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A history of the genetic and molecular identification of genes and their functions controlling insect sex determination

Giuseppe Saccone

Department of Biology, University of Naples Federico II, Via Cinthia 26, 80126, Naples, Italy

A B S T R A C T

The genetics of the sex determination regulatory cascade in *Drosophila melanogaster* has a fascinating history, interlinked with the foundation of the Genetics discipline itself. The discovery that alternative splicing rather than differential transcription is the molecular mechanism underlying the upstream control of sex differences in the *Drosophila* model system was surprising. This notion is now fully integrated into the scientific canon, appearing in many genetics textbooks and online education resources. In the last three decades, it was a key reference point for starting evolutionary studies in other insect species by using homology-based approaches. This review will introduce a very brief history of *Drosophila* genetics. It will describe the genetic and molecular approaches applied for the identifying and cloning key genes involved in sex determination in *Drosophila* and in many other insect species. These comparative analyses led to supporting the idea that sex-determining pathways have evolved mainly by recruiting different upstream signals/genes while maintaining widely conserved intermediate and downstream regulatory genes. The review also provides examples of the link between technological advances and research achievements, to stimulate reflections on how science is produced. It aims to hopefully strengthen the related historical and conceptual knowledge of general readers of other disciplines and of younger geneticists, often focused on the latest technical-molecular approaches.

1. Introduction: a brief history of the *Drosophila melanogaster* genetics and the discovery of the primary sex determination signal

A relevant question in Biology is how the first fertilized cells with very similar sizes and shapes differentiate within a given species to produce either male or female pluricellular organisms. The improvement of microscopy in the first decade of the 19th century and curiosity led researchers to investigate cell contents in some insect species. This resulted in the serendipitous discovery that the number of chromosomes or shape (known as accessory or idio-chromosomes) differs between the two sexes. After decades of debate concerning whether a nucleus exists within each cell rather than being an artefact, Nettie Stevens and her mentor, Edmund Beecher Wilson independently proposed a "causal connection of some kind" between the nuclear chromosomes and insect sexual development and dimorphism (Carey et al., 2022; Wilson, 1905). These researchers developed this hypothesis, while studying males of the mealworm *Tenebrio molitor*, which produce two types of reproductive cells, with either a large X or small Y chromosome. In contrast, females produce only one type of reproductive cell, containing only the X chromosome. Following the discovery of sex chromosomes, the importance of the nucleus in heredity over the influence of the cytoplasm finally emerged, and became consolidated. In parallel, Mendelism (heredity controlled by "modules/particles") and Darwinism (evolution by mutation and natural selection) spread in England, Europe, and the USA.

Thomas Hunt Morgan from Columbia University took years to finally change his belief that chromosomes do not carry Mendelian factors (i.e., genes) (Benson, 2001; Miko, 2008). Morgan and his team found that in flies, such as *Drosophila melanogaster*, inheritable phenotypic variations can also be caused by single-gene mutations that behave like Mendelian factors (F_2 progeny of monohybrid showing a dominant:recessive 3:1 ratio). Some of these mutations showed a special mode of inheritance depending on the parental sex bearing the mutant phenotype. A student of Morgan, Alfred Sturtevant, discovered that these "special" genes showed fixed relative positions (Sturtevant, 1913). Furthermore, he found that linkage among different genes can vary in strength and can be quantified by the frequency of recombination. For the second time in the recent history of biology, after the elucidation of the Mendelian phenotypic and mathematical genotypic patterns, simple calculations led to another great discovery. Genetic mapping based on the frequency of recombination with proximal linked genes was established and paved the way for the future molecular isolation of genes. The ease with which rare X-linked recessive mutations (the first one was *white eye,* instead of red) can be identified in hemizygous male flies (XY) led to the discovery that genes are located on chromosomes. Indeed, when X^wX^w homozygous recessive females (for an X-linked white eye mutation, *white*) were crossed with wild-type red-eyed males (X^+Y), the expected F_1 progeny was composed of red-eyed female flies (X^+X^w daughters' eyes similar to

E-mail address: giuseppe.saccone@unina.it.

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the fathers) and white-eyed male flies (X^wY sons' eyes similar to the mothers), suggesting that the *white* gene was on the X chromosome. Another student of Morgan's, Calvin Bridges, together with Lilian Morgan (the wife of the lab leader and an embryologist who would become an independent and genial geneticist; Keenan, 1983), identified very rarely expected white-eyed daughters and red-eyed sons among many F1 progeny of this special cross (Bridges, 1916). By using microscopy and cytology, these researchers further showed the flies presented chromosomal aneuploidy and deduced that they could reasonably represent X^wX^wY females (with two X chromosomes inherited from the mother in an aberrant egg) and X^+0 males (with X^+ inherited from the father and no X from the mother in another aberrant egg) (Bridges, 1916). This was a formal proof of the chromosomal theory of heredity. Bridges proposed that *Drosophila* primary signal for sex determination is based on the ratio of the X sex chromosome number to autosome ploidy (*X:A* ratio; 2X:2A leads to females, 1X:2A, leads to males and 2X:3A intersexes), suggesting a genic balance mechanism underlying this developmental choice (Bridges, 1916). Similarly, another brilliant student of Morgan's, Mary Stark, discovered that a single sex-linked mutation can induce hereditable tumours in the larval skin of the fly (Stark, 1919). This was another "gift" of knowledge from the emerging *Drosophila* genetics model (with the help of the special heredity of sex-linked traits). - It should be mentioned that Morgan was a generous mentor with a great sense of ethics, as he shared the money received from the Nobel Prize with his students Sturtevant and Bridges (Berg and Singer, 2003). -

Herman Joseph Muller, a part-time student of Morgan's, later flourished as an independent scientist studying the use of X-rays to induce hereditable recessive lethal mutations in *Drosophila*. Muller linked some of these mutations to chromosomal deficiencies that were visible in the giant polytenized larval chromosomes typical of larval salivary glands (Bridges, 1935; Pontecorvo, 1968). These unusually large chromosomes - known as polytene - are produced in cells with secretory functions via repeated rounds of DNA replication without subsequent mitosis. They form sister chromatids packed together with homologous chromatids (unusual somatic pairing typical of meiosis). Each of the euchromatic arms shows a specific banding pattern, following chemical colouration, due to the differential condensation of chromatin, which looks almost like a product bar code. Observing these special chromosomes provided an initial high-resolution view of the *Drosophila* genome, leading to the physical mapping of genes and preliminary studies in the emerging field of eukaryotic genomics (Kaufman, 2017). Chromosomal aberrations can be easily resolved in polytene chromosome preparations from cell lysates. When these changes in genome structure are associated with genetic loci, the loci can be physically mapped with remarkable precision (cytological mapping).

The virtual linear order of genes and the concept of a genetic map derive from genetic and cytological mapping studies (Muller, 1920). Furthermore, Muller demonstrated the usefulness and the danger of X-rays using the elegant *ClB* test. The X-chromosome was again very useful, as recessive lethal mutations induced during spermatogenesis of the grandfather could be quantified by lethality in hemizygous grandsons using this test. This property of X-rays was a game-changing discovery that shifted the focus of genetics from spontaneous to randomly induced genetic mutations and led to a search for additional molecules to improve the mutagenesis approach (Falk, 2010). All three of Morgan's most famous students (Muller, Sturtevant, and Bridges) introduced reductionist empirical approaches to study the chromosomal theory of heredity. However, Muller concentrated on mutations, which were defined then as changes in the heterocatalytic properties of genes while maintaining their autocatalytic (self-replication) properties and reducing heredity to ultramicroscopic material entities (Falk, 2010; Muller, 1927). This idea suggested the future possibility of the direct physical identification and molecular isolation of genes.

2. Methodological and conceptual advances facilitating the molecular characterization of sex determination genes

In the first half of the last century, *D. melanogaster* gradually became a model system used for genetics by developing convenient genetic strategies (Gayon, 2016). -Once single gene mutations were obtained, they were mapped to specific chromosomes with respect to other known genetic markers and maintained in stocks, using balancer chromosomes - which "suppress" recombination with the homologous ones (Miller et al., 2019)(Appendix, Suppl. Fig. 1). Two other valuable features of *Drosophila* as a model for genetic studies are the presence of very large polytene chromosomes in some larval tissues, and the consistency of their chromosomal band and interband patterns. The experimental study of altered chromosomes of mutant strains demonstrated the idea of colinearity of the cytogenetic and linkage genetic maps. Bridges developed more precise illustrations and reliable classifications of these banding patterns, which became an enduring system of reference for future generations of drosophilists (Bridges, 1935). Hereditable mutations are necessary to perform classical genetics studies. Alderson (1965) developed ethyl methane sulfonate (EMS) mutagenesis in *D. melanogaster,* which became a general method for inducing point mutations and performing genetic screening. This method led to new discoveries in fruitfly biology over the next few decades, including the identification of genes involved in processes such as the control of developmental pathways (e.g. wing vein patterns and sex determination), behaviour (e.g. mating), and morphogenesis (e.g. homeotic genes). The serendipitous discovery of the hybrid dysgenesis and the related *P* element in *D. melanogaster* led to the development of a gene transfer technique based on transposons and subsequently to very effective and rapid random mutagenesis screening to isolate corresponding mutated genes (Kidwell et al., 1977; Spradling and Rubin, 1982; Rubin and Spradling, 1982; Spradling et al., 1995; Appendix, Suppl. Fig. 2). This unbiased forward mutagenesis screening continues to be required to search for novel genes involved in specific biological aspects or unpredictable gene interactions (Funato, 2020), even with the availability of recently developed gene editing technologies (Gantz and Akbari, 2018).

The recombinant DNA technology of the last century, which emerged in the 1970s–1980s, led to the isolation and cloning of *Drosophila* genes from specific genomic regions and the connection of molecular mutations with novel phenotypes (Maniatis et al., 1978, 1982; Shendure et al., 2017). Some specific cloning strategies of the 1980s included the microdissection technique of chromosomal bands (from polytenic giant larval chromosomes; Scalenghe et al., 1981) and "chromosome walking", a DNA hybridization-based technique (Bender et al., 1983) (Appendix, Suppl. Fig. 3). The concept underlying this approach is that some mutant strains may harbour structural gene variations with lengths of hundreds of base pairs rather than point mutations. The difference can be observed based on by the different mobilities of some derived restriction fragments (Southern, 2006).

3. Identification and isolation of *transformer (tra) and transformer-2*

Sturtevant made a fundamental discovery for the emerging field of sex determination genetics: a mutation in a single gene could cause complete sexual transformation of *D. melanogaster*, as also suggested by Lebedeff (1934) while studying the heredity of aberrant hermaphroditism in *Drosophila virilis* (Sturtevant, 1945). The availability of a mutant strain carrying X-linked markers was a critical premise for discovering a mutation that reverts XX females into XX males. Sturtevant performed a cross involving females homozygous for an X-linked visible recessive allele (X^aX^a) and wild-type fathers (X^+Y). He expected to observe mutant males (X^aY) and wild-type females (X^+X^a). However, the progeny included not only mutant males but also a consistent number of unexpected wild-type males for the X-linked trait. These males

"wild-type for the *a* gene" showed an unusually larger size than their X brothers, faster emergence from pupae (a trait typical of XX females) and were sterile with rudimentary gonads. Cytogenetic analysis revealed that these larger males had XX karyotype ($X^{+}X^{a}$). They harboured a novel single autosomal recessive mutation, *transformer* gene (*tra*), that transformed XX individuals into XX sterile males, named pseudomales. Crosses with strains carrying balancers - also bearing visible dominant genetic markers on autosomal chromosomes - led to localizing *tra* on the third chromosome (Baker and Ridge, 1980). The cytological mapping of *tra* and deletion analysis in polytenic chromosomes narrowed the region containing the locus to the 73A9-10 interval of chromosome 3, near the *scarlet* mutation. Butler et al. (1986; Rolf Nothiger lab, Zurich, Switzerland) induced chromosomal deficiencies (deletions) covering the *scarlet* (*st*) region by X-irradiation. Crosses between X-irradiated flies from a wild-type strain and mutant *st* flies led to finding individuals showing the *scarlet* phenotype (hence, potentially carrying a deletion of the st^{+} region). Cytological examination of their polytene chromosomes confirmed the presence of gross deficiencies and determined their extent. Furthermore, the authors crossed the *st* mutants with *tra* mutants to select those XX flies with the *tra* phenotype, hence carrying deficiencies of the *transformer* gene (deletions covering both the st^{+} and the tra^{+} genes). Since all the induced deficiencies are homozygous lethal, the corresponding stocks were maintained in heterozygous conditions using a balancer chromosome (see Appendix). Table 1 summarizes the list of *Drosophila* and other insect sex-determining genes, following a chronological order of their cloning (see also Table 2).

Butler et al. (1986) cloned DNA fragments via the microdissection of the 73A9-10 region. They ordered the phage clones in overlapping restriction maps and by "chromosome walking" (Appendix, Suppl. Fig. 3) obtained a 200-Kb continuous DNA region. A Southern blot analysis of wild-type and *tra* mutant strains showed a different DNA restriction length in the *tra* region, suggesting the presence of molecular lesions. To demonstrate that *transformer* gene provides the function for femaleness, transgenic *Drosophila* flies were produced by microinjecting a recombinant *P* transposon containing the wild type genomic locus fragment (Butler et al., 1986). The transgene was able to fully rescue *tra/tra* mutant XX flies, returning them to their original female sexual phenotype. Butler et al. (1986) anticipated that the *tra* gene would be expressed only in XX females, as its function was dispensable in XY males. However, to the authors' surprise, Northern blot analyses showed that the *tra* gene produced transcripts in both sexes, although one was shorter female-specific and one was common in both sexes. This observation suggested that either alternative transcriptional start sites or alternative mRNA processing (discovered a few years earlier) might be the regulatory mechanism underlying the difference. Bruce Baker's team (Stanford University, USA) in the USA (McKeown et al., 1987) published similar data a few months later. These authors anticipated that the non-sex-specific longer *tra* mRNA might be nonfunctional, while the female-specific mRNA would encode the protein with the required function. Boggs et al. (1987) isolated and sequenced *tra* cDNA clones showing that the female-specific transcript, generated by 3′ alternative splice site choice, encodes a 197 amino acids (aa) long serine-arginine-rich putative RNA binding protein. In contrast, the non-sex-specific longer *tra* transcript contains various stop codons throughout the differentially spliced region. As a result, the longest open reading frame found in this non-sex-specific transcript is only 48 aa long, and no full-length Tra protein is produced in XY individuals. In a mutant *Drosophila* strain bearing a deletion of the entire *tra* gene, XY males are normal and fertile, confirming the dispensability of the gene in this sex, at least under laboratory conditions. In the following sections, I will describe that the alternative splicing of an exon introducing a premature termination of translation seems to be an effective ON/OFF regulatory mechanism for sex determination in many other insects. This regulatory mechanism has been evolutionarily conserved for at least 300 million years in lineages of Diptera, Coleoptera and Hymenoptera.

Similar to the findings reported for *transformer*, a mutation in a second gene, *transformer-2* (*tra2*), produced sterile XX pseudomales (Watanabe, 1975; Belote and Baker, 1982). The Nöthiger laboratory applied a cloning approach based on *P* element tagging of the *tra2* gene (Appendix, Suppl. Fig. 2) (Amrein et al., 1988). Goralski et al. (1989), from the Baker laboratory also reported the cloning of *tra2* by using the more traditional strategy of genetic and cytogenetic mapping. They exploited chromosomal deficiencies, polytene chromosome microdissection and a related DNA microcloning technique. The sequencing of *tra2* led to the discovery that Tra2 was also related to known RNA-binding proteins (Amrein et al., 1988). While *Drosophila* Transformer is a serine/arginine-rich splicing factor (RS-type splicing factor) lacking any known structural domains, Tra2 contains an RNA Recognition Motif (RRM), and stretches of serine and arginine (SR-type splicing factor; Best et al., 2014).

4. Identification and isolation of *doublesex (dsx)* and *intersex* (*ix*)

A third autosomal mutation, *doublesex (dsx)*, affects sexual development. However, in contrast to the previous mutations, it transforms both sexes into intersex flies, indicating its bifunctionality (either repressing femaleness in males and maleness in females or promoting proper sexual differentiation in each sex). At the time of its identification, this mutation was considered "mysterious" from a mechanistic point of view (Hildreth, 1965). Hildreth proposed that *dsx* acted prior to the development of either sex, promoting an initial hermaphroditic phenotype skewed in one of two directions "only slightly" by the XX and XY chromosomal composition. It is surprising to discover that during those years, biologists were still asking if genes rather than sex chromosomes were responsible for controlling primary signals for sex determination (Mittwoch, 1969). The *dsx* genomic region was molecularly isolated as overlapping *Drosophila* DNA segments cloned into a lambda phage library. The search for *dsx* was a difficult and time-consuming task - During this genomic walk (Appendix, Suppl. Fig. 3), the authors took advantage of the breakpoints of six inversions, five translocations, and two deficiencies within the *dsx* locus. - When chromosome walking ultimately reached the *dsx* region, chromosomal rearrangements that inactivate the wild-type function were molecularly mapped within the region (Baker and Wolfner, 1988). To identify the salivary chromosome map location of the *dsx* locus, the authors generated some chromosomal rearrangements with breakpoints in and near the *dsx* gene. Furthermore, they exploited a large chromosomal deletion covering part of the *dsx* locus and employed a nearby previously cloned gene (*alpha-tubulin* gene; Mischke and Pardue, 1982), as a molecularly entry site. This marker mapped outside the two breakpoints and was used to start a chromosome walking and ultimately isolate the DNA sequence containing the *dsx* gene.

Burtis and Baker (1989) isolated *dsx* cDNAs and revealed that the male and female transcript isoforms differed in their 3′ ends. The corresponding transcripts encode proteins containing a common N-terminus – with a novel zinc-finger type DNA-binding domain - and sex-specific C-termini. The direct regulation of *dsx* splicing by Tra and Tra2 was confirmed in S2 cells (Ryner and Baker, 1991)(Fig. 1). Burtis and Baker (1989) identified a 13-nt-long putative regulatory sequence that was repeated six times (*dsx* repeat element, *dsx*RE), later identified as a Tra/Tra2 binding site) in the 3′ untranslated region of the female-specific exon. They also found the *dsx*RE conserved in the *D. virilis dsx* orthologue (sharing a common ancestor with *D. melanogaster* 60 million years ago), isolated via low-stringency hybridization. They noted that the 3′ acceptor site of the *dsx* female-specific exon was suboptimal when compared to the *Drosophila* consensus sequence. They proposed that the cis-regulatory *dsx*RE was required in XX individuals to activate the weak 3' splice acceptor site preceding the female-specific exon during pre-mRNA splicing. *In vitro* experiments confirmed the identification of the first example of a splicing enhancer. This *dsx*RE regulatory element is recognized by the Tra/Tra2 protein complex and responsible for *dsx* female-specific

Table 1
List of Drosophila sex determining genes and related orthologues.

