Review

The autoregulatory loop: A common mechanism of regulation of key sex determining genes in insects

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Sex determination in most insects is structured as a gene cascade, wherein a primary signal is passed through a series of sex-determining genes, culminating in a downstream double-switch known as *doublesex* that decides the sexual fate of the embryo. From the literature available on sex determination cascades, it becomes apparent that sex determination mechanisms have evolved rapidly. The primary signal that provides the cue to determine the sex of the embryo varies remarkably, not only among taxa, but also within taxa. Furthermore, the upstream key gene in the cascade also varies between species and even among closely related species. The order Insecta alone provides examples of astoundingly complex diversity of upstream key genes in sex determination mechanisms. Besides, unlike key upstream genes, the downstream double-switch gene is alternatively spliced to form functional sex-specific isoforms. This sex-specific splicing is conserved across insect taxa. The genes involved in the sex determination cascade such as *Sex-lethal* (*Sxl*) in *Drosophila melanogaster*, *transformer* (*tra*) in many other dipterans, coleopterans and hymenopterans, *Feminizer* (*fem*) in *Apis mellifera*, and *IGF-II mRNA-binding protein* (*Bmimp*) in *Bombyx mori* are reported to be regulated by an autoregulatory positive feedback loop. In this review, by taking examples from various insects, we propose the hypothesis that autoregulatory loop mechanisms of sex determination might be a general strategy. We also discuss the possible reasons for the evolution of autoregulatory loops in sex determination cascades and their impact on binary developmental choices.

[Sawanth SK, Gopinath G, Sambrani N and Arunkumar KP 2016 The autoregulatory loop: A common mechanism of regulation of key sex determining genes in insects. *J. Biosci.* **41** 283–294] **DOI** 10.1007/s12038-016-9609-x

1. Introduction

Sex determination is a complex developmental program that involves the fine-tuned action of numerous genes required to direct the developing embryo to either a male or female pathway. It is an essential and universal phenomenon among a majority of metazoans. Organisms show an astounding diversity in the mechanisms regulating sex determination. The primary signal that provides the cue for determining the sexual fate of an organism varies remarkably not only among taxa but also within taxa, suggesting rapid evolution of sex-determining mechanisms. Insects have evolved a variety of primary signals in sex determination pathways. A cascade of genes act upon one another to carry the information from primary signal to terminal differentiation. The three important components of the sex determination cascade are primary signal, key gene and terminal double-switch gene. The primary signal and the key gene vary across different insects but the terminal gene is highly conserved (Graham *et al.* 2003). In the presence or absence of a primary signal, a few splicing regulators interact and lead to regulated splicing in one sex and default splicing in the other. The terminal double-switch gene transcripts so formed are sex-specific and responsible for secondary sexual characters.

Keywords. Autoregulation; convergent evolution; doublesex; sex determination; splicing; transformer

Published online: 6 May 2016

There are many levels of gene regulation in eukaryotes, including the startegy most widely employed by cells, i.e. transcriptional and post-transcriptional regulation. Autoregulatory feedback loops are a kind of gene regulation found in many pathways, wherein a gene regulates its own expression. It forms an important component of post-transcriptional modification machinery. Hence, its behaviour in the course of evolution is of prime interest. Autoregulatory feedback loops can amplify and extend the response of a weak initial signal, and also buffer responses of genes to environmental changes. These loops are important for the coordination of developmental events by predefining precise domains of gene expression and pattern formation (Sonneborn *et al.* 1965; Freeman 2000). Further, they also help in maintaining constant protein concentration, which is independent of cell size and growth parameters. Importantly, autoregulation provides stability to the genetic switches (Becskei and Serrano 2000), and therefore reduces the time necessary to reach the steady state concentration for required proteins (Rosenfeld *et al.* 2002). This mechanism, hence reduces the burden on, and avoids wasting of, cell resources. In this kind of regulation, once the primary signal is triggered, maintaining the effect is not required any longer, thus serving as an excellent memory system. Therefore, it is interesting to study the autoregulatory feedback loop in insect sex determination, where the key genes reported in all insects undergo feedback-loop-based regulation. The gene regulated under the loop (the key gene) acts as splicing regulator and thus splices its own transcript leading to differentially spliced product in the two sexes. The primary signal once triggered is passed on to a terminal gene, generally *doublesex* (*dsx*), which is responsible for sex-specific traits (figure 1).

This review primarily focuses on the positive autoregulatory feedback splicing or, in short, auto-splicing/positive feedback loops, which is one of the most common post-transcriptional regulatory mechanisms seen in genes encoding RNA binding proteins (Damianov and Black 2010; Gates *et al.* 2011; Jangi *et al.* 2014), mainly in genes involved in sex determination. We also review the progress of work done in identifying the autoregulatory loop mechanism present in different insect species and explain their mechanisms (figure 2). A few of the autoregulatory loop mechanisms have been explained here to show that this may be a common mechanism of fixing the primary signal for sex determination in insects. Further, we discuss the possible reasons for the evolution of autoregulatory loops in sex determination cascades and their impact in binary developmental choices.

