Behavioral and genomic sensory adaptations underlying the pest activity of *Drosophila suzukii*

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ABSTRACT

Studying how novel phenotypes originate and evolve is fundamental to the field of evolutionary biology as it allows us to understand how organismal diversity is generated and maintained. However, determining the basis of novel phenotypes is challenging as it involves orchestrated changes at multiple biological levels. Here, we aim to overcome this challenge by using a comparative species framework combining behavioral, gene expression and genomic analyses to understand the evolutionary novel egg-laying substrate-choice behavior of the invasive pest species *Drosophila suzukii*. First, we used egg-laying behavioral assays to understand the evolution of ripe fruit oviposition preference in *D. suzukii* as compared to their closely related species: *D. subpulchrella* and *D. biarmipes*, as well as *D. melanogaster*. We show that *D. subpulchrella* and *D. biarmipes* lay eggs on both ripe and rotten fruits, suggesting that the transition to ripe fruit preference was gradual. Secondly, using two-choice oviposition assays, we studied how *D. suzukii, D. subpulchrella, D. biarmipes* and *D. melanogaster* differentially process key sensory cues distinguishing ripe from rotten fruit during egg-laying. We found that *D. suzukii's* preference for ripe fruit is in part mediated through an aversion to acetic acid and preference for stiff substrates. Lastly, we identified candidate genes involved in *D. suzukii's*

ability to seek out and target ripe fruits. Our results provide detail to the stepwise evolution of pest activity in *D. suzukii*, indicating important cues used by this species when finding a host, and the molecular mechanisms potentially underlying their adaptation to a new ecological niche.

INTRODUCTION

Novel phenotypes can give species the opportunity to occupy a new ecological niche (Mayr, 1960; Moczek, 2008; Muller and Wagner, 1991). Understanding how and when these phenotypes arise is an exciting question in evolutionary biology. Adaptive traits can present as changes to an organism's behavior, physiology, or morphology, and arise through a variety of different molecular mechanisms. Studying the origin and evolution of novel phenotypes provides an opportunity to unravel the molecular genetic basis of new traits. In the context of pest species, adaptation to a new ecological niche can come with damaging environmental, agricultural, and economic consequences. Understanding the basis of novel pest behavior can also shed light on the ecological impact of invasive species.

Unlike the majority of *Drosophila* flies, the species *Drosophila suzukii* prefers to lay its eggs in ripe as opposed to rotten fruit, causing substantial crop damage and leading to economic losses in the fruit industry (Rota-Stabelli et al., 2013). Although originally categorized in Japan and likely to be native to East Asia, within the past decade *D. suzukii* has spread throughout Europe and North America (Adrion et al., 2014; Fraimout et al., 2017; Paris et al., 2020). *D. suzukii* has both physical and behavioral traits that allow it to selectively target ripe fruit as an oviposition substrate (Atallah et al., 2014). Morphologically, *D. suzukii* has evolved an enlarged, serrated ovipositor allowing females to puncture hard surfaces and insert their eggs into ripe fruit. Behaviorally, they have evolved the ability to seek out and selectively target ripe fruits for oviposition through changes in multiple sensory systems (Karageorgi et al., 2017). Thus, the exploitation of the ripe fruit niche by *D. suzukii* requires orchestrated changes at multiple biological levels. Investigating the behavioral and molecular underpinnings of these changes will advance our understanding of the forces and mechanisms that drove *D. suzukii's* evolution into an invasive pest (Karageorgi et al., 2017).

Adaptive shifts in host preference, such as that described for *D. suzukii*, are often mediated through sensory system evolution. More specifically, changes in the function and sequence of chemoreceptor genes – including odorant receptors (ORs), ionotropic receptors

(IRs), and gustatory receptors (GRs) – and mechanosensory receptor genes (MRs) underlie various species-specific host preference differences in insects (Auer et al., 2020; Dekker et al., 2006; Goldman-Huertas et al., 2015; Karageorgi et al., 2017; Mansourian et al., 2018; Vosshall and Stocker, 2007). For example, modifications to sensory receptor genes are linked to the transition to herbivory in *Scaptomyza flava* (Goldman-Huertas et al., 2015), the oviposition preference for morinda fruits (*Morinda citrafolia*) in *D. sechellia* (Auer et al., 2020; Dekker et al., 2006), and the specialization on marula fruit in ancient *D. melanogaster* (Mansourian et al., 2018). Additionally, through knockout experiments in *D. melanogaster*, sensory gene function has been linked to various behaviors relevant to *D. suzukii* pest activity, including preference of acetic acid containing oviposition sites (Joseph et al., 2009), the avoidance of stiff substrates (Jeong et al., 2016; Zhang et al., 2020, 2016), and detection of substances that are at different concentrations in ripe and rotten fruit – such as CO_2, acetic acid, and sugar (Fujii et al., 2015; Kwon et al., 2007; Rimal et al., 2019).

While progress has been made in understanding the evolution of olfactory genes in *D. suzukii* (Keesey et al., 2015; Ramasamy et al., 2016), very little is known about the evolutionary history of other sensory genes in this system, despite *D. suzukii's* oviposition site preference being mediated via multiple sensory systems (Karageorgi et al., 2017). Finally, studies of adaptive sensory gene evolution in *D. suzukii* often focus only on changes to the coding sequence of these genes, despite differential gene expression playing a prominent role in the evolution of adaptive phenotypes (Carroll, 2005; Jones et al., 2012; Phifer-Rixey et al., 2018; Wray, 2007). For example, changes in expression of ORs and odorant-binding proteins in the olfactory organs of *D. sechellia* are thought to in part mediate this species' specialization on morinda fruits (Kopp et al., 2008).

Although the phenotypic and behavioral innovations leading to the pest status of *D. suzukii* are overall well defined, the specific sensory cues used by *D. suzukii* to target ripe fruits as well as the molecular changes accompanying these sensory changes remain unknown. Here we examine these knowledge gaps by investigating the behavioral, genomic, and gene regulatory mechanisms underlying the pest activity in *D. suzukii.*

First, we provide new insights into the stepwise evolution of ripe fruit preference in *D. suzukii* through the in-depth behavioral and genomic examination of two closely related species, *D. subpulchrella* and *D. biarmipes*. Secondly, we identified key sensory cues of ripe and rotten

fruit differentially processed by *D. suzukii, D. subpulchrella, D. biarmipes* and *D. melanogaster* in the context of egg-laying preference. Third, to infer the most recent changes in genome content in *D. suzukii*, we sequenced the genome of the closely related species, *D. subpulchrella,* and performed comparative genomic analysis. Lastly, we identified candidate genes involved in *D. suzukii's* ability to seek out and target ripe fruits through population level analyses of olfactory, gustatory, and mechanosensory receptor genes with differential expression and signatures of positive selection in *D. suzukii* as compared to *D. subpulchrella, D. biarmipes* and *D. melanogaster*. We provide a view, from multiple levels of analysis, of the origin of pest activity in *D. suzukii*, indicating important sensory cues used by this species during egg-laying, and the gene expression and genomic changes potentially underlying this novel pest behavior.

RESULTS AND DISCUSSION

Ripe fruit preference in *D. suzukii* evolved after the split with *D. subpulchrella.*

To clarify the evolutionary history of early fruit stage preference in *D. suzukii*, we first analyzed the oviposition preference of *D. suzukii, D. subpulchrella, D. biarmipes* and *D. melanogaster* for fruits of differing maturation stages, specifically strawberries, a main target fruit of *D. suzukii* (Gong et al., 2016). We determined the ripe vs rotten fruit preference of each species by conducting a two-choice oviposition assay in which female flies were placed in a cage with both a ripe and rotten whole strawberry and allowed to lay eggs overnight. Afterwards, the number of eggs laid in each fruit was counted and an oviposition preference index (OPI) was calculated (Figure 1A, supplementary file 1). As established in previous studies, *D. suzukii* showed a strong preference for ripe fruit (OPI = 0.788 ± 0.039SEM), *D. melanogaster* preferred rotten fruit (OPI = -0.959 ± 0.013SEM), and *D. biarmipes* displayed an intermediate behavior (OPI = -0.276 ± 0.109SEM), with no pronounced preference for either fruit (Karageorgi et al., 2017) (Figure 1B, supplementary file 1). Interestingly, we found that *D. subpulchrella* also displayed an intermediate oviposition behavior (OPI = 0.196 ± 0.138SEM), with no distinct preference for ripe or rotten fruit. It was previously believed that *D. subpulchrella* preferred ripe fruit as oviposition substrates, as *D. suzukii* does (Karageorgi et al., 2017), perhaps because they share the trait of an enlarged ovipositor, and the ability to lay eggs in ripe fruits (Atallah et al., 2014). However, this assumption had not been empirically tested before this study. *D. subpulchrella*'s intermediate preference suggests that the shift to ripe fruit preference in *D.*

suzukii is due to factors beyond the enlargement of the ovipositor and ability to puncture ripe fruits' skin and evolved after the split between *D. subpulchrella* and *D. suzukii*.

D. suzukii's preference for early fruit maturation stages is associated with preference for stiff substrates.

Previous studies indicate that *D. suzukii's* preference for ripe fruit evolved through changes in multiple sensory systems, including the olfactory, gustatory, and mechanosensory systems (Karageorgi et al., 2017; Keesey et al., 2015; Ramasamy et al., 2016). However, the evolutionary history of these sensory changes, as well as the sensory cues involved in *D. suzukii's* oviposition site choice, remain unknown. To investigate these questions, we tested the oviposition preference of *D. suzukii, D. subpulchrella, D. biarmipes* and *D. melanogaster* females for four sensory cues that change over the course of fruit maturation from ripening to rotting: ethanol, sucrose, acetic acid, and stiffness (supplementary file 2). 3-4 females of each species were placed overnight in custom built egg-laying chambers and given the choice between two agarose egg-laying substrates: one control and one containing the experimental substrate (Figure 2A, supplementary file 2). Afterwards, the number of eggs laid on each substrate was counted and the OPI was calculated. The preference for each substrate was tested at three biologically relevant concentrations in the context of fruit maturation and oviposition choice (Dudley, 2004; Karageorgi et al., 2017; Kim et al., 2018; Schwartz et al., 2012).

