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Note

The Gene *doublesex* of the Fruit Fly *Anastrepha obliqua* (Diptera, Tephritidae)

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ABSTRACT

The gene *doublesex* of *Anastrepha obliqua* is composed of four instead of the usual six exons. It is transcribed in both sexes and its primary transcript undergoes sex-specific splicing, producing female Dsx^F and male Dsx^M proteins, which have in common the amino-terminal region but which differ at the carboxyl-terminal region.

THE gene *doublesex* (*dsx*) of Drosophila is the last gene in the genetic cascade that controls sex determination. It is transcribed in both sexes but gives rise to female (Dsx^F) and male (Dsx^M) proteins through sex-specific splicing of its primary transcript (reviewed in Sánchez *et al.* 2005).

In recent years, molecular mechanisms regulating sex determination have received special attention due to their potential use in sterile insect technique (SIT) programs for the control and eradication of insect pests. The genes of the sex determination cascade, like *dsx*, have been proposed as candidates for such purposes (Pannuti *et al.* 2000; Saccone *et al.* 2002). Tephritids have a serious detrimental economic impact on agriculture. Among fruit flies, the gene *dsx* has been characterized in *Bactrocera tryoni* (Queensland fruit fly) (Shearman and Frommer 1998), *B. oleae* (olive tree fly) (Lagos *et al.* 2005), and *Ceratitis capitata* (medfly) (Saccone *et al.* 2002 cited in Pane *et al.* 2002). As in Drosophila, *dsx* in these species encodes male- and female-specific proteins, which are produced by sex-specific splicing of its primary transcript.

Although the biological investigation of some tephritids (Ceratitis and Bactrocera) is already well under way, other species, like the genus Anastrepha, are less extensively analyzed. Considering the potential use of the *dsx* gene in control programs of fruit flies, our objective was to isolate and characterize this gene in *Anastrepha obliqua* (fruit fly), a species of great economic importance on the American continent.

A first step in the isolation of the *A. obliqua dsx* gene (*Aodsx*) was a PCR reaction on *A. obliqua* genomic DNA to amplify the well-conserved cysteine-rich DNA binding domain Dsx-DM domain. The sequence of the amplified fragment corresponded to this putative domain. Next we used the amplified fragment as probe to screen a genomic library from *A. obliqua* that we constructed. A positive phage, Dsx7.1A, was isolated. A total of 4757 bp of its genomic insert were sequenced in the 5′ and 3′ directions, and its conceptual translation was compared to the Dsx protein of *B. oleae*, demonstrating that we isolated the amino-terminal region enclosing the DM domain of the putative *Ao*Dsx protein.

A specific primer was synthesized from the sequence corresponding to the beginning of the putative *Ao*Dsx protein. This primer and an oligo(dT) were used in PCR with cDNA of *A. obliqua* male and female adults separately. A band of ~1.6 kb in males and a band of ~1.5 kb in females were amplified, cloned, and sequenced. Their translation and their comparison with the sequence of the genomic insert of phage Dsx7.1A indicated that we had amplified the genomic sequences corresponding to the amino-terminal region of male and female *Ao*Dsx proteins. None of the other isolated genomic phages carried the 3′ region of the *Aodsx* gene. To determine the molecular organization of this gene, we used the following plan.

First, specific primers from the putative *A. obliqua* exons were synthesized after comparison of the *A. obliqua* and *B. oleae* male and female *dsx*-cDNAs, respectively. These primers were then used to amplify

