOS, M. R., RODRIGUES, A. R. S., SILVA, W. M., SILVA, T. B. M., SILVA, V. R. F., GUEDES, R. N. C. & SIQUEIRA, H. A. A. 2014. Spinosad and the tomato borer Tuta absoluta: a bioinsecticide, an invasive pest threat, and high insecticide resistance. *PloS one,* 9**,** e103235.
CARTER, J. 1984. Viruses as pest-control agents. *Biotechnology and genetic engineering reviews,* 1**,** 375-419.
CARVAJAL, J. I. & MARKOW, T. 2010. Genetic diversity of Drosophila suzukii in San Diego. *Drosophila Information Service,* 93**,** 67.
CHA, D. H., HESLER, S. P., COWLES, R. S., VOGT, H., LOEB, G. M. & LANDOLT, P. J. 2013. Comparison of a synthetic chemical lure and standard fermented baits for trapping Drosophila suzukii (Diptera: Drosophilidae). *Environmental entomology,* 42**,** 1052-1060.
CHA, D. H., HESLER, S. P., PARK, S., ADAMS, T. B., ZACK, R. S., ROGG, H., LOEB, G. M. & LANDOLT, P. J. 2015. Simpler is better: fewer non-target insects trapped with a four-component chemical lure vs. a chemically more complex food-type bait for Drosophila suzukii. *Entomologia Experimentalis et Applicata,* 154**,** 251-260.
CHABERT, S., ALLEMAND, R., POYET, M., ESLIN, P. & GIBERT, P. 2012. Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, Drosophila suzukii. *Biological Control,* 63**,** 40-47.
CHTARBANOVA, S., LAMIABLE, O., LEE, K.-Z., GALIANA, D., TROXLER, L., MEIGNIN, C., HETRU, C., HOFFMANN, J. A., DAEFFLER, L. & IMLER, J.-L. 2014. Drosophila C virus systemic infection leads to intestinal obstruction. *Journal of virology,* 88**,** 14057-14069.
CINI, A., IORIATTI, C. & ANFORA, G. 2012. A review of the invasion of Drosophila suzukii in Europe and a draft research agenda for integrated pest management. *Bulletin of insectology,* 65**,** 149-160.
CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2016. GenBank. *Nucleic Acids Research,* 44**,** D67-D72.
COLAUTTI, R. I., RICCIARDI, A., GRIGOROVICH, I. A. & MACISAAC, H. J. 2004. Is invasion success explained by the enemy release hypothesis? *Ecology Letters,* 7**,** 721-733.
COLLINS, J., CUPERUS, G., CARTWRIGHT, B., STARK, J. & EBRO, L. 1993. Consumer attitudes on pesticide treatment histories of fresh produce. *Journal of Sustainable Agriculture,* 3**,** 81-98.
CORMIER, D., VEILLEUX, J. & FIRLEJ, A. 2015. Exclusion net to control spotted wing Drosophila in blueberry fields. *IOBC-WPRS Bull,* 109**,** 181-184.
COSSENTINE, J., ROBERTSON, M. & BUITENHUIS, R. 2016. Impact of acquired entomopathogenic fungi on adult Drosophila suzukii survival and fecundity. *Biological Control,* 103**,** 129-137.
COSTA, A., JAN, E., SARNOW, P. & SCHNEIDER, D. 2009. The Imd Pathway Is Involved in Antiviral Immune Responses in Drosophila. *PLOS ONE,* 4**,** e7436.
CROSS, J. & BERRIE, A. 2006. The challenges of developing IPM programmes for soft fruit crops that eliminate reportable pesticide residues. *Journal of fruit and ornamental plant research,* 14**,** 49.
CROUCH, J. A., CLARKE, B. B. & HILLMAN, B. I. 2006. Unraveling Evolutionary Relationships Among the Divergent Lineages of Colletotrichum Causing Anthracnose Disease in Turfgrass and Corn. *Phytopathology,* 96**,** 46-60.
CROWDER, D. W. & JABBOUR, R. 2014. Relationships between biodiversity and biological control in agroecosystems: current status and future challenges. *Biological control,* 75**,** 8-17.
CUTHBERTSON, A., COLLINS, D., BLACKBURN, L., AUDSLEY, N. & BELL, H. 2014a. Preliminary Screening of Potential Control Products against Drosophila suzukii. *Insects,* 5**,** 488-498.
CUTHBERTSON, A. G. & AUDSLEY, N. 2016. Further screening of entomopathogenic fungi and nematodes as control agents for Drosophila suzukii. *Insects,* 7**,** 24.
CUTHBERTSON, A. G., BLACKBURN, L. F. & AUDSLEY, N. 2014b. Efficacy of commercially available invertebrate predators against Drosophila suzukii. *Insects,* 5**,** 952-960.
DEDDOUCHE, S., MATT, N., BUDD, A., MUELLER, S., KEMP, C., GALIANA-ARNOUX, D., DOSTERT, C., ANTONIEWSKI, C., HOFFMANN, J. A. & IMLER, J.-L. 2008. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. *Nat Immunol,* 9**,** 1425-1432.

