Chapter I

Introduction
Silkworm has been the source of silk industry since the dawn of human civilization. The earliest silk textile is nearly five thousand years old (Kuhn, 1988). In modern times, *Bombyx* has been used as a model for genetic studies since the birth of genetics as a formal Science in the early 1900's. As early as in 1905, Toyama, one of the founders of silkworm genetics, was breeding genetic hybrids between Thai and Japanese silkworms for improved vigor and silk production (Yokoyama, 1968). He first reported discovery of a chorion mutation that affects the shape and transparency of the eggshell in 1910 (Tazima, 1964), the same year as the publication of Morgan's famous white-eyed mutant of *Drosophila melanogaster*. Although studies with many lepidoptera have made important contributions to genetics, today with more than two hundred mutations mapped, the silkworm stands as the only member of this taxonomic group whose genetic system is established well enough to consider adopting it as a molecular genetic model for solving a broad range of fundamental biological problems.

Investigations to understand embryonic development of the organs like testis, ovary, liver, heart, kidney have been active areas of research using model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans* and mouse. One such area has been molecular basis of sex-determination. There are diverse sex-determining mechanisms present in different animal taxa. This diversity demands evolutionary reason for its occurrence and it is in this respect that the study of sex determination in silkworm, *Bombyx mori*, assumes significance as it has not been investigated from evolutionary and developmental point of view in as much detail as it has been studied in *Drosophila* (Slee and Bownes, 1990), the nematode (Hodgkin, 1990; Villeneune and Meyer, 1990), and the mouse (Singh and Jones, 1984; Eicher and Washburn, 1986; Gubbay *et al.*, 1990). A comparison of sex-determining mechanisms in these model systems raises important questions about convergent evolution and, the extent to which the developmental processes are evolutionarily conserved.
It is now possible to isolate specific genes controlling organ development and differentiation, study their structure in detail, identify and compare the time and site of their expression in the embryo and predict the final product. This was the approach that led to the discovery of the existence of homeotic genes (or Horn genes) in *Drosophila* (Ruddel, 1994; Carvoll, 1995). The Horn genes are known to act as master switches for organ specificity during embryogenesis in *Drosophila*. When studies were extended to other animals like frog, mouse and human, they were also found to have homeotic genes of *Drosophila* type that operated in the similar way. These studies reveal basic similarity in genetic planning of embryogenesis in diverse group of animals. This also suggests that homeotic genes are evolutionarily conserved and function in taxonomically diverse groups of animal systems (Akam, 1989; Quiring et al., 1994). Genetic mechanisms of sex determination and differentiation, therefore needs an in depth study to understand whether they too have evolved from a common genetic program.

**Sex-Determination**

Molecular tools in form of recombinant DNA techniques are being used to discover the similarity and differences in genes causing heteromorphic sex differentiation in various animals. There are predominantly, three kinds of chromosomal sex determining mechanisms. In one, males are heterogametic, like mammals, in the other females are heterogametic like the lepidopteran insects, snakes and birds (Tazima, 1964; Traut and Mosbacher, 1968; Robinson, 1971; Singh, 1972; Bull, 1983; Strunnikor, 1983; Jones and Singh, 1984) and in the third kind, males are heterogametic sex but sex is determined based on the ratio of X-chromosomes and autosomes like *Drosophila* and nematodes. There is yet another mechanism of sex-determination found in turtles, alligators and muggers, where temperature controls the development of a particular sex. For example, turtles develop into females at warm temperature and into males at cool temperature. Alligators and muggers on the other hand develop into females at cool temperatures and males at warm temperatures (Bull, 1980; Deeming and Ferguson, 1988). In the insect, *Apis mellifera*, the sex is chromosomally determined but in a slightly different way: diploid individuals
become female and haploid individuals develop into males (Bull, 1983; Hodgkin, 1992). These examples, thus, indicate operation of different sex-determining mechanisms in the development of heterogametic male and female phenotypes.

**Function and Consequences of Sex**

Sex is a morphological expression of being a male or a female and sexual structures like male and female gametes of complementary nature participate in production of a diploid embryo leading to the development and organization of a diploid male or female individual. These individuals in their subsequent sexual reproduction generate haploid gametes by meiotic cell division. During meiosis, homologous chromosome pairs recombine leading to the production of gametes with parental and recombinant genetic constitution. Thus, the function of sex and sexual reproduction constitutes a genetic mechanism for producing genetically variable gametes and, in turn, resulting in genetically variable offspring required for successful survival under varying natural conditions.