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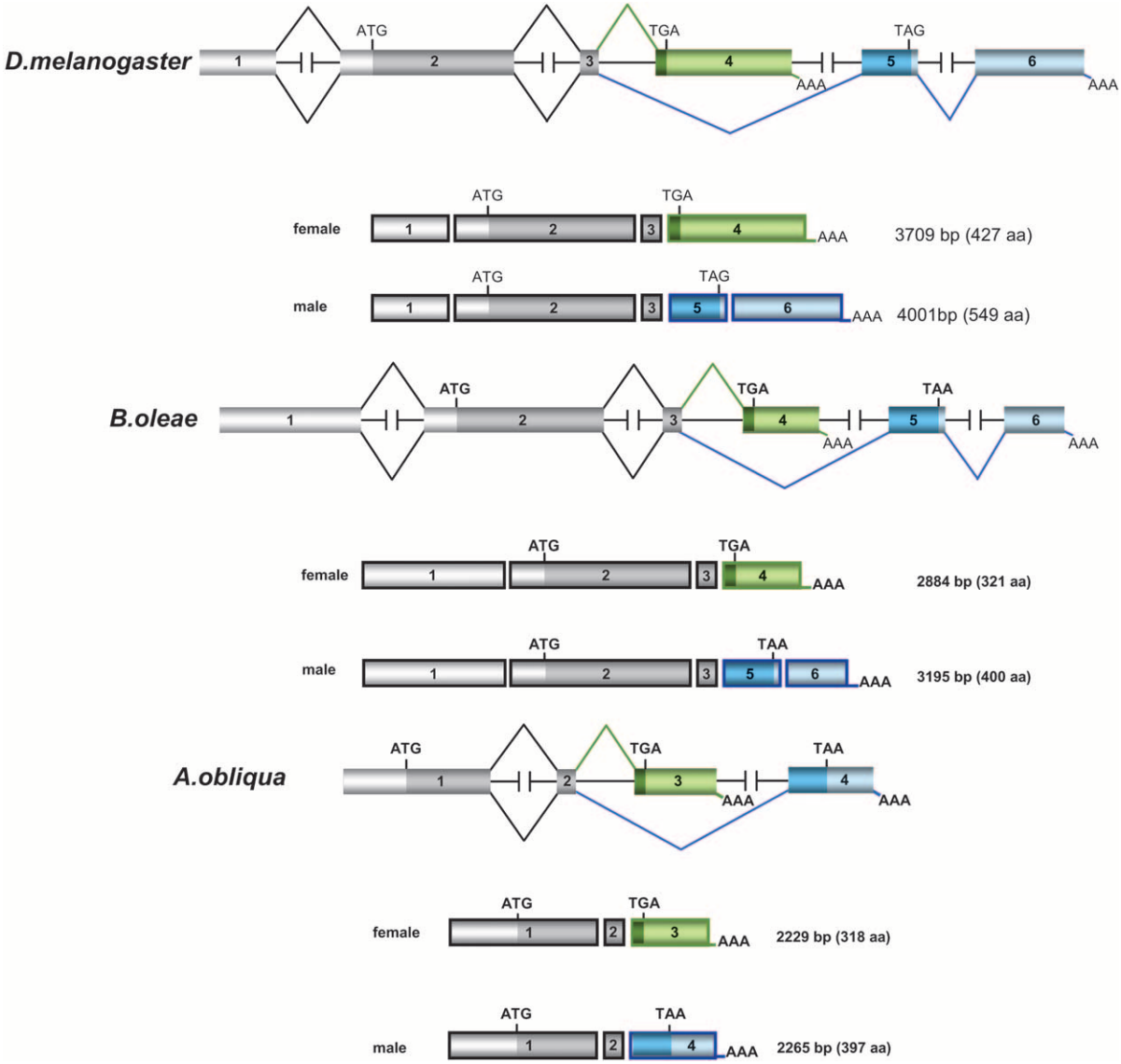


