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Ph.D. Thesis

Molecular cytogenetic analysis of the W sex chromosome in Lepidoptera

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Annotation

An implementation of transgenesis for the development of genetic sexing strains in lepidopteran pest species has been suggested in order to facilitate the field application of the Sterile Insect Technique (SIT). In this thesis, the molecular cytogenetic techniques necessary for the accomplishment of the transgenic sexing strains construction in Lepidoptera are put forward. Furthermore, the first genomic survey of a lepidopteran W chromosome using a blend of cytogenetics and molecular biology is presented here. With my co-authors, we developed a straightforward method to isolate W chromosome-specific DNA sequences in the codling moth, *Cydia pomonella*. We utilized a novel approach, which took advantage of the presence of a female specific sex chromatin consisting solely of the W chromosome, and dissected it from polyploid nuclei by the means of laser microdissection. We developed W painting probes and created a W chromosome-specific plasmid library.

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Declaration

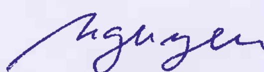
I thereby declare that I prepared this thesis on my own, only with use of relevant references

České Budějovice, 21. 9. 2007 Iva Fuková

Co-authors' report of the contribution of Iva Fuková to the following publications:

Publication no. 1
Fuková I., Nguyen P., Marec F. (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. *Genome* **48,** 1083-1092.

We hereby declare that Iva Fuková contributed to this study to a great extent. She did most experimental works (i.e. complete cytogenetic analysis of the codling moth karyotype using various techniques and molecular differentiation of the W-Z sex chromosomes using GISH and CGH) except location of ribosomal gene cluster, which was done by Petr Nguyen. She also prepared all figures and together with F. Marec prepared the first version of the manuscript.


Petr Nguyen

František Marec

Publication no. 2
Mediouni J., Fuková I., Frydrychová R., Dhouibi M.H., Marec F. (2004) Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). *Caryologia* **57,** 184-194.

We hereby declare that Iva Fuková contributed to this study significantly by successful differentiation of carob moth sex chromosomes using GISH and CGH. Her results further provided basic information about molecular composition of the W chromosome in this species.

Jouda Mediouni

Radmila Frydrychová-Čapková

František Marec

M. H. Dhouibi

Publication no. 3

Fuková I., Traut W., Vítkova M., Nguyen P., Kubíčková S., Marec F. (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. *Chromosoma* **116,** 135-145. Epub 2006.

We declare that Iva Fuková had a major contribution to this study. She did herself or participated in all experimental procedures and data analyses including laser microdissection and DOP-PCR amplification, development and testing of the W-chromosome painting probe, DNA isolations, cloning and sequence analysis of W-chromosome DNA fragments, Southern hybridization analysis of cloned W-chromosome fragments, except sequencing of W-chromosome library that was done in the lab of W. Traut (Lübeck, Germany). She also prepared all tables and figures and together with W. Traut and F. Marec prepared the first version of the manuscript.

Walther Traut | Magda Vítková | Petr Nguyen

Svatava Kubíčková | František Marec

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Marec F., Neven L.G., Fuková I. (2007) Developing transgenic sexing strains for the release of non-transgenic sterile male codling moths *Cydia pomonella*. *In*: Vreysen MJB, Robinson AS, Hendrichs J (eds.) *Area-Wide Control of Insect Pests: From Research to Field Implementation. Springer, Dordrecht, The Netherlands., pp. 103-111* (*in press*).

We declare that Iva Fuková contributed to this study by detail knowledge of the codling moth cytogenetics and particularly the sex chromosomes. She also participated in preparation of molecular tools for the codling moth transgenesis in the lab of Lisa G. Neven (USDA-ARS, Wapato, WA, USA).

František Marec | Lisa G. Neven

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Introduction

An overview of the state of art in molecular cytogenetics

Cytogenetics in model species

One discipline which has shown its utility in "-omic model species" (e.g. humans and *Drosophila*) is cytogenetics. Usage of molecular cytogenetics in medical applications enabled great technological improvements and brought about a number of new techniques in the field. Especially the introduction of non-radioactive labeling methods for **fluorescent *in situ* hybridization** (FISH) allowed both the biological and medical research to probe questions previously left untouched. For example, the detection of homologous chromosomal segments in mammals (Müller et al. 2003), physical mapping of human disease-related genes (Laan et al. 1996) and detection of chromosomal rearrangements (Tyson et al. 2005).

The fluorescent *in situ* hybridization is a widely used method for the detection and localization of specific DNA sequences on chromosome or interphase preparations. Probes derived from various repetitive sequences, genes, single chromosomes or even whole genomes are denatured and hybridized to chromosomes immobilized on a slide. The probe hybridization pattern detection is either direct due to the fluorescent label or indirect by secondary detection and signal amplification of the primary label (e.g. biotin) (reviewed in Joos et al. 1994). The use of FISH is the basis of molecular cytogenetics and it has appropriated a number of variants depending on the conditions, substrate and probes used. A technique developed to accurately assess the size and orientation of DNA probes and to map probes relative to one another is **fiber-FISH** (Heiskanen et al. 1994). This entails using a labeled probe on a DNA substrate that is released from chromatin and is stretched on a glass slide. Fiber-FISH has been used to visualize a probe as small as an Expressed Sequence Tag (EST; ca. 500 ~ 900 bp) (Horelli-Kuitunen et al. 1999). Usually, two probes are used to eliminate false positives; this can be two ESTs in close proximity to each other in the genome or an EST and a Bacterial Artificial Chromosome (BAC) clone containing the EST sequence. The substrate for the probe hybridization needs not to be the whole genomic DNA but it can be individual clones such as BACs or YACs (Yeast Artificial Chromosome) allowing thus precise physical mapping of two sequences present on the BAC (reviewed in Weier 2001).

The probe can also be of any length as it is possible to detect the signal and distinguish it from false positives. In fiber-FISH, the resolution can be as high as 1,000 bp (i.e. two sequences can be distinguished if they are more than 2,000 bases apart on the substrate). In a standard FISH the resolution is in the order of mega bases. In addition, the size of a single-copy probe must be in an

order of magnitude larger than the 100 bp used in fiber-FISH (Heiskanen et al. 1996). A variant of the standard FISH technique used with normal, non-stretched substrates is called **BAC-FISH**. There the probe is a BAC clone, which is hybridized on chromosomes in order to determine its location. Multiple BACs can be labeled with dyes with different emission spectra (**multicolor FISH**) and thus determine their relative position on the chromosomes (Sahara et al. 2007).

The sensitivity of FISH can be further enhanced using specific techniques. Mainly in plant research the **primed *in situ* labeling** (PRINS; e.g. Kubaláková et al. 1997) has been utilized for physical mapping of repetitive sequences. The principle of the method is to perform polymerase chain reaction (PCR) with the target DNA directly on a chromosome preparation using fluorescently labeled nucleotides. An alternative method is **tyramide-FISH** based on the signal amplification by enzymatically deposited biotinylated or fluorescently labeled tyramide molecules. This technique has allowed the detection of single-copy sequences bellow 1 kb in size (Schriml et al. 1999).

In order to identify the individual chromosomes, a number of techniques are routinely employed. The simplest one is characterization according to size and shape of each chromosome. More sophisticated techniques involve the use of various dyes or chromosome treatments. A banding pattern is visible in human metaphase chromosomes using Giemsa staining following digestion with trypsin (i.e. G-banding). Other staining techniques using fluorescent dyes such as DAPI or chromomycin allow chromosomes to be distinguished according to a banding pattern or relative intensity of staining. For example, DAPI has a higher affinity for AT base pairs which allows for heterochromatic regions to be readily distinguished. Also special chromosomes can supply higher resolution. A classical example is *Drosophila* polytene chromosomes. More than three thousand bands can be distinguished on these chromosomes following a simple staining procedure (Sorsa 1988). The polytene chromosomes are, however, limited to several groups of animals and plants (Diptera, Collembola, some Ciliata and few *Phaseolus* and *Vigna* species) (Stalker 1954; Ammermann 1969; Nagl 1969; Cassagnau 1974; Guerra & Carvalheira 1994). FISH can also be used to generate chromosome patterns or landmarks. For example, the differential distribution of probes prepared from repetitive sequences successfully distinguished chromosomes in several plant species (e.g. Ellneskog-Staam et al. 2006; Lan et al. 2006). The BAC-FISH technique mentioned above can also be used to create landmarks for each chromosome.

A field of cytogenetics, which has been flourishing recently, is that of comparative cytogenetics. This begun with the comparison of karyotypes from related species within a genus or a family and has now extended in molecular cytogenetics. The **comparative chromosome painting** (often called **Zoo-FISH**) involves the use of a probe originating from a different species than the substrate material (Scherthan et al. 1994). In most studies, the whole chromosome painting probes utilized are derived from fluorescently labeled flow-sorted or microdissected chromosomes (Telenius

et al. 1992; Guan et al. 1994). As mentioned before, Müller et al. (2003) used the Zoo-FISH with painting probes generated from flow-sorted gibbon and human chromosomes to resolve the conflicting phylogeny in gibbons, Hylobatidae. Two related methods of molecular cytogenetics, **comparative genomic hybridization** (CGH) and **genomic *in situ* hybridization** (GISH), proved to be very powerful techniques for the detection of chromosome duplications and deletions in cancer research (reviewed in Forozan et al. 1997), for identification of inter-species similarities in mammals (Houseal et al. 1995; Toder et al. 1998) and investigations of plant hybrids (Bennett et al. 1992; Schwarzacher et al. 1992).

Cytogenetics in Lepidoptera

Pre-2002

Unfortunately, many cytogenetic methods adopted in humans and plants were unavailable in Lepidoptera. The reasons were related with the biology of Lepidoptera as well as the limited financial support when compared with human biology research. Lepidopteran karyotypes differ substantially from the mammalian karyotypes due to the holokinetic nature of lepidopteran chromosomes. Before the start of this Ph.D., there was no banding or other technique to identify specific lepidopteran chromosomes. Generally, the use of conventional chromosome banding techniques is limited in insects, especially in those orders possessing holokinetic chromosomes such as Lepidoptera and Heteroptera. Furthermore, the lepidopteran chromosomes are numerous (the modal haploid number for Lepidoptera is 31 with some species reaching hundreds; Robinson 1971) and small, about a tenth of those of humans.

Researchers have been working to overcome these limitations. One technique involves using pachytene chromosomes, in which the homologous chromosomes are paired and therefore the number of elements present in nucleus is haploid. In addition, pachytene bivalents are much longer then metaphase chromosomes and thus provide a better resolution. The characteristic chromomere pattern of pachytene chromosomes was utilized for identification of six chromosome pairs out of 28 in the silkworm, *Bombyx mori* (pachytene mapping) (Traut 1976). But this method is not universal and is insufficient for many applications.

Additional cytogenetic landmarks on lepidopteran chromosomes were obtained by the FISH technique with repetitive DNA probes. For example, the telomeric sequences were detected in *B. mori* and several other lepidopteran species (Okazaki et al. 1993; Sahara et al. 1999). In all species examined, telomeres contained the $(TTAGG)_n$ motif which was proposed to be an ancestral insect telomeric sequence as it was widely spread among most insect orders and occurred also in other

arthropods (Sahara et al. 1999). Later the motif was shown to be ancestral not only for insects but for all arthropods (Vítková et al. 2005).

In sex chromosome research, the technique of CGH was adopted to identify the sex chromosomes in lepidopteran model species, *B. mori*, the flour moth, *Ephestia kuehniella*, and the wax moth, *Galleria mellonella* (Traut et al. 1999). The *B. mori* wild-type W sex chromosome was identified for the first time by this method. In a previous study of Kawamura and Niino (1991) the sex chromosome pair was distinguished only in a *B. mori* strain bearing a specific autosome-W chromosome translocation.

Post-2002

In the last few years since this Ph.D. project started, Lepidoptera Cytogenetics has seen some important breakthroughs which are outlined here to allow the readers familiarize themselves with the current research paradigm.

A novel blend of advanced molecular cytogenetic methods and genomics represented by BAC-FISH provided a robust approach for karyotyping (Sahara et al. 2003b; Yoshido et al. 2005a), synteny research and physical mapping (Yasukochi et al. 2006). In *B. mori*, all chromosomes were identified using this method and gross conservation of gene order has been shown between *B. mori* and *Heliconius melpomene*. Most recently, the conserved synteny was also identified between the *B. mori* chromosome 15 and the corresponding chromosome of *Manduca sexta* (Sahara et al. 2007). The chromosome identification by BAC-FISH is, however, currently possible only in the silkworm, *B. mori*, the genomic model species for Lepidoptera. Even though the number of lepidopteran species in which BAC libraries are available is increasing rapidly, these are rarely in the public domain. Moreover, the utilization of BAC-FISH for chromosome identification is neither straightforward nor easy. Very labor-, money- and time-consuming chromosome-specific markers development followed by chromosome-specific BAC clone selection procedure is necessary. More importantly, *B. mori* BACs are highly species-specific and have not hybridized successfully in any species outside the *Bombyx* genus in which the experiment was attempted, requiring the creation and use of species-specific BAC libraries (K. Sahara & F. Marec, pers. comm.).

An interesting question on the role of interstitial telomeric repeats in lepidopteran karyotype evolution was addressed by Rego and Marec (2003) utilizing the tyramide-FISH method. The reduced karyotype of the vapourer moth, *Orgyia antiqua*, consisting of 14 pairs of chromosomes, probably arose by multiple fusions of original chromosomes. The authors supposed that the occurrence of the interstitial telomeric repeats in *O. antiqua* chromosomes represented putative ancestral chromosome breaks, which supported the hypothesis on the *O. antiqua* karyotype evolution. In another model

species examined in the study, *E. kuehniella* mutant bearing a radiation-induced translocation, no interstitial telomeric sequences were detected in the fused chromosome. This suggested that the telomeres were lost before or during the two original chromosomes fused (Traut et al. 1986).

Sex chromosomes

Research on model species

Of special interest in evolution is the study of sex chromosomes, a pair of chromosomes which exhibit differences in the two sexes. The vast majority of higher organisms reproduce sexually but mechanisms of sex determination (i.e. the mechanisms which makes an individual develop as a male or a female) are surprisingly variable. Gender can be determined genetically or be induced by environmental factors. Many animals including vertebrates and insects (and a small number of plants) rely on sex chromosomes as gender determinants. In many taxa (including mammals and Diptera) the male is the heterogametic sex, i.e. the sex which carries two types of sex chromosomes: the X and the Y. Females are the homogametic sex and therefore carry two copies of the X chromosome. In a number of other taxa (e.g. birds, snakes, moths and butterflies) the heterogametic sex is the female and the sex chromosome system is denoted as WZ and ZZ in order to distinguish it from male heterogamety and also to make the point that the Z and the X are not necessarily homologous (Ellegren 2000; Jones & Singh 1985). At times, the number of X and/or Y chromosomes can be more than one (i.e. multiple sex chromosomes as in platypus, *Ornithorhynchus anatinus*; Grützner et al. 2004).

It is hypothesized that the sex chromosomes evolved repeatedly and independently in various taxa from a regular pair of autosomes (Charlesworth 1991; Rice 1996). After one of the homologous chromosomes adopted a dominant sex-determining gene (e.g. the SRY gene on the mammalian Y), recombination was restricted in that region in order to preserve the mutation. Subsequently, the region begun to differentiate due to random drift, further reinforcing the cessation of recombination. One effect of this lack of recombination is that deleterious mutations begun to accumulate including a proliferation of repetitive sequences via transposition. As a result, all non-essential genes were gradually lost from the Y chromosome. The X chromosome, on the other hand, retained its genetic content due to the ongoing recombination in the homogametic sex. The question if this decreased recombination rate has driven a faster rate of evolution on the X is hotly debated. According to Graves (1995), the mammalian sex chromosomes also received genetic material via fusion with parts of autosomes, thus forming neo-X and neo-Y chromosomes. Therefore, new recombining regions were formed which subsequently underwent through a new non-recombining phase as described above. This cycle of addition-attrition was repeated multiple times in mammals (e.g. Skaletsky et al. 2003). In

has long been recognized that the Y degeneration may ultimately lead to its complete loss and hence a X0/XX system (e.g. Orthoptera such as the locust and other grasshoppers; Huxley 1942). In such systems, the actual constitution of the sex chromosome system may be volatile and chromosomal species/races have been found (e.g. in Orthoptera: *Vandiemenalla* sp, Barton et al. 2007; *Podisma sapporensis*, Bugrov 1995).

Most our current knowledge on sex chromosome evolution is derived from the studies on *Drosophila* and mammals. Both *Drosophila* and mammalian sex chromosomes represent an ancient and highly differentiated sex chromosome pair (Carvalho 2002; Graves 2006). The sex determination mechanism in the two is, however, completely different. The dominant male-determining gene located on the Y chromosome (*SRY*) determines the sex in mammals (Sinclair et al. 1990). In *Drosophila*, sex is determined by the ratio between the number of X chromosomes and sets of autosomes. Individuals with a ratio equal to one develop as females and individuals with a ratio of 0.5 are males. The *Drosophila* Y chromosome does contain genes controlling male fertility but is not required for primary sex determination (reviewed in Carvalho 2002).

There are only few plant species with heteromorphic sex chromosomes described including the white campion, *Silene latifolia*, and the common sorrel, *Rumex acetosa*. *Silene* possesses large well-differentiated sex chromosomes. The Y chromosome acts like a dominant male flower determinant in a similar fashion as the mammalian Y chromosome. Sex determination in *R. acetosa* resembles the *Drosophila* X:A system with multiple sex chromosomes necessary for the male flower fertility (reviewed in Vyskot & Hobza 2004). Overall, there is evidence that plant sex chromosomes are rather young compared to those of *Drosophila* or human. The *Silene* Y chromosome is morphologically distinguished from the X but is still euchromatic and shares number of genes with its partner. Homomorphic sex chromosomes can also be found, for example, in papaya, *Carica papaya*. In this species the non-recombining male specific region on the Y chromosome spans only 4-5 Mb (Liu et al. 2004).