Studies on the mating behavior in birds, mammals and other animals suggests the involvement of competition over mates for sexual reproduction. The concept of sexual selection based on competition over mates suggests that both males and females enter into competition in choosing appropriate partners for the act of mating and sexual reproduction (Andersson, 1994; Petrie, 1994; Hasselquist et al., 1996). Since, it is known that the 'Y' is the male determining chromosome in mammals and W the female determining chromosome in lepidoptera, birds and snakes, selection pressure will favour localization of genes on the respective sex determining chromosomes for successful mating and reproduction (Hastings, 1994). Recent studies on genetic information located on Y-chromosome clearly shows that it contains genes for sperm production, body size and tooth development, the traits assumed to be important in male-male contest for female selection (Roldan and Gomendio, 1999). Accordingly, sexual selection might be the main driving force for sex chromosome heteromorphism in heterogametic systems.
Another most pertinent question in this context would be the revelation and understanding of the evolution of molecular mechanisms favoured by sexual selection pressure that function in the development of heteromorphic sex chromosomes.

**Sex-Determination and Dosage Compensation in Heterogametic Systems**

*Bombyx mori* exhibits chromosomal sex-determination with heterogametic females and homogametic males. The heteromorphic chromosomes are designated as W and Z. In contrast to *B. mori*, other species like *C. elegans*, *D. melanogaster*, *M. domestica* and *Homo sapiens*, which also exhibit chromosomal sex determination, have male heterogamety with heteromorphic sex chromosomes designated as the X and Y.

W-chromosome exhibits developmentally regulated partial or complete heterochromatinization depending upon the nature in Lepidopteran species. They share this property with snakes (Ray-Chaudhuri *et al.*, 1971; Singh, 1972), birds (Stefos and Arrighi, 1971) and with the Y-chromosome of different animal groups (John, 1988). It is important to mention here that Z-linked genes in ZZ/ZW are not dosage compensated (Cock, 1964; Johnson and Turner, 1979; Stevens 1997; Lucchesi, 1998). The reason for such fundamental difference in the dosage compensation system of XX/XY and ZZ/ZW system is not clear.

The standard strains of *M. domestica* are heterogametic males with dominant male determiner ‘M’ located on the Y-chromosome which functions to produce maleness by repressing the activity of female determining gene ‘F’. Accordingly, the male determiner is epistatic to female determiner in this system (Schmidt *et al.*, 1997a, 1997b).

Studies on this aspect in the three model organisms i.e., *Drosophila*, *C. elegans*, and humans have shown simultaneous operation of genetic systems controlling both, sex heteromorphism and dosage compensation. In *Drosophila*,
XX individuals are female and XY are male. Y-chromosome of the male does not function in determination of maleness but is mainly required for functional sperm production as it carries fertility genes (Sternwann-Zwicy, 1992). In C. elegans, XX individuals are female in their soma and both male and female in their germ line. 'XO' individuals remain male both in somatic cells and germ cells. In these two systems the ratio of number of the X-chromosomes to the number of sets of autosomes generates the primary signal for development of maleness or femaleness (Hodgkin, 1990). In mammalian system, the genes for maleness are located on Y. The 'XX', 'XO' and 'XY' systems of sex-determination result in inequality of dosage of genes present on the X, as there are two copies of such genes in XX individuals (female) and one copy in XY individuals (male). In spite of such differences in the dosage, the level of product of almost all X-linked genes is found equal in the two sexes. This is achieved by dosage compensation due to silencing of one of the two X-chromosomes.

There are three basic dosage compensation regulatory strategies. In mammals, inactivation of one X-chromosome in females is the mechanism for equalizing the products of X-linked genes in female individuals (Lyon, 1974). In D. melanogaster and C. elegans, X:A ratio is responsible for dosage compensation. The Sxl is the master control gene, involved in determination of sexual phenotype and dosage compensation in Drosophila while Xol1 gene in association with Sdc1 and Sdc2 genes determine sexual phenotype and dosage compensation in C. elegans (Villeneuve and Meyer, 1987; Miller et al., 1988; Nusbaum and Meyer, 1989).

**Mechanisms of Heteromorphic Sex Chromosome Evolution**

First step in chromosomal sex determination and evolution of heteromorphic sex chromosome probably involved acquisition of male (M) and female (F) determining gene(s) at the sex specific locus of the otherwise homomorphic sex chromosome pair. In other words, heteromorphic chromosomes evolved from a pair of homomorphic chromosomes with allelic difference of a single gene at the sex-determining locus (Ohno, 1967; Bull, 1983; Jablonka and
Lamb, 1990). This contrasts with the current status of the mammalian sex determination genetics in which genes causing maleness on Y and femaleness on 'X' are not the alleles of a single gene (Jimnez and Burgos, 1998). Heteromorphic changes in the organization of homologous chromosomes around the sex-determining locus in heterogametic sex is proposed to have been the primary cause of their non-homology in the sex-determining region. The development of such sex-locus-specific non-homology in otherwise homologous sex-chromosome pair prevents genetic recombination between their non-homologous regions. Thus, acquisition of sex determining function and suppression of recombination in the sex determining non-homologous region are the two significant biological reasons for the sex chromosomes to undergo differentiation during evolution (Bull, 1983; Charlesworth et al., 1986; Jablonka and Lamb, 1988; Steinemann 1993), either by Mullers ratchet, sex specific acquisition of Bkm sequences (Singh et al., 1976) or by genetic hitchhiking (Rice, 1987).

