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1 **Correlated Evolution of two Copulatory Organs via a Single Cis-Regulatory Nucleotide**  
2 **Change**

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5  
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17

18 **One Sentence Summary:** We identify one nucleotide substitution in a gene regulatory region  
19 contributing to evolutionary change of two distinct copulatory organs.

20

21 **Highlights:**

- 22 - We identify a gene and 3 substitutions causing genital evolution between species
- 23 - The evolved mutations lie in a pleiotropic enhancer
- 24 - One mutation decreases genital bristle number and increases leg sex comb tooth number
- 25 - This mutation disrupts a binding site for Abd-B in genitals and for another factor in legs

26

27 **SUMMARY**

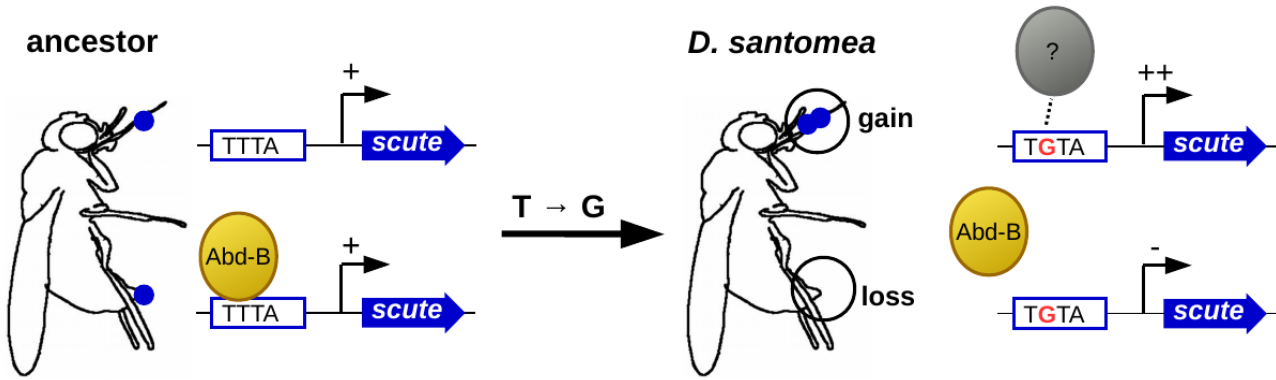
28 **Diverse traits often covary between species [1–3]. The possibility that a single mutation could**  
29 **contribute to the evolution of several characters between species [3] is rarely investigated as**  
30 **relatively few cases are dissected at the nucleotide level. *Drosophila santomea* has evolved**  
31 **additional sex comb sensory teeth on its legs and has lost two sensory bristles on its genitalia.**  
32 **We present evidence that a single nucleotide substitution in an enhancer of the *scute* gene**  
33 **contributes to both changes. The mutation alters a binding site for the Hox protein**  
34 **Abdominal-B in the developing genitalia, leading to bristle loss, and for another factor in the**  
35 **developing leg, leading to bristle gain. Our study suggests that morphological evolution**  
36 **between species can occur through a single nucleotide change affecting several sexually**  
37 **dimorphic traits.**

38 (126 words)

39

40 **Keywords:** pleiotropy, cis-regulatory, sensory organ, *Drosophila*, copulation, genitalia,  
41 hypandrium, sex comb, microevolution, *scute*, *Abd-B*

42 **Graphical Abstract:**



43

44

45 **RESULTS AND DISCUSSION**

46 “Variability is governed by many unknown laws, of which correlated growth is probably the most  
47 important.” [1]

48 Correlated evolution of traits is widespread among taxa [1,2] and can be due to pleiotropy, where a  
49 single locus causally affects several traits [3]. Pleiotropy imposes large constraints on the paths of  
50 evolution [4,5], making it crucial to assess the extent of pleiotropy to understand the evolutionary  
51 process. Empirical studies suggest that many loci influence multiple traits [3,6,7] and current data  
52 cannot reject the idea that all genetic elements have pleiotropic roles [3,8,9]. Several pleiotropic  
53 substitutions have been associated with natural variation [10–13]: most are coding changes and all  
54 underlie intraspecific changes ([www.gephebase.org](http://www.gephebase.org)). Nevertheless it remains unclear whether  
55 pleiotropic mutations contribute also to interspecific evolution, as experimental evidence suggests  
56 that the mutations responsible for interspecies evolution may be less pleiotropic than the mutations  
57 underlying intraspecific variation [14].

58 Here we focused on male sexual bristle evolution between *Drosophila yakuba* and  
59 *Drosophila santomea*, which diverged approximately 0.5-1 million years ago [15] and can produce  
60 fertile F1 females in the laboratory [16], facilitating genetic mapping. We found that hypandrial  
61 bristles – two prominent mechanosensory bristles located on the ventral part of male genitalia in all  
62 *D. melanogaster* subgroup species – are missing in *D. santomea* males (Figure 1). Examination of  
63 many inbred stocks and 10 closely related species revealed that the absence of hypandrial bristles is  
64 a derived *D. santomea*-specific trait (Figure 1, see also Extra Tables). No other genital bristle type  
65 was noticeably variable in number between *D. yakuba* and *D. santomea* (see Extra Figures).

66 We performed whole-genome QTL mapping between *D. santomea* and *D. yakuba* and found  
67 that the left tip of chromosome X explains 44% of the variance in hypandrial bristle number in each  
68 backcross (confidence interval = 7 Mb for the *D. santomea* backcross and 2.6 Mb for the *D. yakuba*  
69 backcross, Figure 2A). Duplication mapping in rare *D. santomea*-*D. melanogaster* hybrid males  
70 narrowed down the causal region to a 84.6 kb region of the *achaete-scute* complex (AS-C) (Figure  
71 2B-C, see also Extra Tables).

72 The AS-C locus contains four genes, but only two, *achaete* (*ac*) and *scute* (*sc*), are required  
73 for bristle formation [17]. Both genes are co-expressed, share *cis*-regulatory elements and act  
74 redundantly to specify bristles [18,19]. The elaborate expression pattern of *ac* and *sc* genes  
75 prefigures the adult bristle pattern and is controlled by numerous *cis*-regulatory elements [18]. We  
76 tested which of the two genes, *ac* or *sc*, contributes to loss of bristles using null mutants in *D.*  
77 *melanogaster*. All *ac*<sup>CAMI</sup> null mutant males had 2 hypandrial bristles (n=15) and *sc*<sup>M6</sup> null mutants  
78 had none (n=15) (Tables S1-2), indicating that *sc* is required for hypandrial bristle development in  
79 *D. melanogaster*.

80 We detected 64 nucleotide differences in the *sc* coding region between *D. yakuba* and *D.*

81 *santomea*, and all were synonymous substitutions, indicating that coding changes in *sc* are not  
82 responsible for the evolved function of *sc*. Using molecularly mapped chromosomal aberrations, we  
83 identified a 5-kb region located > 46 kb downstream of the *sc* promoter that is required in *D.*  
84 *melanogaster* for hypandrial bristle development (Figure S1A, see also Extra Figures, Tables S1-2).  
85 Independently we screened 55 *GAL4* reporter constructs tiling the entire *AS-C* locus and identified  
86 three *GAL4* lines (*15E09*, *054839* and *18C05*) that drive expression in hypandrial bristles (Figures  
87 2C and S1B-E, see also Extra Tables). Only one of these lines, *18C05*, increased hypandrial bristle  
88 number with *UAS-scute* in a *sc* mutant background or in a *sc*<sup>+</sup> background (Figures 2C and S1F-Q,  
89 see also Extra Figures). The 2036-bp *18C05* region is located within the 5-kb candidate region  
90 identified with *ac-sc* structural mutations (Figure 2C), suggesting that *18C05* is a good candidate  
91 region for hypandrial bristle evolution.

92 To test whether loss of hypandrial bristles in *D. santomea* resulted from changes(s) in the  
93 *18C05 cis*-regulatory region, we assayed whether orthologous *18C05* regions from *D.*  
94 *melanogaster*, *D. yakuba* and *D. santomea* driving a *sc* coding region could rescue hypandrial  
95 bristles in a *D. melanogaster sc* mutant. The *D. melanogaster 18C05* enhancer rescued two bristles  
96 in both *sc*<sup>29</sup> and *sc*<sup>M6</sup> mutant backgrounds, indicating that this construct mimics normal levels of *sc*  
97 expression (Figure 3). The *D. yakuba 18C05* enhancer rescued on average 2 hypandrial bristles in  
98 *sc*<sup>M6</sup> and 0.5 bristles in *sc*<sup>29</sup> whereas the *D. santomea 18C05* enhancer rescued significantly fewer  
99 bristles (1.1 in *sc*<sup>M6</sup> and 0 bristles in *sc*<sup>29</sup>, Figure 3). For another measure of *18C05* enhancer activity,  
100 we compared the ability of enhancer-*GAL4* constructs containing the *18C05* region from *D.*  
101 *melanogaster*, *D. yakuba* or *D. santomea* to induce extra bristles in *sc* mutants using the *UAS-GAL4*  
102 system with *UAS-sc*. In this assay the *D. santomea 18C05* region also induced fewer bristles than  
103 the corresponding *D. yakuba* region (Figure 3, GLM-Quasi-Poisson,  $F(19, 509) = 161.7$ ,  $p < 10^{-5}$   
104 for *sc*<sup>29</sup>;  $F(19, 415) = 125.9$ ,  $p < 10^{-5}$  for *sc*<sup>M6</sup>). Together, these results suggest that changes(s) within  
105 *18C05* contributed to hypandrial bristle evolution in *D. santomea*.

106 To narrow down the region responsible for hypandrial bristle loss, we dissected the *18C05*  
107 element from *D. melanogaster*, *D. yakuba* and *D. santomea* into smaller overlapping pieces and  
108 quantified their ability to produce hypandrial bristles with the *GAL4* rescue experiment. For all  
109 three species we found that smaller segments rescued significantly fewer bristles than the  
110 corresponding full region (Figure S2A-B, see also Extra Figures). Thus, transcription factor binding  
111 sites scattered throughout the entire ~2 kb of the *18C05* element are required to drive full  
112 expression in the hypandrial bristle region.

113 Sequence alignment of the *18C05* region from multiple species revealed 11 substitutions and  
114 one indel that are fixed and uniquely derived in *D. santomea*. Among them, seven substitutions  
115 altered sites that are otherwise conserved in the *D. melanogaster* subgroup (Extra Figures). We  
116 tested the effect of these seven *D. santomea*-specific nucleotide changes by introducing them one at  
117 a time or all together, into either a *D. yakuba 18C05* enhancer or into the inferred ancestral enhancer  
118 driving *sc* expression (Figure S2, see also Extra Tables and Extra Figures). The ancestral *18C05*  
119 sequence was resurrected by reverting the *D. santomea*-specific and *D. yakuba*-specific mutations  
120 to their ancestral states and it produced the same number of bristles as the *D. yakuba* construct  
121 (Figure 3, Extra Figures). Four substitutions (*G869A*, *T970A*, *T1008C* and *T1482C*) had no effect,  
122 whether in the *D. yakuba* or in the ancestral background (GLM-Quasi-Poisson,  $p > 0.6$ ). Three  
123 substitutions (*T1429G*, *A1507G* and *T1775G*) decreased the number of rescued bristles in both the  
124 *D. yakuba* and the ancestral sequence, and these effects were highly significant, except for *A1507G*  
125 in the *D. yakuba* background, which was slightly above statistical threshold (using the most  
126 stringent correction method) (Figure 3). These results are consistent with analysis of smaller pieces  
127 of *18C05* and of *18C05* chimeric constructs containing DNA fragments from *D. yakuba* and *D.*  
128 *santomea* (Figure S2C). When combined into the *D. yakuba* background, the seven *D. santomea*-  
129 specific substitutions rescued the same number of bristles as the *D. santomea 18C05* construct  
130 (Figure 3, GLM-Quasi-Poisson,  $p > 0.9$  in *sc*<sup>M6</sup>). We conclude that at least three fixed substitutions

130 within a 350-bp region located 49 kb away from *sc* contribute to the reduction in hypandrial bristle  
131 number in *D. santomea*.

132 Analysis of *18C05-GAL4* and *18C05-GFP* reporter constructs revealed that the *18C05*  
133 region drives expression not only in male genital discs [20] but also in male developing forelegs in  
134 the presumptive sex comb domain [21](Figure 4A-B,D-F). The *18C05-GFP* reporter constructs  
135 drive expression in fewer cells than *sc-GFP* (Fig. 4C), indicating that *sc* expression in the  
136 presumptive sex comb domain is also regulated by cis-regulatory regions outside of *18C05*. Sex  
137 combs are sensory organs used for grasping the female during copulation [22]. They differ in bristle  
138 number between *D. santomea* and *D. yakuba* (Figure 4G-I, see also Extra Figures), and 35% of the  
139 species difference is attributed to the X chromosome [23], where *sc* is located. These results  
140 prompted us to test whether the mutations contributing to hypandrial bristle evolution also affect  
141 sex combs. Significantly more GFP-positive cells were detected in the first tarsal segment at 5h  
142 after puparium formation (APF) with *18C05yakT1775G-GFP* than with *18C05yak-GFP* (GLM-  
143 Poisson, Chi-squared (20,2) deviance = 9.75,  $p = 0.033$ ), suggesting that *T1775G* increases *sc*  
144 expression in the first tarsal segment. Sex comb tooth number was reduced in *sc<sup>M6</sup>* and *sc<sup>6</sup>*  
145 and significantly rescued with several *18C05-sc* constructs (Figure 4J-K). Analysis of *sc<sup>M6</sup>* and *sc<sup>6</sup>*  
146 mutants rescued with the *yak18C05-sc* constructs containing the *D. santomea*-specific substitutions  
147 showed that *T1429G* and *T1507G* have no effect and that *T1775G* increases the number of sex  
148 comb teeth (Figure 4J-K). We conclude that the *T1775G* substitution contributes to both the  
149 increase in sex comb tooth number and the loss of hypandrial bristles.

