AHDB Horticulture research report

Project title:	Viral Pathogens suitable for the Control of Drosophila Suzukii in the UK
Project number:	CP122
Project leader:	Dr Darren Obbard (University of Edinburgh)
Report:	Annual report, December 2016
Previous report:	N/A
Key staff:	Dr Darren Obbard Dr Jerry Cross
Location of project:	University of Edinburgh, Edinburgh and East Malling Research, Kent
Industry Representative:	Marion Regan, Huwe Lowe Farms, Barons Place 215 Willow Wents, Mereworth, Maidstone, Kent, ME18 5NF
Date project commenced:	September 2014
Date project completed (or expected completion date):	September 2017

DISCLAIMER

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

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.

[Name] DARREN ORSARD
[Position] RESEARCH FELLOW
[Organisation] UNIVERSITY OF EDINBURGH
Signature ..... Date 19 Dec '16

[Name]
[Position]
[Organisation]
Signature ..... Date .....

Report authorised by:

[Name]
[Position]
[Organisation]
Signature ..... Date .....

[Name]
[Position]
[Organisation]
Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

- New viruses specific to SWD are being investigated for their potential as commercial control agents

Background and expected deliverables

Drosophila suzukii (Matsummura), also known as the spotted wing drosophila (SWD) is a new pest of soft and stone fruit. Its spread from its native Japan to the fruit growing regions of North America and Europe have prompted an interest in developing new control measures. Conventional crop protection methods have many drawbacks and are difficult to implement within integrated pest management (IPM) programmes. The development of an alternative, IPM compatible biopesticide would be beneficial for growers, consumers, and pest management professionals alike.

The viruses of *Drosophila suzukii* (SWD) offer good potential candidates for the development of a microbe-based bioinsecticide, yet, to date the viruses of *D. suzukii* remain almost completely unstudied. This project seeks to characterise the viral diversity of SWD with the aim of identifying a pathogen suitable for the control of this pest in UK fruit crops. Both cutting-edge genetic techniques and traditional lab based investigations will be employed to identify the viruses infecting SWD, from large samples of wild flies. Viruses will then be isolated and investigated for their interactions with their *Drosophila* host or hosts in the laboratory and field.

Summary of the project and main conclusions

Metatranscriptomic surveys have revealed a diversity of the viruses infecting D. suzukii. So far, we have discovered seven new RNA viruses unique to SWD along with a host of other viruses, which although described first in other fly species, regularly infect British SWD. Techniques have been developed to isolate new viruses, to test their pathogenicity and applicability as biological control agents.

Financial benefits

The impact of this pest on the European horticultural industry has already been substantial, with *D. suzukii* 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. In the Pacific fruit growing regions of the USA, the estimated damage due to *D. suzukii* has been calculated at over €400 million/year (Bolda et al., 2010). With damage estimates for the UK slow to

emerge, it is hard to quantify the level of damage caused in the UK since its establishment here.

A key consideration for UK growers 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 not compatible with residue or resistance management practices. Without IPM compatible products, damage is not limited to that done by the pest itself but also extends to secondary pest damage.

The development of a viral biopesticide specific to SWD would not only offer significant damage savings, but also provide an IPM compatible control product.

Action points for growers

- No action points have been developed for growers from this project so far.

SCIENCE SECTION

Introduction

Drosophila suzukii is closely related to the famous laboratory model organism *Drosophila melanogaster* (Lewis et al., 2005, Kopp, 2006). Some physical 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 (to which the common species name 'Spotted Wing Drosophila' refers) 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* go through three larval instars inside the fruit, feeding on the mesocarp and developing from egg to adult in approximately 8 to 10 days at 25 °C, and from 21 to 25 days at 15 °C (Kanzawa, 1939). Extensive studies examining the life history traits of this species were carried out in Japan in the 1930's (Kanzawa, 1939, Kanzawa, 1935) with further information on oviposition behaviour (Mitsui et al., 2006), life stages, host range and overwintering (Walsh et al., 2011) being provided more recently. A network of monitoring traps has been established in the UK since the first detection of this pest in 2012. 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 (Dr M Fountain *pers comm*). As British records of D. *suzukii* only date back three growing seasons, data on the phenology of the organism are 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.

Pattern of invasion

First described in Japan in 1916 (Kanzawa, 1935), *D. suzukii* was reported to be widely distributed in Japan shortly after (Kanzawa, 1939). The first records of this pest from outside Asia came from Hawaii in the 1980's (Kaneshiro, 1983). Since its detection in the southern states of the USA (Bolda, 2008) and in Spain (Calabria et al., 2012) in 2008, *D. suzukii* has been spreading northwards and was reported for the first time in the UK in 2012 (Harris and Shaw, 2014).

Economic Damage

D. suzukii can cause severe damage to commercial soft fruit crops when a female fly oviposits through the skin (exocarp) of ripening fruit leaving a puncture wound. 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. Where *D. suzukii* has established, substantial (up to 80%) crop loss has been reported on a variety of soft skinned fruit crops (Walsh et al., 2011).

Control

Despite some success developing control programs (Beers et al., 2011, Cuthbertson et al., 2014, Van Timmeren and Isaacs, 2013) effective control of this pest has yet to be universally achieved in practice. This is in part due to the biology of the organism: a short generation time, wide host range and cryptic feeding stages in close-to-harvest fruit combine to hinder conventional control.

Most current control strategies currently include a combination of high volume, short persistence, crop protection product spray programmes and attract-and-kill bait traps. These are suboptimal techniques due to high material costs, a substantial labour investment and the negative impacts associated with such spray regimes. High volume spray programmes run the risk of driving the rapid development of product resistance in target and non-target pests, whilst also having a negative impact on already established integrated pest management (IPM) programmes.

IPM compatible solutions for *D. suzukii* infestation are 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). 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). Trapping is generally environmentally benign and compatible with existing IPM programmes. Placement of traps does, however, pose a large investment in labour time for growers.

