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Intra- and inter-specific communication in *Drosophila suzukii*: From genome to behavior.

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Intra- and inter-specific communication in *Drosophila suzukii*: From genome to behavior.

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Cover: Description of photograph (Female *Drosophila suzukii* resting on Raspberry fruit)
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Abstract

Drosophila suzukii (Diptera: Drosophilidae), an invasive pest from Asia, colonized US and Europe in 2008 and since then has economically damaged multi-million commercial fruit production. With fruit-breaking serrated ovipositor the female lays eggs in the unwounded ripening fruits making them unmarketable. To know the genetic likelihood of *D. suzukii* with other *Drosophila* species its genome and transcriptome were sequenced. The whole genome was mined to understand the origin, speciation and adaptation and was correlated with the ecology of the species. Genomic analyses revealed that *D. suzukii* is adapted to temperate climate and has lower selective pressure and gene-sequencing rate compared to its sibling sp. *D. biarmipes*. From the genomic and ecological studies, one of the objectives was to understand the role of olfaction in host fruit recognition and identify key volatile compound/s involved in female decision-making for oviposition on fresh fruits. Based on gas chromatography mass spectrometer (GC-MS) and GC-electro-antennal detector activity, isoamyl acetate was found to be one of the key compounds involved in the oviposition site selection. The phylogenetic analysis revealed that *D. suzukii* not only possess the full repertoire of genes encoding olfactory receptors activated by isoamyl acetate in *D. melanogaster*, but showed that Or67a (Or67a1 to Or67a5) is even represented by duplicated copies. Another aim was to investigate the pheromone communication in this species. The extraction and identification of cuticular hydrocarbons from the males inherently showed that the species trans-evolved by terminating the production of sex pheromone *cis* vaccenyl acetate (*c*VA), which is used by species throughout *melanogaster* group, but able to smell it with 'fewer' T1 sensilla. Being under highly selective pressure *D. suzukii* has i) scaled-down the size of ejaculatory bulb in males, ii) fewer T1 tricoid sensilla, and iii) comparatively smaller glomerulus, in the antennal lobe (AL), involved in receiving sensory impulses from T1 sensilla when compared to *D. melanogaster*. However *D. suzukii* shares all functional fatty acid synthase (*FAS*) genes responsible to CH production. On applying *D. melanogaster* male equivalent synthetic *c*VA on males of *D. suzukii*, it significantly reduced the mating acceptance in the females, which otherwise increased in *D. melanogaster*. Therefore, by adapting not to produce *c*VA as a sex pheromone *D. suzukii* tend to avoid competition with congeneric species for oviposition. The whole spectrum of the present and future studies would help to understand the evolution of the olfactory code among the closely related species of *Drosophila* and, as a consequence, contribute to develop alternative control methods of *D. suzukii*. Indeed, comparison of *D. suzukii* with sibling species and *D. melanogaster* could shed light on the evolution of ecological innovations and help researchers in understanding what makes a species to be an invasive pest.

Keywords: Drosophila suzukii, Drosophila melanogaster, genome sequence, *cis*-vaccenyl acetate, isoamyl acetate, *Or*67d.

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Dedication

I dedicate my thesis to my late parents for their everlasting encouragement. And also to my brother and sisters for their love and affection throughout my life…

Contents

Manuscripts

Linking genomics and ecology to investigate the complex evolution of an invasive Drosophila pest. *Genome Biology and Evolution*. (5) 745-757.

Olfactory responses of *Drosophila suzukii* female to host plant volatiles. (Manuscript)

From pheromone to behavioral antagonist: *cis*-vaccenyl acetate loss in *Drosophila suzukii* reverses its role in sexual communication. (Manuscript)

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ometto, L., Cestaro, A., Ramasamy, S., Grassi, A., **Revadi, S**., Siozios, S., Moretto, M., Fontana, P., Varotto, C., Pisani, D., Dekker, T., Wrobel, N., Viola, R., Pertot, I., Cavalieri, D., Blaxter, M., Anfora, G., and Rota-stabelli, O. 2013. Linking genomics and ecology to investigate the complex evolution of an invasive Drosophila pest. *Genome Biology and Evolution*. (5) 745-757.

II **Santosh Revadi**, Silvia Vitagliano, Marco Valerio Rossi Stacconi, Suzan Mansurian, Sukanya Ramasamy, Silvia Carlin, Urska Vrhovsek, Paul G. Becher, Valerio Mazzoni, Omar Rota-Stabelli, Sergio Angeli, Teun Dekker, Gianfranco Anfora. Olfactory responses of *Drosophila suzukii* female to host plant volatiles. (Manuscript)

III Teun Dekker, **Santosh Revadi**[*], Suzan Mansourian[*], Sukanya Ramasamy, Sebastien Lebreton, Paul G. Becher, Sergio Angeli, Omar Rota-Stabelli, Gianfranco Anfora. From pheromone to behavioral antagonist: *cis*-vaccenyl acetate loss in *Drosophila suzukii* reverses its role in sexual communication. (* Indicate equal contribution) (Manuscript)

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The contribution of Santosh Revadi to the papers included in this thesis was as follows:

I Generated *Drosophila suzukii* inbred lines (F5 generation) and setting of monitoring traps.

II Conducted behavioural and electrophysiological experiments and wrote the manuscript.

III Standardized behaviour experiment, GC-MS analyses, and electrophysiological experiment and wrote behavioural studies section of the manuscript.

Abbreviations

AL	Antennal lobe
*c*VA	*cis*-vaccenyl acetate
GABA	γ-aminobutyric acid
GC-EAD	Gas chromatography- electroantennal detector
GC-MS	Gas chromatography- mass spectrometry
GOBP	General odorant binding proteins
GR	Gustatory receptors
iGluR	Ionotropic glutamate receptors
IR	Ionotrophic receptors
OBP	Odorant binding proteins
ODE	Odorant deactivating enzyme
OR	Odorant receptors
Orco	Odorant receptor co-receptor
ORN	Olfactory receptor neuron
PBP	Pheromone binding proteins
SSR	Single sensillum recording
7TM	7 Transmemberane domain proteins

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1 Introduction

Insect ability to smell is one of the oldest (Wyatt, 2003) and unique sensory system insects rely-on to find their food, mate and for defense against their enemies (Firestein, 2001). From the blend of volatiles present in the air, insects depend only on the relevant compounds for decision-making, resulting in behavioral response, be it for locating the food or reacting for the opposite sex for mating (Firestein, 2001; Pellegrino and Nakagawa, 2009). In *Drosophila*, the feeding site is the fermenting food that releases several volatile compounds mostly the by-products of microbial fermentation (de Bruyne et al., 2010), detected by bunch of sensory hairs called sensilla basiconica, while for sex attraction, the pheromone *cis* vaccenyl acetate (*c*VA) is detected at a short distance by a specialized hairs called sensilla trichoidea (Hallem et al., 2006).

Based on previous studies on *Drosophila melanogaster*, the aim of the thesis here has been to study the olfactory system from genome to behavior, of the south-east Asian fly *Drosophila suzukii,* the only *Drosophila* pest in *melanogaster* group, that has invaded Europe and United States, devastating multi-million soft skinned fruit production since 2008 (Walsh et al., 2011). In order to have more clear view on the *D. suzukii* origin, speciation and genetic relatedness with other *Drosophila* sp., *D. suzukii* genome and transcriptome were sequenced. Based on the genomic findings, further studies were focused to understand the strategy of the fly discriminating fermenting fruit *versus* fresh unwounded ripening fruit explored for oviposition, reason and role of evolution for speciation and the mating strategies of *D. suzukii* over time, different from the parental species.

1.1 The model

Drosophila is one of the genus with diverse species studied extensively in various disciplines because of its ecological adaptations and higher generation

number (Ometto et al., 2013) and has successfully spread world-wide from Africa 10,000 years ago (David and Capy, 1988). One example is *D. suzukii*, a native pest of South-east Asia now established in US and Europe harming several soft skinned fresh fruits with its serrated ovipositor. Unlike *D. melanogaster*, the cosmopolitan fermenting fruit-feeding (de Bruyne et al., 2010) ancestral species of *D. suzukii*, this species feeds on the fermenting fruits, but lays eggs on unwounded ripening fruits (Mitsui et al., 2010; Ometto et al., 2013; Walsh et al., 2011). Notwithstanding, it also breeds on the fermenting fruits in the absence of congeneric competition (Ometto et al., 2013). *D. suzukii* is known to infest wide host plants ranging from blueberry, strawberry, cherry, raspberry, blackberry, grapes, figs, apricot peach, plums, mulberry and wild fruits (Kanzawa, 1939; Poyet et al., 2014; Rota-Stabelli et al., 2013; Walsh et al., 2011).

1.2 Species description

Unlike most of the close relative species *D. suzukii* is one of the few Drosophilids that lay eggs in the fresh fruits (Fig. 1A). The adults flies commonly referred as Spotted Winged Drosophila (SWD) are 2-3 mm in length, red eyes resembling vinegar flies *D. melanogaster* (Cini et al., 2012; Walsh et al., 2011) (Fig. 1B). The male *D. suzukii* has a conspicuous wing spot on the front edge near to the tip of the forewing (Fig. 1E) (so the name) while female posses a melanized saw-like serrated ovipositor (Fig. 1C) (Cini et al., 2012). Secondly, microscopic observation reveals the split-up sex combs on the forelegs of male while *D. melanogaster* has one sex comb (Kopp, 2011).

D. pulchrella and *D. subpulchrella* are two other sister species known to possess serrated ovipositor and oviposit on the fresh undamaged fruits (Mitsui et al., 2010). Nevertheless, there is no report on the economic damage caused by these species to the fruit production in their native geographical locations. However, *D. subpulchrella* has the ability to lay eggs on unwounded fruits similar to *D. suzukii* tested in the laboratory condition (Atallah et al., 2014). *D. suzukii* hence is the only Drosophilid so-far reported to cause significant crop damage.

1.3 Invasion in America

D. suzukii was first found in California (North America) in 2008 (Hauser, 2011; Walsh et al., 2011) and a year later it was reported in Canada and Florida along the east coast. Later in a year time, there was potential damage reported in strawberry and raspberry cultivation because of SWD (Goodhue et al., 2011;

Hauser, 2011; Steck et al., 2009). Considering the rapid spread and threat, growers monitored the pest with apple cider vinegar, yeast sugar solution, wine and other fermenting fruit traps (Lee et al., 2012). This was the period when large number of scientific groups initiated the work on the management aspects, especially designing the monitoring traps. Between 2011 and 2012, 44 scientific articles were published in various journals mainly focused on the *D. suzukii* management. Few recommendations have comparatively given comprehensive results, but few found to be ineffective because of the masking of the effect of fermentation traps in grapes and other crops (Lee et al., 2012). Nonetheless, the combination of wine and vinegar was found to be attractive in trapping the flies (Cha et al., 2012). A major problem with these food baits is that they get a large part of unspecific trap captures which makes difficult to process traps, especially for non-entomologists who may have difficulties distinguishing *D. suzukii* from other *Drosophila* species.

1.4 *D. suzukii* in Europe

The story of the first detection and spread of *D. suzukii* in Europe was revised by Cini et al. (2012). First adults of *D. suzukii* were caught contemporaneously in Spain (Rasquera Province) (Calabria et al., 2012) and in Italy (Tuscany region) in 2008. A year later it was reported to spread up north to Montpelleir (France) (Calabria 2012). In the same year in Trentino Province (Italy), both first oviposition on wild hosts (*Vaccinium*, *Fragaria* and *Rubus* spp.) and economically important damage on several species of cultivated berries were reported. The European and Mediterranean Plant Protection Organization (EPPO) released the Pest Risk Analysis (PRA) and concluded that *D. suzukii* would prevail in the region because of vast cultivation area and that was impossible to suppress. This called-off for an emergency meeting of scientists represented from ten European countries for discussing effective control steps (Cini et al., 2012). By 2010-2011, the range of *D. suzukii* was further enlarged, invading other regions in Italy and France (Cini et al., 2012), but also spreading to the North and East invading Switzerland (2011), Slovenia (2011), Croatia (2011), Austria (2011), Germany (2012), Belgium (2012), The Netherlands (2012), United Kingdom (2012), Hungary (2013) and Greece (2014) (see EPPO website). Such escalating outbreak has narrowed the benefits because of the high investment on pest management (Cha et al., 2014).

1.4.1 Damage

Although a recent arrival to Europe, *D. suzukii* already caused severe damages in several small fruit growing areas across southern Europe, e.g. on sweet cherries (75 to 90% yield loss), strawberries (80% yield loss), raspberries (80% yield loss), blackberries and blueberries (each 30 to 40% yield loss). Even higher levels of damages were reported for locations in Northern Italy (Trentino) and in France, with up to 100% damage registered on blackberry, raspberry, strawberry and sweet cherry (Cini et al., 2012; Weydert and Mandrin 2013). Furthermore, *D. suzukii* also attacked apricots, currants, figs and grapes. In France it was also reported on apples and peaches although without any economically significant damage (Weydert and Mandrin 2013).

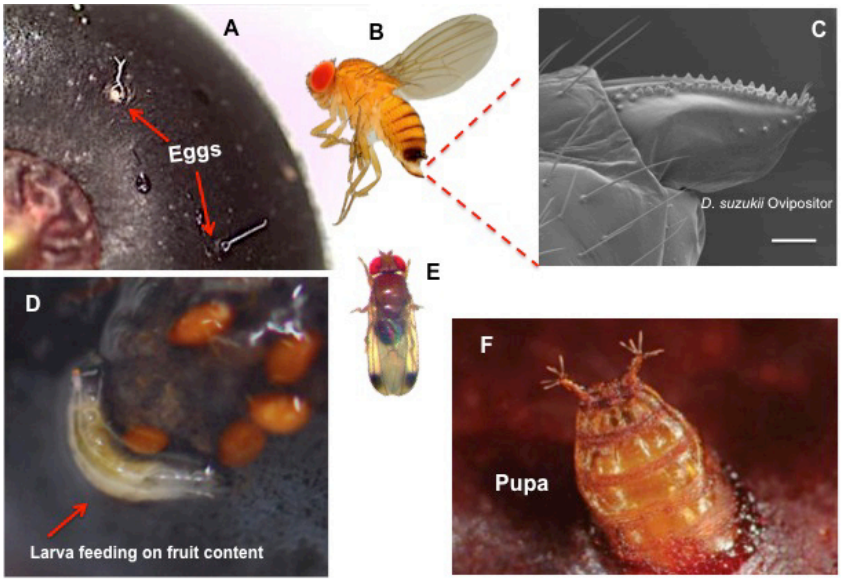

Figure 1. (A) *Drosophila suzukii* eggs on blueberry (cv. Brigitta) collected from the field. (B and C) *D. suzukii* female with serrated ovipositor used to pierce the fruit skin. (D) *D. suzukii* larva feeding on the fruit content. (E) *D. suzukii* male with typical wingspot on the forewing margin. (F) Yellowish brown pupa found on/ inside the fruit.

In most areas, low population levels are observed in spring, but numbers increase fast during summer, with populations reaching their highest level in late autumn (Weydert and Mandrin 2013). Although cherry is considered to be a favoured host, population density in early summer, during the ripening period of cherry, is much lower than those faced by crops maturing later. Thus, summer crops, such as strawberries and other berry crops, are subjected to much higher infestation pressure. Although grapes are not supposed to be a main host for *D. suzukii* (Bellamy et al., 2013), some soft-skinned varieties

may suffer from the extremely high population densities in autumn. High population densities in autumn may in turn lead to higher infestation pressure in spring. An efficient regulation of *D. suzukii* in late-maturing crops presumably also lowers the infestation level in the following spring. It is therefore likely that a range of regulation strategies is needed for early-season and late-season crops. For example, a delay of immigration thanks to perimeter mass trapping might be a suitable strategy in situations of low pest abundance, whereas in situations of high pest abundance this strategy may not provide reliable control. Population dynamics also depend on pedo-climatic conditions: in cooler climates the pest activity starts later while in warmer and drier conditions the population density is drastically reduced during the summer season. In addition, lower temperature will lead to fewer generations per year (Ometto et al., 2013). It is therefore possible that different regulation strategies are necessary for different climatic zones even within the Mediterranean region.

1.4.2 Economic impact

As one of the major ecological, environmental and socio-economical risk at both global and local scale, alien species that become invasive are considered to be the main direct drivers of several detrimental effects on biodiversity, on human and animal health and welfare and on crop production worldwide. Controlling invasive species and repairing the damage they do is estimated to cost European economies at least €12 billion each year.

D. suzukii infestation is an actual major threat for the fruit industry of the concerned countries and can therefore become a model of the economic impact of a new pest at European level. Assessments about the *D. suzukii* economic impact are however relatively scarce at present, and focused on the USA (Bolda et al., 2010; Goodhue et al., 2011). A provisional economic injury level was calculated at >2 adults/ monitoring trap for all crops and farmers are encouraged to apply preventive insecticide treatments when the threshold is reached (Gardeman and Tanigoshi, 2011). De Ros et al. (2012) made a very first evaluation of the pest's economic impact on the five host crops mostly affected by pest infestation. This study is however only focused on Trento Province, Italy, where it was estimated that the 400-ha soft fruit production areas faced losses of around 500,000 EUR in 2010 and 3 million EUR in 2011. As a consequence of infestation and harvesting dynamics, the greater economic impact in Trentino can be ascribed to the blueberry production. However, this estimation took into account the revenue losses of small fruit industry while not considering the management costs of control strategies and other societal consequences of the increase of chemical inputs.

1.5 *D. suzukii* yesterday and today: Evolutionary perspective

D. suzukii was first reported by Kanzawa (1939) as a pest on several fruit species in Japan. It also spread in Asia however, most of the reports published are in Asian languages and so no translation been done on pest status, economic impact and distribution (Dreves, 2011). Out of Asia, it was first spotted in the Hawaiian island in the early 1980's but there was no report of the crop damage or pest spread (Kaneshiro, 1983). Lately in 2008, the species had widespread in USA and also in Europe, significantly affecting several fruit productions (Calabria et al., 2012; Hauser, 2011). *D. suzukii* is the product of several hundred-fold years of speciation of the *Drosophila* genus out-of-Africa (David and Capy, 1988) and still needs detailed studies to understand the behavioral and physiological adaptations in relation to its ecological importance (Ometto et al., 2013). Within *suzukii* subgroup, geographically distributed in Asia, *D. suzukii, D. subpulchrella* and *D. pulchrella* look very much similar to each other with male having dark spot on the forewing (Takamori et al., 2006) and female are attracted to ripening fruits for oviposition (Mitsui et al., 2010). *D. biarmipes* is another *suzukii* subgroup species that posses wing spot in males, but is much alike *D. melanogaster*, attracted to fermenting fruits (McRobert et al., 1997).

D. suzukii being thrived on the fresh fruits in its geographical origin, found similar climatic conditions in US and Europe on the similar soft fruits. However, interestingly, recent report of *D. suzukii* infestation on fruits of woody tree species *Prunus serotina*, a south American exotic tree species in Europe, shows the potential of the species host-switching ability, other than the preferred hosts (Poyet et al., 2014). Considering the economic importance and adaptation of the pest, it is inevitably necessary to study basic biology of the pest ranging from its olfactory system, neural plasticity that governs host selection and its interaction with the host plants.

The genome sequence of *D. suzukii* was made available only before last year (Ometto et al., 2013). In the *suzukii* subgroup, *D. suzukii* and *D. biarmipes* have been sequenced but two other important species *D. pulchrella* and *D. subpulchrella*, which are believed to be sibling species *D. suzukii* are yet to be studied at gene level. According to the recent update, 23 drosophilid genomes have been sequenced including *D. suzukii* (Ometto et al., 2013) and provides excellent opportunity to study the evolution of chemosensation in this model group.

2 Chemosensation in *Drosophila*

Chemical sensing in one of the oldest sensory mechanism ever studied in an organism, be it a unicellular bacteria or a multicellular organisms (Steiger et al., 2011; Wyatt, 2003). Chemical communication can be broadly classified based on smell (olfaction) and taste (gustation). The olfaction is the mode to detect the volatile odours from distance, while gustation depends on the event of coming in contact with the source to detect and react (Vosshall and Stocker, 2007). The mechanism involved in detecting the odour molecules in the air is mediated by the specialized proteins called odorant receptors (OR), while for the taste the proteins are referred to as gustatory receptors (GR) (Clyne et al., 1999; Vosshall et al., 1999).

Drosophila has around 60 ORs encoding 62 receptors including gustatory receptors and ionotropic receptor genes and is being used as a model organism to study neural circuits involved in chemoreception (Fishilevich and Vosshall, 2005; Robertson et al., 2003).

2.1 *Drosophila* olfactory system

The third antennal segment of *Drosophila*, flagellum is primarily involved in olfaction (Vosshall and Stocker, 2007). In addition to antennal flagellum, the maxillary palp are also involved in mediating the olfactory orientation of the fly. Both flagellum and maxillary palp house a compact hair like sensory structures called sensilla, that are the basic functional units of olfactory system (de Bruyne et al., 2001, 1999; Hallem and Carlson, 2004). These sensilla have hydrophobic membrane and allow predominantly lipophilic odour molecules in the air to pass through small pores and enter the aqueous fluid and reach olfactory receptor neurons (ORN). Each sensillum has one to four ORNs (de Bruyne et al., 2001, 1999) and these ORNs have their dendrites suspended in the sensillum lymph while the axon is projected towards the intermediate

information-processing unit of brain called as antennal lobe (AL) (Hallem et al., 2004; Hiroi et al., 2008). Upon contact with the odour molecule, the dendrites pass-on the stimulus information via difference in the membrane potential to AL.

2.1.1 Antenna

In *Drosophila*, there are three major morphological types of sensilla on the antenna, which differ in their shape, size and function *i.e.,* basiconic, tricoid and coeloconic sensilla (Shanbhag et al., 1999; Stocker, 1994). Altogether, *Drosophila* has around 419 sensilla in males and 457 in females. Out of which, around 120 sensilla are tricoid and the remaining are basiconic and coeloconic sensilla (Shanbhag et al., 1999). Basiconics are further divided as large basiconics (AB1, AB2 and AB3) and small basiconics (AB4, AB5, AB6, AB7, AB8, AB9 and AB10) while tricoid are categorized as AT1, AT2, AT3 and AT4 (Riesgo-Escovar et al., 1997).

All basiconic sensilla are involved in detecting odour molecules for locating food (de Bruyne et al., 2001; Hallem et al., 2004; Yao et al., 2005), while tricoid sensilla are involved in detecting short range communicating pheromone *cis* vaccenyl acetate (cVA) in case of *D. melanogaster* (Naters and Carlson, 2007). *c*VA is one of the major cuticular hydrocarbons present on the sexually matured male used for courtship and mating. The third type, coeloconic sensilla, are involved in sensing food odour molecules, acids, humidity and ammonia (Benton et al., 2009; Galizia and Rössler, 2010; Yao et al., 2005).

2.1.2 Maxillay palp

These are secondary olfactory organs of *Drosophila* adjacent to proboscis (de Bruyne et al., 1999). Compared to antenna, maxillary palp has lesser number of sensilla, belonging to only one class, *i.e.,* basiconics (Singh and Nayak, 1985). There are only around 60 basiconic sensilla on the maxillary palp, but sensitive to many odorants (de Bruyne et al., 1999).

2.2 Perireceptor events in olfaction

The definition “perireceptor events” for the first time was coined by Getchell et al., (1984) to define the uptake of the odour molecule, binding with transporting protein, inactivation of odour molecule and eventually receptor activation and transduction. All sensilla, irrespective of their types, function similarly in transforming chemical signal into electrical signal via changes on the membrane potential of the ORNs (Leal, 2013).

Upon contact with the hydrophobic cuticular membrane of the sensillum the odour molecule reaches the entry port, the pore tubules from the surface and is picked by unique protein, called as *Odorant Binding Protein* (OBP), in the aqueous medium around the dendrites of a neuron (Hallem et al., 2006, 2004; Vogt et al., 2003) (Fig. 2). These proteins accordingly transport the odour molecule to their receptors *i.e.,* odorant receptor (OR), gustatory receptors (GR), ionotropic receptors (IR) present on the dendritic membrane (Pelosi et al., 2006). This protein solubilizes the odour molecule after activating the stimulus response and is immediately deactivated by another enzyme *Odorant Deactivating Enzyme* (ODE) (Ishida and Leal, 2005). It is a matter of milliseconds that the odour molecule activates the response channel and is immediately deactivated (Ishida and Leal, 2005).

2.2.1 Odorant Binding Proteins (OBPs)

The discovery of odorant binding proteins in *Antheraea polyphemus,* wild silk moth antenna by Richard Vogt and Lynn Riddiford in 1981 was a milestone in the field of insect olfaction. In 1999 again, the same group confirmed that OBP are universally present across different species (Vogt et al., 1999) including mammals (Tegoni et al., 2000). In fruit fly, 52 genes are believed to be involved in OBP synthesis (Sanchez-Gracia et al., 2009).

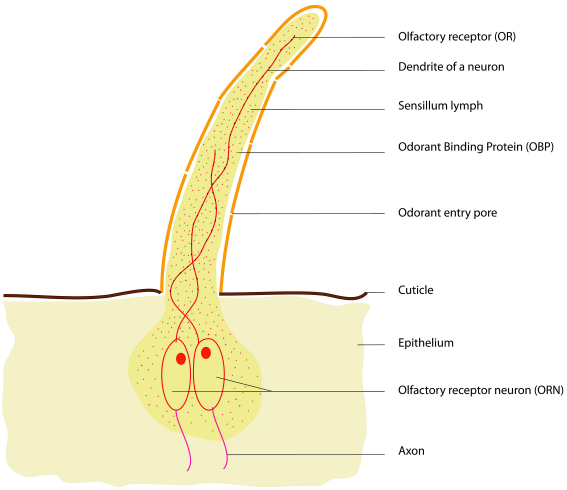

Figure 2. Schematic drawing of an olfactory sensillum represented by an olfactory receptor (black oval dots), dendrite (projecting red line), sensillum lymph (light yellow envelope), pore, OBPs (light pink dots), ORNs and Axon. (Image modified after Sanchez-Gracia et al., 2009).

Once the odorant molecule binds with the OBP it is carried to the olfactory receptors on the dendrite on the ORN. The specificity of the OBP for pheromone and for general odour molecules was unclear until when Smith and his colleagues in 2005 showed that *D. melanogaster* mutant flies lacking LUSH OBP lost their complete sensitivity to detect the pheromone *cis*-Vaccenyl acetate (*c*VA). In case of moths, general odorant binding proteins

(GOBP) are believed to be associated with carrying the odour molecules other than pheromone, to the binding site on the dendrites (Steinbrecht et al., 1996), expressed in male and female antennae in the different class of ORNs (Vogt et al., 1991).

2.2.2 Olfactory receptors (ORs)

Olfactory receptors (ORs) are seven transmembrane domain proteins located on the cell membrane of the neuron (Benton et al., 2006). Insect ORs are composed of novel family of seven transmembrane domain proteins (7TM) and are distinctly different from the ORs of vertebrates (Hansson and Stensmyr, 2011), that is, the insect ORs have inverted topology of 7TM domain proteins with N- terminal inside and C-terminal outside the cell membrane (Benton et al., 2006). The insect OR from fruit fly *D. melanogaster* was first identified by bioinformatic-based approach (de Bruyne et al., 1999; Vosshall et al., 1999). After this discovery, there was a spectacular expansion of knowledge on the insect olfactory system. Now, similar research has been focused on honeybees, ants, beetles and mosquitoes (Leal, 2013). However, the knowledge is still biased towards olfaction of *D. melanogaster* because of the vast available information on the species (Leal, 2013).

Odorant Receptor Co-receptor (ORCo)

ORCo is believed to be the integral part of ORs involved in the localization of ORs to ORN dendrites and enhance specific responses to the odorant without affecting the ligand specificity (Benton et al., 2006). OR83b is the ORCo earlier identified from moths (Krieger et al., 2002) and mosquitoes (Hill et al., 2002) and is claimed to be highly conserved among most of the insects across different orders where it is believed to perform similar function (Larsson et al., 2004).

The odorant-OBP complex arrives at the dendrite membrane, a reception site and then fits in the receptor site as a “key and lock” manner (Leal, 2013). It then generates signals on the dendritic membrane via ion exchange channels and the signals are passed-on to antennal lobes (AL). Eventually, the chemical signal is transformed into appropriate behavioural response (Jacquin-Joly and Merlin, 2004).

To understand the functional classes of receptors expressed in the antenna and maxillary palp, individual receptors were functionally expressed in other organism (Hallem et al., 2006). OR43a was the first odour receptor to be functionally characterized from *D. melanogaster*. The overexpression of OR43a and also as heterologous expression in *Xenopus laevis* oocytes responded for fruit and other natural odours (Dahanukar et al., 2005). After few

years, nearly all the odour receptors of *D. melanogaster* antenna and maxillary palp were characterized (Hallem et al., 2006). The ORs from other insects are expressed in the mutant flies' "empty neuron" system (Hallem et al., 2004; Vosshall et al., 2000) thus characterizing the ORs from which they were derived. It was proved from this technique that generally the ORN express only one functional receptor (Hallem et al., 2004). However, interestingly, some neurons like pb2A, the sensillum present on the maxillary palp, represent two functional receptors genes, OR33c and OR85e (Goldman et al., 2005). Furthermore, the sensitivity of these functional receptor genes is not similar, while OR85e is broadly tuned to smell more compounds and OR33c is narrowly tuned (Goldman et al., 2005).

2.2.3 Gustatory receptors (GRs)

Gustatory receptors were also identified based on bioinformatic-based approach alike olfactory receptors (Clyne et al., 2000). Gustatory receptors are another set of 7TM domain proteins from the divergent protein family like olfactory receptors (Robertson et al., 2003), responsible for taste. They were identified in the labella of *Drosophila* (Clyne et al., 2000). These chemosensory receptors are expressed in different parts of the fly body ranging from antennae, maxillary palp, proboscis, tibiae, tarsi and wing margin of *Drosophila* (Robertson et al., 2003). Similar to olfactory receptors, gustatory receptors have inverted topology with N-terminal inside and C-terminal outside cell membrane (Clyne et al., 2000). There are few exceptional gustatory receptors expressed on the olfactory organ, antenna of *Drosophila,* which innervate pair of the glomeruli and not the suboesophageal ganglion and hence show their partial olfactory function along with taste (Scott et al., 2001; Vosshall et al., 2000). The phylogenetic analyses of GRs and ORs is supportive of the fact that gustatory receptors belong to much older fossil protein family than the olfactory receptors being evolved from it (Robertson et al., 2003; Scott et al., 2001), so it is possible that GRs were earlier also involved in olfaction.

D. melanogaster has 60 OR and GR genes that encode for 62 OR and 68 GR receptor proteins, respectively (Robertson et al., 2003). Following their identification, few GRs were functionally characterized, like GR68a that is expressed only in the male *Drosophila* forelegs, sensitive for pheromone detection (Bray and Amrein, 2003), GR66a for bitter taste expressed in the labium (Wang et al., 2004) and GR5a for trehalose reception (Dahanukar et al., 2001). As mentioned before, of all identified GRs, GR21a and GR63a are expressed in the antennae of the fly (Jones et al., 2007; Kwon et al., 2007; Scott et al., 2001), and function as an olfactory unit innervating the glomeruli (Scott et al., 2001). Following the *in-situ* hybridization of these GRs in *D.*

melanogaster antenna and by electrophysiological assays it was confirmed the involvement of these receptors in CO_2 detection (Jones et al., 2007).

2.2.4 Ionotropic Receptors (IRs)

Other than olfactory receptor and gustatory receptor, recently a novel class of receptor was identified, which also is based on bioinformatics-based approach, the ionotropic receptor (IR), to be a part of chemosensation and belong to ionotropic glutamate receptor (iGluR) protein family (Benton et al., 2009). These receptors are expressed in the sensilla on the fly antenna, sensilla coeloconica in four different types, ac1 to ac4, involved in the detection of acids, ammonia and humidity (Benton et al., 2009; Yao et al., 2005). Other than ORs and GRs on the fly antenna, IRs are found on two different regions of the antenna, *i.e.,* on the arista and sacculus (Foelix et al., 1989; Shanbhag et al., 1995). Based on the genomic analyses, 66 IRs have been identified, of which 16 IRs are found on the antennae and 10 of them are expressed in the coeloconic sensilla on the antenna, either singly or co-expressed with other IRs (Benton et al., 2009; Croset et al., 2010). Like ORCo (OR83b), which is co-expressed in ORs, IR8a and IR25a are broadly co-expressed with many other IRs (Benton et al., 2009).

Both ORs and IRs are involved in the olfaction in *Drosophila* with distinct structure and ligand specificity (Rytz et al., 2013) but the co-existence of both the receptors has so far no proper explanation. The olfactory response neurons of ORs and IRs revealed distinct differences between the two, however, though, both converge the neurons to the antennal lobe (Ai et al., 2010). It is reported that IR neurons are fine-tuned for few odours compared to OR neurons, are less sensitive (Getahun et al., 2012; Yao et al., 2005) and phlegmatic in their response and adaption to the odours compared to ORs (Getahun et al., 2012). While the discriminating difference between ORs and IRs is the response profile for the compounds, strongest IR ligands are weakly or not at all detected by ORs (Silbering et al., 2011; Yao et al., 2005) and *vice versa*, strongest OR ligands are not recognized by the IR receptors (de Bruyne et al., 2001; Hallem et al., 2006).

