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Isolation and characterization of oligomerization domain I and II coding regions of *doublesex* genes in agricultural fruit flies (Diptera: Tephritidae)

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Abstract. *Bactrocera* fruit flies are ranked among the most destructive pests of the worldwide fruit and vegetable trades. Coding regions of two oligomerization domains within *doublesex* (*dsx*) genes were determined in *Bactrocera dorsalis* (oriental fruit fly) and *B. correcta* (guava fruit fly). Resulting sequences revealed a high degree of similarity at both nucleotide and putative amino acid levels in the genus *Bactrocera*. Conservation of the DNA-binding DM motif and several known molecular features within the domains suggest a presence of strong purifying selection on the DSX proteins. Topology of the phylogenetic gene trees and deduced amino acid substitution patterns suggest that the coding region sequences of the two domains are diversified in concert parallel with the species differentiation.

INTRODUCTION

Sex determination in *Drosophila melanogaster* is regulated by the so-called X : A ratio and an RNA-splicing cascade involving sex-specific splicing of *sex-lethal* (*Sxl*), *transformer* (*tra*), and *doublesex* (*dsx*) genes (Cline, 1993; Sanchez, 2008). A recent discovery of an important auxiliary role of *tra-2* in a *tra* autoregulatory loop in *Ceratitis capitata* led to a better understanding of the dipteran sex determination cascade, which could be Y-linked M factor > *tra* > *dsx* (Franz & Willhof, 1996; Salvemini et al., 2009). Although there might be many different master switches in insects other than *Drosophila*, their *dsx* proteins are highly conserved both in structure and function (Saccone et al., 1998, 2002; Shearman, 2002; Oliveira et al., 2009).

In *D. melanogaster*, the male-specific (DSX^M) and female-specific (DSX^F) proteins result from an alternative splicing of the *dsx* mRNA. Both proteins are active but have an opposing regulatory role in controlling the downstream somatic sexual differentiation genes (Baker & Wolfner, 1988; Burtis & Baker, 1989; Cho & Wensink, 1997). The DSX proteins share a common N-terminal region but differ at a sex-specific C-terminal region. Two functionally important DSX domains are responsible for the protein structure and DNA binding activity (An et al., 1996; Zhu et al., 2000). Oligomerization domain I (OD1) is located within a shared 66-amino-acid segment, common to both male and female isoforms (amino acids 39–104; An et al., 1996). The other important domain consists of both sex-specific and non-sex-specific portions. This domain constitutes a second oligomerization unit: $OD2^M$ (male-specific; amino acids 350–427) and $OD2^F$ (female-specific; amino acids 350–412) (An et al., 1996). These two domains have roles in specific DNA sequence recognition and protein oligomerization. In addition, they can interact with other proteins (Erdman et al., 1996; Cho & Wensink, 1997; Bayrer et al., 2005; Yang et al., 2008).

Orthologues of the *dsx* genes have been isolated in non-drosophilid insects such as in *Megaselia scalaris* (Sievert et al., 1997; Kuhn et al., 2000), *Musca domestica* (Hediger et al., 2004), *Anopheles gambiae* (Scali et al., 2005), *Bactrocera tryoni* (Shearman & Frommer, 1998), *B. oleae* (Lagos et al., 2005), *B. dorsalis* (Chen et al., 2008), *Anastrepha obliqua* (Ruiz et al., 2005, 2007), *C. capitata* (G. Saccone et al., cited in Pane et al., 2002), *Bombyx mori* (Ohbayashi et al., 2001), *Apis mellifera* (Cho et al., 2007), and *Nasonia* wasps (Oliveira et al., 2009). Here, two oligomerization domains in the *dsx* gene coding regions in both sexes of *B. dorsalis* and *B. correcta* (Diptera: Tephritidae) were isolated and characterized.

MATERIAL AND METHODS

Genomic DNA extraction

Genomic DNA was extracted from flies obtained from laboratory stocks of *B. dorsalis* (Hendel) and *B. correcta* (Bezzi). Individual flies were homogenized in an extraction buffer (100 mM NaCl, 200 mM sucrose, 100 mM Tris-HC1 (pH 9.1), 50 mM EDTA, 0.5% SDS, and 100 µg/ml proteinase K), treated with RNase A, and purified in phenol : chloroform (Baruffi et al., 1995).

