Male-specific Y-linked transgene markers to enhance biologically-based control of the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae)

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**Abstract**

**Background:** Reliable marking systems are critical to the prospective field release of transgenic insect strains. This is to unambiguously distinguish released insects from wild insects in the field that are collected in field traps, and tissue-specific markers, such as those that are sperm-specific, have particular uses such as identifying wild females that have mated with released males. For tephritid fruit flies such as the Mexican fruit fly, *Anastrepha ludens*, polyubiquitin-regulated fluorescent protein body markers allow transgenic fly identification, and fluorescent protein genes regulated by the spermatocyte-specific β2-tubulin promoter effectively mark sperm. For sterile male release programs, both marking systems can be made male-specific by linkage to the Y chromosome.

**Results:** An *A. ludens* wild type strain was genetically transformed with a piggyBac vector, pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3], having the polyubiquitin-regulated EGFP body marker, and the β2-tubulin-regulated DsRed.T3 sperm-specific marker. Autosomal insertion lines effectively expressed both markers, but a single Y-linked insertion (YEGFP strain) expressed only PUbnlsEGFP. This insertion was remobilized by transposase helper injection, which resulted in three new autosomal insertion lines that expressed both markers. This indicated that the original Y-linked Asβ2tub-DsRed.T3 marker was functional, but specifically suppressed on the Y chromosome. The PUbnlsEGFP marker remained effective however, and the YEGFP strain was used to create a sexing strain by translocating the wild type allele of the black pupae (bp⁺) gene onto the Y, which was then introduced into the bp-mutant strain. This allows the mechanical separation of mutant female black pupae from male brown pupae, that can be identified as adults by EGFP fluorescence.

**Conclusions:** A Y-linked insertion of the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] transformation vector in *A. ludens* resulted in male-specific expression of the EGFP fluorescent protein marker, and was integrated into a black pupae translocation sexing strain (T(YEGFP/bp⁺)), allowing the identification of male adults when used in sterile male release programs for population control. A unique observation was that expression of the Asβ2tub-DsRed.T3 sperm-specific marker, which was functional in autosomal insertions, was specifically suppressed in the Y-linked insertion. This may relate to the Y chromosomal regulation of male-specific germ-line genes in *Drosophila*.

**Background**

A critical component to any prospective field release of a transgenic insect strain is a reliable and robust marking system. Foremost, this is to unambiguously identify the transgenic insects, and to distinguish them from insects in the field, especially in traps that monitor the effectiveness of the release program [1]. For tephritid fruit flies, fluorescent protein markers regulated by the constitutive polyubiquitin (PUB) gene promoter are quite effective since the PUB promoter is active in all cell types throughout development (see [2-4]), and for the Caribbean fruit fly, PUB-DsRed.T3 can be visualized unambiguously and detected by PCR in deceased flies maintained in two types of liquid field traps for up to three weeks [5]. A high priority for SIT programs is evaluating the number of wild females that have mated with released sterile males, which can be achieved by sperm-specific markers. Using the spermatocyte-specific
β2-tubulin promoter [6] to regulate either EGFP or DsRed, fluorescent sperm markers detectable specifically in the female spermathecae, have been developed for several tephritid and mosquito species [7-11]. The Mexican fruit fly, Anastrepha ludens, has been successfully transformed using piggyBac transposon vectors [12], and specifically by those having fluorescent protein marker genes regulated by the Drosophila polyubiquitin and A. suspensa β2-tubulin (Asβ2tub) promoters [13]. In selecting for dual-marked pBXL[PLubnlsEGFP, Asβ2tub-DsRed.T3] transformants, we noted that in autosomal integrations, as determined by segregation analysis, males and females expressed EGFP in the body while only males expressed testis-specific DsRed. However, one line expressed EGFP specifically in males and not in females, suggesting a Y-linked integration, but the expected testis-specific expression of DsRed was not apparent. Here we provide data showing that remobilization of the Y-linked insertion to autosomal sites restores Asβ2tub-DsRed.T3 expression, indicating that Y-specific suppression of the Asβ2-tubulin promoter may be occurring.

Sex-specific fluorescent protein markers, such as those linked to the Y-chromosome (or Z-chromosome in moths), or whose expression is controlled by a sex-specific promoter or intron-splicing mechanism, can be used for sexing strains previous to release [14,15]. This is particularly advantageous for SIT [16] where sterilization and sexing strains previous to release [14,15]. This is particularly advantageous for SIT [16] where sterilization and sexing strains previous to release [14,15].