Extremely variable sex determining mechanisms ranging from environmental or social induction to hermaphroditism and gonochorism with male heterogamety (XY/XX) or female heterogamety (WZ/ZZ) occur in fish (reviewed in Devlin & Nagahama 2002). The sex determination varies even within the same species (e.g. different populations of *Xiphophorus maculatus* possess either XY/XX or WZ/ZZ system; Kallman 1968). The reason for such enormous plasticity in sex determination is not known. In medaka, *Oryzias latipes*, a teleost fish, Matsuda et al. (2002) sequenced ca. 422 kb of a shared region between the X and the Y. Of the genes identified in the region, they found that only three genes were expressed in embryos but only one gene, *DMY*, was expressed exclusively in XY individuals and was only present on the Y.

In platypus, a chain of sex chromosomes has been identified. It consists of five X and five Y chromosomes. The chain arose probably by sequential reciprocal translocation events involving the original sex chromosome pair and four pairs of autosomes, which thus became neo-X and neo-Y chromosomes (Grützner et al. 2004). The platypus X_1 chromosome shows a homology to the human X chromosome and the X_5 chromosome on the other end of the sex chromosome chain shares the candidate sex-determining gene *DMRT-1* with the chicken Z chromosome. This suggested a possible mechanism for the mammalian XY system evolution from ancestral reptile WZ system (Ezaz et al. 2006; Gruetzner et al. 2006) but the exact sex determination mechanism remains unknown.

Research on Lepidoptera

The lepidopteran sex chromosome constitution is of the WZ/ZZ type with heterogametic females. This chromosome sex determination system is rare among insects occurring only in Lepidoptera and their sister order Trichoptera (caddis flies; Marec & Novák 1998). It was suggested that the ancestral sex chromosome system in Lepidoptera is Z0/ZZ as it occurs in caddis flies and basal clades of Lepidoptera (Traut & Marec 1997; Lukhtanov 2000). It was also assumed that the W chromosome arose in a common ancestor of Tischeriina and Ditrysia, the clade comprising more than 98% of the extant lepidopteran species (Traut 1999). There is, however, no suggestion how the Z may have evolved without a partner from an ancestral autosome only system.

The level of sex chromosome differentiation in Lepidoptera ranges from homomorphic sex chromosomes to distinguishable heteromorphic chromosomes differing largely in their size. The variation in size of lepidopteran sex chromosomes is probably much higher than it was previously thought. Recently, Yoshido et al. (2005b) showed a large variability of sex chromosome constitution between geographical populations and related species in genera *Samia* and *Orgyia*. Examples of the extreme W chromosome degeneration leading to its loss and thus to a secondary Z0/ZZ sex chromosome constitution are given in Traut & Marec (1997). The relative simplicity of the W chromosome losses lead to the hypothesis that the sex determination system could be similar to the X:A balance system in *Drosophila* with male promoting factors on the Z chromosome and counteracting female promoting factors on autosomes or in the cytoplasm (Traut & Marec 1996). Suzuki et al. (1998; 1999) surveyed for dosage compensation on known Z-linked genes of *B. mori* and found no dosage compensation, which is in agreement with the hypothesis. Another study undertaken by Johnson and Turner (1979) also showed no dosage compensation in two *Heliconius* species. The three genes in these studies is, however, an extremely low number and cannot rule out an alternative hypothesis. In *Helicoverpa armigera* a Z-linked endosulphan resistance has been hypothesized to be dosage compensated (Daly & Fisk 1998). Furthermore, the W chromosome research in the main lepidopteran model species, *B. mori*, implies a strong female determining role of the W chromosome

(Tazima 1964) and the putative feminization factor (*Fem*) is proposed to be located on the *B. mori* W (Tazima 1944).

Several traits have been proposed to be W-linked in lepidopteran species, e.g. dark wing color in female color morphs in *Papilio glaucus* (Scriber et al. 1996), egg size in *B. mori* (Kawamura 1988) and male-killing factor in *E. kuehniella* (Marec et al. 2001). The attempt to isolate and map any W-linked genes is complicated. The high content of repetitive elements in the W chromosome makes assembly of Whole Genome Shotgun sequencing (WGS) of the chromosome infeasible (Abe et al. 2005). Extensive effort to isolate the *Fem* locus was not successful until recently. In 2007, two research groups independently isolated candidate sex-determining genes coding zinc-finger proteins in *B. mori* (Ajimura et al. 2007; Satish et al. 2007). Their involvement in sex determination pathway still needs to be confirmed. Only one W-linked gene beside the *B. mori* putative sex determining factors was isolated so far. In *Antheraea pernyi*, the W chromosome contains multiple copies of the *period* gene (*perW*), while one copy is located on the Z chromosome (*perZ*) (Gotter et al. 1999).

In *E. kuehniella*, the W chromosome is maintained in a heterochromatic state except during meiosis in oocytes (Traut 1975). In female interphase nuclei, it forms the W-body, a dense heterochromatic structure easily discernible in polyploid nuclei. The W-body was found in more than 80% of more than 230 lepidopteran species investigated (Traut & Marec 1996). The W-body appearance reflects the W chromosome structure, e.g. the W chromosome rearrangements in radiation-induced translocations involving the W chromosome and autosomes or the Z chromosome lead to a disruption of the W-body or change in its shape due to gene expression from the translocated euchromatic part (Marec & Traut 1994; Makee & Tafesh 2006). By ultrastructural autoradiography Guelin (1994) showed that the heterochromatic W chromosome is transcriptionally active during previtellogenesis in *E. kuehniella* nurse cells. This finding supports the retention of active genes in the heterochromatic and highly differentiated W chromosome in *E. kuehniella*. Guelin further speculates about the nature of these genes but her speculations have not been tested so far.

The molecular differentiation of the sex chromosomes has been studied in detail by CGH in a few lepidopteran species only (Traut et al. 1999; Sahara et al. 2003a). The surprising result, displaying the female-specific W sex chromosome highlighted by a male-derived probe, led Traut et al. (1999) suggest a hypothesis on the W chromosome content. According to the hypothesis the lepidopteran W chromosome accumulated female-specific and common interspersed repetitive elements during its degeneration. The representation of these repeats differed, however, in the three species examined in the study. *G. mellonella*, *E. kuehniella* and *B. mori* showed a decreasing proportion of female-specific sequences as the *B. mori* W almost entirely lacked this type of repeats.

Conclusion

Sex chromosome research in Lepidoptera is highly active and an integration of a number of different approaches promises to solve some of the mysteries of sex determination. Indeed, molecular cytogenetics is a very powerful method. With the advent of lepidopteran genomics in a diversity of species and advances in human molecular cytogenetics and molecular biology, however, lepidopteran cytogenetics needs to keep up by utilizing and developing novel methodologies. Only then will we be able to test the various hypotheses put forward for the evolution of sex chromosomes and sex determination.

Outline of research

In order to facilitate research on Lepidoptera sex chromosomes using a cytogenetic approach or general cytogenetic studies, new technologies have to be imported or developed. This was the primary aim of my thesis with two foci: to survey the molecular content of the W chromosome and to show the utility of molecular cytogenetics and molecular biology for enhancing pest management practices.

In chapter one, I present the basic karyotype of the carob moth, *Ectyomyelois ceratoniae*, which served as my training ground in Lepidoptera cytogenetics. The carob moth, also known as the date moth, is a devastating pest of dates in the South Mediterranean and the Middle East. In Tunisia, the carob moth causes staggering economic losses with infestation rates as high as 90% in pomegranates, especially in the south of the country, 75% in pistachios, and 20% in dates (Mediouni & Dhouibi 2007). In this paper, my co-authors and I present a reliable technique for identifying the sex chromosomes of this species. The carob moth, possibly like many other Lepidoptera species, does not have a morphologically distinct sex chromosome pair. We failed to distinguish them in mitosis and meiosis using any of the standard techniques such as pachytene mapping or DAPI staining. I implemented a molecular cytogenetic methodology in order to identify the sex chromosome pair. The methods are based on fluorescent staining of chromosomes by differentially labeled male and female genomic DNA (gDNA; CGH) or labeled female gDNA (GISH) competed with an excess of unlabeled male gDNA (Traut et al. 1999). Single sex probes were also used to estimate the similarity of the male's genome to the content of the W chromosome and make inferences about its content.

In the chapter two, I introduce the codling moth Sterile Insect Technique (SIT) program which drove much of my research and in the third chapter I supply codling moth basic karyotype data and sex chromosome identification methods to be used for genetic sexing strains construction.

The codling moth, *Cydia pomonella*, withstands a high research attention as a key pest of pome fruit world-wide. Repeated cases of resistance against various insecticides (e.g. organophosphates, carbamates, benzoylureas, ecdysone agonists and pyrethroids) and cross-resistance to different insecticides have instigated intensive research on resistance mechanisms and call for a modified pest management and development of alternative control strategies (e.g. Knight et al. 1994; Sauphanor 1998; Reuveny & Cohen 2004).

Sterile Insect Technique (SIT) is a method of biological control, whereby a very large number of sterile insects are released with intention of disrupting the mating and proliferation of the wild population. Its basic premise is the seasonal release of radiation sterilized adult moths. The mating of

sterile males with the wild-type females produces no offspring and therefore leads to a decrease or even the collapse of the field population. The technique was first used in 1954 to control the New World screwworm fly, *Cochliomyia hominivorax*, in the Netherlands Antilles (Baumhover et al. 1955). This program was highly successful as it ultimately resulted in the eradication of *C. hominivorax* from Mexico, Central America and the southern United States (Wyss 2000). In Lepidoptera the SIT was adopted against two pests, the pink bollworm, *Pectinophora gossypiella*, in the USA (Staten et al. 1993) and the codling moth, *C. pomonella*, in Canada (Bloem and Bloem 2000). The SIT was suggested to be implemented in area-wide suppression programs also in other lepidopteran pests (e.g. *Grafolita molesta* (Taret 2007) and *Ectomyelois ceratoniae* (Mediouni & Dhouibi 2007)). The codling moth suppression program was initiated in 1995 in the Okanagan Valley, British Columbia, Canada (Bloem & Bloem 2000). The efficiency of the control can, however, be highly improved by male-only releases using a transgenic sexing strain to eliminate females and such an implementation is described in chapter two.

Finally, in chapter four, I present the first genomic survey of a lepidopteran W chromosome using a blend of cytogenetics and molecular biology. This work was initially driven by two aims: create a cytogenetic painting probe for the W in order to facilitate its identification and develop a set of W-specific markers to be used in molecular sexing. With my co-authors, we developed a straightforward method to isolate W-chromosome specific DNA sequences without any previous sequence data requirements. We utilized a novel approach, which took advantage of the presence of a female specific sex chromatin consisting solely of the W chromosome and dissected it from polyploid nuclei by the means of laser microdissection. We used this material for developing painting probes and creating a W chromosome-specific plasmid library.

Original publications

Chapter one:
Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, Ectomyelois ceratoniae *(Lepidoptera: Pyralidae)*

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Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

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Abstract — This paper reports results of the first cytogenetic investigation carried out in the carob moth, *Ectomyelois ceratoniae* Zeller, the serious polyphagous pest of both stored products and field crops in the Mediterranean basin and Near East regions. Preparations of mitotically dividing spermatogonia and oogonia revealed that the carob moth karyotype consists of 2n = 62 chromosomes. As typical for Lepidoptera, the chromosomes were of a holokinetic type, only slightly differed in their sizes, and displayed no morphological structures including primary constrictions (the centromeres) that could enable us their identification. Metaphase I spermatocytes showed 31 bivalents. Accordingly, 31 chromosome elements were observed in metaphase II spermatocytes. Hence the haploid chromosome number is n = 31. In pachytene complements, two NOR bivalents were observed. In highly polyploid nuclei of the Malpighian tubule cells and silk glands, females showed a heterochromatin body, the so-called sex chromatin or W chromatin, that was absent in male nuclei. This indicated that the carob moth possesses a WZ/ZZ sex chromosome system. However, we failed to identify the sex chromosome bivalent WZ in pachytene oocytes. In order to differentiate the sex chromosomes, we employed genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH). GISH detected the W chromosome by strong binding of the Cy3-labelled, female-derived DNA probe. With CGH, both the Cy3-labelled female-derived probe and Fluor-X labelled male-derived probe evenly bound to the W. This suggested that the W is composed predominantly of repetitive DNA sequences occurring scattered in other chromosomes but accumulated in the W chromosome.

Key words: carob moth, CGH, genomic hybridization, GISH, holokinetic chromosomes, karyotype, sex chromosomes, W chromatin.

INTRODUCTION

The carob moth, *Ectomyelois ceratoniae* Zeller (Pyralidae), is the most important and destructive insect pest attacking dates (*Phoenix dactylifera*) in Tunisia. This polyphagous species also attacks several other host plants both in the storage and field, and yearly causes great economic losses in the Mediterranean basin and Near East regions. The chemical control against carob moth is not efficient because its larvae feed and develop inside fruit. Taken together with the increasing environmental and human health concerns due to harmful effects of insecticides, implementation of alternative and more effective control technologies is required to keep carob moth populations below economic levels. These technologies mainly include biological controls such as the use of natural enemies (DHOUIBI 1989), and genetic control methods, namely the sterile insect technique (SIT) and inherited sterility (LACHANCE 1985; DHOUIBI and ABDERAHMANE 2001).

Successful implementation of SIT programmes against the pink bollworm, *Pectinophora gossypiella*, in the USA (STATEN *et al.* 1993) and the codling moth, *Cydia pomonella*, in Canada (DYCK *et al.* 1993; BLOEM and BLOEM 2000), together with the increasing number of promising data on the applicability of inherited sterility against various lepidopteran pests (*e.g.*, CARPENTER and GROSS 1993; MAKEE and SAOUR 1997; BLOEM *et al.* 2001; CARPENTER *et al.*

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2001; Nguyen Thi and Nguyen Thanh 2001; Ramesh *et al.* 2002) evoked a great interest to adopt these methods also for the suppression of carob moth populations (Dhouibi and Abderahmane 2001). In 1999, a pilot programme for the carob moth control was initiated in a pomegranate field near Tunis, Tunisia. The programme is based on the radiation-induced inherited sterility and consists of releases of partially sterile males and fully sterile females (1000 irradiated insects/hectare/week) during the entire season. Promising results after three years of operation suggest a great potential of this control tactic for the carob moth suppression (Dhouibi *et al.* 2001; Abderahmane *et al.* 2002). However, we lack a convenient genetic sexing technique to release only males. This would improve applicability and cost-efficiency of SIT or inherited sterility to be used in an area-wide control programme (Marec *et al.* 1999).

However, genetic sexing has been documented in only two lepidopteran species: the silkworm, *Bombyx mori* (L.) (Strunnikov 1975; Ohnuma and Tazima 1983; Ohnuma 1988), and the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Marec *et al.* 1999). This genetic sexing is based on the use of balanced lethal strains that can be developed in three steps: (i) induction and isolation of sex-linked recessive lethal mutations (Marec 1990), (ii) induction of translocation of a Z-chromosome segment onto the W chromosome – the so called T(W;Z) translocation (Marec and Mirchi 1990), and (iii) construction of a balanced lethal strain by using a multiple cross-breeding scheme (Marec 1991). However, the main obstacle in developing a similar genetic sexing strain in the carob moth (or another economically important lepidopteran species) is the lack of suitable markers for mutation experiments and absence of basic knowledge of carob moth cytogenetics.

In the present study we report fundamental karyological data in the carob moth and compare both the female and male mitotic and meiotic chromosomes. A particular interest is devoted to the sex chromatin status, chromosome mechanism of sex determination, and identification of sex chromosomes. The latter is crucial namely for the detection of induced T(W;Z) translocations that are required for developing of balanced lethal strains in the carob moth.

MATERIALS AND METHODS

Insects - For cytogenetic studies we used insects taken from the 35th generation of the carob moth mass-rearing colony that was initiated at the Laboratory of Entomology (INAT, Tunis, Tunisia) in 1999, from a field collection of infested dates, and kept at a rate of 10 generations per year. Larvae were reared on an artificial diet based on wheat bran (for details, see Ould Abderahmane 1997). At the Institute of Entomology (České Budějovice, Czech Republic), where all preparations and procedures had been done, the rearing was conducted in a constant temperature room (27 ± 1°C) at a 14L:10D light regime without humidity control.

Preparation of polyploid nuclei - To determine the sex chromatin status of the carob moth, Malpighian tubules and silk glands were dissected out from both sexes (larvae and adults). Highly polyploid nuclei of their cells were stained with 1.5% lactic acetic orcein (for details, see Traut *et al.* 1986) and inspected in a light microscope at a magnification of 200-400x.

Chromosome preparations - In both sexes, mitotic and meiotic chromosomes were prepared from gonads of 4th (males only) to 5th (males and females) instar larvae. Gonads were dissected in a saline solution for *Ephestia* (Glaser 1917: cited by Lockwood 1961). Ovaries were fixed in freshly prepared Carnoy fixative (ethanol, chloroform, acetic acid, 6:3:1) for 10-30 min. Testes were first pretreated for 10 min in a hypotonic solution (0.075 M KCl) and then fixed in Carnoy fixative.

For obtaining spread pachytene nuclei, we followed the protocol of Traut (1976) for pachytene mapping. Briefly, fixed gonads were dissociated in a drop of 60% acetic acid with the help of fine tungsten needles and their cells were then spread on the slide using a heating plate at 45°C. Some preparations were stained and mounted in 2.5% lactic acetic orcein and inspected with phase contrast optics. For fluorescence microscopy, preparations were either stained with 0.5 µg/ml DAPI (4'6-diamidino-2-phenylindole; Sigma-Aldrich) in PBS buffer containing 1% Triton X-100 or with 500 nmol/ml YOYO-1 (Molecular Probes) in PBS buffer. Fluorescent preparations were mounted in DABCO antifade (0.233 g 1,4-diazabicyclo(2.2.2)-octane, DABCO, Sigma-Aldrich; 800 µl sterile H_2O; 200 µl 1 M Tris-HCl, pH 8.0; 9 ml glycerol).