2. Evolution of sex determination systems in insects

Insects show vast diversity in the mechanism of sex determination. Their study offers an opportunity to understand how a basic gene network might have evolved by the addition of new players to the existing ones over a period of time. The primary signal, which varies across different orders of insects, has undergone divergent evolution to suit different triggering factors like environmental and genetic signals (Bull 1983) responsible for the fixation of sex of the embryo. Wilkins' bottom-up theory (Wilkins 1995) suggests that most conserved gene is found at the bottom of the sex determination cascade, while newly added genes are found on top of the cascade. The double-switch gene *doublesex*'s ubiquitous role and its conserved architecture in holometabolus insects at the bottom of the hierarchy signifies common ancestry in all insects. In fact, the only sex-determining gene, which shares similar protein domain across worms, flies and vertebrates is DSX. This is also supported by partial rescue of the phenotype or correct splicing of heterologous *dsx*. *D. melanogaster* and *Caenorhabditis elegans* are highly diverged on the evolutionary time scale, yet *Dmdsx* can partially complement male-specific development in *mab-3* deficient worms (Raymond *et al.* 1998). Thus, *dsx* is the oldest and most highly conserved gene at the bottom of the sex determination cascade.

The second gene in the cascade, which is highly conserved across insects, is *transformer*. *tra* regulates the *dsx* splicing. The marked features of a *tra*-like memory device, *dsx* splicing, conveying signal in an on-off mode, and the presence of *dsx RE-PRE* sites on *dsx* transcripts indicate the close realtionship between *tra and dsx* from an evolutionary point of view. Constant evolutionary pressure might have made *tra* receptive to diverse primary signals as observed in *Drosophila* (as subordinate), *Musca* (as master), and haplodiploid sex determination in *Nasonia*. *csd*in *A. mellifera* is another example of the diverse roles played by *tra* (*fem*), where the duplication of *tra* governs the primary signal for sex determination. Heterologous *Cctra* that feminizes male transgenic *Drosophila* (Pane *et al.* 2005) shows the level of conservedness. Unlike *dsx*, the variegated role of *tra* in sex determination is evident by its non functional role in sex determination in Lepidopetra and a few species of Coleoptera among holometabolus insects (Suzuki 2001, 2010a; Keeling *et al.* 2013)

The addition of master regulatory gene *Sxl* to the cascade found only in *Drosophila* suggests that it has been recruited recently in the pathway. The limited role of *Sxl* in sex determination is also evident from the fact that *Ceratitis* (Saccone *et al.* 1998) and *Musca Sxl* products could not induce a feminizing effect when expressed in *Drosophila*, although they share the same order (Meise *et al.* 1998). The acquisition of an autoregulatory loop by *Sxl* might have relieved *tra* to lose its master regulatory role, and thereby its self-splicing ability. Thus, it can be concluded that in the sex

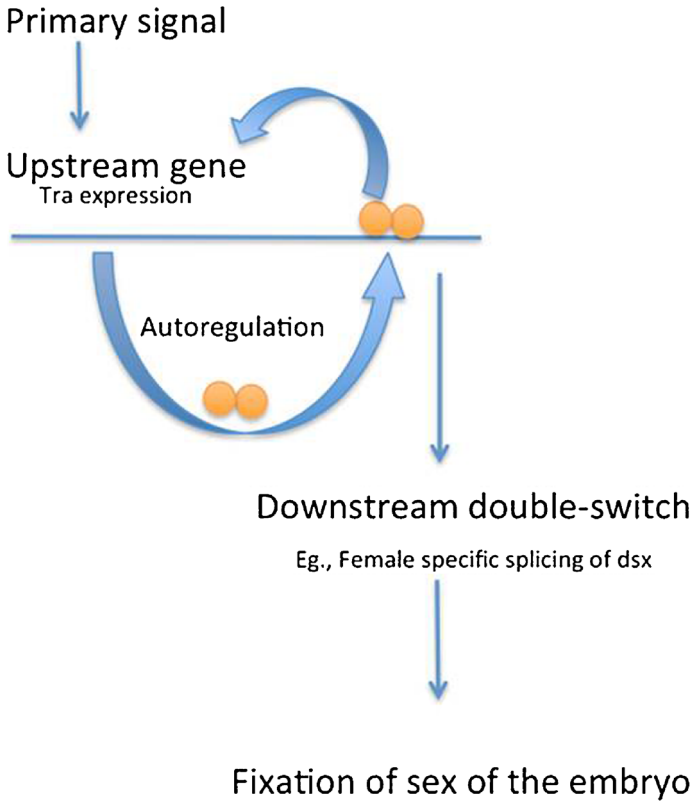

Figure 1. A model of autoregulatory loop mechanism in sex determination cascade.

determination cascade, the order of conservation of genes is reverse, where newly added genes to the pathway are found at the top, and the most conserved ones at the bottom. The executive role of *Sxl* in Drosophilidae lineage only demonstrates that sex-determining genes are evolving at an accelerated pace.

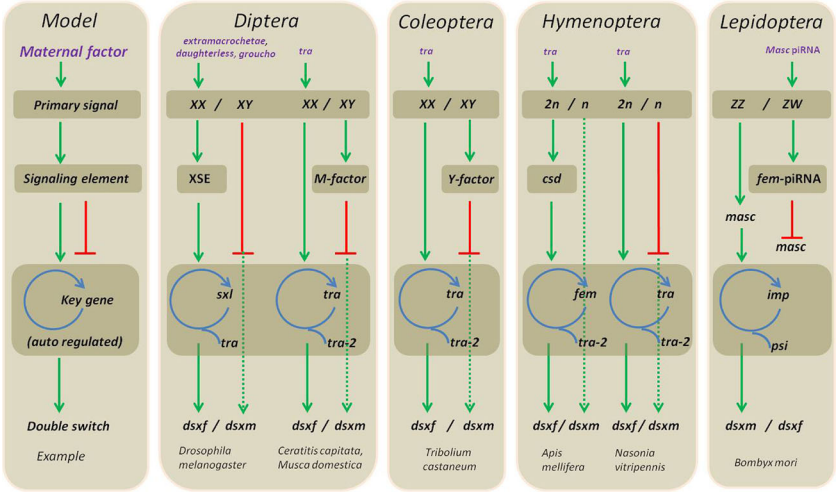

Figure 2. Schematic representation of autoregulatory genes in various insect sex determination pathways.The cascade is segregated in to various hierarchical levels wherein the autoregulation is conserved at the level of 'key gene'among phyla.