DEL FAVA, E., IORIATTI, C. & MELEGARO, A. 2017. Cost-benefit analysis of controlling the spotted wing drosophila (Drosophila suzukii (Matsumura)) spread and infestation of soft fruits in Trentino, Northern Italy. *Pest Management Science*.

DEPRÁ, M., POPPE, J. L., SCHMITZ, H. J., DE TONI, D. C. & VALENTE, V. L. 2014. The first records of the invasive pest Drosophila suzukii in the South American continent. *Journal of Pest Science,* 87**,** 379-383.

DESNEUX, N., DECOURTYE, A. & DELPUECH, J.-M. 2007. The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.,* 52**,** 81-106.

DOBOS, P., HILL, B., HALLETT, R., KELLS, D., BECHT, H. & TENINGES, D. 1979. Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *Journal of Virology,* 32**,** 593-605.

DOSTERT, C., JOUANGUY, E., IRVING, P., TROXLER, L., GALIANA-ARNOUX, D., HETRU, C., HOFFMANN, J. A. & IMLER, J.-L. 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat Immunol,* 6**,** 946-953.

DUVICK, D. N. 1984. Genetic diversity in major farm crops on the farm and in reserve. *Economic Botany,* 38**,** 161-178.

EPPO 2010. Pest Risk Analysis for : Drosophila suzukii. *In:* ORGANISATION, T. E. A. M. P. P. (ed.). http://www.eppo.int/QUARANTINE/Pest_Risk_Analysis/PRAdocs_insects/11-17189_PRA_record_Drosophila_suzukii_final%20.pdf.

ESLIN, P. & PRÉVOST, G. 1998. Hemocyte load and immune resistance to Asobara tabida are correlated in species of the Drosophila melanogaster subgroup. *Journal of Insect Physiology,* 44**,** 807-816.

FARNSWORTH, D., HAMBY, K. A., BOLDA, M., GOODHUE, R. E., WILLIAMS, J. C. & ZALOM, F. G. 2017. Economic analysis of revenue losses and control costs associated with the spotted wing drosophila, Drosophila suzukii (Matsumura), in the California raspberry industry. *Pest management science,* 73**,** 1083-1090.

FERA. 2015. *DROPSA: Strategies to develop effective, innovative and practical approaches to protect major european fruit crops from pests and pathogens* [Online]. Fera Science Ltd (Fera). Available: https://secure.fera.defra.gov.uk/dropsa/ [Accessed 15/06/2015 2015].

FERNÁNDEZ-BRAVO, M. An experimental autoinoculation device to control an invasive Asiatic pest, Drosophila suzukii. Poster session presented at: 47th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology, August 3-7 2014 Mainz, Germany.

FOUNTAIN, M. & MEDD, N. 2015. Integrating pesticides and predatory mites in soft fruit crops. *Phytoparasitica,* 43**,** 657-667.

GABARRA, R., RIUDAVETS, J., RODRÍGUEZ, G. A., PUJADE-VILLAR, J. & ARNÓ, J. 2014. Prospects for the biological control of Drosophila suzukii. *BioControl,* 60**,** 331-339.

GARGANI, E., TARCHI, F., FROSININI, R., MAZZA, G. & SIMONI, S. 2013. Notes on Drosophila suzukii Matsumura (Diptera Drosophilidae): field survey in Tuscany and laboratory evaluation of organic products. *Redia,* 96**,** 85-90.

GERRITSMA, S., HAAN, A. D., ZANDE, L. V. D. & WERTHEIM, B. 2013. Natural variation in differentiated hemocytes is related to parasitoid resistance in Drosophila melanogaster. *Journal of Insect Physiology,* 59**,** 148-158.

GOODHUE, R. E., BOLDA, M., FARNSWORTH, D., WILLIAMS, J. C. & ZALOM, F. G. 2011. Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. *Pest management science,* 67**,** 1396-1402.

GOPAL, M., GUPTA, A., SATHIAMMA, B. & NAIR, C. 2001. Control of the coconut pest Oryctes rhinoceros L. using the Oryctes virus. *International Journal of Tropical Insect Science,* 21**,** 93-101.

GRABHERR, M. G., HAAS, B. J., YASSOUR, M., LEVIN, J. Z., THOMPSON, D. A., AMIT, I., ADICONIS, X., FAN, L., RAYCHOWDHURY, R., ZENG, Q., CHEN, Z., MAUCELI, E., HACOHEN, N., GNIRKE, A., RHIND, N., DI PALMA, F., BIRREN, B. W., NUSBAUM, C., LINDBLAD-TOH, K., FRIEDMAN, N. & REGEV, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotech,* 29**,** 644-652.

GRAHAM, R. I., RAO, S., POSSEE, R. D., SAIT, S. M., MERTENS, P. P. C. & HAILS, R. S. 2006. Detection and characterisation of three novel species of reovirus (Reoviridae), isolated from geographically separate populations of the winter moth Operophtera brumata (Lepidoptera: Geometridae) on Orkney. *Journal of Invertebrate Pathology,* 91**,** 79-87.

