Supporting File S1

Daniel F. Paulo *et al* ., Specific Gene Disruption in the Major Livestock pests *Cochliomyia hominivorax* and *Lucilia cuprina* using CRISPR/Cas9

Content:

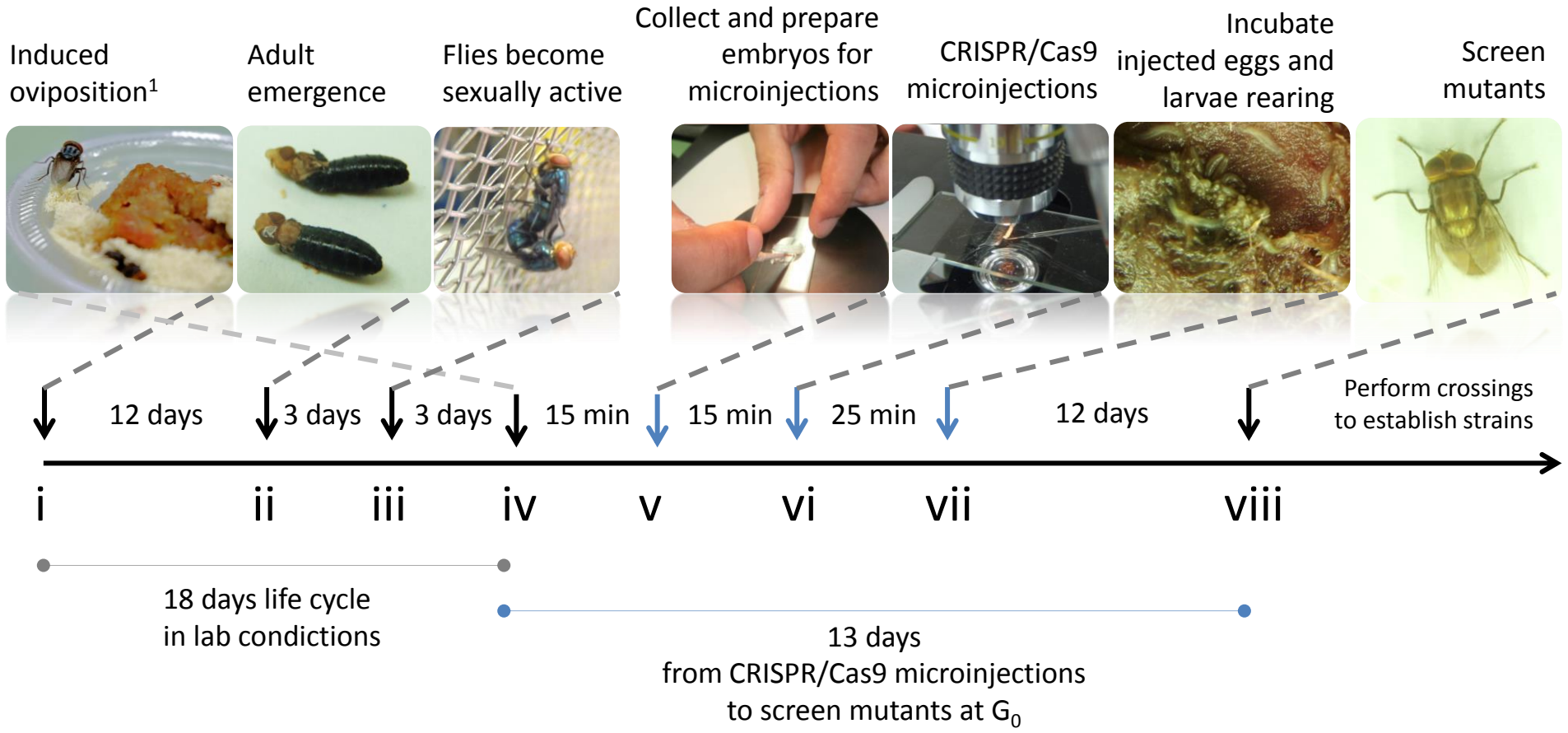
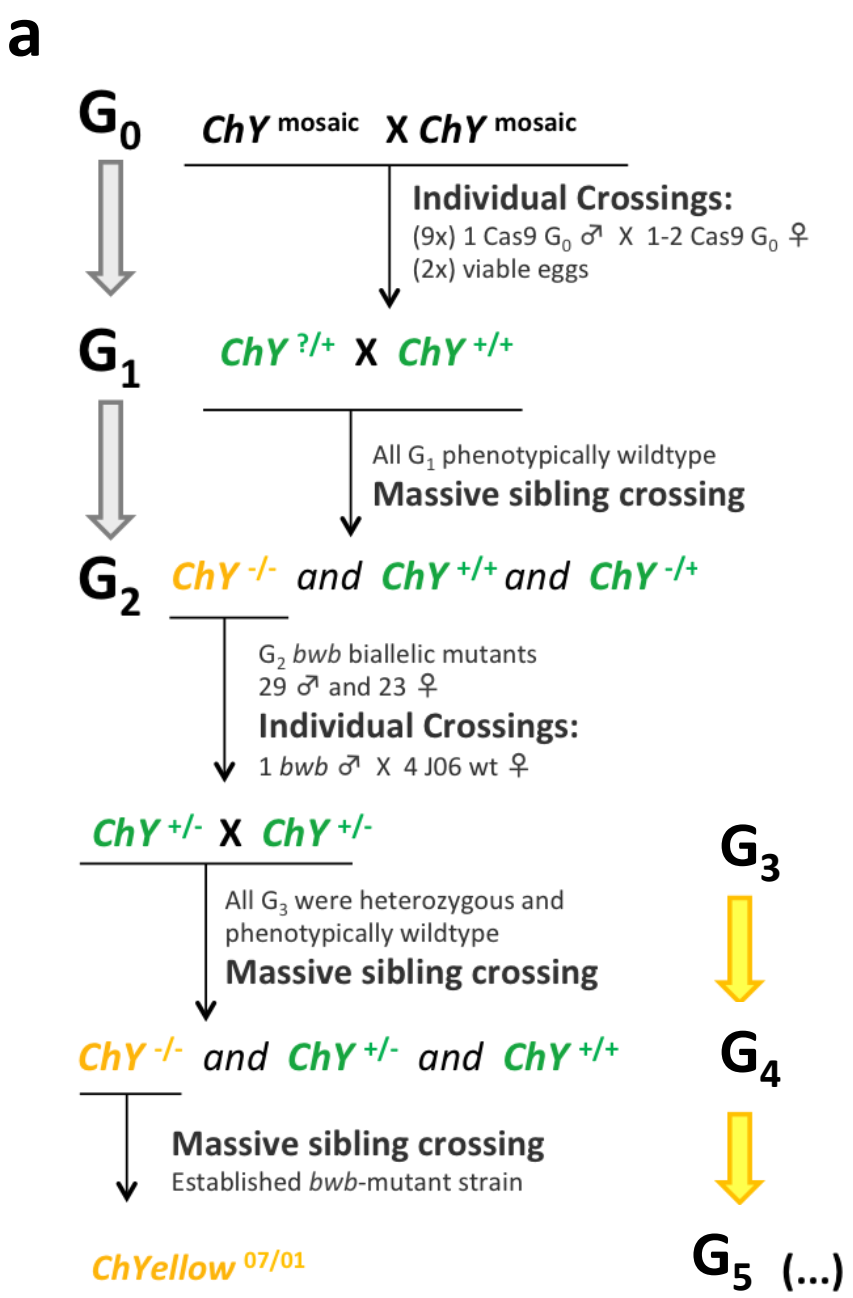