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
Drosophila melanogaster (Drosophilidae; Diptera)	*transformer* (*tra*)	Regulator of female-specific *dsx* and *fru splicing*	RS splicing factor	Mutation, mapping, microdissection and chromosome walking	Low sequence conservation of orthologues among Drosophilidae. Lack of known structural domains.	Sturtevant (1945), Butler et al. (1986), McKeown et al. (1987), Boggs et al. (1987), O'Neil and Belote, 1992.
D. melanogaster (Diptera)	*transformer-2* (*tra2*)	Auxiliary regulator of female-specific *dsx* and *fru splicing;* male-specific germ-line function for fertility	SR- splicing regulator (RRM RNA binding domain)	Mutation, P-element tagging, mapping, microdissection and chromosome walking	High sequence conservation of orthologues in vertebrates	Watanabe (1975), Belote and Baker (1982), Amrein et al. (1988), Goralski et al. (1989).
D. melanogaster (Diptera)	*doublesex* (*dsx*)	Sex-specific transcriptional regulator of multiple genes	DM zinc-finger DNA-binding domain	chromosomal walking, molecular mapping of rearrangements,	Sequence and functional conservation of DM in sexual development of metazoans	Hildreth (1965), Baker and Wolfner (1988), Burtis and Baker (1989).
D. melanogaster (Diptera)	*Sex-lethal* (*Sxl*)	Master regulator of female-specific *tra* and *msl-2* splicing (dosage compensation)	Splicing regulator (RRM RNA binding domain)	*P* element insertion,	Sxl autoregulates positively in XX individuals. Sxl represses dosage compensation in XX individuals by promoting *msl-2* non productive female-specific splicing. Sxl has high sequence but no functional conservation outside of Drosophilidae family.	Muller and Zimmering (1960); Cline (1978), Bashaw and Baker (1995), Maine et al. (1985), Bell et al. (1988).
D. melanogaster (Diptera)	*fruitless* (*fru*)	In sexual differentiation: Male-specific regulator of multiple genes	BTB zinc-finger transcription factor	P element tagging; genomic library screening with Tra/Tra-2 binding site sequence.	The gene has complex expression pattern, encodes many different isoforms, with only few male-specific.	Ito et al. (1996), by Ryner et al. (1996).
Drosophila virilis (Drosophilidae; Diptera)	*Dvdsx*	Likely the same as Dm *dsx*.	DM zinc-finger DNA-binding domain	Genomic library screening (low stringency)	Sequence conservation of tra/tra2 binding sites in the female-specific exon. Default splicing (not specifically regulated) is male-specific	Burtis and Baker (1989).
D. melanogaster (Diptera)	*sisterless-a* (*sis-a*)	Primary sex determining signal	bZIP transcriptional factor	Genetic mapping, deletions, P element complementation		Cline (1986), Erickson and Cline (1993).
D. melanogaster (Diptera)	*sisterless-b* (*sis-b*)	Primary sex determining signal	HLH transcriptional factor	Mapping, transposon insertion, genomic library screening		Cline (1988), Villares and Cabrera (1987).
D. melanogaster (Diptera)	*daughterless* (*da*)	Primary sex determining signal	HLH transcriptional factor	Chromosome walking, P mutagenesis, X-rays mutagenesis		Cline (1978), Caudy et al. (1988).
D. melanogaster (Diptera)	*intersex* (*ix*)	Auxiliary factor of DsxF; activation of vitellogenin genes, female differentiation	transcriptional factor	Spontaneous mutation; cytological and physical localization of intersex, RFLP mapping, phage clones covering the entire region		Baker and Ridge (1980), Garrett-Engele et al. (2002).
D. virilis (Drosophilidae, Diptera)	*DvSxl*	Likely the same as Dm Sxl	Splicing regulator (RRM RNA binding domain)	Genomic library screening (low stringency)	Female-specific expression of Dm and DvSXL at embryonal stages. Early embryonal female-specific DvSxl activation as in Dm (early Promoter).	Bopp et al. (1996), Jinks et al. (2003).
Drosophila pseudobscura (Drosophilidae, Diptera)	*DpSxl*	Likely the same as Dm Sxl	Splicing regulator (RRM RNA binding domain)	Genomic library screening (low stringency)		Penalva et al. (1996).
Chrysomya rufifacies (Calliphoridae, Diptera)	*CrSxl*	Unknown; No-sex-specific regulation.	Splicing regulator (RRM RNA binding domain)	Genomic library screening (low stringency)		Müller-Holtkamp (1995)
Megaselia scalaris (Phoridae, Diptera)	*MsSxl*	Unknown; No-sex-specific regulation.	Splicing regulator (RRM RNA binding domain)	PCR with degenerate primers		Sievert et al. (1997)
Ceratitis capitata (Tephritidae, Diptera))	*CcSxl*	Unknown; non-sex-specifically regulated,	Splicing regulator (RRM RNA binding domain)	cDNA library screening (low stringency)		Saccone et al. (1998)
Musca domestica (Muscidae, Diptera)	*MdSxl*	Unknown; non-sex-specifically regulated,	Splicing regulator (RRM RNA binding domain)	PCR and degenerate primers;		Meise et al. (1998).

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
Sciaridae species (Diptera)	*SoSxl, ScSxl, RaSxl, TpSxl*	Unknown; non-sex-specifically regulated,	Splicing regulator (RRM RNA binding domain)	Genomic library screening (low stringency)		Serna et al. (2004).
Bombyx mori (Lepidoptera)	*BmSxl*	non-sex-specifically regulated; essential function in spermatogenesis	Splicing regulator (RRM RNA binding domain)	PCR and degenerate primers;	Functional study performed by gene targeting by Sakai et al. (2019)	Niimi et al. (2006), Sakai et al. (2019).
Bactrocera oleae (Tephritidae, Diptera)	*BoSxl*	Unknown; non-sex-specifically regulated,	Splicing regulator (RRM RNA binding domain)	PCR and degenerate primers; library screenings		Lagos et al. (2005).
D. virilis (Drosophilidae, Diptera)	*Dvtra*	Likely the same as Dm *tra*; sex-specific regulation	Female-specific RS splicing factor	Genomic library screening (low stringency)	TRA and DvTRA showed 31–36% aa identity	O'Neil and Belote (1992).
C. capitata (Tephritidae; Diptera)	*Cctra*	Master regulator of its own female-specific splicing (novel epigenetic function); possible maternal function of *Cctra*: egulator of female-specific C*cdsx* and C*cfru* splicing;	Female-specific RS splicing factor	Cloning by synteny: genomic library screening (low stringency) using *l3Ah* gene linked to *tra*, Functional analysis by embryonic RNAi	*Cctra* has the novel function of positive autoregulation in XX individuals, analogous to Dm *Sxl*. DmTRA and CcTRA do not shown significant similarly by BLASTp; a clustal alignment revealed only 3 short protein regions (17–56 aa) showing 35–70% identity.	Pane et al. (2002).
Anastrepha species (Tephritidae, Diptera)	*Aotra, Agtra, Asetra, Asotra, Astri-tra, Abtra, Aatra, Altra*	Similar to *Cctra* master function	Female-specific RS splicing factor	PCR and degenerate primers		Ruiz et al. (2007).
Bactrocera oleae (Tephritidae, Diptera)	*Botra*	Similar to *Cctra* master function	Female-specific RS splicing factor	Cloning by synteny: genomic library screening (low stringency) using *l3Ah* gene linked to *tra*	Functional analysis by embryonic RNAi	Lagos et al. (2007).
Apis mellifera (Hymenoptera)	*feminizer*	Similar to *Cctra* master function	Female-specific RS rich protein	Positional cloning by genetic mapping of markers (RAPD, multilocsu fingerprinting) linked to *csd*, chromosome walking	Embryonic RNAi; paralogue of *csd* gene.	Hasselmann et al. (2008).
Lucilia cuprina (Calliphoridae, Diptera)	*Lctra*	Similar to *Cctra* master function	Female-specific RS splicing factor	PCR and degenerate primers	Functional analysis by embryonic RNAi	Concha and Scott (2009).
Musca domestica (Muscidae), Diptera)	*Mdtra*	Similar to *Cctra* master function; maternal function of *Mdtra*	Female-specific RS splicing factor	PCR and degenerate primers pairing with *tra/tra2* binding sites (involved in *Cctra* splicing autoregulation)	Functional analysis by embryonic RNAi	Hediger et al. (2010).
Tribolium castaneum (Coleoptera)	*Tctra*	Similar to *Cctra* master function	Female-specific RS splicing factor	blastn search at NCBI Beetlebase (Apis feminizer aa seq as probe)	Functional analysis by embryonic RNAi	Shukla and Palli (2012).
Anastrepha suspensa (Tephritidae, Diptera)	*Astra*	Similar to *Cctra* master function	Female-specificRS splicing factor	tBLAST search of a EST database	Functional analysis by embryonic RNAi	Schetelig et al. (2012).
Bactrocera jarvisi (Tephritidae, Diptera)	*Bjtra*	Similar to *Cctra* master function	Female-specific RS splicing factor	tBLAST search of an embryonic transcriptome		Morrow et al. (2014).
Bactrocera dorsalis (Tephritidae, Diptera)	*Bdtra*	Similar to *Cctra* master function	Female-specific RS splicing factor	PCR and degenerate primers; tBLAST search of an embryonic transcriptome	Functional analysis by embryonic RNAi	Liu et al. (2015), Peng et al. (2015).
Nasonia vitripennis (Hymenoptera)	*Nvtra*	Similar to *Cctra* master function	Female-specific RS splicing factor	BLAST search of a genome database	Functional analysis by embryonic RNAi; in early haploid embryos maternal *Nvtra* allele is not transcribed; in diploid embryos, the paternal *Nvtra* allele is active from early stages. *Nvtra* maternal mRNA is possibly required to start the female-specific *tra*	Verhulst et al. (2010b).

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
					autoregulation in late diploid embryos.	
Leptopilina clavipes (Hymenoptera: Cynipidae)	*Lc-tra*	Possibly similar to *Cctra/Nvtra* master function	Female-specific RS splicing factor	BLAST search of a genome database	A Lc-tra paralogue is present in this species, lacking sex-specific regulation	Geuverink et al. (2018a).
Asobara tabida (Braconidae)	*At-tra*	Possibly similar to *Cctra/Nvtra* master function	Female-specific RS splicing factor	BLAST search of a genome database	A non-sex-specific maternal At-tra mRNA encoding a different isoform is present	Geuverink et al. (2018b).
Phlebotomus pernicious (Psychodidae)	*Ppetra*	Possibly similar to *Cctra* master function	Female-specific RS splicing factor	BLASTn search of a transcriptome database with the tra/tra-2 binding sites consensus	PpeTRA has very divergent sequence compared to the known TRAs.	Petrella et al. (2019).
Rhodius prolixus, (Hemiptera)	*Rptra*	sex-specific tra splicing	Female-specific RS splicing factor	BLAST analyses of genone/transcriptome databases		Wexler et al. (2019).
Blattella germanica (Blattodea)	*Bgtra*	No sex-specific *tra* splicing; sex-specific biases of typical male and female isoforms; however, *Bgtra* is required for female develoment and for female-specific *Bgdsx* splicing	no-sex-specific RS splicing factor	BLAST analyses of genone/transcriptome databases	RNAi affects female but not male development	Wexler et al. (2019).
Pediculus humanus (Anoplura; body louse)	*Phtra*	No sex-specific *tra* splicing; likely sex-specific biases of typical male and female isoforms	no-sex—specific RS splicing factor	BLAST analyses of genone/transcriptome databases		Wexler et al. (2019).
D. virilis (Diptera)	*Dvtra2*	Likely similar to *Dm tra2*	SR-splicing regulator (RRM RNA binding domain)	Genomic library screening (low stringency)		Chandler et al. (1997).
Musca domestica (Muscidae)	*Mdtra2*	Regulator of female-specific *Mddsx* and *Mdfru* splicing; regulator of *Mdtra* female-specific splicing (*Mdtra* auto regulation).	SR-splicing regulator (RRM RNA binding domain)	PCR and degenerate primers;	the Musca *F* gene is likely homologous to the Medfly *tra* gene and is able to autoregulate itself using Tra/Tra2 binding sites and Mdtra2.	Burghardt et al. (2005).
C. capitata (Diptera)	*Cctra2*	Regulator of female-specific Cc*dsx* and Cc*fru* splicing; regulator of *Cctra* female-specific splicing (*Cctra* auto regulation).	SR-splicing regulator (RRM RNA binding domain)	BLAST in EST database, PCR and degenerate primers;	Embryonic RNAi	Gomulski et al. (2008), Salvemini et al. (2009).
Anastrepha obliqua (Diptera)	*Aotra2*	AoTRA2 protein can partially substitute for the endogenous Dm TRA2 protein. Regulator of dsx splicing.	SR- splicing regulator (RRM RNA binding domain)	nested PCR and using degenerated primers		Sarno et al. (2010).
Sciaridae (Diptera)	Sciara *tra2*	partial activity in promoting female-specific *dsx* splicing in Drosophila transgenic flies	SR- splicing regulator (RRM RNA binding domain)	nested PCR and using degenerate primers		Martín et al. (2011).
Apis mellifera (Hymenoptera)	*Am-tra2*	Novel vital embryonic function; female sex determination and differentiation	SR- splicing regulator (RRM RNA binding domain)	BLAST on genome	RNAi functional studies: novel vital function during the embryogenesis of both sexes; *Am-tra2* is required for *fem* and *Amdsx* female-specific splicing	Dearden et al. (2006)
N. vitripennis (Hymenoptera)	*Nvtra2*	*Nasonia tra* genes also requires TRA2-orthologous proteins to perform positive female-specific autoregulation	SR- splicing regulator (RRM RNA binding domain)	tBLASTn screening with an *A. mellifera* TRA2 amino acid sequence		Geuverink et al. (2017)
B. mori (Lepidoptera)	*Bmtra2*	required for normal testis development	SR- splicing regulator (RRM RNA binding domain)	BLAST search of EST database	Embryonic RNAi; *Bombyx mori tra2* seems not to be involved in controlling *Bmdsx* splicing and sex determination	Niu et al. (2005); Suzuki et al. (2012)
T. castaneum (Coleoptera)	*Tctra2*	female-specific splicing of *Tctra,* as observed for Medfly *tra*, but also for	SR- splicing regulator (RRM RNA binding domain)	BLAST search of genome/transcriptome databases	Embryonic RNAi;	Shukla and Palli (2013)

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
		male-specific *Tctra* splicing				
Nilaparvata lugens (Hemiptera)	*Nltra2*	Required for female development, fertility of both sexes.	SR- splicing regulator (RRM RNA binding domain)	BLAST search of genome databases	cross-talk between the sexual differentiation and wing polyphenism pathways	Zhuo et al. (2017)
Cyclommatus. metallifer (Coleoptera)	*Cmtra2*	Required for female development	SR- splicing regulator (RRM RNA binding domain)	BLAST search of transcriptome database	Pre-pupal RNAi is lethal	Gotoh et al. (2016a)
Bemisia tabaci (Hemiptera, Aleyrodidae),	*Bttra2*	Male genitalia, vitellogenin regulation in females	SR- splicing regulator (RRM RNA binding domain)	BLAST search of transcriptome database	it is expressed at higher levels in haploid males versus diploid females	GuoXie et al. (2018), Xie et al. (2014).
Anopheles gambiae (Diptera)	*Femaleness* (*Fle*)	Required for Anopheles *dsx* and *fru* female-specific splicing, female development and repression of. dosage compensation	Related to Tra2; SR- splicing regulator (RRM RNA binding domain)	BLAST search of transcriptome database	Embryonic and cell line RNAi: female-specific lethality (dosage compensation impairment), masculinization of mosquitoes, induction of male-specific *dsx* and *fru* splicing.	Krzywinska et al. (2021).
B. tryoni (Diptera)	*Btdsx*	Likely similar to Dm *dsx:* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	PCR and degenerate primers; 5′ and 3′ race	Sequence conservation of tra/tra2 binding sites in the female-specific exon.	Shearman and Frommer (1998)
Caenorabditis elegans (Rhabditida, Nematode)	*mab-3*	Male differentiation	DM zinc-finger DNA-binding domain	Mapping, genetic deficiencies (deletions)	This findings showed that sex-determining mechanisms do not differ completely between phyla	Raymond et al. (1998)
Megaselia scalaris (Diptera)	*Msdsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	cDNA library screening (low stringency)		Kuhn et al., 2000
Bombyx mori (Lepidoptera)	*Bmdsx*	Development of abdominal segments, of genital structures, fecundity	DM zinc-finger DNA-binding domain	BLAST, EST database	Bmdsx default splicing is female-specific; Gene editing (Talens, Cas9; Xu et al., 2017)	Ohbayashi et al. (2001), Suzuki et al. (2001), Xu et al. (2017).
C. capitata (Diptera)	*Ccdsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	cDNA library screening (low stringency)	Sequence conservation of tra/tra2 binding sites in the female-specific exon.	Pane et al. (2002), Saccone et al. (2008).
Musca domestica (Diptera)	*Mddsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	PCR and degenerate primers;		Hediger et al. (2004).
Anopheles gambiae (Diptera)	*Agdsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	BLAST, EST database	Absence of Tra/Tra-2 binding elements in the female-specific exon; likely not regulated by TRA/TRA-2 orthologues.	Scali et al. (2005).
B. oleae (Diptera)	*Bodsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	PCR and degenerate primers;		Lagos et al. (2005).
A.obliqua (Diptera)	*Aodsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	PCR and degenerate primers;		Ruiz et al. (2005).
Apis mellifera (Hymenoptera)	*Amdsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing; reproductive organs and gonads development	DM zinc-finger DNA-binding domain	BLAST genome database	Gene editing by Cas9 (Roth et al., 2019.)	Cristino et al. (2006); Roth et al. (2019), Cho et al. (2007).
Anastrepha species (Diptera)	*A sp.- dsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	PCR and degenerate primers;	strong purifying selection	Ruiz et al. (2007).
Nasonia giraulti and N. vitripennis	*Ngdsx, Nvdsx*					Oliveira et al. (2009).
Aedes aegypti (Diptera)	*Aaedsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	BLAST, genome database	Different sex-specific splicing regulation; Absence of Tra/Tra-2 binding elements in the female-specific exon; likely not regulated by TRA/TRA-2 orthologues.	Salvemini et al. (2011).
B. jarvisi (Diptera)	*Bjdsx*	Likely similar to Dm *dsx,* sex-specifically regulated by alternative splicing	DM zinc-finger DNA-binding domain	BLASTm transcriptomics		Morrow et al. (2014).
Sciaridae species (Diptera)		No-sex-specific splicing		PCR and degenerate primers;	Only DSXF isoform expressed in both sexes as	Ruiz et al. (2015).

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
	S. coprophila dsx, S. Ocellaris dsx		DM zinc-finger DNA-binding domain		protein; minor role if any, in sexual differentiation	
Cyclommatus metallifer (Coleoptera)	*Cmdsx*	16 Multiple sex-specific and non-specific-specific isoforms	DM zinc-finger DNA-binding domain	BLAST analyses of transcriptome databases	Only CmDsxB and CmDsxD appear critical for generating sex specific morphologies respectively during male (including mandible length) and female differentiation	Gotoh et al. (2016a)
Gnatocerus cornutus (Coleoptera)	*Gcdsx*	Multiple sex-specific and non-specific-specific isoforms	DM zinc-finger DNA-binding domain	BLAST analyses of transcriptome databases	Gcdsx controls sexually selected weapon traits, such as mandible, genae and horns.	Gotoh et al. (2016b)
Athalia rosae (Hymenoptera)	*Ardsx*	sex-specific splicing	DM zinc-finger DNA-binding domain	BLAST analyses of transcriptome databases	RNAi induced almost complete haploid male-to-female sex reversal; no effects on diploid females.	Mine et al. (2017).
20 ant species (Hymenoptera) *Solenopsis invicta*, *Vollenhovia emeryi*, and *Wasmannia auropunctata*;	*Sinv_dsx, Veme_dsx* and *Waur_dsx,*	As in other insects, female-specific exons are skipped in males, and the male-specific transcripts are more extended at the 3' - terminus. Caste-specific isoforms.	DM zinc-finger DNA-binding domain	BLAST analyses of transcriptome databases	*dsx* genes of ants have evolved under positive selection; overall male-biased expression of *dsx*. *Veme_dsx* and *Waur_dsx,* exon usage between queens and workers for some exons is different. *dsx* evolution seems to correlate with the evolution of eusociality in some ants.	Jia et al. (2018)
Bemisia tabaci (Hemiptera)	*Btdsx*	likely sex-specific splicing; development of genitalia;	DM zinc-finger DNA-binding domain	BLAST analyses of transcriptome databases	RNAi-treated females showed a reduction in fecundity and egg hatching. Contrasting data in the two references.	GuoXie et al. (2018); Singh Brar et al. (2022)
Rhodius prolixus, (Hemiptera)	*Rpdsx*	sex-specific splicing	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Wexler et al. (2019).
Blattella germanica (Blattodea)	*Bgdsx*	sex-specific splicing controlled by *Bgtra*	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases	RNAi: *BgDsx* is necessary for male-specific but not female-specific sexual differentiation	Wexler et al. (2019).
Pediculus humanus (Phthiraptera)	*Phdsx*	No-sex specific splicing	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Wexler et al. (2019).
Zerene cesonia (Lepidoptera)	*Zcdsx*	sex-specific splicing, sexually dimorphic UV colouration of the wings	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases	two distinct *dsx* paralogues	Rodriguez-Caro et al. (2021).
Bicyclus anynana (Lepidoptera)	*Badsx*	sex-specific splicing; BaDSXF represses development of male-specific scent organ	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases	Cas9 Gene editing	Prakash and Monteiro (2020).
Zootermopsis nevadensis (Isoptera; termite)		no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Hodotermopsis sjostedti (Isoptera; termite)	*Hsjo_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Cryptotermes secundus (Isoptera; termite)	*Csex_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Reticulitermes speratus (Isoptera; termite)	*Rspe_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Coptotermes formosanus (Isoptera; termite)	*Cfor_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Macrotermes natalensis (Isoptera; termite)	*Mnat_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Nasutitermes takasagoensis (Isoptera; termite)	*Mtak_dsx*	no sex-specifc splicing, male-specific transcription	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).
Cryptocercus punctulatus (Blattodea;	*Cpun_dsx*	sex-specifc splicing,	DM zinc-finger DNA-binding domain	BLAST analyses of genone/transcriptome databases		Miyazaki et al. (2021).

