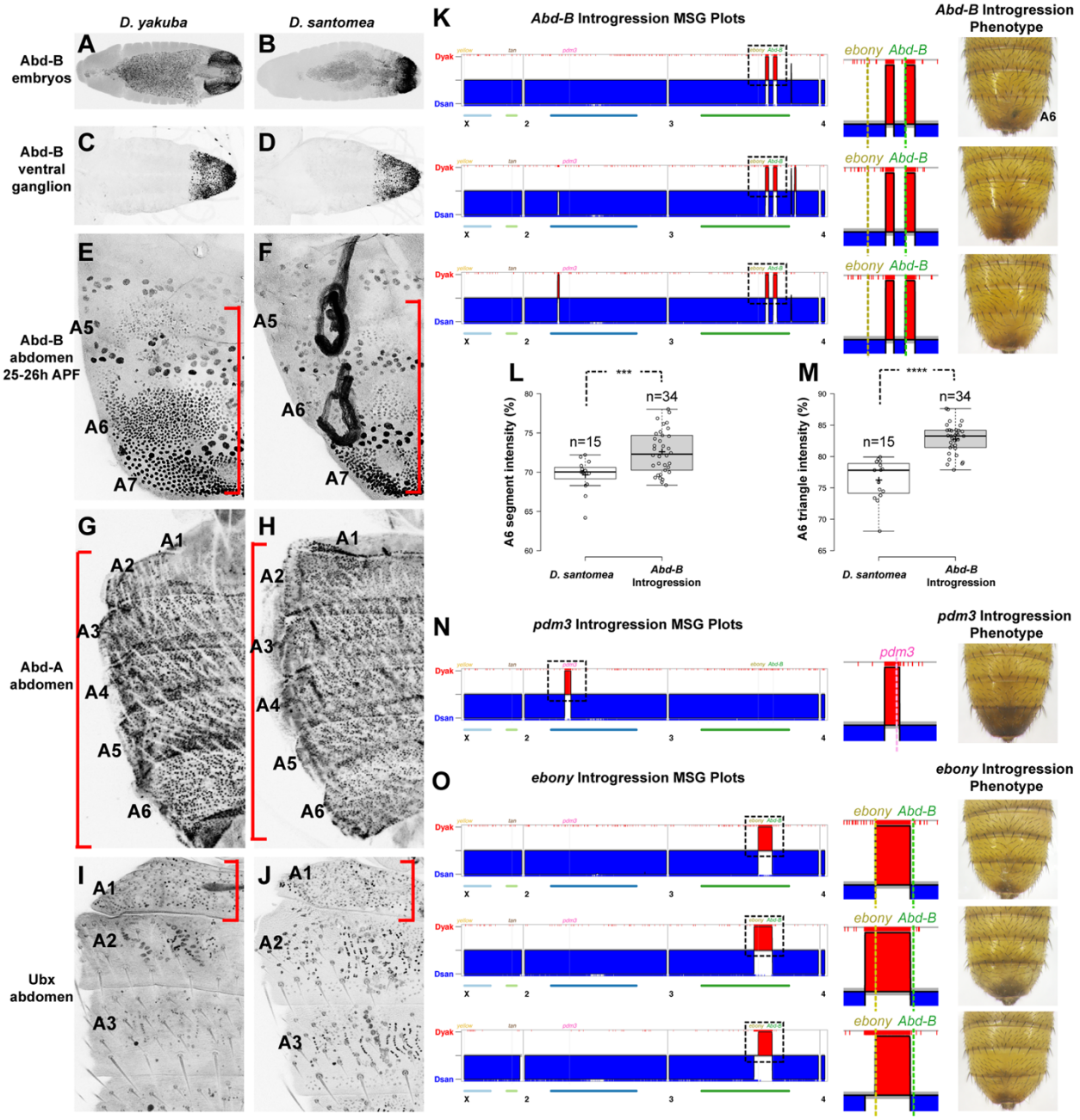
D. yakuba
D. santomea
Abd-B embryos
A
B
Abd-B ventral ganglion
C
D
Abd-B abdomen 25-26h APF
E
F
A5
A6
A7
Abd-A abdomen
G
H
A1
A2
A3
A4
A5
A6
Ubx abdomen
I
J
A1
A2
A3
K
Abd-B Introgression MSG Plots
Abd-B Introgression Phenotype
Dyak
Dsan
ebony Abd-B
A6
L
M
A6 segment intensity (%)
A6 triangle intensity (%)
***
****
n=15
n=34
D. santomea
Abd-B Introgression
N
pdm3 Introgression MSG Plots
pdm3 Introgression Phenotype
pdm3
O
ebony Introgression MSG Plots
ebony Introgression Phenotype

Figure S1. Expression analysis of the Hox genes of the bithorax complex, introgression line maps and phenotypes. Related to Figure 1. (A-F) Abd-B is detected in similar spatial patterns in *D. yakuba* and *D. santomea* late stage embryos (stage 12-13) (A-B), third instar larval brain (C-D), and early pupal abdomens (25-26 hAPF) (E-F). (G-H) Abd-A is expressed similarly between *D. yakuba* and *D. santomea* throughout the mid-late pupal stage abdomen (~68 hAPF). (I-J) Ubx is limited to epithelial nuclei of the A1 abdominal segment in mid-late stage pupae (~68 hAPF) of both *D. yakuba* and *D. santomea*. Red brackets highlight the expressing segments in pupal stages.(K-O) display multiplex shotgun genotype maps [S1] of each introgression line and their corresponding abdominal phenotypes. Plots show multiplex shotgun genotyping results for representative individuals, indicating homozygous *D. santomea* regions in blue, homozygous *D. yakuba* regions in red, and heterozygous regions in white. The positions of genes studied here and the previously mapped QTL [S2] are indicated on each map. (K) The *Abd-B* introgression line is consistently homozygous for two segments, one of which contains the *D. yakuba Abd-B* gene. Individuals from the line contain a variable number of other heterozygous fragments. Dashed box indicates the region of the plot that is zoomed in to the right. A representative adult image is shown beside each map. (L) Box plot showing the average A6 pigmentation of animals containing the homozygous *Abd-B* introgression (and a variable number of other homozygous and heterozygous fragments). (M) Box plot showing the intensity of pigmentation in a small posterior triangular region of the A6 tergite of animals containing the homozygous *Abd-B* introgression (and a variable number of other homozygous and heterozygous fragments). The number of stars in box plots indicates *p* value: ****: $p<0.0001$, ***: $p<0.001$, Student's t-test. (N) Genome map and representative phenotype of the *pdm3* introgression. (O) Genome maps and representative phenotypes of the *ebony* introgression line.

