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Sex-determining mechanisms in insects

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ABSTRACT Sex determination refers to the developmental programme that commits the embryo to either the male or the female pathway. The animal kingdom possesses a wealth of mechanisms via which gender is decided, all of which are represented among the insects. This manuscript focuses on a number of insects for which genetic and molecular data regarding sex determination mechanisms are available. The sex determination genetic cascade of *Drosophila melanogaster* is first discussed, followed by an analysis of the sex determination genes of other dipteran and non-dipteran insects. Representative examples of sex determination mechanisms that differ in their primary signal are also described. Finally, some evolutionary aspects of these mechanisms are discussed.

KEY WORDS: *Drosophila, Apis, Sciarids, Coccids, heterochromatisation, chromosome elimination*

Introduction

Perpetuation by sexual reproduction is the rule within the animal kingdom. Males and females are different at the morphological, physiological, and behavioural levels. This sexual dimorphism results from the integration of two processes: sex determination and sexual differentiation. Sex determination refers to the developmental programme that commits the embryo to either the male or the female pathway. The genes underlying this programme are the *sex determination genes*. Sexual differentiation refers to the expression of the *sex-cytodifferentiation genes* (which are controlled by the *sex determination* genes), the expression of which give rise to the formation of the sexually dimorphic structures that characterise the male and female adults.

The animal kingdom possesses a wealth of mechanisms via which gender is decided (Bull, 1983). This is no more evident than among insects, among which all known types of sex determination mechanism are represented. These mechanisms can be classified into three main categories depending on the origin of the primary, genetic, sex determination signal, which can be zygotic, maternal or environmental.

Sex determination can be based on chromosome differences, one sex being homomorphic and the other heteromorphic for the sex chromosomes. This is seen in *Drosophila*, in which sex determination is based on the ratio between the X and the autosomal chromosomes: females are 2X;2A and males are X;2A (X stands for the X chromosome and A stands for a haploid complement of autosomes). Recently, it has been reported that the X/A signal predicts sexual fate instead of actively specifies it, so that it is the number of X chromosomes rather than the X/A signal what has an instructive role in sex determination (Erickson and Quintero, 2007, and cites therein). In other species, the heteromorphic sex carries the male-determining factors on the Y chromosome. This is true of the tephritid flies (*Ceratitis*, *Bactrocera* and *Anastrepha*) and *Musca*. In some *Musca* populations, the male-determining factor may, instead, be located on one of the autosomes. In all these examples, the female is the homomorphic sex (XX), and the male is the heteromorphic sex (XY). However, there are insects, such as the lepidopterons (butterflies and moths), in which the male is the homomorphic sex (ZZ) and the female the heteromorphic sex (ZW) (the letters Z and W are used to distinguish this system from the XY system) (for a revision on the relationships between ZW and XY sex chromosome systems see Ezaz *et al.*, 2006, and cites therein). In other genera, the chromosomal difference between males and females is based on haploidy/diploidy, e.g., in *Apis*, females are diploid and males are haploid.

In most species, the sex of an individual is fixed at fertilisation, as in *Drosophila, Ceratitis, Bactrocera, Anastrepha* and *Musca*, the chromosomal constitution of the zygote being a direct consequence of the chromosomal constitution of the gametes (Bull, 1983). However, in other species the chromosomal differences determining sex are brought about by the specialised behaviour of sex chromosomes during the first stages of embryonic development. The differential elimination of sex chromosomes as a

Abbreviations used in this paper: CSD, complementary sex determination.

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mechanism of sex determination is seen in *Sciara*, in which all zygotes start with the 3X;2A constitution; the loss of either one or two paternal X chromosomes determines whether the zygote becomes 2X;2A (female) or X0;2A (male). In the coccids, the differential elimination or heterochromatisation of all the paternal chromosomes gives rise to functional diploid or haploid zygotes, which develop into females or males respectively.

Finally, environmental sex determination is also seen among the insects. The sex ratio of some *Sciara* species depends on the temperature at which the embryos develop (see below).

At the gene level, sex can be controlled by a single locus or by multiple loci, the gene or genes involved showing zygotic and/or maternal effects (Bull, 1983). A classic example of sex determination based on a single locus with several alleles - *complementary sex determination* (CSD) - is seen in the honeybee *Apis mellifera*. Heterozygotes at the sex-determining locus are normal fertile females, whereas homozygous diploid zygotes develop into sterile males. Haploids are hemizygous at the sex locus and develop into functional males (see below).

The blowfly *Chrysomya rufifacies* provides an example of maternal sex determination in which no heteromorphism of the sex chromosomes exists, the sex of the zygote being exclusively determined by the genotype of the mother. In this insect, two types of female exist: gynogenic females, which only produce female offspring, and androgenic females, which only produce male offspring. The gynogenic females are heterozygous for the gene *F*, which encodes a maternal factor that accumulates in the oocytes during oogenesis and which imposes female development on the zygotes derived from them. Androgenic females and males are homozygous for the recessive *f* allele, which does not produce the maternal factor.

The present manuscript focuses mainly on the insects with different types of sex determination mechanisms for which genetic and molecular data are available. The sex determination genetic cascade of *D. melanogaster* is discussed in the next section, followed by an analysis of the sex determination genes of other dipteran and non-dipteran insects. Later, representative examples of sex determination mechanisms that differ in their primary signal are discussed. Finally, some evolutionary aspects of these systems are examined.

The sex-determining genetic cascade of *Drosophila melanogaster*

This section briefly reviews the genetic basis of sex determination in *D. melanogaster*, an organism for which a coherent picture of sex development is now emerging. This has been made possible thanks to the combination of sophisticated genetic and molecular techniques available for use with this paradigmatic insect. For detailed information on sex determination in *D. melanogaster*, the interested reader is referred to other reviews (MacDougall *et al.*, 1995; Cline and Meyer, 1996; Schütt and Nöthiger, 2000; Sánchez *et al.*, 2005). For other aspects on sex-determining mechanisms in other organisms see revisions by De Loof and Huybrechts (1998), Lalli *et al.* (2003) and Manolakou *et al.* (2006). This revision focuses on sex determination in the somatic component of the animal. The germ line shows sexual dimorphism, as does the somatic tissue. Cells with the 2X;2A chromosomal constitution follow the oogenic pathway, while XY;2A cells develop into sperm. Sex determination in the germ line is not discussed here; the interested reader is referred to the reviews of Cline and Meyer (1996) and Oliver (2002).

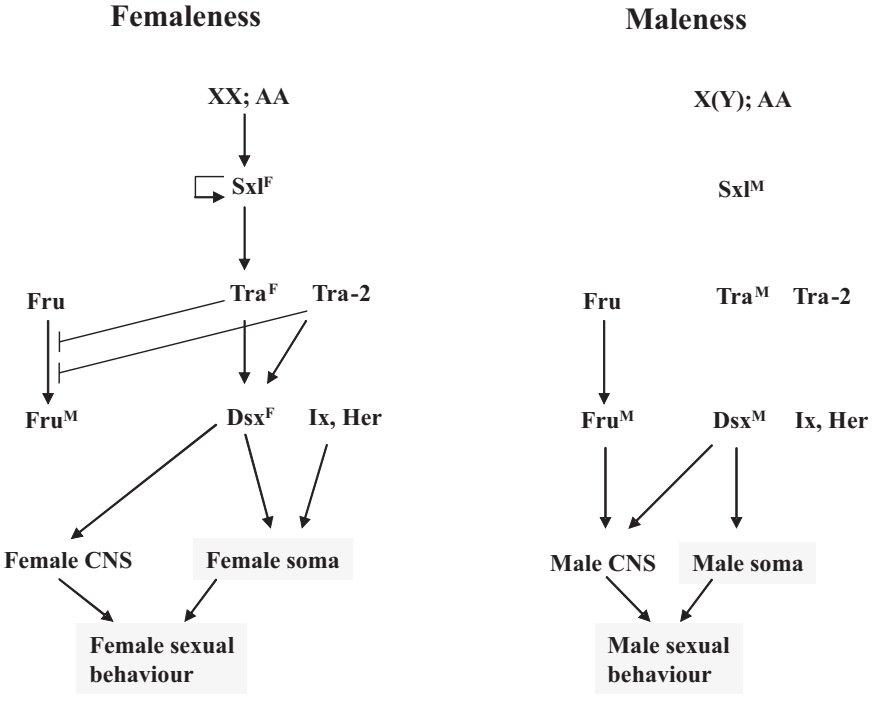

Fig. 1. The sex determination gene cascade in *Drosophila melanogaster*. *Sxl^F and Sxl^M stand for functional female and non-functional truncated male Sxl protein respectively. Tra^F and Tra^M stand for functional female and non-functional truncated male Tra protein respectively. Dsx^F and Dsx^M stand for functional Dsx protein in females and males respectively. Fru^M stands for functional Fru protein in males. In the absence of the X/A signal in males, the default state corresponds to the production of Sxl^M, Tra^M, Dsx^M and Fru^M proteins. Ix and Her proteins are produced in both sexes. CNS, central nervous system. For description of the genes and their regulation, see text.*

In *D. melanogaster*, sex determination is under the control of the gene *Sex lethal (Sxl)* (Cline, 1978; reviewed in Penalva and Sánchez, 2003). The epistatic relationships between *Sxl* and the other sex determination genes *transformer (tra)*, *transformer-2 (tra-2)* and *doublesex (dsx)* have revealed that a hierarchical interaction exists among them (Baker and Ridge, 1980). Their characterisation has shown that sex-specific splicing of their primary transcripts controls their expression during development, the product of one gene controlling the sex-specific splicing of the pre-mRNA of the downstream gene in the cascade (see Fig. 1).

The gene *Sxl*, which is at the top of this cascade, has two promoters, the so-called early and late promoters (Salz *et al.*, 1989) (see Fig. 2A). The early promoter functions only in females and responds to the primary genetic X/A

(ratio of X chromosome to autosomal sets) signal, which is transmitted in 2X;2A (females) but not in XY;2A (males) flies; this controls *Sxl* at the transcriptional level (Torres and Sánchez, 1991; Keyes *et al.*, 1992). *Sxl* is activated in females but not in males (the Y chromosome plays no role in *Sxl* activation); the result is the production of early Sxl protein in females but not in males. Once the activity of *Sxl* is determined, an event that occurs around the blastoderm stage, the X/A signal is no longer needed and the activity of *Sxl* remains fixed (Sánchez and Nöthiger, 1983; Bachiller and Sánchez, 1991). The capacity of *Sxl* to function as a stable genetic switch throughout development and during adult life is owed to a positive autoregulatory function of this gene (Cline, 1984); this constitutes the device memory for sex determination. The biological role of the primary genetic X/A signal is therefore to transiently «switch on» the early *Sxl* promoter, providing females with the early Sxl proteins needed to establish female-specific control of *Sxl* once the late constitutive promoter of this gene begins its function. The identification of a set of genes involved in the initial step of *Sxl* activation indicates that a conventional genetic system is at the basis of the X/A signal. To learn more about the molecular nature of this signal, the interested reader is referred to Cline (1993), Cline and Meyer (1996), and Sánchez *et al.* (1994, 1998, 2005). For a theoretical analysis of the formation of the X/A signal and its effect on *Sxl* activation, see Louis *et al.* (2003).

After the blastoderm stage, the late *Sxl* promoter begins to function in both sexes, and production of the late *Sxl* transcripts persists throughout the remainder of development and adult life. The male transcripts differ from the female by the inclusion of a male-specific exon that places stop codons in the open reading frame of mature mRNAs. This gives rise to truncated, non-functional Sxl proteins. In females, this male-specific exon is spliced out and functional Sxl proteins are produced (Bell *et al.*, 1988; Bopp *et al.*, 1991). Therefore, the control of *Sxl* after the blastoderm stage and throughout development and adult life occurs by the sex-specific splicing of its transcript, such that functional Sxl proteins are only produced in females (Fig. 2A). The autoregulatory function of *Sxl* takes place at the level of splicing: the Sxl protein participates in the female-specific splicing of its own primary transcript (Bell *et al.*, 1991).