2.3 Insect Antennal Lobe (AL)

Antennal lobe is the primary sensory information-processing center in insects composed of smaller morphological and functional units called glomerulus. Antennal lobes are innervated with three different types of neurons; one, that carry electrified signals from the periphery (in the fly, from antenna and

maxillary palp (Singh and Nayak, 1985)) to the glomeruli, referred as olfactory receptor neurons (ORNs), the second is the local interneurons (LN), connecting different glomerulus within the AL, and thirdly, the projection neurons (PN), that receive partially-processed signals from glomerulus and synapse with neurons of complex brain centers of protocerebrum, the mushroom bodies and lateral horn (Roman and Davis, 2001). This sequence of events then leads to the behavioral response in the insect for locating food, mate or escape from enemies. The fruit fly antennal lobe consists of ~43 glomeruli (Laissue et al., 1999) receiving chemical signals from 60 ORs and GRs, respectively (Robertson et al., 2003).

Local neurons/ local interneurons do not protrude outside but connect different glomeruli within the antennal lobe. They necessitudinally switch their function by inhibiting or exciting the response within the antennal lobe by releasing γ-aminobutyric acid (GABA) or acetylcholine enzyme, respectively (Masse et al., 2009). The projection neurons are directly connected with the ORNs in the glomerulus and send the information to mushroom bodies and lateral horn, while most of them are cholinergic (excitatory) few are GABAergic (inhibitory) (Masse et al., 2009). Experimental inactivation of mushroom bodies have shown behavioral response in the insects that is mediated by the lateral horn, however, the signals sent to mushroom bodies help the insect in associative learning for the neural stimulus (Connolly et al., 1996).

3 Pre- and post mating behavior in Drosophila

In genus *Drosophila*, species have radiated from being cosmopolitan and generalist to specialist on one or few preferred hosts. The genus represents large variation in utilization of resources, host specialization, courtship signaling (Mazzoni et al., 2013) and female remating (de Crespigny et al., 2006; Markow and O'Grady, 2005). Researchers have used *D. melanogaster* as a premier model organism to study from basic principles of genetic to behavior to medical implications (Strauch et al., 2014). However, the interspecific diversity within the genus has now opened wide area of interest to study the genetics, sexual selection, speciation, feeding and mating preference among different *Drosophila* species (Remsen and O'Grady, 2002; Ritchie and Gleason, 1995).

For a given species, mating occurs at one particular location at a specific time of a day/year. For this reason, the species has to first find the location either for feeding or for mating or oviposition. Most but not all *Drosophila* species feed and mate on the same substrate *e.g.,* the male *D. nigrospiracula* feed on the same substrate alongside female but for mating the male waits for the arrival of the female on the plant away from feeding site (Markow, 1988), and it is mediated by the chemosensory system of the fly to reach the targeted location. Adult feeding sites are in general the mate-finding sites for most of the *Drosophila* species. The male produced *cis* vaccenyl acetate (*c*VA) is the cuticular hydrocarbon component in the genus *Drosophila* that serves as a sex pheromone and aggregation pheromone (Symonds and Wertheim, 2005). The *Drosophila* genus has very diverse species that feeds various food substrates ranging from fermenting or fresh or poisonous fruits, cacti, flowers, mushrooms and other fungi, sap tree exudates, excretion of land crabs (Higa and Fuyama, 1993; Markow and O'Grady, 2005; Stensmyr et al., 2008). Accordingly, these species have evolved their pre and post mating behavior

including search of the mate and feeding site location. For instance, *D. sechellia, D. nigrospiracula* and *D. pachea* have a specific host preference and uniquely the preferred host is toxic to other Drosophilids. *D. sechellia* feed and oviposit on the fruit of *Morinda citrifolia* and has developed resistance for the toxic compounds produced by the fruit (Dekker et al., 2006; McBride, 2007). *D. sechellia* responds very strongly to high concentration of hexanoate compounds from fruits, while other flies including *D. melanogaster* die or repel for the same concentration (Dekker et al., 2006). Other species *D. pachea* and *D. nigrospiracula* feed on the Senita cactus (*Lophocereus schottii*), an endemic plant species of Sonoran desert (northwestern Mexico and United States) (Kopp, 2012; Lang et al., 2012). The flies feed on the concentrated alkaloids of the plant and have adapted their physiology and food preference only to cactus plant, while other species like *D. nigropsiracula, D. mojavensis* and *D. mettleri* feed on the cactus-soaked soil but not on the actual cactus tissue because of the higher concentration of alkaloids (Kopp, 2012). There are other species of *Drosophila* like *D. carcinophila, D. endobranchia* and *Lissocephala poweilli*, living on land crabs feeding on the microbes and nitrogenous waste compounds (Stensmyr et al., 2008).

Of all the species studied in *Drosophila* till now, *D. suzukii* has a unique importance for it is the only species of economic impact. Indeed, *D. suzukii* feeds on the fermenting fruits along with congeneric *D. melanogaster* and other fermenting fruit-driven species (Mitsui et al., 2010) but for oviposition the species generally switch to fresh fruits, but however, female oviposit also on the fermenting fruits in the absence of congeneric competition. The species has wide host acceptance range and the larvae feed on the fruit pulp and the fruit withers (Walsh et al., 2011). The males are found on/ around the fermenting fruit for a successful mating. The courtship index in male is not much different from *D. melanogaster* except for some minor acoustic signals and male wing display during courtship (Mazzoni et al., 2013). Unlike *D. melanogaster* which is known to mate even in the dark mediated by pheromonal signals, *D. suzukii* is unable to mate in the dark (Fuyama, 1979). The adults prefer to feed and mate preferably in the early morning as compared to the rest of the day (Revadi et al., unpublished data) (Fig. 3).

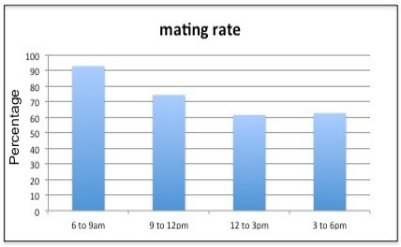

Figure 3. Mating rate of *D. suzukii* female flies tested at different time points (n=100, for each time period).

4 Objectives

The main objective of the thesis was to understand the ecology and communication mechanisms in *D. suzukii* from genome to behavior as a prerequisite to establish potential monitoring and control methods. Based on this, the study objectives were split into-

1. Linking genomics and ecology to understand the evolutionary complex of the pest.

2. Chemical characterization of volatile compounds emitted by the host fruits and perceived by *D. suzukii*, probably involved in the recognition of the oviposition site by gravid females.

3. To investigate from genome to behavioral level the possible role of cuticular hydrocarbons (CHs) involved in mating and species recognition.

5 Summary of Results

5.1 Linking genomics and ecology to understand the evolutionary complex of the pest (Paper I).

For better understanding *D. suzukii* and its peculiar ecology, it was first important to know evolutionary history right from the divergence from its parental species and the rational for switching the reproductive behavior from rotten to fresh fruits. For this reason *D. suzukii* genome and transcriptome was sequenced and annotated. The population collected from Trentino province of Italy was inbred for F_5 generation. The genomic DNA was extracted from 10 inbred flies (5 males + 5 females), while the RNA was extracted from 15 individuals of various life stages, randomly selected from fly culture. The size and contents of the transcriptome and mitochondrial genome (mtDNA) was very much comparable to the already sequenced *Drosophila* sp.

The phylogenetic and dating analyses with transcriptome and the mtDNA genome data of *D. suzukii* was done in comparison with 20 additional *Drosophila* sp. for which the data already exists. Ninety one protein-coding genes extracted from transcriptome of 21 *Drosophila* sp. were plotted on the phylogeny tree (Fig. 4A). Another phylogenetic construction with mtDNA genome of 21 *Drosophila* species was done but did not support the findings of transcriptome-derived phylogeny. *D. eugracilis* was placed as a sister species of *D. ficusphila* in the mtDNA genome derived phylogeny tree, while the same species was placed intermediate between *melanogaster* and *suzukii* group in transcriptome-derived tree (Fig. 4B).

The molecular clock analyses of 21 *Drosophila* species using mtDNA genome dataset and trancriptome dataset put *D. suzukii* and *D. biarmipes* next to each other on the phylogeny tree and shows that speciation occurred between 6-9

Mya (Fig. 5A). However, it should be noted that at the basal root there was a variation in nodes between mtDNA and transcriptomic data.

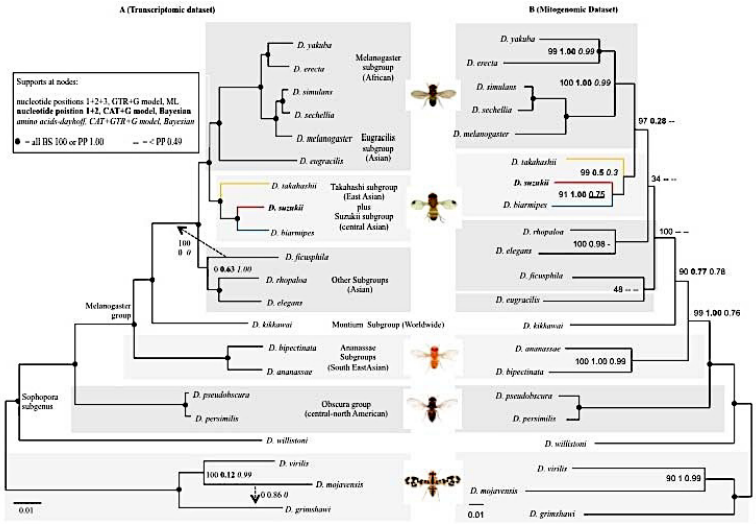

Figure 4. The evolutionary affinities of *Drosophila suzukii* and the other *Drosophila* species inferred from phylogenomic and mitogenomic data. A: Phylogenetic analyses of 91 orthologous nuclear genes (200,475 bp). B: Phylogenetic analyses of 12 mitochondrial genes (11,139 bp). Both datasets support an Asian affinity of *D. suzukii*.

The current distribution of *D. suzukii* and the sister species compiled with climatic model of Asian Tortonion era (late Miocene age) showed the confinement of *D. biarmipes* to the southern equatorial habitat, while *D. suzukii* towards north favored with temperate climatic condition (Fig. 5B). This model also supports the molecular clock analysis of species divergence. The monitoring traps with apple cider vinegar set to catch *D. suzukii* in the alpine region of Trentino (Province in the north Italy) along the altitudinal gradient caught significantly higher number of flies at higher altitude of 600-1000 and > 1000 above mean sea level (AMSL) (Fig. 5C). This also supports the findings of molecular clock analysis and also the climatic model of Tortonian era with current species distribution of *D. suzukii D. biarmipes* and *D. takahashi*. As a known fact, the most preferred host plants of *D. suzukii* also thrive in the temperate climate. The species abundance and activity at higher altitude however suggest that either they breed on the wild host fruits adapted to tolerate colder weather or they migrate to higher altitude to escape higher temperature. It is also possible that at higher altitude the temperature is low and the fermentation of fruit is also delayed and adults can feed on the fruits for longer time.

2336 orthologous genes of five species; *D. suzukii, D. biarmipes, D. takahashi, D. melanogaster* and *D. ananassae* were choosen (*D. biarmipes* and *D. takahashi* were chosen for their close relatedness with *D. suzukii*, *D. melanogaster,* for being widely studied close relative and *D. ananassae*, a randomly chosen far relative) to understand the molecular evolutionary history shared by these species.

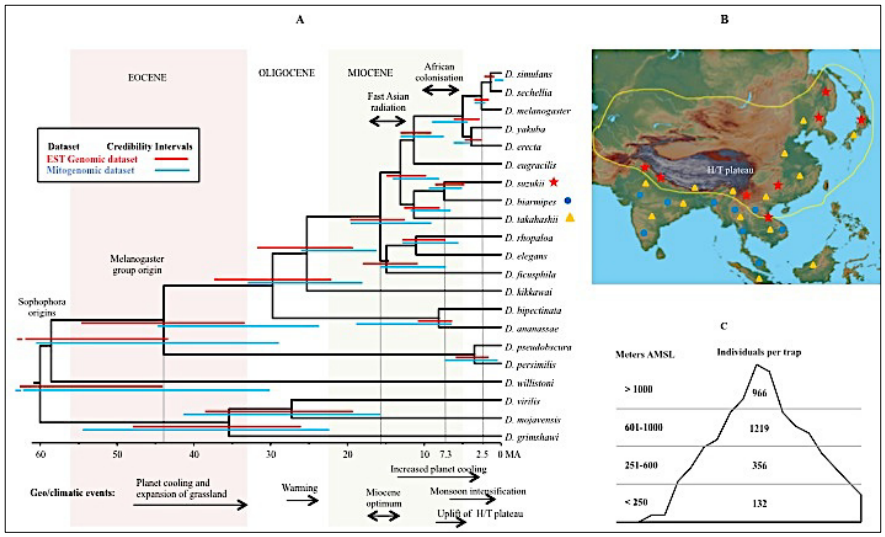

Figure 5. Molecular timetrees, paleoclimate and field trapping of *Drosophila suzukii*. A: Relaxed clock analyses of the *Drosophila* species using both the nuclear and mitochondrial datasets. B: Current endemic geographical distribution of *D. suzukii* (stars) compared to that of *D. biarmipes* (dots) and *D. takahashi* (triangles); yellow line marks the border of temperate (mostly mountainous) forested area during the Tortonian age. C: Annual captures per trap at five different altitudes in the Alps.

D. suzukii genes are characterized by lower rate of molecular evolution (Fig. 6A). Synonymous (dS) and non synonymous (dN) substitution (nucleotide substitution) rates were calculated for all the species (Fig. 6B), and *D. suzukii* has significantly lower rate of substitution compared to its sister sp. *D. biarmipes* and the results show reduced rate of substitution along with *suzukii* branch (Fig. 6B). The molecular analysis showed that *D. suzukii* has the lowest substitution rate among all the five species considered. Based on this result, when the selective pressure was calculated by the ratio dN/dS, *D. suzukii* has significantly lower selective pressure compared to *D. biarmipes*. The results also showed differential selective pressure with significantly larger dN/dS ratio in autosomal genes while lower for X-linked chromosomes (Fig 6C). These results support the current physiology of *D. suzukii*, which is known to undergo

winter reproductive diapause in the temperate climate and as a consequence, has lower number of reproductive generations per year with respect to other related species.

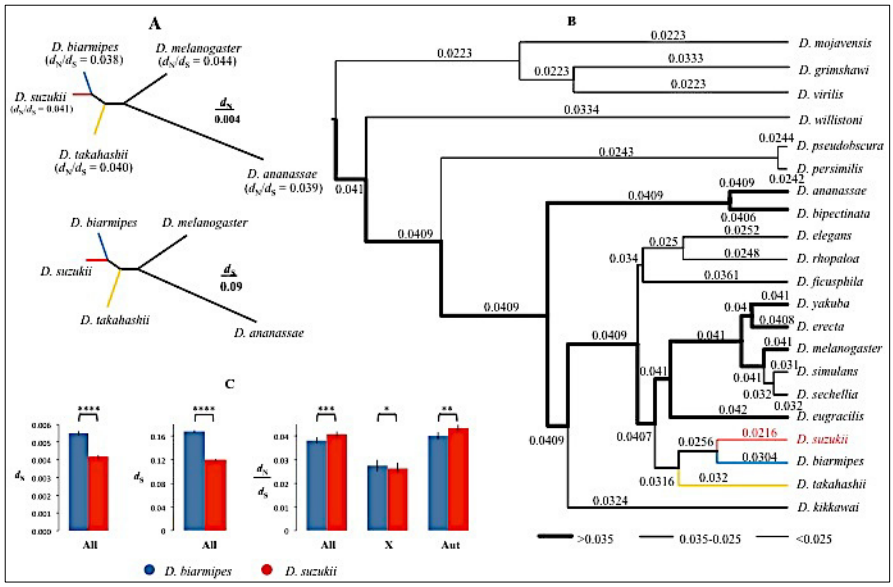

Figure 6. The slowly-evolving genome of *Drosophila suzukii*. (A): Consensus evolutionary analysis of 2,336 orthologous genes in five key species. Upper and lower are respectively the trees derived from analyses of non-synonymous (*d*N) and synonymous (*d*S) substitutions. The *d*N/*d*S for each species is given in parentheses. (B): Branch specific normally modelled mutation rates as optimized by Beast using as initial value a mutation rate of 0.0346 neutral substitutions per base pair per million of year (St. Dev=0.00281). (C): A detailed comparison between the rate of molecular evolution in *D. suzukii* and its sister species *D. biarmipes*, for all genes (All) as well for autosomal (Aut) and X-linked genes (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Wilcoxon test after controlling for gene length).

5.2 Chemical characterization of volatile compounds emitted by the host fruits of *D. suzukii*, probably involved in the recognition of the oviposition site by gravid females (Paper II).

D. suzukii being able to search and locate the undamaged fresh fruits to oviposit, which otherwise is not a generalized behavior for many Drosophilids, was hypothesized to have unusual olfactory capacity to discriminate fermenting and fresh fruits. In the presence of congeneric competition, *D. suzukii* switch to fresh fruits for oviposition and olfaction plays a key role in the recognition of fruits by females. Therefore, it was important to know the sensory modalities of the fly that helps to locate fresh ripening fruits. The prerequisite was to identify the active volatile compounds present in the fresh fruits, which serve as a host for fertile adult female, and five fruit species were chosen based on their susceptibility *viz.,* blackberry, blueberry, cherry, raspberry and strawberry, for testing the attraction of adult females for oviposition, which included behavior and electrophysiological experiments.

Y-tube olfactometer based behavioral experiments with mated *D. suzukii* females showed that all the fruits included in the experiment are equally susceptible for oviposition. Significant difference was recorded when 25 gr of fresh fruits were tested against same weight of fruits wrapped in the odor proof plastic bag. Blueberry was highly attractive fruit in the olfactometer against control, followed by cherry, raspberry strawberry and blackberry (Fig. 7).

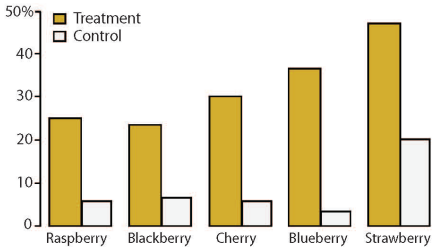

Figure 7. Percentage of flies tested in Y-tube olfactometer with fresh fruit (25g) (Treatment) or fresh fruit enclosed in a transparent plastic bag (Control) (n=100): raspberry (χ^2=12.0; d.f.=1; P<0.001); blackberry (χ^2=8.8; d.f.=1; P<0.01); cherry (χ^2=16.5; d.f.=1; P<0.001); blueberry (χ^2=27.2; d.f.=1; P<0.001); strawberry (χ^2=10.1; d.f.=1; P<0.001). The comparison among fruits was not significant (χ^2=9.1; d.f.=4; P=0.06).

Following behavioral experiments, the headspace collections of fruits (blueberry, blackberry, cheery, raspberry and strawberry) used in the electrophysiological experiment using gas chromatography coupled with electro antennal detector (GC-EAD) (Fig. 8) gave information on 29 key volatile compounds that are antennally sensitive for female *D. suzukii*.

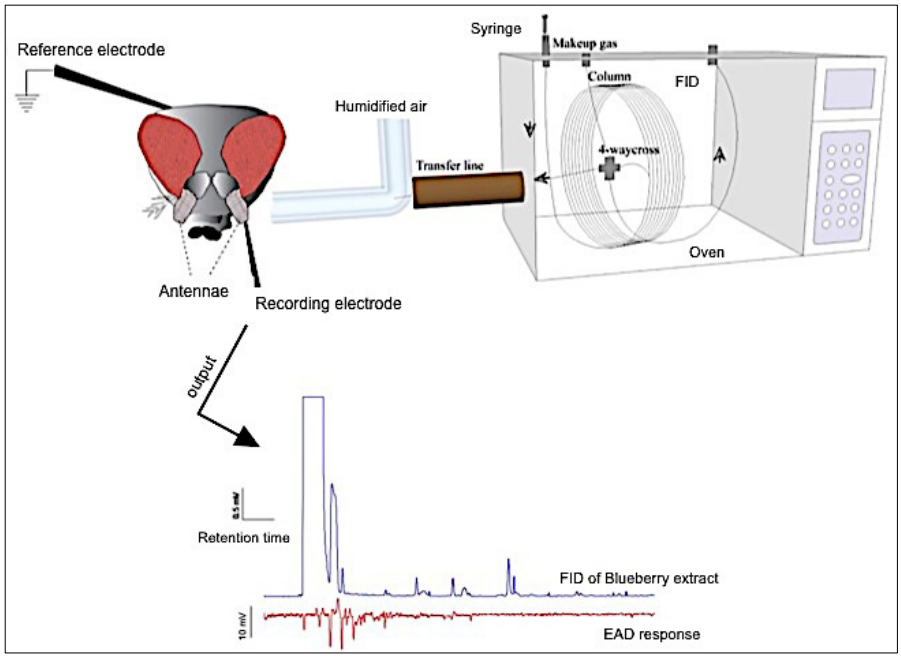

Figure 8. Schematic representation of gas chromatography coupled with electro-antennal detector. The chromatograph of 3 days old mated *D. suzukii* female antenna to headspace extract of blueberry (cv. Brigitta). Volatile compounds eluting from HP 5890 GC, Polar Innowax column.

Of all potential volatile compounds, isoamyl acetate was found in all the fruit extracts identified on GC-MS and consistently elicited antennal response in all EAD runs for all headspace extracts. Based on the electrophysiological findings, isoamyl acetate tested on electroantenogram (EAG) with increasing doses ranging from 1pg/µL to 100µg/ µL, showed significant increase in the response at 10 and 100µg/µL concentration (Fig. 9). This result signified the importance of isoamyl acetate for the mated female. However, the EAG experiment by itself was not stand-alone data that could prove the behavioral significance of isoamyl acetate emitted from fresh fruits. So, it was again tested in the behavioral bioassay in the Y-tube olfactometer, and the results showed that 10µg/µL (χ^2= 4.21; d.f.=1; P<0.05) concentration of isoamyl acetate when loaded on the rubber septa dispenser was significantly attractive to the mated female compared to 1 (χ^2=0.92; d.f.=1; P<0.9) and 100µg/µL (χ^2=0.67; d.f.=1; P<0.57) concentrations, respectively (Fig. 10). The emission rate of isoamyl acetate from 10µg/µL dispensers was comparable to those recorded from fresh fruits, which was at least 100 fold lower than in fermenting substrates (Cha et al., 2012).

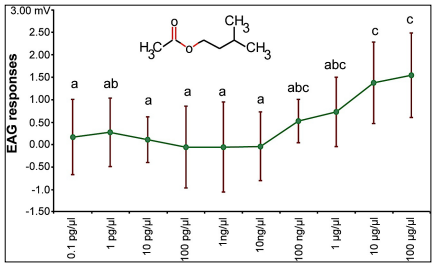

Figure 9. Mean EAG (mV±SD) dose-response curve of mated *D. suzukii* females to increasing doses of isoamyl acetate (n=10) (ANOVA, Tukey test: F=5.30; d.f.=99; P<0.001).

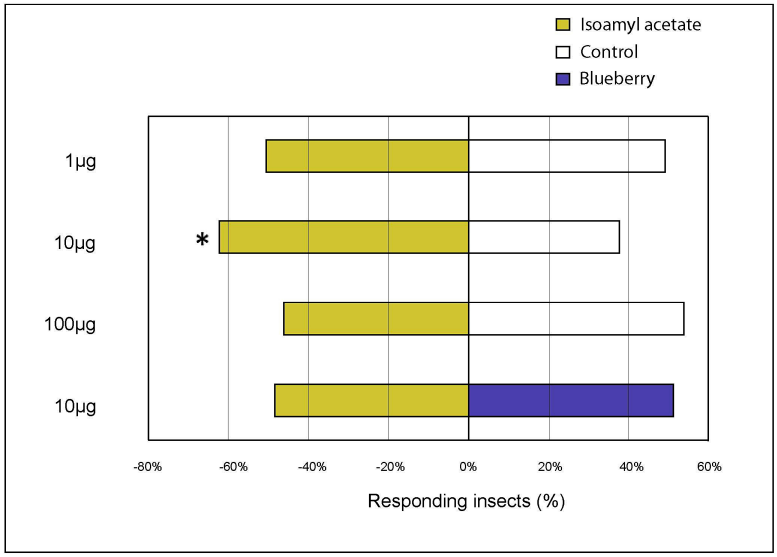

Figure 10. Percentage of flies (n=120) showing preference for isoamyl acetate (1, 10, or 100 µg loaded on a red rubber dispenser) *versus* a solvent control (10 µL hexane). Asterisk indicates significant differences in the insect choice between stimulus and control at different isoamyl acetate loadings per dispenser: 1 µg/µL (χ^2=0.92; d.f.=1; P=0.90); 10 µg/µL (χ^2=4.21; d.f.=1; P<0.05); 100 µg/µL (χ^2=0.67; d.f.=1; P=0.57), ii) Also preference of the flies (n= 100) to 10µL of synthetic isoamylacetate (10 µg/µL) *versus* fresh bluberry fruits (25 g) (χ^2 =0.05; P=0.73).

Therefore, these results showed that isoamyl acetate is a key volatile compound that at optimum concentration in fresh fruits evokes oviposition response in the mated females. Accordingly, in a further Y-tube behavioural bioassay with mated *D. suzukii* female, no significant difference (χ^2=0.05; P=0.73) of attraction was recorded for blueberry (25gr), the most attractive host fruit in the previous experiment (Figure 10: basal bar with intense-yellow

and blue color), and isoamyl acetate deployed at the attractive dosage (10 µL of 10µg/µL concentration per rubber septum).

The gene assembly and phylogenetic analysis supported physiological and behavioural evidences that included *D. suzukii, D. melanogaster* and *D. biarmipes*. It is known that in *D. melanogaster*, at least ten odorant receptors are activated by isoamyl acetate, they are: OR2a, OR9a, OR10a, OR19a, OR42a, OR42b, OR43b, OR47a, OR67a and OR98a (Database of Odorant Receptors: (Galizia et al., 2010)). The orthologues encoding these receptors in *D. suzukii* genome revealed that they are extremely conserved and similar to the orthologues in *D. melanogaster* and *D. biarmipes* as suggested by high bootstrap support and similar branch lengths (Fig. 11). Interestingly, results show that OR67a experience a series of duplications in both *D. suzukii* and *D. biarmipes* (with a nomenclature, OR67a1-OR67a5).

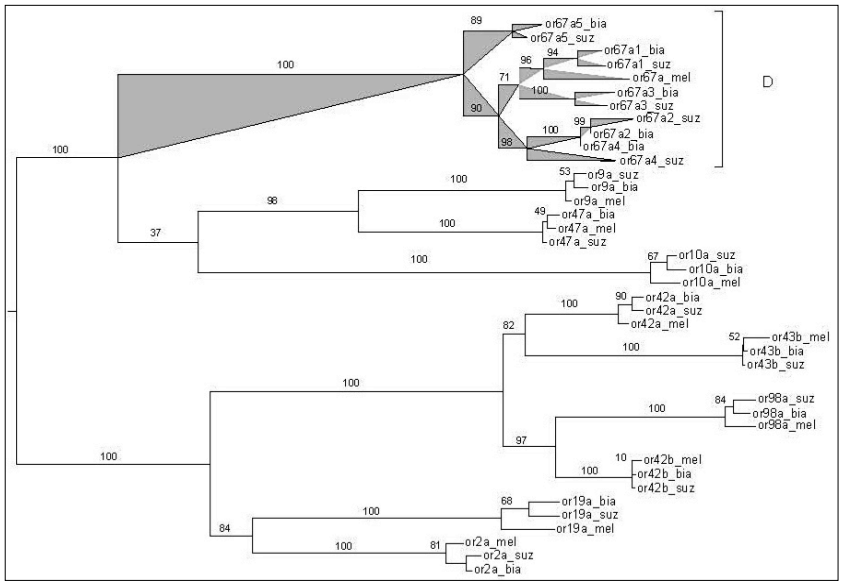

Figure 11. Phylogenetic tree of ten genes encoding a set of olfactory receptors (OR2a, OR9a, OR10a, OR19a, OR42a, OR42b, OR43b, OR47a, OR67a and OR98a) that bind isoamyl acetate in *D. suzukii*, *D. biarmipes* and *D. melanogaster* using Phyml. The numbers specify the bootstrap value, indicating the branch support for each node. The tree is rooted on midpoint. Three types of evolutionary events were studied: gene gain, gene loss and duplications (D).

5.3 To investigate from genome to behavioral level the possible role of cuticular hydrocarbons (CHs) involved in mating and species recognition (Paper III).

The gene assembly and annotation of *D. suzukii* alongside other *Drosophila* species already sequenced and annotated, suggested that *D. suzukii* is unique as it has evolved differently even from its closest related species *D. biarmipes* (Paper I). These results suggested that further mining of the genome of *D. suzukii* for variation in the genes could possibly be pivotal compared to other *Drosophila* species. It was noted that *D. suzukii* has all genes conserved responsible for the biosynthesis of species-specific cuticular hydrocarbons in *Drosophila* (Chertemps et al., 2007; Dallerac et al., 2000). Using solvent extraction and gas chromatography coupled with mass spectrometer the cuticular hydrocarbon (CH) profile of male and female *D. suzukii* were analyzed (Fig. 12A and B). The cuticular hydrocarbon profiles of male and female were isomorphic with minor differences but however, more surprisingly, *D. suzukii* male lacked *cis* vaccenyl acetate (*c*VA), a major CH component in most of the species of the *melanogaster* group, used as sex pheromone. Interestingly, no such compound *c*VA-like was detected in *D. suzukii* males. Tracking back further towards the production site of *c*VA, that is the ejaculatory bulb (EB), the microscopic dissection showed that the ejaculatory bulb in *D. suzukii* is significantly reduced in its size compared to *D. melanogaster* (Fig. 13A).

It was then expected that *D. suzukii* has some evolutionary shifts even in the olfactory circuit, that do not code for *c*VA since the biosynthesis site is diminished completely. In *D. melanogaster*, *c*VA is detected as close contact pheromone by the specialized and abundant sensilla type called trichoid present on the third antennal segment. The T1 sensilla that express OR67d odorant receptor are innervated by the glomerulus DA1 present in the antennal lobe (Kurtovic et al., 2007). In *D. suzukii*, it was noted that the number of trichoid sensilla are more or less equal in number (Fig. 13B), but the T1 sensilla are significantly lesser than in *D. melanogaster*, but have functional OR67d receptor highly conserved. On the contrary *D. suzukii* showed an increase in the number of T4 sensilla that houses OR65a receptors, compared to *D. melanogaster*. In *D. melanogaster*, it is shown that OR65a is involved in suppressing *c*VA-mediated male-male aggression and decreasing receptivity towards recently mated females. In *D. suzukii*, T4 sensilla were found to be highly conserved and sensitive to *c*VA. The results showed that *D. suzukii*

responded to *c*VA prior touch stimulation (sensory recording probe 3-4 mm away), which otherwise in *D. melanogaster*, the response was seen upon close contact (less than 1mm closer) (Fig. 13C).

Upon dissection to look into the antennal lobe (AL) of *D. suzukii*, it was noted that DA1 that receives signals from T1 sensilla was correspondingly reduced significantly in its size compared to *D. melanogaster* (Fig. 14). On the contrary, DL3 that innervates T4 sensilla was significantly enlarged.

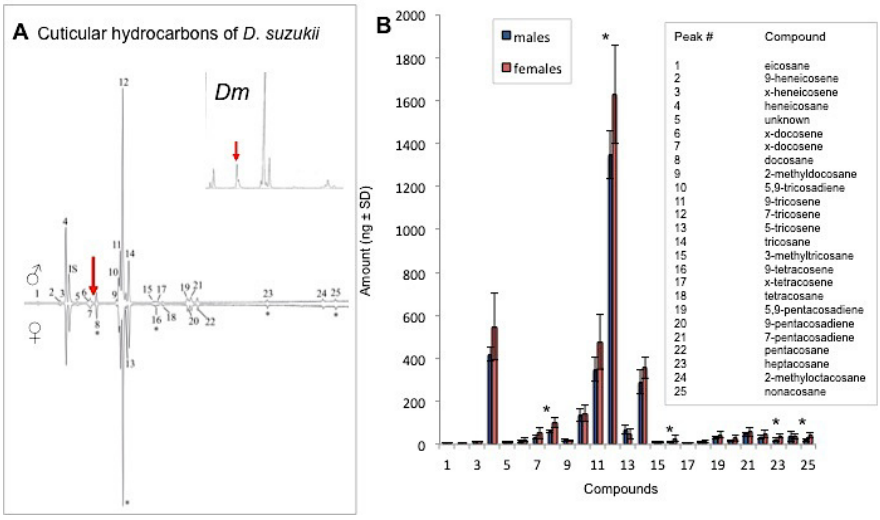

Figure 12. (**A**) Chromatograms showing female and male *D. suzukii* cuticular hydrocarbons. Arrow indicates retention time where *c*VA would elute. Inset: chromatogram of *D. melanogaster* with the arrow indicating *c*VA. ***IS*=** Internal standard (heptadecenyl acetate, 17:OAc), (**B**) comparison of the cuticular hydrocarbon profile of male and female *D. suzukii* (n=6 and n=5, respectively). Stars indicate significant differences between males and females (Mann-Whitney test, $\alpha<0.05$).