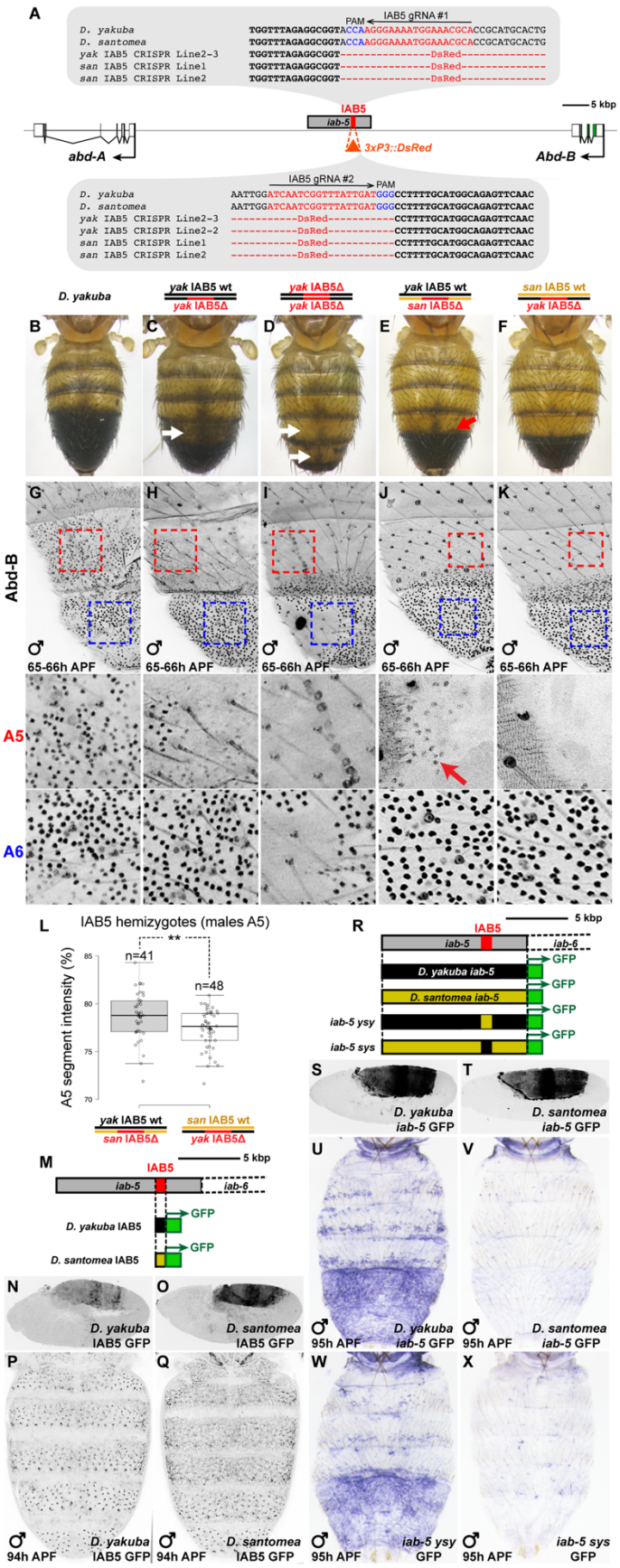
A
IAB5 gRNA #1
PAM
D. yakuba TGGTTTAGAGGCGGTACCAAGGGAAAATGGAAACGCACCGCATGCACTG
D. santomea TGGTTTAGAGGCGGTACCAAGGGAAAATGGAAACGCACCGCATGCACTG
yak IAB5 CRISPR Line2-3 TGGTTTAGAGGCGGT----------DsRed----------
san IAB5 CRISPR Line1 TGGTTTAGAGGCGGT----------DsRed----------
san IAB5 CRISPR Line2 TGGTTTAGAGGCGGT----------DsRed----------
IAB5
iab-5
3xP3::DsRed
5 kbp
abd-A
Abd-B
IAB5 gRNA #2
PAM
D. yakuba AATTGGATCAATCGGTTTATTGATGGGCCTTTTGCATGGCAGAGTTCAAC
D. santomea AATTGGATCAATCGGTTTATTGATGGGCCTTTTGCATGGCAGAGTTCAAC
yak IAB5 CRISPR Line2-3 ----------DsRed----------CCTTTTGCATGGCAGAGTTCAAC
yak IAB5 CRISPR Line2-2 ----------DsRed----------CCTTTTGCATGGCAGAGTTCAAC
san IAB5 CRISPR Line1 ----------DsRed----------CCTTTTGCATGGCAGAGTTCAAC
san IAB5 CRISPR Line2 ----------DsRed----------CCTTTTGCATGGCAGAGTTCAAC
D. yakuba
yak IAB5 wt / yak IAB5Δ
yak IAB5Δ / yak IAB5Δ
yak IAB5 wt / san IAB5Δ
san IAB5 wt / yak IAB5Δ
B
C
D
E
F
Abd-B
G
H
I
J
K
♂ 65-66h APF
A5
A6
L
IAB5 hemizygotes (males A5)
A5 segment intensity (%)
n=41
n=48
**
yak IAB5 wt / san IAB5Δ
san IAB5 wt / yak IAB5Δ
M
IAB5
iab-5
iab-6
5 kbp
D. yakuba IAB5
D. santomea IAB5
GFP
N
D. yakuba IAB5 GFP
O
D. santomea IAB5 GFP
P
♂ 94h APF D. yakuba IAB5 GFP
Q
♂ 94h APF D. santomea IAB5 GFP
R
IAB5
iab-5
iab-6
5 kbp
D. yakuba iab-5
D. santomea iab-5
iab-5 ysy
iab-5 sys
GFP
S
D. yakuba iab-5 GFP
T
D. santomea iab-5 GFP
U
♂ 95h APF D. yakuba iab-5 GFP
V
♂ 95h APF D. santomea iab-5 GFP
W
♂ 95h APF iab-5 ysy GFP
X
♂ 95h APF iab-5 sys GFP

Figure S2. **Analysis of the abdominal pigment patterning function of the *iab-5* region. Related to Figure 2.** (A) Schematic of the *Abd-B* locus displaying sequence confirmation of IABΔ lines. (B-D) Phenotypic comparison among wild type *D. yakuba* (B), IAB5Δ heterozygote *D. yakuba* (C) and IAB5Δ homozygote *D. yakuba* male flies (D). Phenotypically, replacement of IAB5 with an RFP cassette generated consistent defects in the A5 segment of heterozygotes of *D. yakuba* (C). In *D. yakuba* homozygotes, pigmentation was nearly absent in the A5 segment, and sometimes defective in the A6 segment (D). White arrows in (C) and (D) indicate segments with reduced pigmentation. (E, F) Reciprocal hemizygotes containing a wildtype copy of IAB5 from *D. yakuba* (E) or *D. santomea* (F). Red arrow in (E) indicates the gain of pigmentation in the A5 segment compared to (F). (G-K) Antibody staining of Abd-B in each of the genotypes listed in (B-F) at mid-late pupal stages (65-66 hAPF). Middle and bottom panels show the magnified regions outlined in the top panels by red and blue dashed lines in A5 and A6, respectively. In IAB5Δ heterozygotes (H), we see weak or sporadic expression of Abd-B in the A5 segments of pupae. In IAB5Δ homozygotes (I), A5 expression is absent, and some defects in Abd-B expression manifest in A6 segments. Red arrow in (J, middle panel) indicates sporadic expression of Abd-B in the A5 segment of a hemizygote bearing a wildtype copy of the *D. yakuba* IAB5 element. (L) Quantification of the pigment intensity of the A5 segment of IAB5Δ reciprocal hemizygote male progeny (Student's t-test, **: $p<0.01$). This plot contains measurements of the same samples from Figure 2C (plot of A5 pigment area) quantified by an alternate method. (M) Schematic of the 15 kb *iab-5* region, showing the 1kb IAB5 initiator element, which was cloned from *D. yakuba* and *D. santomea* into GFP reporter constructs. (N-Q) IAB5 GFP reporter constructs from *D. yakuba* and *D. santomea*, showing activity in stage 9 embryos (N, O) and late stage (94 hAPF) pupal abdomens (P, Q). (P, Q) Reporter constructs containing only the IAB5 initiator elements from each species showed only low levels of expression in pupal stages. (R) Schematic of the entire 15kb *iab-5* regulatory region GFP reporter constructs from *D. yakuba* and *D. santomea* and the chimeric constructs of the *D. yakuba iab-5* region, with the initiator element IAB5 replaced by *D. santomea* (*iab-5 ysy*), and its reciprocal construct (*iab-5 sys*). (S, T) The activities of *D. yakuba* and *D. santomea* whole *iab-5* regulatory regions in stage 9 embryos. (U-X) *in situ* hybridization of GFP mRNA in *iab-5* GFP reporter transgenic late pupal abdomens (95 hAPF).

