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CYP genes are duplicated in *Drosophila suzukii* and carry transposable elements

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Gene duplication and transposable element (TE) insertions are key drivers of genome evolution and have been linked to increased insecticide resistance in insects. The aim of this study was to characterize the distribution and potential impact of TEs within and near cytochrome P450 monooxygenase (CYP) genes in the invasive pest *Drosophila suzukii* compared with the non-pest *Drosophila melanogaster*. We performed in silico analyses of the CYP gene repertoire and genome architecture in *D. suzukii* and *D. melanogaster*. We identified ten duplicated CYP genes that are exclusive to *D. suzukii* and absent from its closely related species *D. melanogaster*. These duplications are enriched with TE fragments, predominantly Helitrons. Thirty-six percent of TE sequences within CYP genes and their flanking regions carry putative transcription-factor binding sites in *D. suzukii*, indicating a possible role in gene regulation. Building on the proposed model of Helitron-mediated exon shuffling, our findings suggest that these elements contribute to gene rearrangement, thereby potentially enhancing functional diversity. At the genome level, *D. suzukii* harbors a higher overall TE content than *D. melanogaster*, with a relative enrichment within CYP genes. The increased TE content may have enhanced genomic plasticity, thereby facilitating the species' invasive success, rapid population growth, and ability to adapt to diverse habitats, such as native environments and agricultural fields.

KEYWORDS

cytochrome P450 monooxygenases, genome TE content, Helitron, transcription-factor binding site, transposon

Introduction

Cytochrome P450 monooxygenases (CYPs) are a diverse group of isoenzymes that play a fundamental role in the metabolism of endogenous and exogenous compounds (Feyereisen, 2005). Belonging to an ancient and widespread gene family, CYPs are present in virtually all living organisms (Feyereisen, 2005). In insects, they are critical for detoxification processes, contributing to the metabolism of both natural plant

allelochemicals and synthetic insecticides (Scott, 1999; Feyereisen, 2005; Li et al., 2007). Variation in CYP gene expression and copy number has often been linked to the development of metabolic resistance to insecticides in arthropod pest species (Hu et al., 2025).

Resistance to insecticides serves as a valuable model for studying evolutionary phenomena because the selective agent (insecticide) is well understood, and the response to selection (resistance) is typically rapid (McKenzie and Batterham, 1994). For instance, insecticide resistance has been associated with overexpression of *CYP6p3* in *Anopheles gambiae* (Müller et al., 2008), and *CYP6bq9* in the brain of *Tribolium castaneum* (Zhu et al., 2010). In the aphid *Myzus persicae*, resistance is mediated by duplication of *CYP6cy3* and *CYP6g1* (Puinean et al., 2010), whereas in *Drosophila melanogaster* it involves both duplication and overexpression of *CYP6g1* (Daborn et al., 2002; Harrop et al., 2014).

In addition to gene duplication and regulatory changes, the activity of transposable elements (TEs) has emerged as a key mechanism in modulating CYP gene expression and driving the evolution of insecticide resistance. TEs are repetitive DNA sequences capable of moving within and between genomes and this mobility can affect genome structure and function, leading to phenotypic changes through altered gene expression and increases in genome size (Elliott and Gregory, 2015). In Drosophila, TEs - including insertions and TE-derived fragments - have been shown to shape CYP genes regulation and contribute to insecticide resistance (Daborn et al., 2002; Catania et al., 2004; Schlenke and Begun, 2004; Bogwitz et al., 2005; Marsano et al., 2005; Chung et al., 2007; Carareto et al., 2013). For instance, overexpression of *CYP6g1* in *D. melanogaster* is associated with the insertion of the *Accord* retroelement upstream of the gene (Daborn et al., 2002), while in *D. simulans*, the ortholog is overexpressed due to insertion of the *DOC* element in its flanking region (Schlenke and Begun, 2004). Moreover, in *D. melanogaster*, insertion of the *Bari-1* element at the 3' end of *CYP12a4* enhances gene expression (Bogwitz et al., 2005; Marsano et al., 2005). Among these, Helitrons—rolling-circle DNA transposons capable of capturing and reshuffling host gene fragments—represent a particularly dynamic family whose role in CYP evolution is explored in detail in this study.

Drosophila species are best known as model organisms in genetics and evolutionary biology, but a few have become significant agricultural pests. Among them, *Drosophila suzukii* (Matsumura, 1931) is notable as one of the most damaging fruit crop pests worldwide (Walsh et al., 2011; Asplen et al., 2015). Native to Japan, *D. suzukii* has spread throughout Asia; North America (Walsh et al., 2011; Asplen et al., 2015; Cini et al., 2012; Rota-Stabelli et al., 2013); Europe (Kaneshiro, 1983; Leblanc et al., 2009); and South America, where our group first reported its occurrence (Deprá et al., 2014). Females possess a serrated ovipositor that allows them to lay eggs in healthy fruits rather than decaying ones (Walsh et al., 2011; Lee et al., 2015). The resulting perforations provide entry points for pathogens, leading to economic losses of up to 80% in fruit production, mostly berries (Dreves et al., 2009; Hauser, 2011; Escudero et al., 2012), and releasing volatile compounds (Abraham et al., 2015) that attract other drosophilid species (Timmeren and Isaacs, 2013; Joshi et al., 2014; Lasa and Tadeo, 2015). Once established, *D. suzukii* is extremely difficult to eradicate, resulting in increased production costs due to the need for constant monitoring, intensive management, greater insecticide use, and post-harvest fruit sorting. Given its invasive success, broad ecological tolerance, and heavy exposure to insecticides, *D. suzukii* provides an ideal model to investigate how gene duplication and transposable elements contribute to genomic plasticity and adaptive evolution.

Understanding the genetic basis of *Drosophila suzukii*'s adaptability and resistance to control measures is essential for developing effective management strategies. The sequenced genome of this species (Chiu et al., 2013) contains 76 annotated CYP genes (SpottedWingFlyBase, Annotation Release v1), compared with 99 in *D. melanogaster* (FlyBase, Release v3). Since CYPs are among the major metabolic systems in insects capable of mediating resistance to all major classes of insecticides (Scott, 1999; Li et al., 2007), examining these genes—along with their associated TEs and potential regulatory effects—can provide valuable insights into the genetic and molecular mechanisms underlying insecticide resistance and the species' invasive success.