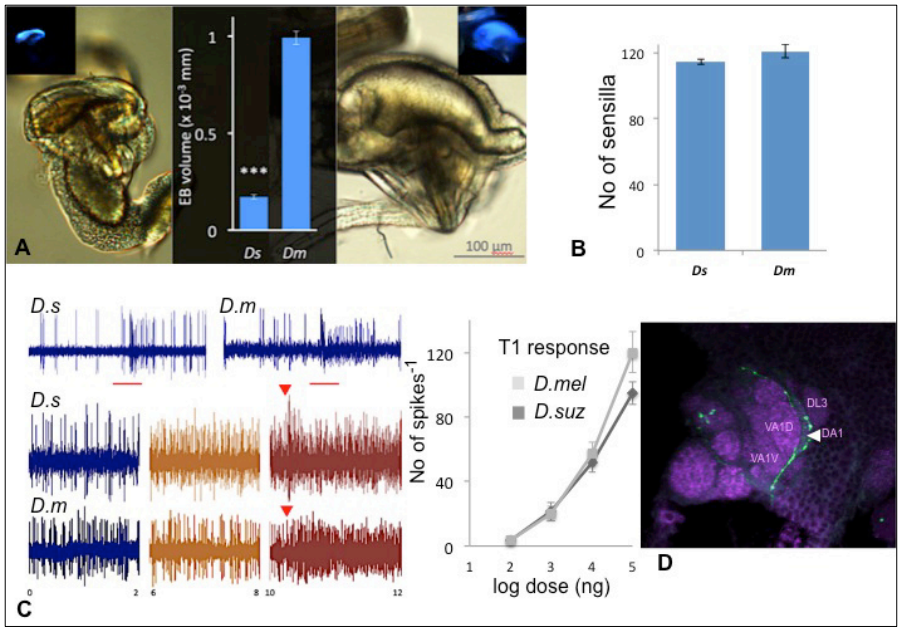

Figure 13. (**A**) Micrographs of the ejaculatory bulb, lateral view, light and autofluorescence microscopy (insets). Overlay: volumetric estimates of the EB of *D. suzukii* (Ds) and *D. melanogaster* (Dm) (n=10 for each species, independent t-test; $p<0.001$). (**B**) Number of sensilla trichoidea and sensilla intermedia in *D. suzukii* and *D. melanogaster* females. (**C**) Top: sample trace of T1 sensilla to 0.5 s 1 µg *c*VA stimulation. Bottom: sample traces of T4 sensilla in *D. suzukii* and *D. melanogaster* to *c*VA, using the 'touch' stimulation, with the time (s) indicated at the bottom of the traces. Blue: before stimulation, orange: just prior to contact, red: contact. Side panel: dose response curves of T1 sensilla in *D. suzukii* and *D. melanogaster* to 0.5 s *c*VA stimulation (red bar). **(D)** Neurobiotin backfill of T1 neuron (a spurious fill of a neighboring AB7 neuron toVM5v is also visible). Letters indicate various anterior trichoid glomeruli. Arrowhead indicates DA1.

It was then hypothesized that in *D. suzukii* *c*VA has a antagonistic role in courting and mating because of the switch in the size of DA1 and DL3 glomerulus compared to *D. melanogaster*. To prove this, when *D. suzukii* virgin males were perfumed with *D. melanogaster* male equivalent synthetic *c*VA, it rapidly brought down the mating rate in four days old males. On the contrary, upon perfuming synthetic *c*VA on *D. melanogaster*, it increased the mating rate in males (Fig. 15 insets). However, upon degradation of *c*VA from *D. suzukii* males over time, the mating rate was found to increase which confirms that *c*VA acts as antagonistic compound in *D. suzukii* mating (Fig. 15). This result invariably supports the previous findings of i) reduced ejaculatory bulb size and termination of *c*VA biosynthesis, ii) reduced number

of T1 sensilla and higher number to T4 sensilla compared to *D. melanogaster* and iii) reduction in the volume of glomeruli DA1 and enlargement of DL3.

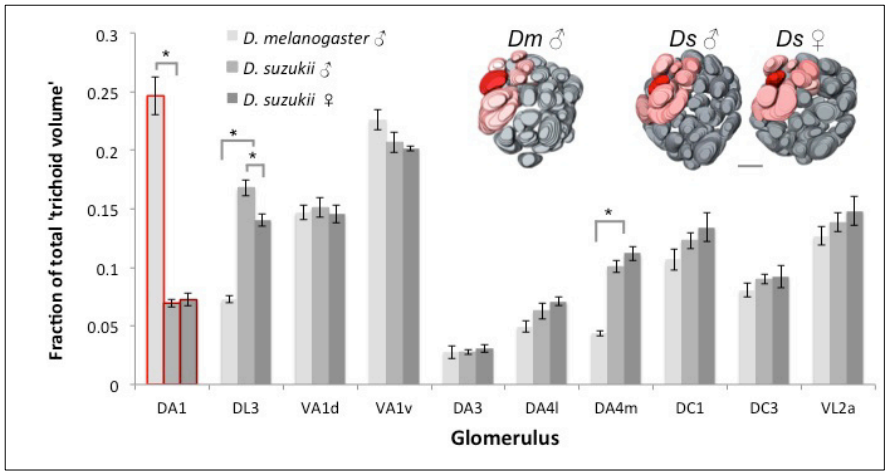

Figure 14. Volume of DA1 and other trichoid glomeruli, relative to the total volume of all glomeruli receiving input from sensilla trichoidea and intermedia. Red outlined bars: DA1 of *D. melanogaster* and *D. suzukii* ♂ and ♀. Insets are reconstructions of the antennal lobes, with in bright red DA1 and in light red other glomeruli that receive input from sensilla trichoidea and sensilla intermedia neurons. Scale bar 20 µm.

The loss of *c*VA production in *D. suzukii* as a sex pheromone and the species' inability of mating in the dark compared to *D. melanogaster* (Fuyama, 1979) lead to the speculation that the wingspot on the forewings of males aid in mating at close range that might have replaced the role of *c*VA in *D. suzukii*. Therefore, two other species of *suzukii* subgroup that have wingspots in case of males, *D. biarmipes* and *D. subpulchrella* were checked for the *c*VA production and glomerulus size (DA1 and DL3). The former species feed and breed on fermenting fruits, while the later breeds on fresh fruits but feeds on the fermenting fruits. The results showed that *D. biarmipes*, which is *D. melanogaster*-like in its feeding habit, has functional ejaculatory bulb that produces *c*VA and also, the species has DA1 and DL3 glomeruli that correspond to the size of *D. melanogaster*-like ratio (Fig. 16). Contrary to this, *D. subpulchrella*, which is *D. suzukii*-like in its oviposition habit, has miniature sized ejaculatory bulb, reduced DA1 and enlarged DL3 similar to *D. suzukii*.

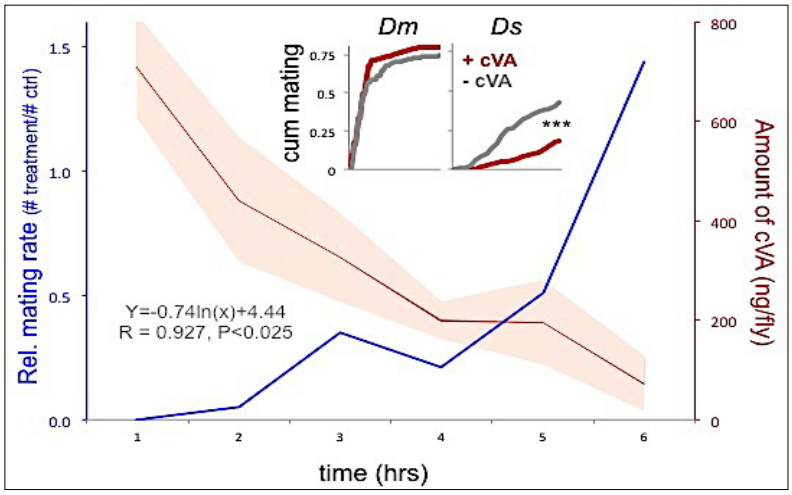

Figure 15. Effect of *c*VA perfuming on mating in *D. suzukii* and *D. melanogaster*. The relative mating rate is increasing (blue line) with a decreasing amount of *c*VA on the male flies. Insets: cumulative mating in *D. melanogaster* and *D. suzukii* in response to the perfuming with *c*VA (+*c*VA, red lines) or hexane (control, - *c*VA, grey lines).

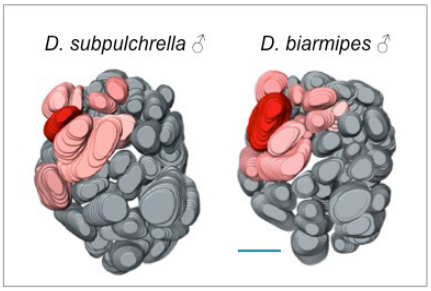

Figure 16. Reconstruction of antennal lobes of *D. subpulchrella* and *D. biarmipes*. In bright red DA1, which received input from T1 neurons, and in light red other glomeruli receiving input from sensilla trichoidea neurons (Scale bar 20 µm).

To verify all these results, oviposition experiment for *D. suzukii* was conducted to observe the behavior of the mated female when the fly encounter blueberry fruit treated with synthetic *c*VA (10µL of 1µg/ µL concentration). Preliminary results show that *D. suzukii* tend to avoid treated fruits compared to hexane treated fruits (control) (data not presented). Our hypothesis is that *c*VA represents for *D. suzukii* a signal of the presence of other Drosophilids in the rotten fruits, which in turn are not suitable for oviposition. So, it is possible that *c*VA or *c*VA-like compound can be used as oviposition deterrent on fruits in combination with other management methods for the effective damage control for *D. suzukii.*

6 Thesis outcome in a blink

6.1 Paper I

1. Protein-coding gene analysis confirmed the phylogenetic relatedness of *suzukii* subgroup with *takahashi* subgroup.
2. *D. biarmipes* and *D. subpulchrella* are two other members of *suzukii* subgroup and are closely related to *D. suzukii*.
3. *D. suzukii* has lower mutation rate and less number of generations per year and thus has significantly lower gene sequencing rate compared to its sibling species *D. biarmipes*.
4. *D. suzukii* has lower selective pressure measured by the ratio dN/dS (Non-synonyms substitution/ synonymous substitution) compared to *D. biarmipes*.
5. *D. suzukii* migrated towards higher altitude to avoid high temperature, survive on alternate hosts and undergo winter diapause.
6. Molecular analysis suggests that *D. biarmipes* and *D. suzukii* got separated (speciation) from each 6 to 9 million years ago.

6.2 Paper II

7. In the Y tube olfactometer based behavioral experiment, blueberry was found to be highly attractive against control (blank) followed by cherry, raspberry, strawberry and blackberry.
8. All fruit types included in the behavioral experiment are equally susceptible for female attraction.
9. Identified 29 antennally active volatile compounds from 5 headspace extracts of blueberry, blackberry, cherry, strawberry and raspberry and their emission rate was calculated.

10. Isoamyl acetate was present in all headspace collections and also elicited EAD response in all GC-EAD runs.
11. 10 µg/µL isoamyl acetate in the rubber septa reproducing the release rate from fresh fruits was significantly attractive to the adult female compared to 1 µg/µL and 100 µg/µL in the Y tube olfactometer.
12. No significant difference was recorded between isoamyl acetate 10 µg/µL and 25gr of blueberry to female *D. suzukii* in the Y tube olfactometer.
13. Alike *D. melanogaster*, *D. suzukii* also has all 10 odorant receptors involved in sensing isoamyl acetate, in addition has multiple copies (5x) of Or67a responsible for isoamyl acetate detection compared to *D. melanogaster*.

6.3 Paper III

14. The cuticular hydrocarbon profile of *D. suzukii* male and female are isomorphic except few minor differences.
15. *D. suzukii* has all genes responsible for biosynthesis of species-specific cuticular hydrocarbon compared to *D. melanogaster*.
16. *D. suzukii* does not produce *c*VA or *c*VA-like cuticular hydrocarbon that mediate courtship and mating in *Drosophila*.
17. *D. suzukii* and *D. subpulchrella* (similar to *D. suzukii* in habitat) have miniature sized non-functional ejaculatory bulb, while another sibling species *D. biarmipes* has functional ejaculatory bulb similar to *D. melanogaster*.
18. Though *D. suzukii* has more or less equal number of sensilla trichoidea compared to *D. melanogaster*, but lesser T1 sensilla and abundant T4 sensilla.
19. T1 sensilla that house Or67d influence *c*VA-mediated behavior like male-male aggression and courtship; T4 sensilla that house O65a suppress *c*VA-mediated behavior.
20. *D. suzukii* and *D. subpulchrella* have smaller DA1 (glomerulus that receive impulses from T1) and bigger DL3 (receive impulses from T4) glomerulus compared to *D. melanogaster* and *D. biarmipes,* that is involved in *c*VA-mediated aggression in males and suppression of aggression, respectively.
21. The reverse sized glomeruli size of DA1 and DL3 suggests that *c*VA has antagonistic role in *D. suzukii* mating and courtship.

22. Perfuming *D. melanogaster* male equivalent *c*VA on *D. suzukii* males significantly reduces mating, but in *D. melanogaster* perfuming males with *c*VA increases mating rate.

23. Sex-specific genes *fruitless*, *sexlethal* and *transformer* are conserved in *D. suzukii* alike *D. melanogaster*.

24. Fruitless genes Fru is translated in both sexes in *D. suzukii* contrary to *D. melanogaster*, where only males posses *fruitless*.

*25. c*VA is a signal for *D. suzukii* for the recognition of substrates already occupied by congeneric species.

7 Conclusion and future prospectives

Drosophila suzukii, an invasive pest reported for the very first time in 2008 in US and Europe and the very next year caused severe damage for the fruit production. The primary focus after invasion was only to find an immediate solution of controlling or decreasing the intensity of loss caused by female oviposition on fresh fruits. In the present work, instead the focus was also given to understand the basic aspects of the pest that included evolutionary origin, speciation and olfactory communications. Based on the existing information on *D. melanogaster*, an attempt was made to understand the differences in the behavior, ecology and physiology of the pest, which is a prerequisite to manage the menace in environmental-safe methods without using hazardous insecticides on the ready-to-harvest staged fruits.

The genome based phylogenetic analysis placed *D. suzukii* in the *suzukii* subgroup close to another fly *D. biarmipes* that resembles *D. suzukii* males with wingspots. *D. subpulchrella* and *D. pulchrella* are two other species with similar habitat as *D. suzukii*, and are believed to be the members of *suzukii* subgroup (Mitsui et al. 2010). But this needs to be confirmed by further genome analyses. *D. suzukii* has undergone many behavioral and physiological changes from its ancestors by adapting for higher altitude temperature. This influenced the fly evolve to tolerate lower temperature by arresting the growth in winter through reproductive diapause. As a result, the species has lesser generations per year and lower gene sequencing rate (Paper I).

Following this study, *D. suzukii* female was tested for the attraction towards its preferred host fruit volatiles in the behavior and electrophysiological assays. Of 29 antennally active volatiles, isoamyl acetate was found highly attractive to mated female at 10µg/ µL concentration with release rate comparable to fresh fruits. For the mated female, the same amount of isoamyl acetate loaded in the rubber septa was found equally attractive to blueberry. The attraction to isoamyl acetate is fine tuned in *D. suzukii* with additional gene duplication

compared to *D. melanogaster*. Isoamyl acetate in addition with other volatile compounds will be a potential combination of compounds to trap gravid females in the field (Paper II).

In the following part the study it was earlier aimed to identify possible role of male pheromone that could potentially be used to attract opposite sex in the field. However, it turned out to be unique studies when it was noted that *D. suzukii* do not produce sex pheromone. With all potential genes conserved that are responsible for the biosynthesis of cuticular hydrocarbons and non-functional small ejaculatory bulb, *D. suzukii* is in the intermediate state beyond which it is not possible to predict if the species will loose the genes responsible for sex pheromone biosynthesis because of selective pressure. However, the antagonistic role of *c*VA on *D. suzukii* mating in the behavioral experiment allows to predict that *D. suzukii* might lose the possible genes of *c*VA production over time. However, based on some preliminary oviposition assays, it is clear that *c*VA acts as deterrent for *D. suzukii* ovipositon. So, it is possible that *c*VA or *c*VA-like compound can be used as oviposition deterrent on fruits in combination with other management methods for the effective damage control.

Based on behavior and physiology, it is also known that *D. pulchrella* and *D. subpulchrella* resemble *D. suzukii*, except for the economic damage of the fruit production. This makes the two closely related species very important to be understood in terms of genome and adaptation, temperature tolerance etc. so that the information can be used in the management of *D. suzukii*. It is also important to know the behavior and electrophysiological responses of these two species to isoamyl acetate as was done for *D. suzukii* for further understanding.

Current control efforts for *D. suzukii* rely heavily on the use of insecticides. Unfortunately, the insecticides which are currently available to growers for control of *D. suzukii* are not very effective, since the use of highly efficient broad spectrum chemicals is being progressively restricted. In particular, organic production is seriously threatened because only few natural insecticides are admitted and their efficacy against *D. suzukii* is either not known or lower than the conventional insecticides (Walsh *et al*., 2011). Furthermore, the fast generation turnover requires many chemical interventions at the ripening stage, which can increase the risk of residues in fruits, promote insect resistance and negatively affect pollinators and other beneficial species. Hence, the uncontrollable wide spread of *D. suzukii* within a short time has made inevitable to combine different pest control methods in order to bring down the pest population densities. Biological control is another important management tool that can be adapted for the pest control. Rossi-Stacconi et. al

(2013) reported the presence of indigenous *Drosophila* pupal parasitoid *Pachycrepoideus vindemiae* in European and Oregon small fruit production areas. The lab experiments confirmed successful parasitization and hence could be mass-produced and released in the field at right time to bring down the population below the threshold level. More extensive efforts with integrated pest management, *D. suzukii* can be effectively control in US and Europe.

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Linking Genomics and Ecology to Investigate the Complex Evolution of an Invasive *Drosophila* Pest

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Abstract

Drosophilid fruit flies have provided science with striking cases of behavioral adaptation and genetic innovation. A recent example is the invasive pest *Drosophila suzukii*, which, unlike most other *Drosophila*, lays eggs and feeds on undamaged, ripening fruits. This not only poses a serious threat for fruit cultivation but also offers an interesting model to study evolution of behavioral innovation. We developed genome and transcriptome resources for *D. suzukii*. Coupling analyses of these data with field observations, we propose a hypothesis of the origin of its peculiar ecology. Using nuclear and mitochondrial phylogenetic analyses, we confirm its Asian origin and reveal a surprising sister relationship between the *eugracilis* and the *melanogaster* subgroups. Although the *D. suzukii* genome is comparable in size and repeat content to other *Drosophila* species, it has the lowest nucleotide substitution rate among the species analyzed in this study. This finding is compatible with the overwintering diapause of *D. suzukii*, which results in a reduced number of generations per year compared with its sister species. Genome-scale relaxed clock analyses support a late Miocene origin of *D. suzukii*, concomitant with paleogeological and climatic conditions that suggest an adaptation to temperate montane forests, a hypothesis confirmed by field trapping. We propose a causal link between the ecological adaptations of *D. suzukii* in its native habitat and its invasive success in Europe and North America.

Keywords: draft genome, genome evolution, population genetics, molecular clocks, Sophophora phylogeny.

Introduction

The genus *Drosophila* is one of the most studied in virtually all fields of biology because of an invaluable combination of reproductive (high fecundity and short generation time) and ecological (wide range of niches and fast adaptability) traits. These features have allowed several *Drosophila* species to expand well outside their ancestral range. A classic example is *Drosophila melanogaster*, whose worldwide distribution is the result of an out-of-Africa expansion approximately 15,000 years ago (David and Capy 1988). A more recent example of this invasiveness is *Drosophila suzukii*, which in only a handful of years has invaded several Western countries from its original Asian distribution. The global spread of *D. melanogaster* has little economic consequence, but the spread of *D. suzukii* is of significant concern.

Unlike most of its close relatives, which lay eggs only on decaying or rotten fruits, *D. suzukii* lays eggs and feeds on unripe and undamaged fruits (Dreves 2011; Walsh et al. 2011; Rota-Stabelli, Blaxter, et al. 2013), and consequently, this species is quickly becoming an economically significant pest of fruit industries. This difference in ecology is reflected in morphological adaptations, such as an enlarged serrated ovipositor (used to break ripening fruits), and must also include additional neurological, lifecycle, and physiological adaptations to finding, and feeding on, unripe food sources. *D. suzukii* is thus a promising model for the study of the origins and bases of behavioral innovation. Understanding the cues by which *D. suzukii* finds its host fruits, and the mechanisms used for invading and

feeding thereon, is a key goal in research programs aiming to devise novel control systems (Cini et al. 2012).

To investigate the evolutionary history behind the switch in the reproductive behavior of *D. suzukii* from rotten to fresh fruit, and to better understand how this species established itself in western countries at such an impressive speed, we sequenced and annotated the genome and transcriptome of *D. suzukii* from an Italian Alpine population. On the basis of the combined results of phylogenetic and clock analyses, comparative genomics, and field observations, we propose a paleoecological scenario to explain the peculiar *D. suzukii* ecological behavior.

Materials and Methods

Specimens and Sequencing

Inbred *D. suzukii* lines were established from individuals collected at approximately 500 m above sea level (asl) in Valsugana, Trento, Italy, and subsequently maintained in the laboratory under standard conditions. Genomic DNA was extracted from 10 siblings of an F_5 inbred generation (five males and five females), whereas total RNA was extracted from 15 unrelated individuals at various developmental stages (five males and five females adults, three larvae, and two pupae). The pooled cDNA library and two short DNA libraries (180 base pairs [bp] and 300 bp) were sequenced at the GenePool Genomics Facility of the University of Edinburgh, using 100 base paired-end sequencing on the Illumina Hiseq2000 platform (proportions were 0.2, 0.4, 0.4 for the cDNA, 180 bp and 300 bp libraries, respectively). The raw data have been deposited in European Nucleotide Archive (study accession ERP001893) and the assembly in the ENA under accession numbers CAKG01000001–CAKG01061569.

RNAseq Assembly

The RNAseq sequencing generated a total of 35.7 million 100 base paired reads. Data quality was evaluated with fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Tallymer (Kurtz et al. 2008). Low quality positions were trimmed using fastx (http:// hannonlab.cshl.edu/fastx_toolkit/) with a threshold of 0.3. We assembled the resulting 30,951,598 read pairs using two distinct approaches. First, we used Oases (Schulz et al. 2012) with k-mers ranging from 25 to 53, obtaining 24,358 contigs (length 100-15,000 bp). In the second approach, we used ABySS (Simpson et al. 2009) with k-mer 45 and obtained 140,736 contigs. The two sets were merged using cd-hit (Li and Godzik 2006) with an identity threshold of 100% and eventually super-assembled using CAP3 (Huang and Madan 1999) using default settings. The final data set consisted of 25,810 putative transcripts with lengths varying from 50 to 16,500 bp.

Nuclear Genome Assembly

Assembly of the nuclear genome was performed using both 180 bp and 300 bp libraries. The 180 bp library generated 67,153,264 100 base read pairs totaling 14.3 gigabases (Gb) and the 300 bp library 51,792,255 100 base read pairs covering 10.4 Gb. The insert sizes of both libraries were close to expectations. We initially partitioned the reads depending on whether they originated from nuclear, mitochondrial, or *Wolbachia* DNA. Nuclear genome assembly was based on reads that were not mappable to a reference database of the genomes of five *Wolbachia* strains (*W. ananassae, W. melanogaster, W. simulans, W. willinstoni*, and *wRi*) or to the *D. melanogaster* mitochondrial DNA (mtDNA). Mapping was performed using Smalt, (http://www.sanger.ac.uk/resources/software/smalt; see Table 3). Reads that passed this screening were further cleaned using sickle (https://github.com/najoshi/sickle) with a quality score cutoff of 25 (phred scale) applied to a sliding window of 40 bp. Following this step, reads had an average length of 93 bases (standard deviation [SD] = 14) and 94 bases (SD = 15) for the 180 and 300 bp libraries, respectively, an average quality value of 35, and spanned a total of 20Gb. Assuming similar genome sizes in *D. suzukii* and *D. melanogaster*, this translates to a coverage of approximately 168-fold. Genome assembly was carried out using ABySS (http://www.bcgsc.ca/platform/bioinfo/software/abyss) with k-mer size ranging from 48 to 64 (Table 4). After quality assessment of the assemblies, we retained as best assembly the one obtained using a k-mer of 64 (Table 4). All contigs longer than 1kb have been submitted to the European Nucleotide Archive at EBI website (http://www.ebi.ac.uk) under ID CAKG01000001-CAKG01061569.

Assembly of Drosophilid Mitochondrial Genomes

All *D. suzukii* reads that matched the *D. melanogaster* mtDNA were assembled using Geneious (http://www.geneious.com), generating 15 contigs, the longest of which (14,736 bp) was identified as the nearly complete *D. suzukii* mtDNA. This fragment covers all genes but lacks the control region, whose length is unknown. To assist our phylogenetic analyses, we also reconstructed the partial mitochondrial genomes of eight additional *Drosophila* species (*D. biarmipes, D. bipectinata, D. elegans, D. eugracilis, D. ficusphila, D. kikkawai, D. rhopaloa*, and *D. takahashi*). The draft genomes and transcriptomes of these species were kindly made available by the Baylor College of Medicine and modENCODE Consortium (https://www.hgsc.bcm.edu/content/drosophila-

modencode-project). For each species, we separately compared the transcriptome and draft genome against the *D. melanogaster* mtDNA using Basic Local Alignment Search Tool (BLAST) (Camacho et al. 2009). We used Geneious to assemble each set of contigs identified by BLAST, using the *D. melanogaster* mtDNA as a reference. We compared by eye the resulting assemblies to the complete mtDNA genome available for 12 other *Drosophila* species (*Drosophila* 12 Genomes Consortium et al. 2007), revealing many putative nuclear mitochondrial DNA (NUMTs). Finally, we retained only the transcriptome-based assemblies. These contained a large number of undetermined sites due to the expected intraspecific mtDNA polymorphism in the source *Drosophila* populations.

Repeat Identification

To the genome of *D. suzukii* and the other eight *Drosophila* species mentioned earlier, we added the (draft) genomes of 12 additional *Drosophila* species (*D. melanogaster, D. ananassae, D. sechellia, D. simulans, D. yakuba, D. erecta, D. pseudobscura, D. persimilis, D. willistoni, D. mojavensis, D. virilis*, and *D. grimshawi*; downloaded from http://flybase.org). In each genome, we automatically annotated repeats using RepeatMasker (http://www. repeatmasker.org/), at default settings, and then used the Repbase database (Jurka et al. 2005) as a reference for *de-novo* identification. We analyzed the entire genome without distinguishing between euchromatin and heterochromatin partitions, as these information are either incomplete or unknown for most of the *Drosophila* species used in this study. We used all fragments irrespective of their length, because the *D. suzukii* genome assembly and some of the other draft genomes contained many contigs shorter than the 200 kb limit recommended (*Drosophila* 12 Genomes Consortium et al. 2007). We quantified the presence and size of repeats as the percentage of repeated sequences over the draft genome size. This approach has the advantage of reducing biases due to the uncertain draft genome size of the different species, which may vary due to the different assembly strategies and/or genome quality levels, and may not reflect the actual genome size. To account for this inaccuracy, we further calculated the percentage of total repeats using two contrasting and conservative estimates of the putative average *Drosophila* genome size (a minimum at 130 Mb and a maximum at 180 Mb).

Orthologous Gene Set Identification

For comparative genomic analyses, we collated data for 21 *Drosophila* species. We downloaded the latest coding sequences (CDS) data sets available for *D. melanogaster* (release 5.43) and *D. ananassae* (release 1.3) from FlyBase, as well the masked alignments of all single-copy orthologues used in the 12 *Drosophila* project available from ftp://ftp.flybase.net/genomes/12_species_analysis/clark_eisen/alignments/all_species.guide_tree.longest.cds.fasta (Drosophila 12 Genomes Consortium et al. 2007). We also downloaded the assembled RNA-Seq data of eight modENCODE *Drosophila* species (https://www.hgsc.bcm.edu/content/drosophila-modencode-project). We identified best-hit homologous sequences between the nine RNA-Seq and two CDS datasets using pairwise BLASTn (optimized using the parameter "-best_hit_overhang 0.15").

Rates of molecular evolution and tests of positive selection were based on the set of orthologous genes identified in *D. melanogaster, D. biarmipes, D. takahashi, D. suzukii*, and *D. ananassae*. To minimize the possibility of spurious matches, we filtered matches to exclude any with less than 60% of the length of either sequence aligned. We produced two lists of putative orthologues sets from this five-species set. In the first ("^{STAR}orthologues"), we identified as orthologous genes the reciprocal best hits between *D. melanogaster* and each of *D. biarmipes, D. takahashi, D. suzukii*, and *D. ananassae* (fig. 6). Using this approach, we identified a total of 2,336 ^{STAR}orthologues quintuplets.

The second, more conservative list, included only those genes found as reciprocal best hits for all pairwise comparisons between the five species (^{WEB}orthologues; fig. 6). This data set included 1,021 ^{WEB}orthologues quintets and by definition is a subset of the ^{STAR}orthologues dataset. All sequences within each orthologue set were oriented based on the *D. melanogaster* sequence and aligned with MUSCLE (Edgar 2004). We then trimmed partial codons at the 5' and 3' ends based on the *D. melanogaster* sequence.

For the orthologue groups used for molecular evolution analyses, we then extracted the portion of the alignments with representation from all taxa. Finally, all alignments were re-aligned using Prank (Loytynoja and Goldman 2008) as implemented in TranslatorX (Abascal et al. 2010), which aligns protein-coding nucleotide sequences based on their corresponding amino acid translations.

We removed from these two data sets all orthologues sets with alignments shorter than 100 bp. The resulting 2,263 ^{STAR}orthologue quintuplets had a mean length ± Standard Error (SE) of 1,335.7 ± 29.0 bp (median = 1,092 bp; mode = 942 bp), corresponding to 69.9 ± 29.5% of the *D. melanogaster* gene length. The 1,007 ^{WEB}orthologue quintuplets had a mean length ± SE of 1,575.1 ± 29.8 bp (median = 1,275 bp; mode = 606 bp) corresponding to 76.4 ± 26.0% of the *D. melanogaster* gene length. We found that the results of our analyses did not change qualitatively when based on ^{STAR}orthologues or ^{WEB}orthologues. Thus, for ease of presentation, and unless specified, we have presented only those obtained using the ^{STAR}orthologues data set.

Analyses of the Rate of DNA and Protein Evolution

Rates of molecular evolution were analyzed for both WEBorthologues and STARorthologues using PAML 4.4 (Yang 2007). We estimated the rate of nonsynonymous substitution, dN (leading to amino acid changes), and synonymous substitution, dS (which should accumulate neutrally), over all branches of the phylogenetic tree using the "free-ratio" model (M0' (Yang 1998); model= 1 and NSsites= 0). This model allows ω= dN /dS, i.e. the level of selective pressure experienced by a gene, to vary among branches of the tree. Following the results of the phylogenetic analysis (see later), the input unrooted tree had the structure (*D. melanogaster*, (*D. ananassae*,(*D. takahashi*,(*D. biarmipes,D. suzukii*)))). We then used PAML to test different models of substitution rates across coding sites (Yang and Nielsen 2000; Yang et al. 2000), with the aim of detecting genes that either evolved at a different rate or underwent positive selection along the *D. suzukii* lineage.

In the first test, we compared models that assumed one or more substitution rates across the phylogeny. The first of such models is the basic "one-ratio" branch model (M0), which assumes a constant ω across the phylogeny (model = 0 and NSsites= 0). Following the manual recommendations, this model was used to get the branch lengths for each gene tree, which were then copied into the tree structure file to be used with the "*branch* and *site*" substitution models. The likelihood of the M0 model was compared with that of a *branch* model that assumed two ω values, one for the *D. suzukii* branch (the so called foreground branch) and one for the rest of the tree (the background branches; model= 2 and NSsites= 0). Subsequently, the value of twice the difference between the two likelihoods ($2\Delta\lambda$) was tested using a χ^2 test with 1 degree of freedom.

