

Generating Cisgenic Sexing Strains in Insect Pests

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Article

Keywords: CRISPR/Cas9, Sex Specific Alternative Splicing, Genetic Sexing Strains, Medfly

Posted Date: April 21st, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6449302/v1>

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Additional Declarations:

Yes there is potential Competing Interest. A patent has been filed on this technology. O.S.A is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. N.P.K is a founder of Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

Table 1 is available in the Supplementary Files section.

Generating Cisgenic Sexing Strains in Insect Pests

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Abstract

Insect pest population control via sterile insect technique severely benefits from separation by sex prior to release. To simplify this process, traditional genetics has been deployed to develop genetic sexing strains (GSSs) for several disease vectors and agricultural pests of vast economic significance, although very few are applied in the field due to associated fitness costs and instability. In this study, we generated a method to engineer cisgenic GSS (CGSS) in insects. We use CRISPR/Cas9-mediated homology-directed repair to seamlessly translocate a sex-specific alternatively spliced intron into a dominant phenotypic gene generating a genetically stable strain that enables sex-sorting by eye. To achieve this feat, we use *Ceratitis capitata* as our model and relied on the sex-specifically spliced intron of the endogenous *transformer* gene, which we seamlessly inserted into the pupal colouration *white pupae* gene. This minimal modification resulted in the generation of a homozygous strain we term IMPERIAL that was phenotypically stable where all female pupae are brown while male pupae are white with overall good fitness. By minimally editing the genome, our CGSS approach can be applied to other pests that may aid more efficient and economically suitable pest control.

Key words: CRISPR/Cas9; Sex Specific Alternative Splicing; Genetic Sexing Strains, Medfly

Main text

Genetic sexing strains (GSSs) have been developed in multiple insect species of economic significance to allow easier male and female separation necessary for efficient population control. Specifically, GSSs are used within sterile insect technique (SIT) programmes which work via frequent releases of sterilised insects into the wild for temporary population control (1-3). Male-only releases, aided by GSS implementation, strongly succour in released fly dispersal and their mating frequency with wild females, thus enhancing SIT success (4-6). Vast efforts have focused on GSS generation in mosquito disease vectors and agricultural fruit fly pests to avoid labour-intensive sex-sorting by eliminating the females from the released population early in the life cycle (7-12).

The traditional GSS approach requires two key attributes: a selectable marker and a Y-chromosome or male-determining locus linkage from which it needs to be expressed (4). A primary example of a traditional GSS is the VIENNA 8 strain developed in the tephritid fruit fly pest *Ceratitidis capitata* (Mediterranean fruit fly or medfly) possessing an increasing threat to the agricultural industry with its expanding global distribution and vast host range (13-14). VIENNA 8, similarly to its predecessor VIENNA 7, relies on a radiation-induced simultaneous translocation of *wp* gene and *temperature sensitive lethal (tsl)* genes onto the Y-chromosome involved in pupal colouration and heat tolerance respectively (15-16). Whilst the former, *wp*, has been characterised in multiple tephritids, the latter, *tsl*, remains to be identified in *C. capitata* and its relatives. As the VIENNA 8 strain has a *wp* and *tsl* double-mutant background, the white-pupaed females die upon embryonic heat exposure, whilst the brown-pupaed males persevere into adulthood (4; 17). Across the *Tephritidae* family, such traditional GSSs have been successfully developed in multiple species, although only those developed in *C. capitata* and *Anastrepha ludens* have been implemented on a SIT facility scale (18-19). Other traditional examples tested on a larger scale include the eye colour phenotype-dependent *Aedes aegypti* GSSs (20).

In parallel to similar GSSs in other tephritid species, the VIENNA 8 strain is infrequently susceptible to phenotype loss via recombination requiring an extra filtering step at SIT facilities (4, 21). Furthermore, they have notable reductions in fertility, thus creating obstacles for mass rearing (18, 22). To elevate GSS fitness, multiple transgenic approaches have been engineered for female exclusion either through a selectable phenotypic marker or female-specific lethality in the medfly and other fruit fly species (23-33). Abundantly, a sex-specific intron from the *transformer (tra)* gene has been implemented in the medfly for female-specific transgene

expression from the autosomes (25-26, 30). Most recently this was completed for female-specific fluorescence marker expression in a Sexing Element Produced by Alternative RNA-splicing of Transgenic Observable reporter (SEPARATOR) system whereby both the *C. capitata* and newly the *A. ludens tra* introns were used (25). While these transgene-based approaches provide exciting alternatives, they still require regulatory authorisation which can be a slow and cumbersome process.

Herein, we wanted to develop a universal method in insects to engineer GSSs that use modern engineering techniques to generate minimal genetic modifications wherein both donor and recipient are derived from the same species which we call Cisgenic GSS (CGSS). We used the medfly as our model system to engineer a non-transgenic CGSS without exogenous elements. Sex-specific expression of the *wp* selectable marker was achieved through sex-specific splicing of the *tra* intron. This was attained through a homology-directed repair (HDR)-dependent knock-in of the *tra* intron seamlessly into the *white pupae (wp)* locus of wild-type Benakeion medfly embryos, feasible due to recent success of CRISPR/Cas9-mediated HDR in the species (34-36).