Studies into the biological control of *D. suzukii* using invertebrate natural enemies have given mixed results. Several studies have shown a resistance of *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 and laboratory (Gabarra et al., 2014, Stacconi et al., 2013). 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. Potential invertebrate predators of *D. suzukii*, all belonging to the taxon Heteroptera, have been identified in lab studies (Malagnini et al., 2014, Cuthbertson et al., 2014) and in field surveys (Arnó et al., 2012), however, no effective strategy for their implementation has yet been reported.

The susceptibility of *D. suzukii* to microbial biological control agents has been tested in a number of studies with varying degrees of success. The susceptibility of *D. suzukii* to entomopathogenic fungi has been demonstrated in the lab for the pathogens: *Bauvaria bassiana, Lecanicillium muscarium* (Cuthbertson et al., 2014) and *Metarhizium brunneum* (strain EAMa 01/58-Su)(Fernández-Bravo, 2014).

The viruses of *D. suzukii* offer an interesting potential source for a microbial biological control agent. Similarly to 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).

Characteristics of viral biological control agents

Entomopathogenic viruses are represented in many of the known virus families with some families of virus 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). 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)).

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 consequently led to huge declines in pest population over the course of 1-3 years. A reduction in crop damage accompanied the reduction in population. 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). A genus of the virus family Parvoviridae, the densoviruses or densonucleosis viruses (DNV's) are another group of viruses with potential use as viral insecticides. These single stranded DNA viruses were first discovered infecting the greater wax moth *Galleria mellonella* by Meynadier et al. (1964). Since that point they have been subsequently isolated from a range of insect taxa (see Maramorosch (2012)). No publications report their isolation from *Drosophila*, however, evidence of their presence has been detected in *Drosophila* transcriptome datasets (Obbard, *pers. comm.*). They have been advocated for the control of Mosquitoes (Carlson et al., 2006, Ledermann et al., 2004) and cockroaches (Jiang et al., 2008) although field studies into their application are yet to be published.

Drosophila virus diversity

Considering its status as a model organism, the full diversity of viruses infecting *D. melanogaster* has only recently been explored (Webster et al., 2015, Webster et al., 2016) and studies examining the viruses of other members of the genus remain relatively rare (Webster et al., 2016). Prior to wide scale metagenomic viral discovery 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).

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, 2016) used next generation sequencing technology to identify more than 50 previously undescribed RNA and DNA viruses associated with *Drosophila* spp. Their survey of over 2000 individual wild *D. melanogaster* 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.

A study by Unckless (2011) has identified a DNA nudivirus infecting wild *Drosophila innubila*. This viruses 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, this virus was found to be relatively common in wild *D. melanogaster* (4.6% prevalence globally) and was shown to be interacting with antiviral immune pathways in its host.

Knowledge of Invertebrate virus diversity, more generally, has recently been massively expanded (Shi et al., 2016). Shi *et al* described 1445 new RNA viruses from a wide diversity of invertebrates, deepening our understanding of the phylogenetic relationships between RNA virus taxa and highlighting the diversity of genome structures employed by viruses across these groups.

Antiviral Immunity in Drosophila

To fight invading pathogens, insects rely solely on an innate immune response, as opposed to the familiar, adaptive, immune response found solely in vertebrates.

The most important antiviral system in insects 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.

Materials and methods

Specimen collection 2016

Further collections of wild Drosophila have been carried out this year in both the UK and Japan.

UK collections were carried out exclusively at the NIAB-EMR research centre, East Malling, Kent. Techniques for trapping live adult flies, as outlined in previous reports, went unchanged and provided adequate catches of a number of species, namely: *D. suzukii, D. melanogaster, D. hydeii, D. subobscura, D. obscura,* and *D. immigrans.* Species were identified using the relevant identification keys (D'Assis Fonseca, 1965, Bächli et al., 2004). Traps were set in five different locations within the grounds of the centre (UK grid: TQ 51 57) and emptied after two three day intervals between 29th August and 11th September. Further to adult collections, approximately 200 larval *D. suzukii* were extracted from wild berries picked from hedgerows

surrounding sweet cherry orchards. All specimens were separated by species and transferred to vials containing an agar plug within three hours of capture. Flies were then transported, alive, on these agar vials to the University of Edinburgh. All flies caught were grouped into pools of between 1 and 30 flies based on their species, geographic location and habitat in which they were caught. All samples were frozen at -80°C immediately after identification.

In May 2016 I was awarded additional funding from the Davis Expedition Fund (University of Edinburgh) to extend my sample collections to the native range of *D. suzukii* in Japan. The aim of this expedition was to investigate the viral diversity infecting *D. suzukii* in its native range: not only to maximise chances of discovering potential control agents but also to elucidate the patterns of virus prevalence and abundance in a recently invasive species.

Japan, thought to be within the native range of *D. suzukii*, and was an excellent destination to investigate these aims as much scientific work here is focused on its control. Japan has dealt with this fly as an agricultural pest since 1916 when damage was first reported. Different prefectures within Honshu have varying amounts of soft fruit production and the local government scientists work relatively independently of those in other prefectures.

Tokyo

The University of Technology and Agriculture, the base for this expedition is located in Fuchu within the city of Tokyo. It has a relatively small plot of cultivated land used to teach agricultural techniques to students. This includes plots of blueberry (*Vaccinium* spp.) bushes, of a number of different varieties, which suffer with some damage by *D. suzukii* (Figure 1). Bait traps were placed in these areas, as well as a nearby mulberry (*Morus* sp.) tree, and provided a steady catch of fruit flies through the duration of the trip.

Additional trapping locations within Tokyo were also exploited: Naganuma Park and Tama Hills, to the west of Fuchu, boasted a large number of wild cherry (*Prunus* subg. *padus*) and mulberry trees, potential host species of *D. suzukii.* A public park and a university run climate research station respectably, both were relatively unsuccessful sampling locations, yielding just a handful of flies each.