Figure S1. Schematic timeline of *Cochliomyia hominivorax* rearing and CRISPR/Cas9 microinjection experiments. The New World Screwworm fly takes 18 days to mature from eggs to adult in laboratory conditions (gray line: i to iv). Females are ready to lay eggs six days after emergence (i and iv). First instar larvae hatch ~12 hrs later, passing through three larval instars before dropping to the ground to pupate. Adults emerge 12 days after oviposition (ii) and females become sexually receptive three days after emergence (iii). For microinjections, fresh screwworm embryos are collected (iv), dissociated, aligned and dehydrated (v). Microinjections of CRISPR/Cas9 cocktail are performed into the posterior end of the pre-blastoderm embryos within the first 40 min of development (vi). After hatching, immatures are reared to develop until adulthood (vii) and emerged injected G_0 adults are subsequently screened for Cas9-induced mutations (viii). This entire experiment takes approximately 13 days to complete (blue line: iv to viii). [1]Photograph by Jorge Herrera.



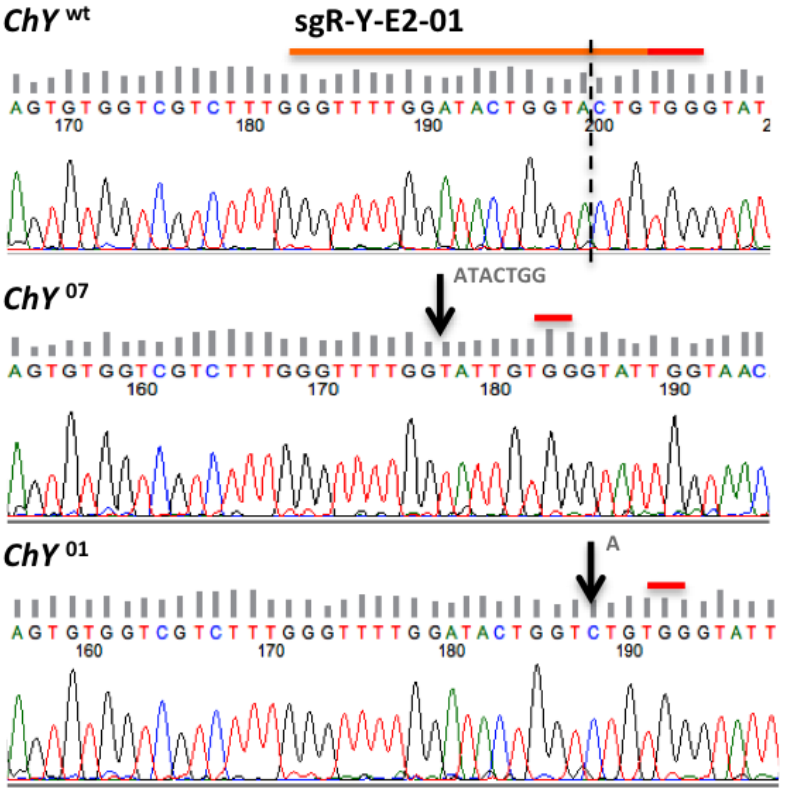


Figure S2. Crossing scheme used to establish the biallelic *bwb*-mutant strain of the screwworm fly. (a) Mosaic *bwb*-mutant flies (Fig. 1B, second column) were obtained after microinjections of Cas9 RNPs against the *ChYellow* loci. Nine individual crossings were made aiming to evaluate the inheritance capability of the Cas9-induced mutations in the *ChY* gene, but only 2 resulted in viable ovipositions. All G_1 flies were phenotypically wildtype, presumably heterozygous for the *bwb* mutation. As the *bwb* mutation is recessive and autosomal in *C. hominivorax*, only mutations in both copies of the *ChY* gene will result in the unpigmented body phenotype. Thus, massive sibling crossings were carried out, and biallelic *bwb* mutant flies were obtained at G_2 generation (Fig. 1B, last two columns). A *bwb* male was randomly selected and backcrossed to wildtype females (yellow arrows). The offspring were let to interbreed freely in cages and the obtained biallelic *bwb*-mutants used to establish the strain *ChYellow*$^{07/01}$, currently being maintained at the COPEG biosecurity plant in Panama-PA. **(b)** Molecular genotyping of *ChYellow*$^{07/01}$.