Collectively, using the intron of *tra*, a master gene of tephritid female sex determination (37-39), results in extremely robust desired phenotypes, rendering it suitable for further use in CGSSs, yet to be developed. The knock-in construct (1167A) was engineered using the endogenous *transformer (tra)* intron, which was placed between the homology arms of each approximately 700 bp in length. The anticipated outcome was female-specific *wp* gene expression, whereby males harbouring two copies of the *tra*-containing *wp* gene would have a white pupae phenotype and females harbouring two copies of the *tra*-containing *wp* gene would have a brown pupae phenotype, which can be distinguished by eye (Fig. 1A). As the *wp* mutation is recessive in nature, the knock-in strain, hereupon named IMPERIAL, was isolated using backcrosses to the irradiation-generated *white pupae* knock-out (*wp*^{-/-}) strain at G0 and G1 (Fig. 1B). We confirmed successful knock-in of the *tra* intron (*wp*^{KI+/-}) in all obtained brown-pupaed G2 females using amplicon sequencing of the integration site. A homozygous *wp*^{KI+/+} line was established at G7 (F0) after crossing sibling white-pupaed males with brown-pupaed females, genotyping all parents and screening the whole progeny at every intermediate generation. To verify the integration in the *wp*^{KI+/+} IMPERIAL strain, genomic DNA from CGSS females was sequenced. The sequencing revealed reads with the expected 1345 bp CRISPR-HDR insertion indicating a seamless intragenic insertion of the *tra* intron into the *wp* gene (fig. S1).

To verify the sex-sorting suitability of the strain, phenotypic pupae colour stability and sex ratios of the IMPERIAL strain were examined alongside the existing VIENNA 8 GSS, where males and females emerge from brown and white pupae accordingly. For five consecutive generations (F2-F6), pupae and adult phenotypes were recorded for IMPERIAL (pupae $n = 4147$; adult $n = 4074$) and VIENNA 8 (pupae $n = 1386$; adult $n = 1227$) strains in parallel, maintained under the same conditions. As anticipated, in the $wp^{KI/+}$ IMPERIAL strain all females emerged from brown pupae and all males emerged from white pupae (Fig. 2A). The reverse phenotypes were universally observed amongst individuals from the VIENNA 8 strain. Altogether, the proportion of adult females in the IMPERIAL strain (mean = 49.8%) exceeded that of the VIENNA 8 strain (mean = 37.4%). Whilst populations within every generation of the IMPERIAL strain adhered to the expected 1:1 male: female sex ratio (chi-square goodness of fit tests), the VIENNA 8 did not at F2, F4 and F5 (Fig. 2A). In sum these results confirm the phenotypic stability of the IMPERIAL strain that, dissimilarly to VIENNA 8, consistently confines to the expected 1:1 male: female sex ratio.

To better understand differences in the *tra* intron-dependent *wp* splicing in males and females of the $wp^{KI/+}$ IMPERIAL strain, we performed reverse-transcription PCR (RT-PCR) on genomic and complementary DNA (cDNA) templates from adult flies (fig. S2). A single female band was amplified from the cDNA template, corresponding to a transcript with the fully spliced-out *tra* intron (37), confirmed by sequencing thereafter. Male cDNA banding entirely consisted of larger fragments from which two unique male isoforms with premature stop codons were isolated via clonal sequencing, both containing sequences of the two male-specific exons. These results are suggestive of functional White pupae protein production in females and ablation of its translation in males. Further investigation into *wp* splicing of the IMPERIAL strain was conducted using RNAseq. As expected, all three female libraries had multiple reads that spanned the junction splicing the *tra* intron. In contrast, the three male libraries had no such reads indicating that the intron is spliced in females and not in males (fig. S3). As expected, the clustering and principal component analyses indicated a close relationship of samples by sex (fig. S4).

The IMPERIAL sexing strain was further compared to its parental wild-type Benakeion and VIENNA 8 strains in terms of general fitness, and thus suitability for larger-scale employment. First, a standard egg-adult survival assay utilising triplicate sibling crosses of 10 males with 20 females was conducted, whereby rates of egg laying, egg hatching, hatched larvae-pupae recovery, pupae-adult recovery, and total egg-adult recovery were assessed (Fig. 2B). Kruskal-

Wallis and sequential Dunn's tests were performed as means of statistical analysis. Notably, egg production within the measured time period was significantly elevated in the IMPERIAL strain compared to both wild-type and VIENNA 8 strains ($p < 0.05^*$). Hatching rate in VIENNA 8 was significantly lower than in wild-type ($p = 0.0036^{**}$), although insignificant reductions in the IMPERIAL strain were also observed ($p = 0.0899$). We recorded the highest larval-pupal survival in the IMPERIAL strain, which was significantly raised compared to VIENNA 8 ($p = 0.0036^{**}$). In line with the earlier phenotypic stability experiments, the pupal-adult recovery rates were significantly lower in the VIENNA 8 strain, compared to both wild-type and IMPERIAL strains ($p < 0.05^*$). Overall egg-adult survival was significantly reduced in VIENNA 8 when assessed against wild-type ($p = 0.0127^*$) and IMPERIAL ($p = 0.0368^*$) strains alike. Egg-adult survival in wild-type and IMPERIAL strains, however, was statistically similar ($p = 0.3274$), indicative of good fitness in the IMPERIAL strain.