Total RNA extraction

The total RNA was isolated from adult *B. dorsalis* and *B. correcta* flies by using Trizol reagent (Gibco/BRL Life Technologies, Gaithersburg, MD, USA) as recommended by the manufacturer.

Primers

Primers (Table 1) were designed according to the alignment of the *dsx* sequences obtained from GenBank: *B. tryoni* (Froggatt), *B. oleae* (Gmelin), and *Ceratitis capitata* (Wiedemann) or otherwise as indicated in Table 1. Refer to supplementary Table S1 for the GenBank accession numbers.

Polymerase chain reaction amplifications

All amplification reactions were performed in a FlexCycler PCR thermal cycler (Analytik Jena, Germany). Amplification reactions contained 10 ng of genomic DNA, 1× *Taq* DNA polymerase buffer A (Vivantis Technologies, Selangor, Malaysia), 200 µM of mixed dNTP, 1.5 mM $MgCl_2$, 4 pmol of both BD and RevBD primers each, and 1 U of *Taq* DNA polymerase (Vivantis Technologies) in a total volume of 20 µl. PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 1

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TABLE 1. Primer sequences.

Primers	Sequence 5' to 3'	References
BD	ATGGTTTCTGAGGATAATTGGAACA	Designed in this study
RevBD	GGCACTGTGGTCATGTGATG	Designed in this study
Btk	CAGCGGCAGTTCGATCTCC	Shearman & Frommer, 1998
Btl	GCACGGTGCCGTATTCATGG	Shearman & Frommer, 1998
Oligo(dT) adapter	$CGGGACTCGTCGACATCGA(T)_{17}$	Shearman & Frommer, 1998
Adapter	CGGGACTCGTCGACATCG	Shearman & Frommer, 1998

min, 60°C for 30 s, 72°C for 1 min; and one cycle of final extension of 72°C for 7 min.

3' RACE PCR amplifications

3' RACE was carried out as described by Frohman et al. (1988). ImProm-II reverse transcription system (Promega, Madison, WI, USA) was used to reverse transcribe ~3–5 µg total RNA from adult flies as recommended by the manufacturer. PCR conditions for 3'RACE were 94°C for 4 min, followed by five cycles of 94°C for 1 min, 60–63°C for 2 min and 72°C for 2 min; 30 cycles of 94°C for 40 s, 58–60°C for 2 min, 72°C for 2 min; and one cycle of final extension of 72°C for 7 min. The first amplification reaction employed a BD-adapter primer pair while subsequent semi-nested PCRs used Btk and Btl adapter primers.

Fragment isolation, cloning, and sequencing

PCR products were purified from 1% agarose gel using the Geneclean II kit (Bio 101 Inc., La Jolla, CA, USA), and then ligated into pGEM-T Easy Vector (Promega). The recombinant plasmids were transformed into DH5α competent cells with blue/white colony screening. Plasmids were isolated as described by Sambrook et al. (1989). All sequencing was performed on both strands using the ABI3730XL sequencer serviced by Macrogen Inc., Seoul, South Korea.

Sequence alignment and phylogenetic tree reconstruction

ClustalW (1.83) was used to align sequences (Thompson et al., 1994). Programs from the PHYLIP package version 3.68 (Felsenstein, 2004) and TREEVIEW program (Page, 1996) were used to reconstruct the phylogenetic trees. Refer to supplementary Table S1 for the molecular sequences in the alignments.

RESULTS AND DISCUSSION

The coding regions of *Drosophila* DSX OD1 orthologues were isolated from the genomic DNA of *B. dorsalis* ($Bd1DSX^M$ OD1 and $Bd1DSX^F$ OD1) and *B. correcta* ($BcDSX^M$ OD1 and $BcDSX^F$ OD1). Since the OD2 coding region of the *dsx* gene is composed of both sex-specific and non-sex-specific parts (An et al., 1996), the DSX OD2 coding regions of the *dsx* orthologues were isolated from 3' RACE cDNA fragments of *B. dorsalis* ($Bd1DSX^M$ OD2 and $Bd1DSX^F$ OD2) and *B. correcta* ($BcDSX^M$ OD2 and $BcDSX^F$ OD2). Longer male cDNA sequences were observed in the DSX OD2 orthologues of both species. This was consistent with previous findings for other Diptera insects such as *B. tryoni* (Shearman & Frommer, 1998), *B. oleae* (Lagos et al., 2005), *A. obliqua* (Ruiz et al., 2005), and *D. melanogaster* (Burtis & Baker, 1989; An et al., 1996). A BLAST search confirmed that these genomic and cDNA fragments were parts of the *dsx* genes by grouping *B. dorsalis* and *B. correcta dsx* with those of other species. The sequence boundaries corresponding to *D. melanogaster* DSX OD1 and OD2 were determined in *B. dorsalis* and *B. correcta* by multiple sequence alignments (Figs 1 and 2). The GenBank accession numbers for this work are FJ185162, FJ176944, FJ185165, and FJ185166.