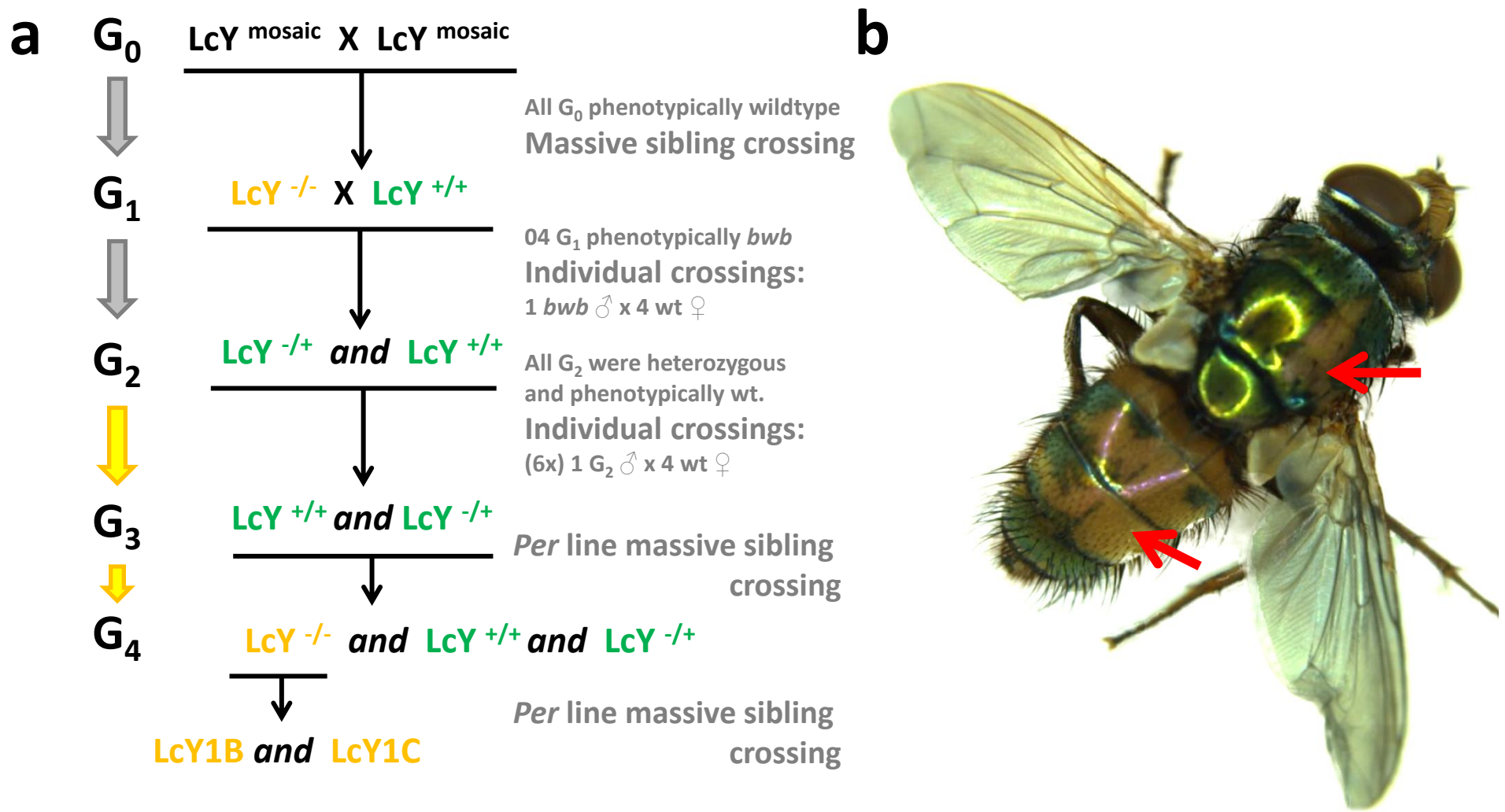


Figure S3. Cas9-mediated disruption of the *LcY* locus in the Australian sheep blowfly, *Lucilia cuprina*. (a) Crossing scheme used to establish the homozygous *bwb*-mutant strains LcY1B and LcY1C of *L. cuprina*. Briefly, all G_0 adults developed from microinjections were let to inbreed freely in cages, and from their offspring 4 adults showed the *bwb*-mutant phenotype. Molecular genotyping confirmed that mutants carried deletions in the *LcY* gene (Figure 2B). Since the *bwb*-mutants are likely to host different mutated alleles, *bwb* males at G_1 were individually backcrossed to wildtype females, followed by a second backcross at G_2. Massive sibling crosses were then carried out at G_3 and any G_4 flies that showed the *bwb* phenotype were inbred to establish the homozygous mutant strains. **(b)** The presence of lightly pigmented body areas in the G_0 mosaic flies (indicated by red arrows) reveals biallelic hits in the *LcY* loci after microinjections using the Cas9 purified protein instead of the Cas9 mRNA.

a

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Drosophila      MFQDKGWI---LVTLITLVTPSWAAYKLQERYSWSQLDFAFPNTRLKDQALASGDYIPQN   57
Bactrocera      MQANLRLTLRNLVAWLCLVAHAQATYKLQERYSWTELDFAFPNQGLKQQALASGDYIPQN   60
Ceratitis       MFANSHLSLRSIVACLCLVAHAQAAYKLQERYSWTQMDFAFPNPGLKQQALASGDYIPQN   60
Lucilia         ----MNCFLFSLLAVVCCVNYAFATYKLQERYSWNQLDFAFPNDQLKQQAIASGDYIPQN   56
Cochliomyia     ----MNCFKFSLLAFACCLSYVSGAYKLQERYSWTQLDFAFPNEQLKQQAIASGDYIPQN   56
Stomoxys        ----MKCFLVGLLTFVLCVQFGYATYKLQERFNWKQLDFAFPNENLKQQALASGDYIPQN   56
Musca           ----MKCLLVGLLTFVLCIHFGNAAYKLQERFNWKQLDFAFPNENLKQQALASGDYIPQN   56
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Drosophila      ALPVGVEHFGNRLFVTVPRWRDGIPATLTYINMDRSLTGSPELIPYPDWRSNTAGDCANS   117
Bactrocera      ALPVGVEHWGNRLFVTVPRWRDGIPATLTYINMDHSVTGSPELIPYPDWRSNTAGDCANS   120
Ceratitis       ALPVGMEHWGNRLFVTVPRWRDGIPATLTYINMDHSATGSPELIPYPDWRSNTAGDCANS   120
Lucilia         ALPVGVEHWGNRLFVTVPRWRDGIPATLTYINMDHSVTGSPALIPYPDWRSNTAGDCANS   116
Cochliomyia     ALPVGVEHWGNRLFVTVPRWRDGIPATLTYINMDHSVTGSPALIPYPDWRSNTAGDCANS   116
Stomoxys        ALPVGVEHWQNRLFVTVPRWRDGIPATLTYINMEHSVTGSPALIPYPDWRSNTAGDCANG   116
Musca           ALPVGVEHWQNPLFVTIPRWRDGIPATLTYINMDHSVTGSPALIPYPDWRSNTAGDCANS   116
                *****:**: * ****:****************::* **** *****************.

Drosophila      ITTAYRIKVDECGRLWVLDTGTVGIGNTTTNPCPYAVNVFDLTTDTRIRRYELPGVDTNP   177
Bactrocera      ITTAYRIKADECGRLWVLDTGTVGIGNTTTNPCPYAVNVFDLQTNTRIRRYVLRADDTNA   180
Ceratitis       ITTAYRIRADECGRLWVLDTGTVGIGNTTTNPCPYAVNVYDLQTNTRIRHYELRAEDTNA   180
Lucilia         ITTAYRIQADECGRLWVLDTGTVGIGNTTTNPCPYAVNVFDLQTNTRIRRYELRADDTNA   176
Cochliomyia     ITTAYRIQVDECGRLWVLDTGTVGIGNTTTNPCPYAVNVFDLQTNTRIRRYELRPDDTNA   176
Stomoxys        ITTAYRIKVDECGRLWVLDTGTIGIGNTTTNPCPYAVNVFDLATHTRIRRYELNPEDTNA   176
Musca           ITTAYRIKVDECGRLWVLDTGTLGIGNTTTNPCPYAVNVFDLATHTRIRRYELRPEDTNA   176
                *******:.************:***************:** *.****:* *   ***