First, we looked at the stiffness preference of the four focal species, using agarose concentration as a proxy. Fruit stiffness decreases throughout fruit maturation and *D. melanogaster* is known to prefer softer substrates, indicative of rotten fruit (Karageorgi et al., 2017; Zhang et al., 2016). When given the choice between 0.25% and 1%, 1.5%, or 2% agarose substrates (supplemented with 75mM sucrose and 1% ethanol), *D. suzukii* consistently preferred the stiffer substrate, while *D. subpulchrella, D. biarmipes* and *D. melanogaster* preferred the softer substrate (Figure 2B, S1A, supplementary file 2, Wilcoxon signed rank test, all P-values <0.01). However, this preference for soft substrates was less pronounced in *D. subpulchrella* as compared to *D. biarmipes* and *D. melanogaster. D. suzukii's* striking divergence from the other three species in stiff substrate preference may suggest that the species-specific targeting of stiff oviposition sites is associated with the early fruit stage preference only exhibited by *D. suzukii.*

Relaxation of rotten fruit oviposition preference in *D. suzukii, D. subpulchrella,* and *D. biarmipes* is associated with an aversion to acetic acid.

Next, we measured the oviposition preference for acetic acid among the four species. Acetic acid is a product of fermentation that increases as fruit rots, and is a known attractive oviposition cue for *D. melanogaster* (Eisses, 1997; Joseph et al., 2009; Jouandet and Gallio, 2015). Experimental substrates were 1% agarose and contained 75mM sucrose, 1% ethanol and either 1.5%, 2.5%, or 3.5% acetic acid, while control substrates contained no acetic acid. *D. melanogaster* showed a strong preference for acetic acid at each concentration tested (Wilcoxon signed rank test, all P-values <0.001), whereas *D. suzukii, D. subpulchrella,* and *D. biarmipes* avoided acetic acid at high concentrations (Figure 2C, S1B, supplementary file 2, Wilcoxon signed rank test at 3.5% acetic acid, all P-values <0.01). This aversion exhibited by *D. suzukii, D. subpulchrella,* and *D. biarmipes* suggests that the avoidance of high acetic acid substrates may have evolved in concert with the relaxation of rotten fruit preference, contributing to *D. suzukii's* ability to target and parasitize early fruit maturation stages (Figure 3).

***D. suzukii* and *D. subpulchrella* do not prefer high sucrose oviposition substrates.**

We next investigated the effect of sucrose on oviposition preference in the four focal species. Sucrose content, along with other sugars, increases during fruit ripening, reaching its peak at fruit maturation, and then decreases as fermentation occurs (Dudley, 2004; Montero et al., 1996). Sucrose is thought to be an attractive oviposition cue for *D. melanogaster* (Schwartz et al., 2012). However, under lab conditions, the sucrose oviposition preference of *D. melanogaster* is not consistent, and has been identified as both an attractive and repulsive oviposition cue (Schwartz et al., 2012; Yang et al., 2008). This trend may be linked to the size of the arena used in egg-laying assays. Flies tested in larger assays prefer sucrose, which may reflect the need for larvae to be on a sucrose containing substrate when they must forage over larger ranges (Schwartz et al., 2012). In our behavioral assays, we used an arena that is large in comparison to arenas used in previous studies testing sucrose preference (Schwartz et al., 2012; Yang et al., 2008). Experimental substrates were 1% agarose and contained 1% ethanol and either 200mM, 400mM or 600mM sucrose, while control substrates contained no sucrose. We found that *D. melanogaster* and *D. biarmipes* have a strong oviposition preference for sucrose (Wilcoxon signed rank test, all P-values <0.01), while *D. suzukii* and *D. subpulchrella* show no

preference or aversion to sucrose, with *D. suzukii* having no preference at each concentration measured (Wilcoxon signed rank test, all P-values >0.05), and *D. subpulchrella* having no preference at 400mM and 600mM sucrose (Wilcoxon signed rank test, all P-values >0.05) (Figure 2D, S1C, supplementary file 2).

Because sugar increases during ripening, high sugar content indicates that rotting will begin imminently, perhaps explaining why *D. melanogaster* chooses high sugar substrates despite the fact that their preferred substrate of rotten fruit contains less sugar than ripe fruit. Similarly, *D. suzukii's* indifference towards sucrose may be associated with their transition towards ovipositing on early fruit maturation stages. While in a two choice scenario *D. suzukii* prefers ripe fruit (higher sugar) over rotten fruit (lower sugar), when given the option of earlier maturation stages, they equally target pre-ripe and ripe fruits for egg-laying (Karageorgi et al., 2017). Sugar is still relatively low during pre-ripe stages (Basson et al., 2010; Montero et al., 1996), suggesting that the loss of sucrose preference in *D. suzukii* could be associated with the selection of early fruit stage oviposition sites.

Oviposition preference for ethanol does not differ among species.

Lastly, we tested the oviposition preference for ethanol containing substrates in each of the four species. As fruit rots the concentration of ethanol increases due to fermentation (Dudley, 2004), and ethanol is a known attractive oviposition cue for *D. melanogaster* (Azanchi et al., 2013; Eisses, 1997; Kacsoh et al., 2013). Experimental substrates were 1% agarose and contained 75mM sucrose and either 3%, 5%, or 7% ethanol, while control substrates contained no ethanol. At lower concentrations, *D. suzukii, D. biarmipes* and *D. melanogaster* preferred ethanol containing substrates (Wilcoxon signed rank test at 3% and 5% ethanol, all P-values <0.05), while *D. subpulchrella* displayed an indifference towards ethanol at each concentration measured (Wilcoxon signed rank test, all P-values >0.05) (Figure S1D). However, using a linear regression approach comparing the difference in preference curve y-intercepts between the four focal species, we found that overall oviposition preference for ethanol did not differ between *D. suzukii, D. subpulchrella, D. biarmipes* and *D. melanogaster* (Figure 2E, see methods for details). As the females from all four species responded similarly to the increasing concentrations of alcohol in the substrate, ethanol is unlikely to be a cue on which *D. suzukii's* relies to target ripe fruits. In contrast to the other species, in the choice-assay experiments, *D. suzukii* had a

marked decrease in the number of eggs laid as ethanol concentration increased (Figure S2D). This may be associated with *D. suzukii's* substantial decrease in survival rate on ethanol containing substrates as compared to *D. melanogaster* (Kim et al., 2018).

Stepwise evolution of behaviors and morphologies associated with *D. suzukii* oviposition site preference.

The species-specific egg-laying behavior of *D. suzukii* has been shown to be associated with the enlargement and strengthening of the ovipositor allowing females to puncture stiff fruit, a trait shared by its sister species, *D. subpulchrella* (Atallah et al., 2014). Previous work established a stepwise model for the evolution of *D. suzukii* as a pest species in relation to *D. melanogaster* and *D. biarmipes*, focusing on ovipositor size and fruit stage preference (Karageorgi et al., 2017). Our work clarifies and builds upon this model with the addition of empirical fruit preference data as well as an in depth analysis of sensory cues used for the discrimination of fruit maturation stages for *D. subpulchrella*, a species representing an intermediate step toward the exploitation of ripe fruit as an egg-laying substrate (Figure 3).

Sequencing and assembly of the *D. subpulchrella* genome.

The results from our phenotypic assays suggesting strongly anchored behavioral differences between species motivated a deeper study of the genetic factors underlying these behavioral differences. In order to apply comparative genomic and genetic approaches, we generated a near-chromosomal level assembly of the *D. subpulchrella* genome using PacBio sequencing (see methods). The genome size is about 276 megabases (Mb), and has a contig N50 of 11.59Mb. Specifically, the longest 6 contigs are 29.67 Mb, 29.50 Mb, 26.21 Mb, 22.17 Mb, 20.23 Mb, and 11.59 Mb, accounting to a total of 139.38 Mb (details, see supplementary table S1). We assessed the gene content of the *D. subpulchrella* genome and found that 98.11% (2746 out of 2799) of the Diptera BUSCO genes are present in the genome, among which, 97.43% (2727) are complete and single-copy genes. When using the 303 BUSCO eukaryota genes, 302 complete genes were found in the genome. This suggests we have assembled a highly complete genome with relatively low levels of redundancy. We then performed three rounds of genome annotation by training the annotation program using MAKER2. In total, we annotated 15,225 genes. Among which, 13,435 genes have reciprocal best hits in *D. suzukii*. This high-quality

genome makes it possible to study the genomic differences and genome evolution of *D. suzukii* and *D. subpulchrella,* and how they compare to other closely related species.

Adaptive evolution of sensory receptors implicated in *D. suzukii* oviposition site preference.

Our behavioral analyses established that the sensory modalities used by *D. subpulchrella* and *D. biarmipes* for egg-laying reflected the stepwise progression toward *D. suzukii*'s ability to use ripe fruit as egg-laying substrate. To identify the genetic changes that lead to *D. suzukii*'s oviposition behavior, we first analyzed the repertoire of *Drosophila* olfactory, gustatory, and mechanosensory receptors for signals of positive selection. Using population level genomic data from over 200 *D. suzukii* females, we performed a McDonald-Kreitman test (MK) to identify candidate odorant receptors (OR), gustatory receptors (GR), ionotropic receptors (IR), and mechanosensory receptors (MR) evolving under positive selection in *D. suzukii* as compared to *D. subpulchrella, D. biarmipes,* and *D. melanogaster* separately (Figure 4). The MK test infers the presence of positive selection by comparing the numbers of fixed and segregating, synonymous and nonsynonymous substitutions in the genomes of a focal population (McDonald and Kreitman, 1991). While there is previous knowledge on ORs evolving under positive selection in *D. suzukii* as compared to *D. melanogaster* (Ramasamy et al., 2016), our analyses have the additional power of population level data, the additional pairwise comparisons of *D. suzukii* to both *D. biarmipes* and *D. subpulchrella*, and the addition of GRs, IRs, and MRs.

We used three independent methods to perform the MK test (see methods), and consistently identified about 3000 genes evolving under positive selection in *D. suzukii*, as compared to the other three focal species. This number is very large compared to positively selected genes in other *Drosophila* species when inferred through whole genome and population level genomic analyses, which range from 500-1000 positively selected genes (Begun et al., 2007; Langley et al., 2012; Zhao and Begun, 2017). Specifically, we found that the d_N/d_S is significantly larger than p_N/p_S, and that both d_N and d_S are large (Figure S3). Since *D. suzukii* recently invaded North American, its evolutionary history suggests that the over-representation of positively selected genes may be caused by the bottleneck of invasion and the subsequent increase of the population size and likely effective population size (Eyre-Walker, 2002; McDonald and Kreitman, 1991). In addition, this trend has been observed in other populations such as between humans and old-world monkeys, where about 35% of amino acid substitutions

have been found to be adaptive (Fay et al., 2001). Given that humans have undergone population size expansion, it may suggest that despite the migration induced evolutionary bottleneck, the adaptive substitutions are still reasonably estimated.

