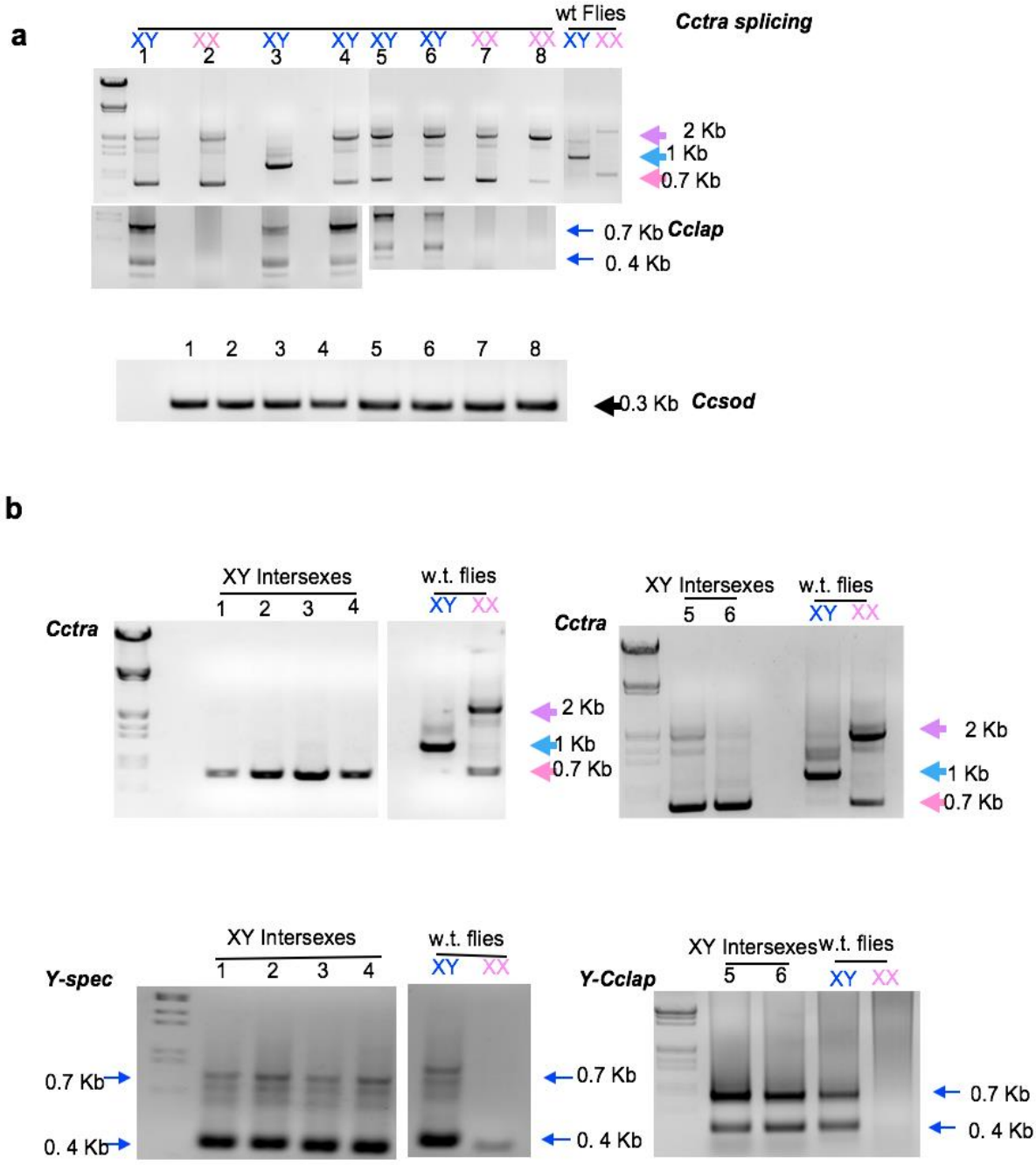
N°	DE+CQ transcripts 4-8h	Medfly	Baylor Genome	Presence in Fam18 Canu	male-specific in 4-8 h Ben embryos	CQ transcripts 0-48h+males	Blastx Ceratitis	Blastx Drosophila	male specific in B. oleae embryos	mapdXY.CQ	mapdXX.CQ	CQ
1a	TRINITY_DN40516_c0_g1_i6	No	(shorter highly related sequences)	multiple	Yes		no similarity	no similarity	no similarity	11118	0	0
1b	TRINITY_DN40516_c0_g2_i3	No	(shorter highly related sequences)	multiple	Yes	Lyra	putative gustatory receptor 59f XP_004526066.1 (E value 2.9)	none	no similarity	10916	0	0
1c	TRINITY_DN40516_c0_g2_i2	No	(shorter highly related sequences)	multiple	Yes	Lyra	putative gustatory receptor 59f XP_004526066.1 (E value 1.7)	zpg	no similarity	10794	0	0
1d	TRINITY_DN40516_c0_g1_i2	No	(shorter highly related sequences)	multiple	Yes	Lyra	no similarity	no similarity	no similarity	10759	0	0
1e	TRINITY_DN40516_c0_g2_i1	No	(shorter highly related sequences)	multiple	Yes		putative gustatory receptor 59f XP_004526066.1 (E value 2.2)	mabiki [Drosophila melanogaster] (E value 7.5)	yes, short and weak	11935	0	0
2	TRINITY_DN38563_c5_g1_i1	No	(shorter highly related sequences)	none	Yes		branchpoint-bridging protein (E value 6e-39)	quaking related 58E-2, isoform A (E value 1e-30)	no similarity	79	0	0
3a	TRINITY_DN40292_c0_g1_i10	No	(shorter highly related sequences)	multiple	Yes		no similarity	no similarity	no similarity	5813	0	0
1f	TRINITY_DN40516_c0_g1_i5	No	(shorter highly related sequences)	none	Yes		no similarity	no similarity	no similarity	10249	0	0