To address this issue, the main aim of this study was to compare the CYP gene repertoire of *D. suzukii* and *D. melanogaster*, focusing on gene structure, the occurrence and distribution of TE insertions within or near these genes, to explore their potential influence on gene structure and TE-derived regulatory elements. We hypothesized that transposable elements (TEs) may contribute to regulatory diversification, potentially playing a key role in the adaptive success and insecticide resistance of *D. suzukii*.

Materials and methods

In silico analysis of CYP genes

CYP gene sequences and structures were retrieved directly from the curated genome annotations available in the Gbrowser databases (SpottedWingFlyBase, SpottedWingFlyBase, 2013, and FlyBase, FlyBase, 1993), using Annotation Release v1 for *D. suzukii* and Annotation Release v3 for *D. melanogaster* (Supplementary Tables S1-S4). Only protein-coding CYP genes were included in the analysis. Because these genes were obtained from assembled annotations rather than from *de novo* searches, no overlapping or ambiguous hits were encountered. As transposable elements (TEs) in flanking regions can provide novel transcriptional regulatory signals, we extracted 10 kb upstream (5′ flanking region) and 10 kb downstream (3′ flanking region) of each CYP gene based on the annotated gene coordinates. The retrieved sequences were visually inspected in Gbrowser to compare genomic features between species.

Gene length comparisons between *D. suzukii* and *D. melanogaster* were performed and visualized in R using the *genoPlotR* package (Guy et al., 2010). In this package, each orthologous gene is plotted side-by-side to allow direct structural

comparison, enabling visualization of local synteny among the species such as exon–intron organization, total gene length, and the positions of TE insertions. All graphical outputs were refined in Inkscape v0.92.1[1].

For phylogenetic context, we incorporated the maximum-likelihood phylogeny generated by Chiu et al. (2013) into our comparative analyses. We further expanded these analyses to include orthologous CYP genes from two sister species of *D. suzukii, Drosophila biarmipes* and *Drosophila takahashii* (Annotation Release 101 for both species), and screening these orthologs for TE insertions (Supplementary Tables S5, S6) using the same RepeatMasker-based pipeline described below to ensure robust cross-species comparisons.

In silico analysis of transposons

To detect the presence of TEs associated with CYP genes, we analyzed each gene sequence and, separately, 10 kb of its 5′ and 3′ flanking regions using the RepeatMasker web server (RepeatMasker Open-4.0 Software, 1996). Searches were conducted with the parameters: *crossmatch, fruit fly*, and GC-level–based matrix. TE classification was assigned according to the highest-scoring match using the *Drosophila* reference library from Repbase (Jurka et al., 2005). Because the "fruit fly" RepeatMasker library is optimized for *D. melanogaster*, it may therefore underestimate lineage-specific or recently diverged TE families in *D. suzukii*.

Based on the RepeatMasker output, we classified TE fragments according to their genomic location relative to CYP genes as: (i) intronic, (ii) 5′ flanking (within 10 kb upstream of the transcription start site), or (iii) 3′ flanking (within 10 kb downstream of the transcription termination site). TE sequences located within CYP genes and 10 kb of its flanking regions were further analyzed to identify putative transcription-factor binding sites (TFBS). Strand-specific predictions were performed using the ConSite web server (ConSite Software, 2004) with the JASPAR CORE Insecta database (Bryne et al., 2008) for *D. melanogaster*, applying a 90% TFBS cutoff score, following the methodology of Carareto et al. (2013).

To evaluate whether the number of TE insertions in CYP genes was proportional to the overall genomic TE composition of the studied species, Illumina reads were obtained from the Sequence Read Archive (SRA): *D. suzukii* – SRR942805 (North American sample; Chiu et al., 2013), and *D. melanogaster* – SRR1738161. Graph-based clustering of NGS reads was performed using RepeatExplorer (Novák et al., 2013) on the Galaxy-based web server, following the pipeline described by Silva et al. (2016). This analysis provided genome-wide estimates of TE content and the relative contribution of different TE superfamilies, including Helitrons, which were then compared with the proportion of TE and Helitron copies overlapping CYP genes.

Statistical analysis

We first compared the size distributions of CYP genes and non-CYP genes within each species. For this purpose, a dataset of 500 additional genes was randomly selected from each genome. For *D. suzukii*, random genes were sampled using BEDTools v2.27.0 (Quinlan, 2014), and their orthologs in *D. melanogaster* were subsequently identified. Median gene lengths were used instead of means due to the asymmetrical distribution of gene sizes. Within each species, CYP gene lengths were compared with the lengths of the 500 randomly selected genes using the Mann-Whitney non-parametric test.

To account for differences in overall genome size and gene-length distributions between species, we normalized CYP gene lengths by the median size of the 500 randomly selected genes in each species. Normalized gene size was calculated as the length of each gene (in base pairs) divided by the species-specific median length of the 500 randomly selected genes. A Wilcoxon signed-rank test was then applied to compare normalized CYP sizes between orthologous CYPs of *D. suzukii* and *D. melanogaster*.

TE enrichment was compared between *D. suzukii* and *D. melanogaster* using four approaches: (1) the frequency of TEs within genes, including their flanking regions; (2) the frequency of TEs within CYP genes and flanking regions; (3) the frequency of TEs in CYP genes versus its frequency in other genes in the genome; and (4) the frequency of Helitron insertions in CYP genes versus its frequency in non CYP genes. The comparisons were performed using Chi-square tests with 1 degree of freedom and Yates' continuity correction, which is usual in these cases. All annotated genes and intergenic regions in each genome were considered for these comparisons. Statistical analyses were conducted using SPSS® version 18, and a significance threshold of $p \leq 0.05$ was applied.

