Molecular basis for Sex determination in the silkworm, *Bombyx mori*

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CERTIFICATE

This is to certify that Mr. **V Sathish** has carried out the research work in the present thesis under my supervision and guidance for a full period prescribed under the PhD ordinance of this university. I recommend his thesis entitled ***"Molecular basis for Sex determination in the silkworm, Bombyx mori"*** for submission for the degree of Doctor of Philosophy of this university.

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DECLARATION

I hereby state that the work presented in this thesis entitled **"Molecular basis for Sex determination in the silkworm, *Bombyx mori*"** has been carried out by me under the supervision of Dr. J. Nagaraju at Centre for DNA Fingerprinting and Diagnostics, Hyderabad and that this work is original and has not been submitted in part or full for any degree or diploma of any other university earlier.

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"Faced with a really difficult-looking problem should one follow the advice of Descartes or of Holmes? Should one proceed step by step from what is easily understood, as Descartes advised, 'starting with what was simplest to know, and rising little by little to the knowledge of the most complex'? It sounds like good advice and on the whole modern science takes it. But the methodical step-by-step strategy does not always work. First steps can particularly be tricky and you have to know in which direction to go. There are times when you need the advice not of Descartes, but of Sherlock Holmes.

You see Holmes, far from going for the easy bits first, would positively seek out those features in a case that were seemingly incomprehensible – 'singular' features he would call them. They can point the way. They can tell you what sort of a problem it is that you are dealing with".

A. G. CAIRNS-SMITH
Seven Clues to the Origin of Life

ABSTRACT

The unusually rapid evolution of sex chromosomes undergo a continuous accumulation-attrition cycle involving several stages which include acquisition of a sex determining function; suppression of recombination in the heterogametic sex; differentiation of both sex chromosomes; translocation of the sex determining function (and other essential genes) to another chromosome and finally, the loss of the heterogametic sex chromosome. Much like XY chromosomes in groups with male heterogamety, the lepidopteran sex chromosomes show various degrees of structural differentiation. Differences between Z and W chromomere patterns range from undetectable to obviously non-homologous. The sex-determining systems consist of parental sex-ratio genes, parental-effect sex determiners, and zygotic sex determiners, which are subject to different selection pressures because of differences in their modes of inheritance and expression. Attempts at identifying a W-linked factor have a long history since the observation of sex in polyploid silkworms- the presence of W *chromosome* ensures that the zygote develops into a female suggesting the existence of an epistatic system of sex determination. Except for the identification of a few W-specific RAPD markers, the molecular structure of W chromosome is not known. Screening and characterization of homologues of genes like *sex lethal* (*sxl), transformer* (*tra), doublesex* (*dsx), fruitless (fru)* and *intersex* (*ix*) which constitute the sex determination cascade of *D. melanogaster,* revealed that they were not found to be located on the W chromosome. A comparative gene expression profiling was our second strategy to screen for a female-specific factor. The default splice state of *dsx* transcript is female and is currently hypothesized to be regulated by a cascade of repressors. A Differential display screen at early embryonic stages yielded a C-x8-C-x5-C-x3-H type zinc finger transcript, which was subsequently found to be encoded by a W-linked gene. Also, two more copies of the gene linked to XXV chromosome, were also identified based on BLAST analysis and Southern hybridization. C-x8-C-x5-C-x3-H type zinc finger motif- containing genes are reported to be involved either in protein-protein interactions or protein-RNA interactions to facilitate the 3'splice site selection. Presence of the transcripts of these genes in the unfertilized embryo and the subsequent ubiquitous expression in all stages and all tissues, suggest a primary role for the genes in the sex determination pathway. Transient RNAi assays targeting all the three transcripts revealed a

possible interaction between the zinc-finger genes in determining the splice state of the downstream gene, *doublesex*, in a direct or indirect manner. Thus a model is proposed for the sex determination pathway in the silkworm involving the three zinc finger motif-containing genes.

INTRODUCTION

Sericulture, or silk production, from the moth, *Bombyx mori* (L.), has a long history- from its discovery around 2,700 B.C., to the passage of the technique through "Silk Road (from Eastern China to the Mediterranean sea) beginning 139 B.C., to spreading to Japan through Korea, and to the west in 300 A. D. and to playing an influential role in local economies of developing nations in modern times (KURIN 2002). Equally long is the history of silkworm biology and genetics. Silkmoths, both the wild species of the family Saturniidae and the domesticated *B. mori,* have been important objects of research in insect physiology and developmental biology (GOLDSMITH and KAFATOS 1984). Their many advantages as model insect system include their large body size, the ease of rearing them, the hormonally controlled radical metamorphic changes they undergo late in the life cycle, and their relatively simple body plan, allowing access to many developmental systems usually of a few related cell types (e.g. silk gland, fatbody, ovarian follicles). In the case of *B. mori,* an additional advantage is that the study of its genetics, pursued because of the commercial importance of that species, is well advanced. In all 28 linkage groups have been defined on the haploid set of 28 chromosomes and more than 400 mutations in over 200 loci have been described and mapped (YAMAMOTO *et al.* 2006; YAMAMOTO *et al.* 2008). With the development of the molecular linkage maps, EST databases, draft genome sequence, and transgenic transformation, and RNAi technologies, *B. mori* contends its position successfully as a model for basic insect science and the biotechnology sector.

After Coleoptera, the order Lepidoptera comprises the most biodiverse group of animals (PASHLEY *et al.* 1993). The family Noctuidae is the largest in the order, and it includes some of the most devastating pests of agriculture, particularly the Heliothines (*Helicoverpa armigera*, *H. zea*, *Heliothis virescens*) and other genera (*Mamestra*, *Spodoptera*, *Trichoplusia* etc.). Although lepidopteran pests of agriculture, forestry and food storage are found in diverse families (*Noctuidae, Pyralidae, Arctiidae, Sphingidae, Tortricidae, Lymantridae, Yponomeutidae*) they all belong to the same suborder, Ditrysia, and are not very divergent phylogenetically. Therefore, the genomic information of the model species *B. mori* should be applicable to most economically important species in Lepidoptera. Hence

comparative studies on behavioral and developmental features at the molecular level between different Lepidoptera and other insects as well would be significant. Particularly, this would enable develop strategies of control against the pest species, especially species-specific ones that avoid damage to other insect species. Some of the new concepts intend to exploit the sex determination mechanisms. Hence studying sex determination mechanisms in the lepidopteran species manifests an all too significant economic angle apart from the exciting inputs gained in towards understanding the evolutionary dynamics of sex determination.

Among the many well studied developmental processes, the biology of sex determination has a special place. Sex determination mechanisms unlike other embryonically initiated pathways are remarkably diverse across the animal kingdom (ZARKOWER 2001). Sex determination is an integral part of reproduction and an essential process for the evolvement and enrichment of the genome. Data so far accumulated by a variety of model organisms have shown a relative economy in the molecular regulation of sex determination and it involved one of the following three mechanisms:

- **Environmental action on the embryo at a crucial stage of development.** To the extent that this interaction is associated with temperature alterations, the process is also described as temperature-dependent sex determination and the developmental stage of sex determination is referred to as the thermosensitive period (TSP). This mechanism is mainly observed in reptiles and fish (GODWIN *et al.* 2003).
- **Genetic action, when at least one specific gene is considered to be the central regulator in a cascade of events leading to the determination of sexual phenotype.** This mechanism is already known to apply in the case of several animals, including invertebrates (insects, worms) and amphibians. Moreover, it is a proposed regulatory mechanism for several species, whose study has so far been limited or led to inconclusive data as to the attempt to detect a single, specific, sex-determining gene (CHARLESWORTH 2002).
- **The presence of distinct sex chromosomes or gonosomes.** The identical pair may be present in both males (birds) and females (mammals) and their major sex-determining gene may be either known (e.g. mammalian SRY) or still suspected (BULL 1987).

Although sex determination has been suggested to promote specific functions at a universal level, such as selective cell proliferation or steroid hormone accumulation, this issue remains debatable. It is generally accepted that environmental sex determination is the ancestral state and that genetic sex determination evolved as a derived condition. It is also recognized that genetic sex determination is evolutionarily highly labile, having evolved into existence on many independent occasions across diverse taxa (SARRE *et al.* 2004).

In Lepidoptera, in contrast to most other animal groups, females are the heterogametic sex. They share this property with Trichoptera (caddis flies), their closest relatives, and with snakes and birds (ENNIS 1976; TRAUT and MAREC 1996). Sex chromosome systems with female heterogamety are referred to as WZ/ZZ and Z/ZZ (alternatively designated WZ and ZO) depending on the presence or absence of a W chromosome. Data from inheritance of irradiated chromosome fragments suggest that the sex is strongly controlled by the presence or absence of a specific region of the W chromosome in *B. mori* (FUJII and SHIMADA 2007).This led to the prediction long ago that the W chromosome carries an epistatic feminizing gene that determines female development. So far, no genes have been assigned to the W chromosome except for *Fem*, the strong determinant of femaleness. By contrast, results obtained in sex chromosome mutants of the Mediterranean flour moth, *Ephestia kuehniella* Zeller, suggest that the W chromosome carries a male killing factor that might serve as a feedback control of sex rather than as a determinant in the female sex differentiation pathway (MAREC *et al.* 2001). However, the absence of a W chromosome in primitive Lepidoptera and frequent secondary loss of the W in advanced Lepidoptera favor balance mechanisms of sex determination with Z-linked male promoting factors instead of W-linked female promoting factors (TRAUT and MAREC 1996).

In search of sex chromosome-specific markers five W-linked RAPDs were identified (ABE *et al.* 1998a; ABE *et al.* 1998b; ABE *et al.* 2000a; ABE *et al.* 1998c; ABE *et al.* 2000b). Using these markers for subsequent cloning and sequencing of W-derived BAC clones, it was revealed that the W chromosome is largely composed of nested, full-length retrotransposable elements. Neither a classical linkage map of the Z chromosome of *B. mori* containing 15 morphological traits dispersed over 50cM (FUJII 1998), nor did the 18 Z-linked RAPD

markers (YASUKOCHI 1998) nor the 16 SSR/ISSR/RAPD marker-based genetic map revealed any sex-determining locus (NAGARAJA *et al.* 2005). The 13 genes identified in a contiguous 320 kb walk of the Z-chromosome are also not of significance in terms of sex determination (KOIKE *et al.* 2003).

Ohbayashi et al. (2001) isolated from *B. mori* a homologue of the *doublesex* (*dsx*) gene that is a downstream member of the sex-determining cascade in *Drosophila melanogaster*. *B. mori doublesex* (*Bmdsx*) is present on autosomes in both sexes and, like *Drosophila,* is alternatively spliced to yield male and female-specific mRNAs (SUZUKI *et al.* 2001). Suzuki et al. (2003; 2005) showed that *Bmdsx* encodes male-specific (BmDSXM) and female-specific (BmDSXF) polypeptides and that the BmDSXF protein has a regulatory function in females that acts repressively in males. These results strongly support the view that *Bmdsx* acts early in the hierarchy of regulatory genes controlling female differentiation in *B. mori.* However, sex-specific splicing is regulated by repression of the default female-specific processing pattern (OHBAYASHI *et al.* 2001) and thus very different from that in *Drosophila,* where the default state is male-specific splicing, and female-specific splicing is under control of positive splicing cofactors (GRAHAM *et al.* 2003). Thus, sex-specific splicing of *Bmdsx* is controlled not by a splicing activator like TRA but instead, by some as yet unidentified splicing repressor, indicating a difference between *Drosophila* and *Bombyx* in the mechanisms of splicing regulation of their respective *dsx* genes. In *Drosophila*, the female-type DSX protein has been reported to interact with a partner protein encoded by the *intersex* (NUNES *et al.*) gene, which activates transcription of the yolk protein gene (*Yp*) (GARRETT-ENGELE *et al.* 2002). Although the WGS assembly of *Bombyx* contains a homolog of *ix*, the protein encoded by the gene did not interact with BmDSX in the yeast two hybrid system (FUJII and SHIMADA 2007). Thus, it is possible that BmDSX interacts with an as yet unidentified factor unrelated to *ix* in order to bring about female-specific regulation. The whole-genome shotgun sequences for *Bombyx* do not contain a homolog of *tra* (FUJII and SHIMADA 2007), which encodes an essential regulator of *dsx*, though a *tra-2* (cofactor of *tra*) homolog was found in the *Bombyx* genome (NIU *et al.* 2005). This is consistent with the hypothesis that *Bmdsx* is regulated by a male-specific splicing repressor. In *Drosophila*, *fruitless* (*fru*) is important for sex determination in the nervous system (GAILEY *et al.* 2006;

KIMURA *et al.* 2005). *Bombyx* genome contains *Bmfru,* which is orthologous to *Drosophila fru*, but it is not known if *Bmfru*, *Bmdsx*, and/or another factor determines sex in *Bombyx* neurons. As is true for *Drosophila fru*, *Bmfru* exists in many isoforms produced by transcription initiation at different promoters and by alternative splicing. Moreover, expression in the adult head is quantitatively different between the sexes, suggesting a sex-dependent function for *Bmfru* in the silkworm brain (FUJII and SHIMADA 2007). In *Drosophila*, control of germ line sex determination is very different from that of somatic cells. Although the mechanisms of germline sex determination in *Drosophila* are not yet fully understood, it is known that *Sxl*, *bam*, *otu*, *ovo*, and *snf* regulate sex determination of the germ line in this species (CASPER and VAN DOREN 2006). Although orthologs of *Sxl*, *otu*, *ovo*, and *snf* can be found in *Bombyx* WGS contig sequences, a recognizable ortholog of *bam*, critically important for determination of female germ cells in *Drosophila*, is absent in *Bombyx*, suggesting that the sexual differentiation pathway in *Bombyx* germ cells of is different from that in *Drosophila* (MITA *et al.* 2004; XIA *et al.* 2004). Using SAGE approach, a novel non-coding RNA was found that is abundantly expressed in cystocytes and later in female germ cells (FUNAGUMA *et al.* 2007). Knock-down of this non-coding RNA via RNAi induced an interesting phenotype- oocyte-nurse cell differentiation was frequently disrupted in RNAi-treated individuals and the genitalia of females were transformed into male-like structures, similar to what is observed for transgenic moths expressing the male form of *Bmdsx* (FUJII and SHIMADA 2007).

Initiating studies on sex determination mechanism and sex chromosomes in *B .mori* our group recorded the following significant observations:

- Identification and characterization of a GATA-binding protein expressed predominantly in the pupal ovary of *B. mori* (PRIYADARSHINI *et al.* 2003).
- Generation of a recombination map of 334.5 cM with 16 Z-linked markers which were distributed throughout the Z chromosome. Four RAPD and four SSR markers that were linked to W chromosome were also identified (NAGARAJA *et al.* 2005).

Questions on the sex determination process in silkworm and evolution of sex determination systems in Lepidoptera would remain unsolved without the identification and characterization of genes present on W chromosome. With the available information on the silkworm homologues of genes in the sex determination pathway, a model for the sex determination pathway was constructed which would help in the search for W-linked sex determining genes.

Analyzing gene expression patterns in early embryonic stages is the first step in studying developmental pathways. In particular, comparing male and female embryonic transcription profiles is advantageous in identifying sex determining genes that may or may not be sex chromosome-linked. With the availability of a diapausing translocated stock [W chromosome with a translocation of a part of 10th chromosome which contains dominant allele ($+^{w2}$) for egg serosal pigmentation] monitoring of differential gene expression in colourless male and coloured female embryos during the early stages of blastulation was made possible (FUJII and SHIMADA 2007). Several different approaches which enable the identification of euchromatin regions of W chromosome and the analysis for the presence of female-specific genes were considered. Apart from the much attempted marker-based approaches, comparative genomic hybridization (CGH), laser micro dissection etc are the preferred methods to identify W-linked genomic regions, while representational differential analysis (KUNTZ *et al.*), RNA fingerprinting, subtractive hybridization, differential display PCR (DDPCR) are RNA-based methods. Since RNA-based methods offer a high throughput and efficient route for the identification of sex-specific sequences, DDPCR was employed for comparing the expression profiles of male and female silkworms and screening for female-specific transcripts in different developmental stages.

The primary outcome of the screening is the identification and characterization of a W-linked gene possessing C-x8-C-x5-C-x3-H type zinc finger motifs which was followed by the simultaneous identification of additional copies of the gene on W chromosome and XV chromosome. This thesis investigates the prospects of the zinc finger genes as the master regulators of the sex determination pathway of silkworm.

REVIEW OF LITERATURE

Sex and the Silkworm

Among the many reasons for renewed interest in Lepidoptera genetics are the many pest species that belong to this insect group and cause considerable losses in agriculture. Therefore, there is a high demand to develop strategies of pest control against these species, especially species-specific ones that avoid damage to other insect species. Historically, only a few species belonging to the order Lepidoptera- a large group even among the insects- have attracted attention in genetic research in the past. Among them were the flour moth *Ephestia kuehniella*, in which A. Kühn et al. (1956; 1966; 1955) studied the ommochrome synthesis pathway; *Papilio dardanus* in which C.A. Clarke and P.M. Sheppard studied Batesian mimicry (1959; 1960a; 1960b; 1962; 1972; 1976; 1961); the psychid moth *Solenobia triquetrella* and related species in which J. Seiler and colleagues studied development of sexual intergrades and evolution of parthenogenesis (1959; 1960; 1965; 1967; 1968; 1969; 1950); and the gypsy moth *Lymantria dispar* in which R. Goldschmidt and colleagues studied intersexuality and sex determination (1942). The saturniid moth *Antheraea pernyi* was studied for circadian rhythm by Reppert et al. (1999; 1996).

Only the economically important silk moth *Bombyx mori* has continuously attracted research for a long time and became an insect model species, second only to *Drosophila melanogaster* in importance (GOLDSMITH *et al.* 2005). The availability of mutations and inbred lines is the single major advantage of working with the domesticated silkworm over other lepidopteran species. More than 400 Mendelian mutations have been described. Large collections are maintained at stock centers in China [>600 strains], Japan [>850 strains], and Korea [>300

strains] (GOLDSMITH *et al.* 2005). Most mutations are spontaneous, discovered during mass-rearing for silk production. One or a few alleles are available for most characters. Stable, irradiation-induced, sex-limited translocations carrying a visible marker on the W or female-determining chromosome have enabled development of "autosexing" strains for discrimination of males and females by egg, larval, or cocoon color and proved useful for studying sex differentiation during early embryogenesis (MAREC *et al.* 2005). Rather than traditional cytogenetics which is limited due to poor chromosome differentiation (TRAUT and MAREC 1996), translocations have provided landmarks for uncovering the causes of spontaneous mutations (GOLDSMITH 1995). The availability of molecular markers for genome-wide mapping and tracking the inheritance of chromosomes and chromosome segments has made it possible to localize these mutations, analyze quantitative trait loci (QTL), and clone the underlying genes. This strong foundation of classical genetics complements modern genomics. The availability of vast amounts of sequence data- including whole-genome shotgun (WGS) sequences of *Bombyx mori* and expressed sequence tag (EST) collections from various Lepidopteran species, developmental stages and tissues – triggered extensive research in Lepidopteran genomics. Also, together with the emergence of various fluorescence *in situ* hybridization (FISH) techniques and the use of pachytene chromosomes, moth and butterfly chromosomes have become amenable to cytogenetics (SAHARA *et al.* 1999; SAHARA *et al.* 2003; SAHARA *et al.* 2007; YOSHIDO *et al.* 2005). Finally, the accelerated progress in silkworm genomics is augmented by speedy assessment of functions of large numbers of genes by genome-wide assays using RNAi and related techniques. Gene silencing in *B. mori* embryos and first instar larvae was first demonstrated by direct injection

of dsRNA corresponding to a 925-bp fragment of the silkworm ortholog of the *Drosophila* white gene, *w3*(QUAN *et al.* 2002).

The biological features of silkmoths and the amount of accumulated background information available on them have encouraged fundamental studies in developmental regulation. Much of the current knowledge about how hormones regulate the lives of all insects had its origin in ligations, transplantations and parabioses in diverse Lepidoptera (NAGY 1995). Most of the insect haemolymph products were first characterized in species of moths. Two best-studied systems, the silk gland and the chorion-producing follicle have brought into light special transcriptional and translational mechanisms of complex gene clusters (HUI 1995; KAFATOS 1995). Many developmental pathways have since been elucidated and promptly subjected to successful comparative analysis. These discoveries illustrate ways that very different organisms are, at a fundamental level, similar to one another. But not all developmental processes are so conservative; an outstanding example is sex determination. Sex determination is a crucial process in developmental biology. Its accurate regulation is a prerequisite for reproductive success and, therefore, the continued survival of a species. Since reproduction is also the function that determines the categorization of specific populations in the same or different species, the analysis of the specific molecular patterns that this process may follow is crucial for the comprehension of the detailed biochemical background mediating and maintaining the phenotypical variety observed at a macroscopic level. Sex determination is an essential process for the evolvement and enrichment of the genome. It has been the subject of many studies in reference to species across the entire animal kingdom. From insects to mammals, there is much to learn from the many

mechanisms employed to determine sexual fate. This is no lost cause, since the study of sex determination and differentiation is only the natural expansion of comparative biology and reproductive physiology in the modern, molecular era.

Sexual Dimorphism and Sex Determination Systems

Sexual dimorphism is established by regulatory hierarchies that dictate the path of sexual development that an initially ambiguous embryo would proceed to. Both the initial signals that trigger this process, which can be either genetic or environmental, and the regulatory pathways that respond to these cues, vary remarkably between species. The variety of primary sex determination cues was appreciated long before the advent of molecular genetics. The two broadest categories are genetic sex determination (GSD), in which the sex of offspring is set by a sex chromosome or an autosomal gene, and environmental sex determination (ESD), in which sex is determined by temperature (as with turtles), local sex ratio (as with some tropical fish), or population density (as with mermithid nematodes) (BULL 1983). Though little is known about the molecular mechanisms of ESD, within the GSD systems many different mechanisms have been uncovered. Dual sex chromosome systems, in which either the female (ZW/ZZ) or the male (XX/XY) is heterogametic, are common, as are systems set by the ratio of the number of X chromosomes to sets of autosomes (X:A). Dimorphic sex chromosome systems independently evolved in animals, mosses, and dioecious plants (LENGELER *et al.* 2002; ZARKOWER 2001). A related but distinct sexual incompatibility system is found in many lower eukaryotes, including algae, protozoans, monoecious plants, and fungi. In these organisms, multiallelic mating-type

(MAT) loci monitor cell interactions for sexual compatibility, and if inbreeding is detected, mating is aborted (FRASER *et al.* 2004).

The long-standing mystery of sex determination and its diversity began by comparisons between distantly related species. A better understanding of sex determination pathways holds lessons about the evolution of development in general.

Invertebrates

Hymenoptera

One such interesting mechanism is the haplodiploid genetic system found in the insect order of Hymenoptera. More than 200, 000 species of ants, bees and wasps are capable of laying both unfertilized eggs, that typically develop into uniparental (originating from one single female parent) haploid males, and fertilized eggs that can give us biparental (originating from two parents, male and female) diploid females. That can be accomplished with several strategies. One of the best understood seems to be single-locus complementary sex determination (sl-CSD), in which sex is determined by multiple alleles at a single locus (EVANS *et al.* 2004; PIENAAR and GREEFF 2003; STAHLHUT and COWAN 2004; ZHOU *et al.* 2006). Heterozygotes at that sex locus develop as females whereas hemizygotes, and the odd case of homozygous diploids (i.e. through matched matings or faulty meiosis), develop as males (**Figure1**).

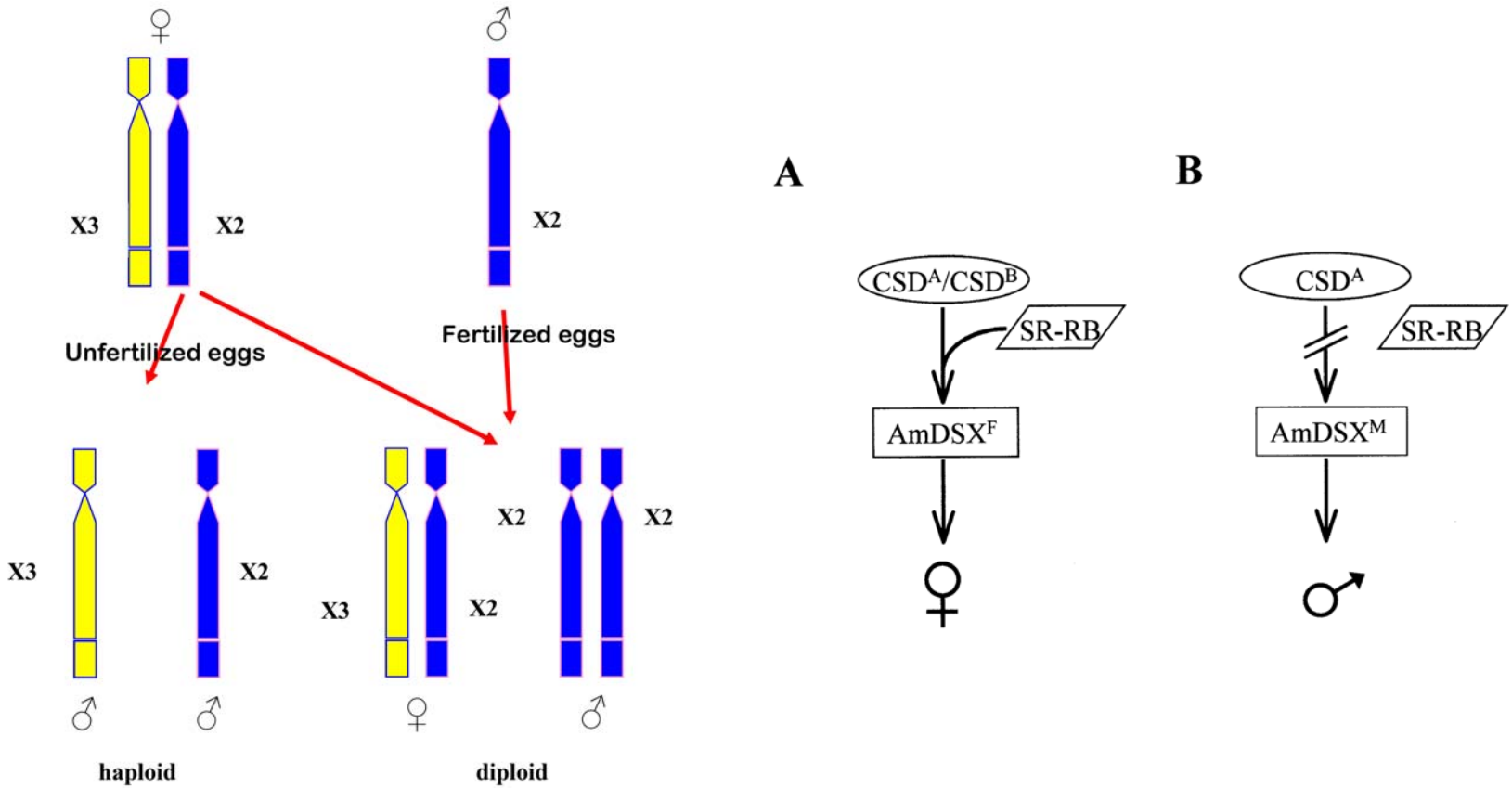


Figure1 Complementary mode of sex determination and the result of inbreeding. Half of the fertilized eggs are homozygous for the sex-determining locus and develop into non-reproducing diploid males. Heterozygotes develop into females. The reproductive haploid males are derived from unfertilized eggs. (A) With heterozygous csd composition in females the heteromeric CSD protein is functional through cooperatively binding of a SR- type protein containing an RNA recognition motif (SR-RB protein) that splices Apis dsx pre-mRNA. $AmDSX^F$ protein is produced that induces female development. (B) With hemi- or homozygous csd composition in males the one CSD protein present is nonfunctional. *Amdsx* is expressed by default to produce the $AmDSX^M$ isoform, which induces male development.

