The gain of *cis*-regulatory activities underlies novel domains of *wingless* gene expression in *Drosophila*.

Shigeyuki Koshikawa[1,2], Matt W. Giorgianni[1], Kathy Vaccaro[1], Victoria A. Kassner[1], John H. Yoder[3], Thomas Werner[4] and Sean B. Carroll[1,*].

1 Howard Hughes Medical Institute, Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin, USA, **2** The Hakubi Center for Advanced Research and Graduate School of Science, Kyoto University, Japan, **3** Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama, USA, **4** Department of Biological Sciences, Michigan Technological University, Houghton, Michigan, USA

* corresponding author

Abstract

Changes in gene expression during animal development are largely responsible for the evolution of morphological diversity. However, the genetic and molecular mechanisms responsible for the origins of new gene expression domains have been difficult to elucidate. Here, we sought to identify molecular events underlying the origins of three novel features of *wingless* (*wg*) gene expression that are associated with distinct pigmentation patterns in *Drosophila guttifera*. We compared the activity of *cis*-regulatory sequences (enhancers) across the *wg* locus in *D. guttifera* and *D. melanogaster* and found strong functional conservation among the enhancers that control similar patterns of *wg* expression in larval imaginal discs that are essential for appendage development. For pupal tissues, however, we found three novel *wg* enhancer activities in *D. guttifera* associated with novel domains of *wg* expression, including two enhancers located surprisingly far away in an intron of the distant *Wnt10* gene. Detailed analysis of one enhancer (the vein-tip enhancer) revealed that it overlapped with a region controlling *wg* expression in wing crossveins (crossvein enhancer) in *D. guttifera* and other species. Our results indicate that one novel domain of *wg* expression in *D. guttifera* wings evolved by co-opting pre-existing regulatory sequences governing gene activity in the developing wing. We suggest that the modification of existing enhancers is a common path to the evolution of new gene expression domains and enhancers.

Introduction

As animals have adapted to diverse habitats, they have evolved many new and different kinds of body parts. One of the major outstanding questions in evolutionary biology is: what kinds of mechanisms underlie the origin of morphological novelties? It is well established that the regulatory genes responsible for the formation and patterning of animal bodies and body parts, the so-called "toolkit" genes for animal development, are shared and highly conserved among most animal phyla. (1-4). The fact that very different forms are generated by similar sets of developmental genes, and a large body of empirical, comparative studies, have led to the general consensus that divergence in the expression and regulation of toolkit genes and the genes they control largely underlies morphological diversity (5-9).

Similarly, several studies have revealed that new features of regulatory gene expression are associated with the evolution of morphological novelties, such as new color pattern elements on insect wings (10-14). How new patterns of regulatory gene expression evolve, however, has been more difficult to elucidate. In principle, new patterns of gene expression may evolve through: i) changes in the deployment of upstream trans-acting regulatory factors; ii) changes in the cis-regulatory sequences of the genes themselves; or iii) a combination of these mechanisms. For example, the novel, male-specific wing spot in *Drosophila biarmipes* and a few close relatives evolved through a combination of changes in the spatial expression of the trans-acting Distal-less (Dll) transcription factor and the evolution of Dll and other binding sites in a cis-regulatory element of at least one pigmentation gene (15, 16). In this case, the Dll protein is said to have been co-opted in the evolution of a new morphological trait.

However, the mechanism underlying the co-option of Dll is not known in this case, nor for any other instances of the co-option of regulatory genes. It is not known, for instance, whether new features of gene expression evolve via the *de novo* origin of enhancers, or through the transposition or modification of existing enhancers. One distinguishing feature shared by most developmental regulatory gene loci is that, like *Dll* (17, 18), they often contain vast cis-regulatory regions harboring numerous independent enhancers. To complicate matters, some of these enhancers may be located far away in other genes. The diversity of enhancers belonging to individual regulatory genes is explicit evidence that gene function has expanded in the course of evolution by accumulating additional enhancers, but understanding how this occurs presents significant experimental challenges. To further our understanding of the molecular basis of gene expression novelties, it is necessary both to identify the novel enhancers in the species of interest, and to ascertain their structural and functional relationships to sequences in other species lacking

the specific domains of gene expression (19).

The Wg protein is a secreted signaling molecule that acts as a morphogen in the development of numerous structures and pattern elements in Drosophila and other animals. (20-23). Here, we have traced the molecular basis of three novel features of *wingless* (*wg*) gene expression in *Drosophila guttifera* that are associated with three distinct features of adult pigmentation. By searching through the *wg* and adjacent loci of both *D. guttifera* and *D. melanogaster,* we found three novel enhancer activities in *D. guttifera*. We show that one of these enhancers, the novel vein-tip enhancer in *D. guttifera,* is nestled within a conserved enhancer in other species. We propose that the new enhancer activity evolved through the modification of the preexisting enhancer.

Results

Novel *wg* expression domains in the *D. guttifera* pupal wing

Regulatory genes coordinate important developmental events, thus their expression patterns are constrained and usually conserved, particularly among closely related species. *wg* expression patterns in larval imaginal discs (wing disc, eye-antennal disc, and leg disc) of *D. melanogaster* and *D. guttifera* adhere to this generality and are essentially identical (Fig. S1). In both species, *wg* expression were virtually identical in the developing wing pouches and the future nota of wing discs (Figs. S1A and S1D), the anterior-ventral parts of antennae, ventral and dorsal sides of eye discs (Figs. S1B and S1E), and anterior-ventral parts of leg discs (Figs. S1C and S1F).

In contrast, in the developing pupal wings of *Drosophila guttifera*, *wg* is expressed in two domains that are not present in *D. melanogaster* pupal wings (14, Fig. 1). Whereas in *D. melanogaster*, *wg* is expressed in cells along the developing wing margin (henceforth "margin") and crossveins (“crossveins”, Fig. 1A, arrows), in *D. guttifera* (Fig. 1B) *wg* is also expressed at the tips of longitudinal veins ("vein tip", asterisks) and in precursors of the campaniform sensilla ("campaniform sensilla", arrowheads). None of the other several species closely related to *D. guttifera* within the *D. quinaria* species group (*D. deflecta, D. nigromaculata, D. palustris and D. quinaria*) exhibited *wg* expression in the developing campaniform sensilla or vein-tips (Fig. S2). Both novel *wg* expression domains correlate with color pattern formation in *D. guttifera* (Fig. 1D), but not in *D. melanogaster* (Fig. 1C).

The *wg* enhancers active in imaginal discs are conserved between species

Our primary task was to identify the enhancers responsible for these novel features of *wg* expression in *D. guttifera*. Because we could not predict where novel enhancers might be located, our approach was to identify functional enhancers across the entire *D. guttifera wg* region and to compare their activity and structure with the homologous regions of the *D. melanogaster wg* region. This approach offered the added benefit of enabling a comparison of the overall organization of the cis-regulatory regions of the *wg* locus of the two species. Because we could not assume that *D. guttifera* enhancers would have the same activity in *D. melanogaster* (the usual host for transgenic methods in Drosophila) as in *D. guttifera*, we constructed reporter genes with DNA from each species and injected them into their species of origin. The *D. melanogaster wg* locus sequence had been determined previously (20, 22, 24). From a draft assembly of the *D. guttifera* genome and PCR amplification, we located the *D. guttifera wg* locus on a 64 kb long contig. We systematically fused 0.3-10kb (average 4.0kb) non-coding segments of each species' *wg* region to an EGFP/DsRed reporter gene (Fig. S3).

We first monitored the larval imaginal discs for reporter protein activity, and we were able to confirm or identify several orthologous *wg* enhancers (Fig. 2) including: i) a previously reported enhancer driving wing pouch expression in the 5' region in both species (Figs 2B and 2E; the *spade*^{flag} (*spd*^{fg}) region in *D. melanogaster*; 25); ii) enhancers active in the eye-antennal discs and leg discs that are located in the 3' region of *wg* gene (Figs 2C and 2F); and iii) an enhancer in the 3' region of the *Wnt6* gene (Figs. 2D and 2G). These results are consistent with a recent survey describing a large collection of imaginal disc enhancers of *D. melanogaster* (Flylight; 26). Because the *Wnt6* expression pattern is mostly similar to that of *wg* (27), and the clustering of four Wnt genes is conserved in the *Drosophila* genus, the loci have been inferred to share regulatory elements (28). We note that the overall position and order of imaginal disc enhancers is largely colinear across the 60-74 kb region in both species (Fig. 2A). This result indicates that there have not been any significant inversions or other rearrangements across the region in either lineage since the two species diverged from a common ancestor approximately 63 million years ago (the divergence between the subgenus *Drosophila* and subgenus *Sophophora*, 29).

The *D. guttifera wg* locus contains has a novel vein-tip enhancer

In our search for enhancers that regulate the *D. guttifera*-specific *wg* expression domains in pupal wings, we identified two enhancers located 3' of the *D. guttifera wg* gene: a crossveins enhancer (gutCV-T) and margin enhancer (gutME), which together account for the conserved *wg*-expression domains (Figs. 3A-C; see also Fig. 1B). We also identified the

orthologous enhancers, melCV and melME, from *D. melanogaster*, which drove reporter expression in the crossveins and wing margin, respectively (Fig. 3D and 3E). Importantly, in addition to the conserved crossvein expression, gutCV-T also drove reporter expression in the developing wing tips, where the wing veins meet the margin, which is part of the novel *wg* expression pattern in *D. guttifera* (Fig. 3B).

The difference in activities between the orthologous melCV and gutCV-T enhancers of *D. melanogaster* and *D. guttifera* could be due to differences in trans-acting regulatory factors expressed in each wing, differences in cis-regulatory sequences between the enhancers, or both. To determine which might be the case, we carried out a simple cis-trans test by introducing the gutCV-T enhancer into *D. melanogaster*. The gutCV-T fragment drove reporter protein expression in both the crossveins and vein-tips in pupal *D. melanogaster* wings (Fig. 3F). This result indicates that the trans-acting factors necessary for the vein-tip expression pattern are present in both species. Thus, the differences in activities between the gutCV-T and melCV enhancers must reside in their cis-regulatory sequences.

We also isolated and tested the orthologous cis-regulatory region from *D. deflecta*, which is one of the most closely related species to *D. guttifera* but does not have *wg* expression in the vein tips (Fig. S2C). This *D. deflecta* crossveins enhancer (defCV) drove reporter protein expression in the wing crossveins in *D. guttifera*, but showed no activity in the vein tips (Fig. 3G). This result indicates that the vein-tip enhancer activity is unique to *D. guttifera*, and that the novel feature of *wg* expression in *D. guttifera* wing vein tips arose through the evolution of cis-regulatory sequences in the *D. guttifera* lineage, after it split off from a common ancestor shared with *D. deflecta*.

The *D. guttifera*-specific vein-tip enhancer is nestled within the crossvein enhancer

The 2.4 kb gutCV-T enhancer, which drove both crossvein and vein-tip expression in *D. guttifera* (Fig. 3B), shares numerous collinear, highly-conserved blocks of sequence with both the 1.8 kb melCV fragment from the *D. melanogaster* locus and the 1.7 kb defCV fragment from the *D. deflecta* locus, which both lack vein-tip activity (Fig. 3D and 3G; Fig. 4A). We considered two possibilities to explain how the novel vein tip expression of gutCV-T may have evolved within the domain of the crossvein enhancer: i) a distinct enhancer element, able to independently drive expression in the vein tips, inserted into the *D. guttifera wg* locus (by chance next to another pupal wing enhancer); or ii) a novel activity arose within the crossvein enhancer that utilized and is dependent upon pre-existing sites in the crossvein enhancer. To attempt to distinguish these

possibilities, we compared the *D. guttifera, D. deflecta,* and *D. melanogaster* sequences and searched for major insertions or regions unique to *D. guttifera*. Indeed, we found that the *D. guttifera* fragment is over 900 bp longer than the orthologous *D. melanogaster* sequence and 600 bp longer than the orthologous *D. deflecta* sequence (Fig. 4A). This size difference is largely due to a region in the less conserved 5' end of the gutCV-T enhancer. This additional sequence did not show any similarity to known transposable elements (when tested by blastn against the NCBI nucleotide collection). To test whether this region might contain a distinct enhancer, we divided gutCV-T into two fragments; the insert-containing 5' 1653bp (gutCVT5) and the 3', highly-conserved 756bp fragment (gutCVT-core) (Fig. 4A). While the gutCVT5 fragment showed no activity, the gutCVT-core fragment drove expression in both the crossveins and vein tips (Figs. 4B and 4C). These results reveal that the novel activity in the gutCVT enhancer arose within the 3' 756bp region.

To examine how this region may have acquired its unique vein tip activity, we compared it in detail with the orthologous *D. deflecta* sequence that lacks vein tip activity. The *D. guttifera* CVT-core region is 83% similar to the orthologous *D. deflecta* region, with many large blocks of identical sequence and just a few small (<10bp) insertions or deletions (Fig. S4). This pattern of sequence homology indicates that the novel domain of *wg* expression in the vein tips of *D. guttifera is* likely due to a small number of nucleotide changes and/or small indels nestled within the well-conserved crossvein enhancer.