Due to a warmer than average spring the fruit trees in this particular area (all locations within Tokyo were at approximately 35.5° latitude and between 50 & 150 MASL) were somewhat advanced compared to previous years. In fact main fruit fall had been approximately two weeks earlier. This negatively affected fruit fly catch and although some *D. suzukii* were caught in these areas, numbers were disappointing.

During this trip The Tokyo University of Agriculture and Technology insect pathology laboratories were used as the primary location for sample storage and fly identification.

Yamagata

In the second week of the expedition we travelled northwards to the cherry growing region of Yamagata. Being considerably further north than Tokyo the fruiting period of *Prunus* spp. here was still ongoing. Yamagata has a large area of cultivated sweet cherry and is famed for its production of cherries specifically. We visited the Yamagata prefecture soft fruit research facility in Sagae, Yamagata. At this research station we were unable to catch a large number of fruit flies however researchers working at the facility were able to provide some flies caught recently emerging from damaged fruit. The reason for this low fly abundance was thought to be the effective use of chemical pest controls at the facility.

Fukushima

The district of Fukushima, despite recent decline, was a fertile and productive region of Japan. Associated heavily with soft fruit production, Fukushima represented another potentially good sampling location for Drosophila. We visited researchers at the 'Agriculture Synthesis Centre Fruit Tree Research Institute' and were able to catch a number of Drosophila species in the institute's fruit growing plots. At no point during our visit to the Fukushima prefecture did we come near the official exclusion zones in the east of the region

Yamanashi

This prefecture is also known for its production of fruit: primarily table grapes and peaches. More mountainous than other areas visited during this expedition, its increased altitude meant certain wild cherry trees were still producing fruit alongside commercial harvested varieties. This combination of fruiting host plants yielded the highest numbers of *D. suzukii* of any location visited.

Figure 1: Netted Blueberry plots, TUAT, Tokyo.

Metagenomic viral discovery

Wild flies collected in September 2015 were submitted to Edinburgh Genomics for total RNA sequencing in late 2015 and samples collected in 2016 were submitted in mid-November of the same year. For metagenomic sequencing, RNA was extracted from all individual samples of wild caught *D. suzukii* using Trizol® (Life Technologies) and DNAse treated (Life Technologies). These samples ranged from single flies through to pools of 30 flies grouped by trap location and date of collection. Quality checks were applied to extracted RNA using Qubit™ and Nanodrop™ appliances as well as running all samples on 1% agarose electrophoresis gels to check for RNA degradation. Aliquots of all samples were mixed at a volume proportional to the amount of RNA per fly present in the sample. This ensured an equal chance of detecting a virus in any individual fly regardless of the size of pool from which it came. RNA-seq was performed on an Illumina next generation sequencing platform. Ribosome depletion was conducted on the samples using RiboZero™. Raw reads were quality-trimmed (sickle version 1.2) (Joshi, 2015) and paired-end sequences were then *de novo* assembled using Trinity (version 2.0.6) (Grabherr et al., 2011). Within these contigs the longest open reading frame was identified, translated and used to query the virus database in the Genbank non-redundant protein database ('nr') (Benson et al., 2013) using blastp (blast version 2.2.28+) (Camacho et al., 2009). Default parameters were used but with an e-value threshold of 10^{-5}. The single 'best' hit for this query was retained. These candidate lists, comprising all the sequences for which the top hit was a virus, were then combined and used to query 'nr' using blastp, again using an e-value threshold of 10^{-5} and retaining the top 20 hits. Sequences for which the top hit was still a virus, and sequences with a blastx hit to viruses but no other blastp hits in 'nr', were treated as putatively of viral origin.

Virus Phylogenies

We inferred the phylogenetic placement of each virus using sequences for viral RNA polymerase, a highly conserved protein coding region in RNA viruses. We used blastp to query the Genbank non-redundant protein database ('nr') and tblastn to query the Genbank Transcriptome Shotgun Assembly database ('tsa_nt') to identify potential relatives for inclusion in the phylogenetic analysis. Additional sequences were sourced from online resources associated with Shi et al. (2016). We aligned protein sequences using BLOSUM (BLOcks SUbstitution Matrix). Consensus alignments were examined and trimmed manually. Alignments between distant relatives remained highly ambiguous and should be considered when viewing resultant phylogenetic trees. Neighbor-Joining consensus trees were then drawn using Geneious™ with a Jukes-Cantor distance model. All trees are presented with transformed branch lengths and show percent support at nodes.

Virus Prevalence

To infer viral prevalence and distribution of viruses in wild flies we used reverse transcription PCR (RT-PCR) to assay for the presence of all newly discovered viruses as well as a selection of viruses from other species of *Drosophila* (Supplementary Table 1). Prevalence values were calculated using a maximum-likelihood method, as described in Webster (2015) using presence or absence values from pools varying sizes. PCR primers were designed using Primer3 (Rozen and Skaletsky, 1999) through the Geneious™.

Results

Metagenomics

In 2015 a total of 866 individual *D. suzukii,* in 88 pools were caught, identified and processed for sequencing. In 2016 fewer *D. suzukii,* 456, were caught but these were from a greater number of traps and therefore a larger number of pools, 146, were processed.

Metatranscriptomic data for 2015's samples were returned in early 2016 and the bioinformatics pathway described above yielded putative viral sequences from which several new RNA viruses were described. No DNA viruses have described in British SWD to-date.

Medway and Teise Viruses

Two viruses related to the family Luteoviridae but more broadly classified as belonging to the 'Luteo-Sobermo Clade' were discovered in both 2015 and 2016 pools of SWD. Luteoviruses possess a single stranded, positive sense, RNA genome. They are non-enveloped viruses with a virion diameter of around 30nm. Recently many luteoviruses have been discovered infecting a broad range of invertebrate taxa including arthropods, chelicerates and crustaceans (Holmes, 2016).