3b	TRINITY_DN40292_c0_g3_i1	No	No	single	Yes	Corvus	none	Lace isoform E (E value 1.7)	yes, short	68	0	0
4a	TRINITY_DN40142_c1_g4_i1	No	(highly related paralogous sequences)	multiple	Yes		histone H2B (E value 6e -43)	histone H2B (E value 2e-42)	no-male specific	872	0	0
5	TRINITY_DN33215_c0_g2_i1	No	(highly related paralogous sequences)	none	No		uncharacterized protein LOC105665391 (E value 5.6)	none	yes, short	3982	0	0
1g	TRINITY_DN40516_c0_g1_i7	No	(shorter highly related sequences)	multiple	Yes		no similarity	no similarity	none	8580	0	0
6	TRINITY_DN40470_c17_g1_i5	No	(highly related paralogous sequences)	none	Yes		cytosol aminopeptidase (E value 2e-23)	Sperm-Leucylaminopeptidase 5 (E value 3e-13)	yes	3182	0	0
4b	TRINITY_DN40142_c1_g1_i5	No	(highly related paralogous sequences)	multiple	No		no similarity	no similarity	yes	274	0	0
4c	TRINITY_DN40142_c1_g4_i2	No	(shorter highly related sequences)	multiple	Yes		histone H2B (E value 9e-30)	histone H2B (E value 1e-29)	no-male specific	336	0	0
7	TRINITY_DN38104_c3_g2_i2	Yes	No	none	No		no similarity	no similarity	none	6253	0	0
8	TRINITY_DN40402_c3_g5_i1	No	(highly related paralogous sequences)	multiple	Yes		no similarity	no similarity	none	16013	0	0
9	TRINITY_DN36540_c0_g1_i1	Yes	No	none	No		no similarity	no similarity	none	11903	0	0
10	TRINITY_DN37671_c9_g1_i1	Yes	No	none	Yes		F-Box/SPRY domain-containing protein (E vaule 9.4)	no similarity	none	11015	0	0