For the majority of species pairwise comparisons between the four focal *Drosophila* species, the sensory receptors analyzed are evolving under positive selection in *D. suzukii* to a greater degree than other regions of the genome (Figure 4, S4). Receptors that are under positive selection in *D. suzukii* in each of the three pairwise species comparisons may underlie *D. suzukii's* novel oviposition behavior. Such genes include *Or22a*, which is involved in host choice evolution in various *Drosophilid* species (Auer et al., 2020; Goldman-Huertas et al., 2015; Mansourian et al., 2018), *Or85a,* a gene implicated in *D. sechellia*'s specialization on morinda fruit (Auer et al., 2020) that is also thought to either be a pseudogene (Hickner et al., 2016) or have changed function in *D. suzukii* (Ramasamy et al., 2016), and *Or85d*, which is linked to the detection of yeast volatiles in *D. melanogaster* (Tichy et al., 2008). Gustatory and ionotropic receptors of interest include *Gr63a*, which is involved in CO_2 detection, a volatile emitted by ripening fruit (Jones et al., 2007; Krause Pham and Ray, 2015; Kwon et al., 2007), and *Ir7a*, an ionotropic receptor linked to acetic acid consumption avoidance in *D. melanogaster* (Rimal et al., 2019). Lastly, mechanosensory receptors of note include *nan*, which is involved substrate stiffness detection during egg-laying in *D. melanogaster* (Jeong et al., 2016; Zhang et al., 2020), *ppk*, a gene involved in the acetic acid attraction during oviposition choice in *D. melanogaster* (Gou et al., 2014), and *Trpγ*, which is also linked to CO_2 detection (Badsha et al., 2012).

Even if the number of genes under positive selection is overestimated, one would expect that there is no bias in which gene categories are enriched for signals of selection. Overrepresentation of signals for positive selection in olfactory, gustatory, and mechanosensory receptors suggest that these genes are at least partly contributing to adaptive evolution in *D. suzukii* (Figure S4).

Differential expression of sensory receptors implicated in *D. suzukii* oviposition site preference.

To further understand the molecular changes associated with *D. suzukii's* egg-laying substrate preference, we sequenced full transcriptomes and analyzed gene expression data from female adult heads of *D. suzukii*, *D. subpulchrella, D. biarmipes* and *D. melanogaster*. We

analyzed expression across all genes and then focused on the same set of sensory receptors included in our MK analysis and determined differential gene expression in *D. suzukii* as compared to the three other focal species (supplementary tables S2-5). To determine the strongest candidate genes, we built upon our positive selection population genomic analysis and curated a final list of genes which exhibit both significant MK tests (in all three species pairwise comparisons), and a significant difference in gene expression (in at least one species comparison). These criteria generated 15 candidate receptor genes potentially underlying *D. suzukii's* oviposition behavior (Figure 5). These genes include previously mentioned receptors *Or85a, Or85d, Gr63a*, and *Trpγ*, strengthening the potential role of CO_2 detection and host fruit specializing in *D. suzukii's* ripe fruit preference. This list also includes *Ir56d*, another receptor implicated in the response to carbonation and CO_2 (Sánchez-Alcañiz et al., 2018). Other ORs implicated are *Or10a* and *Or85f*, which are both involved in response to benzaldehydes in *D. melanogaster* (Rollmann et al., 2010), a major volatile emitted by fruits during ripening (Girard and Kopp, 1998). Lastly, candidate MRs include *wtrw*, which is involved in humidity sensation in *D. melanogaster* (Liu et al., 2007), *trpl*, a cation channel shown to mediate gradual dietary shifts in *D. melanogaster* (Zhang et al., 2013), and *Piezo*, which interacts with the receptor *nan*, mentioned above, to sense substrate stiffness differences in *D. melanogaster* (Zhang et al., 2020). While we focus on behavioral and genomic changes to the peripheral sensory system here, we acknowledge that differences in oviposition choice between species could be due to changes in central-brain processing (Seeholzer et al., 2018), and this would be an interesting direction for future studies on *D. suzukii* oviposition preference.

CONCLUSIONS

Here we present an investigation of the behavioral patterns, sensory modalities, genetic factors and evolutionary forces contributing to the emergence of ripe fruit preference of *D. suzukii*, a newly invasive and rapidly spreading fruit pest. We show that *D. suzukii* differs from closely related species *D. subpulchrella, D. biarmipes* and *D. melanogaster* in discrete and important ways as it pertains to oviposition preference for whole fruit, and common substances associated with fruit maturation and rotting. In contrast to the other *Drosophila* species studied, *D. suzukii* prefers to oviposit in ripe fruit, and has lost the preference for sucrose and acetic acid, while gaining a preference for stiff oviposition substrates. The comparative analysis of species closely

related to *D. suzukii* reveals a stepwise progression toward ripe fruit oviposition preference and provides new insights into the evolutionary history of pest status in this group of species. Additionally, we provided clarity to the egg-laying preference of *D. subpulchrella and* generated a high-quality genome for this species. *D. subpulchrella* is the non-pest sister-species of *D. suzukii*, has an intermediate preference for ripe fruit, and also exhibits an aversion to acetic acid and lack of preference for sucrose. Future comparative studies focused on *D. subpulchrella* may help reveal how *D. suzukii* evolved into an invasive pest, while other closely related species did not. Finally, we analyzed the genomic changes associated with *D. suzukii's* sensory evolution and generated a list of candidate olfactory, gustatory and mechanosensory receptors with signals of both differential gene expression and positive selection. A substantial number of these genes seem to be evolving under positive selection and are differentially expressed in *D. suzukii* as compared to the other species analyzed, despite being recently diverged. Previous work on our candidate genes in *D. melanogaster* and other *Drosophilid* species suggests that changes in CO_2 detection, stiffness differentiation, fermenting fruit volatile sensing, and host choice specialization in part underlie *D. suzukii's* oviposition preference for ripe fruit. Further work is required to truly understand the functional implications of the genetic changes seen in *D. suzukii*, and the results outlined here are a valuable resource for future studies aimed at understanding the behavioral and molecular basis of pest activity in this species.

METHODS

Fly Stocks and husbandry.

All flies were reared on standard cornmeal medium at 24°C, 55% relative humidity, on a 12-hour light-dark cycle (lights on at 8:00am). Egg-laying experiments were conducted under the same conditions. For behavioral assays we used a set a wild type strains: Canton S for *D. melanogaster*, the genome strain raj3* (Chen et al., 2014) for *D. biarmipes*, #NGN6 from Japan for *D. subpulchrella* (Ehime Stock Center), and the genome strain WT3 (WT3, Chiu et al., 2013) for *D. suzukii*. We tested different lines within species and found all the behaviors are consistent. For example, in *D. melanogaster*, w^{1118} and Canton S show the same results, suggesting that the traits tested here are likely to be fixed in each species. For genomic analyses we used the genome reference strain of *D. melanogaster* (BDSC #2057, Adams et al., 2000), the genome strain of *D.*

biarmipes (raj3*) (Chen et al., 2014), the genome strain of *D. suzukii* (WT3, Chiu et al., 2013), and our lab inbred strain of the wild caught line *D. subpulchrella* (Ehime Stock Center #NGN6).

Egg-laying assays.

Whole fruit 2-choice oviposition assay. Flies were collected as virgins and aged for 7-8 days in food vials containing approximately 10 males and 10 females. For each trial 10 females and 5 males were placed in a mesh experimental cage (10x10x10 inch, BugDorm 4F2222) which contained both a whole ripe and whole rotten strawberry without anesthesia using an aspirator. Flies were allowed to lay eggs for 19 hours from late afternoon to the next morning, after which the total number of eggs laid in each fruit was counted, and an oviposition preference index (OPI) calculated as follows: (number of eggs on ripe fruit – number of eggs on rotten fruit)/(number of eggs on ripe fruit + number of eggs on rotten fruit). Ripe strawberries (always of the same variety) were purchased from a local supermarket the day of the experiment, and rotten strawberries (same variety, purchased from the same supermarket) were allowed to rot in a 24°C, 55% relative humidity room for four days prior to the experiment. Only intact fruits without any damage were used in experiments. Between 10 and 12 replicate assays were performed for each species. In total, 45 assays were performed.

Substrate 2-choice oviposition assays. For all substrate choice assays flies were collected as virgins, separated by sex, and aged separately for 3-4 days in food vials. 2-3 days prior to the experiment, males and females were placed in a new food vial supplemented with yeast paste (1.5ml ddH_2O + 1g live active yeast) to mate and produce eggs. For each trial 3-4 females were placed in a custom laser cut egg-laying chamber (see description below) containing an agarose pad of the experimental substrate, and an agarose pad of the control substrate. Flies were inserted through a trap door with an aspirator without the use of anesthesia. Flies were allowed to lay eggs for 19 hours, after which the total number of eggs laid on each agarose pad was counted, and the OPI was calculated as follows: (number of eggs on experimental substrate – number of eggs on control substrate)/(number of eggs on experimental substrate + number of eggs on control substrate). Only assays where flies had laid a total of 10 eggs were included in the final analyses, and between 15 and 32 replicate assays were performed for each species at each concentration point. In total, 905 assays were performed.

Chamber design: Egg-laying chambers for substrate choice assays were custom made using laser cut acrylic plastic. Each chamber contained two separate egg-laying arenas separated by 6 mm of plastic, so two trials could be conducted in one chamber. Each arena contained two wells, into which agarose substrates were poured. Wells measured 32×45×12.7 mm and were divided by 5 mm of plastic. Each arena contained a trap door through which flies could be inserted without the use of anesthesia. A 100×75 mm glass sheet covers the entire chamber to allow light into the chamber, and to prevent flies from escaping.

Sucrose: For sucrose preference assays, experimental substrates were 1% agarose and contained 1% ethanol and increasing concentrations (200mM, 400mM or 600mM) of sucrose (ThermoFisher #S5-3); control substrates were 1% agarose and contained only 1% ethanol.