The potential lethality of these viruses is unknown. Teise, however, has the highest prevalence of any newly described virus in live adult flies (Figure 7), suggesting that it does not cause high levels of mortality in infected flies.

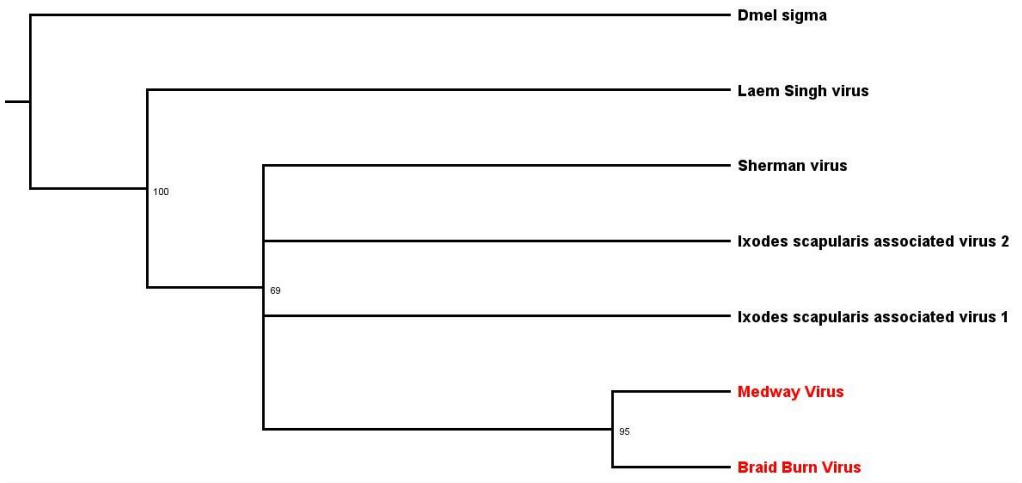

Figure 2. Phylogenetic tree of Medway virus and closest relatives. Viruses highlighted in red are those described here or by publications produced by the Obbard lab (i.e. Webster (2015 & 2016)).

Eccles Virus

Eccles virus is an unclassified virus closely related to a virus found in *D. melanogaster* named as Galbut virus (Webster, 2015). Eccles is again a single stranded RNA virus but it is much less common in wild SWD. We know very little about this virus as it does not have close relatives with which to compare. Isolation and electron microscopy along with further sequencing efforts will tell us more about the nature of this illusive virus.

Deadman's Wood Virus

Deadman's wood virus is a close relative of the *D. melanogaster* virus: Bloomfield virus. Belonging to the family Reoviridae this virus has a double stranded RNA genome divided into 10 segments. Reovirus virions are non-enveloped but have a double capsid structure protecting their genetic material. These newly discovered viruses are closely related to the genus Fijivirus (Figure 3) which contains a number of viruses responsible for plant diseases such as Fiji Disease, Mal de Reo Cuarto and Rice Black streaks. Deadman's Wood virus is relatively rare in adult flies.

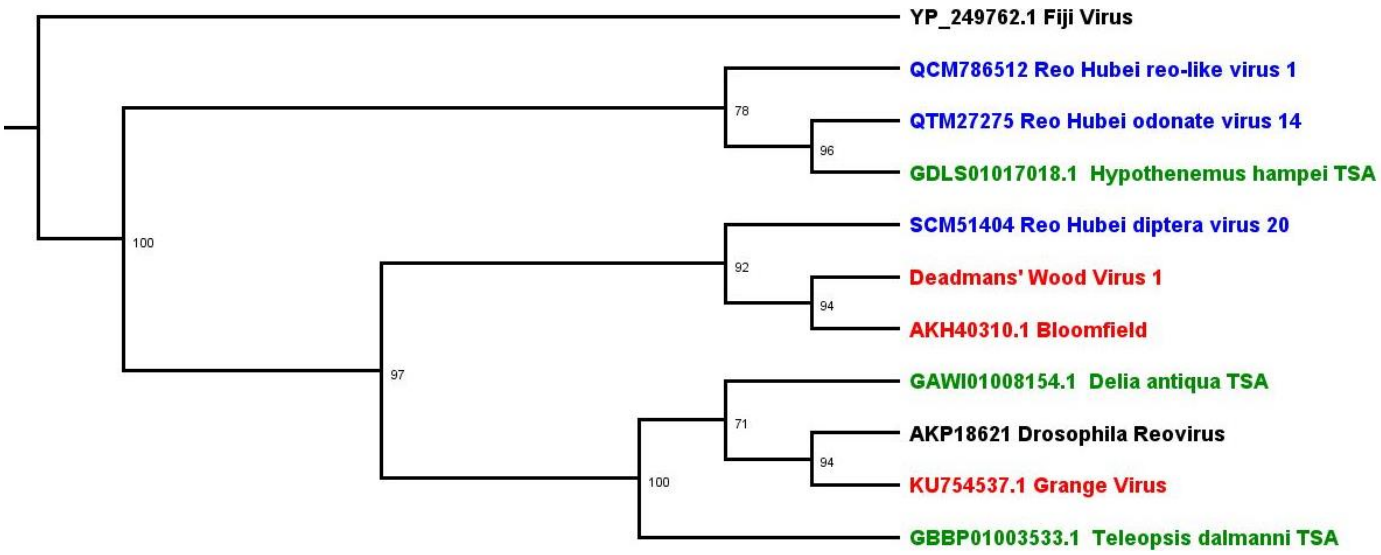

Figure 3. Phylogenetic tree of Deadman's wood virus and closest relatives. Viruses highlighted in red are those described here or by publications produced by the Obbard lab (i.e. Webster (2015 & 2016)), viruses highlighted in blue are those published by Shi et al. (2016) and those highlighted in green represent putative viral sequences discovered in TSA datasets through Blast.