A high degree of sequence conservation was observed in both DSX OD1 and OD2 domains of *B. dorsalis* and *B. correcta* (Table 2). The nucleotide identity of the two domains was the highest in *Bactrocera* (92–99%), less in other genera of tephri-

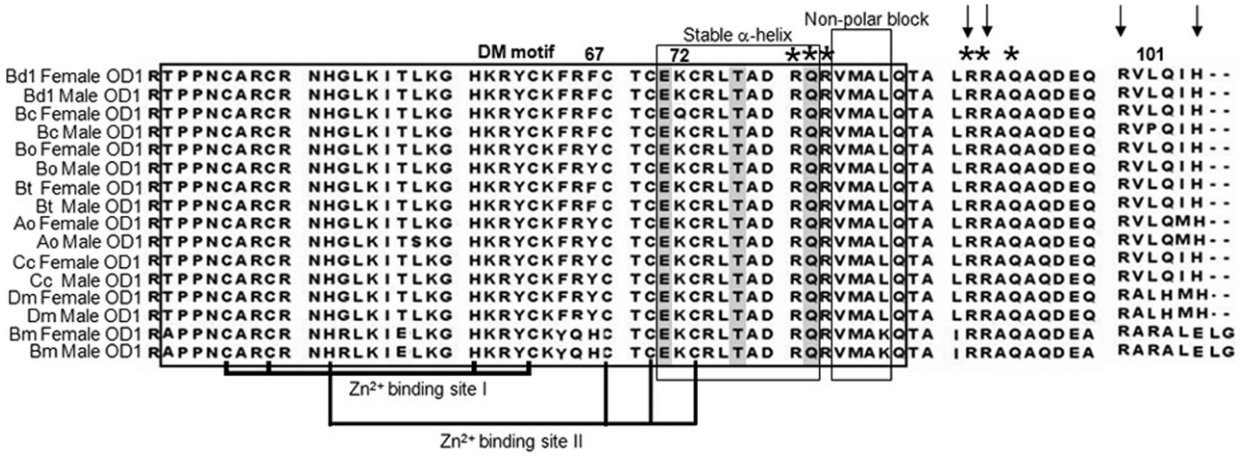

Fig. 1. Aligned putative amino acid sequences of DSX OD1 domain. Regions of DM motif, stable α-helix, and a series of non-polar side chain amino acids are indicated in three blocks. Both Zn^{2+} ion binding sites, CCHC and HCCC are represented as Zn^{2+} binding sites I and II, respectively (Zhu et al., 2000). Asterisks (*) indicate two patches of conserved RQ side chains and arrows illustrate basic amino acids at C-terminal distal end (Narendra et al., 2002). Three amino acids conserved within most of the insects (E, T, and Q) are shaded (Oliveira et al., 2009). Putative amino acid changes in *B. dorsalis* and *B. correcta* are indicated at positions 67, 72, and 101. Positions of putative amino acid residue correspond to those of *D. melanogaster* DSX (An et al., 1996). Refer to supplementary Table S1 for the abbreviation for the species and the GenBank accession numbers.