3. Autoregulatory feedback loops in sex determination pathways

Feedback loops are a major component of biochemical systems. In signalling pathways, different wirings of feedback loops result in a broad spectrum of signal responses (Hafner *et al.* 2012). A transcriptional autoregulatory feedback loop is the simplest network motif built out of a transcription factor regulating its own transcription. In higher organisms many examples of autoregulation have been reported often in the context of key regulatory processes (Kielbasa and Vingron 2008). The function of autoregulatory loops has been extensively studied. It has been shown that a negative autoregulatory circuit approaches its steady state much faster than the non-autoregulatory circuit. In contrast, positive feedback is a mechanism that converts a graded input into a binary response in a eukaryotic gene circuit. Bistability allows cells to maintain either of two distinct gene expression states, providing a mechanism by which past environmental conditions or intercellular signals can be remembered – this way a mixture of responses can be potentially achieved in a single population with genetically regulated ratios. Moreover, since bistable systems are expected to display some degree of hysteresis, their role as buffering mechanisms in preventing noise causing accidental switching between the states has been discussed (Kielbasa and Vingron 2008). The ability of a positive feedback loop system to provide a bistable system may be one of the main reasons for evolution and wiring in sex determination cascades. Therefore, auto-feedback loops could have the capacity to promote the irreversible activation of a specific sex determination pathway, and stabilize the cellular sex switch, contributing to its fixation (Zhang *et al.* 2013).

Most animal species have only two sexes (male and female), which is probably a result of well-coordinated factors involved in sex determination leading to fixation of one of the two sexes. This binary decision evolved long ago, and over time became established especially in more complex creatures. Studies clearly show sex-specific splicing leading to the binary choice in insect sex determination (Salz 2011). Work on sex determination in different insect species belonging to the orders Diptera, Coleoptera, Hymenoptera and Lepidoptera reveal the involvement of 'positive feedback autoregulatory mechanisms' in the regulation of key genes in sex determination. In insect sex determination, some autoregulatory loops like the early promoters of *Sxl* act only during early embryonic stages until the fixation of sex. However, other autoregulatory loops like *tra*/*tra-2* and *Bmimp* act throughout the life cycle of the insect similar to sex-specific alternative splicing of sex determining genes.

3.1 *Diptera*

The order Diptera includes insects belonging to many families such as Drosophilidae (*Drosophila melanogaster*), Tephritidae (*Ceratitis capitata*, *Anastrepha obliqua*, *Bactrocera oleae*), Muscidae (*Musca domestica*), Glossinidae (*Glossina morsitans*), etc. Although Dipterans show a variety of sex determination systems, yet follow a common strategy to achieve this. The cascade includes a primary signal, a key gene and a double-switch. The primary signal among dipterans varies. In *D. melanogaster*, (XX is female, while XY is male) the double dose of X chromosomes determines femaleness and a single dose directs maleness (Erickson and Quintero 2007). In other dipterans, such as *M. domestica* and *C. capitata,* a male-determining Y chromosome is employed to determine male sex determination (Dübendorfer *et al.* 2002; Pane *et al.* 2002). The primary signal is responsible for the differential expression of the key gene between the two sexes. The key gene *Sxl* (*Drosophila*), or *tra* (Tephritidae, Muscidae and Glossinidae), directly or indirectly regulates the splicing of double-switch gene. The terminal gene double-switch shows differential splicing between the two sexes and is responsible for the differentiation of traits between male and female.

In *Drosophila* the key sex-determining gene *Sxl* controls sexual development and dosage compensation (Cline 1978). The expression of *Sxl* can be classified temporally into two phases – establishment and maintenance phase. Establishment phase includes early zygotic stages up to the 14th cycle of embryogenesis, whereas later embryonic stages until adulthood denotes maintenance phase. The expression of *Sxl* is under the control of two different promoters. Early promoter (*Pe*) is active during establishment phase only in females and late promter (*Pl*) is active during maintenance phase in both sexes (Keyes *et al.* 1992). Transcription from the early *Sxl* promoter requires a set of genes including maternally supplied *groucho*, *extramacrochetae*, *daughterless* and *hermaphrodite* genes, autosomal *deadpan*, and X-linked signal elements (XSE) which includes four transcription factors, encoded by the *scute*, *sisA*, *runt* and *unpaired* genes on the X chromosome (Cline 1988; Duffy and Gergen 1991; Jinks *et al.* 2000; Sefton *et al.* 2000 Penalva and Sanchez 2003;). The interplay of these gene products leads to the transient activation of the *Sxl* early promoter (*SxlPe*) (Cline and Meyer 1996; Avila and Erickson 2007). The initial switch (activation of *Sxl*) is determined by the threshold of XSE. The twofold difference of X:A in females initiates transcription of the early *Sxl* protein but not in males as XSE is below the threshold level owing to the fact that they are hemizygous for these X-linked loci (XY) (Salz and Erickson 2010).

