SEX DETERMINATION

Maleness-on-the-Y (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests

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In insects, rapidly evolving primary sex-determining signals are transduced by a conserved regulatory module controlling sexual differentiation. In the agricultural pest *Ceratitis capitata* (Mediterranean fruit fly, or Medfly), we identified a Y-linked gene, *Maleness-on-the-Y* (*MoY*), encoding a small protein that is necessary and sufficient for male development. Silencing or disruption of *MoY* in XY embryos causes feminization, whereas overexpression of *MoY* in XX embryos induces masculinization. Crosses between transformed XY females and XX males give rise to males and females, indicating that a Y chromosome can be transmitted by XY females. *MoY* is Y-linked and functionally conserved in other species of the Tephritidae family, highlighting its potential to serve as a tool for developing more effective control strategies against these major agricultural insect pests.

Tephritidae is a dipteran family comprising 5000 species, dozens of which are invasive and highly relevant pests of fruit crops. *Ceratitis capitata* (Medfly) is one of the most destructive members of this taxon, affecting more than 200 plant species (*1*). Besides pesticides, the most successful method to control Medfly is the sterile insect technique (SIT) (*2*), which involves the continuous mass-release of biofactory-reared, sterilized males that suppress wild populations by mating with wild females. A key determinant to the success of SIT programs has been the translocation of selectable traits to Medfly Y chromosome in genetic sexing strains that enable male selection on a massive scale (*2*). However, the development of similar strains in other Tephritidae pest species using classical genetics has been difficult. Identifying the male-determining factor (M factor) in Medfly and in related pests holds great promise for the development of novel genetic sexing strains using modern genetics (*2*) or even for transforming females into males, thereby increasing the efficiency of insect biofactories.

In insects, widely divergent primary signals of sex determination act via the conserved genetic switch *transformer* (*tra*), which was first characterized in *Drosophila* as a gene regulated by and operating through sex-specific alternative splicing (*3*–*6*). In females, two doses of the X chromosome result in an early zygotic transcriptional burst of the master gene *Sex-lethal* (*Sxl*), which promotes female-specific splicing of *tra* and female differentiation. In males, a single X leads by default to a *tra* transcript that encodes a short nonfunctional TRA polypeptide and male differentiation occurs. In Medfly, as in other non-Drosophilidae species, *Sxl* is not involved in sex determination (*3*). Unlike *Drosophila*, maternal deposition of *Ceratitis capitata tra* (*Cctra*) in developing embryos initiates its positive autoregulatory female-specific splicing, similar to the housefly (*5*, *6*), leading to female differentiation. In Medfly XY embryos, a Y chromosome–linked M factor either directly or indirectly represses *Cctra* function, thus promoting male development (*5*). However, the molecular identity of this Y-linked M factor has remained unknown (*7*, *8*).

We conducted the search for this M factor by: (i) focusing on transcripts in the 4 to 8 hours after egg laying (AEL), the period when male sex determination is first established (*9*); (ii) producing an XX-only embryonic RNA sequencing dataset as a reference; (iii) developing a long-read PacBio sequencing–based male genome assembly from the *Fam18* Medfly strain (*8*) that bears a shorter Y chromosome; and (iv) searching for conservation of putative M factors in another Tephritidae, the olive fruit fly, *Bactrocera oleae*. We identified 19 M candidates that expressed transcripts in mixed-sex embryos but not in XX-only embryos and that were predicted to be Y-linked on the basis of the ratio of genomic reads mapping from female versus male samples (chromosome quotient, CQ) (*10*) (Fig. 1A, table S1, and supplementary text S1). Seven of these transcripts did not map to the *Fam18* male genome assembly and were excluded from further analysis. Sequence similarity searches by nucleotide basic local alignment search tool (BLASTn) showed that 3 out of the remaining 12 transcripts had hits to XY but not to XX embryonic transcripts from *B. oleae*. Furthermore, one of these three Medfly transcripts (DN40292_c0_g3_i1) corresponds to a 0.7-kb sequence we previously identified in a preliminary screen (supplementary text S2). This transcript mapped to a predicted 12-kb long Y-linked contig in the *Fam18* genome assembly. Functional analysis (below) confirmed that this gene is the Medfly M factor and was thus named *Maleness-on-the-Y* (*MoY*). *MoY* is located on the long arm of the Y chromosome in proximity to the centromere (Fig. 1B) in a genomic region that contains nine other transcription units (Fig. 1C and supplementary text S3). *MoY* expression begins at 2 to 3 hours AEL, before embryonic cellularization, peaks at 15 hours, and is undetectable starting at 48 hours until adulthood (Fig. 1D and data S1).