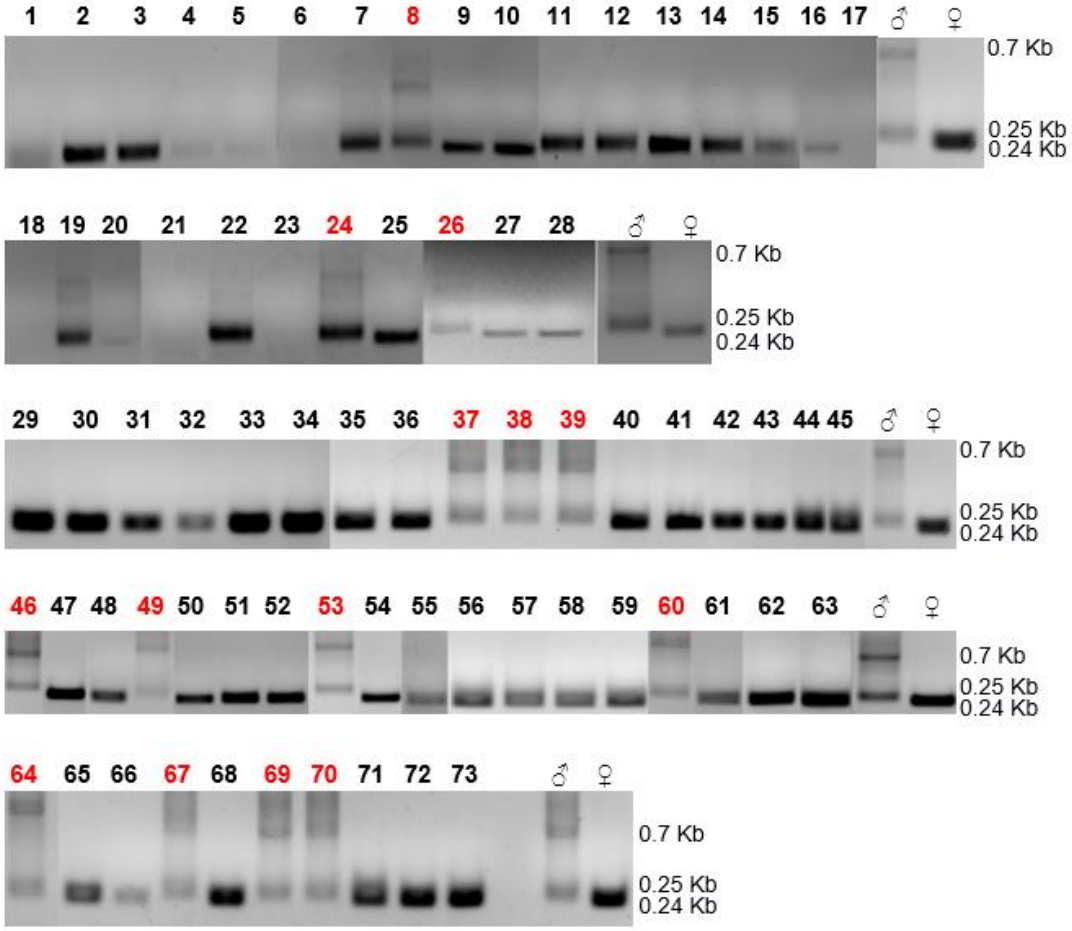
Extended Data Table 1 | List of 19 embryonic (4-8 h) transcripts corresponding to 10 putative Y-linked genes, filtered by DE and CQ analyses. BLASTn analyses showed that most of the 19 transcripts are missing in the currently available medfly genome (NCBI), which however contain paralogous sequences. Only 3 transcripts have 100% corresponding genomic sequences in this assembly. In contrast, BLASTn analyses on the male medfly Canu *Fam18* genome showed that 12 transcripts have corresponding identical sequences, with most present in different contigs suggesting multiple copies. The presence of corresponding transcripts only in the mixed XX/XY but not in the XX-only transcriptome further supported male-specificity for 15 of them. 4 out of 19 transcripts corresponded to 2 previously selected putative Y-linked male-specific genes (*lyra* and *corvus*). BLASTx analysis on protein databases of *C. capitata* and *D. melanogaster* showed some similarity mostly to short stretch of peptidases, transcriptional factors, receptors and histone proteins. The number of *Fam18* male (mapdXY.CQ) and female (mapdXX.CQ) mapped reads and the chromosome quotient value (CQ), calculated as mapdXX.CQ/mapdXY.CQ, are reported.