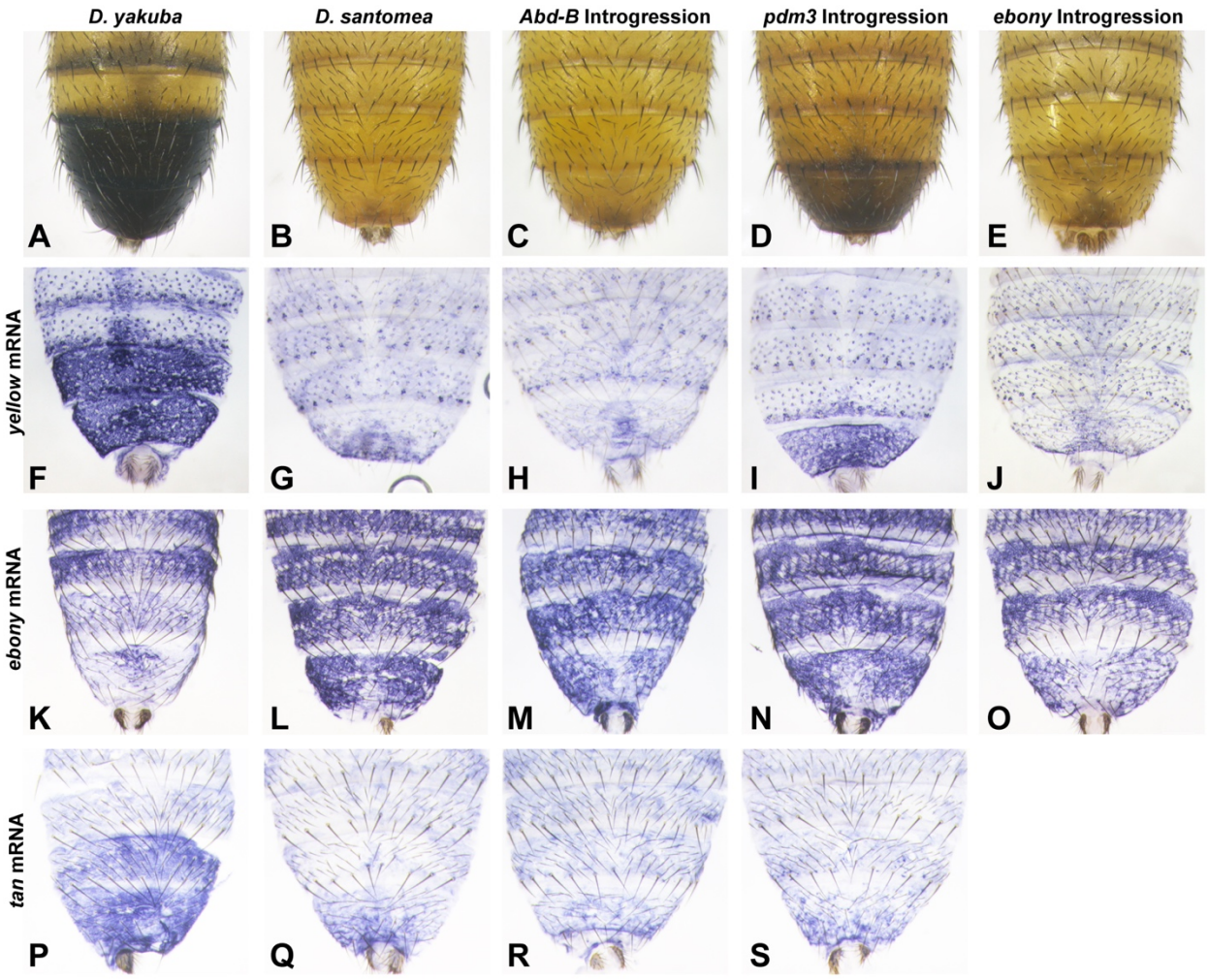

Figure S3. Effects of introgression lines on *yellow*, *ebony* and *tan* expression. Related to Figure 3. (A-E) Phenotypes of *D. yakuba* (A), *D. santomea* (B) the *Abd-B* introgression (C), *pdm3* introgression (D), and *ebony* introgression (E). (F-J) Expression patterns of *yellow* in mid-late pupae. (K-O) Expression patterns of *ebony* at eclosion. (P-S) Expression of *tan* in late stage pupae. The background for each abdomen is labeled at the top of the figure.