The campaniform sensillum and thoracic stripe enhancers are in the distant *Wnt10* region

During our initial search for *wg* enhancer activities in *D. guttifera*, we were puzzled by our inability to find an enhancer for the novel patterns of *wg* expression in the developing wing campaniform sensilla, which contributes several spots to the overall polka-dotted wing pattern (14). Therefore, we expanded our search into adjacent *Wnt* loci just in case they might contain enhancers that regulate *wg* transcription. Using seven additional scaffolds, we extended the region analyzed to include a 174 kb region containing the *Wnt4*, *Wnt6,* and *Wnt10* genes. We were surprised to find two more distinct enhancer activities in the *Wnt10* region, more than 69kb away from the *wg* transcription start site and separated from it by the *Wnt6* locus (Fig. 5A; Fig. S3; see also Fig. 2). One 5kb fragment within the second intron of the *Wnt10* gene (gutCS; Fig. 5A) drove reporter expression in the campaniform sensilla and along the anterior margin of the pupal wing (Fig. 5B, arrowheads). Because *wg* is the only gene in this *Wnt* cluster that is expressed in campaniform sensilla (Fig. S5), we conclude that this enhancer controls *wg*

expression.

A second, partially overlapping 4.3 kb fragment (gutTS, Fig. 5A) drove reporter expression in a series of thoracic stripes (Fig. 5C) that correspond well with the adult thoracic striped pigmentation pattern (Fig. 5D). We were not able to confirm by *in situ* hybridization that this reflects a native *wg* expression domain because gene probes did not yield reliable signals in pupal thoracic body wall tissues. However, we performed RT-PCR on thoracic body wall total RNA to ascertain which *Wnt* genes were active in this tissue. Only *wg* showed strong expression while the other, adjacent *Wnt* genes (*Wnt4, Wnt6, and Wnt10*) exhibited weak or no expression (Fig. S6). These results, and the strong correlation with thoracic pigmentation, indicate that *wg* is expressed in the thorax and regulated by the gutTS enhancer.

Cis-regulatory sequence evolution is partly responsible for novel distant enhancer activities

We next sought to identify the relative contribution of *cis*-acting and *trans*-acting regulatory factors in the evolution of the *D. guttifera* gutCS and gutTS enhancer activities. We conducted reciprocal tests of the *D. guttifera* and homologous *D. melanogaster* sequence in the other species' genetic background. Contrary to the gutCV-T enhancer, the *D. guttifera* CS enhancer was not active in *D. melanogaster* wings, indicating a role for trans-acting factors in enhancer activity in *D. guttifera* (Fig. S7C). In addition, the homologous *D. melanogaster* fragment (45.7% similarity) was not active in either *D. guttifera* or *D. melanogaster*, indicating an additional contribution of cis-regulatory changes in the gutCS enhancer (Fig. S7A and S7B). Taken together, these results indicate that both *cis*-regulatory and *trans*-regulatory changes were responsible for the evolution of the novel *wg* expression domain in campaniform sensilla.

We performed a similar set of reciprocal experiments with the gutTS enhancer and homologous *D. melanogaster* sequence (Fig. S8). The homologous fragment from *D. melanogaster* (46.3% similarity) was inactive in both *D. melanogaster* (Fig. S8A) and *D. guttifera* (Fig. S8B), whereas the *D. guttifera* TS enhancer was weakly active in stripes in the *D. melanogaster* thorax (compare Fig. S8C and Fig. S8D). These results indicate that cis-regulatory changes are largely responsible for the novel activity of the gutTS enhancer and that some, but perhaps not all, of the trans-acting factors involved in regulating the enhancer are deployed in *D. melanogaster*.

Discussion

A large body of comparative studies has shown that changes in the spatiotemporal

expression of toolkit genes and their target genes they regulate correlate with the evolution of morphological traits. In a considerable number of instances, these spatiotemporal changes in gene expression have been demonstrated to involve the modification of enhancers (6, 7, 30-36). However, there are relatively few cases in which the origins of new enhancers have been elucidated, and none involving regulatory genes themselves.

Here, we have shown that three novel domains of *wg* expression in *D. guttifera* are governed by three novel enhancers, respectively (Fig. 6). We found that the evolution of *wg* cis-regulatory sequences within the *D. guttifera* lineage played a role in the gain of each enhancer activity, and that the evolution of trans-acting regulatory factors was also necessary for the activity of two elements (gutCS and gutTS). Detailed analysis of the *D. guttifera* vein-tip enhancer revealed that it evolved within another conserved enhancer, while two other enhancers (the campaniform sensilla and thoracic stripe enhancers) arose within in an intron of the distant *Wnt10* locus. These results bear on our understanding of the mechanisms underlying the evolution of new enhancers and domains of gene expression.

The origin of the vein-tip enhancer via co-option of an existing enhancer

The *D. guttifera* vein-tip enhancer activity was localized within a 756 bp DNA segment that was also active in the developing pupal crossveins. This DNA segment is orthologous to segments of DNA in *D. melanogaster* and *D. deflecta* that were only active in the crossveins. The segments are all collinear, and contain numerous blocks of identical sequence, which suggests that the vein tip enhancer activity evolved within the pre-existing crossvein enhancer. This inference is further supported by the observation that we were unable to separate the two enhancer activities by subdivision of the 756bp fragment.

One explanation for the presence of two inseparable activities in this one fragment is that they share functional sites – i.e., binding sites for common transcription factors. Because both activities appear in the pupal wing, it is likely that they utilize common tissue-specific (wing) and temporal (pupal) inputs. The evolution of a new activity in the vein tips could have arisen through the addition of DNA-binding sites for TFs that were already present active in cells at vein tips. In this scenario, the novel enhancer activity would have resulted from the evolutionary co-option of an existing enhancer.

There is precedent for multifunctional enhancers and for this mechanism of co-option. For example, one enhancer of the *D. melanogaster even-skipped* gene governs two domains of gene expression that are controlled by shared inputs (37). In addition, Rebeiz et al. (19)

demonstrated that a novel optic lobe enhancer of the *Drosophila santomea Neprilysin-1* gene arose via co-option of an existing enhancer. Moreover, it was shown that co-option had occurred in just a few mutational steps. The co-option of existing elements is an attractive explanation for the evolution of novel enhancers because it requires a relatively short mutational path.

The evolution of distant cis-regulatory elements

One surprising property of enhancers is their ability to control gene transcription at promoters located at considerable linear distances away in the genome (38-40). For example, the enhancer that drives *Sonic hedgehog* (*Shh*) expression in the developing amniote limb bud is located in the intron of another gene approximately 1 megabase (Mb) from the *Shh* locus (41, 42). A growing body of evidence indicates that long segments of DNA are looped out in accommodating long-range enhancer-promoter interactions (43, 44). The ability of enhancers to act over such long ranges suggests that new enhancers could evolve at considerable distances from the promoters that they regulate.

Here, we identified two enhancers in an intron of the *D. guttifera Wnt10* gene that control transcription of the *wg* gene from a distance of ~70 kb, and separated by the *Wnt6* locus. Our data suggests that the gutTS enhancer preferentially regulates *wg* transcription and not *Wnt10* or *Wnt6* transcription, although we cannot offer any explanation at present for this preference. The origins of the gutCS and gutTS enhancers are not as clear as the vein tip enhancer. We did not detect any pupal enhancer activity in the orthologous DNA segments of *D. melanogaster*, so we do not have any evidence of enhancer co-option. Nor did we find any obvious insertions in these DNA segments such as a transposon. Nevertheless, the discovery of these novel, distant elements reflects the functional flexibility of cis-regulatory elements and their contribution to the evolution of gene regulation and morphological diversity.

Materials and Methods

Fly strains and genomic DNA

Drosophila melanogaster Canton-S (wild-type) was used for genomic DNA preparation and expression analysis of *Wnt* genes. We obtained *D. guttifera* (stock no.15130-1971.10), *D. deflecta* (15130-2018.00), *D. quinaria* (15130-2011.00), and *D. palustris* (15130-2001.00) from the Drosophila Species Stock Center at University of California, San Diego, and *D. nigromaculata* (strain no. E-14201) from EHIME-Fly, Ehime University, Japan.

Genomic DNA was extracted and purified using a squish method (45) and Genomic tip-20/G columns (Qiagen, Hilden, Germany).

In situ hybridization

Species specific, partial sequences of *Wnt* genes (*Wnt4*, *wg*, *Wnt6* and *Wnt10*) were amplified by PCR from genomic DNA and cloned into the *pGEM-TEasy* vector (Promega, Madison, WI). PCR products re-amplified from the plasmid clones were *in vitro* transcribed to produce DIG-RNA probes (35). Imaginal discs of late 3rd instar larvae and wings of P6 stage pupae (46) were subjected to *in situ* hybridization as described previously (14, 47). Specimens were mounted and imaged under a stereomicroscope SZX-16 (Olympus, Tokyo).

Genomic sequence of the *Wnt* locus

The genome sequence reads of *D. guttifera* were obtained with a Genome Analyzer IIx (Illumina, San Diego, CA), and assembled with CLC workbench (CLC Bio, Aarhus, Denmark). The *Wnt* locus of *D. guttifera* was reconstructed with seven genomic scaffolds and genomic PCR products (Accession no. KP966547, Fig. S3). For the comparison of sequences from multiple species, we used GenePalette software (48). All primers are listed in Table S1.

EGFP/DsRed reporter assay for enhancer activity using transgenic *Drosophila*

For the site-specific integration of transgenes into *D. melanogaster*, the plasmid vector S3aG (36), fly strains VK00006 (cytogenetic location 19E7)(49) and *ZH-attP-51D* (cytogenetic location 51D)(50) were used. *D. guttifera* transgenics were made according to the previously described method (14), using the cloning shuttle vector pSLfa1180fa harboring *DsRed2* or *DsRed.T4*, the *piggyBac* transposon vector *pBac*{*3xP3-EGFPafm*}(51, 52) and the *piggyBac* helper plasmid *phspBac* (53). Fluorescent reporter expression was observed under a stereomicroscope SZX-16 and a confocal laser-scanning microscope FV1000 (Olympus, Tokyo).

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Author contributions

The authors have made the following declarations about their contributions: Conceived and designed the experiments: SK MWG TW SBC. Performed the experiments: SK MWG KV VAK JHY TW. Analyzed the data: SK MWG VAK SBC. Contributed reagents/materials/analysis tools: SK MWG KV VAK JHY TW SBC. Contributed to the writing of the manuscript: SK MWG TW SBC.

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Figure Legends.

Figure 1. Unique *wingless* expression domains in *Drosophila guttifera* pupal wings correlate with adult pigment spots. (*A*) *wg* expression pattern in the pupal wing of *D. melanogaster* visualized by *in situ* hybridization. *wg* is expressed in the developing crossveins and along the wing margin. (*B*) *wg* expression pattern in the pupal wing of *D. guttifera*. *wg* is expressed in the campaniform sensilla (arrowheads), crossveins (arrows) and longitudinal vein tips (asterisks), and along the entire wing margin. (*C*) Adult wing of *D. melanogaster*. (*D*) Adult wing of *D. guttifera*.

Figure 2. Conserved *wg cis*-regulatory elements control similar gene expression patterns in Drosophila imaginal discs. (*A*) Schematic of enhancers plotted on the *wg* locus of *D. melanogaster* and *D. guttifera*. Solid vertical lines connected by horizontal gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. (*B-D*) *D. melanogaster* third instar imaginal discs showing reporter expression with *D. melanogaster* enhancer fragments (EGFP, green). (*E-G*) *D. guttifera* third instar imaginal discs showing very similar reporter expression patterns driven by orthologous *D. guttifera* enhancer fragments (DsRed, magenta). All discs are oriented with anterior to the left and dorsal on top. w: wing disc. ea: eye-antennal disc. l: leg disc. (Magnification: B–G, 200x.)

Figure 3. A novel vein tip enhancer activity *in D. guttifera*. (*A*) Schematic of pupal wing enhancers in *D. guttifera* and *D. melanogaster*. Black bars connected by gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. Inset: Schematic of *wg* expression in the pupal wing that is color-coded for the responsible enhancers. (*B*) *D. guttifera* pupal wing showing reporter expression from the gutCV-T enhancer in the crossveins and vein tips (DsRed, magenta). (*C*) *D. guttifera* pupal wing showing reporter expression from the gutME enhancer (DsRed, magenta) along the wing margin. (*D*) *D. melanogaster* pupal wing showing reporter expression from the melCV enhancer fragment (EGFP, green) in the crossvein. (*E*) *D. melanogaster* pupal wing showing reporter expression from the melME enhancer (EGFP, green) along the wing margin. (*F*) *D. melanogaster* pupal wing showing reporter expression from the gutCV-T enhancer (EGFP, green) in the crossveins and vein tips. (*G*) *D. guttifera* pupal wing showing reporter expression (DsRed, magenta) from the defCV enhancer in the crossveins (asterisks). (Magnification: B–G, 100x)

Figure 4. The *D. guttifera* vein-tip enhancer is nestled within a conserved crossvein enhancer. (*A*) Schematic comparing crossvein enhancer regions in *D. melanogaster, D. guttifera*, and *D. deflecta*. The gutCV-T enhancer (gray bar) aligned with the melCV enhancer (black bar, top) and the defCV enhancer (blue bar, bottom) using GenePalette (gray boxes connected with gray lines indicate sequences of 15 bp or longer with 100% conservation between species) and Vista Browser (50bp sliding window with percent sequence identity indicated, peaks with greater than 80% sequence identity are shaded in pink). Peaks show extent of sequence conservation in a sliding 50bp window. The gutCV-T enhancer was divided into two fragments, gutCVT5 (yellow bar) and gutCVT-core (green bar). (*B*) *D. melanogaster* pupal wing showing absence of reporter expression from gutCVT5 (EGFP, green). (*C*) *D. melanogaster* pupal wing showing reporter expression from the gutCVT-core fragment (EGFP, green) in the crossveins (arrows) and vein tips (asterisks). (Magnification: B and C, 100x.)