The female-specific splicing of late *Sxl* pre-mRNA requires, in addition to the Sxl protein, the function of other genes, such as

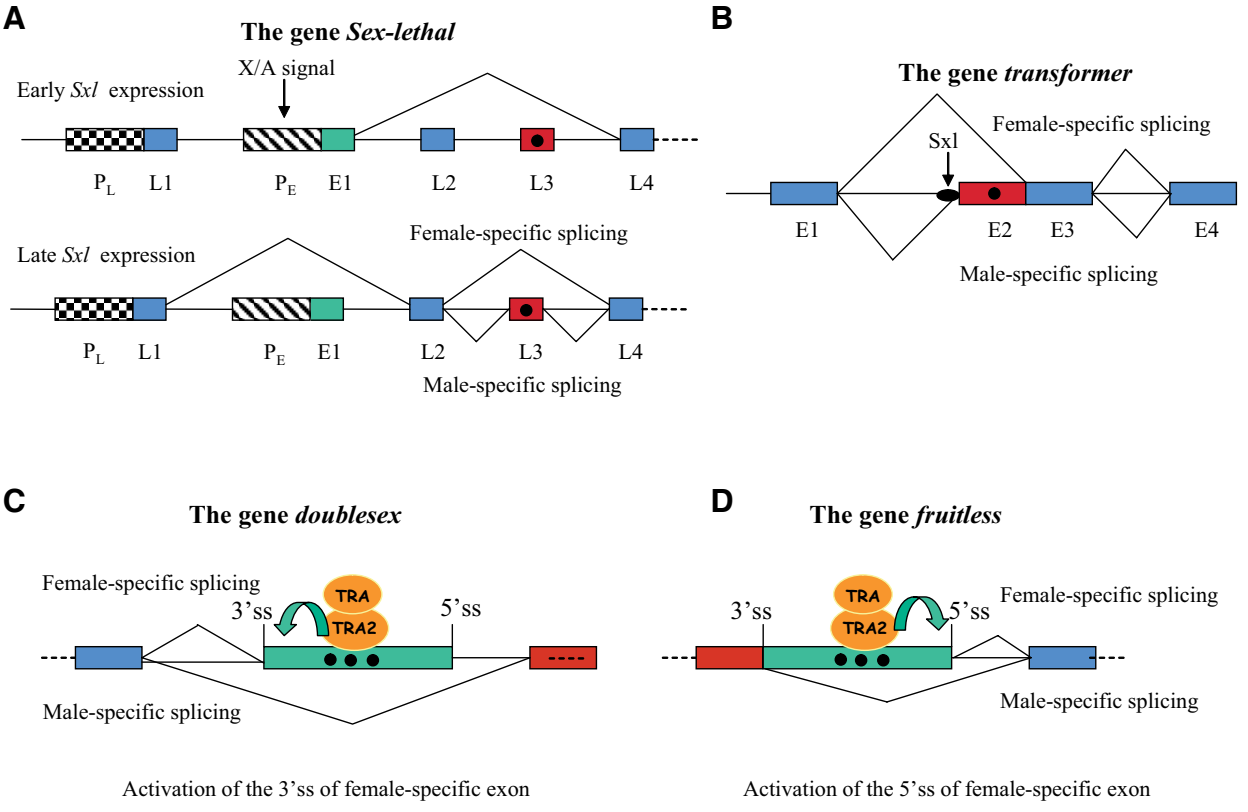

Fig. 2. Sex-specific splicing of *Sxl* (A), *tra* (B), *dsx* (C) and *fru* (D) pre-mRNAs. *Boxes and thin lines represent exons and introns respectively.* **(A)** Sxl *produces two temporally distinct sets of transcripts corresponding to the function of its two early (P_E) and late (P_L) promoters. The X/A signal acts on the early promoter (P_E). The early* Sxl *primary transcripts follow a fixed splicing pattern in which exon L2 and the male-specific exon L3 (in red) are excluded, and in which the early specific exon E1 (in green) is directly spliced to exon L4. Exon L4 and the exons downstream from it are present in both early and late Sxl mRNAs. The dot inside the male-specific exon L3 denotes the presence of translation stop codons.* **(B)** *The black ellipse represents the sequence to which both Sxl and U2AF proteins bind. The binding of the former prevents the binding of the latter. The dot inside the male-specific exon E2 denotes the presence of translation stop codons.* **(C,D)** *The small black balls inside the female-specific exon (red box) denotes the Tra-Tra2 binding sites. This complex activates the 3'splicing site (3'ss) of the female-specific exon in the* dsx *pre-mRNA, whereas it activates the 5'ss (the 5' splicing site) of the female-specific exon in the* fru *pre-mRNA. The blue, red and green boxes represent common and male- and female-specific exons, respectively.*

sans fille (snf) (Albrecht and Salz, 1993; Flickinger and Salz, 1994; Salz and Flickinger, 1996; Samuels *et al.,* 1998), *female-lethal-2-d (fl(2)d)* (Granadino *et al.,* 1990; 1996; Penalva *et al.,* 2000; Ortega *et al.,* 2003), and *virilizer (vir)* (Hilfiker and Nöthiger, 1991; Hilfiker *et al.,* 1995; Niessen *et al.,* 2001). These genes, however, play no role in the splicing pattern of early *Sx/*transcripts (Horabin and Schedl, 1996).

*Sxl*controls the splicing of the pre-mRNA from the downstream gene *tra*. This gene is transcribed in both sexes, but its RNA follows alternative splicing pathways (Fig. 2B). The *tra* pre-mRNA has two alternative 3' splice sites; one is female-specific and the other non-sex specific. When the latter is used, a non-sex-specific transcript is generated that carries a translation stop codon in the open reading frame, leading to the production of a truncated, non-functional Tra protein. In females, approximately half of the *tra* pre-mRNA is spliced differently, the female-specific 3' splice site being used due to the intervention of the Sxl protein. As a result, the stretch containing the termination codon is not included in the mature transcript and synthesis of full length Tra protein occurs (Boggs *et al.,* 1987; Belote *et al.,* 1989; Válcarcel *et al.,* 1993). The genes *fl(2)d* (Granadino *et al.*, 1996) and *vir* (Hilfiker *et al.*, 1995) are also required for female-specific splicing of the *tra* pre-mRNA. Genetic analyses have ruled out a direct role of *snf* in *tra* pre-mRNA splicing (Cline *et al.,* 1999).

The Tra product and the product of the constitutive gene *tra-2* control the sex-specific splicing of pre-mRNA from the gene *doublesex (dsx)*. This gene is at the bottom of the sex determination cascade and is transcribed in both sexes, but gives rise to two different proteins - Dsx^F in females and Dsx^M in males. These two Dsx proteins are transcription factors that control the activity of the final target genes involved in sexual differentiation. Both proteins share the N-terminal domain, which contains a DNA-binding domain (DM domain). However, they differ in their C-terminal domains, which endow these proteins with their specific function (Burtis and Baker, 1989; Hoshijima *et al.,* 1991). The protein finally produced depends on the result of the sex-specific splicing of the primary transcript (Fig. 2C). Male-specific splicing represents the default mode. The incorporation of the female-specific exon instead of the male-specific exon is caused by activation of the weak female-specific splice acceptor site. This activation is exerted by the binding of the Tra-Tra2 complex to the 13-nucleotide repeated sequences (DsxRE), plus the binding of a specific member of the SR family - RBP1 – to the repeats, and finally the binding of dSF2/ASF to the purine-rich element (PRE) in the female-specific exon. This allows the weak female specific 3' splice site to be recognised and used by the generic splicing machinery (Hedley and Maniatis 1991; Hertel *et al.*, 1996; Ryner and Baker, 1991; Tian and Maniatis, 1993).

The Tra product and the product of the constitutive gene *tra-2* control also the sex-specific splicing of pre-mRNA from the gene *fruitless (fru)* (Fig. 2D). This gene, as well as *dsx*, is involved in the male sexual development of the central nervous system (CNS) (Rideout *et al.*, 2007), which is required for male courtship behaviour (Shirangi *et al.,* 2006). It is a complex gene, transcribed in both sexes, with four promoters (P1-P4), which generate multiple types of mRNAs by alternative splicing of the primary transcript. These mRNAs encode different Fru proteins that differ at their C-terminal domain. The Fru proteins have a BTB domain in their N-terminus and, with the exception of some variants, two Zn-finger motifs in their C-terminus. The male-specific FruM protein is generated from the sex-specifically spliced primary transcript from the P1-promoter. In females, the Tra-Tra2 complex controls the sex-specific splicing of the P1-primary transcript: the binding of this complex to their target sites in the female-specific exon promotes its inclusion into the mature mRNA. This exon contains translation-stop codons so that in females no FruM protein is produced. In males, where no Tra protein is available, the female-specific exon is spliced out (Ryner *et al.,* 1996; Heinrichs *et al.,* 1998; Goodwin *et al.,* 2000). The P2-P4 promoters function in neuronal and non-neuronal tissues from embryonic stages onward and encode Fru proteins that exert a vital function in both sexes. The function of P1 promoter is restricted to ~2% of neurons in the CNS, in various regions of the brain and ventral ganglia, from late third instar larva onward (reviewed in Billeter *et al.,* 2002; 2006). To this respect, it is worth mentioning that sexual behaviour of the *Drosophila* male is irreversibly programmed during a critical period extending from shortly before puparium formation into early metamorphosis (Arthur *et al.,* 1998). Sexual behaviour, however, is not reviewed here. The interested reader is referred to Yamamoto *et al.* (1998), O'Kane and Asztalos (1999), Greenspan and Ferveur (2000) and Billeter *et al.* (2002; 2006) and references therein.

The gene *intersex (ix)* is transcribed in both sexes. Its pre-mRNA does not follow sex-specific splicing, indicating that the Ix protein is present in both sexes. Ix interacts with Dsx^F but not Dsx^M, suggesting that Ix and Dsx^F form a complex to control female terminal differentiation (Chase and Baker, 1995; Waterbury *et al.,* 1999; Garrett-Engele *et al.*, 2002).

The gene *hermaphrodite (her)* has a dual function. Its maternal expression is necessary for early *Sxl* activation, although its zygotic expression is necessary for female terminal differentiation and some aspects of male terminal differentiation (Pultz and Baker, 1995; Li and Baker, 1998).

Genes homologous to the *Drosophila* sex determination genes in other dipteran and non-dipteran insects

Major progress has been made in understanding the developmental processes associated with selected model organisms, among which *Drosophila melanogaster* has played a paramount role. Moreover, the past few years have seen a great amount of interest in the evolution of developmental mechanisms at the genetic and molecular levels, and in determining the evolutionary processes by which these mechanisms came into existence. In this respect, sex determination is a process that seems to be exceptionally suitable for comparative study, given the great variety of sex determination mechanisms that exist. Indeed, sex determination has long been of major interest not only as a developmental process but also as an evolutionary problem – a problem that can only be solved once the genetic structures of sex determination pathways have been identified and compared. Molecular genetic technologies now permit such comparisons. In addition, sex determination in the reference system -that of *Drosophila melanogaster*- is understood in fine detail (as reported above), making truly informative comparisons possible. Data on the evolution of sex-determining mechanisms should provide new insight into how sex determination evolved (and so rapidly). Further, the information obtained is likely to be very important in

the study of the evolution of other developmental mechanisms, and how these came into existence.

The search for genes homologous to the sex determination genes of *D. melanogaster* has been undertaken (see Fig. 3). Among the genes that form the X/A signal, *scute* of *D. subobscura* (Botella *et al.,* 1996) and *sisterless-a* of *D. pseudoobscura* and *D. virilis* (Erickson and Cline, 1998) have been characterized. These genes show significant conservation in terms of their structure and function.

The Sex lethal *gene*

The *Sxl* gene has been characterised in different *Drosophila* species, and its structure and sequence organisation determined in *D. virilis* (Bopp *et al.,* 1996) and *D. subobscura* (Penalva *et al.,* 1996). As in *D. melanogaster, Sxl* regulation occurs by sex-specific alternative splicing: the *Sxl* transcripts in males have an additional exon containing stop translation codons. The *Sxl* of *D. virilis*, however, is unusual given the presence in males of an open reading frame, which encodes an Sxl protein, downstream from the last stop codon in the male-specific exon. This is identical to the female Sxl protein except for the first 25 amino acids of the amino terminal region, which are encoded by differentially spliced exons. The male Sxl protein predominantly accumulates in the embryonic ectoderm, suggesting it has a role in the development of the central nervous system (Bopp *et al.,* 1996). Sxl protein has also been detected in the males of other species of the virilis radiation (*D. americana, D. flavomontana*, and *D. borealis*) (Bopp *et al.,* 1996).