150 A bioinformatics search revealed that the *T1775G* substitution is predicted to alter a binding  
151 site for the Hox protein Abdominal-B (*Abd-B*) (Table S3). *Abd-B* is expressed only in the posterior  
152 part of the fly, where it directs the development of posterior-specific structures such as the genitalia  
153 [24]. We found that reducing *Abd-B* expression, using either genetic mutations or RNA interference,  
154 resulted in loss of hypandrial bristles (Figure S3 and Table S4), indicating that normal levels of  
155 *Abd-B* expression are required for hypandrial bristle development. Electrophoretic mobility shift  
156 assays showed that *Abd-B* proteins bind more strongly to a 54-bp fragment of the *18C05* sequence  
157 containing the *D. yakuba*-specific T at position 1775 than the *D. santomea*-specific G at this  
158 position (Figure S4). These results are consistent with the hypothesis that the *T1775G* substitution  
159 decreases *ABD-B* binding, contributing to reduction in *sc* expression levels, and ultimately reducing  
160 the number of hypandrial bristles. Since *Abd-B* is not expressed in developing legs, *T1775G* is  
161 expected to affect binding of other factors to increase sex comb tooth number. Overall, our study  
162 suggests that *T1775G* alters overlapping binding sites for distinct factors in the leg and the genitalia.  
163 All our analyses of the effects of individual substitutions have been carried out in *D. melanogaster*  
164 background. It is thus possible that the *18C05* enhancer represents only part of the effect of the *sc*  
165 locus on bristle divergence.

166 Intriguingly, the two organs affected by substitution *T1775G* – hypandrial bristles and sex  
167 combs – may both aid the male to position himself on top of the female during copulation [22,25].  
168 Genitals are the most rapidly evolving organs in animals with internal fertilization [26]. To our  
169 knowledge, only two other mutations contributing to the evolution of genital anatomy are known.  
170 First, a 61-kb-deletion of a cis-regulatory region of the *androgen receptor (AR)* gene in humans is  
171 associated with loss of keratinized penile spines in humans compared to chimpanzees [27]. Second,  
172 an amino acid change in the *nath10 acetyltransferase* gene which probably appeared recently in  
173 laboratory strains of the nematode *C. elegans*, alters morphology in the presence of some mutations  
174 but not in a wild-type genetic background [10]. Both mutations appear to be pleiotropic: the *AR*  
175 deletion is associated with loss of facial vibrissae in humans and the *nath10* mutation affects egg  
176 and sperm production as well. The paucity of known mutations responsible for genital evolution  
177 makes it currently difficult to propose general rules for the causes of rapid genital evolution. Our  
178 results are reminiscent of Mayr's pleiotropy hypothesis [28], which posits that certain characters  
179 may evolve arbitrarily as a result of selection on other traits due to pleiotropic mutations. In our

180 case, whether the evolutionary change in sex comb tooth number or in genital bristle number has  
181 any effect on fitness is unknown.

182 We report here the first experimental evidence for a cis-regulatory substitution between  
183 species with pleiotropic effects. Given the large number of bristle types regulated by *sc* (>100 in  
184 adult flies), it is possible that no cis-regulatory mutation in *sc* can affect only one bristle type. Our  
185 results challenge the idea that cis-regulatory enhancers are strict tissue-specific modules underlying  
186 evolutionary changes in targeted traits [29]. Even though cis-regulatory mutations may affect  
187 several tissues, it is probable that they still tend to be less pleiotropic than coding changes. Our  
188 results are thus compatible with the idea that cis-regulatory changes tend to have fewer pleiotropic  
189 effects than coding changes on average. Enhancer sequences evolve rapidly, with rapid turn over of  
190 individual binding sites while maintaining transcriptional output over millions of years by  
191 compensatory mutations [30]. Since pleiotropic mutations can have deleterious off-target effects,  
192 we propose that evolution of pleiotropic sites within enhancers should trigger the subsequent  
193 selection of compensatory mutations in cis, thus contributing to rapid divergence of cis-regulatory  
194 sequences. Overall, our results suggest that pleiotropic cis-regulatory mutations may play a more  
195 important role in evolution than previously thought.

196

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217

## 218 AUTHOR CONTRIBUTIONS

219 J.R.D. found that *D. santomea* lacks hypandrial bristles and that the trait difference is X-linked,  
220 D.L.S. genotyped flies with MSG, A.Y., I.N. and V.C.O. performed the QTL mapping experiment,  
221 D.R.M. made the *D. santomea-D. melanogaster* hybrids, I.N. dissected them, O.N. did all other fly  
222 crosses and dissected them, O.N., I.N., R.S. and A.E.P. phenotyped >3000 males for hypandrial  
223 bristles, O.N. phenotyped all other bristles, O.N. and M.L. did EMSA, O.N. and I.N. constructed  
224 the plasmids, O.N. performed immunostainings and microscopy, A.E.P. performed all statistical  
225 analyses with feedback from O.N. and M.L., D.R.M. collected wild flies, V.C.O. supervised

226 research, performed bioinformatics sequence analysis and wrote the paper with O.N. All authors  
227 provided feedback on the text.

228

## 229 **DECLARATION OF INTEREST**

230 The authors declare no competing interests.

231

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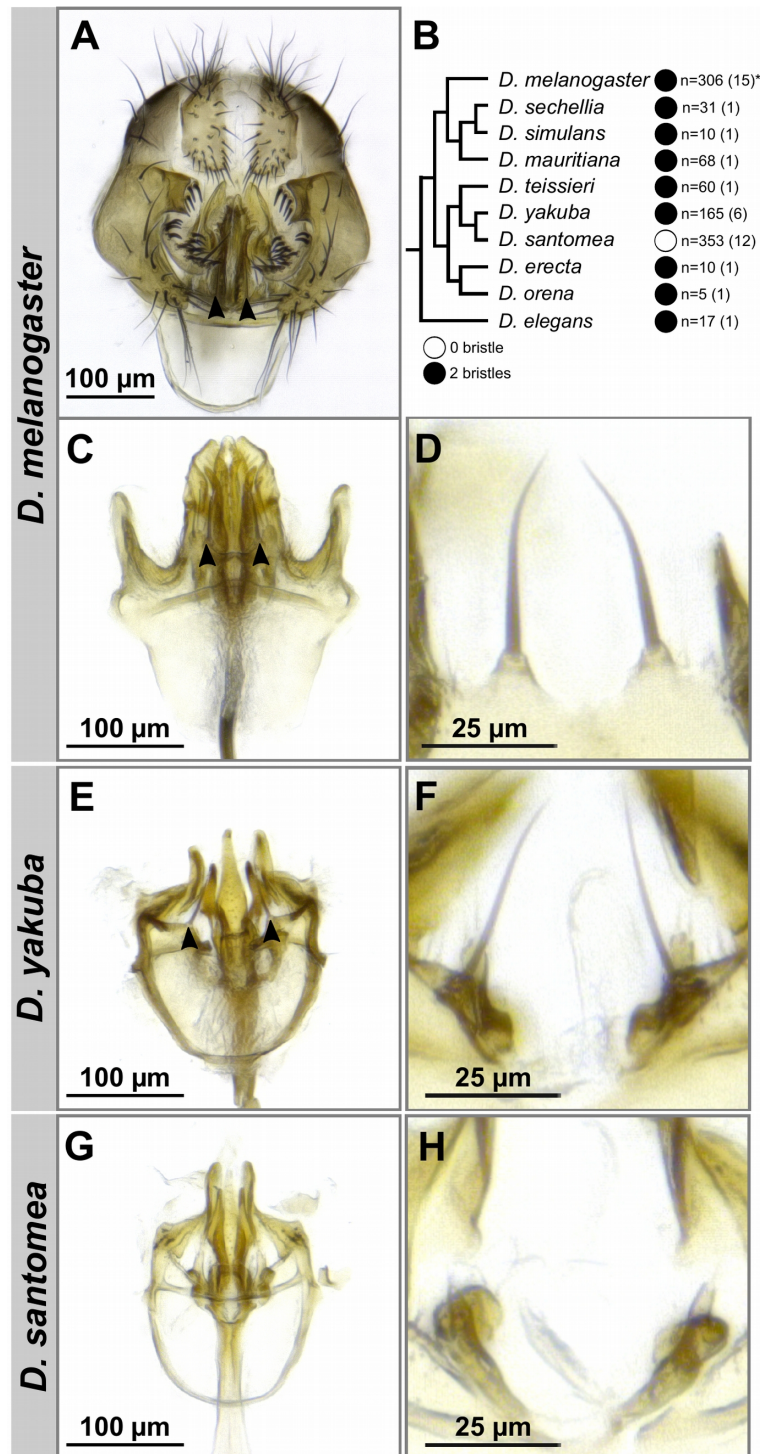
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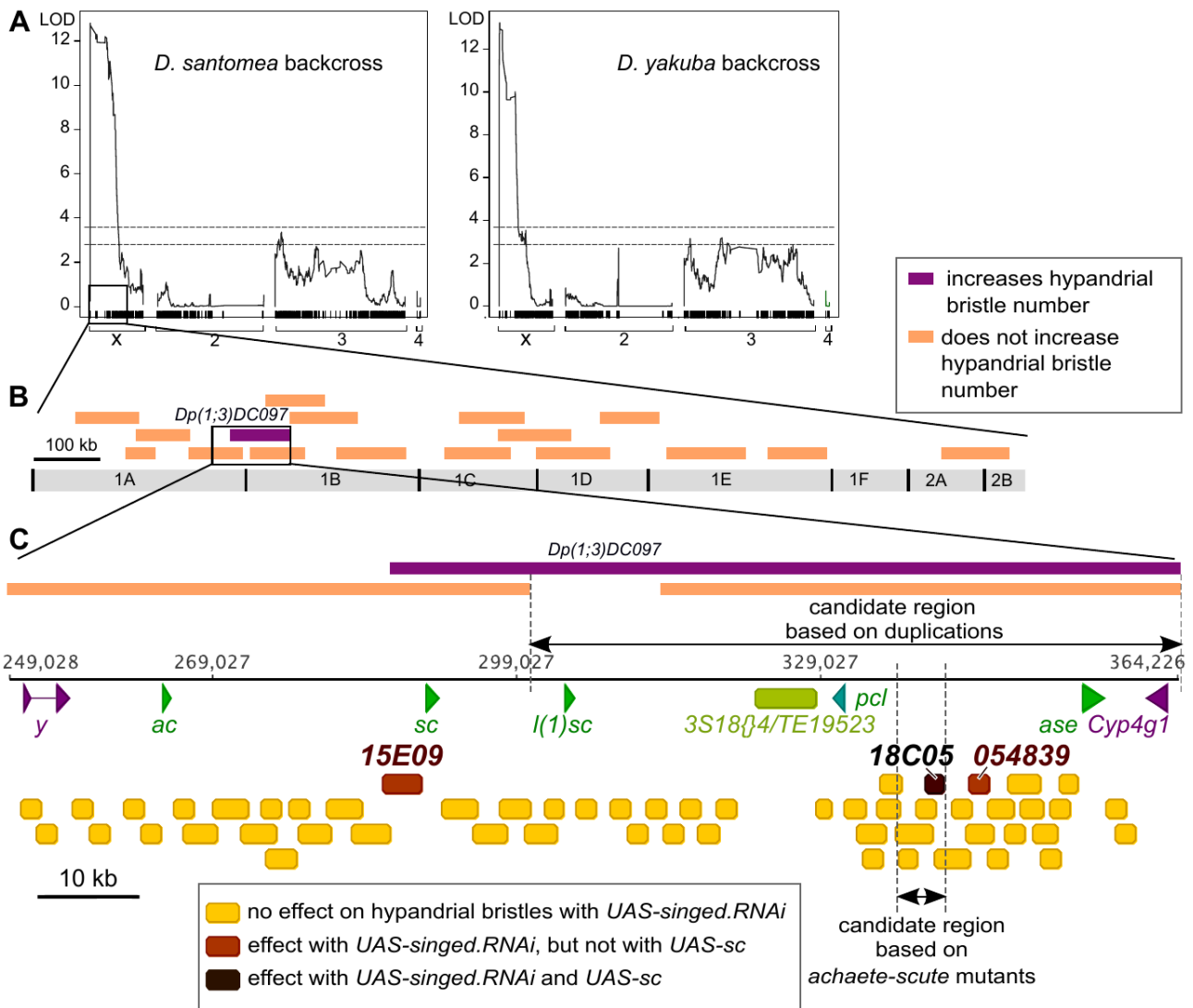
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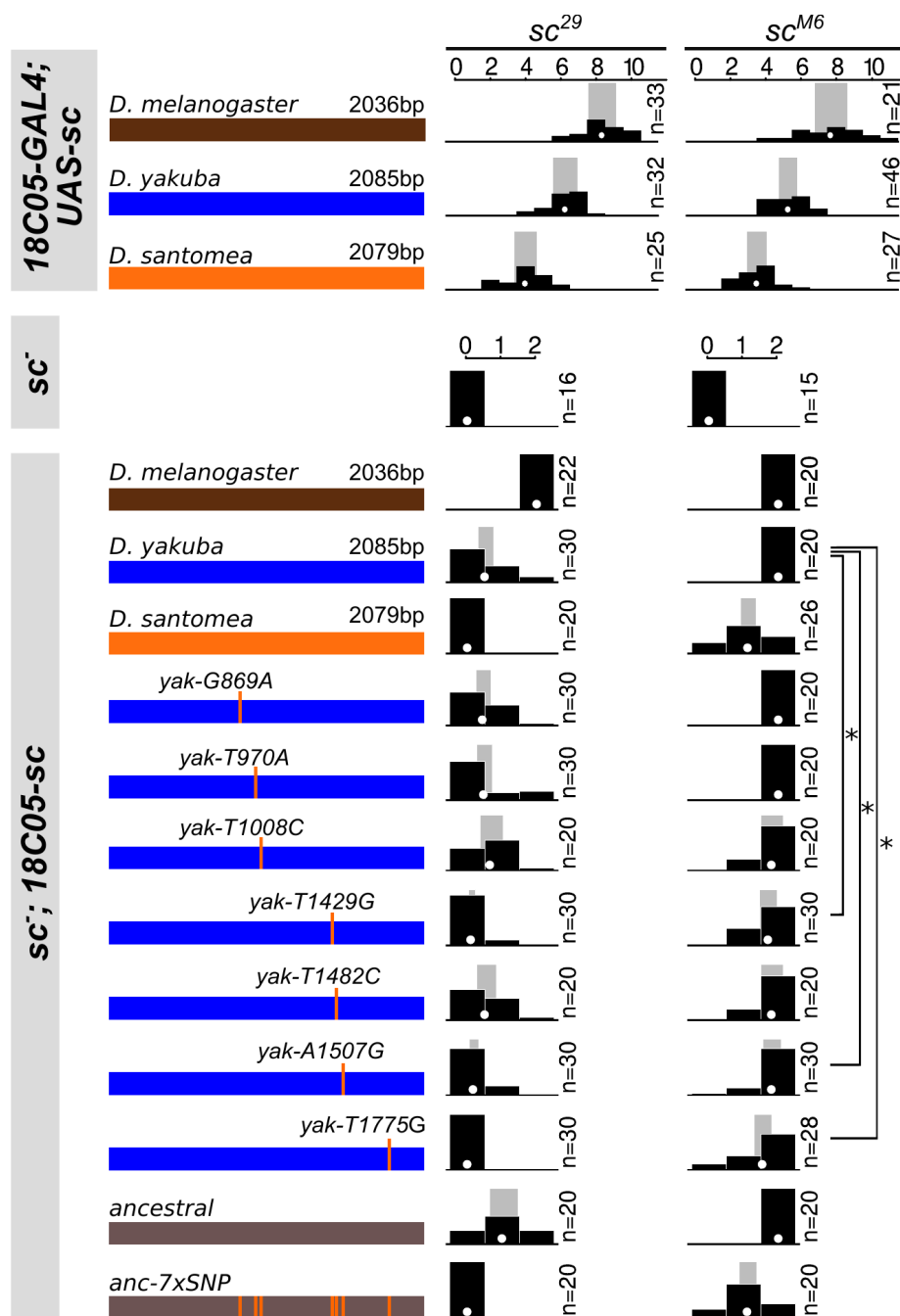