Figure 5. The distant *Wnt10* region contains two novel and distinct *wg* enhancers in *D. guttifera*. (*A*) Schematic showing the location of two enhancer fragments in the second intron of *Wnt10*. (*B*) *D. guttifera* pupal wing showing reporter expression driven by the gutCS enhancer (DsRed, magenta) in the campaniform sensilla (arrowheads). (*C*) *D. guttifera* pupal thorax showing a striped reporter expression pattern driven by the gutTS enhancer (DsRed, magenta). (*D*) Stripes of black pigmentation on the thorax of an adult *D. guttifera*. (Magnification: B, 80x.; C, 50x.; D, 32x.)

Figure 6. Three novel *wg* enhancers drive *D. guttifera*-specific pigmentation patterns. The genomic organization of the *D. guttifera Wnt* region is shown with colored shapes corresponding to enhancers from this study. The pupal expression domains of each enhancer are mapped by their respective color onto the pigmentation patterns of the adult animal.

Figure S1. *wg* expression patterns in imaginal discs are conserved between two *Drosophila* species. *In situ* hybridizations with third instar larval imaginal discs. (*A*) *D. melanogaster* wing disc. (*B*) *D. melanogaster* eye-antennal disc. (*C) D. melanogaster* leg disc. (*D*) *D. guttifera* wing disc. (*E*) *D. guttifera* eye-antennal disc. (*F*) *D. guttifera* leg disc. All discs are oriented with anterior to the left and dorsal on top.

Figure S2. *wg* expression in longitudinal vein tips and campaniform sensilla of the pupal wing is unique to *D. guttifera*. *In situ* hybridization for the *wg* gene is shown in pupal wings of various species. (*A*) *Drosophila melanogaster*. (*B*) *D. guttifera*. (*C*) *D. deflecta*. (*D*) *D. nigromaculata*. (*E*) *D. palustris*. (*F*) *D. quinaria*. *D. melanogaster* belongs to *melanogaster* species-group of the subgenus *Sophophora*, while the other species belong to the *quinaria* species group of the subgenus *Drosophila*. All probes are species-specific.

Figure S3. Map of the Wnt region of *D. melanogaster* and *D. guttifera*. Vertical bars connected with black lines indicate sequences of 40bp or longer with 100% sequence conservation between species. Red lines indicate conserved but inverted sequences. Numbered horizontal solid bars indicate DNA fragments tested by transgenic reporter assays. *D. melanogaster* fragments were tested in transgenic *D. melanogaster* using phiC31 integration. *D guttifera* fragments were tested in transgenic *D. guttifera* using the *piggyBac* transposon.

Figure S4. The novel activity of the *D. guttifera* gutCV-T enhancer arose within a conserved enhancer. Sequence alignment of the gutCV-T enhancer and the orthologous defCV enhancer (Serial Cloner v2.6.1, local alignment, and word size=15 were used). Mismatches are marked with a #. Red bars indicate identical sequence matches >10bp with the melCV-core enhancer from *D. melanogaster*. The extent of collinear sequence conservation (with no significant rearrangements) between the two species indicates that a small change or an accumulation of small changes is responsible for the new enhancer activity in the vein tips.

Figure S5. Expression patterns of four *Wnt* genes in pupal wings of *D. guttifera* visualised by *in situ* hybridizations. (*A*) *Wnt4*. (*B*) *wingless*. (*C*) *Wnt6*. (*D*) *Wnt10*.

Figure S6. *wingless* is the predominant *Wnt* gene expressed in the pupal thorax. *Wnt* genes expressed in the pupal thorax (top) and embryo (middle) were detected by RT-PCR and reaction products profiled by gel electrophoresis. Control reactions from genomic DNA are shown at the bottom

Figure S7. *Cis*- and *trans*-regulatory changes are responsible for the novel campaniform sensillum expression of *wg* in *D. guttifera*. (*A*) In the *D. melanogaster* pupal wing, the melCS enhancer shows no restricted expression (EGFP, green). (*B*) In the *D. guttifera* pupal wing, the

melCS enhancer shows no expression (DsRed, magenta). (*C*) *D. melanogaster* pupal wing, the gutCS enhancer shows no restricted expression (EGFP, green). (*E*): *D. guttifera* pupal wing, the gutCS enhancer drives reporter expression in the campaniform sensillum (DsRed, magenta).

Figure S8. *Cis*- and *trans*-regulatory changes are responsible for the striped expression of *wg* in the pupal thorax of *D. guttifera*. (*A*) *D. melanogaster* pupal thorax, the melTS fragment shows no stripe expression (EGFP, green). (*B*) *D. guttifera* pupal thorax, the melTS fragment shows no stripe expression (DsRed, Magenta). (*C*) *D. melanogaster* pupal thorax, the guts enhancer is expressed in incomplete stripes (EGFP, green). (*D*) *D. guttifera* pupal thorax, the gutTS enhancer drives full stripe expression which corresponds to the adult pigmentation pattern (DsRed, magenta).

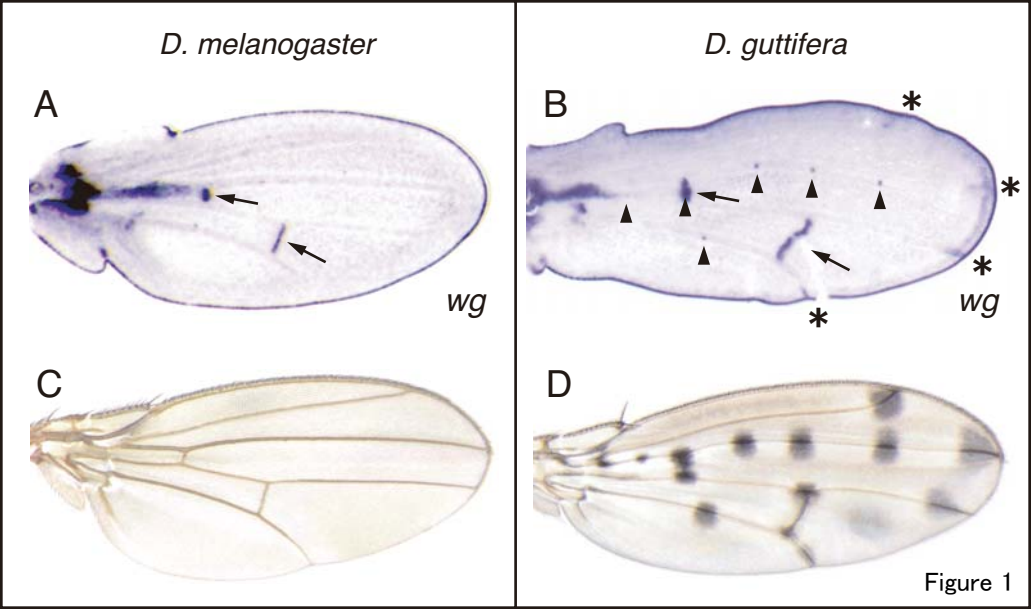
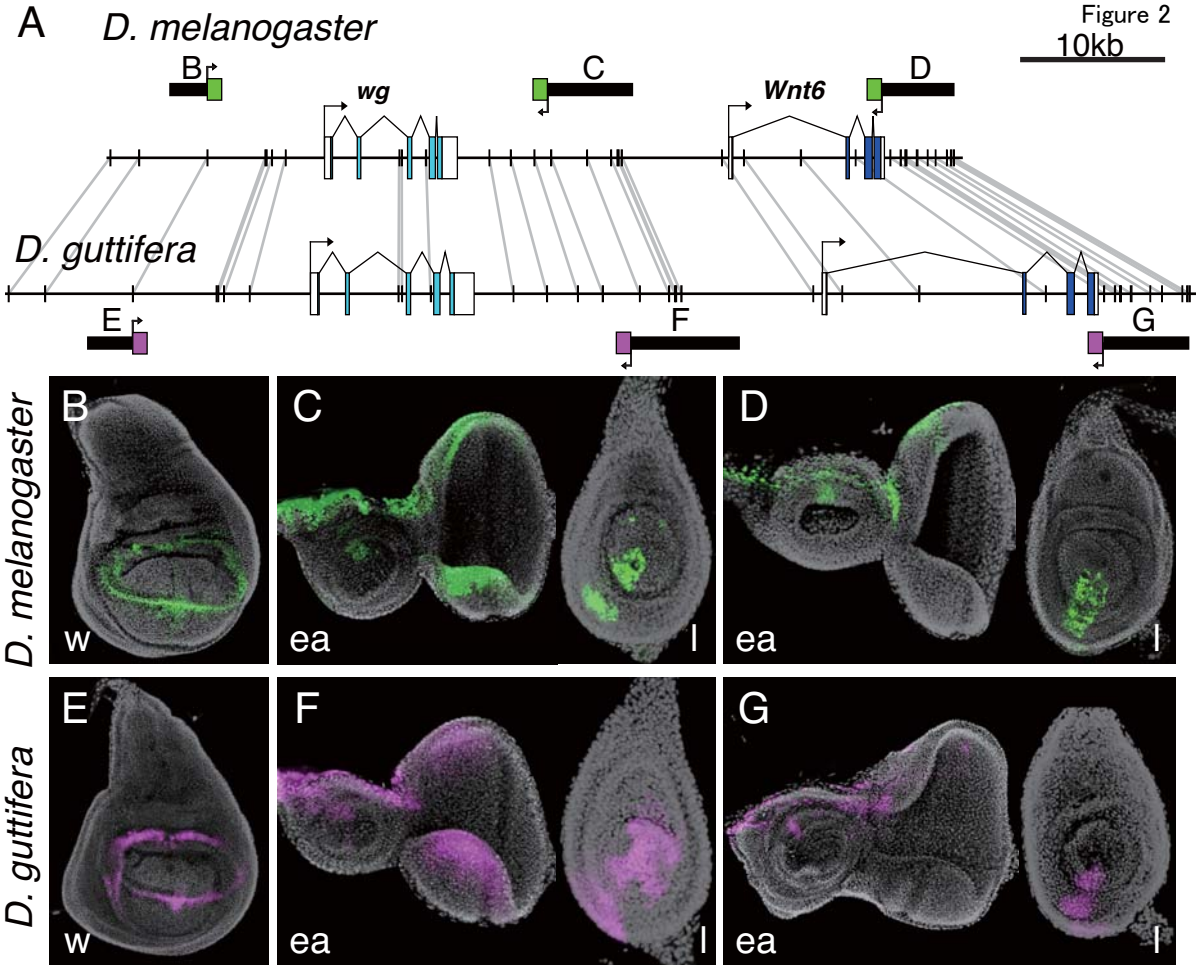


Figure 1


A
D. melanogaster
10kb
B
wg
C
Wnt6
D
D. guttifera
E
F
G
D. melanogaster
B
w
C
ea
l
D
ea
l
D. guttifera
E
w
F
ea
l
G
ea
l