Methods. From YEGFP irradiated pupae, 900 adult males were screened, from which five potential lines were selected where all females had the mutant color phenotype, in addition to green fluorescence observed in four other autosomal insertion lines, was not observed in the Y-linked line (Fig. 1A-C). The structural integrity of the Asβ2tub-DsRed.T3 vector construct in YEGFP was verified by PCR sequencing (see Additional file 2), indicating that this was not due to a mutation or rearrangement. Therefore, to determine whether suppression of Asβ2tub-DsRed.T3 was due to a chromosomal position effect the vector was re-mobilized by injection of phsp-pBac transposase helper plasmid into 832 embryos from the YEGFP hemizygous line. Of these, 40 G0 surviving males were individually crossed to three wild type females, resulting in three G1 lines where adult males expressed both thoracic EGFP and testis-specific DsRed fluorescence (Fig. 1D-F), whereas the remaining 37 fertile matings expressed only EGFP. Segregation analysis of crosses to wild type indicated that the DsRed fluorescent lines resulted from remobilization into autosomal loci. In addition to PCR transgene sequencing in the YEGFP and ME8 autosomal line, derived from the vector remobilization in YEGFP (see Additional file 2), this verifies the functional integrity of the original Y-linked vector insertion, and suggests that Y chromosome suppression of Asβ2-tub-DsRed.T3 expression had occurred. Transposon vector remobilizations typically result in local insertions (or ‘hops’) into sites within the same linkage group (which facilitates transposon mutagenesis strategies) [23]. It is not unlikely that local hops occurred in this remobilization as well, which would not have been recognized if Asβ2-tubulin promoter suppression was a general attribute of Y linkage, and not limited to a specific locus (or loci).

Translocation Y-EGFP/bp+ strain development. To create a black pupae sexing strain marked with male-specific PLubnlsEGFP expression to identify released males in traps, the YEGFP strain was used as a host strain for a bp+ translocation induced by γ-irradiation as described in Methods. From YEGFP irradiated pupae, 900 adult males were screened, from which five potential lines were selected where all females had the mutant black pupae (bp) phenotype, and all males had the brown pupae (bp+) wild type phenotype, in addition to green fluorescence observed under epifluorescent optics (Table 1; Figure 2).

Evaluation of the Y(YEGFP/bp+) strains. Life fitness parameters for the five translocation strains were evaluated by observing the survival of 1,000 embryos through life stages from larval hatching to adulthood. Overall survival from the egg stage to adulthood was 17.6% in
Figure 1 Y-linked and autosomal fluorescent marker expression in *A. ludens* transformed with pBXL{pubnlsEGFP, Asβ2tub-DsRed.T3}.

The brightfield (BF; A, D, G) and epifluorescent EGFP (GFP2; B, E, H), and DsRed (TXR; C, F, I) phenotypes of: a YEGFP male (left) and female (right) shown in panels A, B, and C; an autosomal insertion (unmapped) strain male (left) and female (right) shown in panels D, E, and F; and testes from a YEGFP and autosomal insertion strain male shown in panels G, H, and I. See Methods for details on epifluorescent microscopy and filter sets.
line T(YEGFP/bp+)-1 to 36.4% in line T(YEGFP/bp+)-4, which was comparable to 38.1% survival in the Tapachula-7 control strain already being mass-reared for SIT programs. Line T(YEGFP/bp+)-3 had a similar survival rate of 33.4% (Table 2). The integrity of translocation strains can often be compromised by recombination, especially between sequences within the translocated autosome. When this occurs in sequences proximal to the centromere, the mutant and WT alleles can be exchanged resulting in a breakdown of the sexing system. To assess such recombination in the T(YEGFP/bp+) lines, they were maintained without selection for four generations and then screened for an exchange of the bp+ and bp- phenotypes in males and females. In the T(YEGFP/bp+)-1 and -2 lines recombinant individuals were not detected, while the T(YEGFP/bp+)-3, -4 and -5 lines exhibited 0.28% (1 male bp-), 0.23% (1 female bp+) and 1.74% (4 male bp-; 2 female bp+) recombinant frequencies, respectively. These frequencies are considerably higher than the 0.05% frequency for Tapachula-7 [21], and is most likely a function of the distance between the bp allele and translocation breakpoint, which is expected to increase with distance [24]. Since the strains exhibiting recombinants were also the most highly viable, induction of an inversion in this region to suppress recombination, as has been achieved for the medfly VIENNA-8 translocation sexing strain [24], may be considered. Selection of additional translocation lines having strong viability and minimal recombination is also feasible.