**Figure 1. *D. santomea* lost hypandrial bristles.**

(A) *Drosophila melanogaster* male genitalia. (B) Phylogeny of the *Drosophila melanogaster* species subgroup. All species of have two hypandrial bristles (black circles) except *Drosophila santomea*, which lacks hypandrial bristles (white circle). n: number of scored males, with the number of scored strains in parentheses. Asterisk indicates that 4 males out of 306 had three hypandrial bristles. (C-H) Light microscope preparations of ventral genitalia (C,E,G) and hypandrial bristles (D,F,H) in *D. melanogaster* (C-D), *D. yakuba* (E-F) and *D. santomea* (G-H). Hypandrial bristles are indicated with arrowheads on A, C and E.



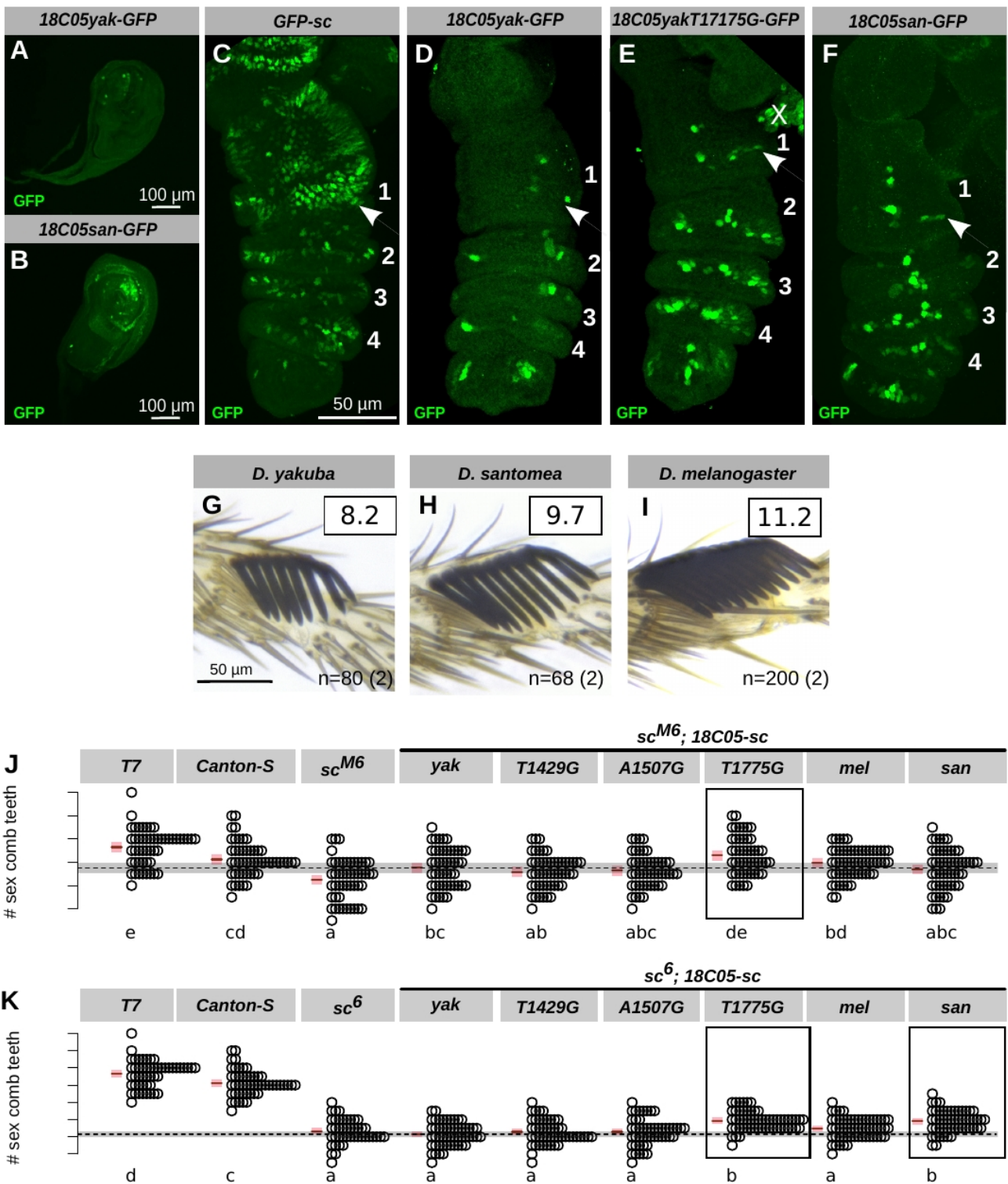
387 **Figure 2. Mapping of the cis-regulatory element involved in hypandrial bristle evolution.**  
 388 (A) QTL analysis of hypandrial bristle number in a *D. santomea* backcross (left) and a *D. yakuba*  
 389 backcross (right). On the y-axis are the LOD profiles from a Haley-Knott regression analysis. The  
 390 x-axis represents physical map position in the *D. yakuba* genome. Ticks represent recombination  
 391 informative markers. Dotted lines represent the 1% (top) and 5% (bottom) significance thresholds.  
 392 (B) Schematic representation of the left tip of chromosome X and of 19 duplicated fragments of  
 393 chromosome X that were tested for their effect on hypandrial bristle number in *D. santomea*-*D.*  
 394 *melanogaster* hybrid males. All duplications had no significant effect (orange) except  
 395 *Dp(1;3)DC097* (purple), which significantly increased hypandrial bristle number. (C) Genomic  
 396 organization of the AS-C locus in *D. melanogaster*. Arrows indicate the coding regions of *yellow*  
 397 (*y*), *achaete* (*a*), *scute* (*sc*), *lethal of scute* (*l(1)sc*), *pepsinogen-like* (*pcl*), *asense* (*ase*) and  
 398 *cytochrome P450-4g1* (*Cyp4g1*) genes. The light green box represents the insertion of a  
 399 *3S18{4}/TF9523* natural transposable element. Boxes indicate cis-regulatory elements whose  
 400 corresponding *GAL4* reporter lines have been tested. Expression of *UAS-singed.RNAi* with 52  
 401 *GAL4* lines (yellow boxes) has no effect while it results in singed hypandrium bristles with *15E09*-,  
 402 *18C05*- and *054839-GAL4*. Extra hypandrial bristles are found with *UAS-sc* and *18C05-GAL4*  
 403 (dark brown box) but not with *15E09*- and *054839-GAL4* (light brown boxes).  
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**Figure 3. Three *D. santomea*-specific substitutions in *18C05* contribute to the loss of hypandrial bristles.**

Rescue of the hypandrial bristle loss of *sc*<sup>29</sup> (left column) and *sc*<sup>M6</sup> (right column) *D. melanogaster* mutants by expression of either *GAL4* with *UAS-sc* or *sc* driven by *18C05* sequences from *D. melanogaster* (brown), *D. yakuba* (blue) and *D. santomea* (orange). Seven *D. santomea*-specific substitutions (vertical orange bars) were introduced into either the *D. yakuba* region (blue) or the ancestrally reconstructed *18C05* region (grey). Distribution of hypandrial bristle number (black histogram), together with mean (white dot) and 95% confidence interval (grey rectangle) from a fitted GLM Quasi-Poisson model are shown for each genotype. Note that for a given rescue construct, *18C05-GAL4 UAS-sc* produces more hypandrial bristles than *18C05-sc*, probably due to the amplification of gene expression caused by the *GAL4/UAS* system. n: number of scored individuals. \*:  $p < 0.05$



420 **Figure 4. *D. santomea*-specific substitution *T1775G* contributes to increase in sex comb tooth**  
 421 **number.**

422 (A-F) GFP staining (green) in T1 leg discs of late L3 larvae (A-B) and in 5h APF pupal legs (C-F)  
 423 in *D. melanogaster* containing 18C05 reporter transgenes or *sc*-GFP. Genotype is indicated on top  
 424 of each panel. Tarsal segments are numbered. Arrowheads point to the presumptive sex comb  
 425 regions. "X" indicates non-leg tissue. (G-I) Leg sex comb in *D. yakuba* (G), *D. santomea* (H) and  
 426 *D. melanogaster* (I). Average sex comb tooth numbers per leg are shown in squares. n: number of  
 427 scored individuals, with the number of scored strains in parentheses. (J-K) Sex comb tooth number  
 428 in wild-type (T7 and Canton-S), *sc*<sup>M6</sup> (J) and *sc*<sup>6</sup> (K) mutants rescued with different 18C05-sc

429 constructs. Each circle represents one male raised at 25°C. Mean (brown line) and 95% confidence  
430 interval (pink rectangle) from a fitted GLM Quasi-Poisson model are shown. Letters indicate the  
431 results of all-pairwise comparisons after Holm-Bonferroni correction. Two genotypes are  
432 significantly different from each other ( $p < 0.05$ ) when they do not share a letter. For easier  
433 comparison, the horizontal dashed line and the surrounding grey line indicate the mean and 95%  
434 confidence interval for *sc<sup>+</sup>;18C05yak-sc*. Transgenic constructs with sex comb tooth number  
435 significantly different from *18C05yak-sc* are shown in boxes in J-K. On average *D. santomea* males  
436 have about 1 extra tooth per sex comb compared to *D. yakuba* (G-H). The substitution *T1775G*  
437 produces on average 0.5 extra sex comb tooth per leg, which is more than expected. It is possible  
438 that the *D. melanogaster* background, where all our rescue constructs were tested, amplifies the  
439 effect of the tested substitutions, especially since *D. melanogaster* males have more sex comb teeth  
440 than *D. santomea* or *D. yakuba*.



## 441 STAR\*METHODS

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## 443 CONTACT FOR REAGENT AND RESOURCE SHARING

444 Further information and requests for resources and reagents should be directed to and will be  
445 fulfilled by the Lead Contact, Virginie Courtier-Orgozo ([virginie.courtier@normalesup.org](mailto:virginie.courtier@normalesup.org)).

446

## 447 EXPERIMENTAL MODEL AND SUBJECT DETAILS

448 The origin of all the fly strains used can be found in KRT Table, Table S1 and Extra Tables. All flies  
449 were cultured on standard cornmeal–agar medium in uncrowded conditions at 25°C unless stated.  
450 We used *Canton-S* as a wild-type *D. melanogaster* strain. Transgenic constructs were integrated into  
451 the *attP2* landing site in *D. melanogaster* *w<sup>1118</sup>* by BestGene Inc. Hybrid males between *D. yakuba*  
452 and *D. santomea* were obtained by collecting 20 virgin females with 20 males from each stocks and  
453 crossing them reciprocally in both directions. At least 10 such crosses were made and flipped every  
454 4-5 days for several weeks. For QTL mapping, *D. yakuba* *yellow[1]* virgin females were crossed *en*  
455 *masse* to *D. santomea* SYN2005 males to generate F1 hybrid females, which were subsequently  
456 backcrossed, separately, to both parental strains. Genitalia of backcross males were isolated for  
457 dissection and the remaining carcass was stored at -20 °C for subsequent sequencing library  
458 preparation.