Figure 1.—The molecular organization of the *A. obliqua dsx* gene and its comparison with *dsx* of *D. melanogaster* and *B. oleae*. Exons (boxes) and introns (lines that when broken indicate that the length of the intron remains unknown) are not drawn to scale. The numbers inside the boxes indicate the number of the exon. The beginning and the end of the ORF are indicated by ATG and TGA or TAA, respectively. The longest cDNA variant is shown. We constructed the genomic library from *A. obliqua* using the λ-DASH II/*Eco*RI vector kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The screening of the *A. obliqua* genomic library was performed using the PCR fragment amplified from genomic DNA, using degenerated primers for the DM region found in Dsx proteins. Hybridization and identification of positive clones were performed using the protocols described by Maniatis *et al.* (1982). The 5′-RACE was performed using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) following the manufacturer's instructions. To determine the exon/intron junctions of *Aodsx*, the BD Genome Walker universal kit (BD Biosience) was used following the manufacturer's instructions. The 5′-RACE product and all PCR and RT-PCR products were subcloned in TOPO-TA cloning vector (Stratagene). Sequencing was performed using an automatic 377 DNA sequencer (Applied Biosystems, Foster City, CA). The accession numbers for the ORFs of Dsx^F and Dsx^M are AY948420 and AY948421, respectively. For the sequences of primers, see supplemental data at http://www.genetics.org/supplemental/.

genomic DNA of *A. obliqua*. Amplified fragments were obtained only with primers from the putative exon 3 and exon 4 and with primers from putative exon 5 and exon 6. The sequences of these fragments were compared with the male and female cDNA sequences mentioned above. The first amplified genomic fragment contained an intron of 126 bp, and the second one contained only exon sequences, indicating that exons 5 and 6 form a unique exon in the *Aodsx* gene. The Genome Walker kit methodology was next applied to determine the exon/intron junctions through genomic walking on *A. obliqua* genomic DNA. The sequences of the genomic fragments that were generated were compared with the *A. obliqua* male and female cDNA sequences found previously. In this way, the exon/intron junctions were unambiguously determined.