Injection Mix	Karyotypes of injected embryos	Injected embryos	Adults	XY males	XX females	XY females	XX males	XY intersexes	XX intersexes
#1: dsRNA *MoY*	XX/XY	1217	96	16*	59	**14**	0	**7**	0
#2: dsRNA *MoY*	X X; *wp/wp* X Y-*wp*$^{+}$; *wp*, A-Y	260	10	2* (XY-*wp*$^{+}$)	**7**	**1** (XY-*wp*$^{+}$)	0	0	0
#3: CRISPR/Cas9 vs *MoY*	XX/XY	250	32	7*	18	**2**	0	**5**	0
#4: linear 5 Kb *MoY* DNA	XX/XY	310	28	16	3°	0	**3**	0	**6**
#5: *MoY* 5 Kb plasmid	XX/XY	190	16	8	5°	0	**1**	0	**2**
#6: his-MOY protein	XX	428	31	-	25	-	0	-	**6**
#7: dsRNA *BoMoY*	XX/XY	550	24	6	10	**3**	0	**5**	0
#8: dsRNA *BdMoY*	XX/XY	540	41	16	17	**4**	0	**4**	0

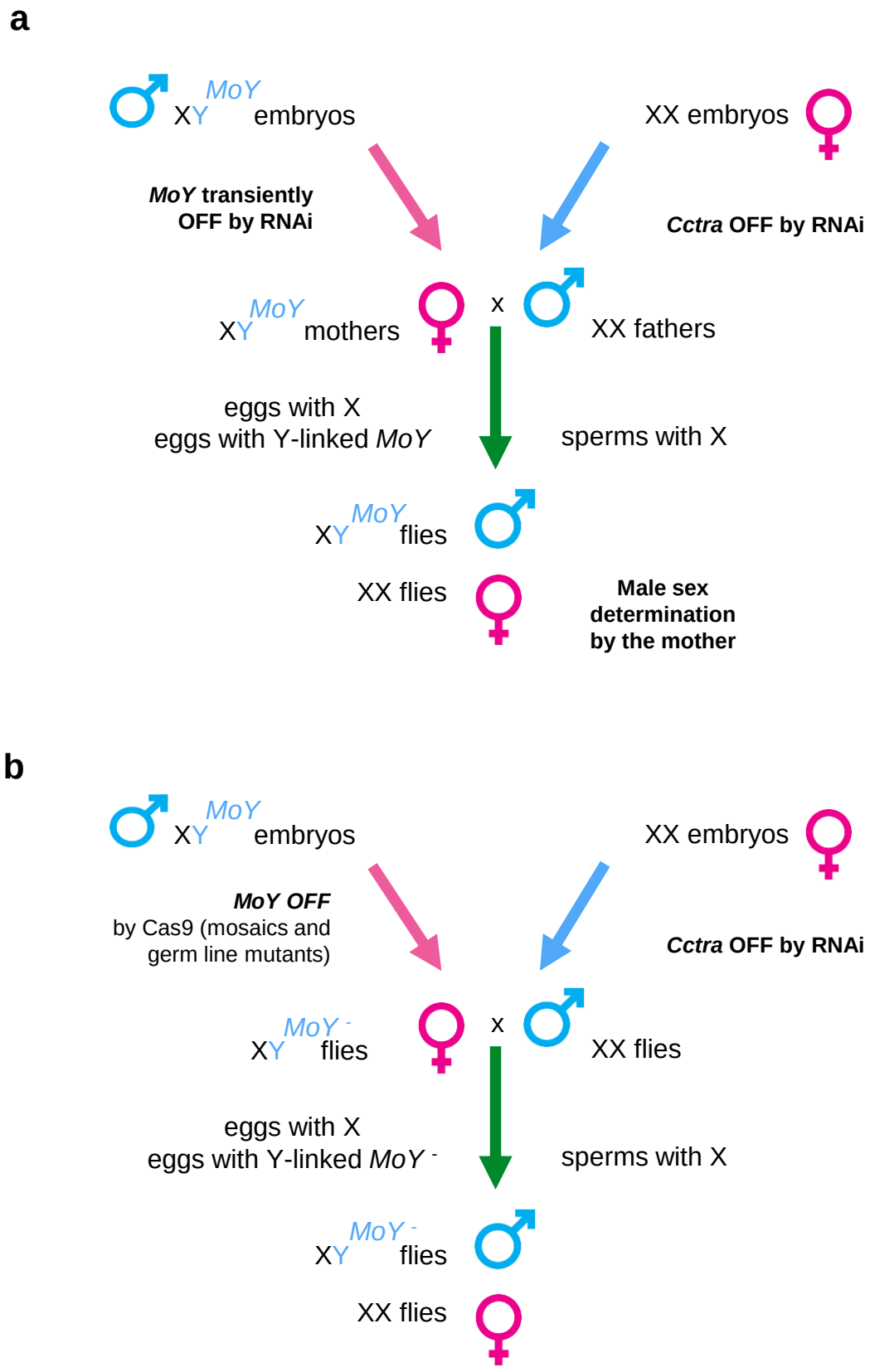
Extended Data Table 2 | *MoY* is necessary (#1, #2 and #5) and sufficient (3#, 4# and 6#) for male sex determination and functionally conserved in *Bactrocera* species (#7-#8). Medfly embryos injections at 0-1 h AEL of *MoY* dsRNA (#1-#2), DNA (#3, #4), Cas9 RNP (#5), and protein (#6). Embryos injections of *MoY* orthologues dsRNA in *Bactrocera oleae* and *B. dorsalis* (8#, 9#). In red are indicated numbers of flies showing partial or apparently full sexual transformations. In injection set 2#, a Y-marked brown pupae strain, carrying a white pupae recessive mutation on an autosome was used (reciprocal autosome-Y chromosome translocation). The male flies marked with * in strongly female-biased progenies (#1, #2 and #5) were assigned to XY karyotype without molecular analyses, considering them as *MoY* RNAi or Cas9 escapers. Similarly, the female flies marked with ° in male-biased progenies (#3 and #4) were assigned to XX karyotype without molecular analyses. Injection set 1# reports data from 3 biological replicates reported in Extended Data Table 2 .