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
subsocial woodroach)						
D. pseudobscura	*Dps-ix*	Likely partner of dsxF to control female differentiation	Putative transcriptional factor	BLAST in genome/ transcriptome databases		Siegal and Baker (2005)
D. virilis	*Dvix*	Likely partner of dsxF to control female differentiation	Putative transcriptional factor	BLAST in genome/ transcriptome databases		Siegal and Baker (2005)
Glossina morsitans	*Gmix*	Likely partner of dsxF to control female differentiation	Putative transcriptional factor	BLAST in genome/ transcriptome databases		Siegal and Baker (2005)
Megaselia scalaris	*Mgix*	Likely partner of dsxF to control female differentiation	Putative transcriptional factor	BLAST in genome/ transcriptome databases	Expression of *M. scalaris* Ix in transgenic *ix Drosophila* rescued *ix* female-specific function	Siegal and Baker (2005)
Anopheles gambiae	*Agix*	Likely partner of DsxF to control female differentiation	Putative transcriptional factor	BLAST in genome/ transcriptome databases		Siegal and Baker (2005)
Maruca vitrata (Lepidoptera)	*Mvix*	Likely partner of DsxF to control female differentiation	Putative transcriptional factor	low stringency hybridization screening of pupal cDNA library	a novel female-specific transcript found only in pupae; *Mvix* is expressed at higher levels in females. MvIx protein can partially replace the *Drosophila* endogenous Ix	Cavaliere et al. (2009)
B. mori (Lepidoptera)	*Bmix*	Likely partner of DsxF to control female differentiation; development of the imaginal disc, including the wing, antenna, and leg	Putative transcriptional factor	BLAST in genome/ transcriptome databases	Novel testis-specific spliced transcripts; CRISPR/Cas9 female mutants were sterile and had irregular external genitalia	Arunkumar and Nagaraju (2011) Xu et al. (2019)
Bemisia tabaci (Hemiptera)	*Btix*	adult female reproduction *Btix* controls vitellogenic expression in females.	Putative transcriptional factor	BLAST in genome/ transcriptome databases	Knocking down by larval feeding RNAi affected nd the eclosion rate of the progeny, the length and reproduction of females, no female-to-male sexual transformations were observed,	Liu et al. (2020a)
Oncopeltus fasciatus (Hemiptera)	*Ofix*	adult female reproduction	Putative transcriptional factor	BLAST in genome/ transcriptome databases	RNAi at nymphal stages affected male and female genital morphological structures; partial sexual transformations of females into males;	Aspiras et al. (2011), Ewen-Campen et al. (2011).
Nilaparvata lugens (Hemiptera)	*Nlix*	pleiotropic roles in embryogenesis and development of the reproductive system.	Putative transcriptional factor	BLAST in genome/ transcriptome databases	RNAi at nymphal stages affected adult genital structures	Zhang et al. (2021)
D. melanogaster (Diptera)	*Fruitless* (*fru*), also known as *satori* (*sat*)	Regulated by tra/tra2, male sexual behaviour	BTB zinc-finger transcription factor	*P* transposon tagging and screening based on behavioural tests; genomic library screening by short radioactive probe (tra/ tra2 binding site)	*FruM* regulates target genes controlling neuronal projection morphogenesis or encoding key cell surface molecules	Ito et al. (1996), by Ryner et al. (1996).
21 Diptera species, including various Drosophiilae. C. capitata. Bactrocera dorsals, B. cucurbitae and *Bombyx mori*	Various names	Possible function in male sexual behaviour	Putative BTB zinc-finger transcription factor	Low stringency hybridisation using a Dm FRU (BTB protein-protein-binding domain) radiative probe	Fragment of the FRU gene encoding BTB protein-protein-binding domain	Davis et al. (2000).
Anopheles gambiae (Diptera)	*Agfru*	Possible function in male sexual behaviour	BTB zinc-finger transcription factor	BLAST analyses of genone/transcriptome databases	Likely involved in controlling male sexual differentiation as in Drosophila; sex-specific splicing	Gailey et al. (2006).
Nasonia vitripennis	*Nvfru*	Possible function in male sexual behaviour	BTB zinc-finger transcription factor	PCR and degenerate primers;		Bertossa et al. (2009).
Schizocerca gregaria (Orthoptera)	*Sgfru*	highest expression in the testes, male accessory glands and in the brain	BTB zinc-finger transcription factor	BLAST analyses of ESTs databases	RNAi, reduced male copulation frequency and testes weight	Boerjan et al. (2011).

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Table 1 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
Ceratitis capitata (Diptera)	*Ccfru*	Likely male courtship behaviour	BTB zinc-finger transcription factor	Davis et al. (2000) isolated a genomic fragment;	RNAi; XX males show male mating behaviour, are fertile and expesss male-specific Ccfru transcripts. Sex-specific splicing likely controlled by CcTRA/ Cctra-2	Salvemini et al. (2009).
Musca domestica (Diptera)	*Mdfru*	Likely male courtship behaviour	BTB zinc-finger transcription factor	PCR and degenerate primers; 5′ and 3′ race	*fru* sex-specific trascnritps in the brain.	Meier et al. (2013).
Aedes aegypti (Diptera)	*Aaefru*	Likely female feeding behaviour	BTB zinc-finger transcription factor	BLAST analyses of genone/transcriptome databases	*fru* sex-specific transcripts in the brain; Cas9 gene editing caused reduced female-specific feeding behaviour rather than affecting male-specific mating.	Salvemini et al. (2013), Basrur et al. (2020).
Bombyx mori (Lepidoptera)	*Bmfru*	function in male mating	BTB zinc-finger transcription factor	BLAST analyses of genone/transcriptome databases	Cas9 gene editing caused normal male courtship behaviour but no mating.	Xu et al. (2020).

alternative splicing (Hedley and Maniatis, 1991; Inoue et al., 1992; Tian and Maniatis, 1992, 1993, 1994; Hertel et al., 1996; Lynch and Maniatis, 1995, 1996; Hertel and Maniatis, 1998). In XY individuals, in the absence of the TRA protein, *dsx* followed a male-specific default splicing pattern, with the male-specific exons being "stronger" and, hence, preferred by the spliceosome machinery. Tra2 seems more capable than TRA of binding with high specificity to the *dsx* repeat sequence in the absence of other factors (Hedley and Maniatis, 1991); not surprisingly, this interaction requires the Tra2 RRM (Tian and Maniatis, 1993). This specific binding to the repeat element is likely to involve interactions among Tra, Tra2, and other SR proteins, such as Rbp1 (Lynch and Maniatis, 1996). Erdman and Burtis (1993) showed that Dsx isoforms share a novel zinc finger-related DNA binding domain in their common region. The DSXF isoform activates the transcription of yolk genes (*vitellogenin* genes, *vg*) in the fat bodies and ovarian follicle cells by binding a specific enhancer (Burtis et al., 1991). Many other potential gene targets of DsxF and DsxM were identified at a genome-wide level via DamID chromatin profiling (Luo et al., 2011), analyses of *in vivo* occupancy, binding site prediction, and evolutionary conservation (Clough et al., 2014). However, we still have limited knowledge of how the sexual differentiation of *Drosophila* is carried out by the genetic network downstream of the regulatory cascade during development, including the underinvestigated metamorphosis (Lebo et al., 2009; Robinett et al., 2010). A *dsx*-controlled male-specific trait of *Drosophila melanogaster* has been the subject of intensive and fruitful evolutionary studies (Kopp et al., 2000; Williams et al., 2008). In females, *dsxF* activates *bric-a-brac* gene (*bab*), which represses male-specific pigmentation of A5 and A6 segments (Kopp et al., 2000). In the posterior abdomen of males, the repression of *bab* is exerted by *dsxM*, and it requires the integration between the homeotic (*abdominal B* and *abdominal A*) and sexual differentiation regulatory inputs (*dsx*). Comparative genetic studies between 15 *Drosophila* species, also analysing the presence/absence of male pigmentation, led to a model of the evolution of a *dsx*-controlled genetic circuit under sexual selection involving *bab* and homeotic genes (Kopp et al., 2000).

In the first half of the last century, diploid intersexes were described in *D. simulans, D. virilis* and *D. pseudobscura* carrying mutations in single genes (Sturtevant, 1920; Lebedeff, 1934, 1939; Dobzhansky and Spassky, 1941). In *D. melanogaster*, XX intersexes can also be caused by spontaneous mutations in the *intersex* (*ix*) gene which is required for female, but not male, somatic sexual development (Baker and Ridge, 1980; Chase and Baker, 1995).

Genetic epistasis studies indicated that *ix* acts parallel to or downstream of *dsx* in the sex determination hierarchy (Baker and Ridge, 1980). The *ix* gene was localized to the cytological region 47E-47F11- 18 using complementation tests with deficiencies and loss-of-function alleles (Chase and Baker, 1995). Garrett-Engele et al. (2002; Baker lab, USA) cloned the gene using the RFLP mapping, genomic phages (covering a 65 Kb region), cDNA phages corresponding to different genes present in the region, and performed *P*-element-mediated germline transformation experiments. The *ix* gene produces only one splice form in all tissues of both sexes, is expressed at higher levels in females and encodes a protein similar to DNA-binding transcription factors. Ix physically interacts with DsxF to promote female-specific expression of yolk genes and cooperates for the female-specific differentiation of sexually dimorphic cuticular traits.

5. Identification and isolation of *fruitless*

In *Drosophila*, the development of male sexual behaviour and sexual orientation requires a male-specific isoform produced from the *fruitless* gene by sex-specific alternative splicing, which is also controlled by Tra/ Tra2 (Gailey et al., 1991; Demir and Dickson, 2005). The molecular cloning of the *fruitless* gene was achieved by Ito et al. (1996), using *P* transposon tagging and screening based on behavioural tests, and by Ryner et al. (1996), using an ingenious novel molecular approach. The hypothesis of Ryner et al. (1996) was that similar to *dsx*, other *tra/tra2* regulated genes exist and should contain various copies of the splicing regulatory element. They used the short 13 nt Tra/Tra2 binding element as a radioactive probe to screen a *Drosophila* genomic library. The authors identified a second genomic locus corresponding to the *fruitless* gene, in addition to the *dsx* locus itself. The *fruitless* gene encodes a BTB zinc-finger transcription factor regulating a series of developmental genes involved in the control of cell identity and neuronal connectivity as well as the functional properties of neurons in adulthood (Hall, 1994; Basrur et al., 2020). Male flies with a mutant form of *fruitless* court other males. The artificial expression of the male-specific *fruitless* isoform in females triggers male singing behaviour and their sexual orientation toward other females (Demir and Dickson, 2005). In females, Tra and Tra2 promote the female-specific splicing of *fru* primary transcripts derived from an alternative P1 promoter, preventing Fru protein translation (Ryner et al., 1996; Heinrichs et al., 1998; Usui-Aoki et al., 2000). In males, due to the absence of Tra, default splicing produces male *fru* transcripts (*fruM*), which are translated into male Fru protein isoforms (collectively referred to as FruM) (Billeter et al., 2006; Salvemini et al., 2010; Yamamoto and Koganezawa, 2013). Cachero et al. (2010) found that *fru* likely controls sex-specific anatomical differences in the *Drosophila* brains, including neuronal projections. The authors used the

Table 2
List of master and novel intermediate sex determining genes in insects.

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
Bombyx mori (Lepidoptera)	BmPSI	Regulation of Male-specific splicing of Bmdsx	Splicing factor and transcriptional regulator	male- and female-specific cell lines of *B. mori*		Suzuki et al. (2008).
Bombyx mori (Lepidoptera)	*Masculinizer (Masc)*	regulation of male-specific *Bmdsx* splicing	CCCH-type zinc finger domains (ZFs)	Differential expression analysis of sexed embryos	The two CCCH-type zinc finger domains in the Masc protein are dispensable for masculinization;	Kiuchi et al. (2014).
Bombyx mori (Lepidoptera)	*BxRBP1 and BxRBP3*	recognize sex-specific *Bmdsx* exons	RNA-binding proteins	Yeast three-hybrid screening; RNA affinity chromatography and UV cross-linking in cell nuclear extracts		Zheng et al. (2019).
Nilaparvata lugens (Hemiptera)	*Nlfmd*	Female development, female-specific dsx splicing, female-specific embryonic vital function	RNA-binding protein	BLAST of embryonic transcriptome and RNAi functional screening		Zhuo et al. (2021).
Apis mellifera (Hymenoptera)	*Complimentary sex determining locus (csd)*	Possibly involved in splicing regulation of feminizer (*Apis Cctra* orthologue)	Possibly RNA-binding protein	Positional cloning	*Csd* locus highly polymorphic, Haploids (one *csd* copy) develop as males, diploids (two different *csd* alleles) develop as females; rarely diploids (two copies of the same *csd* allele) develop as larger males.	Beye et al. (2003).
Bombyx mori (Lepidoptera)	*Feminizer* (*Fem*)	piRNA targeting *Masc* mRNAs		RNA-seq of male and female embryos, differentially expressed transcripts.		
Anopheles stephensi (Diptera)	*Gene Unique to the Y (GUY1*	male-specific function in controlling dosage compensation; not yet shown that GUY1 controls *dsx* splicing	Unknown, novel short protein of 56 aa	Chromosome quotient analysis (male and female genomic sequencing, quantitative comparison)	GUY1 transgene on autosomes induces female-specific lethality (impaired dosage compensation); transgenic males are more competitive.	Criscione et al. (2013), idem (2016), Qi et al. (2019)
Aedes aegypti (Diptera)	*Nix*	Distantly related to *Drosophila transformer-2,*	RNA-binding protein	Chromosome quotient analysis (male and female genomic sequencing, quantitative comparison)	*Nix* knockout by (CRISPR)–Cas9 resulted in largely feminized genetic males, showing *dsx* and *fru* female-specific mRNAs.	Hall et al. (2015).
Anopheles gambiae (Diptera)	*Yob*	vital for male survival, male dosage compensatio; male-specific Agdsx splicing	Unknown, novel short protein of 56 aa	Transcriptomics of sexed embryos, mapping on a *Y*-specific scaffold (Krzywinski et al., 2004) differential expression analysis,	*Yob* is of the same length and similar secondary structure, but nonconserved primary sequence with the respect of Aedes GUY1.	Krzywinska et al. (2016).
Aedes albopictus (Diptera)	*Aal-Nix*	*Nix* orthologue	RNA-binding protein	BLAST of genome/ transcriptome databases	CRISPR/Cas9- mediated knockouts of *AalNix in vivo* and in the *Ae. albopictus* cell line lead to a shift of *dsx* and *fru* splicing towards the female isoforms.	Gomulski et al. (2018), Liu et al. (2020b).
Musca domestica (Diptera)	*Musca domestica* male determiner (*Mdmd*)	*Mdmd* represses the *Mdtra* female-specific splicing	paralogue of nucampholin, a spliceosome-associated protein	RNA-seq differential expression analyses of male and female embryos	*Mdmd* Cas9-targeting caused a complete sex reversal of genotypic males (*M/m*) into fertile females (in a *Musca* strain with homomorphic sex chromosomes)	Sharma et al. (2017).
Ceratitis capitata (Diptera)	*Maleness-on-the-Y (/MoY)*	*MoY* represses directly or indirectly the *Cctra* female-specific splicing	Novel short protein of 70 aaa	differential expression analyses of embryonic mRNAs (partially sexed embryos; XX versus XX/XY) combined with the CQ approach and PacBio genomic sequencing	*MoY* is necessary and sufficient for male sex determination in the Medfly; functional analyses by embryonic injections of dsRNA, genomic MoY DNA recombinant MOY protein, and Cas9-MoY-sgRNA.	Meccariello et al. (2019).
Bactrocera oleae (Diptera)	*BoMoY*	Bo*MoY* represses directly or indirectly the Bo*tra* female-specific splicing	Orthologue of Medfly *MoY*	tBLASTn analyses of transcriptomes/genomes	*BoMoY* is necessary for male sex determination in the olive fly; embryonic RNAi.	Meccariello et al. (2019).
Bactrocera dorsalis (Diptera)	*BdMoY*	Bd*MoY* represses directly or indirectly the Bd*tra* female-specific splicing	Orthologue of Medfly *MoY*	tBLASTn analyses of transcriptomes/genomes	*BdMoY* is necessary for male sex determination in the oriental fly; embryonic RNAi.	Meccariello et al. (2019).
Zeucodacus cucurbitae, Bactrocera jarvisi, B. latifrons, B.	*ZcMoY, BjMoY, BlMoY, BtMoY, BcMoY, BzMoY*		Orthologues of Medfly *MoY*	tBLASTn analyses of transcriptomes/genomes	*MoY* is Y-linked in *B. jarvisi* and *B. troni,* and likely also in the other species.	Meccariello et al. (2019).

(*continued on next page*)

Table 2 (*continued*)

Species name	Gene name	Gene function	Protein function	Method of discovery	Comments	References
tryoni,B. correcta, B. zonata.						
Bactrocera dorsalis	*miRNA-1-3p*	Autosomal gene, male-biased, repressor of *Bdtra* autoregulation, during embryogenesis,	Non coding microRNA	differential analysis of small RNA libraries in embryos before and after male sex determination	The male-bias of miRNA-1-3p could be due to the activities of the male determining *BdMoY* gene. Functional analyses by CRISPR/Cas9 and by agomiR, antagomiR, and CRISPR/Cas9 have shown that *miRNA-1-3p* over expression fully masculinises XX individuals, while its repression fully feminizes XY.	Peng et al. (2020).
Nasonia vitripennis (Hymenoptera)	*wasp overruler of masculinization (wom)*	Transcribed only from the paternal allele in diploid embryos; won activates *Nvtra* in diploid emrbyos inducing female sex determination.	DNA-binding protein of the P53 family; transcriptional ctivator of Nvtra	mRNA-seq-based differential expression analyses of haploid and diploid embryos at 2 and 5 h after egg laying,	parental RNA interference (pRNAi) of *wom*, induces diploid eggs to develop as fertile males (instead of females), which expressed male-specific *Nvtra* mRNAs.	Zou et al. (2020).

analogy of the jumper switches in an electronic circuit to produce differences in a broadly conserved network. Several putative target genes of FruM (some involved in neuronal projection morphogenesis or encoding key cell surface molecules) have been identified using a variety of approaches. However, we are still far from understanding the basis of courtship in the brain of the male fly (Nojima et al., 2014; Sato and Yamamoto, 2020).

Pan and Baker (2014) have uncovered some other exciting aspects of the FruM functions in building up innate sexual behaviour and the possibility for the male fly lacking this gene function to learn from male peers through social experience (Peng et al., 2021). The most straightforward idea to explain this phenomenon proposes the transformation of a time-consuming learned courtship to a robust innate behaviour, presumably by building up a similar neuronal network during the development that encodes this complex phenotype (Pan and Baker, 2014; Peng et al., 2021). Is social learning or is FruM-genetically induced sexual behaviour evolutionarily conserved in other species? Furthermore, how ancient are they?

There are multiple *fruM* transcripts encoding potentially five male-specific FruM isoforms different in their C-terminus (FruAM-FruEM). FruBM isoform recruits histone deacetylases and heterochromatin binding proteins to ~130 target sites on *Drosophila* polytene chromosomes (Sato and Yamamoto, 2020). This observation revealed the complexity of FruBM modulation of a "genetic orchestra", which ultimately leads to the formation of sexually dimorphic neural structures. For example, one well-established FruBM transcriptional target is the axon guidance protein gene *robo1* (Sato and Yamamoto, 2020).

Concerning gross brain differences between *Drosophila* sexes, a male-specific neuronal cluster (P1) coexpresses *fru* and *doublesex* and is the potential trigger for male-type courtship behaviour (Yamamoto, 2008).

6. Identification of *Sex-lethal* (*Sxl*) as the master switch for *Drosophila* sex determination and dosage compensation

The identification of different classes of mutations in the same gene, *Sex-lethal* (*Sxl*), causing either female-specific (loss of function) or male-specific (gain of function) lethality as well as sexual transformation when lethality is overcome (Muller and Zimmering, 1960; Cline, 1978), led to the discovery of a link between sex determination and dosage compensation (Lucchesi and Skripsky, 1981; Cline, 1984). Indeed, counting X chromosome numbers is required in *Drosophila* for establishing sex determination and, later on, for equalizing levels of X-linked encoded gene products, despite a 2X or 1X composition in female and male cells, respectively. Once the dosage compensation is active in XY larvae hyper-transcribing X-linked genes, the *X:A* ratio is equalized in XY and XX. Sánchez and Nöthiger (1983) proposed that the *X:A* signal irreversibly sets the ON/OFF activity status of *Sxl* in the blastoderm stage, which epigenetically "memorizes" this developmental choice (Sánchez and Nöthiger, 1983; Bachiller and Sánchez, 1991). Their results with X0 clones in XX individuals were compatible with the idea that *Sxl* is the only gene that responds to the *X:A* signal, which was later confirmed.