Squash chromosome preparations were made only from testes of early 5th instar male larvae. After fixation testes were briefly macerated in a drop of 50% acetic acid with fine tungsten needles in order to release clusters of spermatocytes, then covered with a silicone-coated coverslip and squashed. The coverslip was then removed by using dry ice or liquid nitrogen and slides were immediately dehydrated in an ethanol series (70%, 80%, and 100%; 30 second each) and air-dried. Preparations were then stained either with 2.5 lactic acetic orcein or 0.1 µg/ml DAPI.

Comparative genomic hybridization (CGH) and genomic in situ hybridization (GISH). Whole genomic DNAs were isolated separately from female and male larvae of the carob moth by phenol-chloroform-isoamyl alcohol extraction according to the standard procedure. Label-

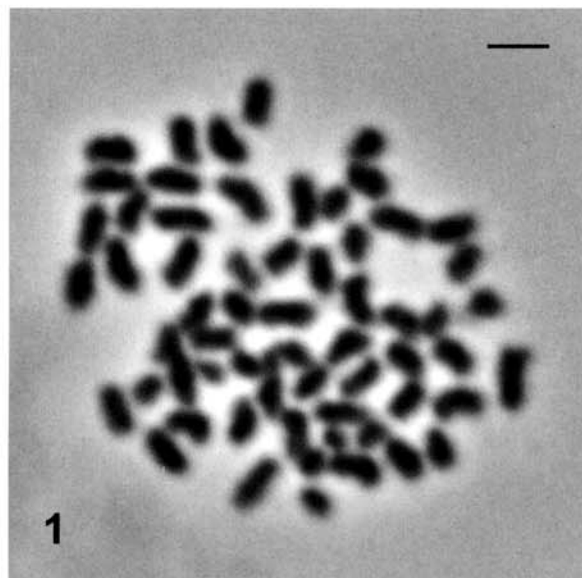


Fig. 1 — Squashed mitotic spermatogonium of the carob moth, *Ectomyelois ceratoniae*, showing 62 metaphase chromosomes, stained with orcein. Scale bar = 2 µm.

ling was done by nick translation using the Bionick Labeling System (GibcoBRL, Life Technologies Inc., Karlsruhe, Germany). DNA of females was directly labelled with Cy3-dCTP (red), DNA of males with FluorX-dCTP (green) (Amersham Life Science, Arlington Heights, Illinois).

Spread chromosome preparations were made from pachytene cells of 5th instar female larvae as described above. After spreading on a heating plate and air-drying, slides were passed through an ethanol series (70%, 80%, and 100%; 30 second each) and stored at –20°C till further use.

CGH was carried out essentially following the procedure described in TRAUT *et al.* (1999) with slight modifications. After removal from the freezer, chromosome preparations were passed through the ethanol series and air-dried. Denaturation was done at 69°C for 3 min 45 sec in 70% formamide in 2x SSC buffer. The probe cocktail for one slide (10 µl; 50% formamide, 10% dextran sulphate, 2x SSC) contained 500 ng of labelled female genomic DNA, 500 ng of labelled male genomic DNA, 5 µg of unlabelled sonicated competitor DNA from males, and 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich). Hybridization was for three days at 37°C. A stringent wash for 5 min at 62°C in 0.1x SSC containing 1% Triton X-100 (Sigma-Aldrich) was followed by washes at room temperature, counterstaining with 50 ng/ml DAPI in PBS buffer for 5 min and mounting in DABCO antifade.

The procedure for GISH was the same as for CGH, except the probe cocktail. For one slide, it contained 500 ng of labelled female genomic DNA, 5 µg of unlabeled sonicated male genomic DNA, and 25 µg of sonicated salmon sperm DNA in 10 µl of hybridization solution (50% formamide, 10% dextran sulfate, 2x SSC).

Microscopy and image processing - Preparations were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images of nuclei and chromosomes were recorded with a cooled CCD camera (F-View, Soft Imaging System GmbH, Münster, Germany). In CGH and GISH preparations, images were captured separately for each fluorescent dye, then pseudocoloured (light blue for DAPI, green for FluorX, and red for Cy3) and superimposed with the aid of an image processing program, Adobe Photoshop Version 5.0.

RESULTS

Carob moth karyotype - Squash preparations of spermatogonia of the carob moth males showed 62 mitotic metaphase chromosomes of a holokinetic type (Fig. 1). They were rod-shaped and lacked primary

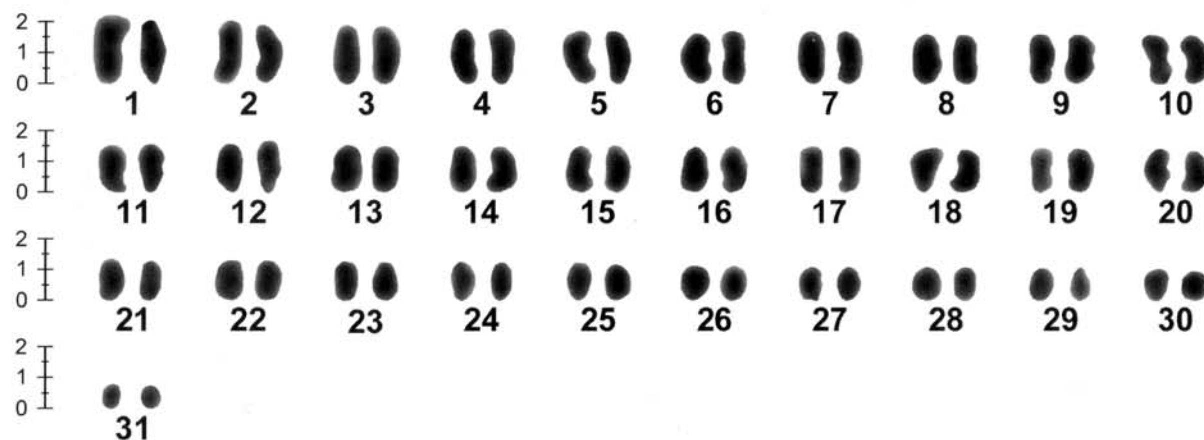
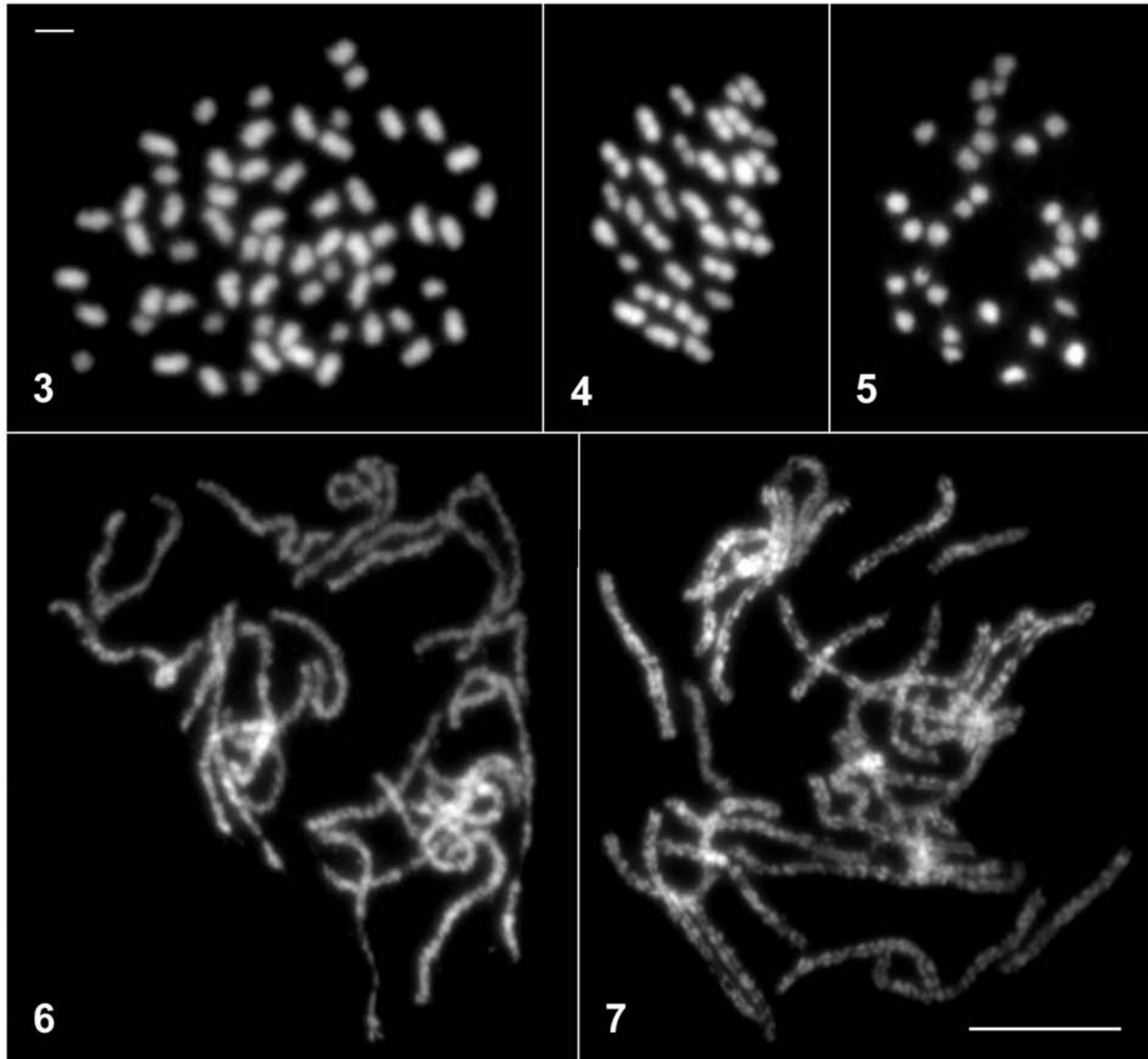


Fig. 2 — Karyotype of the carob moth, *Ectomyelois ceratoniae*, constructed from a mitotic spermatogonial metaphase, stained with orcein. Scales (left column) = 2 µm.



Figs 3-7 — Chromosome complements of the carob moth, *Ectomyelois ceratoniae*. Fluorescent images, stained with DAPI. **Fig. 3.** Squashed mitotic spermatogonium showing 2n=62 metaphase chromosomes. **Fig. 4.** Squashed meiotic spermatocyte showing n=31 metaphase I bivalents. **Fig. 5.** Squashed meiotic spermatocyte showing n=31 metaphase II chromosomes. **Fig. 6.** Squashed pachytene spermatocyte showing 31 bivalents. **Fig. 7.** Spread pachytene oocyte showing 31 bivalents with preserved chromomere/interchromomere pattern. **Figs 3-5.** Scale bar = 2 µm. **Figs 6-7.** Scale bar = 10 µm.

constrictions (the centromeres) as well as other structural landmarks that would enable us identification of individual chromosomes. Therefore, in the karyotype constructed, chromosomes were ordered only according to their sizes (Fig. 2). The largest chromosomes were about 2 µm long (pairs no. 1 and 2), the smallest about 0.7 µm long (pair no. 31).

A detailed analysis of DAPI stained chromosomes in both sexes confirmed the above findings. Mitotic spermatogonia showed regularly 62 rod-shaped chromosomes (Fig. 3). In metaphase I spermatocytes, 31 bivalents were observed (Fig. 4). Typically, the metaphase I bivalents were oriented perpendicularly to the equatorial plane, and most of them displayed a clear gap separating two homologous chromosomes. In accordance with the number of metaphase I bivalents, metaphase II complements consisted of 31 relatively small, spherical chromosomes (Fig. 5). In squash preparations, most pachytene spermatocytes displayed clumps of bivalents without any morphological landmarks or chromomeres. The latter were evidently dissociated by hypotonic treatment used. In a few well squashed nuclei, 31 pachytene bivalents were found (Fig. 6). Spread preparations of pachytene oocytes, prepared without hypotonic treatment, showed 31 bivalents

with the chromomere/interchromomere pattern (Fig. 7) that is typical for the pachytene stage (*cf.* TRAUT 1976).

The use of another fluorescent dye, YOYO-1 that stains both DNA and RNA (*cf.* WEEKS *et al.* 2001), revealed two spherical nucleoli in spread pachytene nuclei of both sexes (Fig. 8). One nucleolus was large, associated with an interstitial region of a mid-sized bivalent, the other was much smaller and located at the terminal segment of a shorter bivalent.

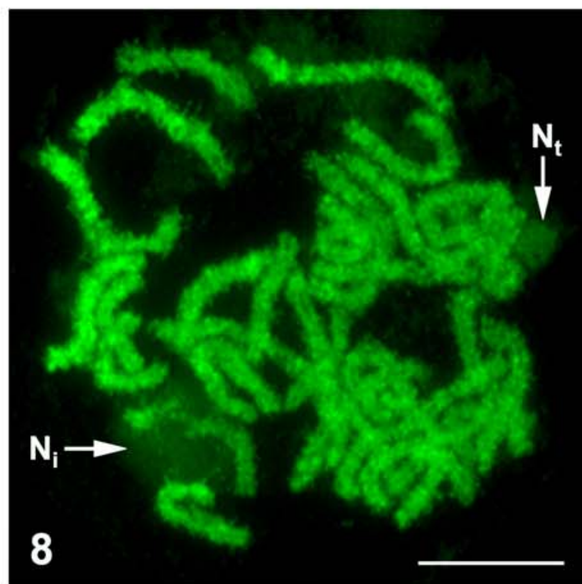


Fig. 8 — Spread spermatocyte pachytene nucleus of the carob moth, *Ectomyelois ceratoniae*, stained with YOYO-1. N_i, a conspicuous nucleolus carried by a bivalent with interstitial NOR; N_t, a small nucleolus carried by a bivalent with terminal NOR. Scale bar = 10 μm.

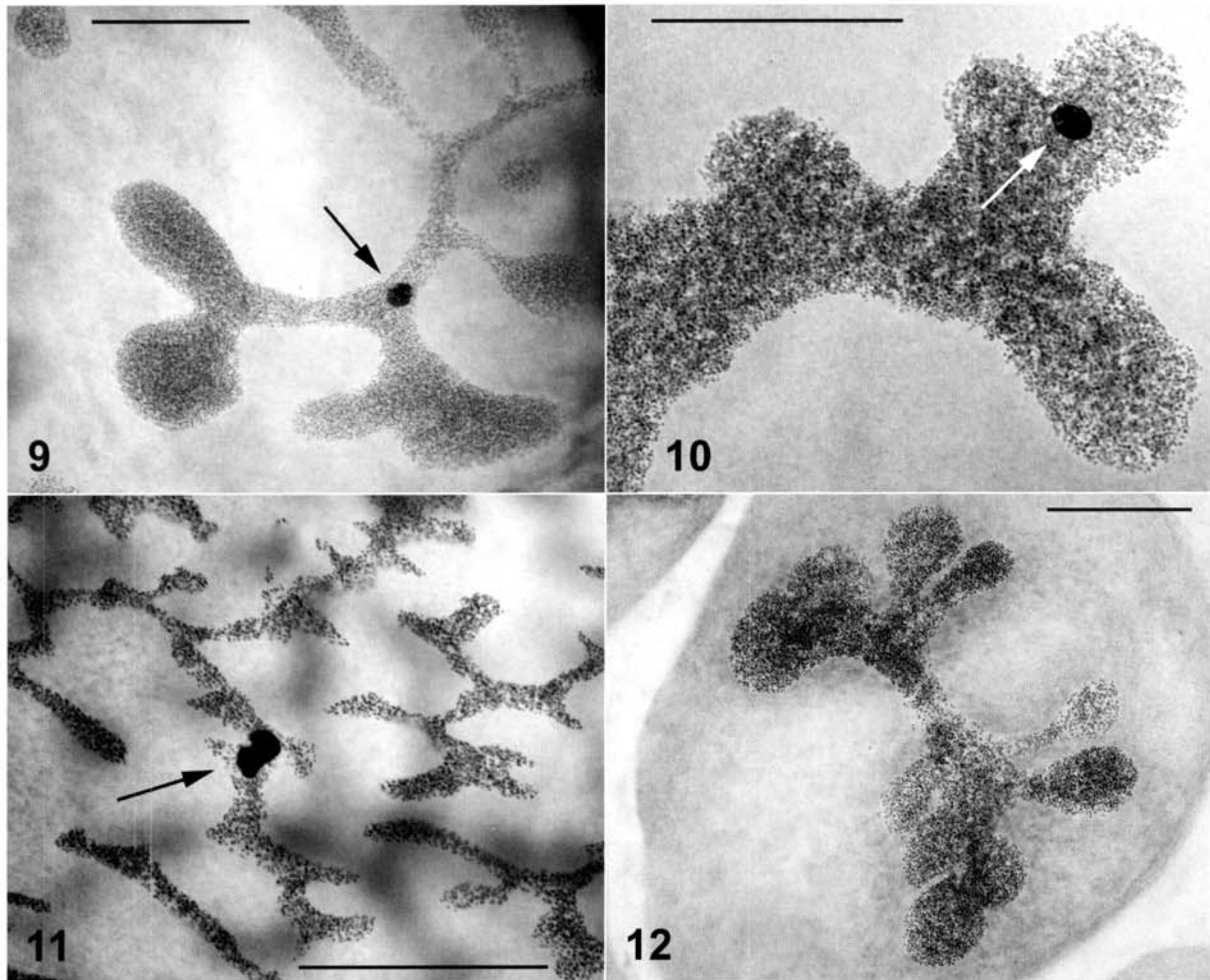
Sex chromatin status and sex chromosome system - In preparations of Malpighian tubules from adult females, lobbed nuclei were observed. Their large size indicated a high level of polyploidy. Each nucleus displayed a single, spherical heterochromatin body (Figs. 9 and 10). Similar bodies were observed in highly branched nuclei of female silk glands (Fig. 11). In contrast, somatic polyploid nuclei of males displayed uniform texture of dense, fine chromatin grains without any heterochromatin (Fig. 12). We concluded that the heterochromatin body in female somatic nuclei represents the sex chromatin (W-chromatin) which is typical for female Lepidoptera and is formed by multiple copies of the W sex chromosome (review: TRAUT and MAREC 1996). The presence of W-chromatin suggested a WZ/ZZ (female/male) sex chromosome system in the carob moth.