During the maintenance phase, after cycle 14 of embryogenesis, the early promoter shuts off and only the late promoter remains active in both the sexes. The SXL protein produced from the *SxlPe* can now guide splicing of the transcripts formed from the *Sxl* late promoter. Unlike the early promoter, *Sxl* transcription occurs equally in both sexes

at the late promoter. In females, the early SXL protein formed in establishment phase alternatively splices the late *Sxl* pre-mRNA excluding the male-specific exon, which harbours the in-frame stop codon. The so-formed functional late SXL protein along with other known key players like *sansfille* (*snf*), *female-lethal(2)d (fl(2)d*), *virilizer* (*vir*) and *partner of sansifille* (*PPS*) (Albrecht and Salz 1993; Flickinger and Salz 1994; Johnson *et al.* 2010) splices its own pre-mRNA, thus forming postive autoregulatory loop (Bell *et al.* 1991). The autoregulatory loop is maintained throughout the adult life cycle. In males, due to the absence of early SXL protein, the pre-mRNA undergoes default splicing, which includes male-specific exon, thus leading to a truncated non-functional protein. The female functional SXL protein splices *tra* in the female mode, thus carrying the signal further down the cascade.

In other dipterans (e.g. *M. domestica*, *C. capitata*, *B. oleae* and *A.obliqua*), *Sxl* is not an intermediary between the primary signal and *tra* (Meise *et al.* 1998; Saccone *et al.* 1998; Lagos *et al.* 2005; Ruiz *et al.* 2007). The *Sxl* homologue shows no sex-specific difference in the expression of mRNAs and protein isoforms irrespective of the primary signal, proving that there is no role for *Sxl* in somatic sex determination outside Drosophilids.

Transformer (*tra*) directly regulates the splicing of the terminal gene of the cascade. It is the second most highly conserved gene after *dsx* in the cascade. Tra is a splicing factor and belongs to the family of SR-type proteins, rich in arginine and serine dipeptides, called RS domain (Boggs *et al.* 1987). Other domains like the dipteran domain and hymenopteran domain are unique to Diptera and Hymenoptera respectively. The absence (Drosophilidae) and presence (Tephritidae, Muscidae and Glossinidae) of the putative autoregulation domain in *tra* suggests the operation of the autoregulatory loop in the above-mentioned insects except *Drosophila*. The *tra* undergoes sex-specific splicing and functional *tra* is formed only in females. In *Drosophila* females, active SXL protein excludes exon 2 of *tra*, which harbours several stop codons. In males, tra undergoes default splicing in the absence of functional SXL protein and retains exon 2 to produce truncated TRA protein (Boggs *et al.* 1987). Non-drosophilids show similar splicing pattern as that of drosophila *tra*. In females the maternal *tra* in zygote excludes the exon carrying the in-frame stop codon from zygotic *tra,* which yields functional TRA protein (Dübendorfer and Hediger, 1998). In females, forced splicing of *tra* is maintained throughout the life cycle by virtue of the autoregulatory loop (Jablonka and Lamb 1995; Gabrieli *et al.* 2010; Pane *et al.* 2002; Morrow *et al.* 2014). In males, a hypothetical M factor (masculanizer) on Y or autosomal chromosome inactivates maternal TRA (Schmidt *et al.* 1997). The *tra* undergoes default splicing, which results in non-functional truncated protein. The RNAi-based knockdown experiment of *tra* (*Cctra, Bdtra* and *Mdtra*) in embryo resulted in masculinized females and intersex, which has two X chromosomes (Pane *et al.* 2002; Liu *et al.* 2015). Thus *tra*, like *Sxl* in *Drosophila,* acts as the key regulatory gene for sex determination in other dipterans.

Interestingly, in the case of *M. domestica* a dominant female-determining factor ($Mdtra^{D}$) establishes feminizing activity even in the presence of male-determining M factor. $Mdtra^{D}$ is an allelic variant of the *Mdtra* (*M. domestica transformer*) gene (Hasselmann *et al.* 2008). There exist other alleles of *Mdtra*, such as $Mdtra^{man}$, which is a loss of function allele. $Mdtra^{man}$ in homozygous condition results in significantly lower levels of TRA protein and leads to male-specific splicing of the conserved *Mddsx* gene, and individuals develop into males even in the absence of *M factor*.

The downstream binary switch *doublesex* is responsible for male or female somatic sexual development (Saccone *et al.* 1996; Shearman and Frommer 1998; Kuhn *et al.* 2000; Hediger *et al.* 2004; Lagos *et al.* 2005; Ruiz *et al.* 2005; Scali *et al.* 2005). All dipteran insects have *dsx* at the bottom of the cascade (Saccone *et al.* 1996; Shearman and Frommer 1998; Kuhn *et al.* 2000; Ohbayashi *et al.* 2001; Hediger *et al.* 2004; Lagos *et al.* 2005; Ruiz *et al.* 2005; Scali *et al.* 2005). The protein DSX framework across many insects shows similar design, a Zinc finger module N-terminal DNA binding domain and oligomerisation domain. *dsx* undergoes sex-specific splicing, where the C terminal end differs between the sexes. Functional TRA protein splices *dsx* in the female mode. In males, due to lack of the active TRA, protein dsx is spliced in the default mode. Analogous to *dsx*, the *Mddsx* (*M. domestica*), *Ccdsx* (*C. capitata*), *Bodsx* (*B. oleae*) and *Aodsx* (*A. oblique*) have TRA binding sites (Saccone *et al.* 1998; Geuverink and Beukeboom 2014). This organization involves the retention of female-specific exon in XX embryos by a splicing enhancer complex which includes TRA.