### Discussion

Here we report the creation of an A. ludens transgenic line with a piggyBac transformation vector that includes fluorescent protein markers useful for identifying released males in the field and wild females that have mated with the released males. Notably, the vector insertion site is Y-linked, so that a sexing line could be created by translocating the wild type allele for the bp mutation onto the Y chromosome, allowing the separation of black pupal (bp-) females from brown pupal (bp+) males during rearing.

Use of pupal color markers in Y-translocation strains has been an efficient means of creating sexing strains in tephritid flies [18,19]. Recessive mutations resulting in pupal phenotypes exhibiting darker or lighter coloration than wild type are relatively common, and translocations of their wild type allele to the male-specific Y chromosome are straightforward to create and select. Relatively inexpensive rice sorters can then be used to efficiently separate large numbers of wild type male pupae from mutant females. One drawback is that, typically, pupal markers do not confer an adult phenotype (or one that is easily identifiable), so that identification of released males

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**Table 1 F2 progeny of YEGFP/bp+ translocation males**

| Lines          | Pupae | F2 adults* |  |  |  |  |  |
|----------------|-------|------------|---|---|---|---|---|
|                | bp+   | bp-        | bp+ | male | female | bp+ | male | female |
| T(YEGFP/bp+)-1 | 57    | 46         | 51  | 0   | 0     | 40  | 88.35 |
| T(YEGFP/bp+)-2 | 34    | 30         | 21  | 0   | 0     | 19  | 62.50 |
| T(YEGFP/bp+)-3 | 66    | 51         | 62  | 0   | 0     | 45  | 91.45 |
| T(YEGFP/bp+)-4 | 73    | 57         | 40  | 0   | 0     | 18  | 44.62 |
| T(YEGFP/bp+)-5 | 39    | 25         | 35  | 0   | 0     | 13  | 75.00 |

* adults emerging from indicated pupal phenotypes
depends upon the use of fluorescent powders that can be unreliable (due to loss from grooming or transfer to wild males), and a health risk to workers involved in rearing and release [22]. Thus, the male-specific Y-linked fluorescent protein transgene marker provides a reliable means of identifying released male adults in traps, a secondary means of verifying pupal sex if cuticle coloration is ambiguous, and a rapid means of identifying putative recombinants (having an EGFP/bp- phenotype). If Y-linked fluorescence is detectable in embryos or early stage larvae, it may be eventually useful as a means to select males by automated fluorescence-based sorters early in development [9], thereby eliminating females previous to rearing to the pupal stage, which is costly and inefficient.

The Y-linked transformant line was originally selected during a previous transformation experiment, where both the polyubiquitin-regulated EGFP body color marker and the β2-tubulin-regulated sperm marker were easily identifiable and distinguishable in autosomal integrations [13]. However, while the Y-linked PUbnlsEGFP marker was strongly expressed and reliably detected in males, the Asβ2tub-DsRed marker was not visibly detectable, which we presume is the result of suppressed transcription since its sequence integrity has been verified. This is unfortunate since it eliminates the ability to identify females that have mated with the transgenic males by identifying fluorescent sperm stored in their spermathecae. However, remodeling of the Y-linked integration to autosomal sites restored Asβ2tub-DsRed expression, which may be similarly achieved in T(YEGFP/bp-*) strains by a local remodeling of the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] vector to the translocated autosome, thereby maintaining male-specificity. Alternatively, an autosome carrying the vector transgene could be crossed into the translocation line, thus providing both fluorescent markers.

Beyond an unusual phenomenon, the Y-specific suppression of the Asβ2tubulin promoter may, nevertheless, have important implications for how the male germ-line is regulated by the Y chromosome in tephritids. Position effect variegation (PEV), resulting from suppression of gene expression typically affecting euchromatic genes positioned proximal to or within heterochromatin, is well documented [25]. Differential promoter regulation by PEV is less well established, but evidence exists in D. melanogaster for the Y chromosome having a general suppressive effect on PEV [26–28], and for specific regions of the Y chromosome having a positive trans-activator function specifically for transcription of male germ-line genes [29]. If this type of activity occurs in mexfly, it is conceivable that the transgene vector integration may have disrupted Y-activation of the Asβ2-tubulin promoter, but if so, other germ-line genes (including the native A. ludens β2-tubulin gene) also should have been affected resulting in diminished fertility, which was not apparent. Remobilization of the transgene could have also resulted in local hops within the Y, with the expectation that a site or region-specific position effect on the original insertion would be less effective in some remobilized Y-linked lines, which was also not apparent. Thus far, the specific suppression of a Y-linked β2-tubulin gene promoter, or any other promoter, is a unique observation. It will be important to determine whether this is the result of a gene expression regulatory function that is specific to a particular Y-linked locus or region, or a chromosome-wide effect for the chromosome, and whether other male germ-line specific genes are similarly affected.