Drosophila      NTFIANIAVDIGKNCDDAYAYFADELGYGLIAYSWELNKSWRFSAHSYFFPDPLRGDFNV   237
Bactrocera      NTFIANVAVDIGKSCDDAFAYFSDELGYGLVVYSWEQNKSWRFSAHSYFFPDPLRGDFNI   240
Ceratitis       NTFVANIAVDIGKSCDDAFAYFSDELGYGLIVYSWEQNKSWRFSAHSYFFPDPLRGDFNI   240
Lucilia         NTFIANIAVDIGKSCDDAFAYFSDELGYGLIAYSWEQNKSWRFSAHSFFFPDPLRGDYNI   236
Cochliomyia     NSFIANIAVDIGKSCDDAFAYFSDELGYGLIAYSWEQNKSWRFSAHSFFFPDPLRGDYNI   236
Stomoxys        NTFIANIAVDIGKSCDDAFAYFSDELGYGLISYSWEQNKSWRFSGHSYFFPDPLRGDYNI   236
Musca           NTFIANIAVDIGKSCDDAFAYFSDELGYGLIAYSWEQNKSWRFSGHSYFFPDPLRGDYNI   236
                *:*:**:******.****:***:*******: **** *******.**:*********:*:

Drosophila      AGINFQWGEEGIFGMSLSPIRSDGYRTLYFSPLASHRQFAVSTRILRDETRTEDSYHDFV   297
Bactrocera      AGLNFQWGEEGIFGMSLSPIRSDGYRTMFFSPLASHRQFAVSTRILRDESRVEDSFHDFV   300
Ceratitis       AGLNFQWGEEGIFGMALSPIRSDGYRTMFFSPLASHRQFAVSTRILRDESRVEDSYHDFV   300
Lucilia         AGLNFQWGEEGIFGMSLSPLRSDGFRTMYFSPLASHRQFAVSTRILRDESRVEDSFHDFV   296
Cochliomyia     AGLNFQWGEEGIFGMSLSPLRSDGYRTMYFSPLASHRQFAVSTRILRDESRVEDSFHDFV   296
Stomoxys        AGLNFQWGEEGIFGMALSPLRSDGFRTMYFSPLASHRQFAVSTRILRDESRVEDSYHDFI   296
Musca           AGLNFQWGEEGIFGMALSPIRSDGYRTMYFSPLASHRQFAVSTRILRDETRVEDSYHDFL   296
                **:************:***:****:**::********************:*.***:***:

Drosophila      ALDERGPNSHTTSRVMSDDGIELFNLIDQNAVGCWHSSMPYSPQFHGIVDRDDVGLVFPA   357
Bactrocera      VLDERGPNSHTTSRVMSDDGIELFNLIDQNAVGCWHSSMPYQPQFHGVVDRDDVGLVFPA   360
Ceratitis       ALDERGPNSHTTSRVMSDDGVELFNLIDQNAVGCWHSSMPYTSQFHGVVDRDDVGLVFPA   360
Lucilia         ALDERGPNAHTTSHVMSDDGIELFNLIDQNAVGCWHSSMPYSPQFHGIVDRDDVGLVFPA   356
Cochliomyia     ALDERGPNSHTTSHVMSDDGIELFNLIDQNAVGCWHSSMPYSPQFHGIVDRDDVGLVFPA   356
Stomoxys        ALDERGPNSHTTARVMSDDGVELFDLIDQNAVGCWHSSMPYSPQFHGIVDRDDVGLVFPA   356
Musca           ALDERGPNSHTTARVMSEDGVELFNLIDQNAVGCWHSSMPYSPQFHGIVDRDDVGLVFPA   356
                .*******:***::***:**:***:***************  ****:************

Drosophila      DVKIDENKNVWVLSDRMPVFLLSDLDYSDTNFRIYTAPLATLIENTVCDLRN-NAYGPPN   416
Bactrocera      DVKIDENKNVWVLSDRMPVFLISDLDYNDVNFRIYTAPLSALIDNTVCDIRN-NAYGPSN   419
Ceratitis       DIKIDENKNVWVLSDRMPVFLITELDYNDVNFRIYTAPLSSLIENTVCDIRN-SAYGPSN   419
Lucilia         DVIIDENKNVWVLSDRMPVFLLAELDYNDVNFRIYTAPLSTLIEGTVCDQR-SAVYGPSN   415
Cochliomyia     DVIIDETKNVWVLSDRMPVFLLAELDFSDVNFRIYTAPLSTLIEGTVCDQR-SAVYGPSN   415
Stomoxys        DVKIDENKNVWVLSDRMPVFLLSELDYNDINFRIYTAPLSTLIEGTVCDQRTSSVYGPPN   416
Musca           DVKIDENKDVWVLSDRMPVFLLSELDYNDVNFRIYTAPLSTLIEGTVCDQR-SNVYGPHN   415
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Drosophila      TVSI---------------------------PKQAVLPMGPPLYTKQYRPVLPQKPQTS   448
Bactrocera      AVGSTSFYATPTSTNSVFGTQLGFVGNKVYGPPNPVGLPKSQTLFTKQYLPPLSIKPSIQ   479
Ceratitis       SVGSTSFYGTPTSTNNIFGPQLNLGGNKVYGPPNPVSVPKSQTRFNKPYLPPLPTKPTLQ   479
Lucilia         AVS---------------------------------IPKQPPVYNKHYLTPLPQKPIHG   441
Cochliomyia     AVS---------------------------------IPKQNPLYNKQYVPSLTQKPSQG   441
Stomoxys        SVA--------------------------------AVKPQFPLYTKQYLPPAPAKPTII   443
Musca           SVA--------------------------------AVKPLHPIYPKTYLNPTAK-----   437
                :*.                                :      : * *

Drosophila      WA-SSPPPPSRTYLPANSGNVVSSISVSTNSVGPAGVEVPKAYIFNQ-HNGINYET--SG   504
Bactrocera      ADVPRVAPPSRNYLPPLMGTGYST---------TQRSDPAKAYVFN---NGLSYETGIGG   527
Ceratitis       ADIPRVAPPSRNYLPPLMGTGYST---------TQRSDAAKAYVFN---NGLSYEAGVGG   527
Lucilia         TVIQPIAHPTPNYLPSYA--------------SQKVDVPKTFVYNQ-QNGLTYEAS-NG   484
Cochliomyia     TYIQHAALPSPTYLSSYA--------------SQKVDVPKTFVYNQ-QNGLTYEAS-NG   484
Stomoxys        QHHVPISSARPTYLPPYSG-------------SQRLNVPSAFVYNQ-HNGLSYEAS-NG   487
Musca           PSYVAIPSSRPSYLPPYSG-------------SQRPNVPNAFLYNQQHNALTYDAA-NG   482
                          .**                        :  .::::*   *.:.*::  .*

Drosophila      PHLFPTHQPAQP-----GGQDGGLKTYVNARQSGWWHHQHQG     541
Bactrocera      PHLFPL-------------HSEGLKNYVTTRNSGWWTH----     552
Ceratitis       PHLFPL-------------HSEGLRNYVSARTSGWWLEH---     553
Lucilia         PHLFPPIQL---TAHTSVSQPDGLKNYVTARSSNWWRHHHH-     522
Cochliomyia     PHLFPPLQL---THQ--TVQPDGLKNYVSARSSNWWRHHHPX     521
Stomoxys        PHLFPAIAHIQQIQHTA--QPEGLGSYATSRNTPWWKRQ---     524
Musca           PHLFPAIAQIQQIQHPAPAAREGLGSYATSRSVPWWQQHH--     522
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b