We also explored adult longevity with virgin males and females restricted to separate husbandry under regular lab conditions (Fig. 2C). Highest survival was observed in wild-type females, whilst the shortest longevity belonged to VIENNA 8 females. Pairwise comparisons via log-rank tests were performed between strains, and the combinations of strain and sex (tables S1 and S2). Altogether, VIENNA 8 had significant reductions in longevity compared to both wild-type ($p = 0.0022^{**}$) and IMPERIAL ($p = 0.0048^{**}$) strains. Wild-type Benakeion and IMPERIAL strains with the same genetic background, on the other hand, did not have a significant difference between one another ($p = 0.3920$). Amongst females, significant differences were recorded for VIENNA 8 against both wild-type ($p < 0.0001^{****}$) and IMPERIAL ($p < 0.0001^{****}$) strains, while male comparisons between strains were statistically insignificant. These results are suggestive of comparable longevity of the males and importantly females from the IMPERIAL strain with wild-type.

A delay in white-pupaed female development has been documented in the VIENNA 8 strain (40). To determine whether similar issues occur in the males of the IMPERIAL strain because of their white-pupaed phenotype, we compared pupal eclosion times from age-matched egg collections by sex. The development times were significantly different by strain and sex (nested ANOVA, $F = 178.6$, $p < 0.0001^{****}$) (Fig. 2D). The time for wild-type males (mean = 18.60 days) and females (mean = 18.75 days) to reach adulthood were statistically similar to IMPERIAL males (mean = 18.51 days) and females (mean = 18.59 days) (table S3). Flies from the VIENNA 8 strain were significantly slower to eclose, with means of 20.11 and 24.00 days for males and females

accordingly. Whilst there may be differences in acclimation of the VIENNA 8 to a newer laboratory environment in comparison to the other two strains, there was a significant difference between VIENNA 8 males and females ($p < 0.0001^{****}$), which is absent in both wild-type ($p = 0.94854$) and IMPERIAL ($p = 0.99781$) strains. The observed trends strongly indicate that the IMPERIAL strain does not experience a developmental discrepancy between sexes which is present in the current VIENNA 8 GSS.

The mating preferences of females towards the IMPERIAL, Benakeion and VIENNA 8 strains were assessed through simultaneous allowing of mating between males from all three strains with females from the irradiation-generated *wp*^{-/-} strain. Due to the recessive nature of the *wp* mutation, the father(s) were easily 'revealed' through pupal colour and corresponding adult phenotype screening (Table 1). The most common parentage belonged to wild-type male(s) (36.93%), followed by equal parentage by IMPERIAL male(s) (28.41%) and VIENNA 8 male(s) (28.41%). We also observed offspring cohorts from single mothers with mixed strain paternity at a low frequency (6.25%). This data indicates that the pupal colour of the IMPERIAL males does not disadvantage their mating success in respect to brown-pupaed VIENNA 8 males.

Here, we describe an entirely novel CRISPR/Cas9-generated CGSS method with proof of concept for the major agricultural pest *C. capitata* using the *wp* gene involved in pupal pigmentation (16). Although multiple robust next-generation GSS approaches have been established in the medfly to date (24-26, 33), the herein-established IMPERIAL strain is the first to exclusively encompass endogenous elements. Our approach uses the sex-specific intron of *tra*, a gene responsible for female-specific fate induction in the *Tephritidae* fruit fly family and beyond, making it an appealing target for cross-species application (41-42). Importantly, the Y-chromosome-independent GSS approach highlighted herein is time-effective as it only requires comprehensible cross completion for line establishment. Via CRISPR/Cas9-mediated HDR, we integrated the *tra* intron into the *wp* gene to achieve its expression in females exclusively, resulting in a brown-pupae phenotype. Due to sex-specific splicing (fig. S2), males in the strain do not translate *wp* transcripts successfully. Specifically, this is caused by the inclusion of early termination codons upon transcription leading to a white-pupae phenotype. Given that no foreign DNA is inserted into the genome, our approach may be easier to gain regulatory approvals for release, but this remains to be determined.

Characterisation of the IMPERIAL strain revealed that its fitness is comparative to its ancestral wild-type strain from which it was generated. This included survival during development and upon

adulthood, as well as eclosion time comparison between males and females (Fig. 2). Despite statistically similar egg-adult survival, however, the IMPERIAL strain egg hatching rates was insignificantly reduced. In all above-mentioned assessments the VIENNA 8 strain performance indicated greater fitness costs than in the herein generated strain, although mating competitiveness was similar between the two GSSs (Table 1). To explore the fitness of the IMPERIAL strain in further detail, and hence its suitability for SIT implementation, larger scale trials need to be performed. Among all males and females screened in the process of line characterisation in this study, no reverse phenotypes were observed, suggestive of vast line stability. Furthermore, similarly to wild-type flies, the males and females are consistently distributed in equal proportions in the IMPERIAL strain (Fig. 2A). This highlights the limited effect of the white-pupae phenotype on the male survival to adulthood in our GSS, opening a possibility for its efficient facility-based rearing. In theory, the sex-sorting of this strain can be performed at pupal developmental stage using automated machinery, already employed, and optimised at SIT *C. capitata* facilities. Thus, with its stability and cisgenic nature, our system could be an advantageous and cost-effective alternative for currently used strategies in SIT-mediated population control.