Beult Virus

Beult virus belongs to an interesting group of insect viruses call the Negeviruses. This group contains other previously described Drosophila viruses: Muthill, Marsac, Bofa, Buckhurst and Boutonnet viruses (see Figure 4). It is rare in SWD and the pathogenicity is unknown. Virions in this group are small, 25-30nm, and contain a single stranded, positive sense genome of around 9Kb in length.

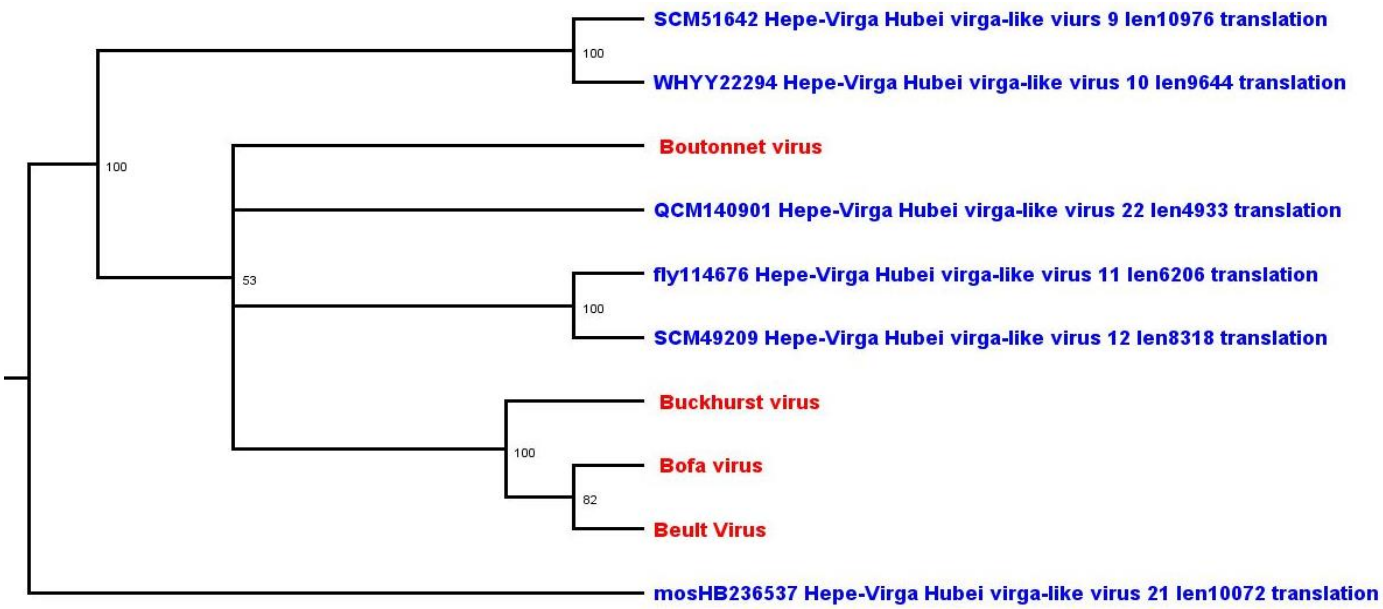

Figure 4. Phylogenetic tree of Beult virus and closest relatives. Viruses highlighted in red are those described here or by publications produced by the Obbard lab (i.e. Webster (2015 & 2016)), viruses highlighted in blue are those published by Shi et al. (2016).

Ditton Virus

Belonging to the Bunyaviridae, Ditton virus is another virus with a single stranded RNA genome, however, these viruses have a negative-sense tripartite genome. It's virions are likely enveloped and around 100nm in diameter. The Bunyavirus clade contains a diverse slection of ecologies, with some certain clades of virus infecting plants, vertebrates, insects, crustaceans, myriapods and spiders. Ditton virus has few close relatives (Figure 5), even given the recently elucidated diversity in the clade (Holmes, 2016), making it of potential interest for further evolutionary study.

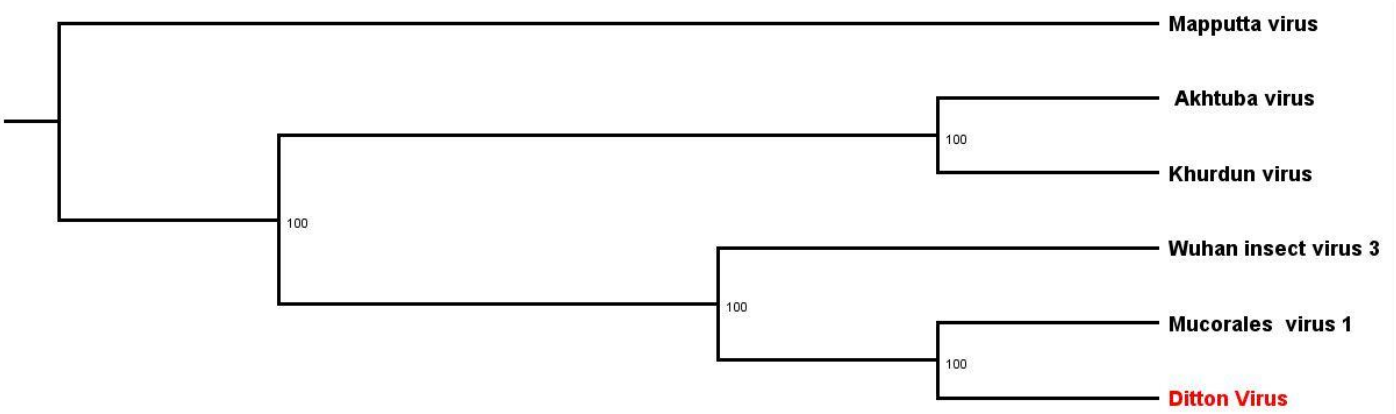

Figure 5. Phylogenetic tree of Ditton virus (red) and closest relatives.