GRANADOS, R. R. 1980. Infectivity and mode of action of Baculoviruses. *Biotechnology and Bioengineering,* 22**,** 1377-1405.

GRASSI, A., ANFORA, G., MAISTRI, S., MADDALENA, G., DE CRISTOFARO, A., SAVINI, G. & IORIATTI, C. Development and efficacy of Droskidrink, a food bait for trapping Drosophila suzukii. VIII Workshop on Integrated soft fruit production, 2014.

GUINDON, S. & GASCUEL, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic biology,* 52**,** 696-704.

HABAYEB, M. S., CANTERA, R., CASANOVA, G., EKSTRÖM, J.-O., ALBRIGHT, S. & HULTMARK, D. 2009. The Drosophila Nora virus is an enteric virus, transmitted via feces. *Journal of invertebrate pathology,* 101**,** 29-33.

HABAYEB, M. S., EKENGREN, S. K. & HULTMARK, D. 2006. Nora virus, a persistent virus in Drosophila, defines a new picorna-like virus family. *Journal of General Virology,* 87**,** 3045-3051.

HARRIS, A. & SHAW, B. 2014. First record of Drosophila suzukii (Matsumura) (Diptera, Drosophilidae) in Great Britain. *Dipterists Digest,* 21**,** 189-192.

HENIKOFF, S. & HENIKOFF, J. G. 1992. Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 10915-10919.

HUANG, Z., KINGSOLVER, M. B., AVADHANULA, V. & HARDY, R. W. 2013. An antiviral role for antimicrobial peptides during the arthropod response to alphavirus replication. *J Virol,* 87**,** 4272-80.

HUGER, A. M. 2005. The Oryctes virus: Its detection, identification, and implementation in biological control of the coconut palm rhinoceros beetle, Oryctes rhinoceros (Coleoptera: Scarabaeidae). *Journal of Invertebrate Pathology,* 89**,** 78-84.

HUNTER-FUJITA, F. R., ENTWISTLE, P. F., EVANS, H. F. & CROOK, N. E. 1998. *Insect viruses and pest management*, John Wiley & Sons Ltd.

HUSZAR, T. & IMLER, J. L. 2008. Chapter 6 Drosophila Viruses and the Study of Antiviral Host-Defense. 72**,** 227-265.

JACKSON, T. 2009. The Use of Oryctes Virus for Control of Rhinoceros Beetle in the Pacific Islands. *In:* HAJEK, A., GLARE, T. & O'CALLAGHAN, M. (eds.) *Use of Microbes for Control and Eradication of Invasive Arthropods.* Springer Netherlands.

JOSHI, J. & VRIELING, K. 2005. The enemy release and EICA hypothesis revisited: incorporating the fundamental difference between specialist and generalist herbivores. *Ecology Letters,* 8**,** 704-714.

JOUSSET, F.-X., BERGOIN, M. & REVET, B. 1977. Characterization of the Drosophila C virus. *Journal of General Virology,* 34**,** 269-283.

JOUSSET, F., CROIZIER, G. & THOMAS, M. 1972. Existence in Drosophila of 2 groups of picornavirus with different biological and serological properties. *Comptes rendus hebdomadaires des séances de l'Académie des sciences. Série D: Sciences naturelles,* 275**,** 3043.

KACSOH, B. Z., LYNCH, Z. R., MORTIMER, N. T. & SCHLENKE, T. A. 2013. Fruit Flies Medicate Offspring After Seeing Parasites. *Science,* 339**,** 947-950.

KACSOH, B. Z. & SCHLENKE, T. A. 2012. High hemocyte load is associated with increased resistance against parasitoids in Drosophila suzukii, a relative of D. melanogaster. *PloS one,* 7**,** e34721.

KANESHIRO, K. Drosophila (Sophophora) suzukii (Matsumura). Proc Hawaiian Entomol Soc, 1983. 179.

KANG, Y. & MOON, K. 1968. Drosophilid fauna of six regions near the demilitarized zone in Korea. *Korean Journal of Zoology,* 11**,** 65-68.

KANZAWA, T. 1935. Research into the fruit-fly Drosophila suzukii Matsumura (preliminary report). *Yamanashi Prefecture Agricultural Experiment Station Report*.