Outside the genus *Drosophila*, *Sxl* has been characterised in the dipterans *Chrysomya rufifacies* (blowfly) (Müller-Holtkamp, 1995), *Megaselia scalaris* (the phorid fly) (Sievert *et al.,* 1997, 2000) and *Musca domestica* (the housefly) (Meise *et al.,* 1998), in the tephritids *Ceratitis capitata* (Medfly) (Saccone *et al.*, 1998) and *Bactrocera oleae* (olive fly) (Lagos *et al.,* 2005) (all of which belong to the suborder Brachycera), and in *Sciara ocellaris* (Ruiz *et al.,* 2003), *Sciara coprophila, Rynchosciara americana* and *Trichosia pubescens* (Serna *et al.,* 2004), which belong to the suborder Nematocera. *Sxl* has been also characterised in the lepidopteron *Bombyx mori* (Niimi *et al.,* 2006). The *Sxl* gene of these species is not regulated in a sex-specific fashion, and

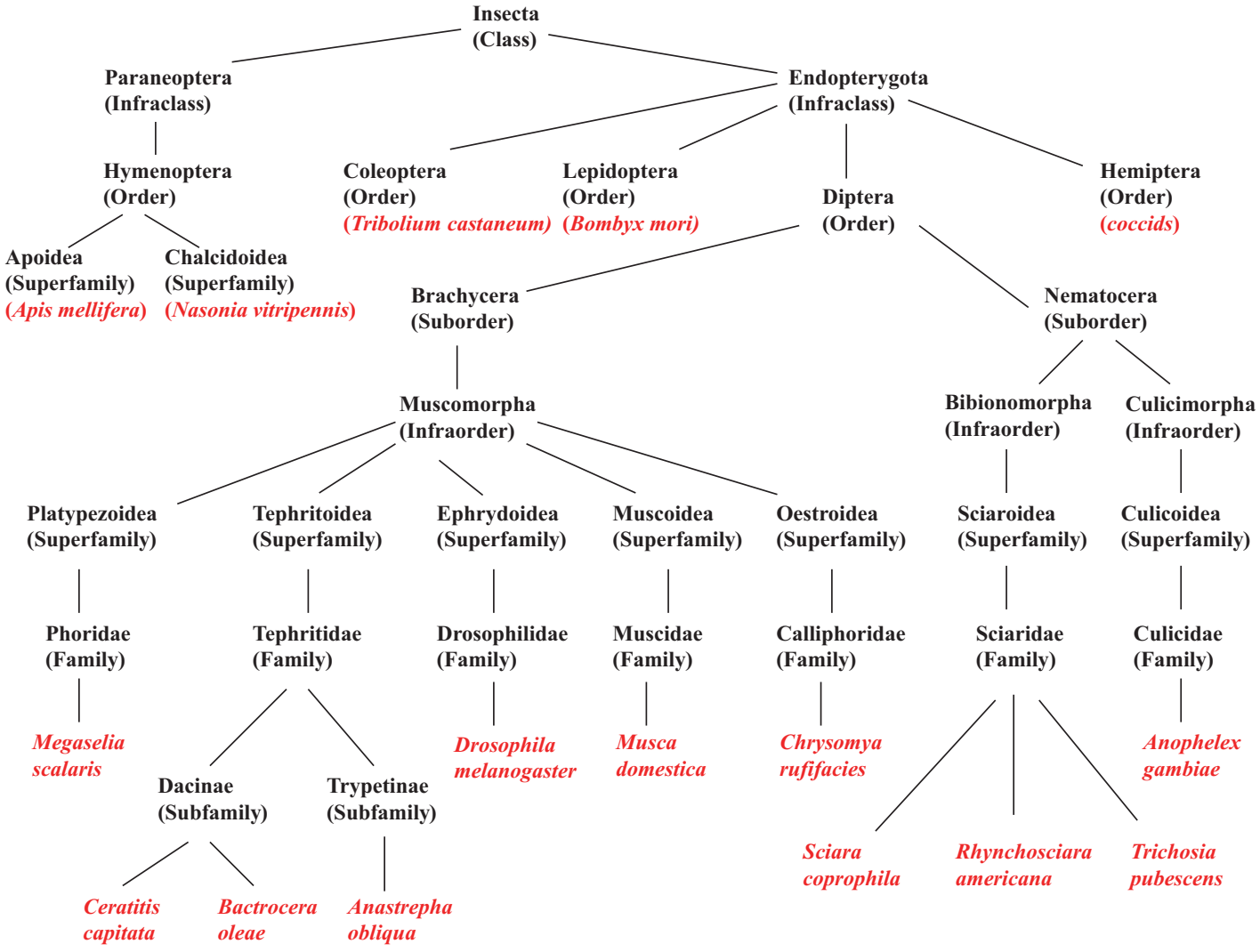

Fig. 3. Classification of the insects mentioned in this article. *The species representing a given phylogenetic lineage in the figure is highlighted in red.*

therefore the same *Sxl* transcript encoding the functional Sxl protein is found in both males and females. Thus, in the non-drosophilids, *Sxl* does not appear to play the key discriminating role in sex determination that it plays in *Drosophila*. This further suggests that *Sxl* has been co-opted to become the master regulatory gene in sex determination during the evolution of the *Drosophila* lineage.

Questions naturally arise regarding the role of *Sxl* in the non-drosophilids. It has been proposed that outside the drosophilids, the primary or even exclusive function of *Sxl* is to modulate gene activity through inhibition of mRNA translation in both sexes (Saccone *et al*., 1998). This suggestion is based on the following observations. First, *Sxl* controls dosage compensation in *Drosophila* through the regulation of translation of the mRNA of gene *msl-2* (Kelley *et al*., 1997). Second, Sxl protein accumulates at many transcriptional active sites in the polytene chromosomes of females (Samuels *et al*., 1994). And third, ectopic expression of *Ceratitis capitata* (Saccone *et al*., 1998) and *Musca domestica* (Meise *et al*., 1998) Sxl protein in *Drosophila melanogaster* is lethal in both sexes, presumably by interfering with certain cellular functions since *Drosophila, Ceratitis*, and *Musca* Sxl proteins have conserved RNA-binding domains. In *Drosophila melanogaster,* the function of gene *Sxl* is only needed in females since males lacking this function are viable and fertile (Cline, 1978).

In the sciarid flies, the Sxl protein has been found in polytene chromosome regions of all actively transcribing chromosomes, co-localising with RNA polymerase II but not with RNA polymerase I. This was observed in both sexes (Ruiz *et al*., 2003; Serna *et al.,* 2004). Moreover, comparison of the different Sxl proteins showed their two RNA-binding domains to be highly conserved (Serna *et al.,* 2004). These results agree with the proposition that, in the non-drosophilids, *Sxl* might work as an inhibitor of translation of mRNAs (Saccone *et al.,*1998). However, the alternative, non-mutually exclusive possibility that Sxl is a general splicing factor cannot be ruled out since both functions are exerted through its two RNA-binding domains. Nevertheless, all the results point to the idea that the ancestral *Sxl* gene was involved in general non-sex-specific gene regulation at the splicing and/or translational levels. Therefore, during the phylogenetic lineage that gave rise to the drosophilids, evolution modified the co-opted *Sxl* gene to convert it into a specific splicing and/or translation inhibitor for controlling sex determination and dosage compensation, profiting from certain properties of the recruited gene that are maintained in the evolved *Drosophila Sxl* gene.

The transformer *gene*

The *tra* gene of *D. simulans, D. mauritiana, D. sechellia*, *D. erecta* (O'Neil and Belote, 1992; Kulathinal *et al.,* 2003), *D. hydei* and *D. virilis* (O'Neil and Belote, 1992) has also been characterised. Its comparison with *tra* of *D. melanogaster* revealed an unusually high degree of divergence, yet heterologous genes can rescue *tra* mutations in *D. melanogaster* (O'Neil and Belote, 1992).

Outside the drosophilids, the gene *tra* has been characterised in the tephritids *Ceratitis capitata* (Pane *et al*., 2002), *Bactrocera oleae* (Lagos *et al.,* 2007) and *Anastrepha obliqua* (Ruiz *et al*., 2007a). The putative Tra protein of these tephritids is larger than that of the drosophilids due to an extra tract of amino acids in the N-terminal domain. As in the drosophilids, the gene *tra* is constitutively expressed in both sexes and its primary transcript shows sex-specific alternative splicing. However, whereas in the drosophilids *Sxl* regulates *tra*, in the tephritids this gene appears to have an autoregulatory function that produces functional Tra protein specifically in females. The gene *tra* in the tephritids have male-specific exons that contain translation stop codons. The incorporation of these exons into the mature *tra* mRNA in males determines that in these a truncated, non-functional Tra protein is produced. In females, the male-specific exons are spliced out because of the presence of Tra protein. The presence of putative Tra-Tra2 binding sites in the male-specific exons and in the surrounding introns suggests that the tephritid Tra and Tra2 proteins form a complex, whose binding to their target sequences prevents the male-specific exons from becoming incorporated into mature *tra* mRNA. The introduction of *Ceratitis* (Pane *et al*., 2005) or *Bactrocera* (Lagos *et al*., 2007) *tra*-dsRNA into *Ceratitis* or *Bactrocera* embryos, respectively, results in the destruction of endogenous *tra* function in both species and the subsequent complete transformation of females into fertile males. Moreover, the *Ceratitis tra-cDNA* encoding the putative full-length Tra protein can strongly transform *Cctra* transgenic *Drosophila melanogaster* males into females though this transformation appears to be incomplete (Pane *et al*., 2005). Pane *et al*. (2002) proposed that in *Ceratitis* the gene *tra* plays a key regulatory role, acting as the memory device for sex determination via its autoregulatory function.

The Tra protein in the tephritids *Ceratitis, Bactrocera* and *Anastrepha* shows a dual splicing role. On one hand it behaves as a splicing activator of *dsx* pre-mRNA -the binding of Tra to the female-specific exon promotes the inclusion of this exon into the mature mRNA. On the other hand, Tra acts as a splicing inhibitor of its own pre-mRNA -the binding of Tra to the male-specific exons prevents the inclusion of these exons into the mature mRNA. It has been proposed that the Tra2-ISS binding sites, which have been found in the splicing regulatory region of the *tra* pre-mRNA, but not in *dsx* pre-mRNA, of the tephritids provide the distinguishing marker for the dual splicing function of the Tra-Tra2 complex in tephritids (Ruiz *et al.,* 2007a).

The transformer-2 *gene*

The gene *tra-2* of *D. virilis* has been characterised (Chandler *et al.,* 1997). It encodes a set of protein isoforms analogous to those of *D. melanogaster*, and can rescue *tra-2* mutations in this species. Outside the genus *Drosophila*, *tra-2* has been characterised in the housefly *Musca domestica* (Burghardt *et al*., 2005). The injection of *Musca tra-2* dsRNA into *Musca* embryos results in the complete transformation of genotypically female embryos into fertile adult males, highlighting the role of *tra-2* in *Musca* sex determination. This gene is transcribed in both sexes and its function is required for the female-specific splicing of *Musca dsx* pre-mRNA. Genetic data suggest that *tra-2* participates in the autocatalytic activity of gene *F* (Burghardt *et al.,* 2005), the key sex-determining gene in the housefly (Dübendorfer *et al.,* 2002).