Results and discussion

Overall prevalence of TE insertions in CYP genes

Among the 76 CYP genes annotated for *D. suzukii*, 42 contained transposon sequences within or near the genes (Figure 1; Table 1; Supplementary Tables S1, S2). In *D. melanogaster*, 41 of the 91 genes analyzed harbored such insertions (Figure 1; Table 1; Supplementary Tables S3, S4). Although *D. suzukii* has fewer CYP genes overall, we detected a higher number of TE fragments in these genes (140 vs. 136). Most of this difference is mainly attributable to a markedly higher number of Helitron elements in *D. suzukii* (118 fragments; 84% of all TE insertions, Table 1) compared with *D. melanogaster* (32 fragments; 24%, Table 1). Other TE subclasses also differed in proportion between species (Table 1), with *D. melanogaster* showing 51% of LTR retrotransposons (69 fragments). Nevertheless, the absolute difference in total TE fragment counts between the two species is primarily explained by the excess of Helitron insertions in *D. suzukii* (Figure 1; Table 1).

1 http://gitlab.com/inkscape/inkscape.

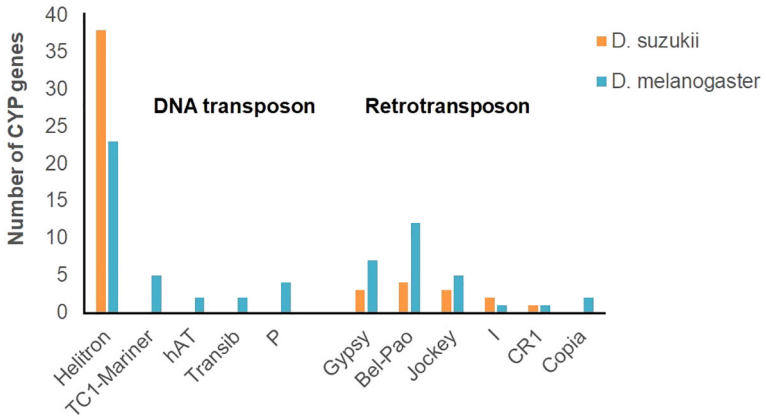

FIGURE 1
Number of CYP genes with fragments of transposable elements.

In flanking regions, TEs were detected both upstream and downstream of CYP genes, and were also detected within introns in both species (Figure 2). *D. suzukii* displayed more TE insertions in the 5′ flanking regions, where most promoter sequences are located. In contrast, *D. melanogaster* showed more TE insertions within introns, mainly due to a single gene, *CYP307a2*, which harbors 30 TE fragments in its intronic regions (Supplementary Table S3). In this gene, retroelements predominate across all regions (5′, 3′, and introns). The *D. suzukii* ortholog of *CYP307a2* contains seven intronic insertions (Supplementary Table S1), where DNA transposons were the most abundant in all regions of this gene (5′, 3′, and introns). No TE insertions were detected within annotated exons.

When comparing CYP genes with TE insertions in *D. suzukii* and *D. melanogaster*, we observed that some *D. suzukii* genes were longer than their orthologs in *D. melanogaster* (Figures 3A–J). Specifically, ten of the 36 *D. suzukii* genes containing TE insertions had additional exons and introns, organized as repetitive conserved blocks, compared with their *D. melanogaster* counterparts. In general, gene organization is highly conserved among species of the same order; therefore, exon and intron annotations are well supported and consistent with the phylogenetic relationships of the species (Rewitz et al., 2007). To further explore this pattern, we also examined two sister species of *D. suzukii* with available genome sequences, *D. biarmipes* and *D. takahashii*. In all four species, exon and intron annotations are confirmed at the transcript level (Chiu et al., 2013; Graveley et al., 2010; *Drosophila biarmipes* Annotation Release 101; *Drosophila takahashii* Annotation Release 101).

Helitron elements contribute to CYP gene length variation

Helitron insertions account for only a small proportion of the total length of each longer CYP gene in *D. suzukii*, ranging from 0.43% to 5.63%, except *CYP4e2*, which contains 1,403 bp of Helitron sequence in a gene of 7,998 bp (17.54%) (Supplementary Table S1). These results indicate that, although Helitrons are consistently present in longer genes, they represent only a minor contribution to overall gene length. This suggests that Helitrons may have contributed to gene lengthening through rearrangements such as exon shuffling rather than by adding new sequences. In this view, Helitron activity could have facilitated structural reorganization of CYP genes, consistent with their known role in mediating exon capture and recombination events.

Analysis with genoPlotR, which compares gene and genome maps, revealed conserved exon structures across orthologous genes in *D. suzukii*, *D. biarmipes*, *D. takahashii*, and *D. melanogaster* (Figures 3A–J). The genes *CYP12a4*, *CYP12e1*, *CYP6a18*, *CYP6a20*, *CYP6a21*, *CYP6a23*, *CYP6d5*, and *CYP4e2* of *D. suzukii* contain at least one *Helitron* fragment in the intron region.

The *CYP4e2* gene in *D. suzukii* is larger than its *D. melanogaster* ortholog but contains only one fewer exon compared with the *D. biarmipes* ortholog (Figure 3H). Interestingly, even with one fewer exon, the *D. suzukii CYP4e2* remains longer than its *D. biarmipes* counterpart. A Helitron fragment located between exons six and seven is present in *D. suzukii* but absent from *D. biarmipes*, suggesting that the loss of this exon in *D. suzukii* may have resulted from the Helitron insertion.

In contrast, the *CYP4c3* gene in *D. suzukii* contains two Helitron insertions in the 3′ flanking region in a positive orientation (5’-3’) (Figure 3I). Its sister species *D. takahashii* carries a single Helitron insertion between exons five and six in a negative orientation (3’-5’). The structural differences observed in *D. suzukii CYP4c3* could be explained by the presence of these two Helitrons and the rolling-circle recombination mechanism associated with this TE superfamily, whereby exons nine to eleven may have arisen through exon shuffling involving exons six to eight.