Embryonic RNA interference (eRNAi) by injecting double-stranded RNA (dsRNA) into embryos 0 to 1 hours AEL resulted in loss of male-specific *Cctra* transcripts in 8-hour AEL embryos (Fig. 2, A to C, and tables S2 and S3). We observed a switch to the female-specific *Cctra* splicing in 3-day-old XY larvae from injected embryos and in XY adult intersexes (fig. S1). Among adults, 38% (14 of 37) of the molecularly karyotyped XY individuals displayed complete phenotypic feminization, and 19% (7 of 37) were intersex (Fig. 2D and fig. S2). Individuals were phenotypically classified as intersex if they displayed a mix of male- and

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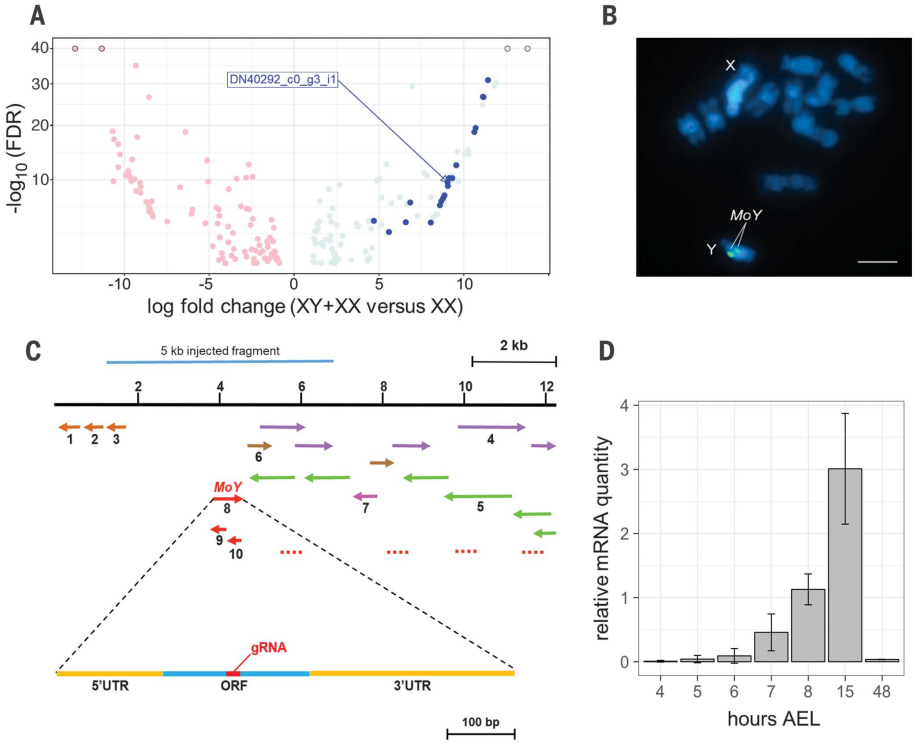