The latest reiteration of the traditional GSSs, VIENNA 8, possesses a heat sensitivity component via the *tsl* gene (15). As its wild-type copy is expressed from the Y-chromosome in an autosomally-mutant *tsl* (*tsl*^{-/-}) background, only males are tolerant to heat (4). To improve our system further, a heat-inducible component can be added to the IMPERIAL strain. This will additionally allow for a thorough investigation of the fitness costs in VIENNA 8 observed in this work. Once *tsl* locus is characterised in full, alternative iterations of the *tra* intron for functional male-specific splicing, or entirely different sex-specific introns (37, 43) can be used for its expression.

Data availability

Complete plasmid sequence is available at Addgene.org (#218233). The raw data used for figure generation is provided in the Source data files. The sequencing data are available at NCBI under BioProject PRJNA1189200 (Reviewer link <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1189200?reviewer=8am21kg9tm2eskhv6ls27knsd>) The herein-generated knock-in strain is available upon request from A.M.

Disclaimer

This material was made possible, in part, by a Cooperative Agreement from the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS). It may not necessarily express APHIS' views.

Acknowledgements

This work was supported by cooperative agreements (23-8130-1007-IA and AP23PPQS&T00C108 and AP19PPQS&T00C237 and AP22PPQS&T00C188) between the United States Department of Agriculture (USDA)—Animal and Plant Health Inspection Service (APHIS)— Plant Protection and Quarantine (PPQ) and Imperial College London and University of California—San Diego, respectively. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture, an equal opportunity employer. J.M. is supported by funding that was provided by the European Union's Horizon Europe Research and Innovation Programme REACT (Grant agreement 101059523).

Author Contributions

A.M and O.S.A conceived the project and directed the research with contributions from E.B. J.L and N.P.K designed the construct and J.L performed the cloning. A.M and O.S.A designed the experiments. A.M performed the germline transformation. S.D performed medfly and molecular validation experiments. S.D maintained the strains with the help of J.M. S.D, A.M and O.S.A analysed the data. J.L prepared samples for sequencing; I.A performed the sequencing and analysis. All authors contributed to writing and editing of the manuscript and approved the final article.

Competing interests

A patent has been filed on this technology. O.S.A is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. N.P.K is a founder of Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

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Figure legends

Fig. 1 Design behind the cisgenic IMPERIAL strain.

(A) A simplified diagram showcasing the IMPERIAL strain generation and its underlying mechanism. The knock-in, mediated via homology-directed repair was performed into the Benakeion wild-type strain. Due to the presence of premature stop codons in the male-specific exons, the males are phenotypically white-pupaed, whilst in females, gene rescue occurs resulting in a brown-pupae phenotype (E1-E4, Exons 1-4; LHA, left homology arm; MFS, Major Facilitator Superfamily; RHA, right homology arm; *tra*, transformer; *wp*, white pupae). (B) Graphic summary of IMPERIAL strain establishment via outcrosses to the irradiation-generated homozygous recessive *white pupae* mutant (*wp*^{-/-}) strain (KI, knock-in).

Fig. 2 Characterisation of the cisgenic IMPERIAL sexing strain.

(A) Stack graphs displaying pupal colour and adult phenotypes in IMPERIAL and VIENNA 8 strains for five consecutive generations (F2-F6). Chi-squared test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$. (B) Bar charts showing egg-adult survival of the IMPERIAL strain compared to both its parental wild-type Benakeion, and VIENNA 8 strains, completed in biological triplicates. Egg-adult survival was measured using 5-hour collections of eggs and their subsequent hatching rates, hatched larval-pupal recovery rates, and pupal-adult recovery rates. Bar levels represent mean values, whilst individual replicate values are shown with dots. Dunn's test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$. (C) Survival curves (Kaplan-Meier) of adult males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains. The 95% confidence intervals are displayed using pale shading for each test group. (D) Proportion of eclosing males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains measured daily from age-matched triplicate 24-hour egg collections. Dots represent mean values of the replicates and standard error is indicated using whiskers. (A-D) were constructed in RStudio. Source data is included in the source data file.

Table legends

Table 1 Mating preference of females towards wild-type, IMPERIAL and VIENNA 8 strains.

Males from the three tested strains were collectively released to mate with females from the recessive homozygous *white pupae* mutant (*wp*^{-/-}) strain in 3 repeats. Adult progeny from individual females was scored by pupal colour and corresponding adult phenotypes.