The occurrence of positive selection was tested by the *branch-site* test, which aimed at detecting positive selection affecting a few sites along the *D. suzukii* foreground branch. In this test (*branch-site* model A, test 2 (Yang et al. 2005), ω can vary both among sites in the protein and across branches on the tree (model= 2, NSsites = 2). As for the branch model, we used tree structures with branch lengths estimated by model M0. The null model fixed ω2 = 1 (fix_omega= 1, omega=1), where as the positive selection model allowed ω2 > 1 (fix_omega= 0, omega= 1). The likelihood ratio test had 1 degree of freedom. To account for multiple testing, we also estimated the false discovery rate (FDR) of each test using the q value approach (Storey 2002) implemented in R (R Development Core Team 2009). We note that the reciprocal best-hit approach is prone to miss genes with high sequence divergence, including those that underwent particularly intense divergent adaptive evolution. Thus, we could have missed targets of positive selection among our sequenced genes.

Codon Usage Analysis

We inferred preferred codons and codon usage bias in *D. melanogaster, D. ananassae, D. takahashi, D. biarmipes*, and *D. suzukii* in the genes of the ^{STAR}orthologues groups with more than 30 codons. We estimated codon bias using the effective number of codons, *Nc* (Wright 1990), and the frequency of optimal codons, *Fop* (Ikemura 1981): Stronger synonymous codon usage bias is identified by larger *Fop* values and lower *Nc* values. Both indices were calculated using the program CodonW (http://codonw.sourceforge.net). Putative optimal (preferred) codons were identified as those that were significantly over-represented in the 5% of genes with highest and lowest usage frequencies (supplementary table S1). Base composition affected synonymous codon usage, as shown by the strong correlation between GC and GC3 (GC in the third codon position) content and both *Nc* and *Fop* (Spearman correlation, $P<10^{-16}$). To remove the potential noise due to this correlation, we estimated a version of the effective number of codons, *Nc*', which accounts for background nucleotide composition (Novembre 2002). We also used in our analyses the residuals of the regression between GC3 content and *Fop* and *Nc*.

Transcriptome and mtDNA Data Sets for Phylogenetic Analyses

We assembled a data set of 91 orthologous genes from the transcriptomes of 21 *Drosophila* species including *D. suzukii*. Strict orthology within the complete set of *D. melanogaster* genes (*Drosophila* 12 Genomes Consortium et al. 2007) and the other 20 transcriptomes was assessed using the reciprocal best BLAST hits method. We first identified single copy ^{WEB}orthologues between *D. melanogaster, D. biarmipes, D. bipectinata, D. elegans, D. eugracislis, D. ficusphila, D. kikkawai, D. rhopaloa, D. takahashi*, and *D. suzukii*. We identified the masked alignments of all these ^{WEB}orthologues in the 12 *Drosophila* alignments (*Drosophila* 12 Genomes Consortium et al. 2007), thus selecting 97 groups of putative orthologues. A few of these were removed after manual inspection revealed that they contained incomplete, frame-shifted, and/or dubiously assembled sequences, leaving 91 highly reliable orthologue groups. These were aligned using TranslatorX and concatenated into a superalignment of 200,475bp, which was further inspected by eye and corrected for the correct frame of codons (inclusion of partial stop codons that altered the frame) and minor errors that escaped the first manual inspection.

We translated this alignment into amino acids and selected conserved regions using Gblocks (Castresana

2000) with parameters 1:11, 2:17, 3:8, 4:10, and 5:half). We retained 90% of the sites, totaling 60,757 amino acids. The final nucleotide alignment of 182,271 bp, perfectly corresponding to the amino acid alignment, was used for further sequence analyses excluding third codon positions.

A mitochondrial genome alignment was constructed by extracting CDS from available and newly assembled (see earlier) mtDNA. The 12 CDS genes were checked for their correct codon frame and concatenated. We also excluded third codon positions from the mtDNA data set for further sequence analyses.

Phylogenetic Analyses

We performed Bayesian and maximum likelihood (ML) analyses on both the transcriptomic and the mitochondrial genomic datasets. For the Bayesian analyses, we used PhyloBayes3 (Lartillot et al. 2009) setting two independent runs until the maxdiff was less than 0.1. We calculated the 50% majority rule consensus trees by pulling sampled trees after a burn-in that minimized the maxdiff statistic in PhyloBayes3. Maximum Likelihood analyses were performed using Phyml (Guindon et al. 2010) on 100 non-parametric bootstrapped replicates. In all cases, a discrete gamma distribution (with four rate categories) was used to model among site rate variation. We performed three main experiments on both datasets using different dataset treatments and models of replacement:

1. ML analyses on nucleotide alignments using all the three codon position and a single nt-general time reversible (GTR) model for all codon positions (nucleotides positions 1+2+3, GTR+G, ML in fig. 1).
2. Bayesian analyses on nucleotide alignments using the CAT model after exclusion of the third codon position (nucleotide positions 1+2, CAT+G, Bayesian).
3. Bayesian analyses on the corresponding amino acid alignments using a six category Dayhoff recoding and the CAT+GTR model (amino acids-Dayhoff, CAT+GTR+G, Bayesian).

Molecular Clock Analyses

We performed two different molecular clock analyses. We first used PhyloBayes (Lartillot et al. 2009) on both the transcriptomic and mitochondrial genomic datasets at the nucleotide level. We employed a CIR process clock model and a GTR+G model of replacement on both datasets using the fixed tree topology of Figure 1A. We constrained four nodes as in Prud'homme et al. (2006) using their suggested biogeographical calibrations. To account for uncertainty in bio-geographical constrains, we allowed both minima and maxima to be soft, thus allowing the posterior dates to be sampled outside the set bounds (Yang and Rannala 2006). We employed a root prior of 80 Myr ago with a permissive SD of 40 Myr and assumed a birth-death process along all nodes. We modeled replacement using CAT and the clock using CIR as in Rota-Stabelli, et al. (2013b). In a second approach, we used BEAST (Drummond and Rambaut 2007) without constraining internal nodes and the random local clock but only a normally distributed root prior centered at 80 Myr with SD 20 Myr. We assumed the initial mutation rate of 0.0346 (SD=0.00281) suggested in Obbard et al. (2012). Because mutation rate refers to unconstrained sites, we used only the 4-fold degenerate sites of the genomic data set for the BEAST analysis.

Field Monitoring and Trapping

Field trapping for tests of distribution by altitude were carried out between 15 April (week 14) and 31 October (week 43) 2011. Forty sites across Trento Province were chosen representing both agricultural and natural ecosystems. Traps were deployed on a large-scale altitudinal gradient and assigned to four altitudinal ranges (<250m asl [n=10], 250-600m asl [n=10], 600-1,000 m asl [n=10], >1,000 m asl [n=10]). At each trapping site, we placed, in a shady spot, one plastic transparent bottle with multiple small lateral holes (diameter between 5 and 10 mm) containing 250 ml of apple cider vinegar as bait. Weekly, traps were checked, insects collected, and vinegar replaced. Weekly captures of *D. suzukii* in each trap were averaged per altitudinal range.

Results

Genome, Transcriptome, Mitogenome, and *Wolbachia* Sequencing

We sequenced and assembled a draft genome and transcriptome of *D. suzukii* from an Italian Alpine population. The draft genome was sequenced to high depth (an average of 80x coverage) and comprises 49,558 contigs spanning a total of 160 Mb. The draft transcriptome contains 25,810 unique sequences. Both the size of the genome and its repetitive element contents are comparable with that of *D. melanogaster* and other sequenced *Drosophila* (fig. 4). We also assembled the nearly complete mitochondrial genome for *D. suzukii* (~15 kb), whose size and gene content is similar to that of other sequenced *Drosophila*. Finally, we extracted and assembled the genome of a *Wolbachia* endosymbiont (*wSuzi*, 1.3 Mb) harbored by the Italian *D. suzukii* population. Preliminary analyses based on several genes identify *w*Suzi as closely related to *w*Ri from *D. simulans* Riverside (Klasson et al. 2009). A more detailed characterization of *w*Suzi is presented in Siozios et al. (2013).

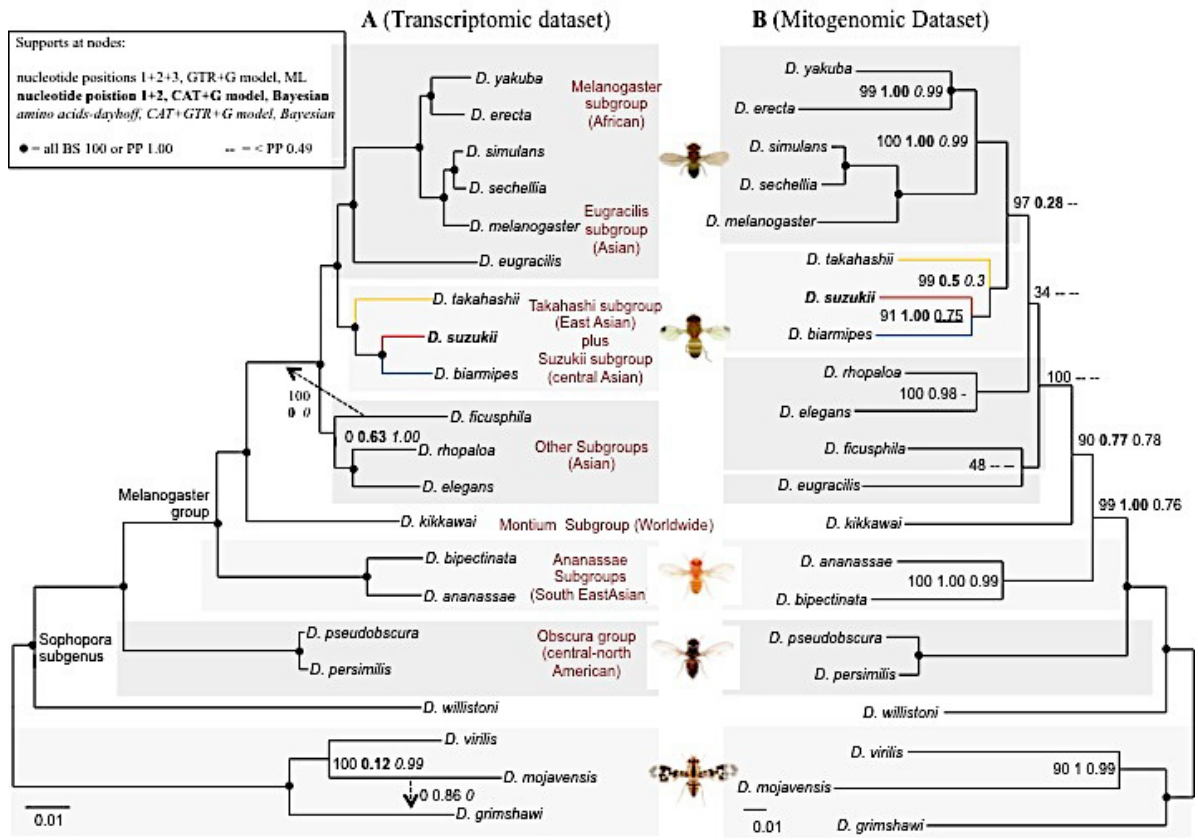

Figure 1. The evolutionary affinities of *D. suzukii* and the other *Drosophila* species inferred from phylogenomic and mitogenomic data. (**A**) Phylogenetic analyses of 91 orthologous nuclear genes (200,475 bp). (**B**) Phylogenetic analyses of 12 mitochondrial genes (11,139 bp). Both data sets support an Asian affinity of *D. suzukii*. *Drosophila* images from Prud'homme and Gompel, used by permission.

Molecular Phylogenetics Using Transcriptomic and mtDNA Data Sets

We used data from the *D. suzukii* genome to conduct a comprehensive multi-locus phylogenetic and dating analysis in the context of genome data from 20 additional *Drosophila* species. We conducted two separate analyses using two distinct datasets.

In the first analysis, we used 91 protein-coding genes extracted from the transcriptomes of the 21 species, covering more than 200,000 nucleotides (fig. 1A). We analyzed the aligned data both as nucleotides, excluding third codon positions to exclude likely saturated positions or characters associated with synonymous substitutions, and as amino acid sequences. We also employed different phylogenetic frameworks (Bayesian and ML) and both homogenous and more sophisticated heterogeneous models such as CAT+GTR on a Dayhoff recoded dataset (Rota-Stabelli, Lartillot, et al. 2013). All analyses converged on a tree that supported a sister relationship between the *suzukii* and *takahashii* subgroups, and *D. eugracilis* as sister of the *melanogaster* subgroup (fig. 1).

In a second analysis, we reconstructed a phylogeny from the mitochondrial genomes of the 21 *Drosophila* species. We assembled nearly complete mitochondrial genomes for eight additional *Drosophila* species for which whole transcriptome shotgun data were available. Phylogenetic analyses using the same set of experimental procedures used for the transcriptome dataset failed to support most of the findings of the genome-derived transcriptome tree (fig. 1B).

Molecular Clocks and (Paleo) Ecological Analyses

We performed molecular clock analyses using both the transcriptome and the mtDNA datasets (fig. 2A, see Materials and Methods for details). Despite some discrepancies for the ages of nodes closer to the root, the two datasets converged in supporting divergence of *D. suzukii* from *D. biarmipes* in a period between 9 and 6 Mya (*i.e.,* the Tortonian).

To link these clock analyses with the current distribution of *D. suzukii* in Asia, we mapped the current known distribution of *D. suzukii* and their sister species onto a previously compiled climatic model of the Asian Tortonian (fig. 2B). The current distribution of *D. suzukii* extends over the Tortonian montane temperate forests, whereas *D. biarmipes* is confined to a more equatorial southern habitat. To investigate a possible

preference for temperate climate in *D. suzukii* (see Discussion), we monitored the distribution of *D. suzukii* Italian populations along a gradient of altitude over 1 year (fig. 2C). *D. suzukii* preferentially inhabits higher, more temperate altitudes, although the majority of human activity and fruit sources are concentrated at lower altitudes.

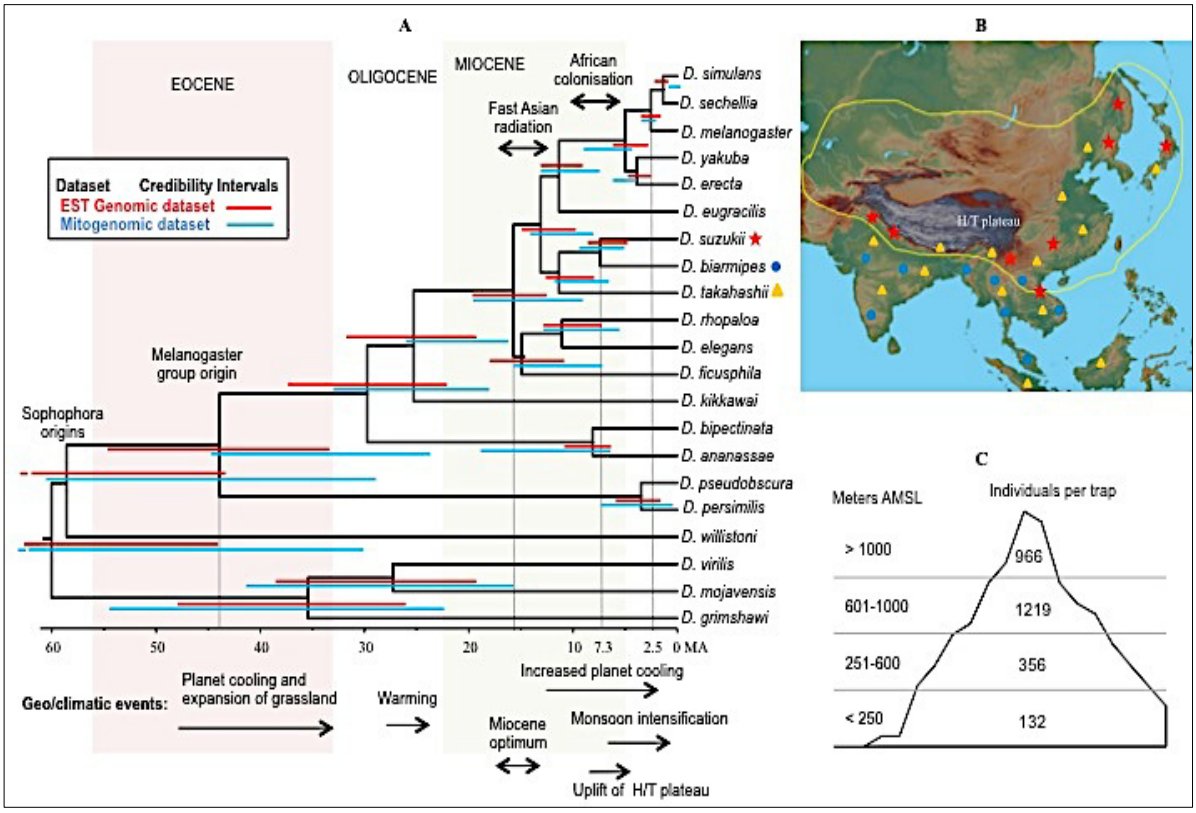

Figure 2. Molecular timetrees, paleoclimate, and field trapping suggest a montane temperate origin of *D. suzukii*. (A) Relaxed clock analyses of the *Drosophila* species using both the nuclear and mitochondrial data sets of figure 1. *D. suzukii* is predicted to have diversified toward the late Miocene (Tortonian) simultaneous with an increased uplift of the Himalayan/ Tibetan (H/T) plateau and an intensification of the monsoon cycles. Most speciation events (Asian radiation) within the *melanogaster* group happened just after the mid Miocene climatic optimum in concomitance with further temperature decrease. (B) Current endemic geographical distribution of *D. suzukii* (stars) compared with that of *D. biarmipes* (dots) and *D. takahashi* (triangles); yellow line marks the border of temperate (mostly mountainous) forested area during the Tortonian age, the current area being similar but restricted toward the North East. These distributions suggest that *D. suzukii* speciated from *D. biarmipes* by adapting to more temperate mountainous environment. Some species distribution taken from Markow and O'Grady (2005). (C) Annual captures per trap at five different altitudes in the Alps confirm a montane/ forest optimum for *D. suzukii,* despite greater food resources from fruit production below 600 masl.

Reduced Rate of Molecular Evolution and Reduced Effective Population Size in *D. suzukii*

We explored the patterns of molecular evolution of the *D. suzukii* genome by studying a set of 2,336 orthologous genes from five key species carefully chosen to illuminate key points in its evolutionary history. *D. suzukii* genes are characterized by a slow rate of molecular evolution (fig. 3A; supplementary table S2). Both synonymous (dS) and non-synonymous (dN) substitutions rates are significantly lower compared with those of its sister species *D. biarmipes* (fig. 3B), consistent with a reduction in substitution rate along the *D. suzukii* branch. This finding is reinforced by a molecular clock analysis that showed that *D. suzukii* has the lowest substitution rate among the *Drosophila* species considered (fig. 3C).

We next examined whether in *D. suzukii* the low substitution rate was accompanied by different levels of selective pressure compared with its close relative. The level of overall genomic selective pressure, as measured by the ratio dN /dS, is on average significantly lower in *D. suzukii* than in *D. biarmipes* (fig. 3B). Interestingly, there is a significantly larger dN /dS in autosomal genes of *D. suzukii* compared with those of *D. biarmipes*, whereas the opposite is true for X-linked genes dN/dS (fig. 3B), consistent with a difference in levels of selective pressure between autosomes and the X chromosome.

To obtain a broader picture of the evolutionary processes, we further analyzed the codon usage in these five species (table 4 and supplementary table S1). In many organisms, synonymous codons are used with different frequencies, leading to codon usage bias. Such bias can be under weak selection ($|Nes| \approx 1$), and is maintained by the concurrent action of selection, drift, and mutation. Thus, in principle, codon usage bias should be stronger in species with larger effective population size, *Ne*, compared with species with lower *Ne*. Both the effective number of codons, *Nc* (Wright 1990), and the frequency of optimal codons, *Fop* (Ikemura 1981), are significantly different between *D. suzukii* and *D. biarmipes* (*P*< 10e-15; supplementary table S3) and are consistent with less codon usage bias in the former. Because GC and GC3 (GC in the third codon position) content are significantly correlated to codon usage bias measures in *D. suzukii* and *D. biarmipes* (Spearman's rho> 0.68 for GC and rho< -0.65 for GC3, *P*< 10e-15, for both species), we repeated the comparative analyses while correcting for compositional bias. Codon usage bias measures *Nc* and *Fop* do not differ significantly between *D. suzukii* and *D. biarmipes* when correcting for GC or GC3 (*P*> 0.139, for both comparisons). The modified version of *Nc*, which accounts for background nucleotide composition, *Nc*' (Novembre 2002), is significantly larger in *D. suzukii* than in *D. biarmipes* (*P*= 6 X 10e-11), suggesting less codon usage bias in the former.

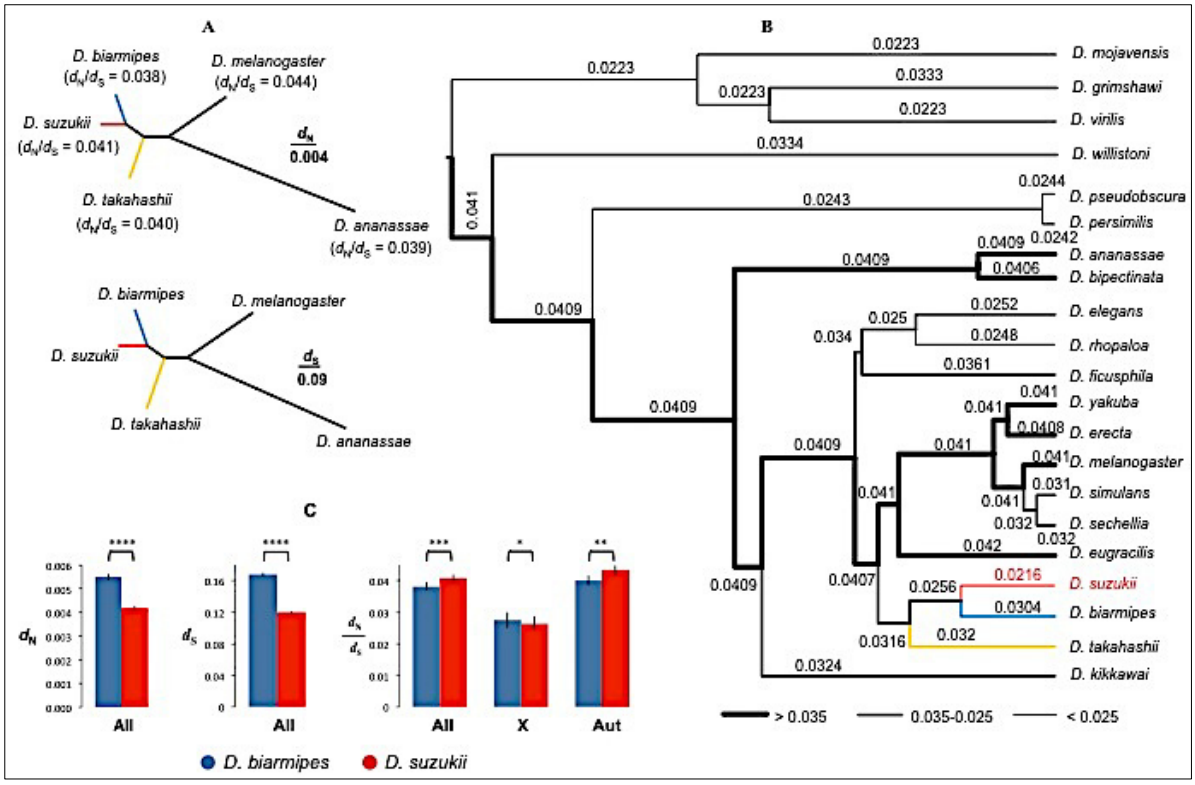

Figure 3.The slowly evolving genome of *D. suzukii* can be linked to reduced number of generations per year due to winter sexual (female) diapause. (A) Consensus evolutionary analysis of 2,336 orthologous genes in five key species. Upper and lower are, respectively, the trees derived from analyses of non-synonymous (dN) and synonymous (dS) substitutions. The dN/ dS for each species are given in parentheses. (B) Branch-specific normally modeled mutation rates as optimized by BEAST using as initial value a mutation rate of 0.0346 neutral substitutions per base pair per million of year (SD= 0.00281). Branch thickness is proportional to the rate. *D. suzukii* is clearly characterized by the lowest rate. Other slower evolving genomes are those of the *virilis–repleta* radiation and of the *pseudobscura* group, which are also preferentially distributed in a temperate/ holoartic environment (North American and Central American plateaus). (C) A detailed comparison between the rate of molecular evolution in *D. suzukii* and its sister species *D. biarmipes*, for all genes (All) as well for autosomal (Aut) and X-linked genes (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, Wilcoxon test after controlling for gene length; see also table 3).

The analysis of the rate of molecular evolution at the single-gene level revealed only few genes that evolved at different rates in the *D. suzukii* branch compared with the rest of the phylogenetic tree (*D. melanogaster*, *D. ananassae*, [*D. takahashi*, {*D. biarmipes*, *D. suzukii*}]) or where *branch-site* models detected the occurrence of positive selection specifically affecting sites along the *D. suzukii* branch (tables 1 and 2).

Discussion

The Evolutionary Affinities of *D. suzukii* and the Sister Group of the *Drosophila* Subgroup

Analyses based on 91 nuclear protein-coding genes (fig. 1A) confirmed a sister relationship between the *suzukii* and *takahashii* subgroups (Yang et al. 2012). The *melanogaster* subgroup was found to be closely related to *D. eugracilis*, a new hypothesis of Sophophora evolution that was extremely robust to various dataset treatments (exclusion of third codon positions in nucleotide sequences and translation into the corresponding amino acid sequences) and experimental procedures (use of homogenous and heterogeneous substitution models in both a Bayesian and ML framework; see legend of fig. 1A). Not all relationships were well resolved using this large dataset. The placement of *D. ficusphila* is dataset and model dependent, but the use of the sophisticated CAT+GTR model coupled with Dayhoff recoding of the amino acid dataset (performed to reduce possible systematic errors in phylogenomic analyses; Rota-Stabelli, Lartillot, et al. 2013) points toward its grouping with *D. rhopaloa* and *D. elegans*.

To corroborate our phylogenetic results, we further analyzed an mtDNA dataset, which failed to support all the findings of the nuclear gene set tree (fig. 2B). This is most likely because of a lack of phylogenetic signal in the mtDNA. Thus, the apparently robust bootstrap support (97%) against the sister relationship *D. eugracilis-melanogaster* subgroup vanishes when highly saturated third codon positions are excluded, or when an amino acid dataset was employed, indicating that signal contradicting the nuclear phylogeny carried by mitochondrial genomes is concentrated in unreliable third codon positions and/or

Table 1. Top 10 Genes identified as putative target of positive selection along the *D. suzukii* branch

D. melanogaster orthologue	P[a]	q-value[b]	P_2 [c]	ω_2 [d]	Function and phenotype (sex, neuron, thorax)
Cyp4d20	$7x10^{-13}$	$8x10^{-10}$	0.0026	15.2	Predicted electron carrier activity. Protein with features of Cytochrome P450. Expression at moderate levels in the following post-embryonic organs or tissues: adult head, adult eye, adult heart, adult spermathecae, adult carcass
endos	0.00085	0.27948	0.0200	999.0	Predicted sulfonylurea receptor binding activity. Involved in regulation of meiotic cell cycle, mitotic spindle organization, oogenesis, water homeostasis and response to nutrient. Phenotypically relevant in egg, oocyte and follicle cell.
Ptp4E	0.00103	0.29554	0.0048	999.0	Predicted transmembrane receptor protein tyrosine phosphatase activity. Involved in motor axon guidance, central nevous system development, and open tracheal system development. Phenotypically relevant in ventral nerve cord.
T48	0.00134	0.33996	0.0084	999.0	Unknown molecular function. Phenotypically relevant in ventral furrow.
CG15626	0.00157	0.36001	0.0030	693.7	Unknown molecular and biological function.
Cyp4aa1	0.00199	0.39060	0.0024	999.0	Predicted electron carrier activity. Involved in insecticide catabolic and hormone metabolic processes
Osi20	0.00205	0.39060	0.0078	747.8	Unknown molecular and biological function. Phenotypically relevant in trichogen cell
CG13397	0.00271	0.47676	0.0024	200.4	Predicted alpha-N-acetylglucosaminidase activity. Protein domains suggest involvement in carbohydrate metabolic process
yemalpha	0.00351	0.52651	0.0022	135.4	DNA binding activity. Involved in female meiosis. Phenotypically relevant in oocyte
toe	0.00358	0.52651	0.0123	70.3	Predicted molecular function in sequence-specific DNA binding transription factor activity. Involved in compound eye development, and negative regulation of transcription from RNA polymerase II promotor. Phenotypically relevant in scutum and scutellum (mesothoracic tergum) development.

[a]Likelihood ratio test probability based on branch-site models of codon evolution, with *D. suzukii* set as foreground branch.

[b]Proportion of false positives (FDR) of the test.

[c]Proportion of sites under positive selection estimated in the foreground branch (*D. suzukii*) by the branch-site model A.

do estimated for the sites under positive selection in the foreground branch (*D. suzukii*) by the branch-site model A.

synonymous substitutions. Overall, our phylogenetic analyses reveal that the African *melanogaster* subgroup evolved from within a rapid Asian radiation, identifying *D. eugracilis* as a key intermediate species to polarize evolutionary traits of the melanogaster subgroup. With respect to the placement of *D. suzukii* in the phylogeny, our analyses suggest that this species is the sister taxon of *D. biarmipes*. *D. subpulchrella*, another little-studied fly in the *suzukii* subgroup, has been reported to have feeding behavior similar to that of *D. suzukii* (Mitsui et al. 2010). This species, is however, thought to be most closely related to *D. pulchrella* (hence its name), which is sister to the *suzukii*+ *biarmipes* clade (Yang et al. 2012), suggesting independent acquisition of unripe fruit feeding. It will be important to explore the relationships of *D. subpulchrella* using genome-scale data.

Rate of Molecular Evolution Suggests Winter Diapause

Our results indicate that gene sequences evolve at a significantly lower rate in *D. suzukii* than in its sister species *D. biarmpes* (fig. 3 and table 1). We hypothesize that the low substitution rate of *D. suzukii* could be due to an idiosyncratic, low mutation rate, and/ or because the species has a reduced number of generations per year compared with its relatives. The reproductive ecology of the species supports the second hypothesis, as in its distributional range *D. suzukii* reproduces only during the warm season and is able to over-winter as sexually immature, cold tolerant females (Mitsui et al. 2010). Our genomic evidence supports the hypothesis that *D. suzukii* has a winter sexual diapause and thus had a reduced number of generations since its last common ancestor with *D. biarmipes*.

Table 2. Top 10 Genes Evolving at a Significantly different rate along the *D. suzukii* Branch.

D. melanogaster orthologue	P[a]	q-value[b]	ω_{FB} [c]	ω_R [d]	Function and phenotype (sex, neuron, thorax)
ran	$6x10^{-10}$	0.000001	0.0752	0.0001	GTP and protein binding activity. Involved in regulation of meiotic spindle organization, cell cycle, cell shape, cell adhesion and actin filament organization. Phenotypically relevant in photoreceptor cell R7, meitotic spindle, karyosome, ommatidium and pigment cell.
mtm	$1x10^{-9}$	0.000002	0.1639	0.0190	Phosphatidylinositol-3-phosphatase activity. Involved in mitotic cell cycle, chromosome segregation and response to wounding. Phenotypically relevant in sessile hemocyte and embryonic/ larval hemocyte.
wcd	$4x10^{-8}$	0.00003	0.3814	0.0782	Unknown molecular function. Involved in ribosome biogenesis and neuroblast proliferation and female germ-line stem-cell division. Phenotypically relevant in trichogen cell and mesothoracic tergum.
I(3)72Dn	$9x10^{-8}$	0.00005	0.4781	0.1330	Unknown molecular function. Involved in ribosome biogenesis and neurogenesis. Phenotypically relevant in mesothoracic tergum.
CG9135	$7x10^{-7}$	0.00031	0.2774	0.0150	Predicted guanyl-nucleotide exchange factor activity. Unknown biological function. Phenotypically relevant in mesothermic tergum.
CG8562	$3x10^{-6}$	0.00105	0.3100	0.0624	Predicted metallocarboxypeptidase activity. Protein domains suggest involvement in proteolysis.
Oatp33Ea	0.00001	0.00175	0.2131	0.0556	Predicted organic anion transmembrane transporter activity.
Ibk	0.00001	0.00175	0.0001	0.0435	Involved in chaeta morphogenesis and oogenesis.
Iid	0.00001	0.00175	0.1231	0.0421	Histone acetyltransferase and demethylase activity. Involved in chromatin organization, and histone acetylation and demethylation. Phenotypically relevant in mesothermic tergum, imaginal disc, and embryonic/ larval optic lobe.
dome	0.00001	0.00270	0.0186	0.0886	Transmembrane signalling receptor activity and protein heterodimerization activity. Involved in blastoderm segmentation, hindgut morphogenesis, border follicle cell migration, long-term memory, JAK-STAT cascade, open tracheal system development, and compound eye morphogenesis. Phenotypically relevant in spiracle, integumentary specialization, embryonic hindgut, and compound eye.