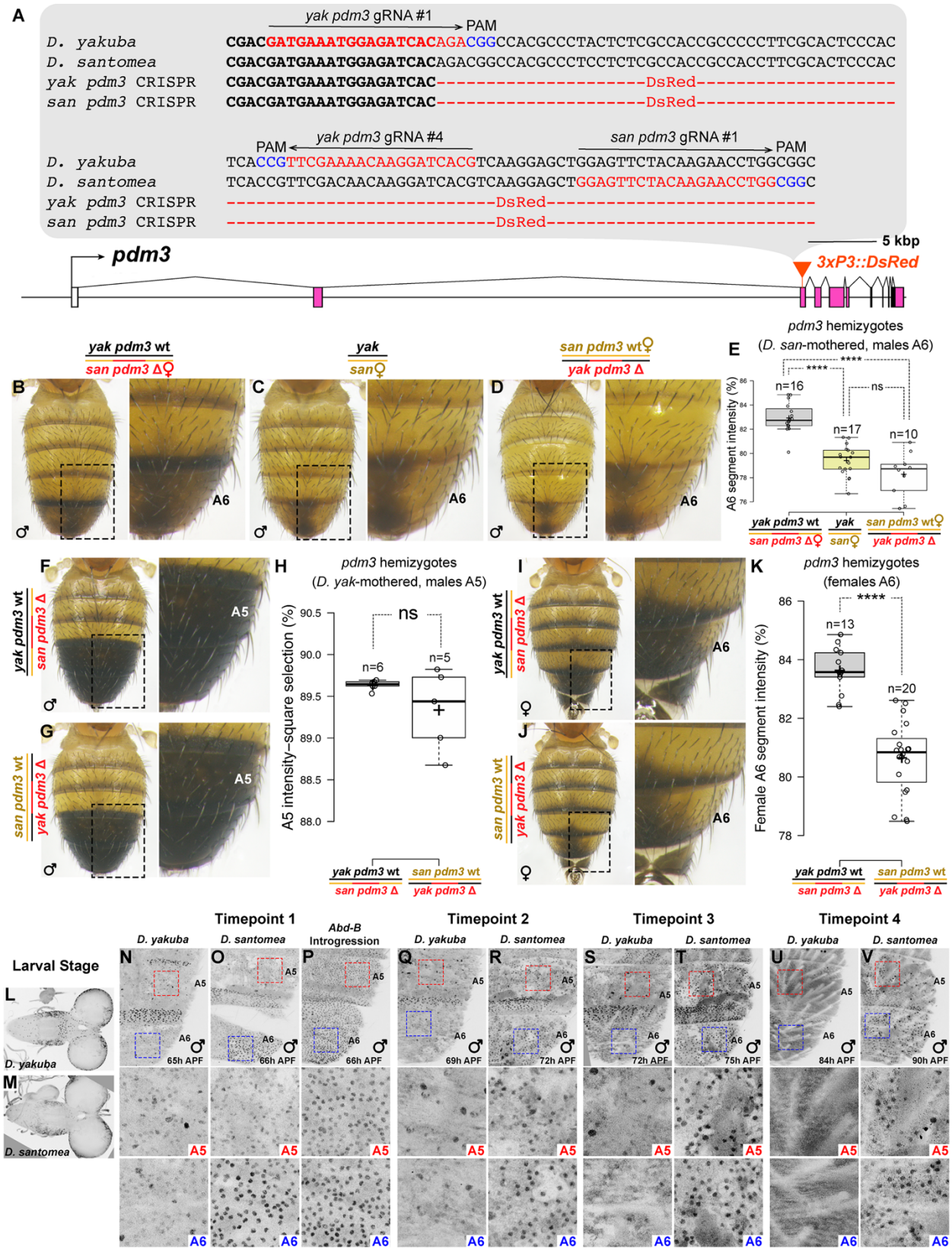
A
yak pdm3 gRNA #1
PAM
D. yakuba CGACGATGAAATGGAGATCACAGACGGCCACGCCCTACTCTCGCCACCGCCCCCTTCGCACTCCCAC
D. santomea CGACGATGAAATGGAGATCACAGACGGCCACGCCCTCCTCTCGCCACCGCCACCTTCGCACTCCCAC
yak pdm3 CRISPR CGACGATGAAATGGAGATCAC----------DsRed----------
san pdm3 CRISPR CGACGATGAAATGGAGATCAC----------DsRed----------
PAM
yak pdm3 gRNA #4
san pdm3 gRNA #1
PAM
D. yakuba TCACCGTTCGAAAACAAGGATCACGTCAAGGAGCTGGAGTTCTACAAGAACCTGGCGGC
D. santomea TCACCGTTCGACAACAAGGATCACGTCAAGGAGCTGGAGTTCTACAAGAACCTGGCGGC
yak pdm3 CRISPR ----------DsRed----------
san pdm3 CRISPR ----------DsRed----------
5 kbp
pdm3
3xP3::DsRed
yak pdm3 wt
san pdm3 Δ♀
B
A6
♂
yak
san♀
C
A6
♂
san pdm3 wt♀
yak pdm3 Δ
D
A6
♂
pdm3 hemizygotes
(D. san-mothered, males A6)
E
A6 segment intensity (%)
n=16
n=17
n=10
****
****
ns
yak pdm3 wt
san pdm3 Δ♀
yak
san♀
san pdm3 wt♀
yak pdm3 Δ
F
yak pdm3 wt
san pdm3 Δ
A5
♂
G
san pdm3 wt
yak pdm3 Δ
A5
♂
H
pdm3 hemizygotes
(D. yak-mothered, males A5)
A5 intensity−square selection (%)
90.5
90.0
89.5
89.0
88.5
88.0
n=6
n=5
ns
yak pdm3 wt
san pdm3 Δ
san pdm3 wt
yak pdm3 Δ
I
yak pdm3 wt
san pdm3 Δ
A6
♀
J
san pdm3 wt
yak pdm3 Δ
A6
♀
K
pdm3 hemizygotes
(females A6)
Female A6 segment intensity (%)
86
84
82
80
78
n=13
n=20
****
yak pdm3 wt
san pdm3 Δ
san pdm3 wt
yak pdm3 Δ
Larval Stage
L
D. yakuba
M
D. santomea
Timepoint 1
Timepoint 2
Timepoint 3
Timepoint 4
D. yakuba
D. santomea
Abd-B
Introgression
D. yakuba
D. santomea
D. yakuba
D. santomea
D. yakuba
D. santomea
N
O
P
Q
R
S
T
U
V
A5
A6
♂
65h APF
66h APF
66h APF
69h APF
72h APF
72h APF
75h APF
84h APF
90h APF

Figure S4. Enhanced suppression of pigmentation by *pdm3* in *D. santomea*. Related to Figure 4. (A) Schematic of the *pdm3* locus. The orange triangle indicates the position of a *3xP3*::*DsRed* cassette that replaced a portion of the second coding exon of *pdm3* to generate a null mutation. Sequence confirmation of the insertion is presented in the alignment. (B-E) Phenotypes of male hybrids from the *D. santomea*-mothered *pdm3* reciprocal hemizygosity test crosses. (B) *D. santomea*-mothered hybrid bearing a wildtype *D. yakuba pdm3* allele. (C) Wild-type *D. santomea*-mothered hybrid. (D) *D. santomea*-mothered hybrid bearing a wildtype *D. santomea pdm3* allele. (E) Quantification of A6 pigmentation in genotypes shown in (B-D). The plot in (E) is identical to Figure 4A, but additionally shows quantification of the *D. santomea*-mothered hybrid phenotype. (F-H) Phenotypes of male hybrids from the *D. yakuba*-mothered *pdm3* reciprocal hemizygosity test crosses. (F) *D. yakuba*-mothered hybrid male bearing a wildtype *D. yakuba pdm3* allele. (G) *D. yakuba*-mothered hybrid male bearing a wildtype *D. santomea pdm3* allele. (H) Quantification of A5 pigmentation phenotype. (I-K) Phenotypes of female hybrids from the *pdm3* reciprocal hemizygosity test crosses. (K) Quantification of A6 segment pigment intensity of the female hybrids. *p* values were calculated by Student's t-test (****: $p<0.0001$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$; ns = not significant). In each panel showing abdominal phenotypes, the right image displays the magnified area outlined in the left image by black dashed lines. (L-V) Time course of Pdm3 expression, showing the third instar larval brain (L, M), and different time points of pupal development of the *D. yakuba* (N, Q, S, U), *D. santomea* (O, R, T, V) and *Abd-B* introgression (P) abdomen. In (N-V), the middle and bottom images show magnified regions of A5 and A6 body segments outlined in the top images in red and blue, respectively. Timepoint 1:65-66 hAPF, Timepoint 2: 69-72 hAPF, Timepoint 3: 72-75 hAPF, Timepoint 4: 84-90 hAPF.