Bruce Baker carried out epistasis analyses with existing sex determination mutations to establish the order of gene activity (Andrew et al., 2019). Baker et al. (1987) and Baker and Wolfner (1988) proposed a hierarchical scheme of *Drosophila* sex determination in which the primary signal, the *X:A* ratio, activates the *Sxl* gene only in XX individuals beginning in embryogenesis, which in turn activates *transformer* and, thus female-specific *dsx* functions. *Sxl* and *tra* remain inactive in XY individuals, allowing *dsx* to express its default male-specific functions (Fig. 1). Unlike mammalian sex determination, in which diffusible

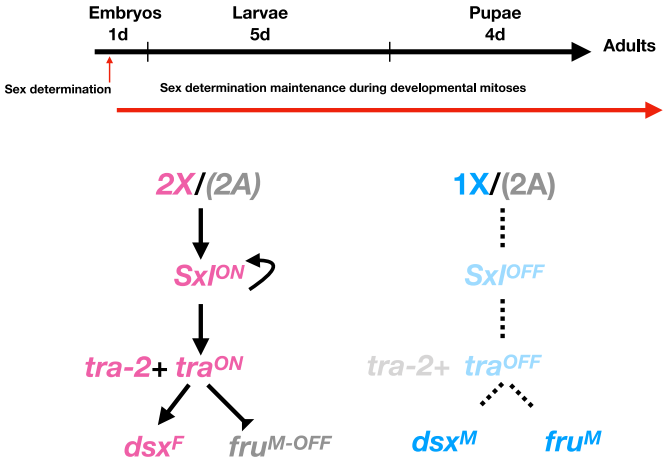

Fig. 1. *Drosophila* sex determination genetic regulatory cascade. *Drosophila* female sex determination (top) is established at 2–4h after egg laying, and the regulatory genes are continuously active (red arrow) during all development and adulthood. The primary signal is shown as 2X or 1X if the *XSE* model is correct, and, in parentheses, the contribution of the autosomes if the *X:A* ratio model is correct. The 2X/(2A) primary signal activates the master gene *Sxl*, which remains active by setting up its auto-regulatory function. In turn, *Sxl* activates *tra,* which promotes the expression of DsxF isoform with the assistance of the auxiliary *tra2* function. DsxF induces female differentiation and represses male differentiation and expression of *fru*M isoform. The 1X/(2A) primary signal is insufficient to activate *Sxl*. In the absence of its function, *tra* is switched off, and by default, *dsx* and fru express male-specific isoforms promoting sexual differentiation and brain development promoting male courtship behaviour.

substances such as hormones play critical roles, *Sxl*, *tra* and *dsx* act cell autonomously, and nearby somatic clones. Neat borders can show the opposite sex, indicating no waning of the effect on the sex choice of the cells (Morgan, 1916; Cline, 1979; Baker and Ridge, 1980; Sanchez and Nothiger, 1982; Wieschaus and Nothiger, 1982). Nagoshi et al. (1988) molecularly confirmed that in XX individuals, *Sxl* controls the female-specific splicing of *tra* and that *tra* together with *tra2* promotes the female-specific splicing of *dsx*. In contrast, *Sxl* and *tra* are switched OFF in XY individuals, and *dsx* follows a male-specific splicing pattern; all three events occur by default (Fig. 1).

Tom Cline - Princeton University and the University of California-Berkeley, USA - performed classical genetics studies on *Sex-lethal* and described in a seminal article the "Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state" (Cline, 1984). His studies attracted considerable curiosity about the intriguing genetic mechanism by which *Sxl* could respond to the two X chromosome doses, establishing and maintaining female sex determination via a self-propagating response (Cline, 2005). The establishment and maintenance of *Sxl* in a female activity state early in embryogenesis depends on the positive feedback function of *Sxl* products promoting female-specific *Sxl* splicing and maintaining female sex determination during development and fly adult life (Cline, 1984; Keyes et al., 1992; Salz and Erickson, 2010).

Previous cytogenetic studies placed *Sxl* in a specific cytogenetic interval (8F5–7Al) of the *Drosophila melanogaster* X chromosome (Nicklas and Cline, 1983). The serendipitous isolation of a female-specific lethal mutation among the progeny of a cross between male wild-type flies and females from a laboratory stock led to the hypothesis that the mutation was a mutant *Sxl* allele caused by *P* element transposon insertion. A dysgenic cross mobilizing the *P* transposon inherited from the father likely generated this *Sxl* insertion (Maine et al., 1985). A radioactive *P* element probe hybridized to polytene chromosomes of the mutant stock at two closely linked sites (6F and 7A) consistent with the known location of *Sxl*. The phage genomic library obtained with DNA from this mutant *Sxl* strain was screened with the *P* element probe, and the flanking regions of the insertion were found to contain the *Sxl* sequences. Salz et al. (1989) from the Paul Schedl laboratory (Princeton University, USA) performed extensive Northern blotting analyses of *Sxl,* discovering transcripts of different lengths in the two sexes, similar to *transformer* gene expression patterns.

Bell et al. (1988; Schedl laboratory) isolated and sequenced clones from cDNA libraries from sexed adult flies and revealed that the *Sxl* female-specific transcripts are generated by exon-skipping, and encode an RNA binding-type protein. Longer male-specific clones contained an additional exon introducing a premature stop codon in the *Sxl* open reading frame. Bell et al. (1988) found that SXL was similar to other known RNA-binding ribonucleoproteins. The SXL protein contains two copies of the RRM that is widely conserved in plant and animal species (Shepard and Hertel, 2009). Furthermore, these authors proposed that the alternative sex-specific splicing of *Sxl* pre-mRNA was the basis for both the binary ON/OFF state of *Sxl* in the two sexes. They suggested that the splicing-based positive autoregulatory function of *Sxl* is required to maintain female sex determination from embryonic stages until adulthood (Bell et al., 1991).

Sakamoto et al. (1992) performed cell line transfection experiments showing that the SXL protein is required to repress default male-specific *Sxl* splicing. This model was confirmed by Horabin and Schedl (1993) using transgenic flies and mutant DNA *Sxl* constructs. Furthermore, Sxl antibodies (abs) confirmed the female-specific expression of the full length protein from very early stages of embryogenesis before cellularization and showed that maternal *Sxl* mRNAs are not translated (Bopp et al., 1991, 1993). It is still unclear why untranslated maternal *Sxl* mRNAs exist in the eggs. Furthermore, the cross-reactivity of the antibodies with orthologous SXL proteins in other *Drosophila* species revealed sex-specific expression and led to the proposal that *Sxl* is a conserved master gene for sex determination (Bopp et al., 1996). Bopp et al. (1996) also mentioned preliminary data showing a lack of sex-specific SXL expression in a distantly-related dipteran species, *Musca domestica*, confirmed two years later by Meise et al. (1998). *In vitro* experiments in human HELA cells transfected with *Drosophila* genetic constructs allowed Valcárcel et al. (1993) to show that SXL antagonizes the constitutive splicing protein U2AF to promote the female-specific splicing of *transformer*. Sosnowski et al. (1989) showed that female-specific *tra* splicing regulation (alternative 3' splice site) is based on a splice site blockage mechanism exerted by Sxl protein. Lallena et al. (2002) showed in *Drosophila* cell lines that Sxl inhibits the splicing of Spf45-dependent substrates, such as the *Sxl* pre-mRNA itself, by interacting with this splicing factor.

7. The primary signal for *Drosophila* sex determination: *X:A-XSE*

The primary signal for *Drosophila* sex determination was first described as the X chromosome-to-autosome ratio (*X:A*) (Bridges, 1916, 1921). Two X-linked genetic elements that appear to be important components of this *X:A* signal are *sisterless-a* (*sis-a*) (Cline, 1986) and a region of the *achaete-scute* complex (AS-C) known as *sisterless-b* (*sis-b*) (Cline, 1988), which corresponds to the *scute* (*sc*) gene (Belote et al., 1985a; Torres and Sánchez, 1989; Torres and Sánchez, 1991). The genetic manipulation of these genes generates sex-specific lethal phenotypes due to misregulation of *Sxl* (Cline, 1988; Torres and Sánchez, 1989), indicating that *sis-a* and *sis-b* are strong numerator elements in the *X:A* ratio. This primary signal acts in early XX embryos to activate the master gene *Sex-lethal* (*Sxl*), also using a maternally-provided gene product from the *daughterless* gene (*da*) (Cline, 1978). Parkhurst et al. (1990) serendipitously discovered, while investigating *Drosophila* embryonic morphogenesis, that overexpressing the *hairy (h)* pair-rule segmentation gene from a transgene resulted in female-specific lethality. The authors reasoned that such sex-specific lethality resembled loss-of-function mutations in the *Sxl* gene, impairing female-specific dosage compensation. Furthermore, they noted that *hairy* encodes a Helix-Loop-Helix transcription factor and acts as a negative regulator of *achaete (AS-C T5)* during bristle patterning, where *achaete* also encodes an HLH protein. Additionally, the maternally provided Daughterless (Da) protein encodes an HLH protein, as *sis-b* (Villares and Cabrera, 1987). When overexpressed, the H protein can antagonize other HLH proteins needed for *Sxl* transcription (e.g., Da; Caudy et al., 1988), or it can prevent the action of zygotic chromosome-counting elements (*sis-a, sis-b*). Parkhurst et al. (1990) finally demonstrated that *achaete (AS-C T5)* corresponds to *sis-b* and that *hairy* overexpression prevents normal *sis-b* function in the activation of *Sxl* in XX embryos. They also proposed a mechanistic model of the *X:A* ratio in which a threshold content of Da/Sis-b heterodimers is responsible for *Sxl* activation from an early female-specific promoter (*SxlPe*). Erickson and Cline (1993) cloned *sis-a* by *P* element tagging, revealing that this gene encodes a bZIP DNA-binding transcriptional regulator, consistent with the proposed model. Hence, *Drosophila* sex determination is established zygotically during the first 2–4 h of embryogenesis by either or not transcriptionally activating the master gene *Sxl* in XX embryos and XY embryos, respectively. The XX embryos (2 h old) use the early SXL female-specific protein, produced after *SxlPe activation,* to initiate the female-specific splicing and hence autoregulation of *Sxl*. Two hours later (in 4 h old embryos) a promoter switch and a non-sex-specific transcription of *Sxl* starts from the *SxlP-late*. XX embryos maintain *Sxl* positive autoregulation. In XY embryos, *Sxl* is spliced in the male-specific mode in the absence of early Sxl protein and they follow a male genetic sex determination program by default. In this conventional titration model, the embryo reads the value of the *X:A* ratio by measuring the dose of X-linked numerator gene products with the respect to autosomal denominator proteins (such as *deadpan, dpn*) to establish the appropriate ON or OFF activity state of the master sex-determining gene *Sxl* (Cline, 1993; Louis et al., 2003; Parkhurst et al., 1990; Parkhurst and Ish-Horowicz, 1992; Younger-Shepherd

et al., 1992). The male or female dose of X chromosome is defined by the collective concentration of proteins produced by the X-linked counting elements (HLH and ZIP classes of transcription factors; Cline, 1993). Proper assessment XSE concentrations by *SxlPe* depends on numerous protein cofactors present in equal amounts in XY and XX embryos. These cofactors include the zygotically expressed autosomal *dpn*, but various maternally supplied proteins are thought to play the predominant quantitative roles in defining the effective *X:A* ratio (Younger-Shepherd et al., 1992). An alternative model proposes that the primary sex determination signal is mainly an X chromosome-counting process (X-linked signalling elements, *XSE*), rather than an *X:A* signal, minimizing the role of the masculinizing autosomal contribution Erickson and Quintero (2007) and the influence of dosage compensation system controlled by the Male-Specific Lethal protein complex (MSLs, Erickson, 2016). Mahadeveraju et al. (2020) discovered that early zygotic expression of the segmentation *runt* gene, (an autosomal gene known to be required for *Sxl* activation in some regions of the embryos; Torres and Sánchez, 1992) seems to help amplifying the difference between female and male *XSE* signals During early embryogenesis, Runt antagonizes the Groucho protein, a known maternal repressor of *SxlPe*. However, the *X:A* ratio model is still alive because it still partially explains why haploid cells develop toward femaleness (monitored as early activation of *Sxl* from the *SxlPe*) and XX:AAA triploid animals develop as sexual mosaics, supporting the idea of a contribution from the autosomes (such as the autosomal gene *dnp*) in repressing *Sxl*, at least in some cells (Erickson an Quintero, 2007; Salz and Erickson, 2010).

Two critical questions are whether there is a continuing need for the function of the sex determination gene hierarchy throughout adulthood to ensure the sex-specific regulation of target genes and whether this regulation is reversible or epigenetically fixed during early development. The use of a *Drosophila melanogaster* temperature-sensitive allele of *tra2* allowed Belote et al. (1985b) to show that vitellogenin protein synthesis in adult females is reversible and continuously dependent on the expression of the DsxF isoform. Li and Handler (2017) replicated this mutation in *Drosophila suzukii* by introducing the same temperature-sensitive amino acid mutation in the *transformer-2* orthologue via gene editing and found that temperature shifts in adulthood also affect the *vg* expression, suggesting a continuous requirement of Tra2 (and indirectly of DsxF). Arthur et al. (1998) used a heat shock-controlled transgene expressing TRA (normally female-specific) in *Drosophila* transgenic flies at various developmental times (in XX mutant for *tra* and in XY). They found that, in contrast, innate sexual behaviour is irreversibly programmed during the critical period of puparium formation due to the activity or inactivity of a single regulatory gene.

8. Molecular isolation of *Sxl* orthologues

Low-stringency hybridization of genomic libraries and the cross-reactivity of Sxl antibodies with orthologous proteins led to the discovery that the sex-specific regulation of *Sex-lethal* and hence its function in sex determination are conserved in the genus *Drosophila* (Bopp et al., 1996; Penalva et al., 1996; Jinks et al., 2003).

Whether *Sxl* and other regulatory genes of the *Drosophila* sex determination cascade are partially conserved in more distantly dipteran species attracted the interest of several laboratories, mainly based in Europe (Nöthiger and Steinmann-Zwicky, 1985). Rolf Nöthiger (Zurich University), and a few other European colleagues, founded a group of scientists (named as the Nothiger group, which is still very active) investigating the evolution of sex determination and organized regular meetings to exchange unpublished data and ideas (Nöthiger and Steinmann-Zwicky, 1985).

A joint FAO-IAEA program was established more than three decades ago to regularly organize meetings to promote molecular genetics research of sex determination and transgenesis in *Ceratitis capitata*, and related species in the Tephritidae family, which are major agricultural pests. The key aim of the program was to improve the sterile insect technique and widen its applicability (Ashburner, 1995; Franz and Robinson, 2011; Handler and O'Brochta, 1991; Horn and Wimmer, 2000; O'Brochta and Atkinson, 2004; Robinson, 2002; Vreysen et al., 2021; Wimmer, 2003). For fruitfly species damaging fruit crops, genetic research and the application of gene transfer techniques developed in *Drosophila* have been inspired by potential biotechnological applications (Louis et al., 1988; O'Brochta and Handler, 1988).

Various preliminary attempts to clone *Drosophila Sxl*-related sequences in *C. capitata* by low-stringency hybridization in genomic libraries led to false positives, likely because of repetitive sequences included in the *Sxl* probe (Sidén-Kiamos et al., 1993; Saccone, G. and Polito, LC, unpub. res.). Using a shorter *Drosophila Sxl* probe containing two RNA-binding Motifs whose sequences were likely more conserved led Müller-Holtkamp (1995) to isolate the first non-Drosophilidae *Sxl* orthologue (*CrSxl*) in the blowfly *Chrysomya rufifacies*. In this species, the primary sex determination signals for maleness or femaleness are based on the maternal genotype and remain to be molecularly clarified. Compared to *Drosophila Sxl, CrSxl* showed 82% nucleotide and 88% amino acid identities in the most conserved region. However, RT–PCR and cDNA sequence analyses showed that *CrSxl* mRNAs with an identical structure were present in adult flies of both sexes, suggesting that the *Sxl* orthologue could not be involved in sex determination in this species. Other studies led to cloning *Sxl* orthologues by PCR and degenerate primers approach. Sievert et al. (1997, 2000) cloned *Sxl*-orthologous sequences (*MsSxl*) in the phorid fly *Megaselia scalaris*.

Another cloning approach was based on low-stringency hybridization of *Drosophila Sxl* probes to screen genomic and cDNA libraries. Saccone et al. (1998) and Meise et al. (1998) isolated *Sxl* cDNA orthologous sequences (as cDNA libraries are less complex than genomic ones, this strategy further reduced the risk of false positives) in the Medfly and in the housefly *Musca domestica*. These authors showed that 1) the cDNAs of the *Sxl*-orthologous sequences were identical in the two sexes and, as observed for *CrSxl*; 2) a *Drosophila* antibody recognized the SXL-orthologous proteins; 3) the Cc and MdSxl proteins were highly conserved but expressed embryos and adults of both sexes, in contrast to *Drosophila*, where *Sxl* is expressed only in XX individuals from early developmental stages; and 4) forced *CcSxl* or *MdSxl* overexpression from a transgene in *D. melanogaster* flies ultimately resulted in lethality in both XY and XX animals but not in a sexual transformation of XY individuals into females (modifying *tra* splicing as DmSxl does) or in a male-specific lethality (by repressing dosage compensation system as DmSxl usually does in XX individuals) (Bashaw and Baker, 1995; McDowell et al., 1996). Serna et al. (2004) isolated *Sxl* orthologues of species belonging to Sciaridae. All studies concluded that the *Sxl* orthologues are not master regulators of sex determination in *Ceratitis capitata*, *Musca domestica* or Sciaridae species.

Niimi et al. (2006) isolated *Sxl* orthologous cDNAs from embryonic stages in the phylogenetically distant lepidopteran *Bombyx mori*. Lagos et al. (2005) isolated an *Sxl* orthologue in the olive fly *B. oleae*, followed by genomic and cDNA library screening. In all of these species, *Sxl* orthologues lacked sex-specific splicing. This lack of sex-specific regulation of *Sxl* orthologues was subsequently described in many other insect species outside the Drosophilidae family, strongly suggesting a parallel lack of a conserved master function in sex determination (Cline et al., 2010; Zhang et al., 2014).

Ruiz et al. (2013) investigated the potential differences between *Drosophila* SXL proteins performing sex-specific functions and those orthologues lacking them. They performed biochemical studies with recombinant chimeric SXL proteins from *D. melanogaster* and *Sciara*. They concluded that the sex-specific properties of the extant *Drosophila* Sxl protein depend on its global structure rather than on a specific domain.

Recently, Sakai et al. (2019) induced in *B. mori Sxl* loss-of-function mutations by gene targeting, showing that it plays important roles in spermatogenesis in this species. This is an interesting observation in

light of the essential additional function that *Sxl* has in the *Drosophila* female germline, suggesting its more ancestral function in cells dedicated to gametogenesis. During the evolution of *Drosophila* ancestor species, *Sxl* likely acquired a second novel developmental function in female sex determination through the expansion of the sex-specific regulatory network, alteration of the protein sequence and likely feedback from the downstream-regulated *transformer* gene (Bopp, 2010; Cline et al., 2010; Siera and Cline, 2008; Zhang et al., 2014).

Based on the available data, we can conclude that *Sxl* orthologues of non-Drosophilidae species do not show sex-specific expression, nor control *tra* female-specific splicing (in those species in which *tra* is conserved), and hence do not play a key role in sex determination (Traut et al., 2006). CRISPR/Cas9 will help answer the question concerning which functions *Sxl* exerts in all these species and if they are conserved. We will see in the next paragraph that during the evolution of insects, in certain species or taxa in the Diptera, Hymenoptera and Coleoptera, upstream regulators other than *Sxl* have been recruited to control *tra*. Similarly, upstream regulators other than *tra* have been recruited to control *dsx* sex-specific splicing regulation (e.g. in mosquitoes and *Bombyx mori*).