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## 461 METHOD DETAILS

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### 463 Genotyping of backcross males for QTL mapping

464 The carcass of each male was crushed in a 1.5-ml Eppendorf tube with a manual pestle in 180 µl of  
465 Qiagen Tissue Lysis buffer. DNA of individual flies was extracted using Qiagen DNeasy Blood &  
466 Tissue extraction kit (cat #69506). A Multiplexed Shotgun Genotyping sequencing library was made  
467 from 189 *D. santomea* backcross males and for 181 *D. yakuba* backcross males as described  
468 previously [31]. The list of barcodes used in this study can be found in Mendeley  
469 (<http://dx.doi.org/10.17632/xjvz2m8z6r.1>) or in the Extra Data files, within the names of the  
470 individuals that were sequenced. *D. yakuba* and *D. santomea* parental genome sequences were  
471 generated by updating the *D. yakuba* genome sequence *dyak-4-chromosome-r1.3.fasta* with  
472 Illumina paired-end reads from *D. yakuba* *yellow[1]* and *D. santomea* SYN2005 (sequenced by  
473 BGI) using the *msgUpdateParentals.pl* function of the MSG software package. The resulting  
474 updated genome files are *dsan-all-chromosome-yak1.3-r1.0.fasta.msg.updated.fasta* and *dyak-4-*  
475 *chromosome-r1.3.fasta.msg.updated.fasta*. Ancestry was estimated for all backcross progeny using  
476 MSG software ([github.com/YourePrettyGood/msg](https://github.com/YourePrettyGood/msg)). Ancestry files were reduced to only those  
477 markers informative for recombination events using the script *pull\_thin\_tsv.py*  
478 ([github.com/dstern/pull\\_thin](https://github.com/dstern/pull_thin)). Markers were considered informative when the conditional  
479 probability of being homozygous differed by more than 0.05 from their neighboring markers.

480

### 481 QTL mapping

482 QTL mapping was performed using the R/*qtl* package version 1.4 [32,33]. The thinned posterior  
483 genotype probabilities were imported into R/*qtl* using the R function *read.cross.msg.1.5.R*  
484 ([github.com/dstern/read\\_cross\\_msg](https://github.com/dstern/read_cross_msg)). QTL mapping was performed independently on each  
485 backcross population. We performed genome scans with a single QTL model (“scanone”) using the

486 Haley-Knott regression method [34] which performs well with genotype information at a large  
487 number of positions along the genome. The genome-wide 5% and 1% significance levels were  
488 determined using 1,000 permutations. One QTL peak above the 1% significance level was found for  
489 both backcrosses. To check for additional QTL, we built a QTL model with this single QTL using  
490 the “fitqtl” function and scanned for additional QTL using the “addqtl” function. A second QTL was  
491 found on chromosome 3 for both backcrosses. When introduced into a new multiple QTL model,  
492 refined and fitted to account for possible interactions, a third significant QTL was found. Based on  
493 the full three-QTL model, no additional significant QTL were found with the function “addqtl”: the  
494 highest LOD score for a fourth QTL reached only 1.8 and 1.2 for the *D. yakuba* backcross and the  
495 *D. santomea* backcross, respectively. Various three-QTL models with different interactions between  
496 loci were assessed. Positive significant interaction was detected between the QTL on chromosome 1  
497 and both QTLs on chromosome 3. The interaction between the two QTLs on chromosome 3 was  
498 not significant. For the three-QTL model with interactions between the QTL on chromosome 1 and  
499 both QTLs on chromosome 3, we computed the LOD score of the full model and the estimated  
500 effects of each locus. The 2-LOD intervals were calculated using the “lodint” function with  
501 parameter drop of 2. Analysis of F1 hybrid males is consistent with a large effect of the X  
502 chromosome on hypandrial bristle number: male F1 hybrids carrying a *D. yakuba* X chromosome  
503 have on average 1.9 hypandrial bristles (n=34) while reciprocal hybrid males possessing the *D.*  
504 *santomea* X chromosome have none (n=29) (Extra Tables). Note that few informative markers are  
505 found on the right arm of chromosome 2, suggesting the presence of an inversion between parental  
506 lines. In both backcrosses the large-effect QTL is estimated to cause a decrease of  $0.9 \pm 0.1$  bristles  
507 between a *D. yakuba* hemizygote and a *D. santomea* hemizygote male (Extra Data Files). The QTL  
508 peak is at position 46,886 and 221,928 for the *D. santomea* and *D. yakuba* backcross, respectively.  
509 The AS-C locus is at position 179,000-290,000.

510

#### 511 Duplication Mapping in *D. santomea*-*D. melanogaster* hybrids

512 We used a set of *D. melanogaster* duplication lines to test overlapping parts of chromosome X for  
513 their effect on hypandrial bristle number [35]. Each line contains a fragment of the chromosome X  
514 inserted into the same attP docking site on chromosome 3L using  $\Phi$ C31 integrase, allowing direct  
515 comparison between fragments. Each duplication was used to screen for complementation of the  
516 loss of function allele(s) from *D. santomea*. We exploited the fact that rare *D. santomea*-*D.*  
517 *melanogaster* hybrid males can be produced by crossing *D. melanogaster* females carrying a  
518 compound X chromosome with *D. santomea* males [36]. The resulting hybrid males carry a *D.*  
519 *santomea* X chromosome. We first created a *D. melanogaster* stock whose genotype is *TM3, Sb[1]*  
520 *Ser[1]/Nup98-96[339]* by crossing *Nup98-96[339]/TM3, Sb[1]* with *Df(3R)D605/TM3, Sb[1]*  
521 *Ser[1]*. We then performed three successive crosses at room temperature in glass vials: (a) *C(1)RM,*  
522 *y[1] w[1] f[1] ; +/+  $\times$  +/+; TM3, Sb[1] Ser[1]/Nup98-96[339]*, (b) *C(1)RM, y[1] w[1] f[1] ; TM3,*  
523 *Sb[1] Ser[1]/+  $\times$  +/Y; Dp(1,3)/Dp(1,3)*, (c) *C(1)RM, y[1] w[1] f[1] ; TM3, Sb[1] Ser[1]/Dp(1,3)*  
524 *D. melanogaster* females  $\times$  *D. santomea* males. The same procedure was followed for 21  
525 duplication lines and progeny was obtained for 17 of them. Hybrid males from the last cross were  
526 sorted in two pools, the [*Sb*<sup>-</sup>, *Ser*<sup>-</sup>] males who carried the duplication and the [*Sb*<sup>+</sup>, *Ser*<sup>+</sup>] males  
527 which were used as controls which carried no duplication but the balancer chromosome *TM3 Sb[1]*  
528 *Ser[1]*. In *D. melanogaster*/*D. santomea* hybrids, dominant markers are not always fully penetrant.  
529 A few progeny males exhibited [*Sb*<sup>+</sup>, *Ser*<sup>-</sup>] or [*Sb*<sup>-</sup>, *Ser*<sup>+</sup>] phenotypes; they were considered as  
530 control individuals carrying the balancer chromosome *TM3, Sb[1] Ser[1]*. Males were stored in  
531 ethanol until dissection. Duplication mapping narrowed down the causal region to a 84.6 kb region  
532 (*DC097*) of the *achaete-scute* complex (AS-C) (Figure 2.B-C, see also Extra Tables, GLM-Poisson,  
533  $\text{Chisq}(17,478)=398.44$ ,  $p = 10^{-4}$ ).

534

#### 535 Examination of Hypandrial Bristle Phenotypes

536 Male genitalia were cut with forceps and then hypandria were dissected with fine needles or forceps  
537 Dumont #5 (112525-20, Phymep) in a drop of 1x PBS. For *D. melanogaster* in order to see the  
538 hypandrial bristles better we removed the aedeagus by holding the aedeagal apodem with forceps  
539 and gently pushing the hypandrium upwards with an other forceps until it separated. Hypandria  
540 were mounted in DMHF (Dimethyl Hydantoin Formaldehyde, Entomopraxis). Before dissection,  
541 males were sometimes stored at -20°C in empty Eppendorf tubes or in glycerol:acetate:ethanol  
542 (1:1:3) solution. For analysis of non-hypandrial bristles, males were stored at -20°C in  
543 glycerol:acetate:ethanol (1:1:3) solution. We never stored these males in empty tubes because we  
544 found that such a storage procedure can break and remove external bristles (but, as far as we know,  
545 hypandrial bristles were not affected by such a procedure, maybe because hypandrial bristles are  
546 relatively internal and protected by the epandrium). Furthermore, we never observed a single socket  
547 devoid of shaft on the male hypandrium, indicating that hypandrial bristles cannot be accidentally  
548 cut or lost with our experimental protocol. 3D projection images of the preparations were taken at  
549 500X magnification with the Keyence digital microscope VHX 2000 using optical zoom lens VH-  
550 Z20R/W.

551

#### 552 Examination of Other Bristles

553 Since genitalia are the most rapidly evolving organs in animals with internal fertilization [26], we  
554 compared the number of genital bristles between two strains of *D. yakuba* and two strains of *D.*  
555 *santomea*. We found no difference between *D. yakuba* and *D. santomea* in any genital bristles  
556 except for anal plate and clasper bristles, where a slightly significant interspecific variation was  
557 detected (Extra Figures). The loss of hypandrial bristles in *D. santomea* is thus the major change in  
558 genital bristles between *D. santomea* and *D. yakuba*. Genitalia were dissected in 1X PBS, hypandria  
559 were removed and the epandria were mounted in 99% glycerol. Gentle pressure was applied on the  
560 cover-slip with forceps to flatten the preparations in order to see all bristles. Pictures were taken at a  
561 500X magnification with a digital microscope VHX 2000 (Keyence) using lens VH-Z20R/W.

562 Bristles were counted on the images.

563 For sex comb preparations, prothoracic legs were dissected at the coxa with forceps Dumont #5 and  
564 were mounted in DMHF (Dimethyl Hydantoin Formaldehyde, Entomopraxis). Images of the sex  
565 combs were taken at 1000x magnification with the Keyence digital microscope as written above.  
566 Sex comb teeth were counted on the images with Image J [37].

567

#### 568 Analysis of *scute* coding sequence

569 The *scute* coding sequence (CDS) of *D. melanogaster* iso-1 was retrieved from FlyBase. We  
570 blasted the updated genome sequences of *D. yakuba* yellow[1] and *D. santomea* SYN2005 (see  
571 above) with *D. melanogaster* *scute* coding region and retrieved only one locus in each species. The  
572 *scute* coding region was then annotated with Geneious and no intron was found, as in *D.*  
573 *melanogaster*.

574

#### 575 Screening *as-GAL4* lines for expression in the hypandrium

576 The *as-GAL4* lines were ordered from VDRC [38] and Bloomington Stock Center (Extra Tables).  
577 Two lines were not available (*GMR1509* and *VT054822*) so we created new transgenic lines for  
578 these regions, named *GMR15X09-GAL4* and *VT054822b-GAL4* (see below). Because screens are  
579 easier on adults than on genital discs, and also because the exact developmental stage and location  
580 of hypandrial bristle development are unknown [39], we decided to look for GAL4-triggered  
581 phenotypes in adult males. As a readout of GAL4 expression, we tested various UAS lines (*UAS-*  
582 *mCD8-GFP*, *UAS-yellow* in a yellow mutant background, *UAS-sc.RNAi*, *UAS-achaete.RNAi*, *UAS-*  
583 *forked.RNAi*, *UAS-singed.RNAi*) (lines are listed in KRT Table, see also Extra Tables) together  
584 with *DC-GAL4*, which drives expression in the dorso-central thoracic bristles [40]. To enhance  
585 RNAi potency we also used *UAS-Dicer-2* [40]. With *UAS-mCD8-GFP* and *UAS-yellow* the change

586 in fluorescence or color was hardly visible. The most penetrant bristle phenotype was obtained with  
587 *UAS-Dicer-2 UAS-singed.RNAi<sup>105747</sup>* at 29 °C (Extra Tables). Therefore this line was chosen for  
588 screening all the *as-GAL4* constructs.

589 Five *as-GAL4* males of each *as-GAL4* line were crossed to five *Dcr2; UAS-*  
590 *singed<sup>105747</sup>.RNAi/CyO* virgin females. Crosses were kept at 29 °C. The non-curly males (*Dcr2;*  
591 *UAS-singed<sup>105747</sup>.RNAi/+; +/-as-GAL4*) were collected for dissection and kept at -20 °C. Hypandrium  
592 dissection and image acquisition were performed as indicated above. For each *as-GAL4* line at least  
593 5 genitalia were examined (Extra Tables).

594 To test whether the *15E09*, *18C05* and *054839* enhancer-*GAL4* drive expression in the  
595 hypandrial bristle region in absence of *sc*, we crossed five *sc<sup>29</sup>; UAS-scute (III)* females with five  
596 males of each respective *GAL4* line, as well as five *sc<sup>M6</sup>/FM7 ; UAS-scute (III)* females with five  
597 males of each respective *GAL4* line. Of the three *GAL4* lines, only *18C05* could induce hypandrial  
598 bristles with *UAS-sc* in a *sc* mutant background. The *18C05-GAL4* line produced approximately 10  
599 bristles, where normally only two develop, which may reflect the amplification of gene expression  
600 that is inherent to the *UAS-GAL4* system. These results suggest that only *18C05* drives sufficiently  
601 strong expression in the hypandrial region to alter bristle patterning.