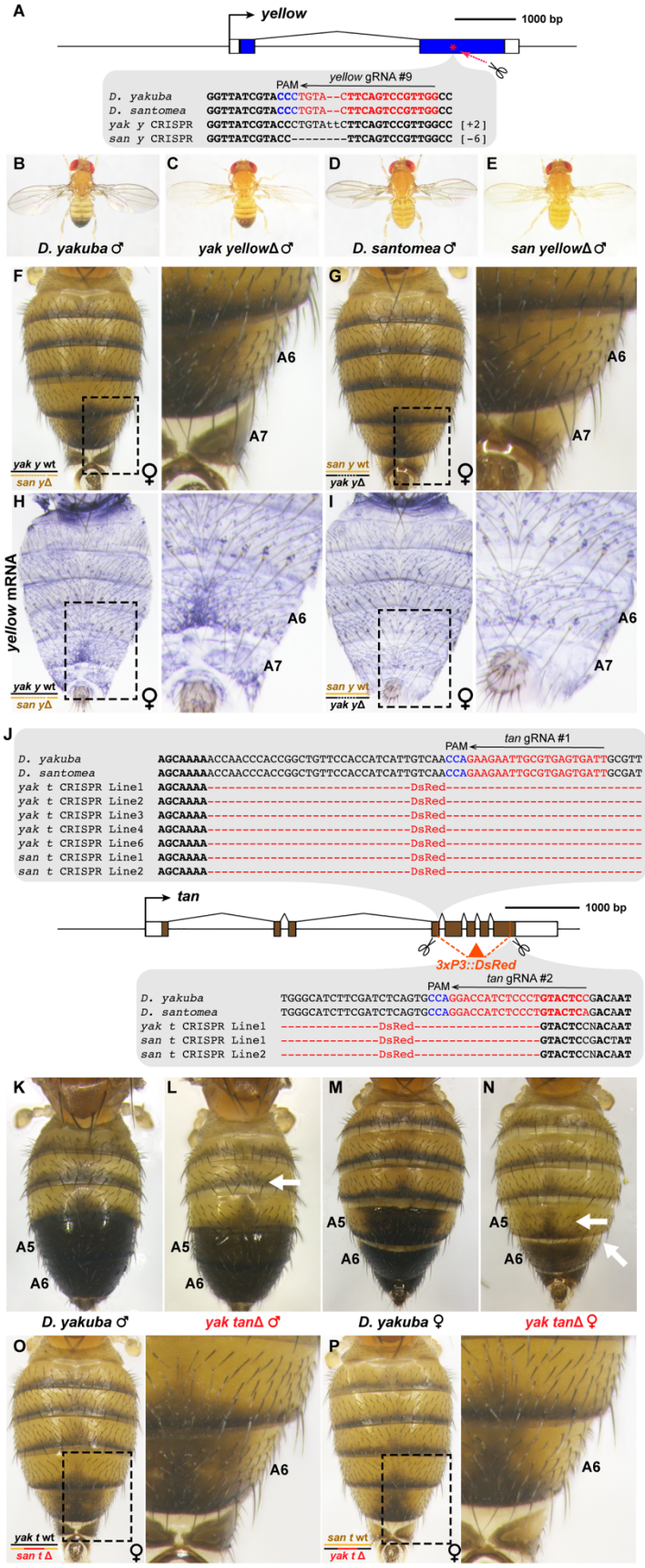
A
yellow
1000 bp
yellow gRNA #9
PAM
D. yakuba GGTTATCGTACCCTGTA--CTTCAGTCCGTTGGCC
D. santomea GGTTATCGTACCCTGTA--CTTCAGTCCGTTGGCC
yak y CRISPR GGTTATCGTACCCTGTAttCTTCAGTCCGTTGGCC [+2]
san y CRISPR GGTTATCGTACC--------TTCAGTCCGTTGGCC [-6]
B
D. yakuba ♂
C
yak yellowΔ ♂
D
D. santomea ♂
E
san yellowΔ ♂
F
yak y wt
san yΔ
A6
A7
G
san y wt
yak yΔ
A6
A7
H
yellow mRNA
yak y wt
san yΔ
A6
A7
I
san y wt
yak yΔ
A6
A7
J
tan gRNA #1
PAM
D. yakuba AGCAAAAACCAACCCACCGGCTGTTCCACCATCATTGTCAACCAGAAGAATTGCGTGAGTGATTGCGTT
D. santomea AGCAAAAACCAACCCACCGGCTGTTCCACCATCATTGTCAACCAGAAGAATTGCGTGAGTGATTGCGAT
yak t CRISPR Line1 AGCAAAA----------DsRed----------
yak t CRISPR Line2 AGCAAAA----------DsRed----------
yak t CRISPR Line3 AGCAAAA----------DsRed----------
yak t CRISPR Line4 AGCAAAA----------DsRed----------
yak t CRISPR Line6 AGCAAAA----------DsRed----------
san t CRISPR Line1 AGCAAAA----------DsRed----------
san t CRISPR Line2 AGCAAAA----------DsRed----------
tan
1000 bp
3xP3::DsRed
tan gRNA #2
PAM
D. yakuba TGGGCATCTTCGATCTCAGTGCCAGGACCATCTCCCTGTACTCCGACAAT
D. santomea TGGGCATCTTCGATCTCAGTGCCAGGACCATCTCCCTGTACTCAGACAAT
yak t CRISPR Line1 ----------DsRed----------GTACTCCNACAAT
san t CRISPR Line1 ----------DsRed----------GTACTCCGACTAT
san t CRISPR Line2 ----------DsRed----------GTACTCCNACAAT
K
A5
A6
D. yakuba ♂
L
A5
A6
yak tanΔ ♂
M
A5
A6
D. yakuba ♀
N
A5
A6
yak tanΔ ♀
O
yak t wt
san t Δ
A6
P
san t wt
yak t Δ
A6

Figure S5. **Reciprocal hemizygosity tests for *yellow* and *tan* uncover phenotypic contributions to *D. santomea* pigmentation. Related to Figure 5.** (A) Schematic of the *yellow* locus, showing the location of a guide RNA used to generate the CRISPR/Cas9 mutants (red star). Sequence alignment displays confirmation of non-homologous end joining events. (B-E) Phenotypes of *yellow* mutations induced in *D. yakuba* (C) and *D. santomea* (E). (F-G) Phenotypes of the female hybrids from the *yellow* reciprocal hemizygosity test crosses. (H-I) *In situ* hybridization of female samples from reciprocal hemizygosity test crosses, showing the accumulation of *yellow* mRNA at a mid-late pupal stage (74 hAPF). (J) Schematic of the *tan* locus, showing the locations of guide RNAs used to replace exons with a *3xP3::RFP* cassette (orange triangle) to generate a null mutant. Alignments display sequence confirmation of the insertion. (K-N) Phenotypes of *D. yakuba tan* mutant males (L) and females (N), compared to wildtype *D. yakuba* (K, M). (O-P) Reciprocal hemizygote phenotypes of *tan* in females. (O) Female hemizygote bearing a wildtype *D. yakuba tan* allele. (P) Female hemizygote bearing a wildtype *D. santomea tan* allele. Hemizygote females with a functional *D. yakuba tan* allele (O) showed stronger pigmentation than hemizygote females with a functional *D. santomea* allele (P). In all multi-image panels, the images on the right side of each panel show magnified views of the regions outlined in the images to the left.