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475 **Supplementary materials**
476 **Generating Cisgenic Sexing Strains in Insect Pests**
477

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Materials & Methods

Plasmid design and construction

We used Gibson enzymatic assembly to build the 1167A plasmid, which contains *C. capitata* *MFS transporter* Exon 3 (LOC101451947), the *tra* intron and Opie2-DsRed outside the homology arms. A pre-existing plasmid containing *piggyBac* flanks, with an Opie2 promoter regulating DsRed, was linearised by NdeI and KpnI to clone 1167A. The *MFS transporter* Exon3 was amplified into two fragments from *C. capitata* genomic DNA using primer pairs 1167A.c1F and c2R, as well as 1167A.c5F and c6R (table S4). The *tra* intron was amplified from 795H1 (Addgene #205482) using primer pair 1167A.c3F and c4R, then inserted inside of the *MFS* exon 3 coding sequence (table S4).

***C. capitata* maintenance**

All fly stains were reared under standard lab conditions described previously (1). A carrot-based diet was provided for larval development (2) and a 1:1 yeast: glucose mix was given to adult flies. The Benakeion wild-type strain was supplied by the Saccone Lab (University of Naples “Federico II”), whilst irradiation-generated *white pupae* *-/-* (*wp**-/-*) (3) and VIENNA 8 D53- strains were obtained from the FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture (Seibersdorf, Austria).

***C. capitata* germline transformation**

Microinjections of the 1167A plasmid were performed into embryos of the wild-type Benakeion strain. The plasmid (250 ng/μl) was injected alongside a pre-assembled ribonucleoprotein (RNP) complex of Cas9 protein (200 ng/μl) (PNA Bio) and pre-synthesized gRNA-*wp* (100 ng/μl) (Synthego) (4).

***wp*^{KI/+} line establishment**

The cross scheme for G0-G3 line generation is summarized in Fig. 1. The injected G0s were reciprocally crossed to the characterised irradiation-generated *wp**-/-* strain (3). The resulting G1 progeny was separated by pupal colour, and all males emerging from white pupae were backcrossed in pools to the females from the irradiation-generated *wp**-/-* strain. At G2, the brown pupae were isolated, and all 10 female adults from the same parental G1 cross were collectively crossed to five sibling white-pupaed males. After mating, genomic DNA (gDNA) was individually extracted from G2 brown-pupaed females using an altered phenol-chloroform protocol (5). The

knock-in site was amplified using Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs®) with genome-specific 1167A_F and 1167A_R primers (table S4) designed in Geneious Prime 2023.1.2. To confirm the initial integration, the PCR products were purified via Monarch® PCR & DNA Cleanup Kit (New England Biolabs®) and analysed via Oxford Nanopore sequencing (Full Circle Labs).

Over the course of the following 4 generations (G3-G6) multiple sibling crosses were performed in parallel and the parental genotypes were verified. Hereby three possible alleles were differentiated: 1) the irradiation-generated *wp*^{-/-}; 2) the intron-less *wp*^{-/-} with indels and 3) the *tra* intron knock-in (fig. S5). For this, a multiplex 3-primer PCR was designed using a forward (1167A_F) binding upstream of the integration site, a reverse (1167A_R) downstream of the integration site and a second reverse (8kb_B_R) binding to the irradiation-generated *wp*^{-/-} inserted sequence (fig. S5).

Verification of genome integration site in the homozygous strain

To verify the insertion site in the *wp*^{KI/+} IMPERIAL strain, we conducted Oxford Nanopore genomic DNA sequencing. Genomic DNA was extracted from four knock-in adult males and four knock-in adult females using the Blood & Cell Culture DNA Midi Kit (Qiagen).

PCR *tra* intron splicing confirmation

In parallel, single males and females were separately collected for gDNA and RNA extractions. gDNA was extracted as detailed above. RNA was extracted via an adapted TRIzol® (Ambion)-chloroform-based protocol (6). cDNA was synthesised from total RNA using Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (ThermoFisher) according to the instructions provided by the manufacturer. Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs®) was used for PCR amplification from gDNA and cDNA templates using the 1167_F_V1 splicing and 1167A_R primer pair (table S4). Bands amplified from cDNA of the IMPERIAL strain were purified using a Monarch® DNA Gel Extraction Kit (New England Biolabs®) and Sanger sequenced (Genewiz Inc.). Male cDNA PCR reaction was additionally subjected to Sanger sequencing post-PCR product cloning using the StrataClone PCR Cloning Kit (Agilent) whereby different isoforms were isolated.

RNA sequencing

To confirm the sex-specific expression of the *wp* gene, we performed Illumina RNA sequencing. Total RNA was extracted from mature *wp*^{KI/+} adult males and females from the IMPERIAL strain in three biological replicates (six samples) using the miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen), following the manufacturer's protocol. Genomic DNA was removed using the gDNA eliminator column included with the kit. RNA integrity was tested with the RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies).