In honeybees, for example, the sex locus has recently been identified as the csd (complementary sex determiner) gene that encodes an SR protein (Arginine-Serine rich protein) (BEYE *et al.* 2003). The initial observation that csd function was required only in females and that its product is nonfunctional when derived from only one allele was followed by the suggestion of three possible models. First, that different allelic CSD proteins form active heterodimers. Second, those CSD proteins derived from the same allele form homomers, with two homomer species in females and one in males. And third, that merely the existence of different alleles is required in females for csd to complete its function. However, it should be noted that sl-CSD has been known to exhibit an evolutionary pressure against species with higher rates of inbreeding, due to one of its major faults. In most cases, mating leads to the creation of offspring with two different alleles at the sex locus (diploid females). However, a mating in such populations has higher probability of a union between a male and a female that share the same allele, a condition also known as a matched mating. In matched matings half the diploid offspring are predicted to turn out homozygous at the sex locus and develop as males rather than females, whereas diploid males in species with sl-CSD are generally sterile, unable to mate or not viable. Such is the example of the honeybee, where homozygous diploid males created from inbreeding are eaten by the workers.

Dipterans (*Drosophila melanogaster*)

Taking things a step further, the focus would fall on Dipterans and *Drosophila melanogaster*, one of the model organisms in which the sex determination pathway has been elucidated in the greatest detail. Here the choice between male and female development is made by one

single switch gene by the name of sex-lethal (*sxl*) in response to the ratio of X chromosomes to autosomes (X:A ratio) (ZARKOWER 2001) (**Figure2**).

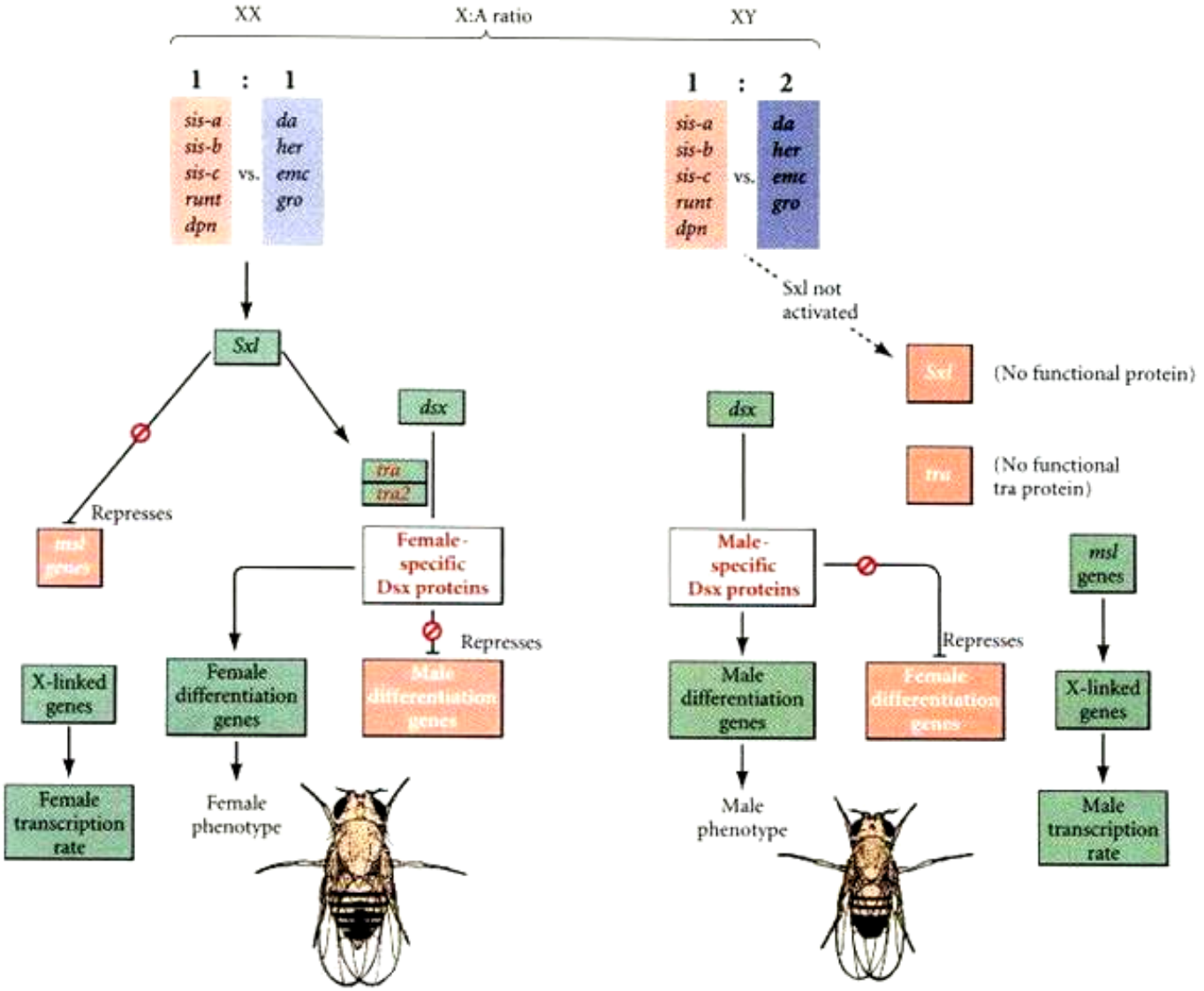


Figure2 Regulation cascade for *Drosophila* somatic sex determination. Arrows represent activation, while a block at the end of a line indicates suppression. The *msl* loci, under the control of the *Sxl* gene, regulate the dosage compensatory transcription of the male X chromosome.

The latter is communicated early in development through the delicate balance between the dose-sensitive X chromosome numerator elements (those include genes such as *sis-a*, *sis-b*, *runt* and less so *sis-c*) and the autosomal denominators (such as *dpn*) in conjunction with the maternally derived products of the *da* gene and the more recently studied *emc*, *groucho*, *her* and *snf*. It seems that the feminizing effect of the numerator elements is measured against the masculinizing denominators, with the maternally derived products of the rest of the genes acting as point of reference (MANOLAKOU *et al.* 2006). All this takes place early in development, leading to the activation of the *sxl* gene through an "early" promoter in females. This early form of the SXL protein, absent in males, then orchestrates a specific splicing of the mRNA produced through the activation of the "mature" promoter in females. In males, standard splicing of the *sxl* mRNA leads to a non-functional protein. It is only in females, through an autoregulatory feedback loop, that *sxl* manages to keep itself in an active state through this sex-specific splicing. Once the SXL active state has been established, it then goes on to regulate a series of other proteins that control female development, once again through the process of alternative splicing, leading finally to the two alternative products of the *doublesex* gene (*dsx*), DSXF and DSXM. A series of gene interactions take it from there and establish the development of the appropriate sex. Still, it is interesting to note, that the Y chromosome, present in males, takes no part in this entire process, and that its sole use is to help in the successful completion of the process of spermatogenesis later on in the differentiation of the male germ line (HACKSTEIN 1987; LIFSCHYTZ 1987; ZHANG and STANKIEWICZ 1998).

Nematodes (*Caenorhabditis elegans*)

Another model organism that uses a single gene switch and the subsequent hierarchy of gene pathways to determine sex is the nematode *C. elegans*. Here again the animal's sexual fate depends on the X: A ratio, and there isn't even a Y chromosome present in males to later on interfere with the germ line (**Figure3**). However, *C. elegans* worms are special in that the choice lies between males with one X chromosome and hermaphrodites with two (HODGKIN 1987; HODGKIN *et al.* 1994).

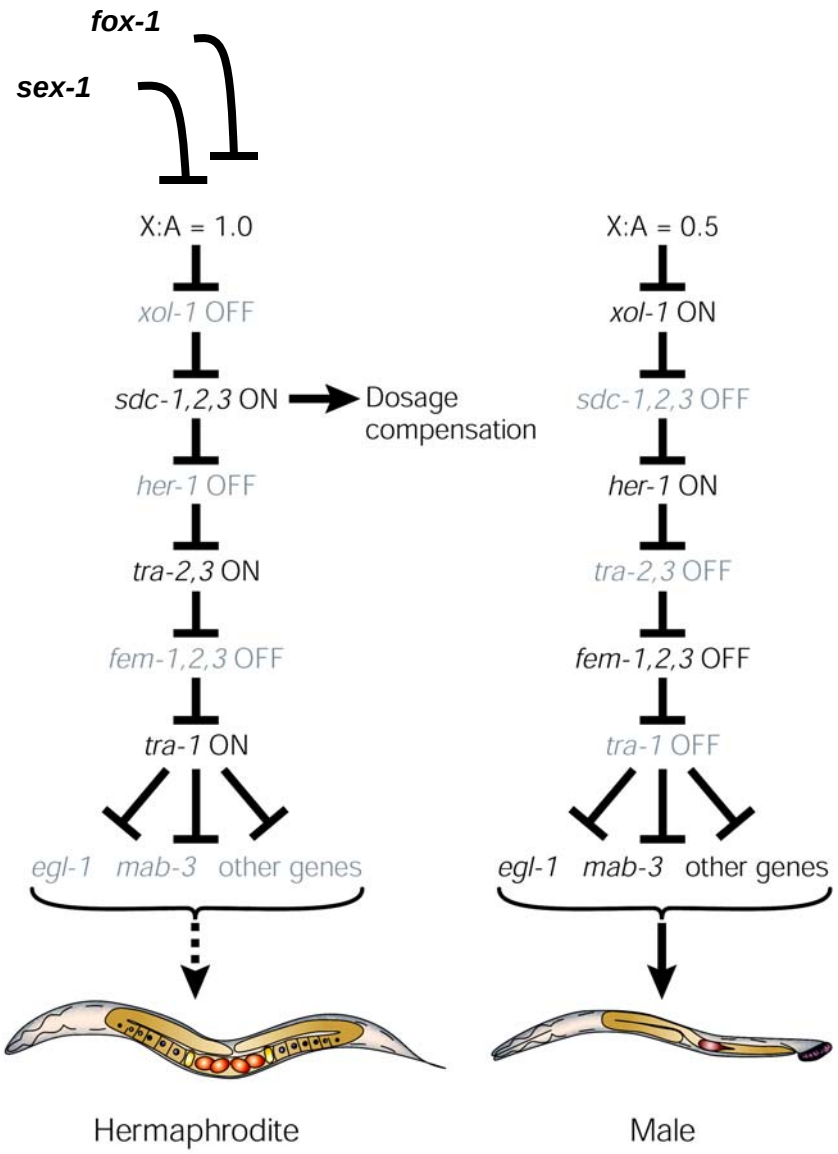


Figure3 In *C .elegans*, the X-signal elements, such as the SEX-1 and FOX-1 proteins, control the levels of XOL- 1 and help determine sex. In XX hermaphrodites, the XO-lethal 1 (xol-1) gene is repressed, which permits, through a series of negative regulatory interactions, the TRA-1 protein to be active. Active sdc-1, -2 and -3 repress her-1. They also control sex determination and activate dosage compensation, leading to hypotranscription of X-chromosome-linked genes. Active TRA-1 represses the expression of egg-laying defective 1 (egl-1), male abnormal 3 (mab-3), and other unidentified genes, allowing female somatic development to occur. (Hermaphrodites in *C. elegans* are somatically female but produce a limited amount of sperm, so they are self-fertile as well as cross-fertile). Right-hand side: In XO males, xol-1 is active, which leads to post-translational inactivation of TRA-1. So, egl-1, mab-3 and other genes are expressed and direct male differentiation.

As before, the X: A ratio is communicated with the help of several "X-signal elements", such as the SEX-1 (signal element on X) protein that acts on the level of transcription and the FOX-1 (feminizing locus on X) protein that acts posttranscriptionally (SKIPPER *et al.* 1999). These two, among others that have yet to be deciphered, manage to suppress the levels of the XOL-1 (XO lethal) key protein, or what we could call the *C. elegans* sex switching gene. From there on, it is a matter of tracking down a pathway of inhibitory genes, to result at the TRA-1 (transformer) protein, free to act in hermaphrodites and regulate several other genes. This pathway in fact involves several groups of gene products, some of which retain their active state in males and others in hermaphrodites. One possible model incorporating these interactions includes the interaction between HER-1 and the TRA-2 receptor in males, which allows the FEM proteins to inhibit TRA-1 from acting as a transcription factor. However, the *C. elegans* hermaphrodites pose an interesting issue. These are specialized females which in the fourth and final larval stage (L4) produce around 300 sperm, to use for self-fertilization when there are no males available. This requires a careful regulation of the switching between the male and female differentiation of the same germ cells without the benefit of the usual sex determination pathway, since the "male" genes that normally regulate spermatogenesis are inactive anyway. Instead, a new series of genes take over in a specific stage of development and act in place of the HER-1 protein to inhibit *tra-2* and allow spermatogenesis to take place till the end of the L4 stage. Once this is over and at the onset of adult life, a new series of genes take their place, *tra-2* is once again active, and the adult hermaphrodite is free to continue with oogenesis for the rest of its life (DE BONO and HODGKIN 1996; HODGKIN 1999).

Vertebrates

Reptiles

The different species of reptiles present a considerable variety of sex determination patterns. For instance, most snakes possess a ZZ/ZW pattern of sex chromosomes, similar to that of the model mechanism for sex determination in birds (JANZEN and PHILLIPS 2006; RIVAS and BURGHARDT 2005). The study of lizards has led to more complex findings, with different species having either a ZZ/ZW sex chromosome pair or a XX/XY system, similar to that observed in mammals (EZAZ *et al.* 2005). On the other hand, many species of reptiles, including most terrestrial turtles and all crocodilians and sea turtles examined to this date, have no discernible sex chromosomes, nor is their sex determined by the presence or absence of specific genes (CREWS 2003; FERGUSON-SMITH 2007). In these organisms, it is the temperature of the environment in a specific period of incubation that can determine whether the animal in question will turn into a male or a female. Indeed, studies have shown that there seem to be no significant differences in the expression of sex-related genes. Instead, there is a specific period of incubation, which is generally considered to lie in the middle third of development, during which the temperature of the eggs controls quite accurately their sexual fate (**Figure4**).

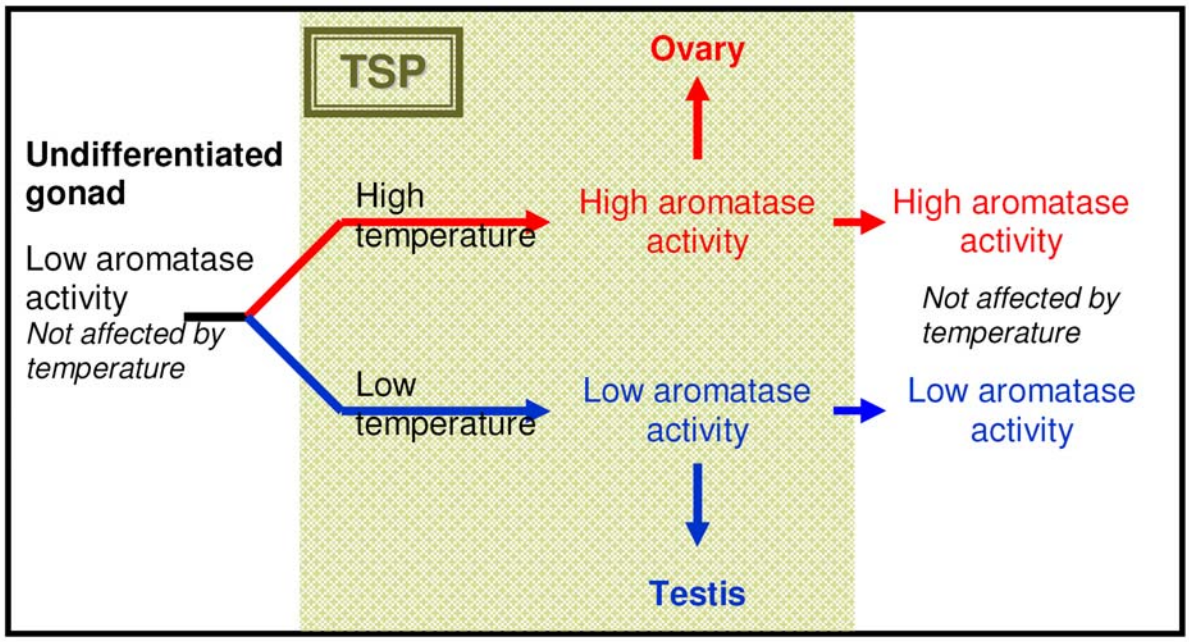


Figure4 Aromatase activity levels during the thermosensitive period (TSP) are regulated by the temperature of the environment and control gonadal differentiation. Changes in the environment temperature before and after TSP do not seem to affect sex.

This particular period is also known as the thermosensitive period (TSP). It is during this period that a very specific enzyme enters into the equation. Aromatase, a cyt450 enzyme responsible for the conversion of androgens into estrogens is common among many organisms. In reptiles, while steroidogenesis begins very early, prior even to the thermosensitive period, aromatase activity remains universally low (BELAID *et al.* 2001; DORIZZI *et al.* 1996; DOURNON *et al.* 1990; KUNTZ *et al.* 2003; PIEAU *et al.* 1998). With the onset of the thermosensitive period however, aromatase activity seems to increase in certain temperatures, which vary for each species. For example, in marine and freshwater turtles, higher temperatures cause an exponential increase of aromatase activity, whereas in lower temperatures aromatase activity remains low. The different levels of aromatase activity then guide the differentiation of the indifferent gonad into an ovary or testis. Once the thermosensitive period is over and the fate of the gonad has been established, further changes in temperature seem to have no effects. Interestingly, a number of genes originally described as part of the genetic regulation of sex development in men and other mammals have also been detected in reptiles. For instance, in the sea turtle *Lepidochelys olivacea*, several genes so far related to mammalian sex determination are expressed, including DAX1 (*dosage-sensitive sex reversal 1*), DMRT1 (*doublesex- and mab-3 related transcription factor 1*) and SOX9 (*SRY related HMG box 9*) (FERGUSON-SMITH 2007). In particular, DAX1 is a known regulator of gonadal development in mice and other mammals, considered to be an "anti-testis" gene. Although this may approach may prove to be too simplified, in reptiles, the gene is not differentially expressed in response to temperature variation during the TSP, therefore, its role in reptile sex determination is unclear. The gene is also expressed in crocodilians with temperature-dependent sex

determination, such as *Alligator mississippiensis*. Whether this gene could indeed be a target for androgen or estrogen-related actions following the TSP remains unknown. As far as DMRT1 is concerned, the gene was initially related to sex determination in *D. melanogaster*, due to the presence of a domain compatible to the sex determinant gene *dsx*. Subsequent research, however, has proven the gene's expression in several other species as well, including birds, fish and reptiles. In alligators, such as *A. mississippiensis*, the gene is expressed exclusively in the gonads of males. Moreover, its expression appears to precede that of SOX9, another testis-specific gene conserved in a vast number of species, ranging from reptiles to mammals. The latter gene is originally expressed in the bipotential gonad of reptile embryos, but following the TSP, it remains active only in males, making it a candidate gene for sex steroid-induced regulation. In alligators, SOX9 is also related to increased AMH (Anti- Müllerian Hormone) levels, but, contrary to mammals, AMH induction chronologically precedes that of SOX9 (WESTERN *et al.* 1999a; WESTERN *et al.* 1999b). In the case of lizards, an attempt has also been made to examine sexual dimorphism in the brain. The first results from these experimental series show distinct differences in estrogen receptor expression and progesterone concentrations in specific areas of the central nervous system, a finding that may imply that aromatase regulation is only the first step in a sequence of several more complex sex-specific/dimorphic genetic phenomena that still remain to be examined. Finally, it has recently been suggested that aromatase may also be regulated by secondary parameters, other than temperature. This has been described for instance, in the case of Prostaglandin E2, which appears to be associated with increased aromatase action (MORRISH and SINCLAIR 2002; SINCLAIR *et al.* 2002; VALLELEY *et al.* 2001).

Amphibians

The thermosensitivity of the gonads has been demonstrated not only in reptiles, but also in several fish and some amphibians (DOURNON *et al.* 1990). These tend to combine a genotypic sex determination mechanism- either male heterogamety, female heterogamety or polygenic. The result is a phenomenon known as sex reversal, where the effects of temperature may go against the genotypic directions, allowing the existence of animals in genotypic and phenotypic sex discordance. In particular, male or female heterogamety has been described in various species of anurans and urodeles. Sex chromosomes of various types may be present, following both the XY/XX and WZ/ZZ pattern that usually apply to mammals and birds, respectively (ANANIAS *et al.* 2007; MIURA 2007; OGATA *et al.* 2008). The exact mechanism by which temperature regulates sex determination in amphibians is not yet deciphered, but it does not seem to apply to the TSP-aromatase regulation model of reptiles. Hormonal action may also act in the process of acquisition of sexual phenotype, either independently or in conjunction with temperature variation. Gene studies in amphibian sex determination are not as extensive as in other animal models. Of the various genes so far associated with sex determination in other species, amphibians appear to express DMRT1 (NICULITA-HIRZEL *et al.* 2008; YOSHIMOTO *et al.* 2008). However, it is not yet clear whether this is a downstream product in the sex differentiation cascade or a factor with a more central role in sex determination.

Fish

As with the insects, yet another huge group in the animal kingdom, the fish are represented by only relatively few, specific model organisms, each of which has been

considered representative of the reproductive physiology of several other closely related species. Among the mechanisms observed, one may refer to a) the presence of true hermaphrodites, a strategy usually associated with lower evolutionary levels (e.g. the previously described model of invertebrates-nematodes) b)temperature-dependent sex determination, with a process similar to the one known to be characteristic of most reptiles and c) sex chromosomes (BULMER 1987; CHARLESWORTH 2004; CONOVER and KYNARD 1981; MATSUDA 2003; NAGAHAMA *et al.* 2004). The latter may follow either the XY/XX or the ZW/ZZ pattern. Contrary to mammals, the sex determining genes have not yet been described in fish, although some candidacies have been proposed. It might also be possible that, instead of a common, uniform gene pattern for all fish, different genes will be proven to be the major sex determinants in every species. According to some researchers, it might also be possible to assume a number of competing genes in every species, with environmental and/or hormonal parameters regulating their relative priority in sex determination in every birth. Of the various model organisms available for study, the four characteristic examples are the atlantic salmon, the platyfish, the medaka and the zebra fish. The atlantic salmon (*Salmo salar*) was, until recently, an organism within unknown genetic sex determinants. However, recent data has detected the candidate sex-determining locus of this species as part of chromosome 2. For this reason, this large metacentric chromosome is now regarded as the sex chromosome of this species. Research has now turned to the detailed study of the region, in an attempt to identify the exact position and structure of the single sex-determining gene, which has been proposed to exist within the aforementioned locus. The genome of platyfish (*Xiphophorus maculatus*) may contain any of three sex chromosomes, namely X, Y and

W. This allows significantly more combinations in the population than those observed in other species, applying to the "traditional" principle of only two sex chromosome types available (ZW and XY pairs, respectively). Of all the combinations, WX, WY and XX develop as females, while XY and YY become males. No specific sex-determining gene has been described so far, although the W chromosome is considered a major candidate for its position, since its presence coincides with female phenotype regardless of the type of the second sex chromosome. However, some genes, previously described in other species and associated to reproductive physiology and development, are also found in this and other fish species. These include SOX family members, such as SOX9 and DMRT1. On the other hand, classical hormonal regulators of sex differentiation, such as anti-Mullerian hormone (AMH) have not yet been identified in fish. DMRT1 has been shown to be particularly important for sex determination in the teleost medaka, *Oryzias latipes*. The sex determining system of the medaka is male heterogametic, i.e. it follows the XX/XY principle known from mammalian reproduction. Although some similarities with genes of the mammalian sex chromosomes may exist, the major sex determinant of mammals, i.e. SRY (sex determining region of the Y chromosome) is missing. Consequently, another, previously unknown, sex-determining gene must be present in the medaka genome. Indeed, in the Y chromosome of the fish a new gene has been detected, bearing six exons and a DM domain. The latter is a major characteristic of genes involved in sex determination in invertebrates, such as *doublesex* and *mab-3* in *D. melanogaster* and *C. elegans*, respectively. This new gene was named DMY (DM domain of the Y chromosome) and it is homologous to DMRT1 gene, which is conserved in various species (CHARLESWORTH 2004; ZHANG 2004). Although a lot of information is still

missing, it appears that in the male, DMY and DMRT1 operate in procession as strong determinants of gonadal development. In the female, the role of aromatase is once again central, although its induction, in this case, may be a genetic rather than temperature-related event. Other genes' expression has also been detected exclusively in females, such as FIGa (factor in the germ line a), but their correlation with aromatase induction remains to be proven (MATSUDA 2005; MATSUDA 2008; OTAKE *et al.* 2006). Finally, sex determination in the zebra fish is considered to be a genetic phenomenon, but the details of the process are still under examination. Of particular interest are recent data, proving the expression of two sex-related genes in the zebra fish. These are a) *vasa*, a gene family expressed exclusively in the gonads of several species, including *D. melanogaster*, mice and fish and b) *FtzF1*(fushi tarazu factor 1), a gene originally described in Drosophila and known to encode the steroidogenic factor 1 (SF1) in mammals, thus regulating sex steroid production (SCHARTL 2004; VOLFF and SCHARTL 2002).

Birds

In birds however, females are the heterogametic sex, carrying one copy of each of the so called Z and W sex chromosomes, whereas males are homogametic ZZ. The Z and W chromosomes have no relation to the mammalian X and Y, and in fact seem to have evolved from different pairs of autosomes (MIZUNO *et al.* 2002). And this is part of the reason it is not yet certain which of the two carries the genetic trigger for sex determination. To this day, there are two major theories under investigation. Sex may depend on Z chromosome dosage, according to the example of *D. melanogaster* and *C. elegans*. One candidate gene for this theory is the DMRT1, which is located on Z

chromosomes, escapes dosage compensation and is expressed specifically in the gonads, and is thus capable of linking the number of Z chromosomes with gonadal differentiation (SMITH and SINCLAIR 2004). On the other hand, sex may be determined by the feminizing presence of the W chromosome, following the example of Y in eutherian mammals. There are two different mechanisms that are being studied and can support this theory. One includes the FET1 gene, which is located on W, does not have a Z homologue and is expressed almost exclusively in the female urogenital system (PACE and BRENNER 2003). The other includes the ASW gene, also known as WPKCI, and its Z homologue ZPKCI, since it has been proposed that the products of those two genes are capable of dimerisation, with a ZPKCI homodimer acting as a testis factor and a WPKCI/ZPKCI heterodimer preventing this effect (CLINTON and HAINES 2001) (**Figure5**).