Larkfield Virus

Larkfield virus only appears in the second year metagenomic surveys. It belongs to the family Totiviridae which to-date contains one other virus of wild drosophila (Obbard, *unpublished*). The totiviruses are double stranded RNA viruses typically with a non-enveloped, icosahedral virion of around 40nm in diameter. The viruses most closely related to Larkfield virus are all insect viruses, including two more well known viruses of Campodromus ants (Figure 6).

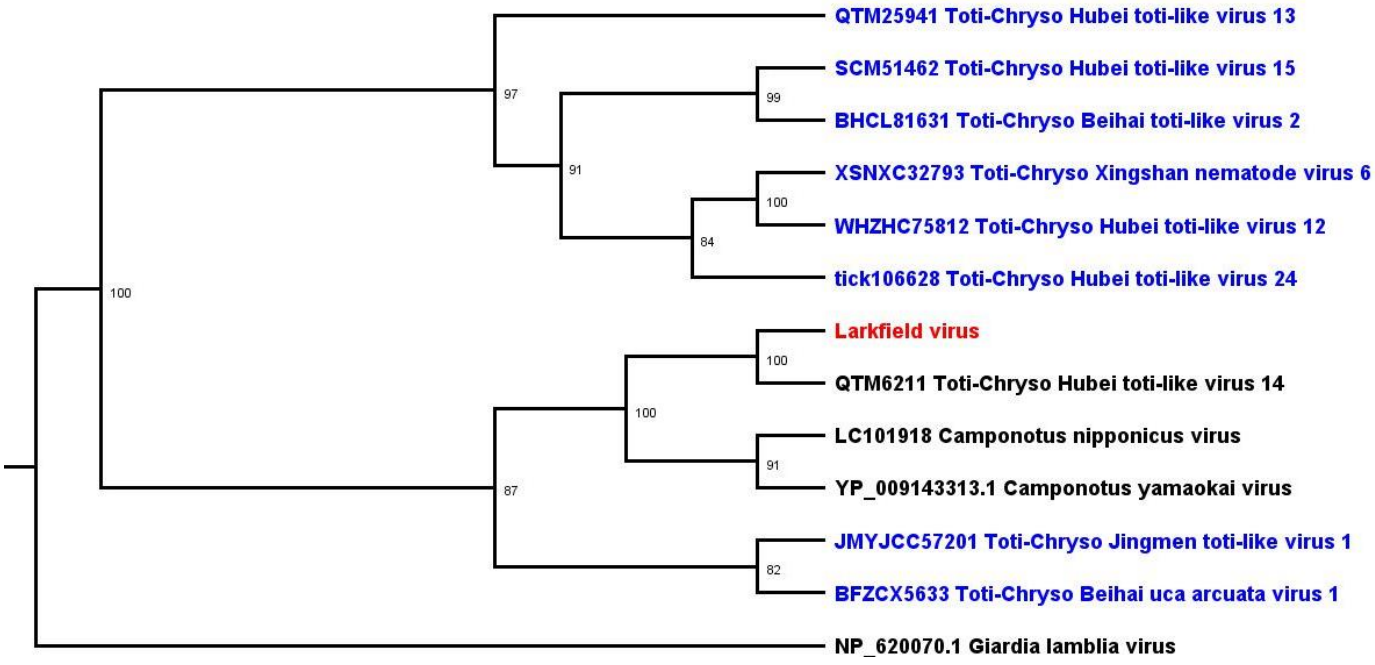

Figure 6. Phylogenetic tree of Deadman's wood virus and closest relatives. Viruses highlighted in red are those described here or by publications produced by the Obbard lab (i.e. Webster (2015 & 2016)), viruses highlighted in blue are those published by Shi et al. (2016) and those highlighted in green represent putative viral sequences discovered in TSA datasets through Blast.

Virus Prevalence

Teise virus is shown to be the most common of the new viruses so far analysed, appearing in 42% and 25% of Japanese and UK flies, respectively (Figure 7). It also seems to occur in pools of other Drosophila species, including *Drosophila immigrans*. Beult virus is the only other newly discovered virus detectable in Japanese adult flies. As all viral primers were designed from the sequences discovered in UK flies the true diversity of Japanese *D. suzukii* viruses will only be elucidated through further metagenomic studies, currently underway.