In order to confirm the above finding, we examined pachytene oocytes stained with lactic acetic orcein following the procedure of Traut (1976) for pachytene mapping. This technique was successfully used to identify sex-chromosome bivalents in a number of lepidopteran species according to the heterochromatic thread of the W chromosome (*e.g.*, MAREC and TRAUT 1994; TRAUT and MAREC 1997). However, in the carob moth we failed to recognize the WZ bivalent even in exceptionally well spread pachytene oocytes (Fig. 13). Most bivalents showed pairs of chromomeres indicating homology between the two homologous chromosomes. Microscopic observations confirmed the presence of two NOR-bivalents in the pachytene complement of carob moth. No bivalent showed a continuous heterochromatic thread or at least a clear block of heterochromatin that would enable us to identify the WZ bivalent.

W chromosome identification by GISH and CGH - The use of orcein staining and phase contrast microscopy did not disclose the sex chromosome bivalent in carob moth oocytes. This suggested that the W and Z sex chromosomes are not enough differentiated morphologically. Therefore, we employed two methods of molecular cytogenetics, genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH). The methods can differentiate the heterologous W chromosome provided that its DNA composition differs sufficiently from the Z chromosome (TRAUT *et al.* 1999; SAHARA *et al.* 2003a,b).

In pachytene oocytes, GISH regularly detected a bivalent, in which one chromosome thread was highlighted with red signals of the Cy3-labelled female genomic probe (Fig. 14). The red-labelled thread was well seen even in compact, unspread pachytene nuclei (not shown). In most pachytenes, the highlighted chromosome was not homogeneously labelled but showed strongly labelled segments alternating with weakly labelled ones or even interrupted with gaps which were almost without hybridization signals (Fig. 15). Since the sex-chromatin body in interphase nuclei was also deeply stained with the female genomic probe (Fig. 16), we concluded that GISH detected the sex-chromosome bivalent WZ and the red-labelled thread represented the W chromosome. GISH identified the W chromosome even among metaphase chromosomes of mitotically dividing oogonia (Fig. 17). A detailed analysis of the WZ bivalent confirmed binding of the female-derived probe over most of the W chromosome length but the intensity of fluorescent signals varied, probably reflecting differences in DNA composition (Figs. 18-20). The W appeared as the slightly longer partner in the WZ bivalent, twisting along the Z chromosome (Fig. 20). The Z appeared less stained with the probe than the autosomes (Fig. 19).

In CGH, Cy3-labelled DNA from females and FlourX-labelled DNA from males in the presence of an excess of sonicated, unlabelled male-derived DNA stained all chromosomes yellowish due to collocation of red and green signals. In pachytene oocytes, the WZ bivalent stood out from the autosome bivalents by stronger binding of both probes to the W chromosome (Fig. 22), though not distinguishable in the DAPI image (Fig. 21). However, the W-chromosome labelling was



Figs 9-12 — Highly polyploid somatic nuclei of the carob moth, *Ectomyelois ceratoniae*, stained with orcein. **Fig. 9.** A lobbed nucleus of the Malpighian tubule cell from adult female, showing a sex-chromatin body (arrow). **Fig. 10.** A detailed image of a part of the Malpighian tubule cell nucleus from adult female, showing a spherical, deeply stained sex-chromatin body (arrow). **Fig. 11.** A part of highly branched nucleus of the silk gland cell from adult female showing a sex-chromatin body (arrow). **Fig 12.** A lobbed nucleus of the Malpighian tubule cell from male larva without sex chromatin. Scale bars = 50 µm.

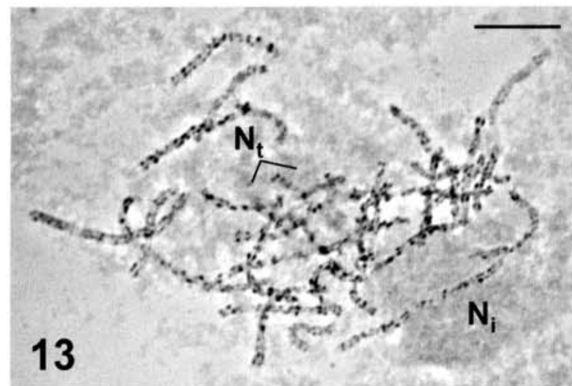
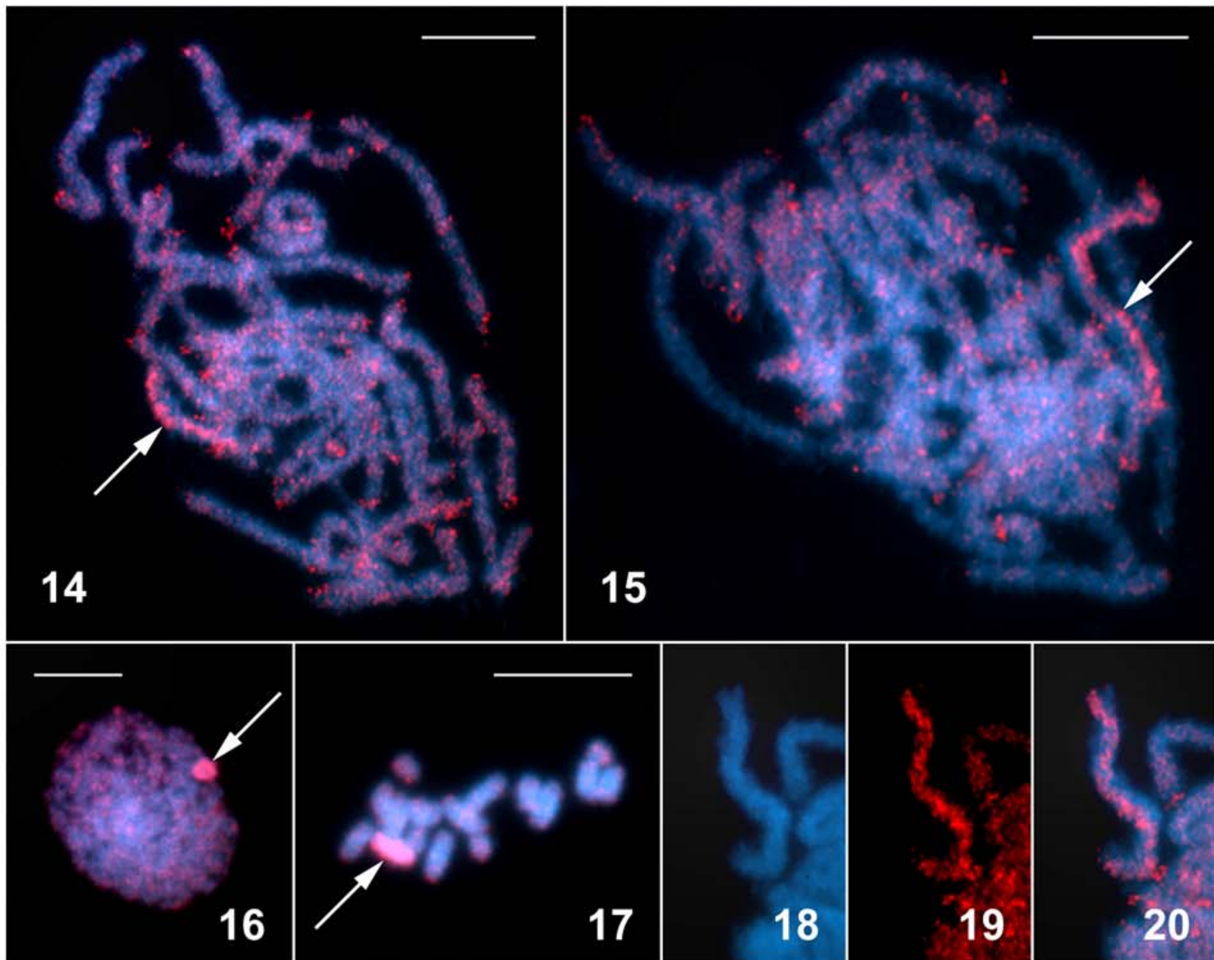


Fig. 13 — Spread pachytene oocyte of the carob moth, *Ectomyelois ceratoniae*, showing 31 bivalents, stained with orcein. Note characteristic chromomere/interchromomere pattern. N_i, a conspicuous nucleolus carried by a bivalent with interstitial NOR; N_t, a small nucleolus carried by a bivalent with terminal NOR. Scale bar = 10 µm.

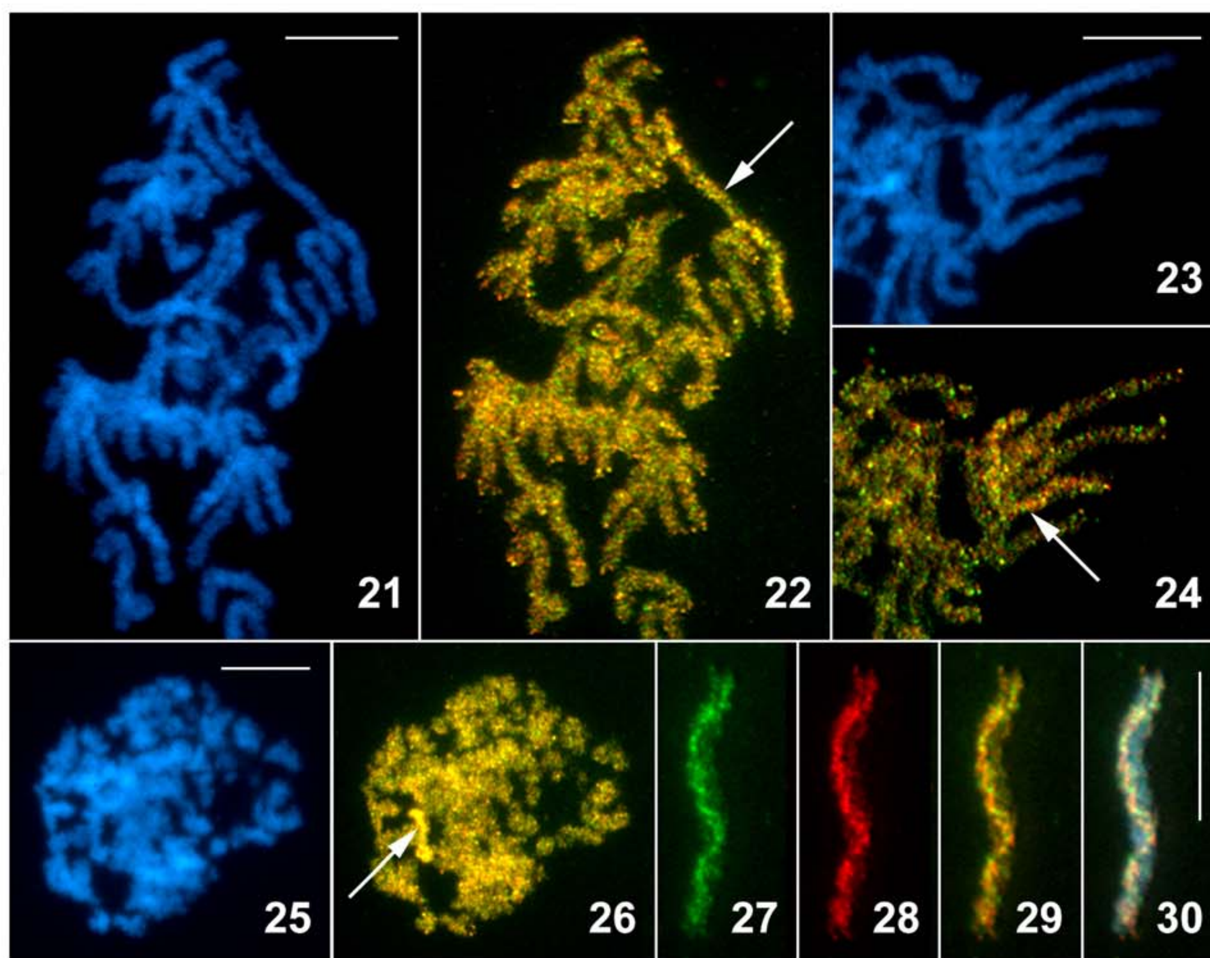


Figs. 14-20 — Genomic *in situ* hybridization (GISH) in females of the carob moth, *Ectomyelois ceratoniae*; blue, chromatin counterstained with DAPI; red signals, Cy3-labelled female genomic probe. **Figs. 14-15.** Pachytene complements; the WZ bivalent is identified by pronounced binding of the female-derived probe to the W chromosome (arrows). **Fig. 16.** Interphase nucleus showing a sex-chromatin body strongly labelled with the female-derived probe (arrow). **Fig. 17.** A part of the mitotic metaphase chromosome set showing the W chromosome highlighted with the female-derived probe (arrow). **Figs. 18-20.** A pachytene WZ bivalent. **Fig. 18.** DAPI image. **Fig. 19.** Signals of female-derived probe. **Fig. 20.** Composite image: DAPI and female-derived probe. Scale bars = 10 µm.

not as intense as in GISH preparations, making thus the WZ bivalent less distinct from the autosome pairs in a number of pachytene nuclei (Figs. 23 and 24). On the other hand, the W chromatin was easily identified by CGH in nuclei of nurse cells, where it remained compact with aggregate hybridization signals (Figs. 25 and 26). A detailed analysis of the WZ bivalent at the pachytene stage revealed the same labelling pattern in the W chromosome with both the male- and female-derived probes. The Z chromosome, in contrast, did not show enhanced binding of either probe (Figs. 27-30).

DISCUSSION

We showed here that the karyotype of carob moth (*Ectomyelois ceratoniae*) consists of 31 pairs of chromosomes (2n = 62), which is in the rank of most common haploid chromosome numbers in species of the order Lepidoptera, n = 28-32 (SUOMALAINEN 1969; ROBINSON 1971; FISK 1989). The chromosomes are of a holokinetic type, only slightly differ in their sizes, and display no morphological structures including primary constrictions (the centromeres). Similar to another pyralid moth, *Ephestia kuehniella* (n = 30), two autosomal NOR-bivalents were observed in pachytene complements (*cf.* MAREC and TRAUT 1993). On the contrary, a single NOR-bivalent was reported in several other lepidopterans. For example, an autosome bivalent carries the NOR in *Bombyx mori* with n = 28 (TRAUT 1976; SAHARA *et al.* 2003b). One NOR-bivalent was also observed in



Figs. 21-30 — Comparative genomic hybridization (CGH) in females of the carob moth, *Ectomyelois ceratoniae*; blue, chromatin counterstained with DAPI; green signals, FluorX-labelled male genomic probe; red signals, Cy3-labelled female genomic probe. **Figs. 21-22.** Pachytene complement. **Fig. 21.** DAPI image. **Fig. 22.** CGH image; the WZ bivalent is identified by binding of both the male- and female-derived probes to the W chromosome (arrow). **Figs. 23-24.** A part of pachytene nucleus. **Fig. 23.** DAPI image. **Fig. 24.** CGH image; the WZ bivalent shows a faint identification by binding of both the male- and female-derived probes to the W chromosome (arrow). **Figs. 25-26.** Nurse cell nucleus. **Fig. 25.** DAPI image. **Fig. 26.** CGH image; the W is labelled with both the male- and female-derived probes (arrow). **Figs. 27-30.** A pachytene WZ bivalent. **Fig. 27.** Signals of male-derived probe. **Fig. 28.** Signals of female-derived probe. **Fig. 29.** CGH image with both the male- and female-derived probes. **Fig. 30.** Composite image: DAPI and both probes. Scale bars = 10 μm.

moths of the genus *Orgyia* (Lymantriidae) with low chromosome numbers due to multiple fusions. It was an autosome bivalent in *O. antiqua* (L.) with n = 14 (TRAUT and CLARKE 1997), whereas *O. thyellina* Butler with n = 11 showed the NOR located in the WZ bivalent (TRAUT and CLARKE 1996).

Our study also showed that the carob moth possesses a WZ/ZZ chromosome system of sex determination, which is common in 'advanced' Lepidoptera, the Ditrysia (TRAUT and MAREC 1997), and females are the heterogametic sex. In polyploid somatic nuclei of females, multiple copies of the W chromosome formed a single heterochromatin body (the so-called sex chromatin or W chromatin) that is characteristic for females of most Ditrysia (TRAUT and MAREC 1996) and Tischeriina (LUKHTANOV 2000). The sex chromatin was absent in male nuclei. In *E. kuehniella*, the model species of Lepidoptera cytogenetics, the sex-chromosome bivalent WZ can be easily identified in pachytene oocytes according to the heterochromatic thread of the W chromosome, frequent incomplete pairing due to the lack of homology between the W and Z, and characteristic twisting of the longer Z along the shorter W (MAREC and TRAUT 1994). Similar characteristics, in particular heterochromatinization of the W can be applied for the recognition of WZ bivalents in a number of other lepidopterans (TRAUT and MAREC 1997). However, the carob moth appears to be an exception. In this species, we failed to identify the WZ bivalent using

pachytene mapping because of the absence of distinct morphological differences between W and Z and lack of W-chromosome heterochromatinization in the pachytene stage. Only the use of two advanced methods of molecular cytogenetics, GISH and CGH, enabled us to identify the WZ bivalent. With GISH, the W chromosome was strongly labelled with the female-derived genomic probe in interphase nuclei and both mitotic and meiotic chromosome plates, indicating its high molecular differentiation from the Z chromosome. However, CGH revealed that the W chromosome is differentially stained with both the male- and female-derived probes. This strongly suggests that the W is composed predominantly of repetitive DNA sequences occurring scattered in other chromosomes but accumulated in the W chromosome.