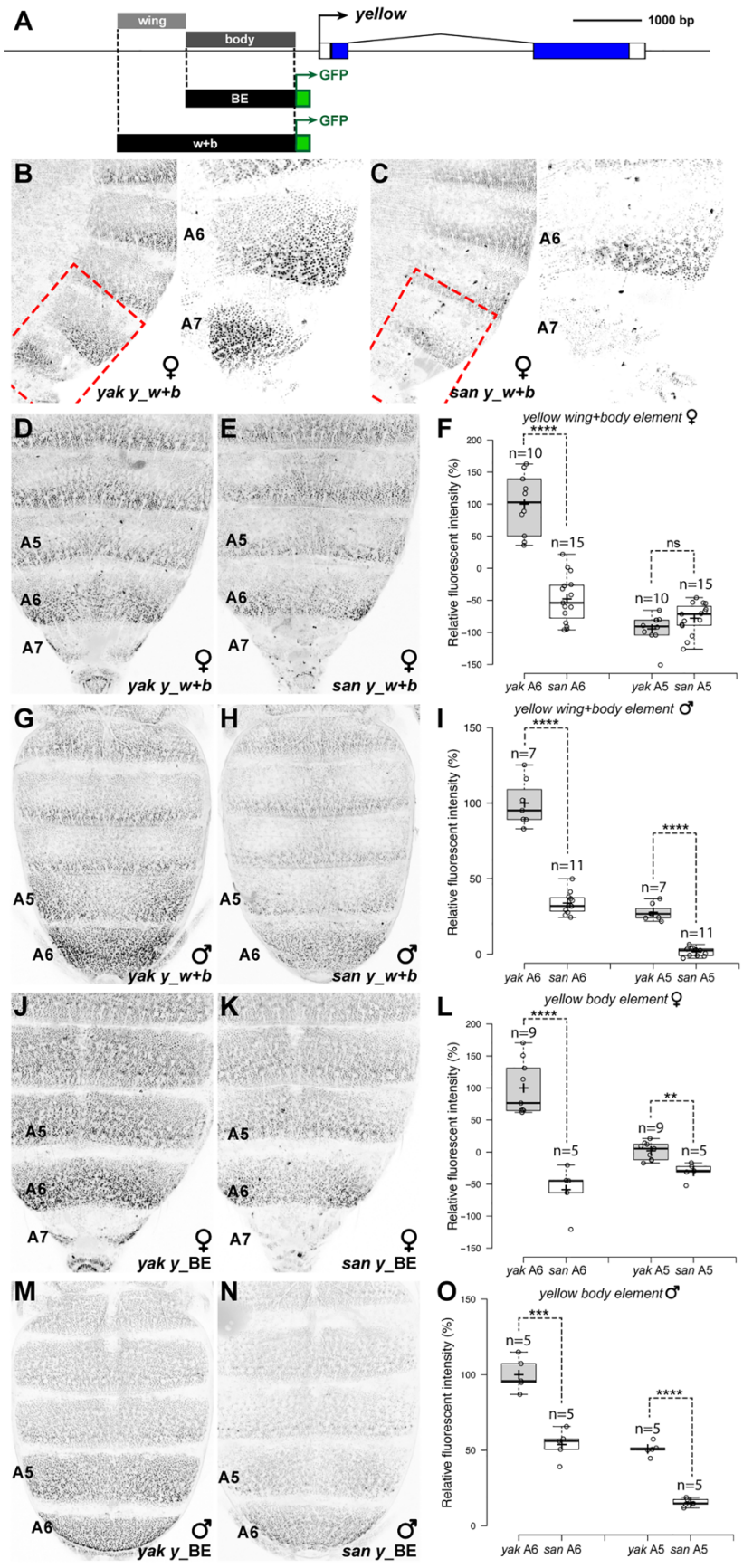
A
wing
body
yellow
1000 bp
GFP
BE
GFP
w+b
B
A6
A7
yak y_w+b
C
A6
A7
san y_w+b
D
A5
A6
A7
yak y_w+b
E
A5
A6
A7
san y_w+b
F
yellow wing+body element ♀
Relative fluorescent intensity (%)
****
n=10
n=15
ns
n=10
n=15
yak A6 san A6
yak A5 san A5
G
A5
A6
yak y_w+b
H
A5
A6
san y_w+b
I
yellow wing+body element ♂
Relative fluorescent intensity (%)
****
n=7
n=11
****
n=7
n=11
yak A6 san A6
yak A5 san A5
J
A5
A6
A7
yak y_BE
K
A5
A6
A7
san y_BE
L
yellow body element ♀
Relative fluorescent intensity (%)
****
n=9
n=5
**
n=9
n=5
yak A6 san A6
yak A5 san A5
M
A5
A6
yak y_BE
N
A5
A6
san y_BE
O
yellow body element ♂
Relative fluorescent intensity (%)
***
n=5
n=5
****
n=5
n=5
yak A6 san A6
yak A5 san A5

Figure S6. Identification of cis-regulatory mutations in the *yellow* regulatory region. Related to Figure 5. (A) Schematic of the *yellow* locus, showing the positions of the wing and body enhancer elements upstream of the transcription start site and GFP reporter constructs containing either the body element ("BE") or the wing+body element ("w+b"). (B-F) Comparison of *D. yakuba* (B, D) and *D. santomea* (C, E) *yellow* wing+body reporter activities in females at a late stage of pupal development (91 hAPF). Expression from the *D. santomea* w+b reporter was reduced in A6 and the small triangular A7 body segment. In (B, C), the right images show the magnified views of the regions outlined in images to the left. Both lateral (B, C), and dorsal (D, E) views are shown, and quantifications of A5 and A6 segment intensity are shown in (F). (G-I) Comparison of the *D. yakuba* (G) and *D. santomea* (H) *yellow* wing+body reporter activities in males at a late stage of pupal development. Boxplot in (I) shows quantification of A5 and A6 segment intensities. (J-O) Comparison of the *D. yakuba* (J, M) and *D. santomea* (K, N) *yellow* body element reporter in females (J, K) and males (M, N) at a late stage of pupal development is quantified in (L, O). This element drove similar differences in expression compared to the w+b element. *p* values were calculated by Student's t-test (****: $p<0.0001$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$; ns = not significant).

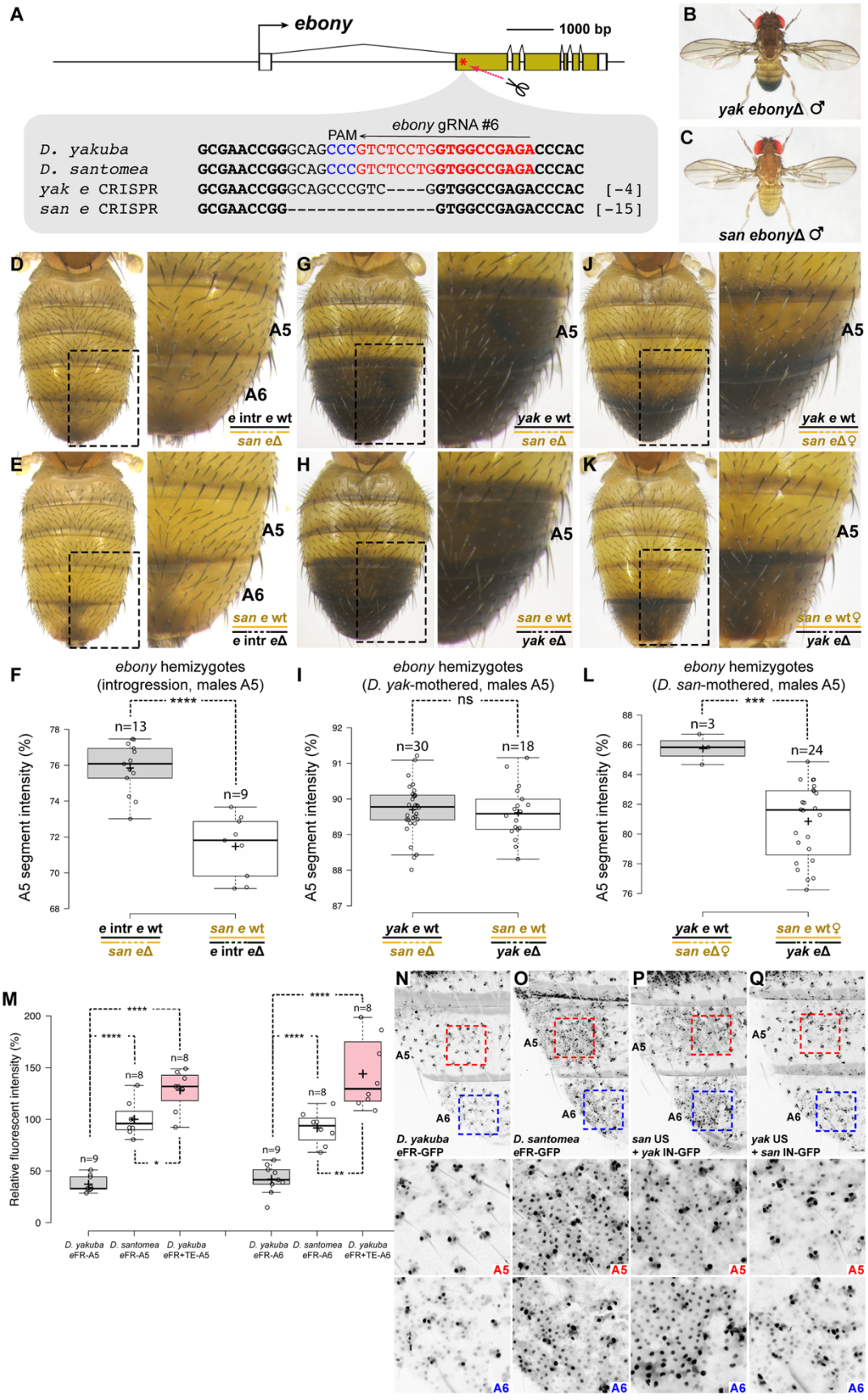
A
ebony
1000 bp
ebony gRNA #6
PAM
D. yakuba GCGAACCGGGCAGCCCGTCTCCTGGTGGCCGAGACCCAC
D. santomea GCGAACCGGGCAGCCCGTCTCCTGGTGGCCGAGACCCAC
yak e CRISPR GCGAACCGGGCAGCCCGTC----GGTGGCCGAGACCCAC [-4]
san e CRISPR GCGAACCGG----------GTGGCCGAGACCCAC [-15]
B
yak ebonyΔ ♂
C
san ebonyΔ ♂
D
A5
A6
e intr e wt
san eΔ
G
yak e wt
J
yak e wt
san eΔ♀
E
san e wt
e intr eΔ
H
san e wt
yak eΔ
K
san e wt♀
yak eΔ
F
ebony hemizygotes
(introgression, males A5)
****
n=13
n=9
A5 segment intensity (%)
e intr e wt
san eΔ
san e wt
e intr eΔ
I
ebony hemizygotes
(D. yak-mothered, males A5)
ns
n=30
n=18
yak e wt
san eΔ
san e wt
yak eΔ
L
ebony hemizygotes
(D. san-mothered, males A5)
***
n=3
n=24
yak e wt
san eΔ♀
san e wt♀
yak eΔ
M
Relative fluorescent intensity (%)
D. yakuba eFR-A5
D. santomea eFR-A5
D. yakuba eFR+TE-A5
D. yakuba eFR-A6
D. santomea eFR-A6
D. yakuba eFR+TE-A6
N
D. yakuba eFR-GFP
O
D. santomea eFR-GFP
P
san US + yak IN-GFP
Q
yak US + san IN-GFP