Fig. 1. *MoY* gene is Y-specific and transiently transcribed in embryos. (**A**) Volcano plot of 195 differentially expressed transcripts at 4 to 8 hours AEL. Pink dots indicate log fold change (mixed XX and XY samples versus XX samples) less than 0 (biased in the XX sample), light-blue dots represent log fold change > 0 (biased in the mixed sample), and dark-blue dots indicate 19 transcripts with a CQ value of 0 (putatively Y-linked). The transcript corresponding to *MoY* (DN40292_c0_g3_i1) is also shown. (**B**) Fluorescence in situ hybridization of *MoY* (green signals) on mitotic chromosomes stained with 4′,6-diamidino-2-phenylindole (blue); signals (one for each sister chromatid) locate *MoY* on the long arm of the Y chromosome near the centromere (scale bar, 5 μm). (**C**) A scheme of a 12-kb Y-linked genomic contig (contig00013010) containing the *MoY* transcript and other flanking transcriptional units (supplementary text S3). Also shown is the 5-kb region used for injections and the *MoY* guide RNA target site for Cas9. 5′UTR, 5′ untranslated region; 3′UTR, 3′ untranslated region; ORF, open reading frame. (**D**) Relative transcript expression of *MoY* during Medfly embryogenesis compared with housekeeping genes. For reference, Medfly cellularization occurs at ~9 hours AEL.