The doublesex *gene*

Outside the drosophilids, the gene *dsx* has been characterised

in the dipterans *Megaselia scalaris* (Sievert *et al.*, 1997; Kuhn *et al.*, 2000), *Musca domestica* (Hediger *et al.,* 2004), and *Anopheles gambiae* (Scali *et al.*, 2005), in the tephritids *Bactrocera tryoni* (Shearman and Frommer, 1998), *Bactrocera oleae* (Lagos *et al.*, 2005), *Ceratitis capitata* (Saccone *et al.*, cited in Pane *et al.*, 2002) and *Anastrepha obliqua* (Ruiz *et al.*, 2005; 2007b), and in the lepidopteran *Bombyx mori* (Ohbayashi *et al.*, 2001; Suzuki *et al.,* 2001). The molecular organisation of the *dsx* ORF varies among these insects, but in all cases *dsx* encodes male- and female-specific RNAs that encode putative male- and female-specific Dsx proteins sharing the N-terminal region but differing in their C-terminal regions (as in *Drosophila*). The protein finally produced depends on the result of the sex-specific splicing of the primary transcript. In all the dipteran insects, putative Tra-Tra2 binding sites are found in the female-specific exon, suggesting that, like in *Drosophila*, male-specific splicing represents the default mode and that female-specific splicing requires Tra protein (which would only be present in females). Unlike in the dipteran insects, the lepidopteran *Bombyx dsx* does not have a weak female-specific splice site, and does not carry the putative Tra–Tra2 binding sequences. This suggests that the female-specific splicing of *dsx* pre-mRNA is the default-splicing mode in *Bombyx*. In the lepidopterans, the heterogametic sex is female (ZW) and the homogametic sex is male (ZZ), unlike in the dipterans that show sexual heteromorphic chromosomes: the homogametic sex is female (XX) and the heterogametic sex is male (either XY or X0).

The fruitless *gene*

The gene *fru* of *D. simulans, D. yakuba, D. pseudoobscura, D. virilis* and *D. suzukii* (reviewed in Billeter *et al.*, 2002) and that have *Anopheles gambiae* and *Tribolium castaneum* (Gailey *et al.*, 2006) has been characterised. In all the cases, the gene *fru* show a conserved molecular structure and the male-specific FruM protein arise by conserved mechanisms of sex-specifically activated and alternative exon splicing, which is controlled by *transformer*. Moreover, the ectopic expression of *A. gambiae* Fru^{MC} in *D. melanogaster* was sufficient to rescue the muscle of Lawrence (MOL)-less phenotype–fru^{3} mutant males and to induce the formation of a MOL-like muscle in females (Gailey *et al.*, 2006). The MOL in *D. melanogaster* is under the control of *fru* gene (reviewed in Billeter *et al.,* 2002, and cites therein).

The intersex *gene*

The gene *ix* has been characterised in the dipterans *D. virilis* and *Megasela scalaris*, and in the lepidopteran *B. mori* (Siegal and Baker, 2005). The Ix proteins appear to have a conserved organisation. The amino-terminal region is rich in glutamine, proline, glycine and serine residues, a feature of known transcription regulators. The carboxy-terminal region contains a high proportion of polar amino acids and two conserved phenylalanine residues, which appear to be specific to the Ix proteins. The *D. virilis* and the *M. scalaris ix* genes fully restore the *ix* function in otherwise *ix* mutant *D. melanogaster* flies, suggesting that these Ix homologs can interact with the *Drosophila*—Dsx^{F} protein just as *Drosophila* Ix protein does. However, the *B. mori ix* gene only partially rescues female sexual development of *Drosophila ix* mutant females. This suggests that Dsx^{F} and Ix proteins co-evolved, and that sufficient time has elapsed since the moment of separation of the lepidopteran and dipteran lineages to allow the accumulation of divergence between the Dsx^{F} and Ix proteins of these species (Siegal and Baker, 2005).

The complementary sex determination system (CSD)

In some hymenopteran insects, such as wasps, sawflies, ants and bees, gender is regulated by haploidy/diploidy: males are haploid and develop from unfertilised eggs, whereas females develop from fertilised eggs. In these insects, the sexual pathway followed by the zygote depends on a primary signal based on the presence of either one or two different alleles of a single gene at the so-called *complementary sex determiner (csd)* locus. Males are hemizygous and females are heterozygous at this locus. Infertile diploid males may arise when this locus is homozygous (Cook, 1993).

The work of Beye *et al.* (2003) provided a breakthrough in the understanding of the genetic basis underlying this haploid/diploid sex determination mechanism. These authors isolated the *csd* gene of the honeybee *A. mellifera*. This gene encodes an arginine-serine rich (SR) protein, with structural similarity to the dipteran *transformer* genes. The gene *csd* is transcribed in both sexes but has a highly variable region -responsible for the allelic variation that characterises this locus- giving rise to the production of different Csd isoforms. It is thought that allelic heterozygosity produces two functional Csd protein isoforms capable of forming a complex that determines female development, whereas hemizygosity, or allelic homozygosity, generates a single Csd protein isoform that cannot generate a functional complex, thus determining male development (Beye *et al.*, 2003; Beye, 2004) (see Fig. 4).

The CSD system does not apply to other hymenopteran insects, such as *Nasonnia vitripennis*, which belong to the parasitoid wasp group Chalcidoidea, because homozygous diploids develop into females (reviewed in Beukeboom *et al.,* 2007 and cites therein). A new model has been put forward to explain sex determination in these parasitoid hymenopterans (Beukeboom *et al.,* 2007). The MEGISD model (for Maternal Effect Genomic Imprinting Sex Determination) proposes that the sex of the zygote depends on the activity of the *zygotic sex determiner (zsd)* gene, whose function determines female development. A *maternal effect gene (msd)* causes imprinting of the *zsd* gene during oogenesis so that the female-inherited *zsd* allele is not active in the zygote. Consequently, haploid zygotes develop as males because they carry the imprinted *zsd* allele inherited from the mother. Diploid zygotes develop as females because the paternally inherited *zsd* is not imprinted and then becomes expressed. For more details see Beukeboom *et al.* (2007).

Sex determination mechanisms based on X chromosome elimination: the sciarid system

In most of the species, the sex of an individual is fixed at fertilisation. However, in some cases the chromosomal differences determining gender are brought about by specialised behaviour of the X chromosomes during the first stages of embryonic development. This is the case of the dipteran families Cecydomyiidae (White, 1973; Stuart and Hatchett, 1991) and Sciaridae (DuBois, 1933; Metz, 1938), which belong to the subor-

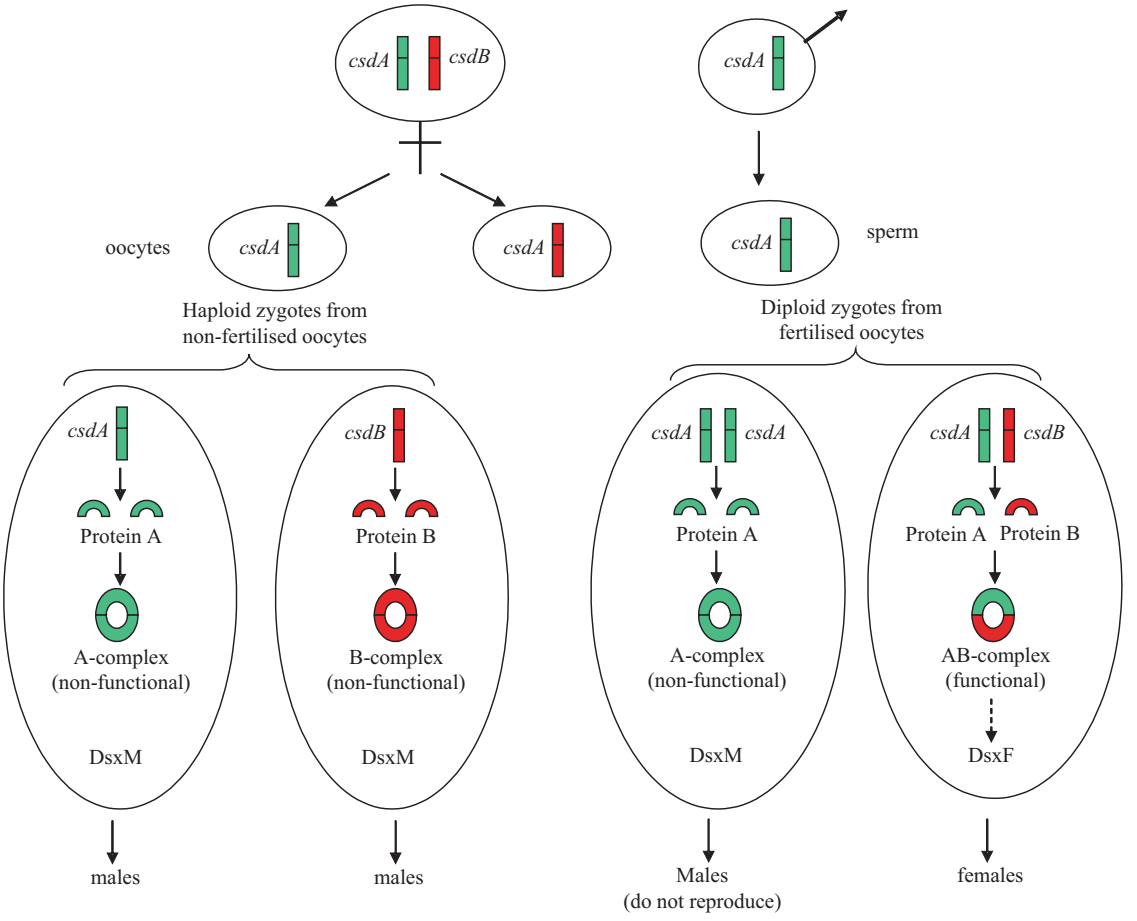

Fig. 4. Sex determination in the honeybee *Apis mellifera*. *The green and red segments represent the chromosomes carrying the* complementary sex determination (csd) *locus.* CsdA *and* csdB *stand for two alleles of this locus, which encode two Csd protein isoforms, protein A and B respectively. Modified from Beye (2004).*

der Nematocera.

Since sciarid males are X0;2A and females are XX;2A (Metz and Schmuck, 1929), the primary genetic signal that triggers sex determination appears to be the ratio of X chromosomes to sets of autosomes (X/A ratio), as in *Drosophila*. There is, however, a fundamental difference in the way the X/A chromosomal signal is formed. In *Drosophila*, embryos are either 2X;2A or XY;2A, a direct consequence of the chromosomal constitution of the gametes: oocytes are X; A, and sperm are either X;A or Y;A. In sciarids, oocytes are X;A and sperm are 2X;A (see Fig. 5). Consequently, *Sciara* embryos start development with two sets of autosomes and three X chromosomes (3X;2A), two of which are sister chromatids of paternal origin (Metz, 1938). When the zygotic nuclei reach the egg cortex, one paternal X chromosome is eliminated in the somatic cells of embryos destined to be females (2X;2A) and two are eliminated in those destined to become males (X;2A) (DuBois, 1933; Perondini *et al.,* 1986). Therefore, in the formation of the X/A chromosomal signal in sciarids an "imprinting" process must occur in one of the parents, which determines that the chromosomes to be eliminated are of paternal origin. Historically, the term "imprinting" was coined to describe selective identification of paternal chromosomes in sciarids (Crouse, 1960).

Among the sciarids there are some species in which the females produce offspring of only one sex - the unisexual (or monogenic) species. These females are either androgenic (male producers) or gynogenic (female producers). Some species are bisexual (digenic or amphigenic), i.e., the progeny is composed of a mixture of males and females. The sex ratio (number of males vs. females) of the progeny of each female, however, is highly variable, deviating from 1:1. Nevertheless, at the level of the whole population, the sex ratio follows a normal distribution around this value (Metz, 1938).

In the case of the monogenic species *Sciara coprophila*, gynogenic and androgenic females differ in the presence of a special X' chromosome in the former: these females are X'X, whereas androgenic females are XX (see Fig. 6). The XX females produce only X0 males, which do not receive the X' chromosome. The gynogenic females produce both gynogenic and androgenic females. The system functions as if regulated by a pair of alleles, the X'X and XX females being produced in a 1:1 ratio (Moses and Metz, 1928; Metz and Schmuck, 1929; Metz, 1938; Gerbi, 1986). There is an inversion in the X' chromosome that prevents its recombination with the homologous X chromosome, thus retaining in the X' the factor for female production (Crouse, 1960). Thus, the elimination of the paternally-derived chromosome is maternally controlled. The gynogenic females produce two classes of oocytes carrying either the X' or the X chromosome. Both contain the maternal factor that controls X-chromosome elimination, thus these two types of oocytes are pre-determined to eliminate one X chromosome of the two inherited from the father. The X'- and X-oocytes contribute to the production of the gynogenic and androgenic females (respectively) of the following generation. The XX androgenic females produce a unique class of X-oocytes without the maternal factor involved in X chromosome elimination; therefore these oocytes are pre-determined to eliminate the two X chromosomes inherited from the father. Consequently, the X-oocytes from the androgenic females contribute to the production of X0 males of the following generation.