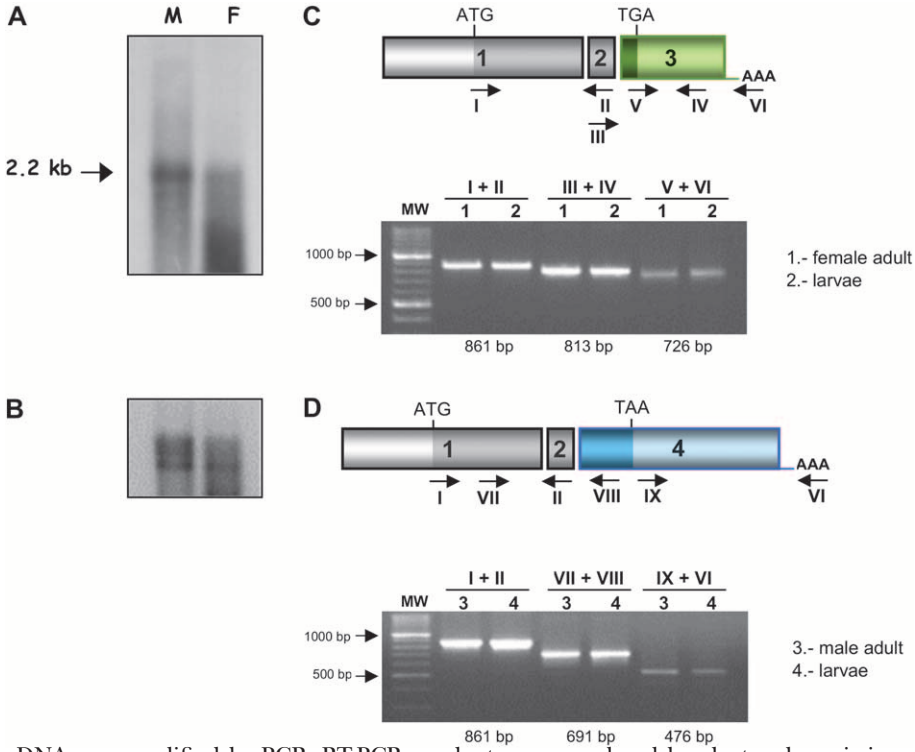


Figure 2.—Expression of the gene *dsx* of *A. obliqua*. (A) Northern blots of total RNA from males (M) and females (F) adults. The Northerns were hybridized with a riboprobe produced from the *Aodsx* male cDNA. Hybridization with the *D. melanogaster* rDNA probe (pDm238) (Roiha *et al.* 1981) was used as a loading control (B). Overlapping RT-PCR analyses from total RNA of female (C) and male (D) adults or a mixture of male and female larvae. (C and D, top) The molecular organization of the corresponding cDNAs is shown. The locations of the primers are shown by arrows and identified by Roman numerals (for the sequences of primers see supplemental data at http://www.genetics.org/supplemental/). Ten micrograms of total RNA from larvae (a mixture of males and females) and adults (males and females separately) were reverse transcribed with the Superscript II RNase H⁻ reverse transcriptase (Invitrogen, San Diego) using primer VI and following the manufacturer's instructions. Ten percent of the synthesized cDNA was amplified by PCR. RT-PCR products were analyzed by electrophoresis in agarose gels, and the amplified fragments were subcloned using the TOPO TA-cloning kit (Invitrogen) following the manufacturer's instructions. These were then sequenced using an automatic 377 DNA sequencer (Applied Biosystems) from the universal forward and reverse primers.

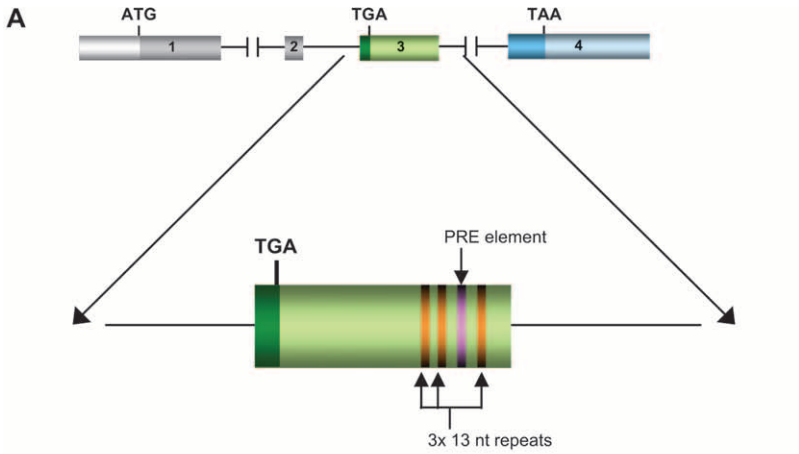
Second, to determine the beginning of the *Aodsx* transcription unit, 5′-RACE analysis was performed. The 5′-RACE product ran as a clear band on the gel, indicating that most likely there are no further exons 5′ of exon 1. Thus, the *Aodsx* gene lacks the intron located in the 5′-UTR region of the gene *dsx* of Drosophila and Bactrocera. In addition, it indicated that the transcription start site would lie 638 bp upstream of the beginning of the ORF. Therefore, the sizes of the complete male and female cDNAs are 2265 and 2229 bp, respectively.

The molecular organization of *Aodsx* gene and its comparison with that of the *dsx* gene of *Drosophila melanogaster* and of *B. oleae* is shown in Figure 1. The *Aodsx* gene is composed of four instead of six exons: the first two are common to both sexes, whereas exon 3 is female specific and exon 4 is male specific. Exon 1 of *Aodsx* corresponds to the fusion of exons 1 and 2, and exon 4 of *Aodsx* corresponds to the fusion of male-specific exons 5 and 6.

Northern blots (Figure 2, A and B) and overlapping RT-PCRs (Figure 2, C and D) of male and female *A. obliqua* adults and of a mixture of male plus female larvae of different developmental stages indicated that a single *dsx* transcript of ~2.2 kb is present in males and in females, in agreement with the size of the male and female cDNAs determined previously, and confirmed that the gene *dsx* is transcribed in both sexes, producing two different spliced mRNAs, one in each sex, during larval development and in adult life. The RT-PCR analyses revealed also that females have four *dsx* cDNA variants, differing in the length of their 3′-UTR and corresponding to four different polyadenylation sites in the female-specific exon (see below). These variants are 2229 bp (found in female larvae and adults), 1860 bp (found in adult females), and 1759 and 1701 bp (found in female larvae). Only the biggest variant contains the 13-nucleotide repeats (dsxRE) and the purine-rich element (PRE) element involved in splicing regulation (see below). Two variants of 2265 and 2223 bp, differing at their 3′-UTR, were found in males. The former is present in larvae and adults, and the latter is present in adults (data not shown).