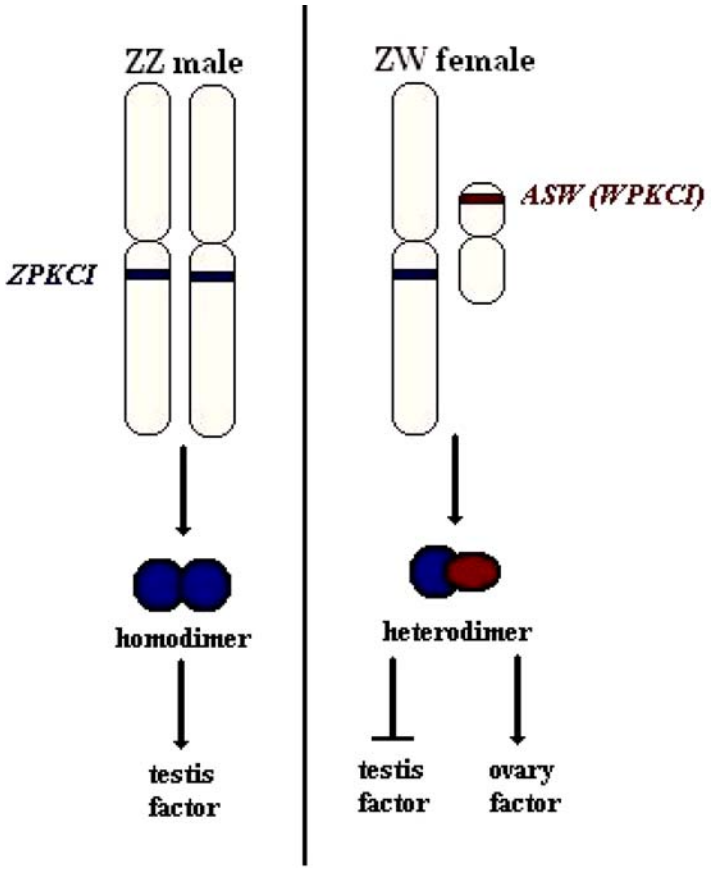


Figure5 The role of ZPKCI and ASW (WPKCI) in ZW sex determination. According to one theory, the ZPKCI proteins form homodimers in ZZ males that stimulate a factor required for the differentiation of the testes. Whereas in ZW females, the ASW (also known as WPKCI) proteins form heterodimers with ZPKCI that may prevent the activation of that factor or stimulate directly the differentiation of ovaries.

One way to discern between the two theories would be to look into different combinations of Z and W chromosomes. Indeed, ZW aneuploidy has been studied in an effort to better understand how things work. It turns out that ZZZ animals develop testes but are infertile, ZWW animals die early in embryonic development, but ZZW combinations manifest as intersexual: the animals appear female on hatching, but slowly turn into males at sexual maturity (ELLEGREN 2001). It is still possible, thus, that a combination of the above is in fact applied.

Mammals

Marsupials

In marsupials, a set of X and Y chromosomes related to those found in eutherian mammals are present. The basic marsupial Y chromosome is the smallest of any mammal but retains its ability to turn the undifferentiated gonads into testes (MARSHALL GRAVES and SHETTY 2001). However, the differentiation of the embryonic testis does not also control all aspects of sex differentiation. The formation of the mammary glands and scrotum develops before gonadal differentiation takes place and is independent of gonadal hormones. In fact, it appears to be under the control of genes located on the X chromosome. So it happens that XXY animals have testes, but a pouch with mammary glands has replaced their scrotum, whereas XO animals have no testes, but an empty scrotum in place of a pouch. These X-linked genes have yet to be identified, but already the autosomal SOX9 has been reported of being expressed in the scrotum and mammary primordial before birth (RENFREE and SHORT 1988; SHARMAN 1969; SHARMAN *et al.* 1970). Sex determination in mammals has been more extensively studied than in any

other species, most probably due to its direct relevance to human physiology and pathophysiology. A large number of genes have already been described and many more are expected to be added in the process, since the relevant research constantly reveals new players in the complex network of reactions related to sex determination (**Figure6**).

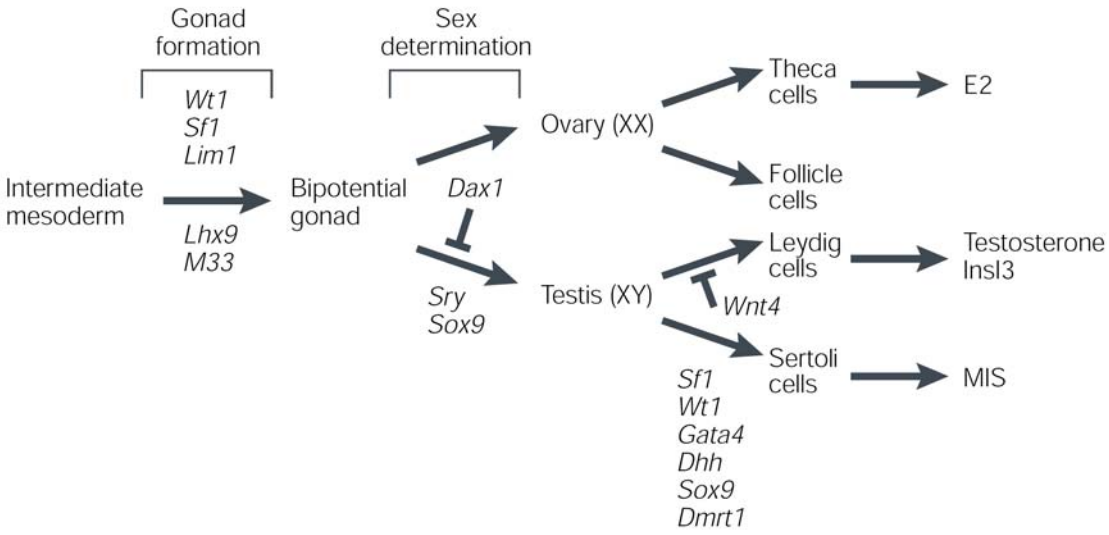


Figure6 In mammals, the testis is necessary and sufficient to initiate male development and, accordingly, sex-determining genes act in the testis, which then trigger sexual development elsewhere in the body through hormones. Gonad formation requires the *Wilms tumour 1* (*Wt1*), *steroidogenic factor 1* (*Sf1*), *LIM* homeobox protein 1 (*Lim1*, also called *Lhx1*), LIM homeobox protein 9 (*Lhx9*) and polycomb group M33 genes, among others, and initially results in a sexually indifferent, or bipotential gonad. The bipotential gonad is committed to testis or ovary differentiation by the action of the sex-determining region gene on the Y chromosome (*Sry*) and SRY-box containing gene 9 (*Sox9*) in XY embryos. In XX embryos, lack of *Sry* allows ovary development. No ovary-determining gene comparable to *Sry* has been found, but *DAX1* (nuclear receptor subfamily 0, group B, member 1) seems to act as an 'antitestis' gene, and Wnt4 (wingless-related MMTV integration site 4) suppresses Leydig-cell differentiation in the ovary, among other functions. This period of development is sex determination. Once sex is determined, gonad differentiation occurs. In males, several genes, including Sf1, Wt1, Gata4 (GATA-binding protein 4), Dhh (desert hedgehog), *Sox9* and *Dmrt1* (*dsx* and *mab-3*-related transcription factor 1), direct the differentiation of the different cell types of the testis. The testis secretes the hormones Müllerian inhibiting substance (MIS, also called anti-Müllerian hormone or AMH) from the Sertoli cells, and testosterone and Insl3 (insulin-like 3) from the Leydig cells. Both MIS and testosterone suppress female differentiation and support male differentiation, respectively, elsewhere in the body.

Even in the common, bipotential gonad, the expression of several genes is considered crucial for subsequent development and normal sexual dimorphism. These include, among others, *WT1* (*Wilm's tumor 1*), *FtzF1/SF1* (*Fushi tarazu factor 1/steroidogenic factor 1*) and *Lim1* (ZARKOWER 2001). Absence of any of these products at this stage, especially WT1, is inconsistent with further gonadal development and may also cause other malformations, e.g. affecting the adrenal gland and renal buds. Genes of the *wnt* family, such as wnt4, may also participate in the regulation of epithelial organization and epithelial mesenchymal interactions in the area of the gonadal primordium. SRY expression is the major sex-determining signal, since it is prerequisite for normal testis formation. Its role mimics that of a molecular switch, since its peak expression is limited in a specific time period that is still considered sufficient to induce male-type differentiation of the reproductive system, via downstream gene action. The latter refers to several genes, including sox family members, *SF1* (sex steroid regulation) and transcriptional factors, such as GATA4. Sox family genes share a common HMG box, similar to that observed in SRY, which is considered necessary for their action at a molecular level (SWAIN and LOVELL-BADGE 1999). The fact that members of the group have been detected in various species of vertebrates, such as fish (SOX9) and all mammals (e.g. SOX2 and SOX14 in monotremes) further emphasizes their importance for genetic sex determination. The observation of this gene family's evolutionary conservation adds further credit to the multistage model of sex chromosome evolution described above, since sox3 has been proposed as the autosomal ancestor of SRY, which places it among the chronologically first sex-related genes in the common evolutionary history of all vertebrates. In the female embryo, the Y chromosome is not present and,

therefore, SRY is not expressed. The genetic cascade regulating female reproductive system differentiation is not as extensively studied as in men, but *DAX1* (and its regulatory system, including genes such as *Wnt4* and *SF1*) is generally considered as a significant player in this process, which is how it came to acquire the rather simplistic description of the "antitestis gene". Sex steroid production regulation is also important for the establishment of a normal female phenotype and it is mediated via SF1 expression and aromatase enzyme complex induction. Two relatively recently described genes with a potential role in sex determination and differentiation are *DMRT1* and *Stra8* (stimulated by retinoic acid gene 8) (RAYMOND *et al.* 1999). The first has been already discussed in previous units as a conserved sex-related gene, bearing a DM domain originally studied in nematodes. In humans, XY sex reversal in cases of 9p chromosome deletions have been attributed to impaired action of *DMRT1* or its homologue, *DMRT2*. Still, their exact involvement in the sex determination circuit has not been clarified. *Stra8*, on the other hand, is exclusively expressed in female germ cells and its presence signals their sexual gradual differentiation, in an anterior to posterior direction. However, it has not yet been established whether the gene's product directly induces sex determination towards the female pathway, or rather acts a simple marker of this phenomenon, without active participation in the process *per se*.

Sex chromosomes systems in Lepidoptera

Sex chromosomes stand out from the rest of the chromosome complement by being structurally different, having a different behaviour in meiosis, sometimes being visible as heterochromatic bodies in interphase nuclei, and by having an associated phenotype,

female or male. Sex chromosome systems with female heterogamety are referred to as WZ/ZZ and Z/ZZ (alternatively designated WZ and ZO) depending on the presence or absence of a W chromosome. In Lepidoptera, in contrast to most other animal groups, females are the heterogametic sex. They share this property with Trichoptera (caddis flies), their closest relatives, and with snakes and birds. They are predominantly WZ/ZZ systems, but Z/ZZ, $W_1 W_2$ Z/ZZ and $WZ_1 Z_2$ / $Z_1 Z_1 Z_2 Z_2$ systems do occur. Correct distribution of multiple sex chromosomes in meiosis is ensured as they form a single multivalent (TRAUT and MAREC 1996; TRAUT *et al.* 2007).

Achiasmatic Female Meiosis

With respect to crossing-over, Lepidoptera display the reverse situation of the *Drosophila* case: males have crossovers, females do not. Male meiosis follows the conventional cytogenetic scheme with typical chiasma figures, crosses and rings in diplotene and diakinesis. Female meiosis, however, is achiasmatic (ENNIS 1976; MAREC and TRAUT 1994). This is consistent with not having crossovers. In early prophase, the conventional sequence of leptotene, zygotene and pachytene are visible but no chiasmata are formed. At metaphase I, therefore, the homologues are not held together by chiasmata but so-called 'elimination chromatin' can be seen between the paired homologues. The WZ bivalent of many species is recognized at this stage by the asymmetric pattern of the paired chromosomes. In a *Bombyx mori* strain with a W to which a large segment of chromosome 2 was translocated, the WZ bivalent was identified as an asymmetric bivalent in pachytene. It is generally assumed that homologues recognize each other and finally synapse because of their sequence identity. Hence W-Z pairing could be

explained as a consequence of either non-availability of other partners for either of them or presence of hypothetic recognition sequences – possibly only at one end – conserved in the W chromosome in spite of extensive degeneration of most other sequences. Full-length synapsis of the non-homologous sex chromosomes poses no genetic problem, however. Chromosome integrity is not at risk since there is no intrachromosomal recombination in female meiosis. Even when two W chromosomes are paired to one Z chromosome in W1W2Z/ZZ systems, sex chromosome separation appears to be precise. Sex chromosome multivalents behave like a single Mendelian factor in meiosis (RASMUSSEN 1976; RASMUSSEN 1977).

Sex Chromatin in Interphase Nuclei

Females but not males of the majority of moth and butterfly species display a heterochromatic body in interphase nuclei (RATHJENS 1974). Thus, like mammals, Lepidoptera possess sex chromatin. The source, however, is different in the two taxa. Although present in females of mammals as well as those of Lepidoptera, sex chromatin of Lepidoptera occurs in the heterogametic sex and, therefore, is neither a heterochromatic X or Z chromosome nor associated with dosage compensation.

Composition of the Sex Chromosomes

Lepidopteran chromosomes are small, holocentric, and numerous; the extent to which they are evolutionarily conserved is a major unsolved question. . The silkworm Z chromosome is one of the larger chromosomes cytogenetically, with 17 loci on the classical recombination map (YOSHIDO *et al.* 2005). Some of these encode traits found in

other lepidopteran species that theoretically could contribute to prezygotic reproductive isolation. These are Late maturity (Lm, 1–2.0), a major locus affecting body size, voltinism, and number of molts ; Giant egg (Ge, 1–14.0); and period, whose homolog is also sex linked in *Antheraea pernyi*, as is its ortholog in the fruit fly, where it regulates circadian rhythm, mating behavior, and fertility (FUJII 1998). Additionally, a QTL controlling a difference in egg-laying behavior between *B. mori* and *B. mandarina* has been found on the Z chromosome. Yasukochi et al. (2007; 2006) found that the genes *tpi* (*triose phosphate isomerase*) and *apterous* were located in the Z chromosome of *B. mori* and a very distantly related species, the nymphalid butterfly *Heliconius melpomene*. *tpi* maps to the Z chromosome also in the tiger swallow tail butterfly *Papilio glaucus* and the crambid moth *Ostrinia nubilalis*. Koike et al. sequenced a 320-kb segment of the Z chromosome using three overlapping BAC clones. Within this segment, a region covering ~100 kb that contains four putative muscle proteins, *Bmkettin*, *Bmtitin*, *Bmtitin2* and *Bmprojectin*, and a pleiotrophin-like protein, *Bmmiple*, forms a small microsyntenic cluster with an 80-kb region on the third chromosome of *Drosophila*; other portions do not have synteny to fruit fly chromosomes (KOIKE *et al.* 2003). This evidence suggests that the Z chromosome evolved through multiple rounds of assembly of ancestral chromosome fragments so that there is no longer strong synteny with the Diptera.

Somatic tissues of *B. mori* females contain a densely staining heterochromatic body formed by the W chromosome, a characteristic of the Ditrysia, the evolutionary clade to which most present-day Lepidoptera belong (TRAUT *et al.* 2007). There have been several approaches to analyze DNA composition of the W chromosome. Observations that a

whole chromosome was painted by fluorescent probes with comparative genomic hybridization or genomic in situ hybridization reinforced the notion that it was the W chromosome, and might consist largely of repeated sequences (FUKOVA *et al.* 2005; FUKOVA *et al.* 2007; VITKOVA *et al.* 2007). Direct molecular confirmation came from the work of Abe et al. (1998a; 1998b; 2000a; 1998c; 2000b), who first identified nucleotide sequences on the W chromosome as female-specific RAPD markers. All deduced sequences of W-chromosome-specific RAPD markers with one exception show similarity to amino acid sequences of retrotransposable elements. The three major groups of LTR retrotransposons are well represented on the W chromosome, including gypsy-Ty3 (Kabuki), Ty1-copia (Yokozuna), and Pao (Kamikaze, Yamato, and BEL). There are also full-length copies of BMC1, a non-LTR retrotransposon LINE-1 element (FUJII and SHIMADA 2007). Sequences originating from the W chromosome of *B. mandarina* are also composed of nested retrotransposons. Interestingly, some *B. mori* W chromosome-linked RAPD primers do not amplify bands in *B. mandarina*, suggesting that the two W chromosomes diverged after the species separated. These results contrast with findings on the Z and other autosomes, in which many non-LTR retrotransposons are found, but they are truncated to small fragments largely spanning the region from the 3' UTR to the poly(A) tail; moreover, there are few LTR retrotransposons. The presence of many complete or long retrotransposable elements exist on the W compared with other chromosomes could basically be accounted for by three factors. First, the accumulation of retrotransposable elements on the W chromosome may not be deleterious because it carries very few, if any, functional genes, and so inserted elements are not efficiently eliminated. Second, the lack of crossing over in females means that the W chromosome is

recombinationally isolated from the Z and other autosomes; this could lead it to a relatively slow rate of evolution. Finally, because cells undergo more divisions in spermatogenesis than in oogenesis, higher mutation rates are predicted for the Z and autosomes. All these factors can promote the accumulation of retrotransposable elements on the W.

Very few genes have been found linked to the W chromosome. In *B. mori*, a female determining factor, *fem* and a giant-egg trait factor, *esd* are considered to be associated with the W chromosome but their location has not been identified yet (TRAUT and MOSBACHER 1968; TRAUT *et al.* 2007). Results obtained from sex chromosome mutants of *Ephestia kuehniella* suggest the presence of a male-killing factor in the W chromosome (MAREC *et al.* 2001). In *Papilio glaucus*, the dark morph trait is transmitted maternally and, therefore, considered W-linked, but W-linkage is disrupted in some crosses, presumably due to a Z-linked suppressor (ANDOLFATTO *et al.* 2003). In *Antheraea pernyi*, several copies of the otherwise Z-linked period gene have been detected, one of them, *perW*, produces a truncated protein while another one is the source of an antisense RNA (GOTTER *et al.* 1999; SAUMAN *et al.* 1996).

Sex Determination: the Primary Signal

In female-heterogametic systems, the distribution of sex chromosomes to the eggs decides upon the sex chromosome constitution of the zygote and subsequently sexual development of the embryo. ZZ embryos develop into males, WZ embryos (or ZO embryos in Z/ZZ systems) into females. Since only 1 of the 4 nuclei that result from

female meiosis, is becoming the female pronucleus (the egg nucleus), the orientation of the WZ bivalent or the position of the Z univalent, respectively, in the metaphase I spindle determines the sex chromosome constitution of the egg (TRAUT *et al.* 2007). Although the female-heterogametic system has been conserved in all species so far investigated, the role of sex chromosomes in sex determination has not. WZ systems are not different from XY sex chromosome systems in this respect. Theoretically, the primary sex determining signal in a WZ/ZZ system may be generated by (1) female determining gene(s) on the W chromosome while male development is the default; thus presence of the W causes female development, absence of the W male development; or (2) male promoting gene(s) on the Z acting against female promoting gene(s) located on autosomes or a female promoting maternal effect (which may be coded for by a gene on any chromosome including the W). This, in effect, is then a Z-counting mechanism: a single Z chromosome causes female development, 2 Z chromosomes male development. Although we have no precise knowledge of the genes involved in generating the primary sex determining signal, both the 'dominant W' and the 'recessive Z' systems do occur in Lepidoptera (TRAUT and MAREC 1996). Thus it cannot be decided *a priori* whether sex determination in a WZ/ZZ species depends on a 'dominant W' or a Z-counting mechanism. *B. mori*, on the other hand, represents the 'dominant W' system. The W chromosome carries a female-determining factor, *fem*. This has been inferred from polyploids and aneuploids. Whenever a W chromosome is present, a female develops from the embryo. In its absence a male individual develops. A couple of deletions and translocations of W chromosomal segments in *B. mori* are known that do not include *fem* (ABE *et al.* 2008; ABE *et al.* 2005). The female-determining function, hence, maps to the

remaining segments of the W, but the precise location of *fem* is not yet known nor has *fem* been isolated to reveal its molecular structure. Extensive hybridization experiments between Japanese and European races of the gypsy moth, *Lymantria dispar*, have been performed in the second and third decade of the past century by Richard Goldschmidt (1942). Basically, European females crossed with Japanese males produced WZ intersexes and ZZ males (F1 hybrid intersexes). The reciprocal crosses, Japanese females crossed with European males, and their daughters backcrossed to European males, produced WZ females and ZZ intersexes (F2R hybrid intersexes). The results were interpreted as showing the effect of strong female- and male-promoting genes in the Japanese but weak female- and male-promoting genes in the European races. Female- and male-promoting activities are well balanced within each race, while inter-race crosses produce the imbalances seen in the development of intersexes. The Z chromosomes carry the male-promoting genes, but whether they act against a zygotic or a maternal female-promoting effect cannot safely be decided.

Sex Determination: the Sex-determining Pathway

In Drosophila, the primary sex-determining signal, briefly called the X:A ratio (ratio of X chromosomes to autosome sets), initiates a cascade of gene actions which ultimately regulate the development of sex-specific somatic characters, sex-specific behaviour, and dosage compensation of X-chromosomal genes. This well-investigated sex-determining pathway of Drosophila had once been considered a paradigm of insect sex determination. But during the last 2 decades, it has become evident that only parts of the cascade are conserved in non-drosophilid insects (ZARKOWER 2001). Among Lepidoptera, only *B.*

mori has been investigated with respect to sex-determining cascade genes. The orthologue of *Drosophila sxl* gene is conserved in *Bombyx*, but the function is not (NIIMI *et al.* 2006; TRAUT *et al.* 2006). The *sxl* transcript sex-specifically spliced into two isoforms, but only in the germline, indicating that it cannot be a master switch for sex determination in the silkworm. Its function in *B. mori* is unknown. Of the next lower level, *tra-tra2*, only *tra2* has an identified orthologue in *Bombyx* (NIU *et al.* 2005). The orthologue of *Drosophila tra* gene, however, could not be identified yet. This is presumably due to its rapid sequence evolution which had previously been seen even among *Drosophila* species. Thus we do not know whether this level of the cascade is functionally conserved. Presumably it is not since the *dsx* transcript of the next lower level of the cascade lacks the characteristic binding motif for TRA-TRA2. Finally, the *dsx* gene at the bottom of the cascade is conserved, structurally as well as functionally. The transcript of *B. mori dsx*, *Bmdsx*, is sex-specifically spliced into female- and male-specific forms of mRNA. The regulation of splicing, though, differs between *Drosophila* and *Bombyx dsx* (OHBAYASHI *et al.* 2001). Besides absence of the TRA-TRA2 binding motif, the female-specific 3' splice site is not weak, and the female form – not the male form – is the default splice variant in *Bombyx*, as shown with *in vitro* splicing reactions (SUZUKI *et al.* 2003). This means that the sex-specific splicing of *Bmdsx* is controlled not by a splicing activator such as the *Drosophila* TRA protein, but by an unknown splicing repressor. Thus at least, the last step of the cascade is conserved in Lepidoptera, as it is in all other insect species investigated so far. The vitellogenin gene is a direct target of the BmDSX protein. Suzuki et al. demonstrated that transgenic male silkworms carrying female-type *Bmdsx* cDNA express vitellogenin, which is scarcely produced by normal

males. By contrast, transgenic female silkworms expressing the male-type *Bmdsx* mRNA show repressed expression of vitellogenin, accelerated expression of pheromone- binding protein mRNA, and abnormal morphology of several genital organs (SUZUKI *et al.* 2005). This indicates that *Bmdsx* affects not only the vitellogenin gene but also pheromone-binding protein genes and morphogenesis of genital organs, although whether the mechanisms are the same as those for the vitellogenin gene is unknown. The finding that the female-type *Bmdsx* expression barely induced sex reversal in the transgenic animals suggested the need for another molecule or female differentiation. In *Drosophila*, the female-type DSX protein interacts with a novel protein encoded by the *intersex* (NUNES *et al.*) gene (BELOTE and BAKER 1983; GARRETT-ENGELE *et al.* 2002); together they activate transcription of the yolk protein gene (*yp*). One could speculate that an ortholog of *ix* also interacts with BmDSX-F and regulates female differentiation in the silkworm. The WGS assembly of *B. mori* contains two homologs of *ix*, although the *Drosophila* genome contains only one *ix* gene. The function of the two silkworm genes should be investigated to understand their roles in sexual differentiation.

Dosage Compensation

It is well known that in metazoan organisms with heterogametic (XY) sex chromosomes such as mammals, *D. melanogaster*, and *C. elegans*, dosage compensation mechanisms allow equal amounts of gene expression per cell from the female and male X chromosomes. Mutations affecting the dosage compensation mechanism lead to lethality in the fruit fly, indicating its importance Similarly, the double dose of Z chromosomal genes in males and the single dose in females may create a problem if correct gene

expression is dosage dependent. Therefore it is interesting to see whether there is a dosage compensation mechanism in Lepidoptera, similar to those in mammals, *Drosophila* or *Caenorhabditis*. In two *Heliconius* species, *H. melpomene* and *H. elato*, the activity of the Z-linked enzyme 6-phosphogluconate dehydrogenase was twice as high in male as in female tissues (JIGGINS *et al.* 2001; NAISBIT *et al.* 2002). Suzuki et al. (1999) first reported lack of gene dosage compensation in *Bombyx mori* at the mRNA level. They showed that there is twice as much mRNA from the Z-chromosome-linked genes, *T15.180a* and *Bmkettin*, in males than in females. Koike et al. (2003) quantified the mRNA levels of 13 Z-chromosome-linked genes around the *Bmkettin* locus and revealed that most genes expressed more abundant mRNA in males than in females, with only a few exceptions. Similarly, in another Z-linked gene, *period*, in *Antheraea pernyi*, neither the amount of transcript nor that of protein was dosage compensated (GOTTER *et al.* 1999). Thus, experimental evidence shows that there is no dosage compensation of sex-linked genes in Lepidoptera. The lepidopteran Z appears to contain no genes whose correct functioning is dosage dependent, or else, if it is, the function has to be sex-specific. It is suggested that the Z chromosome has evolved through a process of genome shuffling to carry only genes whose products are required at higher levels in males. Perhaps genes that must be expressed in equal amounts in the two sexes have already translocated onto autosomes or acquired gene-specific regulatory mechanisms. In light of these evolutionary differences between flies and moths, it is of interest to look in the *B. mori* genome for homologs of the genes required for dosage compensation in *D. melanogaster*. The major genes are *mof*, *mle*, *msl-1*, *msl-2*, and *msl-3*. EST analyses and the WGS assembly contain putative orthologs for *mof*, *mle*, and *msl-3* (XIA *et al.* 2004).

mof encodes a histone acetyltransferase needed for chromatin remodeling, *mle* codes for a helicase, and *msl-3* encodes a nonsex-specific protein, as does *msl-1*. *msl-1* and *msl-2*, which is sex-dependently spliced to produce a male-specific functional protein, are probably missing from the *B. mori* genome. The proteins coded by these genes compose a complex with X-encoded RNA molecules roX1 and roX2 called a compensasome. The lack of these molecules may explain the lack of gene dosage compensation. It is believed that mammalian orthologs of MOF, MLE, and MSL-3 compose a complex for transcriptional regulation other than dosage compensation, which suggests that *D. melanogaster* recently utilized these proteins to regulate sex-linked genes transcriptionally.