9. Molecular isolation of *transformer* orthologues

The first orthologues of *Drosophila melanogaster transformer* were isolated by low-stringency hybridization in genomic libraries of four *Drosophila* species sharing a 60 million-year-old common ancestor (O'Neil and Belote, 1992). This study revealed an unusually high level of *tra* sequence divergence (31–36% amino acid identity). It showed that *D. virilis tra* (*Dvtra*) was sex-specifically regulated and that a *Dvtra* transgene was capable of partially rescuing a *D. melanogaster tra* mutation. The rapid nonsynonymous evolution of the *tra* gene gives rise to the puzzling notion that an important developmental gene shows very low functional constraint in its sequence (McAllister and McVean, 2000). O'Neil and Belote (1992) suggested that the identification of these DNA sequences conserved in Drosophilidae *tra* orthologues could have aided in approaching *tra* cloning in more distantly related species by PCR and designing degenerate primers, but this was not the case. This challenge was confirmed by the isolation of *tra* false positive in the Medfly and solved by Pane et al. (2002; Polito Lab, Naples, Italy), which used a syntenic approach. They took advantage of the tight conserved linkage of *tra* and *(3)*73Ah genes overlapping in their 3′UTRs (Boggs et al., 1987). This physical overlap of the two transcriptional units was conserved in *D. virilis* (O'Neil and Belote, 1992). The previous molecular characterization of the *Drosophila l(3)73Ah* gene had also revealed very high protein sequence conservation in vertebrates, suggesting its broad conservation in many other dipteran species (Irminger-Finger and Nöthiger, 1995). The *Drosophila l(3)73Ah* gene was used as a probe to screen a *C. capitata* cDNA library and isolate an highly-conserved orthologue (Pane et al., 2002). This cDNA clone was subsequently used to screen a *Ceratitis* genomic library, leading to the isolation of a region containing the *Ceratitis l(3)73Ah* gene. DNA sequencing of the downstream region of this gene led to the identification of a second transcriptional unit that showed sex-specific alternative splicing and potentially encoded a serine/arginine-rich protein (Pane et al., 2002). Despite the very low sequence similarity of the gene to TRA, the authors concluded that the *Ceratitis* gene corresponded to the *Drosophila tra* orthologue (*Cctra*) based on its linkage to Cc*l(3)*73Ah, sex-specific splicing, similar amino acid composition and a functional study. The two linked *Ceratitis* genes showed conservation of the overlap between their respective 3′UTRs, as observed in *Drosophila*. Medfly *tra* showed sex-specific alternative splicing by exon skipping (instead of the 3′ alternative splice sites observed in *Drosophila*; Fig. 2) (Pane et al., 2002). A longer open reading frame (429 aa, versus the 199 aa long DmTRA) was found within female cDNAs. In contrast, male cDNAs contained additional male-specific exons introducing premature stop codons, similar to *Drosophila tra* OFF regulation and encoding only truncated

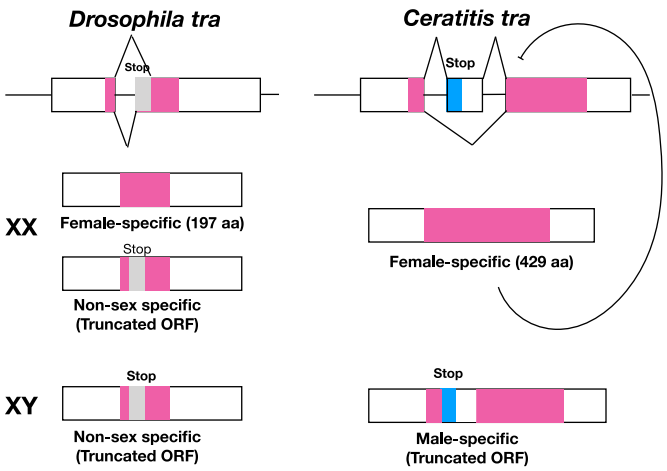

Fig. 2. *Drosophila* and *Ceratitis transformer* splicing regulation. In *Drosophila,* the *tra* pre-mRNA has two alternative 3′ acceptor sites. The use of the first 3′ acceptor site, which is non-sex-specific, leads to specific mRNAs having a stop codon in the ORF. Using the second 3′ acceptor site, which is female-specific, leads to a female-specific mRNA encoding a 197 aa long TRA protein. *Sxl* protein binds to *tra* pre-mRNA and represses the use of the first 3′ acceptor site promoting *tra* female-specific splicing. In XY individuals, by default, only the non-functional truncated Tra isoform is produced, leading to a *tra* OFF state. In *Ceratitis* XX females, *transformer* regulation is based on female-specific exon skipping. CcTra and CcTra2 promote this exon skipping by binding to cis-regulatory elements (Tra/Tra2 binding sites) in the male-specific exons and around them. The *CctraF* mRNAs encodes a 429 aa long CcTra protein required for female-specific *Cctra* splicing (Pane et al., 2002). In *Ceratitis* XY, *MoY* represses the establishment of female-specific *Cctra* autoregulation, leading to the inclusion of a stop containing male-specific exon in the final mRNAs, which encode truncated CcTra male-specific protein isoforms.

CcTra isoforms (Fig. 2). Furthermore, CcTra was rich in serine and arginine but lacked significant similarity to DmTRA or other known proteins. These observations suggested functional conservation of Tra female-specific function in Medfly. Pane et al. (2002) took advantage of the RNA interference technique to examine the functional conservation of Medfly *transformer* (Fire et al., 1998). Transient embryonic RNAi targeting *Cctra* was sufficient for the development of strongly male-biased progeny (85%), including intersex individuals (14%) and a few females (1%). The male progeny also included XX fertile individuals, confirming the absence of key fertility factors on the Y chromosome of Medfly (Willhoeft and Franz, 1996). These XX fertile males expressed, as expected, male-specific transcripts of the *Ceratitis doublesex* orthologue (*Ccdsx)* and, surprisingly, *Cctra*. How does the transient depletion of *Cctra* mRNA during embryogenesis permanently shift the *Cctra* splicing pattern in XX embryos? In one meeting organized in Naples (Italy) in 2000, a discussion (based on my recollection) about the preliminary *Cctra* data from Medfly with Daniel Bopp (Nothiger lab, Zurich), who was studying sex determination evolution in *D. melanogaster* and *Musca domestica*, led to the idea that RNAi targeting *Cctra* affects its regulation in XX males (explaining the presence of male-specific *Cctra* mRNAs) and that the gene was therefore able to autoregulate itself. There were two other examples of gene autoregulation for sex determination at the time: *Drosophila Sxl* (Bell et al., 1991) and the *Musca domestica F* factor, for which elegant classical genetics studies were previously performed (Dubendorfer and Hediger, 1998). Additional evidence supporting this hypothesis was provided by the presence of maternal *Cctra* mRNA (which may initiate the positive feedback loop in XX embryos), and especially by the almost serendipitous discovery of clusters of *Tra/Tra2* binding sites within the *Cctra* genomic region containing male-specific exons (Pane et al., 2002). The finding that *Ceratitis tra2* is also required for the female-specific splicing of *Cctra* pre-mRNA offered further support to the autoregulatory model (Salvemini et al., 2009).

Therefore, the transient depletion of either *Cctra* or *Cctra2* maternal and zygotic mRNAs in early embryos during the zygotic activation of gene expression affects only XX individuals, blocking the establishment of the female-specific *Cctra* autoregulatory loop. The RNAi-mediated reduction/absence of either *Cctra* or *Cctra*-2 mRNAs in early embryos leads *Cctra* to follow a default male-specific splicing pattern producing nonfunctional mRNAs (Pane et al., 2002). Hence *Cctra* is a master gene for female sex determination able to maintain the *Ceratitis* female sex choice in XX individuals or to stay OFF by default in XY males. Transient transcriptional repression of *Cctra* by embryonic dCas9 targeting, also induced a stable shift to male-specific splicing in XX individuals, which develop as fertile males (Primo et al., 2020). This autoregulatory mechanism is very similar to the one performed by the *Sxl* master gene in *Drosophila* (Pane et al., 2002). It was clear that during evolution, "masters can change, but slaves remain" (Graham et al., 2003).

Interestingly, a BLASTp analysis of CcTra (Blosum45) to search the *Drosophila* protein database failed to identify any similarity (Saccone et al., 2011). The Clustal alignment of CcTra/DmTra amino acid sequences identified only 35% identity over a short region of 56 aa and overall 18% identity. Despite this lack of sequence conservation, the ectopic expression of the CcTra protein in transgenic *Drosophila* flies partially rescued endogenous *tra* function, including the control of female-specific *dsx* and *fru* splicing, which was a very surprising finding (Pane et al., 2005). Similarly, the other Tephritidae *Anastrepha obliqua* Tra protein when ectopically expressed in *Drosophila* feminized flies (Ruiz et al., 2010). Ruiz et al. (2007) isolated Medfly *transformer* orthologues in various *Anastrepha* species (Tephritidae) by PCR, using a combination of *Cctra*-specific and degenerate primers and confirmed conservation of the sex-specific alternative splicing regulation.

The discovery of a novel and additional key autoregulatory function of *transformer* in the Medfly female sex determination revealed a striking analogy with the splicing autoregulation of the D. *melanogaster* master gene *Sex-lethal* (Pane et al., 2002; Graham et al., 2003). Various other researchers interested in developmental and evolutionary genetics of sex determination, especially in insect species of economic or medical relevance, started the search for *Cctra* orthologues.

Lagos et al. (2007) used the same syntenic approach adopted in Medfly to isolate *transformer* in another Tephritidae species, the olive fly *Bactrocera oleae* and found *Botra* to be a functional *Cctra* orthologue. As the *(3)73Ah* and *tra* orthologues are not tightly linked in *Lucilia cuprina* (Calliphoridae) or *Musca domestica* (Muscidae), the syntenic cloning approach was unsuccessful. Indeed, Concha and Scott (2009) isolated the Medfly *tra* orthologue in *L. cuprina* (*Lctra*) by PCR using the few conserved amino acid stretches among TRAs of Drosophilidae, *Ceratitis* and *Bactrocera* to design a list of degenerate primers. Embryonic *Lctra* RNAi led to the masculinization of XX individuals and male-specific splicing of both *Lctra* and *Lcdsx*, as in the Medfly. The presence of maternal *Lctra* mRNAs suggested that also, in this species, female-specific splicing could be initiated in XX embryos by maternal Tra protein.

Hediger et al. (2010) conceived a novel strategy based on the ideas that the *Musca domestica F* autoregulating gene corresponded to *Mdtra* (Burghardt et al., 2005; Dubendorfer and Hediger, 1998; Dubendorfer et al., 2002) and that it contained Tra/Tra2 binding sites, as the *Cctra* gene. The authors used the sequence of the *Tra/Tra2* cis-regulatory elements present in *Mddsx* to design partially degenerate primers. A *Musca* genomic clone that contained a cluster of nine Tra/Tra2 binding sites was isolated by PCR. The molecular characterization of this region revealed the identification of the *Musca* orthologue of the Medfly *tra* showing similar gene structure and female-specific alternative splicing by exon skipping (Hediger et al., 2010). The female-specific mRNA encodes a full-length MdTra protein, while the male-specific ones encode truncated MdTra peptides. Ectopic transient expression of MdTra in genotypic transgenic males was sufficient to start a positive feedback loop causing a strong male-to-female transformation (Hediger et al., 2010). *Mdtra* and *Mdtra2* embryonic RNAi induced phenotypic masculinization of genotypic females and male-specific splicing of both *Mdtra* and *Mddsx*, confirming the model of a positive *Mdtra* autoregulation in females. *Musca* flies homozygous for the partial loss-of-function allele of *F*, F^{man}, develop as males. The introduction of a MdTra transgene in this F^{man} mutant strain led to the complete rescue of the female phenotype, suggesting that *Mdtra* acts at the level of *F* or downstream (Hediger et al., 2010). The observation that in the F^{man} mutant strain, the *Mdtra* gene has small deletions removing various copies of the Tra/Tra2 binding sites, led to the conclusion that the *F* gene likely corresponds to *Mdtra* (Hediger et al., 2010).

The *Anastrepha tra*, *Lctra* and *Mdtra* genes showed conservation of all the structural, regulatory and functional key features present in the Medfly *tra* gene and absent in the *Drosophila* gene*:* 1) a conserved female-specific exon skipping splicing (rather than *Dmtra* 3' alternative splice acceptor sites), 2) Tra/Tra2 binding sites in the male-exon genomic region, 3) a lack of protein sequence similarity to DmTra (and a relatively high sequence similarity to CcTRA; approximately 50–57% identity and 60–70% similarity) and 5) a novel positive female-specific autoregulatory function. Embryonic *tra* RNAi in *B. oleae, L. cuprina* and *M. domestica* led to full masculinization of genotypically female individuals, showing male-specific *tra* mRNAs, as observed in the Medfly. These functional data confirmed the evolutionary conservation of the key epigenetic function of *Cctra* orthologues in maintaining female sex determination of other dipteran species (Saccone et al., 2011; Verhulst et al., 2010a).

In the honeybee *Apis mellifera*, haploid embryos develop as males and diploid ones as females. One of the females becomes a queen, depending on nutrition and cell size (Haydak, 1970). Because of the high polymorphism at the sex-determining locus in various bee populations, egg formation will likely result in heterozygous individuals which develop as females (Beye, 2004). Inbreeding leads to diploid males because of homozygosity at the sex-determining locus. Worker bees remove these males from the hive at the larval stage shortly after hatching. Beye et al. (2003) used a positional cloning strategy to clone the sex-determining region (see a next paragraph on primary sex-determining signals) and found the *complementary sex determiner* gene (*csd*) (Table 2). Flanking markers have been determined for a 360 kb long fragment by physical mapping (Beye et al., 1999) and used to obtain a fine-scale genetic mapping (Hasselmann et al., 2001; Appendix). Results further indicated that *csd* is not sex-specifically regulated but encodes a serine-arginine rich protein showing significant BLASTp similarity to a 30 aa long *Ceratitis* Tra region. Hasselmann et al. (2008) extended the cloning and sequencing of the sex-determining region of *Apis mellifera* and searched for additional genes potentially involved in sex determination. These authors found a *csd*-paralogue, *feminizer*, 12 kb upstream of the *csd* locus. Similarly, to the dipteran *tra* gene, female-specifically spliced mRNAs of *fem* encode a full-length Fem protein. In contrast, the male-specific ones contain a stop codon truncating the open reading frame. The Csd and Fem show 64% protein identity over a 420 amino acid long region and a conserved 30-amino acid motif shared with Medfly Tra (Beye et al., 2003; Gempe et al., 2009). The *fem* locus is the ancestrally conserved progenitor gene from which *csd* arose (Hasselmann et al., 2008). Differently from Medfly and *Musca*, no maternally provided *fem* transcripts have been detected, suggesting that a different *fem* activation mechanism operates in the diploid honeybee. Gempe et al. (2009) transiently expressed the Fem protein in male embryos by injecting Fem-encoding artificial mRNAs and observed a partial switch from male- into female-specific splicing pattern of the endogenous *fem* mRNAs. These data suggested that *fem* is sufficient to transactivate the endogenous *fem* gene. Furthermore, they supported the model of a positive autoregulation of *fem* based on female-specific splicing, in which the *Apis tra2* gene is also required. Embryonic RNAi (Hasselmann et al., 2008) or Cas9-targeting of *Apis fem* causes honeybees to profoundly switch sex toward maleness and to control small-size polyphenism of female workers (McAfee et al., 2019; Roth et al., 2019). Furthermore, these masculinized *fem* mutants (genotypically diploid

females) displayed the male *dsx* transcripts (Roth et al., 2019).

With the emergence of next-generation sequencing (NGS) technologies in the last two decades, identifying orthologous genes has become a generally quick and easy task, achieved by simply performing *in silico* analyses. The development of FASTA and BLAST bioinformatic tools to search for DNA and protein sequence similarity (Altschul et al., 1990; Lipman and Pearson, 1985) gave rise to new possibilities for investigating gene and protein functions.

Medfly *tra* orthologues have been found by sequence similarity, for example, in the coleopteran *Tribolium castaneum* and other Tephritidae major pests, such as *Anastrepha suspensa*, *Bactrocera jarvisi and Bactrocera dorsalis* (Diptera, Nematocera) (Schetelig et al., 2012; Shukla and Palli, 2012; Morrow et al., 2014; Liu et al., 2015; Peng et al., 2015). However, the use of *Drosophila tra* nucleotide or protein sequences for BLAST analyses fails to identify non-Drosophilidae orthologues (Saccone et al., 2011).

Schetelig et al. (2012) performed embryonic RNAi targeting of *tra* and *tra2* orthologues in *Anastrepha suspensa* and observed the production of XX pseudomales expressing male-specific *Astra* mRNAs, similar to the findings in Medfly. These data also supported the conservation of Medfly *tra* autoregulation in this Tephritidae species. The cloning of *tra* in Phlebotominae sand flies based on a BLASTp search failed because of the very low sequence similarity (Petrella et al., 2019). A second attempt required the approach based on Tra/Tra2 binding sites used for *Musca domestica tra* cloning. The authors performed an *in silico* BLASTn searches of Phlebotominae transcriptomic databases using the 13 nucleotide long Tra/Tra2 binding site consensus (Petrella et al., 2019). In all these species, *tra* showed a conserved sex-specific splicing regulation, strongly suggesting *tra* functional conservation.

Wexler et al. (2019) isolated *tra* orthologues in more distantly related hemimetabolous species *in silico*. Only one species (*Rhodius prolixus,* Hemiptera) showed the conservation of sex-specific *tra* splicing with a premature male-specific stop codon resulting in the truncation of the *tra* coding sequence, as observed in the holometabolous insect orders Diptera, Coleoptera, and Hymenoptera. The Blattodea *Blattella germanica Bgtra* gene produces identical full-length transcripts in both sexes (likely the same BgTra protein). However, RNAi targeting *Bgtra* affected *Bgdsx* female-specific splicing, as in other insects, indicating a *Bgtra* female-specific function despite the lack of sex-specific regulation.

The primary signal for haplo-diploid sex determination in the hymenopteran *Nasonia vitripennis* is based on the Maternal Effect Genomic Imprinting Sex Determination (MEGISD) model, which proposes a primary instructor gene is maternally silenced in unfertilized eggs (Beukeboom et al., 2007). In contrast, fertilized eggs receive a nonsilenced paternal allele of the instructor gene. Verhulst et al. (2010b) isolated in *N. vitripennis* a *transformer* orthologue (*Nvtra*) in a genome database. *Nvtra* is also sex-specifically spliced and likely able to autoregulate positively in diploid females. In fertilized diploid *Nasonia* eggs, *Nvtra* is transcriptionally activated during very early embryogenesis by the upstream instructor gene. The maternal input of *Nvtra* could also contribute to start *Nvtra* female-specific splicing (Verhulst et al., 2010a). In these fertilized eggs *Nvtra* produces female-specifically spliced functional mRNAs encoding a full-length NvTra protein. In contrast, the maternal *Nvtra* allele is not transcribed in haploid (unfertilized) eggs because of maternal imprinting of the upstream instructor gene. In these haploid eggs, the zygotic *Nvtra* gene is transcribed slightly later. It produces male-specific non-functional mRNAs (encoding a truncated NvTra), leading to a default male-specific *Nvdsx* splicing and male development. Geuverink et al. (2018a), found in the genome of *Leptopilina clavipes* (Hymenoptera: Cynipidae) a sex-specifically regulated *tra* orthologue and a *tra* paralogue lacking the genomic region coding for male-specific exon, which could be also involved in sex determination. Another hymenopteran parasitoid *Asobara tabida* showed a conserved sex determination cascade with *tra* and *dsx* sex-specifically regulated (Geuverink et al., 2018b). Differently from *Nasonia* and other hymenopteran species, *At-tra* and *At-tra2* amRNAs are maternally provided, suggesting that the sex determination of *A. tabida* differs from the MEGISD mechanim (Geuverink et al., 2018b).

The isolation of the *Ceratitis capitata tra* orthologue led to the discovery of its ancestral epigenetic (autoregulatory) master function in female sex determination outside Drosophilidae. Furthermore, the conservation of the *tra* > *dsx* regulatory module was also found to be widely conserved (see following paragraphs). These conclusions have been supported by the isolation of *tra* orthologues and their functional analyses in insect species belonging to Diptera, Coleoptera, and Hymenoptera.

10. Molecular isolation of *transformer-2* orthologues

Drosophila transformer-2 orthologues were first isolated in our species (*Hstra2*) by Dauwalder et al. (1996) via a BLAST similarity search of human Expressed Tag Sequences (ESTs), and in *D. virilis* (*Dvtra2*) by Chandler et al. (1997), via the low-stringency hybridization of a *D. melanogaster* probe on a genomic library. Chandler et al. (1997) confirmed the structural conservation of the gene (85% aa identity in the RRM). They showed that a *Dvtra2* transgene expressed in mutant *Dmtra2 D. melanogaster* was capable of regulating the female-specific splicing of *dsx* and properly feminizing XX individuals, rescuing the endogenous mutation.

The first non-Drosophilidae insect *tra2* orthologues were isolated in *Musca domestica* (*Mdtra2*) by Burghardt et al. (2005), using PCR with degenerate primers, and in the lepidopteran *Bombyx mori* (*Bmtra2*) by Niu et al. (2005), via *in silico* BLAST analysis of expressed tag sequences. Embryonic transient RNAi led to the full masculinization of genotypically female *Musca* individuals into fertile males, giving rise to two hypotheses: 1) *Mdtra2* is involved in the autoregulation of the *Musca F* gene determining femaleness; and 2) the *F* gene is homologous to the Medfly *tra* gene and can autoregulate likely using conserved Tra/Tra2 binding sites. Both hypotheses were later confirmed by Hediger et al. (2010). In contrast, Suzuki et al. (2012) suggested that *Bombyx mori tra2* is not involved in controlling *Bmdsx* splicing and sex determination.

The *Ceratitis tra2* gene was independently isolated by Gomulski et al. (2008), via a BLAST search of ESTs, and by Salvemini et al. (2009), using PCR with degenerate primers. As predicted by Pane et al. (2002), the embryonic transient RNAi knockdown of *Cctra2* led, to male-specific splicing of *Cctra* (and also of *Ccdsx*) and full masculinization of XX individuals, supporting the model of *Cctra* autoregulation (Salvemini et al., 2009; Pane et al., 2002). In contrast, a genetic construct constitutively expressing *tra2* dsRNA in *Drosophila* transgenic flies only partially masculinized XX individuals, (Fortier and Belote, 2000). As *Drosophila tra* lacks autoregulation, the RNAi targeting *tra2* is likely less effective in switching off the gene and in promoting male-specific of *dsx* in all cells during development.

The approach of PCR and degenerate primers was successfully used in various studies. Sarno et al. (2010) isolated *tra2* orthologues in *Anastrepha obliqua* (*Aotra2*). Sarno et al. (2011) showed that in transgenic *Drosophila*, the AoTra2 protein can partially substitute for the endogenous Tra2 protein. Similarly, Martín et al. (2011) isolated *tra2* orthologues in two Sciaridae species (suborder Nematocera) and expressed the corresponding proteins in *Drosophila*, again showing partial activity in promoting female-specific *dsx* splicing and likely less efficient interaction with the endogenous TRA protein.