602

### 603 Cloning of enhancers into pBPGUw and pBPSUw

604 Enhancers were cloned into the *GAL4* reporter vector pBPGUw using the same strategy as  
605 in [38,41]. Enhancer sequences were amplified by Phusion® High Fidelity Polymerase (New  
606 England Biolabs) in two steps reaction using the primers and templates (Extra Tables). PCR  
607 products and vectors were purified by Nucleospin Gel and PCR Clean-Up Kit (Machery-Nagel).  
608 Clones were purified by E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek). All *GAL4* constructs were  
609 cloned using the Gateway® system (ThermoFisher Scientific). The enhancer fragments were first  
610 ligated into *KpnI* and *HindIII* restriction enzyme site of the vector pENTR/D-TOPO (Addgene)  
611 (Extra Tables). Recombination into the destination vector pBPGUw was performed using LR  
612 clonase II enzyme mix (Invitrogen) and products were transformed into One Shot® TOP10  
613 (Invitrogen) competent cells. Recombinant clones were selected by ampicillin resistance on Amp-  
614 LB plates (100 µg/ml)

615 The pBPSUw vector was constructed by replacing the *GAL4* cassette of pBPGUw by *scute*  
616 CDS. The *scute* CDS was amplified from *D. melanogaster iso-1* with Scute-CDS-Rev and Scute-  
617 CDS-For primers and ligated into pGEM-T Easy (Promega). The *sc-CDS* insert was cut out using  
618 *KpnI* and *HindIII* and cloned into *KpnI* and *HindIII* sites in pBPGUw, thus replacing *GAL4*. The  
619 vector was named pBPSUw where “S” stands for *scute*. *18C05* sequences from *D. melanogaster*, *D*  
620 *yakuba* and *D. santomea* were cloned into pBPSUw and tested in rescuing hypandrial bristles in *sc*  
621 mutants as written above. We found that *18C05* from *D. melanogaster* rescued two hypandrial  
622 bristles in both *sc<sup>29</sup>* and *sc<sup>M6</sup>* mutants. *D. santomea 18C05* enhancer rescued fewer hypandrial  
623 bristles on average than the *D. yakuba 18C05* region (Figure 3., bristle number for *D. yakuba*  
624 *18C05* in *sc<sup>29</sup>* is significantly different from 0 (Exact-Poisson,  $p < 10^{-16}$ ) and bristle number for *D.*  
625 *santomea 18C05* in *sc<sup>M6</sup>* is significantly different from 2 (Exact-Poisson,  $p = 0.0008$ )).

626 The *18C05* full length sequences were amplified by PCR from *D. melanogaster iso-1* (BL2057), *D.*  
627 *melanogaster T-7*, *D. yakuba Ivory Coast* and *D. santomea SYN2005* with the primers described in  
628 Extra Tables. The PCR products were cloned into pBPSUw as described above. Three different *D.*  
629 *melanogaster 18C05* sequences were tested with *UAS-sc* in the hypandrium in *sc<sup>29</sup>* and *sc<sup>M6</sup>*. *GMR-*  
630 *18C05* (BL2057) was obtained from the Janelia Farm collection [41] and *18C05\_BL2057* and  
631 *18C05\_T7* were cloned in this study. Hypandrial bristle number was found to be significantly  
632 higher for *GMR-18C05* than for *18C05\_BL2057* and *18C05\_T7* in both backgrounds (GLM-Quasi-  
633 Poisson,  $F(2, 63) = 16.88$ , both  $p < 10^{-6}$  for *sc<sup>29</sup>*;  $F(2, 58) = 20.9$ ,  $p < 10^{-10}$  and  $p < 10^{-5}$  for *sc<sup>M6</sup>*).

634 The *GMR-18C05* fragment is inserted in the expression vector 3'-5' compared to the *D.*  
635 *melanogaster* genome sequence. In contrast, the *18C05\_BL2057* and *18C05\_T7* are cloned 5'-3'. All

636 the *18C05* constructs we made were inserted in the same orientation, 5'-3'. *GMR-18C05* and  
637 *18C05\_BL2057* are the same sequences (from *D. melanogaster* Bloomington Stock Center Strain  
638 #2057), but cloned in opposite directions. *18C05\_T7* contains the *18C05* sequence of *D.*  
639 *melanogaster* T.7 strain. Comparing bristle number between *GMR-18C05-GAL4* and  
640 *18C05\_BL2057-GAL4* shows that the orientation of the cis-regulatory region has an effect on bristle  
641 number.

642 The *18C05-chimera-pBPSUw* constructs were cloned using Gibson Assembly [42] by fusing  
643 together different lengths of *18C05* sequences from *D. yakuba* Ivory Coast and *D. santomea*  
644 SYN2005. The different chimeras are described in Extra Tables. Cloning primers were designed  
645 using NEBuilder Tools (<http://nebuilder.neb.com/>). Primer sequences and templates used in PCR  
646 are listed at Extra Tables. To assemble the *18C05* fragments in pBPSUw (Extra Tables), the vector  
647 was linearized by *AatII* and *FseI* restriction enzymes (New England Biolabs Inc.). After digestion  
648 thermosensitive alkaline phosphatase (FastAP, ThermoFisher Scientific) was added to the reaction  
649 to prevent self-ligation of the plasmid. PCR products and the linearized plasmid were isolated from  
650 1% agarose gels and spin column purified. Gibson Assembly was performed as in [42], except that  
651 the assembly reactions were incubated at 37 °C for 10 minutes and then 3 hours at 50 °C in a PCR  
652 machine. 2 µl of assembly mixtures were transformed into NEB® 10-beta (New England Biolabs  
653 Inc.) competent cells and ampicillin-resistant colonies were selected on 100 µg/ml Amp-LB plates.  
654 The Gibson Assembly Master-mix was prepared according to [42], its components were purchased  
655 from Sigma-Aldrich.

656 The *18C05-yakubaSNP-pBPSUw* constructs were cloned by Gibson Assembly as described  
657 above, except for *18C05yakT1008C* and *18C05yakT1482C* sequences, which were synthesized and  
658 cloned by GenScript® (Extra Tables). The *18C05-ancestral* sequences were synthesized and cloned  
659 by GenScript® into pBPSUw *AatII* and *FseI* sites, except for the *18C05\_AncG869A*,  
660 *18C05\_AncT670G* and *18C05\_Anc-7SNP* sequences, which were cloned by us by Gibson Assembly  
661 into pBPSUw *AatII* and *FseI* sites using the *18C05\_Ancestral\_Gibson\_forward* and  
662 *18C05\_Ancestral\_Gibson\_reverse* primers (Extra Tables).

663 All transgenic constructs were integrated into the *attP2* landing site in *D. melanogaster* *w<sup>1118</sup>* by  
664 BestGene Inc. The *T1775G* substitution affects nucleotide position 447,055 in the Dm6 reference  
665 assembly.

#### 666 Genomic DNA preparations for sequencing the *18C05* region

667 Genomic DNA was isolated with Zymo Research Quick-DNA™ Miniprep Plus Kit from 3  
668 males and 3 females from the *D. yakuba*, *D. santomea* and *D. teissleri* lines listed in the summary of  
669 the alignment of *18C05* sequences available at <https://doi.org/10.6084/m9.figshare.6972707>. *18C05*  
670 sequences were amplified with San-Yak\_lines\_sequencing-For and San-Yak\_lines\_sequencing-Rev  
671 primers (Extra Figures) using Phusion® High Fidelity Polymerase (New England Biolabs).  
672

#### 673 Sequence Analysis

674 Geneious software was used for cloning design and DNA sequence analysis. Nucleotide positions  
675 are given according to the alignment of *D. yakuba* Ivory Coast *18C05* sequence with *D. santomea*  
676 SYN2005 *18C05* sequence. The *18C05ancestral* sequence of *D. yakuba* and *D. santomea* was  
677 reconstructed in Geneious based on the *18C05* sequence alignment of multiple *Drosophila* species  
678 available at <https://doi.org/10.6084/m9.figshare.6972707>. Manual parsimony reconstruction of all  
679 the ancestral nucleotides was unambiguous, except for one position (766, indel polymorphism),  
680 where the sequence is absent in the *simulans* complex and in *D. santomea*, while it is present in *D.*  
681 *teissleri* and polymorphic in *D. yakuba*. For this position we chose *D. teissleri* as the ancestral  
682 sequence. The *18C05* sequences of *D. melanogaster* subgroup species were retrieved by BLAST  
683 from the NCBI website. Transcription Factor (TF) binding sites in *18C05* were predicted using the  
684 JASPAR CORE Insecta database (<http://jaspar.genereg.net> [43]). 25-60 bp sequences of *18C05*  
685

686 were scanned with all JASPAR matrix models with 50-95% Relative Profile Score Thresholds to  
687 test for sensitivity and selectivity [43] (Table S3). For TFs which were absent in JASPAR (Scute),  
688 we used Fly Factor Survey [44] to analyze their putative binding affinities to the probe. As *sc* cis-  
689 regulatory region is known to contain binding sites for Scute itself [45], we looked for Scute  
690 binding sites in *18C05*, *15E09* and *054839*. Two putative Scute binding sites (consensus motif  
691 *CAYCTGY*, Fly Factor Survey [44] were found in *15E09* and *054839* but not in *18C05*. Given the  
692 present results, we cannot exclude the involvement of *15E09* and *054839* in the evolution of  
693 hypandrial bristle evolution in *D. santomea*. In this paper, we decided to focus on the *18C05*  
694 enhancer, whose effect could be studied in a *sc* mutant background.

695

#### 696 Abd-B homeodomain (Abd-B-HD) purification and EMSA

697 The Abd-B-HD-pGEX-4T-1 plasmid [46] (kindly provided by Sangyun Jeong) was transformed  
698 into BL21 (DE3) chemically competent cells. Protein expression was induced by 0.1 mM IPTG  
699 (isopropyl- $\beta$ -D-thiogalactopyranoside, Sigma Aldrich). Recombinant protein was purified from 500  
700 ml of bacterial culture as described in Frangioni [47] except that proteins were eluted into 50 mM  
701 Tris-HCl, pH 8.0, 500 mM NaCl, 10mM reduced glutation (Sigma-Aldrich, G-4251) and 5%  
702 glycerol. Concentrations and purity of the protein were determined by SDS-PAGE and Qubit 2.0  
703 Fluorometer (Life Technologies). Protein aliquots of 20  $\mu$ l were snap-frozen in liquid nitrogen and  
704 stored at -80 °C.

705 The HPLC-purified biotinylated and non-labelled oligonucleotides (Sigma-Aldrich) were used  
706 in PCR to obtain 54 bp probes *yak* and *san* (*san=yakT1775G*) from *18C05yak-pBPSUw* and  
707 *18C05yakT1775G-pBPSUw* plasmid templates. Oligonucleotides are listed in Extra Tables. PCR  
708 products were column-purified.

709 We then used electrophoretic mobility shift assay (EMSA) to test whether the purified Abd-B  
710 homeodomain (ABD-B-HD) can bind directly to a 54-bp fragment of *18C05* with the T1775G site  
711 at position 13 containing either T (*yak* probe) or G (*san* probe). In each binding reaction, 20 fmol of  
712 probes were mixed with the purified ABD-B HD ranging from 0-1.25  $\mu$ g (0  $\mu$ g, 0.75  $\mu$ g, 1  $\mu$ g and  
713 1.25  $\mu$ g) in binding buffer containing 10mM TRIS pH 7.5, 50 mM KCl, 0.5 mM DTT, 6.25 mM  
714 MgCl<sub>2</sub>, 0.05 mM EDTA, 50 ng/ $\mu$ l Salmon Sperm DNA (Sigma Aldrich) and 9.00% Ficoll 400  
715 (Sigma Aldrich). The competition assay was performed by adding 9 pmol of unlabeled probes (450-  
716 fold excess) to the binding reaction. The reaction mixtures were incubated at 22 °C for 30 min and  
717 run on a non-denaturing 6% polyacrylamide gel (Invitrogen) in 0.5X TBE (Eurofins).

718 Labeling reactions were carried out with LightShift™ Chemiluminiscent EMSA Kit  
719 (ThermoFisher Scientific) according to the provider instructions with the following modifications:  
720 after electrophoresis, gels were blotted overnight in 20X SSC using the TurboBlotter Kit (GE  
721 Healthcare Life Sciences) and cross-linking of the probe to the membrane UV-light was performed  
722 at 254 nm and 120 mJ/cm<sup>2</sup> (UV stratalinker® 2400, STRATAGENE). Chemiluminescence stained  
723 membranes were exposed to a CDD camera (FUJIFILM, LAS-4000) for 50x 10 sec exposition time  
724 increments. The last images were used for quantification and were never saturated according to  
725 LAS 4000 software.

726 To quantify the binding affinity of Abd-B-HD to the probes, the fractional occupancy (ratio of  
727 bound/(free+bound) probe) was calculated for three replicate experiments (Figure S4E) using the  
728 intensity values of the bands measured in ImageJ [37]. The mean fractional occupancy was  
729 significantly lower with *D. santomea* probes than with *D. yakuba* probes (ANCOVA,  
730  $F(1,15)=10.58$ ,  $p = 0.005$ ). We found that ABD-B-HD binds both *D. yakuba* and *D. santomea* DNA  
731 (Figure S4B-D). ABD-B-HD binding to the *D. yakuba* probe always resulted in a stronger shift than  
732 to the *D. santomea* probe. Furthermore, the *D. santomea* cold probe did not compete as efficiently  
733 as the *D. yakuba* cold probe to prevent formation of the *D. yakuba* DNA-ABD-B-HD complex (U-  
734 test,  $p=0.05$ ).