Figure S7. Evolution at *ebony* contributed to the pigmentation pattern of *D. santomea*. Related to Figure 6. (A) Schematic of the *ebony* locus, indicating the location of a sgRNA for CRISPR/Cas9 mutagenesis by non-homologous end joining. Alignment shows sequence confirmation of edits. (B, C) Phenotypes of CRISPR/Cas9 induced *ebony* mutations in *D. yakuba* (B) and *D. santomea* (C). (D-F) Phenotypes of reciprocal hemizygosity test for *ebony* carried out in male hybrids of *D. santomea* and the *ebony* introgression line (which contains a *D. yakuba ebony* gene in a *D. santomea* background). (D) Male hemizygote bearing a wildtype copy of *ebony* from *D. yakuba* carried on the introgressed genome fragment. (E) Male hemizygote bearing a wildtype copy of the *D. santomea ebony* gene. (F) Quantification of A5 segment intensity of *ebony* introgression x *D. santomea* hemizygotes. Note that these are the same samples for which A6 segment intensity is quantified in Figure 4D. (G-I) Reciprocal hemizygosity test for *ebony* in *D. yakuba* mothered hybrids. (G) *D. yakuba* mothered hybrid bearing a wildtype copy of *ebony* from *D. yakuba.* (H) *D. yakuba* mothered hybrid bearing a wildtype copy of *ebony* from *D. santomea*. (I) Quantification of A5 segment intensity in hybrids reveals that male reciprocal hemizygotes from *D. yakuba* mothers with alternative *ebony* alleles produced similarly pigmented A5 and A6 segments. (J-L) Phenotypes of male hybrids from the *D. santomea*-mothered *ebony* reciprocal hemizygosity test crosses. (J) *D. santomea* mothered hybrid bearing an intact *D. yakuba ebony* allele. (K) *D. santomea*-mothered hybrid bearing an intact *D. santomea ebony* allele. (L) Box plot of A5 segment intensities of *D. santomea*-mothered progeny revealed that in this background hemizygotes with a *D. yakuba* functional *ebony* allele displayed darker A5 body segments than hemizygotes with a functional *D. santomea ebony* allele. The combined effects of the *D. santomea ebony* and X chromosome produce male A5 segments that are two-fold lighter than would be expected if *ebony* and alleles on the X chromosome were to act strictly additively (4.8 units, P<3e-5 by ANOVA). In each panel showing abdomen phenotypes, the right image displays the magnified region outlined in the left image. (M-Q) Analysis of *ebony* full regulatory GFP reporters. (M) Quantification of A5 and A6 segment fluorescent intensities of *D. yakuba e*FR-GFP, *D. santomea e*FR-GFP, and the *D. yakuba e*FR-GFP with the *D. santomea* helitron element inserted (*D. yakuba e*FR-GFP+TE). (N-Q) *ebony* reporters localize changes to its 5' regulatory region. (N) Activity of *D. yakuba e*FR-GFP reporter. (O) Activity of *D. santomea e*FR-GFP reporter. (P) Chimeric reporter containing the upstream region from *D. santomea* fused to the intron of *D. yakuba.* (Q) Chimeric reporter containing the upstream region of *D. yakuba* fused to the intron of *D. santomea*. The chimeric construct bearing the 5' region of *D. santomea* drove heightened expression in the A5 and A6 segments (P). The chimera containing the *D. yakuba* 5' regulatory region drove background epithelial expression (Q), showing only upregulation in the bristles, which is driven by a separate enhancer element of the *ebony* 5' regulatory region. From (N) to (Q), the middle and bottom images show the magnified regions outlined in the top images by red (A5) and blue (A6) dashed lines. All *p* values were calculated by Student's t-test (****: $p<0.0001$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$; ns = not significant).

Protein	*D. san* nsam	*D. yak* nsam	*D. san* fixed	*D. yak* fixed	*D. san* poly	*D. yak* poly	Shared poly
Yellow	32	57	0	0	1	3	1
Tan	32	58	0	0	2	6	0
Ebony	32	59	1	2	3	8	0
Abd-B-PB	32	61	0	0	6	4	0
Abd-B-PF	32	61	0	0	2	2	0
Pdm3	32	62	0	0	13	31	2

Table S1. Summary of amino-acid altering differences between *D. yakuba* and *D. santomea* populations. **Related to STAR Methods.** *D. san=D. santomea*; *D. yak=D. yakuba*; nsam=sample size; fixed=fixed difference between species samples; poly=polymorphic within species; Shared=polymorphisms shared by both species.

Tested Gene	Forward Primer	Reverse Primer	Notes
yak/san yellow	TTYGCCGTMTCCACGAGGAT	taatacgactcactataggAKGCCGTTGTGCTGGTTGAA	A T7 promoter was appended to the reverse primer.
yak/san ebony	AGCTATCGCCAGATGAACGAG	taatacgactcactataggGTCTTGAAAACGCTCACCGTCTC	A T7 promoter was appended to the reverse primer.
yak/san tan	GACGGAGACCCTGAATCACTAC	taatacgactcactataggGTTTTGCCGCTGCGCAAGAGCTC	A T7 promoter was appended to the reverse primer.
GFP	atttaggtgacactatagaCCACCATGGTGAGCAAGGGCGAGG	taatacgactcactataggTTAGCGTCTTCGTTCACTGCTGCG	A T7 promoter was appended to the reverse primer, while an SP6 promoter was appended to the forward primer.

Table S2. Primers for *in situ* hybridization. Related to STAR Methods. Lowercase letters indicate promoters appended for *in vitro* transcription.