A similar example of a lepidopteran species with problematic identification of sex chromosomes is *B. mori*, in which the W chromosome was first recognized only when marked with a conspicuous translocation (KAWAMURA and NIINO 1991). The wild-type W was identified only recently by using CGH (TRAUT *et al.* 1999), GISH using female-derived genomic probes and FISH with W chromosome-derived bacterial artificial chromosome (BAC) probes (SAHARA *et al.* 2003b). Based on the comparison of hybridization signal intensities in CGH experiments, SAHARA *et al.* (2003a) suggested that the W chromosomal DNA in Lepidoptera consists of two components: (i) repetitive DNA common to females and males, *i.e.*, present in autosomes and/or Z chromosomes, and (ii) repetitive DNA exclusively or preponderantly present in the female genome, *i.e.*, W-specific DNA. The second component was not evident in *B. mori*, while present in two pyralids, *E. kuehniella* and *Galleria mellonella* (L.). Our results suggest that the carob moth possesses a type of molecular differentiation of the W similar to that of *B. mori*.

In conclusion, there is an interest to develop a genetic sexing system in the carob moth, based on sex-linked recessive lethal mutations (STRUNNIKOV 1975; MAREC *et al.* 1999), to improve applicability of radiation-induced inherited sterility for the suppression of carob moth populations (ABDERAHMANE *et al.* 2002). However, this appears to be extremely difficult task in the pest, in which no sex-linked visible markers are available. In particular, it is virtually impossible to isolate a T(W;Z) translocation that is crucial for the construction of a balanced lethal strain (MAREC and MIRCHI 1990). MAREC and TRAUT (1994) showed in *E. kuehniella* that the T(W;Z) translocation causes the formation of deformed or fragmented W chromatin bodies in highly polyploid somatic nuclei of females. Since carob moth females exhibit a distinct W chromatin body (this study), the female-specific sex chromatin could serve as a cytological marker to isolate a collection of induced structural mutants of the W chromosome as proposed by MAREC and TRAUT (1994). From this collection, lines possessing the required T(W;Z) translocations could be then selected in the next generation, by using cytogenetic techniques such as CGH or GISH which can visualize the W chromosome in pachytene oocytes of the carob moth (this study). These techniques were already successfully used to disclose the actual structure of mutant W chromosomes in other lepidopterans (see MAREC *et al.* 2001; SAHARA *et al.* 2003b).

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Chapter two:

Developing transgenic sexing strains for the release of non-transgenic sterile male codling moths Cydia pomonella

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Developing Transgenic Sexing Strains for the Release of Non-Transgenic Sterile Male Codling Moths *Cydia pomonella*

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ABSTRACT Sterile insect releases for management of lepidopteran pest populations are based on bisexual releases. Male-only releases of codling moth *Cydia pomonella* (L.) have never been tested in the field, due to the lack of efficient ways to separate males from females or produce only males. Recently, a new approach for the development of genetic sexing strains in Lepidoptera has been proposed. It is based on the construction of transgenic females carrying a dominant conditional lethal mutation (DCLM) in the female-determining W chromosome. Such a transgenic sexing strain would be propagated under permissive conditions. Under restrictive conditions, the W-linked DCLM would kill all females while allowing mass-rearing and release of radiation-sterilized, but non-transgenic males. This paper describes the principle of the proposed transgenic approach and discusses its benefits, environmental safety, and other potential concerns. The aim is to develop transgenic sexing strains in the codling moth. Appropriate molecular tools for codling moth transgenesis are already available and germ-line transformation in this species has been successfully accomplished. Significant progress has also been made in codling moth cytogenetics. In particular, data on the codling moth sex chromosomes and their identification, obtained using advanced molecular cytogenetic methods, will facilitate the development of genetic sexing strains in this pest.

KEY WORDS codling moth, transgenesis, W chromosome, genetic sexing, cytology, dominant conditional lethal mutation

1. Introduction

In Lepidoptera, area-wide programmes integrating the sterile insect technique (SIT) have been successfully implemented against two species: containment of the pink bollworm *Pectinophora gossypiella* (Saunders) in the USA (Staten et al. 1993), and suppression of the codling moth *Cydia pomonella* (L.) in Canada (Dyck et al. 1993, Bloem and Bloem 2000, S. Bloem et al., this volume). The use of inherited sterility against various lepidopteran pests is also a very promising approach (Carpenter and Gross 1993, Makee and Saour 1997, Bloem et al. 2001, Carpenter et al. 2001, Nguyen Thi and Nguyen Thanh 2001, Seth and Sharma 2001, Carpenter et al. 2005). The success of these programmes and the potential use of inherited sterility have encouraged research activities aimed at improving its efficiency and applicability worldwide.

Current programmes using SIT and/or inherited sterility for the population control of lepidopteran pests rely on bisexual releases, but there are reasons to believe that the release of sterile males only would bring significant improvement to these technologies (Marec et al. 2005). On the other hand, recent results involving field-cage experiments with the cactus moth *Cactoblastis cactorum* (Berg), suggest that sterile females have a positive

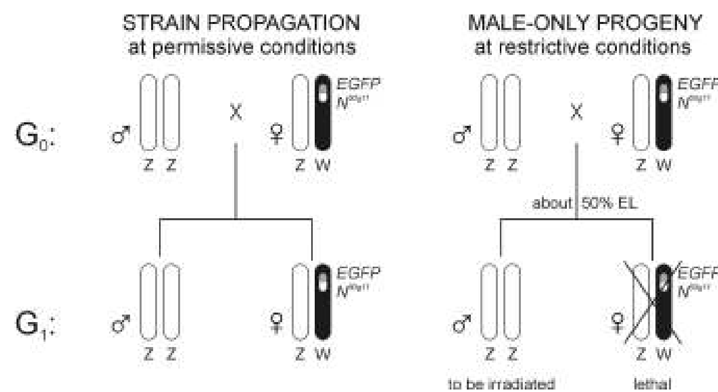

Figure 1. *Theoretical scheme to generate a genetic sexing strain based on the use of transgenic females that have an insert in their W chromosome containing the enhanced green fluorescent protein marker gene* (EGFP) *and a conditional dominant lethal mutation of the* Drosophila Notch *gene* (N^{60g11}). *If kept under restrictive conditions (low temperature), the strain produces only males that can be irradiated and released (G_0 and G_1: generations, Z and W: sex chromosomes, EL: embryonic lethality (Modified after Marec et al. (2005)).*

impact on population suppression (Hight et al. 2005). However, caution is required with the interpretation of these data as field-cage tests cannot reflect all factors involved in field programmes. This has been well demonstrated in programmes using the SIT against the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann). Although field-cage tests were inconclusive, the use of genetic sexing strains, enabling mass-production and release of males only, has resulted in enormous economic benefits by decreasing release costs and increasing the efficiency of the sterile males in comparison with bisexual releases (Robinson 2002, Rendon et al. 2004). Obviously, large-scale field tests are needed to compare the efficiency of male-only with bisexual releases for the control of lepidopteran pests.

A convenient genetic sexing system to produce male-only progeny in key lepidopteran pests is not yet available. Sophisticated genetic sexing strains such as those constructed in the Mediterranean fruit fly (reviewed by Robinson 2002), cannot be developed in any lepidopteran pest because their sex chromosome system is different to that in fruit flies. In moths and butterflies, the chromosome mechanism of sex determination is of the WZ/ZZ type. Females are the heterogametic sex with a pair of WZ sex chromosomes, while homogametic males have a ZZ pair (Traut 1999). This means that sexing strains constructed on a similar principle as in the Mediterranean fruit fly would eliminate male but not the female progeny.

Recently, a new approach for the development of genetic sexing strains in Lepidoptera has been proposed (Marec et al. 2005), based on the construction of transgenic females carrying a dominant conditional lethal gene in the female-determining W chromosome. The main advantage of this approach is that the males are not transgenic and, therefore, could be released as in a normal area-wide integrated pest management (AW-IPM) programme using the SIT.

Based on this principle, it is intended to develop transgenic sexing strains in the codling moth *C. pomonella*. A detailed analysis of the codling moth karyotype with a particular focus on the identification and characterization of sex chromosomes is available (Fukova et al. 2004, 2005), and successful and stable germ-line transformation has also been

achieved (Ferguson et al. 2004). The principle of the transgenic approach and the molecular tools available for codling moth transgenesis are described below and data on codling moth cytogenetics are summarized. Also included is a discussion as how the sex chromosomes could be used to develop transgenic sexing strains.

2. Mendelian Approaches to Genetic Sexing in Lepidoptera

In the silkworm *Bombyx mori* (L.), several mutant strains have been constructed, in which wild-type alleles of autosomal marker genes coding for visible traits such as egg colour, larval phenotype or cocoon colour, are translocated onto the W chromosome while their recessive mutant alleles remain located on autosomes. The visible traits then exhibit sex-limited inheritance. This makes possible the separation of males and females according to the sex-specific phenotype during embryonic, larval, and pupal development, respectively (Nagaraju 1996). However, in lepidopteran pests convenient selectable marker genes are not available (Robinson 1971).

An alternative approach has been to use female killing systems based on balanced lethal strains as proposed by Strunnikov (1975) for the silkworm *B. mori*. To date, balanced lethal strains have been developed in two species, the silkworm and the Mediterranean flour moth *Ephestia kuehniella* Zeller (reviewed by Marec et al. 2005). Briefly, the balanced lethal-2 strain of the latter species produces males, which are heterozygous, in *trans*, for two sex-linked recessive lethal mutations. Sexing is achieved when balanced lethal-2 males are outcrossed to wild-type females. Such crosses produce almost exclusively male progeny, while the female progeny die during embryogenesis because they are hemizygous for one of the lethal mutations (Marec 1991, Marec et al. 1999).

This scheme has several drawbacks. Firstly, the development of balanced lethal strains is laborious and difficult due to lack of the sex-linked markers needed to detect and effectively rear insects with lethal mutations and the T(W;Z) translocations required for the construction of balanced lethal strains (Marec 1991). Secondly, the maintenance of two different colonies, a wild-type strain and a balanced lethal strain (Fig. 3 in Marec et al. 1999), and routine checking of the balanced lethal strain to prevent colony breakdown due to genetic recombination or contamination, represent serious obstacles for a mass-rearing facility. Finally, an additional sex separation is indispensable before crossing balanced lethal males with wild-type females to produce male-only progeny for irradiation and release. Considering these disadvantages, this technology is not applicable for mass-rearing.

3. Principle of the Transgenic Approach to Genetic Sexing

Theoretically, a dominant conditional lethal mutation (DCLM) located in the female-determining W chromosome would be a simple and efficient selective mechanism for the development of genetic sexing strains in Lepidoptera, easily applicable in a mass-rearing facility. The DCLM would be inherited exclusively by mutant females, whereas males would be wild-type. The strain would be propagated under permissive conditions, and the female progeny eliminated under restrictive conditions. However, it would be very difficult to induce a DCLM in the W chromosome since it is largely heterochromatic and probably genetically inert in most lepidopteran species (Traut 1999). Therefore, it would be better to try to identify a DCLM in the gene-rich Z chromosome (or in an autosome) and then translocate it onto the W chromosome (Fig. 2 in Marec et al. 2005). Unfortunately, no DCLM has been reported to date in any lepidopteran species and a labour-intensive and long-term study would be required to obtain a desirable mutation in a particular pest.

In order to circumvent this problem, Marec et al. (2005) proposed that genetic sexing in Lepidoptera, based on W-linked DCLMs, can

be accomplished through the use of transgenesis. Recently, successful and stable germ-line transformation has been achieved in several lepidopteran species using the transposable element *piggyBac* (Peloquin et al. 2000, Tamura et al. 2000).

The key requirement under this proposal is the insertion of a DCLM of a known gene, which is conserved in insects and expressed during embryogenesis, into the W chromosome (Fig. 1). For this purpose, a mutant allele of the *Notch* gene, N^{60g11}, has been chosen. The N^{60g11} allele was originally isolated in *Drosophila melanogaster* (Meigen). It encodes a truncated form of the *Notch* protein, which causes the death of heterozygous *Drosophila* embryos kept below 20°C (Fryxell and Miller 1995). Thus, N^{60g11} is dominant, sensitive to cold temperature and seems to be well suited for genetic sexing. A plasmid construct required for germ-line transgenesis is already available in the laboratory of L. Neven. It contains the *piggyBac* transposon with N^{60g11} in tandem with the enhanced green fluorescent protein (EGFP) marker gene from the jelly fish *Aequorea aequorea* (Forskal), under the *B. mori Actin A3* promoter (Fig. 3 in Marec et al. 2005).

A transgenic strain would be reared at temperatures above 20°C in order to maintain the large production colony. For male production, eggs would be held at a temperature below 20°C for a specified length of time to kill female embryos. The eggs would then be returned to permissive conditions, where only male larvae would hatch. This step could be easily checked because no larva expressing the enhanced green fluorescent protein (i.e. transgenic female larva) should hatch. The male-only progeny would be kept at normal temperature until adulthood. After completing their development the adult males would be irradiated and released (Fig. 2).

The transgenic approach has several advantages: (1) the sexing process is simple and can be carried out easily on large numbers of eggs, (2) the process does not require any additional technology in a mass-rearing facility, (3) any escaped females would die if temperatures dropped below 20°C, (4) the released males would not be transgenic, and (5) any genetic background can be introduced through mating males with the transgenic females. As the males are not transgenic, they would not be negatively affected by pleiotropic effects of the transgene used for the elimination of females and they should not trigger any regulatory concerns related to their release.

Nevertheless, there is a concern about this proposed transgenic approach related to the possibility of inserting a transgene into the largely heterochromatic W chromosome as the inserted transgene could be silenced by neighbouring heterochromatin. In addition, there is no evidence that the transgene will be expressed and exhibit the desired temperature lethality in female embryos. However, these are testable research questions that can be addressed.

4. Genetic Transformation in the Codling Moth

The groundwork for the creation of stably transformed codling moth has been established in the laboratory of L. Neven, where the first successful germ-line transformation has been achieved in this species (Ferguson et al. 2004). The molecular tools available for the development of transgenic sexing strains in the codling moth and the current progress are summarized as follows: (1) codling moth eggs are suitable for DNA injection as they attach firmly to the collecting device and have a transparent chorion, (2) the *piggyBac* transposon has been identified as an effective transposable element to insert foreign DNA into moth embryos, (3) the enhanced green fluorescent protein was found to be a useful marker for identifying transformants; however, it is not ideal due to excessive autofluorescence of the codling moth in the range used to visualize the enhanced green fluorescent protein. Therefore, it would be worthwhile to replace the enhanced green fluorescent protein with another fluorescent marker such as DsRed2 (Horn et al. 2002), (4) the *B. mori Actin A3*

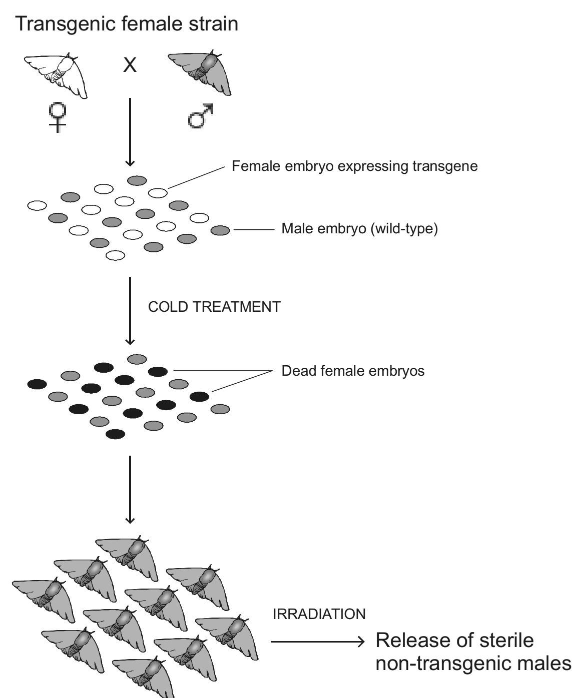

Figure 2. *Transgenic genetic sexing approach to a non-transgenic release of sterile male Lepidoptera (for details, see text).*

promoter was found to function in the codling moth; however, it sometimes exhibited a chimeric fluorescence pattern and therefore, it would be better to replace it with the artificial *3xP3* promoter that supports the enhanced green fluorescent protein expression specifically in the eyes of all life stages (Thomas et al. 2002), (5) an appropriate lethal gene, the dominant cold-sensitive allele N^{60g11}, has been identified. A plasmid construct containing the *piggyBac* transposon with N^{60g11} in tandem with the enhanced green fluorescent protein marker gene under the *Actin A3* promoter is available, and (6) eggs from transgenic lines heterozygous for the enhanced green fluorescent protein and N^{60g11} were exposed to 12°C for two days and 50% of the embryos died and none of the survivors, either larvae or adults, expressed the enhanced green fluorescent protein. The wild-type and enhanced green fluorescent protein controls showed a normal egg hatch and development. This indicates that those embryos carrying the N^{60g11} transgene died, confirming the lethality of this gene at low temperatures (L. Neven, unpublished).

Based on the above it can be concluded that the development of transgenic genetic sexing strains in the codling moth according to the proposed scheme is technically feasible. However, there is still the need to determine experimentally the overall behaviour of the transgene.