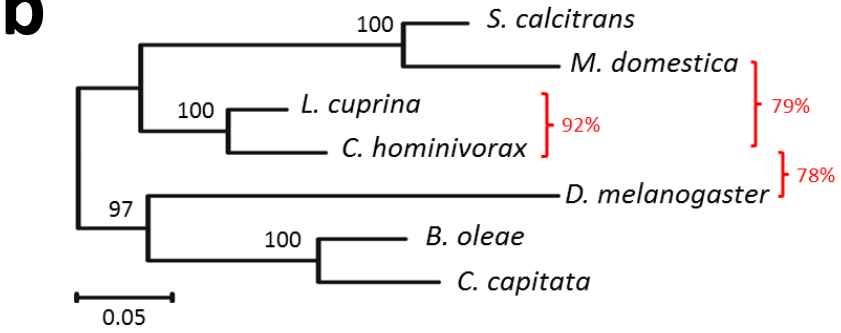

Figure S4. Orthology analysis of the *yellow* gene found in Schizophora species. (a) Multiple sequence alignment of yellow protein from *Drosophila melanogaster* (NP_476792.1), *Bactrocera oleae* (XP_014092322.1), *Ceratitis capitata* (XP_004521097.1), *Lucilia cuprina* (XP_023305007.1), *Cochliomyia hominivorax* (M.J.Scott, unpublished), *Stomoxys calcitrans* (XP_013102593.1) and *Musca domestica* (XP_011290952.1). Major Royal Jelly Protein (MRJP; pfam03022) conserved domain is highlighted in the yellow box **(b)** Evolutionary relantionship of yellow protein reconstructed by the Maximum Likelihood method under the JTT+G+I substitution model. Node supports were estimated under 100 replicates of bootstrap. Sequence identity between key species are shown in red.

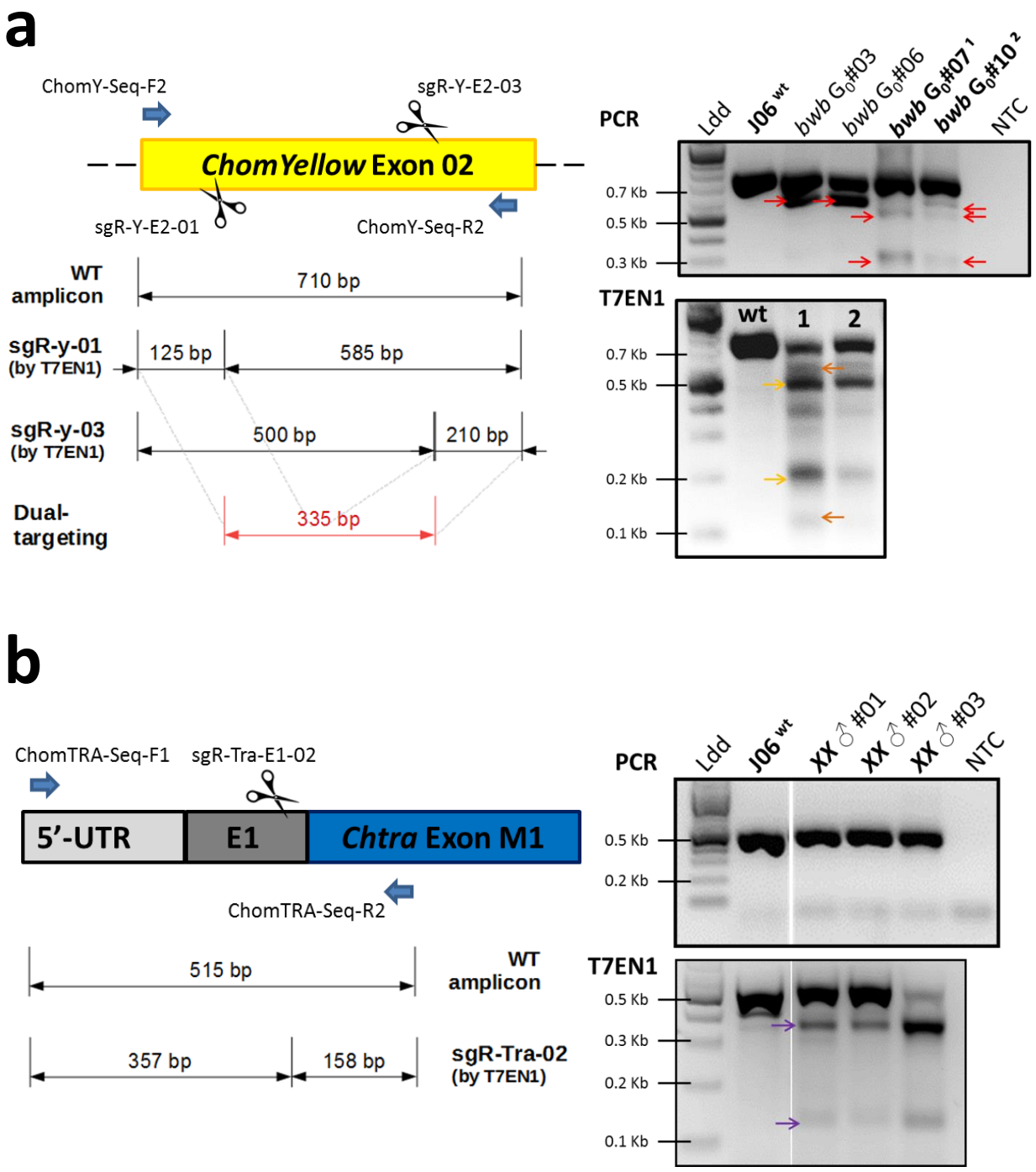

Figure S5. T7 endonuclease 1 assay (T7EN1) of Cas9 targeted sites in the screwworm genome. (a) Schematic of CRISPR strategy used to knockout the *ChY* loci (leftmost), showing the positions of each sgRNAs (scissors), PCR genotyping primers (blue arrows), the PCR amplicon size for wildtype (wt) and the expected band migration after the T7EN1 assay. (rightmost) PCR amplifications of *ChY* targeted site on J06 wt and selected *bwb* mosaic flies obtained at G_0 (above). Putative medium to large deletions (red arrows) were detected in these amplifications, presumably due to the *dual-targeting* approach (see alleles in Figure 1C and S5). Only crosses made with the male flies #07 and #10 resulted in viable G_1 eggs (see Results), thus these two flies were submitted to the T7EN1 assay (bellow), which revealed the presence of indels at the specific Cas9 targeted sites of the used sgRNAs: sgR-Y-E2-01 (orange arrows) and sgR-Y-E2-03 (yellow arrows). **(b)** (leftmost) Schematic of CRISPR strategy used to knockout the *Chtra* locus and expected band migration for wt and after T7EN1. (rightmost) PCR amplifications of *Chtra* targeted site (above) and results obtained by T7EN1 assay (bellow) for the intersexed flies showed in Figure 3B. Band migration patterns consistent with the expected Cas9 cleavage using the sgRNA sgR-Tra-E1-02. Other abbreviations used: Ldd = Ladder; NTC = non-template control; Kb = Kilo bases.