[a] Likelihood ratio test probability based on branch models of codon evolution, with *D. suzukii* set as foreground branch.
[b] Proportion of false positives (FDR) of the test.
[c] ω estimated for the focal (*D. suzukii*) branch.
[d] ω estimated for the rest of the phylogenetic tree.

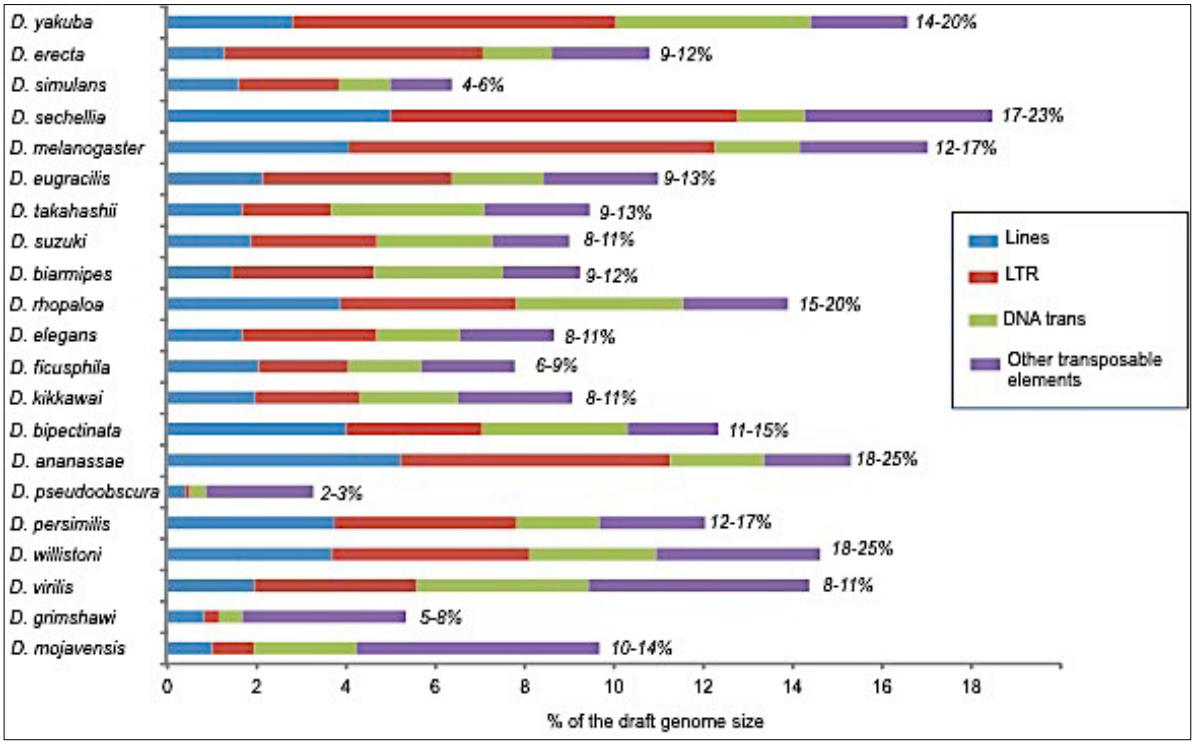

Figure 4: Repeated elements in *D. suzukii* genome. The distribution and number of repeats in *D. suzukii* is similar to that of sister species *D. biarmipes* and *D. takahashii*, thus making sense of their phylogeny. In most other cases there is not a similarity between closely relates species, see for example *D. yakuba* and *D. erecta*.

Reduced Effective Population Size Affects the Efficiency of Positive Selection in *D. suzukii*

The level of overall genomic selective pressures, as measured by the ratio dN/dS, is lower in *D. suzukii* than in *D. biarmipes* (fig. 2B). This result is consistent with more relaxed selection along the *D. suzukii* lineage, possibly because this species has a smaller effective population size, *Ne*, than *D. biarmipes*. A reduced *Ne* would allow the fixation of larger number of slightly deleterious non-synonymous mutations (Charlesworth 2009), as further supported by the observation that *D. suzukii* has a lower frequency of optimal codons and a lower codon usage bias than *D. biarmipes* (Table 4 and supplementary Table S3). The alternative possibility that larger dN/dS values correspond to pervasive positive selection in the *D. suzukii* genome (i.e., increased fixation of beneficial mutations) is not supported by our data. First, the fixation of favorable alleles in multiple genes would lead to a high dispersion in the distribution of dN across the genome (Presgraves 2005), whereas the variance in dN is significantly lower in *D. suzukii* than in *D. biarmipes* (2.9x 10e-5 *vs*. 4.4x 10-5, F test $P< 10e-15$). Second, only a few genes were detected as significant targets of positive selection in *D. suzukii* (Table 1). Thus, the most likely explanation for the low substitution rate of *D. suzukii* is a reduced number of generations per year and a smaller *Ne* compared with its relatives. It is unlikely that the reduced *Ne* is a direct consequence of the bottleneck associated with a colonization of Europe, as the invasion took place only few generations ago (the first record dates back to 2008, Cini et al. 2012), and thus the genome-wide pattern of substitutions should represent that of the ancestral population. We propose instead that the low substitution rate reflects the ecology and evolutionary history of this species. The winter diapause, we suggest, explains both the low substitution rate and the reduced selection efficiency in *D. suzukii* compared with *D. biarmipes*. Overwintering diapause results in recurrent population size bottlenecks, particularly for males, and thus in a lower effective population size *Ne*, and lower selection efficiency in removing slightly deleterious non-synonymous mutations, as indicated by its genome-wide higher dN/dS.

The hypothesis that males undergo more severe bottlenecks than females is supported by the discrepancy in levels of selective pressure between the autosomes and the X chromosome. As males contain only one copy of X (and two of the autosomes), sex-biased population size changes would alter relative levels of X-linked and autosomal *Ne*, namely by decreasing *Ne* of autosomes 2-fold relative to X chromosome in males. We indeed observed a significantly larger dN

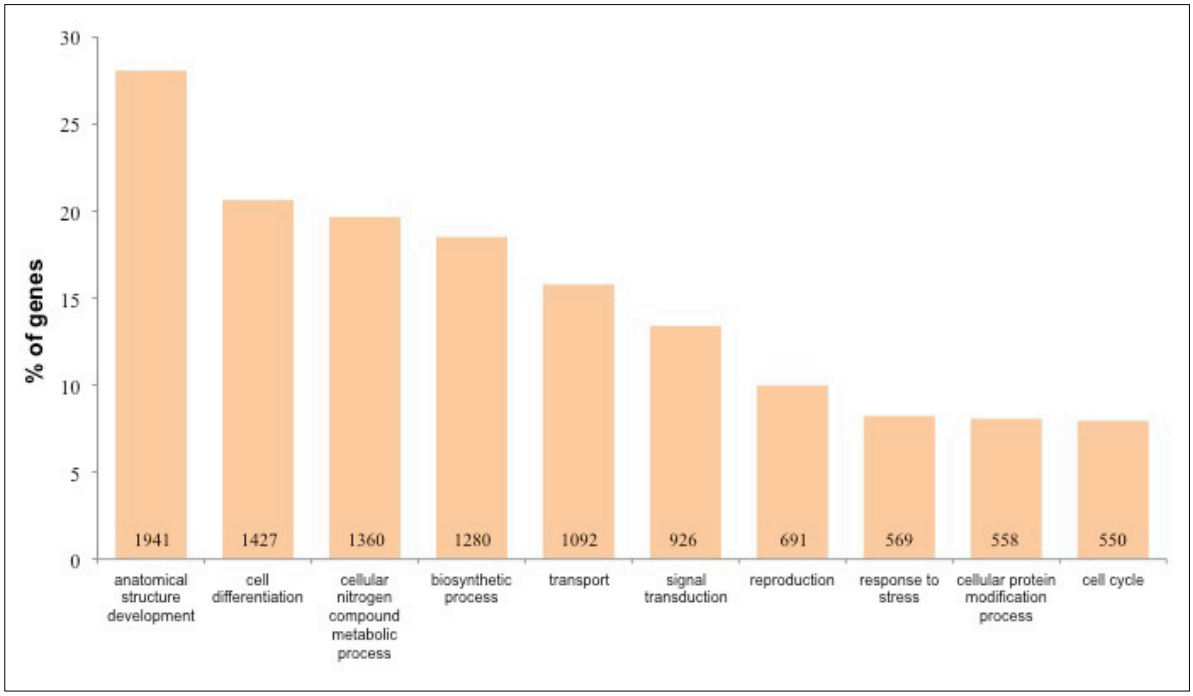

Figure 5. Putative function of the *D. suzukii* genes. *D. suzukii* genes obtained through RNASeq sequencing were blasted against D. melanogaster genes. A total of 8,137 reciprocal best hits were retained as putative orthologues, representing 74% of the D. melanogaster annotated genes. Assuming conserved synteny and chromosomal organization between *D. suzukii* and *D. melanogaster*, we could verify that chromosomes were evenly covered by our RNAseq gene sequences (chromosome 2: 72.2%, chr. 3: 76.4%, chr. X: 73.7%; exception is chr. 4: 53.8%). Putative function was assigned based on gene ontology (GO) terms of the *D. melanogaster* genes using the web tool available at http://go.princeton.edu. Only categories representing more than 7% of the total GO terms in the list are shown (the actual number of genes is given within the bars).

/dS in autosomal genes of *D. suzukii* than of *D. biarmipes*, whereas in X-linked genes, dN/dS was lower in *D. suzukii* than in *D. biarmipes* (fig. 3B). If we assume that levels of dN/dS are a proxy for effective population size, the X/autosome ratio of *Ne* values, *Ne*X/*Ne*A, seems to be higher in *D. suzukii* than in *D. biarmipes*, possibly leading to differences in the efficiency of selection on the X and autosomes between the two species. One hypothesis to explain this observation is a difference in the efficiency of purifying selection in removing recessive deleterious mutations in hemizygous males, a phenomenon which can often lead to a faster-X effect (Charlesworth et al. 1987; Vicoso and Charlesworth 2009a). Thus, the bottlenecks associated with the winter diapause of *D. suzukii* could be directly responsible for the relative difference in *Ne*X/*Ne*A between the two species (Pool and Nielsen 2007). Other factors that may have affected the differences in the ratio *Ne*X/*Ne*A between *D. suzukii* and *D. biarmipes* include different recombination rates (Vicoso and Charlesworth 2009b) and variance in male reproductive success due to sexual competition among males (Andersson 1994; see Mank et al. 2010 for a review). Additional genetic and behavioral studies will be necessary to disentangle these forces and evaluate their role in the evolution of *D. suzukii.*

Paleobiology and Adaptation to Temperate Ecology

The presence of a winter diapause in *D. suzukii* may be an adaptation that is relevant to the switch in ecology of the species. Relaxed clock studies of both nuclear and mitochondrial genomes (fig. 2A) converged on a scenario in which *D. suzukii* diverged from *D. biarmipes* approximately 9–6 Mya, towards the end of the Miocene (Tortonian). Climate modeling has shown that, during the Tortonian, the ecology of region between North India, Indochina, and the Chinese coasts (delineated by the yellow line in fig. 2B) was characterized by extended montane temperate forests. Toward the present, forests reduced in extent to the North and East and alternated with scrublands or tropical forests (Pound et al. 2011). The present endemic distribution of *D. suzukii* extends over this region, whereas *D. biarmipes* is endemic to a more equatorial, southern habitat. The distribution of the two species suggests that speciation of *D. suzukii* was accompanied by adaptation to temperate habitats, through the increase uplift

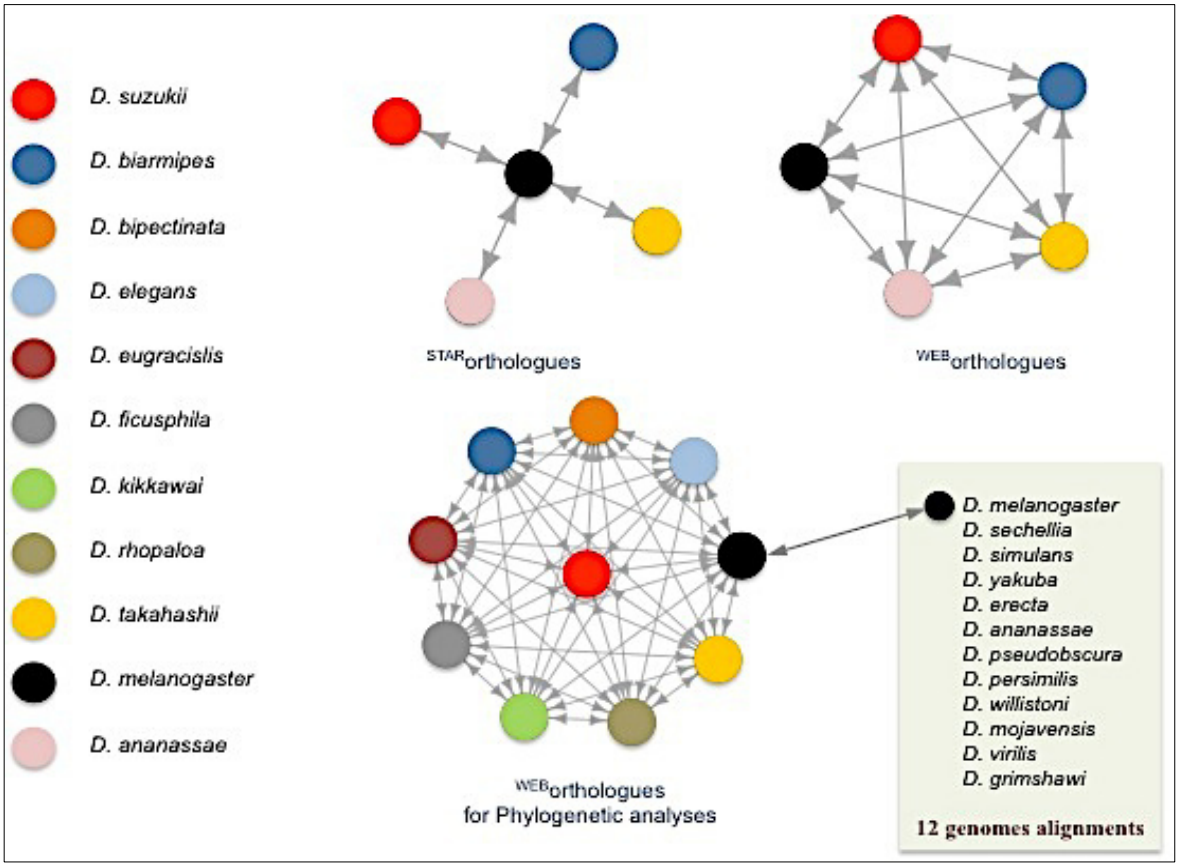

Figure 6. Orthologues search. Putative orthologues were identified using a reciprocal best-hit approach across *Drosophila* species. See Methods for details.

of the Tibetean plateau and the concomitant intensification of the monsoon regime in the Tortonian (Zachos et al. 2001).

A strong preference for montane temperate climate in current invasive populations is supported by the results of trapping surveys. Although extensive soft fruit production is concentrated below 600m asl in the surveyed Trentino Province of North Italy, the majority of the captures we made were at higher altitudes (fig. 2C). The proposal that *D. suzukii* originated in a temperate, montane ecology is congruent with its current life habit. In temperate forests, fruit production, and thus the availability of rotting fruit, is highly seasonal, whereas in the tropics fruiting, and thus the production of rotted food sources, is near continuous (Willson 1991; Ting et al. 2008). For a species occupying a temperate ecosystem, ovipositing in fresh fruit is required to access food. Growing larvae can then accelerate decay and fermentation to provide food for the adult stage. Overwintering diapause bridges the winter months when fruits are scarce if not absent, and low temperatures limit both fermentation and fly activity.

Preadaptations Suggest Invasive Success

An innate predisposition to temperate climates might also explain why *D. suzukii* was able to invade European and North American regions so rapidly. *D. melanogaster* has also invaded temperate climates, but the colonization originated from an ancestral tropical African range and was accompanied by local adaptation (Ometto et al. 2005), whereas invading populations of *D. suzukii* are likely to have already had many traits adaptive in the newly occupied range. Genes under positive selection (Table 1) and those with fast evolutionary rates (Table 2) are good candidates for loci involved in such adaptations. We found no evidence for a significant overrepresentation of Gene Ontology-defined functional classes in these gene sets, but many of the identified genes are phenotypically linked (through their *D. melanogaster* orthologues) with biology of the genitalia, the neuronal system and (particularly and unexpectedly) with the mesothoracic tergum. The genetic and neurological bases of the adaptive behavioral and lifecycle traits of *D. suzukii* may hold the keys to understanding the origin of a novel behavioral repertoire and lead to strategies for control of this pest in western countries. Because the current hypothe

Table 3: *D. suzukii* genome cleaning statistics. Number of reads in libraries and number of reads that blast against different W*olbachia* genomes and *D. melanogaster* mtDNA.

Genome	NCBI accession	*n* reads (180 bp)	*n* reads (300 bp)
D. suzukii initial reads	ERA an	134,306,528	103,584,510
W. simulans	NZ_AAGC00000000.1	126,346	218,494
W. melanogaster	NC_002978.6	161,996	288,266
W. ananasse	NZ_AAGB00000000.1	77,484	259,026
W. willistoni	NZ_AAQP00000000.1	115,601	197,998
W. wRi	NC_012416.1	162,071	288,803
all Wolbachia		399,088	683,606
D. melanogaster mtDNA	NC_001709	59,342	129,646
D. suzukii reads after cleaning*		124,381,960	96,007,805

Columns "180bp" and "300bp" indicates the number of matching reads for the tow libraries. * After the quality checks reads had an average length of 93 bp (sd14) for the 180bp library and of 94bp (sd 15) for 300 bp library, and an average quality value of 35.

Table 4: Genome assembly statistics. Abyss trials with different k-mer size.

K-mer size	N contigs	n:200	n: N50	N80	N50	N20	Max	Sum(MBp)
48	1,399,155	93,256	7,826	1,369	4,756	18,300	208,969	185.3
54	1,200,237	105,190	9,204	1,283	4,445	15,559	169,965	195.7
64	961,286	131,597	12,820	1,089	3,565	11,309	169,947	209.6

n:200 is the number of contigs shorter than 200 bp, n:N50 is the number of contigs longer than the median, N80 is the size of the 80 percentile, N50 is the median contig size, N20 is the size of the 20 percentile, sum is the overall contigs size in millions of base pairs.

sis of the phylogeny within the *suzukii* subgroup has not yet been confirmed by whole-genome phylogenetics, we cannot exclude the possibility that *D. subpulchrella* is sister to *D. suzukii* rather than *D. pulchrella*. Under this scenario, some adaptations currently modeled as arising within *D. suzukii* may in fact be shared with *D. subpulchrella*. Genome sequencing of *D. subpulchrella* will clarify this question. Our evolutionary analyses of the *D. suzukii* genome suggest an intriguing causal link between adaptation to temperate environments and its particular biology. The genetic bases of adaptation to temperature could be a key factor to develop new pesticides or containment strategies for this pest.

Acknowledgments

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Supplementary table

Table S1. Codon usage in Drosophila. Darker colors identify synonymous codons used at higher frequency among the [STAR]orthologues.

D. melanogaster= Dm

D. ananassae= Da

D. takahashi= Dt

D. biarmipes= Db

D. suzukii= Ds

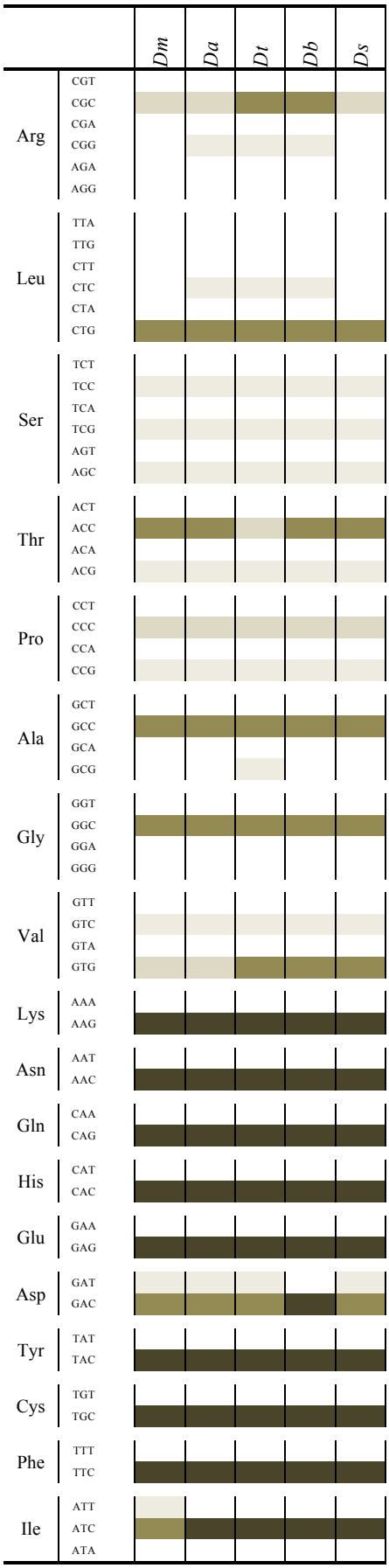

Table S2. Total branch length (*tot*) and rate of synonymous (d_S) and non-synonymous substitution (d_N) across ^{WEB}orthologues along the *Drosophila biarmipes* and *D. suzukii* lineages.

	D. biarmipes			***D. suzukii***			***P*** [b]	
	n [a]	**mean**	**SD**	***n***	**mean**	**SD**	**raw**	**res_L**
tot	1002	0.1092	0.0479	1002	0.0789	0.0372	1×10^{-49}	0.0076
d_N	1002	0.0048	0.0056	1002	0.0037	0.0046	3×10^{-9}	0.0004
d_S	1002	0.1628	0.0765	1002	0.1169	0.0575	6×10^{-51}	0.0376
d_N/d_S	999	0.0352	0.0662	989	0.0365	0.0648	0.5799	0.0058

[a] Exceedingly large d_N/d_S values corresponding to $d_S = 0$ were ignored in the analysis. SD = standard deviation.

[b] Wilcoxon test probability calculated for the raw data and after correcting for gene length (res_L; we used the residuals of the correlation between gene length and the statistic of interest).

Table S3. Mean (Standard deviation, SD) codon usage and GC content in ^{STAR}orthologues..

	Fop [a]		***Nc*** [b]		***Nc'*** [c]		**GC** [d]		**GC3** [e]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
D. suzukii	0.668	0.093	43.9	6.7	50.2	4.7	56.6	4.0	72.3	8.9
D. biarmipes	0.745	0.097	41.0	7.1	49.3	4.8	58.3	4.0	76.9	9.1
D. takahashi	0.724	0.094	42.0	6.6	49.5	4.8	57.5	4.0	74.9	8.9
D. melanogaster	0.613	0.086	45.9	6.5	50.9	4.4	55.6	3.7	69.3	8.2
D. ananassae	0.662	0.098	45.6	7.2	50.9	4.4	55.8	4.2	70.0	9.4

[a] Frequency of the optimal codon.

[b] Number of effective codons.

[c] Number of effective codons when accounting for background nucleotide composition.

[d] Percentage of GC content across genes.

[e] Percentage of GC content in the third codon position across gene

Olfactory responses of *Drosophila suzukii* females to host plant volatiles

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Abstract

Drosophila suzukii, an endemic pest of South-east Asia, has invaded the EU and US. Unlike most closely related sibling species, its serrated ovipositor permits ovipositing in undamaged fresh fruits. There is an urgent need for complementing existing management techniques with novel, environmentally safe ones. Here we identify volatiles from host plants, which potentially are involved in its host recognition and oviposition behaviour. We show that mated *D. suzukii* females are attracted to the volatiles emitted from intact fruits. Using gas chromatography coupled mass spectrometry (GC-MS) and GC-electroantennographic detection (EAD) we identified an antennally active suite of compounds released from ripe fruits. In the olfactometer bioassay, mated *D. suzukii* females were significantly attracted to one of the most consistently elicited EAD-active volatile, isoamyl acetate, tested at the rate of 10 µg of synthetic compound loaded in the rubber septa with the release rate comparable to that of fresh fruits. In addition, a genomic survey showed that *D. suzukii* not only possess the full repertoire of genes encoding odorant receptors activated by isoamyl acetate in *D. melanogaster*, but shows that one of them, OR67a, is even represented by five duplicated copies. The results indicate that *D. suzukii* uses olfactory cues to select oviposition sites. The identification of behaviourally-active volatiles emitted by host fruits of *D. suzukii* may therefore aid in the development of selective and efficient synthetic lures and also synergize the existing monitoring traps. As a close relative of *D. melanogaster,* the par-excellence model organism, *D. suzukii* provides a unique opportunity to understand the physiological mechanisms involved in the shift of this species from rotten to ripe fruits for oviposition.

Key words: spotted wing drosophila, semiochemicals, kairomones, isoamyl acetate, olfactory receptors

Introduction

Most drosophilid flies feed on fermenting fruits. However, inasmuch as fruit preference is not ancestral in these fungophilic species (Begon, 1982), fruit flies repeatedly adapted to many different ecological peculiarities, to which their olfactory system seems adapted (Stensmyr et al. 2003; Dekker et al. 2006; Ibba et al. 2010). *Drosophila sechellia* for instance is attracted for oviposition to n-caproic acid released from Indian mulberry, *Morinda citrifolia* (Dekker et al. 2006; Higa and Fuyama, 1993; Jones, 2005), as well as its esters (Dekker et al. 2006; Ibba et al. 2010). In contrast, insects with a wider host range detect the oviposition substrate discriminating and responding to several key volatiles (Pellegrino and Nakagawa, 2009) and do not depend on the presence or absence of a particular compound (Bruce et al. 2005).

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), member of *Drosophila melanogaster* group and native of South-east Asia, is a pest of fresh fruits first identified in Japan by Matsumura in 1931 on cherry fruit. *D. suzukii* is one of the few Drosophilid with serrated ovipositor, which enables it to oviposit in unwounded fresh fruits thereby making them unmarketable. This ability may not be unique of *D. suzukii*: two of its closest related and geographical proximal sister species, *D. subpulchrella* and *D. pulchrella,* also possess a serrated ovipositor, whose biological significance is however still largely unknown; indeed these species have not been reported as pests. *D. suzukii* is highly polyphagous and, at present, infests various soft skinned fruits including cherry, blueberry, blackberry, strawberry, raspberry, apricot and grapes and the potential of the pest infesting other crops is not clear yet (Walsh et al. 2011; Cini et al. 2012). Recently, *D. suzukii* invaded western countries and is now a threat to both European and American fruit industry (Walsh et al. 2011; Cini et al. 2012; Rota-Stabelli et al. 2013).

The perspectives of using conventional tools in combating *D. suzukii* are poor. Insecticides efficacy is in-

deed limited as larvae are protected within the fruit and very frequent sprays are required to have any effect, which conflicts with the preharvest interval requirements, as well as with common IPM protocols (Cini et al. 2012). New monitoring and control measures are therefore sorely needed. Among the environmentally safe monitoring strategies those based on the interferences with the insect olfactory communication are often the most effective in terms of management and are long lasting (Heuskin et al. 2011). In particular, monitoring of *D. suzukii* is now mainly relying on cup traps containing fermenting baits such as vinegar, wine and other sweet solutions, which likely serves as food cue for the fly. Although fermented baits, such as vinegar, ubiquitously trap Drosophilids (Zhu et al. 2003; Becher et al. 2010), as well as many other dipterans (Qian et al. 2013), lepidopterans (Knight et al. 2011), and coleopterans (Kirkendall et al. 2008; Kanga and Somorin 2011), their broad attractiveness for non-targeted Drosophilid complicates their use. Notably, small and young male *D. suzukii* sometimes lack their unique wing spot (Hauser, 2011), which could lead to misidentification and necessarily requires expertise in identifying *D. suzukii* with other Drosophilid. So, 'fine-tuning' the traps with oviposition attractants may reduce misidentification and complement the effectiveness of the trap and pest control (Landolt et al. 2012a). Accordingly, a bunch of studies has been focusing in the selection of the most electrophysiological and behavioural active volatiles in the headspace of vinegar and wine baits, and a subset of only four compounds was shown, in specific environmental conditions, to be more attractive and selective to *D. suzukii* than the original bait (Landolt et al. 2012a, 2012b; Cha et al. 2013, 2012)

Beside its economic relevance, *D. suzukii* is a close relative of the model organism *D. melanogaster*, and offers prospects for addressing some longstanding questions in the field of insect olfaction. *D. suzukii,* being one of the Drosophilid concerning fruit infestation, would show relevant differences in its ability to discriminate and prefer volatiles of fresh fruits over fermenting fruits (Mitsui et al. 2006) for oviposition.
We therefore aim at identifying volatiles characteristic for ripe fruits that may be at the basis of *D. suzukii*'s evolutionary shift for ovipositional preference towards fresh fruits over fermenting fruits. Identification of attractive compounds released from the oviposition site may thus represent a step forward in the improvement of synthetic chemical lures.

We have evaluated the responses of mated *D. suzukii* females for the odour released by fresh and ripe host fruits from the main host plants in behavioural assays (Y-tube olfactometer). The volatile compounds emitted by the fresh fruits were extracted and identified (GC-MS). Comparative electrophysiological analyses (GC-EAD) of *D. suzukii* to the extracted compounds were performed. A single component, isoamyl acetate, the most consistent EAD-active compound present in all fruit extracts, which approximated the release rates by fresh fruits, was used in further laboratory bioassays. A genomic survey was carried out in order to ascertain whether *D. suzukii* possesses the putative repertoire of the most important olfactory receptors (ORs) normally activated by isoamyl acetate in *D. melanogaster.*

Material and methods

Insect rearing

D. suzukii population collected in Trento Province was reared on a standard *Drosophila* semi-artificial diet (*Drosophila* species stock center, https://stockcenter.ucsd.edu/info/food_cornmeal.php,2013) at the temperature of 23-25°C, relative humidity (R.H.) of 65±5% and 16L:8D photoperiod. To obtain virgins, newly-eclosed flies were aspirated and sexed between 0830 to 1230 hr from the tubes with the larval diet. Two-day-old adult females were starved in a vial containing water soaked cotton swab for more than 12 hr, mated with same aged males on the third day (for 3 hr), and then used in all experiments. Fruits were picked on the day of experiment and kept at room temperature (25°C) 1 hr before conducting the experiment.

Dispensers

The synthetic isoamyl acetate (purity >97%; Sigma-Aldrich, Milan, Italy) was loaded in red rubber septa (Wheaton, 20 mm straight plug stopper, Millville, NJ, USA) at 1, 10, and 100 µg per septum. Solutions were prepared using hexane (>99% purity, Sigma-Aldrich) as solvent. Before analysis, dispensers were kept 1 hr in a climatic chamber (25 ± 2°C and 60 ± 5% R.H.) in order to allow solvent evaporation and to equilibrate.

Y-tube olfactometer bioassays

I) Testing host fruits against D. suzukii.

The responses of mated *D. suzukii* adults to host fruit volatiles were investigated using a dual choice Y-tube olfactometer (stem, 30 cm; arm length, 20 cm; arm angle, 60°; internal diam., 4 cm) (Mazzoni et al. 2009). Each arm of the Y-tube was connected to a pyrex glass bulb (250 mL). One chamber held the test material (25 g of fresh and ripe fruits), the other chamber served as the control, holding the same amount of fruits wrapped in a transparent odor proof plastic bag (Toppits, Melitta, Sweden). Airflow was maintained from each cylinder through the olfactometer arms, using an air pump with the airflow adjusted with a flow meter to 250 mL/min. The incoming air was passed through activat-

ed charcoal and humidified with bubbled distilled water. Flies were introduced singly into the olfactometer at the entrance of the main stem, and were observed until they reach the fruit source at the end of an arm or until 5 min had elapsed. Flies reaching the fruit source were considered as 'choice' while flies walking back and forth from one arm to another 'without reaching' fruit source were excluded. Flies that did not choose a side arm within 5 min were recorded as 'no choice'. Flies were retrieved from the olfactometer irrespective of their choice after 5 min. The fruit samples were randomly assigned at the beginning of the bioassays, and they were reversed after having tested five individuals in order to minimize any spatial effects. After each day of trials during which approximately 20 flies had been released, the Y-tube was washed with detergent, rinsed with distilled water and absolute ethanol, and baked overnight at 200°C. Either each fruit sample or odour dispenser were replaced after five single insect releases. Treatments tested in a day were randomly chosen. The responses of 100 flies per fruit species were tested. The experiments were conducted in a laboratory at a temperature of 23±1°C, a relative humidity of 60±10%, 1,000 lux during experimental hour. The fruits tested were raspberry (cv. Heritage), blackberry (cv. Dirksen), cherry (cv. Kordia), blueberry (cv. Brigitta) and strawberry (cv. Elsanta).

