The characterisation of sex determination genes in Drosophila Melanogaster has shown that the product of a gene controls the sex-specific splicing of the pre-mRNA from the downstream gene in the genetic cascade (reviewed in Sánchez et al., 2005). Sex-lethal (Sxl) is at the top of this cascade and acts as the memory device for female sexual development via its auto-regulatory function: its product controls the splicing of its own pre-mRNA (Bell et al., 1991). In addition, Sxl controls the splicing of the pre-mRNA from the downstream gene transformer (tra) (Boggs et al., 1987; Belote et al., 1989). The Tra product and the product of the constitutive gene transformer-2 (tra-2) (Goralski et al., 1989; Amrein et al., 1990) control the sex-specific splicing of the pre-mRNA of the gene doublesex (dsx), which is transcribed in both sexes (Burtis and Baker 1989). In females, the Tra-Tra2 complex binds to the female-specific exon (see Fig. 2A) and directs the splicing of the dsx pre-mRNA according to the female mode, giving rise to the female DsxF protein that promotes female sexual development. In males, in which no functional Tra protein is available, the female DsxF protein that promotes female sexual development.

Genes homologous to the sex determination genes of D. melanogaster have been sought in other insects (reviewed in Sánchez et al., 2008; Verhulst et al., 2010; Gempe and Beye 2010). In the tephritid fruit flies, the Sxl gene is not regulated in a sex-specific fashion (Saccone et al., 1998; Lagos et al., 2005) so that in the tephritids, Sxl does not appear to play the key discriminating role in sex determination that it plays in Drosophila. As in the drosophilids, the tephritid tra (Pane et al., 2002; Lagos et al., 2007; Ruiz et al., 2007) and tra-2 (Salvemini et al., 2009; Sarno et al., 2010) genes are expressed in both sexes. The tra primary transcript shows sex-specific alternative splicing. However, whereas in the drosophilids Sxl regulates tra, in the tephritids this gene appears to have an auto-regulatory function that produces functional Tra protein specifically in females (Pane et al., 2002; Lagos et al., 2007; Ruiz et al., 2007) so that it plays the key regulatory memory device for sex determination (Pane et al., 2002). A similar role for tra has been observed in the dipterans Musca domestica (Muscidae) (Hediger et al., 2010) and in Lucilia cuprina (Calliphoridae) (Concha and Baker 1997). The injection of the respective tra-2 dsRNA into Musca (Burghardt et al., 2005), Ceratitis (Salvemini et al., 2009) and Anastrepha (Sarno et al., 2010) results in the destruction of endogenous tra-2 function in these species and the subsequent male-specific splicing of the endogenous tra and dsx pre-mRNAs, leading to the transformation of genotypically female embryos into adult pseudomales. This highlights the role of tra-2 in Musca, Ceratitis and Anastrepha sex determination.
The present paper studied whether the *Anastrepha* Tra2 protein shows conserved sex-determination function in *Drosophila*; i.e., whether *Anastrepha* Tra2 is able to substitute the endogenous *Drosophila* Tra2 protein in the control of sex determination in this species.

**Results and Discussion**

The rationale of the experiment was to express the transgenic AoTra2 protein in *Drosophila* XX pseudomales lacking the *tra-2* gene function and checked whether these pseudomales showed feminisation. The GAL4-UAS system was used to express the AoTra2 protein in *Drosophila*.

The systemic expression of AoTra2 with the ubiquitous-expression *da-GAL4* or *hs-GAL4* drivers was found to be lethal to both male and female flies. The same lethality has been observed in *Drosophila* males and females that ectopically express their own Tra2 protein (Qi et al., 2006). Therefore, the *m-GAL4* local expression driver was used. This driver expresses GAL4 in agreement with the expression domain of the gene *rotund* (*rn*), which is expressed in imaginal discs as well as in the embryonic and larval central nervous systems (CNS) (St. Pierre et al., 2002). The expression of *rn* in the tarsal region of the foreleg imaginal disc commences during the early third larval instar, but is no longer evident in the late third instar, though in the *m-GAL4* line persists the tarsal expression (St. Pierre et al., 2002). This *m-GAL4* driver allowed the expression of the AoTra2 protein in the foreleg basitarsus, a well-characterised sexually dimorphic region of *Drosophila*. The expression of AoTra2 by the *m-GAL4* driver was also lethal to both males and females. This might be due to the expression of AoTra2 in the embryonic and/or larval CNS, as mentioned above.