735

### 736 Abd-B RNAi and clonal analysis

737 To test whether *Abd-B* is required for hypandrial bristle development, we reduced *Abd-B* expression  
738 using either genetic mutations or RNAi. Two *UAS.Abd-B-RNAi* lines (#51167 and #26746) were  
739 crossed with 3 different *GAL4* lines, *GMR18C05-GAL4*, *NP5130-GAL4* and *NP6333-GAL4*.  
740 Crosses were kept at 29 °C and the hypandrium phenotype was examined in 10-50 F1 males (Table  
741 S4). Using the genitalia *GAL4* drivers *esg-GAL4<sup>NP5130</sup>* [48] and *NP6333* [49] to express *Abd-*  
742 *B.RNAi<sup>51167</sup>*, we obtained 20 males out of 100 with developed hypandrium, among which two  
743 aberrant hypandrial bristle phenotypes were found, either bristle size reduction or bristle loss  
744 (Figure S3A-F, n=9/11 for *NP5130*, n=8/9 for *NP6333*, Table S4). Smaller bristles might arise from  
745 a delay in *sc* expression during development [50]. Since *Abd-B* null mutations are lethal [51], we  
746 produced mitotic mutant clones for two null mutations, *Abd-B<sup>M1</sup>* [51] and *Abd-B<sup>D18</sup>* [52]. *Abd-B*  
747 mutant mitotic recombinant clones were induced by the FLP/FRT system [53] using *Abd-B<sup>M1</sup>* and  
748 *Abd-B<sup>D18</sup>* null mutations. To induce clones, ten *yw hsflp122; FRT82B hs-CD2 y<sup>+</sup> M(3) w<sup>123</sup>/TM2*  
749 virgin females were crossed to ten *y; FRT82B Abd-B<sup>M1</sup> red[1] e[11] ro[1] ca[1]/TM6B* or *y;*  
750 *FRT82B Abd-B<sup>D18</sup>/TM3* males (stocks were kindly provided by Ernesto Sánchez-Herrero). Crosses  
751 were flipped every 24 hours and F1 progeny were heat-shocked at 38 °C for 1 hour at different  
752 stages of larval development: 24-48, 48-72, 72-98 and 96-120 hours after egg laying [54]. From  
753 both crosses, a total of 82 F1 males (Table 4) with the genotype of *yw hsflp122; FRT82B hs-CD2 y<sup>+</sup>*  
754 *M(3) w<sup>123</sup>/FRT82B Abd-B<sup>M1</sup> red[1] e[11] ro[1] ca[1]* and *yw hsflp122; FRT82B hs-CD2 y<sup>+</sup> M(3)*  
755 *w<sup>123</sup>/FRT82B Abd-B<sup>D18</sup>* were examined. Hypandria were mounted and bristle clones were screened  
756 as described above. Most of the resulting males showed extreme transformation of the genitalia  
757 (Figure S4G-H, O-P) but 12 males out of 82 had analyzable hypandrium (twelve males for *Abd-B<sup>M1</sup>*  
758 and two males for *Abd-B<sup>D18</sup>*). Among them, 6 males were devoid of one or both hypandrial bristles  
759 (Figure S4I-N, Table S4). When hypandrial bristles were present, most of them were heterozygous  
760 for the *Abd-B* mutation according to the visible markers associated with somatic recombination.  
761 Together, our results suggest that *Abd-B* is required for hypandrial bristle development.

762

### 763 Immunostaining

764 For leg disc stainings the larvae were fed on freshly prepared Formula 4-24<sup>®</sup> Instant Drosophila  
765 Medium, Blue (Carolina) and staged by the presence of blue staining in their gut [55]. Larvae were  
766 chosen with the most clear gut, indicating a developmental stage of 1-6 hours before pupa formation  
767 [56]. Head parts of the larvae were cut and fixed in 4% PFA in PBS pH 7.4 for 20 minutes at room  
768 temperature. For pupal leg preparations the anterior part of the pupae were cut and fixed in 4% PFA  
769 in PBS pH 7.4 for 50 minutes at room temperature. Following fixation, samples were washed three  
770 times for 5 minutes in PBS containing 0.1% Tween20 and then permeabilized in TNT buffer (TRIS-  
771 NaCl buffer containing 0.5% Triton X-100) for 10 minutes. Samples were washed in 5% BSA in  
772 TNT for up to 5 hours at room temperature and then incubated with rabbit anti-GFP primary  
773 antibodies (Thermofisher #A6455) diluted in 1:1000 in TNT overnight at 4 °C and rinsed in TNT  
774 three times for 10 minutes at room temperature. Then, samples were washed in 5% BSA in TNT for  
775 up to 5 hours at room temperature and incubated with donkey Anti-Rabbit Dylight<sup>®</sup> 488  
776 (Thermofisher) secondary antibodies diluted 1:200 in TNT overnight at 4 °C. After washing the  
777 preparations in TNT for 5 minutes DNA was stained in 1µg/µl DAPI solution (Sigma-Aldrich) for  
778 30 minutes at room temperature. The preparations were finally washed in TNT three times for 5  
779 minutes and the imaginal discs and pupal legs were dissected in PBS and mounted in Vectashield<sup>®</sup>  
780 H-1000. Images were acquired using Spinning Disc CSU-W1. Number of GFP-positive cells were  
781 counted in the z-stack using ImageJ [37] in a blind fashion regarding the genotypes using  
782 randomized file names.

783

### 784 **QUANTIFICATION AND STATISTICAL ANALYSIS**

785

786 Since bristle number is a classical type of count data, we performed statistical analysis using  
787 generalized linear models (GLM) and generalized linear mixed models (GLMM) where bristle  
788 number, the response variable, is assumed to follow a Poisson distribution [57–59]. All statistical  
789 analyses were performed using R 3.4 [60]. GLM were fitted with the function `glm()` ("stats" core  
790 package 3.5.0) and GLMM with the function `glmer()` ("lme4" package 1.1-14 [61] with the  
791 parameter "family" taken to be "Poisson". We tested differences in bristle number by comparing two  
792 wild-type stocks of *D. yakuba* with two wild-type stocks of *D. santomea*. We tested the difference  
793 between species, using a GLMM of the Poisson type (GLMM-Poisson) where the number of  
794 bristles was the response variable, species was a fixed effect to test and stock a random effect. For  
795 all other analyses, we tested differences in bristle number between genotypes using GLM of the  
796 Poisson type (GLM-Poisson) where the response variable was bristle number and genotype, a  
797 categorical variable, was the fixed effect. When we noticed important differences between residual  
798 deviance and residual degrees of freedom, we also fitted a quasi-likelihood model of the type  
799 "quasi-Poisson" (GLM-Quasi-Poisson) which allows for a model of the Poisson type, but where the  
800 variance can differ from the mean and is estimated based on a dispersion parameter (see for  
801 example [59] p. 225). For each model, in order to retain the model that fitted best to the data,  
802 analysis of deviance was performed using the `anova.glm()` with "test = Chisq" for GLM-Poisson  
803 and "test = F" for GLM-Quasi-Poisson. When needed, we performed multiple comparisons using  
804 the `glht()` function and the "Holm" adjustment parameter ("multcomp" package 1.4-7 [62]) which  
805 performs multiple comparisons between fitted GLM parameters and yields adjusted p-values  
806 corrected according to the Holm-Bonferroni method [63,64] also performed an exact Poisson test (R  
807 function "poisson.test") to test sample mean to a reference value assuming a Poisson distribution.  
808 Mean and 95% confidence intervals were directly extracted from the fitted GLM and transformed  
809 using `exp(coef())` and `exp(confint.default())`.

810 For EMSA data, response curves were compared between yak probe and san probe using an  
811 ANCOVA after natural log transformation. The unlabeled san 450x responses were compared  
812 between yak probe and san probe using a one-sided Mann-Whitney U-test.

813

#### 814 DATA AVAILABILITY

815 Sequences were deposited into GenBank (accession numbers MG460736-MG460765). Source data  
816 for Bristle Number, QTL mapping analysis, EMSA and immunostaining are available in BioRxiv  
817 and at Mendeley: <http://dx.doi.org/10.17632/xjvz2m8z6r.1>. Additional Data Figures and Data  
818 Tables are available in BioRxiv and at Figshare: <https://doi.org/10.6084/m9.figshare.6972707> and  
819 <https://doi.org/10.6084/m9.figshare.6972740>, respectively.

820

821



REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
rabbit anti-GFP primary antibody	ThermoFisher	#A6455
donkey Anti-Rabbit Dylight® 488	ThermoFisher	#SA5-10038
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Dimethyl Hydantoin Formaldehyde	Entomopraxis	N/A
Paraformaldehyde	Sigma-Aldrich	#158127-5G
Dapi	Sigma-Aldrich	#D9542-1MG
Vectashield® H-1000	Vector Laboratories	#H-1000
reduced glutation	Sigma-Aldrich	#G-4251
Abdominal-B-HD protein	This paper	N/A
<b>Critical Commercial Assays</b>		
Qiagen DNeasy Blood & Tissue extraction kit	Qiagen	#69506
Nucleospin Gel and PCR Clean-Up Kit	Machery-Nagel	#740609
E.Z.N.A.® Plasmid Mini Kit I	Omega Bio-tek	#D6942-01
Quick-DNA™ Miniprep Plus Kit	Zymo Research	#D4069
LightShift™ Chemiluminiscent EMSA Kit	ThermoFisher Scientific	#20148
TurboBlotter Kit	GE Healthcare Life Sciences	#10416314
<b>Deposited Data</b>		
Raw and analyzed data	This paper	Mendeley: <a href="http://dx.doi.org/10.17632/xjvz2m8z6r.1">http://dx.doi.org/10.17632/xjvz2m8z6r.1</a> (DOI: 10.17632/xjvz2m8z6r.1)
Genitalia Bristle Number	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972707.v1">https://doi.org/10.6084/m9.figshare.6972707.v1</a>
Alignment of 18C05 sequence from <i>D. santomea</i> SYN2005 and <i>D. yakuba</i> (Ivory Cost)	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972707.v1">https://doi.org/10.6084/m9.figshare.6972707.v1</a>
Twelve substitutions are fixed in <i>D. santomea</i> 18C05	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972707.v1">https://doi.org/10.6084/m9.figshare.6972707.v1</a>
Sex comb tooth number in <i>D. yakuba</i> and <i>D. santomea</i> and <i>D. melanogaster scute</i> mutants	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972707.v1">https://doi.org/10.6084/m9.figshare.6972707.v1</a>
Hypandrial bristle number in pure species and F1 hybrids	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972740.v3">https://doi.org/10.6084/m9.figshare.6972740.v3</a>
Test of various <i>UAS</i> -reporter constructs with <i>DC-GAL4</i>	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972740.v3">https://doi.org/10.6084/m9.figshare.6972740.v3</a>

<i>Achaete-scute GAL4</i> lines and their hypandrial bristle phenotype with <i>Dcr2</i> ; <i>UAS-singed.RNAi</i> <sup>10574</sup>	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972740.v3">https://doi.org/10.6084/m9.figshare.6972740.v3</a>
Cloning strategy	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972740.v3">https://doi.org/10.6084/m9.figshare.6972740.v3</a>
18C05_D. san_A1200.4	This paper	GenBank #MG460738
18C05_D. san_car1490.3	This paper	GenBank #MG460742
18C05_D. san_BS14.1	This paper	GenBank #MG460740
18C05_D. san_51.7.3_1560	This paper	GenBank #MG460737
18C05_D. san_OBAT_1200.13	This paper	GenBank #MG460744
18C05_D. san_Quija650.37	This paper	GenBank #MG460747
18C05_D. san_Quija650.22	This paper	GenBank #MG460746
18C05_D. san_Quija650.14	This paper	GenBank #MG460745
18C05_D. san_C1350.14	This paper	GenBank #MG460741
18C05_D. san_B1300.13	This paper	GenBank #MG460739
18C05_D. san_Rain42	This paper	GenBank #MG460748
18C05_D. san_Field3.4	This paper	GenBank #MG460743
18C05_D. san_STO4	This paper	GenBank #MG460749
18C05_D. san_SYN2005	This paper	GenBank #MG460750
18C05_D. yak_15.6.8	This paper	GenBank #MG460759
18C05_D. yak_LP1	This paper	GenBank #MG460761
18C05_D. yak_2.22.1	This paper	GenBank #MG460756
18C05_D. yak_4.23.1	This paper	GenBank #MG460757
18C05_D. yak_4.32.1	This paper	GenBank #MG460758
18C05_D. yak_Tai18E2	This paper	GenBank #MG460765
18C05_D. yak_Ivory Coast	This paper	GenBank #MG460760
18C05_D. yak_Tai18E2 (NCBI)	This paper	GenBank #MG460765
18C05_D. yak_PB 3.1.3	This paper	GenBank #MG460763
18C05_D. yak_PB 3.4.1	This paper	GenBank #MG460764
18C05_D. yak_PB1.4.21	This paper	GenBank #MG460762
18C05_D. yak_5.3.1	This paper	GenBank #MG460755