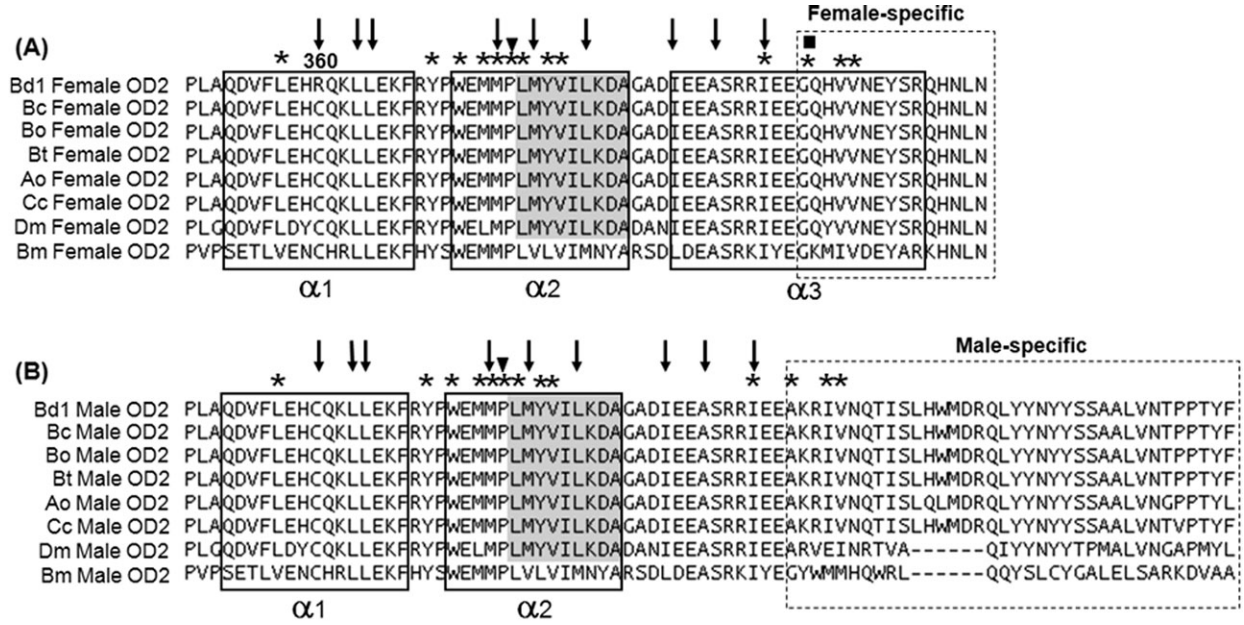

Fig. 2. Aligned putative amino acid sequences of DSX^F OD2 domain (A) and DSX^M OD2 domain (B). The highlighted molecular features of the following are summarized from the indicated references. The regions of α1, α2, and α3 of the UBA-like folds are indicated in three successive blocks. Dash-lined blocks represent sex-specific portions of the OD2. Asterisks (*) indicate the positions of amino acids in the non-polar core of the dimerization surface and arrows illustrate the positions of a hydrophobic minicore (Bayrer et al., 2005). A triangle (▾) indicates at a proline kink at position 375 and the square (■) indicates mutation G398D which is associated with an intersexual development (Erdman et al., 1996). There is only one amino acid change, C360R, is observed in *B. dorsalis* DSX^F OD2 sequence. The amino acid patch (LMYVILKDA), which is mostly conserved in most Diptera, is shaded (Oliveira et al., 2009). Some of the molecular features indicated in the DSX^M OD2 domain were from the study of females (Bayrer et al., 2005). Positions of putative amino acid residue correspond to those of *D. melanogaster* DSX (An et al., 1996). Refer to supplementary Table S1 for the abbreviation for the species and the GenBank accession numbers.

tids (81–89%), and dropped even more so in compared to *D. melanogaster* (45–77%). A known molecular analysis of the *Anastrepha* fruit flies indicated that a strong purifying selection acting on the *dsx* gene has led to the preservation of the functional structure of the DSX proteins (Ruiz et al., 2007). However, our results revealed a substantial difference at the nucleotide level even between fruit flies in the same family.

The topology of the phylogenetic trees (Fig. 3A and B) indicated that all *Bactrocera* fruit flies are in one clade. The bootstrap values that isolated the fruit flies into the following clades: genus of *Bactrocera*, other genera of the Tephritidae (*A. obliqua* and *C. capitata*), and those of more distantly-related insect species (*D. melanogaster* and *B. mori*) were very high. The specific clustering and phylogenetic relationships of the two DSX domains were congruent with the taxonomic classification. Thus, it can be inferred that the diversification of the coding regions of DSX OD1 and OD2 domains has changed in parallel concert with the differentiation of the species.