Ethanol: For ethanol preference assays, experimental substrates were 1% agarose and contained 75mM sucrose and increasing concentrations (3%, 5%, and 7%) of ethanol (ThermoFisher #BP2818); control substrates were 1% agarose and contained only 75mM sucrose.

Acetic Acid: For acetic acid preference assays, experimental substrates were 1% agarose and contained 75mM sucrose, 1% ethanol and increasing concentrations (1.5%, 2.5%, and 3.5%) of acetic acid (ThermoFisher #A465-250); control substrates were 1% agarose and contained only 75mM sucrose and 1% ethanol.

Agarose: For agarose/stiffness preference assays, experimental substrates contained 75mM sucrose, 1% ethanol and increasing concentrations (1%, 1.5%, and 2%) of agarose (Lonza SeaKem LE Agarose #50001); control substrates were 0.25% agarose and contained 75mM sucrose and 1% ethanol.

Statistics. All statistical analyses were performed using R (RStudio version 1.2.1335). For the whole fruit oviposition assays, a Wilcoxon signed rank test was used with the null hypothesis set to 0, signifying no preference. For the substrate gradient preference oviposition assays, a linear regression approach was performed for each substrate using the lme4 package in R to find the

overall preference difference between species across the concentrations tested. To determine if the preference curve across the three concentrations differed significantly between the four focal species, we used the lm function in R with the response term of OPI and predictor terms of the cross of concentration and species. The reference group was manually manipulated to perform pairwise comparisons between the preference curves of each species using the relevel function. Effectively, this sets each of the four different species as the baseline OPI response to the concentration tested and compares this baseline to the other species analyzed. P-values refer to the difference between pairwise comparisons of the y-intercepts of each species' preference curve across the three concentrations. For supplemental egg number analyses, a linear regression approach was also used, as described above. The P-values represent the level of significance for the difference between the slope of each species' regression and 0. Error bars in all figures are mean ± standard error of the mean (SEM) unless otherwise noted.

Genomic analysis

D. subpulchrella inbred line generation. To generate an inbred line for PacBio sequencing, *D. subpulchrella* flies (Ehime Stock Center #NGN6) were inbred via sib-mating for ten generations to generate the strain denominated "*D. subpulchrella* 33 F10 #4".

D. subpulchrella genome library preparation and sequencing.

DNA extraction and Sequencing: We extracted DNA from adult females following the protocol of Chakraborty et al. (Chakraborty et al., 2016). The DNA was sheared using 20 plunges of 21-gauge needle and size selected using the 30kb lower cutoff size on Blue Pippin size selection system (Sage Science). 30kb SMRTbell template library was prepared from the size selected DNA and was sequenced on 4 SMRTcells of Pacific Biosciences Sequel I platform. We also sequenced this sheared DNA sample with 150 bp paired-end library on Illumina Hiseq 4000. All sequencing was performed at UCI GHTF.

Genome assembly: We generated 45.3 Gb of long reads, in which 50% of the sequence is contained within sequences longer than 33.8kb (the longest sequence is 160 kb) and 149.40 million 150 bp paired-end Illumina reads. The reads were corrected and assembled with canu v1.7 using genomeSize=220M (Koren et al., 2017). The assembly was polished twice with arrow

(smrtanalysis v5.1.0) using the long reads and twice with Pilon using the Illumina reads (Walker et al., 2014). The size of the final assembly was 276 Mb, 50% of which is contained within reads that are 11.59 Mb or longer (assembly contig N50 11.59 Mb).

D. subpulchrella genome annotation.

To evaluate the genome assembly quality, we used BUSCO (Simão et al., 2015) to estimate the proportion of the 2799 Diptera orthologous genes and the 303 eukaryotic genes that were completely or partially assembled in the genome. We then used the MAKER2 (Holt et al., 2011) genome annotation pipeline for genome annotation. To improve the annotation quality, we trained the HMM for three times before using it for the final annotation (Zhao and Begun, 2017) After that, we used OrthoMCL (Li et al., 2003) to find homologous genes between *D. subpulchrella* and the other *Drosophila* species. For multiple-copy genes, we assigned their orthologous genes by using reciprocal best hits through BLASTP.

McDonald-Kreitman test for positive selection.

A high quality population genome dataset was aligned to the *D. suzukii* genome using bowtie2. After that we called bi-allelic SNPs using ANGSD version 0.920 (Korneliussen et al., 2014). On average the coverage of locations with SNPs is 178. We used bi-alleles that met the following criteria: minimum mapping quality of 30, minimum allele frequency of 0.05, and a minimum coverage larger than 10. We then created alternative references using the set of SNPs. We re-extracted the coding sequence of each gene from alternate references, then re-aligned using PRANK with the –codon function for each *D. suzukii* gene and their orthologous gene in *D. melanogaster*, *D. biarmipes*, and *D. subpulchrella.* We only carried out MK tests for genes that showed at least one variant in each of four categories, polymorphic, fixed, synonymous, and nonsynonymous (Begun et al., 2007). For genes that passed the above criteria we carried out unpolarized McDonald–Kreitman tests using the MK.pl script (Begun et al., 2007), and a version of our own python scripts independently, followed by a Fisher's exact test for each gene within each species pairwise comparison. A third method of MK test using SNPs directly from individuals was also used for confirming the results. For each gene, we estimated the proportion of adaptive amino acid fixations (α) (Smith and Eyre-Walker, 2002) and the Direction of Selection (DoS) index (Stoletzki and Eyre-Walker, 2011).

Lists of ORs, GRs, IRs and MRs were compiled using FlyBase assigned gene groups for *D. melanogaster* and their orthologous genes in the other three species inferred from OrthoMCL or BLASTP. We did not analyze the full list of sensory receptors listed on FlyBase due to the short length of some of proteins, as short proteins cannot be reliably analyzed using the MK test often due to lack of polymorphisms.

Simulations were used to compare the average degree of adaptive evolution occurring in receptor gene groups to the genome-wide average. We randomly sampled gene sets of identical size to the receptor gene set of interest for each separate species comparison (*D. suzukii – D. melanogaster, D. suzukii – D. biarmipes,* and *D. suzukii – D. subpulchrella*) and computed the mean Fisher's exact test P-value for that random gene set. Simulations were run 10,000 times for each set of interest, and P-values represent the proportion of the simulated distribution of means below the observed mean P-value for the receptor gene set of interest.

RNA library preparation, and sequencing.

We generated head transcriptomes from female *D. suzukii, D. melanogaster, D. biarmipes,* and *D. subpulchrella.* All individuals were mated and precisely 3-days-old. Female flies were very briefly anesthetized with CO_2 and heads were collected with a clean razor blade. Dissections were performed within a 1-hour window always at the same circadian zeitgeber time (ZT1-ZT2; with light turning on at 8 am, and the dissections being performed between 9 am and 10 pm). We collected 3 biological replicates for each species, each sample contained 15 heads. Dissected heads were immediately transferred into a low retention Eppendorf tube containing 100 μL TRIzol (Invitrogen) and RNA was extracted immediately post dissection.

All RNA extractions were performed according to TRIzol manufacturer protocol and immediately followed by a DNase treatment using the TURBO DNase from Invitrogen. RNA quality was assessed by a Bioanalyzer run of an Agilent Eukaryote Total RNA Pico chip while RNA quantity was measured with a Nanodrop One (ABI). About 1 μg total RNA was used for library preparation. Libraries were fragmented and enriched for mRNA using NEBNext Poly(A)$^+$ Magnetic Isolation Module (NEB #E7490) and prepared using NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7765) and single indexing from the NEBNext Multiplex Oligos kit (NEB #E7555L) following manufacturer protocol including beads size selection for 200 bp inserts. Library quality was first assessed on Agilent D1000 ScreenTapes for Tapestation and

then by Qubit and Agilent Bioanalyzer. Finally, 150 bp paired-ends libraries were sequenced on an Illumina Nextseq500 platform.

Identification of differentially expressed sensory receptor genes.

Adaptors and low-quality bases from RNA-seq reads were trimmed using Trimmomatic (Bolger et al., 2014) using the setting LEADING:1 TRAILING: 1 SLIDINGWINDOW:20:25 and MINLEN:36. Bowtie2 (Langmead and Salzberg, 2012) was then used to align reads to the reference genome of the species being analyzed. Gene and transcript expression levels (TPM: Transcripts per million) were then quantified using StringTie (Pertea et al., 2015). We then obtained a list of homologous genes of *D. suzukii, D. biarmipes,* and *D. subpulchrella* using OrthoMCL and reciprocal best hits. To compare gene expression between replicates from different species we used TMM (trimmed mean M-values) normalized TPM values. TMM normalization was implemented with the R package edgeR (Robinson et al., 2009). We then conducted a Log_2 transformation across all replicates and calculated a P-value using a Student's *t*-test between *D. suzukii* and each of the other focal species to determine if each gene is differential expressed between two species. We then manually checked the expression patterns of the sensory receptor genes inferred by homology with *D. melanogaster* sensory receptor genes extracted from FlyBase (Thurmond et al., 2019).

DATA AVAILABILITY

All single-molecule sequence data have been deposited to the NCBI Sequence Read Archive (SRA) and can be found under BioProject accession PRJNA557422. The *D. subpulchrella* genome assembly has been deposited in the NCBI Assembly database under accession JACOEE000000000. The genomes and SNPs used in the MK analysis, and raw behavioral data can be found at the GitHub page https://github.com/LiZhaoLab/D.suzukii_genomes_and_analysis.