3.2 *Hymenoptera*

Sex in the honeybee (*Apis mellifera*) is determined by heterozygosity at the *complementary sex determiner* (*csd*) locus (Beye *et al.* 2003). *csd* is a recently evolved gene in the honeybee lineage by virtue of gene duplication of an ancestral gene *'fem'*, and both of these paralogs are considered as orthologs of *tra* with substantive sequence divergence (Gempe *et al.* 2009). Honeybees that are heterozygous at the *csd* locus develop into females, whereas those that are homozygous or hemizygous develop as males. CSD proteins determine sexual fate in *A. mellifera*, by controlling alternative splicing of the downstream gene *feminizer* (*fem*). Heterozygosity at *csd* activates *fem* to produce female-specific mRNA and initiating the autoregulatory feminizing loop. However, CSD can direct *fem* splicing only during a narrow

window in early embryogenesis. Analogous to the late SXL protein, the female-specific FEM splices its own pre-mRNA. Female-specific *fem* guides pre-mRNA to its own splice form, when ectopically expressed in males, suggesting the operation of positive autoregulatory loop by female-specific *fem*. Thus, the *csd* gene acts as a primary signal of sex determination in the honeybee during early embryogenesis and later this signal is maintained through a positive autoregulatory loop. However, for the determination of maleness, no signal is required. The male variant of *fem* is formed by default in the absence of functional CSD, which contains a stop codon in exon 3 that results in truncated protein (Gempe *et al.* 2009).

A. mellifera transformer - 2 (*Amtra-2*), an ortholog of *tra-2*, is an important component of the splicing complex that participates in female-specific splicing of both the *fem* and *A. mellifera doublesex* (*Amdsx*) transcripts in the honeybee (Nissen *et al.* 2012). AmTRA-2 interacts with functional CSD to promote female-specific splicing of the *fem* pre-mRNAs, and is thus an important component for developing a positive *fem* autoregulatory loop. Interestingly, AmTRA-2 protein is also necessary for the splicing of male *fem* mRNAs in the presence of inactive CSD, which leads to default male-specific *Amdsx* splicing (Nissen *et al.* 2012).

In the parasitoid wasp *Nasonia vitripennis,*as in *Apis*, diploids develop as females and haploids as males. However, in spite of some similarities in sex determination cascades with other insects, the *N. vitripennis* sex determination pathway is unique in many ways. In this species, sex is not determined by CSD as observed in many hymenopterans. Although the primary gene involved in the sex determination pathway is not yet identified, *tra* of *Nasonia* (*Nvtra)* proves to be a crucial gene whose maternal supply is essential for female development (Verhulst et al. 2010a, b). The presence of ample amounts of the female form of *Nvtra* mRNA or protein from the maternal contribution is required to maintain the female-specific splicing pattern of *Nvtra*. Analogous to *Apis*, sex determination in *N. vitripennis* is dependent on the paternally inherited genome in maintaining the *Nvtra* sex determination autoregulatory loop. Maternal input of *Nvtra* mRNA/protein autoregulates embryonic splicing of *tra,* only if a paternally inherited genome is present, as observed in fertilized eggs. In unfertilized eggs, *Nvtra* remains transcriptionally silent and fails to autoregulate *tra*, leading to default *Nvtra* and *Nvdsx* splicing, and finally to male-specific development. This precise role of the paternal genome in *Nasonia* sex determination pathway constitutes a novel and unique mechanism in the way *tra* is regulated (Beukeboom and van de Zande 2010).

Nvtra produces three splice forms in males and none of these code for any functional protein, while in females, a functional protein is synthesized as a result of splicing of the second exon which results in excluding a stop codon. RNAi-based knockdown of *Nvtra* in female pupae resulted in diploid males rather than female progeny, suggesting the requirement for a maternal TRA supply and thereby the existence of autoregulation of maternally supplied *Nvtra* transcripts in female development (Beukeboom and van de Zande 2010). These observations suggest that the NvTRA protein product promotes female-specific splicing of *Nvtra* transcripts by regulating its own levels. Both *fem* of *A. mellifera* and *Nvtra* of *N. vitripennis* act as splicing inhibitors of their own pre-mRNA splicing and as splicing activators of *dsx* splicing. The only differences are the nature of the primarysignal regulating these genes (for *Apis* it is CSD, while for *Nasonia* it is yet to be discovered). The most unique feature of Hymenopterans is the dependence of *fem*/*tra* on the parental inherited genome for the sustenance of an autoregulatory loop.