The comparison of *Aodsx* mRNA molecular organization between males and females suggests that in *A. obliqua* the male-splicing pathway represents the default mode. First, the putative female-specific amino acid region is skipped over in males. And second, the female-specific exon 3 contains three putative dsxRE targets for the Tra-Tra2 complex as well as for the PRE inserted between dsxRE targets 2 and 3 (Figure 3). The dsxRE elements are highly conserved in the different species (Figure 3). In addition, the female-specific exon contains four polyadenylation sequences, whose function determines the four *dsx*-mRNAs variants found in *A. obliqua* females.

The conceptual translation of the male and female *Aodsx* mRNAs shows that they encode two polypeptides of 397 and 319 amino acids, respectively. Their comparison with the Dsx proteins of the other insects is presented in Figure 4 and Table 1. The number of



GCCAACATGT CGTAAACGAG TACTCCCGTC AACATAATCT CAATATTTAT GACGGGGGTG AGCTGCGCAG
TACGACGAGG CAATGTGGAT GATAATTTTT AACGTAATTA CCGAGCGGGT TATGTTTTGA GTAAAACTAA
ATTAATTTAA TTTAAAAGTT TAATAAAGCG TCATTTGAAT CGAATGAAAA ATAATATAGG AAATATGGTT
ATGAACTATC AAAACAAATG AAAGCAATA AAATAATTCA GTTGAACCAA TTTTTGGCTG AATCCTATTT
CGTGTAATCA AATTAAAAAC TATTGAAATA TTTGTTTATG TTTTAAATAT GCAAATATCT GAAAATTTGG
CATCCTTCGA AAAGTGAAAG TAAAATAATG TTTTCAAAAA TTAACTTAAC TTCAATCAAC AATCCCATGG
GAAAAATATC TTCAATCAAC ATACATAAAT AAAGGGCGAC ATTCGCATGA TTCTACAATC AAAAAGCCCA
TCAGGACAAC TTCTGCAATC AACTTTGAAG CCCTTCCGTA ATTCCACATC ATGTCGAGGC ACGAAATATG
AAGTTTAACA GCTCTGCAAT CAACATACCC AAGATATCCA CGGCAATAGC GTTAAAGGGC AGGCAATTAT
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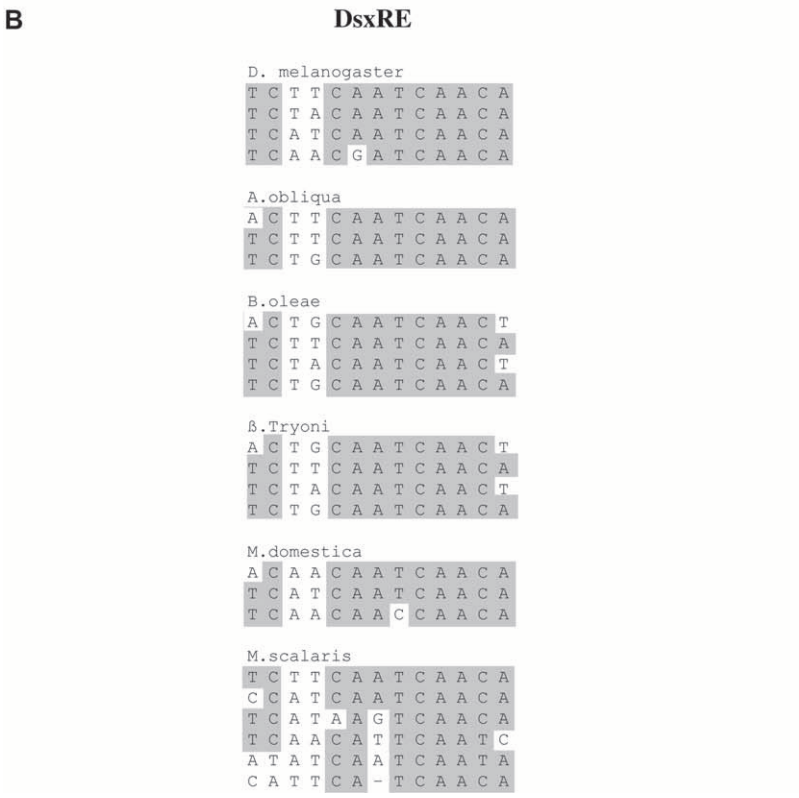