Based on the amino acid similarity between more distantly-related species and the tephritid consensus, the DSX OD1 domain seems to have relatively higher similarity (95.59% *D. melanogaster* and 76.47% *B. mori*) than that of the OD2 (87.5% *D. melanogaster* versus 52.08% *B. mori*). A DM motif, which is found in the sex-determination pathway of organisms across the phyla, was also uncovered in the DSX OD1 region (Raymond et al., 1998). All the Dipterans included in this study have identical zinc modules, a stable α-helix region, and a non-polar block (VMAL) within the DM motif (Zhu et al., 2000; Narendra et al., 2002). The three highly conserved amino acids (E, T, and Q) shared among *dsx* from a broad spectrum of insect taxa are also present in residues 71–80 of the stable α-helix (Fig. 1) (Zhu et al., 2000; Oliveira et al., 2009). Based on the ability of *D. melanogaster* DSX^M to restore a male-specific feature in *Caenorhabditis elegans* (Raymond et al., 1998) and the analyzed amino acid conservation, it can be inferred that the DSX OD1 is more conserved than the OD2.

The conservation of certain amino acid was detected in the common region of DSX OD2. Fig. 2A and B showed a nine amino acid stretch (LMYVILKDA) and the three α-helices that make up the UBA-like fold in *B. dorsalis* and *B. correcta*. These

TABLE 2. Nucleotide and putative amino acid similarity indicated by the multiple alignment analysis.

Regions of *B. dorsalis* and *B. correcta dsx* genes	In the genus *Bactrocera*		Among different genera of Tephritidae		Among different families of Diptera	
	Nucleotide level	Amino acid level	Nucleotide level	Amino acid level	Nucleotide level	Amino acid level
DSX OD1(common to both sexes)	92–96%	96–100%	81–83%	93–98%	75–76%	92–93%
DSX^M OD2 (252 bp)	97–99%	100%	86–88%	95–98%	45–47%	73%
DSX^F OD2 (189 bp)	97–98%	98–100%	86–89%	98–100%	76–77%	87–88%