**Methods**

**Insect strains.** The black pupae (bp-) mutant strain was originally isolated from A. ludens flies mass-reared at the MOSCAFRUT facility. The pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] transgenic strains were created as previously described [13], with the YEGFP strain having a Y-linked integration based on segregation analysis. Transgenic flies were screened by epifluorescence microscopy for DsRed (TXR filter: ex: 560/40; em: 610 LP) and EGFP (GFP2 filter; ex: 480/40, em: 510 LP)
fluorescence. The wild type Chiapas strain was originally collected from infested fruit in the state of Chiapas, Mexico, and the genetic sexing strain “Tapachula-7” was created as described [21].

Plasmids. The pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] piggyBac transformation vector (plasmid #389) used to create the YEGFP strain was described previously (see Additional file 1) [10,13]. The piggyBac transposase helper plasmid, phsp-pBac, used to remobilize pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] in YEGFP, was described previously [30].

pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] remobilization. Remobilization of the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] vector in YEGFP /bp+ translocation strain. Pupae from the YEGFP /bp+ strain were γ-irradiated with 30 Gy using Cobalt60, with newly eclosed males crossed to homozygous bp+/bp+ mutant females. Phenotypic wild type (brown) F1 males, having the genotypes YEGFP /bp+ /bp+ or T(YEGFP, bp+bp+); D(bp+)/bp+ , were backcrossed to bp+/bp+ females in single pair matings, with F2 T(YEGFP, bp+bp+) translocation lines identified by those having all males eclosing from brown pupae (bp+bp+) (expected in all lines), but where all females eclosed from black pupae (bp−bp−), versus black and brown female pupae generated from non-translocation males. F2 females inheriting the D(bp+) autosome from translocation males were lethal due to aneuploidy, and thus only bp+/bp+ females survived. Male-specific expression of PUbnlsEGFP also indicated that the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] Y-linked insertion was not deleted by the translocation, and these lines were designated as T(YEGFP/bp+).

Life fitness test. All translocation lines were inbred with approximately 1,000 eggs from each line put on artificial diet in groups of 100 eggs, with larvae and pupae collected and recorded [31]. Pupae were sexed by pupal color that was verified after adult eclosion. The same procedure was applied as a control to the Chiapas wild type and Tapachula-7 strains. Statistical analysis was carried out comparing the YEGFP translocation strains, the Tapachula-7 strain and the wild type A. ludens strain by analysis of variance (ANOVA) and Tukey-Kramer tests [32].

PCR analysis. To verify the integrity of the Asβ2tub-DsRed.T3 marker transgene in autosomal and Y-linked vector integrations, genomic DNA from the autosomal ME8 and Y-linked T(YEGFP, bp+) lines was isolated for PCR reactions using the primer pair P15 (GGTGGAG CTCCTAGCTTTGT TTC) / MFS-10 (ACGACCCCGT- GAGTCAAATGAGCC) and Platinum Taq polymerase (Invitrogen). PCR was performed on both genomic samples and the control AH389 vector plasmid using the following conditions: 1 min at 95°C; 5 cycles of 15 s at 94°C, 20 s at 65°C (-2°C/cycle), 2.5 min at 72°C; 30 cycles of 30 s at 94°C, 45 s at 56°C, 2.5 min at 72°C; and 3 min at 72°C. All 2.4 kb fragments were subcloned in pCR4 vector (Invitrogen) and sequenced at Macrogen using the oligos M13F, M13R and P17 (CCCTCCGAGGGGAATTCAGC). Multiple sequence alignments were performed in Geneious 7.1 (Biomatters, Ltd.) using the standard Geneious Alignment algorithm.

Additional material

Additional file 1: Schematic (to scale) of the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] transformation vector.

Additional file 2: Integrity of Asβ2tub-DsRed.T3 marker transgene. A multiple sequence alignment of PCR sequenced transgene vector fragments from genomic DNA from the Y-linked YEGFP and autosomal ME8 translocation lines, and the pBXL[PUbnlsEGFP, Asβ2tub-DsRed.T3] plasmid vector. This verifies the integrity of the marker transgene in the two transformant lines based on 100% identity among the sequences.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JSM, AMH, MFS, and CSZ-C conceived of the study, its design and coordination, and JSM wrote the manuscript, for which the final draft was read and approved by all authors.

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