KANZAWA, T. 1939. Studies on Drosophila suzukii Mats. *Studies on Drosophila suzukii Mats.*
KATZOURAKIS, A. & GIFFORD, R. J. 2010. Endogenous viral elements in animal genomes. *PLoS genetics,* 6**,** e1001191.
KEANE, R. M. & CRAWLEY, M. J. 2002. Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology & Evolution,* 17**,** 164-170.
KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28**,** 1647-1649.
KELLY, D. W., PATERSON, R. A., TOWNSEND, C. R., POULIN, R. & TOMPKINS, D. M. 2009. Parasite spillback: a neglected concept in invasion ecology? *Ecology,* 90**,** 2047-2056.
KIM, V. N., HAN, J. & SIOMI, M. C. 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol,* 10**,** 126-139.
KIRCHER, M., SAWYER, S. & MEYER, M. 2011. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic acids research,* 40**,** e3-e3.
KISS, B., KIS, A. & KÁKAI, Á. 2016. The rapid invasion of spotted wing drosophila, Drosophila suzukii (Matsumura)(Diptera: Drosophilidae), in Hungary. *Phytoparasitica,* 44**,** 429-433.
KOONIN, E. V., DOLJA, V. V. & MORRIS, T. J. 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Critical reviews in biochemistry and molecular biology,* 28**,** 375-430.
KOPP, A. 2006. Basal relationships in the Drosophila melanogaster species group. *Molecular Phylogenetics and Evolution,* 39**,** 787-798.
KOYAMA, S., SAKAI, C., THOMAS, C. E., NUNOURA, T. & URAYAMA, S.-I. 2016. A new member of the family Totiviridae associated with arboreal ants (Camponotus nipponicus). *Archives of Virology,* 161**,** 2043-2045.
KOYAMA, S., URAYAMA, S.-I., OHMATSU, T., SASSA, Y., SAKAI, C., TAKATA, M., HAYASHI, S., NAGAI, M., FURUYA, T., MORIYAMA, H., SATOH, T., ONO, S.-I. & MIZUTANI, T. 2015. Identification, characterization and full-length sequence analysis of a novel dsRNA virus isolated from the arboreal ant Camponotus yamaokai. *Journal of General Virology,* 96**,** 1930-1937.
L'HÉRITIER, P. & TEISSIER, G. 1937. Une anomalie physiologique héréditaire chez la Drosophile. *CR Acad. Sci. Paris,* 231**,** 192-194.
L'HERITIER, P. H. 1948. Sensitivity to CO2 in Drosophila[mdash] A review. *Heredity,* 2**,** 325-348.
LANGMEAD, B. & SALZBERG, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature methods,* 9**,** 357-359.
LAVRINIENKO, A., KESÄNIEMI, J., WATTS, P. C., SERGA, S., PASCUAL, M., MESTRES, F. & KOZERETSKA, I. 2017. First record of the invasive pest Drosophila suzukii in Ukraine indicates multiple sources of invasion. *Journal of Pest Science,* 90**,** 421-429.
LE, S. Q. & GASCUEL, O. 2008. An improved general amino acid replacement matrix. *Molecular biology and evolution,* 25**,** 1307-1320.
LEACH, H., VAN TIMMEREN, S. & ISAACS, R. 2016. Exclusion Netting Delays and Reduces Drosophila suzukii (Diptera: Drosophilidae) Infestation in Raspberries. *J Econ Entomol*.
LEE, J. C., BURRACK, H. J., BARRANTES, L. D., BEERS, E. H., DREVES, A. J., HAMBY, K. A., HAVILAND, D. R., ISAACS, R., RICHARDSON, T. A. & SHEARER, P. W. 2012. Evaluation of monitoring traps for Drosophila suzukii (Diptera: Drosophilidae) in North America. *Journal of economic entomology,* 105**,** 1350-1357.
LEE, J. C., SHEARER, P. W., BARRANTES, L. D., BEERS, E. H., BURRACK, H. J., DALTON, D. T., DREVES, A. J., GUT, L. J., HAMBY, K. A. & HAVILAND, D. R. 2013. Trap designs for monitoring Drosophila suzukii (Diptera: Drosophilidae). *Environmental entomology,* 42**,** 1348-1355.
LEE, K.-Z. & VILCINSKAS, A. 2017. Analysis of virus susceptibility in the invasive insect pest Drosophila suzukii. *Journal of Invertebrate Pathology,* 148**,** 138-141.