II) Testing Isoamyl acetate against D. suzuki:

With the same set-up and duration, an olfactory bioassay with synthetic isoamyl acetate was carried out with mated *D. suzukii* females. In this bioassay, one chamber of the Y-tube held the test compound, loaded on the previously described red rubber dispensers, at increasing doses (1, 10, or 100 µg of isoamyl acetate per septum); the other chamber served as control, holding a red rubber septum loaded with 10 µl of hexane. The solvent was allowed to evaporate for 1 hr before starting the experiments. A total of 120 mated female flies were tested per dose. A chi-square test was used to compare the number of individuals that chose the odour source versus those that chose the control arm within each test fruit and in the isoamyl acetate bioassay. Differences among fruits were evaluated by contingency table analysis based on Chi-square. Both Chi square were Yates corrected.

III) Testing Isoamyl acetate and blueberry against D. suzukii

Another olfactometer bioassay was performed with synthetic isoamyl acetate (10 µg per septum) and fresh blueberry. A similar experimental protocol was followed as before. In total, 100 mated females were tested for the choice between isoamyl acetate and blueberries (25g). Chi square analysis was performed to evaluate the differences in the female choice.

Chemical analyses

Volatiles were collected from the headspace of freshly picked ripe fruits. Fruits weighing 200 g were placed in a 25 x 38 cm polyacetate bag for collection of volatiles (Anfora et al. 2009). Air from the headspace of the bag was pulled out at 150 mL/min through a sorbent cartridge (75 mg Super Q; Sigma-Aldrich) connected to a vacuum pump through teflon tubes. Charcoal-filtered air was pushed simultaneously in the bag by the same pump to maintain a constant pressure. Collections were done for 24 hr in a climatic chamber at 25±2°C, 60±10% R.H., 16L: 8D photoperiod and 1,000 lux during the light period. Three collections from different groups of each fruit species were carried. Volatiles were eluted from the sorbent cartridge by solvent desorption at room temperature using 500 µl of hexane (>99% purity, Sigma-Aldrich). Collections were reduced to 50 µl using a slow stream of nitrogen and stored in 2-mL vials at -20°C until used for GC-EAD, GC-MS and oviposition experiments.

Other fruit samples of the 5 fruit species were prepared for chemical quantification, extracting the headspace volatiles in a similar manner as described above. Three collections were done for each species. After eluting each sample with 500 µL of hexane 0.5 µg nonyl acetate (≥99% purity) was added as an internal standard (Bengtsson et al. 2001) and the collected extracts were reduced to 50 µL as described above.

Chromatographic analyses were done with a Trace GC Ultra gas chromatography coupled with a TSQ Quantum XLS Tandem mass spectrometry (Thermo Electron Corporation, USA) and equipped with a PAL Combi-xt autosampler (CTC Analytics AG Zwingen, Switzerland). The separation module consisted of a ZB Wax PEG capillary column (30m × 0.25mm I.D. × 0.25 µm film thickness, Phenomenex, Bologna, Italy) programmed to increase from 60°C (held for 3 min) at 8°C min^{-1}, to 220°C (held for 10 min) and finally to 250°C at 10°C min^{-1} for 5 min. Helium was used as the carrier gas at a flow-rate of 1.2 mL min^{-1}. The temperature of the transfer line was 250°C. The electron impact energy was 70eV and the filament current was 50 µA.

The putative identities of the compounds were characterized by comparison with synthetic standards taking into account their GC retention indices and with mass spectra compared with Wiley mass spectra database. The most abundant compound in each extract was quantified by comparing its peak areas to that of the internal standard (Faccoli et al. 2011).

GC-EAD experiments

Two µl of the concentrated fruit extracts were injected in an Hewlett-Packard 5890 GC in splitless mode, with a polar Innowax column (30 m x 0.32 mm; J & W Scientific, Folsom, CA, USA) programmed from 60°C

(hold 3 min) at 8°C/min to 220°C (hold 7 min) with helium as the carrier gas and interfaced with the EAG apparatus (GC-EAD) (Riolo et al. 2012). The GC column effluent was combined with nitrogen make-up gas and then a 1:1 ratio between the flame ionization detector (FID) and an antenna of *D. suzukii* female. A glass capillary indifferent electrode filled with Kaissling solution containing 5g/l polyvinylpyrrolidone K90 was inserted in the severed fly's head. The different electrode was a similar capillary, brought into contact with the distal end of fly's antenna. Compounds eluting from the capillary column were delivered to the antenna through a glass tube (12 cm × 8 mm) by a charcoal-filtered and humidified air stream. The antennal signal and the FID signal were amplified and recorded simultaneously using Syntech software (Kirchzarten, Germany). Samples from fruit extracts were tested on different *D. suzukii* females. A compound was considered electrophysiologically active when it elicited at least five antennal responses that were different from background noise as described in Anfora et al. (2009).

EAG dose response curve

Based on the GC-MS and consistent GC-EAD results in all fruit extracts, synthetic isoamyl acetate was used to record EAG dose-response curves on *D. suzukii* mated females. A standard EAG apparatus (Syntech), as previously described, was used. EAG response to increasing doses of isoamyl acetate (concentrations ranging from 0.1 pg/μL to 100 μg/μL) in hexane solutions were recorded for the antennal activity. Aliquots (10 μL) of each solution were adsorbed on a piece (1.5 cm^2) of filter paper (Albet® 400), inserted in a Pasteur pipette; stimuli were applied in ascending order. The solvent was allowed to evaporate for 10 min before starting the experiments. Ten mated females were tested for each dose. Before and after each recording, EAG responses to solvent (hexane) and to a common plant volatile Z3-hexen-1-ol (1 μg/μL in hexane, Sigma Aldrich) as standard stimuli (Germinara et al. 2011) were recorded. EAG responses were analysed with EAG2000 software (Syntech), and evaluated by measuring the maximum amplitude of negative deflection (mV) elicited by a given stimulus and then subtracting the amplitude of the response to the hexane control.

Parametric one-way ANOVA followed by the Tukey post-hoc multiple comparison test was used to assess the effect of isoamyl acetate dosage on the amplitude of *D. suzukii* female antennal responses; homogeneity of variance had been determined previously with Levene's test (Statistica® 9, Statsoft *Inc.* Tulsa, Oklahoma, US).

Release rate of dispensers

Isoamyl acetate collection. SPME samples were taken under static conditions to estimate the releasing rate of dispensers loaded with isoamyl acetate used in behavioural experiments. Volatiles were adsorbed from the headspace on a fiber coated with polydimethyilsiloxane (100 μm; Supelco, Bellefonte, PA). Chemical analyses were performed on the GC-MS described above. Confirmation of the isoamyl acetate was obtained by comparing its retention time with that of the synthetic standard (Sigma-Aldrich; purity≥97%).

Estimation of collection efficiency. The SPME technique was used for a quantitative headspace sampling of the isoamyl acetate. The method for calculating the recovery capacity of the SPME-fiber and the optimal SPME recovery time was described in Anfora et al. (2005). Four dosages between 1 and 1000 ng of synthetic isoamyl acetate were diluted in 2 μL of hexane in 10 mL vials. The solvent was allowed to evaporate for 10 min before starting the experiments. Following an equilibration period of 10 min, the SPME fiber was exposed in the vial for 1 hr. The amount of isoamyl acetate collected from the headspace was calculated by comparison of the GC areas obtained by direct injections of known amounts of synthetic isoamyl acetate. The amount of isoamyl acetate emitted by rubber septa was therefore corrected according to the recovery efficacy of the synthetic compound.

Collection of the effluvia from dispensers. Headspace collection from dispensers has been described in Anfora et al. (2008). Rubber septa were placed in a 10 mL vial. The solvent was allowed to evaporate for 10 min before starting the experiments. The SPME collection started after 10 minutes of equilibration and lasted 60 minutes. The SPME fiber was then injected into a GC-MS.

Gene assembly and phylogeny

Apart from *D. melanogaster*, *D. biarmipes* was chosen for the comparative genomics in this study, since it is the only genome available, predicted as the closest relative of *D. suzukii* (Ometto et al. 2013). We mined the recently released draft genome and transcriptome of D. suzukii (Accession number: CAKG00000000.1, Ometto et al. 2013) in search of odorant receptors (OR) OR2a, OR9a, OR10a, OR19a, OR42a, OR42b, OR43b, OR47a, OR67a and OR98a. These genes were chosen because of their highest mean positive responses to isoamyl acetate in single cell recording (SSR) experiments and excitatory responses at antennal lobe level (de Bruyne et al. 2001; Hallem et al. 2004; Hallem and Carlson 2006, see also the DoOR database: Galizia et al. 2010). For comparative reasons we mined the D. biarmipes draft genome (Accession number: AFFD00000000.2). We used the above D. melanogaster OR orthologs extracted from FlyBase

(www.flybase.org: Pierre et al. 2014) as query to T-BLASTN D. suzukii and D. biarmipes genomes; contigs with e-values higher than 1e-10 in the blast searches were pulled and assembled individually using Geneious (Geneious 5.6.5). The ten putative ORs of D. suzukii and D. biarmipes were translated into amino acid using the universal code, aligned with those of D. melanogaster using Muscle (Edgar, 2004), and the resulting alignment processed for phylogenetic inference with Phyml (Guindon and Gascuel, 2003) using the LG (Le and Gascuel, 2008) model of sequence evolution plus a discrete Gamma distribution using four categories (Yang, 1996), and repeating the search on 100 bootstrap pseudoreplicates. Three types of evolutionary events were inspected on the tree topology: gene gain, gene loss and duplications.

Results

Y-tube olfactometer bioassays

Testing host fruits against D. suzukii:

Results are summarized in Figure 1. One fruit type was tested each time in the Y-tube olfactometer. Flies choosing the treatment arm generally remained on the fruits, whereas flies choosing the control arm were hyperactive often observed to move back and forth in the olfactometer. All fruit types included in the experiment were significantly attractive for *D. suzukii* female. Blueberry (χ=27.2; d.f.=1; P<0.001) was significantly more attractive compared to control, followed by cherry (χ=16.5; d.f.=1; P<0.001), raspberry (χ=12.0; d.f.=1; P<0.001), strawberry (χ=10.1; d.f.=1; P<0.001) and blackberry (χ=8.8; d.f.=1; P<0.01). However, there was no significant differences among the fruit types recorded (χ=9.1; d.f.=4; P=0.06).

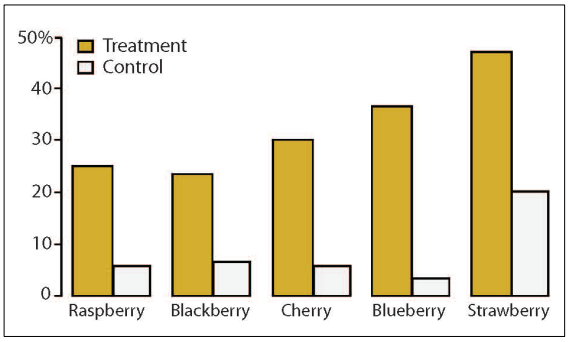

Figure 1. Percentage of flies choosing one of the arms in a Y-tube olfactometer that was either holding fresh fruit (25g) (Treatment) or fresh fruit enclosed in a transparent plastic bag (Control) (n=100): raspberry (χ^2=12.0; d.f.=1; P<0.001); blackberry (χ^2=8.8; d.f.=1; P<0.01); cherry (χ^2=16.5; d.f.=1; P<0.001); blueberry (χ^2=27.2; d.f.=1; P<0.001); strawberry (χ^2=10.1; d.f.=1; P<0.001). The comparison among fruits was not significant (χ^2=9.1; d.f.=4; P=0.06).

Electrophysiological analyses

Compounds identified from five fruit types are shown in Table 1. Analyses of Super Q extracts revealed 91 compounds, of which 29 compounds elicited consistent antennal responses in female *D. suzukii*. Of 29, ten compounds belongs to esters group, six are alcohol, five monoterpenes, acids and aldehydes two each, ketone, aromatic, irregular terpenoids and sesquiterpenes one each. In a total of 29 EAD active compounds, raspberry shared 20 EAD active compounds, 15 in blueberry, 10 in blackberry, 14 in strawberry and 10 in cherry. Female antenna responded to several odour compounds with low relative quantity (Relative Quantities are relative to the most abundant compound, set at a value of 100, in each species - RQ) in all fruits. (*Z*)-3-hexen-1-ol gave a strong antennal response (0.20±0.09) in spite of low RQ (0.8). Similarly, (*Z*)-3-hexenyl acetate (RQ 1.3; EAD 0.15±0.06) and linalool gave strong antennal response (RQ 4.1; EAD 0.17±0.08) with low RQ. In blueberry, ethanol (RQ 1.7; EAD 0.2±0.09) gave a strong response followed by linalool (RQ 11.9; EAD 0.11±0.06) and ethyl acetate (RQ 51; EAD 0.11±0.06). In blackberry, ethyl acetate (RQ 25.1; EAD 0.15±0.08) elicited strong response followed by ethyl hexanoate (56.5 ± 13.4 ng/hr; EAD 0.11±0.04). Ethyl acetate gave a strong response in strawberry (RQ 15; EAD 0.46±0.15) followed by isoamyl acetate (RQ 12.7; EAD 0.2±0.07), methyl hexanoate (RQ 99.4; EAD 0.16±0.06), ethyl hexanoate (80.9 ± 17.7 ng/hr; 0.15±0.07) and then by (*Z*)-3-hexenyl acetate (RQ 4.8; EAD 0.1±0.03). In case of cherry, compounds that elicited strong antennal response were (α)-ionone (RQ 6.4; EAD 0.2±0.08), methyl salicylate (RQ 12.4; EAD 0.15±0.06) followed by (Z)-3-hexen-1-ol (RQ 95.5; EAD 0.1±0.04). Ethyl hexanoate with highest RQ of 100 elicited varying EAD responses in blueberry (0.003±0.001), blackberry (0.11±0.04) and strawberry (0.15±0.07). While most of the monoterpenes in raspberry, (α)-phellandrene (RQ 62.3; EAD 0.003±0.001), Beta-phellandrene (RQ 30.7; EAD 0.001±0.001), limonene (RQ 11.3; EAD 0.004±0.002) and p-cymene (RQ 4.8; EAD 0.002±0.001) gave fairly weak antennal responses in spite of higher RQ. Beta-phenylethanol consistently elicited low antennal response in raspberry (RQ 9.5; EAD 0.03±0.008), blueberry (RQ 3.2; EAD 0.06±0.0.03) and strawberry (RQ 0.1; EAD 0.02±0.008). Acetic acid gave a fairly higher response in raspberry (RQ 1; EAD 0.09±0.03) and blueberry (RQ 0.9; EAD 0.09±0.07) but the response was lower in blackberry (RQ 2.4; EAD 0.02±0.07). Interestingly, isoamyl acetate gave a fairly consistent antennal response in all fruits; raspberry (RQ 1.6; EAD 0.06±0.02), blueberry (RQ 21.4; EAD 0.09±0,02), blackberry (RQ 5.2; EAD 0.05±0.02), strawberry (RQ

12.7; EAD 0.2±0.07) and cherry (RQ 13.6; EAD 0.03±0.007).

Table 1: Relative quantities of volatile compounds collected in the headspace of bagged fresh mature fruits belonging to raspberry (cv. Heritage), blackberry (cv. Dirksen), cherry (cv. Kordia), blueberry (cv. Brigitta) and strawberry (cv. Elsanta) and antennally active in GC-EAD experiments on Drosophila suzukii mated females.

Fruit	Raspberry		Blueberry		Blackberry		Strawberry		Cherry	
Compounds[c]	RQ[a]	EAD	RQ[a]	EAD	RQ[a]	EAD	RQ[a]	EAD	RQ[a]	EAD
Acids										
acetic acid	1.0	0.09±0.03	0.9	0.09±0.07	2.4	0.02±0.007				
hexanoic acid	6.1	0.08±0.05								
Alcohols										
ethanol	2.2	0.05±0.01	1.7	0.2±0.09	5.6	0.06±0.01				
hexanol			0.7	0.15±0.05			3.6	0.07±0.02	59.1	0.09±0.05
(Z)-3-hexen-1-ol	0.8	0.20±0.09	0.6	0.009±0.002	0.4	0.008±0.004			95.5	0.10±0.04
1-octanol	0.4	0.08±0.02	0.4	0.06±0.03						
1-octen-3-ol	1.9	0.04±0.01	1.8	0.16±0.05						
β-phenyl ethanol	9.5	0.03±0.008	3.2	0.06±0.03			0.1	0.02±0.008		
Aldehydes										
(E)-2-hexenal							3.0	0.07±0.04	100	0.07±0.002
nonanal	1.1	0.09±0.04	0.7	0.004±0.001					7.1	0.008±0.002
Ketones										
2-heptanone							2.8	0.13±0.04	86.4	0.09±0.02
Esters										
ethyl acetate	100	0.35±0.07	51.0	0.11±0.06	25.1	0.15±0.08	15.0	0.46±0.15		
hexyl acetate							23.9	0.09±0.03		
isoamyl acetate	1.6	0.06±0.02	21.4	0.09±0.02	5.2	0.05±0.02	12.7	0.20±0.07	13.6	0.03±0.007
ethyl butanoate	0.7	0.002±0.001	1.1	0.002±0.001						
ethyl hexanoate	9.1	0.002±0.001	100	0.003±0.001	100	0.11±0.04	100	0.15±0.07		
ethyl octanoate							1.8	0.04±0.01	4.9	0.009±0.004
methyl hexanoate							99.4	0.16±0.06		
methyl octanoate							3.6	0.08±0.02		
(Z)-3-hexenyl acetate	1.3	0.15±0.06			5.8	0.009±0.003	4.8	0.10±0.03	30.6	0.005±0.001
Aromatics										
methyl salicylate	0.4	0.09±0.03	23.0	0.09±0.02	4.4	0.03±0.007			12.4	0.15±0.06
Norisoprenoids										
(α)-ionone									6.4	0.20±0.08
Monoterpenes										
α-phellandrene	62.3	0.003±0.001								
β-phellandrene	30.7	0.001±0.001								
limonene	11.3	0.004±0.002								
p-cymene	4.8	0.002±0.001								
(±)-linalool	4.1	0.17±0.08	11.9	0.11±0.06	3.1	0.07±0.03	15.0	0.08±0.03		
Sesquiterpenes										
(E)-caryophyllene	0.8	0.05±0.01	5.0	0.08±0.06	1.0	0.007±0.002	0.2	0.03±0.005		

[a]RQ: Relative Quantities are relative to the most abundant compound (set at a value of 100) in each species.

[b]EAD: Mean ± SD antennal responses (mV) elicited by compounds contained in headspace extracts. The average amount of the most abundant compound collected from 200 g of freshly picked mature fruits: raspberry) 274.3 ± 97.4 ng/hr of ethyl acetate; blueberry) 29,8 ± 8.7 ng/hr of ethyl hexanoate; blackberry) 56.5 ± 13.4 ng/hr of ethyl hexanoate; strawberry) 80.9 ± 17.7 ng/hr of ethyl hexanoate; and cherry) 26.6 ± 11.0 ng/hr of (E)-2-hexenal. [c]Compounds were identified by comparison of their mass spectra with Wiley mass spectra database and of their retention indices with authentic standards.

Biological activity of isoamyl acetate

Isoamyl acetate elicited antennal response from all fruits we tested on GC-EAD: for this reason we performed specific EAG analyses on this likely key compound of fresh fruit. Analysis of the EAG responses to increasing doses of isoamyl acetate showed a significant dose-response relationship ($F=5.30$; d.f.=99; $P<0.001$) (Figure 2). In particular, mated *D. suzukii* females showed significant increases in their EAG response at 10 and 100 µg/µL concentration of isoamyl acetate.

In the Y-tube olfactometer behavioural bioassays, isoamyl acetate showed significant attractiveness to mated *D. suzukii* females when loaded on rubber septa dispensers at the dosage of 10 µg ($\chi^2=4.21$; d.f.=1; $P<0.05$). Conversely, *D. suzukii* was not attracted to isoamyl acetate deployed at dosages of 100 µg ($\chi^2=0.67$; d.f.=1; $P=0.57$) and 1 µg ($\chi^2=0.92$; d.f.=1; $P=0.90$) (Figure 3). The release rate of rubber septa loaded with 1, 10 and 100 µg isoamyl acetate was 0.5±0.1 ng/hr, 2.4±0.6 ng/hr and 9.8±1.6 ng/hr, respectively.

In a similar Y-tube behavioural bioassay with mated *D. suzukii* female, no significant difference ($\chi^2=0.05$; $P=0.73$) of attraction was recorded for blueberry (25g), the most attractive host fruit in the previous experiment (Figure 1), and isoamyl acetate deployed at the attractive dosage (10 µg per rubber septum).

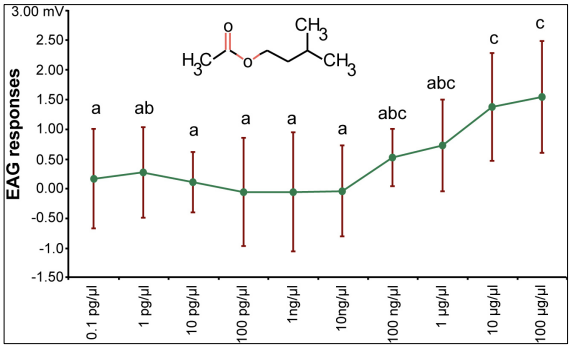

Figure 2. EAG dose-response curve (mean [mV]±SD) of *D. suzukii* mated females to isoamyl acetate (0.1 pg/µL to 100 µg/µL tested on filter paper) (n=10). Responses with the same letter are not significantly different (ANOVA, Tukey test: F=5.30; d.f.=99; P<0.001).

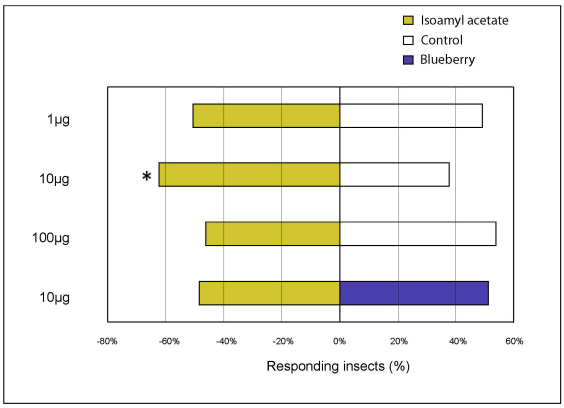

Figure 3. Percentage of flies (n=120) showing preference for isoamyl acetate (1, 10, or 100 µg loaded on a red rubber dispenser) *versus* a solvent control (10 µL hexane). Asterisk indicates significant differences in the insect choice between stimulus and control at different isoamyl acetate loadings per dispenser: 1 µg (χ^2=0.92; d.f.=1; P=0.90); 10 µg (χ^2=4.21; d.f.=1; P<0.05); 100 µg (χ^2=0.67; d.f.=1; P=0.57), ii) Also preference of the flies (n= 100) to synthetic isoamylacetate (10 µg) *versus* fresh bluberry fruits (25 g) (χ^2=0.05; P=0.73).

A complete set of isoamyl acetate specific odorant receptors in *D. suzukii*

According to literature, isoamyl acetate activates with high specificity at least ten odorant receptors (OR) in *D. melanogaster*: OR2a, OR9a, OR10a, OR19a, OR42a, OR42b, OR43b, OR47a, OR67a and OR98a (Database of Odorant Receptors: Galizia et al. 2010). We have identified all the orthologs encoding these receptors in *D. suzukii* genome (Ometto et al. 2013), retrieved and annotated their full length sequence and nearly full length or partial sequences for the others (due to genes being placed at scaffold boundaries. Phylogenetic analysis revealed that these receptors in *D. suzukii* are extremely conserved and similar to the orthologs in *D. melanogaster* and *D. biarmipes* as suggested by high bootstrap support and similar branch lengths (Figure 4). Interestingly, results show that OR67a experience a series of duplications in both *D. suzukii* and *D. biarmipes* (with a nomenclature, OR67a1-OR67a5).

Discussion

A specific and selective attractant blend for a reliable monitoring of the *D. suzukii* presence is the first step for a successful integrated pest management. At present, *D. suzukii* management is very much based on insecticides, several other conventional methods (Van Timmeren and Isaacs, 2013), as well as baits of fermenting products (e.g., wine and vinegar, Cha et al. 2013, 2012). Fermentation baits-based traps as a food attractant catch other Drosophilids in general (Wu et al. 2007; Landolt et al. 2012a), but they could possibly be more efficient if complemented with oviposition attractants specific for *D. suzukii*. Becher et al. (2012) reported that *D. melanogaster* is more attracted to fermenting yeast *Saccharomyces cerevisiae* compared to 'non-fermenting' grape juice or growth media. On the contrary, gravid *D. suzukii* females, unlike *D. melanogaster,* are likely attracted to the odours produced by fermenting fruits for feeding but prefers to find undamaged ripening fruits for oviposition (Mitsui et al. 2006; Walsh et al. 2011). Hence, identifying the cues by which *D. suzukii* finds the host fruits is crucial to understand the olfactory circuit and devise novel monitoring and control systems.

It is evident from the olfactometer results that all fruits included in the experiment are significantly attractive to egg-laying female *D. suzukii*. Such results indicate that *D. suzukii* uses olfactory cues to select oviposition sites. Furthermore, the absence of a significant difference in attractiveness across different fruit types may be an indication of the fact that *D. suzukii* is capable of infesting a wide range of hosts (Bellamy et al. 2013) for oviposition and larval feeding (Cini et al. 2012; Lee et al. 2011; Walsh et al. 2011). Similar results were reported recently by Bellamy et al. (2013) through multiple choice oviposition assays by individual *D. suzukii* females, where they show no difference in choosing blueberry, raspberry, blackberry, cherry and strawberry extracts. It may be only subtle differences in the bouquet that cause differential attractiveness in flies. Possibly, similarities in the odour profile (based on the GC-EAD results) across fruit types are decisive in attraction.

The previous literature reporting EAD-active compounds for *D. suzukii* used headspace extracts from wine and vinegar (Cha et al. 2013, 2012). Here we identified EAD-active compounds from the host plants of *D. suzukii*. We identified 29 active volatile compounds through GC-EAD from commercial varieties of blueberry, blackberry, raspberry, cherry and strawberry that elicited electrophysiological response in female *D. suzukii*. EAD active compounds include esters, alco-

hols, acids, aldehydes, ketones, aromatics, monoterpenes, sesquiterpenes and irregular terpenoids. While most of the monoterpenes were detected only in the raspberry extract (except linalool in blueberry, blackberry and strawberry) had fairly higher RQ but gave weaker EAD response. Some of these compounds were reported to be repellent in other insects (Jaenson and Pålsson, 2006). Both the absolute amounts and relative ratios of compounds released varied substantially across different fruit types. Regardless of the amount released, most of the esters and volatile alcohols from ripening fruits were found to be EAD-active. A number of the EAD active compounds identified are known to elicit electroantennographic responses from other fruit fly species (e.g., Zhu et al. 2003). Ethyl hexanoate, was one such compound generally present in fruit extract and Drosophila feeding substrate (Stensmyr et al. 2003) was present in all fruit types (except cherry) and elicited notable EAD responses. Female adult *D. suzukii* responded also to few key volatiles like ethanol, acetic acid and Beta-phenyl ethanol, which are major byproducts of fermentation. These are also present in the fresh fruits at lower concentration and are attractive to a diverse set of insect species (Zhu et al. 2003).

Within *D. suzukii biology,* fruits infested by the larvae collapse and undergo decay thus providing feeding site for the adult flies (Beers et al. 2011). The females use fermenting fruits as a mate finding and feeding site, whereas ripening fruit for oviposition (Ometto et al. 2013). Generally, fermenting fruits attract Drosophilids with two major volatile components: ethanol and acetic acid (Zhu et al. 2003; Stökl et al. 2010; Becher et al. 2012). Our GC-EAD and GC-MS results show ethanol and acetic acid in small amount in raspberry, blueberry, and blackberry extracts. However, both acetic acid and ethanol were not detected in the fresh cherry fruit, which is consistent with similar reports (Mattheis, 1992; Sun et al. 2010). Yet, cherry is the primary host fruit for *D. suzukii* (Kanzawa, 1939). This may indicate that the ethanol+acetic acid based mixes, commonly used for attracting *D. suzukii* (Landolt et al. 2012b) will attract the flies as feeding attractant but bears little relevance for oviposition-site searching. Mixtures that mimic suitable oviposition sites therefore likely require other compounds.

Ethyl acetate was another abundant volatile compound recorded in all fresh fruits except cherry and elicited strong electrophysiological response. It is a highly volatile ester and is attractive to several insect species (Larry and Hengchen, 1991; Stensmyr et al. 2003). Interestingly, Cha et al. (2012) demonstrated that a mixture of acetic acid and ethanol by itself was strongly attractive to *D. suzukii,* while ethyl acetate along with acetic acid and ethanol proved to be deterrent. Based on the responses of female *D. suzukii* on GC-EAD, ethyl acetate needs further behavioural tests with different concentrations similar to isoamyl acetate in order to elucidate its ecological/ behavioural role for *D. suzukii*.

Isoamyl acetate, ubiquitous in the odour bouquet of both ripening, ripe and early fermenting fruits and eliciting a response in many Drosophilids (Stökl et al. 2010), was considered a key compound for *D. suzukii* as (i) it was present in the headspace of all fruits, (ii) always induced relative EAD response across runs in mated female. Hence its relevance as a oviposition cue for *D. suzukii* was evaluated in the olfactometer. The attraction in the olfactometer was significant at 10 μg concentration than at 1 and 100 μg, respectively. Interestingly, the release rate from rubber septa at 10 μg is comparable to that we have recorded from fresh fruits and, in accordance, also from Y-tube bioassay there were no statistical differences in attraction recorded between isoamyl acetate and blueberries, pointing out the crucial role of the release rate for the discrimination between unwounded and fermenting fruits. As a matter of fact, isoamyl acetate is also one of the major compounds produced in the fermentation process of fruits but at a rate of about 100-folds higher than in fresh fruit (Cha et al. 2012; Zhu et al. 2003). Both the absolute and the relative amount of ubiquitous plant volatiles was shown as one of the critical factors mediating the recognition and the species specificity of the host-plant profiles (Bruce et al. 2005; Tasin et al. 2010), and as such we expect that both the amount of isoamyl acetate and the mixture in which it is embedded is of significance in oviposition stimulation.

Our genomic survey seems to fit with behavioural and electrophysiological results since it indicates that *D. suzukii* not only possess the complete putative full repertoire of genes encoding odorant receptors normally activated by isoamyl acetate in *D. melanogaster*, but also shows that one of them, OR67a (Hallem and Carlson, 2006) underwent a series of duplications. Within a general pattern of balanced birth and death process of evolution (Robertson and Kent, 2009), indeed, OR67a gene duplications may have an adaptive role in *D. suzukii* biology such as an increase affinity for fresh fruit. Counter intuitively, the same duplications are shared with sister species *D. biarmipes*, suggesting that an affinity for fresh fruit is not unique to *D. suzukii* but was already present in the common ancestor with *D.*

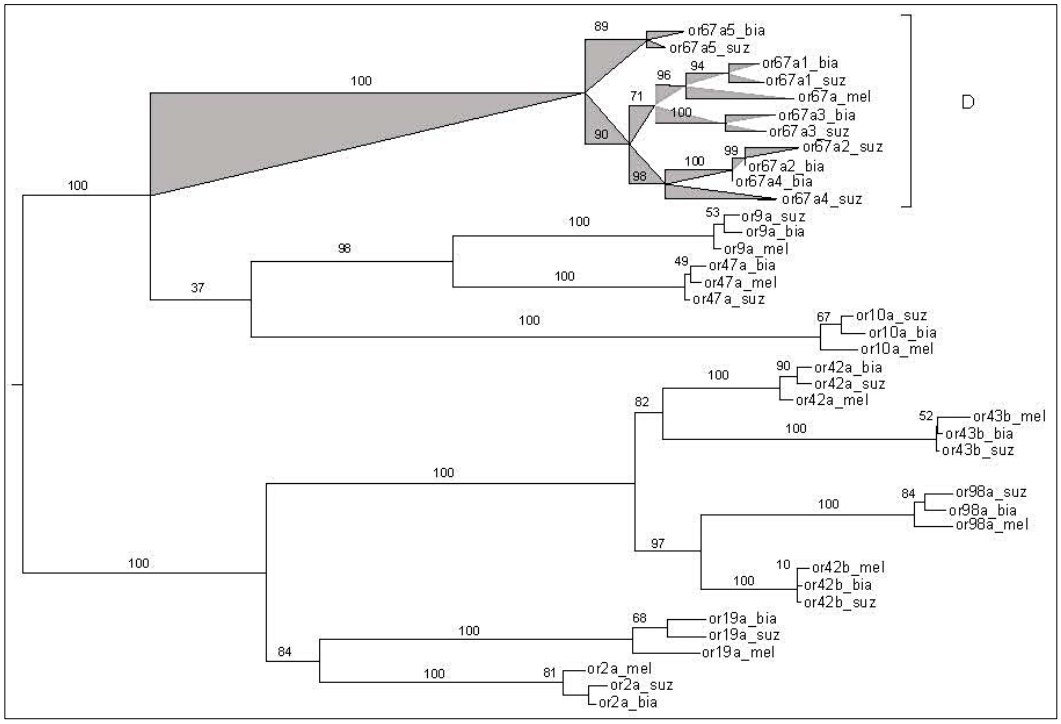

Figure 4. Phylogenetic tree of ten genes encoding a set of olfactory receptors (OR2a, OR9a, OR10a, OR19a, OR42a, OR42b, OR43b, OR47a, OR67a and OR98a) that bind isoamyl acetate in D. suzukii, D. biarmipes and D. melanogaster using Phyml. The numbers specify the bootstrap value, indicating the branch support for each node. The tree is rooted on midpoint. Three types of evolutionary events were studied: gene gain, gene loss and duplications (D).

biarmipes. We know virtually nothing of the biology of *D. biarmipes*, nor we know where these genes are expressed: further behavioural, functional characterization, and expression studies are required to assess the fate of the duplication event in *D. suzukii*. OR67a duplicates are however good candidates to explain fruit odour perception in *D. suzukii* females; these genes may become targets of functional characterisation by orthologous expression in empty neuron systems (Dobritsa et al. 2003) aimed at identifying the most effective agonist or antagonist compounds for field applications.