The *in silico* approach by homology search led to isolate *tra2* orthologues in hymenopteran, coleopteran and hemipteran species. Embryonic RNAi of *Apis tra2* (*Am-tra2*; Dearden et al., 2006) showed its conserved function in *fem* splicing autoregulation of diploid females and in *Am-dsx* female-specific splicing (Nissen et al., 2012). Furthermore, *Am-tra2* seems to have a novel vital function during the embryogenesis of both sexes (Nissen et al., 2012), as also described for *tra2* orthologues in the red flour beetle *Tribolium castaneum* (Shukla and Palli, 2013) and in the stag beetle *Cyclommatus metallifer* (Coleoptera; Gotoh et al., 2016a).

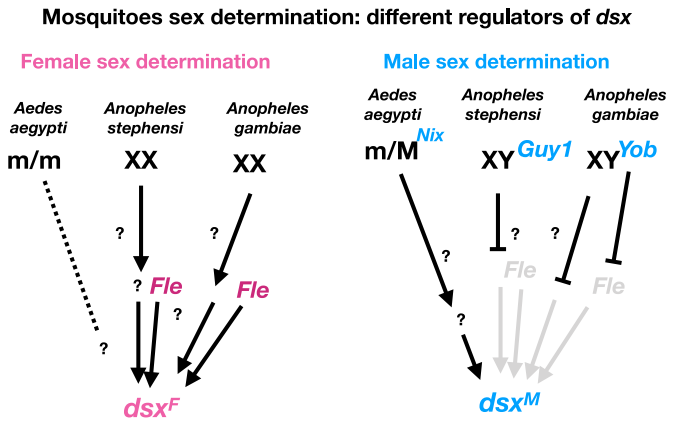

Fig. 3. Mosquitoes' sex determination pathway. In mosquitoes, different male-determining factors (*Nix*, *Guy1* and *Yob*) lead to the expression of male-specific DsxM isoform by alternative splicing. The direct upstream regulators of *dsx* seem to be different from Tra. It is unclear if *dsx* in mosquitoes has a default sex-specific splicing pattern in one of the two sexes or if its splicing requires regulators in each sex. In *A. gambiae* and *A. stephensi* the *fle* gene is expressed in both sexes but is required in XX individuals for female-specific *dsx* splicing. *Fle* encodes a Tra2-and Nix-related putative splicing regulator, suggesting its direct involvement in sex-specific *dsx* splicing. In XY individuals of the two species, *fle* function is somehow repressed by the male-determining *Yob* and *Guy1* genes. In *Aedes aeygpti*, sex chromosomes are homomorphic (*m/m* and *M/m*). The male-determining *M* chromosome carries the *Nix* gene, which promotes male-specific *dsx* splicing, likely by direct interactions with *dsx* pre-mRNA. In *Anopheles stephensi* and *A. gambiae*, the Y-linked genes *Guy1* and *Yob* promote promote female-specific *dsx* splicing with the help of *Fle*.

The *tra2* homologue in *N. vitripennis* (*Nvtra*) was isolated using as a BLAST query the *A. mellifera* Tra2 amino acid sequence (Geuverink et al., 2017). The *Nasonia tra* gene, as *Apis fem*, also requires NvTra2 to perform *Nvtra* positive female-specific splicing autoregulation. Parental RNAi of *Nvtra2* showed that maternally provided transcripts are crucial for the timely activation of female sex determination mediated by *Nvtra* in *N. vitripennis* (Geuverink et al., 2017).

Shukla and Palli (2013) isolated the *tra2* orthologue from the beetle *Tribolium castaneum* (*Tctra2*). Embryonic RNAi showed that *Tctra2* is necessary not only for the female-specific splicing of *Tctra,* as observed for Medfly *tra*, but surprisingly also for male-specific *Tctra* splicing. In the sweet potato whitefly, *Bemisia tabaci* (Hemiptera, Aleyrodidae), *Bttra2* expresses two alternatively spliced isoforms during development, and it is expressed at higher levels in haploid males versus diploid females (GuoXie et al., 2018; Xie et al., 2014). RNAi of *Bttra2* affected vitellogenin gene expression in females and genitalia development in males.

In the coleopteran *C. metallifer*, RNAi of *Cmtra2* during prepupal development was lethal and it was not possible to examine its phenotypic effects on adult sexually dimorphic traits (Gotoh et al., 2016a). Zhuo et al. (2017) isolated *tra2* orthologue (*Nltra2*) in the *Nilaparvata lugens* (Hemiptera). Gene repression at the nymph stages by RNAi caused genotypic females to develop into infertile pseudo males containing undeveloped ovaries and males to develop their normal phenotype but be sterile. A maternal RNAi of *Nltra2* (dsRNA injections in adult females) resulted in long-winged females rather than short-winged ones (male progeny remained short-winged). Zhuo et al. (2017) proposed a cross-talk between the sexual differentiation and wing polyphenism pathways.

Krzywinska et al. (2021) performed a BLAST screening in *Anopheles gambiae* embryonic transcriptomes for Tra2-related proteins, isolating *femaleless* (*fle*). *Fle* encodes a Tra2 distantly related splicing factor, similar to the male determining *Aedes aegypti Nix* (Hall et al., 2015). The authors showed by *fle*-RNAi that is necessary for the *Anopheles dsx* female-specific, which was previously isolated and found to be sex-specifically regulated by Scali et al. (2005; see next paragraph). *Fle* is required also for *Anopheles fruitless* female-specific splicing and to repress dosage compensation (Krzywinska et al., 2021) (Fig. 3).

Based on current data, we can conclude that most of the *tra2* orthologues are required for *tra* female-specific autoregulation (at least in those species having *Cctra* functional orthologues) and *dsx/fru* female-specific splicing regulation.

11. Molecular isolation of *doublesex* orthologues

Wilkins (1995) proposed that genetic pathways such as those determining sex in *D. melanogaster* and *C. elegans* evolve in reverse order via the sequential acquisition of novel genetic switches, each of which reverses the action of the previous one. Indeed, Raymond et al. (1998) serendipitously found that in the nematode *C. elegans*, the male sexual regulatory gene *mab-3* is related to *Drosophila dsx*, revealing the striking finding of very deep evolutionary functional conservation of the zinc finger DNA-binding domain (DM) in sex determination. Over the last three decades evolutionary studies have supported this model, showing that *dsx* is a conserved regulatory gene for sexual differentiation in different phyla, including vertebrates (Kopp, 2012; Matson and Zarkower, 2012). DM-containing genes have even upstream sex-determining functions in different vertebrate species (Zhao et al., 2015). On the contrary Tra/Tra2 are conserved only at the level of different insect orders (Verhulst et al., 2010b), and primary sex-determining signals seem to be conserved at the lower taxon level of families or even species (e.g. in *Musca domestica*; Dubendorfer et al., 2002).

Drosophila melanogaster doublesex has conserved structure, sex-specific regulation and likely sex-specific functions in *D. virilis* (Burtis and Baker, 1989). The PCR/degenerate primers approach was used for cloning of *dsx* orthologues in a list of Tephritidae species (*Bactrocera tryoni, Bactrocera oleae, Anastrepha obliqua*), in *Musca domestica*, and in few Sciaridae species. Shearman and Frommer (1998) isolated the first non-Drosophilidae *dsx* orthologue in the Tephritidae species *Bactrocera tryoni* (Queensland fruit fly). Northern blotting and cDNA cloning/sequencing showed that *Btdsx* produces sex-specifically spliced mRNAs encoding highly conserved Dsx isoforms. As observed in *Drosophila*, the BtDsx sex-specific isoforms differ in their C-termini. More striking and interesting was the finding the Tra/Tra2 repeat elements clustered in the *Btdsx* female-specific exon, as observed for *D. melanogaster dsx*, strongly indicating evolutionary conservation of the upstream Tra/Tra2 splicing regulators in Tephritidae. The evolutionary conservation of these *dsx* features was confirmed in orthologues of many other dipteran species, including *Megaselia scalaris* (Kuhn et al., 2000; low-stringency hybridization on cDNA libraries), *Musca domestica* (Hediger et al., 2004), and various other Tephritidae species, including *Bactrocera oleae* (Lagos et al., 2005), *Anastrepha obliqua* (Ruiz et al., 2005), *Bactrocera dorsalis* (Chen et al., 2008), *C. capitata* (Pane et al., 2002; Saccone et al., 2008; low-stringency hybridization) and *B. jarvisi* (Morrow et al., 2014; *in silico*). All *dsx* orthologues showed the conservation of a cluster of Tra/Tra2 binding sites within the female-specific exon coupled with the 3' alternative splice site regulation of sex-specific exons. The first functional evidence of the evolutionary conservation of the *transformer>doublesex* regulatory module was provided by Pane et al. (2002) in Medfly. These authors showed in XX masculinized individuals induced by *Cctra*-specific RNAi a shift to the male-specific pattern of *Ccdsx* splicing, similarly to the effects of a *Cctra2* RNAi (Salvemini et al., 2009). Comparing DmDsx and CcDsx was useful to clone the *Musca domestica dsx* orthologue by PCR (Hediger et al., 2004). The embryonic RNAi knockdown and interspecific transgenic studies of *Mddsx* in transgenic Drosophila flies have suggested the structural and functional conservation of the *Drosophila* orthologue. However, the MdDsxM protein does not affect the normal female development of *Drosophila* XX flies. In contrast, the Medfly male-specific CcDsxM isoform can partially masculinize *Drosophila* transgenic XX flies (a transgene controlled by a

heat shock promoter) but to a greater extent than MdDsxM, likely because of its higher protein similarity to the DmDsxM protein (Saccone et al., 2008). Chen et al. (2008) isolated *B. dorsalis dsx* (*Bddsx*; by PCR), injected dsRNA into adult abdomens of both sexes and observed effects only in females. These individuals showed a reduction of vitellogenin synthesis, delayed ovary development, a reduction in the number of mature eggs and deformed ovipositors. The sex ratio of their offspring was not biased (Chen et al. (2008).

Alvarez et al. (2009) used the binary UAS-GAL4 system to drive the two *Anastrepha obliqua* (Tephritidae) sex-specific Dsx isoforms in transgenic *Drosophila* flies and observed partial sexual transformation in various sexually dimorphic regions. The authors noted that the greater evolutionary divergence among Dsx proteins between *Musca* and *Drosophila* than between *Drosophila* and the Tephritids could explain the reduced ability of MdDsxM to masculinize *Drosophila* XX individuals. Ruiz et al. (2007) isolated *dsx* orthologues in ten Anastrepha species closely related to *Anastrepha obliqua* using PCR and various combinations of *Aodsx*-specific primers. The authors found that most of the nucleotide changes in the common regions of the homologous DsxF and DsxM DNA sequences were synonymous, suggesting strong purifying selection. Ruiz et al. (2015) isolated *dsx* orthologues in two Sciaridae species and found they produce two alternatively spliced transcripts encoding DsxF- and DsxM-homologous proteins, which occurred simultaneously in both sexes. Furthermore, a rabbit antibody showed that DsxF was present at similar amounts in Sciara males and females. In contrast, the DsxM protein was not detected (likely because of a very low expression level). The authors presented a novel model of *dsx* function in Sciaridae species that could explain these major differences from other dipteran species (Ruiz et al., 2015).

Most of the *dsx* studies performed in the last two decades used *in silico* BLAST approaches to identify orthologues in ESTs, genomic and transcriptomic databases. Scali et al. (2005) found that *Anopheles gambiae dsx* (*Agdsx*) has sex-specific splicing regulation and protein conservation of the two sex-specific isoforms (Fig. 3). In this distantly related dipteran species, they found some structural differences, leading the authors to speculate that novel splicing factors other than Tra/Tra2 could be involved. Salvemini et al. (2011) found that the sex-specific alternative splicing of *Aedes aegypti* (*Aaedsx*) had some relevant differences when compared to the *Anopheles* and *Drosophila dsx* regulation. Ohbayashi et al. (2001) described *Bombyx mori doublesex* orthologue sex-specific alternative splicing regulation. Suzuki et al. (2001) used an *in vitro* splicing assay, and *HeLa* cell line nuclear extracts, to show that *Bmdsx* has female-specific default splicing and regulated male-specific splicing, likely controlled by novel upstream male-specific regulators (Shukla et al., 2011; Gopinath et al., 2016). Suzuki et al. (2003) developed transgenic *Bombyx mori* moths ectopically expressing BmDsxF isoform in ZZ males, but no phenotypic feminization was observed. These transgenic males expressed *vitellogenin* genes and showed a reduction in the expression of a male-biased gene (a pheromone-binding protein gene). Transgenic strains of *Bombyx mori* expressing TALENs or Cas9 were used by Xu et al. (2017) to target and mutagenize *Bmdsx*, which resulted in females with mutant abdominal segments. Furthermore, *Bmdsx*-induced mutations affected external genitalia and fecundity in both males and females.

Kijimoto et al. (2012) have shown by larval *RNAi* that *Onthophagus taurus* (Coleoptera) *dsx* (*Otdsx*) controls essential aspects of horn development, including differences between sexes and morphs (large males and small ones, caused by suboptimal nutrition). Male-specifically spliced *Otdsxm* transcripts were preferentially expressed during horn development in large morphs. Larval *Otdsx*-RNAi led to substantially reduced horn development in the large but not the small males. Treated males also showed malformed genitalia. In contrast, *Otdsx*-RNAi-treated female larvae develop small ectopic horns as adults, while this sex is usually hornless. Hence, while *Otdsxm* promotes horn development in large males, *Otdsxf* inhibits horn formation in females. In conclusion, *Otdsx*-RNAi caused in both sexes a default development of a small-horned phenotype (Kijimoto et al. (2012). These authors investigated a closely related species, *O. sagittarius*, sharing a common ancestor with *Onthophagus taurus* ~5 million of years ago (Mya), showing a radically divergent pattern of horn development. This species shows a reversed sexual dimorphism, with females having single long medial thoracic and head horns absent in males. Also, *O. sagittarius dsx* (*Osdsx*) is sex-specifically spliced encoding isoforms highly similar to those encoded by *Otdsx* (Kijimoto et al. (2012). *Osdsx*-RNAi reduced thoracic horn size in females and induced a small but conspicuous protrusion in the male prothorax. These contrasting effects of RNAi in the two sexes can be explained by concluding that *Osdsxm* inhibits the growth of the thoracic horn, whereas *Osdsxf* promotes growth. The contrasting effects of RNAi in the two coleopteran species are likely due to the reverted sex-specific functions of *dsx* in regulating thoracic horn growth in *O. sagittarius* compared with *O. taurus*. Ito et al. (2013) studied *Td-dsx* developmental function in the Japanese rhinoceros beetle *Trypoxylus dichotomus* (Coleoptera, Scarabaeidae, Dynastinae), which has sexually dimorphic exaggerated horns on the head and prothorax. These males have acquired horns independently from *Onthophagus*. *Td-dsx* produces sex-specific and common transcripts which are expressed in horn-forming regions at the prepupal stage (Ito et al., 2013). RNAi-treated male larvae developed into males showing a reduction of the head horn and disappearance of the thoracic one. RNAi-treated female larvae developed into females showing a small horn on top of the head (Ito et al., 2013). The male genitalia were severely affected and transformed into female-like ones, whereas the female genitalia were less affected. Interestingly, these sex-specific isoforms of *Td-dsx* have different regulatory functions for the head and prothoracic horns. Other subtle sexually dimorphic traits of this species were affected by *dsx*-RNAi. Ito et al. (2013) concluded that *dsx* has essential roles in regulating widely conserved pre-existing sex-specific traits and controlling horn development as an evolutionary novelty in beetles. The *dsx* gene might also have played a critical role during the evolutionary transition from sexually monomorphic to dimorphic characteristics.

Similar *dsx* RNAi functional studies in the sexually dimorphic coleopteran *Cyclommatus metallifer* (Gotoh et al., 2016a), showed partial sexual transformations of females into males like those observed with *Cmix*-RNAi, when targeting few of the multiple *dsx* isoforms. In the sexually dimorphic broad-horned beetle *Gnatocerus cornutus* (Tenebrionidea, Tenebrionidae, Coleoptera) *dsx* (*Gtdsx*) produces sex-specific and non-sex-specific isoforms. Knockdown of all *dsx* isoforms resulted in intersex phenotype of weapon traits both in male and female. In treated-females mandibles became longer, genae (the “cheeks”) became wider, and pair of horns-primordia started to develop between the eyes. In treated-males, mandibles became shorter and genae became narrower (Gotoh et al., 2016b).

Jia et al. (2018) isolated the *dsx* orthologues of various ant species (Hymenoptera) describing novel sex- and caste-specific alternative splicing and found that the *dsx* evolution seems to correlate with the evolution of eusociality observed in some ants. Cristino et al. (2006) isolated the *dsx* orthologue (*Amdsx*) from the hymenopteran *Apis mellifera*, showing conservation of sex-specific alternative splicing regulation (Cho et al., 2007). *Amdsx* mutants induced by Cas9 also develop intersex reproductive organs and male-like gonads (Roth et al., 2019). Oliveira et al. (2009) reported the identification of *dsx* orthologues of *Nasonia giraulti* (*Ngdsx*), and of *N. vitripennis* (*Nvdsx*), regulated by sex-specific splicing. Phenotypically variable *Nasonia* gynandromorphic mutants expressed both sex-specific NvDsx isoforms, suggesting *dsx* functional conservation (Oliveira et al., 2009). Wang et al. (2022) silenced *Nvdsx* in male pupae by RNA interference. They observed pheromonal feminization in resulting adult males, which can no longer attract females from a distance. Furthermore wild-type males courted these differently-smelling treated males. Also, the hymenopteran sawfly, *Athalia rosae*, uses a haplodiploid mode of reproduction. Fertilized eggs develop into diploid females, whereas unfertilized eggs develop into haploid males. The *dsx* orthologue *Ardsx* in this species produces

sex-specifically spliced mRNAs (Mine et al., 2017). Interestingly, the knockdown of *Ardsx* in haploid males caused almost complete male-to-female sex reversal, but the resulting eggs were infertile (Mine et al., 2021). On the contrary, RNAi did not affect the female differentiation of diploid individuals.

Price et al. (2015) performed an extensive phylogenetic analysis of the *dsx* orthologues from 30 insect orders. They found the presence of dsx sex-specific splicing in several of them. Furthermore, chelicerates and crustaceans express two *dsx* paralogous genes, which lack sex-specific alternative splicing. Zhuo et al. (2018) isolated the *doublesex* orthologue of the brown planthopper (BPH), *Nilaparvata lugens* (Hemiptera*)* - one of the most devastating rice pests in many Asian countries - and observed sex-specific alternative splicing of the transcripts. RNAi knockdown showed that *Nldsx* is required for proper somatic development during male development and mating behaviour. Wexler et al. (2019) isolated *dsx* orthologues in species belonging to three hemimetabolous insect orders and found *dsx* sex-specific splicing regulation in two of them (in the hemipteran *R. prolixus* and in the blattodean *B. germanica*). No sex-specific splicing regulation was detected in the *Pediculus humanus* (Phthiraptera), suggesting that some insects may have secondarily lost sex-specific *dsx* splicing. Despite the presence of male and female isoforms in the blattodean *B. germanica* and the hemipteran *N. lugens*, *dsx* RNAi knock-down strongly feminized genotypically males and had no apparent effects in females (Wexler et al., 2019; Zhuo et al., 2018).

GuoXie et al. (2018) investigated the *dsx* homologue in the hemipteran *B. tabaci* (B-biotype), reported the absence of sex-specific splicing and found that RNAi mediated silencing resulted in malformed genitalia only in males. Contrary to the study of GuoXie et al. (2018), Singh Brar et al. (2022) found in *B. tabaci* AsiaII-1 that the pre-mRNA of *Btdsx* is sex-specifically spliced and that RNAi-mediated knockdown in the expression of *Btdsx* results in the development of deformed genitalia in females, whereas the male genitalia were unaltered. *Btdsx* RNAi knockdown resulted also in up-regulation of *vitellogenin* (*Bmvg*) and *vitellogenin receptor* (*Bmvgr*) genes in males and their down-regulation in females. Treated females showed a reduction in fecundity and egg hatching. No effects were visible on sexual differentiation of both sexes, and neither sex ratio distortion was observed (Singh Brar et al., 2022).

Following the identification of *dsx* orthologues in termites (Isoptera), Miyazaki et al. (2021) found that only some species maintain sex-specific splicing regulation, while male-specific *dsx* transcription is an acquired trait in eusocial termite species. The authors proposed that sex-specific alternative splicing of *dsx* would have been acquired early in the evolution of insects, and secondarily lost in some hemimetabolous insects, including termites.

Rodriguez-Caro et al. (2021) described in the butterfly *Zerene cesonia* (Southern Dogface; Lepidoptera) two distinct *dsx* paralogues (*ZcdsxF* and *ZcdsxM)*. The two genes are expressed in a sex-specific manner by differential transcription and associated with the sexually dimorphic UV colouration of the wings. This novel finding points to the evolutionary flexibility of *dsx* sex-specific regulation and the stability of *dsx* in controlling sexual development as well as the phenomenon of mimicry observed in other butterfly species (Kunte et el al., 2014). The isolation of upstream transcriptional regulators of the *ZcdsxF* and *ZcdsxM* genes will be of great interest in understanding different evolutionary routes of sex-determining genetic networks.