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AUTHOR CONTRIBUTION

S.M.D, L.Z., and N.S. conceived the study. S.M.D performed the behavioral analyses with the help of M.K. S.M.D generated the genome strain of *D. subpulchrella* and performed RNA sequencing library construction. M.C. and J.J.E. sequenced the *D. subpulchrella* genome. A.A., K.M.L., and J.C.C. generated the *D. suzukii* population genome data. L.Z., S.M.D., A.G., J.P., and N.S. performed the computational analysis. S.M.D. and L.Z. wrote the manuscript with the input from all authors.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURES

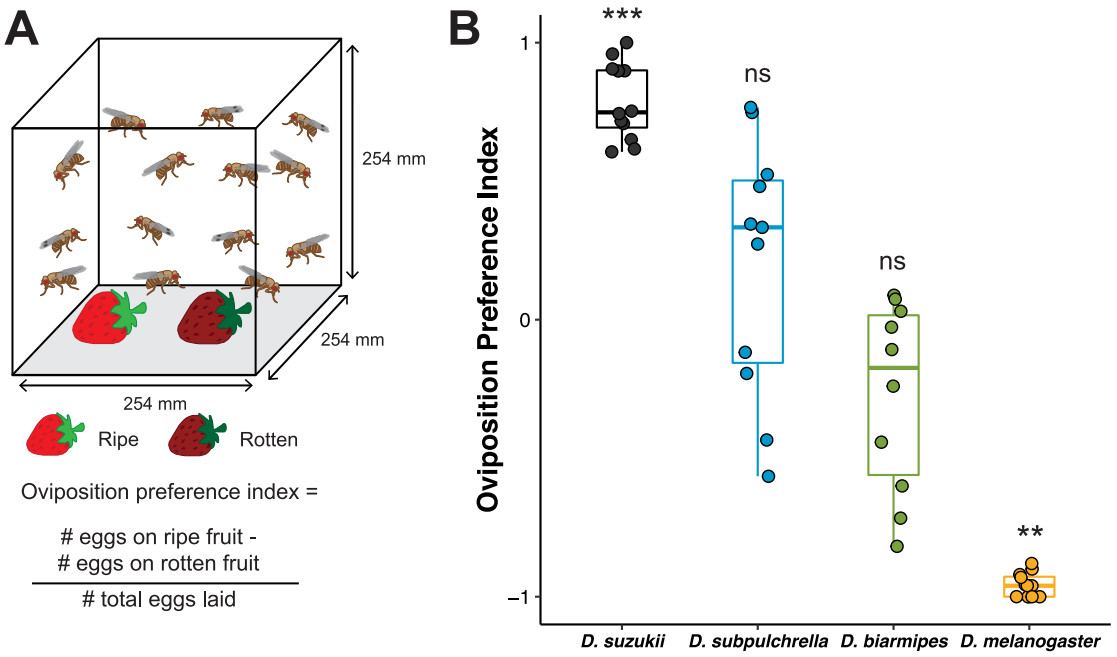

Figure 1. The evolution of ripe fruit preference in *D. suzukii* occurred gradually, with *D. subpulchrella* and *D. biarmipes* as intermediates.
A. Schematic of two-choice oviposition preference assay. 10 females and 5 males were placed in a cage with a ripe and rotten strawberry for 18 hours. Number of eggs on each fruit was then counted. B. Oviposition preference for ripe fruit (positive OPI) of four species in the *melanogaster* species group. *D. suzukii* prefers to oviposit in ripe fruit, *D. subpulchrella* and *D. biarmipes* show no preference for either fruit, and *D. melanogaster* prefers rotten fruit. Each data point represents one experimental trial and data dispersion is represented by a boxplot. Preference P-values were calculated using a Wilcoxon signed rank test against a theoretical value of 0 (no preference).

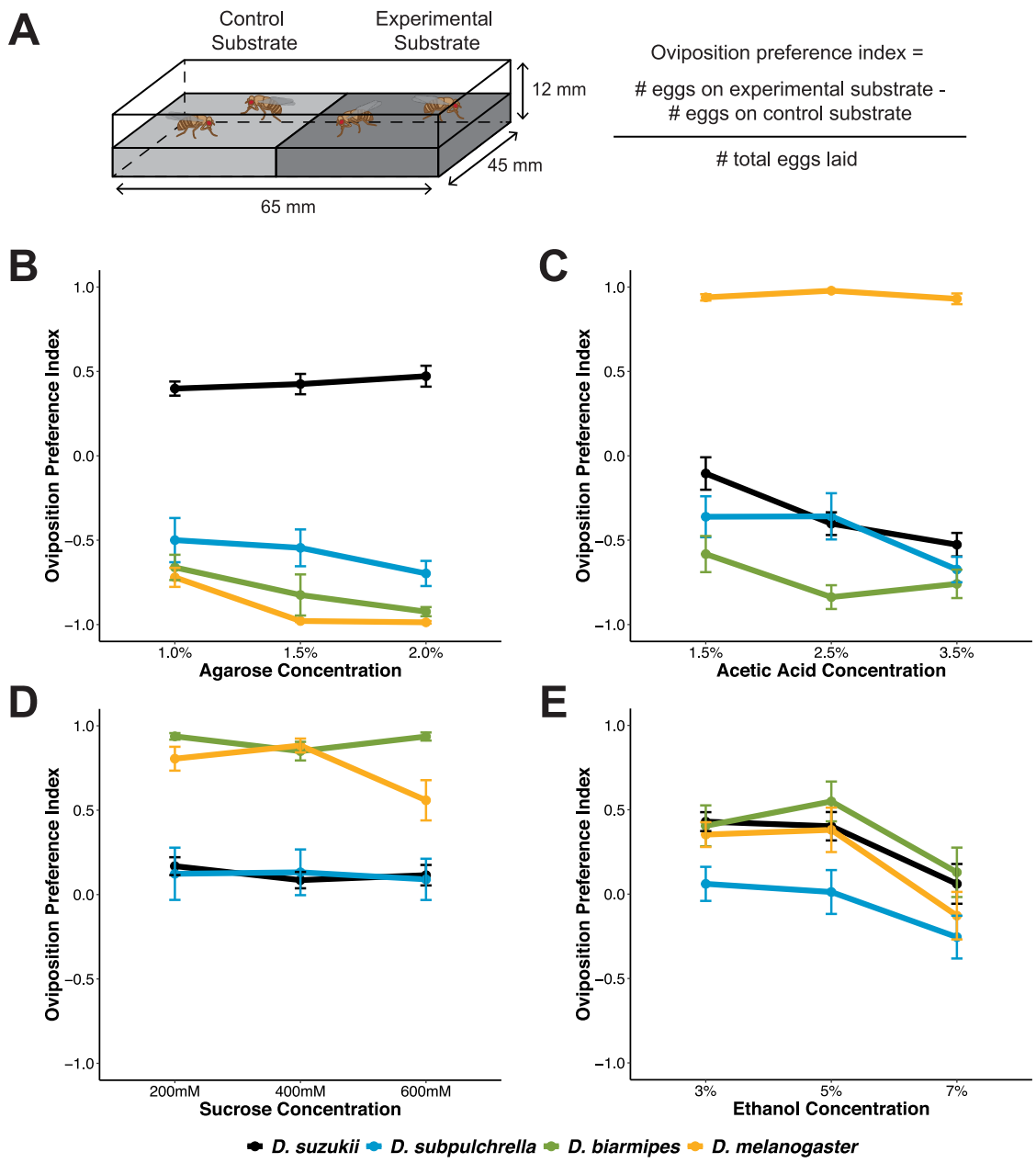
A
Control Substrate
Experimental Substrate
12 mm
45 mm
65 mm
Oviposition preference index =
eggs on experimental substrate - # eggs on control substrate
total eggs laid
B
C
D
E
Oviposition Preference Index
1.0
0.5
0.0
−0.5
−1.0
1.0%
1.5%
2.0%
Agarose Concentration
1.5%
2.5%
3.5%
Acetic Acid Concentration
200mM
400mM
600mM
Sucrose Concentration
3%
5%
7%
Ethanol Concentration
D. suzukii
D. subpulchrella
D. biarmipes
D. melanogaster

Figure 2. Oviposition preference for substrates associated with fruit maturation differ among focal species.
A. Schematic of egg laying chamber for the substrate gradient two-choice oviposition preference assay. 3-4 females were placed in an arena with a choice between two substrates for 18 hours. Number of eggs on each substrate was then counted. Experimental substrate was varying concentrations of either ethanol, sucrose, acetic acid, or agarose. $n \geq 15$ for each species at each concentration. P-values calculated through linear regression analysis through pairwise comparisons of the y-intercepts of each species' preference curve across the three concentrations (see methods). Data points are mean ± SEM.

B. Stiffness preference separated *D. suzukii* from the other three species, as it was the only species that consistently prefers stiffer oviposition substrates. There were significant differences in the preference curves of *D. suzukii* and *D. subpulchrella* (*)*, D. suzukii* and *D. biarmipes* (**)*, and D. suzukii* and *D. melanogaster* (***).

C. *D. melanogaster* had a strong oviposition preference for acetic acid at each concentration measured, while *D. biarmipes, D. subpulchrella,* and *D. suzukii* had an aversion to acetic acid containing substrates. There were significant differences in the preference curves of *D. suzukii* and *D. biarmipes* (**)*, D. suzukii* and *D. melanogaster* (***)*, D. subpulchrella* and *D. melanogaster* (***)*,* and *D. biarmipes* and *D. melanogaster* (***).

D. *D. suzukii* and *D. subpulchrella* did not show an aversion to or preference for sucrose at any concentration measured, while *D. biarmipes* and *D. melanogaster* prefer sucrose containing substrates. There were significant differences in the preference curves of *D. suzukii* and *D. biarmipes* (***), *D. suzukii* and *D. melanogaster* (***), *D. subpulchrella* and *D. biarmipes* (**), and *D. subpulchrella* and *D. melanogaster* (**).

E. Ethanol oviposition preference did not differ between *D. suzukii, D. biarmipes*, or *D. melanogaster*, with all species showing a preference for ethanol at 3% and 5%, and a neutral response to ethanol at 7%. *D. subpulchrella* displayed a neutral response to ethanol at each concentration measured. There were no significant differences in preference curves between any two species.