To prevent embryonic lethality a strategy was followed that allowed the temporal control of AoTra2 expression under the *m-GAL4* driver. For this purpose the GAL4/GAL80 system was used. The GAL80 protein inhibits GAL4 function. GAL80 is temperature sensitive, with 18°C the most permissive temperature and 29°C the most restrictive (McGuire et al., 2003). *Drosophila* XX pseudomales mutant for *tra-2* and carrying *Aotra-2-UAS* together with *rn-GAL4* and *Tub-GAL80ts* were produced. The cross producing these pseudomales was performed at 18°C, and several two-days egg collections were made. Three days later each collection was transferred to 29°C environment. By this time the larvae had hatched, which were maintained at this temperature for the rest of their development. This treatment eliminated the embryonic lethality caused by the expression of the AoTra2 protein. Even under these conditions, the males and females expressing the AoTra2 protein were lethally affected, probably because of an excess of this transgenic protein affecting the development of the larval CNS.

Finally, since at 25°C *GAL80* retains some function (McGuire et al., 2003), it was reasoned that if XX pseudomales mutant for *tra-2* and carrying *Aotra-2-UAS*, *m-GAL4* and *Tub-GAL80ts* can develop at that temperature, the GAL4 from the *m-GAL4* driver ought not to be completely inhibited. Consequently a certain amount of AoTra2 protein ought to be produced (less than at 29°C) but which might not be lethal. Thus, these pseudomales might be able to survive to adulthood. This was the case. Fig. 1 shows the effect of expressing the AoTra2 protein on the sexually dimorphic development of the foreleg basitarsus in XX pseudomales and in their brother XY males, both mutant for *tra-2* and carrying *Aotra2-UAS*, *m-GAL4* and *Tub-GAL80ts*. The foreleg basitarsus contained several transversal rows, the last one forming the sex comb structure (SC) in males and in XX pseudomales mutants for *tra-2* (Fig. 1C). This is composed of dark, thick bristles, and is rotated to lie parallel to the proximal-distal leg axis. Females lack the sex comb (Fig. 1B). A significant reduction (P<0.0001, one-way ANOVA) was seen in the number of bristles forming the sex comb structure (SC) in males and in XX pseudomales mutants for *tra-2* (Fig. 1C).

![Fig. 1. Effect of the Aotra2 transgene on the somatic development of Drosophila.](image)

(A) Size (number of bristles) of the sex comb in the Aotra2 transgenic *Drosophila* flies. The bars in the histograms represent the 95% confidence limits. The sample size is given in parenthesis underneath the histogram. XX pseudomales: *ywTDAo#1*/*; *tra-2*/*Df(2R)*; *Tub-GAL80ts*, *w*+/*; *rn-GAL4*, *w*+/*). XY males: *ywTDAo#1/Y; *tra-2*/*Df(2R)*; *Tub-GAL80ts*, *w*+/*; *rn-GAL4*, *w*+/*). (Cross 1 in Materials and Methods). (B) Foreleg basitarsus of wild type female. LTR stands for last transversal row. (C) Foreleg basitarsus of XX pseudomale mutant for *tra-2* at 18°C. SC stands for sex comb. (D,E) Foreleg basitarsus of XX pseudomales mutant for *tra-2* at 25°C.
The presence of endogenous *Drosophila* Dsx protein is expected to cause this reversion of the male towards the female phenotype. The analysis of the *Drosophila* dxF pre-mRNA splicing in transgenic *Drosophila* XX pseudomales mutant for tra-2 and expressing the AoTra2 protein confirmed this expectation. The inducible hs-GAL4 driver was used to express the AoTra2 transgene. XX pseudomales yw/w; Df(2R)trix,tra2[-]/tra-2B; Aotra2/hs-GAL4 were produced at 25°C. After the hatching of the adults the flies were divided into two populations; one was maintained at 25°C (control flies) and one subjected to heat-shock pulses (experimental flies) to induce the expression of the Aotra2 transgene. At 25°C, the transgenic lines did not express the female dxF mRNA isoform (Fig. 2B). After the heat shocks, however, these transgenic lines expressed the female dxF mRNA isoform (Fig. 2B). Two amplicons could be detected. The smaller one (646 bp) corresponded to the female Drosophila dxF gene, the larger one (758 bp) was to be expected if the intron 3 were retained (Fig. 2B). Two amplicons could be detected. The smaller one (646 bp) corresponded to the female dxF mRNA. The larger amplicon (758 bp) was to be expected if the intron 3 were retained (Fig. 2B). The cloning and sequencing were not the consequence of the heat-shocks since their brothers (males yw/Y; Df(2R)trix,tra2[-]/tra-2B; Aotra2/hs-GAL4) expressing the AoTra2 transgene did not express the female dxF mRNA isoform (Fig. 2C). Negative controls for all these PCR reactions produced no amplicons (see Materials and methods). Thus, the *Anastrepha* Tra2 protein is able to promote the female-specific splicing of the *Drosophila* dxF pre-mRNA.