LEWIS, R. L., BECKENBACH, A. T. & MOOERS, A. Ø. 2005. The phylogeny of the subgroups within the melanogaster species group: likelihood tests on COI and COII sequences and a Bayesian estimate of phylogeny. *Molecular phylogenetics and evolution,* 37**,** 15-24.
LIU, S., VIJAYENDRAN, D. & BONNING, B. C. 2011. Next generation sequencing technologies for insect virus discovery. *Viruses,* 3**,** 1849-1869.
LONGDON, B., OBBARD, D. J. & JIGGINS, F. M. 2009. Sigma viruses from three species of Drosophila form a major new clade in the rhabdovirus phylogeny. *Proceedings of the Royal Society of London B: Biological Sciences***,** rspb20091472.
LONGDON, B., WILFERT, L., OSEI-POKU, J., CAGNEY, H., OBBARD, D. J. & JIGGINS, F. M. 2011. Host-switching by a vertically transmitted rhabdovirus in Drosophila. *Biology letters***,** rsbl20110160.
LUCKOW, V. A. & SUMMERS, M. D. 1988. Trends in the development of baculovirus expression vectors. *Nature Biotechnology,* 6**,** 47-55.
LUE, C.-H., L. MOTTERN, J., WALSH, G. C. & BUFFINGTON, M. L. 2017. New record for the invasive spotted wing drosophila, Drosophila suzukii (Matsumura, 1931)(Diptera: Drosophilidae) in Anillaco, western Argentina. *Proceedings of the Entomological Society of Washington,* 119**,** 146-150.
LUO, C., JONES, C., DEVINE, G., ZHANG, F., DENHOLM, I. & GORMAN, K. 2010. Insecticide resistance in Bemisia tabaci biotype Q (Hemiptera: Aleyrodidae) from China. *Crop Protection,* 29**,** 429-434.
MAIER, C. T. 2012. First detection and widespread distribution of the spotted wing drosophila, Drosophila suzukii (Matsumura)(Diptera: Drosophilidae), in Connecticut in 2011. *Proceedings of the Entomological Society of Washington,* 114**,** 329-337.
MALAGNINI, V., ZANOTELLI, L., TOLOTTI, G., PROFAIZER, D. & ANGELI, G. Evaluation of predatory activity of Orius laevigatus (Fieber) and O. maiusculus Reuter towatds Drosophila suzikii (Matsumura) under laboratory conditions. VIII Workshop on Integrated soft fruit production, 2014. 120.
MANDURIC, S. 2017. Drosophila suzukii–experiences from the fly's northernmost inhabited region (from the first record to two years after the detection). *IOBC-WPRS Bulletin,* 123**,** 150-156.
MATSUMURA, S. 1931. 6000 Illustrated Insects of Japan-Empire, Tokyo. *Sphyracephala quadriguttata (Walk.) figured***,** 371.
MAZZI, D., BRAVIN, E., MERANER, M., FINGER, R. & KUSKE, S. 2017. Economic Impact of the Introduction and Establishment of Drosophila suzukii on Sweet Cherry Production in Switzerland. *Insects,* 8**,** 18.
MILLER, B., ANFORA, G., BUFFINGTON, M., DALTON, D. T., MILLER, J. C., WIMAN, N. G. & WALTON, V. M. 2015. Seasonal occurrence of resident parasitoids associated with Drosophila suzukii in two small fruit production regions of Italy and the USA.
MITSUI, H., BEPPU, K. & KIMURA, M. T. 2010. Seasonal life cycles and resource uses of flower-and fruit-feeding drosophilid flies (Diptera: Drosophilidae) in central Japan. *Entomological Science,* 13**,** 60-67.
MITSUI, H., TAKAHASHI, K. H. & KIMURA, M. T. 2006. Spatial distributions and clutch sizes of Drosophila species ovipositing on cherry fruits of different stages. *Population Ecology,* 48**,** 233-237.
MOHAN, K. & PILLAI, G. 1993. Biological control of Oryctes rhinoceros (L.) using an Indian isolate of Oryctes baculovirus. *International Journal of Tropical Insect Science,* 14**,** 551-558.
MORTON, R. 1993. Evolution of Drosophila insecticide resistance. *Genome,* 36**,** 1-7.
MOSCARDI, F. 1999. Assessment of the application of baculoviruses for control of Lepidoptera. *Annual review of entomology,* 44**,** 257-289.
NAGAYAMA, S. & OKAMOTO, H. 1940. List of fruit insect pests in Korea. *Ann Agr Exp St Gov Gen Chosen,* 12**,** 195-247.
NAKAMOTO, M., MOY, RYAN H., XU, J., BAMBINA, S., YASUNAGA, A., SHELLY, SPENCER S., GOLD, B. & CHERRY, S. 2012. Virus Recognition by Toll-7 Activates Antiviral Autophagy in Drosophila. *Immunity,* 36**,** 658-667.
NARANJO-LÁZARO, J. M., MELLÍN-ROSAS, M. A., GONZÁLEZ-PADILLA, V. D., SÁNCHEZ-GONZÁLEZ, J. A., MORENO-CARRILLO, G. & ARREDONDO-BERNAL, H. C. 2014.