Unlike what was previously observed in *D. suzukii*, the *CYP12a4* and *CYP12e1* orthologs in *D. biarmipes* and *D. takahashii* lack transposon insertions (Figures 3A, B). However, in *D. melanogaster*, the *CYP12a4* ortholog contains the *BARI* element in the 3’ flanking region (Figure 3A), as previously annotated (Bogwitz et al., 2005). The biological functions of *CYP12a4* and *CYP6a20* have been reported as insecticide

TABLE 1 Transposable element fragments belonging to subclasses and orders in *CYP* genes and flanking regions.

Class		*D. suzukii*	*D. melanogaster*
Class I (retrotransposon)	LTR	15 (11%)	69 (51%)
	non-LTR	7 (5%)	14 (11%)
Class II (DNA transposon)	Subclass 1	0 (0%)	19 (14%)
	Subclass 2	118 (84%)	32 (24%)
	TOTAL	140 (100%)	136 (100%)

Long Terminal Repeat (LTR) = Gypsy and Bel-Pao superfamilies.
Non-LTR = RTE, I, Jockey, and CR1 superfamilies.
Subclass 1 = TC1-Mariner, hAT, Transib, P, PIF-Harbinger, and Zator superfamilies.
Subclass 2 = Helitron superfamily.

responses and aggressive behavior, respectively - two key traits that contribute to the ecological success and invasive potential of insects.

Little or no similarity was observed for the *CYP6w1* gene annotated in scaffold 2 (Figure 3J). However, the same gene annotated on scaffold 8 showed high similarity to its orthologs. BLAST searches performed at NCBI showed high sequence identity with the *CYP6d2* genes of the sister species *D. biarmipes* (89%) and *D. takahashii* (87%). The *D. suzukii CYP6d2* gene is absent from Gbrowser (SpottedWingFlyBase, 2013) but is predicted by the NCBI genome browser. On the other hand, the *CYP6d5* gene is annotated in two scaffolds (99 and 1273) (Figure 3G). Both paralogs display high similarity to each other and the *D. biarmipes* ortholog, suggesting a duplication event in *D. suzukii* that may have been facilitated by Helitron-mediated insertion and transposition.

To determine whether the observed genomic size increase and TE insertions were specific to CYP genes in *D. suzukii* or also occurred in other gene families, we randomly selected 500 additional genes from each genome for comparison. We visually inspected 124 genes that were longer in *D. suzukii* than in their *D. melanogaster* orthologs. Among these longer *D. suzukii* genes, 45 carried a total of 249 *Helitron* copies, whereas in *D. melanogaster*, 41 genes carried 110 *Helitron* copies (Supplementary Table S7).

To allow for a fair comparison not influenced by the overall genome size differences between species, we compared CYP gene lengths with the median length of 500 randomly selected genes within each species. In *D. melanogaster*, CYPs were significantly smaller (median = 2,117 bp) than random genes (median = 5,603 bp; $p = 0.025$, Mann-Whitney). In *D. suzukii*, however, CYPs (median = 8,032 bp) did not differ significantly in size from random genes (median = 6,325 bp; $p = 0.526$).

Because CYP size differences could be influenced by overall genome size rather than TE-mediated arrangements, we normalized CYP lengths to the median size of the 500 random genes from each species. After normalization, CYPs in *D. melanogaster* were proportionally shorter (median ratio = 0.38) than in *D. suzukii* (median ratio = 1.27; $p = 0.002$, Wilcoxon). These results indicate that, on average, CYP genes in *D. melanogaster* are 38% shorter than the typical genes in its genome, whereas in *D. suzukii* they are 27% longer. Therefore, CYPs remain proportionally larger in *D. suzukii* than in *D. melanogaster*, even after normalization for genome size differences. This normalization was necessary to account for differences in overall genome size and structure between species, allowing a direct evolutionary comparison of relative gene length rather than absolute values, which can be biased by genome expansion or contraction.

Transposons are enriched in putative TFBS

Transposable elements (TEs) often carry transcription-factor binding sites (TFBS), and these sequences are preferentially retained within genes because they can contribute to transcriptional regulation (Jordan et al., 2003; Feschotte, 2008). Such retention likely reflects a byproduct of TE transposition combined with the host-level selection. We therefore searched for putative TFBS in all sequences identified within the CYP genes of *D. suzukii* (88 TFBS) and *D. melanogaster* (140 TFBS) (Figure 4; Supplementary Table S8). Of these, 35 of the 88 TFBS in *D. suzukii* were located within Helitrons, whereas only 5 of the 140 TFBS in *D. melanogaster* were found in Helitrons. Across all

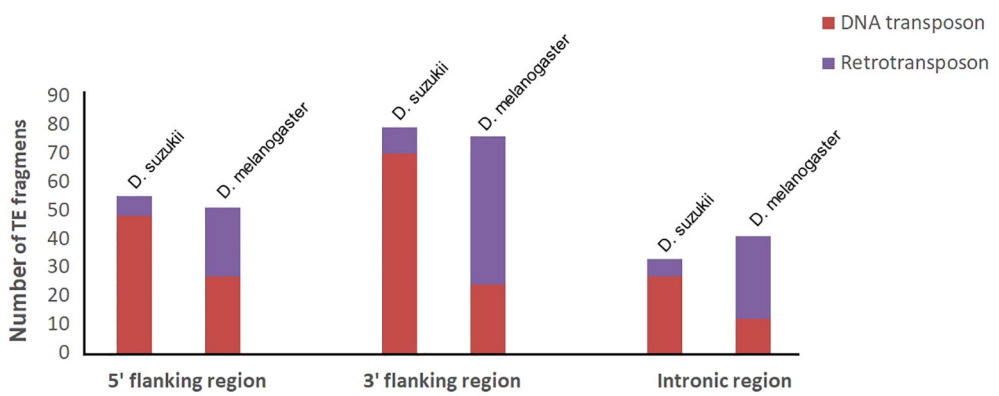

FIGURE 2
Insertion position (5' and 3'-flanking region, and intron region) of transposable elements in *CYP* genes of *Drosophila suzukii* and *Drosophila melanogaster*.