Male (ID# 27026-27028) and female (ID# 27023-27025) libraries with three replicates each were sequenced to approximate depth of 20M paired end reads (table S5). The reads were aligned with STAR (<https://github.com/alexdobin/STAR>) to the EGII-3.2.1 genome assembly (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_905071925.1/), into which *traF* intron was inserted at the white pupae locus (GCA_905071925.1_EGII-3.2.1_genomic.traF-intron.fna). To generate a more complete annotation file we transferred the Ccap_2.1 annotations (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_000347755.4/) to the EGII-3.2.1 genome by aligning Ccap_2.1 transcript sequences with BLAT and parsing the alignments to generate a GTF file (Supplemental File 1), which was used for all subsequent analysis steps. Gene abundances were quantified with featureCounts (<https://subread.sourceforge.net/featureCounts.html>), count data (table S6, source data) were converted to TPM (table S7, source data) and FPKM (table S8, source data) values and combined using Perl scripts. Gene annotations were downloaded from EnsemblMetazoa using the BioMart tool (https://metazoa.ensembl.org/Ceratitis_capitata_gca000347755v4/Info/Index) and added to the quantification data (tables S6-S8, source data). TPM values were used to perform PCA and clustering analyses in R to identify possible sample outliers. Replicates for each sex clustered together as expected and displayed high correlations between each other without obvious outliers (Fig. S4, table S9, source data). To visualize splicing of the *traF* intron within the white pupae locus (LOC101451947), BAM files produced by STAR were imported into IGV (<https://igv.org/doc/desktop/>).

Stability assay

From G3 until G7 all pupae were separated by colour and corresponding adults were scored by sex. When the homozygosity of the IMPERIAL strain was verified at G7 (F0), alongside the VIENNA 8 D53- strain, for 5 consecutive generations (F2-F6) eggs were collected five days after eclosion and raised under regular conditions until pupal stage of development. Brown and white pupae were then separated, and the sex of all adults from each pool was recorded.

Egg-adult survival assay

Sibling crosses of 10 males and 20 females were set up in triplicates simultaneously for the IMPERIAL, VIENNA 8 D53-, and wild-type Benakeion strains. Egg numbers and their hatching rates were determined as described previously (7). Specifically, five days after eclosion, all eggs laid within a 5-hour period were collected and unhatched eggs were counted twice four days apart using Fiji (8). Pupal and adult recovery rates were determined thereafter.

Adult longevity assay

Age-matched adults from the wild-type Benakeion, IMPERIAL and VIENNA 8 D53- strains were separated by sex upon eclosion and placed into cages of 10 individuals. Three male and three female replicates were set up simultaneously for each strain; and maintained under standard conditions thereafter. Daily, dead flies were counted and removed from the cages for 30 consecutive days.

Mating-preference assay

90 females from the irradiation-generated *wp*^{-/-} strain (3) were simultaneously placed together with 15 males from each of the Benakeion, IMPERIAL and VIENNA 8 D53- strains for a total of a 1:2 male: female ratio. The experiment was repeated 3 times. The flies were left to mate for 4 full days, after which females were separated into individual small cages. Upon oviposition, eggs from all females which oviposited were separately collected and reared normally until pupation. In the cases where white and brown pupae were present, they were separated by colour. Adults eclosing from both mixed and brown pupae-only collections were screened by sex.

Eclosion assay

24-hour egg collections were made from sibling crosses of 10 males and 20 females of the IMPERIAL, VIENNA 8 D53-, and wild-type Benakeion strains, set up in parallel triplicates. The offspring were reared under normal conditions until pupation, whereby IMPERIAL and VIENNA 8 pupae were sorted by colour. The eclosing adults were therein scored by sex every day.

Figure generation and statistical analyses

All statistical analysis and plot generation was performed in RStudio. Chi-squared tests were used for sex ratio and pupal colour analysis. Egg-adult survival was assessed using Kruskal-Wallis and Dunn's tests. Longevity data was plotted and analysed using survival and survminer packages.

Eclosion rates were assessed using nested ANOVA by strain, with sex added in as an extra factor. Pairwise comparisons were conducted via a *post hoc* Tukey HSD test thereafter. Microsoft PowerPoint and Inkscape 1.3.2 (9) were used to create construct and fly-centred diagrams.

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Supplementary Figure legends

Fig. S1 Oxford Nanopore genome sequencing of the *white pupae* genomic site.

Diagrams depicting the nanopore sequencing reads (top grey) aligning to the *white pupae* genomic location (blue) with the *tra* intron inserted in the IMPERIAL strain.

Fig. S2 *white pupae* is spliced sex-specifically in the IMPERIAL strain.

(A) The diagram showing the *white pupae* gene in the genome of the *transformer* (*tra*) intron IMPERIAL knock-in (KI) strain with forward and reverse primers labelled as F and R, accordingly (table S4). (B) The annotated electrophoresis gel of the PCR products using primers from (A). The PCR was performed on genomic and complementary DNA templates from wild-type and IMPERIAL adults. The DNA ladder and the negative control were run in the first and tenth wells, respectively.

Fig. S3 RNA sequencing alignments to the *wp* genomic locus.

A zoomed-out genome browser view of the *white pupae* genomic locus and gene structure (blue bars on bottom). RNAseq reads were aligned (light grey bars) for the female samples (27023-27025) and the male samples (27026-27028). The inserted *tra* intron is indicated in dotted red box.

Fig. S4 Medfly RNAseq Clustering and Principal Component Analysis.

Fig. S5 *white pupae* genotyping for homozygous IMPERIAL strain generation.

Diagrams depicting the genotyping strategy to distinguish the three possible alleles, used at every generation from G2 till G7.

Supplementary Table legends

Table S1 Statistical analysis of adult longevity by strain.