Sciara ocellaris is a digenic species in which the sex of the offspring is also determined by the female parent through the production of oocytes that contain - or do not contain - the maternal factor for X chromosome elimination (Liu, 1968). In this species the offspring are composed of male and female individuals, but the sex ratio varies widely (Metz, 1938; Davidheiser, 1943; Mori *et al.*, 1979). It has been shown that this variation depends on temperature: at 18-20°C, the sex ratio distribution, although variable, shows a median at approximately 50%, but at 24-29°C the sex ratio moves towards the production of more females. This change in sex ratio is not caused by a higher mortality among males, but by a transformation of male into females (Nigro, 1995; Campos *et al.*, 1996; Nigro *et al.*, 2007). This increase in female production is due to an increase in the number of embryos that eliminate one instead of the two paternally-derived chromosomes. Temperature-shift experiments have shown that the temperature-sensitive period for the determination of the final sex ratio is from the mid-pupa stage to the emergence of the adult females (Nigro, 1995; Nigro *et al.*, 2007), the period during which oogenesis takes place (Berry, 1941). It should be recalled that in *Sciara* females the number of oocytes is fixed during the early larval stages and that no further mitosis occurs nor are new oocytes produced in the pupal/adult stages (Berry, 1941).

Spontaneous deviations and mistakes in X-chromosome elimi-

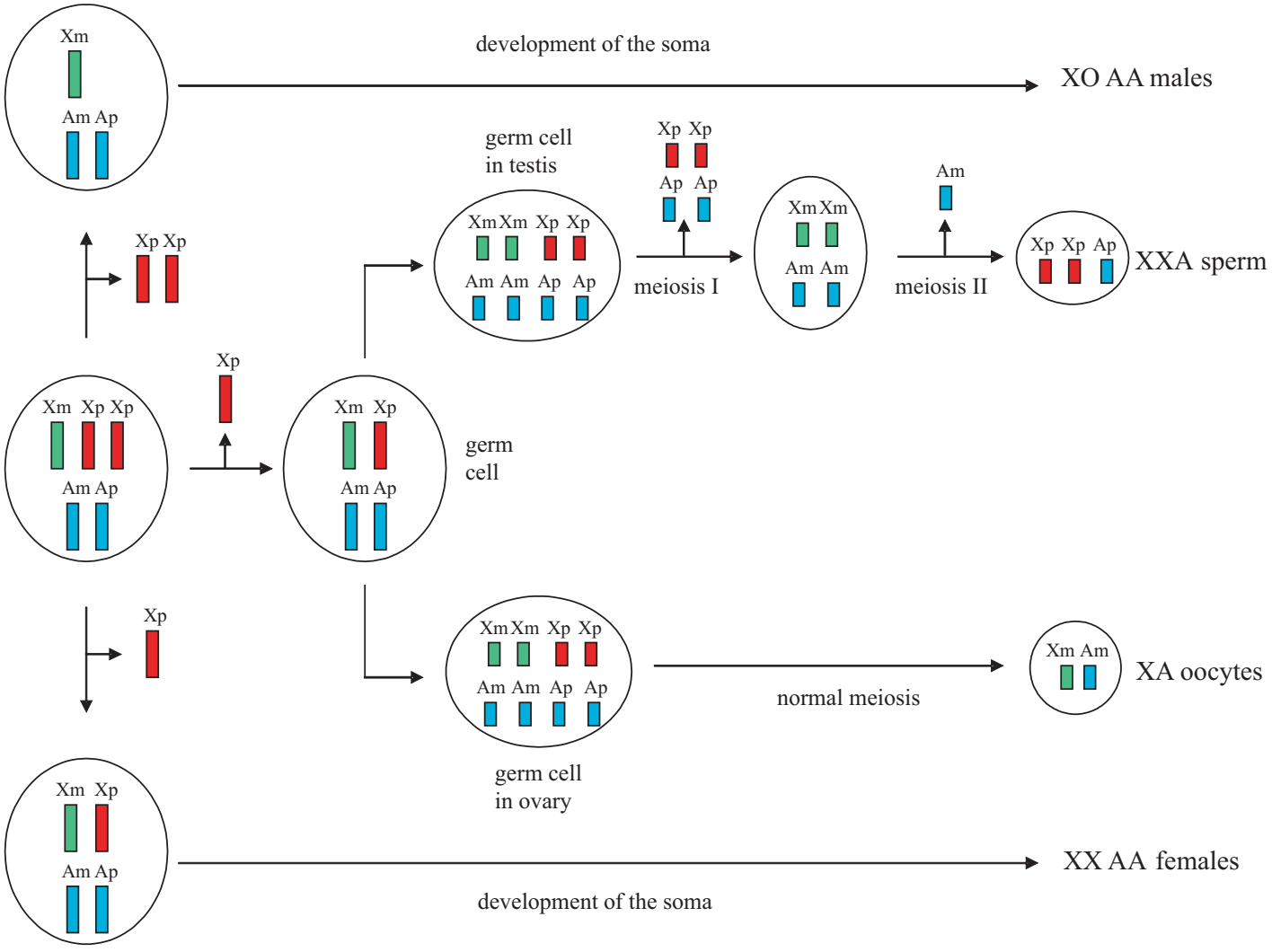

Fig. 5. Diagram of chromosome behaviour in sciarid flies. *X stands for the X chromosome and A for the autosomes; m and p denote maternal or paternal origin. The pole cells, which are set apart at the 4th cleavage (DuBois, 1932; Berry, 1941; Perondini* et al., *1986), do not eliminate X chromosomes at the same time as the nuclei in the somatic regions of the embryos. Elimination of one paternally-derived X chromosome occurs later, at the beginning of germ band segmentation; just one of the two paternal X chromosomes is eliminated in both male and female embryos (Berry, 1941; Rieffel and Crouse, 1966; Perondini and Ribeiro, 1997; Perondini, 1998). Meiosis is highly specialised in males, showing elimination of the paternally derived genome (1st division), and the non-disjunction of the two X chromatids and elimination of one chromatid of each autosome during the 2nd division. The chromosomes undergoing elimination during both meiotic divisions are segregated and discarded in a cytoplasmic bud (Esteban* et al., *1997). The end result is that the functional spermatids and ensuing sperm are of a unique type and contain a haploid set of autosomes and two X chromosomes, all of maternal origin (Gerbi, 1986; Fuge, 1994; Esteban* et al., *1997; and references therein). The paternal X chromosomes that are eliminated in each generation are maternal in the previous generation; i.e., the imprinting mark that determines the origin of the chromosomes that are eliminated is erased (or modified) in each generation. Modified from Sánchez and Perondini (1999) and from Goday and Esteban (2001).*

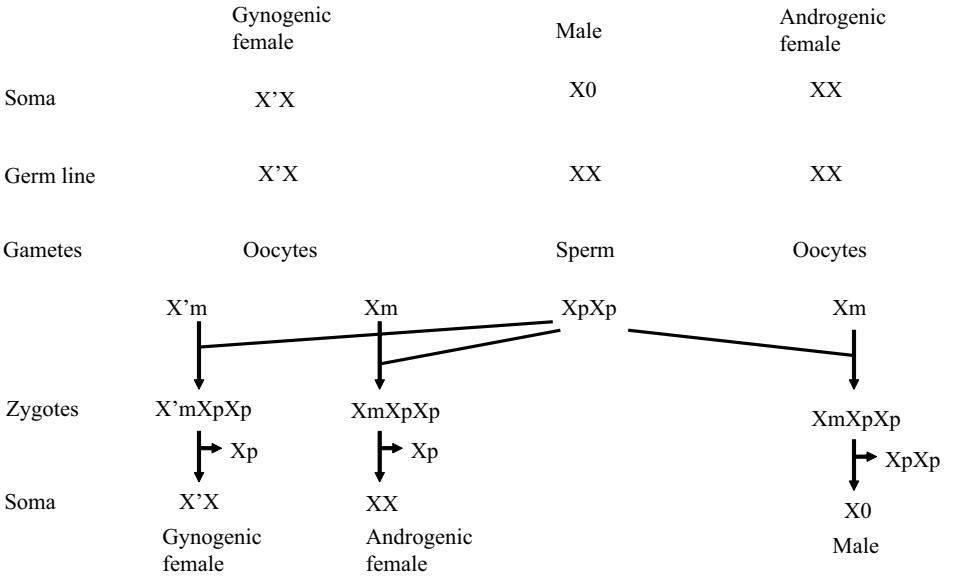

Fig. 6. The production of gynogenic and androgenic females, as well as males, in *Sciara coprophila*. *The maternal and paternal origin of the chromosomes is denoted by m and p respectively.*

nation can occasionally occur in the sciarid chromosomal system (for details see Sánchez and Perondini, 1999). Two basic types of error are seen (see Fig. 7): 1) Quantitative errors, involving the number of paternally-derived X chromosome that are eliminated (i.e., some zygotic nuclei of embryos derived from oocytes predetermined to eliminate one X chromosome end up eliminating the two X-paternally derived chromosomes instead). These quantitative errors produce gynandromorphs - individuals with some portions of the body typically male (X0) and others typically female (XX). 2) Qualitative errors, in which the X chromosome eliminated is the one inherited from the mother rather than either of the two inherited from the father. This type of error leads to mosaic flies, either male or female, bearing a mixture of tissues with the normal chromosome set plus tissues patroclinous for the X chromosome (see Fig. 7).

Errors in X-chromosome elimination have been experimentally induced by the UV irradiation of embryos (Perondini *et al.*, 1987; Guatimosin and Perondini, 1994). The UV doses used did not penetrate more than a few micrometers into the cytoplasm and therefore had little effect on egg survival. Two stages of development were irradiated: 1) the early cleavage stage, when only the cytoplasm of the insect egg was affected by the UV radiation (the nuclei remained deep in the endoplasm and were thus shielded), and 2) the syncytial blastoderm stage, by which time the nuclei have migrated to the periplasm and thus both the cytoplasm and the nuclei were UV targets. Irradiation during the cleavage state produced gynandromorphs, i.e., individuals with quantitative errors, while irradiation during the syncytial blastoderm stage produced both gynandromorphs and mosaics, i.e., individuals with quantitative and qualitative errors. Both types of errors were reversed after photoreactivation treatment of the UV-irradiated embryos (Guatimosin, 1996).

The conclusions drawn on the mechanism of X-chromosome elimination derived from the analysis of spontaneous or induced errors are summarised below (for more details see Sánchez and Perondini, 1999; and references therein).

1) The mechanism of X-chromosome elimination is similar in monogenic and digenic species.

2) A maternal factor is produced during oogenesis, which collects in the oocyte and then governs the elimination of the X chromosome in the developing zygote.

3) The embryo eliminates either one or two Xp chromosomes independently of the number of X chromosomes initially present in the zygote.

4) There are two independent mechanisms involved in the elimination of X-chromosomes. One regulates the number of X to be eliminated, and resides in the cytoplasm, the other identifies the X to be eliminated, and seems to be nuclear.

5) Among the embryos produced by gynogenic mothers, errors more frequently occur in XXX zygotes that develop into androgenic females than in X'XX zygotes that become gynogenic females.

6) Errors are rare in XXX embryos produced by androgenic females.

We have proposed a model for the control of differential X-chromosome elimination in sciarid flies, based on the following assumptions:

1) A chromosomal factor (CF) binds to the paternal X chromosome causing its elimination. This factor is produced in limiting amounts and at similar concentrations in both male and female embryos. Whether CF is maternal or zygotic is irrelevant.

2) A maternal factor (MF) determines the number of paternal X chromosomes to be eliminated. This maternal factor interacts with CF, inactivating it, so that the CF-MF complex cannot interact with the paternal X chromosome. Therefore, the number of X chromosomes eliminated depends on the amount of free CF, which in turn depends on the amount of MF.

3) Imprinting, in relation to the identification of the X chromosome to be eliminated, occurs in the maternal chromosomes and not in the paternal counterparts. The imprinted state is manifested by the inability of the maternal X chromosome to bind CF; consequently, this chromosome cannot be eliminated.