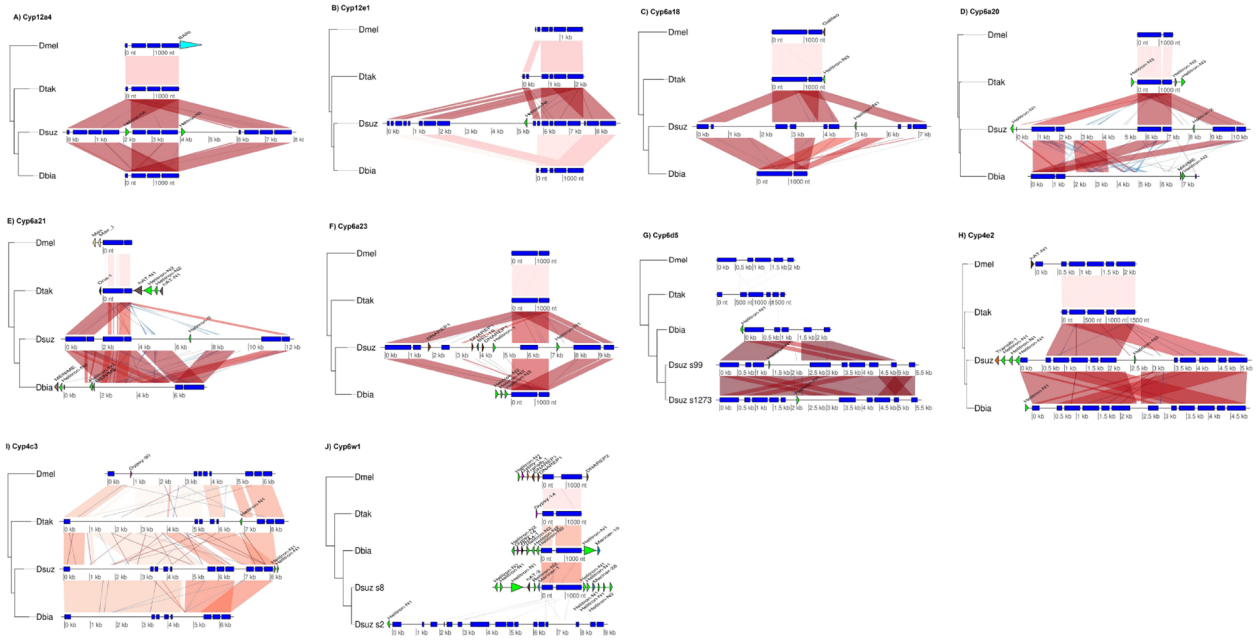

FIGURE 3
(A–J) Comparative analysis showing similarity between the CYP genes in Drosophila species. The intensity of red boxes between genes highlights the closest sequence above for which genes are denoted. Triangles represent the multiple transposable element insertions and their orientation. The phylogeny on the left is the phylogeny from Chiu et al. (2013), which was inferred by maximum-likelihood methodology. Genes are scaled to real length, except for flanking regions. Dmel, Drosophila melanogaster; Dtak, Drosophila takahashii; Dsuz, Drosophila suzukii; Dbia, Drosophila biarmipes.

TE fragments located within CYP genes and their flanking regions in *D. suzukii*, 51 out of 140 (36%) carried at least one predicted TFBS (Supplementary Table S8). Although no explicit false-positive control or background frequency analysis was implemented, confidence scores for each predicted TFBS are provided in Supplementary Table S8.

As different classes of TEs were present in the CYP genes (Table 1), a broad diversity of TFBS motifs was expected (Thornburg et al., 2006). However, we detected little variation in the TFBS classes across TE families (Figure 4 and Supplementary Table S8). Although *D. suzukii* has a larger overall proportion of TE sequences in its genome (35.94%) compared with *D. melanogaster* (15.96%) (Table 2), the highest number of TFBS was found in TE fragments located within CYP genes of *D. melanogaster* (Supplementary Table S8). This is likely explained by the greater total base pair coverage of TEs within CYP genes and their flanking regions in *D. melanogaster* (76,813 bp; Supplementary Table S3) compared with *D. suzukii* (47,421 bp; Supplementary Table S1). It is important to note that these base pair values refer only to TE content in CYP genes and should not be confused with the genome-wide TE proportions reported in Table 2.

For both species, the putative TFBS *Hunchback* and *CF2-II* (Chorion factor 2) are consistently overrepresented (Figure 4). These proteins belong to the C2H2 zinc finger class of transcription factors. *Hunchback* is strongly expressed early in development (Nüsslein-Volhard and Wieschaus, 1980; Lehmann, 1988), whereas *CF2-II* is expressed later in embryogenesis (Shea et al., 1990). TE insertions in flanking regions and introns of CYP genes may therefore influence expression by harboring putative TFBSs. In this study, flanking regions were defined as ±10 kb upstream and downstream of each gene, encompassing proximal regulatory zones where transposon-derived enhancers or silencers are most likely to act. This, in turn, suggests that TEs may play an important role in facilitating adaptation to different environments in both species (Jordan et al., 2003; Feschotte, 2008; Shea et al., 1990; Thornburg et al., 2006). However, these regulatory implications remain hypothetical. Future functional and transcriptomic experiments will be required to confirm whether these TE-associated TFBSs have effects on CYP gene regulation or adaptive phenotypes.

TE content in *Drosophila* genomes

To place these findings in a genomic context, we next examined overall TE content in the genomes of *D. suzukii* and *D. melanogaster*. Approximately 36% of the assembled *D. suzukii* genome consists of TE sequences, compared with 16% in the genome of *D. melanogaster* (Table 2).