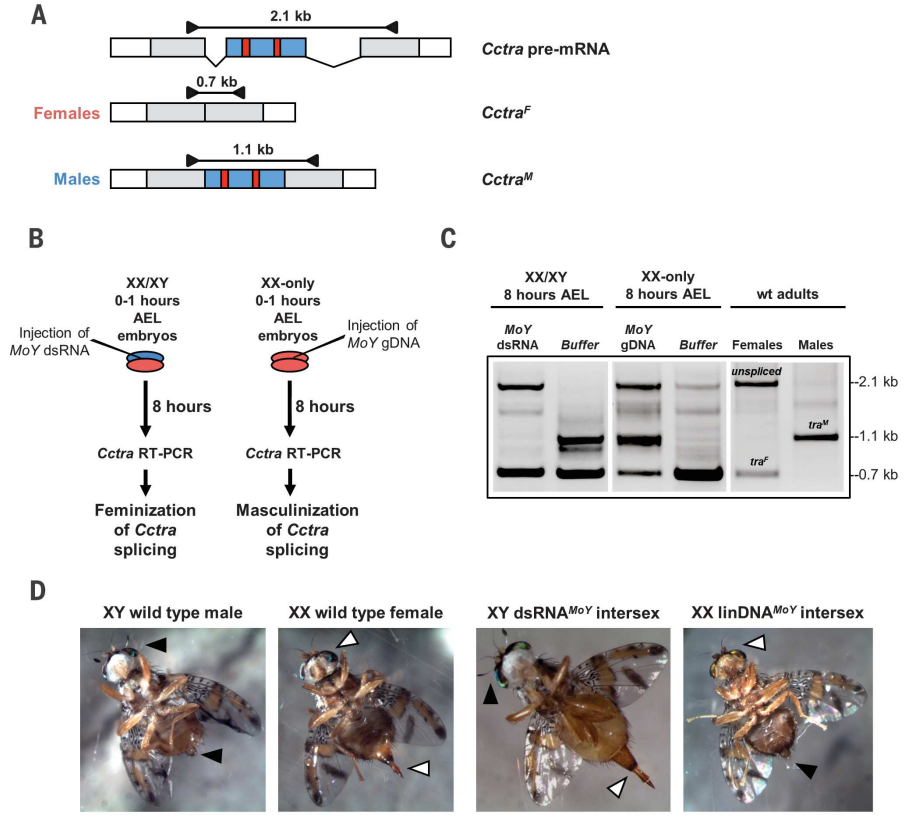

Fig. 2. *MoY* is necessary and sufficient for male sex determination. (**A**) Scheme of *Cctra* sex-specific transcripts. Coding regions, the male-specific exon, and stop codons are shown in gray, blue, and red, respectively. Black arrows indicate primers for reverse transcription polymerase chain reaction (RT-PCR). (**B**) Schematic overview of embryonic *MoY* injection experiments and expected effects on *Cctra* splicing after 8 hours. (**C**) RT-PCR analysis showing splicing patterns of *Cctra* in embryos injected at 8 hours AEL. (Left) Transient *MoY*-eRNAi (at 0 to 1 hours AEL) depletes mixed-sex embryos of the male-specific *Cctra* isoform at 8 hours AEL. (Middle) Injection of *MoY* gDNA is sufficient to instruct male-specific splicing of *Cctra* in XX-only embryos. The 2.1-kb *Cctra* amplicon is the unspliced transcript, detectable only in females (*5*). (Right) Wild-type male and female flies for reference; black and white colors inverted in the gel photo for clarity. (**D**) Representative photos of Medfly wild-type males and females and Medfly intersexes. The XY intersex from *MoY*-eRNAi has a female ovipositor (white arrowhead) and a male-like head with orbital bristles (black arrowhead). The XX intersex from *MoY* linear gDNA (linDNA; PCR fragment) injections has male genitalia (black arrowhead) and a female-like head, without orbital bristles (white arrowhead).

female-specific traits (Fig. 2D). To evaluate the fertility of XY females and to test whether a Y chromosome can be maternally transmitted, crosses were established to XX males that were generated by eRNAi targeting *Cctra* (*5*) (table S4 and fig. S3A). These crosses made it possible to demonstrate that the maternal transmission of the Y chromosome determined the male sex of the progeny. Among the 14 recovered XY females, one was fertile and transmitted the Y chromosome to one son. In a second experiment of *MoY*-eRNAi, using a strain displaying sex-specific pupal colors (*Vienna8*), we found another XY female that transmitted the Y chromosome (*2*) (table S2). This work demonstrates that the two

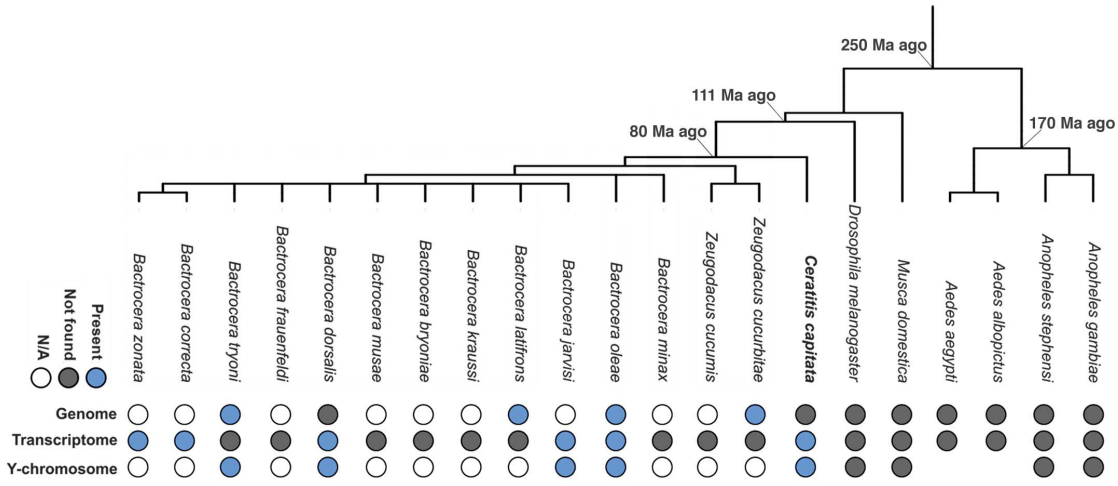

Fig. 3. *MoY* is conserved in Tephritidae species. A search of Medfly *MoY* orthologs was performed in 14 Tephritidae species and in 6 other dipteran species, in which primary sex determining signals have been molecularly characterized. *MoY* orthologs were discovered in the genomes and/or transcriptomes of eight Tephritidae species (blue circles). Y-linkage was confirmed by genomic PCR in four of these (Fig. 4A and supplementary text S4 to S12). The lack of detection of *MoY* (dark gray circles) in the remaining six Tephritidae species is likely the result of limited sequencing coverage. Divergence times are indicated as million years ago (Ma ago). N/A, not applicable.