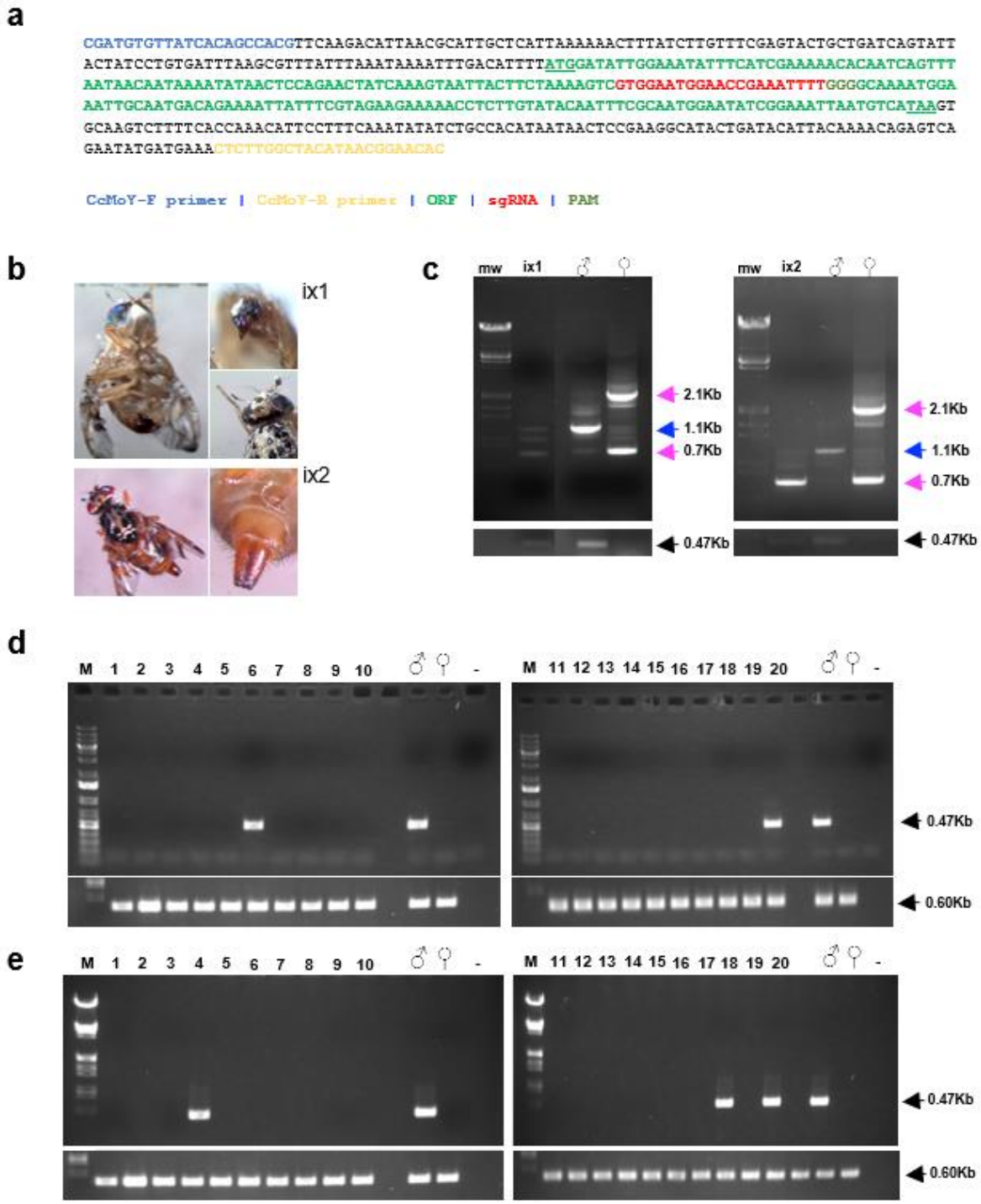
Extended Data Fig. 1 | Transient embryonic silencing of *MoY* leads to *Cctra* female-specific splicing in XY larvae. a, RT-PCR analyses of *Cctra* in 8 larvae hatched from *MoY* dsRNA-injected embryos showing *Cctra* female-specific transcripts (2 Kb and 0.7 Kb cDNA bands) in 4 out of 5 XY individuals (lanes 1, 4, 5 and 6). No effect was observed on female-specific *Cctra* splicing pattern in 3 XX larvae (lanes 2, 7 and 8) and on male-specific splicing pattern of 1 XY (lane 3) larvae. Sex-specific *Cctra* transcripts were amplified from adult flies as a reference for gel migration of the corresponding cDNA bands (female-specific 2.1 Kb and 0.7 Kb; male-specific 1.1 Kb). Individual larvae were molecularly karyotyped using a Y-derived transcript from *Cclap* pseudogene (Salvemini et al., 2011). *Ccsod* transcripts were used as a positive control and as negative control for genomic DNA contamination. **b,** RT-PCR analyses of *Cctra* carried out in 6 out of 7 adult intersex XY flies showed either female-specific transcripts (1-4) or a mix of male-specific and female specific ones (5-6). Molecular karyotyping of XX/XY individuals by RT-PCR on Y-specific transcribed sequences (transcribed repetitive Y-linked sequence pY114–related by YF/YR primers; lanes 1-4; Anleitner and Haymer, 1992) and Y-linked *Cclap* (lanes 5-6) confirmed the expected XY karyotype of the intersexes, indicating partial feminization. Sex-specific *Cctra* and Y-specific *Cclap* transcripts were amplified from adult flies as a reference for gel migration of the corresponding cDNA bands.