Evolution of the Lepidopteran Sex Chromosome System

Lepidoptera share female heterogamety with the closely related Trichoptera (caddis flies). Hence, female heterogamety has arisen in the common ancestor of the two orders, which together form the monophyletic group of Amphiesmenoptera (FRIEDLANDER 1983; KJER *et al.* 2001; PASHLEY *et al.* 1993). The earliest certain trichopteran and lepidopteran fossils date from 180–185 and 190 mya, respectively (TRAUT *et al.* 2007). Female heterogamety in this group, thus, exists for more than 190 mya. All caddis flies investigated so far have a Z/ZZ sex chromosome system and do not display sex chromatin. This is not different from the situation in basic lineages of Lepidoptera. Therefore, it can be assumed that the lepidopteran W chromosome came into being after Lepidoptera had split from the common phylogenetic tree. A larger data set on the distribution of the W chromatin among different families of Lepidoptera indicates that the

W chromosome came into being at the common root of Ditrysia and Tischeriina, together containing 98% of the extant Lepidoptera species (TRAUT and MAREC 1996). The earliest evidence for a Ditrysian species is from 97 mya (KRISTENSEN 1970). Thus the female heterogametic system already had a long history before the lepidopteran W arose. As the simplest hypothesis to account for the change from a Z/ZZ to a WZ/ZZ sex chromosome constitution one may assume a fusion between an autosome and the original Z chromosome, giving rise to a neo-W neo-Z sex chromosome constitution that is in fact our present day WZ pair of the majority of Lepidoptera species. But other chromosome rearrangements cannot be excluded. Chromosome losses and fusion events have evidently contributed to the evolution of the sex chromosomes in more recent times. Sporadic losses of the W chromosome have turned the sex chromosome system formally back to a Z/ZZ system, though of course the Z chromosome of the more advanced Lepidoptera may have only partial homology to the ancestral Z, which is common to both, Trichoptera and basic Lepidoptera.

It is generally agreed that the evolution of a differential pair of sex chromosomes starts from a pair of homologous chromosomes and requires 2 conditions: (1) possession or acquisition of the sex determining function, i.e. a dominating role in the generation of the primary sex determining signal, and (2) establishment and extension of a non-recombining region. The consequences then are molecular and morphological differentiation between the originally homologous proto-X and proto-Y chromosomes. The evolution is most dramatic in the sex-specific sex chromosome, the Y chromosome in XY/XX systems and the W in WZ/ZZ systems. Decay of genes and accumulation of

repetitive sequences like transposons is facilitated in these chromosomes by constant heterozygosity and cannot be repaired or cleaned by recombination. Several mechanisms have been described that act under these conditions and promote the process: Muller's ratchet (CHARLESWORTH 1978; MULLER 1964), selective sweeps by genetic hitchhiking (RICE 1987), and background selection (CHARLESWORTH 1994). Considering those preconditions, the female-heterogametic sex chromosome system as well as suppression of crossover in female meiosis, had already been established when Lepidoptera branched off from the common ancestral Trichoptera-Lepidoptera lineage. Both, Trichoptera and basic Lepidoptera, possess the Z/ZZ (ZO) sex chromosome system, hence, no trace is left in these groups of the hypothetical proto-W. But even in the new W chromosome that arose in the Lepidoptera lineage, genetic erosion has advanced rather far, as the few genes known from the W chromosomes and the massive invasion of repetitive elements can tell. Even the sex determining function seen in *Bombyx* (*fem*) is certainly absent in species with Z/ZZ sex chromosome systems that have secondarily lost the W chromosome. How much of that W chromosome has been conserved in present-day W chromosomes besides the telomeric sequences is unknown. Even among related species, the change in W chromosome sequence composition is considerable, as a comparative FISH study among 4 pyralid moths has shown (VITKOVA *et al.* 2007). So it may be impossible to trace the W chromosome back to its origin, from its present-day sequence composition. The proto-Z chromosome, however, may have been conserved throughout Trichoptera and Lepidoptera even though fusion events with autosomes have taken place. Conserved synteny of Z chromosomal genes in Lepidoptera indicates the stability of the sex chromosome system in Lepidoptera. The sex chromosome pair has not been

substituted by another pair of chromosomes in the species considered. But whether the syntenic genes belong to the ancestral Z chromosome or to a later acquisition, e.g. to the presumed addition of an autosome to the Z in connection with the birth of the lepidopteran W chromosome, has yet to be determined.

High throughput analysis of differential gene expression

Elucidation of the changes in gene expression associated with biological processes is a central problem in biology. Although a gene must not necessarily be up- or down-regulated to play a key role in a certain process, screening for differentially expressed genes is one of the most straightforward approaches to unravel the molecular basis of a biological system. The difficulty of isolating differentially expressed genes, particularly low-abundance ones, has been experienced. To fully recognize this, it is important to take into account the complexity of a cell's transcriptome. A eukaryotic cell contains ~15, 000–30, 000 distinct mRNAs with a prevalence ranging from one to several thousands in a total mass of ~100, 000 mRNAs. About 50% of the transcript population is made up of a relatively small number (some hundreds) of abundant transcripts, representing only 1% of the different mRNA species. The other half contains the 'rare' mRNAs (LIEVENS *et al.* 2001; WAN *et al.* 1996). Not surprisingly, the difficulty of fishing out a gene responsible for a specialized function in a certain biological program often originates from the fact that the gene is expressed at low levels whereas the bulk of a cell's mRNA is made up of highly abundant transcripts.

Differential hybridization

In traditional screening methods, such as differential hybridization, the hybridization pattern of the total content of a cDNA library is compared between two samples. The fact that the abundant transcripts are also displayed implies high redundancy of non-relevant clones and thus very low labor efficiency. This problem has been solved partly by normalization and subtraction; even then, many interesting low-abundance differentially expressed genes are missed because of the low amplification of the hybridization signal (BOLL *et al.* 1986; MILLER *et al.* 1999; SHIGEMOTO *et al.* 1987; WAN and ERLANDER 1997). Other drawbacks are the limitation to pairwise comparisons and the fact that the techniques are mainly qualitative because of the relative insensitivity of the hybridization.

Differential display

With the availability of PCR, low-abundance transcripts could be amplified (MILLER *et al.* 1999; WAN and ERLANDER 1997). One of the first differential screening methods that used this possibility was the differential display technique described by Liang and Pardee (LIANG and PARDEE 1995; LIANG and PARDEE 1998; LIANG and PARDEE 2001). By combining 3' anchored oligo primers and short 5' arbitrary primers, subsets of the transcriptome are amplified, the resulting cDNA fragments are separated on a denaturing polyacrylamide gel and visualized autoradiographically. Original statistics indicated that 80 primer combinations would be sufficient to cover the whole transcript mass. The expected advantages were numerous: the method would be fast, producing band patterns in 2 days; it was based on simple, well established and widely accessible techniques, making it easily applicable for most researchers; compared with previous methods the

sensitivity had been increased dramatically, resulting in a good detection of low-abundance genes; both induced and repressed genes could be detected and more than two samples could be compared, making it highly versatile; furthermore, only a small amount of starting material was needed. Once a candidate band has been eluted from the gel, been reamplified and cloned, gene expression analysis tools, such as northern blot, RT–PCR or RNase protection, are applied to confirm the expression pattern and to attribute it to the correct clone.

Beyond differential display

Almost simultaneously with differential display, a conceptually similar technique was developed and published, called RNA fingerprinting by arbitrarily primed PCR (RAP–PCR) (PERUCHO *et al.* 1995; TORTOLA *et al.* 1998; WELSH *et al.* 1995). It differs from differential display principally in the use of an arbitrary primer both for first-strand cDNA synthesis and subsequent PCR amplification steps. After differential display had been introduced, more methods using PCR were developed. A large number of techniques involved the generation of a gel profile to display differences between different mRNA samples, but, in contrast to differential display, tried to evade the use of arbitrary primers and so circumvent problems that originated from mismatch priming during amplification. Instead, restriction sites were used to generate a subset of cDNA fragments that differed in size. Amplification following restriction enzyme digestion was done with primers that matched previously ligated adaptors. Eventually, one or more selective bases were added to the 3' ends of the primers to further reduce the subset of

cDNA fragments that will be displayed, such as in cDNA-amplified fragment length polymorphism [cDNA-AFLP] (BOTTON *et al.* 2008; FUKUDA *et al.* 1999). The kinetics of gene expression revealed by cDNA–AFLP were similar to those of northern blot analysis, rendering the displayed expression pattern quantitative. Other more sophisticated methods include a selection step to end up with only one restriction fragment per mRNA, hence completely ruling out redundancy. In the restriction enzyme analysis of differentially expressed sequences (READS), this aim was obtained through special adaptor design that only allows amplification of the most 3' restriction fragments (PRASHAR and WEISSMAN 1999). An extension of this method was the 'ordered differential display' that utilized the PCR suppression effect for selection of 3' end restriction fragments of cDNA. In two other methods, the 3' end was selected by biotinylated oligo primers. In gene expression fingerprinting (GEF) an additional restriction enzyme digestion was performed after PCR amplification (SHMELKOV *et al.* 2001) and, in the cDNA–AFLP-based transcript profiling method, the second digestion occurred before amplification.

Other types of techniques improved and refined the traditional technology of cDNA library construction and screening. Representational difference analysis (KUNTZ *et al.*), for example, merged the advantages of subtractive hybridization with the power of PCR amplification (HUBANK and SCHATZ 1994; LANCASHIRE *et al.* 2007; LISITSYN *et al.* 1993; SUNG *et al.* 2005). Suppression subtractive hybridization (SSH) combined normalization and subtraction in a single procedure. In both techniques, the final product is an amplified pool of cDNAs, enriched for species that are specific for the tester sample (HUANG *et al.*

2007; NEMETH *et al.* 1998; VILLALVA *et al.* 2001). In addition, in the case of SSH, the abundance of cDNAs is equalized. This pool is usually cloned and differentially screened. Still other similar methods that generate enriched pools of cDNAs include linker capture subtraction [LCS] (YANG and SYTKOWSKI 1996), rapid subtraction hybridization [RaSH] (BOUKERCHE *et al.* 2007; JIANG *et al.* 2000), enzymatic degrading subtraction [EDS] (ZENG *et al.* 1994) and selective amplification via biotin- and restriction-mediated enrichment [SABRE] (LAVERY *et al.* 1997; SCHIBLER *et al.* 2001).

Serial Analysis of Gene Expression

Advances in molecular and computational biology have led to the development of powerful, high-thoughput methods for the analysis of differential gene expression. These tools have opened up new opportunities in disciplines ranging from cell and developmental biology to drug development. A completely different sequence-based approach to identify differentially expressed genes is followed by serial analysis of gene expression [SAGE] (HUANG *et al.* 2005). In this method, very short (10–14 bp) cDNA tags are generated by restriction digestion, amplified by PCR and ligated, after which the resulting concatemers are sequenced. The tags are long enough to identify the corresponding genes unequivocally and the frequency of the tags is a measure of their expression level. This method is very fast (with an automated sequencer more than 1000 transcripts can be analyzed in one 3 h run) and straightforward because it does not imply selection of mRNAs to create displayable subpopulations, nor does it depend on tricky procedures, such as normalization or subtraction. Although regarded as one of the most cost-effective methods, the limitation of SAGE is that the corresponding gene can be

identified only for the tags deposited in gene banks, making its efficiency dependent on the complexity of available databases. Variants have been published that circumvent some drawbacks of SAGE [tandem arrayed ligation of expressed sequence tags (TALEST) and massively parallel signature sequencing (MPSS)].

Expressed Sequence Tags

Large collections of partial sequences of expressed genes called Expressed Sequence Tags (ESTs) are built by single-pass sequencing of random cDNAs. These are used to discover new genes at only a fraction of the cost of genomic sequencing and, in addition, they facilitate the identification of coding regions in genomic sequences. The ESTs recovered from a certain cell type indicate what kinds of genes are expressed. Although the redundancy of a sequence gives an idea regarding the expression level, this approach (referred to as 'digital northern' or 'electronic subtraction') cannot be used routinely as an expression analysis tool (CHEN *et al.* 2006; MADDEN *et al.* 2003; NIE *et al.* 2005; YANG *et al.* 2007).

Microarray

After a method was found to grid large numbers of cDNA clones at high density, thus permitting their efficient screening by differential hybridization, the usefulness of large-scale sequencing projects has been extended from just a source of new genes to a tool for high-throughput transcriptome analysis (AHMED 2006; SEBAT *et al.* 2003; TARCA *et al.* 2006). Mega-scale reverse northern approaches are now possible thanks to high-speed robotic printing of cDNAs. Originally, cDNAs were spotted on nylon membranes and

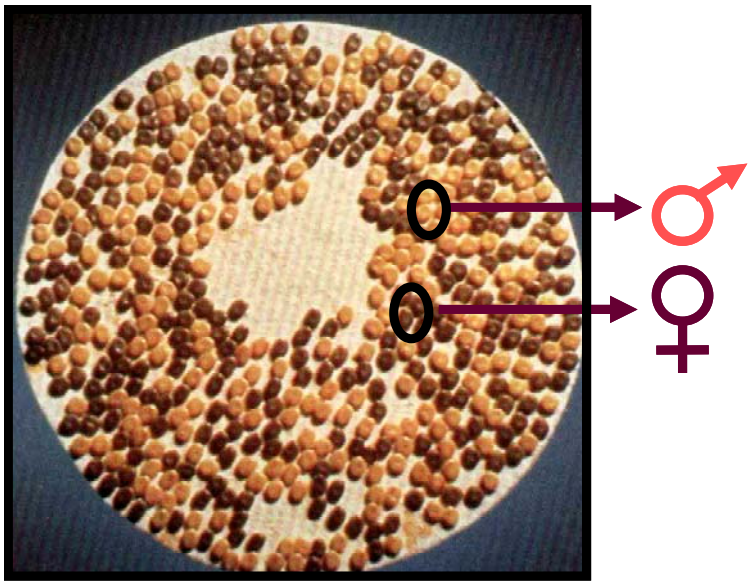
hybridized with traditional methods. With the current microarray technology thousands of clones are displayed on just a couple of square centimeters of glass support and are hybridized in microvolumes with fluorescently labeled cDNA probes, resulting in improved screening sensitivity. Alternatively, the DNA targets can be synthetic oligonucleotides synthesized in situ on silicon. These oligomers are highly gene specific, thus greatly reducing cross-hybridization. The power of this technology is self-evident: data can be collected for large numbers of genes in one experiment and genome-wide expression patterns can be observed. However, major limitations reside in the questionable sensitivity of the probes and in the relatively costly and time-consuming collection of the set of (unique) sequences. The thoroughness of the preceding EST sequencing defines the success, because only genes can be analyzed for which sequences are available. However, a feasible approach for organisms that lack extensive EST collections is to use anonymous clones from cDNA libraries; clones with interesting expression patterns can be sequenced after the expression analysis.

Although microarrays undoubtedly allow high-throughput screening, the efficiency depends on the method used to collect the arrayed sequences. Except for model organisms the brute-force approach of simple EST sequencing of genuine cDNA pools, which are unbiased for a certain cell type, seems rather unwise in view of the number of different mRNAs present in a cell. Several routes can be taken first to enrich the cDNA pool, in order to efficiently screen differentially expressed genes with microarrays. Combinations of microarrays with RDA (ANDERSSON *et al.* 2003; WELFORD *et al.* 1998), SSH (GARDMO *et al.* 2002; VAN DEN BERG *et al.* 2004) or differential display (FUKUSAKI

et al. 2001; HUI *et al.* 2003) have proven very successful and, recently, another method has been described that supplies a normalized pool of so-called 'open reading frame-expressed sequences tags' [ORESTES] (NUNES *et al.* 2004; SAKABE *et al.* 2003). Large-scale sequencing and microarray analysis will probably take the lead for genome-wide studies of gene expression. However, for tackling more limited biological questions, open-ended techniques, such as differential display and cDNA–AFLP-based transcript profiling, will no doubt remain equally useful in the post-genomic future.

Marked embryo sex-limited stock

A translocation stock JPSL where, due to sex-limited pigmentation of the embryos sex separation is possible was used as the source of RNA (**Figure7**) (FUJII and SHIMADA 2007). As early as 30 hours post oviposition, the cells forming the serosal layer at the blastula stage synthesize pigments which endow the embryo with the dark colouration. Kynurenine monoxygenase is an enzyme involved in pigment synthesis and the gene coding for the enyzme is present on the chromosome 10. As a result of translocation of the chromosome 10 fragment harbouring the gene on to W chromosome, only the female embryos show colouration.



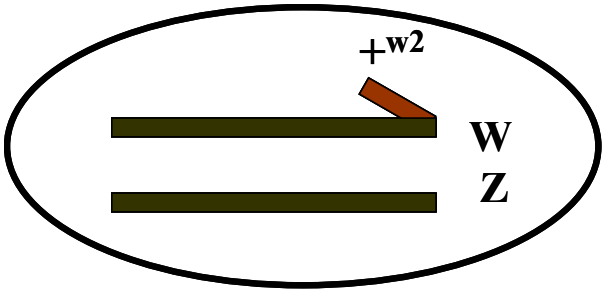


Figure7 Eggs of silkworm stock carrying W chromosome translocated with a part of 10th chromosome, which contains dominant allele ($+^{w2}$) for egg serosal pigmentation.

Marked Sex Limited Larvae

A radiation hybrid stock Pure Mysore Sex-limited, where sex-limited larval markings were present was used as the source of RNA and for RNA interference assays (NAGARAJA *et al.* 2005). The female larvae possess markings in the cephalic and thoracic segments while the male larvae lack the same and thus sex separation is made possible at early IV instar stage (**Figure8**).

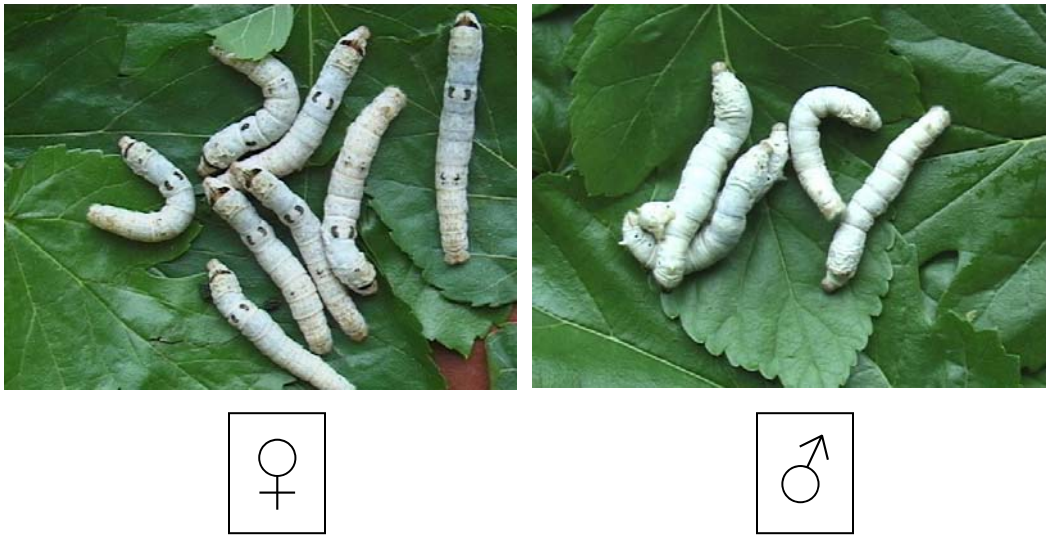


Figure8 Sex-limited PMSL larvae. Female larvae possess crescent-shaped markings in the cephalic and thoracic segments while the male larvae lack the same.

DNA extraction

DNA extraction was performed on male and female larvae frozen in liquid nitrogen using the method of Nagaraja and Nagaraju (1995). Briefly, the whole body of the larvae except midgut was ground in liquid nitrogen using a pestle and mortar. Extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA and 1% SDS) and proteinase K (100 µg/mL) was added to the ground tissue and incubated at 37°C for 2 h with occasional swirling. The DNA was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) and once with chloroform. The supernatant DNA was ethanol-precipitated, resuspended in TE (10 mM Tris-HCI, 1 mM EDTA, pH 8.0) buffer and incubated at 37°C for 1 h after addition of RNase A (100 µg/mL). DNA was re-extracted with phenol-chloroform and ethanol-precipitated as described earlier. The genomic DNA was quantified on 0.8% agarose gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, final pH 8.0).

RNA Extraction

RNA from fresh or frozen unfertilized eggs, male and female embryos and different tissues –head, silkgland, midgut, fatbody, ovary, testis- at different developmental stages was extracted using TRIZOL® Reagent (Invitrogen Corporation, USA) according to the manufacturer's instruction [Chomczynski and Sacchi method (1987)]:

> Tissue samples were homogenized in TRIZOL Reagent with its volume being 1 ml per 50-100 mg of tissue using dounce homogenizer. The homogenized samples were incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. To the homogenate 0.2 ml of chloroform per 1 ml of

TRIZOL reagent was added, mixed vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. The samples were centrifuged at 12,000 × g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube and the RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol in a ratio of 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Samples were then incubated at 15 to 30°C for 10 minutes and centrifuged at no more than 12,000 × g for 10 minutes at 2 to 8°C. After removing the supernatant, the RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The sample was then vortexed and centrifuge at 7,500 × g for 5 minutes at 2 to 8°C. The RNA pellet was briefly air-dried (5-10 minutes) and dissolved in RNase-free water (Ambion, Inc, USA) and then incubated for 10 minutes at 55 to 60°C. The redissolved RNA was stored in aliquots at -70°C until further use.

Differential Display PCR

Genomyx HEIROGLYPH mRNA profile kit including the set of 12 3’ double-anchored oligo dT primers (AP1-AP12) and 5’ arbitrary primer sets 1 and 2 (four primers each; ARP1-ARP8) (Beckman Coulter Inc, USA) was employed for performing differential

display PCR. The sequences of the primers are listed in the table1&2. Differential Display PCR was performed according to manufacturer's instructions:

- ✶ DNase I treatment of RNA

  For 50 µg total RNA, 10 µl RNase inhibitor (1 U/µl), 1 µl RNase-free DNaseI (10 U/µl), 5 µl 0.1 M Tris-Cl pH 8.3, 5 µl 0.5 M KCl and 5 µl 15 mM MgCl2 were added, mixed and incubated for 30 minutes at 37 °C. The DNase treatment was followed by phenol extraction and precipitation by incubation for 30 min at -80 °C with 5 µl sodium acetate and 200 µl 100 % ethanol and centrifugation at 12,000 × g for 10 minutes at 2 to 8°C. After removing the supernatant, the RNA pellet was washed once with 200 µl of 75% ethanol and dissolved in 20 µl of RNase-free water. Concentration of RNA was estimated and run on a denaturing gel to check for integrity.

  - ❖ cDNA synthesis

    For each RNA twelve reactions were set up (one tube for each double-anchored oligo(dT) primer set – AP1 to AP12. DNA-free RNA was diluted to 0.2 µg/µl with RNase-free water. A core mix consisting of 4 µl 5 x reverse transcriptase buffer, 2 µl DTT (0.1 M), 1.6 µl 4dNTP mix (250 µM), 200 ng RNA, 2 µl AP oligo (10 pmol/µl), with H_2O to 19 µl was prepared, mixed and incubated for 5 min at 65 °C and for 10 min at 37 °C. After 1 minute of quick chill on ice, 1 µl Superscript II reverse transcriptase (200 U/µl; Invitrogen, USA) was added to the core mix and incubated for 50 min at 42 °C. The reverse transcriptase was inactivated by incubation for 5 min at 95 °C. The

synthesized cDNA was used immediately for PCR amplification or stored in aliquots at -20 °C for later use.

❖ Amplification by Polymerse Chain Reaction (PCR)

All PCRs were performed in triplicates and always run in the same thermal cycler [model 9700 (Perkin Elmer, USA)] to minimize variations. Core mix for all PCR reactions which contain the same 3' AP oligo primer was prepared and aliquoted 18 µl where, for each 20 µl PCR reaction the composition is 9.2 µl H2O, 2 µl 10 x PCR reaction buffer, 1.6 µl dNTP mix (25 µM), 2 µl 3' AP oligo primer, 2 µl cDNA and 0.2 µl *Taq* DNA polymerase (5 U/µl). To the core mix 2 µl (2 pmol/µl) of 5' arbitrary primers ARP was added and since there are 12 different 3' AP primers and 8 5' ARP primers, the number of reactions set in triplicates are 8*12*3 = 288. Reactions with RNA as templates, also in triplicates formed the negative controls. PCR was run with the following thermal conditions: 40 cycles of 94 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 2 minute and 1 cycle of 72 °C for 10 minutes. The PCR reactions were run on 3% Metaphor Agarose or 6% PAGE gel or stored at -20 °C till further use.

❖ Metaphor® Agarose Gel

MetaPhor is an intermediate melting temperature agarose with which DNA fragments differing in size by 2%, in the range of 200 bp to 800 bp, could be resolved by submarine gel electrophoresis. MetaPhor agarose gels (2% to 4%) approximate the resolution of polyacrylamide gels (4% to 8%). Hence the DDPCR products were run in 3% Metaphor gels and 6% denaturing

polyacrylamide gel as well. DDPCR products were run on 3% Metaphor agarose gels in 0.5x TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA) at 5V/cm for 3 hours, stained with ethidium. bromide, and visualized under UV light and documented using the GelDoc2000 system (Bio-Rad, USA).

❖ 6% Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

The PCR products were also analyzed on a 6% denaturing PAGE (7 M urea, 1 x TBE): after a prerun for 30 min at 60 W, 3.5 µl of the PCR reaction, denatured by mixing with 2 µl formamide loading buffer, incubating 3 min at 95 °C and chilling on ice, was loaded onto the gel together with a labelled size marker and run until xylene cyanol dye nearly reaches the bottom. The run would be approximately 4 hours at 10 V/cm. The gel was silver stained for visualization and the method followed was a modification of the standard silver staining method for DNA as devised by Bassam et al (1991): the gel was rinsed once in distilled water and the DNA in the gel was fixed by incubating in 10% Ethanol for 10 minutes. After rinsing once in distilled water, the gel was incubate in 1% Nitric Acid for exactly 3 minutes. Nitric acid was decanted and the gel was rinsed 2 times in distilled water. It was incubated 2 times for 5 minutes in distilled water followed by 15 minutes incubation in 0.2% silver nitrate. The gel was subsequently rinsed 3 times with distilled water and incubated for 2 min in fresh distilled water. Developing solution (3% (w/v) sodium carbonate, 0.05 % (w/v) formaldehyde in H_2O) was added and shaken briefly, until brown or black precipitate formed

in the solution. Fresh developing solution was added after decanting the used solution. Incubation was continued until desired intensity of bands and background is reached and protection from fluorescent light was ensured all the while. When desired intensity is reached, i.e. best balance between band intensity and background, developing solution was decanted and stop solution was added immediately. The gel was incubated in stop solution [3% (w/v) acetic acid in H_2O] for 5 minutes before proceeding to the next step of PCR fragment isolation or preserving overnight in the gel preserving solution [5 % (w/v) glycerol, 35 % (v/v) methanol in H_2O].

❖ Isolation of PCR fragment

The bands specific to females were cut out with a clean razor blade and soaked in 100 µl H_2O for 10 minutes at room temperature. Then it was boiled for 15 minutes, spun in a microcentrifuge and the supernatant containing the DNA tube was precipitated by incubating at -70 °C for at least 30 min with 10 of 3 M sodium acetate, 5 µl glycogen (10 mg/ml), 400 µl 100 % Ethanol. spin, wash with 85 % ethanol and redissolved in 10 µl H_2O.

❖ Reamplification

Using the appropriate AP-ARP primer combination each gel eluted fragment was reamplified in a 20 µl PCR reaction containing 4 µl of the isolated DNA, 2 µl 10 x PCR reaction buffer, 1.6 µl dNTP mix (250 pmol/µl), 2 µl AP primer and 2 µl ARP primer in 8.2 µl H_2O, 0.2 Taq polymerase (5 U/µl). The PCR conditions used were same as that of the DDPCR reactions. PCR products were run on a 1.5 % agarose gel and isolate fragment of expected

size. When there was no visible PCR product, 1 µl of the first reamplification was used as a template for a second round of reamplification. The isolated PCR fragment was cloned in TA vector (Invitrogen, USA).