3.3 *Coleoptera*

In *Tribolium castaneum*, sex is determined by the XX/XY sex chromosome system and includes known genetic components such as *transformer* (*Tctra*), *transformer-2* (*Tctra-2*) and the highly conserved binary switch *doublesex* (*Tcdsx)*.A positive autoregulatory loop at the level of *tra* is also observed in the coleopteran *T.castaneum*. The maternal supply of *tra* transcript is crucial for the initiation of the positive autoregulatory loop, which eventually directs the female developmental pathway. (Shukla and Palli 2012). *Tctra* produces two splice forms in males (encoding truncated proteins) and one in females (encoding a functional protein). Maternally deposited *Tctra* mRNA is translated to TcTRA protein only in females. TcTRA protein then assists in the splicing of its pre-mRNA transcribed from the zygotic genome ensuring constant supply of TcTRA in females, leading to positive autoregulatory feedback loop as well as sex-specific splicing of *Tcdsx*. In males the translation of maternally supplied *Tctra* transcript is inhibited by an unknown component from Y chromosome, which eventually prevents *tra* autoregulation. Knockdown studies of *Tctra* in pupa or adults, which led to all male progeny, and the existence of multiple putative binding sites for TRA/TRA-2, ISS sequences and RBP1 binding sites in adjacent intron and male-specific exon, signifies the role of *Tctra* in autoregulatory loop and in the maintenance of its female-specific splice form (Shukla and Palli 2012).

3.4 *Lepidoptera*

The genetic and molecular mechanism of sex determination in *B. mori* is substantially different from other insect orders, which can be attributed to difference in sex chromosomes, the actual molecular mechanism and the genes involved in

the pathway. The major genetic difference in *B. mori* when compared to the insects of other orders is the presence of heterogametic sex chromosome system in females. Interestingly, it is the only model organism where a single non-coding RNA (piRNA) acts as the primary signal for sex determination. The splicing mechanism of the conserved binary switch gene (*Bmdsx*) involves a splicing inhibitor complex in males of *B. mori,* whereas in female dipterans, a splicing enhancer complex is recruited. In spite of so many variations in *B. mori* sex determination system, the key gene is still known to be involved in positive autoregulatory loop, similar to that observed in other insects studied. However, it is important to note that in all insect orders except Lepidoptera, the autoloop is maintained in females, whereas in *B. mori* it is in males that the positive autoloop is observed in sex determination.

The silkworm has female heterogametic sex chromosome system, where males are ZZ and females are ZW (Suzuki 2010b). The search for the key sex determining factor on the W chromosome has been hampered by the fact that it is constituted mostly of repetitive sequences and transposons. Similar to that of the *D. melanogaster* sex determination pathway, *B. mori* exhibits evolutionarily conserved *dsx* at the base of the pathway, which undergoes sex-specific splicing (Cho *et al.* 2007). However, the absence of a *tra* orthologue in *B. mori*, and the RNAi-based knockdown of *Bmtra-2* failing to alter sex-specific splicing of *Bmdsx* pre-mRNA, indicate that the mechanism of sex-specific splicing at the *dsx* differs significantly between *D. melanogaster* and *B. mori*. The other remarkable difference lies at the top of the pathway in *B. mori*, where a piRNA (processed from precursor '*fem*') originating from the W chromosome has been shown to be acting as the primary signal. This 29 nucleotides long piRNA negatively regulates a CCCH-type zinc finger gene *Masculinizer* (*Masc*) on the Z chromosome. *Masc* is reported to be involved in dosage compensation and is required for the male-specific splicing of *doublesex* (*Bmdsx*) (Kiuchi *et al.* 2014). *B. mori* homologs of *IGF-II mRNA-binding protein (Bmimp)* and *P-element somatic inhibitor* (*Bmpsi*) play pivotal roles in the sex determination pathway (Suzuki *et al.* 2008). *Bmpsi* is expressed in both females and males, while one of the two splice variants of *Bmimp* is specifically expressed in male tissues (Suzuki *et al.* 2010b). The protein product of the male-specific isoform of *Bmimp* (IMP^M) is known to physically interact with BmPSI *in vitro*, and is probably involved in male-specific splicing of *Bmdsx* by acting as splicing inhibitors, promoting the skipping of exons 3 and 4 of *Bmdsx* pre-mRNA. In ZZ individuals, *Masc* expression is not under the regulation of *fem* and hence the levels remain much higher than ZW animals. The higher level of MASC protein enhances the expression of BmIMP to a level that is sufficient to impose the male-specific splicing of *Bmimp* pre-mRNA and subsequently *Bmdsx*, leading to male development (Suzuki *et al.* 2014).

In *B. mori* males, *Bmimp* is alternatively spliced in males, producing $BmImp^M$ in lower amounts, when compared to the non-sex-specific *Bmimp*. $Bmimp^M$ is produced as a result of retention of exon 8, which is mediated by the autoregulation of its own protein product. This autoregulation involves the binding of BmIMP protein to a 22 nt A-rich sequence in the intronic region between exon 7 and exon 8 of *Bmimp* pre-mRNA, suppressing the general splicing machinery (Sakai *et al.* 2015). Hence, the $Bmimp^M$ autoregulation could be presumed as a memory device in *Bombyx* sex determination, similar to *Drosophila Sxl* and *tra* of several dipterans and hymenopteran insects (Suzuki *et al.* 2014).