Two basic evolutionary pathways termed conformational and structural have been proposed to be the cause of origin and development of sex chromosome heteromorphism. According to conformational pathway, a change in chromatin conformation in the region of heterogametic loci is assumed to be the initial evolutionary reason for sex chromosome heteromorphism. The structural pathway proposes that structural change such as an inversion or a translocation involving originally homomorphic sex chromosomes was the initial event in sex chromosome heteromorphism (Haaf and Schmid, 1989). Singh et al., have proposed an alternative model for rapid evolution of heteromorphic sex chromosomes in snakes, which entails the involvement of a transposon like element to be the primary cause of conformational heteromorphism leading to isolation and degeneration of heteromorphic sex chromosomes (Singh et al., 1976).

Developments in molecular methodologies have provided impeccable molecular tools to study the physical organization of genes and a class of repetitive sequences called satellite DNA in order to understand the role of these sequences in regulating the structural and functional integrity of the
genes. During the past 25 years, Singh and his group have carried out extensive study in a variety of animal system using Bkm satellite DNA as a probe to find out the role of such elements in sex determination in heterogametic animals. Cumulative evidence resulting from these studies clearly indicates a definite and decisive role for this satellite DNA in assigning heteromorphic status to W and the Y- chromosomes.

**Clues from the Silkworm**

Silkworm shows female heterogamety i.e., ZZ/ZW system. The main aim of present study was to find out whether Bkm like sequences have played any role in the origin and evolution of W chromosome in the silkworm. For this purpose, Bkm 2(8), a DNA marker developed and characterized as a reliable genetic probe by Singh and his group (1995), was used to screen the silkworm genome to identify, isolate and characterize the related sequences on its W-chromosome. This was decided in the light of following findings:

1. Bkm sequences are highly conserved (Singh et al., 1981; Singh and Jones, 1982).

2. Bkm is preferentially associated with the sex chromosomes of Drosophila (Singh et al., 1981) and Snakes (Singh et al., 1979, 1980, 1981; Singh and Jones, 1982; Jones and Singh, 1984).

3. Bkm is predominantly associated with the Sxr region of the mouse Y-chromosome, which is necessary and sufficient to convert a female into a male mouse (Epplen et al., 1982; Singh and Jones, 1982; Singh et al., 1984, 1994).

4. Bkm sequences (GATA)_n are predominantly located along the length of the snake W-chromosome which remains highly condensed in all somatic cells but undergoes extensive decondensation in the germ cells in response to sex and tissue specific Bkm-binding proteins (BBP).
Though the highly conserved component of Bkm sequences is a tetra nucleotide repeat GATA, no other simple repeat is as consistently associated with the sex chromosome as are the Bkm repeats. Therefore, it was considered appropriate to screen silkworm genomic library with Bkm for isolating sex-specific (or W-chromosome specific) Bkm sequences in this system. This molecular approach demonstrated genomic distribution of (GATA)$_n$ with no preferential localisation on autosomes or on sex-chromosomes in B. mori. In an alternative approach, we also performed experiments to identify novel sex chromosome specific genes whose expression was restricted to the gonads.

There has been no study to identify and localize sex specific genes on sex chromosomes of B. mori or any other lepidopteran insect. The reasons could be many, but one of them appears to be the failure of mutational approach through classical genetic methods. Modern DNA techniques, however, allow alternative approaches to this problem, and one of these would be to use sex specific heterologous gene probes from other organisms like Drosophila, mouse and human and identify their counterparts in the silkworm genome.

Singh and his group have also developed a testis specific probe, $P_{\phi 2}$, from human testis cDNA library. This gene is highly conserved and is predominantly expressed in the testis. It is localized on the X-chromosome region Xp11.23. In this study, $P_{\phi 2}$ has also been utilized to isolate its homologue in the silkworm and characterize the gene. Present study describes identification, isolation, and molecular characterization of a gene, which is Z-chromosome specific in silkworm and may be involved in the complex pathway of sex-determination, differentiation and spermatogenesis.