Cloning

The reamplified products were cloned by the TA Cloning® method into the plasmid vector, pCR®II-TOPO® exploiting the non template-dependent terminal transferase activity of *Taq* polymerase, which adds a single deoxyadenosine (A) to the 3´ ends of PCR products (YASUDA 1996; ZHOU *et al.* 1995). The linearized pCRII-TOPO vector is supplied with a single, overhanging 3´ deoxythymidine (T) residue which allows PCR inserts to ligate efficiently with the vector. For any subsequent cloning, pCR II-TOPO was the vector of choice.

Sequencing

Sequencing of all the clones were performed by the cycle sequencing/chain termination method on a Thermal Cycler model 9700 (Perkin-Elmer) and analyzed on an ABI Prism 3100 Genetic Analyzer. The thermal conditions for cycle sequencing are 96^{o} C for 1 minute, 25 cycles of 96 ^{o} C for 15 seconds and 60 ^{o} C for 4 minutes.

Reverse transcription (RT)

First strand synthesis by reverse transcription for any particular RNA sample was primed by oligo in general. A core mix consisting of 4 µl 5 x reverse transcriptase buffer, 2 µl DTT (0.1 M), 1.6 µl 4dNTP mix (250 µM), 200 ng RNA, 2 µl oligo (10 pmol/µl), with

H_2O to 19 µl was prepared, mixed and incubated for 5 min at 65 °C. After 1 minute of quick chilling on ice, 1 µl Superscript II reverse transcriptase (200 U/µl; Invitrogen, USA) was added to the core mix and incubated for 60 min at 42 °C. The reverse transcriptase was inactivated by incubation for 10 min at 70 °C. The synthesized cDNA was used immediately for PCR amplification or stored in alquots at -20 °C for later use.

PCR

Primers for amplifying specific targets with specific annealing temperatures are listed in the table4&5. All reactions were carried out in thermal cycler model 9700 (Perkin Elmer, USA)]. Core mix for each 20 µl PCR reaction is composed of 9.2 µl H_2O, 2 µl 10 x PCR reaction buffer, 1.6 µl dNTP mix (25 µM), 2 µl each of 5' and 3' primers (for specific targets), 2 µl cDNA or 50 ng genomic DNA and 0.2 µl *Taq* DNA polymerase (5 U/µl). Reactions with RNA as templates formed the negative controls. PCR was run with the following thermal conditions: 40 cycles of 94 °C for 30 seconds, x °C for 30 seconds, 72 °C for 2 minutes and 1 cycle of 72 °C for 10 minutes. The PCR reactions were run on 1.5% Agarose gels in TAE buffer or stored at -20 °C till further use.

Table1: Anchored primers used for differential display PCR reactions

Serial No.	Primer	Sequence
1	AP1	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTGA-3′
2	AP2	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTGC-3′
3	AP3	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTGG-3′
4	AP4	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTGT-3′
5	AP5	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTCA-3′
6	AP6	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTCC-3′
7	AP7	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTCG-3′
8	AP8	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTAA-3′
9	AP9	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTAC-3′
10	AP10	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTAG-3′
11	AP11	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTAT-3′
12	AP12	5′-GTAATACGACTCACTATAGGGCTTTTTTTTTTTCT-3′

Table2: Arbitrary primers used for differential display PCR reactions

Serial No.	**Primer**	**Sequence**
1	AP1	5'-AGCGGATAACAATTTCACACAGGACGACTCCAAG-3'
2	AP2	5'-AGCGGATAACAATTTCACACAGGAGCTAGCATGG-3'
3	AP3	5'-AGCGGATAACAATTTCACACAGGAGACCATTGCA-3'
4	AP4	5'-AGCGGATAACAATTTCACACAGGAGCTAGCAGAC-3'
5	AP5	5'-AGCGGATAACAATTTCACACAGGAATGGTAGTCT-3'
6	AP6	5'-AGCGGATAACAATTTCACACAGGATACAACGAGG-3'
7	AP7	5'-AGCGGATAACAATTTCACACAGGATGGATTGGTC-3'
8	AP8	5'-AGCGGATAACAATTTCACACAGGATGGTAAAGGG-3'

Nucleic acid hybridizations

❖ Southern hybridization

A standard Southern hybridization protocol was followed where the genomic DNA analyzed was from silkworm of the Daizo strain (SOUTHERN 2006). This strain was used due to the availability of its whole genome sequence and hence the possibility of an informed choice of restriction enzyme for digestion. Genomic DNA from male and female silkworm was digested with the use of *Bgl*II (NEB, USA): at a final concentration of 1U/µg, male and female genomic DNA was incubated with *Bgl*II enzyme (10,000 U/ml) along with 1x NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9) at 37^{o}C for 16 hours. Each individual digested genomic DNA (25 µg) was then loaded onto a 0.8% agarose gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, final pH 8.0) for overnight electrophoresis at 55 V. After capillary gel transfer under alkaline conditions (0.4 M NaOH, 1 M NaCl) to the Hybond N^{+} nylon membrane (Amersham, USA), 10 ng of purified probe was radioactively labelled with α-^{32}P-dCTP by PCR. The radioactive probe was added to 20 ml of hybridization solution [5X SSC, 5X Denhardt's solution (0.1% w/v bovine serum albumin, 0.1% w/v Ficoll, 0.1% w/v polyvinylpyrrolidone), 0.5% SDS] at a concentration of ~1 × 106 cpm/ml. Membrane was placed in the probe/hybridization solution, and hybridization took place overnight at 45°C. After hybridization, the membranes were washed three times in 2× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 60°C for 10 minutes and then once in 0.2× SSC, 0.1% SDS at

60°C for 5 minutes. The radioactive membrane was then exposed to PhosphorImager (Amersham Biosciences, USA) screens. After exposure, the PhosphorImager screen was scanned and results were analyzed with ImageQuant 5.0 software (Amersham Biosciences, USA).

RNA Ligase mediated-Rapid Amplification of Complementary cDNA Ends (RLM-RACE)

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction-based technique which facilitates the cloning of full-length cDNA sequences when only a partial cDNA sequence is available (LIU and GOROVSKY 1993). While the classical RACE can yield complete sequences of cDNA ends, the procedure frequently results in the exclusive amplification of truncated cDNA ends, undermining efforts to generate full-length clones. RLM-RACE permits ligation of the synthetic RNA adapter only to full-length RNA. Additionally, RLM-RACE selects for full length, first strand cDNA synthesis since any first strand cDNA molecules that do not extend all the way to the 5' end of the adapter will not yield product in the PCR. Together, these features insure that only true 5' ends of transcripts are amplified. 5' and 3' RLM-RACE analysis was performed starting with 10 µg of total RNA, using the First-Choice® RLM-RACE kit (Ambion, USA) according to the manufacturer's instructions.

- 5' RACE
  - Total RNA was treated with Calf Intestine Alkaline Phosphatase (CIP), to remove free 5'-phosphates from molecules such as ribosomal RNA,

fragmented mRNA, tRNA, and contaminating genomic DNA. For a 20 µl reaction, 10 µg total RNA was added with 2 µL 10X CIP buffer (10 mM ZnCl2, 10 mM MgCl2, 100 mM Tris pH 8.0), 2 µL Calf Intestine Alkaline Phosphatase (1U/µL) and 20 µL nuclease-free water and incubated at 37^{o} for 1 hour. The cap structure found on intact 5' ends of mRNA is not affected by CIP.

- The RNA is then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. A 10 µL reaction containing 5 µL of CIP-treated RNA, 1 µL 10x TAP buffer [0.5 M sodium acetate (pH 6.0), 10 mM EDTA, 1% β-mercaptoethanol, and 0.1% Triton X-100], 2 µL of TAP enzyme (5U/ µL) and 2 µL nuclease-free water was incubated at 37^{o} C for 1 hour. The reaction was terminated and extracted with 150 µL of phenol:chloroform, then with 150 µL of chloroform, precipitated with 150 µL of isopropanol and washed with 500 µL of 75% ethanol. The pellet was suspended in 11 µL of nuclease-free water.
- A 45 base RNA adapter oligonucleotide is ligated to the RNA population using T4 RNA ligase. A 10 µL ligation reaction was set up with 5 µL of CIP/TAP-treated RNA, 1 µL of RNA adapter (0.3 µg/µL), 1 µL of 10X RNA Ligase Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM Dithiothreitol and 1 mM ATP), 2 µL of T4 RNA Ligase (2.5 U/µL) and 4 µL of nuclease-free water and incubated at 37^{o} C for 1 hour. The adapter cannot ligate to dephosphorylated RNA because these molecules lack the

5'-phosphate necessary for ligation. During the ligation reaction, the majority of the full length, decapped mRNA acquires the adapter sequence as its 5' end.

- A random-primed reverse transcription reaction, followed by a two-step nested PCR with adapter primers (refer table3) and gene-specific primers was performed to amplify the 5' end of a specific transcript. The details of the inner and outer gene-specific primers used in the nested PCR are listed in the table4. The thermal conditions for nested PCR are as follows: 1 cycle of 94^{o}C for 3 minutes, 35 cycles of 94^{o}C for 30 seconds, 60 ^{o}C for 30 seconds and 1 cycle of 72 ^{o}C for 30 seconds, and 72 ^{o}C for 10 minutes.

❖ 3’ RACE

The RLM-RACE Kit can also be used to amplify and clone sequence at the 3' end of an mRNA using the 3' RACE technique. First strand cDNA is synthesized from total RNA using the 3' RACE adapter (refer table3). The reaction conditions for the reverse transcription reaction are as described earlier. The cDNA is then subjected to PCR using one of the 3' RACE primers which are complimentary to the anchored adapter, and a gene-specific primer. The thermal conditions for PCR are as follows: 1 cycle of 94^{o}C for 3 minutes, 35 cycles of 94^{o}C for 30 seconds, 60 ^{o}C for 30 seconds and 1 cycle of 72 ^{o}C for 30 seconds, and 72 ^{o}C for 10 minutes.

Table3: Standard adapter oligos and primers used in RLM-RACE reactions

Serial No.	Primer	Sequence	Concentration
1	5' RACE Adapter	5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3'	(0.3 µg/µL)
2	3' RACE Adapter	5'-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3'	(0.3 µg/µL)
3	5' RACE Outer Primer	5'-GCTGATGGCGATGAATGAACACTG-3'	10 µM
4	5' RACE Inner Primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'	10 µM
5	3' RACE Outer Primer	5'-GCGAGCACAGAATTAATACGACT-3'	10 µM
6	3' RACE Inner Primer	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	10 µM

Table4: Gene-specific primers used in RLM-RACE, RT-PCR and genomic PCR*

Serial No.	Primer	Sequence	Annealing temperature (t_a)
1	10 F	AGGCTCAATGTATGTGATG	58
2	10 R	ACGAGGACGAGGAGACAC	58
3	10 3' F	TCACGCCACAGTGCACGAG	58
4	10 5' R	TCTCTTCGACGTCATTAG	58
5	601 F	GATGTATCTATGTATTCTTCATTGACGA	60
6	526 F	AAGGTTCATCCACCATTACCTAAC	60
7	Sx2 R	ACCTTTGGTTTTAGCTTGAGG	60

*The details of usage of appropriate primer combinations have been are presented in the results section.

Table5: Control primers and universal primers used in the study

Serial No.	Primer	Sequence	Annealing temperature (t_a)
1	β-actin F	5′-CACTGAGGCTCCCCTGAAC-3′	60
2	β-actin R	5′- GGAGTGCGTATCCCTCGTA-3′	60
3	ie-1 F	5′-CCAAACGACTATGACGCAAATT-3′	60
4	ie-1 R	5′-TTGTTAAATTGGCCCACCAC-3′	60
5	T7 promoter primer	5′-GTAATACGACTCACTATAGGGC-3′	60
6	M13-Reverse primer	5'-GCGGATAACAATTTCACACAGGA-3'	60

RNA interference

Transient knock down of gene expression was brought about by injection of double stranded RNA into late IV instar larvae and also by ingestion along with feed from as early as the hatched larva stage through the V instar stage. Expression analysis of targets of the knock down at different developmental stages by RTPCR was performed to assess the extent of knock down.

- Preparation of double-stranded RNA (dsRNA)

a) Template preparation

Gene-specific sequences cloned in pCRII-TOPO vector served as template for *in vitro* transcription with the T7 and SP6 promoter sequences present on the vector backbone driving the reaction. PCR with T7 and SP6 primers generated linear templates for the *in vitro* transcription. Core mix for a 20 µl PCR reaction is composed of 9.2 µl H2O, 2 µl 10 x PCR reaction buffer, 1.6 µl dNTP mix (25 µM), 2 µl each of T7 and SP6 primers, 50 ng of plasmid DNA and 0.2 µl Taq DNA polymerase (5 U/µl). PCR was run with the following thermal conditions: 40 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 2 minutes and 1 cycle of 72 °C for 10 minutes. The PCR reactions were checked on 1.5% Agarose gels.

b) *In vitro* transcription

Two independent reactions were set up using MEGAscript® T7 and SP6 *in vitro* transcription kits (Ambion, USA) in order to generate sense and antisense RNA. A 20 µl reaction contains 2 µl of 10X reaction buffer and 2 µl

each of the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP), 1 µg linear template and 2 µl enzyme mix. The qxconcentrations of individual rNTPs were 75 mM in case of T7 reaction and 50 mM in case of SP6 reaction. The reaction mix is then incubated at 37^{o}C for 6 hours. After ending the reaction with 15 µl Ammonium Acetate stop solution, 115 µl of nuclease-free water was added, mixed thoroughly and then extracted with an equal volume of acidic phenol/chloroform followed by another round of extraction with an equal volume of chloroform. The aqueous phase was recovered and the RNA was precipitated by adding 1 volume of isopropanol, mixed well and incubated for 15 minutes at –20°C before centrifuging at 4°C for 15 minutes at 12,000x g to pellet the RNA. The RNA pellet was resuspended in 5 µl of annealing buffer [1 mM Tris-Cl (pH 7.5), 1 mM EDTA] and the yield was estimated spectrophotometrically.

c) Annealing

To anneal, equimolar quantities of sense and antisense RNAs were mixed and small aliquots of the mixture were heated in a 150-ml beaker of boiling water for 1 minute. Then the beaker was removed from the heat source and allowed to cool for 18 hours to room temperature. The dsRNA was checked for integrity by running 3-5 µg of RNA on a native agarose gel and then stored frozen at –20°C.

❖ dsRNA injection experiments

The dsRNA in annealing buffer was ethanol-precipitated and suspended in injection buffer (0.1 mM sodium phosphate, pH 6.8; 5 mM KCl). Apart from

an untreated control group of fifty individuals, each test group including one injected with non-specific dsRNA, consisted of a total of 8 individuals including 4 males and 4 females. Each individual of the test groups were injected with two doses of 20 µg of dsRNA targeting one or more mRNA species- one at Day 1 of V instar larval stage and another at Day 1 of pupal stage.

❖ dsRNA feeding experiments

Administering dsRNA through the oral route along with feed was also shown to yield similar results as that of direct injection into the larval/pupal haemocoel. On a small area on the ventral surface of a fresh mulberry leaf, dsRNA containing solution was smeared and allowed to dry. The larvae were fed with these dsRNA-coated leaves before they continued to feed on normal leaves. As with the injection experiments, an untreated control group consisted of 50 individuals. Each test group including one fed with non-specific dsRNA, consisted of 24 individuals including 12 males and 12 females. From the day of hatching to the first day of V instar stage, dsRNA treatment regime was spread over 6 doses of gradual increase in quantity proportionate to the body weight. At the hatched larva stage and at the I instar stage, a batch of 6 individuals in each group were fed with 500 ng of dsRNA; in II and III instar larvae, a batch of 4 individuals in each group were treated with 2 µg and 10 µg of dsRNA; in the IV instar stage, 25 µg of dsRNA was administered to 2 larvae and 50 µg of dsRNA per larva in the V instar stage.

Sequence Analysis

❖ BLAST (Basic Local Alignment Search Tool) analysis

DNA sequence similarity searches using the BLASTN and BLASTX algorithms and position-specific iterated BLAST (PSI-BLAST) were performed with default parameters of by interrogating the National Center for Biotechnology Information (NCBI) website (http://ncbi.nlm.nih.gov/). The Smith-Waterman algorithm, using the Blosum62 score matrix, a gap opening penalty of 11 and a gap extension penalty of 1 was used to align low homology sequences (ALTSCHUL *et al.* 1997; GERTZ *et al.* 2006).

❖ CLUSTALW

Multiple sequence alignments were performed using the ClustalW program with default parameters of gap opening penalty of 10, a gap extension penalty of 0.05 and weight transition value of 0.5, available at the GenomeNet database resources of the Kyoto University Bioinformatics Centre [http://align.genome.jp/] (THOMPSON *et al.* 2002).

❖ Contig assembly program (CAP3)

Sequences obtained out of DDPCR and RACE analyses obtained were assembled using the CAP3 program available at Pôle Bioinformatique Lyonnais [http://pbil.univ-lyon1.fr/cap3.php] (HUANG and MADAN 1999).

❖ Gene Machine

In order to find potential coding regions and deduce gene structure for long stretches of silkworm genomic DNA, GeneMachine available at the National Human Genome Research Institute (NHGRI) website

(http://genome.nhgri.nih.gov/genemachine/) was used (MAKALOWSKA *et al.* 2001). It is an integrated tool intended to perform both comparative and predictive gene identification techniques in a single run. The output is returned in ASN.1 format and can then be viewed using NCBI's Sequin tool. BLAST searches are also performed in order to see whether a previously-characterized coding region corresponds to a region in the query sequence. A suite of Perl programs and modules are used to run MZEF, GENSCAN, GRAIL2, FGENES, RepeatMasker, Sputnik, BLASTX and BLASTN. The results of these runs are then parsed and written into ASN.1 format. The resulting ASN.1 file can then be opened using NCBI Sequin, in essence using Sequin as both a workbench and a graphical viewer.

❖ Promoter prediction

The neural network promoter prediction tool available at the Berkeley Drosophila Genome Project site (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict promoter boxes with default parameters (REESE 2001).

❖ Kaikobase and SilkDB

Sequence information regarding whole genome, EST etc were accessed from the Japanese silkworm database called the Kaikobase (http://sgp.dna.affrc.go.jp/KAIKO/) and the Chinese database, SilkDB (http://silkworm.genomics.org.cn/).

Results

Identification of female-specific transcripts

Differential screening for sex-specific transcripts through comparison of male and female transcriptomes is a straight forward approach to study sexual development. Differential display analysis is a powerful tool for such a screening and is much favoured even in this era of high throughput analysis using micro array etc. Sex-specific transcripts identified at early embryonic development are likely candidates influencing sexual development. Hence a scheme of differential transcript analysis across early embryonic stages of male and female development would have a high success rate of identifying sex-specific transcripts. The sex-limited JPSL strain, where only female embryos are marked is of strategic importance for such an analysis since it paves way for sex separation at early embryonic stages. The choice of embryonic stage for differential screening, where sex-specific transcripts are at detectable levels is critical, as it would reflect upon the probability of identifying particularly those which constitute the sex determination pathway. The *Bombyx* homologue of *doublesex* being the downstream most gene in the sex determination hierarchy, would invariably be a target of these genes. Thus the window of development appropriate for screening was identified based on the earliest zygotic expression of *Bmdsx* which is at 96 hours post oviposition (hpo). Differential display PCR was performed on RNA from male and female embryos which were at 56 hours and 78 hpo (**Figure9**).

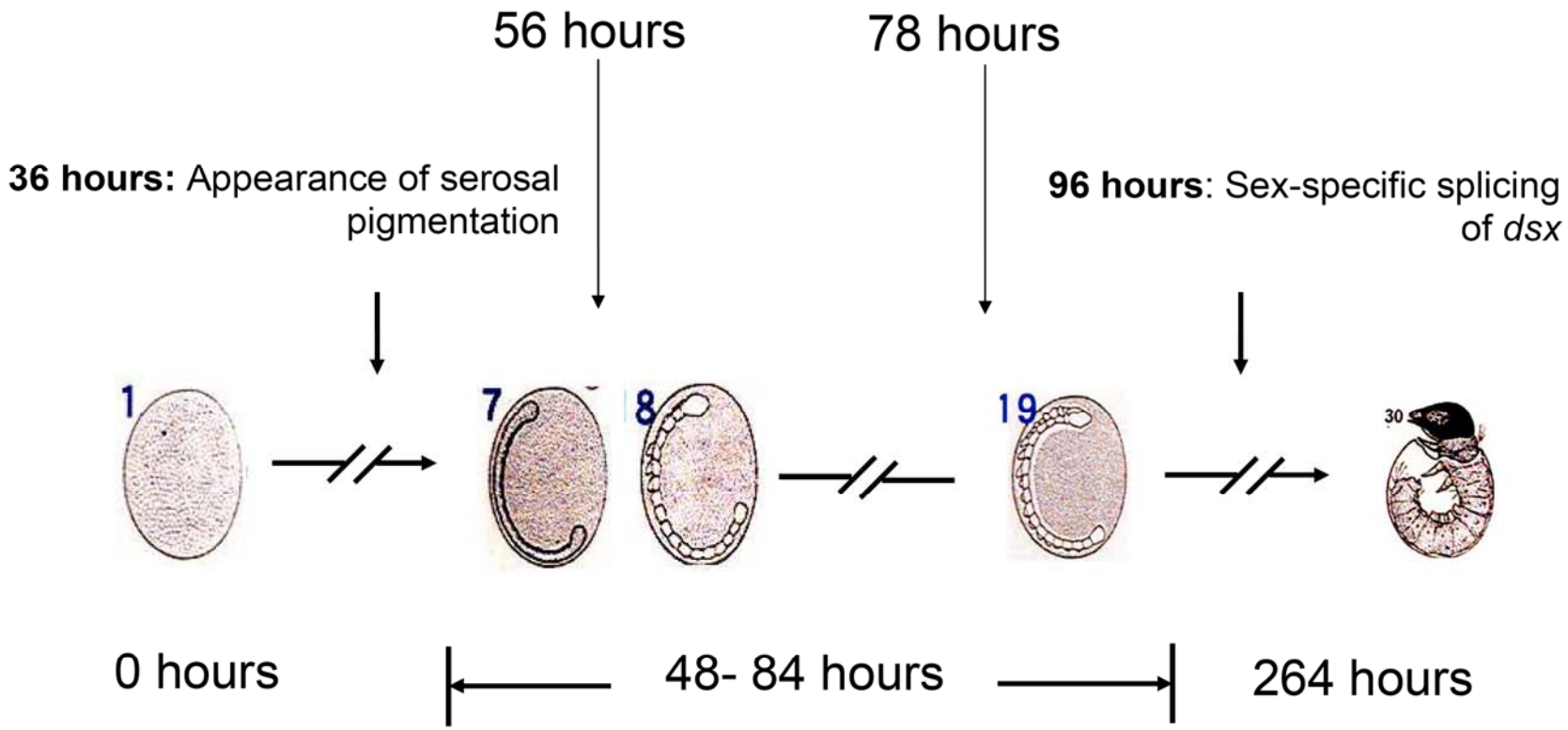


Figure9 The embryonic stages chosen for differential display analysis were 56 hours and 78 hours post oviposition (hpo). These two stages fall well within the developmental window of 48-84 (hpo) within which factors regulating *doublesex* and hence sex determination would possibly be present. Since multiple factors constituting the sex determination cascade has always been the norm, screening of two stages at the extreme ends of the time window permits for high success rate of identifying sex determining genes.

A total of 45 female-specific amplicons were obtained which were cloned in TA vector for downstream sequence analysis. Female-specificity of these 45 amplicons was tested with the specific primers designed based on the sequences obtained and one of them, picked up by the primer pair of AP8/ARP6 showed female-specificity (**Figure10a&b**). This sequence was subjected to further analysis.

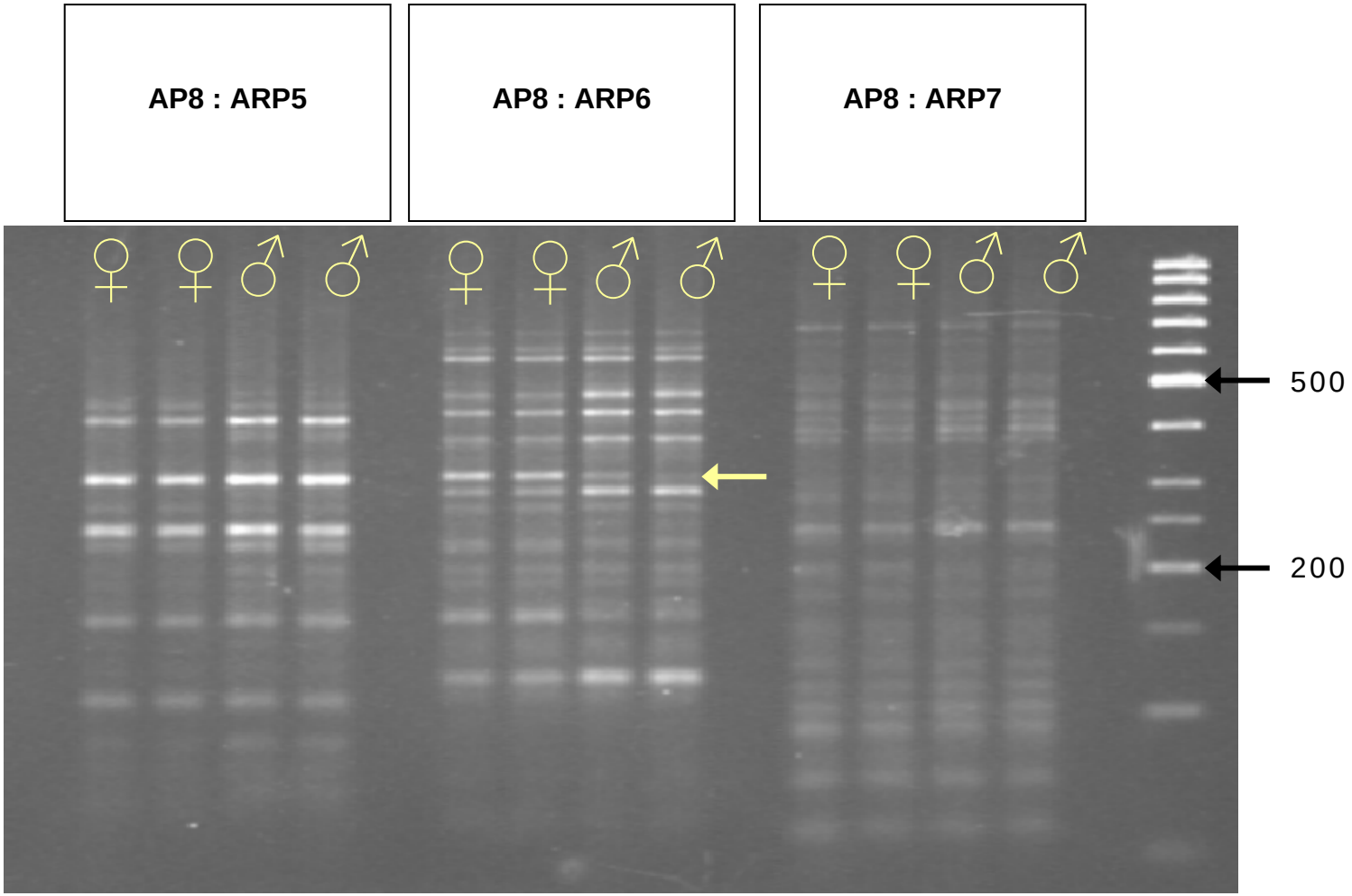


Figure10a Differential display PCR profile obtained with different combinations of anchored primer (AP) and arbitrary primer (ARP). All amplifications have been run in duplicates.