5. Codling Moth Cytogenetics

Until recently, very little was known about the genetics of the codling moth (Robinson 1971, Benz 1991), especially about their sex chromosomes. The only cytogenetic study (Ortiz

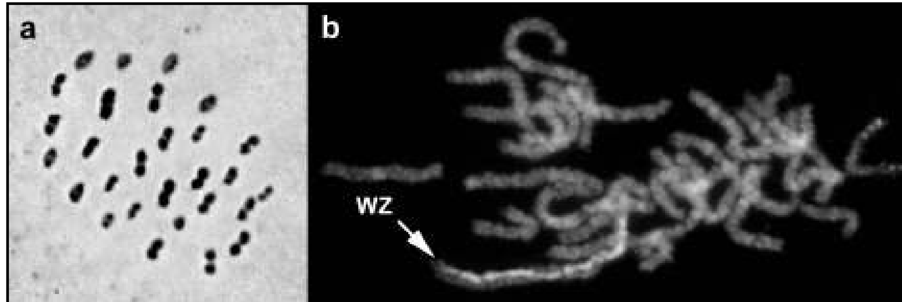

Figure 3. *Photograph showing codling moth chromosomes, (a) squashed spermatocyte, stained with lactic acetic orcein, showing meiotic metaphase I with n=28 bivalents and (b) fluorescent image of spread pachytene oocyte stained with genomic in situ hybridization (GISH) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). The WZ bivalent (arrow) is recognized by prominent hybridization of the Cy-3 labelled, female-derived probe to the W chromosome.*

and Templado 1976) reported that the number of metaphase I bivalents in males was n = 28. To fill this gap, a detailed analysis was made of codling moth chromosomes with a particular focus on the sex chromosomes (Fukova et al. 2005). This study confirmed that the codling moth karyotype consists of 2n = 56 chromosomes in mitotic metaphase, corresponding to n = 28 bivalents in metaphase I spermatocytes (Fig. 3a). Females are heterogametic with a WZ sex chromosome pair and males are homogametic with two Z chromosomes. While the Z chromosome is composed of euchromatin and resembles an autosome, the W chromosome consists largely of heterochromatin.

To develop transgenic sexing strains in the codling moth, a DCLM (transgene), has to be inserted into the W chromosome. Theoretically, the probability of the transgene inserting into the W chromosome is dependent on the size of this chromosome relative to the rest of the genome. In the codling moth, the W chromosome is one of the two largest chromosomes, comprising about 4% of the female genome. This should make it a reasonable target for transgenesis with the probability of insertion of 1 in 25 (if only females are included), or with the overall probability of 1 in 50 (since both female and male embryos are injected). However, since the W chromosome is mainly composed of heterochromatin, silencing of the transgene expression is highly likely. Suggestions as to how to overcome this problem are discussed in Marec et al. (2005). To further characterize the codling moth W chromosome advanced methods of molecular cytogenetics i.e. genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH) were employed. GISH detected the W chromosome as evidenced by strong binding of the Cy3-labelled, female-derived DNA probe (Fig. 3b). With CGH, both the Cy3-labelled female-derived probe and Fluor-X labelled male-derived probe evenly bound to the W chromosome. This suggests that the W chromosome is composed mostly of repetitive DNA sequences that are also distributed on other chromosomes but have accumulated in the W chromosome (Fukova et al. 2005). Finally, W-specific probes were prepared by laser micro-dissection of the W chromatin followed by amplification using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) and DOP-PCR labelling. The probes stained the entire W chromosome with a high specificity. DNA fragments of the micro-dissected W chromatin were cloned and sequenced. The W chromosome sequence analysis revealed no homology to any DNA

sequenced so far (Fukova et al. 2004). Several cloned sequences were found to originate exclusively from the W chromosome. These unique sequences can be very useful as molecular markers of the W chromosome in codling moth transgenesis.

6. Conclusions

The proposed transgenic approach may represent a straightforward method for generating genetic sexing strains in any lepidopteran pest to be controlled using SIT or inherited sterility. The codling moth, which is considered to be one of the best candidates for these technologies (Marec et al. 2005), was chosen to examine this approach. As reported here, all the basic research tools required for the development of transgenic sexing strains in this species are available. Future research will include generating transgenic lines, mapping the inserted transgene, and identifying and characterizing lines with the transgene located in the W chromosome.

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Chapter three:
Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes

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Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes

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Abstract: We performed a detailed karyotype analysis in the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), the key pest of pome fruit in the temperate regions of the world. The codling moth karyotype consisted of $2n = 56$ chromosomes of a holokinetic type. The chromosomes were classified into 5 groups according to their sizes: extra large (3 pairs), large (3 pairs), medium (15 pairs), small (5 pairs), and dot-like (2 pairs). In pachytene nuclei of both sexes, a curious NOR (nucleolar organizer region) bivalent was observed. It carried 2 nucleoli, each associated with one end of the bivalent. FISH with an 18S ribosomal DNA probe confirmed the presence of 2 clusters of rRNA genes at the opposite ends of the bivalent. In accordance with this finding, 2 homologous NOR chromosomes were identified in mitotic metaphase, each showing hybridization signals at both ends. In highly polyploid somatic nuclei, females showed a large heterochromatin body, the so-called sex chromatin or W chromatin. The heterochromatin body was absent in male nuclei, indicating a WZ/ZZ (female/male) sex chromosome system. In keeping with the sex chromatin status, pachytene oocytes showed a sex chromosome bivalent (WZ) that was easily discernible by its heterochromatic W thread. To study molecular differentiation of the sex chromosomes, we employed genomic in situ hybridization (GISH) and comparative genomic hybridization (CGH). GISH detected the W chromosome by strong binding of the Cy3-labelled, female-derived DNA probe. With CGH, both the Cy3-labelled female-derived probe and Fluor-X labelled male-derived probe evenly bound to the W chromosome. This suggested that the W chromosome is predominantly composed of repetitive DNA sequences occurring scattered in other chromosomes but accumulated in the W chromosome. The demonstrated ways of W chromosome identification will facilitate the development of genetic sexing strains desirable for pest control using the sterile insect technique.

Key words: CGH, codling moth, FISH, GISH, genomic hybridization, heterochromatin, holokinetic chromosomes, karyotype, NOR, rDNA, SIT, sex chromosomes.

Résumé : Les auteurs ont effectué une analyse caryotypique détaillée du carpocarpse, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), le principal insecte nuisible des fruits à pépins des régions tempérées. Le caryotype du carpocarpse compte $2n = 56$ chromosomes de type holokinétique. Les chromosomes ont été classifiés en 5 groupes selon leur taille : très grands (3 paires), grands (3 paires), moyens (15 paires), petits (5 paires), et ponctuels (2 paires). Chez les noyaux en pachytène des deux sexes, un curieux bivalent NOR a été observé. Il porte deux nucléoles, chacun associé à une extrémité du bivalent. Des analyses FISH avec une sonde d'ADN ribosomique 18S ont confirmé la présence de deux amas de gènes d'ARNr aux extrémités opposées du bivalent. Conformément, deux chromosomes homologues portant des NOR ont été identifiés en métaphase mitotique, chacun de ceux-ci montrant un signal aux deux extrémités. Chez les noyaux somatiques hautement polyploïdes, les femelles présentaient une grande masse hétérochromatique, la soi-disant chromatine sexuelle ou chromatine W. Ce corps hétérochromatique était absent des noyaux des mâles, ce qui suggère un système de chromosomes sexuels WZ/ZZ (femelle/mâle). Chez les oocytes en pachytène, un bivalent de chromosomes sexuels (WZ) était facilement identifiable en raison de son brin W hétérochromatique. Afin d'étudier la différenciation moléculaire des chromosomes sexuels, les auteurs ont employé l'hybridation génomique in situ (GISH) et l'hybridation génomique comparée (CGH). L'approche GISH a permis de détecter le chromosome W en raison de sa forte hybridation avec une sonde d'ADN femelle marquée au Cy3. En analyse CGH, tant la sonde femelle marquée au Cy3 qu'une sonde mâle marquée au Fluor-X ont hybridé également au chromosome W. Ces méthodes d'identification du chromosome W faciliteront le développement de souches de sexage, lesquelles seraient souhaitables afin de faciliter le contrôle de ce ravageur à l'aide d'insectes stériles.

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Mots clés : CGH, carpocarpse, FISH, GISH, hybridation génomique, hétérochromatine, chromosomes holokinétiques, caryotype, NOR, ADNr, SIT, chromosomes sexuels.

[Traduit par la Rédaction]

Introduction

Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a well known insect thanks to its larva, which is the familiar apple worm. Its origin is Eurasian, but during the past 200 years, it dispersed around the world following the spread of cultivation of apples and pears. Presently, it is the key pest of most pome fruit (apple, pear, and quince) and some walnut orchards in temperate regions of the world, including North America, South America, South Africa, Australia, and New Zealand (Barnes 1991). As a result of its economic impact, it has received considerable attention from both growers and researchers. Besides conventional chemical control, various biological methods have been developed and used to keep codling moth populations low and minimize damage caused by their larvae (Falcon and Huber 1991; Calkins 1998).

Codling moth is also considered to be one of the best candidates of lepidopterous species for the application of the sterile insect technique (SIT) (Proverbs et al. 1982; LaChance 1985) or a related suppression strategy, radiation-induced inherited sterility (IS) (Anisimov et al. 1989; Bloem et al. 1999, 2001). In 1992, an area-wide SIT program against codling moth was initiated in the Okanagan Valley, British Columbia (Dyck and Gardiner 1992). Within several years of operation, codling moth infestation had been eliminated in most treated orchards, while the use of insecticides had been drastically reduced (Bloem and Bloem 2000). Successful implementation of the program in British Columbia, along with increasing codling moth resistance problems and public pressure to decrease insecticide applications, resulted in demands for the expansion of codling moth SIT in other countries and continents.

In the current SIT program against codling moth in the Okanagan, both sterile males and females are released. Notwithstanding the success of this program, there is a considerable potential to increase the efficiency of both the SIT and IS technologies by removing females from the release population. In a mass-rearing facility, such as the one near Osoyoos in the Okanagan with a production capacity of 15 million codling moths per week (L. Tomlin, personal communication 2004), this can only be achieved through the use of genetic sexing strains, which would only produce males for irradiation and release. For the efficiency of SIT programs, the benefits of a male-only population release, as compared with those of a bisexual population release, have been clearly documented in the medfly *Ceratitis capitata* (Wiedemann) (Rendón et al. 2004). However, such a genetic sexing strain is not yet available in the codling moth.

In 2 other Lepidoptera, the silkworm *Bombyx mori* (L.) (Strunnikov 1975) and the Mediterranean flour moth *Ephestia kuehniella* Zeller (Marec et al. 1999), a genetic sexing system based on the construction of balanced lethal (BL) strains has been developed. However, this system is not easily applicable under mass-rearing conditions because of the need to maintain two strains (BL and wild type) that have to be crossed to produce the male-only progeny. Recently, a new scheme for genetic sexing in Lepidoptera has been proposed (Marec et al. 2005). The scheme is based on the construction of transgenic females carrying a dominant conditional lethal gene in the female-determining chromosome W, which appears technically feasible and more practical than the use of BL strains. Following this scheme, we intend to develop transgenic sexing strains in the codling moth. This requires basic knowledge of the codling moth genome and appropriate molecular tools for transgenesis.

Unfortunately, very little is known about the genetics of the codling moth (Robinson 1971; Benz 1991), which makes the development of genetic sexing strain(s) difficult. There is a lack of suitable markers for mutation experiments. Three autosomal recessive mutations kept earlier in St. Petersburg, Russia, pink eyes, light wings, and dark moth, had been lost (A.I. Anisimov, personal communication 1997). The only visible marker currently available is a golden moth, which is reared in the Codling Moth Mass Rearing Facility, Ossoyos, British Columbia (L. Tomlin, personal communication 2004). However, this mutation is also autosomal recessive (Hutt and White 1975), and thus of no use as a marker of sex chromosomes. In addition, no published data are available on codling moth sex chromosomes. The only cytogenetic study, that of Ortiz and Templado (1976), only reported the number of chromosomes in males: $2n = 56$ (or $n = 28$). To fill this gap, we present a detailed analysis of the codling moth karyotype. A particular interest is devoted to the identification and molecular differentiation of sex chromosomes. We also discuss the potential of codling moth sex chromosomes for the development of transgenic sexing strains.

Materials and methods

Codling moth colony

We established a laboratory colony of the codling moth from a sample of the laboratory strain provided by A. Alešová (Prague, Czech Republic) in June 2002. The strain has been maintained in the laboratory of I. Hrdý at the Institute of Organic Chemistry and Biochemistry ASCR (Prague) since 1991. It originated from a wild population collected in Krym, Russia, and adapted to an artificial diet at the Codling Moth Mass Rearing Station (Simfropol, Krym, Russia) in 1961. For research purposes we named the strain Krym-61.

Codling moth larvae were reared on an artificial diet (see below) in a constant-temperature room under nondiapausing conditions (27±1 °C ; 16 h light : 8 h dark regime), without humidity control. Emerged moths were placed in plastic boxes with a piece of sponge soaked with water where they mated and laid eggs. Egg collections were treated with 1% peracetic acid for 30 min to sterilize the egg surface, then washed with tap water and placed for hatching on top of the artificial diet.

The artificial diet was prepared as follows. Agar (100 g), wheat germ (800 g), barley germ (125 g), and dried yeast (125 g) were mixed and boiled in 5.6 L of water for 30 min.

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Then the diet was supplemented with ascorbic acid (25 g), citric acid (25 g), methylparaben (8.5 g), vitamin B-complex (0.35 g), and sorbic acid (7 g).

Chromosome preparations

In both sexes, spread preparations of mitotic and meiotic chromosomes were made from gonads of 5th instar larvae as described in Mediouni et al. (2004). Some preparations were stained and mounted in 2.5% lactic acetic orcein and inspected with phase-contrast optics. For visualization of nucleoli, spread pachytene bivalents were stained with $AgNO_3$ following the protocol of Howell and Black (1980). Briefly, 2 drops of 50% $AgNO_3$ in water and 1 drop of 2% gelatine in 1% formic acid were mixed on a cover slip. The cover slip was then turned onto the preparation. The slide was incubated on a heating plate at 40 °C until the colour of the preparation changed to light brown. The preparation was washed with tap water and air dried. For fluorescence microscopy, preparations were either stained with 0.1–0.5 µg DAPI (4′,6-diamidino-2-phenylindole)/mL (Sigma-Aldrich, St. Louis, Mo.) in PBS (phosphate-buffered saline) buffer containing 1% Triton X-100 (Sigma-Aldrich) or with 4 nmol YOYO-1/L (Molecular Probes Inc., Eugene, Ore.) in PBS buffer containing 1% Triton X-100. Fluorescent preparations were mounted in anitfade based on DABCO (Sigma-Aldrich) (for composition, see Mediouni et al. 2004).

Squash preparations were made only from the testes of 5th instar male larvae. Testes were pretreated for 10 min in a hypotonic solution (75 mmol KCl/L), stained without fixation in 2.5% lactic acetic orcein for 1 h, transferred on a slide into a drop of lactic and acetic acids (1:1), covered with a coverslip, and squashed.

For fluorescence in situ hybridization (FISH) techniques (see below: rDNA–FISH, genomic in situ hybridization (GISH), and comparative genomic hybridization (CGH)), spread chromosome preparations were obtained from the gonads of 5th instar larvae as described in Mediouni et al. (2004). The preparations were passed through an ethanol series (70%, 80%, and 100%; 30 s each) and stored at –20 °C until further use.

Preparation of polyploid nuclei

Malpighian tubules (from larvae and adults) and silk glands (from larvae) were dissected out in a saline solution, briefly fixed, and stained with 1.5% lactic acetic orcein. Highly polyploid nuclei in these cells were inspected in a light microscope at a magnification of 200×–400× for the presence of sex chromatin (see Traut and Marec 1996).

FISH with 18S rDNA probes

Unlabelled 18S rDNA probes were generated by PCR using 2 primers, 18S-Gal forward (5′-CGATACCGCGAATGGCTCAATA-3′) and 18S-Gal reverse (5′-ACAAAGGGCAGGGACGTAATCAAC-3′), custom-made by Generi Biotech (Hradec Králové, Czech Republic). The primers were designed according to the complete 18S rDNA sequence of the wax moth *Galleria mellonella* (L.) (Whiting 2002; GenBank accession No. AF286298), using Lasergene analysis software (DNAStar Inc., Madison, Wis.). PCR was done in a Mastercycler® ep Gradient S (Eppendorf AG, Hamburg, Germany). Reactions were carried out in 25-µL reaction volumes containing 1× Ex *Taq* buffer and 2 U *TaKaRa Ex Taq* HS DNA polymerase (Takara, Otsu, Japan), 200 µmol dNTP/L mix, 500 nmol each primer/L, and 100 ng of template genomic DNA extracted from codling moth adults by a standard procedure (see below). An initial denaturation period of 3 min at 94 °C was followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, and by a final extension step of 5 min at 72 °C. The PCR product showed a single band of about 1650 bp on a 1% agarose gel. The band was cut out from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and used as a template for PCR amplification of 18S rDNA probes. The probes were labelled with biotin-14-dATP by nick translation using a Bionick Labeling System (Invitrogen, Life Technologies Inc., San Diego, Calif.).

For FISH, we used the procedure described in Sahara et al. (1999) with slight modifications. Chromosome preparations were digested with 100 µg/mL RNase A in 2× SSC buffer for 1 h at 37 °C and washed twice for 15 min in 2× SSC. Denaturation of chromosomes was done at 68 °C for 3 min and 45 s in 70% formamide in 2× SSC. The probe cocktail for one slide (10 µL; 50% formamide, 10% dextran sulphate, 2× SSC) contained 15 ng of biotinylated 18S rDNA probe and 15 µg of sonicated salmon sperm DNA (Sigma-Aldrich). Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, Pa.), one round of amplification with biotinylated anti-steptavidin (Vector Labs. Inc., Burlingame, Calif.), and Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 µg DAPI/mL and mounted in DABCO antifade.

Comparative genomic hybridization (CGH) and genomic in situ hybridization (GISH)

Genomic DNA was isolated separately from female and male adults by standard phenol–chloroform extraction. Labelling was done using a Nick Translation System (Invitrogen) or a Nick Translation Mix (Roche Diagnostics GmbH, Mannheim, Germany). Female DNA was labelled with Cy3-dCTP (red), and male DNA with FluorX-dCTP (green) (Amersham Life Science, Arlington Heights, Ill.).