Isoamyl acetate is also released by microbe-infested fruits (Witzgall et al. 2012), such as through the epiphytic community on fruits surface as well as in fermenting substrates. According to our protocol, we however do not know what would be the relative ratio of isoamyl acetate emission between fresh fruits and the natural community of microbes on their surface.

The identification of the most behaviourally-active volatiles emitted by hosts of *D. suzukii* other than isoamyl acetate and those responsible for the different susceptibility of fruits to oviposition, might help the process of the development of more selective and powerful attractant lures. Further research is hence required to test isoamyl acetate activity in the presence of other EAD-active compounds from the host fruit. Apart from the monitoring and control, the species provides a unique opportunity to unravel the changes in the olfactory circuitry that accompany the shift from rotten to ripe for oviposition. The whole spectrum of the present and future studies would help to understand the evolution of the olfactory code among the closely related species of *Drosophila* and, as a consequence, contribute to alternative control methods of *D. suzukii*. Indeed, comparison of *D. suzukii* with sibling species and *D. melanogaster* could shed light on the evolution of morphological, behavioural and physiological innovations (as shown in other *Drosophila* flies, Dekker et al. 2006) and help researchers in understanding what makes a species to be an invasive pest.

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From pheromone to behavioral antagonist: *cis*-vaccenyl acetate loss in *Drosophila suzukii* reverses its role in sexual communication.

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Abstract

Pheromones are often species specific and play an active role in species recognition and the speciation process. The *Drosophila* pheromone *cis*-11-octadecenyl acetate (*c*VA), however, does not seem to fit such a role: it is used by species throughout the *melanogaster* group (Dahanukar & Ray, 2011; Symonds & Wertheim, 2005), and therefore does not seem to convey species identity. Instead, *c*VA has a generic function in assessing sex, mate quality, mating status, and social interactions (Dahanukar & Ray, 2011; Dickson, 2008). In spite of the primal role of *c*VA signaling in *Drosophila*, we report here that *Drosophila suzukii*, an invasive species that oviposits in undamaged soft fruit, does not produce *c*VA. In fact, its production site, the ejaculatory bulb (Guiraudie-Capraz et al., 2007), was atrophied. Yet, T1 sensilla, which express Or67d neurons and are essential in *c*VA-mediated behaviors (Kurtovic et al., 2007; Liu et al., 2011; Stockinger et al., 2005; Weng et al., 2013), were fully functional. T1's were though rare in *D. suzukii*, and the corresponding antennal lobe glomerulus, DA1, minute. As in *D. melanogaster* the behavioral response to *c*VA is determined by the input balance from Or67d neurons (driving *c*VA-mediated behaviors) and Or65a neurons (modulating *c*VA-mediated behaviors), we hypothesized that the shift in glomerular balance observed in *D. suzukii* would reverse *c*VA's role in mating from an aphrodisiac sex pheromone to an inhibitor of sexual behavior. Indeed, perfuming *D. suzukii* males with the same levels of *c*VA found in *D. melanogaster* strongly reduced mating, whereas perfuming *D. melanogaster* males with *c*VA did not negatively affect mating. In *D. suzukii*, *c*VA has thus evolved from an generic aphrodisiac sex pheromone signal to a heterospecific signal that disrupts mating, a saltational shift mediated through offsetting the input balance that is otherwise highly conserved in congeneric species.

Keywords: Drosophila suzukii, *cis* vaccenyl acetate, Drosophila melanogaster, or67d.

Introduction

Melanogaster-clade species generically use male-produced *c*VA as sex pheromone (Symonds & Wertheim, 2005). The underlying *c*VA is arguably the best studied pheromone communication system, from production to detection, and from processing to resulting muscle output (Dahanukar & Ray, 2011) (Yu et al., 2010). Different from 'typical' sex pheromone in insects, *c*VA acts at short range and not only fulfills a role in orientation and sexual attraction, but also most prominently in a variety of sexual and social behaviors in *Drosophila melanogaster*: increases mate acceptance by females (Kurtovic et al., 2007; Weng et al., 2013), reduces attractiveness of newly mated females, reduces male-male courtship, while increasing aggression between males (Liu et al., 2011; Wang & Anderson, 2010). *c*VA is a sex pheromone found throughout and basal to the *melanogaster* group species, such as in the *obscura* and *immigrans* group species (Symonds & Wertheim, 2005), underlining its primal role in *Drosophila*.

The fact that all *melanogaster* group species studied thus far use *c*VA as volatile sex pheromone (Symonds & Wertheim, 2005) would preclude a role of *c*VA in species recognition, a function that is otherwise typical for insect sex pheromone (Symonds & Elgar, 2008). Moths in particular are well known for their species-specific pheromone blends, which are used to discrim-

inate between conspecific and heterospecifics (Löfstedt 1993). Whereas males are finely tuned to their conspecific female-produced pheromone, they often also display acute sensitivity to pheromones of other, often neighboring, species. In the latter case, such pheromones elicit responses on sensory neurons that mediate behavioral antagonism, and typically disrupt orientation to conspecifics (Baker, 2008).

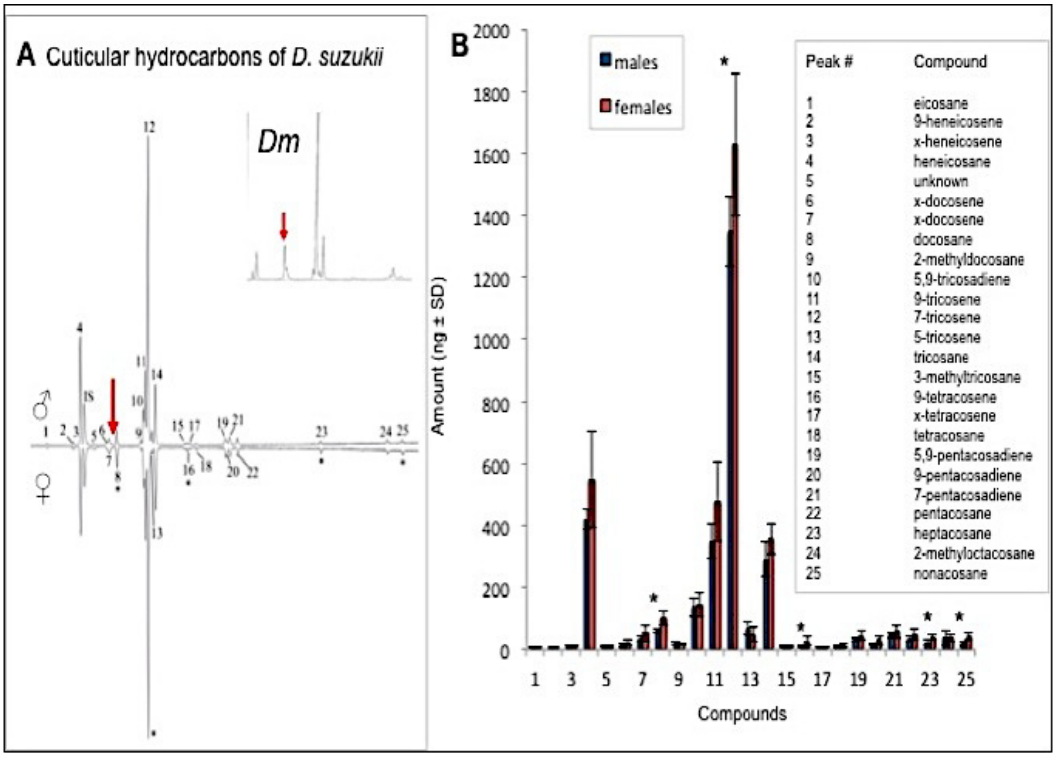

Figure 1 (A) Chromatograms showing female and male *D. suzukii* cuticular hydrocarbons. Arrow indicates retention time where *c*VA would elute. Inset: chromatogram of *D. melanogaster* with the arrow indicating *c*VA (see also Supplementary Fig. S1). Note the dominance of tricosenes in both sexes. Tricosenes are more abundant in male *D. melanogaster*'s CH profile. ***IS***=internal standard (heptadecenyl acetate, 17:OAc). X= unknown double bond position. (B) Comparison of the cuticular hydrocarbon profile of male and female *D. suzukii* (n=6 and n=5, respectively). Stars indicate significant differences between males and females (Mann-Whitney test, $\alpha<0.05$).

Perhaps due to the complex role *c*VA fulfills in a range of sexual and social behaviors, *Drosophila* evolution of the signal to convey species-specificity may be constrained. Besides, other, non-volatile *Drosophila* pheromones fulfill a function in species-recognition instead (Ferveur, 2005). These are non-volatile pheromones, produced by oenocytes and embedded in the cuticular hydrocarbons, and sensed through the taste sensilla on the legs and proboscis (Billeter et al., 2009, 2012; Miyamoto & Amrein, 2008).

However, in spite of the evolutionary constraints *c*VA may have, here we report how this conserved pheromone has undergone a radical functional reversal in *Drosophila suzukii*, a *melanogaster* group species. *D. suzukii* does not produce *c*VA. Here we describe the changes that are at the basis of the inversion of the role of *c*VA from a broadly used sex pheromone to a behavioral antagonist in this species.

Results and Discussion

Using gas chromatography coupled mass spectrometry (GC-MS), we analyzed the cuticular hydrocarbon (CH) profile of *D. suzukii*. Unlike the CH profile of *D. melanogaster*, *D. suzukii*'s CH profile was isomorphic, with just small quantitative differences between sexes (Fig.1A, B). This was also true for its sibling species *D. biarmipes* and *D. subpulchrella* (Supplementary Fig. S1 B, C). We note that *D. suzukii* and *D. biarmipes* lacked the desaturase *desat2* and the elongases *eloF*, which have been implied in the biosynthesis of species-specific and sexual dimorphic CH profiles in *Drosophila* (Chertemps et al., 2007; Fang et al., 2009; Wicker-Thomas & Chertemps, 2010) (Supplementary Fig. S4). However, more striking was the lack of *c*VA in the chemical profile of *D. suzukii* (Fig. 1A, arrow, verified with *D. suzukii* from the USA- Supplementary Fig. S1D). The lack of *c*VA

in *D. suzukii* casts the question of whether this pheromone might have been replaced by another compound similar to *c*VA. However, no other *c*VA-type compounds (including the C20 variant present in some species (Symonds & Wertheim, 2005)) were found in *D. suzukii* cuticular extraction (Fig1A, B and Supplementary Fig. S1D). In fact, the ejaculatory bulb, the production site of *c*VA (Brieger & Butterworth, 1970), was dramatically reduced in volume in *D. suzukii* compared to that of *D. melanogaster* (7.0x, p<0.001, n=10, unpaired t-test, Fig. 2A). In fact, conserved homologues of corresponding genes for *c*VA production, *elo*68 and *desat1*, were nevertheless present in the genome of *D. suzukii* (Table 1 and Supplementary Fig. S3 A1, A2, B), as were miRNA-124 binding sites upstream of *transformer*, a factor involved in *c*VA production in male *D. melanogaster* (Weng et al., 2013) (Supplementary Fig. S4D).

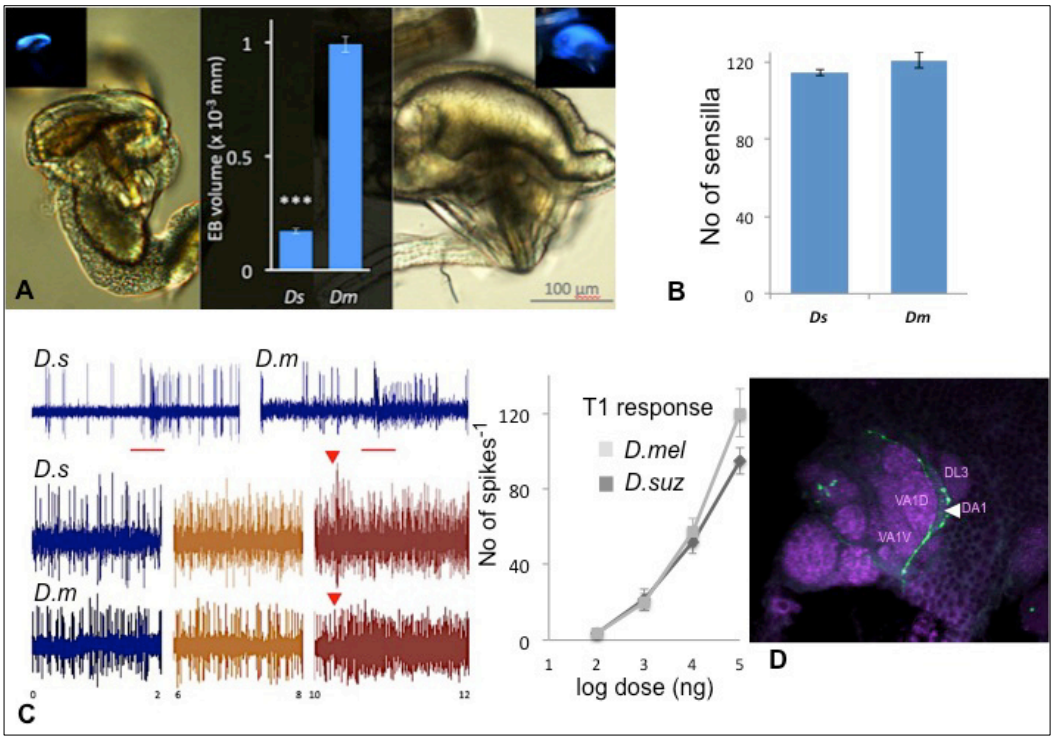

Figure 2 (A) Micrographs of the ejaculatory bulb, lateral view, light and autofluorescence microscopy (insets). Overlay: volumetric estimates of the EB of *D. suzukii* (Ds) and *D. melanogaster* (Dm) (n=10 for each species, independent t- p<0.001). (B) Number of sensilla trichoidea and sensilla intermedia in *D. suzukii* and *D. melanogaster* females. (C) Top: sample trace of T1 sensilla to 0.5 s 1 µg cVA stimulation. Bottom: sample traces of T4 sensilla in *D. suzukii* and *D. melanogaster* to *c*VA, using the 'touch' stimulation (Van der Goes van Naters & Carlson, 2007), with the time (s) indicated at the bottom of the traces. Blue: before stimulation, orange: just prior to contact, red: contact. Side panel: dose response curves of T1 sensilla in *D. suzukii* and *D. melanogaster* to 0.5 s *c*VA stimulation (red bar). (D) Neurobiotin backfill of T1 neuron (a spurious fill of a neighboring AB7 neuron toVM5v is also visible). Letters indicate various anterior trichoid glomeruli. Arrowhead indicates DA1.

We then determined how loss of *c*VA might have influenced its corresponding olfactory circuitry. In *D. melanogaster*, the trichoid sensillum T1 and its cognate Or67d-expressing neuron (Kurtovic et al., 2007) are key in *c*VA-mediated behaviors. T1 project to a large glomerulus, DA1 (Couto et al., 2005; Stockinger et al., 2005). T1s are the most abundant sensillum type in *D. melanogaster* (Shanbhag et al., 1999). Although T1s were fully functional (Fig. 2C), and its receptor, Or67d, highly conserved (Table 1 and Fig. 5), T1 sensilla were rare in *D. suzukii* antenna: we identified only around 7-10 per individual, compared to 55-60 found in *D. melanogaster* (two-tailed independent t-test, p<0.0001 Fig. 3, Supplementary Fig. S2).

At close range *c*VA also induces responses in Or65a expressing OSNs housed in antennal trichoid T4 (Couto et al., 2005; Ejima et al., 2007; Van der Goes van Naters & Carlson, 2008). Or65a neurons counteract behavior induced through Or67d OSNs, reduces *c*VA-mediated male-male aggression (Liu et al., 2011), as well as male attraction to recently-mated females (Ejima et al., 2007). In *D. suzukii* Or65a was highly conserved (Table 1), and T4 sensilla were abundantly present and responded to *c*VA touch stimulation (Van der Goes van Naters & Carlson, 2008) (Fig. 2C). The neurons may be slight-

ly more sensitive to *c*VA in *D. suzukii* than in *D. melanogaster*, responding prior to contact, unlike in *D. melanogaster* (less than 1mm distance). The cognate antennal lobe glomerulus, DL3, which receives input form Or5a neurons, was enlarged in *D. suzukii* (two-tailed independent t-test $p<0.001$, Fig. 3) and 19% larger in male than female *D. suzukii* (two-tailed independent t-test, $p=0.021$). Other glomeruli innervated by sensilla trichoidea and sensilla intermedia neurons (Couto et al., 2005; Shanbhag et al., 1999) were similar in volume between the two species (except for DA4m) and sexually isomorphic (Fig. 3). The total number of trichoid sensilla was similar between species (Fig. 2B). Or65a and DL3 provide sensilla context dependent suppression of *c*VA responses in *D. melanogaster*, putatively by suppressing output of DA1 via antennal lobe inhibitory interneuronal connections (Liu et al., 2011). We therefore hypothesized that the reversal of glomerular volume ratios in *D. suzukii*'s *c*VA circuit (DA1/DL3 from 3.3 in *D. melanogaster* to 0.41 in *D. suzukii*, Fig. 3) would favor Or65a-mediated behavioral responses to *c*VA, generating opposite behavioral outputs to those observed in *D. melanogaster*. We tested this by applying *c*VA to male *D. suzukii* cuticle with doses equivalent to those found on male *D. melanogaster*, and assaying the effect on conspecific courtship behavior. Because mating rates are low in *D. suzukii*, and because applied *c*VA rapidly decreases over time on the cuticle (Fig. 4, red line), we grouped virgin males and females to ensure sufficiently high courtship and mating rates in the bioassay. As we predicted, application of *c*VA on male *D. suzukii* strongly suppressed mating (Fig. 4 insets, Kaplan Meier estimator, $Z=4.45$, $p<0.001$, $n=150$). In contrast, perfuming male *D. melanogaster* with *c*VA did not affect the mating rate (Fig. 4 insets, Kaplan Meier, $Z=0.33$, $p=0.36$, $n=110$, (Kurtovic et al., 2007; Weng et al., 2013)). Following an initially near to complete shutdown of mating in *D. suzukii*–when *c*VA doses were high, the mating inhibition slowly dissipated with time (Fig. 4, blue line, the relative mating rate of perfumed or non perfumed flies). This was significantly correlated with a gradual loss of *c*VA on the cuticle (Fig. 4, Pearson's $r=0.93$, $t=4.38$, $p<0.025$). The down regulation of T1 sensilla expression, the volume decrease of DA1 and the suppression of mating in *c*VA-perfumed *D. suzukii* support a model in which Or65a and DL3 suppress Or67d-mediated behaviors similar to observed in *D. melanogaster* (Ejima et al., 2007; Liu et al., 2011; Wang & Anderson, 2010). Apparently, in *D. suzukii* the balance of DA1 and DL3 input and output shifted though evolution, resulting in a chronic suppression of *c*VA-induced behaviors in this species, when compared to other melanogaster group species. This effectively has reversed the role of *c*VA from a pheromone to a heterospecific signal that inhibits mating.

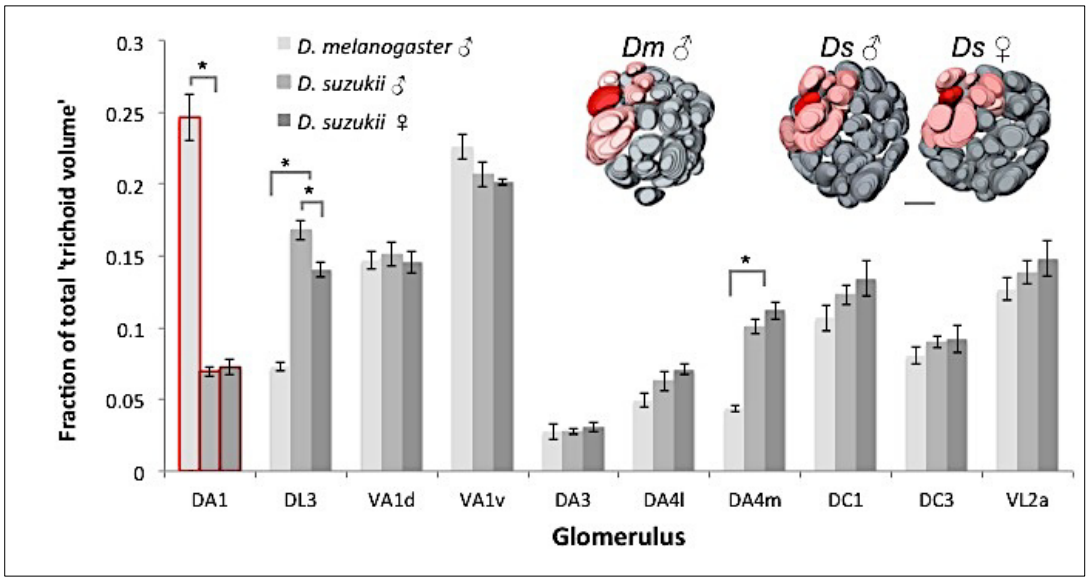

Figure 3 Volume of DA1 and other trichoid glomeruli, relative to the total volume of all glomeruli receiving input from sensilla trichoidea and intermedia. VL2a, which receives input from coeloconic Ac4a neurons, is included as it is part of the *fru* circuitry. Red outlined bars: DA1 of *D. melanogaster* and *D. suzukii* ♂ and ♀. Insets are reconstructions of the antennal lobes, with in bright red DA1 and in light red other glomeruli that receive input from sensilla trichoidea and sensilla intermedia neurons. Scale bar 20 μm.

The concurrent miniaturization of DA1 volume and the behavioral shift in response to *c*VA is reminiscent of observations in *Drosophila*'s coding of general odor preference (Dekker et al., 2006; Ibba et al.,

2010) and preference coding of pheromones in moths (Kárpáti et al., 2010). In these studies changes in relative glomerular volume were associated to shifts in olfactory preference. Increased glomerular volume is associated with an increased preference for the ligand of these glomeruli. In the present study, however, we observed the opposite: a severe reduction in glomerular volume converts attraction into inhibition, suppressing the cascade of behaviors associated with its ligand.

What factors underlie the reduced T1 expression and diminution of DA1 are unknown. An important factor in driving sexual behaviors in *D. melanogaster* is the transcription factor *fruitless* (*fru*). A male-specific splicing variant of *fru*, FruM, causes sex-specific neuronal growth, targeting and corresponding behavior (Cachero et al., 2010; Datta et al., 2008; Demir & Dickson, 2005; Ruta et al., 2010; Stockinger et al., 2005). Our gene annotations show nevertheless that the *fru* region, spanning 100kb of genomic DNA, contains all putative exons to build the various isoforms found in *D. melanogaster*, including FruM (Supplementary Fig. S4, (Stockinger et al., 2005)). Other well-known genes involved in sexual dimorphism of the olfactory circuitry, such as *sexlethal* (*sxl*, (Billeter et al., 2006; Rideout et al., 2011), *transformer* (*tra*, (Billeter et al., 2006; Fernández et al., 2010), and *doublesex* (*dsx*, (Billeter et al., 2006; Rideout et al., 2011) were also well conserved in *D. suzukii* (Supplementary Fig. S4). However, we noted that the volume of DA1 is sexually isomorphic in *D. suzukii* (Fig. 3). This contrasts with *D. melanogaster*, where Fru causes a substantial dimorphism in volume and behavior between males and females (Demir & Dickson, 2005; Stockinger et al., 2005). Similarly, the two other glomeruli receiving input from sensory neurons in the *fru* circuitry, VA1v and VL2a, were also sexually isomorphic (Fig. 3). However, DL3 was 19% enlarged in male compared to female *D. suzukii*, whereas DL3 is isomorphic in *D. melanogaster* (Stockinger et al., 2005). This may indicate an altered expression of Fru in the olfactory circuitry of *D. suzukii* males and females. This notion of an altered expression pattern in *D. suzukii* is substantiated by the observation that, contrary to *D. melanogaster*, Fru is translated in brains of both sexes of *D. suzukii*, whereas in *D. melanogaster* only in males (Yamamoto et al., 2004).

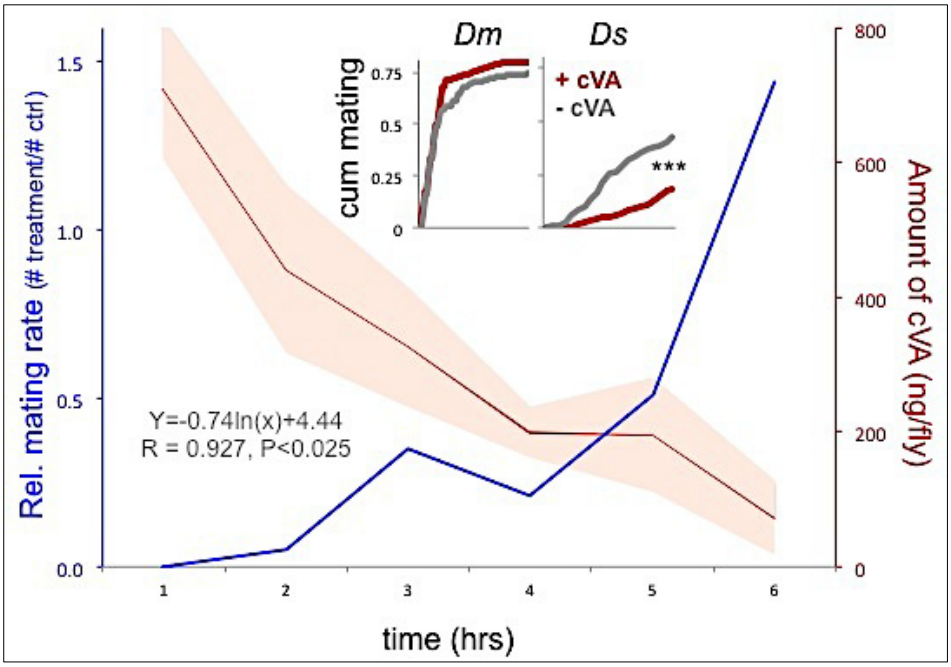

Figure 4 Effect of *c*VA perfuming on mating in *D. suzukii* and *D. melanogaster*. The relative mating rate is increasing (blue line) with a decreasing amount of *c*VA on the male flies. Insets: cumulative mating in *Dm* and *Ds* in response to the perfuming with *c*VA (+*c*VA, red lines) or hexane (control, -*c*VA, grey lines).

The loss of *c*VA as *D. suzukii*'s sex pheromone may be an adaptation associated with its new niche, or reflect this species' higher reliance on visual cues (males have a conspicuous wing spot) over olfactory cues in sex discrimination. It may also be simply the result of drift, although the presence of *c*VA throughout the *melanogaster* group may render this a less likely scenario. We therefore compared *D. suzukii* to the other two *suzukii* group species, *D. subpulchrella* and *D. biarmipes*, males of which also have conspicuous wing spots (Rota-Stabelli et al., 2013). Whereas both species were found to have sexually monomorphic CH profiles (Supplementary Fig. S1),

D. biarmipes, a species that oviposits on rotten fruit, produced large amounts of *c*VA, whereas *D. subpulchrella*, a species with a similar ovipositor and oviposition preference as *D. suzukii*, lacked *c*VA. As expected, the relative volume of DA1 in *D. subpulchrella* was much reduced and comparable to DA1 in *D. suzukii*, whereas that of *D. biarmipes* was similar to *D. melanogaster* (Fig. 6). Apparently, loss of *c*VA is not associated with the use of a conspicuous wing spot. Instead, our data suggests that the loss co-occurs with the shift to fresh fruit. Although this study does not exclude the existence of other volatile pheromones in *D. suzukii*, the CH extracts and the 'relictual' size of the ejaculatory bulb

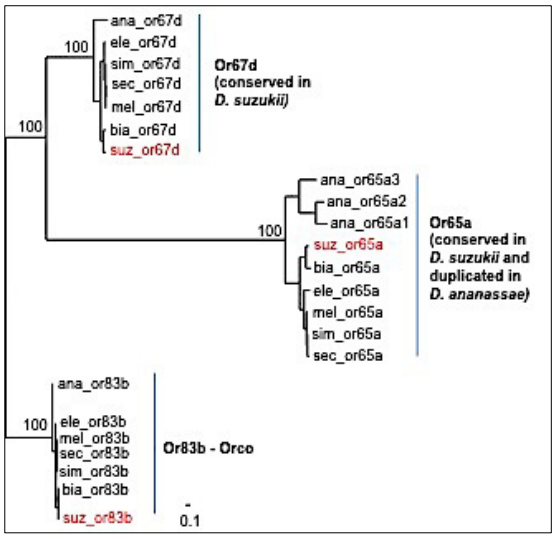

Figure 5 *c*VA odorant receptors are conserved in *D. suzukii*. Phylogenetic tree of Or67d and Or65a in *D. suzukii* and other *Drosophila*. The tree is rooted with the Or coreceptor Orco. Abbreviations and supports are as in Supplemental Fig. S3.

indicate otherwise. Furthermore, we show that in spite of the fact that *c*VA signaling fulfills a primal role and is highly conserved in the *melanogaster* group, it can rapidly evolve to serve a radically opposite behavioral and ecological function, mediated by offsetting the balance of sensory input.

Materials and methods

Flies

D. suzukii colony was established from locally collected specimen in Valsugana, Trentino Province, Italy, whereas the US strain of *D. suzukii* was derived from a colony established by D. Walsh (Washington State University, Prosser, WA, USA). *D. biarmipes* (14023-0361.02) and *D. subpulchrella* (14023-0401.01) were obtained from the San Diego stock center. Flies were reared in a quarantine facility on a standard cornmeal-yeast-agar medium at 21°C under L:D=12:12.

Behavioral assays

Sexes were separated within 1 hr post emergence and placed in groups of 5 (females) and 7 (males). They were placed in standard food vials, and flipped after 3 days to new food vials. After 4 days 7 males were placed into the vial containing 5 females. We used groups to increase the mating incidence of *D. suzukii*, which has a lower mating rate compared to *D. melanogaster*. The higher ratio of males to females offset any potentially negative effect of the shaking on 'availability' of male mates, although no clear negative effects of the procedure of perfuming flies (shaking) were observed. No individuals died during the course of the experiment. Since individuals could not be reliable recognized and followed during the course of the experiments, we scored mating rates only.

Perfuming flies

Three ml vials were coated on the inside with a total of 50 µg *c*VA. The procedures were roughly similar as described in (Billeter et al., 2009). Fifty µl hexane with or without 1 µg of *c*VA was pipetted in a 3 ml glass screw cap vial. The hexane was slowly evaporated while the vial was placed in a horizontal position and slowly rotated. Seven males were briefly immobilized on ice (1 min), and placed in a 3 ml treatment or control vial. Vials, coated with *c*VA or hexane treated only and containing 7 flies, were placed on a rotator and rotated at 4500 rotations/min for 2 minutes, in a cycle of 6 s on, 4 s off. In *D. melanogaster* *c*VA deposits are mostly found on the abdominal segments. However, perfuming the fly with *c*VA results in more equal distribution of pheromone across the body. We therefore increased the total amount of *c*VA slightly to compensate for lower local concentrations on the fly (see Fig. 3). After shaking, treatment (+*c*VA) and control (-*c*VA) male flies, both shaked on the rotator, were introduced into the food vial containing 5 virgin females. Matings were noted every 15 min. As *D. suzukii* exhibits low mating rates compared to *D. melanogaster*, we observed the mating behavior for both species during 5 consecutive hours.