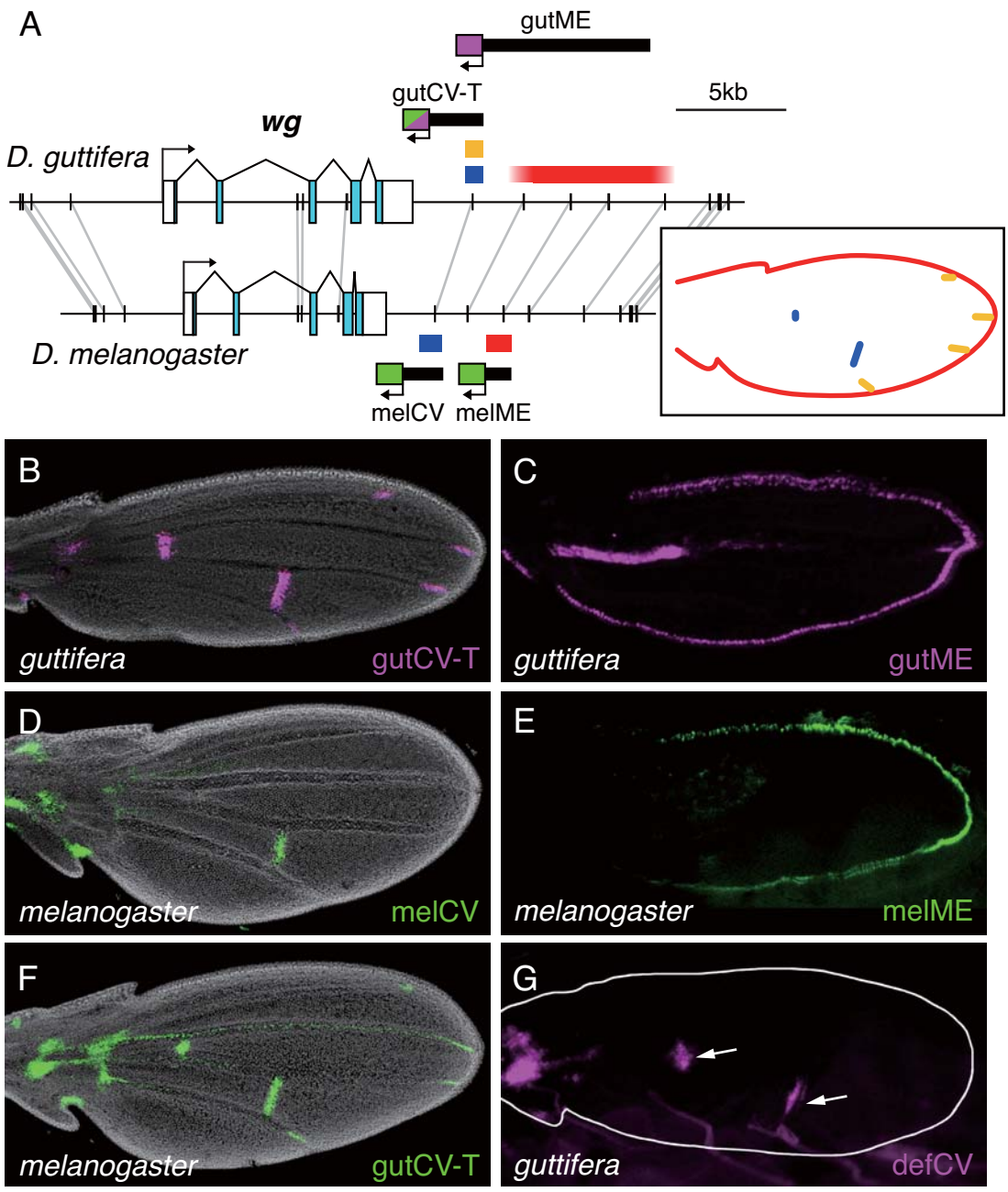
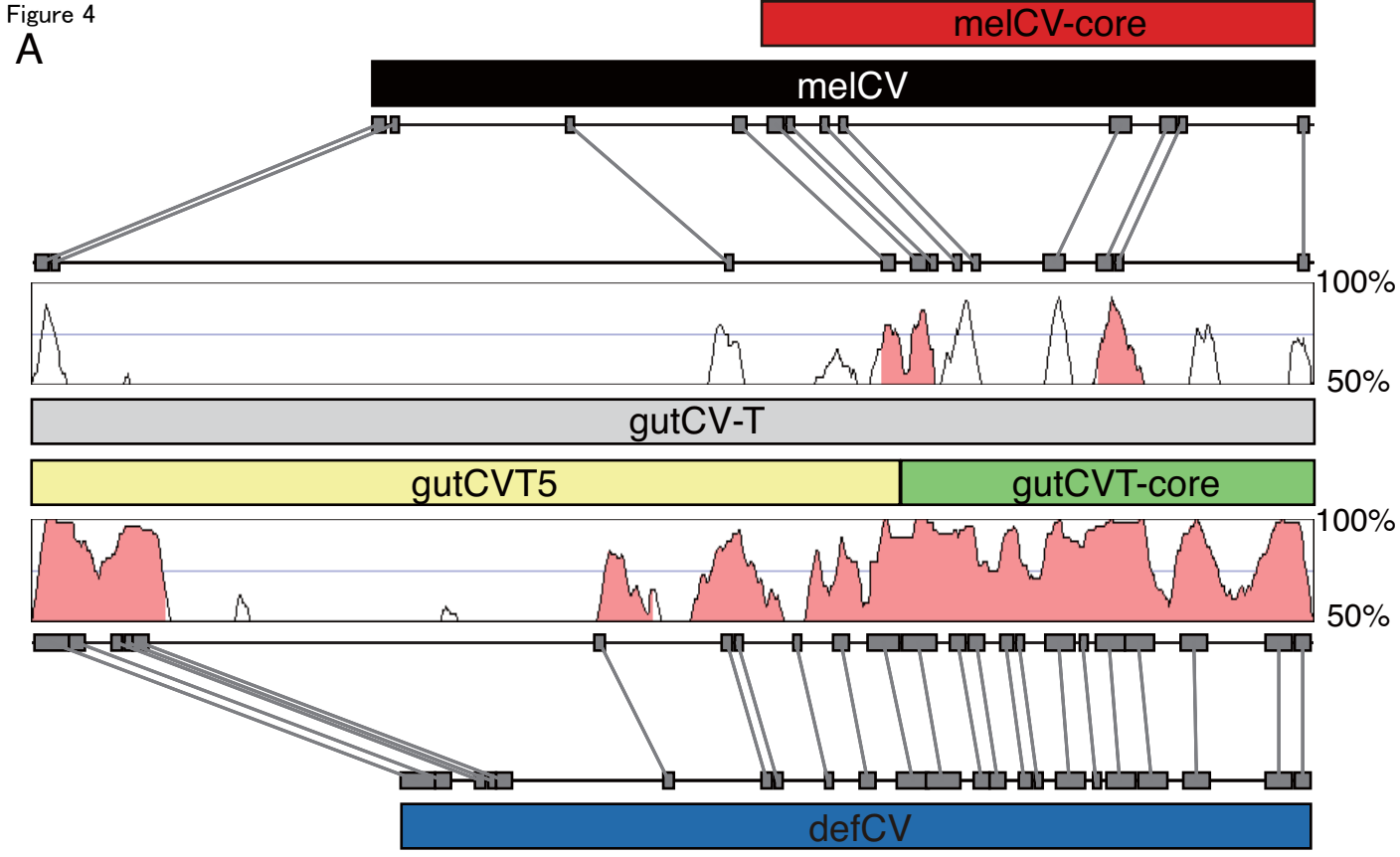

A
gutME
gutCV-T
5kb
wg
D. guttifera
D. melanogaster
melCV
melME
B
guttifera
gutCV-T
C
guttifera
gutME
D
melanogaster
melCV
E
melanogaster
melME
F
melanogaster
gutCV-T
G
guttifera
defCV

Figure 3

Figure 4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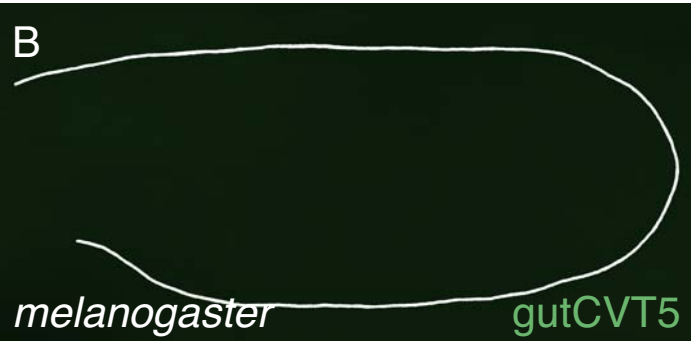
melCV-core
melCV
100%
50%
gutCV-T
gutCVT5
gutCVT-core
100%
50%
defCV


B
melanogaster
gutCVT5

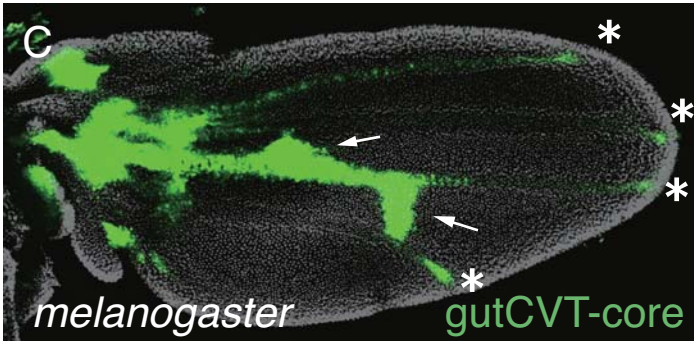
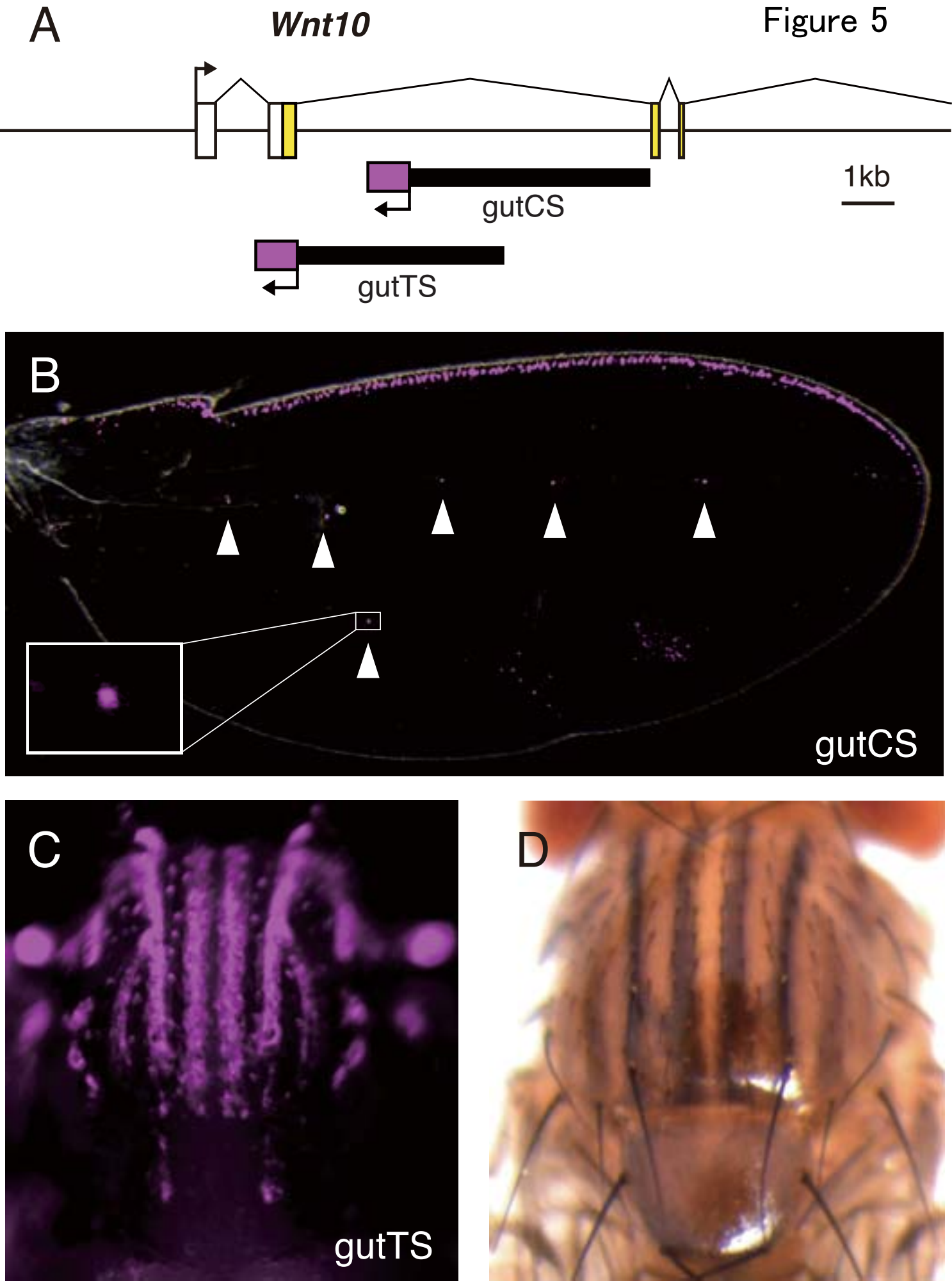