695 *P*-values for strain-by-strain pairwise log-rank test comparisons for adult longevity with Bonferroni
696 corrections.

697

698 **Table S2 Statistical analysis of adult longevity by strain and sex.**

699 *P*-values for pairwise log-rank test comparisons by sex and strain for adult longevity with
700 Bonferroni corrections.

701

702 **Table S3 Statistical analysis of pupal eclosion times by strain and sex.**

703 *P*-values for pairwise comparisons of *post hoc* Tukey HSD test after nested ANOVA by strain and
704 sex.

705

706 **Table S4 Primer summary.**

707 Sequences of primers used for construct cloning and cisgenic strain characterisation.

Figures

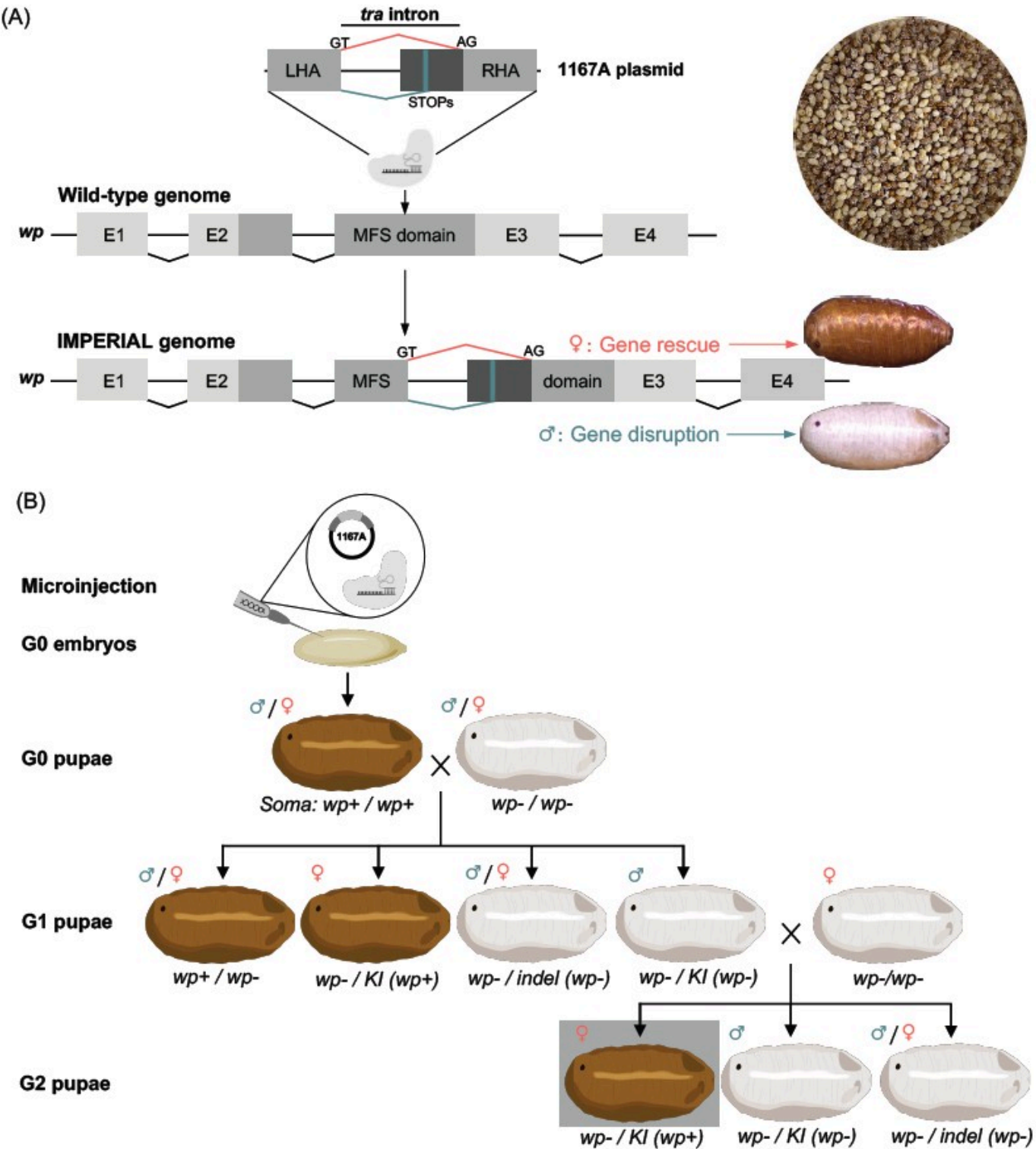


Figure 1

Design behind the cisgenic IMPERIAL strain.

(A) A simplified diagram showcasing the IMPERIAL strain generation and its underlying mechanism. The knock-in, mediated via homology-directed repair was performed into the Benakeion wild-type strain. Due to the presence of premature stop codons in the male-specific exons, the males are phenotypically whitepupaed, whilst in females, gene rescue occurs resulting in a brown-pupae phenotype (E1-E4, Exons 1-4; LHA, left homology arm; MFS, Major Facilitator Superfamily; RHA, right homology arm; tra, transformer; wp, white pupae). (B) Graphic summary of IMPERIAL strain establishment via outcrosses to the irradiation generated homozygous recessive white pupae mutant (wp^{-/-}) strain (KI, knock-in).