Extended Data Fig. 2 | Molecular karyotyping of 73 G0 females obtained from embryonic *MoY* RNAi. Sexing by PCR karyotyping was performed on genomic DNA from a small wing fragment dissected from each of 73 females from injection set #1 (Table 1). The presence of the Y chromosome (as 0.7 Kb and 0.250 Kb bands) was detected in 14 out of 73 adult females (in red) using *CcYF/CcYR* primers[31]. In the remaining 59 adult females, a slightly smaller band was detected indicating the absence of the Y (0.24 Kb) [31].



Extended Data Fig. 3 | In *Ceratitis capitata*, artificial transient female heterogamety (XY) and male homogamety (XX) are compatible with fertility and male sex determination. a, XY mothers were obtained by transient *MoY* embryonic RNAi. XX fathers were obtained by transient *Cctra* RNAi (see Methods). *MoY* gene can be transmitted by the mother and determines the male sex in the progeny. **b,** Maternal transmission of a Y chromosome carrying a *MoY* Cas9-induced null allele.



Extended Data Figure 4 | CRISPR-induced partial feminization of XY individuals. a, *MoY* coding region sequence, Cas9 target site, and primers used for PCR. ATG and STOP codon are underlined. **b**, 2 adult intersexes developed after embryonic CRISPR-Cas9 injections: ix1 shows male-head and malformed ovipositor; ix2 shows male-head and female ovipositor. **c**, RT-PCR of *Cctra* and of *MoY* (0.47 Kb) for karyotyping: both intersexes shown in figure b are XY and express a mix of male and female *Cctra* transcripts. **d**, Molecular karyotype analysis on the 20 G_0 female flies developed from Cas9-gRNA embryonic injections. The sexing by PCR karyotyping was performed on genomic DNA from adult females injected with the ribonucleoprotein Cas9/*MoY*-gRNA complex, using primers CcMoY-F and CcMoY-R. Two females (numbers 6 and 20) were found to be positive for *MoY*, showing a complete feminization due to the injection of the ribonucleoprotein Cas9/*MoY*-gRNA complex, causing a knock-out of the *MoY* gene. **e**, Molecular karyotype analysis on 21 G_1 female flies born from female 6 from Cas9-*MoY* injected embryos (Table 1 - Figure 3). Sexing by PCR karyotyping was performed on genomic DNA from the 21 females born from female 6 (see Table 1), using primers CcMoY-F and CcMoY-R. Three females (numbers 4, 18 and 20) were found to be positive to *MoY*, thus inherited the knock-out *MoY* gene from the mother. *CcSOD* PCR on genomic DNA, used as positive control, shows that the genomic DNA of all samples was amplifiable in both d and e panels.

a

```
ACTTCTAAAAGTCGTGGAATGGAACCGAAATTTTGGGGCAAAATGG   wt MoY
ACTTCTAAAAGT-------------------------CAAAATGG   L-1
ACTTCTAAAAGTCGTGGAATGGAACC---------GGGCATAATGG   L-2
ACTTCTAAAAGTCGTGGAA------C---------GGGGCAAAAAGG  L-3
ACTTCTAAAAGTCGTGGAATGGAACC---------GGGCATAATGG   L-4
ACTTCTAAAAGT-------------------------CAAAATGG   ix-3
ACTTCTAAAAGTCGTGGAATGGAACCGA----------CAAAATGG   ix-4
ACTTCTAAAAGTCGTGGAATGGAACC---------GGGCATAATGG   ix-4
ACTTCTAAAAGTCGTGGAA------C---------GGGGCAAAAAGG  ix-4
ACTTCTAAAAGTCGTGGAATGGAACCG-------GGT-CAATA-GG   ix-4
ACTTCTAAAAGTCGTGGAATGGAACCGAAAT-----GGCAAAATGG   ix-5
ACTTCTAAAAGTCGTGGAATGGAACC----------GGCAAAATGG   Fem-4
ACTTCTAAAAGTCGTGGAATGGAACCGAAAT----GGGCAAAATGG   Fem-10
```