C
melanogaster
gutCVT-core

Figure 5



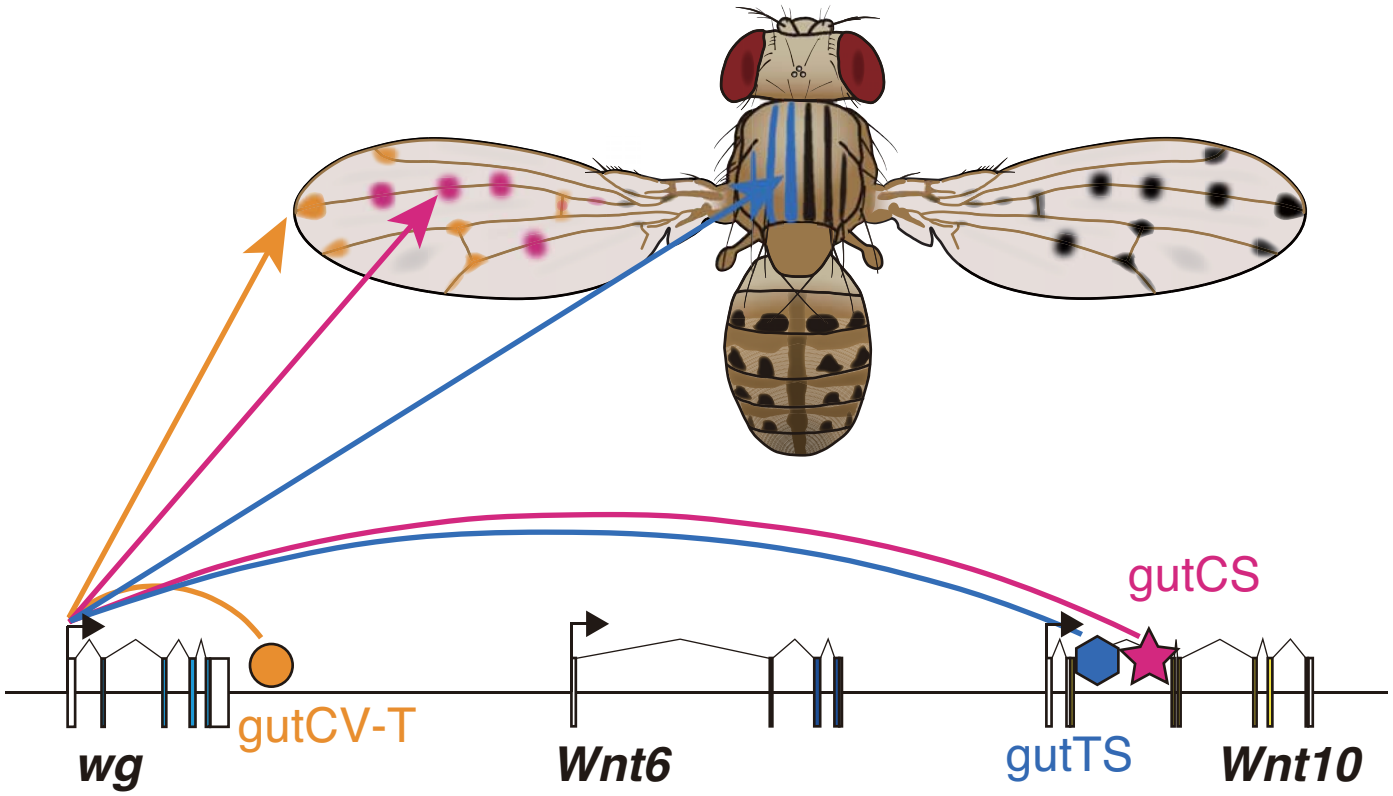


Figure 6

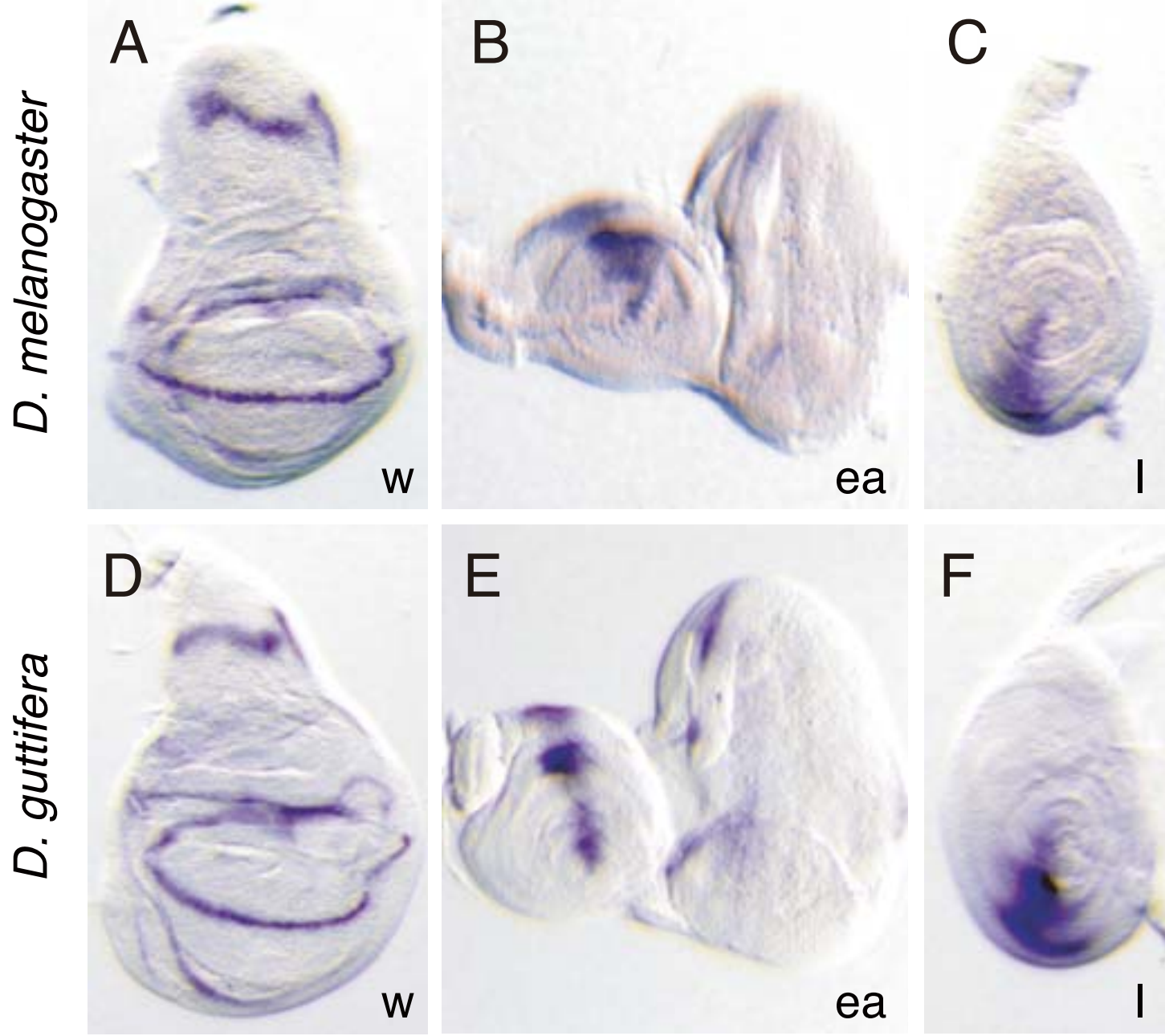


Figure. S1

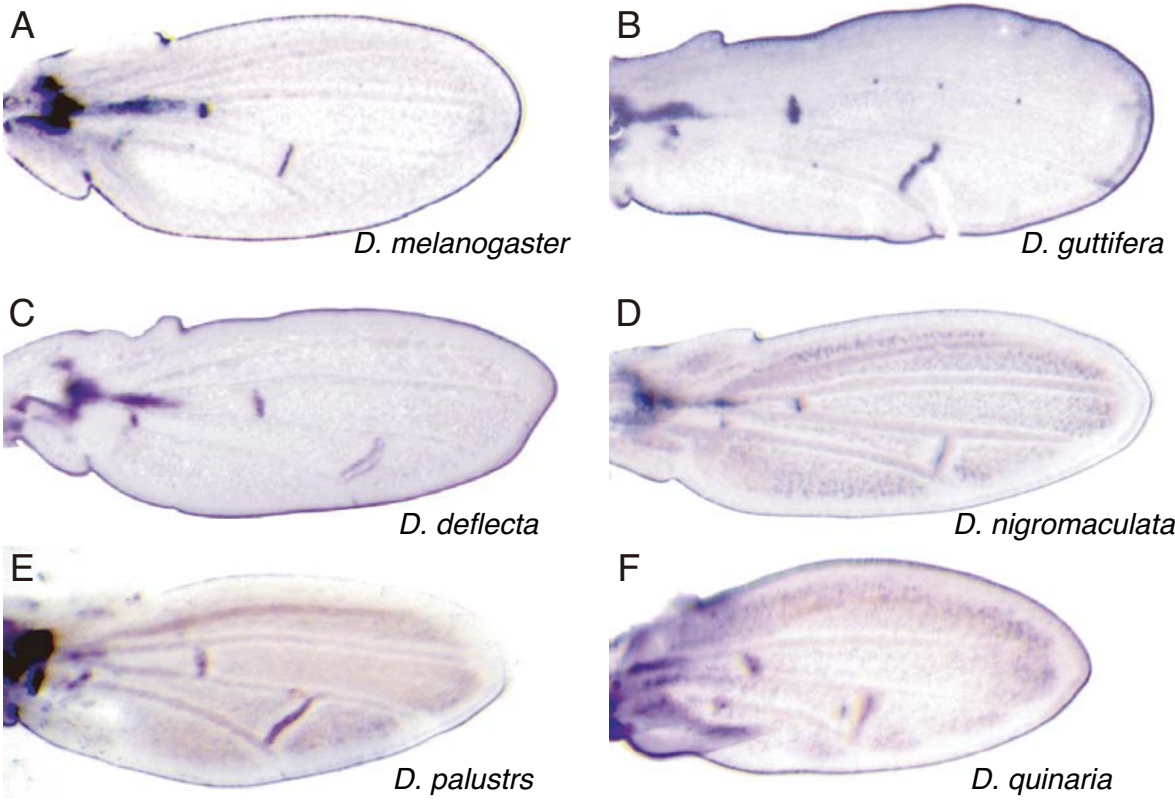
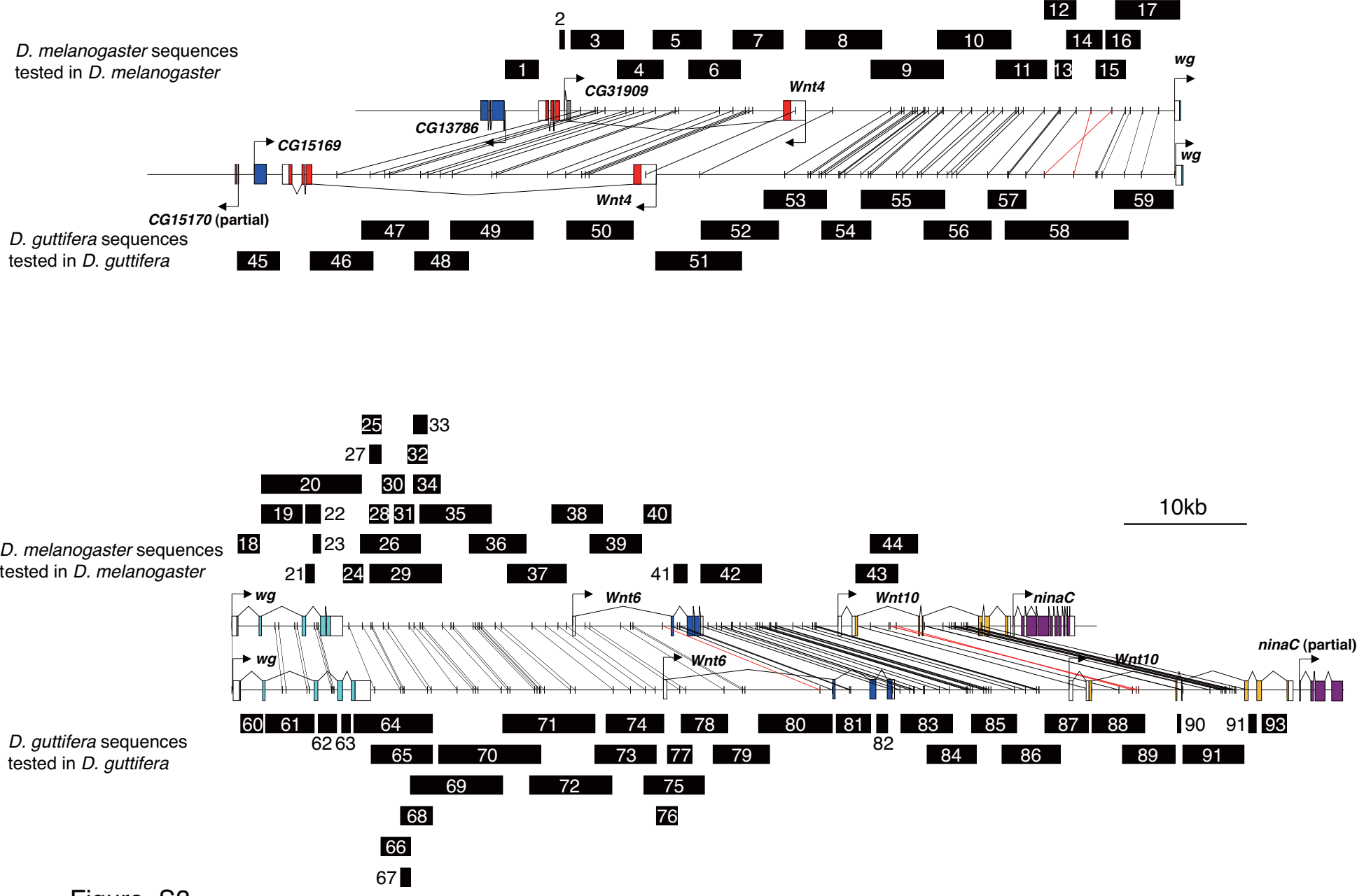

A
D. melanogaster
B
D. guttifera
C
D. deflecta
D
D. nigromaculata
E
D. palustrs
F
D. quinaria