Three genome assemblies are currently available for *D. suzukii*. The first two were generated from North American samples (SRA096061; Chiu et al., 2013) – the same genomic data used in the present study – and from European samples (ERP001893; Ometto et al., 2013), both sequenced on the Illumina HiSeq2000 platform. Chiu et al. (2013) used automated homology comparison against 6,003 *D. melanogaster* TEs. In contrast, Ometto et al. (2013) applied a homology-based approach with RepeatMasker and the Repbase Insect library, estimating ~11% TE content in *D. suzukii* and ~17% in *D. melanogaster*. A third, near-chromosome-level

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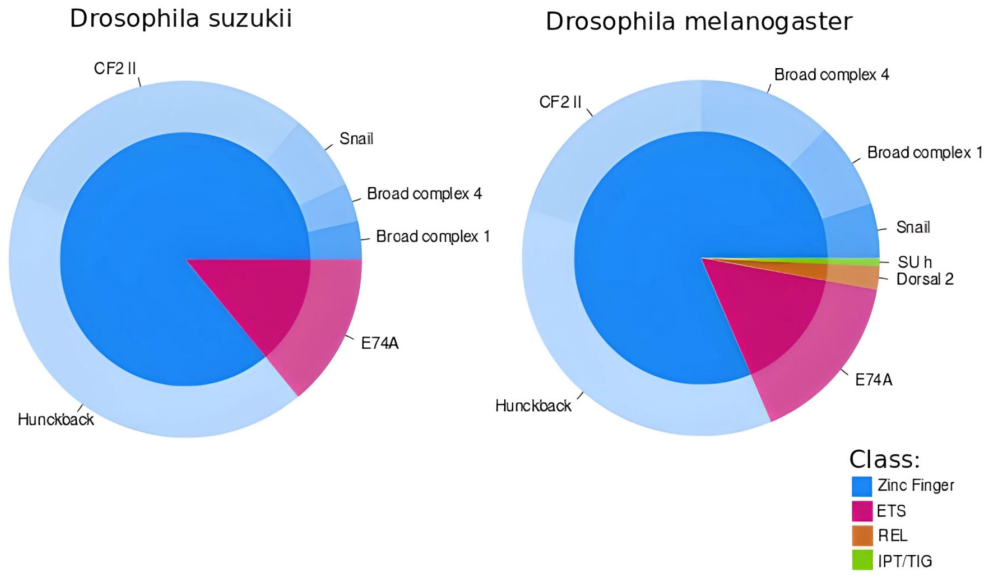

FIGURE 4
Putative TFBS predicted for transposable elements inserted in *CYP* genes of *Drosophila suzukii* and *Drosophila melanogaster*.

assembly was produced by Paris et al. (2020) using PacBio long-read sequencing, which revealed that ~35% of the *D. suzukii* genome consists of repetitive sequences. However, the relative contribution of TE superfamilies was not determined. Differences among these studies likely reflect not only the methodologies used but also sequencing technology, which can affect the assembly completeness and thus TE content estimates.

In this study, we used RepeatExplorer, which integrates two complementary strategies for TE annotation: (1) homology-based searches against the Repbase library, and (2) *de novo* clustering to identify repetitive structures and patterns in the genome. This combined approach provides broader coverage, whereas earlier studies relying solely on homology-based annotation likely underestimated TE content. Supporting this explanation, Sessegolo et al. (2016), using the *de novo* pipeline dnaPipeTE (Goubert et al., 2015), estimated TE content at ~31% for *D. suzukii* and ~12% for *D. melanogaster* - values comparable to our results (~36% and ~16%, respectively). The primary difference is technical: dnaPipeTE requires local installation, while RepeatExplorer is web-based, but both rely on similar principles.

In the two species (Table 2), retrotransposons are the most abundant TE class, consistent with previous evidence that class I elements predominate in *Drosophila* genomes (Drosophila 12 Genomes Consortium, 2007). Among DNA transposons, Helitrons are the most abundant in both species and represent the second-largest TE category in *D. suzukii*. Actual TE content may be even higher, since RepeatExplorer tends to detect medium-to-high copy number and relatively recent TE insertions. In contrast, older, more diverged copies may not pass through similarity filters. Nevertheless, the clustering approach makes RepeatExplorer a fast and effective tool for initial TE analysis of Illumina data (Novák et al., 2013).

Comparing TE distribution between genes and intergenic regions revealed striking interspecific differences. In *D. suzukii*, TEs were found in 8.6% (10044 in 116977) of all annotated genes, whereas in *D. melanogaster*, 41.6% (35013 in 84181) of genes contained at least one TE insertion ($p < 0.001$). Moreover, in *D.*

TABLE 2 Genomic TE content in Drosophila suzukii and Drosophila melanogaster.

Class		*D.suzukii*	*D. melanogaster*
Class I (retrotransposon)	*Copia*	0.05%	0.37%
	Bel-Pao	4.67%	2.67%
	Gypsy	9.85%	5.44%
	LINE	7.00%	4.92%
	Kiri	0.02%	0.00%
	Outcast	0.02%	0.00%
Class II (DNA transposon)	*Tc1-mariner*	0.83%	0.30%
	hAT	0.76%	0.07%
	Transib	0.49%	0.16%
	PiggyBac	0.27%	0.00%
	CACTA	0.22%	0.00%
	PIF-Harbinger	0.05%	0.00%
	P	0.00%	0.37%
	Helitron	7.27%	0.45%
	Maverick	4.26%	0.00%
	Unknown	0.19%	1.21%
	TOTAL	35.94%	15.96%

suzukii, 1.0% (103) of all TE copies and 1.0% (68) of all Helitron copies in the genome were located within CYP genes, while in *D. melanogaster* these proportions were 0.2% (87 TE copies) and 0.2% (19 Helitron copies), respectively. These differences in TE and Helitron proportions within CYP genes were statistically significant ($p < 0.001$).

Helitron distribution also differs between species. In *D. suzukii*, 95.6% of Helitrons are located in intergenic regions, whereas in *D. melanogaster*, 85.9% occur within genes. It should be noted that our methodology does not distinguish between complete Helitron elements and fragmented copies, which may contribute to differences observed between species. Nonetheless, fragmented copies likely represent remnants of once-intact Helitrons that were active earlier in the evolutionary history of these species. For this reason, our interpretations regarding the structural influence of Helitrons refer to their historical activity rather than current transposition or exon-shuffling events. This pattern may reflect a species-specific distribution of Helitrons, suggesting potential differences in TE dynamics, and does not alter the conclusion that *D. suzukii* harbors proportionally more Helitrons in intergenic regions.