18C05_D. teis_(Mt. Selinda)	This paper	GenBank #MG460753
18C05_D. teis_(SDSC#14021-0257.01)	This paper	GenBank #MG460754
18C05_D. mel_BL2057	This paper	GenBank #MG460736
18C05_D. sim_w501	This paper	GenBank #MG460752
18C05_D. sim_M252	This paper	GenBank #MG460751
<b>Experimental Models: Organisms/Strains</b>		
<i>D.melanogaster</i> 3870	Tony Long UC Irvine, RVC 3. Collected from Riverside, California, USA in 1963	N/A
<i>D.melanogaster</i> 3844	Tony Long UC Irvine, BS1. Collected from Barcelona, Spain in 1954	San Diego Stock Center #14021-0231.60
<i>D.melanogaster</i> 3841	Tony Long UC Irvine, BOG1. Collected from Bogota, Colombia in 1962	San Diego Stock Center #14021-0231.59
<i>D.melanogaster</i> 3852	Tony Long UC Irvine, KSA2. Collected in 1963	San Diego Stock Center #14021-0231.64
<i>D.melanogaster</i> 3864	Tony Long UC Irvine, KI2. Collected from Israel in 1954	San Diego Stock Center # 14021-0231.68
<i>D.melanogaster</i> T.7	Tony Long UC Irvine, Collected from Taiwan in 1968	San Diego Stock Center #14021-0231.07
<i>D.melanogaster</i> T.4	Tony Long UC Irvine, Collected from Kuala Lumpur, Malaysia in 1962	San Diego Stock Center #14021-0231.04
<i>D.melanogaster</i> 3875	Tony Long UC Irvine, VAG1. Collected from Athens, Greece in 1965	San Diego Stock Center #14021-0231.69
<i>D.melanogaster</i> 3886	Tony Long UC Irvine, Wild 5B. Collected from Red Top Mountain, Georgia in 1966	N/A
<i>D.melanogaster</i> T.1	Tony Long UC Irvine, Collected from Ica, Peru in 1956	San Diego Stock Center #14021-0231.04
<i>D.melanogaster</i> 3839	Tony Long UC Irvine, BER1. Collected from Bermudas in 1954.	San Diego Stock Center # 14021-0231.58
<i>D.melanogaster</i> 3846	Tony Long UC Irvine, CA1. Collected from Cape Town, South Africa.	San Diego Stock Center #14021-0231.62

<i>D.melanogaster</i> Sam	Tony Long UC Irvine, DSPR line. originally from TFC Mackay Sam; ry506	N/A
<i>D.melanogaster</i> iso-1 y[1]; Gr22b[iso-1] Gr22d[iso-1] cn[1] CG33964[iso-1] bw[1] sp[1]; LysC[iso-1] MstProx[iso-1] GstD5[iso-1] Rh6[1]	Bloomington Stock Center	Bloomington Stock Center #2057
<i>D.melanogaster</i> Canton-S	Roger Karess	Kyoto DGGR #105666
<i>D.melanogaster</i> dor[4]/C(1)RM, y[1] w[1] f[1]	Bloomington Stock Center	Bloomington Stock Center #35
<i>D.melanogaster</i> Nup98-96[339]/TM3, Sb[1]	Bloomington Stock Center	Bloomington Stock Center #4951
<i>D.melanogaster</i> Df(3R)D605/TM3, Sb[1] Ser[1]	Bloomington Stock Center	Bloomington Stock Center #823
<i>D.melanogaster</i> DC002 w1118; Dp(1;3)DC002, PBac{DC002}VK00033	Bloomington Stock Center	Bloomington Stock Center #30213
<i>D.melanogaster</i> DC003 w1118; Dp(1;3)DC003, PBac{DC003}VK00033	Bloomington Stock Center	Bloomington Stock Center #30214
<i>D.melanogaster</i> DC004 w1118; Dp(1;3)DC004, PBac{DC004}VK00033/TM6C, Sb1	Bloomington Stock Center	Bloomington Stock Center #30215
<i>D.melanogaster</i> DC006 w1118; Dp(1;3)DC006, PBac{DC006}VK00033/TM6C, Sb1	Bloomington Stock Center	Bloomington Stock Center #30217
<i>D.melanogaster</i> DC097 w1118; Dp(1;3)DC097, PBac{DC097}VK00033/TM6C, Sb1	Bloomington Stock Center	Bloomington Stock Center #31440
<i>D.melanogaster</i> DC098 w1118; Dp(1;3)DC098, PBac{DC098}VK00033	Bloomington Stock Center	Bloomington Stock Center #31441
<i>D.melanogaster</i> DC007 w1118; Dp(1;3)DC007, PBac{DC007}VK00033/TM6C, Sb1	Bloomington Stock Center	Bloomington Stock Center #30218
<i>D.melanogaster</i> DC008 w1118; Dp(1;3)DC008, PBac{DC008}VK00033	Bloomington Stock Center	Bloomington Stock Center #30745
<i>D.melanogaster</i> DC009 w1118; Dp(1;3)DC009, PBac{DC009}VK00033	Bloomington Stock Center	Bloomington Stock Center #30219
<i>D.melanogaster</i> DC012 w1118; Dp(1;3)DC012, PBac{DC012}VK00033	Bloomington Stock Center	Bloomington Stock Center #30222
<i>D.melanogaster</i> DC099 w1118; Dp(1;3)DC099, PBac{DC099}VK00033	Bloomington Stock Center	Bloomington Stock Center #30749
<i>D.melanogaster</i> DC013 w1118; Dp(1;3)DC013, PBac{DC013}VK00033	Bloomington Stock Center	Bloomington Stock Center #30746
<i>D.melanogaster</i> DC014 w1118; Dp(1;3)DC014, PBac{DC014}VK00033	Bloomington Stock Center	Bloomington Stock Center #31434
<i>D.melanogaster</i> DC400 w1118; Dp(1;3)DC400, PBac{DC400}VK00033	Bloomington Stock Center	Bloomington Stock Center #30795
<i>D.melanogaster</i> DC019 w1118; Dp(1;3)DC019, PBac{DC019}VK00033	Bloomington Stock Center	Bloomington Stock Center #30223
<i>D.melanogaster</i> DC436 w1118; Dp(1;3)DC436, PBac{DC436}VK00033/TM6C, Sb1	Bloomington Stock Center	Bloomington Stock Center #33487
<i>D.melanogaster</i> DC401 w1118; Dp(1;3)DC401, PBac{DC401}VK00033	Bloomington Stock Center	Bloomington Stock Center #30796
<i>D.melanogaster</i> DC-GAL4 yw ; DC-GAL4 , UAS-GFP /TM6B UAS-forked.RNAi <sup>33200</sup>	V. Stamatakis (Pat Simpson lab) Vienna Stock Center	N/A VDRC #33200

<i>D.melanogaster</i> UAS-singed.RNAi <sup>105747</sup>	Vienna Stock Center	VDRC #105747
<i>D.melanogaster</i> UAS-forked.RNAi <sup>24632</sup>	Vienna Stock Center	VDRC #24632
<i>D.melanogaster</i> UAS-ac.RNAi <sup>100647</sup>	Vienna Stock Center	VDRC #100647
<i>D.melanogaster</i> UAS-singed.RNAi <sup>32579</sup>	Vienna Stock Center	VDRC #32579
<i>D.melanogaster</i> UAS-forked.RNAi <sup>103813</sup>	Vienna Stock Center	VDRC #103813
<i>D.melanogaster</i> UAS-sc.RNAi <sup>105951</sup>	Vienna Stock Center	VDRC #105951
<i>D.melanogaster</i> yw; UAS-y y[1] w[1118]; P{w[+mC]=UAS-y.C}MC1	Vienna Stock Center	Bloomington Stock Center #3043
<i>D.melanogaster</i> yw;UAS-y TM3/pnr-GAL4	Mark Rebeiz.	N/A
<i>D.melanogaster</i> UAS-mCD8-GFP GFP transgene on second chromosome	Veronique Brodu	N/A
<i>D.melanogaster</i> w,UAS-Dcr2 ; Pin/CyO	Bloomington Stock Center	Bloomington Stock Center #24644
<i>D.melanogaster</i> UAS-scute	Bloomington Stock Center	Bloomington Stock Center #51672
<i>D.melanogaster</i> GFP-sc GFP inserted at the <i>scute</i> locus by CRISPR-mediated homologous recombination, which produces Scute protein with GFP sequence fused at the N terminus.	F. Schweisguth, [65]	N/A
<i>D.melanogaster</i> yw;UAS-Abd-B.RNAi <sup>51167</sup>	Bloomington Stock Center	Bloomington Stock Center #51167
<i>D.melanogaster</i> yw; UAS-Abd-B.RNAi <sup>26746</sup>	Bloomington Stock Center	Bloomington Stock Center #26746
<i>D.melanogaster</i> yw; NP5130-GAL4	Kyoto DGGR	Kyoto DGGR #109126
<i>D.melanogaster</i> yw; NP6333-GAL4	Kyoto DGGR	Kyoto DGGR #113920
<i>D. simulans</i>	Collected by J. R. David from Marrakech, Morocco in 2010	N/A
<i>D. mauritiana</i>	Collected by J. R. David from Mauritius Island in 1985	N/A
<i>D. sechellia</i> GFP w[1] ; pBac(3xP3-EGFPafm)::MCS::(pW8 mini-white)	San Diego Species Stock Center	San Diego Stock Center #14021-0248.32
<i>D. yakuba</i> Ivory Coast	D. L. Stern, Collected from Ivory Coast in 1955	San Diego Stock Center #14021-0261.00
<i>D. yakuba</i> 15.6.8, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 110m in 2009	N/A
<i>D. yakuba</i> yellow[1]	San Diego Species Stock Center	San Diego Species Stock Center #14021-0261.05
<i>D. yakuba</i> 4.23.1, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 1070m in 2009	N/A
<i>D. yakuba</i> LP1, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 0m in 2009	N/A
<i>D. yakuba</i> 2.22.1, Isofemale stock	Collected by D. R. Matute	Given by D. Matute. Isofemale stock, collected in São Tomé at altitude 1250m in 2009 by D. Matute

<i>D. yakuba</i> PB1.4.21, Isofemale stock	Collected by D. R. Matute	Given by D. Matute. Isofemale stock, collected in Bioko at altitude 1300m in 2009 by D. Matute
<i>D. santomea</i> SYN2005, Mix of six isofemale lines	Given by D. Matute. collected by J. Coyne at the field station Bom Sucesso (elevation 1,150 m) in 2005	N/A
<i>D. santomea</i> STO.4	D. L. Stern, Collected in São Tomé in 1998	San Diego Stock Center #14021-0271.00
<i>D. santomea</i> Quija 650.22, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 650m in 2009	N/A
<i>D. santomea</i> Quija 650.37, Isofemale stock	Given by D. R. Matute, collected in São Tomé at altitude 650m in 2005 by Lucio Primo Monteiro under the supervision of Daniel Lachaise.	N/A
<i>D. santomea</i> Quija 650.14, Isofemale stock	Given by D. R. Matute, collected in São Tomé at altitude 650m in 2005 by Lucio Primo Monteiro under the supervision of Daniel Lachaise	N/A
<i>D. santomea</i> BS14.1, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 1150m in 2009	N/A
<i>D. santomea</i> CAR1490.3, Isofemale stock	Collected by D. R. Matute collected in São Tomé at altitude 1490m in 2009	N/A
<i>D. santomea</i> B1300.13, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 1300m in 2009	N/A
<i>D. santomea</i> OBAT1200.3, Isofemale stock	Collected by D. R. Matute collected in São Tomé at altitude 1200m in 2009	N/A
<i>D. santomea</i> A1200.4, Isofemale stock	Collected by D. R. Matute	Given by D. Matute. Isofemale stock, collected in São Tomé at altitude 1200m in 2009 by D. Matute
<i>D. santomea</i> C1350.14, Isofemale stock	Collected by D. R. Matute	Given by D. Matute. Isofemale stock, collected in São Tomé at altitude 1350m in 2009 by D. Matute