This model is compatible with the basic features of the sciarid chromosomal system, some of which are outlined here (the interested reader can find a more detailed discussion in Sánchez and Perondini, 1999):

1) Sex is controlled by a maternal factor – the above-mentioned MF.

2) Gynogeny and androgeny. The X' chromosome carries a constitutive MF allele. Gynogenic X'X females only produce oocytes with high levels of MF, the embryos derived from these develop into females (i.e., one Xp chromosome is eliminated). In contrast, XX androgenic females carry a weak (or amorphic) MF allele, so they only produce oocytes with low levels of MF (or none at all) and thus give rise to embryos that develop into males (i.e.,

the two Xp chromosomes are eliminated).

3)Quantitative errors in X-chromosome elimination: production of gynandromporphs. The increased frequency of gynandromorphs after UV-irradiation of embryos destined to be females is explained by the destruction of MF. Consequently, a larger amount of free CF will be available to bind the two - instead of one - Xp chromosomes, producing the X0 cells of gynandromorphs. Since MF is located in the cytoplasm, these gynandromorphs can be induced at both the cleavage and syncytial blastoderm stages.

4) Qualitative errors in X-chromosome elimination: production of male and female mosaics. The production of these mosaics is explained in that UV irradiation modifies the imprinting mark of the maternal X chromosome, which then binds with CF and is eliminated. These mosaics are only produced when UV irradiation occurs at the syncytial blastoderm stage, because it is during this stage when the nuclei reach the periphery of the egg and become exposed. The generation of mosaics argues in favour of the imprinting of the maternal, rather than the paternal, X chromosome.

The question arises as to whether it is necessary to assume the existence of two factors, MF and CF, instead of only one, MF, as proposed by de Saint-Phalle and Sullivan (1996). In their one-factor model, it is assumed that the maternal factor promotes, rather than prevents, the segregation of the Xp chromosome. This maternal factor would directly interact with the Xp chromosome to promote separation of its sister chromatids, whereas neither the Xm nor the autosomes need this separation factor for normal segregation. This implies that imprinting, in relation to the identification of the X chromosome to be eliminated, occurs in the Xp chromosome. The imprinted state in this case is manifested by the requirement of this chromosome to bind the separation factor. This model does not, however, explain the existence of mosaic sciarids.

Sex determination mechanisms based on chromosome elimination or heterochromatisation: the coccid system

The coccids (scale insects) belong to the order Homoptera. Sex determination in primitive coccids is usually decided by the XX (female) – X0 (male) sex chromosome mechanism. However, in some groups the sex of an individual is not fixed at fertilisation - as in the sciarids. The differential inactivation and/or elimination of chromosomes as a mechanism of sex determination has been

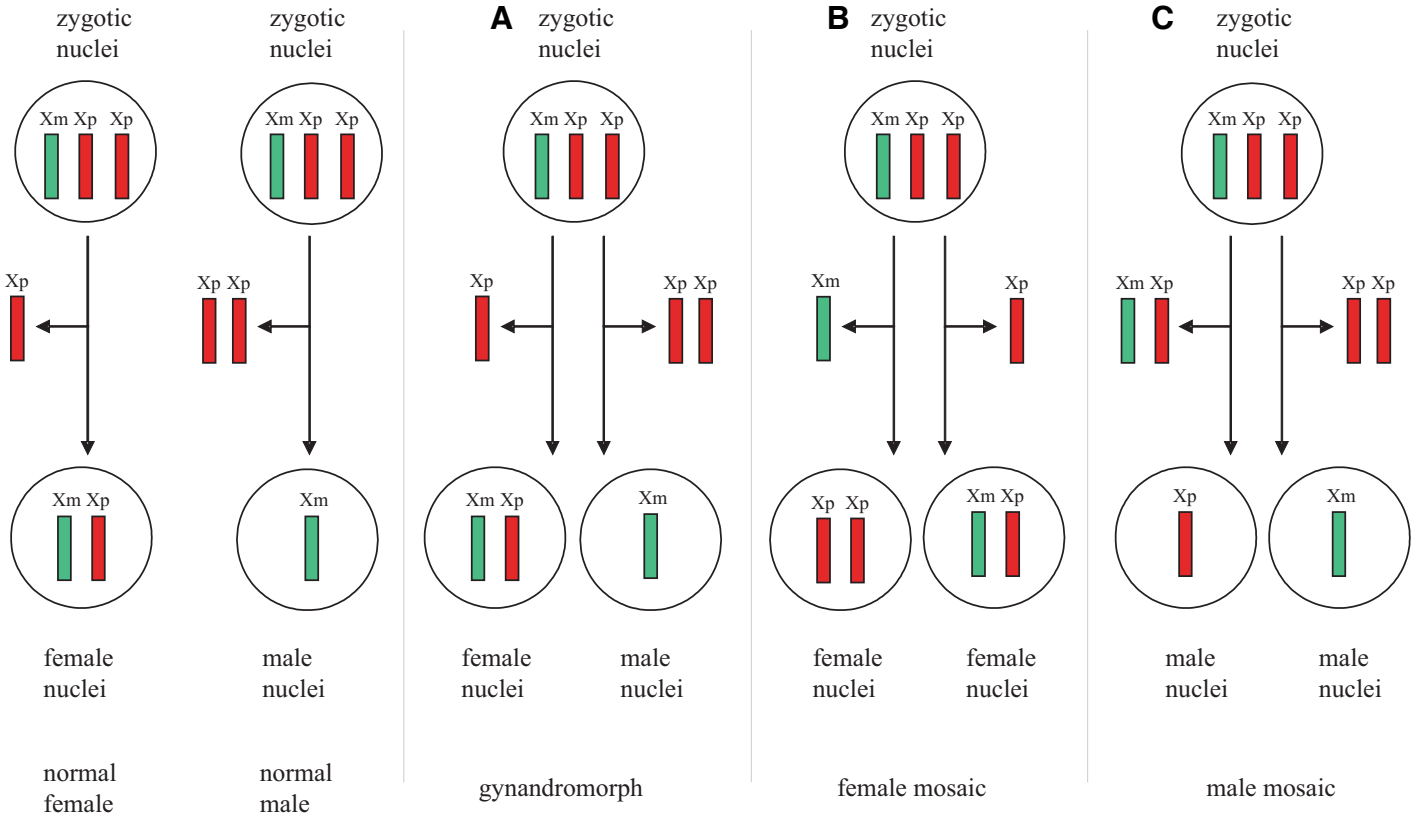

Fig. 7. The type of errors in X chromosome elimination which give rise to gynandromorphs and mosaic sciarid flies. *The maternal and paternal origin of the chromosomes is denoted by m and p respectively.* **(A)** *Gynandromorphs originated by a quantitative error because two instead of one Xp chromosome being eliminated in some nuclei of an embryo destined to be female.* **(B)** *Female mosaics originated by elimination in some nuclei of the Xm chromosome instead of one Xp chromosome. The result is that the XpXp cells in which this happens are patroclinous for the X chromosomes.* **(C)** *Male mosaics originated by elimination in nuclei with one Xp plus the Xm chromosomes. The result is that the cells Xp0 in which this happens are patroclinous with respect to the X chromosome. In both cases, the error is qualitative because the Xm chromosome is abnormally eliminated (modified from Sánchez and Perondini, 1999).*

reported (Brown and Nur, 1964; White, 1973; Brown and Chandra, 1977; Miller and Kosztarab, 1979; Herrick and Seger, 1999; and references therein). Among the different sexual chromosome systems found in the coccids, the two most paradigmatic are discussed: the lecanoid and the diaspidid systems (Fig. 8). Reproduction in both cases is purely sexual.

Sex determination in the lecanoid coccids is based on a functional haploidy/diploidy mechanism. At the cleavage stage, immediately following fertilisation, all the chromosomes of the embryo are euchromatic. Later, the chromosomes inherited from the father become heterochromatic in embryos destined to be males. In the female embryo, none of the chromosomes become heterochromatic. Thus, females have two functional chromosomal complements, while males are structurally diploid but functionally haploid. In the females, both sets of chromosomes remain active not only during development but also in oogenesis, (the orthodox system). In the males, the heterochromatic set remains inactive. Spermatogenesis is characterised by inverse meiosis and the lack of chromosome pairing and genetic recombination. The first meiotic division is equatorial (disjunction of the sister chromatids), while the second is reductional (disjunction of the maternal and paternal homologs). The result is the formation of the expected four nuclei, two of which are euchromatic and two of which are heterochromatic. Only the euchromatic nuclei undergo spermiogenesis to form functional sperm. The heterochromatic nuclei do not form sperm and disintegrate.

The Comstockiella have a similar mechanism, which differs from the former in that one chromosome of the heterochromatic set remains heterochromatic during spermatogenesis; the remainder of the heterochromatic set is eliminated before prophase. This single heterochromatic chromosome is later eliminated so that the sperm only transmit maternal chromosomes (reviewed in Brown, 1977; Miller and Kosztarab, 1979; Herrick and Seger, 1999; and references therein).

Sex determination in the diaspidid coccids is also based on a haploid/diploid mechanism. In the female embryo both chromosomal complements (one inherited from the mother and one from the father) are functional. In embryos destined to be males, all the chromosomes inherited from the father are eliminated, rendering them structurally and therefore functionally haploid. In the females, oogenesis is orthodox. Spermatogeneis, however, is unorthodox since the paternal chromosomes are absent and the second meiotic division does not occur. Sperm cells carrying a set of the maternal chromatids are therefore produced.

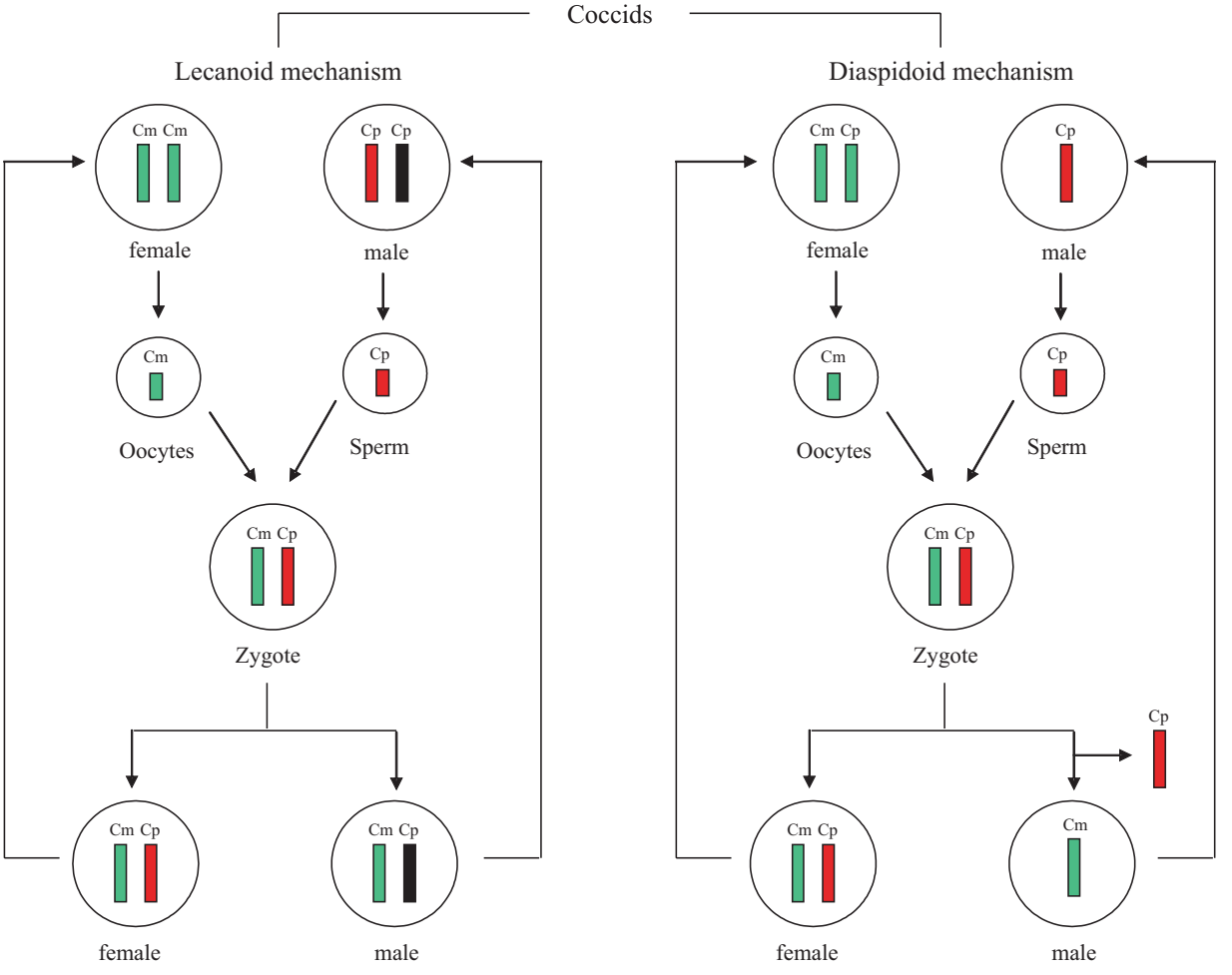

Fig. 8. The chromosome cycle in lecanoid and diaspidid coccids. *Each box represents a haploid chromosome complement (C) inherited from the mother (Cm) or from the father (Cp). The black colour in the lecanoid mechanism symbolises the heterochromatic, genetically inert, chromosomal state.*

The following summarises some features of the lecanoid and diaspidid mechanisms relevant to sex determination. For further details see Brown and Nur (1964), White (1973), Brown and Chandra (1977), Miller and Kosztarab (1979), Herrick and Seger (1999) and references therein.