4. Role of autoregulation of sex determining genes in germline

An additional loop is reported in somatic cells from the gonads of female *D. melanogaster*. The presence of TRA binding sites on pre-mRNA of *Sxl* suggests that TRA stimulates the SXL positive autoregulatory feedback loop. This event is carried over to the other neighbouring germ cells non-autonomously by cell-to-cell interactions (Horabin 2005). Thus, *tra* indirectly forms a positive autoregulatory loop via SXL in females. In *D. melanogaster* somatic tissues, TRA-2 along with TRA is responsible for the differential splicing of *dsx* and *fruitless* (*fru*) (Belote and Baker 1983). In male germinal cells TRA-2 is required for the differential splicing of *exuperantia* (*exu*) and *alternative-testes-transcript* (*att*), which are involved in spermatogenesis (Hazelrigg and Tu 1994; Madigan *et al.* 1996). In male germ cells, *tra-2* undergoes differential splicing, giving rise to two isoforms TRA-2^{179} and TRA-2^{226}(Amrein *et al.* 1990; Mattox *et al.* 1990). Retention of intron 3 results in the formation of TRA-2^{179}, and its exclusion gives rise to TRA-2^{226}(Mattox and Baker 1991; Mattox *et al.* 1996). The splicing in or out of intron 3 is dictated by the amount of TRA-2^{226} present in the cell by the negative autoregulatory loop. Accumulation of TRA-2^{226} results in the repression of the splicing of intron 3, which carries the in-frame stop codon, resulting in the formation of truncated protein, which lacks RS domain. TRA-2^{226} is required for male fertility. Thus, TRA-2 controls the splicing of intron 3 by negative autoregulatory loop (figure 2).

5. The mechanism of *tra* autoregulation

Functional TRA (in Tephritids) and SXL (in *D. melanogaster*) are produced only in females due to the initiation and maintenance of autoregulatory feedback loop. The basic plan in the autoregulation/female-specific splicing of *tra* pre-mRNA is to splice out the inherent stop codon containing part/complete exon(s). In Tephritids, the

mechanism of *tra* autoregulation involves TRA/TRA-2 binding sites, RBP1 binding sites, and TRA-2 -ISS (TRA-2 binding intronic silencer sequence). TRA protein along with RBP1, TRA-2 and an unknown SR protein from nuclear extract form a splicing complex in females. For the *dsx*-pre-mRNA splicing, this complex acts as an activator and promotes splicing (Hoshijima *et al.* 1991; Tian and Maniatis 1993) and for the *tra* pre-mRNA splicing, the TRA/TRA-2 complex eliminates the stop codon containing male-specific exons in females. The binding of functional TRA/TRA-2 protein dimer complex to its binding sites near the splice sites of the exon to be skipped, hinders the general splicing machinery, so that the splice site becomes unrecognized, resulting in the splicing out of that particular exon. Thus, TRA/TRA-2 protein complex functions as a splicing inhibitor complex in case of its own pre-mRNA splicing, just like the BmPSI/BmIMP in *B. mori*, whose function is to skip the inclusion of exon 3 and 4 of *Bmdsx* transcripts in males (Suzuki *et al.* 2010b). This silencing complex of TRA is responsible for its autoregulation in generating the active, full-length protein in females. Thus TRA complex exhibits a dual role in the splicing of *dsx* and *tra* pre-mRNAs. The only difference in the silencing/activating complex is the presence of a TRA-2 -ISS binding element in the *tra* pre-mRNA, to which an extra TRA-2 protein binds and makes the silencer complex (Ruiz *et al.* 2007). Thus, the presence of TRA-2-ISS element in the *tra* pre-mRNA suggests the key requirement for its autoregulation. The *Drosophilatra* pre-mRNA lacks such TRA-2-ISS element along with TRA binding sites, which is evident by the absence of *tra* autoregulation. This autoregulation of *tra* at the level of splicing in insects ensures female-specific expression of active TRA protein and maintenance of protein levels by a positive feedback loop.

6. Convergent evolution of autoregulatory loops in sex determination

The long-standing biological mystery of sex determination and diversity was studied by comparing distant and close animal relatives, which givesa better understanding of the evolution of sex determination genes and pathways. As mentioned in this review, sex determination in insects display an array of different mechanisms to produce mainly two sexes – either male or female, and this holds true for all metazoans, suggesting the existence of sexual dimorphism in the last common ancestor of the bilaterians, a vast clade of animals that excludes sponges, placozoans, ctenophores, cnidarians, and acoel flatworms (Peterson and Eernisse 2001). Studying insects from different orders shows conservedness at the level of terminal double-switch gene *dsx* and the key gene *tra* to some extent. Interestingly, another important aspect, which seems to unify many insects, in the way in which sex is determined, is the presence of positive autoregulatory feedback loops involving the key genes of the sex determination pathways (e.g. *Sxl, tra* and *Bmimp*).

Alternate splicing helps an organism to code for multiple mRNAs from a single gene and this mechanism is widely employed in sex determination pathways of various insect species. Similar to alternative splicing, we speculate that positive autoregulatory feedback loops would have co-evolved parallely in sex determination cascades to arrive at a binary decision, which is crucial for sex determination. However, alternative splicing-based gene regulation exists both at upstream (key gene) and downstream genes in sex determination cascade, contrary to autoregulation mechanism, which is known to act only at upstream (key genes) genes (figure 2). This convergence is observed at a specific position in which the loop operates and we do not see this loop functioning at the level of double-switch or any other downstream players of sex determination cascades.

The sex-specific genetic makeup of chromosomes differs significantly in case of *B. mori,* where females are heterogametic. However, the situation in Diptera, Hymenoptera and Coleoptera is reversed, which is evident by heterogametic males. The autoloop is exhibited at the level of *Bmimp* in *B. mori* males, which is a rather unique scenario, as autoloops are always found in the female sex of other insect orders. Interestingly, a unifying aspect related to positive autoloops in sex determination of insects is that they are always present in the homogametic sex irrespective of the sex chromosome system of that insect (male or female heterogametic system), signifying the convergent evolution of positive autoloops in sex determination systems of insects. From the above observation, we speculate that genes involved in sex determination pathway may not exhibit autoloop mechanism in the case of insects lacking sex chromosomes (having homomorphic chromosomes).