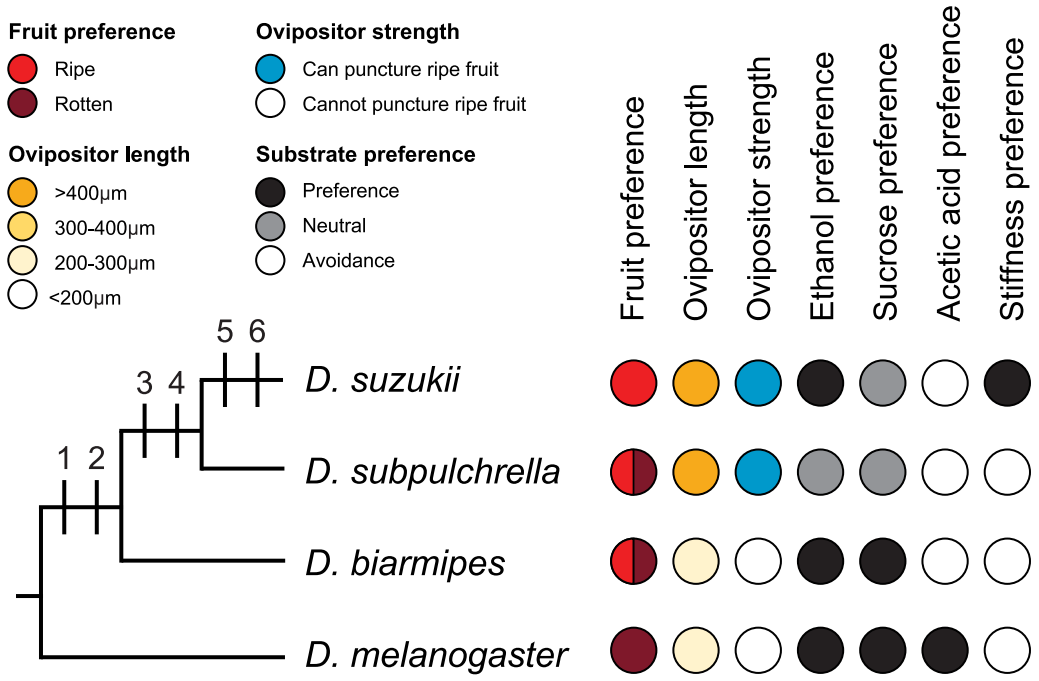

Figure 3. Proposed evolutionary history of pest-like traits in *D. suzukii.*
The traits acquired step-wise leading to the specialization on ripe fruit in this model are: 1. Relaxation of rotten fruit preference, 2. High acetic acid aversion, 3. Enlarged ovipositor and ability to puncture ripe fruits, 4. Loss of sucrose preference, 5. Ripe fruit specialization, 6. Preference for stiff substrates. Ovipositor strength and length data from Atallah et al. 2014.

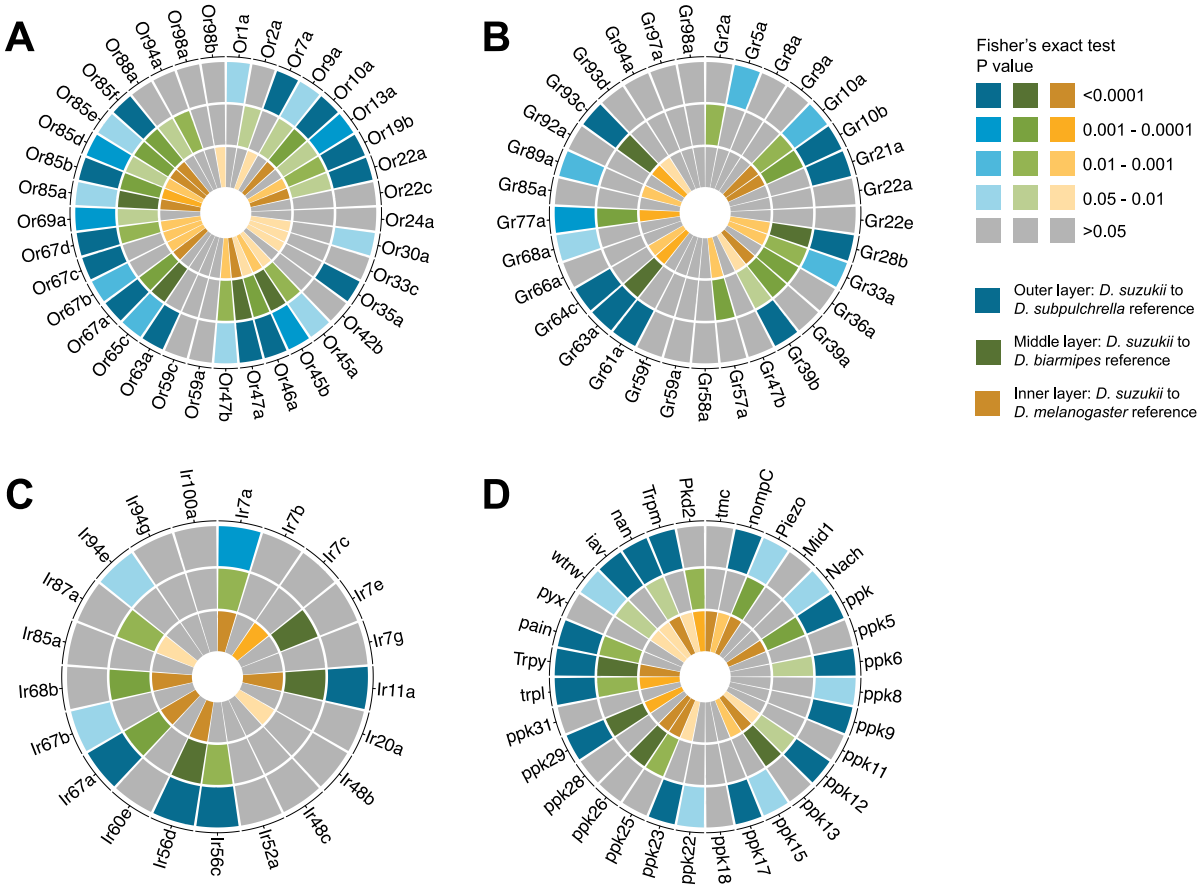

Figure 4. McDonald-Kreitman test for sensory receptors evolving under positive selection in *D. suzukii,* as compared to *D. subpulchrella*, *D. biarmipes,* and *D. melanogaster.*
Odorant receptors (A), gustatory receptors (B), divergent ionotropic receptors (C), and mechanosensory receptors (D) undergoing adaptive evolution in *D. suzukii*. MK test conducted using population data from >200 *D. suzukii* genomes. P-values calculated using Fisher's exact test.

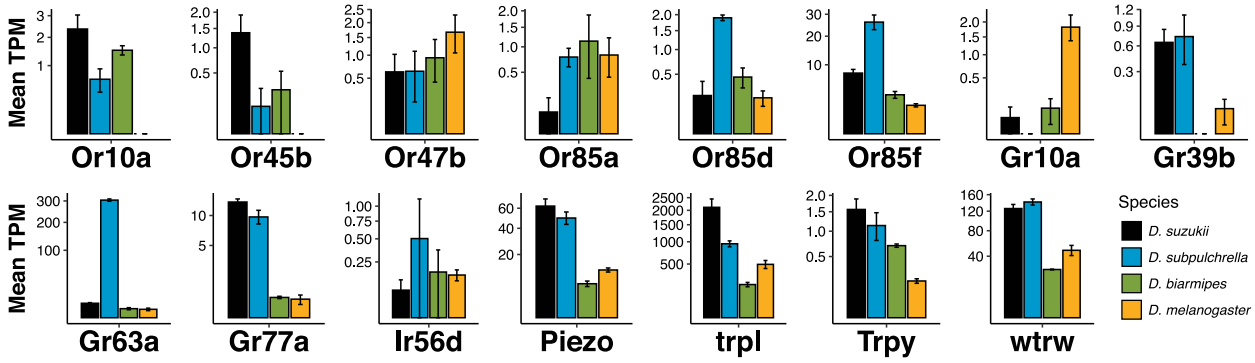

Figure 5. Gene expression of candidate sensory receptors potentially underlying *D. suzukii's* novel oviposition behavior.
Fifteen candidate ORs, GRs, IRs, and MRs were identified as both evolving under positive selection in D. *suzukii* compared to *D. subpulchrella*, *D. biarmipes,* and *D. melanogaster* and significantly differentially expressed ($p < 0.05$) in at least one species as compared to *D. suzukii* in the gene expression analysis. The y-axes are mean transcripts per million (TPM) normalized between all species and each y-axis is square root transformed. P-values listed in supplementary tables S2-5 and calculated using species pairwise *t*-tests. Histogram bars are mean ± SD.

SUPPLEMENTARY FIGURES & TABLES FOR

Behavioral and genomic sensory adaptations underlying the pest activity of *Drosophila suzukii*

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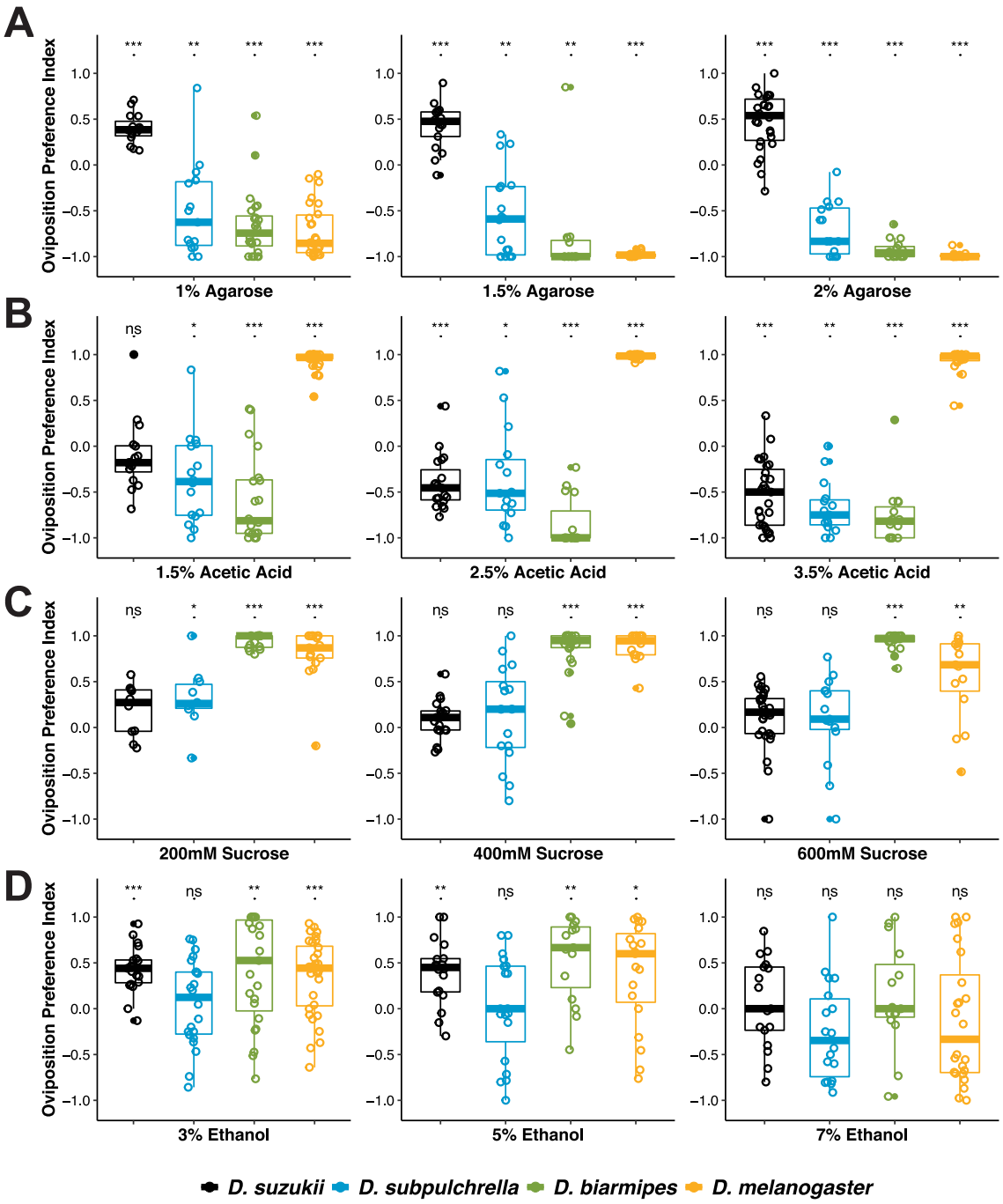