Coccid chromosomes are holocentric. After irradiation of male parents, all of the chromosome fragments in their eventual male offspring are heterochromatic; after irradiation of eventual mothers, all are euchromatic. Therefore, there cannot be a single locus or restricted region on each chromosome that regulates the induction of heterochromatisation (Brown and Nelson-Rees 1961; Nur 1990).

The sex ratio of the offspring of female coccids can fluctuate widely and is subject to environmental influence (Nelson-Rees, 1960). Aging the females before allowing them to mate alters the sex ratio in favour of males – a phenomenon not caused by any differential increase in the mortality of female zygotes. Rather, this is explained by changes in sexual dichronism (i.e., the deposition of male and female embryos at different times during oviposition), the pattern of which can be altered by maternal aging (James, 1937, 1938; Brown and Bennett, 1957; Nelson-Rees, 1960).

Some parthenogenic coccids are known to produce male or female embryos depending upon whether or not the heterochromatisation of one chromosomal set occurs. Nevertheless, the proportion of male to female embryos is very low (about 5% males and 95% females are produced); abnormal or degenerating embryos are often observed, which are almost certainly males. Since heterochromatisation naturally occurs in these coccids, no prior passage of the chromosomes through spermatogenesis is required (Nur, 1963).

In experimentally produced haploid embryos and haploid mosaics, the single chromosome set inherited from the father first undergoes heterochromatisation but later becomes euchromatic. The maintenance of the paternally-derived heterochromatic chromosomes requires the presence of the maternally-derived euchromatic chromosome set (Nur, 1962; Chandra, 1963). However, the suppression of heterochromatisation requires no interaction between homologous chromosomes. When a chromosome is lacking from either set in an embryo, its homologue in the other set shows the typical features of the set to which it belongs (Chandra, 1962).

Together, all this evidence suggests that the genome of the mother determines the heterochromatisation (lecanoid) or elimination (diaspidid) of the inherited paternal chromosomes in coccid embryos. In addition, both the lecanoid and diaspidid mechanisms have an associated imprinting process to distinguish between the maternal and paternal chromosomes. This chromosome behaviour is similar to that seen with respect to the elimination of the paternal X chromosome in sciarid flies. Hence, it is proposed here that the model for the control of differential X-chromosome elimination in the sciarids mentioned above can be applied to the heterochromatisation or elimination of paternal chromosomes in coccids. According to this model, heterochromatisation or elimination is controlled by a maternal factor, with the maternally-derived chromosomes imprinted so that they do not suffer either fate. For the sake of simplification, it is assumed that the maternal factor exerts its effect through direct interaction with the paternally-derived chromosomes to promote their heterochromatisation or elimination. From the molecular point of view, the female therefore produces two classes of oocyte, one with and one without maternal factor. The first will give rise to males whereas the second will produce females. The imprinted state could mean that the maternally-derived chromosomes do not bind the maternal factor, or that they bind it with much lower affinity than do the paternally-derived chromosomes. In zygotes developing in the latter scenario, the maternally- and paternally-derived chromosomes compete in terms of binding the maternal factor -which is preferentially bound to the paternal set. If the binding of the maternal factor follows a co-operative mechanism, the heterochromatisation or elimination of the paternally-derived chromosomes would be favoured whenever the amount of maternal factor is limited.

According to the proposed model, the females produce two classes of oocyte, those which contain maternal factor – and which are pre-determined to heterochromatise or be eliminated, and those without maternal factor, which are pre-determined not to permit any chromosome to follow either such course. The effect of the temperature or the age of the mother on the offspring sex ratio is explained by a change in the ratio of oocytes with and without maternal factor.

The existence of parthenogenic coccids that produce some male embryos supports the assumption of the model that imprinting occurs in the females, and that the imprinted state of the maternally derived chromosomes is made manifest in their incapacity to become heterochromatic. If heterochromatisation requires the chromosomes to pass through spermatogenesis, then the formation of parthenogenetic male embryos would not be expected - in contrast to that observed. Such male embryos carry only maternally-derived chromosomes. Since all of them are imprinted they should all compete equally for binding with the maternal factor, although this binding would be very much impeded because of the imprinting mark. This explains the low frequency with which parthenogenetic male embryos are seen. The probability that a complete haploid set of chromosomes should become heterochromatic is very low, and in the majority of cases the embryo would be aneuploid. Consequently, the frequency of male offspring derived from parthenogenic females is very small, and abnormal and degenerating embryos are frequently observed.

Two temporal relationships may exist between heterochromatisation (or elimination) and sex determination in coccids: either sex determination precedes heterochromatisation or heterochromatisation occurs prior to sex determination. The first relationship implies that the gender of the embryo is determined first: if it is male, heterochromatisation of a haploid set of chromosomes ensues; if it is female, no heterochromatisation occurs. But in this scenario no difference in the primary genetic signal for sex determination can exist between the diploid embryos that will become female, and those diploid embryos that will develop along the male pathway. It could be argued that the females could produce two classes of oocytes depending on whether or not they carry a male sex-determining factor. In this case, the embryos derived from oocytes carrying the proposed factor would develop into males and heterochromatisation would follow. However, this cannot explain the existence of male embryos produced by parthenogenic coccids. On the other hand, heterochromatisation (or elimination) could occur during the first

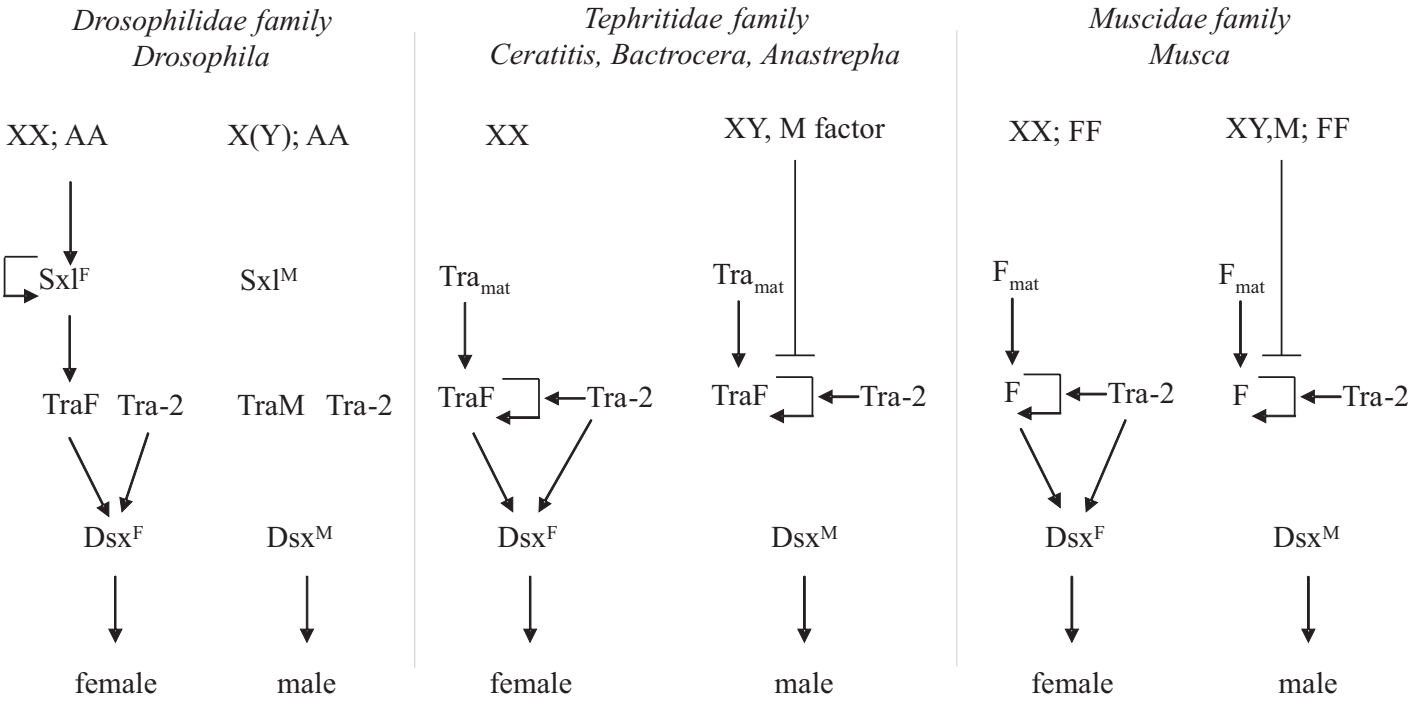

Fig. 9. The sex determination gene cascade in *Drosophila, Ceratitis, Bactrocera, Anastrepha* and *Musca*. *Tra_{mat} and F_{mat} indicate maternal Tra and F product respectively. The production of Dsx^M protein is the default state. The scheme for the Tephritidae is modified from Pane* et al. *(2002), and that for the Muscidae from Burghardt* et al. *(2005).*

cleavage divisions, well before the first developmental (including sex determination) decisions have to be taken (at the blastoderm stage). Thus, heterochromatisation (or elimination) would precede sex determination and would depend on a maternal factor, whereas sex determination would depend on a zygotic chromosomal signal - haploidy or diploidy - which is transmitted after heterochromatisation occurs.

The sex-determining genetic cascade: evolutionary aspects

The characterisation in other insects of the genes homologous to those of the sex determination cascade in *D. melanogaster* (see above) allows the evolution of this genetic cascade to be analysed.

The Dsx proteins of different insects show a high degree of conservation, suggesting the presence of strong purifying selection acting on *dsx* to preserve the mechanism of action of the Dsx proteins (Ruiz *et al.,* 2007b). This further indicates the important function that *dsx* has in controlling sexual development in insects.

The degree of conservation of Dsx proteins contrasts with the degree of variability of the Tra proteins. These show an unusually high degree of evolutionary divergence, yet the RS motifs (protein regions with many arginine-serine dipeptides) are conserved. The different degree of variability in the Dsx and the Tra proteins might be due to the less constrained structural requirements of Tra proteins with respect to the ability to perform their function. The RS domains appear to be the major functional part of the Tra protein, and the presence of just 10 - 20% of RS dipeptides in the Tra protein seems to be sufficient to bestow functionality (Kulathinal *et al.,* 2003). Therefore, the Tra proteins probably underwent high rates of neutral evolution whenever they maintained the appropriate levels of RS dipeptides.

Comparison of the Sxl proteins of dipteran species has shown their two RNA-binding domains (RBD) to be highly conserved, whereas the N- and C-terminal domains show significant variation. The great majority of nucleotide changes in the RBDs appear to be synonymous, indicating that purifying selection is acting on them (Serna *et al*., 2004). These results support the contention that the main modifications that invest *Drosophila* Sxl protein with its functional specificity are located in its terminal domains, outside the well-conserved RNA-binding domains (Meise *et al.,* 1998; Saccone *et al.,* 1998; Serna *et al*., 2004). For further studies on the phylogeny of *Sxl* see Traut *et al.* (2006b).