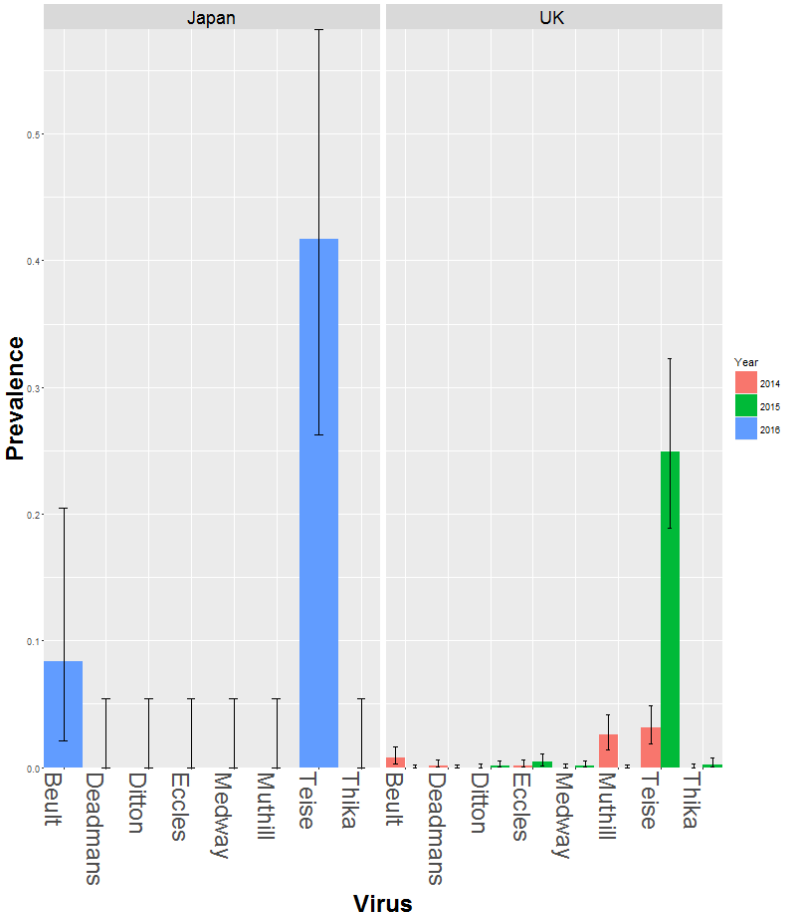

Figure 7. Prevalence scores of D. suzukii viruses, including Muthill and Thika viruses: discovered in other British Drosophila species but detected in D. suzukii. Japanese figures are calculated based on 2016 samples, whilst UK samples span 2014 and 2015 collections. Values are maximum likelihood estimates with 2 log-likelihood intervals.

Discussion

Further viral discovery has continued to discover and describe new species of virus infecting *D. suzukii.* Seven new viruses from a range of viral taxa have been described and analysed phylogenetically. Pathogenicity of these viruses will be assessed if isolation of these viruses is successful. We can make some inferences about the lethality of these viruses based on their prevalence. Viruses that are very common in adult wild flies that were alive at the point of capture in bait traps are probably not particularly lethal. Viruses that cause appreciable mortality in flies are also probably likely to kill larvae before they eclose as this represents the majority of the fly's life cycle. Many more larvae have been sampled in 2016 than in previous years and have now been submitted to RNA sequencing. Proceeding with the above metagenomic discovery in larvae will highlight if there is indeed any difference in the virus diversity between the different life stages.

Information about the prevalence of viruses infecting *D. suzukii* and other British *Drosophila* species has been obtained through large RT-PCR surveys. Most viruses appear to be fairly rare and host specific, infecting only one or two species of fly, with some exceptions. Further work is needed to include more viruses and more fly species in this presence-absence matrix and complete the picture of virus ecology in the British fauna. Analysis on this matrix will also provide interesting information about the host switching of these viruses and determine the extent to which phylogenetic signal shapes the presence of viral infection across this taxa. Samples from the native range of the pest will shed light on how much of a shift in virus diversity has been experienced by *D. suzukii* during its invasion of Northern Europe and may unearth new lethal viruses suitable for control applications.

Conclusions

- Further large numbers of wild *Drosophila suzukii* have been successfully sampled and their viruses surveyed metagenomically.
- Seven viruses, new to science, have been discovered infecting *D. suzukii*.
- Assays confirming virus presence by RT-PCR have allowed an estimate of prevalence for newley discovered and previously discovered viruses.
- Further samples from the UK and Japan have been submitted for metatranscriptomic analysis.
- Virus isolation protocols are being tested with the DNA Nudivirus of *D. melanogaster*, Kallithea Virus.

Knowledge and Technology Transfer

- 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

Glossary

DNA virus: A virus in which genomic sequence is made up of DNA (Deoxyribonucleic acid).

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.

Open reading frame: A stretch of sequence uninteruped by a 'stop codon'. 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.

RNA virus: A virus in which genomic sequence is made up of RNA (Ribonucleic acid).

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

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.

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*.

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

BEERS, E. H., VAN STEENWYK, R. A., SHEARER, P. W., COATES, W. W. & GRANT, J. A. 2011. Developing Drosophila suzukii management programs for sweet cherry in the western United States. *Pest Management Science,* 67**,** 1386-1395.

BENSON, D. A., CAVANAUGH, M., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2013. GenBank. *Nucleic acids research,* 41**,** D36-D42.
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.
BRUN, G. & PLUS, N. 1980. The viruses of Drosophila. *The genetics and biology of Drosophila,* 2**,** 625-702.
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.
CAMACHO, C., COULOURIS, G., AVAGYAN, V., MA, N., PAPADOPOULOS, J., BEALER, K. & MADDEN, T. L. 2009. BLAST+: architecture and applications. *BMC bioinformatics,* 10**,** 421.
CARLSON, J., SUCHMAN, E. & BUCHATSKY, L. 2006. Densoviruses for control and genetic manipulation of mosquitoes. *Advances in virus research,* 68**,** 361-392.
CARTER, J. 1984. Viruses as pest-control agents. *Biotechnology and genetic engineering reviews,* 1**,** 375-419.
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.
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.
CUTHBERTSON, A., COLLINS, D., BLACKBURN, L., AUDSLEY, N. & BELL, H. 2014. Preliminary Screening of Potential Control Products against Drosophila suzukii. *Insects,* 5**,** 488-498.
D'ASSIS FONSECA, E. 1965. A short key to the British Drosophilidae (Diptera) including a new species of Amiota. *Transactions of the Society for British Entomology,* 16**,** 233-244.
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.
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.
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.
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.