Figure S1. Oviposition preference at each concentration point.
Species specific oviposition preference at each separate concentration tested of agarose (A), acetic acid (B), sucrose (C) and ethanol (D). Each data point represents one experimental trial and data dispersion is represented by a boxplot. Preference P-values were calculated using a Wilcoxon signed rank test against a theoretical value of 0 (no preference).

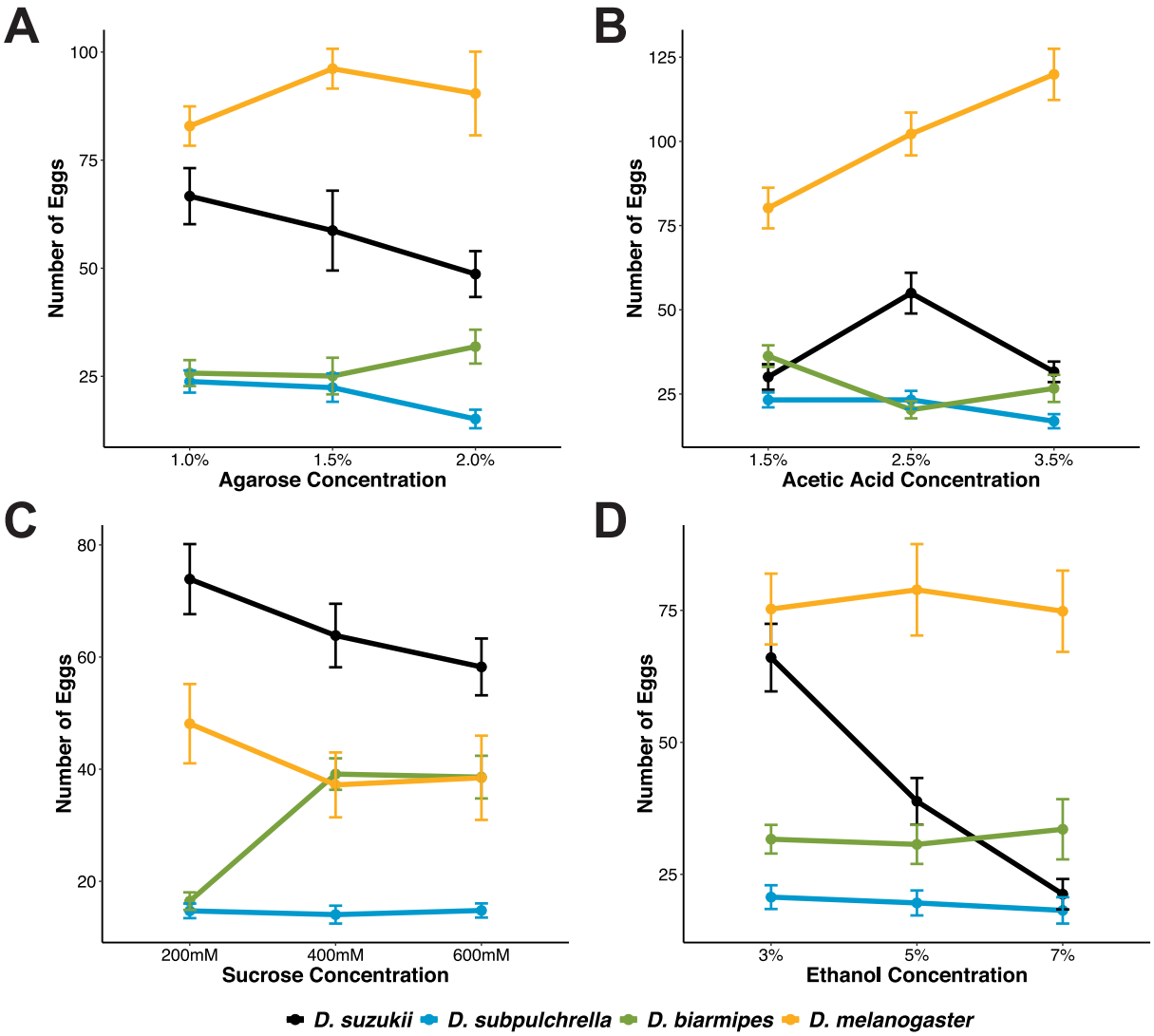

Figure S2. Concentration specific egg number variation differs among focal species. Average total number of eggs laid per trial for the data presented in Figure 2 varies in a species-specific manner. Some species alter their egg laying amount as concentration increased, while others were unaffected. A. *D. suzukii* laid significantly less eggs as agarose concentration increased (*). B. *D. melanogaster* laid significantly more eggs as acetic acid concentration increased (***). C. Increasing sucrose had a significant effect on the number of eggs laid by *D. biarmipes* (**) and *D. suzukii* (*). D. *D. suzukii* laid significantly less eggs as ethanol concentration increased (***). P-values calculated through linear regression analysis through pairwise comparisons of the y-intercepts of each species' egg number curve across the three concentrations (see methods). Species not mentioned did not lay a significantly different number of eggs at different concentrations. Data points are mean ± SEM.

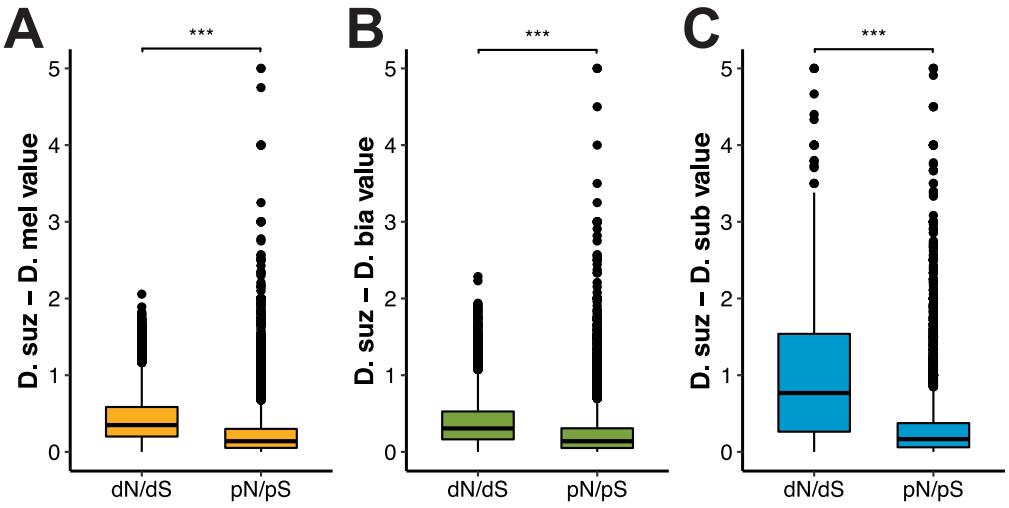

Figure S3. Distribution of d_N/d_S and p_N/p_S from genome wide McDonald-Kreitman test. d_N/d_S is significantly larger than p_N/p_S across the genome in all three species pairwise comparisons: *D. suzukii* to *D. melanogaster* (A), *D. suzukii* to *D. biarmipes* (B), and *D. suzukii* to *D. subpulchrella* (C). Y-axes have a cutoff of 5. However, all values were included for statistical analyses and P-values were calculated through pairwise *t*-tests.