7. Maternal contribution and autoregulatory loops in sex determination

In almost all metazoans there is a lag between fertilization and zygotic genome activation. During this time, the embryo depends on the maternal mRNAs and proteins deposited in the egg during oogenesis (Tadros and Lipshitz 2009). This maternal contribution plays a very important role during various phases of embryonic development until its zygotic genome is activated (e.g. anterior/posterior axis determination in *Drosophila*, sex determination in insects, etc.).

The following is the summary of a few examples from this review describing the importance of maternal input in autoregulatory loops in sex determination (figure 2):

1. Diptera: In *D. melanogaster*, initial activation of *Sxl* early promoter in females requires maternal contribution

in the form of *groucho*, *extramacrochetae*, *daughterless* proteins, along with XSE proteins from two doses of X chromosome. This initial activation of early promoter leads to turning ON of the late *Sxl* promoter. The splicing of *Sxl* in female is maintained by an autoregulatory feedback loop where SXL splices its own pre-mRNA in female and the subsequent gene in the pathway *tra*. The maternally contributed *tra* plays a pivotal role in initiating positive autoregulatory loop in *C. capitata,* which later on is maintained throughout the lifetime of the insect by zygotic *tra*. Similarly, in *M. domestica*, it is the maternally deposited TRA that ensures autoregulatory *tra* loop in females, which is disrupted in the presence of yet to be identified *Mfactor* in males.

2. A slight and interesting variation of *tra* autoregulation is observed in the hymenopteran *N. vitripennis*, where maternally contributed *tra* can direct positive autoregulation loop only in the presence of paternal genome.
3. Maternal *tra* is again a prerequisite in starting autoregulatory loop in coleopteran *T. castaneum*.

Based on the above examples it is quite evident that maternal input of *tra* mRNA/protein in the eggs during oogenesis retains the capacity to initiate *tra* autoregulation. This *tra* autoloop has been demonstrated in almost all examined species except *D. melanogaster*, *A. mellifera* and *B. mori*, suggesting maternal contribution of *tra* to eggs to be an ancestral mechanism, and that all exceptions from this system have evolved recently.

4. In *B. mori*, one male isoform of $Bmimp^{M}$ displays autoregulation, is required for male-specific splicing of *dsx* gene and eventually male development. In females, maternal supply of *Masc* piRNA along with BmAgo3 prevents *Bmimp* autoregulation by down regulating its activator *Masc* by generating more piRNAs against *Masc*via a ping-pong mechanism.

The above examples allow us to speculate that in the species where females are homogametic (e.g. XX), the maternal factor contributes in a positive manner in maintaining autoregulatory loops of the gene involved. However, in case of females being heterogametic (e.g. ZW), there seems to be a suppression of autoregulatory loop to promote female sex determination. Since we have limited information related to female heterogametic system in insects, further study might help us understand the correlation between maternal factor and female sex chromosome composition in insects.

8. Conclusions

In the context of insect sex determination, *tra* happens to be the most prominent gene involved in the autoregulation mechanism, which is evident by its maintenance through the positive autoregulatory loop in all well-studied insect models except *D. melanogaster* and *B. mori*. In the case of *D. melanogaster*, *Sxl* plays the crucial role of key gene by undergoing positive autoregulatory loop and epigenetically maintaining female developmental pathway. Previous work involving *Sxl* and its target *tra* in *D. melanogaster* (Siera and Cline 2008) predicted that the regulatory functional redundancy might have facilitated the evolutionary transition from *tra* to *Sxl* as the positively autoregulated master sex switch. The *tra* gene represents the ancestral state, whereas *Sxl* appears to be a recent evolutionary acquisition in the *Drosophila* pathway, which has forced *tra* to be a mere downstream transducer. However in *B. mori*, no *tra* homolog is found and its function of splicing *Bmdsx* is taken over by *Bmimp,* a RNA binding protein present on the Z chromosome that undergoes positive autoregulation in males.

In insect sex determination, the mechanism of autoregulation seems to be conserved at the level of the key gene (figure 2: *Sxl*, *fem*, *tra* and *Bmimp*). The importance of this kind of autoregulation at the level of splicing is evident by its conservation across insect phyla. Studies involving various insects strongly suggest that only one gene and particularly the gene which is just upstream of the binary switch *dsx* shows a positive autoregulatory loop (*tra, fem* and *Bmimp*) with the exception of the situation observed in *D. melanogaster*. Once activated, the autoregulatory loop takes control of the production of functional proteins and maintains their required levels throughout the development, aiding in the establishment of sex. Since the gene under loop controls the downstream gene and its own RNA, the effect of temporary loss of function of the gene (under loop) by means of RNA interference will have a greater effect than any other gene in the cascade. Thus, the autoregulatory loop mechanism may provide an opportunity to design molecular tools for the development of sterile insect technology.

Acknowledgements

This work is sincerely dedicated to late Dr J Nagaraju (demise 31 December 2012). SKS and GG are recipients of senior research fellowships from the Council of Scientific and Industrial Research, India. We thank Prof Leonard Rabinow for his critical review of the manuscript. We acknowledge the financial support from the Indo-French Center for the Promotion of Advanced Research (IFCPAR award 4903-4).

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MS received 05 November 2015; accepted 15 April 2016

Corresponding editor: Stuart A Newman