FIGURE 3.—The female-specific exon of *A. obliqua dsx*. (A) Distribution of the 13 nucleotide repeats and the PRE, which are marked in orange and pink, respectively, in the sequence. Polyadenylation signals are in blue and the translational stop codon (TGA) is in red. (B) Comparison of the dsxRE elements present in the female-specific exon in different species. The shading indicates identical amino acids.

amino acids for the non-sex-specific and male-specific regions varies among the species, whereas the female-specific region shows conservation, except for *Bombyx mori*, which is composed of more amino acids. The degree of similarity is higher for the female-specific than for the non-sex-specific and the male-specific regions. The similarity is higher between the dipteran species than between the dipteran species and the lepidopteran

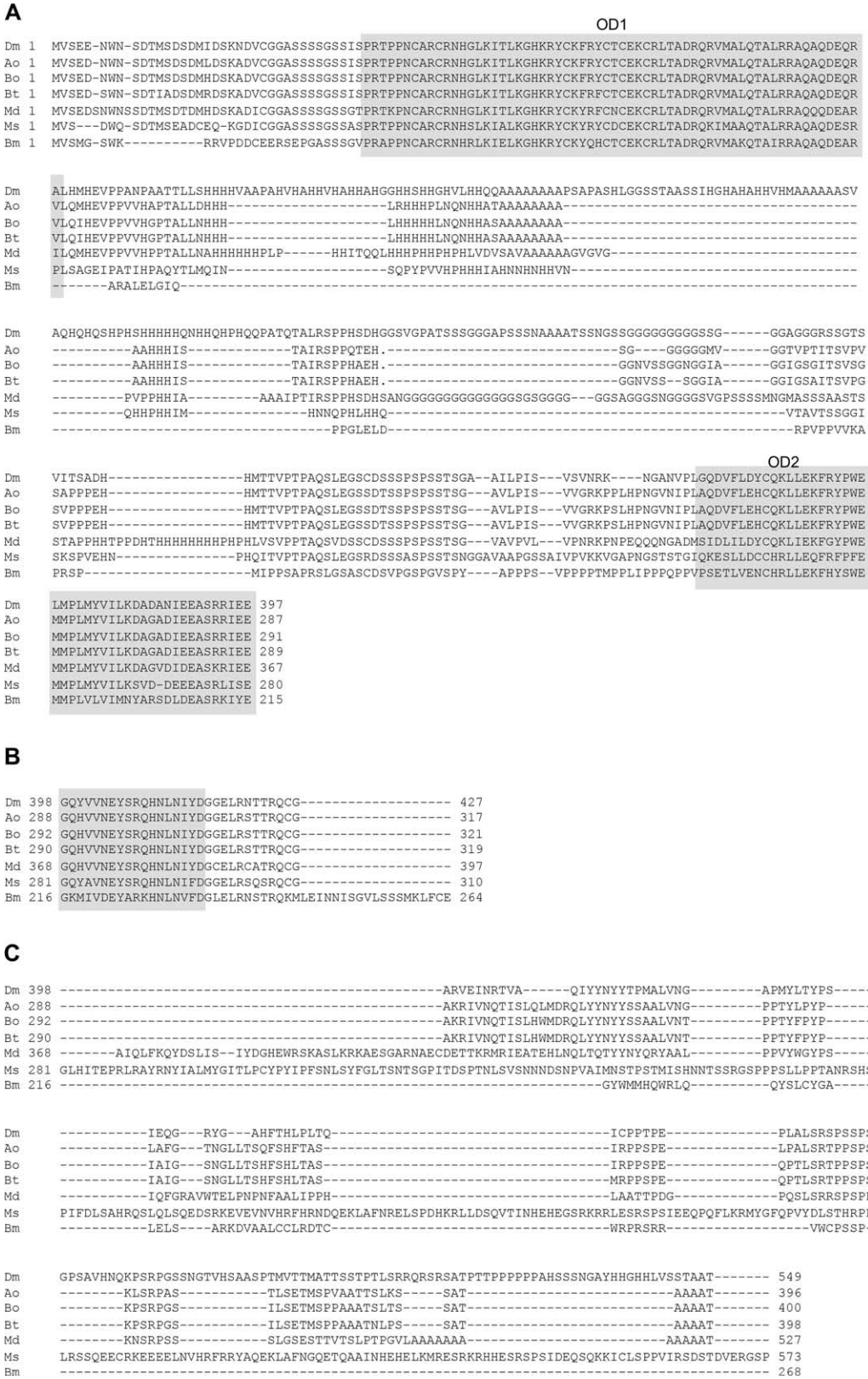

Figure 4.—Comparison of the Dsx predicted polypeptides in *D. melanogaster* (Dm) (Burtis and Baker 1989), *A. obliqua* (Ao) (this work), *B. oleae* (Bo) (Lagos *et al.* 2005), *B. tryoni* (Bt) (Shearman and Frommer 1998), *Musca domestica* (housefly) (Md) (Hediger *et al.* 2004), *Megaselia scalaris* (phorid fly) (Ms) (Sievert *et al.* 1997), and *B. mori* (silkworm) (Bm) (Ohbayashi *et al.* 2001; Suzuki *et al.* 2001). (A) Sequence common to both sexes; (B) female-specific sequence; and (C) male-specific sequence. The DNA-binding domain OD1 and the oligomerization domain OD2 are shaded. Gaps were introduced in the alignments to maximize similarity. The comparison of protein sequences was performed using ClustalW (1.82).