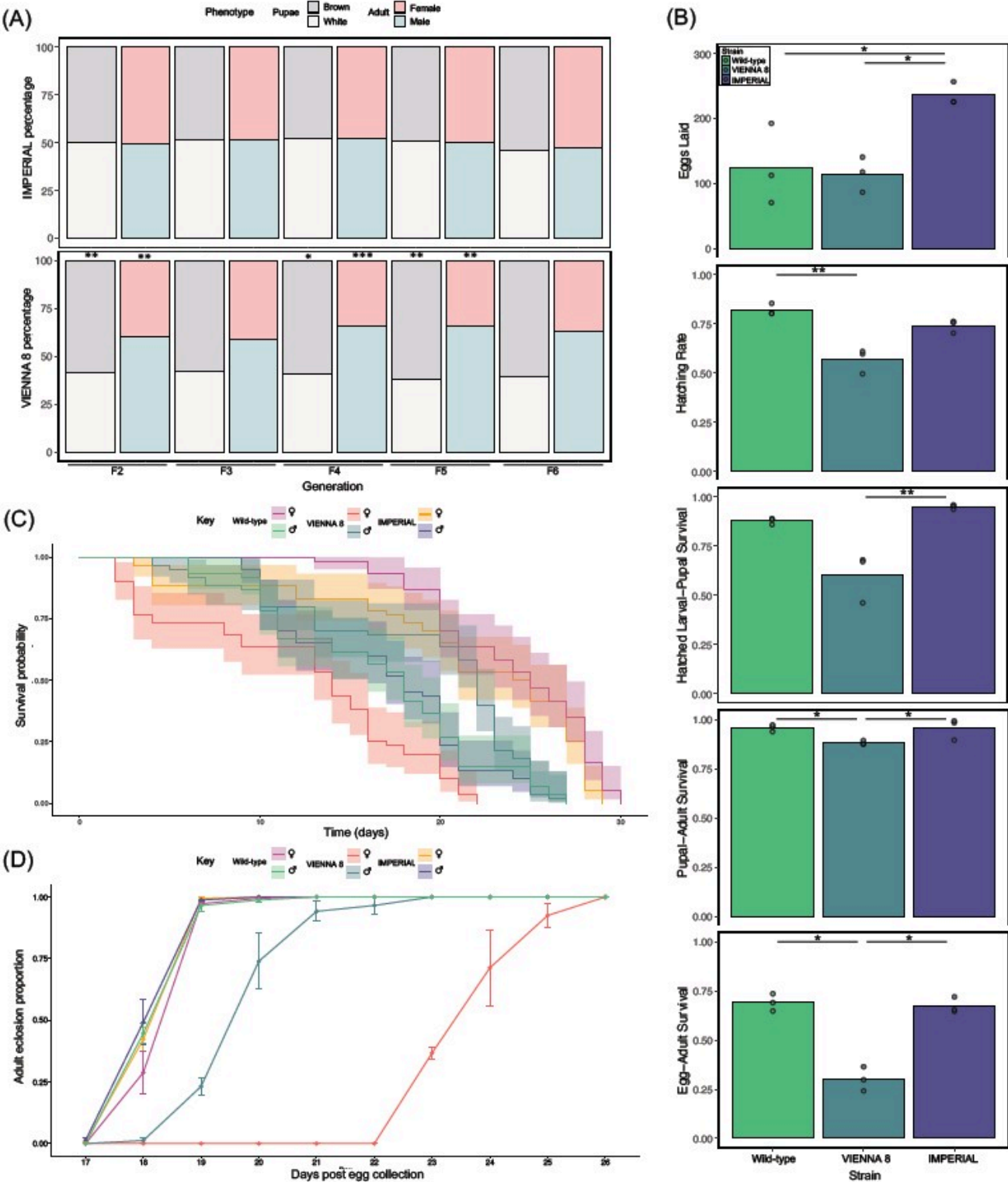


Figure 2

Characterisation of the cisgenic IMPERIAL sexing strain.

(A) Stack graphs displaying pupal colour and adult phenotypes in IMPERIAL and VIENNA 8 strains for five consecutive generations (F2-F6). Chi-squared test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$. (B) Bar charts showing egg-adult survival of the IMPERIAL strain compared to both its parental wild-type Benakeion, and VIENNA 8 strains, completed in biological triplicates. Egg-adult survival was measured using 5-hour collections of eggs and their subsequent hatching rates, hatched larval-pupal recovery rates, and pupal-adult recovery rates. Bar levels represent mean values, whilst individual replicate values are shown with dots. Dunn's test significance levels are indicated as follows: $p < 0.05 = *$, $p < 0.01 = **$. (C) Survival curves (Kaplan-Meier) of adult males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains. The 95% confidence intervals are displayed using pale shading for each test group. (D) Proportion of eclosing males and females from IMPERIAL, VIENNA 8 and wild-type Benakeion strains measured daily from age-matched triplicate 24-hour egg collections. Dots represent mean values of the replicates and standard error is indicated using whiskers. (A-D) were constructed in RStudio. Source data is included in the source data file.

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