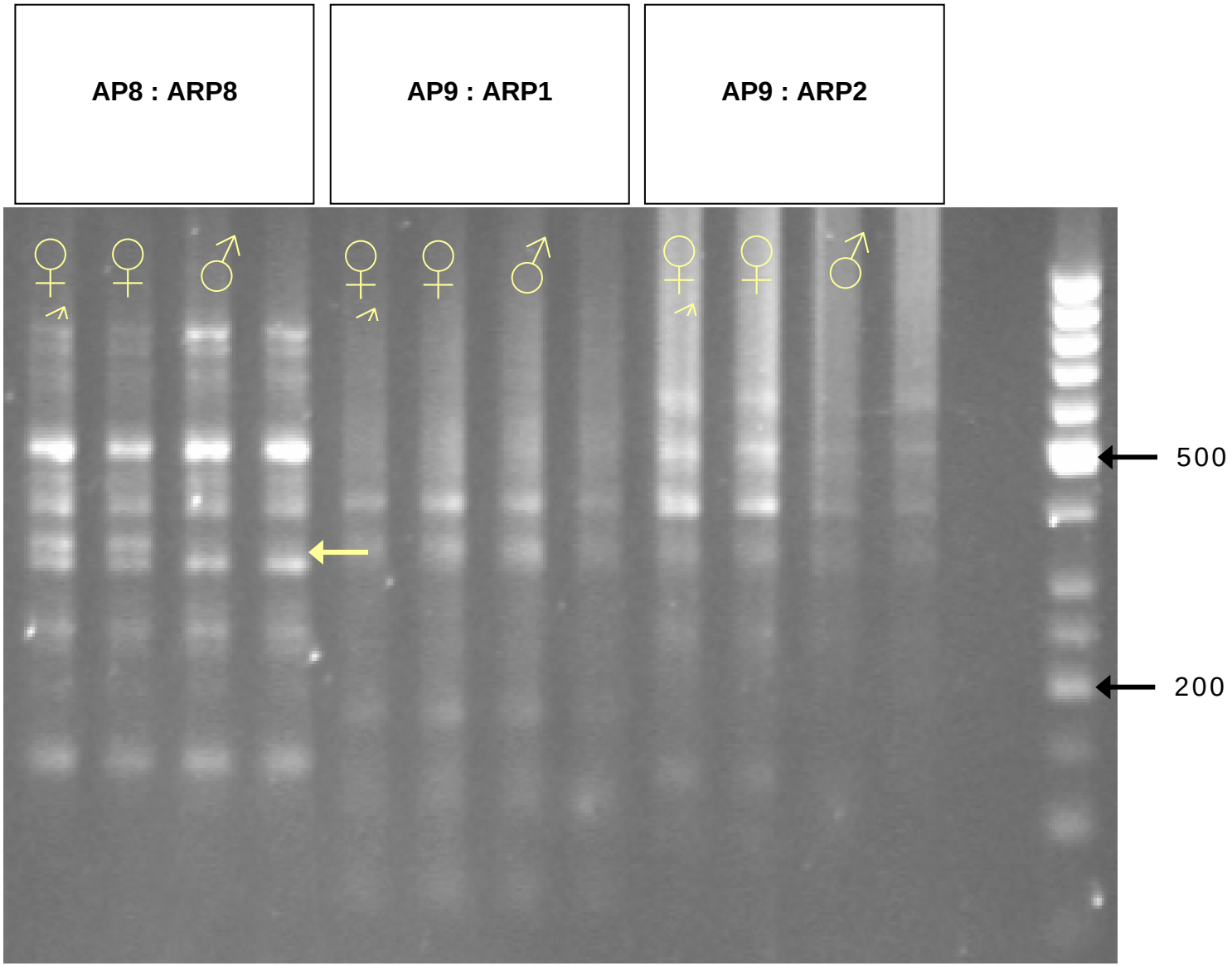


Figure10b Differential display PCR profile obtained with different combinations of anchored primer (AP) and arbitrary primer (ARP). All amplifications have been run in duplicates.

Sequence analysis of the female-specific transcript

A nucleotide BLAST analysis (blastn) of the female-specific sequence against the silkworm Whole Genome Database (WGS) returned three contigs, two from the 3x WGS Japanese assembly and one from the 6x WGS assembly from Southwest Agricultural University, China. Both the Japanese contigs [contig20427 (Accession: BAAB01044259.1), contig643677 (Accession: BAAB01174178.1) were internal to the longer Chinese contig Ctg000783 (Accession: AADK01000783.1), with their sequence information essentially being redundant. Thus there were two copies of the female-specific sequence on Ctg000783 across a 9 Kb region (**Figure11**). Further analysis showed that the copies were present in opposite orientation. The sequenced genome being male, the few mismatches between the female-specific sequence with the WGS contig suggested the presence of a third copy of W chromosome origin (**Figure12**). A BLAST analysis of the WGS-derived contig Ctg000783 against the physical map data using the Silkmap tool suggested their linkage to chromosome XXV (**Figure13**).

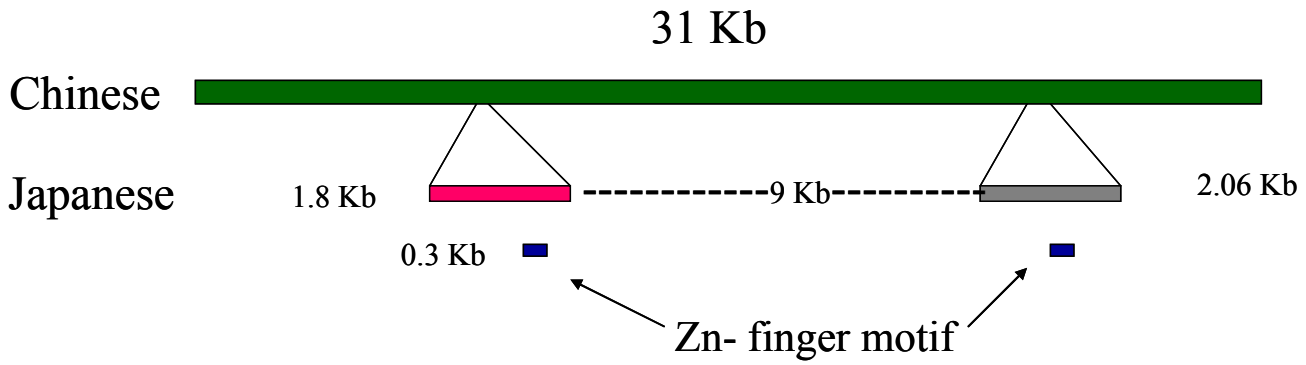


Figure11 BLASTing the female-specific sequence against the 3x Japanese and the 9x Chinese WGS of the male silkworm genome, picked up a 31 KB (Chinese) and two smaller contigs (Japanese) internal to the Chinese contig (2.06 KB and 1.8 KB).

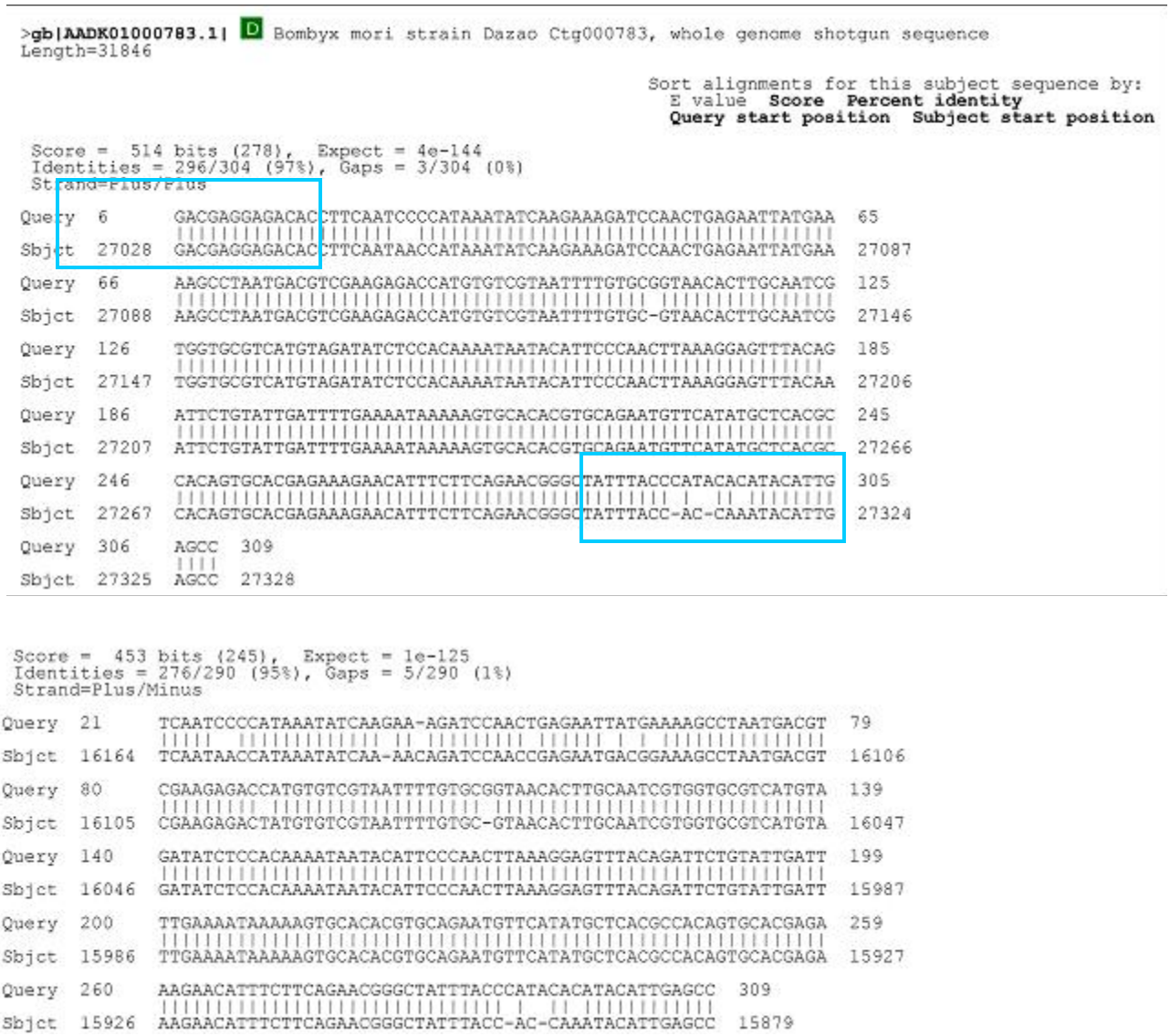


Figure12 The two zinc finger motifs present on Ctg000783 show the few mismatches with the female-specific sequence. The match orientation of the two copies are opposite as revealed (Plus/Plus; Plus/Minus). The blue boxes represent the region where primers for the female-specific sequence bind.

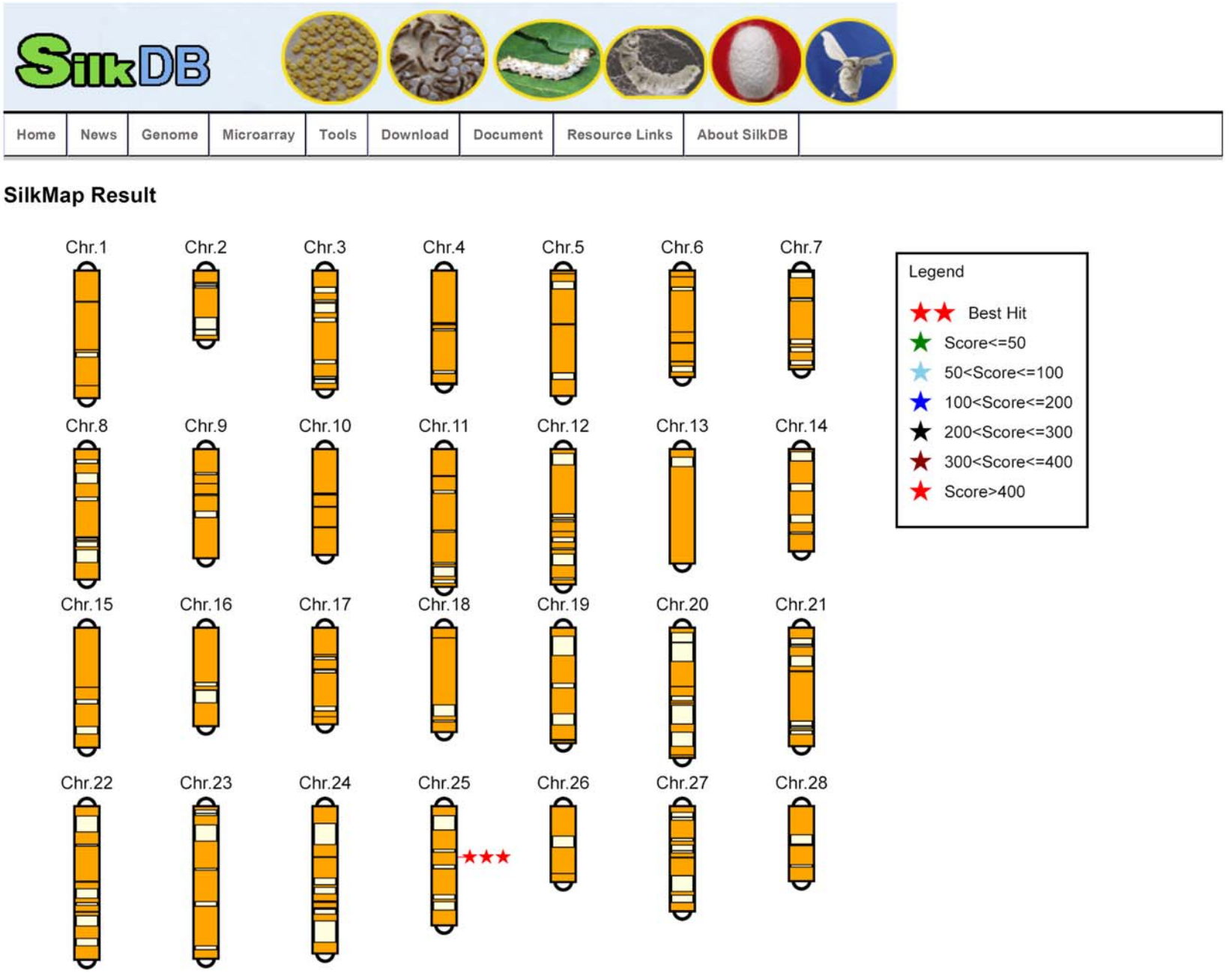


Figure13 BLAST analysis of contig Ctg000783 against the *Bombyx* genome physical map data revealed its linkage to chromosome 25. The red stars mark the best hit with a score of more than 400.

A translated BLAST analysis of the sequence against a translated database of non-redundant sequences (tblastx) revealed that it contained two C-x8-C-x5-C-x3-H type zinc finger motifs in tandem (**Figure14**).

```
>ref|XM_624643.2| UG PREDICTED: Apis mellifera similar to zinc finger CCCH-type containing
10 (LOC552267), mRNA
Length=2151

 GENE ID: 552267 LOC552267 | similar to zinc finger CCCH-type containing 10
[Apis mellifera] (10 or fewer PubMed links)

 Score = 79.0 bits (166),  Expect = 1e-12
 Identities = 30/74 (40%), Positives = 42/74 (56%), Gaps = 0/74 (0%)
 Frame = +3/+1

Query  90   MCRNLVRNTCNRGASCRYLHKIIHSQLKGVYKFCIDFENKKCTRAECSYAHATVHEKERF  269
            +CR+ +RN C+RG  C+YLH+          Y FC DF+N  C    C + H T  E++RF
Sbjct  100  VCRDFLRNVCHRGKRCKYLHERSEDDPIDEYTFCHDFQNGMCNWPGCKFLHCTESEEKRF  279

Query  270  FRTGYLPPNTLSHI  311
              TG LP + LS +
Sbjct  280  RATGELPAHILSRL  321


>ref|NM_001008144.1| UG Xenopus tropicalis zinc finger CCCH-type containing 10 (zc3h10),
mRNA
 gb|BC081358.1| UG Xenopus tropicalis zinc finger CCCH-type containing 10, mRNA
(cDNA clone MGC:89691 IMAGE:7026471), complete cds
Length=2853

 GENE ID: 493507 zc3h10 | zinc finger CCCH-type containing 10
[Xenopus (Silurana) tropicalis] (10 or fewer PubMed links)

 Score = 54.2 bits (112),  Expect = 9e-11
 Identities = 18/41 (43%), Positives = 26/41 (63%), Gaps = 0/41 (0%)
 Frame = +3/+2

Query  171  KGVYKFCIDFENKKCTRAECSYAHATVHEKERFFRTGYLPP  293
            K  + FC DF+NK+C R  C + H T  ++E + +TG LPP
Sbjct  338  KNEFVFCHDFQNKECVRLNCRFIHGTKDDEEHYKKTGELPP  460


 Score = 37.3 bits (75),  Expect = 9e-11
 Identities = 13/29 (44%), Positives = 18/29 (62%), Gaps = 0/29 (0%)
 Frame = +3/+2

Query  63   EKPNDVEETMCRNLVRNTCNRGASCRYLH  149
            E+   V E +CR+ +RN C RG  CR+ H
Sbjct  215  EEAAAVVEHVCRDFLRNVCKRGKRCRFKH  301
```

Figure14 A translated BLAST (tblastx) analysis of the female-specific sequence revealed the presence of two C-x8C-x5-C-x3-H type zinc finger motifs in tandem. C-x8-C-x5-C-x3-H zinc finger motifs are known to be involved in nucleic acid -protein , protein-protein interactions.

Establishing W-linkage of the female-specific DDPCR sequence

RT-PCR and Genomic PCR with primers specific to the female-specific sequence and also to the two WGS-derived copies added evidence to the hypothesis that a minimum of three putative C-x8-C-x5-C-x3-H zinc finger genes are present with one of them linked to the W chromosome (Z1) and two to chromosome XXV (Z2 & Z3) [**Figure15a&b**].

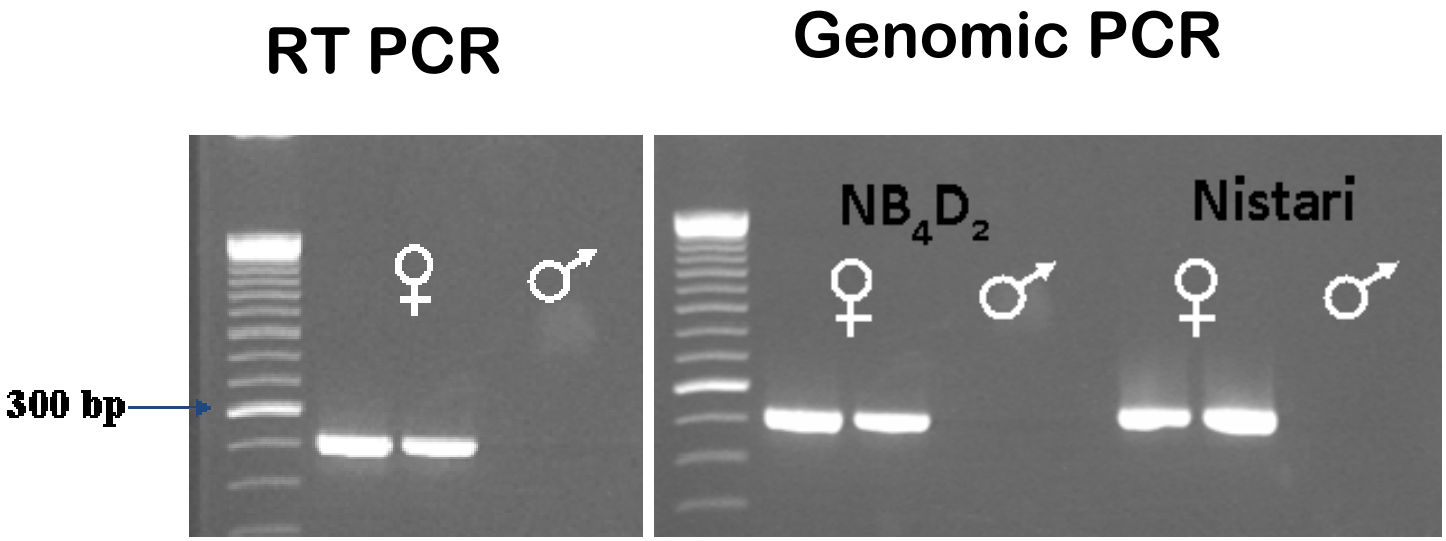


Figure15a The putative female- specific C-x8-C-x5-C-x3-H type zinc finger gene is linked to W chromosome as revealed by PCR with specific primers on genomic DNA from divergent strains of NB_4D_2 and Nistari.

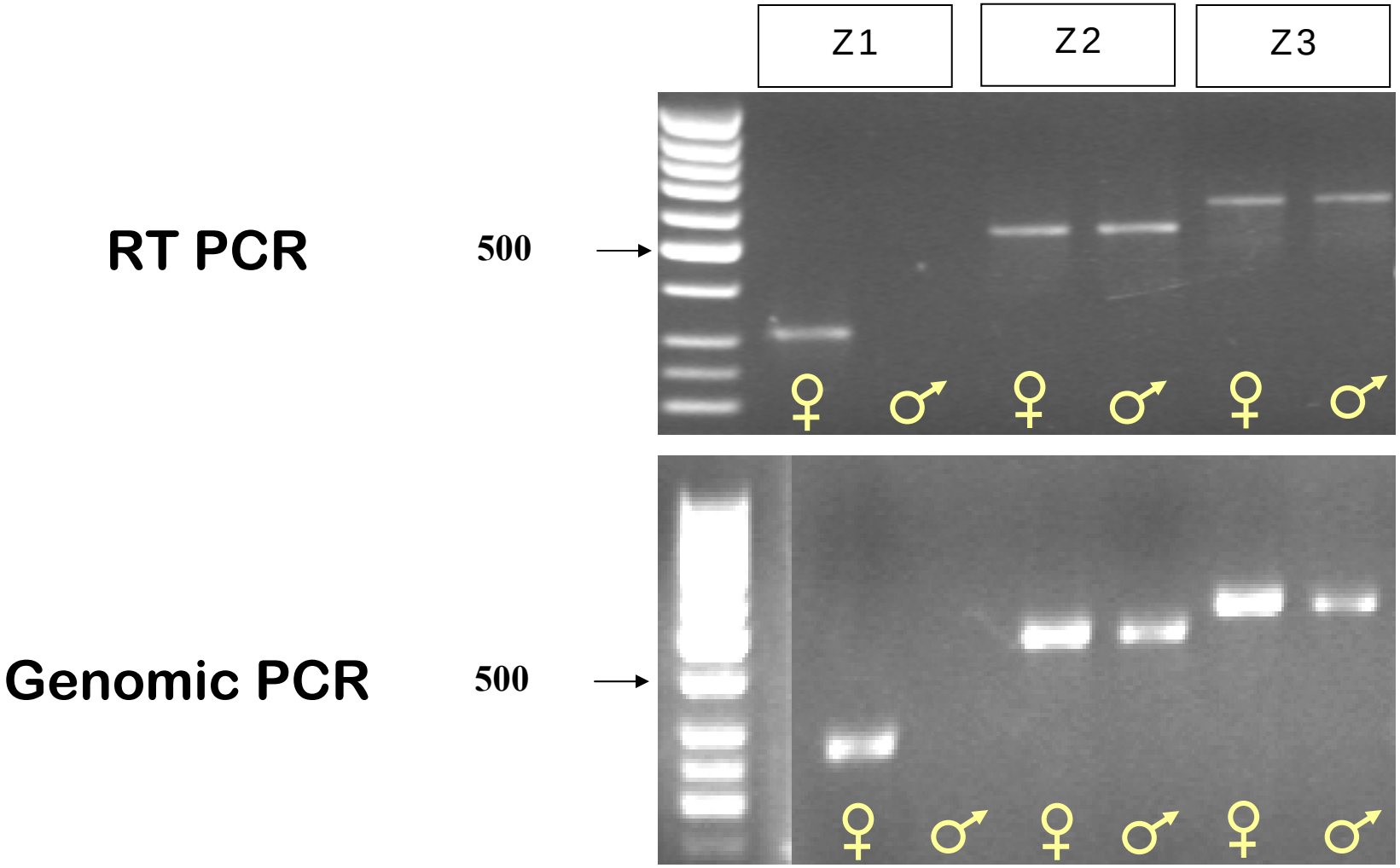


Figure15b The putative female- specific C-x8-C-x5-C-x3-H type zinc finger gene linked to W chromosome has 2 copies Z2 and Z3 on autosome as revealed by RTPCR and genomic PCR with specific primers.

Southern hybridization using a 327 bp fragment which possesses the two C-x8-C-x5-C-x3-H zinc finger motifs confirmed the PCR results showing two bands common to both male and female and one female-specific band (**Figure16**). An additional female-specific band of negligible intensity suggested the presence of more than one W-linked copy.

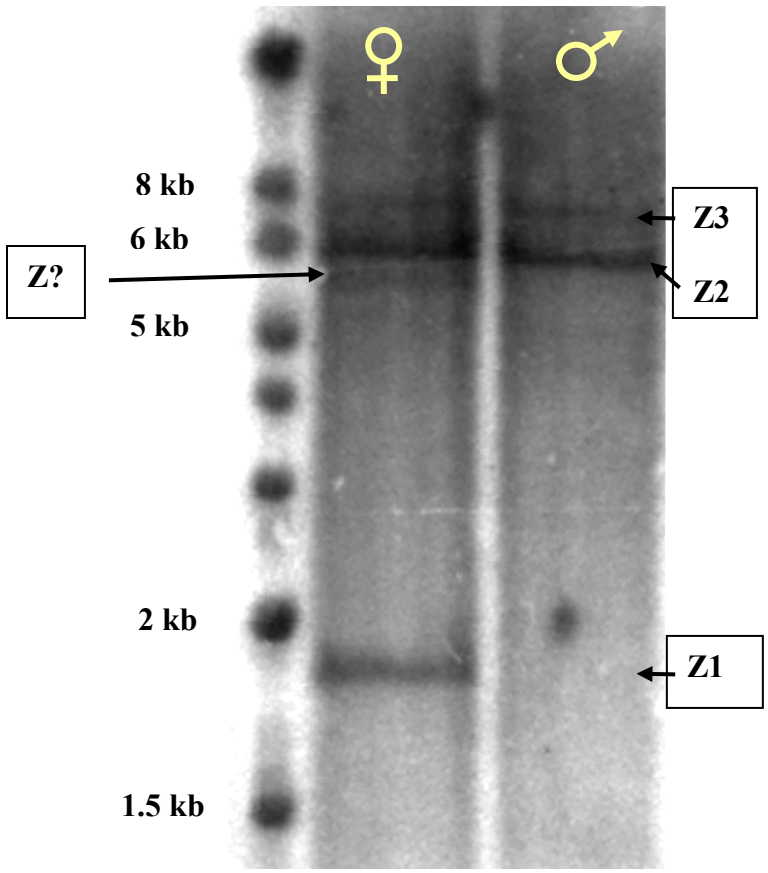


Figure16 Southern hybridization confirms the presence of 3 copies of the C-x8-C-x5-C-x3-H type zinc finger gene Z1, Z2 and Z3. There apparently are more than one W-linked zinc finger copy as the additional female-specific band Z? shows.