Comparative genomic hybridization was carried out following the procedure in Traut et al. (1999) with slight modifications. After removal from the freezer, chromosome preparations were passed through the ethanol series and air dried. Denaturation of chromosomes was done at 68 °C for 3 min and 45 s in 70% formamide in 2× SSC buffer. The probe cocktail for one slide (10 µL; 50% formamide, 10% dextran sulphate, 2× SSC) contained 300 ng of labelled female genomic DNA, 300 ng of labelled male genomic DNA, 3 µg of unlabelled sonicated competitor DNA from males, and 25 µg of sonicated salmon sperm DNA. Hybridization was carried out for 3 days at 37 °C. A stringent wash for 5 min at 62 °C in 0.1× SSC containing 1% Triton X-100 was followed by washes at room temperature, counterstaining with 0.1 µg DAPI/mL in PBS buffer for 5 min, and mounting in DABCO antifade.

The procedure for GISH was the same as that for CGH, except for the probe cocktail. For one slide, the cocktail contained 300 ng of labelled female genomic DNA, 3 µg of unlabelled sonicated male genomic DNA, and 25 µg of

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sonicated salmon sperm DNA in 10 μL of hybridization solution.

Microscopy and image processing

Preparations were observed in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black-and-white images were recorded with a cooled F-View CCD camera and captured with AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). In FISH preparations, images were captured separately for each fluorescent dye and then pseudocoloured (light blue for DAPI, green for FluorX, and red for Cy3) and superimposed with Adobe Photoshop, version 6.0.

Results

Codling moth karyotype

Preparations from larval testes confirmed the chromosome number reported by Ortiz and Templado (1976). Mitotic spermatogonia showed 56 mostly rod-shaped chromosomes (Fig. 1*a*), that lacked primary constrictions (the centromeres), which are typical for holokinetic chromosomes in Lepidoptera (Wolf 1996). In metaphase I spermatocytes, 28 bivalents were observed. They were oriented perpendicularly to the equatorial plane, with each homologous chromosome directed towards the opposite pole (Fig. 1*b*). Metaphase II complements showed 28 oval chromosomes (Fig. 1*c*). We concluded that the karyotype of codling moth males consists of $2n = 56$ ($n = 28$) chromosomes. The chromosomes were classified into 5 groups according to their sizes (Fig. 2): extra large (3 pairs), large (3 pairs), medium (15 pairs), small (5 pairs), and dot-like (2 pairs). The largest chromosomes (pair no. 1) were about 3 μm long; the smallest one about 0.5 μm long (pair nos. 27 and 28). A comparison of male and female preparations (see later) revealed that the two largest chromosomes, standing out by their size in spermatogonial metaphases (see Fig. 1*a*) and forming the largest metaphase I bivalent (see Fig. 1*b*), most probably represent a ZZ pair of the sex chromosomes.

Nucleolar organizing region (NOR) chromosomes and localization of rDNA

In spread preparations of pachytene nuclei of both sexes, a curious bivalent was observed after staining with orcein using phase-contrast microscopy. This was often found, approximately at the pachytene stage, displaying a horseshoe shape with both ends immersed into a weakly visible mass (Fig. 1*d*). Silver staining revealed that this mass represents a nucleolar material, which is associated with the NOR bivalent (Fig. 1*e*). The use of a fluorescent dye, YOYO-1, which stains both DNA and RNA (compare with Weeks et al. 2001), disclosed that the NOR bivalent carries 2 spherical nucleoli, each associated with one end of the bivalent (Fig. 3*a*). In some pachytene nuclei, the NOR bivalent appeared as though it were opening up in both terminal regions. The ends of both homologous chromosomes took no pairing. Then each end displayed a nucleolus, and therefore a total of 4 nucleoli were observed. This observation indicated that the terminal association of prominent nucleolar structures obstructed precise synapsis (Fig. 3*b*). A similar phenomenon was observed in NOR bivalents of the flour moth *Ephestia kuehniella* (Marec and Traut 1993).

FISH experiments with the 18S rDNA probe confirmed that the codling moth genome has 2 clusters of rRNA genes located at opposite ends of a chromosome pair. In mitotic metaphases, 2 obviously homologous chromosomes showed strong hybridization signals at both ends (Fig. 3*c*). According to their size, the chromosomes were classified into the most numerous group of medium chromosomes. In pachytene nuclei, 2 prominent clusters of hybridization signals were associated with both ends of the NOR bivalent (Fig. 3*d*). The signals were arranged in spheres. This feature was similar to that in the nucleoli seen in YOYO-1-stained preparations. Since rRNAs processed in the nucleoli were digested by RNase A, we believe that the signals reflect the distribution of rRNA genes, but not transcribed rRNAs, that could also hybridize with the probe. Thus, the signal distribution suggests that the rDNA forms a kind of network in the nucleoli to facilitate transcription (compare with Schwarzacher and Wachtler 1993; Wachtler and Stahl 1993).

Sex chromatin status and sex chromosome system

Preparations of the Malpighian tubules from larvae showed large oval nuclei. Each female nucleus displayed a single, spherical heterochromatin body (Fig. 4*a*). Similar bodies were observed in branched nuclei of the silk glands from female larvae and in lobbed nuclei of the Malpighian tubules from adult females (not shown). No heterochromatin was observed in somatic polyploid nuclei of males (Fig. 4*b*). The heterochromatin body in female somatic nuclei represents the sex chromatin (W chromatin), which is typical in Lepidoptera females and is formed by multiple copies of the W chromosome (see review by Traut and Marec 1996). The presence of the sex chromatin body in codling moth females and its absence in males suggested a WZ/ZZ (female/male) sex chromosome system.

In spread preparations from oocytes without a hypotonic treatment, pachytene bivalents showed a typical chromomere–interchromomere pattern. Autosomal bivalents displayed pairs of chromomeres that were characteristic of the synapsis of homologous chromosomes. In keeping with the sex chromatin status, a sex chromosome bivalent (WZ) was discernible due to its heterochromatic W thread and characteristic twisting of the Z chromosome along the shorter W (Fig. 5*a*; compare with Marec and Traut 1994). In addition, the WZ bivalent was always the longest bivalent. In metaphase oogonia, 56 chromosomes were regularly observed, as they are in spermatogonia. Among them, 1 of the 2 largest chromosomes was deeply stained with DAPI (Fig. 5*b*), indicating a high content of A–T base pairs. This heterologous element was obviously the W sex chromosome, consisting largely of heterochromatin. Accordingly, the 2 largest chromosomes standing out in metaphase spermatogonia most probably represented 2 homologous Z chromosomes (see Figs. 1*a* and 2).

Molecular differentiation of sex chromosomes by GISH and CGH

Recently, it has been shown that GISH and CGH can differentiate the heterologous W chromosome from other chromosomes in Lepidoptera because its DNA composition differs from that of the Z chromosome (Traut et al. 1999;

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Fig. 1. Chromosome preparations of testes of codling moth larvae stained with either orcein (*a–d*) or $AgNO_3$ (*e*). (*a*) Spread mitotic metaphase showing 56 chromosomes. Arrows indicate putative Z chromosomes. (*b*) Squashed meiotic metaphase I showing 28 bivalents. Arrow indicates the putative sex chromosome pair ZZ. (*c*) Squashed meiotic metaphase II showing 28 chromosomes. (*d*) Spread pachytene spermatocyte displaying grey mass of nucleolar material (arrowhead) adjacent to the NOR bivalent (arrow). (*e*) A pachytene NOR bivalent (arrow) with both ends entering deeply stained mass of nucleolar material (arrowhead). Bar = 3 μm (*a–c*); bar = 10 μm (*d* and *e*).

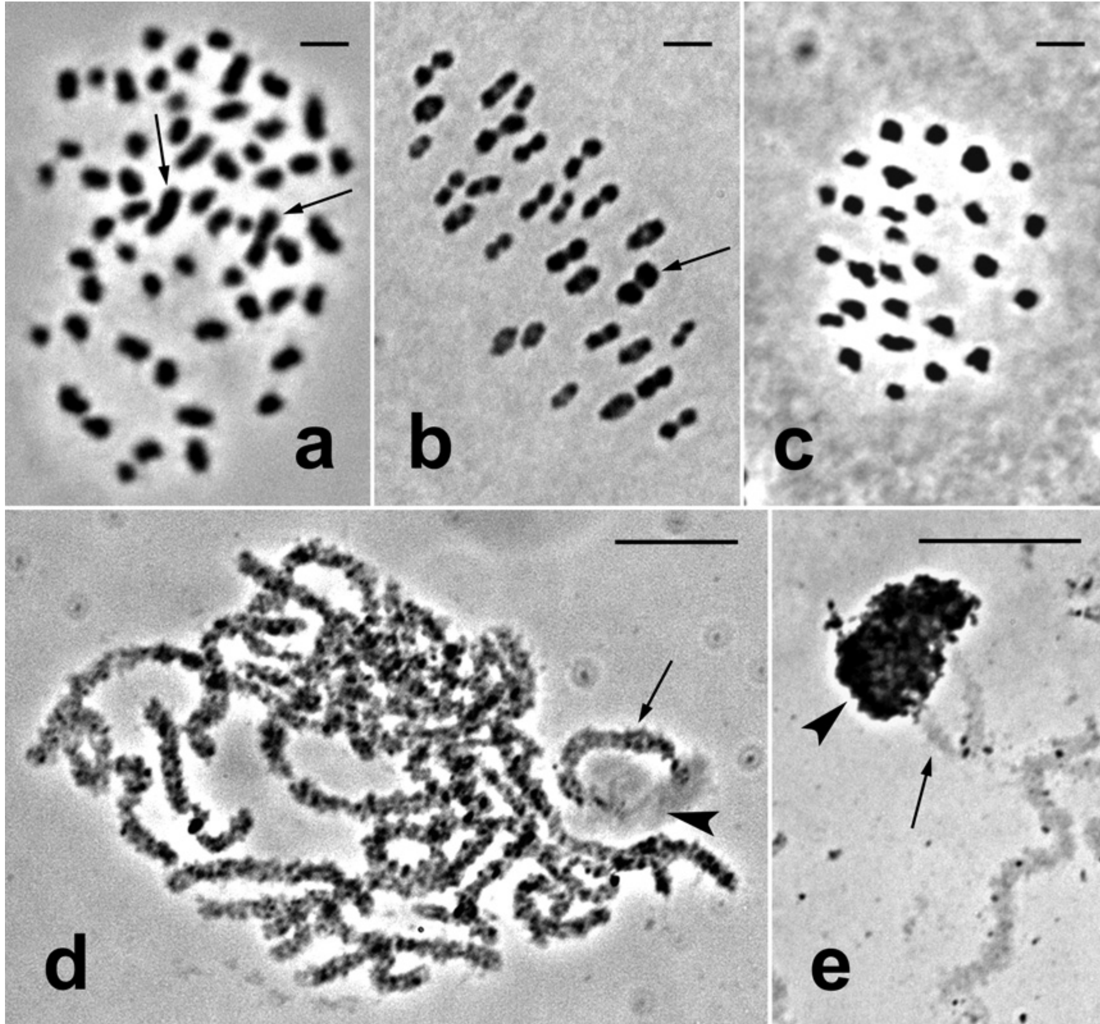

Fig. 2. Codling moth male karyotype derived from the spermatogonial metaphase shown in Fig. 1*a*. Chromosomes are assembled into 5 classes according to their sizes: EL, extra large; L, large; M, medium; S, small; D, dot-like. ZZ, a sex chromosome pair. Vertical bar = 3 μm.

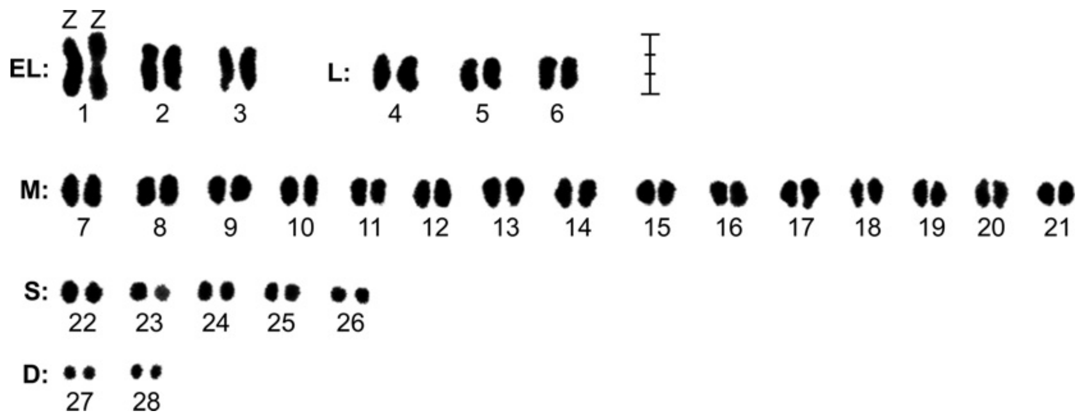

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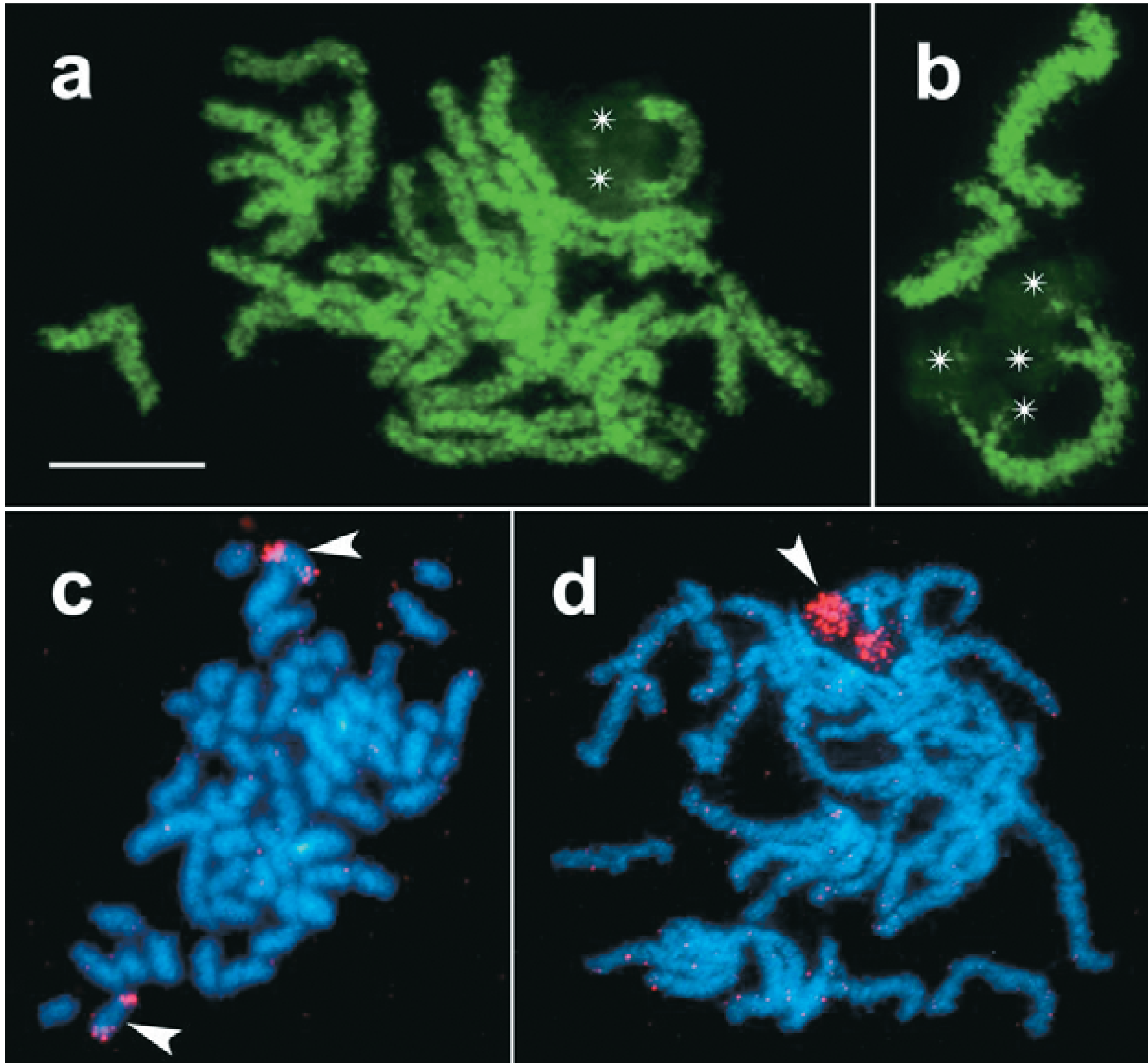
Fig. 3. Localization of nucleolar organizer regions (NORs) in chromosomes of codling moth males either by staining with YOYO-1 (*a* and *b*; green) or by FISH with 18 rDNA probes (*c* and *d*; red signals). Chromosomes are counterstained with DAPI (blue). (*a*) Spread pachytene complement. Note 2 spherical nucleoli (asterisks) at the ends of the NOR bivalent. (*b*) NOR bivalent with incompletely paired terminal segments showing 4 nucleoli (asterisks), each associated with one end of the homologous chromosomes. (*c*) Spermatogonial metaphase with strong hybridization signals at both ends of 2 NOR chromosomes (arrowheads). (*d*) Pachytene complement showing a NOR bivalent with clusters of hybridization signals at both ends (arrowhead). Bar = 10 µm.



Sahara et al. 2003*a*, 2003*b*). Here we used these methods to confirm the identification of the W chromosome and the WZ bivalent.