Construct	Forward Primer	Reverse Primer	Restriction Sites
yak/*san* IAB5 core element	ATAGATCTGGTCTAGAGCCCGGG CGAATTCGCCggcgcgccCAATTGCC CAGGTATCTCCA	GGATCCGCTAGCTTCCGCGGTTG CGATCGCTTcctgcaggTTCCACTTC CGAACTTGGTC	*Asc I*/*Sbf I*
upstream region of *yak*/*san e*FR	TTCCGggcgcgccGAGCAACCCTTTT TATAAGCGATG	TTGCCcctgcaggCCTGCTCTTAMAG CCSCTGCAATTAC	*Asc I*/*Sbf I*
intron region of *yak*/*san e*FR	CATCAATGTATCTTAactagtCTGCG AGCGCCGTTTACAAGTACA	CACACTTATTACGTGactagtAGCTG CTGCTCCTCGAAGATGCGG	*Spe I*
yak/*san yellow* wing+body element	TTCCGggcgcgccCTCCTCCATGGTG GTGGAACTA	TTGCCcctgcaggACGACTGGTGGCC ATAATAAGTC	*Asc I*/*Sbf I*
yak/*san yellow* body element	TTCCGggcgcgccGCTTTCCGCCCAA GTTGAAGTG	TTGCCcctgcaggCGGGTAATCAGGT GGCTTATGC	*Asc I*/*Sbf I*

Table S3. Primers for Cloning Transgenic GFP Reporter Constructs. Related to STAR Methods. Lowercase letters indicate restriction sites added to primers for cloning purposes.

Name	Primer	Notes
iab-5-F-*Asc I*	GGGCGAATTCGCCggcgcgccCRTTTTCCGTTTTATTGCGA	For amplifying *yak/san iab-5* fragment 3
iab-5-R-*Sbf I*	TTGCGATCGCTTcctgcaggCGGCCGATGAAAGCAGTCCGCCAG	For amplifying *yak/san iab-5* fragment 1
yak-iab-5-int-F3	CATGCTGCCATATTGCCAGAAC	For amplifying *yak iab-5* fragment 1
yak-iab-5-int-R3	GTTCTGGCAATATGGCAGCATG	For amplifying *yak iab-5* fragment 2
iab-5-whole MidF	GATGAGATTCAAGTGGCTGCTTTC	For amplifying *yak/san iab-5* fragment 2
iab-5-whole MidR	GAAAGCAGCCACTTGAATCTCATC	For amplifying *yak/san iab-5* fragment 3
san-iab-5-intF6	GTCAATTAGCTGGTGCCAGTGTG	For amplifying *san iab-5* fragment 1
san-iab-5-intR6	CACACTGGCACCAGCTAATTGAC	For amplifying *san iab-5* fragment 2
*san*EndSeqR1	CTGACGAAATTCCGACGGGAG	For amplifying *san iab-5* pre-fragment 1
yaksan-iab-5-int-F5	GTCTTCCATGTCTACGCCTGTTTG	For amplifying *san iab-5* pre-fragment 1
YS*eb*US-chim-F	CTAGAGCCCGGGCGAATTCGCCggcgcgccGATAAGGATTAGTWATATATGRRC	External forward primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region (Restriction site *Asc I*)
YS*eb*US-chim-R	TCCGCGGTTGCGATCGCTTcctgcaggCCTGCTCTTACAGCCGCTGCAATTAC	External reverse primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region (Restriction site *Sbf I*)
yaksan-chim-hltr-F1	GCGCTATTAAAGGTGTACTTGCTCG	Internal primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region
yaksan-chim-hltr-R1	CGAGCAAGTACACCTTTAATAGCGC	Internal primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region
yaksan-chim-hltr-F2-3	CAATTGAAATGATAAATCCGCTCATTATTCTTGAACTCAC	Internal primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region
yaksan-chim-hltr-R2-3	GTGAGTTCAAGAATAATGAGCGGATTTATCATTTCAATTG	Internal primer for overlap extension PCR to clone *yak e*FR+TE-GFP construct upstream region

Table S4. Primers for Cloning Transgenic GFP Reporter Constructs by Overlap Extension PCR or Infusion Cloning. Related to STAR Methods.

ebony 5	GAAATTAATACGACTCACTATAGGTTCGCCCGCTCGTTCATCGTTTTAGAGCTAGAAATAGC
ebony 6	GAAATTAATACGACTCACTATAGGTCTCGGCCACCAGGAGACGTTTTAGAGCTAGAAATAGC
IAB5 1	GAAATTAATACGACTCACTATAGGTGCGTTTCCATTTTCCCTGTTTTAGAGCTAGAAATAGC
IAB5 2	GAAATTAATACGACTCACTATAGGATCAATCGGTTTATTGATGTTTTAGAGCTAGAAATAGC
pdm3-san 1	GAAATTAATACGACTCACTATAGGAGTTCTACAAGAACCTGGGTTTTAGAGCTAGAAATAGC
pdm3-san 2	GAAATTAATACGACTCACTATAGCGTTTGCCGCCCATCTGAAGTTTTAGAGCTAGAAATAGC
pdm3-yak 1	GAAATTAATACGACTCACTATAGATGAAATGGAGATCACAGAGTTTTAGAGCTAGAAATAGC
pdm3-yak 4	GAAATTAATACGACTCACTATAGCGTGATCCTTGTTGTCGAAGTTTTAGAGCTAGAAATAGC
yellow 2	GAAATTAATACGACTCACTATAGGTTTTGGACACTGGAACCGGTTTTAGAGCTAGAAATAGC
yellow 9	GAAATTAATACGACTCACTATAGGCCAACGGACTGAAGTACAGTTTTAGAGCTAGAAATAGC
tan 1	GAAATTAATACGACTCACTATAGGAATCACTCACGCAATTCTTCGTTTTAGAGCTAGAAATAGC
tan 2	GAAATTAATACGACTCACTATAGGGGAGTACAGGGAGATGGTCCGTTTTAGAGCTAGAAATAGC
sgRNAR	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Table S5. Primers for generation of gRNAs. Related to STAR Methods. gRNA target sequences are underlined. gRNA-specific primers were combined with sgRNAR for PCR and the cleaned amplicon was used for in vitro transcription.

IAB5 left arm forward	CTACACAAAAACACGCAAGGC
IAB5 left arm reverse	ACCGCCTCTAAACCACGACTC
IAB5 right arm forward	CCTTTTGCATGGCAGAGTTCAAC
IAB5 right arm reverse	GATTCTTTTCATGGGGCGAAACAA
pdm3 left arm forward	GATCCTTCTGCGAACTGGGT
pdm3 left arm reverse	GAACTCCAGCTCCTTGACGT
pdm3 right arm forward	GAAAGCCAAACAGCAGCACA
pdm3 right arm reverse	ACACACACACTCACCTTGGG
tan left arm forward	GAATGTTTAAGTAAAGACGCGATGG
tan left arm reverse	GCAGGGCAAGTACAACGTCAAG
tan right arm forward	GGCTTCGATAAGATCAGAAAGTTC
tan right arm reverse	TGACCCACTACAGAACAGCC

Table S6. Genomic target sequences for primers used to construct homologous recombination plasmids. Related to STAR Methods. Primers were extended 3' with sequences specific for the plasmids used in Gibson assembly.

Supplemental References

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S2. Carbone, M. A., Llopart, A., DeAngelis, M., Coyne, J. A., and Mackay, T. F. C. (2005). Quantitative trait loci affecting the difference in pigmentation between Drosophila yakuba and D. santomea. Genetics *171*, 211–225.