Analysis of structure of Z1, Z2 and Z3 genes and protein prediction

Using the integrated gene prediction tool, GeneMachine, on scaffold000788 (retrieved from SilkDB) which contains the autosomal contig 000783, the gene structure for Z2 and Z3 was analyzed for an arbitrary estimate of the gene and transcript length. This analysis predicted a longer Z2 gene of size approximately 7.5 Kb and a much smaller Z3 gene of size approximately 1.8 Kb. Further to analyzing the gene structure, adapting the RNA ligase-mediated method, 5' and 3' RACE were performed for the three genes. Primers for 5' and 3' RACE were specifically designed in and around the C-x8-C-x5-C-x3-H zinc finger motif: a) 10 F and 10 5' R formed the nested primer combination for 5' extension of Z1 cDNA while 10 R and 10 3' F were the 3' extension nested primers; b)for Z2, 526F and 10 3' F formed the 3' extension primers while the same nested combination of 10 F and 10 5' R was used albeit at a lower annealing temperature of 55^{o}C; c) 10 5' R and Sx2R were used for 5' extension of Z3 and 601 F and 10 3' F were used for 3' extension.Z1 and Z2 transcripts were of similar size while Z3 mRNA is much smaller. Z1 codes for a transcript of size 2,163 bp, Z2 codes for a transcript of size 1,959 bp and the size of the transcript coded for by Z3 is 850 bp. Reconstructing the gene structure based on RACE analysis, the WGS contig sequence and W-linked genomic sequence obtained by PCR, the size of the genes were arrived at: Z1 was 13.841 Kb, Z2 was 13.897 Kb and Z3 was 2.676 Kb. Both Z1 and Z2 show a similar splicing pattern with a total of 11 and 12 exons constituting the transcript respectively. Between the two genes 9 exons are common spanning the entire open reading frame (ORF) [**Figure17**].

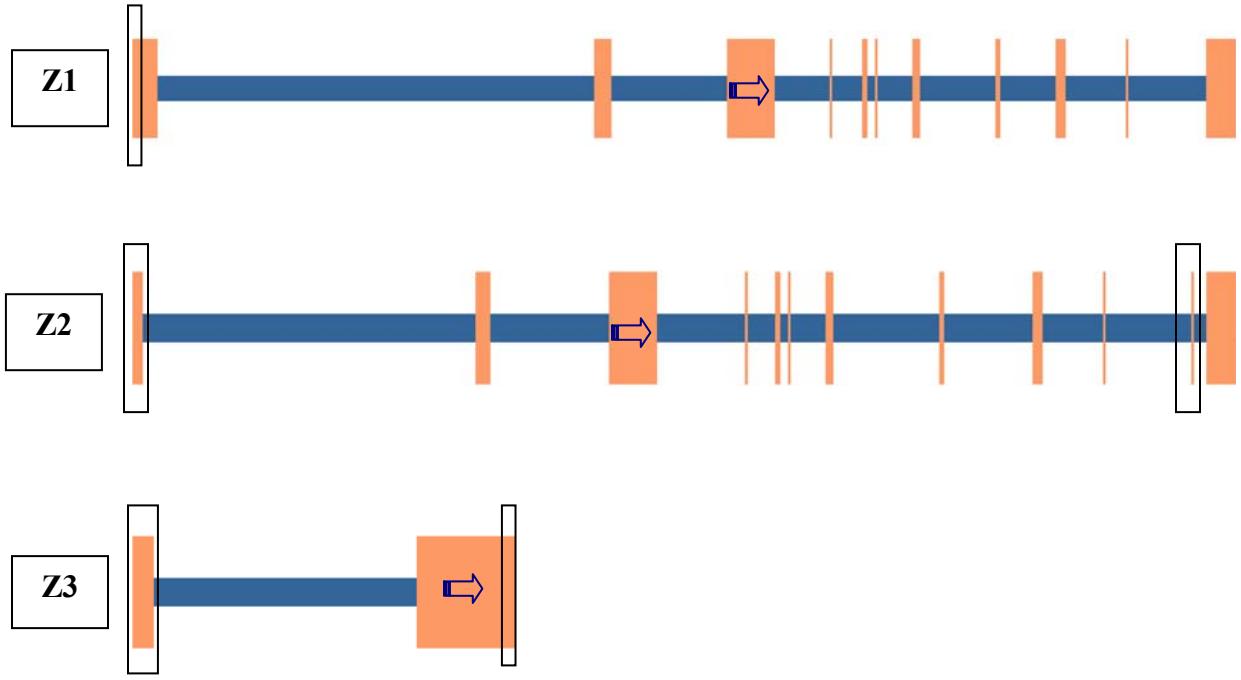


Figure17 Gene structure of the W-linked zinc finger (Z1) shows 11 exons, while autosome-linked Z2 has 12 exons and autosome-linked Z3 possesses 2 exons. C-x8-C-x5-C-x3-H zinc finger motif region in all the three genes is represented by block arrow which is also the high similarity core region. The unique regions in each of the zinc finger genes are represented in black boxes. Exons are represented by orange blocks and the intervening purple strip represents introns.

At DNA level, homology between Z1 and Z2 is about 90% and the difference is 14 residues among the total 360 amino acid residues at protein level. Among Z1 and its autosomal copies, Z3 is probably the most ancient with its splicing pattern being different from Z1 and Z2 and is constituted by only 2 exons translating into a protein of 232 amino acid residues (**Figure18a&b**).

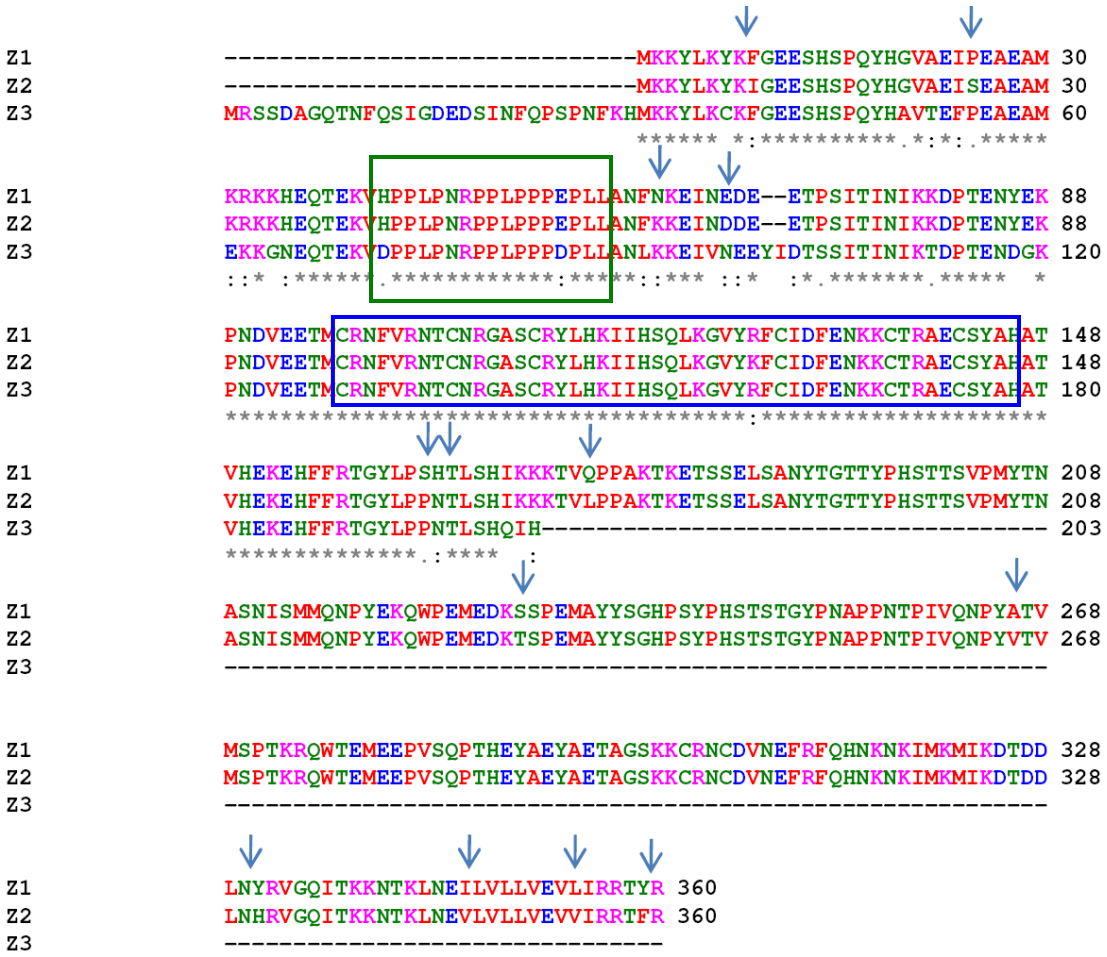


Figure18a ClustalW alignment of the conceptual translation of ORFs of the W-linked zinc finger (Z1) and the autosome-linked zinc fingers Z2 &Z3. Z1 and Z2 possess almost identical amino acid sequence except for the few largely synonymous substitutions denoted by arrows. The red box represents a proline-rich region and the blue box marks the C-x8-C-x5-C-x3-H twin motifs

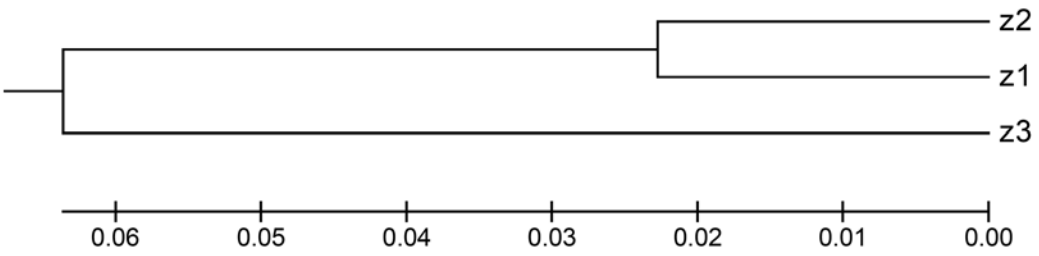


Figure18b. The evolutionary relationship was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.14993842 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 180 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (TAMURA *et al.* 2007).

Expression profile Analysis of the zinc finger genes

Analysis of the expression profile of the three zinc finger genes across different developmental stages and in different tissues suggested the possibility of the zinc finger genes being involved in sex determination. In the unfertilized egg and early embryonic stage of 6 hpo, Z1 and Z3 were expressed while 24 hpo was the earliest stage at which Z2 expression was detected (**Figure19**). The zinc finger gene transcripts Z1 and Z3 thus appear to be maternally supplied. A dip in the levels of Z1 and Z3 transcripts from 0-24 hpo suggests that the maternal supply is being exhausted before the zygotic transcription is activated at 24 hpo. Hence Z1 and Z3 could probably influence the expression of Z2.

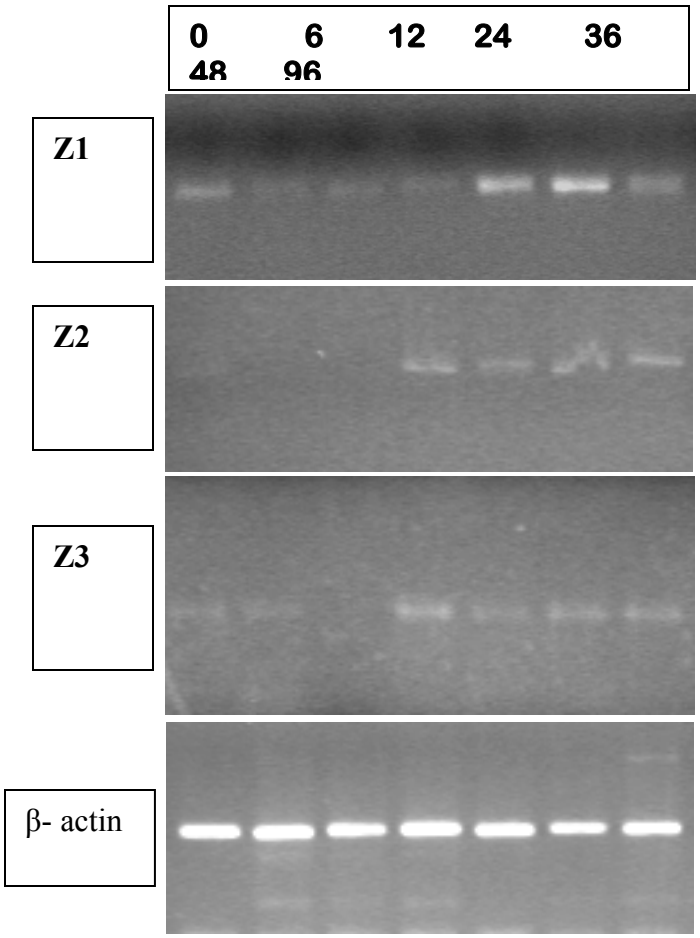


Figure19 The early embryonic expression profile show that Z1 and Z3 are maternally supplied and zygotic transcription of all the three genes Z1, Z2 and Z3 kickstart at 24 hours post oviposition. RTPCR was performed with RNA fro single embryos.

The ubiquitous expression of Z1 in all tissues at all developmental stages examined is the hallmark of a master regulator gene acting higher up in the sex determination hierarchy (**Figure20**). Expression of Z2 and Z3 were also detected in several tissues and at different stages of development though not as consistent as Z1. Since there is no definite pattern of expression of Z2 and Z3 with respect to Z1 expression genes in brain, silk gland, fatbody, midgut, ovary/testis, suggests that their functional role in development is not restricted to sex determination alone.

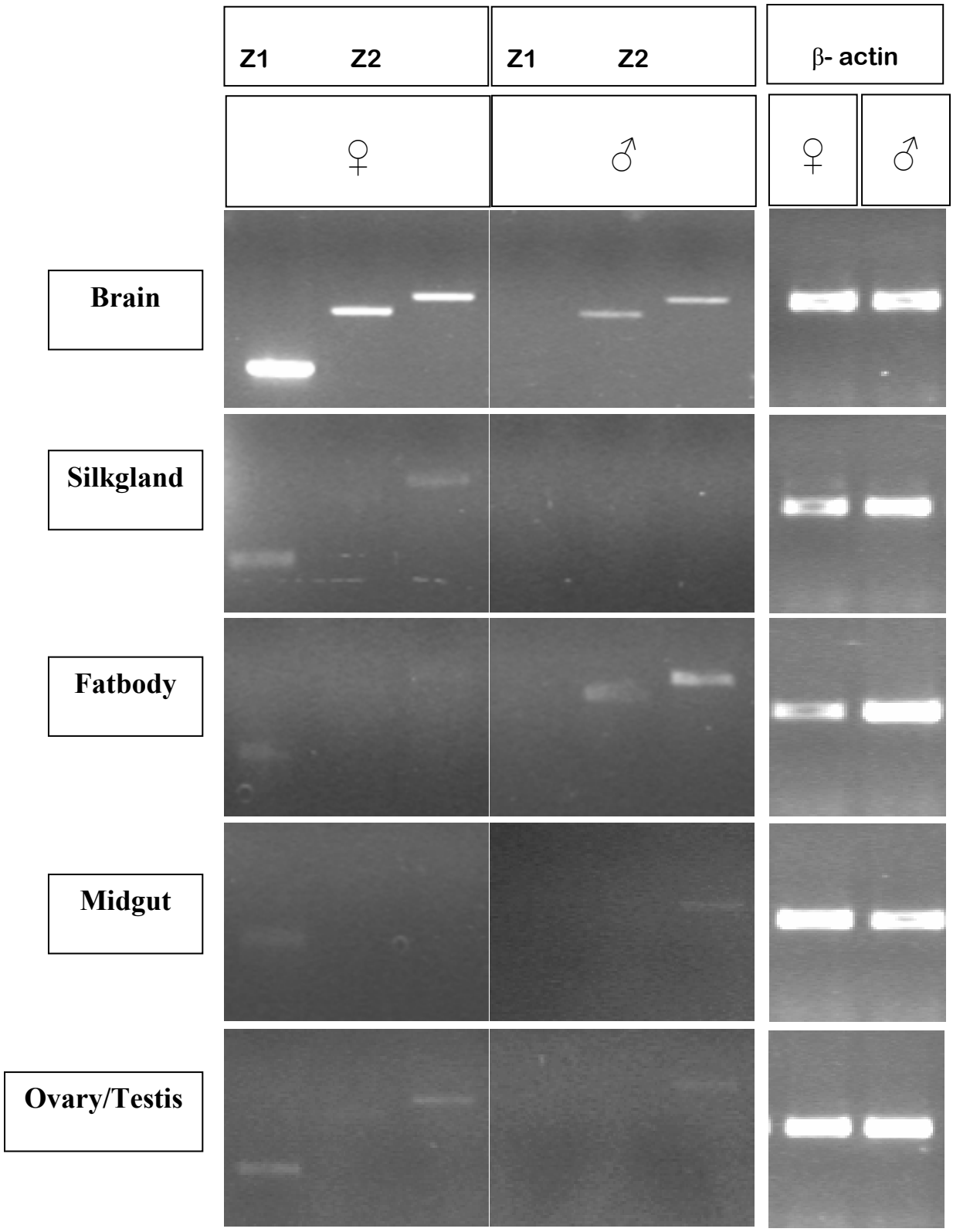


Figure20 Expression profiles of Z1, Z2 and Z3 in different tissues at the V instar larval stage. Ubiquitous pattern of expression was observed of Z1.While Z2 expression is limited to only brain, ovary and fat body, Z3 is expressed in almost as many tissues as Z1.

Functional Analysis by RNA interference

In order to explore the possibility of the zinc finger genes being involved in sex determination, RNA interference assays were performed: dsRNA was administered in two doses by injection into the haemocoel. Primarily, when all the three genes were knocked down targeting the zinc finger motif, the offsprings of dsRNA-treated parents were not viable (**Figure21**). Zygotic development after fertilization was observed in those eggs only for 2 days – apparent by the appearance of serosal pigmentation- beyond which the development stopped and they failed to hatch. Though no significant morphological or anatomical defect was observed in the RNAi-treated parents, at molecular level, aberration in the splicing pattern of *dsx* was observed in males but not in females (**Figure22**). In RNAi-treated males both male and female isoforms of *dsx* were found to be present and a corresponding increase in the levels of *vitellogenin* transcript- a downstream target of *dsx*- was also observed.

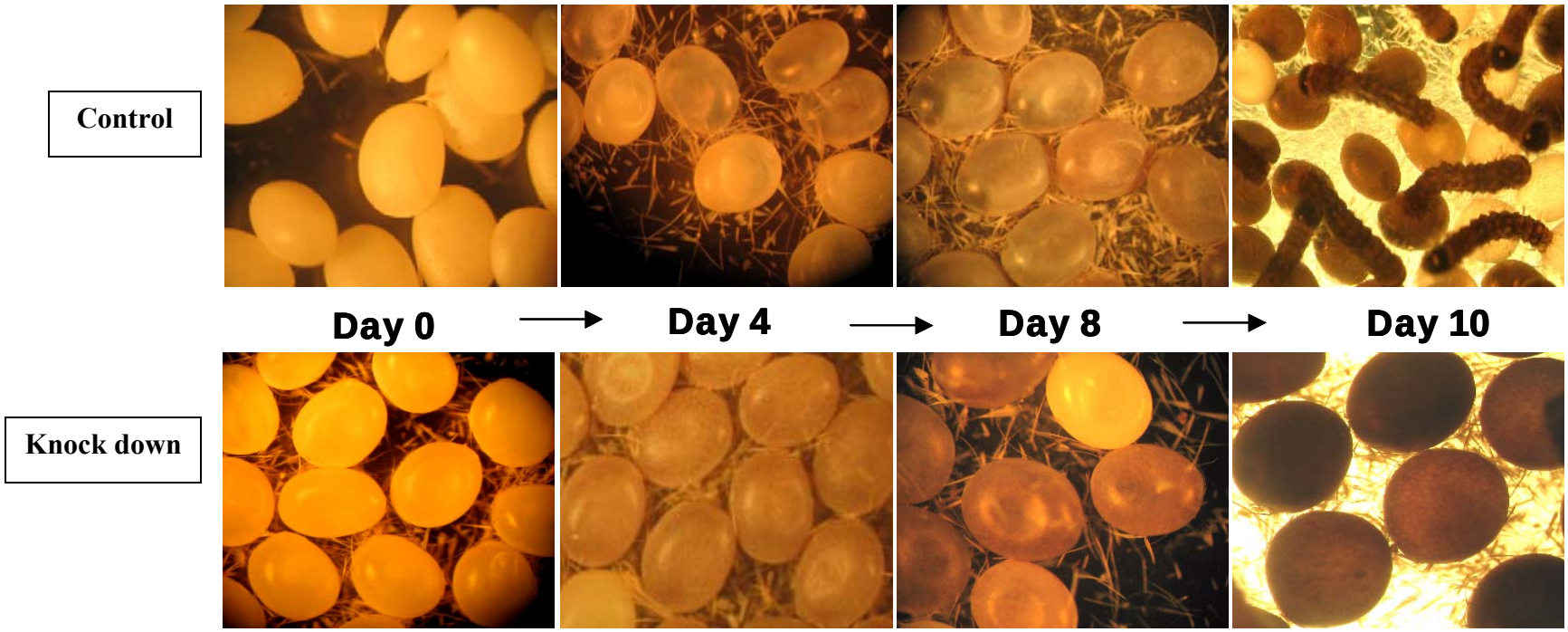


Figure21 G1 embryos of the dsRNA-treated parents abruptly stop development at Day 2 and lethality ensues as against the embryos of non-specific dsRNA-treated parents which are viable and hatch after 10 days. "Control" represents dSRNA treatment for a non-specific target which is ie-1 in this case. "Knockdown" represents drRNA treatment targeting the C-x8-C-x5-C-x3-H zinc finger motif and hence all the three genes.

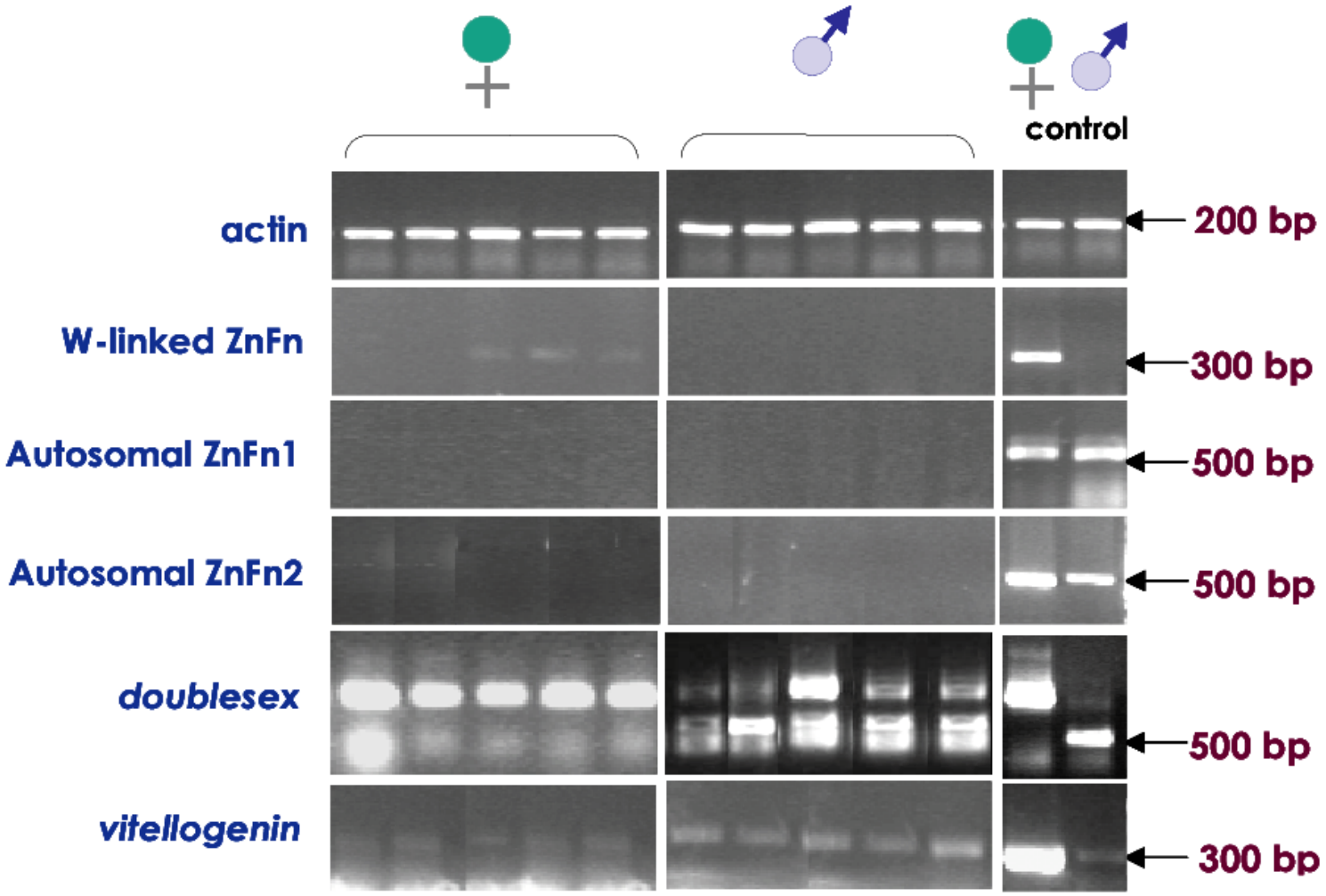


Figure22 Expression profile reveals a partial femaleness effected in males by RNAi targeting the common zinc finger domain. Upregulation of vitellogenin expression is also observed in males displaying aberrant splicing of *doublesex*.

To analyze the effect of knocking down either all three genes or only Z1 and Z2 or Z3 alone early during development, dsRNA was administered in progressively increasing concentrations orally up to the V instar stage. The knock down strategy adapted was in the above combination primarily to a) test for consistency of effects observed on knocking down all the three genes, b) because of the high sequence similarity between Z1 and Z2, c) to establish the role of Z3 beyond sex determination.

Table6: Increasing doses of double-stranded RNA from 500 ng to 50 µg were administered orally along with feed from hatched larvae stage to V Instar stage:

Dev. Stage	Untreated control	Qty of dsRNA administered *per os*	Non-specific dsRNA	Common - domain [Z1, Z2 & Z3]	W- linked Zn1 & Autosomal Z2	Autosomal Z3
Day0	50	500 ng/ 6 larvae	24	24	24	24
I instar	46	500 ng/ 6 larvae	23	18	20	19
II instar	43	2 ug/ 4 larvae	20	14	19	16
III instar	42	10 ug/ 4 larvae	20	10	16	10
IV instar	42	25 ug/ 2 larvae	18	8	16	9
V instar	42	50 ug/ larva	18 (8/10)	8 (3/5)	12 (7/5)	9 (4/5)
P4 spinning	42	--	18 (8/10)	8 (3/5)	9 (5/4)	7 (3/4)
Adult	42	--	18 (8/10)	5 (2/3)	9 (5/4)	4 (1/3)

Table6 shows that continual dsRNA administration till the V Instar stage results in a high rate of lethality among the specific dsRNA-treated individuals as observed against non-specific dsRNA-treated and untreated control larvae. The phenotypic abnormalities observed were ambiguous nevertheless significant.