Whereas the expression of *Anastrepha* Tra2 protein in the XX pseudomales produced their feminisation, its expression in their XY normal brothers mutant for tra-2 did not affect their normal male development. This different effect is explained by the presence of Tra protein in the XX pseudomales and its absence in XY normal males. It should be remembered that the female-specific dxF pre-mRNA splicing mode requires the binding of the Tra-Tra2 complex to the female-specific exon 4 (see Fig. 2A). Therefore, the *Anastrepha* Tra2 protein supplies this Tra2 protein that was expressed since the production of any greater amount is lethal. Alternatively, the interaction between the endogenous *Drosophila* Tra2 protein and the transgenic *Anastrepha* Tra2 protein might be affected such that the *Drosophila*Tra2-*Anastrepha*Tra2 complex is less efficient than the *Drosophila*Tra2-**Drosophila**Tra2 complex at inducing the female-specific splicing of the endogenous *Drosophila* dxF pre-mRNA, as it has been suggested for the interaction between the *Anastrepha* Tra and the *Drosophila* Tra2 proteins (Ruiz and Sánchez 2010). In this context, it is worth mentioning firstly the high degree of divergence resembling the location of the sex comb (Fig. 1D). In other cases, a sex comb-like bristle was found between the female-like bristles that form the last transversal row (arrow in Fig. 1E). The sex comb size of pseudomales raised at 18°C was the same as the sex comb of their XY brothers whether raised at 18 or 25°C (Fig. 1A).

The feminisation produced by the *Anastrepha* Tra2 protein was, however, partial, indicating that the function of this protein in *Drosophila* was incomplete. There are two possible explanations for this. It might be due to an insufficient quantity of *Anastrepha* Tra2 protein being produced in the *Drosophila* transgenic flies; it was necessary to restrict the amount of *Anastrepha* Tra2 protein that was expressed since the production of any greater amount is lethal. Alternatively, the interaction between the endogenous *Drosophila* Tra2 protein and the transgenic *Anastrepha* Tra2 protein might be affected such that the *Drosophila*Tra2-**Drosophila**Tra2 complex at inducing the female-specific splicing of the endogenous *Drosophila* dxF pre-mRNA, as it has been suggested for the interaction between the *Anastrepha* Tra and the *Drosophila* Tra2 proteins (Ruiz and Sánchez 2010). In this context, it is worth mentioning firstly the high degree of divergence.

**Fig. 2.** Effect of the AoTra2 transgene on the splicing of *Drosophila* dxF pre-mRNA. (A) Molecular organisation of *Drosophila* dxF pre-mRNA showing the male and the female splicing pattern. Exons are represented in boxes and introns by dotted lines. The red bars in exon 4 represent the specific binding sequences for the Tra-Tra2 complex. (B) RT-PCR analyses of total RNA from *D. melanogaster* XX pseudomales mutant for tra-2 and either not expressing (25°C) or expressing (HS) the AoTra2 transgene. HS indicates heat shock treatment. TDAo#2: XX pseudomale yw/w; tra-2B/Df(2R)trix,tra2[-]; TDAo#2: hs-GAL4, w[+]; TDAo#3: XX pseudomale yw/w; tra-2B/Df(2R)trix,tra2[-]; TDAo#3: hs-GAL4, w[+] (Crosses II and III in Materials and Methods). (C) RT-PCR analyses of total RNA from *D. melanogaster* XY males mutant for tra-2 (brothers of the XX pseudomales). As a control, the splicing of the primary transcript of gene rp49 that codes for the constitutive ribosomal protein 49 was monitored.
between the Anastrepha and the Drosophila Tra2 proteins (Sarno et al., 2010); secondly, the incomplete feminisation of Drosophila XX pseudomales mutant for tra and expressing the Ceratissi Tra protein (Pane et al., 2005); and thirdly, the incomplete feminisation of Drosophila XX pseudomales mutant for tra-2 and expressing either the human (Dauwalder et al., 1996) or the Sciara (Martin et al., 2011) tra-2 ortholog, whereas Drosophila virilis tra-2 gene can fully replace the endogenous tra-2 function of Drosophila melanogaster for normal female sexual development (Chandler et al., 1997). Collectively, these results suggest that the interaction between the Tra and Tra2 proteins of different species might be impeded as a consequence of changes accumulated in these proteins after the drosophilids separated from the other dipteran phylogenetic lineages, thus suggesting that the Tra and Tra2 proteins co-evolved to exert their functions in sex determination.