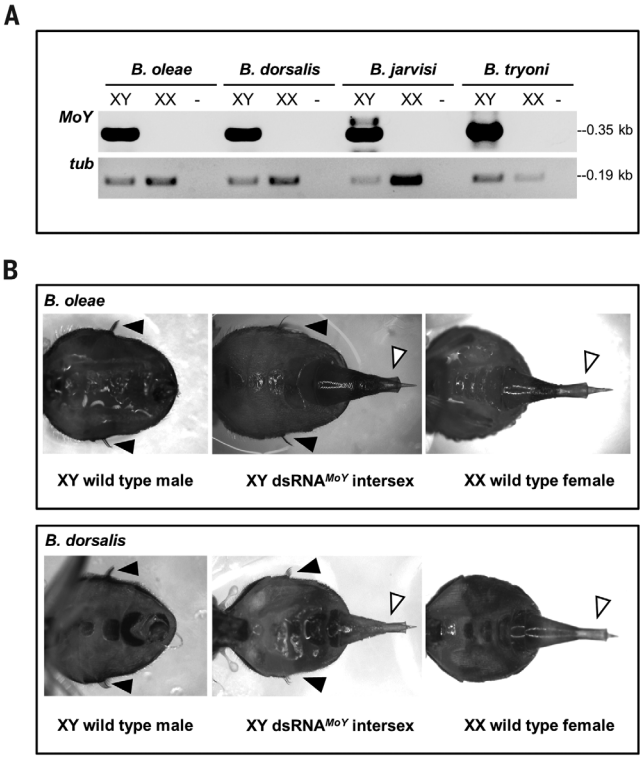

Fig. 4. *MoY* is Y-linked in *Bactrocera* species and functionally conserved. (**A**) PCR on male and female genomic DNA showing Y-linkage of *MoY* orthologs in *B. oleae*, *B. dorsalis*, *B. jarvisi*, and *B. tryoni*. Positive control: beta tubulin (*tub*). Black and white colors are inverted for clarity. (**B**) *B. oleae* (*Bo*) and *B. dorsalis* (*Bd*) male and female wild-type abdomens (left and right, respectively) and abdomens of intersex XY flies (middle), after *BoMoY*- and *BdMoY*-eRNAi (see also fig. S10). In both species, XY intersexes show the presence of female-specific characteristics (ovipositor, white arrowhead) together with male-specific characteristics (abdominal lateral bristles, black arrowheads).

sexes of an animal species defined by each karyotype can be reciprocally switched in both directions while maintaining fertility. This highlights, in both sexes, a resilience of Medfly somatic and gonadal development to perturbations in sex-determination signaling and suggests that the Y chromosome has no major detrimental effects on the development and fertility of XY females.

To further evaluate the role of *MoY* in sex determination, loss-of-function alleles were generated using Cas9 ribonucleoproteins (*11*) targeting the MOY coding sequence (table S2 and fig. S4A). Indels near the single-guide RNA target site were induced in the genomes of four G_0 XY larvae and three G_0 XY adult intersexes (fig. S5A). 50% (7 of 14) of the XY individuals (table S2) were transformed either into phenotypic females (2 of 7) or intersexes (5 of 7) (figs. S4, B to D, and S5B). One XY female crossed to XX males was fertile and produced female-only G_1 offspring composed of 3 XY and 18 XX flies (table S5 and figs. S3B and S4, D and E). Two of these XY G_1 females were analyzed and both showed *MoY* frameshift-inducing deletions resulting in truncated MOY proteins (fig. S5A).