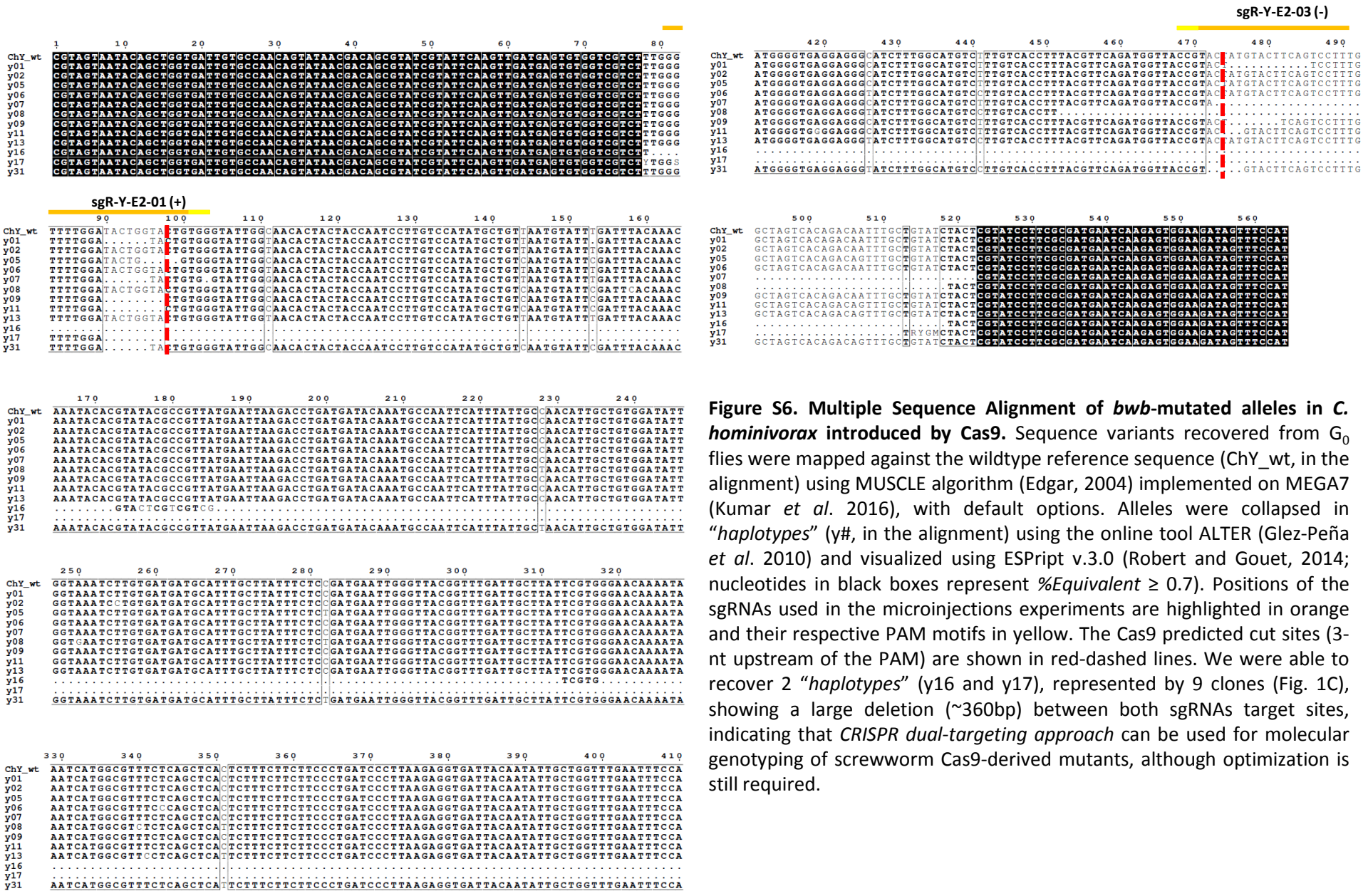

Figure S6. Multiple Sequence Alignment of *bwb*-mutated alleles in *C. hominivorax* introduced by Cas9. Sequence variants recovered from G_0 flies were mapped against the wildtype reference sequence (ChY_wt, in the alignment) using MUSCLE algorithm (Edgar, 2004) implemented on MEGA7 (Kumar *et al*. 2016), with default options. Alleles were collapsed in "*haplotypes*" (y#, in the alignment) using the online tool ALTER (Glez-Peña *et al*. 2010) and visualized using ESPript v.3.0 (Robert and Gouet, 2014; nucleotides in black boxes represent *%Equivalent* ≥ 0.7). Positions of the sgRNAs used in the microinjections experiments are highlighted in orange and their respective PAM motifs in yellow. The Cas9 predicted cut sites (3-nt upstream of the PAM) are shown in red-dashed lines. We were able to recover 2 "*haplotypes*" (y16 and y17), represented by 9 clones (Fig. 1C), showing a large deletion (~360bp) between both sgRNAs target sites, indicating that *CRISPR dual-targeting approach* can be used for molecular genotyping of screwworm Cas9-derived mutants, although optimization is still required.