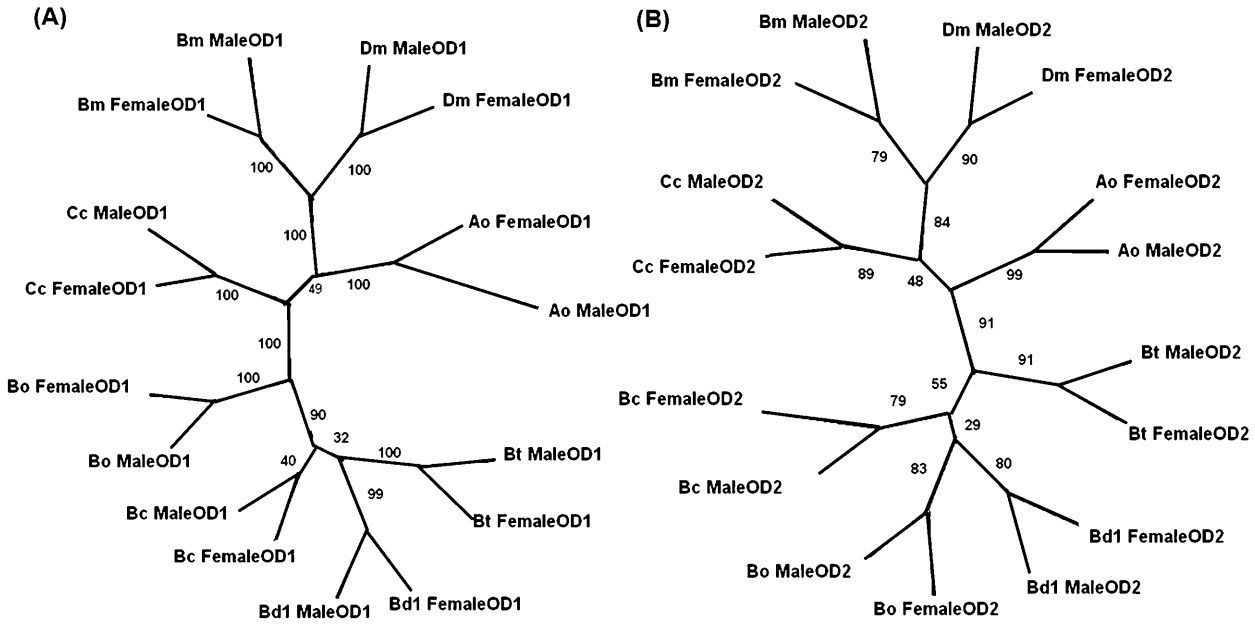

Fig. 3. A 50% majority-rule consensus UPGMA phylogenetic tree based on both male and female sorted nucleotide sequence variable sites of DSX OD1 (A) and the common region of DSX OD2 (B) coding region sequences. The bootstrap values (100 replicates) are indicated on each node. Genetic distances of individual branches are not indicated. Refer to supplementary Table S1 for the abbreviation for the species and the GenBank accession numbers.

molecular features suggest the presence of a ubiquitin regulatory component. (Bayrer et al., 2005; Yang et al., 2008; Oliveira et al., 2009).

Despite the conservation of the DSX proteins in different species, these proteins cannot act as substitute for one another. Examples are the ectopic expressions of *M. domestica* (Hediger et al., 2004), *C. capitata* (Saccone et al., 2008), and *A. obliqua* (Alvarez et al., 2009) DSX proteins in *D. melanogaster*. This might be due to the accumulation of minor differences between these species. All three alterations, Y67F, K72Q, and L101P in the DSX OD1 (Fig. 1) are located within the identified zinc module and the disordered C-terminal tail important for DNA recognition and affinity (Erdman & Burtis, 1993; Zhu et al., 2000). An amino acid substitution at Y67F was found in both sexes of *B. dorsalis*, *B. correcta*, and *B. tryoni*. This amino acid change was identified as being tolerant of amino acid substitutions (Zhang et al., 2006). Likewise, the amino acid changes at both positions 72 and 101 in OD1 can be different in males and females of *B. correcta*, because they might be selectively neutral polymorphisms. L and P amino acid residues at position 101 are both nonpolar in the C-terminal tail of OD1. The basic polar nature of K and polar nature of Q may be selectively neutral, because position 72 is closely flanked by two more important cysteine residues of the Zn^{2+} binding site II. In addition, there is an amino acid substitution, C360R, in Bd1DSX^{F} OD2. Bayrer et al. (2005) studied a crystal structure of the CTD^{F} and it was revealed that the side chain of C360 is one of the centers for a hydrophobic minicore. However, whether the positively charged side chain of C360R has any effects on the oligomerization of the DSX protein or not needs to be biochemically determined.

This work on the sequencing and phylogenetic analysis of two important domains of the *doublesex* genes in two different *Bactrocera* species suggests that one objective of future studies on sex determination should be to develop their original genetic tools such as RNAi, promoter, DNA primers, and a probe system from genes such as *dsx*, *tra*, and *tra-2*. This might lead to the development of genetic sexing strains which could result in more effective programs for controlling species of *Bactrocera*, using sterile insect technique (SIT) (Handler, 2002).

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TABLE S1. The molecular sequences used in the alignments and their GenBank accession numbers.

Name of the domain	Species	GenBank accession numbers
Bd1 DSX OD1 or OD2	*B. dorsalis* (this work)	*Bd1dsx*^{m}: FJ185162 *Bd1dsx*^{f}: FJ176944
Bc DSX OD1 or OD2	*B. correcta* (this work)	*Bcdsx*^{m}: FJ185165 *Bcdsx*^{f}: FJ185166
Bo DSX OD1 or OD2	*B. oleae*	*Bodsx*^{m}: AJ547622 *Bodsx*^{f}: AJ547621
Bt DSX OD1 or OD2	*B. tryoni*	*Btdsx*^{m}: AF029676 *Btdsx*^{f}: AF029675
Ao DSX OD1 or OD2	*A. obliqua*	*Aodsx*^{m}: AY948421 *Aodsx*^{f}: AY948420
Cc DSX OD1 or OD2	*C. capitata*	*Ccdsx*^{m}: AF434935 *Ccdsx*^{f}: AF435087
Dm DSX OD1 or OD2	*D. melanogaster*	*Dmdsx*: AE014279
Bm DSX OD1 or OD2	*B. mori*	*Bmdsx*^{m}: AB048544 *Bmdsx*^{f}: AB048543