Susceptibility of Drosophila suzukii Matsumura (Diptera: Drosophilidae) to Entomophatogenic Fungi. *Southwestern Entomologist,* 39**,** 201-203.
O'BRIEN, S. J. & EVERMANN, J. F. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. *Trends in Ecology & Evolution,* 3**,** 254-259.
O'GRADY, P., BEARDSLEY, J. & PERREIRA, W. 2002. New records for introduced Drosophilidae (Diptera) in Hawai'i. *Bishop Museum Occasional Papers,* 69**,** 34-35.
OKADA, T. 1976. New distribution records of the drosophilids in the Oriental Region. *Acta Dipterologica,* 8**,** 1-8.
PARADKAR, P. N., TRINIDAD, L., VOYSEY, R., DUCHEMIN, J.-B. & WALKER, P. J. 2012. Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway. *Proceedings of the National Academy of Sciences,* 109**,** 18915-18920.
PARSHAD, R. & DUGGAL, K. 1965. Drosophilidae of Kashmir, India. *Drosophila Information Service,* 40**,** 44.
PENG, F. T. 1937. On some species of Drosophila from China. *Annotationes Zoologicae Japonenses,* 16**,** 20-27.
PENG, H.-Y., ZHOU, X.-M., SHENG, R.-J., CHEN, S.-W., LIU, J.-X., CHEN, X.-W., YANG, C.-H. & XIE, T.-E. 2000. Development of Dendrolimus punctatus wenshanensis Cytoplasm Polyhedrosis virus(DpwCPV) Insecticide. *Virologica Sinica,* 15**,** 148-155.
PENG, H., CHEN, X., JIANG, Y., SHEN, R., ZHOU, X. & HU, Z. 1998. Controlling Dendrolimus punctatus with Trichogramma dendrolimi carrying cytoplasmic polyhedrosis virus. *Chinese Journal of Biological Control,* 14**,** 111-114.
PHILLIPS, B. L., KELEHEAR, C., PIZZATTO, L., BROWN, G. P., BARTON, D. & SHINE, R. 2010. Parasites and pathogens lag behind their host during periods of host range advance. *Ecology,* 91**,** 872-881.
PIOTROWSKI, W., ŁABANOWSKA, B., WIECZOREK, W. & ZAJKOWSKI, P. The spotted wing drosophila, Drosophila suzukii-new pest in Europe and in Poland. Ecofruit. 17th International Conference on Organic Fruit-Growing: Proceedings, 15-17 February 2016, Hohenheim, Germany, 2016. Fördergemeinschaft Ökologischer Obstbau eV (FÖKO), 214-219.
PLUS, N., CROIZIER, G., VEYRUNES, J. C. & DAVID, J. 1976. A Comparison of Buoyant Density and Polypeptides of Drosophila P, C and A Viruses. *Intervirology,* 7**,** 346-350.
POURMIRZA, A. A. 2005. Local variation in susceptibility of Colorado potato beetle (Coleoptera: Chrysomelidae) to insecticide. *Journal of economic entomology,* 98**,** 2176-2180.
POYET, M., ESLIN, P., CHABRERIE, O., PRUD'HOMME, S., DESOUHANT, E. & GIBERT, P. 2017. The invasive pest Drosophila suzukii uses trans-generational medication to resist parasitoid attack. *Scientific Reports,* 7.
POYET, M., HAVARD, S., PREVOST, G., CHABRERIE, O., DOURY, G., GIBERT, P. & ESLIN, P. 2013. Resistance of Drosophila suzukii to the larval parasitoids Leptopilina heterotoma and Asobara japonica is related to haemocyte load. *Physiological Entomology,* 38**,** 45-53.
PRICE, J. F., LIBURD, O. E., ROUBOS, C. R. & NAGLE, C. A. 2009. Spotted wing Drosophila in Florida berry culture. *University of Florida, Florida Cooperative Extension Service. Publication ENY-861. Disponível em http://edis. ifas. ufl. edu/in839 (acessado 15 agosto 2014)*.
R CORE TEAM 2017. R: A Language and Environment for Statistical Computing.
ROSSI STACCONI, M. V., BUFFINGTON, M., DAANE, K. M., DALTON, D. T., GRASSI, A., KAÇAR, G., MILLER, B., MILLER, J. C., BASER, N., IORIATTI, C., WALTON, V. M., WIMAN, N. G., WANG, X. & ANFORA, G. 2015. Host stage preference, efficacy and fecundity of parasitoids attacking Drosophila suzukii in newly invaded areas. *Biological Control,* 84**,** 28-35.
ROZEN, S. & SKALETSKY, H. 1999. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics methods and protocols***,** 365-386.
SABIN, L. R., HANNA, S. L. & CHERRY, S. 2010. Innate antiviral immunity in Drosophila. *Current opinion in immunology,* 22**,** 4-9.
SELJAK, G. 2011. Spotted wing Drosophila, Drosophila suzukii (Matsumura), a new pest of berry-fruit in Slovenia. *Sad,* 22**,** 3-5.
SHARIF, M. M., HEJAZI, M. J., MOHAMMADI, A. & RASHIDI, M. R. 2007. Resistance status of the Colorado potato beetle, Leptinotarsa decemlineata, to endosulfan in East Azarbaijan and Ardabil provinces of Iran. *Journal of Insect Science,* 7**,** 31.