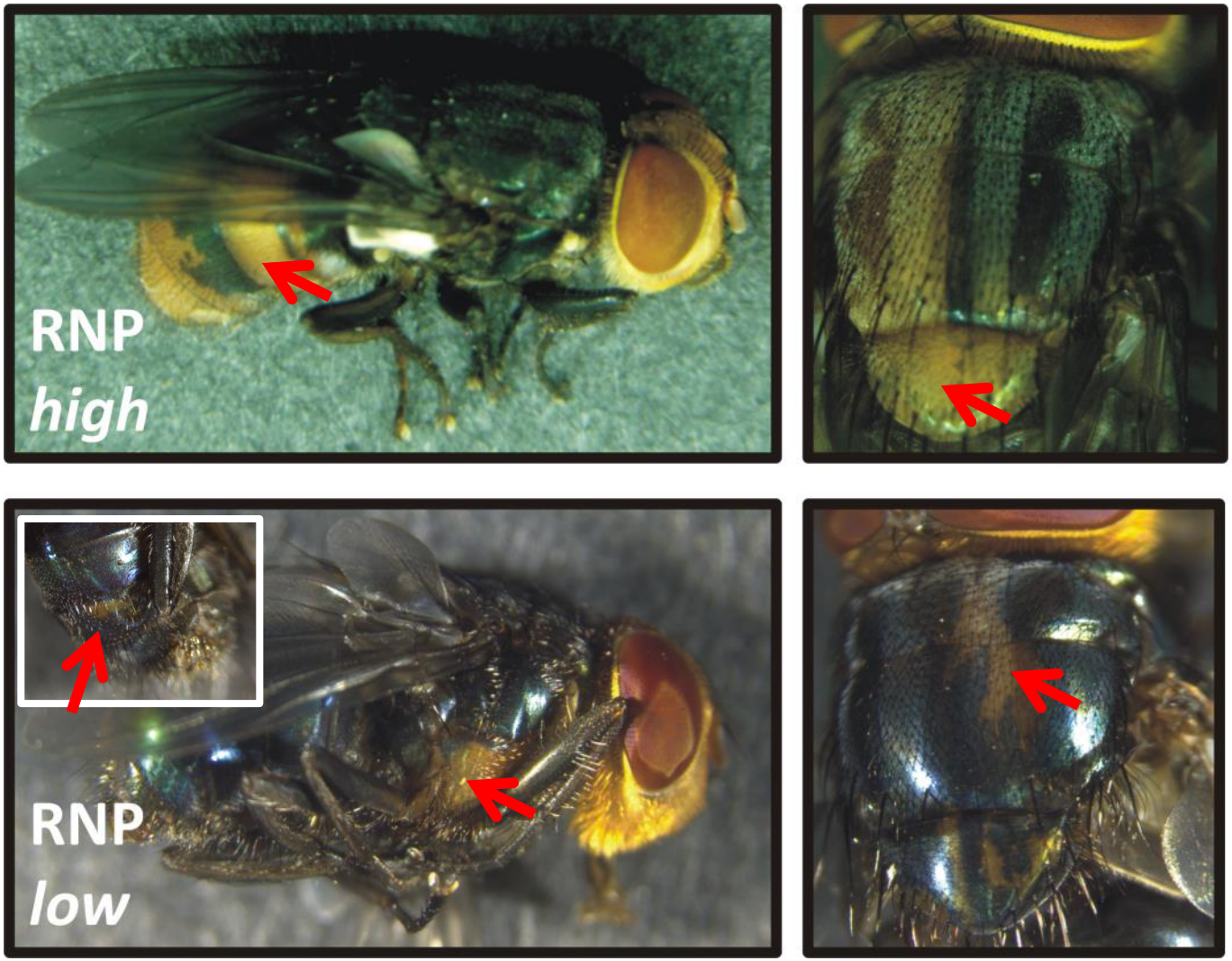

Figure S7. Phenotypic variation between mosaic *bwb* screwworm flies. Adult mutant phenotypes of individuals injected with the high (500 ng/ul) concentration of the RNPs frequently showed a larger unpigmented body area than those injected with the low (360 ng/ul) concentration (indicated by red arrows).

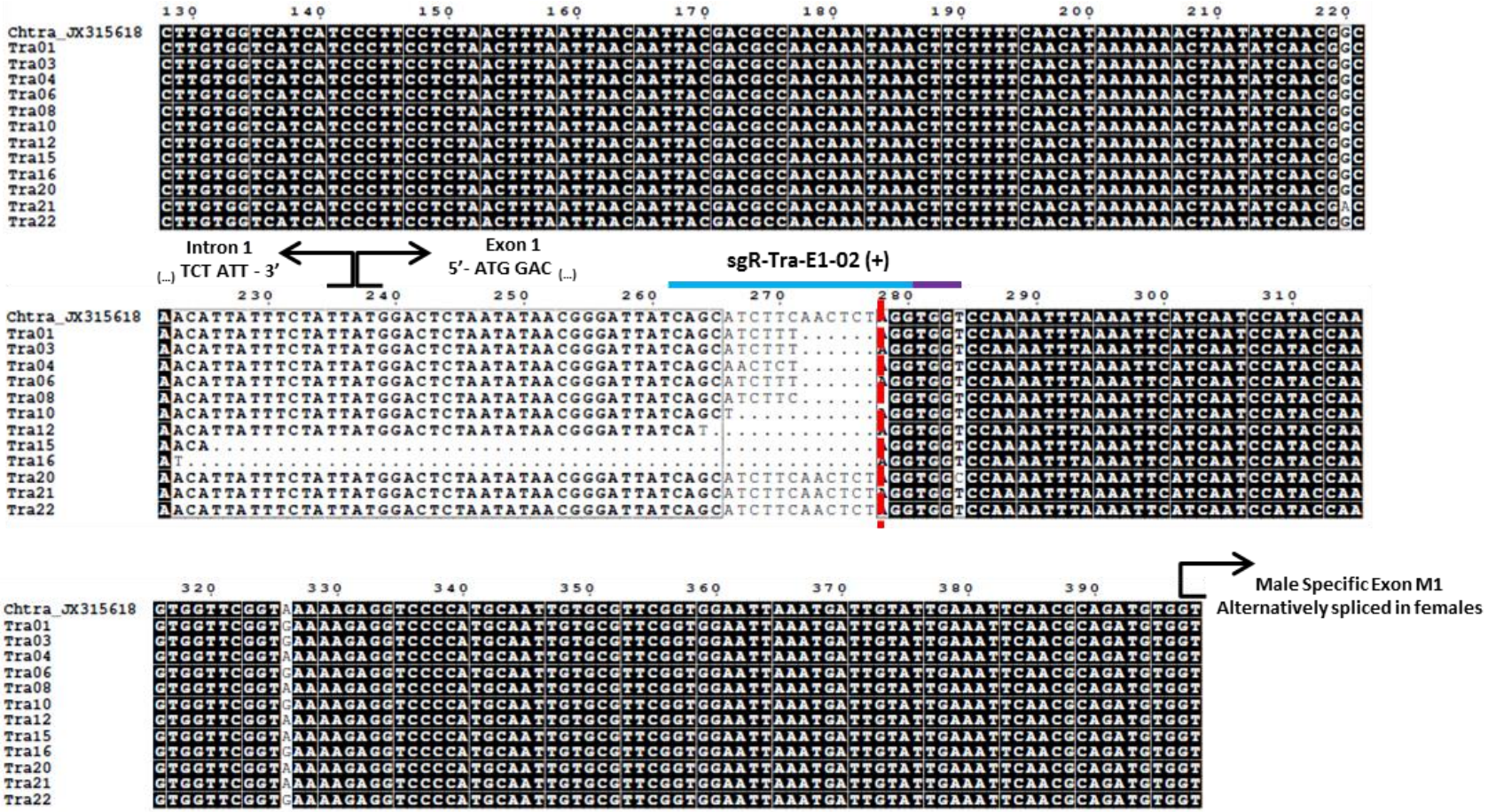

Figure S8. Multiple Sequence Alignment of *tra*-mutated alleles in *C. hominivorax* genome introduced by Cas9. Sequence variants recovered from intersexed G_0 flies (Fig. 3B) were mapped against the wildtype reference sequence (Chtra_JX315618, in the alignment) using MUSCLE algorithm (Edgar, 2004) implemented on MEGA7 (Kumar *et al*. 2016), with default options. Alleles were collapsed in "*haplotypes*" (TRA#, in the alignment) using the online tool ALTER (Glez-Peña *et al*. 2010) and visualized using ESPript v.3.0 (Robert and Gouet, 2014; nucleotides in black boxes represent *%Equivalent* ≥ 0.7). Position of the sgRNA used in the microinjections experiments is highlighted in blue and the PAM motif in purple. The Cas9 predicted cut sites (3-nt upstream of the PAM) are shown in red-dashed lines. Black arrows indicate the genomic position of *Chtra* Intron 1, Exon 1 and the start of the male specific exon M1.