B. mori. Among the dipterans, it is higher among the tephritids *A. obliqua*, *B. oleae*, and *B. tryoni*. The number of amino acids and the similarity of the OD1 and OD2 domains are very high among all species, as expected, since these domains endow the Dsx proteins with the capacity to interact with other proteins and with DNA (An *et al.* 1996; Cho and Wensink 1997).

In summary, the gene *dsx* of *A. obliqua* is transcribed during development and in adult life in both sexes but its primary transcript undergoes sex-specific splicing,

TABLE 1

Comparative analysis of Dsx proteins

Species	No. of amino acids (% similarity)				
	Non-sex-specific region	Female-specific region	Male-specific region	OD1 domain	OD2 domain
D. melanogaster	397	30	152	63	64
A. obliqua	287 (65.2)	30 (100)	109 (53.9)	63 (100)	64 (100)
B. oleae	291 (67)	30 (100)	109 (53.2)	63 (100)	64 (100)
B. tryoni	289 (66.5)	30 (100)	109 (54.6)	63 (100)	64 (100)
M. domestica	367 (71.5)	30 (93)	160 (52.6)	63 (93.6)	64 (96.8)
M. scalaris	280 (56.4)	30 (96.6)	293 (57.9)	63 (96.8)	63 (89)
B. mori	215 (44)	49 (90)	53 (18.4)	63 (93.6)	64 (89)

To allow for a more accurate comparison of the Dsx proteins, they were divided into three regions: non-sex-specific, female-specific, and male-specific regions. In addition, the OD1 and OD2 domains were also compared. The percentage of similarity refers to the identical plus conservative amino acids. The Dsx proteins of *D. melanogaster* were used as reference.

producing the female Dsx^F and male Dsx^M proteins. This sex-specific regulation makes *dsx* a good candidate to be used in the future for the development of molecular tools that can improve the SIT technique to control the Anastrepha pests.

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