Figure. S2


D. melanogaster sequences tested in D. melanogaster
D. guttifera sequences tested in D. guttifera
CG31909
Wnt4
CG13786
CG15169
CG15170 (partial)
wg
wg
Wnt4
2
3
5
7
8
10
12
17
14
16
1
4
6
9
11
13
15
53
55
57
59
47
49
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18
23
26
36
39
44
21
24
29
37
41
42
43
10kb
wg
wg
Wnt6
Wnt6
Wnt10
Wnt10
ninaC
ninaC (partial)
60
61
62
63
64
71
74
78
80
81
82
83
85
87
88
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91
93
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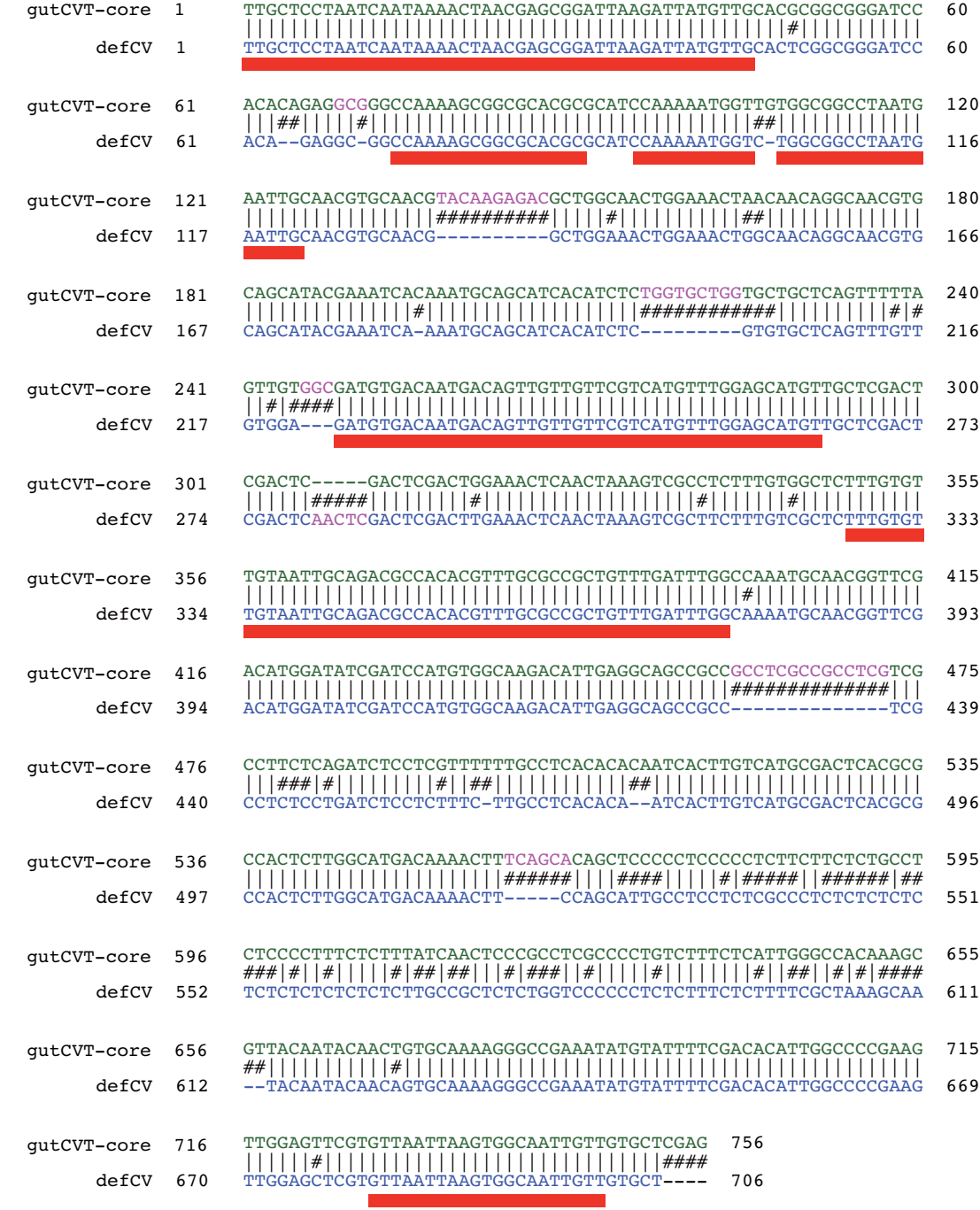
67

Figure. S3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defCV 1 TTGCTCCTAATCAATAAAACTAACGAGCGGATTAAGATTATGTTGCACTCGGCGGGATCC 60
gutCVT-core 61 ACACAGAGGCGGGCCAAAAGCGGCGCACGCGCATCCAAAAATGGTTGTGGCGGCCTAATG 120
defCV 61 ACA--GAGGC-GGCCAAAAGCGGCGCACGCGCATCCAAAAATGGTC-TGGCGGCCTAATG 116
gutCVT-core 121 AATTGCAACGTGCAACGTACAAGAGACGCTGGCAACTGGAAACTAACAACAGGCAACGTG 180
defCV 117 AATTGCAACGTGCAACG----------GCTGGAAACTGGAAACTGGCAACAGGCAACGTG 166
gutCVT-core 181 CAGCATACGAAATCACAAATGCAGCATCACATCTCTGGTGCTGGTGCTGCTCAGTTTTTA 240
defCV 167 CAGCATACGAAATCA-AAATGCAGCATCACATCTC---------GTGTGCTCAGTTTGTT 216
gutCVT-core 241 GTTGTGGCGATGTGACAATGACAGTTGTTGTTCGTCATGTTTGGAGCATGTTGCTCGACT 300
defCV 217 GTGGA---GATGTGACAATGACAGTTGTTGTTCGTCATGTTTGGAGCATGTTGCTCGACT 273
gutCVT-core 301 CGACTC-----GACTCGACTGGAAACTCAACTAAAGTCGCCTCTTTGTGGCTCTTTGTGT 355
defCV 274 CGACTCAACTCGACTCGACTTGAAACTCAACTAAAGTCGCTTCTTTGTCGCTCTTTGTGT 333
gutCVT-core 356 TGTAATTGCAGACGCCACACGTTTGCGCCGCTGTTTGATTTGGCCAAATGCAACGGTTCG 415
defCV 334 TGTAATTGCAGACGCCACACGTTTGCGCCGCTGTTTGATTTGGCAAAATGCAACGGTTCG 393
gutCVT-core 416 ACATGGATATCGATCCATGTGGCAAGACATTGAGGCAGCCGCCGCCTCGCCGCCTCGTCG 475
defCV 394 ACATGGATATCGATCCATGTGGCAAGACATTGAGGCAGCCGCC--------------TCG 439
gutCVT-core 476 CCTTCTCAGATCTCCTCGTTTTTTGCCTCACACACAATCACTTGTCATGCGACTCACGCG 535
defCV 440 CCTCTCCTGATCTCCTCTTTC-TTGCCTCACACA--ATCACTTGTCATGCGACTCACGCG 496
gutCVT-core 536 CCACTCTTGGCATGACAAAACTTTCAGCACAGCTCCCCCTCCCCCTCTTCTTCTCTGCCT 595
defCV 497 CCACTCTTGGCATGACAAAACTT-----CCAGCATTGCCTCCTCTCGCCCTCTCTCTCTC 551
gutCVT-core 596 CTCCCCTTTCTCTTTATCAACTCCCGCCTCGCCCCTGTCTTTCTCATTGGGCCACAAAGC 655
defCV 552 TCTCTCTCTCTCTTGCCGCTCTCTGGTCCCCCCTCTCTTTCTCTTTTCGCTAAAGCAA 611
gutCVT-core 656 GTTACAATACAACTGTGCAAAAGGGCCGAAATATGTATTTTCGACACATTGGCCCCGAAG 715
defCV 612 --TACAATACAACAGTGCAAAAGGGCCGAAATATGTATTTTCGACACATTGGCCCCGAAG 669
gutCVT-core 716 TTGGAGTTCGTGTTAATTAAGTGGCAATTGTTGTGCTCGAG 756
defCV 670 TTGGAGCTCGTGTTAATTAAGTGGCAATTGTTGTGCT---- 706
Similarity : 633/756 (83.73 %)

Figure. S4

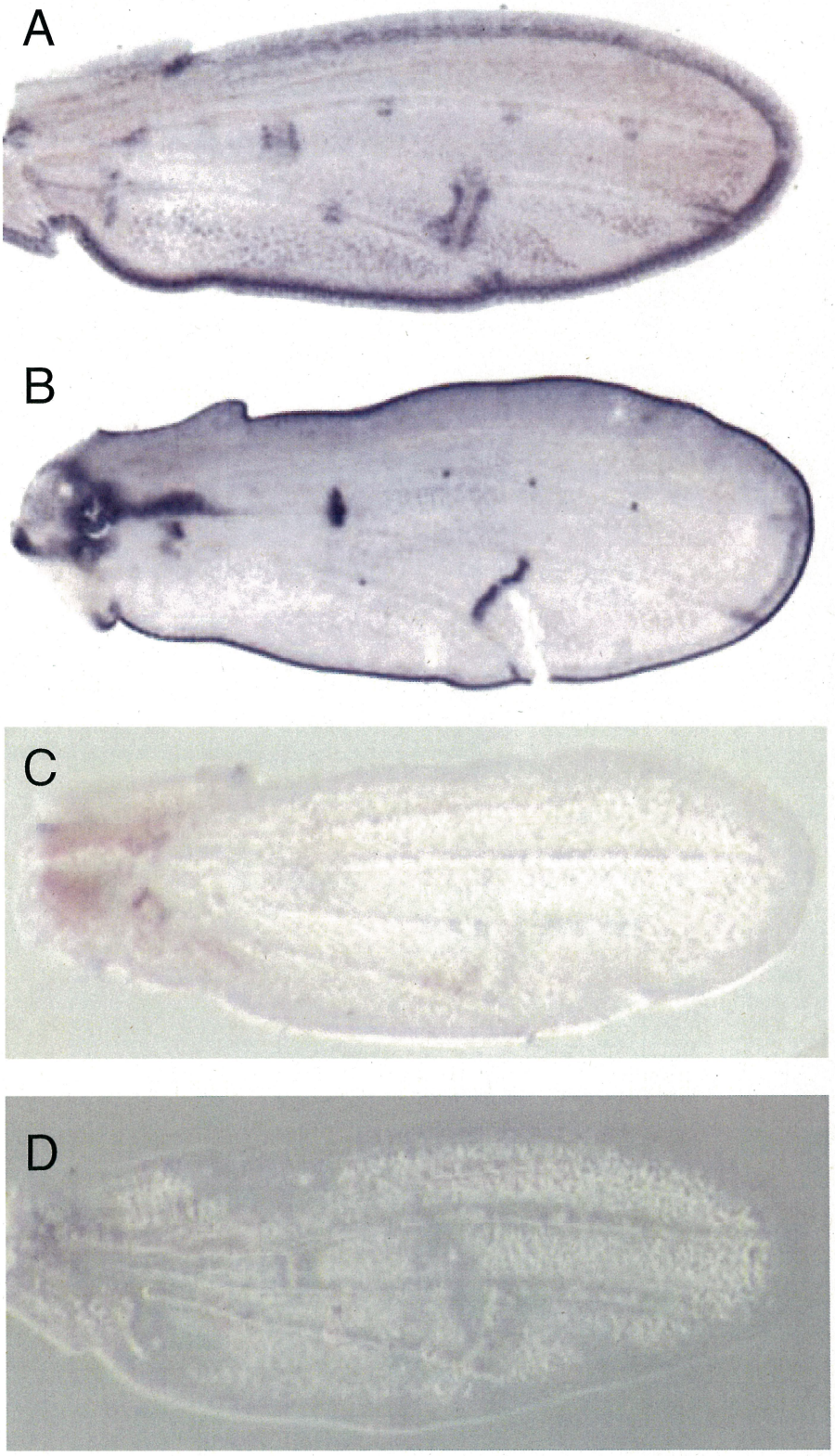

Figure. S5

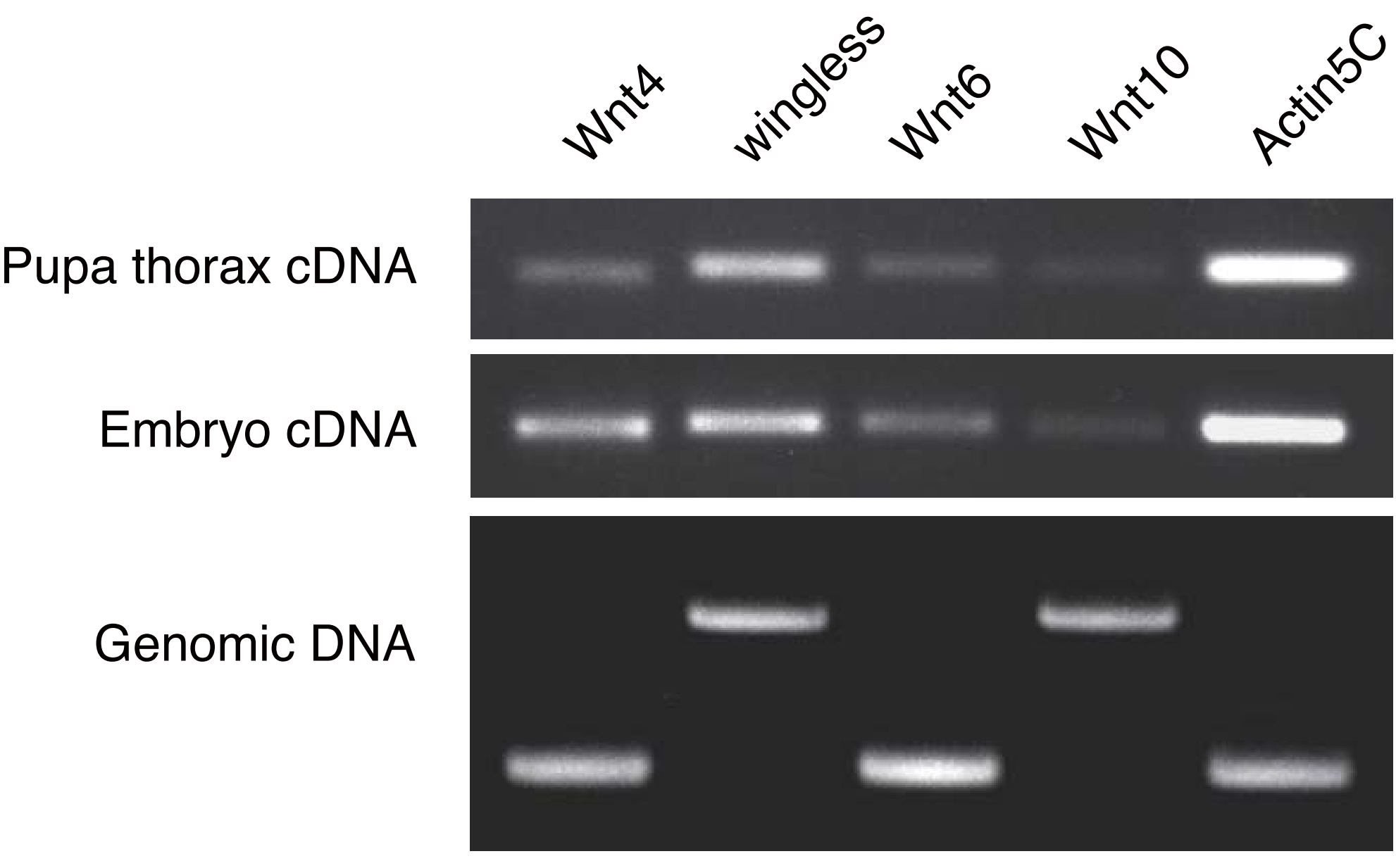
Wnt4
wingless
Wnt6
Wnt10
Actin5C
Pupa thorax cDNA
Embryo cDNA
Genomic DNA