Table S1. Sequences of the single guide RNAs (sgRNAs) used in this study. The sgRNAs were designed by using the standalone version of CRISPOR tool (Concordet and Haeussler 2018). Potential off-targets (see alignments bellow) were evaluated in the context of *C. hominivorax* draft assembly (A.C.M. Junqueira, unpublished) and the complete genome sequence of *L. cuprina* (Anstead *et al*. 2015). We only considered sgRNAs that have potential off-targets with more than 3 mismatches in total, with the maximum amount of mismatches present on the 5 – 10 first bases of the sgRNA directly upstream to PAM motif, which constitute the so called "*seed*" region (Andersson *et al*., 2015; Zheng et al., 2016). Syntheses of sgRNAs were performed via PCR as described by Bassett and Liu (2014), using the primers sgR-Specific-T7-FWD (5'- GAA ATT AAT ACG ACT CAC TAT A(**GG**) **[*specific sgRNA sequence without PAM*]** TTG GGT TTT AGA GCT AGA AAT AGC -3') and sgR-Universal-REV (5'- AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC -3').

Species	Target site	Guide Name	Direction	Protospacer + PAM motif	Length	Potential off-targets for 0-1-2-3-4 mismatches	mitSpecScore
Cochliomyia hominivorax	*Yellow* (*ChY*)	sgR-Y-E2-01	+	(GG)TTTTGGATACTGGTACTG TGG	20 nt	0-0-0-0-4 [a]	89
		sgR-Y-E2-03	-	AAGGACTGAAGTACATAGTA CGG	20 nt	0-0-0-0-5 [b]	69
	transformer (*Chtra*)	sgR-Tra-E1-02	+	CCATGCAATTGTGCGTTCGG TGG	20 nt	0-0-0-0-3 [c]	92
Lucilia cuprina	*Yellow* (*LcY*)	Lc-y-sgRNA2	+	AGCATAGGGGCAAGGAT TGG	17 nt	0-0-0-1-1 [d]	100
		Lc-y-sgRNA1	+	TGTTTGTAACGGTTCCCAGG TGG	20 nt	0-0-0-0-1 [e]	100

a

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sgR-Y-E2-01     GGTTTTGGATACTGGTACTG
off-target-01   GGTTTTGGtTACTGGTggaG
off-target-02   GGTaTTaGATACTGGTACgt
off-target-03   GtTTTTGGATAagGaTACTG
off-target-04   GGTTTTGGAacCTGtTcCTG
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b

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sgR-Y-E2-03     AAGGACTGAAGTACATAGTA
off-target-01   AAcGACTGAAcTACATAaaA
off-target-02   gAGGACTGAAGTAtATAtTt
off-target-03   AAGGACTGAAtTACAgtGTt
off-target-04   AAGGACTGAcGTACAatGTt
off-target-05   AAGGACaGAAGaAaAaAGTA
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c

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sgR-Tra-E1-02   CCATGCAATTGTGCGTTCGG
off-target-01   CCAaGCAATTGTGCGaTgGt
off-target-02   taATGCAATTGTGCcaTCGG
off-target-03   tCATcCAATTGTGtGTTCaG
```

d

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Lc-y-gRNA2      AGCATAGGGGCAAGGATTGG
off-target-01   AaCATAGGGGCAAGGcTTtG
off-target-02   AGCcTAaGGGCcAGGtTTGG
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e

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Lc-y-sgRNA1     TGTTTGTAACGGTTCCCAGG
off-target-01   TGTTTtTAAgGGTTaCCAGc
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Table S2. Specifications of the genotyping primers used in this study. Primers used to sample the allele variants introduced by the Cas9 RNPs on the screwworm and blowfly targeted loci. PCRs were followed by molecular cloning and Sanger sequencing. Sequenced reads were analyzed by CrispRVariants package v.1.8.0 (Lindsay et al. 2016).

Species	Targeted site	Primer Name	Sequence (5' → 3')	Length	Ta	Amplicon Size
Cochliomyia hominivorax	***Yellow* (*ChY*)** 5'-end Exon 2 (Figure 1A)	ChomY-Seq-F2	GCC CTA ATA CCG TAC CCC GA	20 nt	59.4°C	710 bp
		ChomY-Seq-R2	AGA AGA ATG CCA GCA ACC GA	20 nt		
	***transformer* (*Chtra*)** 5'-UTR to Intron M1 (Figure 3A)	ChomTRA-Seq-F1	GTC AGC AGC AAA GAT CTG TCA	21 nt	51.2°C	515 bp
		ChomTRA-Seq-R2	AAT CCC AAC AGT ATG CTT	18 nt		
Lucilia cuprina	***Yellow* (*LcY*)** 5'-end Exon 2	Lc-y-g1F	CTT ACA TCA ATA TGG ATC ACA GTG TAA CC	29 nt	55.6°C	283 bp
		Lc-y-g1R	AAT GTA TTG GCA TTT GTA TCA TCA GCT C	28 nt		
	3'-end Exon 1 (Figure 2A)	Lc-y-g2F	GAA CGT TAT AGT TGG AAT CAG TTG GAT T	28 nt	52.2°C	431 bp
		Lc-y-g2R	GAA TCC TCA TCA TCA ATA ATT GTA TCA GAC A	31 nt		

Backcrossing to *bwb* strain	G0 ♂ founder generated with:	
	Cas9 360ng/ul	Cas9 500ng/ul
Cage 01	56 / 139 (40.3%)	Infertile eggs
Cage 02	0 / 120 (00.0%)	11 / 83 (13.3%)
Cage 03	0 / 131 (00.0%)	78 / 96 (81.3%)
Cage 04	47 / 125 (37.6%)	Infertile eggs
Cage 05	78 / 173 (45.1%)	126 / 126 (100%)
Cage 06	71 / 144 (49.3%)	117 / 121 (96.7%)
Cage 07	32 /173 (18.5%)	120 / 120 (100%)
Cage 08	77 / 163 (47.2%)	0 / 112 (00.0%)
Cage 09	85 / 178 (47.8%)	0 / 145 (00.0%)
Cage 10	31 / 147 (21.1%)	118 / 118 (100%)
Avg ± SEM	**38.4 ± 4.3%**	**81.9 ± 14%**

Table S3. Effect of Cas9 RNPs concentration on germline transmission of *bwb* mutation. For each microinjection experiment using a low (360ng/ul) or high (500ng/ul) concentration of Cas9 protein, 10 mosaic males were randomly selected and tested for their founder habilities by individually backcrossing them with virgin *bwb* females. The G1 offspring were screened for the presence of biallelic *bwb* mutants, revealing germline transmission of the mutated allele.