SHELLY, S., LUKINOVA, N., BAMBINA, S., BERMAN, A. & CHERRY, S. 2009. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. *Immunity,* 30**,** 588-598.
SHI, M., LIN, X.-D., TIAN, J.-H., CHEN, L.-J., CHEN, X., LI, C.-X., QIN, X.-C., LI, J., CAO, J.-P., EDEN, J.-S., BUCHMANN, J., WANG, W., XU, J., HOLMES, E. C. & ZHANG, Y.-Z. 2016. Redefining the invertebrate RNA virosphere. *Nature,* advance online publication.
SIDORENKO, V. 1992. New and unrecorded species of Drosophilidae from Soviet Far East (Diptera, Brachycera). *Spixiana,* 15**,** 93-95.
SINHA, R., STANLEY, G., GULATI, G. S., EZRAN, C., TRAVAGLINI, K. J., WEI, E., CHAN, C. K. F., NABHAN, A. N., SU, T. & MORGANTI, R. M. 2017. Index switching causes "spreading-of-signal" among multiplexed samples in Illumina HiSeq 4000 DNA sequencing. *bioRxiv***,** 125724.
SMITH, G. E., SUMMERS, M. & FRASER, M. 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and cellular biology,* 3**,** 2156-2165.
SORRENTINO, R. P., MELK, J. P. & GOVIND, S. 2004. Genetic Analysis of Contributions of Dorsal Group and JAK-Stat92E Pathway Genes to Larval Hemocyte Concentration and the Egg Encapsulation Response in Drosophila. *Genetics,* 166**,** 1343.
STACCONI, M. R., GRASSI, A., DALTON, D., MILLER, B., OUANTAR, M., LONI, A., IORIATTI, C., WALTON, V. & ANFORA, G. 2013. First field records of Pachycrepoideus vindemiae as a parasitoid of Drosophila suzukii in European and Oregon small fruit production areas. *Entomologia,* 1**,** 3.
STANKOVIĆ, S., ZABEL, A., KOSTIC, M., MANOJLOVIC, B. & RAJKOVIC, S. 2004. Colorado potato beetle [Leptinotarsa decemlineata (Say)] resistance to organophosphates and carbamates in Serbia. *Journal of Pest Science,* 77**,** 11-15.
STASKAWICZ, B. J., AUSUBEL, F. M., BAKER, B. J., ELLIS, J. G. & JONES, J. D. G. 1995. Molecular genetics of plant disease resistance. *Science,* 268**,** 661.
SUKHORUCHENKO, G. I. & DOLZHENKO, V. I. 2008. Problems of resistance development in arthropod pests of agricultural crops in Russia. *EPPO bulletin,* 38**,** 119-126.
SUN, X.-L. & PENG, H.-Y. 2007. Recent advances in biological control of pest insects by using viruses in China. *Virologica Sinica,* 22**,** 158-162.
TENINGES, D. 1968. Mise en evidence de virions Sigma dans les cellules de la lignée germinale mâle de Drosophiles stabilisées. *Archiv für die gesamte Virusforschung,* 23**,** 378-387.
TENINGES, D., BRAS, F. & DEZÉLÉE, S. 1993. Genome Organization of the Sigma Rhabdovirus: Six Genes and a Gene Overlap. *Virology,* 193**,** 1018-1023.
THOMPSON, J. D., GIBSON, T. J. & HIGGINS, D. G. 2002. Multiple Sequence Alignment Using ClustalW and ClustalX. *Current Protocols in Bioinformatics.* John Wiley & Sons, Inc.
TIWARI, S., MANN, R. S., ROGERS, M. E. & STELINSKI, L. L. 2011. Insecticide resistance in field populations of Asian citrus psyllid in Florida. *Pest management science,* 67**,** 1258-1268.
TODA, M. 1991. Drosophilidae (Diptera) in Myanmar (Burma) VII. The Drosophila melanogaster species-group, excepting the D. montium species-subgroup. *Oriental Insects,* 25**,** 69-94.
TOKARZ, R., WILLIAMS, S. H., SAMEROFF, S., LEON, M. S., JAIN, K. & LIPKIN, W. I. 2014. Virome analysis of Amblyomma americanum, Dermacentor variabilis, and Ixodes scapularis ticks reveals novel highly divergent vertebrate and invertebrate viruses. *Journal of virology,* 88**,** 11480-11492.
TORCHIN, M. E., LAFFERTY, K. D. & KURIS, A. M. 2001. Release from Parasites as Natural Enemies: Increased Performance of a Globally Introduced Marine Crab. *Biological Invasions,* 3**,** 333-345.
UNCKLESS, R. L. 2011. A DNA virus of Drosophila. *PloS one,* 6**,** e26564-e26564.
VAN MIERLO, J. T., OVERHEUL, G. J., OBADIA, B., VAN CLEEF, K. W., WEBSTER, C. L., SALEH, M.-C., OBBARD, D. J. & VAN RIJ, R. P. 2014. Novel Drosophila viruses encode host-specific suppressors of RNAi. *PLoS pathogens,* 10**,** e1004256.
VAN REGENMORTEL, M. H., FAUQUET, C. M., BISHOP, D. H., CARSTENS, E., ESTES, M., LEMON, S., MANILOFF, J., MAYO, M., MCGEOCH, D. & PRINGLE, C. 2000. *Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses*, Academic Press.

VOGT, H., BAUFELD, P., GROSS, J., KOPLER, K. & HOFFMANN, C. 2012. Drosophila suzukii: eine neue bedrohung für den Europäischen obst-und weinbau–bericht über eine internationale tagung in trient, 2, Dezember 2011. *Journal für Kulturpflanzen,* 64**,** 68-72.

WALSH, D. B., BOLDA, M. P., GOODHUE, R. E., DREVES, A. J., LEE, J., BRUCK, D. J., WALTON, V. M., O'NEAL, S. D. & ZALOM, F. G. 2011. *Drosophila suzukii* (Diptera: Drosophilidae): Invasive Pest of Ripening Soft Fruit Expanding its Geographic Range and Damage Potential. *Journal of Integrated Pest Management,* 2**,** 1-7.

WARNES, G. R., BOLKER, B., BONEBAKKER, L., GENTLEMAN, R., HUBER, W. & LIAW, A. 2016. gplots: Various R Programming Tools for Plotting Data. 2016; R package version 3.0. 1. *Reference Source*.