Figure. S6

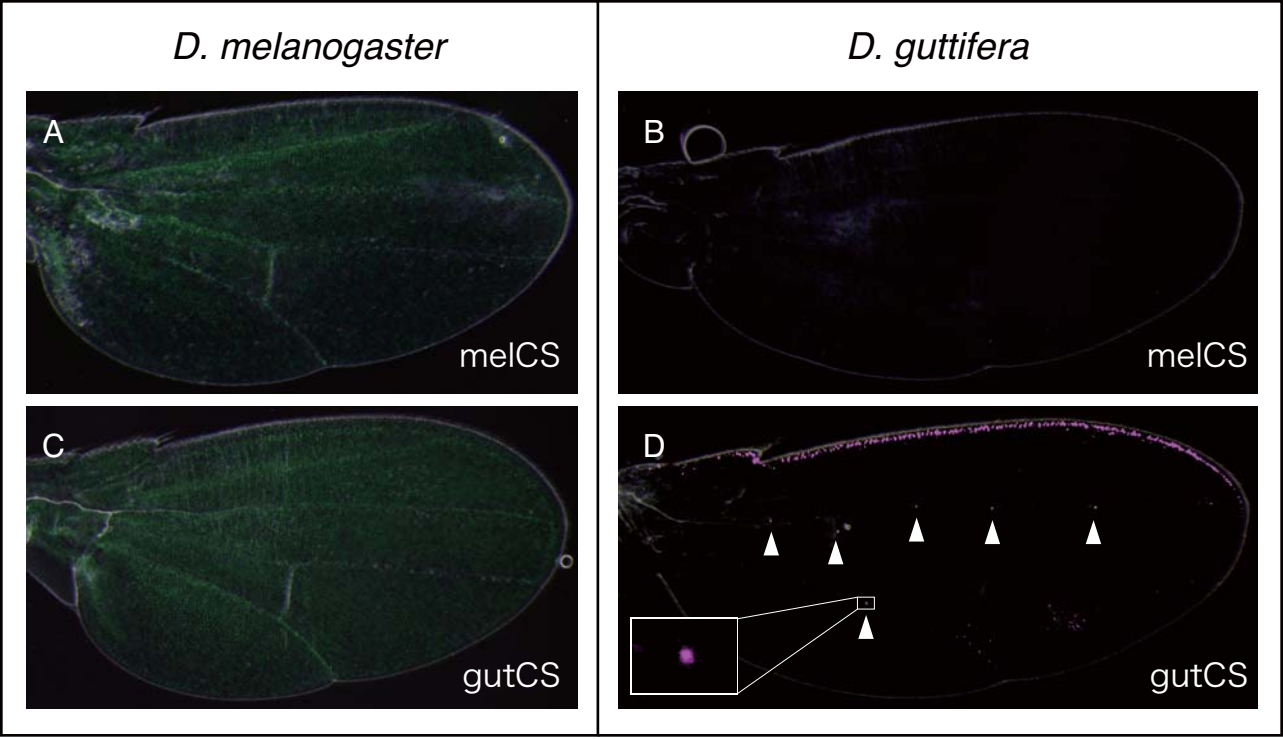

Figure. S7

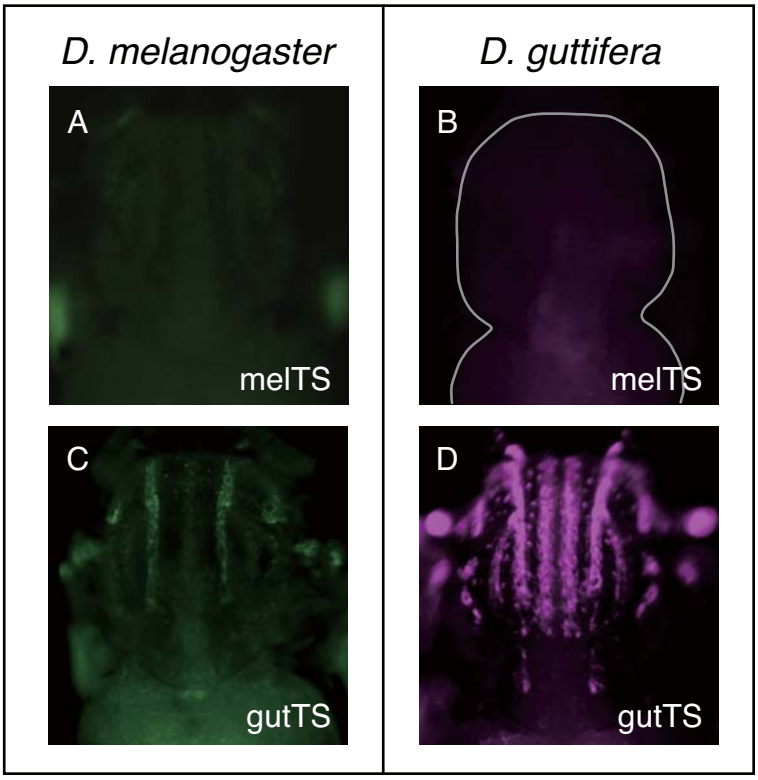

Figure. S8

Table S1. Primers used in the study.

Primers for enhancer screening (Figure S3)	Restriction site	Fragment in FigS3	Host species	Template species	Cloning ststem
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ATCACCTGCAGGCAAAACTGAAATGAAATATAAAGTTTCAC	SbfI	1	melanogaster	melanogaster	S3aG
TCATGGCGCGCCCTGGCAGACATAAAGTATTCGAAATT	AscI	2	melanogaster	melanogaster	S3aG
ATCACCTGCAGGAGTTTTCTGACTGTAGGGTCTATT	SbfI	2	melanogaster	melanogaster	S3aG
TCATGGCGCGCCGACAGGTGGGATTTGAAAAATGTA	AscI	3	melanogaster	melanogaster	S3aG
ATCACCTGCAGGCAAAAGGATGCGCTTTTTATGATGAA	SbfI	3	melanogaster	melanogaster	S3aG
TCATGGCGCGCCCGAAAACCGCAAGACAAACGT	AscI	4	melanogaster	melanogaster	S3aG
AACACCTGCAGGCGTGGCAGCATTTGATTTGTTTAGA	SbfI	4	melanogaster	melanogaster	S3aG
TCATGGCGCGCCGCGAAACGCAATCAACATTAAAAGT	AscI	5	melanogaster	melanogaster	S3aG
AACGCCTGCAGGGGGTATTTTAATTCAATTTGTGGGCT	SbfI	5	melanogaster	melanogaster	S3aG
ACTAGGCGCGCCGTTTGACATTAAACGCGTGTTAATTTC	AscI	6	melanogaster	melanogaster	S3aG
AACGCCTGCAGGCATCGCCATGTGGCACCA	SbfI	6	melanogaster	melanogaster	S3aG
ACTTGGCGCGCCTCATAAAACGCAGCCATCAAAAACGACA	AscI	7	melanogaster	melanogaster	S3aG
AACGCCTGCAGGGAGTCAATCTAGAGCTACTCCCCAT	SbfI	7	melanogaster	melanogaster	S3aG
ACTTGGCGCGCCATCTGAATCATCGACAGCCGA	AscI	8	melanogaster	melanogaster	S3aG
AACGCCTGCAGGCAAAACATCACGTAAACAATTGAGCA	SbfI	8	melanogaster	melanogaster	S3aG

ACTAGGCGCGCCCAGCGTGGCTAATTAGCACAA AscI 9 melanogaster melanogaster S3aG

AACGCCTGCAGGCAAAAGATAAGAGCAGCGCCATA SbfI 9 melanogaster melanogaster S3aG

ACTTGGCGCGCCCGGTAATATGAGCAGTAATAAAATTTGA AscI 10 melanogaster melanogaster S3aG

AACGCCTGCAGGCCTAAGAGGTTCTAATTGAGATGA SbfI 10 melanogaster melanogaster S3aG

ACTTGGCGCGCCAGTTTTGGATGTTTTATCGCTTGATT AscI 11 melanogaster melanogaster S3aG

AACGCCTGCAGGAAAGGAACTAACTGTTCATCGCACA SbfI 11 melanogaster melanogaster S3aG

TCTTGGCGCGCCCTAATATGTGTAAACCACGTTAGT AscI 12 melanogaster melanogaster S3aG

AACGCCTGCAGGACAGGTGATAATTATATAATTTGTGAC SbfI 12 melanogaster melanogaster S3aG

TCTTGGCGCGCCGAATTCTCGTGAACCTCCCAGCA AscI 13 melanogaster melanogaster S3aG

AACGCCTGCAGGGAATTCCCCGATCTTCCAGATACTC SbfI 13 melanogaster melanogaster S3aG

TCATGGCGCGCCAAAACTGTGAAATGCTGTGAAAAGAACG AscI 14 melanogaster melanogaster S3aG

AACGCCTGCAGGTTGTTTGGCTACCGATGGCTTGGA SbfI 14 melanogaster melanogaster S3aG

AAGGAAAAAAGGCGCGCCCCGATTCTCCAATTCTCGAAT SbfI 15 melanogaster melanogaster S3aG

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AAGGAAAAAAGGCGCGCCCTGGAACAAGGGCAGCCACTTG AscI 16 melanogaster melanogaster S3aG

AAGGAAAAAAGGCGCGCCCCGGGGTTCCAGGTTCCAGG SbfI 16 melanogaster melanogaster S3aG

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melanogaster melanogaster S3aG
TCTTGGCGCGCCCTGAAAGAAAATGAGCCAGAAAAC AscI 18
melanogaster melanogaster S3aG
AAGGAAAAAAGGCGCGCCCCTTCACTGCAGGCCCT AscI 19
melanogaster melanogaster S3aG
AAGGAAAAAACCTGCAGGGCGAGTGCCAACACCAGT SbfI 19
melanogaster melanogaster S3aG
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AAGGAAAAAACCTGCAGGGATTCGGGTTCAAGTTCTC SbfI 21
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AAGGAAAAAAGGCGCGCCGCCCATAACAAGTAATAAAAT AscI 21
melanogaster melanogaster S3aG
AAGGAAAAAACCTGCAGGGATTCGGGTTCAAGTTCTC SbfI 22
melanogaster melanogaster S3aG
AAGGAAAAAAGGCGCGCCGCACTCCTGTCGCATCTC AscI 22
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AAGGAAAAAAGGCGCGCCGCACTCCTGTCGCATCTC AscI 23
melanogaster melanogaster S3aG
AACGCCTGCAGGGCCAAGTGGAATTCATTGGTAC SbfI 24
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AAGGAAAAAAGGCGCGCCGGCCAACGAGCGTGTAAATA AscI 25
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ATATGTCGACGACATTGCTCCTAATCAATAAAACTAA SalI 26
melanogaster melanogaster S3aG
ACTAGGCGCGCCCTCACGCCTCGAAACAATTGC AscI 26 melanogaster
melanogaster S3aG

AAGGAAAAAACCTGCAGGCAGCGGATCGATGGTTTG SbfI 27
melanogaster melanogaster S3aG
AAGGAAAAAAGGCGCGCCCATCCTTGTGGTGAGCCTG AscI 27
melanogaster melanogaster S3aG
AACGCCTGCAGGGCAGCGGATCGATGGTTTG SbfI 28 melanogaster
melanogaster S3aG
ACTAGGCGCGCCGCGAACGCTGCATAATGGAA AscI 28 melanogaster
melanogaster S3aG
AACGCCTGCAGGGCAGCGGATCGATGGTTTG SbfI 29 melanogaster
melanogaster S3aG
ACTAGGCGCGCCCTCACGCCTCGAAACAATTGC AscI 29 melanogaster
melanogaster S3aG
ATCGCCTGCAGGACACGCATGTAAACATTGCCATT SbfI 30
melanogaster melanogaster S3aG
ACTTGGCGCGCCTTGTTATCTTTCTCACAATCAAGGT AscI 30
melanogaster melanogaster S3aG
ATCGCCTGCAGGGGTGCGCCAACTTTGTTTAACT SbfI 31
melanogaster melanogaster S3aG
ACTTGGCGCGCCCAAAGCAACAAATTGAAACTAAATACTT AscI 31
melanogaster melanogaster S3aG
AACGCCTGCAGGGAACAGGTGCTGAGCAAATG SbfI 32 melanogaster
melanogaster S3aG
ACTTGGCGCGCCGCGAATGAAGACGTTTCGTGA AscI 32
melanogaster melanogaster S3aG
AACGCCTGCAGGAAGTATTTAGTTTCAATTTGTTGCTTTG SbfI 33
melanogaster melanogaster S3aG
ACTTGGCGCGCCCATCCTTGTGGTGAGCCTGA AscI 33 melanogaster
melanogaster S3aG
AACGCCTGCAGGAAGTATTTAGTTTCAATTTGTTGCTTTG SbfI 34
melanogaster melanogaster S3aG
ACTTGGCGCGCCGCGAATGAAGACGTTTCGTGA AscI 34
melanogaster melanogaster S3aG
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melanogaster melanogaster S3aG
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AACGCCTGCAGGGCGTCGCGTGGCGTAGACT SbfI 39 melanogaster
melanogaster S3aG
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melanogaster melanogaster S3aG
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melanogaster melanogaster S3aG
ATCACCTGCAGGGCCTAGTAGTTGCAGCTTGTTA SbfI 44
melanogaster melanogaster S3aG
ACTTGGCGCGCCGCAACGAAATGGGGTACAGTATTA AscI 44
melanogaster melanogaster S3aG