Chemical analysis

Pheromones were extracted from the fly cuticle by leaving individual flies in 100 µl of hexane for 5 min at room temperature. Hundred ng of heptadecenyl acetate was added as an internal standard. These extracts were analyzed on a gas chromatograph coupled with a mass spectrometer (GC-MS; 6890 GC and 5975 MS, Agilent technologies Inc., Santa Clara,

CA, USA). Extracts were concentrated to ca. 10 µl and 2 µl were injected into a HP-5MS silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness; Agilent technologies Inc.) that was temperature- programmed from 50 (2 min) - 8°C/ min- 300°C.

Compounds of interest were identified based on their mass spectrum, retention time, comparison with already published works on *D. melanogaster* cuticular hydrocarbons (Everaerts et al., 2010) and injection of synthetic corresponding compound (for *c*VA). They were quantified by peak integration and comparison with the response of the internal standard.

Sensory physiology

Using a strong airflow flies were pushed head first into a truncated pipette tip. The pipette tip was cut distally from the head and the fly was gently pushed forward until the head protruded from the narrow end. The pipette tip was placed on a wax surface on a microscope slide, and using a glass micropipette the right antenna was gently bent backwards and stably positioned on a cover slip. The fly was placed under a microscope (Olympus BX51W1), with a magnification ≤1500 x. Via a glass tube a 1 L/min charcoal purified and humidified airflow was constantly blown over the fly head. Tungsten microelectrodes, sharpened in a KNO_2-solution, were used for recording of action potentials of antennal sensory neurons. A motor-controlled micromanipulator (Märzhauser DC-3K, Wetzlar, Germany) equipped with a piezo unit (Märzhauser PM-10) was used for fine positioning. A reference electrode was inserted into the eye with a manually controlled micromanipulator (Narishige MM33, Tokyo, Japan). Touch stimulation was performed using an additional piezo unit to move the electrode at micrometer scale toward the sensillum. A glass electrode drawn to a sharp tip was dipped briefly in a 1 µg/µl *c*VA solution, air dried and used for stimulation. After A/D conversion (Syntech IDAC PCI card), spikes were visualized and stored on a PC. Analysis was done using AutoSpike 3.2 software (Syntech, Kirchzarten, Germany).

Counts of sensilla trichodea

Antennae were mounted using spacer rings (Secure-Seal TM imaging spacers, Sigma-Aldrich) and Vectashield mounting medium Hard Mount (Vector Laboratories, Burlingame, CA). High-resolution confocal scans (Zeiss LSM 510 confocal microscope, Carl Zeiss, Jena, Germany) using a 20x objective, were made through the antennae. The contrast of the relatively thick walled trichoid sensilla permitted identification and counting on the entire antennal surface.

Ejaculatory bulb

D. suzukii and *D. melanogaster* adult males were collected 2 days after emergence. For each species, a total of 10 males were selected. After brief anaesthetization in the freezer, individuals were dissected in phosphate buffered saline (PBS). The abdomen was clipped and immersed in 5% KOH for 5 hr to remove soft tissues and expose intact hard cuticular structures, washed in distilled water and partly dehydrated in 70% ethanol. Afterwards, the ejaculatory bulb (EB) (Bairati, 1968; Chen, 1984) was separated from the rest of the male genitalia and mounted on a glass slide with glycerin. Observations were made with microscope (Leica LMD7000, Wetzlar, Germany). The EB dimensions were measured by Leica LMD7000 microscope with Leica Application Suite Image Analysis Software. Width and height and depth of the gland were measured (n=10). As individuals within and among species differed in size and in order to obtain values comparable from one animal to the others, the values of EB width were normalized by the body length of each individual according to the formula: EB width/body length x 100. Volume was calculated by assuming a sphere ($4/3*\pi*\{w/2\}*\{h/2\}*\{d/2\}$). All measurements were averaged among the 10 observations and two-tails unpaired t-test was used for statistical comparison.

Immunocytochemistry

We verified antennal lobe projection patterns of T1 neurons in *D. suzukii* using anterograde-neurobiotin (Molecular Probes, Carlsbad, CA, USA) backfills. Neurobiotin is readily taken up by neurons and transported throughout the neuron, including its axonal targets in the antennal lobes. A glass microelectrode with a 0.25 M KCl + 2% neurobiotin was placed over the tip of a T1 neuron. Neurobiotin was allowed to diffuse into the sensillum and taken up by the neuron for 1 hr. Preparations were then fixed in 0.1 M PBS with 0.25% Triton-X+4% formaldehyde for 3.5 hr at 4°C, dissected, washed 3x with PBS containing 0.25% Triton-X (PBST), and incubated with fluorescein-avidin. Ten percent mouse α-synapsin antibody (Hybridoma, Univ. of Iowa, Iowa, IA, USA) was included to identify targeted glomeruli in the AL. After 24 hr at room temperature on the rotator brains were washed 3x with PBST, and incubated with anti-mouse conjugated with Alexafluor 546 (Molecular Probes). After another 24 hr on the rotator at RT, brains were washed 3x with PBST and mounted in Vectashield Hard Mount (Vector Laboratories, Burlingame, CA), 0.12 mm thick, using spacer rings (Secure-Seal TM imaging spacers, Sigma-Aldrich). The above-described technique for mouse α-synapsin antibody staining was also used for overview stain-

ings and reconstructions of antennal lobes of *D. suzukii*, *D. biarmipes*, and *D. subpulchrella*.

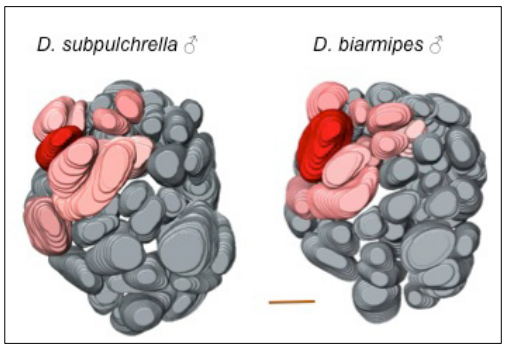

Figure 6 Reconstructions of the antennal lobes of *D. subpulchrella* and *D. biarmipes*. In bright red DA1, which received input from T1 neurons, and in light red other glomeruli receiving input from sensilla trichoidea neurons. Note: the small volume of DA1 in *D. subpulchrella*, which was comparable to *D. suzukii* (see Fig. 3).

Confocal microscopy and reconstructions

Whole-mount brains were viewed in a Zeiss LSM confocal microscope (Carl Zeiss, Jena, Germany) equipped with a 40x, 1.4 oil-immersion DIC objective lens. Structures labelled with Fluorescein Avidin were excited with an Argon laser at 488 nm with detection of reflected light in the range of 505-515 nm. Alexa 546-labeled structures were excited with a HeNe laser at 543nm and detected using a 560 nm long pass filter. Stacks of 50-200 confocal images were scanned and the images were stored at a size of 1024 x 1024 pixels. The three-dimensional reconstructions, volumetric measurements of the glomeruli were done using AMIRA 3.0 software (Visage Imaging, Berlin, Germany). In every optical section the contours of glomeruli were demarcated by hand (*i.e.*, image segmentation) and interpolated. Volumes were obtained from Amira, based on reconstructed images.

	*Desat*F	*Desat*1	Or65a	Or67d	*Elo*68a	*Elo*C2*	*Elo*P
D. suzukii	0.013/0.288 (0.044)	0.008/0.124 (0.062)	0.033/0.140 (0.234)	0.024/0.181 (0.135)	0.016/0.114 (0.1447)	0.034/0.121 (0.280)	0.045/0.142 (0.317)
D.biarmipes	0.014/0.115 (0.124)	0.005/0.07 (0.074)	0.037/0.166 (0.225)	0.021/0.156 (0.136)	0.026/0.113 (0.233)	not present	0.043/0.075 (0.575)

Table 1 Olfactory receptor, desaturase and elongase genes are conserved and under purifying selection. Values are Ka/Ks (dN/dS) with the actual value of the ratio (omega) in brackets. Values have been calculated using http://services.cbu.uib.no/tools/kaks under a Maximum Likelihood framework providing input trees according to trees from Fig. 5 and Supplementary Fig. S3 and 5. In all cases *D. suzukii* has an omega substantially lower than 1, suggesting purifying selection and therefore conserved function. *values obtained using topology: (((*mel*1, (*ere*1, *ere*2)), (*bia*, (*suz*1, *suz*2))), *ana*); similar result were obtained when using an alternative topology: ((((*mel*1, *ere*1), (*bia,suz*1)), *ana*), (*suz*2, *ere*2)); compare with the tree of Supplemental Fig. S4.

Bioinformatics and phylogenetics.

Orthologs searches and assembly. We downloaded various type of genome data (gene, transcript and protein sequences) for odorant receptors and other cVA related genes (*Desat, Elongase, Fruitless, Transformer*) of *D. melanogaster, D. simulans, D. sechellia, D. erecta,* and *D. anannassae* from Flybase (Marygold et al., 2013) and OrthoDB (Waterhouse et al., 2013) repositories. For *D. biarmipes* and *D. suzukii*, the protein sequences of *D. melanogaster* were used queries to identify the corresponding orthologs through exhaustive blast searches. First, TBlastN (BLOSUM 62 matrix with an e-value threshold of 10^{-5}) (Altschup et al., 1990) was applied for preliminary search of all the gene families. For the elongases superfamily alone, orthologs were searched using HMMER (http://hmmer.janelia.org/ v3.1b1, using an e-value threshold of 10^{-5}). The profile "hmmbuild" was constructed from GNS1/SUR4 HMM profile from the PFAM database (Punta et al., 2012), the result of which was run against *D. suzukii* and *D. biarmipes* genomes using "hmmsearch". The output was manually checked for all the positive hits, specifically looking for the presence of elo-specific domain *HXHH* and hydrophobic transmembrane regions. For *Fruitless*, we underwent a complex manual curation directly from *D. suzukii* genome contigs, using the 11 main isoforms of *D. melanogaster* to construct putative exons in *D. suzukii*. This is because *Fru* gene spans over a long genome region, contains long introns, and codes for 15 different isoforms; these

isoforms are unlikely to be present in transcriptomic data or being correctly annotated in gene models.

Phylogenetics.

For each of the gene families (Desaturases, Elongases, Fruitless and Odorant receptors) we constructed nucleotide datasets and correct frame was further checked. The translated protein datasets were aligned using MUSCLE v3.8.31 (Edgar, 2004), and preliminary tree searches were done to assess the actual orthology of genes. These preliminary analyses lead to recognition of few wrong orthologies (false positives due to gene loss), and characterization of new orthologs in not annotated genomes. After this manual curation, a second round of MUSCLE alignment using option–refine was done followed by phylogenetic reconstruction using PHYML (Guindon et al., 2010) and employing 100 non-parametric bootstrap replicates and the LG+G model (Le & Gascuel, 2008) of replacement.

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Competing interests: the authors declare no conflicting interests.

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Supplementary material

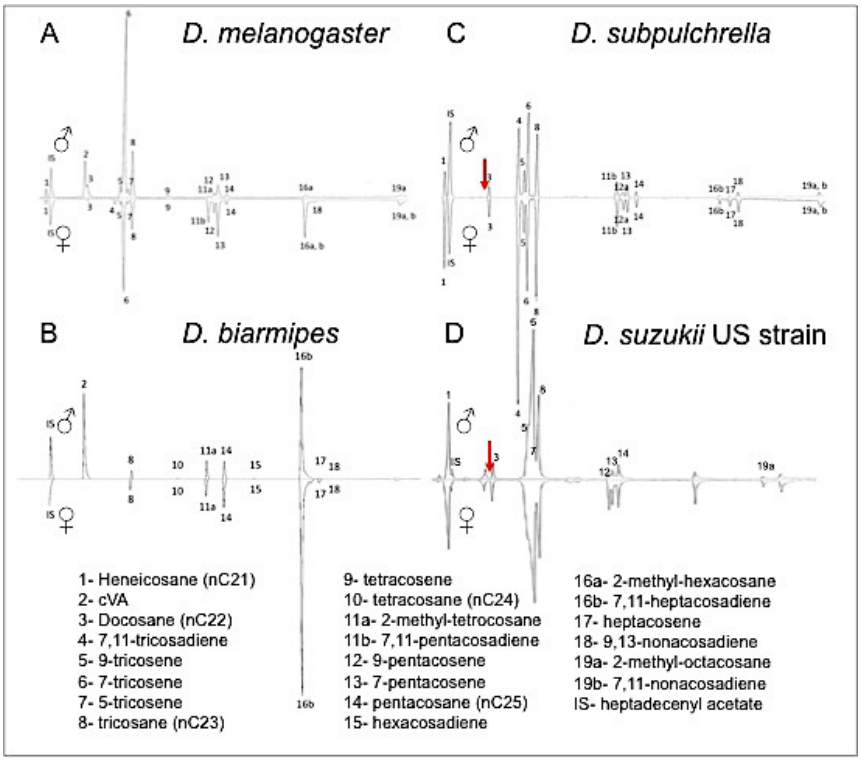


Figure S1. *Drosophila melanogaster*, (B) *D. biramipes*, (C) *D. subpulchrella* and (D) *D. suzukii* (US strain) males and females (n=2 or 3 for each). Numbers refer to compounds listed at the bottom of the figures. *c*VA is peak 2 in 2A and 2B. Red arrows in *D. subpulchrella*'s and *D. suzukii*'s chromatograms indicate where *c*VA would have eluted. *c*VA is not detected in the male CH profiles of either species.

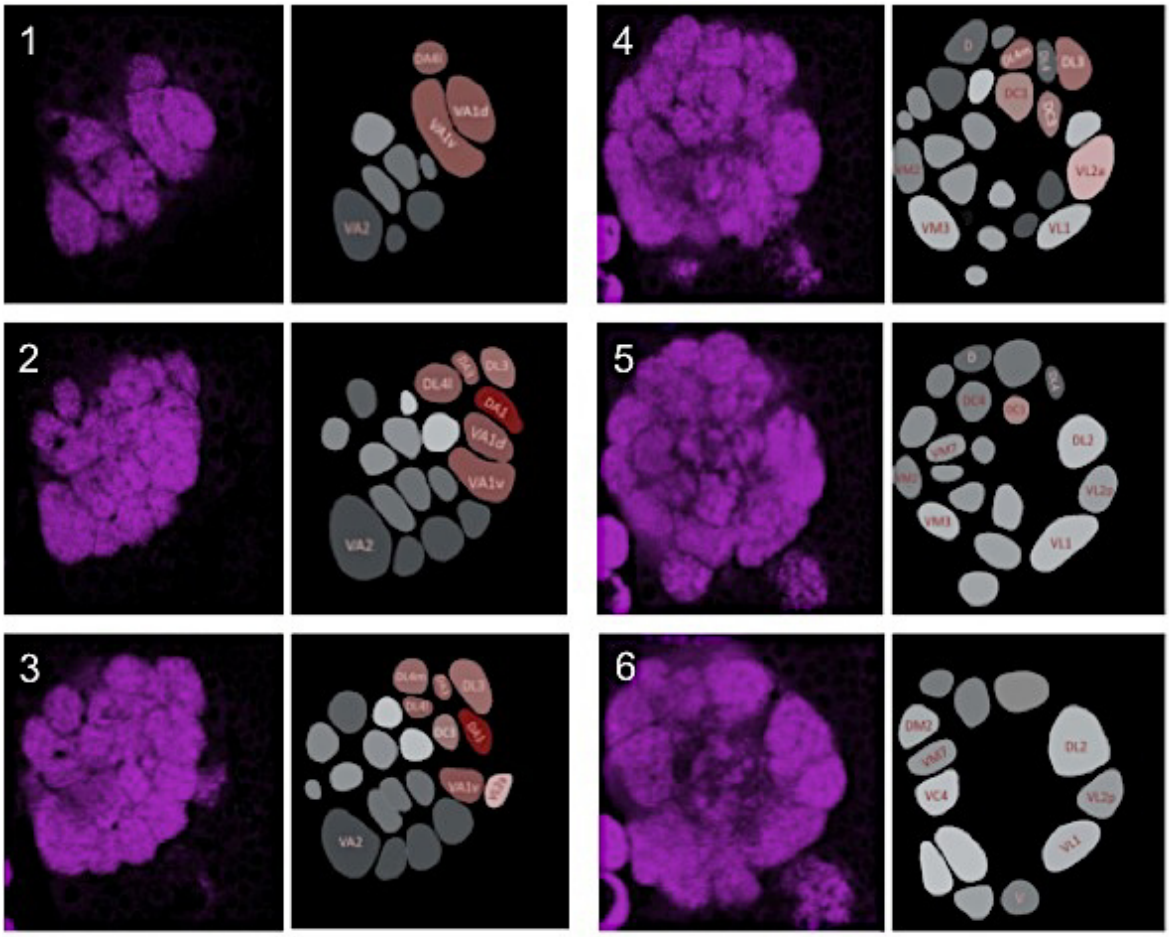
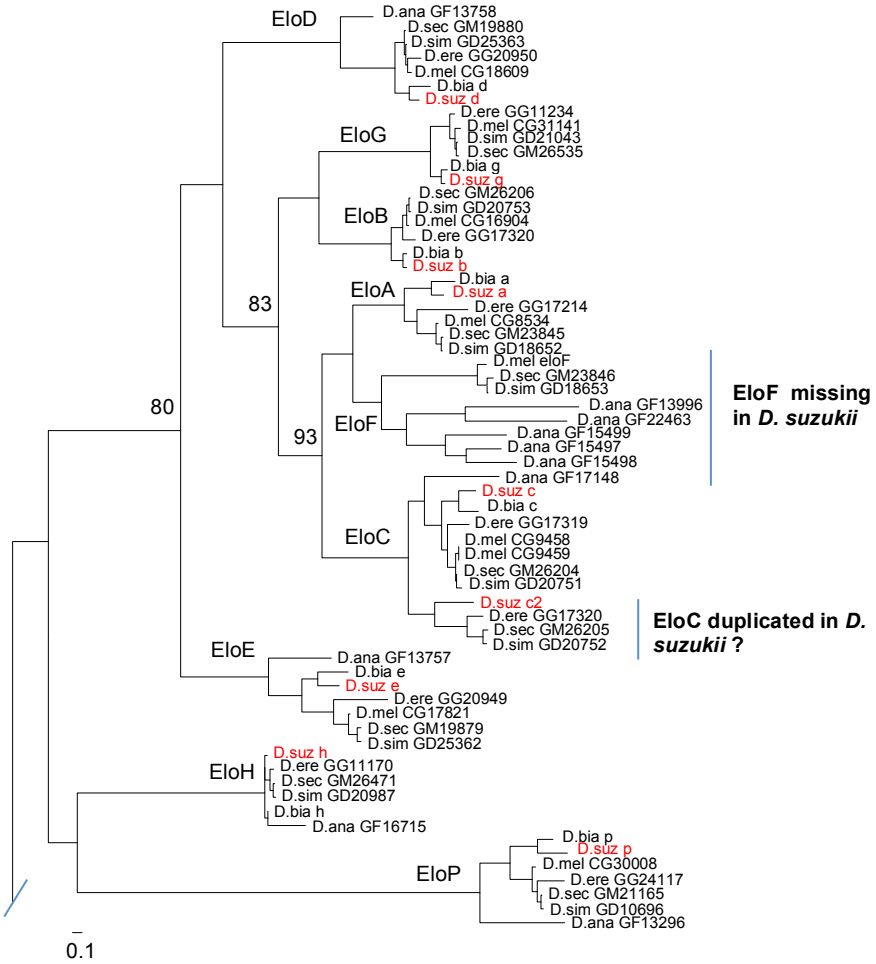


Figure S2. Confocal stack (α- synapsin background staining) and corresponding reconstructed images of a male *D. suzukii* brain antennal lobe. Images are arranged from anterior (1) to posterior (6). Corresponding putative homologous glomeruli of *D. melanogaster* glomeruli are indicated.


EloD
D.ana GF13758
D.sec GM19880
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D.ere GG20950
D.mel CG18609
D.bia d
D.suz d
EloG
D.ere GG11234
D.mel CG31141
D.sim GD21043
D.sec GM26535
D.bia g
D.suz g
EloB
D.sec GM26206
D.sim GD20753
D.mel CG16904
D.ere GG17320
D.bia b
D.suz b
83
EloA
D.bia a
D.suz a
D.ere GG17214
D.mel CG8534
D.sec GM23845
D.sim GD18652
80
93
EloF
D.mel eloF
D.sec GM23846
D.sim GD18653
D.ana GF13996
D.ana GF22463
D.ana GF15499
D.ana GF15497
D.ana GF15498
EloF missing in D. suzukii
EloC
D.ana GF17148
D.suz c
D.bia c
D.ere GG17319
D.mel CG9458
D.mel CG9459
D.sec GM26204
D.sim GD20751
D.suz c2
D.ere GG17320
D.sec GM26205
D.sim GD20752
EloC duplicated in D. suzukii ?
EloE
D.ana GF13757
D.bia e
D.suz e
D.ere GG20949
D.mel CG17821
D.sec GM19879
D.sim GD25362
EloH
D.suz h
D.ere GG11170
D.sec GM26471
D.sim GD20987
D.bia h
D.ana GF16715
EloP
D.bia p
D.suz p
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D.ere GG24117
D.sec GM21165
D.sim GD10696
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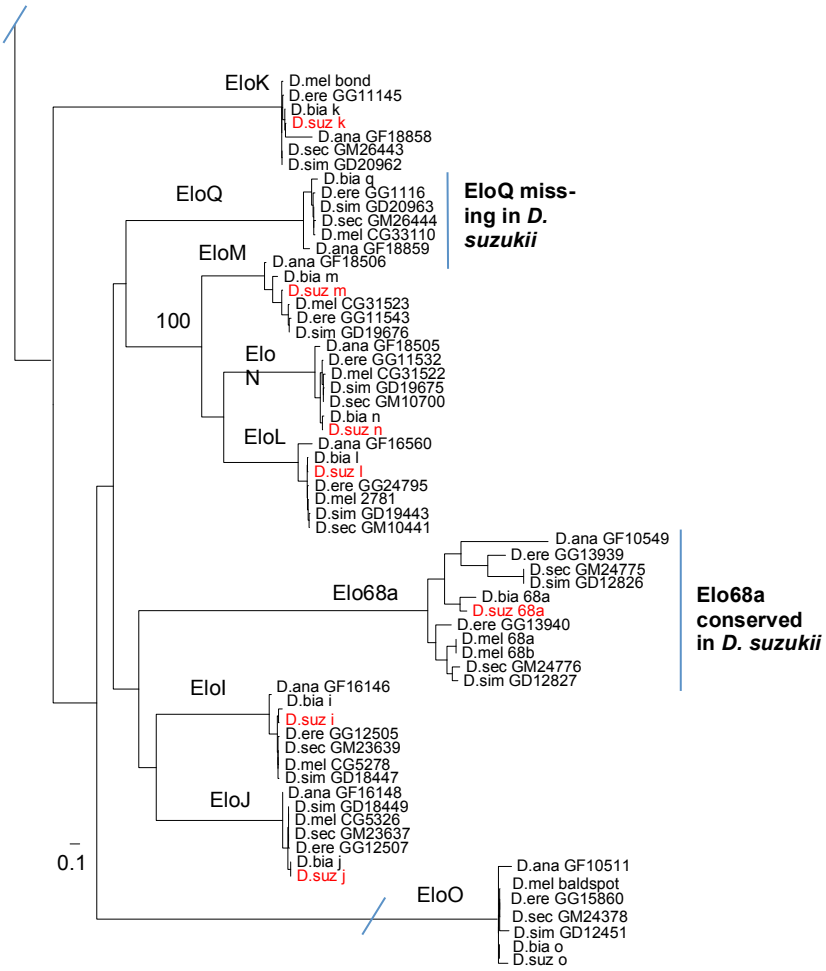
0.1

(S3 A1)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D.mel bond
D.ere GG11145
D.bia k
D.suz k
D.ana GF18858
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D.sim GD20962
EloQ
D.bia q
D.ere GG1116
D.sim GD20963
D.sec GM26444
D.mel CG33110
D.ana GF18859
EloQ missing in D. suzukii
EloM
D.ana GF18506
D.bia m
D.suz m
D.mel CG31523
D.ere GG11543
D.sim GD19676
100
Elo N
D.ana GF18505
D.ere GG11532
D.mel CG31522
D.sim GD19675
D.sec GM10700
D.bia n
D.suz n
EloL
D.ana GF16560
D.bia l
D.suz l
D.ere GG24795
D.mel 2781
D.sim GD19443
D.sec GM10441
D.ana GF10549
D.ere GG13939
D.sec GM24775
D.sim GD12826
Elo68a
D.bia 68a
D.suz 68a
D.ere GG13940
D.mel 68a
D.mel 68b
D.sec GM24776
D.sim GD12827
Elo68a conserved in D. suzukii
EloI
D.ana GF16146
D.bia i
D.suz i
D.ere GG12505
D.sec GM23639
D.mel CG5278
D.sim GD18447
EloJ
D.ana GF16148
D.sim GD18449
D.mel CG5326
D.sec GM23637
D.ere GG12507
D.bia j
D.suz j
0.1
EloO
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D.ere GG15860
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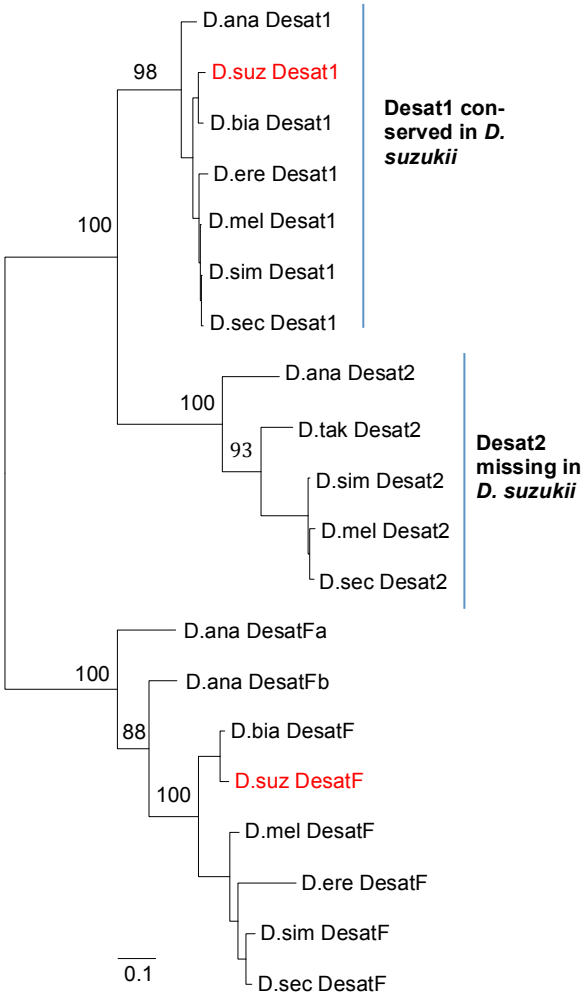
D.bia o
D.suz o

(S3 A2)



(S3 B)

Figure S3. The complex phylogeny of Elongase and desaturase genes, which have implied in cVA production (Wicker-Thomas & Chertemps, 2010). (A1 and A2) Phylogenetic tree of elongase genes of *Drosophila*. A comprehensive bioinformatic analysis of orthologs in various Drosophila species reveals a complex scenario in which *elo*F and elo68a are part of a large family of elongases. The naming of these putative genes is done using alphabetic letters from A to Q, leaving the F for *elo*F. Most of these putative elongases are conserved throughout the species tested. *Elo*68a, which is directly involved in the loss of *c*VA production, is present in *D. suzukii*, but *elo*Q is missing in *D. suzukii* and *elo*C is likely duplicated. These genes are possible candidates involved in the loss of *c*VA production in *D. suzukii*. Interestingly, monomorphic *D. suzukii* and *D. biarmipes* lack *elo*F. *Elo*F is active in hydrocarbon pathway of the *D. melanogaster* subgroup females (Wicker-Thomas & Chertemps, 2010). The same gene is not expressed (although present in its genome) in the also monomorphic D. simulans (Jallon and David, 1987). (B) Phylogenetic tree of desaturase genes in *Drosophila*. The genome of *D. suzukii* contains a conserved copy of *desat*F, involved in CH biosynthesis in males. *D. suzukii* and *D. biarmipes* have lost *desat*2, which is involved in female hydrocarbon biosynthesis (Wicker-Thomas & Chertemps, 2010). Species code used in the tree: D.ana= *Drosophila ananassae*; D.bia= *D. biarmipes*; D.suz= *D. suzukii*; D.mel= *D. melanogaster*; D.ere= *D. erecta*; D.sim= *D. simulans*; D.sec= *D. sechellia*. Values at nodes are bootstrap support from the analysis of 100 pseudo-replicates in PHYML, using LG+G model.

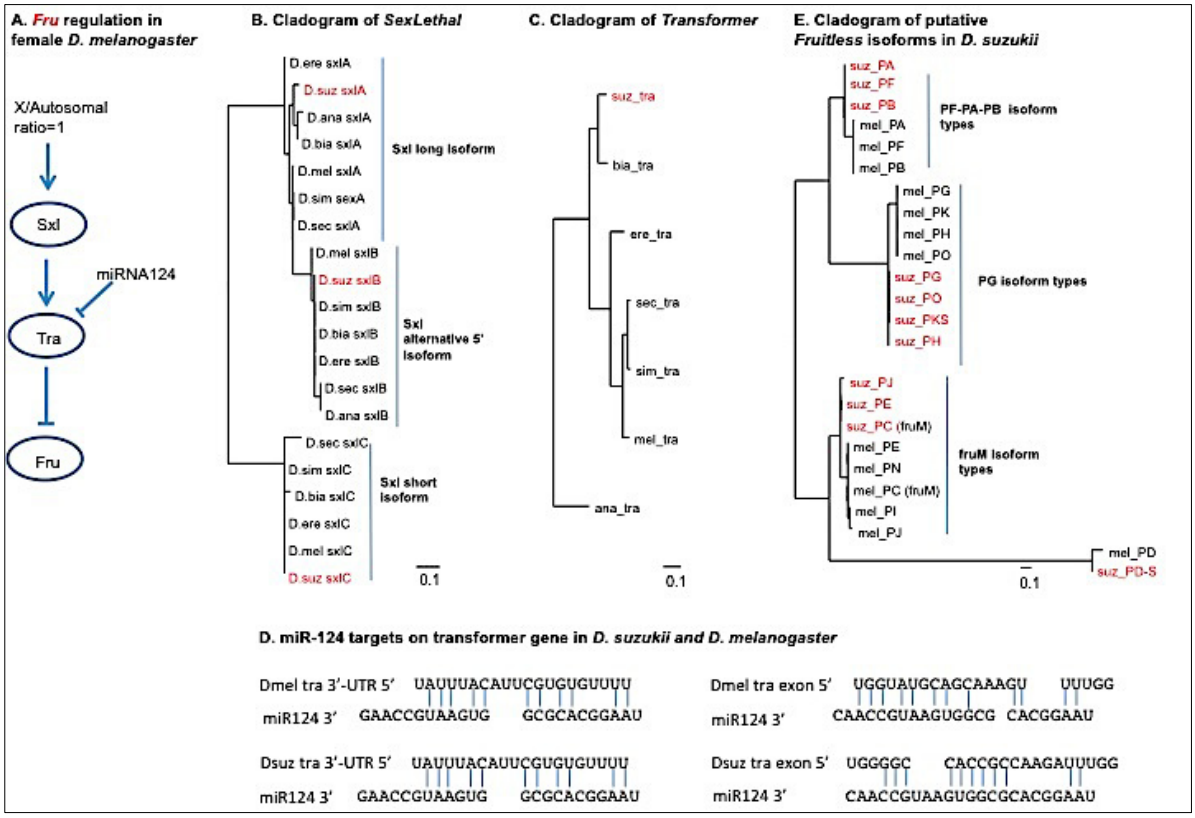


Figure S4. Fruitless/ cVA signaling pathway is conserved in *D. suzukii*. (A) *SexLethal* is present with both its two main isoforms in *D. suzukii*. (B) Transformer is also conserved and capable of both main female and male isoforms in *D. suzukii*. One of the binding sites of miRNA124 is variable in *D. suzukii*, but still capable of binding miRNA (see panel E). (C) Cladogram of possible Fruitless isoforms in *D.suzukii* compared with known isoforms in *D. melanogaster*. The comparison has been done by aligning all known *Fruitless* gene region. Cladogram shows that *D. suzukii fru* gene region contains all putative exons to build all *D. melanogaster* isoforms. We did not explore the mRNA in *D. suzukii*, due to low coverage of RNAseq libraries available. Results are preliminary and the *D. suzukii* putative isoforms should be validated by proper mRNA expression data. (D) Cladogram of possible isoforms in accordance with the phylogeny of species. This is due to shared synapomorphies of closely related species (or apomorphies of that group). (E) *Transformer* gene of *D. suzukii* contains both miRNA-124 binding sires. Left shows the binding site 3' UTR of *Transformer* in D. melanogaster (upper) and *D. suzukii* (lower). Right panel shows the binding site at the last *Transformer* exon.