WEBSTER, C. L., LONGDON, B., LEWIS, S. H. & OBBARD, D. 2016. Twenty five new viruses associated with the Drosophilidae (Diptera). *bioRxiv*.

WEBSTER, C. L., WALDRON, F. M., ROBERTSON, S., CROWSON, D., FERRARI, G., QUINTANA, J. F., BROUQUI, J.-M., BAYNE, E. H., LONGDON, B. & BUCK, A. H. 2015. The Discovery, Distribution, and Evolution of Viruses Associated with Drosophila melanogaster. *PLoS Biol,* 13**,** e1002210.

WOLFE, L. M. 2002. Why alien invaders succeed: support for the escape-from-enemy hypothesis. *The American Naturalist,* 160**,** 705-711.

WOLTZ, J., DONAHUE, K., BRUCK, D. & LEE, J. 2015. Efficacy of commercially available predators, nematodes and fungal entomopathogens for augmentative control of Drosophila suzukii. *Journal of applied entomology,* 139**,** 759-770.

ZAMBON, R. A., NANDAKUMAR, M., VAKHARIA, V. N. & WU, L. P. 2005. The Toll pathway is important for an antiviral response in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 7257-7262.

ZAMOJSKA, J., WĘGOREK, P. & MRÓWCZYŃSKI, M. 2011. Changes in the Colorado potato beetle (Leptinotarsa decemlineata Say) susceptibility level to pyrethroids and the pest resistance mechanisms to deltamethrin. *Journal of Plant Protection Research,* 51**,** 294-299.

ZEDDAM, J.-L., CRUZADO, J. A., RODRIGUEZ, J. L., RAVALLEC, M. & SUBILETE, E. C. 2003. A cypovirus from the South American oil-palm pest Norape argyrrhorea and its potential as a microbial control agent. *BioControl,* 48**,** 101-112.

ZHU, Y., CHEN, H., FAN, J. & WANG, Y. 2000. Genetic diversity and disease control in rice. *Nature,* 406**,** 718.

Appendices

Supplementary table 1. Known Drosophila viruses infecting *D. suzukii*

Name	Accession	Original Host	Taxon	Genome	Sample(s)	Ref.
Galbut	KP714099.1/KP714100.1	Dmel	Partitiviridae	dsRNA	UK2014, UK2015, UK2016, Japan2016	Webster et al. 2015
Chaq virus	KP714088.1	Dmel	Unclassified	-ssRNA?	UK2014, UK2015, UK2015	Webster et al. 2015
Dimm sigma virus	JF311401.1	Dimm	Rhabdoviridae	-ssRNA	France2013, UK2014, UK2016, Japan2016	Longdon et al. 2015
Corseley virus	KU754520.1	Dsub	Tombus-noda	+ssRNA	UK2014, UK2015	Webster et al. 2016
La Jolla virus	KP714073.1	Dmel	Iflaviridae	+ssRNA	France2013, UK2014, UK2015, UK2016	Webster et al. 2015
Muthill virus	KU754517.1	Dimm	Virgavirus	+ssRNA	UK2014, UK2015, Japan2016	Webster et al. 2016
Brandeis virus	MF953177	Dmel/Dsuz	Virgavirus	+ssRNA	UK2014, UK2016, France2013	Webster et al. 2016
Kinkell virus	KU754510.1	Dsus	Iflavirus	+ssRNA	UK2014, UK2015, UK2016	Webster et al. 2016
Dimm Nora virus	KF242511.1	Dimm	Noravirus	+ssRNA	UK2014, UK2015, UK2016, Japan2016	van Mierlo et al. 2014
Dmel Nora virus	JX220408.1, NC_007919.3, KP970094.1, KP970098.1, KP970100.1, KP970105.1	Dmel	Noravirus	+ssRNA	France 2013	Habayeb et al. 2006
Thikka virus	KP714072.1	Dmel	Picornavirales	+ssRNA	France2013, UK2015, UK2016, Japan2016	Webster et al. 2015
Prestney Burn virus	KU754507.1	Dsub	Sobemovirus	+ssRNA	UK2014, UK2015, UK2016, Japan2016	Webster et al. 2016
Buckhurst virus	KU754516.1	Dobs	Negevirus	+ssRNA	UK2015, UK2016, Japan2016	Webster et al. 2016
Drosophila A virus	NC_012958.1	Dmel	Picornavirales	+ssRNA	France2013, UK2014, UK2016, Japan2016	Brun & Plus 1980
Motts Mill virus	KP714076.1,KP714077.1	Dmel	Sobemovirus	+ssRNA	UK2015, UK2016, Japan2016	Webster et al. 2015
Craigies Hill virus	KP714085.1, KP714084.1	Dmel	Nodavirus	+ssRNA	Japan2016	Webster et al. 2015
Dkikkawai virus	SRR346732	Diptera	Fisavirus	+ssRNA	Japan2016	Webster et al. 2015
Bloomfield virus	SRR2063773	Dmel	Reoviridae	dsRNA	UK2014, UK2015, UK2016	Webster et al. 2015