Materials and Methods

**Flies and crosses**

Drosophila flies were cultured on standard food. For the description of the mutant alleles and GAL4 constructs see Lindsay and Zimm (1992) and FlyBase. The crosses to produce the flies were: Cross I: Females yw TDAo#1; tra-2B/CyO; Cy; TDAo#2[w+] /MKRS.Sb and males w; Y; D(2R)trix,tra-2B/CyO; m-GAL4[w+] /MKRS.Sb Cross II: Females yw; tra-2B/CyO; Cy; TDAo#2[w+] /MKRS.Sb and males w; Y; D(2R)trix,tra-2B/CyO; hs-GAL4[y+] /MKRS.Sb Cross III: Females yw; tra-2B/CyO; Cy; TDAo#3[w+] /MKRS.Sb and males w; Y; D(2R)trix,tra-2B/CyO; hs-GAL4[y+] /MKRS.Sb

**Morphological analysis**

Flies used for the analysis of adult forelegs were kept in a mixture of ethanol:glycerol (3:1) for several days. They were then macerated in 10% KOH at 60°C for 15 min, thoroughly washed with water and mounted in Faure’s solution for inspection under a compound microscope.

**Production of the Aotra2 transgene Drosophila melanogaster lines (TDAo)**

For the construction of the Aotra2 transgene, the tra-2 ORF of A. obliqua was amplified by RT-PCR. The PCR reaction was performed using primers PRtra2-1Ao(5’AGAATTGAAATGTTCCACG) and PRtra2-2Ao (5’CATATTTTATAGCCGTAACG). The resulting amplicon was cloned in pGEMTEasy (Promega), following manufacturer’s instructions. The inserted fragment was then cut with EcoRI and cloned in the EcoRI site of pUAST. The microinjections for generating the TDAo (UAS::Aotra-2cDNA transgenic: D. melanogaster lines were performed by Genetic Services (Sudbury, MA, USA). To ascertain that each transgenic line was carrying the correct transgene, PCR on genomic DNA with primers PRtra2-1Ao and PRtra2-2Ao was used to amplify the whole transgene and the amplicons were cloned in pGEMTeasy (Promega), following manufacturer’s instructions and sequenced. Three transgenic lines were produced: TDAo1 (with the Aotra2-transgene inserted in the X chromosome), and TDAo2 and TDAo3 with the Aotra2-transgene inserted in the third chromosome. TDAo stands for pUAS::Aotra-2cDNA.

**Analysis of the splicing of dsex pre-mRNA in transgenic Drosophila melanogaster lines**

The effect of Anastrepha Tra2 protein on the splicing control of Drosophila dsex pre-mRNA was studied in transgenic Drosophila XX flies mutant for tra-2 and expressing the Anastrepha Tra2 protein. The inducible hs-GAL4 driver was used to express the Aotra2 transgene. XX pseudomales of genotype yw/w; tra-2B/Df(2R)trix,tra2-; TDAo2/hs-GAL4 and of genotype yw/w; tra-2B/Df(2R)trix,tra2-; TDAo2/hs-GAL4 and of respective XY brothers of genotype yw/Y; tra-2B/Df(2R)trix,tra2-; TDAo2/hs-GAL4 and of phenotype yw/Y; tra-2B/Df(2R)trix,tra2-; TDAo2/hs-GAL4 were clones in PRtra2-1Ao and expressing the Aotra2 transgene. Two 3 hours heat shock pulses (37°C) per day for two consecutive days with recovery at 25°C between pulses were given to the experimental flies. The effect of transgenic Aotra2 protein on Drosophila dsex pre-mRNA splicing was analysed by RT-PCR on total RNA from adults, which was prepared using the Ultraspec-II RNA isolation kit (Biotecx) following the manufacturer’s instructions. Reverse transcription reactions were performed with an oligo-dT. Two percent of the synthesised cDNA was amplified by PCR, and one percent of this PCR was used for a second round of amplification, in a total volume of 50 µl. The primers used were: dsex1 (5’CATCGGGAAACATCGGTGATC3’) and dsexF1 (5’AACGGGTGTCATCATCGGAC3’).

The PCR conditions were 95°C, 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 20 seconds each cycle, plus an extension step at 72°C for 7 minutes. In all cases, PCR reactions with RNA samples were performed to guarantee there was no contamination with genomic DNA (negative controls of PCR reactions). The amplicons were cloned in pGEMT-easy (Promega), following manufacturer’s instructions.

**DNA sequencing**

It was used an automated 377 DNA sequencer (Applied Biosystems) and the primers forward M13 (-20) and reverse M13 reverse (-24).

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**References**


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