1. Silencing Zn1, Zn2 & Zn3 targeting the zinc finger motif:
    - When dsRNA-treated females were mated with dsRNA-treated males, the subnormal number of offsprings produced was inviable (normal egg count~350).
    - When dsRNA-treated females were mated with normal males, the subnormal number of eggs laid was again found to be inviable.
    - Normal females were mated with dsRNA-treated males laid normal number of eggs which were inviable.
2. Silencing Zn1& Zn2 targeting the 5' UTR region common to both genes:
    - Except for when both the parents were dsRNA-treated, the eggs laid were viable irrespective of the count.
3. Silencing Zn3 targeting the unique 5' UTR:
    - Irrespective of the mating combination with respect to dsRNA treatment, the eggs laid were inviable and subnormal in count.

Though these results are obvious pointers to the nature of the zinc finger genes as essential developmentally regulated genes, they also present a strong possibility of all the zinc finger genes acting in a co-operative manner to regulate the sex determination pathway. Since, any aberration in the expression of genes involved in sex determination

leads to lethality is an established fact. Also comparison of expression profiles of the *dsx* gene- the downstream most gene in the sex determination pathway- and the sex differentiation genes regulated by *dsx* allowed the significant observation that female-specific isoforms of *dsx* appeared in males where all the 3 zinc finger genes were knocked down. In all other cases no such aberration was observed. The phenotypic aberrations and the splice pattern aberrations of the downstream double switch dsx, observed only in the individuals where all the three zinc finger genes were knocked down suggest a role for each one of them in the sex determining pathway.

Discussion

Characteristics of C-x8-C-x5-C-x3-H zinc finger genes

Zinc finger domains are relatively small protein motifs that bind one or more zinc atoms, and which usually contain multiple finger-like protrusions that make tandem contacts with their target molecule. They are now recognised to bind DNA, RNA, protein and/or lipid substrates (BROWN 2005; GAMSJAEGER *et al.* 2007; HALL 2005; KLUG 1999; MATTHEWS and SUNDE 2002). Zinc finger-containing proteins function in gene transcription, translation, mRNA trafficking, cytoskeleton organization, epithelial development, cell adhesion, protein folding, chromatin remodelling and zinc sensing, to name but a few (LAITY *et al.* 2001). Zinc-binding motifs are stable structures, and they rarely undergo conformational changes upon binding their target. The C-x8-C-x5-C-x3-H zinc finger motif has been found in proteins from organisms ranging from man to yeast (BREWER *et al.* 2004; CUTHBERTSON *et al.* 2008; HUANG *et al.* 2002; TENLEN *et al.* 2006). Proteins containing C-x8-C-x5-C-x3-H zinc finger domains include zinc finger proteins from eukaryotes involved in cell cycle or growth phase-related regulation, e.g. human TIS11B (butyrate response factor 1), a probable regulatory protein involved in regulating the response to growth factors, and the mouse TTP growth factor-inducible nuclear protein, which has the same function (HUDSON *et al.* 2004; LAI and BLACKSHEAR 2001). Another protein containing this domain is the human splicing factor U2AF 35 kD subunit, which plays a critical role in both constitutive and enhancer-dependent splicing by mediating essential protein-protein interactions and protein-RNA interactions required for 3' splice site selection (PACHECO *et al.* 2006; SOARES *et al.* 2006). It has been shown that different CCCH-type Zinc finger proteins interact with the 3'-untranslated region of

various mRNA (CARBALLO *et al.* 1998; LAI *et al.* 1999). This type of zinc finger is very often present in two copies.

C-x8-C-x5-C-x3-H zinc finger genes as regulators of sexual development

Several C-x8-C-x5-C-x3-H zinc finger proteins, including PIE-1, MEX-1, MEX-5/6 and POS-1, have been identified in genetic studies as maternal factors that are important for the differentiation of germ cells in *C. elegans* (GUEDES and PRIESS 1997; MELLO *et al.* 1996; REESE *et al.* 2000; SCHUBERT *et al.* 2000; TENENHAUS *et al.* 2001). Particularly, PIE-1 is an essential regulator of *C. elegans* germ cell fate that segregates with the germ lineage by inhibition of transcription or activation of protein expression from maternal RNAs. Three C-x8-C-x5-C-x3-H type zinc-finger genes, *moe-1*, *moe-2* and *moe-3,* are a group related by function and by nucleotide sequence (SHIMADA *et al.* 2002). Identified in *C. elegans* MOE products have redundant functions that are collectively crucial for oocyte maturation in the nematode. At the cellular level, MOE proteins play crucial roles in the regulation of P granules and in events that control the cell cycle. *Zfcth1* gene encodes a protein with two putative CCCH zinc fingers which is essential for oocyte maturation in the zebra fish, *Danio rerio* (TE KRONNIE *et al.* 1999). XC3H-4 is an ovary-specific gene with four zinc finger motifs found in *Xenopus* and there are no known orthologues (DE *et al.* 1999). *Zfp36l2* is an mRNA-binding and destabilizing protein, functions in the physiological control of female fertility at the level of early embryonic development in mouse (RAMOS *et al.* 2004). Thus C-x8-C-x5-C-x3-H type zinc-finger genes have been found to play crucial roles in sexual development across the animal

kingdom. As evident from their function as transcription factors or splicing factors, they have been recruited from other pathways into playing regulators of sexual development.

Gene Duplication and Sexual development

Genes involved in sexual development are often the targets of natural and/or sexual selection and in general originate as duplicates of autosomal genes (VOLFF *et al.* 2003; ZHOU *et al.* 2008). A fact which points to the crucial role gene duplication plays in the evolution of sex-determination systems. Such a Darwinian positive selection may lead to functional changes of the target genes during evolution. In ascomycete fungi the primary role of pheromones is the control of initial recognition between male cells and female organs of opposite mating type. The gene encoding putative pheromone precursor in *Cryphonectria parasitica Mf2*, is present in duplicates (CHOI *et al.* 2005). A duplication in the brachyceran dipteran (fly) lineage after its separation from nematoceran dipterans (mosquito), generated *Sxl* and CG3056 and *Sxl* adopted the new function of sex determiner in *Drosophila*, whereas the other, CG3056, continued to serve some or all of the yet-unknown ancestral functions (TRAUT *et al.* 2006). Hence the genomes of *D. melanogaster*, *D. erecta*, and *D. pseudoobscura* contain 2 paralogous genes, *Sxl* and CG3056, which are orthologous to the single *Sxl* gene present in *Anopheles*, *Apis*, *Bombyx*, and *Tribolium*. In the fish *Oryzias latipes*, *DMRT1* homologue is located on an autosome in a gene cluster together with its paralogues, *DMRT 2* and *DMRT 3*. The Y chromosome-specific region contains a duplicated copy of the autosomal *DMRT1* gene, named *DMRT1Y* (LUTFALLA *et al.* 2003; MATSUDA *et al.* 2003). This is the only functional gene in this chromosome segment and is expressed during male embryonic and

larval development and in the Sertoli cells of the adult testes. In the zebra fish, *Danio rerio*, the *SOX9a/SOX9b* gene pair arose by duplication of a single *SOX9* gene (KLUVER *et al.* 2005). *SOX9a* is considered to play a role in testis development, while *SOX9b* may be important for ovary development. Both proteins are known to have similar DNA-binding and transactivation properties in spite of different expression profiles. In *X. laevis*, *DM-W* is considered to have emerged on the W chromosome following the duplication of *DMRT1*, as the dominant-negative gene for the testis-determining *DMRT1* (YOSHIMOTO *et al.* 2008). No *DM-W* orthologue is identified as yet in the genome of *X. tropicalis*, which is related to *X. laevis*. Mammalian *Dmrt7* is considered to have originated by duplication and divergence of another *Dmrt* gene, which plays a critical role in a male-specific chromatin transition between pachynema and diplonema during meiotic prophase (KAWAMATA *et al.* 2007; KAWAMATA and NISHIMORI 2006). *SCML1*, an X-linked gene with preferential expression in testis, has arisen out of a recent duplication has acquired testis-specific function in primates, as compared to the ancient copies *SCML2* and *SCMH1* which have diverse functions (WU and SU 2008). As evident from these examples, gene duplication is a norm in the evolution of genes involved in early development, particularly in sexual development. The C-x8-C-x5-C-x3-H zinc finger genes of silkworm present such a scenario with the W-linked gene Z1 probably playing the key role. An interesting feature in the evolutionary history of many genes encoded by sex chromosomes in vertebrates, particularly mammals is the degeneration and loss of one functional copy among duplications, as has occurred dramatically on the mammalian X and Y chromosomes. The case of zinc finger genes is in stark contrast with that of mammals, where three functional copies are retained. All the genes are

presumably maintained in a functional form because they serve a role in mating or they function in other roles that provide a survival benefit to the organism.

A model for sex determination pathway

Lethality of G1 embryos on knock down of either all 3 zinc finger genes or only Z3 and the presence of Z1 and Z3 in the unfertilized eggs suggests a role for these genes in regulating early zygotic transcription. Also, supply of Z1 and Z3 maternally to the eggs, points probably to an early developmental role where a feedback loop regulates the expression in the G1 embryos (**Figure23a&b**). Hence, a mechanism of sex determination by maternal effect could probably be existing in *B. mori*. The phenomenon of sex determination by a maternal effect is not new and is reported earlier in dipteran species such as *Sciara coprophila* (CROUSE 1960a; CROUSE 1960b) and *Chrysomya rufifacies* (ULLERICH 1977). A perfect example of the maternally supplied transcript regulating its own expression in the G1 zygote is the *daughterless* (*da*) in *Drosophila* (CLINE 1976; CLINE 1980). The two well-described requirements for *da* function in the ovary are: in the germline for progeny sex determination (CRONMILLER and CLINE 1987) and in the somatic ovary for follicle formation (CUMMINGS and CRONMILLER 1994). In the germline *da* mRNA is produced and eventually concentrated into the oocyte for the maternal sex determination function. DA forms a heterodimer with another bHLH protein SISB to activate transcription of *sxl*. The autoregulatory function is brought about by a direct interaction of DA protein with its own regulatory sequences. Also, negative cis-acting sequences downregulate *da* transcription, thus preventing autoregulatory *da* expression from escalating to produce deleteriously high levels of DA protein. Another example of

maternal factors influencing the sex of the zygote is the sex determination system of the common housefly, *Musca domestica*. In *Musca* female development requires the continuous activity of the sex-determining gene F from early embryogenesis until metamorphosis (HILFIKER-KLEINER *et al.* 1994). To activate F in embryogenesis, two conditions must be met: There must be no male-determining M factor in the zygotic genome, and the egg must be preconditioned by F activity in the maternal germ line. The medfly *Ceratitis capitata* presents yet another model of autoregulation (PANE *et al.* 2002). It contains a gene *Cctra*, with structural and functional homology to the Drosophila melanogaster sex-determining gene transformer *tra*. In XX embryos, a maternal *Cctra* mRNA provides full-length CcTRA protein that initiates a positive feedback regulation. This protein drives a female-specific splicing of the zygotically transcribed *Cctra* pre-mRNA so that new CcTRA protein can be produced. The newly synthesized protein controls the maintenance of *Cctra* autoregulation and the female-specific splicing of *Ccdsx* pre-mRNA. . In XY embryos, *Cctra* autoregulation is impaired by the male determining M factor. The expression profile of the W-linked zinc finger Z1 in silkworm allows for hypothesizing a similar mechanism of autoregulation of its expression in the zygote. In this case, the presence of W chromosome results in the activation of Z1 gene by the maternally supplied Z1 protein and thus triggers the female-specific pathway. This results in the female-specific splicing of *dsx* and initiate female differentiation. The absence of W would leave the maternal Z1 products with no target to regulate and hence the cascade of events results in male development. Z3 could probably be a cofactor working in cooperation with Z1.

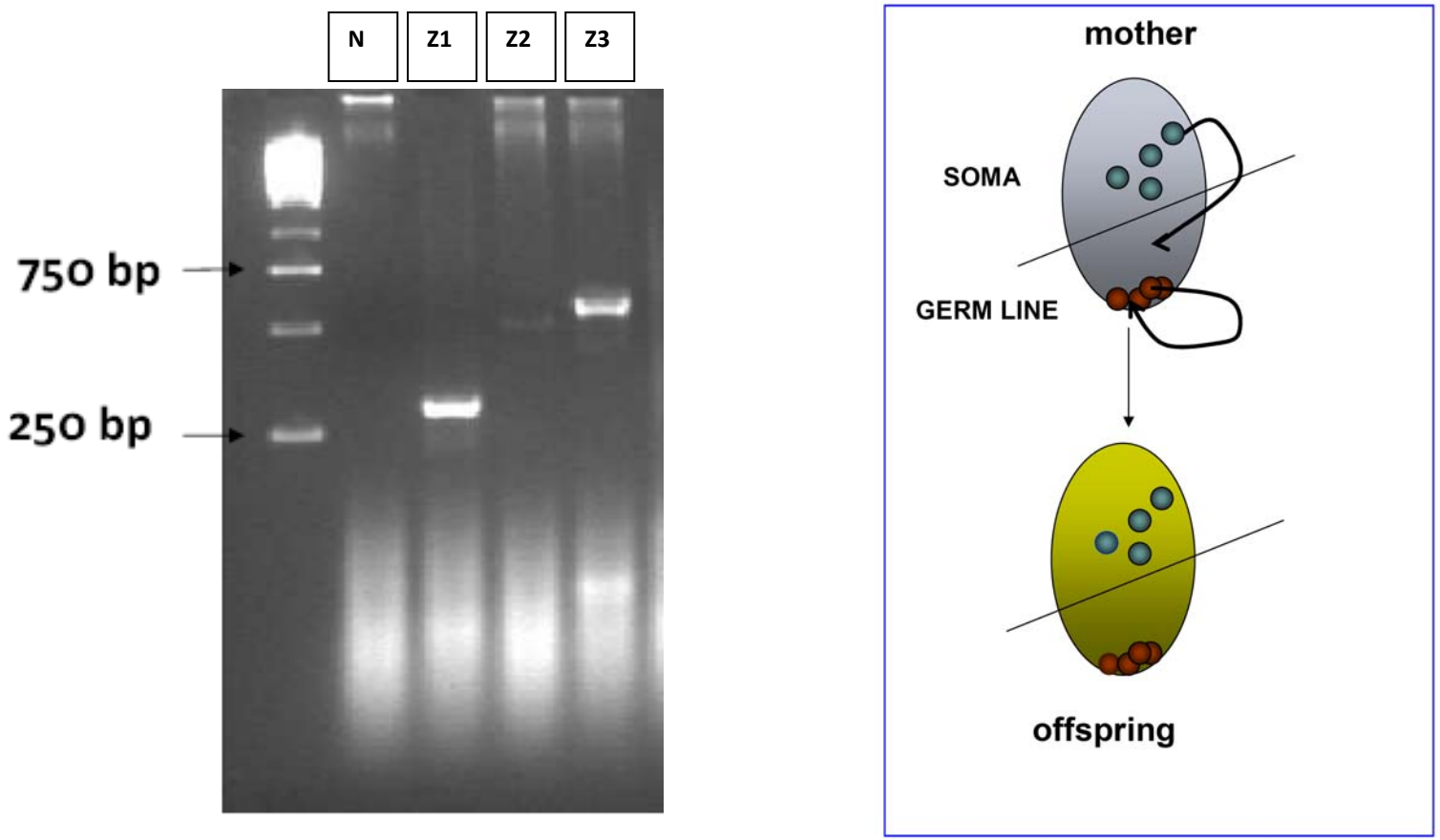


Figure23 a) Expression profile of all the three zinc finger genes in unfertilized eggs shows that Z1 and Z3 are supplied maternally. N, negative control; Z1, W-linked zinc finger transcript; Z2 & Z3, autosome-linked zinc finger transcripts. **b)** Schematic showing maternal supply of Z1 and Z3 transcripts from mother to the offspring though the gonads or transcription in the primordial germ cells or pole cells.

Ubiquitous expression of the primary signal is the hallmark of a cell autonomous sex determination system. Expression of W-linked Z1 gene is indeed ubiquitous and hence qualifies to be the primary signal. The molecular evidence of splicing alteration in males where all 3 zinc fingers were silenced brings Z2 and Z3 into picture their involvement in sexual development. Thus phenotypic and molecular (splicing) aberrations in zinc finger gene-silenced individuals, maternal supply of zinc finger transcripts to offspring, and the presence of a male-specific dsx splicing repressor allows for building a putative model for the sex determination pathway in silkworm (**Figure24**). The primary sex determining signal which triggers the cascade of gene interaction could probably be the W-linked zinc finger gene. While the autosome-linked Z2 and Z3 also constitute the pathway occupying either the immediate downstream or parallel in hierarchy to the W-linked zinc finger. Probably acting antagonistically, they could be repressing each other either at the transcriptional or translational level. The novel male-specific *dsx* splicing repressor *BmPSI*, *Bombyx* homolog of P-element somatic inhibitor (PSI), adds an additional level to the sex determination cascade at which the zinc finger genes could act. This gene may be regulated by the zinc finger genes either in co-operativity or by only one of the zinc finger genes either at the transcriptional or translational level. Another possibility is that the zinc finger genes might interact with *BmPSI* and regulate the splicing of *dsx* in a direct manner.

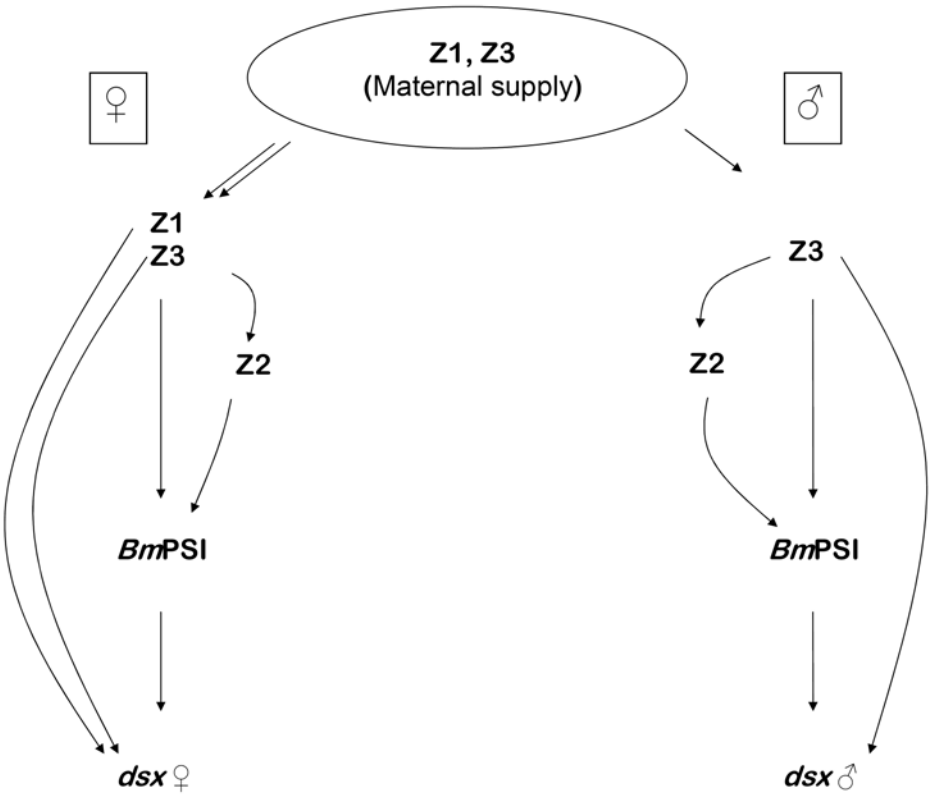


Figure24 Schematic representing a putative model for the sex determination pathway in silkworm. Maternally supplied Zn1 protein completes the autoregulatory loop by regulating its own expression which is absent in the male because of the absence of W chromosome. The other maternally supplied zinc finger gene Zn3 regulates the expression of BmPSI in cooperation with only Zn2 in male while in female the W-linked gene Zn1 is also involved. Zn2 which is expressed later in the fertilized embryo is regulated by Zn3. Alternatively, the zinc finger genes may also act directly on the downstream *dsx* by way of a positive or negative interaction with BmPSI, at the transcriptional or translational level (represented by long arrows).

Evolution of the C-x8C-x5-C-x3-H zinc finger genes

Search for orthologues of the C-x8C-x5-C-x3-H zinc finger genes in the non-redundant database including sequenced genomes, ESTs did not retrieve any. Analysis of Lepidopteran genomes – *Bombyx mandarina* (Japanese strain), *Philosamia Cynthia ricini*, *Antheraea assama*, *Antheraea pernyi*, *Antheraea proylei*, *Antheraea roylei*, *Antheraea mylitta*, *Antheraea yamamai* and *B. mori* - closely related to *Bombyx* was performed by PCR with specific primers for Z1, Z2 and Z3 yielded an interesting picture (**Figure25**).

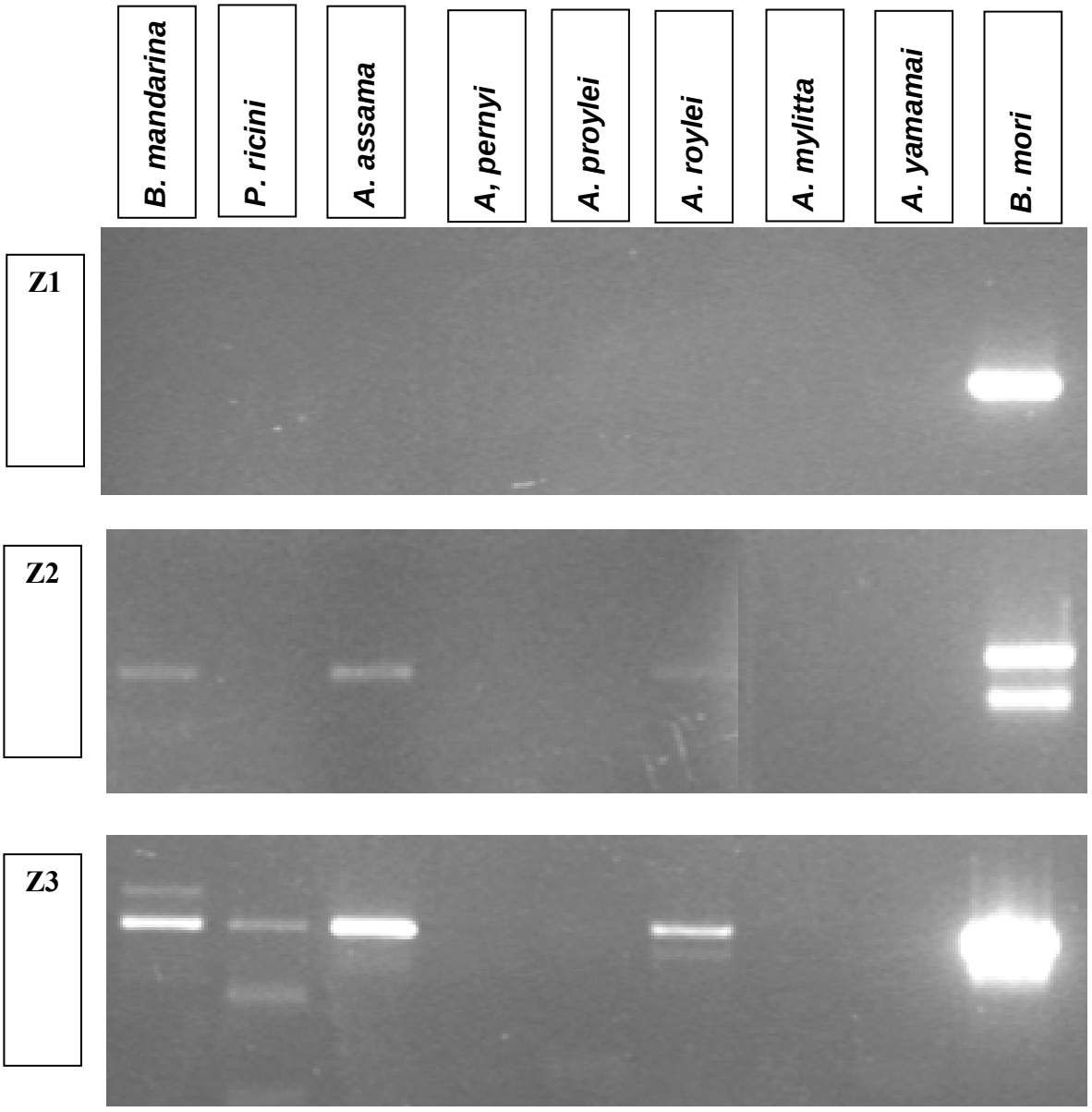


Figure25 Presence of Z1, Z2 and Z3 in the lepidopteran species closely to *B. mori* was examined. Z1 copy amplified exclusively in *B. mori* while Z2 and Z3 were found to be present in *B. mandarina* (Japanese strain), *A. assama* and *A. roylei*. Only Z3 is amplified in *Philosamia cynthia ricini*.

The W-linked gene Z1 was found to be present in *B. mori* alone while Z2 was present in *B. mandarina*, *A. assama* and *A. roylei* and Z3 was present in *B. mandarina*, *P. c. ricini*, *A. assama*, *A. roylei*, Sequence phylogeny shows Z3 to be the ancient of the three zinc finger genes and this is consistent with its presence in several of the species examined. Conservation of Z3 could be the result of the largely limited interchromosomal rearrangements as observed throughout a large section of the Lepidoptera resulting in a high degree of conserved synteny when compared to the pattern seen in the Diptera (PRINGLE *et al.* 2007). An accelerated molecular divergence of the lepidopteran W chromosome in the absence of meiotic recombination as observed among Pyralid moths and the secondary losses of the W chromosome resulting in sporadically occurring Z/ZZ sex chromosome systems in ditrysian families could be attributed for the absence of the W-linked Z1 gene in all the species examined except *B. mori* (TRAUT and MAREC 1996; VITKOVA *et al.* 2007). A related observation made was the presence of Z2 and Z3 in *A. roylei* but not in *A. proylei* and *A. pernyi* which is consistent with an earlier phylogenetic analysis revealing *A. proylei* closer to *A. pernyi* than to *A. roylei* (ARUNKUMAR *et al.* 2006). After being recruited to function as a sex determining factor, the ancestral C-x8-C-x5-C-x3-H zinc finger gene has been subjected to positive selection with further duplications fine tuning a newly evolved sex determination system.

Conclusion

As with other developmental genes, the use of alternative splicing of *dsx* in regulating insect sex differentiation is proposed to be an ancient feature present in the common ancestor of holometabolous insects (CHO *et al.* 2007). In contrast to *dsx*, which is at the bottom of the sex determination pathway, a diverse array of upstream genes and signals in the sex-determination cascade is used, a fact which is in strong support of the postulation that genetic sex-determination pathways evolve in reverse order from the final step in the hierarchy up to the first (POMIANKOWSKI *et al.* 2004; WILKINS 1995). Particularly the primary sex determination signal is found to be subject to frequent changes. Among flies, epistatic maleness and maternal effect systems exist besides *Drosophila's* X-counting system. In Lepidoptera, the primary signal has changed even under the cover of a conserved female-heterogametic sex chromosome system. A transition to a new sex determination system is thought to occur frequently when individuals carry a mutation which is selectively advantageous over those without the mutation. The complex picture presented by the silkworm sex determination pathway and also the sex chromosome system in Lepidoptera fit such a description. The C-x8-C-x5-C-x3-H zinc finger genes could thus have originated from an ancestral gene with a constitutive role early during development. As there had probably been a selective advantage of employing this DNA/RNA/protein-binding transcriptional or splice regulator in the sex determination pathway, it is enticing to propose that the zinc finger genes have carved a niche for themselves in the Lepidopteran genome evolution.

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