AACGGGTACCTTCTCGATCAGCCGTACTAATGAT KpnI 45 guttifera guttifera pBac

TCAACCGCGGGAAAATGAAATAAAATAAAAAGTTTCACATAAAC SacII 45 guttifera guttifera pBac

AACGGGTACCCTGCAAAAGAGAATAAACGGTTTCAA KpnI 46 guttifera guttifera pBac

TCAACCGCGGTATCCATCAACACTTTGGTTTTATAGT SacII 46 guttifera guttifera pBac

AACGGGTACCTTTATCTATTGTATCAAAACGACAAAAATTTGA KpnI 47 guttifera guttifera pBac

TCAACCGCGGATGTGTCGTAAAGATTTTTATTGCATTTTTATG SacII 47 guttifera guttifera pBac

AACGGGTACCGCGTAACCGCAAGACAAACGT KpnI 48 guttifera guttifera pBac

TCAACCGCGGCCTGGCAGCATTTGATTTGTTTAGA SacII 48 guttifera guttifera pBac

AACGGGTACCGCAAAACGCAATCAACATTAAAACTTTT KpnI 49 guttifera guttifera pBac

TCAACCGCGGCGGTATTTTAATTCAATTTGTTGTCATC SacII 49 guttifera guttifera pBac

AACGGCTAGCTCATAAAACGCAGCCATCAAAACGA NheI 50 guttifera guttifera pBac

TCAACCGCGGGAGTAATTTAACAGTTATTGTGAATATGCT SacII 50 guttifera guttifera pBac

AACGGCTAGCCTCGTTCGGCACTCGACT NheI 51 guttifera guttifera pBac

TCAACCGCGGCTCGAAATCCGCTTTAATTGAATCA SacII 51 guttifera guttifera pBac

AACGGCTAGCAAGTCTAAACATTTTTACAGACACCT NheI 52 guttifera guttifera pBac

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TCAACCGCGGTCGATAAATTTTCAAGTTGCCAATAATAAATT SacII 53 guttifera guttifera pBac

AACGGCTAGCTGCGATATGAAGATATTAAGACATGAAT NheI 54 guttifera guttifera pBac

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AACGGCTAGCCAGTAATATGAGCAGTAATAAAATTTGAAT NheI 55 guttifera guttifera pBac

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AACGGCTAGCAGTTTTGGATGTTTTATCGCTTGATT NheI 56 guttifera guttifera pBac

ACTACCGCGGAAAGGAACTAACTGTTCAATCGCA SacII 56 guttifera guttifera pBac

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AACGGCTAGCGAAAGAGAGTGAAAAAGCAGAGAGAAT NheI 61 guttifera guttifera pBac

AACGGCTAGCAGAARGTGAATCTGCACAACAACGAG NheI 62 guttifera guttifera pBac

AACGGGTACCCATGCCRTGGCATTTGCACTC KpnI 62 guttifera guttifera pBac

TCAACCGCGGAAACTTACAATTCAAAACTATTACTTGTTTTA SacII 63
guttifera guttifera pBac
AACGGCTAGCGTAAAGAAGAAAATGAAATAGAAAGTGGA NheI 63
guttifera guttifera pBac
TCAACCGCGGAAGATTATACACACGTGTCTGTAAG SacII 64 guttifera
guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera pBac
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guttifera guttifera pBac

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TCAACCGCGGTGTTTAGAAATTTTAAGTTTCTCGATAAATG SacII 73 guttifera guttifera pBac

AACGGCTAGCAAAATGAAATTAAACGCGCGCTTTAATCA NheI 73 guttifera guttifera pBac

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AACGGCTAGCCCGCGACGTATCTTTATTTTGC NheI 78 guttifera guttifera pBac

ATATCTCGAGTCGGCCAATTGCCAAAAATTAATGCA XhoI 79 guttifera guttifera pBac

TCAACCGCGGGAGATTATGACAACTTAATAGCTACAGA SacII 79 guttifera guttifera pBac

TCAACCGCGGTCCATTGAGGCCTATAACGACA SacII 80 guttifera guttifera pBac

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TCAACCGCGGAAGTGCAACTAAATATGTAACTACTACAA SacII 81 guttifera guttifera pBac

AACGGCTAGCGTGGGAGGCAGAAAGGATAAC NheI 81 guttifera guttifera pBac

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AACGGCTAGCGGAAAATGGAGCAAAAAGAATGCTT NheI 82 guttifera guttifera pBac

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AACGGCTAGCCAATTAAACAATTCATTTATCTTAATTTGTCTG NheI 83 guttifera guttifera pBac

TCAACCGCGGTGACTTTCCCATAAATTAACACAATTTTATTGT SacII 84 guttifera guttifera pBac

AACGGCTAGCGACTGCATTAAAAATCAACTTAATTTCA NheI 84 guttifera guttifera pBac

TCAACCGCGGGTTAATAAAAAACACATAATTGCGTATGTT SacII 85 guttifera guttifera pBac

AACGGCTAGCGTTTACAAACCACAGCACGCA NheI 85 guttifera guttifera pBac

TCAACCGCGGAGCGCCACATCAACGTCATAA SacII 86 guttifera guttifera pBac

AACGGCTAGCACACTTGTTACACTTCAAAGGACTT NheI 86 guttifera guttifera pBac

TCAACCGCGGGTGATGGTGATTCATGGCAATTG SacII 87 guttifera guttifera pBac

AACGGCTAGCAAACATTTTGTAGAACACATTGAAGAAAT NheI 87 guttifera guttifera pBac

TCAACCGCGGAAGTGAGGCGAGCGTATCTTATA SacII 88 guttifera guttifera pBac

AACGGCTAGCTGCCTAACTGGCTTAGATAGCTAA NheI 88 guttifera guttifera pBac

TCAACCGCGGGCCTAGTAGTTGCAGCTTGTTA SacII 89 guttifera guttifera pBac

AACGGCTAGCGGCATATAAATAGACAGTTTGAATTTATTA NheI 89 guttifera guttifera pBac

TCAACCGCGGAAATAAATCTGCTTCTAATGCGAAAATG SacII 90 guttifera guttifera pBac

AACGGCTAGCACATCAGCATAAAAAGCATAAATAAAGAAAG NheI 90 guttifera guttifera pBac

AACGGGTACCGAGTTCCCCAGGTTTCCAC KpnI 91 guttifera guttifera pBac

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AACGGCTAGCAGAAGTCGAAATGGGCAAGATATTAG NheI 92 guttifera guttifera pBac

TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT SacII 93 guttifera guttifera pBac

AACGGCTAGCAAAAATTTAACAAAGAATGTGGGGTTAGATA NheI 93 guttifera guttifera pBac

"Primers for cis/trans test (Figure 3, 4, 5, S7, S8)"	Fragment name	Host species	Template species	Cloning system
TCAACCGCGGAAAAAAAGTGATCGTGCTACATGTGT	SacII	gutCV-T	guttifera	guttifera pBac
AACGGCTAGCAGCACAACAATTGCCACTTAATTAACA	NheI	gutCV-T	guttifera	guttifera pBac
AACGCCTGCAGGAAAAAAAGTGATCGTGCTACATGTGT	SbfI	melCV	melanogaster	melanogaster S3aG
ACTAGGCGCGCCCTCACGCCTCGAAACAATTGC	AscI	melCV	melanogaster	melanogaster S3aG
AACGCCTGCAGGTAAAAAAGTGATCGTGCTACATGTGT	SbfI	gutCV-T	melanogaster	guttifera S3aG
TCTTGGCGCGCCCTCGAGCACAACAATTGCCACT	AscI	gutCV-T	melanogaster	guttifera S3aG
TCAACCGCGGATAAAAAGTGATCGTGCTACATGTGT	SacII	defCV	guttifera	deflecta pBac
ATATCTCGAGGACATTGCTCCTAATCAATAAAACTAA	XhoI	defCV	guttifera	deflecta pBac
TCTGGCTAGCTGCCAATTTATCGATCAACACGCT	NheI	gutME	guttifera	

guttifera pBac
ATATCTCGAGGACATTGCTCCTAATCAATAAAACTAA XhoI gutME guttifera
guttifera pBac
AACGCCTGCAGGAAGTATTTAGTTTCAATTTGTTGCTTTG SbfI melME
melanogaster melanogaster S3aG
ACTTggcgcgccGCGAATGAAGACGTTTCGTGA AscI melME melanogaster
melanogaster S3aG
AACGCCTGCAGGAAAAAAAAGTGATCGTGCTACATGTGT SbfI gutCVT5
melanogaster guttifera S3aG
TCTTGGCGCGCCAGACCGCGACGATGCGAT AscI gutCVT5
melanogaster guttifera S3aG
AACGCCTGCAGGGACATTGCTCCTAATCAATAAAACTAA SbfI gutCVT-core
melanogaster guttifera S3aG
TCTTGGCGCGCCCTCGAGCACAACAATTGCCACT AscI gutCVT-core
melanogaster guttifera S3aG
TCAACCGCGGGACATTGCTCCTAATCAATAAAACTAA SacII gutCVT-core
guttifera guttifera pBac
AACGGCTAGCAGCACAACAATTGCCACTTAATTAACA NheI gutCVT-core
guttifera guttifera pBac
ATCACCTGCAGGGCCTAGTAGTTGCAGCTTGTTA SbfI melCS
melanogaster melanogaster S3aG
ACTTGGCGCGCCGCAACGAAATGGGGTACAGTATTA AscI melCS
melanogaster melanogaster S3aG
ATATCTCGAGGCCTAGTAGTTGCAGCTTGTTA XhoI melCS guttifera
melanogaster pBac
TCAACCGCGGGCAACGAAATGGGGTACAGTATTA SacII melCS guttifera
melanogaster pBac
ATCACCTGCAGGGCCTAGTAGTTGCAGCTTGTTA SbfI gutCS
melanogaster guttifera S3aG
ACTTGGCGCGCCGGCATATAAATAGACAGTTTGAATTTATTA AscI gutCS
melanogaster guttifera S3aG
TCAACCGCGGGCCTAGTAGTTGCAGCTTGTTA SacII gutCS guttifera
guttifera pBac
AACGGCTAGCGGCATATAAATAGACAGTTTGAATTTATTA NheI gutCS
guttifera guttifera pBac
ATCACCTGCAGGGAGTCTCTCATCTATCCTAAGAC SbfI melTS

melanogaster melanogaster S3aG
ACTTGGCGCGCCGGCAAAATGCATTTTAATTGGCTGA AscI melTS
melanogaster melanogaster S3aG
ATATCTCGAGGAGTCTCTCATCTATCCTAAGAC XhoI melTS guttifera
melanogaster pBac
TCAACCGCGGGCAACGAAATGGGGTACAGTATTA SacII melTS guttifera
melanogaster pBac
ATCACCTGCAGGAAGTGAGGCGAGCGTATCTTATA SbfI gutTS
melanogaster guttifera S3aG
ACTTGGCGCGCCGGCATATAAATAGACAGTTTGAATTTATTA AscI gutTS
melanogaster guttifera S3aG
TCAACCGCGGAAGTGAGGCGAGCGTATCTTATA SacII gutTS guttifera
guttifera pBac
AACGGCTAGCTGCCTAACTGGCTTAGATAGCTAA NheI gutTS guttifera
guttifera pBac

Primers for in situ hybridization	Gene	Target species
CACGTCCAAGCGGAGATGCG	wg	melanogaster
GGCGACGGCATGTTCGGGTG	wg	melanogaster
CACGTTCAGGCGGAGATGCG	wg	"guttifera, deflecta, nigromaculata, palstris, quinaria"
GGCGATGGCATATTGGGATGATG	wg	"guttifera, deflecta, nigromaculata, palstris, quinaria"
CGAACACTTTATATCGGAGCA	Wnt4	guttifera
GAGTCATGTCGCAATATTTCGG	Wnt4	guttifera
GCCATTCGCGATGCGATG	Wnt6	guttifera
CTAGAGGCATGTGTTGACCTC	Wnt6	guttifera
GCCGTGTCCAATAACATGGAGT	Wnt10	guttifera
CCTGTATATCCGCTCCTAGAT	Wnt10	guttifera

Primers for RT-PCR	Gene	Template species
GAGCAGCAACTGTTGCTGTC	Wnt4	guttifera
GCCAATCCTTTGTTCACATTGATTC	Wnt4	guttifera
GAGTGCAAATGCCACGGCAT	wg	guttifera
GGCTCCAGATAGACAATATCCTT	wg	guttifera
GCCATTCGCGATGCGATG	Wnt6	guttifera

AATTATGTTCATGACTCTGCCGAG	Wnt6	guttifera
GTTATCGGGAAAGTGCTTTTGC	Wnt10	guttifera
CTTCAGCACTTTGCCAACAATGT	Wnt10	guttifera
ATGTGTGACGAAGAAGTTGCT	Act5C	guttifera
TAGATGGGCACAGTGTGG	Act5C	guttifera