In the swallowtail butterfly *Papilio polytes*, *doublesex* controls the development of a female-limited Batesian mimicry. The mimetic-form in females resembles the unpalatable butterfly *Pachliopta aristolochiae* (Kunte et al., 2014). Nishikawa et al. (2015) identified in *P. polytes* a single ~130-kb autosomal inversion, including *dsx*, between mimetic (*H*-type) and non-mimetic (*h*-type) chromosomes. The authors applied at larval stages an electroporation-mediated small interfering RNA (siRNA) incorporation targeting *Ppdsx*. They demonstrated that female-specific *Ppdsx* isoforms expressed from the inverted *H* allele (*dsx* (*H*)) promote mimetic colouration patterns but also inhibit non-mimetic patterns. The authors concluded that appearance of the non-mimetic pattern on the wings of treated mimetic females suggests that other genes preset the pigmentation pattern and that *dsx*(*H*) merely selects the pigmentation processes.

The damselfly *Ischnura senegalensis* (Odonata) displays a surprising diversity of colour patterns, including intrasexual polymorphisms. Females are either a gynomorph for female-specific colour or andromorph for male-mimicking colour and predominantly express a longer *doublesex* (*Isdsx*) isoform. In contrast, males express a short isoform (Takahashi et al., 2019, 2021). A larval electroporation-mediated RNA interference (RNAi) targeting the *Isdsx* common region affected males and female andromorphs. It reduced melanization and changed their colour pattern into one of the gynomorphic females. However, female gynomorphs were not affected by the treatment. By contrast, RNAi against the *Isdsx* long isoform produced no effects, indicating the importance of the *Isdsx* short isoform for body colour masculinization in males and andromorphic females.

Prakash and Monteiro (2020) used CRISPR/Cas9 to target the butterfly *Bicyclus anynana dsx* orthologue and show that the female-specific splicing isoform is required to repress the development of a male-specific scent organ used for chemical communication during courtship. Baral et al. (2019) analyzed patterns of exon-level molecular evolution and protein structural homology of *doublesex* from 145 species of four insect orders covering 350 million years of divergence. They found that male-specific regions of Dsx evolved faster than female-specific regions in Lepidoptera, Diptera, and Coleoptera. In contrast, this trend was reversed in Hymenoptera. The peculiar structural and functional partitioning of the *dsx* coding sequence may explain its contrasting functions in producing critical adaptations that are, in parts, sex-limited, polymorphic, developmentally conserved, and rapidly evolving across closely related species.

Previous studies have concluded that *dsx* acquired the sex-specific splicing regulation before the divergence of Pterygota and that in hemimetabolous species, *doublesex* has sex-specific isoforms but is not required for female differentiation. The question of how *doublesex* evolved its essential function in female development still needs to be answered.

Chikami et al. (2022) investigated more ancestral regulatory patterns of *doublesex* in the apterygote insect *Thermobia domestica,* belonging to Zygentoma, the sister group of Pterygota (winged insects). The authors found that the *T. domestica doublesex* (*Tddsx*) expresses sex-specific isoforms. Functional RNAi experiments showed that it is necessary for male differentiation of morphology and female upregulation of the *vitellogenin* gene (*vg*). These data suggest that *doublesex* may have already played some role in female biochemical differentiation in the common ancestor of Pterygota (Chikami et al., 2022). The authors proposed that *doublesex* acquired its function in female morphogenesis through a change in the protein structure rather than the emergence of the female-specific exon.

Based on the available data, most insect *dsx* orthologues have a conserved sex-specific splicing regulation. The sex-specific isoforms play at least some biochemical and highly evolving developmental functions in the sexual differentiation of different species. Hence these data support the idea of an hourglass evolutionary model with *dsx* being a regulatory keystone. At the same time, sex-determining signals and downstream target genes diverge rapidly, defining and expanding sex-specific identity into new tissues (Hopkins and Kopp, 2021).

12. Isolation of *intersex* orthologues

Rescuing the *D. melanogaster intersex* null mutant bearing transgenes expressing orthologous *ix* sequences from Diptera and Lepidoptera species suggested its functional evolutionary conservation (Arunkumar and Nagaraju, 2011; Cavaliere et al., 2009; Siegal and Baker, 2005). Cavaliere et al. (2009) prepared a cDNA phage library from pupae of the legume pod borer, *Maruca vitrata* (Lepidoptera). They performed a low

stringency hybridization screening with a probe derived from *B. mori ix*. They isolated the *ix* orthologue (*Mvix*) and detected by Northern blot analysis two alternatively spliced isoforms common in both sexes and a novel female-specific transcript found only in pupae. As *Drosophila ix*, *Mvix* is expressed at higher levels in females. Cavaliere et al. (2009) found *in B. mori* ESTs *ix* transcripts in ovaries and testes, suggesting germ-line functions. MvIx protein can partially replace the *Drosophila* endogenous Ix in controlling sexually dimorphic cuticular structures.

Subsequent *ix* cloning studies took advantage of DNA/RNA sequence databases available in the various investigated species. Siegal and Baker (2005) isolated *Drosophila ix* orthologues in two *Drosophila* species (*D. pseudobscura* and *D. virilis*), three other dipteran species (*Glossina morsitans*, *Megaselia scalaris* and *Anopheles gambiae*), the lepidopteran *Bombyx mori*, the hymenopteran *Apis mellifera*, and in other 12 metazoan species. Expression of *M. scalaris* Ix orthologous proteins in transgenic ix mutant *Drosophila* strains rescued of *ix* female-specific function. This rescue was not observed with *B. mori* or *Mus musculus* Ix proteins. This finding suggested a conserved ancestral function of *ix*, mainly in dipteran species.

Arunkumar and Nagaraju (2011) described in the other lepidopteran *B. mori ix* testis-specific spliced transcripts (in male pupae and adults) encoding a truncated protein of 72 aa. Xu et al. (2019) performed the first direct functional study of an *intersex* orthologue in this species by CRISPR/Cas9. They found that *Bmix* female mutants were sterile and had irregular external genitalia. *B. mori ix* mutants of both sexes showed defective development of the imaginal disc, including the wing, antenna, and leg. Liu et al. (2020a) described additional *ix* orthologues in eight insect species (six Hymenoptera and two Diptera). They found that knocking down by larval feeding RNAi the expression of the *Btix* gene in *B. tabaci* affected adult female reproduction, and the eclosion rate of the progeny. It also shortened the body length of female progeny. The expression of the *vitellogenin* gene (*Btvg*) decreased after silencing *Btix,* suggesting that BtIx activates the orthologous *Btvg* target gene in females together with the female-specific BtDsxF. However, no female-to-male sexual transformations were observed, suggesting divergent evolution of pleiotropic developmental *ix* functions.

RNAi knockdown of the hemipteran *Oncopeltus fasciatus ix* gene at nymphal stages affected male and female genital morphological structures (Aspiras et al., 2011; Ewen-Campen et al., 2011). Similar *ix* functional studies in the sexually dimorphic coleopteran *Cyclommatus metallifer* (Gotoh et al., 2016a), showed partial sexual transformations of females into males like those observed with *Cmdsx*-RNAi. Gotoh et al. (2016a) proposed that, like *Drosophila* Ix, also CmIx interacts with CmDsxF to specify female differentiation. Zhang et al. (2021) isolated the planthopper *N. lugens ix* (Hemiptera) and showed by RNAi that the gene plays pleiotropic roles in embryogenesis and development of the reproductive system.

Based on the available data, we can conclude that many insect *Ix* orthologues have a conserved role as a protein partner of DsxF in controlling the sexual development of females, including genitalia structures. In the Hemiptera, *ix* seems to play additional non-sex-specific functions in embryogenesis and genitalia development.

13. Molecular isolation of *fru* orthologues

Davis et al. (2000) used genomic library screening and PCR with degenerate primers to isolate portions of a *fru*-orthologous gene from Drosophilidae species and in other distantly related insects. The lepidopteran *Bombyx mori* and three Tephritidae species *(Ceratitis capitata*, *Bactrocera dorsalis* and *B. cucurbitae*), showed Fru protein sequence conservation of the BTB-Zn finger DNA binding domain. Bertossa et al. (2009) isolated (by PCR) the *fru* orthologue of the hymenopteran *Nasonia vitripennis* (*Nvfru*). They confirmed the *Nvfru* sex-specific regulation by alternative splicing, found two new C2H2 zinc finger domains and suggested its functional conservation. Salvemini et al. (2009) provided the first experimental indication that outside of Drosophilidae species, in *Ceratitis capitata*, *tra2* orthologue continues to control sex-specific splicing of the *fru* and is likely involved in Medfly male mating behaviour. They performed a *Cctra2* RNAi study observing the complete masculinization of XX Medfly individuals, which expressed *CcfruM* mRNAs.

Gailey et al. (2006) identified a *Drosophila fru* orthologue in the genome sequence of *Anopheles gambiae* and provided the first evidence of the evolutionary conservation of *fru* sex-specific splicing. Salvemini et al. (2013) isolated *in silico* the *Aedes aegypti fru* orthologue, which showed sex-specific splicing. The authors compared the sequences of the sex-specifically regulated exons between *Aedes dsx* and *fru* genes to find conserved cis-regulatory elements either similar to the *Drosophila* ones or novel. The authors proposed a more complex model of sex-specific splicing regulation of the two genes, involving positive and negative upstream splicing regulators in both sexes (hence the absence of *dsx* or *fru* splicing default splicing). The *fru* gene shows very complex transcriptional activity in all investigated species (Salvemini et al., 2010).

Meier et al. (2013) showed that *Mdtra/Mdtra2* control the sex-specific splicing of *Mdfru*, which is confined to neural tissues in the brain and involved in the development of *Musca* male courtship behaviour. Basrur et al. (2020) performed gene-specific mutagenesis of this *Aaefru* gene using CRISPR/Cas9. They revealed that in mosquitoes, *fruitless* controls female-specific feeding behaviour rather than sex-specific mating behaviours. Furthermore, human body odour attracted wild-type females, and these mutant male mosquitoes had a partially feminized olfactory behaviour. Xu et al. (2020) induced the loss of the lepidopteran *Bmfru* by using CRISPR/Cas9, which completely blocked mating, but males displayed normal courtship behaviour, again suggesting partial functional divergence.

Boerjan et al. (2011) identified *in silico fru* orthologous EST sequences of the desert locust *Schizocerca gregaria* (Orthoptera). They showed that RNAi knockdown in the third and fourth nymphal stage induced a significantly lower cumulative copulation frequency and testes weight. In the other orthopteran species, *Grillus bimaculatus fru* gene showed no sex-specific splicing (Watanabe, 2019). In contrast to *Drosophila*, the Fru protein distribution was similar in both sexes' brains. These data suggest that the gene is not involved in the sex determination of neuronal circuitry in hemimetabolous insects (Watanabe, 2019).

Pan and Baker (2014) have uncovered some other exciting aspects of the FruM functions in building up innate sexual behaviour and the possibility for the male fly lacking this gene function to learn from male peers through social experience (Peng et al., 2021). The most straightforward idea to explain this phenomenon proposes the transformation of a time-consuming learned courtship to a robust innate behaviour, presumably by building up during the development of a similar neuronal network that encodes this complex phenotype (Pan and Baker, 2014; Peng et al., 2021). Is this learning or FruM-genetically induced sexual behaviour evolutionarily conserved in other species? Furthermore, how ancient is it?

There are various *fruM* transcripts encoding potentially five male-specific FruM isoforms different in their C-terminus (FruA-FruEM). FruBM isoform recruits histone deacetylases and heterochromatin binding proteins to ~130 target sites on *Drosophila* polytene chromosomes. This observation revealed the complexity of FruBM modulation of a "genetic orchestra", which ultimately leads to the formation of sexually dimorphic neural structures. For example, one well-established FruBM transcriptional target is the axon guidance protein gene *robo1* (Sato and Yamamoto, 2020). Concerning gross brain differences between *Drosophila* sexes, a male-specific neuronal cluster (P1) coexpresses *fru* and *doublesex* and is the potential trigger for male-type courtship behaviour (Yamamoto, 2008).

Based on the available data, we can conclude that *Drosophila fruM* spliced isoform is evolutionarily conserved in various dipteran and hymenopteran species but not in Orthoptera or Lepidoptera. *Drosophila* FruM mutant males can overcome the lack of an innate program to reproduce by learning the courtship behaviour from their male peers

(Pan and Baker, 2014). In some insect groups, this social learning became likely more relevant. In other insects, upstream regulators different from *fruM* have been recruited to build the neuronal network needed to embed and program this behaviour.

14. Different upstream regulators of *dsx* in different insect species: the first level of divergence

The sex-determining pathways in different groups of insects diverge at a level upstream to *dsx* (Lepidoptera, Hemiptera; Fig. 4) or *tra* (Diptera, Coleoptera, Hymenoptera; Fig. 5). The challenges in identifying novel upstream regulators required strategies other than those based on the sequence similarity of *Drosophila* sex-determining genes (Salvemini et al., 2013). Suzuki et al. (2008) used male- and female-specific cell lines of *B. mori* to establish a minigene splicing assay, to search for *Bmdsx* regulatory sequences and for sex-specific proteins that were able to bind to *Bmdsx* those elements and found BmPsi protein (Table 2). Kiuchi et al. (2014) performed a differential expression analysis of sexed *B. mori* embryo transcriptomes. They identified *Masculinizer (Masc), a* Z-linked gene encoding a zinc finger DNA binding protein/KH-domain RNA-binding protein, controlling the regulation of male-specific *Bmdsx* splicing and promoting the vital process of dosage compensation for Z-linked genes. However, Kiuchi et al. (2019) showed that the DNA binding domains seem unnecessary for MAasc masculinizing activity.

Zheng et al. (2019) identified two novel RNA-binding proteins in *Bombyx mori* by yeast three-hybrid screening: BxRbp1 and BxRbp3, which recognize sex-specific *Bmdsx* exons (Table 2). Furthermore, these authors found that *Masc* stimulates the expression of BxRbp3 in ZZ individuals, promoting male-specific *Bmdsx* splicing. RNA affinity chromatography and UV cross-linking in cell nuclear extracts led these authors to identify a previously known *Bmdsx* regulator, the non-sex-specific BmPSI protein (Suzuki et al., 2008). Yang et al. (2021) underlined that the RxRbp proteins could be the transducers of the sex-determining primary signal, similarly to Tra/Tra2 in *D. melanogaster* (Fig. 4). A long-noncoding RNA *Bmdsx-AS1* encoded by the antisense strand of the *Bmdsx* gene locus, displays nucleotide complementary at intron 3-exon 4 junction of *BmDsx* and causes enhancement of male-specific isoform of *Bmdsx* (Xu et al., 2019b). Over-expressing *Bmdsx-AS1* in female cells induces male-specific splicing of *Bmdsx*.

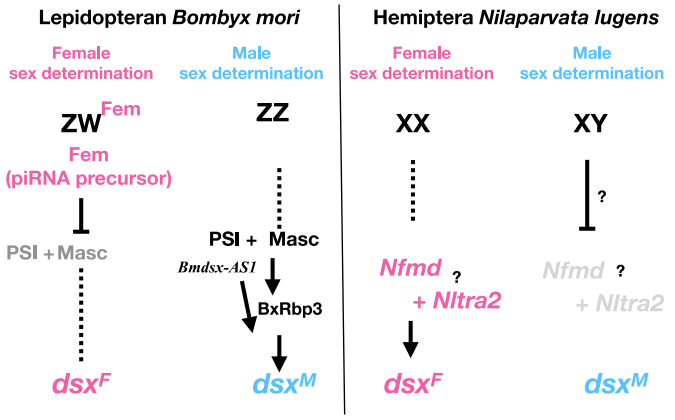

Fig. 4. Lepidopteran and hemipteran different upstream regulators of *dsx*. As in mosquitoes, the lepidopteran *Bombyx mori dsx* is regulated by different upstream genes, such as *Masculinizer*, the auxiliary PSI protein, the antisense Bmdsx-AS1, and the splicing factor RNABxRbp3. In ZW, a W-linked piRNA precursor encoded by Fem, represses Masc, leading to a default female-specific dsx splicing, while ZZ embryos, Masc/PSI/Bmdsx-AS1, promotes male-specific dsx splicing (assisted by RNABxRbp3) and male differentiation. In the Hemiptera *Nilaparvatya lugens*, female-specific splicing of *Nldsx* is promoted by *Nfmd* (and *Nfld2*), and *Nltra2*. In XY individuals, perhaps a Y-linked gene represses *Nfmd* activity leading to *Nldsx* male-specific splicing.

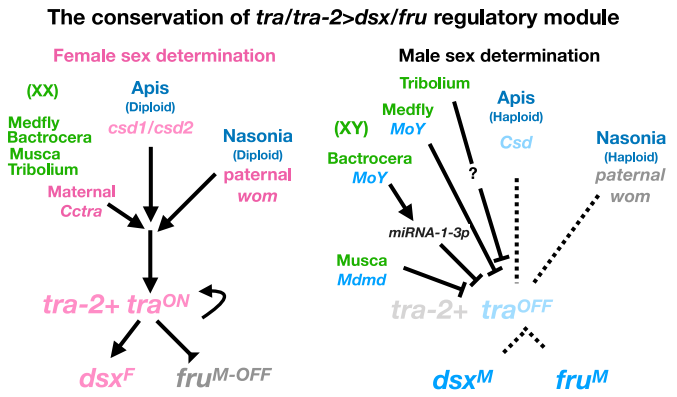

Fig. 5. The evolutionary functional conservation of the Medfly auto-regulatory *transformer* gene upstream to *dsx* and *fru*. In dipteran species, such as *Musca domestica* and Tephritidae, Y-linked male determining genes (*Mdmd*, *MoY*) promotes male-specific *dsx* and *fru* splicing either directly or indirectly, by blocking the establishment of *tra* positive autoregulation induced by the maternal *tra* signal. In XX embryos, *tra* activates itself and maintains its autoregulation, leading to a female-specific DSX isoform and *fru* non productive splicing. In Apis, diploid embryos having an heteroallelic *csd* genotype promotes female-specific *tra* (known as *fem*) and *dsx*, either directly or indirectly, while haploid embryos by default switch off *tra* and expresses male-specific *dsx* and *fru* isoform. In diploid *Nasonia* embryos, a paternally inherited autosomal *wom* allele activates transcriptionally *tra,* which starts its autoregulation and promotes female sexual differentiation by *dsx*. In haploid embryos, *tra* remains inactive and by default male-specific DSX and FRU are expressed.

Yuzawa et al. (2020) identified a direct interaction between *Masc* and *Bmdsx-AS1* by RNA immunoprecipitation assay, which suggests that the male-specific splicing of *Bmdsx* is regulated via the Masc-*Bmdsx-AS1* complex.

Through an impressive effort, Zhuo et al. (2021) identified two novel splicing regulators of *Nldsx* in the hemipteran *Nilaparvata lugens* (Table 2). The authors sequenced the early embryonic transcriptome, selected 200 candidate genes encoding putative splicing factors rich in serine and arginine and performed a large-scale screen using larval RNAi. The knockdown of one of these genes, *Nlfmd* (Female determinant factor), led to the partial masculinization of XX females. These intersexes showed a shortened ovipositor and male-like external genitalia. Interestingly, *Nlfmd* produces female-specific and non-sex-specific isoforms via alternative splicing by exon skipping. Female-specific NlFmd is 613 aa long, and the non-sex-specific form is shorter (449 aa). NlFmd shows very low similarity to the hymenopteran protein *A. mellifera* Feminizer (AmFem) over a very short region, conserved among AmFem and CcTra (Hasselmann et al., 2008). Similar to Tra/Fem, the NlFmd protein is rich in serine and arginine and has a proline-rich region but lacks a predicted RNA binding domain (hence, classified as an RS-type protein; Long and Caceres, 2009). The second novel splicing regulator, NlFmd2, does contain two RNA binding domains (RRM), affects *Nldsx* splicing and interacts with NlFmd in a transfected cell line experiment (Fig. 4).

14.1. Identification of different primary sex-determining signals: the second level of divergence

As earlier noted, the first non-drosophilid sex-determining, molecularly isolated signal was the *csd* gene in *Apis mellifera* that has essential functions in regulating haplo-diploid sex determination (Hunt and Page, 1994; Beye et al., 1994, 2003). RAPD and multilocus fingerprinting markers linked to *csd* have been used to start a chromosome walking (Beye et al., 2003). The *csd* gene was confirmed to be highly polymorphic (11–19) alleles in wild honeybee populations (Hasselmann and Beye, 2004; Beye et al., 2013). Heteroallelic diploid individuals (statistically favoured) develop as females, while haploid individuals ("homoallelic") develop as males (Beye et al., 2003). The "heteroallelic"

Csd proteins derived from the heterozygous *csd* genotype induce female sex determination by promoting the female-specific splicing of the primary transcripts of the *fem* gene, either directly or indirectly. It is still not clear what is the molecular mechanism by which the heteroallelic *csd* genotype of diploid individuals promotes female-specific splicing of *fem.* On the other hand, the hemiallelic *csd* genotype (in haploid individuals or rarely homoallelic *csd* genotype in diploid ones) lets *fem* express by default male-specific mRNAs.