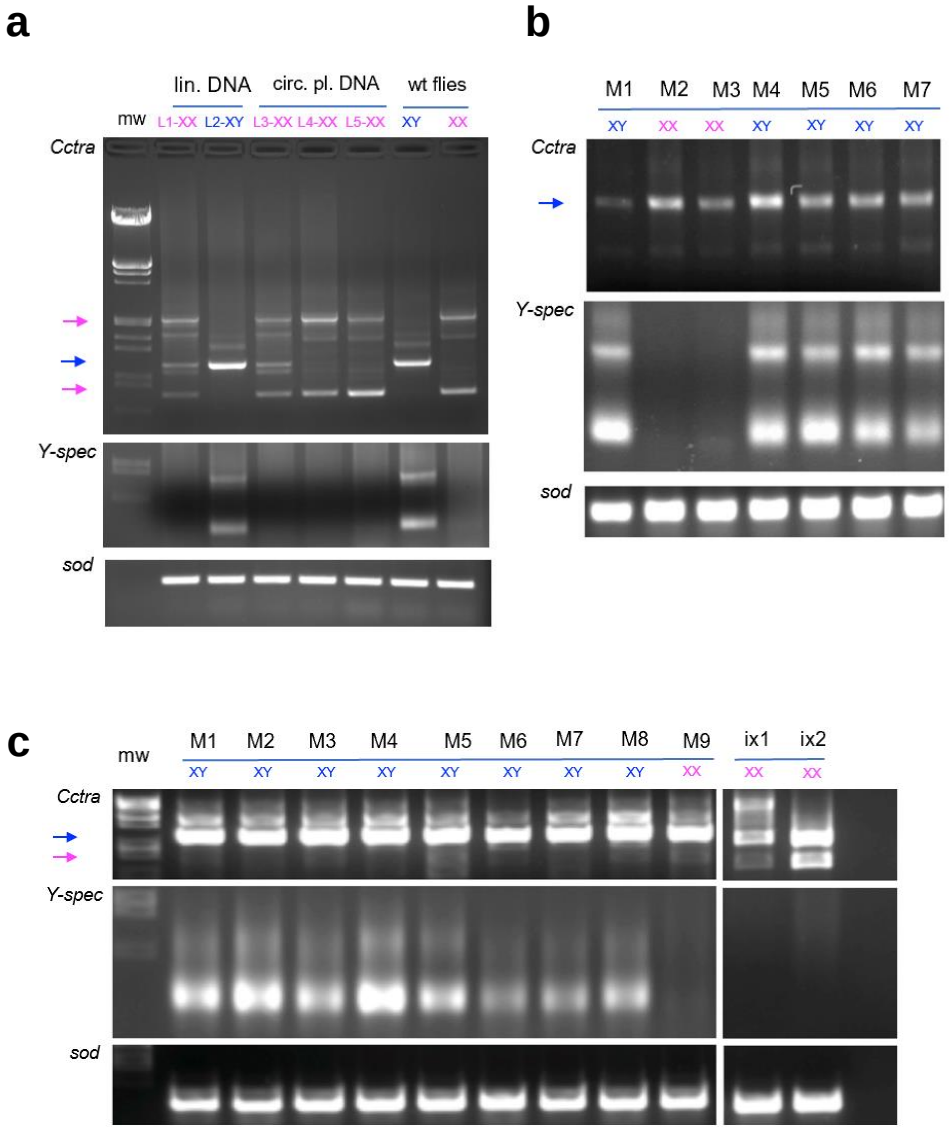
4 G0 XY larvae

3 G0 XY Intersexes

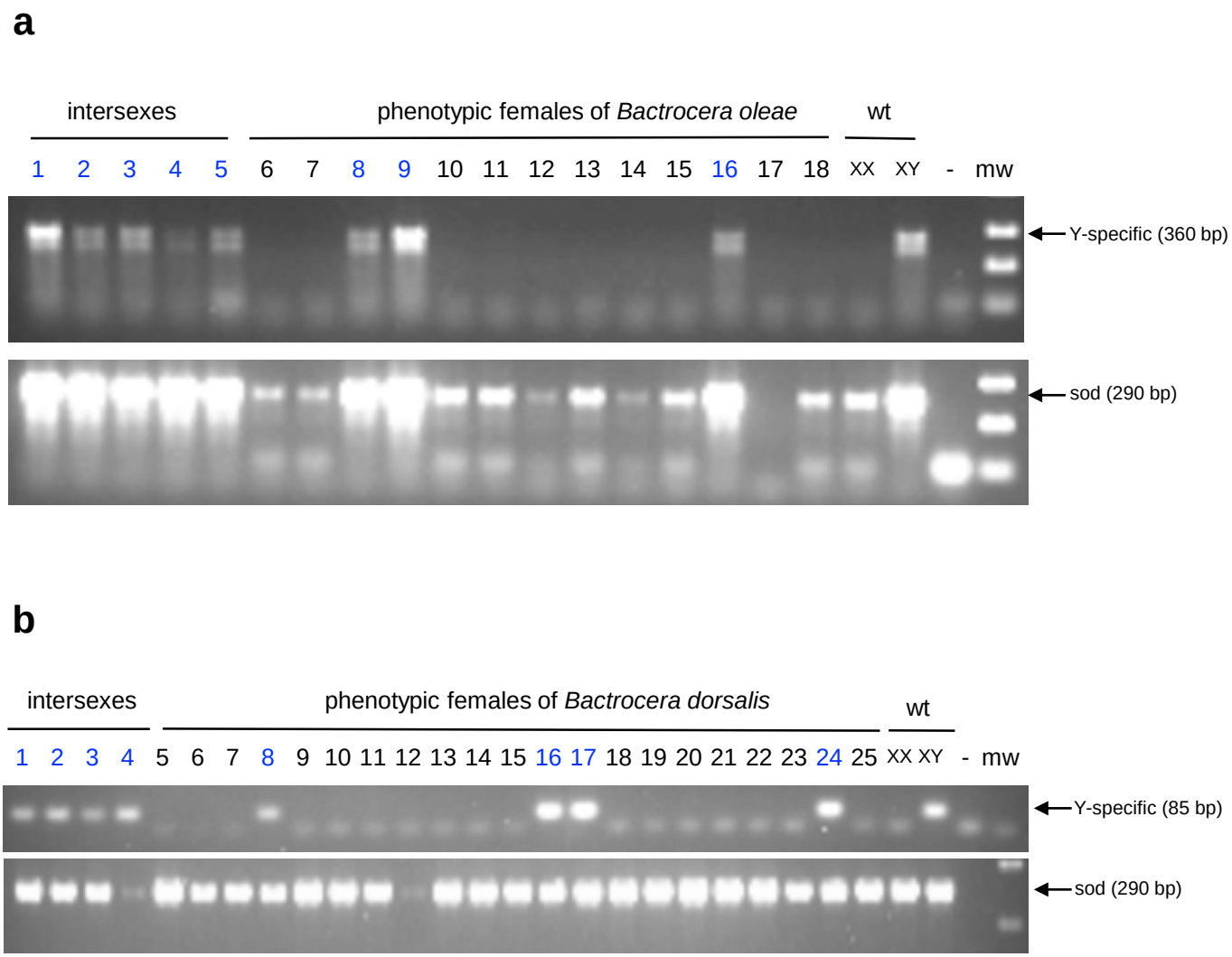
2 G1 XY females

b

Extended Data Figure 5 | CRISPR-Cas9–induced disruption of *MoY* causes complete male-to-female transformation of XY individuals. a, Injection of Cas9 ribonucleoparticles targeting the putative coding region of *MoY* (see Fig. 1d; Table 1, #5) induced indels in proximity to the PAM site, as detected in DNA from a pool of G_0 larvae and from 3 adult G_0 intersexes (see also Extended Data Fig. 12). One intersex is shown with feminization of the head region (no male-specific setae). Two G_1 XY females (female 4 and 20, (born from a full feminized G_0 XY *MoY*-CRISPR*ant* mother and a XX "special" father), showed deletions in *MoY* of respectively 10 bp and 4 bp, causing frameshift mutations (Table 1, #3). **b**, Cas9-induced intersexual phenotypes. Additional three intersexes showed the presence of male bristles on the head (left column) and deformed genitals similar to ovipositors (right column). Indels mutations in the targeted *MoY* region in 3 adult intersexes (ix3, ix4 and ix5) are shown in Fig. 4



Extended Data Fig. 6 | Molecular masculinization of XX larvae following embryos injection with *MoY* genomic DNA. a, **b**, **c**, RT-PCR of *Cctra*, the Y-linked *Cclap* (b) and *Ccsod* as positive control (c) on individuals from injected embryos (Extended Data Table 3, injection set 3# and 4#)(mw: molecular marker). **a,** L1 and L3 larvae, respectively from injected embryos with linear (Lin.) or plasmidic (circ. = circular)) *MoY* DNA showed XX karyotype (lack of Y-specific *Cclap* cDNA bands) and a male-specific *Cctra* band (blue arrow; 1.1 Kb), in