<i>D. santomea</i> Rain42, Isofemale stock	Collected by D. R. Matute	Given by D. Matute. Isofemale stock, collected in São Tomé at altitude 1240m in 2009 by D. Matute
<i>D. santomea</i> Field3.4, Isofemale stock	Collected by D. R. Matute in São Tomé at altitude 1250m in 2009	N/A
<i>D. teissieri</i>	Collected by J. R. David in Mt Selinda, Zimbabwe in 1970	N/A
<i>D. teissieri</i> #14021-0257.01	San Diego Stock Center	San Diego Stock Center # 14021-0257.01
<i>D. orena</i> , Isofemale stock	Collected by J. R. David in 1975 in Cameroon	N/A
<i>D. erecta</i>	Collected by D. Lachaise in La Lopé, Gabon in 2005	N/A
<i>D. elegans</i>	B. Prud'homme, Collected in Hong-Kong.	San Diego Stock Center # 14027-0461.03.
<i>D. melanogaster</i> <i>sc</i> <sup>M6</sup> <i>sc</i> [M6]/ <i>FM7i</i> , <i>P</i> { <i>w</i> [+ <i>mC</i> ]= <i>ActGFP</i> } <i>JMR3</i>	Bloomington Stock Center	#52668
<i>D. melanogaster</i> <i>ac</i> <sup>CAM1</sup> <i>y</i> [1] <i>P</i> { <i>w</i> [+ <i>mW.hs</i> ]= <i>GawB</i> } <i>CG32816</i> [ <i>NP6014</i> ] <i>ac</i> [ <i>cam1</i> ]	Bloomington Stock Center	#36540
<i>D. melanogaster</i> <i>sc</i> <sup>6</sup> <i>sc</i> [6] <i>w</i> [ <i>a</i> ]	Bloomington Stock Center	#108
<i>D. melanogaster</i> <i>ase</i> <sup>1</sup> <i>Df</i> (1) <i>ase-1</i> , <i>sc</i> [ <i>ase-1</i> ] <i>pn</i> [1]/ <i>C</i> (1) <i>DX</i> , <i>y</i> [1] <i>f</i> [1]	Bloomington Stock Center	#104
<i>D. melanogaster</i> <i>sc</i> <sup>5</sup> <i>y</i> [1] <i>sc</i> [5]	Bloomington Stock Center	#178
<i>D. melanogaster</i> <i>ac</i> <sup>1</sup> <i>y</i> [1] <i>ac</i> [1] <i>w</i> [1118]; <i>P</i> { <i>w</i> [+ <i>mC</i> ]= <i>GAL4-ac.13</i> }1	Bloomington Stock Center	#8715
<i>D. melanogaster</i> <i>sc</i> <sup>1</sup> <i>y</i> [1] <i>sc</i> [1]	Bloomington Stock Center	#176
<i>D. melanogaster</i> <i>ac</i> <sup>sbm</sup> <i>ac</i> [ <i>sbm</i> ]	Given by P. Simpson	N/A
<i>D. melanogaster</i> <i>ac</i> <sup>Hw-1</sup> <i>Df</i> (1) <i>sc</i> 10-1, <i>sc</i> [10-1]/ <i>y</i> [1] <i>ac</i> [ <i>Hw-1</i> ]	Bloomington Stock Center	#109
<i>D. melanogaster</i> <i>sc</i> <sup>29</sup> <i>In</i> (1) <i>sc</i> [29], <i>sc</i> [29] <i>w</i> [ <i>a</i> ] <i>eag</i> [ <i>sc</i> 29]	Bloomington Stock Center	#1442
<i>D. melanogaster</i> <i>ac</i> <sup>1</sup> <i>sc</i> <sup>1</sup> <i>y</i> [1] <i>ac</i> [1] <i>sc</i> [1] <i>pn</i> [1]	Bloomington Stock Center	#4596
<i>D. melanogaster</i> <i>sc</i> <sup>H</sup> <i>C</i> (1) <i>DX</i> , <i>y</i> [1] <i>f</i> [1]; <i>T</i> (1;4) <i>sc</i> [ <i>H</i> ], <i>sc</i> [ <i>H</i> ]	Bloomington Stock Center	#4055
<i>D. melanogaster</i> <i>sc</i> <sup>9</sup> <i>In</i> (1) <i>sc</i> [9], <i>sc</i> [9] <i>w</i> [ <i>a</i> ] <i>f</i> [1] <i>Bx</i> [1]	DGRC Kyoto Stock Center	#102028
<i>D. melanogaster</i> <i>sc</i> <sup>S2</sup> <i>T</i> (1;2) <i>sc</i> [ <i>S2</i> ], <i>y</i> [+] <i>sc</i> [ <i>S2</i> ]: <i>cn</i> [1] <i>M</i> (2)53[1]/+; <i>CyO</i>	Bloomington Stock Center	#3333
<i>D. melanogaster</i> <i>sc</i> <sup>7</sup> <i>Df</i> (1) <i>B</i> / <i>In</i> (1) <i>sc</i> [7], <i>In</i> (1) <i>AM</i> , <i>sc</i> [7] <i>ptg</i> [4]	Bloomington Stock Center	#723
<i>D. melanogaster</i> <i>ac</i> <sup>3</sup> <i>sc</i> <sup>10-1</sup> <i>In</i> (1) <i>ac</i> [3], <i>sc</i> [10-1] <i>ac</i> [3] <i>w</i> [1] <i>sable</i> [1]/ <i>FM7i</i> , <i>P</i> { <i>w</i> [+ <i>mC</i> ]= <i>ActGFP</i> } <i>JMR3</i>	Bloomington Stock Center	#36541
<i>D. melanogaster</i> <i>sc</i> <sup>4</sup> <i>In</i> (1) <i>sc</i> [4], <i>y</i> [1] <i>sc</i> [4] <i>ABO-X</i> [1]	Bloomington Stock Center	#789

<i>D. melanogaster</i> sc <sup>8</sup> T(1;3)sc[260-15], sc[260-15]/FM6 B[1] dm[1] sc[8] y[31d]	Bloomington Stock Center	#842
<i>D. melanogaster</i> ac <sup>1</sup> sc <sup>19</sup> Df(1)sc[19]/y[1] ac[1]; Dp(1;2)sc[19]/In(2L)Cy, S[2] Cy[1]	Bloomington Stock Center	#3822
<i>D. melanogaster</i> VT054793-GAL4	Vienna Drosophila Research Center	VT054793
<i>D. melanogaster</i> VT054794-GAL4	Vienna Drosophila Research Center	VT054794
<i>D. melanogaster</i> VT054795-GAL4	Vienna Drosophila Research Center	VT054795
<i>D. melanogaster</i> VT054796-GAL4	Vienna Drosophila Research Center	VT054796
<i>D. melanogaster</i> VT054798-GAL4	Vienna Drosophila Research Center	VT054798
<i>D. melanogaster</i> VT054799-GAL4	Vienna Drosophila Research Center	VT054799
<i>D. melanogaster</i> GMR14C10-GAL4	Janelia Research Campus	GMR14C10
<i>D. melanogaster</i> GMR15B10-GAL4	Janelia Research Campus	GMR15B10
<i>D. melanogaster</i> GMR15C11-GAL4	Janelia Research Campus	GMR15C11
<i>D. melanogaster</i> GMR15X09-GAL4	This paper	N/A
<i>D. melanogaster</i> VT054805-GAL4	Vienna Drosophila Research Center	VT054805
<i>D. melanogaster</i> GMR15A01-GAL4	Janelia Research Campus	GMR15A01
<i>D. melanogaster</i> GMR14C12-GAL4	Janelia Research Campus	GMR14C12
<i>D. melanogaster</i> GMR15A04-GAL4	Janelia Research Campus	GMR15A04
<i>D. melanogaster</i> GMR15C10-GAL4	Janelia Research Campus	GMR15C10
<i>D. melanogaster</i> GMR15E07-GAL4	Janelia Research Campus	GMR15E07
<i>D. melanogaster</i> GMR15E09-GAL4	Janelia Research Campus	GMR15E09
<i>D. melanogaster</i> GMR13D04-GAL4	Janelia Research Campus	GMR13D04
<i>D. melanogaster</i> GMR13C08-GAL4	Janelia Research Campus	GMR13C08
<i>D. melanogaster</i> GMR12H02-GAL4	Janelia Research Campus	GMR12H02
<i>D. melanogaster</i> GMR13B12-GAL4	Janelia Research Campus	GMR13B12
<i>D. melanogaster</i> VT054820-GAL4	Vienna Drosophila Research Center	VT054820
<i>D. melanogaster</i> VT054821-GAL4	Vienna Drosophila Research Center	VT054821
<i>D. melanogaster</i> VT054822b-GAL4	This paper	N/A
<i>D. melanogaster</i> VT054823-GAL4	Vienna Drosophila Research Center	VT054823
<i>D. melanogaster</i> VT054824-GAL4	Vienna Drosophila Research Center	VT054824
<i>D. melanogaster</i> VT054825-GAL4	Vienna Drosophila Research Center	VT054825
<i>D. melanogaster</i> VT054826-GAL4	Vienna Drosophila Research Center	VT054826



<i>D. melanogaster</i> VT054827-GAL4	Vienna Drosophila Research Center	VT054827
<i>D. melanogaster</i> VT054828-GAL4	Vienna Drosophila Research Center	VT054828
<i>D. melanogaster</i> VT054829-GAL4	Vienna Drosophila Research Center	VT054829
<i>D. melanogaster</i> VT054831-GAL4	Vienna Drosophila Research Center	VT054831
<i>D. melanogaster</i> VT054832-GAL4	Vienna Drosophila Research Center	VT054832
<i>D. melanogaster</i> GMR18G09-GAL4	Janelia Research Campus	GMR18G09
<i>D. melanogaster</i> VT054833-GAL4	Vienna Drosophila Research Center	VT054833
<i>D. melanogaster</i> GMR18E07-GAL4	Janelia Research Campus	GMR18E07
<i>D. melanogaster</i> VT054834-GAL4	Vienna Drosophila Research Center	VT054834
<i>D. melanogaster</i> GMR19D04-GAL4	Janelia Research Campus	GMR19D04
<i>D. melanogaster</i> VT054835-GAL4	Vienna Drosophila Research Center	VT054835
<i>D. melanogaster</i> VT054836-GAL4	Vienna Drosophila Research Center	VT054836
<i>D. melanogaster</i> GMR18C05-GAL4	Janelia Research Campus	GMR18C05
<i>D. melanogaster</i> GMR19B11-GAL4	Janelia Research Campus	GMR19B11
<i>D. melanogaster</i> VT054838-GAL4	Vienna Drosophila Research Center	VT054838
<i>D. melanogaster</i> GMR18G07-GAL4	Janelia Research Campus	GMR18G07
<i>D. melanogaster</i> VT054839-GAL4	Vienna Drosophila Research Center	VT054839
<i>D. melanogaster</i> GMR18F05-GAL4	Janelia Research Campus	GMR18F05
<i>D. melanogaster</i> VT054840-GAL4	Vienna Drosophila Research Center	VT054840
<i>D. melanogaster</i> VT054841-GAL4	Vienna Drosophila Research Center	VT054841
<i>D. melanogaster</i> GMR19A06-GAL4	Janelia Research Campus	GMR19A06
<i>D. melanogaster</i> VT054842-GAL4	Vienna Drosophila Research Center	VT054842
<i>D. melanogaster</i> GMR18E10-GAL4	Janelia Research Campus	GMR18E10
<i>D. melanogaster</i> VT054843-GAL4	Vienna Drosophila Research Center	VT054843
<i>D. melanogaster</i> GMR20B05-GAL4	Janelia Research Campus	GMR20B05
<i>D. melanogaster</i> VT054845-GAL4	Vienna Drosophila Research Center	VT054845
<i>D. melanogaster</i> VT054846-GAL4	Vienna Drosophila Research Center	VT054846
<i>D. melanogaster</i> VT054839mel-BL2057-GAL4	This paper	VT054839mel- BL2057
<i>D. melanogaster</i> VT054839yak-GAL4	This paper	N/A
<i>D. melanogaster</i> VT054839san-GAL4	This paper	N/A

<i>E. coli</i> One Shot® TOP10	Invitrogen	#C404003
<i>E. coli</i> NEB10-beta	New England Biolabs	#C3019H
<i>E. coli</i> BL21 (DE3)	Nicolas Joly	N/A
<b>Oligonucleotides</b>		
Primers	This paper	Figshare: <a href="https://doi.org/10.6084/m9.figshare.6972740.v3">https://doi.org/10.6084/m9.figshare.6972740.v3</a>
<b>Recombinant DNA</b>		
pBPGUw	Addgene, [66]	#17575
pBPSUw: GAL4 cassette of pBPGUw replaced by <i>scute</i> CDS.	This paper	N/A
15X09-pBPGUw	This paper	N/A
VT054822b-pBPGUw	This paper	N/A
VT054839yak-pBPGUw	This paper	N/A
VT054839san-pBPGUw	This paper	N/A
18C05_T7-pBPGUw	This paper	N/A
18C05_BL2057-pBPGUw	This paper	N/A
18C05Yakfull-pBPGUw	This paper	N/A
18C05Sanfull-pBPGUw	This paper	N/A
18C05AmelBL-pBPGUw	This paper	N/A
18C05BmelBL-pBPGUw	This paper	N/A
18C05CmelBL-pBPGUw	This paper	N/A
18C05ABmelBL-pBPGUw	This paper	N/A
18C05BCmelBL-pBPGUw	This paper	N/A
18C05Asan-pBPGUw	This paper	N/A
18C05Bsan-pBPGUw	This paper	N/A
18C05Ayak-pBPGUw	This paper	N/A
18C05Byak-pBPGUw	This paper	N/A
18C05Cyak-pBPGUw	This paper	N/A
VT054839mel-BL2057-pBPGUw	This paper	N/A
VT054839yak-pBPGUw	This paper	N/A
VT054839san-pBPGUw	This paper	N/A
18C05_SSSY-pBPSUw	This paper	N/A
18C05_YYSS-pBPSUw	This paper	N/A
18C05_SSYS-pBPSUw	This paper	N/A
18C05_SYYY-pBPSUw	This paper	N/A
18C05_YYYS-pBPSUw	This paper	N/A
18C05_SSYY-pBPSUw	This paper	N/A
18C05_YYSY-pBPSUw	This paper	N/A
18C05_YSYY-pBPSUw	This paper	N/A
18C05_YSSY-pBPSUw	This paper	N/A
18C05yakT970A-pBPSUw	This paper	N/A
18C05YakT1429G-pBPSUw	This paper	N/A
18C05yakA1507G-pBPSUw	This paper	N/A
18C05yakt1775G-pBPSUw	This paper	N/A
18C05_Anc-pBPSUw	This paper	N/A
18C05_AncT1008C-pBPSUw	This paper, GeneScript	N/A
18C05_AncT1429G-pBPSUw	This paper, GeneScript	N/A
18C05_AncT1482C-pBPSUw	This paper, GeneScript	N/A
18C05_AncA1507G-pBPSUw	This paper, GeneScript	N/A
18C05_AncT1775G-pBPSUw	This paper, GeneScript	N/A
18C05_yakG869A-pBPSUw	This paper, GeneScript	N/A
18C05_yakT1008C-pBPSUw	This paper, GeneScript	N/A

18C05_yakT1482C-pBPSUw	This paper, GeneScript	N/A
18C05_AncG869A-pBPSUw	This paper, GeneScript	N/A
18C05_AncT670G-pBPSUw	This paper, GeneScript	N/A
18C05_Anc-SNPall-pBPSUw	This paper, GeneScript	N/A
Abd-B-HD-pGEX-4T-1	S. B. Carroll, [46]	N/A
<b>Software and Algorithms</b>		
Nebuilder Tools	New England Biolabs	<a href="https://nebuilder.neb.com/">https://nebuilder.neb.com/</a>
Jaspar	[43]	<a href="http://jaspar.genereg.net">http://jaspar.genereg.net</a>
R 3.4	[60]	<a href="https://cran.r-project.org/">https://cran.r-project.org/</a>
ImageJ	[37]	<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
Geneious	Biomatters Ltd.	<a href="https://www.geneious.com/download/">https://www.geneious.com/download/</a>

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