Helitron elements and CYP gene evolution in *D. suzukii*

Metabolic resistance mediated by cytochrome P450 monooxygenases (CYPs) is an important adaptive trait in many insect species (Scott, 1999) and a common mechanism by which insects develop resistance to pesticides (Feyereisen, 1999). Transposable elements (TEs) are often found within or near resistance genes, providing indirect evidence of their involvement in the generation of adaptive genome changes (Catania et al., 2004; Chen and Li, 2007; Chung et al., 2007; Carareto et al., 2013; Casacuberta and González, 2013). Barbara McClintock (1984) first proposed that TE activation in response to stress could induce mutations that help organisms adapt to new environmental conditions.

In this study, we examined TEs associated with CYP genes in the highly invasive *D. suzukii* genome. We documented CYPs with varying TE contents, including TEs carrying putative transcription-factor binding sites (TFBS) and structural changes potentially mediated by rolling-circle transposons of the Helitron superfamily. We also found that the *D. suzukii* genome contains roughly twice the TE content of *D. melanogaster*, with Helitrons representing the most abundant subclass of class II DNA transposons in both species (Table 2).

In all CYP genes analyzed, TE fragments were located exclusively in flanking regions and introns, which is consistent with the view that TEs are generally tolerated in non-coding regions. However, TE insertions near genes can also create new regulatory networks (Feschotte, 2008), and changes in a gene-regulation network are thought to be very important during adaptive evolution (Casacuberta and González, 2013). In *D. suzukii*, TE insertions occurred predominantly (88%) in the 5' flanking regions of CYP genes. Previous studies have shown that TE insertions in 5' untranslated regions confer insecticide resistance – for example, in *Drosophila CYP6g1*, where the upstream *ACCORD* retroelement carries specific transcriptional enhancers (Daborn et al., 2002; Chung et al., 2007; Schmidt et al., 2010). *D. melanogaster* and *D. simulans* CYP genes harbor multiple TE insertions, many from the *Helitron* superfamily, which also carries putative TFBS (Carareto et al., 2013; review in Thomas and Pritham, 2015). These findings are consistent with previous hypotheses proposing that TEs may be gradually co-opted for host gene regulation (Chung et al., 2007; Feschotte, 2008).

The acquisition of new *cis*-regulatory elements via TE insertions provides opportunities for adaptation to novel environmental challenges (Casacuberta and González, 2013). Several LTR retrotransposons containing *cis*-regulatory motifs are highly expressed in response to specific stimuli (Kumar and Bennetzen, 1999), and these motifs often match those required for the activation of stress-response genes (Grandbastien et al., 2005). In our dataset, TE fragments carried putative TFBS involved in fly development, including *Hunchback* (embryo patterning) and *CF2-II* (cell differentiation). This suggests that CYPs may be particularly permissive to TE insertions because such sequences can act as donors of transcriptional regulatory signals, potentially altering gene expression at different developmental stages. Similar TFBS have been reported in TE sequences *in silico* (Babu et al., 2006; Thornburg et al., 2006; Carareto et al., 2013). Thus, TEs carrying TFBS may influence gene regulation and contribute to adaptation in *Drosophila* (Feschotte, 2008).

Beyond regulatory effects, TEs can also mediate structural genomic changes such as insertions, excisions, retrotranspositions, and exon shuffling. These processes can lead to exonization or intronization of TE sequences, and in some cases to exaptation, where TE-derived sequences acquire new functional roles. If beneficial, such insertions can be retained in the host genome. Feyereisen (1999) proposed two mechanisms by which CYP genes can evolve insecticide resistance: (1) structural changes in specific CYPs, such as exon gain or loss, and (2) increased gene expression. Exon shuffling, as first proposed by Gilbert (1987), is one route by which novel exons can arise. In our study, ten CYP genes (Figures 3A–J) displayed structural changes involving conserved blocks of exon gain, each associated with at least one Helitron insertion.

*Helitron*s – subclass 2 of Class II DNA transposons (Wicker et al., 2007) – are known to mediate exon shuffling, transduplication, and the introduction of novel regulatory elements (Morgante et al., 2005; Pritham and Feschotte, 2007; Thomas et al., 2014). These elements transpose via a rolling-circle mechanism that displaces a single DNA strand. A loop is formed before cleavage and reintegration elsewhere in the genome. They have a remarkable ability to capture and duplicate gene segments, and their transposition can include flanking sequences (Kapitonov and Jurka, 2007). While Helitrons are well studied in plants – especially maize, where they have captured and redistributed numerous genes (Lal et al., 2009; Barbaglia et al., 2012) – their role in *Drosophila* remains less explored.

Repetitive elements within introns may act as recombination hotspots, thereby promoting exon shuffling (Gilbert, 1987). In maize, most *Helitron* copies have incorporated gene segments, facilitating their amplification and dispersal throughout the genome (Yang and Bennetzen, 2009). A striking example outside *Drosophila* comes from Palmer amaranth (*Amaranthus palmeri*), where Helitron-mediated amplification of the *EPSPS* gene cassette confers glyphosate resistance (Molin et al., 2017). Our observations in *D. suzukii* CYP genes are consistent with such a mechanism, suggesting that Helitron insertions are associated with increased gene length.

We propose a hypothetical example of Helitron-mediated gene capture in Figure 5, illustrating how, if a Helitron bypasses its termination signal, strand displacement could continue through adjacent gene regions until a new signal is encountered, capturing and mobilizing those sequences (Kapitonov and Jurka, 2007; Grabundzija et al., 2016). For instance, in *CYP12a4* (Figure 3A) and *CYP6a20* (Figure 3D), the intronic arrangement, orientation, and high sequence similarity of exons support the possibility of Helitron-mediated capture during transposition (Figure 5A). Further studies should aim to experimentally validate Helitron-mediated gene capture in *D. suzukii* through long-read sequencing and transcriptomic analyses to confirm the presence of chimeric transcripts.

Previous studies (Li et al., 2007) have documented genomic alterations leading to CYP overexpression in insecticide resistance. Mishra et al. (2018) reported that several longer CYP genes – *CYP6w1*, *CYP6a20*, *CYP6a21*, *CYP6d5* – were significantly upregulated under insecticide exposure in *D. suzukii*, with responses varying between populations. Functional assays, such as CRISPR/Cas9-mediated knockouts, will be important for testing whether *Helitron* insertions affect gene expression and adaptive traits of this pest species.