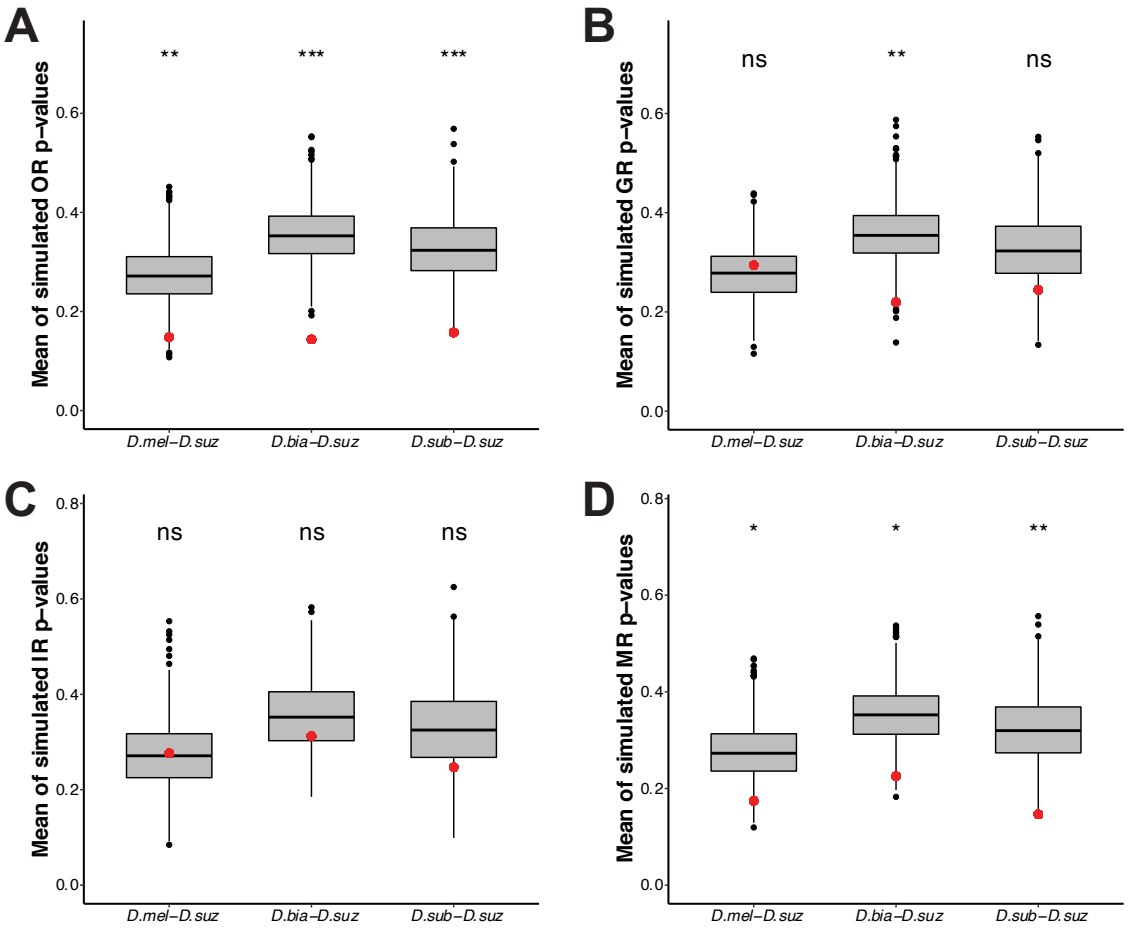

Figure S4. Sensory receptor genes are evolving under positive selection to a greater extent than the genome as a whole.
To generate distributions of simulated mean p-values for ORs (A), GRs (B), IRs (C) and MRs (D) for each species comparison, a random group of genes of the same number as the group of sensory receptor gene groups was selected 10,000 times, and the mean of the random gene group was plotted. Red dots represent the actual mean P-value for the sensory receptor gene group within each species comparison. Significance labels represent the proportion of the distribution lower than the actual mean P-value (red dot).

Table S1. Properties of the chromosome-level assembly of the *D. subpulchrella* genome. The scaffolds listed in the table have at least 6 genes that are reciprocal best hits of *D. melanogaster* genes. Scaffold names starting with "alt_" denote alternate haplotype contigs.

***D. subpulchrella* scaffold name**	**Length (Mb)**	***D. melanogaster* chromosome homolog**	**Best hit gene number with homologous chromosome**
Seq405	29.50	3L	1985
Seq361	22.17	2R	1807
Seq355	20.23	3R	1686
Seq407	29.67	X	1572
Seq360	26.21	2L	1532
Seq354	11.59	3R	794
alt_Seq3	1.79	2L	106
Seq23	2.26	4	56
alt_Seq10	0.75	2R	46
Seq24	2.16	2R	41
alt_Seq14	0.76	2L	14
alt_Seq417	0.47	2L	11
Seq15	4.47	X	9
alt_Seq9	0.08	3R	7
Seq36	1.62	2L	6

Table S2. OR gene differential expression P-values.
OR genes that were included in the MK test were analyzed for differential expression in *D. suzukii* as compared to *D. melanogaster, D. biarmipes*, and *D. subpulchrella*, separately. P-values were calculated through pairwise *t*-tests on Log_2 transformed and normalized mean TPM between *D. suzukii* and the other species.

Gene Name	*D. mel - D. suz*	*D. bia - D. suz*	*D. sub - D. suz*
Or10a	0.031	0.076	0.058
Or13a	0.065	0.053	0.531
Or19b	0.282	0.632	0.662
Or1a	0.010	0.500	0.519
Or22a	0.194	0.459	0.765
Or22c	0.506	0.080	0.310
Or24a	0.272	0.939	0.913
Or2a	0.414	0.725	0.465
Or30a	0.506	0.106	1.000
Or33c	0.157	0.277	0.529
Or35a	0.136	0.283	0.220
Or42b	0.196	0.632	0.338
Or45a	0.084	0.123	0.414
Or45b	0.058	0.292	0.030
Or46a	0.069	0.155	0.335
Or47a	0.405	0.005	0.169
Or47b	0.019	0.162	0.739
Or59a	0.355	0.141	1.000
Or59c	0.876	0.022	0.016
Or63a	0.039	0.024	0.059
Or65c	0.031	0.070	0.026
Or67a	1.000	0.026	0.467
Or67b	0.087	0.060	0.060
Or67c	1.000	0.681	0.241
Or67d	0.639	0.144	0.519
Or69a	0.135	0.088	0.787
Or7a	0.543	0.792	0.385
Or85a	0.054	0.105	0.014
Or85b	0.171	0.083	0.584
Or85d	0.288	0.039	0.007
Or85e	0.843	0.092	0.053
Or85f	0.059	0.968	0.001
Or88a	0.042	0.042	0.529
Or94a	0.296	0.289	0.519
Or98a	0.351	0.028	0.029
Or98b	0.782	0.926	0.285
Or9a	0.021	0.976	0.037

Table S3. GR gene differential expression P-values.
GR genes that were included in the MK test were analyzed for differential expression in *D. suzukii* as compared to *D. melanogaster, D. biarmipes*, and *D. subpulchrella*, separately. P-values were calculated through pairwise *t*-tests on Log_2 transformed and normalized mean TPM between *D. suzukii* and the other species. NA – Not Applicable, gene is not expressed in either of the species in the comparison.

Gene Name	*D. mel - D. suz*	*D. bia - D. suz*	*D. sub - D. suz*
Gr10a	0.001	0.320	0.519
Gr10b	0.569	0.006	0.644
Gr21a	0.392	0.468	0.021
Gr22a	NA	0.331	NA
Gr22e	NA	0.500	0.519
Gr28b	0.073	0.504	0.787
Gr2a	0.059	0.802	0.266
Gr33a	0.092	0.675	0.520
Gr36a	0.585	0.537	0.519
Gr39a	0.369	0.019	0.834
Gr39b	0.036	0.037	0.372
Gr47b	0.007	0.002	0.003
Gr57a	NA	NA	NA
Gr58a	NA	NA	NA
Gr59a	0.506	0.395	0.519
Gr59f	0.956	0.500	0.463
Gr5a	0.784	0.234	1.000
Gr61a	0.364	0.350	0.103
Gr63a	0.103	0.858	0.000
Gr64c	0.267	0.005	0.934
Gr66a	0.003	0.000	0.004
Gr68a	0.344	0.839	1.000
Gr77a	0.009	0.000	0.354
Gr85a	0.049	0.003	0.005
Gr89a	0.124	0.500	0.275
Gr8a	0.622	0.002	0.017
Gr92a	0.074	0.057	0.424
Gr93c	0.994	0.044	0.328
Gr93d	0.924	0.113	0.909
Gr94a	0.510	0.486	0.439
Gr97a	0.006	0.013	0.000
Gr98a	0.544	0.500	0.519
Gr9a	0.506	0.611	0.028

Table S4. IR gene differential expression P-values.
IR genes that were included in the MK test were analyzed for differential expression in *D. suzukii* as compared to *D. melanogaster, D. biarmipes*, and *D. subpulchrella*, separately. P-values were calculated through pairwise *t*-tests on Log_2 transformed and normalized mean TPM between *D. suzukii* and the other species. NA – Not Applicable, gene is not expressed in either of the species in the comparison.

Gene Name	*D. mel - D. suz*	*D. bia - D. suz*	*D. sub - D. suz*
Ir100a	0.131	0.016	0.127
Ir11a	0.371	0.279	1.000
Ir20a	0.282	0.500	NA
Ir48b	NA	0.500	NA
Ir48c	0.824	0.360	0.200
Ir52a	0.109	0.091	0.141
Ir56c	0.946	0.338	0.519
Ir56d	0.032	0.415	0.418
Ir60e	0.116	0.016	0.029
Ir67a	NA	NA	0.334
Ir67b	0.316	0.277	0.010
Ir68b	0.273	0.976	0.489
Ir7a	0.853	0.445	0.276
Ir7b	NA	NA	0.519
Ir7c	0.142	0.902	0.670
Ir7e	NA	0.500	NA
Ir7g	0.157	0.262	0.278
Ir85a	0.652	0.001	0.532
Ir87a	0.881	0.168	0.519
Ir94e	0.172	0.046	0.519
Ir94g	NA	0.362	NA

Table S5. MR gene differential expression P-values.
MR genes that were included in the MK test were analyzed for differential expression in *D. suzukii* as compared to *D. melanogaster, D. biarmipes*, and *D. subpulchrella*, separately. P-values were calculated through pairwise *t*-tests on Log_2 transformed and normalized mean TPM between *D. suzukii* and the other species. NA – Not Applicable, gene is not expressed in either of the species in the comparison.

Gene Name	*D. mel - D. suz*	*D. bia - D. suz*	*D. sub - D. suz*
iav	0.008	0.002	0.060
Mid1	0.049	0.775	0.049
Nach	0.101	0.084	0.257
nan	0.239	1.000	0.857
nompC	0.068	0.149	0.112
pain	0.001	0.036	0.581
Piezo	0.077	0.005	0.149
Pkd2	0.465	0.000	0.307
ppk	0.163	0.545	0.057
ppk11	0.328	0.751	0.456
ppk12	0.932	0.200	0.576
ppk13	NA	0.043	NA
ppk15	0.084	0.003	0.350
ppk17	0.027	0.688	0.276
ppk18	0.329	0.792	0.051
ppk22	0.637	0.001	0.255
ppk23	0.506	0.013	0.083
ppk25	0.204	0.008	0.030
ppk26	0.506	0.717	0.953
ppk28	0.137	0.084	0.344
ppk29	0.866	0.053	0.150
ppk31	0.011	0.133	0.024
ppk5	1.000	0.000	0.329
ppk6	0.506	0.084	0.277
ppk8	0.591	0.946	0.195
ppk9	0.506	0.294	0.039
pyx	0.506	0.013	0.021
tmc	0.506	0.076	0.037
trpl	0.010	0.001	0.018
Trpm	0.078	1.000	0.343
Trpy	0.041	0.649	0.914
wtrw	0.145	0.001	0.001