HARRIS, A. & SHAW, B. 2014. First record of Drosophila suzukii (Matsumura) (Diptera, Drosophilidae) in Great Britain. *Dipterists Digest,* 21**,** 189-192.
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.
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.
JIANG, H., ZHOU, L., ZHANG, J.-M., DONG, H.-F., HU, Y.-Y. & JIANG, M.-S. 2008. Potential of Periplaneta fuliginosa densovirus as a biocontrol agent for smoky-brown cockroach, P. fuliginosa. *Biological Control,* 46**,** 94-100.
JOSHI, N. 2015. FJ (2011) Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.20)[Software].
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.
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.*
KIM, V. N., HAN, J. & SIOMI, M. C. 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol,* 10**,** 126-139.
KOPP, A. 2006. Basal relationships in the Drosophila melanogaster species group. *Molecular Phylogenetics and Evolution,* 39**,** 787-798.
LEDERMANN, J. P., SUCHMAN, E. L., BLACK, W. C. & CARLSON, J. O. 2004. Infection and pathogenicity of the mosquito densoviruses AeDNV, HeDNV, and APeDNV in Aedes aegypti mosquitoes (Diptera: Culicidae). *Journal of economic entomology,* 97**,** 1828-1835.
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.
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.
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.
MARAMOROSCH, K. 2012. *Viral insecticides for biological control*, Elsevier.
MEYNADIER, G., VAGO, C., PLANTEVIN, G. & ATGER, P. 1964. Virose d'un type inhabituel chez le lépidoptère Galleria mellonella L. *Rev. Zool. agric. appl,* 63**,** 207-208.
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.
MOSCARDI, F. 1999. Assessment of the application of baculoviruses for control of Lepidoptera. *Annual review of entomology,* 44**,** 257-289.
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.
ROZEN, S. & SKALETSKY, H. 1999. Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics methods and protocols***,** 365-386.
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.
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.
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.
UNCKLESS, R. L. 2011. A DNA virus of Drosophila. *PloS one,* 6**,** e26564-e26564.
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.
VAN TIMMEREN, S. & ISAACS, R. 2013. Control of spotted wing drosophila, Drosophila suzukii, by specific insecticides and by conventional and organic crop protection programs. *Crop Protection,* 54**,** 126-133.
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.
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. *bioRxiv***,** 021154.

Appendices

Supplementary Table 1. Viruses tested for by RT-PCR in different pool of wild flies from four different species.

Virus	Dmel EIKST	Jap Larva	Jap Adult	Jap Larva pools	Jap Adult pools	Dsuz 2015	Dsuz 2014	JapDimm	Dsuz France 2014	Dros Alt flat mix	Dsub 2015	Dimm 2015
Eridge Virus	N	N	N	N	N	N	N	N	N	N	N	N
Hermatage Virus	N	N	N	N	Y	N	N	N	N	N	N	N
Dimm Iridovirus	N	N	N	N	N	N	N	N	N	N	N	N
Newington Virus	?	?	?	?	N	N	N	N	N	N	N	N
Dimm Nora	N	N	N	N	N	N	N	N	N	Y	N	Y
American Noda Virus	N	N	N	N	N	N	N	?	N	N	N	N
Berkeley virus	N	N	N	N	N	N	N	?	N	N	N	N
Bofa Virus	N	N	N	N	N	N	N	?	N	N	N	N
Charvil Virus	N	N	N	N	N	N	N	?	N	N	N	Y
Drosophila-associated Bunyavirus-like	Y	N	N	N	N	N	N	?	N	N	N	N
Kilifi Virus	Y	N	N	N	N	N	N	?	N	N	N	N
Takaungu Virus	N	N	N	N	N	N	N	?	N	N	N	N
Vesanto Virus	N	N	N	N	N	N	N	?	N	N	N	N
Viltain Virus	N	N	N	N	N	N	Y	N	N	N	N	N
Buckhurst Virus (Dobs)	N	N	N	N	N	N	N	N	N	N	Y	N
Grom Virus	N	N	N	N	N	N	N	N	N	N	Y	N
Lye Green Virus	N	N	N	N	N	N	N	N	N	N	N	N
Machany Virus	N	N	N	N	N	N	N	N	N	N	N	N
Pow Burn	N	N	N	N	N	N	N	N	N	N	Y	Y
Withyham Virus	N	N	N	N	N	N	Y	N	N	N	N	N
Cherry Gardens Virus	N	N	N	N	N	N	N	N	N	N	Y	Y
Dsub Fisa	N	N	N	N	N	N	N	N	N	N	Y	N
Grange Virus	N	N	N	N	N	N	N	N	N	N	Y	N
Presney Burn Virus	N	N	N	N	N	N	N	N	N	N	Y	N
Corseley Virus	N	N	N	N	N	N	Y	N	N	N	N	N
Braid burn virus	N	N	N	N	N	N	N	N	N	N	N	N
Craigmillar Virus	N	N	N	N	N	N	N	N	N	N	N	N
Kinkell Virus	N	N	N	N	N	N	N	N	N	Y	N	N
Beult (Dsuz Ngewotan) Virus	N	N	Y	N	N	N	Y	Y	Y	N	N	N
Brandeis Virus	N	N	N	N	N	N	N	N	N	N	N	N
Deadmans Virus (Dsuz Boolmfield)	N	N	N	N	N	N	Y		N	N	N	N
Eccles Virus (Dsuz Glabut)	Y	N	N	N	N	Y	Y	Y	N	N	N	N
Muthill Virus	N	N	N	Y	N	N	Y	Y	N	N	N	N
Teise Virus (Dsuz Motts Mill)	N	N	Y	Y	Y	Y	Y	Y	Y	N	N	Y
Blackford Virus	N	N	N	N	N	N	N	N	N	N	N	N
Empeyrat	N	N	N	N	N	N	N	N	N	N	N	N
La Tardoire Virus	N	N	N	N	N	N	N	N	N	N	N	N
Marsac Virus	N	N	N	N	N	N	N	N	N	N	N	N
Soudat Virus	N	N	N	N	N	N	N	N	N	N	N	N
Tartou Virus	N	N	N	N	N	N	N	N	N	N	N	N
Mito virus (new)	N	N	N	N	N	Y	N	?	N	?	?	N
Dsuz_wuhan	N	N	N	N	N	Y	N	?	N	?	?	N
Dsuz_c.nipp		N	N	N	N	N	N	?	N	?	?	?