In our comparative analysis of closely related *Drosophila* species (*D. suzukii, D. melanogaster, D. biarmipes*, and *D. takahashii*), the ten longer CYP genes revealed a largely conserved exon–intron organization across species, aligned with phylogenetic relationships (Figures 3A–J). However, *D. suzukii* displayed Helitron insertions absent from its sister species, contributing to gene length variation (*CYP4e2* and *CYP6d5)*. Although *D. suzukii* has fewer CYP genes than does *D. melanogaster*, previous work shows that longer genes can generate greater functional novelty than large gene families (Grishkevich and Yanai, 2014), in part because gene length is positively correlated with the number of splice variants (Kopelman et al., 2005). Gene lengthening, often driven by TE insertions (Grishkevich and Yanai, 2014), may thus contribute to adaptive structural changes. Our findings suggest that Helitrons could be a vehicle for such changes in *D. suzukii* CYP genes through their combined transposition and recombination activities.

Conclusion

In the CYP gene family of *D. suzukii*, we identified variations in the length of ten genes. Transposon sequences were present in intronic regions as well as in upstream and downstream flanking regions, with the Helitron superfamily representing the most frequent TE insertion in these genes.

We further examined the genomic TE content of *D. suzukii* and *D. melanogaster* by combining next-generation sequencing (NGS)

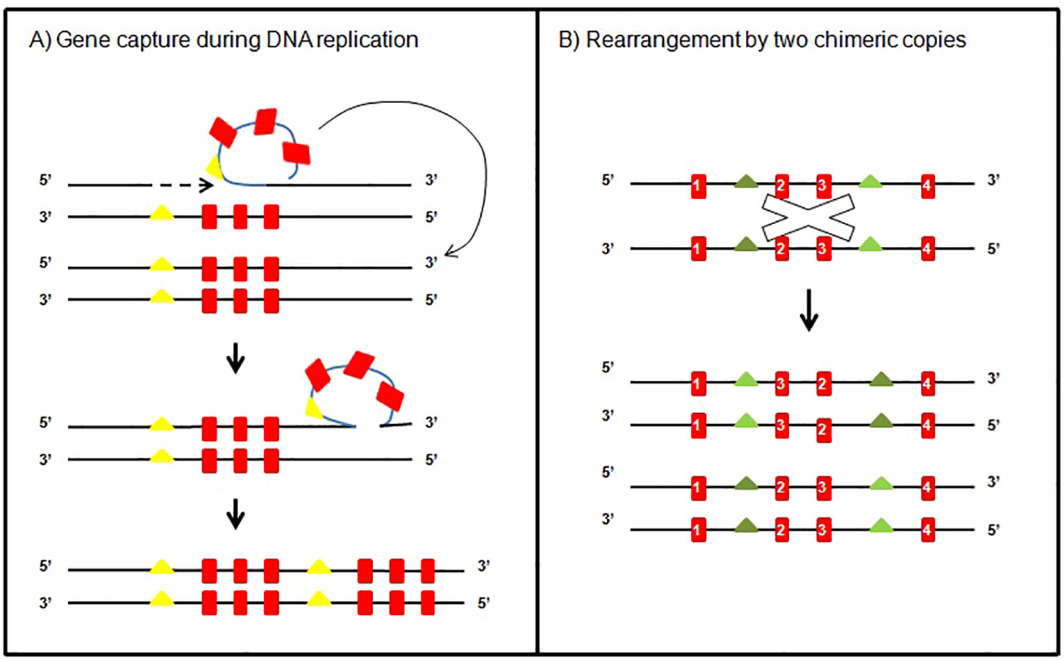

FIGURE 5
Hypothetical exon shuffling by rolling-circle transposon: **(A)** a longer gene formed by *Helitron* during its transposition; **(B)** the hole of two *Helitron* copies rearranging due to the similarity in the sequences.

reads with graph-based clustering to estimate repeat content. This approach enabled us to assess the presence and distribution of TEs, including Helitrons, and to explore their potential association with longer CYP genes, putative transcription-factor binding sites (TFBS), and patterns of TE abundance in *D. suzukii*.

While Helitrons represent the most abundant subclass of class II (DNA transposons) at the genome level in both *D. suzukii* and *D. melanogaster*, class I (retrotransposons) remain overall more abundant than class II elements in both species. Within CYP genes, however, *D. suzukii* shows a striking enrichment of Helitron fragments (84%), whereas *D. melanogaster* contains only 24% Helitron fragments. Thus, although Helitrons are the dominant subclass among DNA transposons genome-wide, they are disproportionately represented within CYP genes of *D. suzukii*, suggesting a lineage-specific pattern of Helitron this species.

Our findings support the view that TEs can play a significant role in adaptation. We observed structural changes in CYP genes – such as exon gain and loss events – suggesting that TEs may influence both gene architecture and regulatory functions. Notably, Helitron elements were enriched in putative TFBS, potentially affecting gene expression in response to environmental pressures. Differences in TFBS composition between TE fragments of *D. suzukii* and *D. melanogaster* further suggest a role for TEs in species-specific regulatory adaptations.

Studying the role of the *Helitron* superfamily within a genomic context is essential for understanding the adaptive mechanism that may have contributed to the evolution and pest status of *D. suzukii*. Future research should investigate the timing of *Helitron* insertions relative to gene divergence events, which will help to clarify the evolutionary dynamics of this element in *D. suzukii*. Such investigations may also shed light on the genetic factors underlying the species' successful colonization and insecticide resistance. Ultimately, this knowledge could advance our understanding of TE mobility, genome size evolution, and the genetic basis of adaptation – providing both theoretical insights and practical applications for pest management, including comparisons between native and invasive populations.

Taken together, our results highlight Helitrons as a major driver of structural and regulatory diversification in CYP genes of *D. suzukii*, potentially underpinning its rapid adaptation and invasive success.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

PB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SC-J: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing, Validation. VV: Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing. MD: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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