addition to the female specific ones (pink arrows, 2 Kb and 0.7 Kb). No effect is observed in XY larvae. L4 and L5 XX larvae are escapers of the masculinization induced by the *MoY* plasmid, possibly because of quantitative variability in the manual embryos injections. Some minor *Cctra* different bands visible in distinct lanes are likely due to variable amplification of intermediates of *Cctra* splicing. **b**, Two out of 7 adult males (Extended Data Table 3, *MoY* DNA linear fragment embryos injections set 3#,), showed XX karyotype but male-specific *Cctra* splicing product (blue arrow; 1.1 Kb), indicating apparently full molecular masculinization. Molecular karyotyping (Y-spec) and positive control (sod) are also shown. Similar molecular analysis and karyotyping were performed on the remaining 12 males from the same injection set 3# (data not shown). **c**, One of 9 adult males from *MoY* DNA plasmid embryos injection set 4# (Extended Data Table 3) showed XX karyotype and male-specific *Cctra* splicing, suggesting again full molecular masculinization. Two phenotypic XX intersexes showed a mix of male- and female-specific *Cctra* products.



Extended Data Figure 7 | Molecular karyotyping of intersexes and G0 females from *MoY* embryonic RNAi in *B. oleae* (a) and *B. dorsalis* (b). Adult flies from injections sets 7# and 8# were molecularly karyotyped by PCR with Y-specific primers (see mat. and meth.). **a,** 5 *B. oleae* intersexes and 3 females were found to be XY. XX and XY are positive controls (the female and male Bo adults, respectively).**b,** 4 *B. dorsalis* intersexes and 4 females were found to be XY. XX and XY are positive controls (the female and male Bd adults, respectively).