Next, we investigated whether *MoY* is sufficient for male sex determination. A 5-kb genomic fragment, encompassing the *MoY* locus and flanking regulatory regions (Fig. 1C), was injected as a linear polymerase chain reaction (PCR) product or as circular plasmid into embryos (table S2). Male-specific *Cctra* splicing was induced in XX individuals at embryonic, larval, and adult stages (Fig. 2C and fig. S6) and led to partial or full masculinization of up to 75% of XX flies (9 of 12) (table S2, Fig. 2D, and figs. S6 and S7). Similarly, microinjection of MOY recombinant protein into XX-only embryos led to partially masculinized flies (showing either male-specific orbital bristles or male genitalia) in 19% (6 of 31) of the emerged XX adults (table S2 and fig. S8).

Ceratitis MoY DNA and protein sequences showed no significant BLAST hits to the National Center for Biotechnology Information databases, suggesting novelty or high sequence divergence. In contrast, tBLASTn (basic local alignment search tool of translated nucleotide databases using a protein query) searches of available genomic or transcriptomic datasets from 14 Tephritidae species spanning 111 million years of evolution (*12*) identified putative MOY orthologs in eight of them (Fig. 3), with an average amino acid sequence similarity to *Ceratitis* of 41 to 57% (supplementary text S4 to S12). The most conserved portion is located in the N-terminal region, where a consensus hexapeptide KXNSRT occurs (fig. S9). We confirmed by PCR of male and female genomic DNA (gDNA) the Y-linkage of *MoY* orthologs in four out of the eight species, namely *B. oleae*, *B dorsalis*, *B. tryoni*, and *B. jarvisi*, whose fly samples were available (Fig. 4A). *MoY*-eRNAi orthologs in *B. oleae* and *B. dorsalis* led to feminization of XY flies (57 and 33%, respectively), confirming the functional conservation of *MoY* (table S2, Fig. 4B, and fig. S10).

Here, we demonstrate that *MoY* is the Y-linked M factor in *C. capitata* because it is both necessary and sufficient to initiate male development during embryogenesis. How *MoY* suppresses female-specific *Cctra* splicing and whether this regulation is direct or indirect remain unclear. MOY does not bear any similarity to known proteins, in contrast

to M factors of *Aedes* and *Musca*, which are related to splicing factors (*13*, *14*). This finding suggests that *MoY* is a newly emerged M factor. *MoY* is functionally conserved in various Tephritidae species, in contrast to previous studies conducted on other insects whose M factors diverged rapidly, even within the same species (*13*–*16*). Our finding that masculinization through transient *MoY* misexpression in Medfly XX embryos does not lead to lethality as in other species (*15*, *16*) suggests that *MoY* is not involved in dosage compensation. However, it is not clear from the present data whether Medfly has such regulatory mechanisms to equalize expression of X-chromosome genes in both sexes. From a translational perspective, these features, including *MoY* conservation and its ability to fully masculinize XX individuals, make *MoY* a promising candidate for transferring to other important Tephritidae pests established genetic control strategies such as SIT, and for future development of emerging methods such as male-converting gene drive (*17*–*20*).

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SUPPLEMENTARY MATERIALS

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Data S1 to S4

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Maleness-on-the-Y (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests

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Making males in a fruit fly pest

The Mediterranean fruit fly or Medfly (*Ceratitis capitata*) is a global and highly destructive fruit pest. Meccariello *et al.* identified the master gene for male sex determination on the Y chromosome of Medfly and named it *Maleness-on-the-Y* (*MoY*) (see the Perspective by Makki and Meller). Flies of each sex were transformed into the other sex by genetic manipulation, and crosses of transformed files generated male and female progeny. *MoY* is functionally conserved in the olive fruit fly and in the invasive oriental fruit fly. This discovery has potential for insect genetic control based on mass release of sterile males and future strategies based on gene drive.

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