Sxl does not appear to play a key discriminatory role in the control of sex determination outside the drosophilids. In *Ceratitis,* this role appears to be played by the gene *tra*, whereas in the housefly *Musca* it is played by *F*. The model proposed for *Ceratitis* sex determination (Pane *et al*., 2002) and that for the housefly (Dübendorfer *et al*., 2002) show similarities (see Fig. 9).

The gene *tra* of *Ceratitis*, which plays a key regulatory role as the device memory for sex determination (via its autoregulation function) shows maternal expression (Pane *et al*., 2002). The latter authors proposed that *tra* mRNA (or its protein) provided by the oocyte supplies the embryo with the Tra protein needed to impose female-specific splicing of the initial zygotic *tra* pre-mRNA. This would produce the first zygotic functional Tra protein and consequently the establishment of *tra* autoregulation. In this scenario the XX embryos follow female development. In XY embryos, however, the M factor present in the Y chromosome

would prevent the setting up of the *tra* autoregulation system. Consequently, these embryos would not produce functional Tra protein and develop as males. The *Bactrocera* (Lagos *et al.,* 2007) and *Anastrepha* (Ruiz *et al.,* 2007a) *tra* genes show a similar molecular organisation and the same expression pattern (including their maternal expression) to those found in *Ceratitis*,. These results suggest that the *tra* device memory mechanism, as well as the M factor mechanism for preventing the establishment of *tra* autoregulation, were likely present in the common ancestor of the *Ceratitis*, *Bactrocera* and *Anastrepha* phylogenetic lineages.

In the housefly, the gene *F* plays the key role for female sex determination. It shows maternal expression and its maternal product is needed to activate the zygotic function of *F*, which appears to show autoregulation (Dübendorfer and Hediger, 1998). The gene *tra-2* is required for this autoregulation (Burghardt *et al.*, 2005). In XY zygotes, the presence of the masculinising factor M in the Y chromosome would prevent the establishment of *F*- autoregulation and cause male development (Dübendorfer *et al.*, 2002).

Together, these results support the model of Wilkins (1995) who proposed that the evolution of the sex-determining cascades were built bottom up, with the genes at the bottom being more conserved than the more upstream genes in the cascade (for a theoretical analysis of this model see Pomiankowski *et al.*, 2004). Indeed, the *dsx-tra(or F)-tra2* elements at the bottom of the cascade, and their relationships, have been found conserved in all the dipterans so far analysed. This suggests they represent the ancestral state (which still exists in the Tephritidae and Muscidae lineages) with respect to the extant cascade found in the more evolved Drosophilidae lineage, in which *tra* is another component of the sex determination gene cascade regulated by *Sxl*. Thus, in the phylogenetic lineage that gave rise to the drosophilids, evolution co-opted for the *Sx*/gene, modified it, and converted it into the key gene controlling sex determination.

Chromosome heterochromatisation or elimination and sex determination in coccids: evolutionary aspects

In primitive coccids, gender is determined by the conventional XX/X0 system, and in more evolved coccids – lecanoids, comstockiellids and diaspidids - on a haploid/diploid system. It appears that the evolutionary sequence is lecanoid-comstockiellid-diaspidid (Brown and McKenzie, 1962; for a theoretical analysis see Haig, 1933; Herrick and Seger, 1999 and references therein). There are two possible evolutionary scenarios for the primary sex determination signal in coccids. In one, the primary genetic signal that determines gender in both primitive and lecanoid/diaspidid coccids is the same. During the evolution of the lecanoid/diaspidid systems the only thing that changed was the appearance of a mechanism that brings about either diploidy or haploidy – whether structural or functional – of the embryo; the primary genetic signal

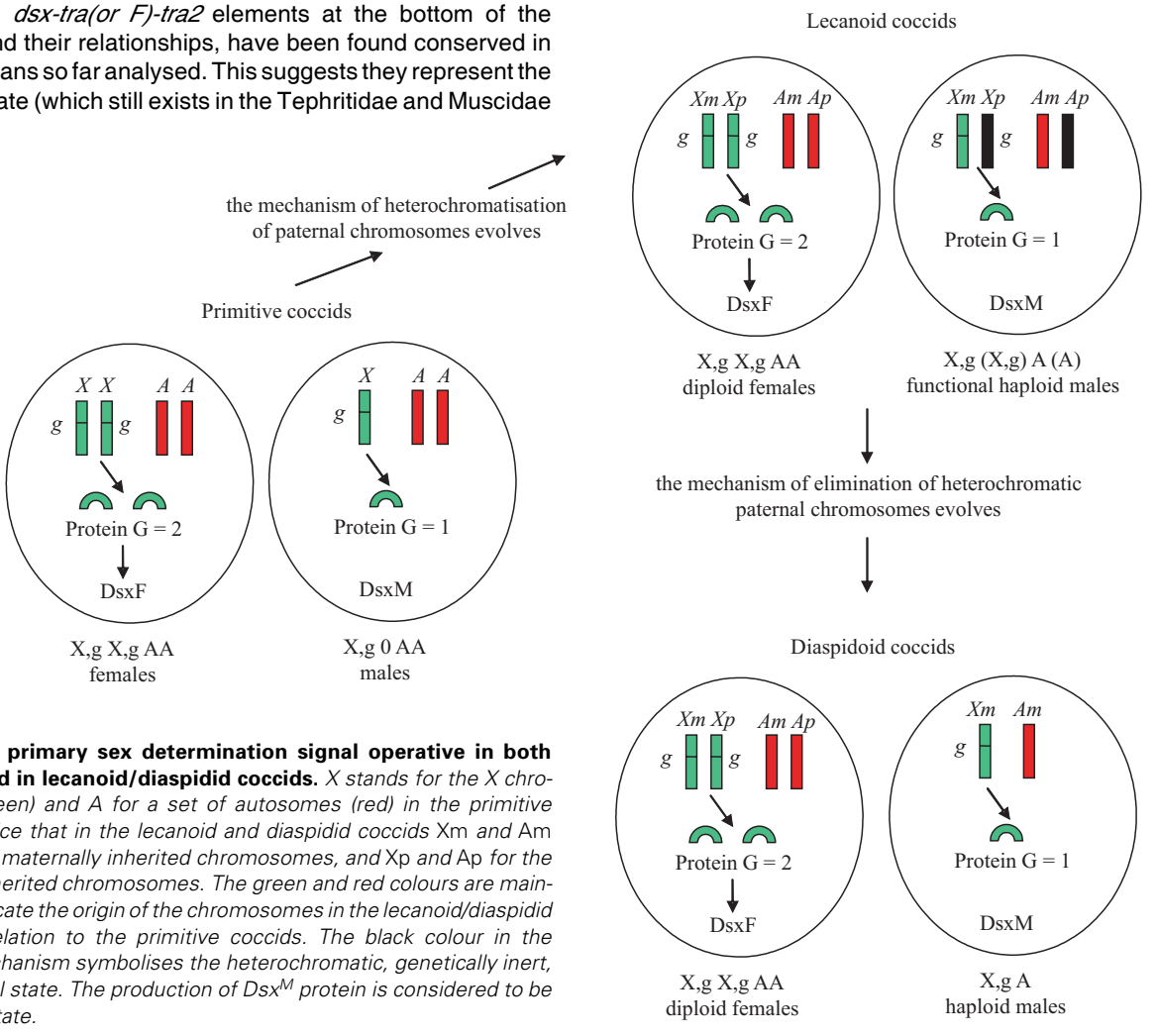

Fig. 10. The primary sex determination signal operative in both primitive and in lecanoid/diaspidid coccids. *X stands for the X chromosome (green) and A for a set of autosomes (red) in the primitive coccids. Notice that in the lecanoid and diaspidid coccids* Xm *and* Am *stand for the maternally inherited chromosomes, and* Xp *and* Ap *for the paternally-inherited chromosomes. The green and red colours are maintained to indicate the origin of the chromosomes in the lecanoid/diaspidid coccids in relation to the primitive coccids. The black colour in the lecanoid mechanism symbolises the heterochromatic, genetically inert, chromosomal state. The production of* Dsx^M *protein is considered to be the default state.*

determining gender remained the same. In the second scenario, the sex-determining mechanism of primitive coccids could not operate with the haploid/diploid strategy evolved in the lecanoid/diaspidid coccids, so a new primary genetic signal evolved. The question arises as to whether the putative primary genetic signal operating in primitive coccids is compatible with the haploid/diploid mechanism operating in lecanoid/diaspidid coccids.

The XX/X0 system of primitive coccids can be explained by four possible mechanisms depending on the primary signal determining the gender of the embryo.

1) In the first, the primary signal is formed by the ratio of X chromosomes to sets of autosomes, with XXAA being females and X0AA males. This mechanism could not operate in the lecanoid/diaspidid coccids because both haploid (male) and diploid (female) embryos have the same female X/A signal. Consequently, only females would be produced.

2) In the second, the primary genetic signal results from a balance between female-determining factors located on the X chromosome and male-determining factors with an autosomal location. This is the "genic balance system of sex determination" proposed by Bridges for *Drosophila* (Bridges, 1925). This hypothesis considers gender as a quantitative characteristic with continuous variation under the control of two opposing polygenic signals, whose component factors each have a small effect. The opposing action of these sets of signals would determine the gender of the embryo, according to the stechiometric assumption that two doses of the female-determining factor (2X chromosomes) outweigh the effect of two doses of the male-determining factor (2 sets of autosomes), leading to females being produced. However, two doses of male-determining factor (2 sets of autosomes) outweigh the effect of one dose of female-determining factor (1X chromosome), leading to male development. This mechanism could not operate in the lecanoid/diaspidid coccids since diploid (female) and haploid (male) embryos have the same ratio of female- and male-determining factors, thus only females could be produced.

3) In the third, the absolute number of X chromosomes determines the primary genetic signal. In this scenario, 2X and 1X embryos will develop as females and males respectively, irrespective of the number of autosomal sets (see Fig. 10). From a genetic point of view, the simplest situation is when a single X-linked gene (*g*) controls sex determination so that the amount of G product dictates the sexual development of the zygote. Thus, XX (two doses of *g*) embryos will have twice the amount of G product as X0 (one dose of *g*) embryos. The former will follow the female pathway whereas the latter will follow the male pathway. This mechanism could operate in the primitive coccids and in the lecanoid and diaspidid coccids whenever two conditions are fulfilled. The first is that either gene *g* is not subject to dosage compensation, or it exerts its function prior to the establishment of dosage compensation (if such a process exists). The second is that gene *g* has only one functional allele. Let us assume that the sex-determining gene *g* has two alleles: a functional and a non-functional allele, and that the presence of the functional allele determines female development and its absence causes male development. In this scenario, X0 embryos, or lecanoid/diaspidid haploid embryos, carrying the functional allele would develop as females, just like XX embryos homozygous or heterozygous for the functional allele. On the other hand, XX embryos, or lecanoid/diaspidid diploid embryos homozygous for the non-functional allele would develop as males.

4) In the fourth mechanism, the primary genetic signal is given by a *complementary sex determiner (csd)* locus, similar to the one found in the honeybee. This mechanism is *a priori* reconcilable with both primitive and lecanoid/diaspidid coccids whenever a high number of alleles exist in the population (see Fig. 4). In this scenario, allelic homozygosity, which causes diploid embryos to develop as males, would be infrequent. Notice that the *csd* locus in the honeybee works in a haploid/diploid scenario. This is also the situation found in the lecanoid/diaspidid coccids. With respect to the primitive coccids, the X chromosome is in the haploid condition in males, and in the diploid condition in females. However, the existence of some parthenogenic coccids known to produce female embryos (see above) makes this mechanism less plausible because these females are homoallelic for the putative *csd* locus, and then they should develop as males instead of as females.

In conclusion, the only sex-determining mechanisms compatible with the XX/X0 system of primitive coccids and the haploid/diploid system of more evolved lecanoid/diaspidid coccids is that in which gender depends on the number of copies of an X-linked gene. However, certain conditions are necessary.

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