No sex-specific regulation of *csd* was detected by RT-PCR and sequencing cDNAs from both sexes. Surprisingly, the Csd proteins showed high sequence similarity to a 13 amino acid-long region of Medfly Tra and were rich in serine and arginine, similar to Medfly and *Drosophila* Tra proteins. The *csd* gene evolved from a recent gene duplication event from an ancestral copy of the *fem* gene in the honeybee lineage (Hasselmann et al., 2008).

Microinjections of *csd*-dsRNA in fertilized (future diploid females) and non-fertilized Apis eggs (future haploid males), led to observing an effect only in female diploid larvae. They had a complete developmental switch from morphologically female to male gonads. Similar experiments microinjecting *csd*-siRNA affected only diploid bees leading to a profound masculinization of the head development (Hasselmann et al., 2008). Also, CRISPR/Cas9 targeting of *csd* induced a masculinized morphology in diploid mutant drones with typical male characteristics and smaller testes (Wang et al., 2021).

Koch et al. (2014) performed a comparative analysis of *fem/csd* in different hymenopteran species. They proposed that various *csd* paralogues originated independently and repeatedly from *feminizer* orthologues through gene duplications in bumblebee, honeybee, and ant lineages. It will be of great interest to investigate if these *csd* paralogues act as similar primary signals which emerged by convergent evolution.

In the last two decades, novel bioinformatic approaches were used to identify other master genes controlling insect primary sex-determining signals, often embedded in heterochromatic regions of sex chromosomes (Table 2). Some failed attempts were also helpful in redirecting efforts (Appendix). However, only the recent development of mRNA-seq and long-read DNA sequencing technology led to the exploration of transcriptional activity within heterochromatic regions. For those species harbouring male-determining factors on highly heterochromatic Y chromosomes, access to related sequences is hampered by the difficulty imposed by its repetitive nature. - It is difficult to clone repeat-rich DNA sequences, assemble and analyze them *in silico*. - To circumvent the problem, Carvalho et al. (2001) compared unmapped sequences from the *Drosophila* genome project to the nonredundant protein database (by BLASTx) and discovered some novel Y-linked genes. A similar approach in *Anopheles gambiae* failed to identify any relevant Y-linked genes (Krzywinski et al., 2006). A differential hybridization of an *A. gambiae* phage genome library, using total genomic DNA of males or females as probes, led to the isolation of various Y-linked markers and Y-linked BAC clones. The study also serendipitously identified a 48-kb-long unmapped scaffold (Krzywinski et al., 2004) containing the *Maleness* factor, *Yob,* discovered 12 years later (Krzywinska et al., 2016).

A novel bioinformatic approach, the chromosome quotient (CQ), was developed by Hall et al. (2013) in Anopheles mosquitoes by sequencing genomic DNA from males and females and by aligning reads to candidate reference gene sequences (embryonic transcriptomes). Autosomal genomic sequences are present in both sexes, and X-linked sequences will be twice the amount in XX versus XY individuals. In contrast, Y-linked sequences will be present exclusively in XY individuals. Hall et al. (2013) isolated Y-linked genes in *Anopheles stephensi* and A*nopheles gambiae* and attempted to isolate the primary sex-determining signals on the Y chromosomes. Criscione et al. (2013) compared high-throughput sequences of male and female genomic DNA and RNA samples. They discovered a Y-specific gene, *GUY1* (*Gene Unique to the Y*), encoding a novel protein of only 56 amino acid residues in *An. Stephensi.* The ectopic expression of GUY1 in transgenic mosquitoes leads to female-specific lethality, because of its involvement in controlling dosage compensation mechanisms (Qi et al., 2019). This protein likely corresponds to the *Maleness* factor in this species (Criscione et al., 2016) (Fig. 3).

To identify the Medfly male-determining gene promoting either directly or indirectly the unproductive male-specific *tra* splicing (Fig. 2), Salvemini et al. (2014) applied a combination of PCR-based suppression subtractive hybridization (SSH), mirror orientation selection (MOS) and differential screening hybridization (DSH) techniques. The idea was to isolate subtracted PCR cDNA fragments derived from mRNAs expressed in mixed Medfly XX/XY embryos but not in XX embryos during the narrow developmental window (8–10 h after egg laying, AEL) in which male sex determination occurs (Gabrieli et al., 2010). However, Salvemini et al. (2014) identified only one Y-linked pseudogene of unclear function, if any, and proposed *in silico* differential sexed embryonic genomic and transcriptome analyses as a future alternative strategy to clone the Medfly *M* factor.

A rather indirect and hypothesis-driven strategy for obtaining information on Medfly sex determination is based on previous information in the reference insect species *D. melanogaster*. This species requires the phosphorylation of Tra, Tra2 and the general Rbp1 factor by the Lammer kinase *doa* (*darkener of apricot*) for proper female sex determination (Du et al., 1998). The monoclonal Ab104 antibody recognizes a highly conserved phospho-epitope shared by several major SR proteins in vertebrates and invertebrates (Zahler et al., 1993). Saccone et al. (2014) speculated that in the Medfly *C. capitata* and the housefly *Musca domestica,* male-determining factors could influence the phosphorylation of SR proteins (likely including *Cctra/Cctra2* and *Mdtra/Mdtra2*). Male-specific phosphorylated SR proteins were detected in adult flies of Medfly but not in those from *M. domestica*. These data supported the hypothesis for *C. capitata* of their role (still to be confirmed) in male sex determination by post-translational regulation.

The mosquito *Aedes aegypti* has homomorphic sex chromosomes, and the *M* locus determines the male sex in this species. Hall et al. (2015) sequenced male and female genomes of *Aedes aegypti*, obtained a draft assembly, mapped genomic reads from each sex and identified a series of contigs possibly corresponding to the *M* locus region. They filtered out only one contig, which intriguingly contained a new gene, *Nix*, a distant homologue of *Drosophila transformer-2* encoding a putative splicing factor with two RNA binding domains (of RRM type). CRISPR/Cas9 mutagenesis and ectopic expression of *Nix* (embryos transient *Nix* expression from a plasmid) showed that it is necessary and sufficient for male sex determination, controlling the sex-specific splicing of downstream *Aaedsx* and *Aaefru* (see also Aryan et al., 2020)(Fig. 3). *Nix* is structurally and functionally conserved in the genome of another mosquito, *Aedes albopictus* (Gomulski et al., 2018; Liu et al., 2020a).

Krzywinska et al. (2016) performed a transcriptomic analysis of sexed *A. gambiae* embryos. They conducted mapping on a *Y*-specific scaffold (previously isolated by Krzywinski et al., 2004) to identify the *Maleness* factor, *Yob.* This gene corresponded to one of the Y-linked genes previously isolated by Hall et al. (2013) in *A. gambiae* and encodes a novel short 56 aa-long protein with a similar helical secondary structure to GUY1 of *A. stephensi* but no sequence similarity (Criscione et al. (2013). *Yob* expression starts during embryogenesis and leads -directly or indirectly - to male-specific splicing of the *Agdoublesex* gene (Fig. 3). *Yob* is vital for male survival because it likely also controls dosage compensation in XY individuals (Krzywinska et al., 2016).

Sharma et al. (2017) performed RNA-seq differential expression analyses of male and female embryos. They found a *Musca domestica* male determiner (*Mdmd*) that encodes a protein with homology to Nucampholin, a spliceosome-associated protein. Nucampholin is required for the assembly of the exon junction complex (EJC) on pre-mRNAs. *Mdmd* is a paralogue of the *Musca* autosomal *nucampholin* gene. Sharma et al. (2017) proposed that *Mdmd.* after duplication and sequence divergence, underwent neofunctionalization during *M. domestica* species evolution. *Mdmd* seems to be absent in other closely related Muscidae species. Targeted *Mdmd* disruption using CRISPR/Cas9

technology caused a complete sex reversal of genotypic males into fertile females because of a shift from male to female expression of the downstream genes *transformer* and *doublesex*.

Meccariello et al. (2019) identified the male-determining factor *Maleness-on-the-Y* (*MoY*) in Medfly via differential expression analyses of embryonic mRNAs combined with the CQ approach and PacBio genomic sequencing (in a Medfly strain with a shorter Y chromosome). *MoY* encodes a short protein of unknown biochemical function that is necessary (RNAi or Cas9 targeting experiments) and sufficient (embryos injections of genomic *MoY* DNA, or recombinant MoY protein) for the male-specific splicing of *Cctra* and hence for male sex determination (Meccariello et al., 2019). Full sex reversals of XX into fertile males and of XY into fertile females were observed, underlying the developmental flexibility of Medfly with the respect to the sex chromosomes.

In the Medfly, female sex determination is maternal (*Cctra* female-specific activation and autoregulation), while male sex determination is zygotic (*MoY* switches off *Cctra*). A homology search was performed using MoY protein sequence (tBLASTn) on genomic/transcriptomic insect databases. MoY orthologous proteins were found only in species of the Tephritidae family (*Bactrocera* genus). Functional analysis by embryonic RNAi of *MoY* orthologues in *B. oleae* and *B. dorsalis* led to the development of XY females (Meccariello et al., 2019). This functional conservation of male-determining *MoY* activity in other Tephritidae indicated that the same sex-determining primary signal could be evolutionarily stable for more than 60 million years, similarly to what is observed for the XSE primary signal among Drosophilidae (Jinks et al., 2003). In contrast, *Musca domestica* shows different male-determining factors in different populations indicating fast evolution (Dubendorfer et al., 2002; Sharma et al., 2017). The *MoY* gene corresponded to one of a dozen Y-linked transcribed sequences isolated years earlier via the CQ approach overlapping with embryonic transcriptomics, and it was considered to be noncoding and likely nonfunctional (Meccariello et al., 2019, sup mat.). The previous transcript was also overlooked because of various preconceptions, such as the lack of a potential open reading frame similar to any protein domain and the expectation of an *M* factor encoding a splicing regulator. The same gene was identified again among dozens of *Maleness* candidates from in-depth differential expression analyses - including extensive transcriptomic/genomic data production and analyses (Meccariello et al., 2019). Three novel *M* candidates were chosen almost randomly for the first round of embryonic RNAi analyses. By chance, the first one tested corresponded to the *M* factor and was named *MoY*. - This not-linear research path is similar to the one that led to the *Yob* identification in *Anopheles gambiae* (see a previous paragraph).-

In the lepidopteran silk moth, *Bombyx mori* ZW individuals develop as females and ZZ as males (Fig. 4). Abe et al. (1998 and 2005) identified RAPD markers on the female determining W chromosome. Kiuchi et al. (2014) used these markers to genotype the sexes of single embryos, perform deep sequencing of RNAs (RNA-seq) and identify differentially expressed transcripts. They found a contig/RNA expressed only in ZW embryos that did not show homology with any known sequence or encode any known protein domains. This contig corresponded to a noncoding RNA, a precursor of a piRNA, required for female sex determination of *B. mori*, and was named as *Feminizer* (*Fem*) (Fig. 4). The authors found a second key gene for sex determination, *Masc*, which is a *Fem* target. The *Masc* masculinizing function is repressed in ZW individuals by *Fem*. This study offered the first example of a sex-determining pathway controlled by the presence or absence of a piRNA (Marec, 2014; Whitworth and Oliver, 2014; Yang et al., 2021). However, Yang et al. (2021) underlined the lack of complete female-to-male reversal when *Fem* was repressed with a specific piRNA inhibitor and suggested that an additional F-factor other than *Fem* may exist, composing the primary sex-determining signal of *B. mori*.

Peng et al. (2020) proposed that miRNAs could be involved in *B. dorsalis* male sex determination. They performed a differential analysis of small RNA libraries from different embryonic stages during which sex determination occurs. They identified a miRNA, *miRNA-1-3p*, produced from an autosomal gene that shows higher expression in XY embryos than in X embryos at 7 h. The authors showed that it targets *Bdtra* transcripts, blocking the establishment of *Bdtra* autoregulation (Fig. 5). The CRISPR/Cas9-mediated knockout of *miR-1-3p* induced the sex reversal of XY individuals into phenotypic females that expressed female-specific splice variants of *Bdtra* and *Bddsx*. Furthermore, overexpression of *miR-1-3p* (with agomir) induced full masculinization of XX individuals. The authors proposed that *miR-1-3p* was required for male sex determination in early embryogenesis in *B. dorsalis* as an intermediate male determiner under the control of the Y-linked male-determining factor (likely *BdMoY*), which would promote the male-biased expression of this small RNA. Whether *miR-1-3p* has similar functions in Medfly and other Tephritidae species is still unknown.

In diploid *Nasonia* wasps, *Nvtra* produces female-specific mRNAs encoding a functional NvTra protein and male-specific mRNAs encoding truncated nonfunctional isoforms (Verhulst et al., 2010a). As described in a previous section, maternal imprinting of the upstream regulator prevents the early zygotic transcription of *Nvtra* in unfertilized eggs, which develop as haploid males. Likely because of the absence of early NvTra protein expression, transcription of *Nvtra* will result in male-specifically spliced mRNAs. In contrast, fertilized eggs receive a non-silenced paternal allele of the upstream regulator. This event leads to early transcription of *Nvtra* and production of female-specifically spliced mRNAs, starting a positive feedback loop. Recently, Zou et al. (2020) identified the upstream regulator of *Nvtra* in diploid embryos. The gene was named *wasp overruler of masculinization* (*wom*) and is transcribed only from the paternal allele. *Wom* encodes a chimeric protein containing a DNA binding domain of the P53 family fused with a dystrophin-like protein-derived duplication of a second gene (Zou et al., 2020). Its identification was achieved by mRNA-seq-based differential expression analyses of haploid and diploid embryos at 2 and 5 h after egg laying, the period during which sex determination occurs. The *wom* and *Nvtra* genes show strongly biased expression in diploid embryos (future females), with peaks at 5 h (*wom*) and 6 h (*Nvtra*), respectively. In the context of sex determination genetics, the *wom* gene provided the first example of a female instructor gene evolved after gene duplication with a parent-of-origin effect. This study also suggested that, within Hymenoptera, different genes and genetic mechanisms have evolved independently to activate *tra/fem* autoregulation only in diploid individuals (Fig. 5). *Apis mellifera* and many other related species evolved *csd* from *fem*. *Nasonia* evolved a non-imprinted paternal *wom* allele.

15. Concluding remarks

We have a deeper understanding of a part of the genetic networks controlling sex determination, especially in insect species. In the last two decades, we have gained a better understanding of the molecular evolution shaping these genetic pathways and the emergence and degeneration of sex chromosomes. The genetics of the sex determination regulatory cascade in *Drosophila melanogaster* was a key reference point for starting evolutionary studies in other insect species three decades ago by using sequence homology-based molecular approaches. Furthermore, when the *Drosophila* transposon-based gene transfer technique and RNAi were translated into other insect species, it led to the investigation of *in vivo* gene functional conservation (Handler et al., 1993; Handler and Harrell, 1999; Loukeris et al., 1995; Spradling and Rubin, 1982; Zwiebel et al., 1995; Pane et al., 2002; Wimmer, 2003).

A widespread partial evolutionary conservation of the *Drosophila* genetic pathway emerged from these studies (Bopp et al., 2014; Dubendorfer et al., 2002; Sánchez, 2008; Nagaraju and Saccone, 2010; Saccone et al., 2014; Verhulst et al., 2010a). However, understanding the genetic mechanisms underlying sex determination in insects was biased towards what was already known in *Drosophila*. It soon became apparent that other, more effective approaches were needed to work our way along the sex determination cascade in different species with

divergent primary signals (Beye et al., 2003; Nagaraju and Saccone, 2010; Saccone et al., 2014). The molecular strategies of differential hybridization and random amplification were used to search for novel genes involved in the sex determination of insect species, with only partial success (Bopp et al., 2014; Salvemini et al., 2014; Krzywinski et al., 2004; Sánchez, 2008). Then, in the last two decades, the study of insect sex determination genetics and sex chromosomes in curiosity-driven projects has added translational value to the final goals (Alphey, 2014; Bernardini et al., 2014; Burt and Crisanti, 2018; Lutrat et al., 2019; Kopp and Saccone, 2020).

Many investigated insect species are of economic, medical or veterinary relevance. Some of them are beneficial species used in biological control (*Nasonia vitripennis*, a model for parasitoid biological control) or are helpful for commercial applications (the silk moth *Bombyx mori* and the honeybee *Apis mellifera*). However, most other studied species are major agricultural pests (Tephritidae such as Medfly, olive fly, Mexican fruit fly and Oriental fruit fly) or vectors of human and other animal diseases (such as mosquitoes, sandflies, screwworms and houseflies). The increase in available research funding for this area of inquiry, together with the emergence of next-generation sequencing (NGS), novel bioinformatic tools, and gene editing technologies (TALENs, zinc finger nucleases and CRISPR/Cas9), allowed this research area to flourish, leading to novel findings concerning the upstream regulators of sex-determining genetic pathways in three different insect orders (Diptera, Hymenoptera, and Lepidoptera). FAO-IAEA promoted the use and improvement of the sterile insect technique for decades as a species-specific eco-friendly alternative to pesticides (Franz and Robinson, 2011; Bourtzis et al., 2020; Vreysen et al., 2021). The need to obtain male-only progeny of various pest insects from mass-rearing facilities has led to the isolation of sex-specifically regulated genes involved in sex determination; it is also helpful to adopt approaches such as sexually transforming karyotypically female XX embryos into adult males, as shown in Medfly and *Aedes aegypti* (Aumann et al., 2020; Aryan et al., 2020; Li and Handler, 2019; Meccariello et al., 2019; Pane et al., 2002; Salvemini et al., 2009), selectively killing females by manipulating the larval diet (Fu et al., 2007; Heinrich and Scott, 2000; Kandul et al., 2019, 2020, 2021; Schetelig et al., 2016; Thomas et al., 2000), manipulating the sex ratio (Meccariello et al., 2021), and using CRISPR/Cas9 to either target specific genes to produce sterile males and kill females (Kandul et al., 2019) or to target a gene necessary for female differentiation via a supermendelian hereditary mechanism (i.e., gene drive) (Kyrou et al., 2018) and integrating selectable markers on one sex chromosome (Condon et al., 2007; Zhang et al., 2018).

One of our next challenges will be isolating new essential master sex determination genes in other insect species and families. The second one will be understanding the biochemical and developmental functions of novel short male-determining proteins, such as Guy-1, Yob and MoY, respectively, in *Anopheles stephensi*, *A. gambiae* and Tephritidae species. Recent advances in the prediction of protein folding, protein-protein interactions, and protein-nucleic acid interactions offer additional tools for addressing these questions. Three other critical questions concerning sex determination and sexual differentiation remain unanswered even in the *Drosophila* model system: which genes shape sexually dimorphic phenotypic traits, and how do they do this? How did they evolve? Which genes shape the mating behaviour of insects? Future investigations will be required to understand the cis- and trans-regulatory elements involved in *tra* and *dsx* sex-specific splicing and their evolution, as well as the recruitment of differentiation genes under the control of Dsx and Fru. EvoDevo studies have started to shed light and propose mechanisms concerning the origin and diversification of a new sex-specific trait, such as the sex combs on the male foreleg of some *Drosophila* species (Hopkins and Kopp, 2021; Rice et al., 2019).

We have limited knowledge of the so-called splicing code problem, including the exons' prediction and alternative use (Baralle and Baralle, 2018). Other questions to be investigated are related to the temporal stages in which sex determination and sexual differentiation are still reversible and the extent of reversibility during development. The female-specific transcription of vitellogenin genes in *Drosophila* continuously depends on DsxF and the upstream regulatory network (Belote et al., 1985b; Li and Handler, 2017). On the contrary, innate sexual behaviour is irreversibly set before metamorphosis (Arthur et al., 1998). Are these genetic and epigenetic features conserved in other insect species? Another expanding area of research is related to how X-linked genes are equalized in their expression in XX and XY individuals in non-Drosophilidae species. The genetic and molecular components (Male-specific lethal complex) of dosage compensation discovered in *Drosophila* are not conserved in insect species of different families but the genetic regulatory mechanism of X-linked or Z-linked genes is present and executed by other molecules to be identified (Scott, 2021).

Additional broader questions are waiting to be addressed. The emergence of population genomics applied to insects in the wild will likely improve our understanding of the evolutionary success of sexual reproduction in nature (McDonald et al., 2016, Neiman et al., 2018). Analyses of the evolutionary cytogenetics of sex chromosomes and different dosage compensation mechanisms will take advantage of improvements in the genomic assemblies of heterochromatic and highly repetitive regions. We will likely gain a better understanding of the structure, function, emergence and degeneration of sex chromosomes (Beukeboom and Perrin, 2014; Gopinath et al., 2017; Kaiser and Bachtrog, 2010; Katsuma et al., 2019; Rosin et al., 2022; Scott, 2021; Traut et al., 2007; Krzywinska et al., 2021).

Future generations of insect molecular geneticists will be aided by novel genetic technologies and artificial intelligence, which will contribute to answering these and other complex questions related to the genetics of sex determination and sexual differentiation in the frame of the emerging systems biology field (Clough and Oliver, 2012; i5K Consortium, 2013; Bachtrog et al., 2014; Hopkins and Kopp, 2021).

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Appendix. Supplementary data

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