With GISH, the W chromosome was easily identified by strong binding of the female genomic probe in all mitotic and meiotic chromosome stages, as well as in interphase nuclei available to spread preparations of ovaries. Molecular differentiation of the W chromosome in mitotic oogonia confirmed that it is indeed 1 of the 2 largest chromosomes (Fig. 6*a*). We estimated that it comprises about 4% of the female genome. In pachytene oocytes, the use of labelled female-derived probe under the excess of unlabelled male genomic DNA highlighted almost the entire W chromosome except for short segments in both subtelomeric regions, which appeared less stained and (or) even interrupted with unlabelled gaps (Fig. 6*b*).

With CGH, both the Cy3-labelled (red) female-derived probe and Fluor-X labelled (green) male-derived probe evenly bound to the W chromosome, resulting in a yellow colouring of the W chromosome thread (Fig. 6*c*). A detailed analysis of the WZ bivalent at the pachytene stage revealed the same labelling pattern in the W chromosome with both the male- and female-derived probes (Figs. 6*d*, 6*e*, and 6*f*). The W chromosome was homogeneously labelled over most of its length except for a few unlabelled gaps. However, similar weakly stained gaps were also seen on counterstained images showing DAPI-highlighted heterochromatin of the W chromosome (Fig. 6*d*). This suggested that these gaps are

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Fig. 4. Highly polyploid nuclei of Malpighian tubule cells in codling moth larvae, stained with orcein. (*a*) A female nucleus showing a deeply stained W chromatin body (arrow). (*b*) A male nucleus of the Malpighian tubule cell without W chromatin body. Bar = 50 µm.

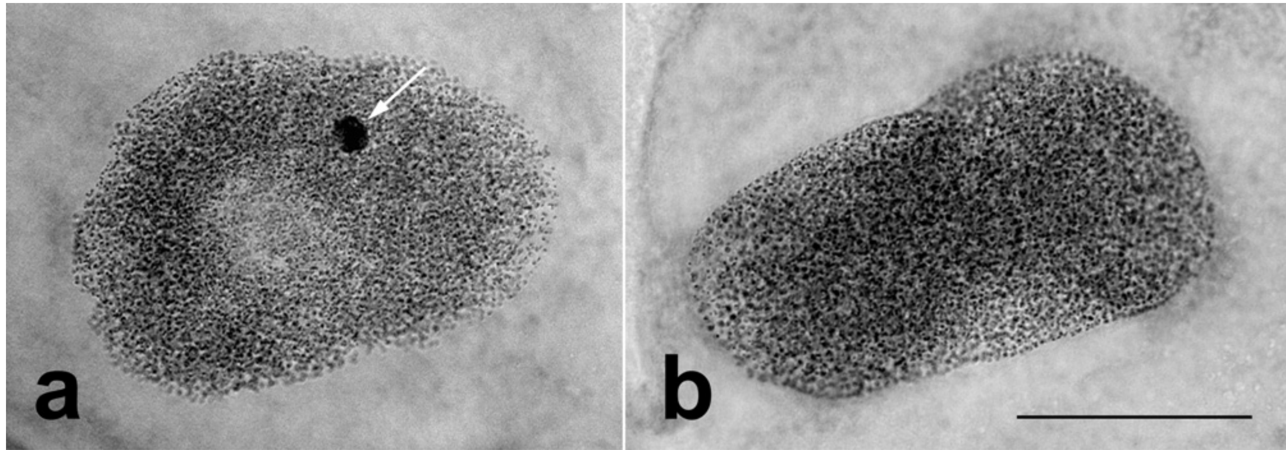
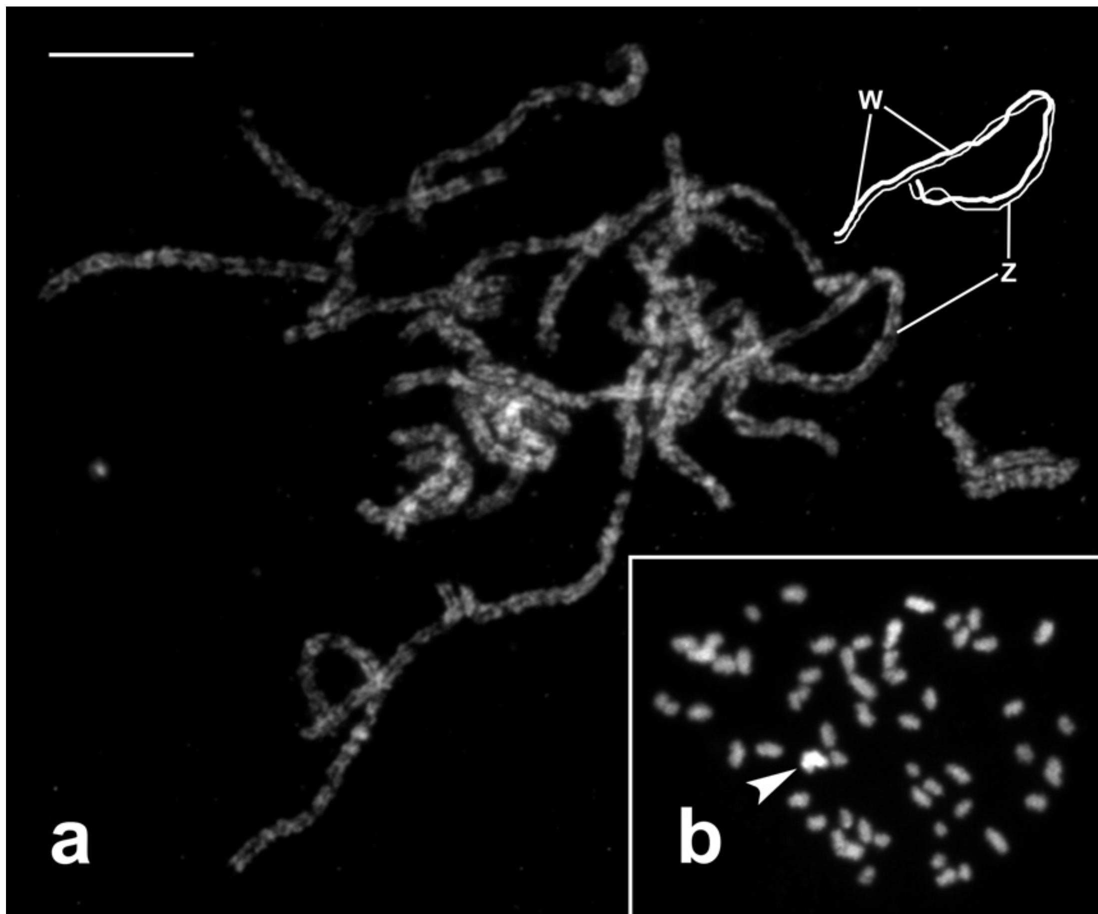


Fig. 5. Chromosome complements of codling moth females. Fluorescent images, stained with DAPI. (*a*) Spread pachytene nucleus with preserved chromomere–interchromomere pattern. The WZ bivalent (see a schematic drawing) was identified according to the heterochromatic thread of the W chromosome, weakly highlighted with DAPI. (*b*) Spread mitotic metaphase with a deeply stained W chromosome (arrowhead). Bar = 10 µm.



artefacts of the spreading procedure rather than undifferentiated regions of homology with the Z chromosome. In contrast with W, the Z chromosome did not show enhanced binding of either probe.

Discussion

Chromosomes in Lepidoptera are generally small and numerous and lack differentiating features, mainly because of

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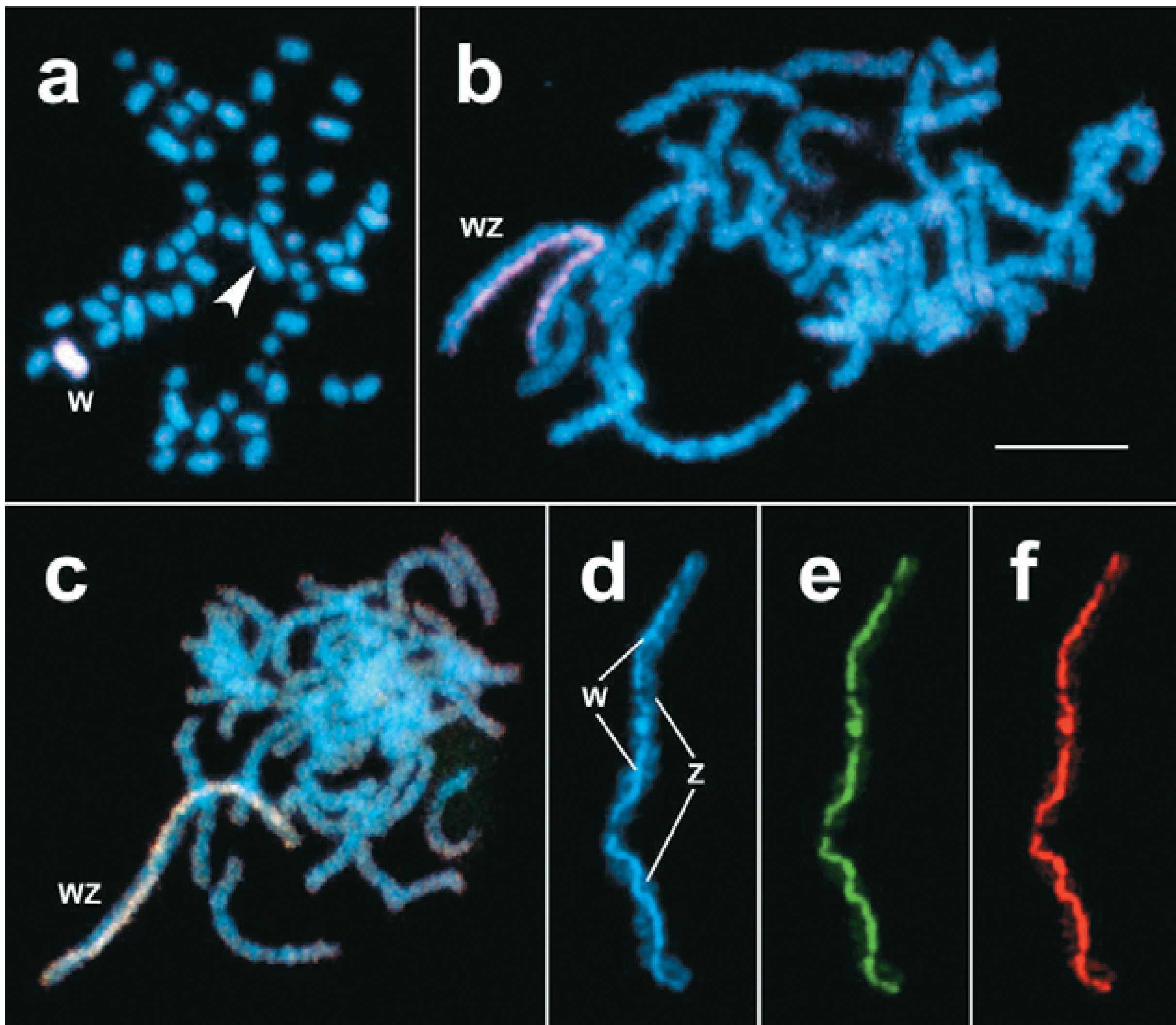
Fig. 6. Genomic in situ hybridization (GISH; *a–b*) and comparative genomic hybridization (CGH; *c–f*) in codling moth females. Chromosomes were counterstained with DAPI (blue). Female-derived probes were labelled with Cy3 (red), male-derived probes with Fluor-X (green). (*a*) Mitotic metaphase with a W chromosome strongly highlighted by female-derived probe. Arrowhead indicates the Z chromosome. (*b*) Pachytene complement. The WZ bivalent was recognized by prominent hybridization of the female-derived probe to the W chromosome. (*c*) Pachytene complement. The WZ bivalent shows a strong binding of both female- and male-derived probes to the W chromosome. (*d–f*) A detailed analysis of the pachytene WZ bivalent stained with CGH. (*d*) DAPI image. (*e*) Signal from male-derived probe. (*f*) Signal from female-derived probe. Bar = 10 μm.



their holokinetic structure. Most species are reported to have haploid numbers close to 30 (n = 28–32) (Suomalainen 1969; Robinson 1971). A karyotype of the codling moth with 28 chromosome pairs ($2n$ = 56) does not differ from the general characteristics of lepidopteran species. The modal, and possibly also ancestral, chromosome number appears to be n = 31 for Lepidoptera, and a typical karyotype structure shows a gradual decrease in chromosome size (reviewed by De Prins and Saitoh 2003). For the family Tortricidae, a slightly lower modal number, n = 30, was determined (Robinson 1971). This number was reported for most of about 27 tortricid species that have been karyotyped. This number is conserved in the subfamily Tortricinae, except for one species with n = 29 (summarized by Ortiz and Templado 1976; Harvey 1997). Meanwhile, in the subfamily Olethreutinae, to which the codling moth belongs, the haploid number of chromosomes varied from n = 22 to n = 28 (Ortiz and Templado 1976; Emelianov et al. 2004). When we gave consideration to conspicuous differences in the size of individual chromosomes shown in this study, chromosome rearrangements, especially fusions, participated in the karyotype evolution of the codling moth ancestors.

Our study revealed that the diploid genome of the codling moth has 2 pairs of NORs constituted by rDNA clusters, which are responsible for the formation of 4 nucleoli. This number of NORs/nucleoli could be an ancestral character of the Lepidoptera, since it occurs in species with modal or close to modal chromosome numbers, such as *Ephestia kuehniella* with n = 30 (Marec and Traut 1993) and *Ectomyelois ceratoniae* Zeller with n = 31 (Mediouni et al. 2004). However, in these species, the NORs are located in 2 different bivalents, whereas in the codling moth, 1 bivalent

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carries 2 NORs located at the opposite ends of the bivalent. This bivalent might have arisen through the fusion of 2 ancestral NOR bivalents. In *B. mori*, a single NOR bivalent was also reported (Traut 1976; Sahara et al. 2003*b*). In the case of *B. mori*, the bivalent displays only 1 nucleolus in an interstitial position. It is possible to indicate a fusion event of 2 different NOR chromosomes through their terminal NORs.

Our study also showed that the codling moth has a WZ/ZZ (female/male) sex chromosome system, which is common in lepidopteran insects (Traut and Marec 1996, 1997; Lukhtanov 2000). In both females and males, the sex chromosomes were the largest elements in the chromosome complement. According to Ortiz and Templado (1976), 1 large chromosome pair was found in each karyotyped species of the family Tortricidae. Recently, it was also reported for the larch budmoth, *Zeiraphera diniana* (Guenée), with $n = 28$ (Emelianov et al. 2004). The large chromosome pair might represent a sex chromosome pair, as it does in the codling moth.

Basic characteristics of W and Z chromosomes in the codling moth are similar to those found in a number of other lepidopterans (compare with Traut and Marec 1997). The codling moth W chromosome appears to consist completely of heterochromatin and is DAPI-positive, which indicates a high content of A–T base pairs. On the other hand, the Z chromosome consists of euchromatin, and it is not differentiated by DAPI staining from the autosomes. In spite of the apparent lack of homology, the WZ pair forms a regular bivalent in pachytene oocytes. This constitution is typical in lepidopteran females (compare with Marec and Traut 1994).

Here we showed that the codling moth W chromosome can be easily identified with GISH or CGH at all mitotic and meiotic chromosome stages and even in interphase nuclei on spread preparations of ovaries. Results obtained from these experiments allowed us to draw the following conclusions: (*i*) The easy differentiation with GISH implies that the gross DNA composition of the W chromosome is thoroughly different from that of the Z chromosome and autosomes; and (*ii*) the labelling pattern with CGH showed that the W was evenly differentiated by both the female and male genomic DNA probes. This result suggests an accumulation of repetitive sequences and transposons occurring scattered in Z and autosomes but accumulated in the W chromosome. It also suggests a low number of W-specific DNA sequences. A similar deduction was done for the W chromosomes of *B. mori* (Sahara et al. 2003*a*) and *Ectomyelois ceratoniae* (Mediouni et al. 2004). In the two species, the female- and male-derived probes also bound evenly to the W chromosome, but differentiation of the W from the Z chromosome was much weaker than it was in the codling moth. A high level of molecular differentiation of the W chromosome was found in *Ephestia kuehniella* and *G. mellonella*, but in contrast with the codling moth, this was mainly as a result of a strong binding of the female-derived probes, indicating the presence of W-specific DNA sequences (Traut et al. 1999; Sahara et al. 2003*a*).

In conclusion, the codling moth W chromosome is one of two largest chromosomes, which makes it a good target for insertion of a transgene to develop a genetic sexing strain for codling moth SIT according to the proposal of Marec et al. (2005). On the other hand, the W chromosome consists of heterochromatin, which may reduce the probability of an insertion event and (or) silence the expression of the transgene. Such silencing of genes, placed into the neighbourhood of heterochromatin, is well known in *Drosophila* as so-called position–effect variegation (Schotta et al. 2003). Although there are no reports of a similar phenomenon in Lepidoptera, this has to be taken into account when designing plasmid constructs for codling moth transgenesis.

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Chapter four:
Probing the W chromosome of the codling moth, Cydia pomonella*, with sequences from microdissected sex chromatin*

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RESEARCH ARTICLE

Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin

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Abstract The W chromosome of the codling moth, *Cydia pomonella*, like that of most Lepidoptera species, is heterochromatic and forms a female-specific sex chromatin body in somatic cells. We collected chromatin samples by laser microdissection from euchromatin and W-chromatin bodies. DNA from the samples was amplified by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) and used to prepare painting probes and start an analysis of the W-chromosome sequence composition. With fluorescence in situ hybridization (FISH), the euchromatin probe labelled all chromosomes, whereas the W-chromatin DNA proved to be a highly specific W-chromosome painting probe. For sequence analysis, DOP-PCR-generated DNA fragments were cloned, sequenced, and tested by Southern hybridization. We recovered single-copy and low-copy W-specific sequences, a sequence that was located only in the W and the Z chromosome, multi-copy sequences that were enriched in the W chromosome but occurred also elsewhere, and ubiquitous multi-copy sequences. Three of the multi-copy sequences were recognized as derived from hitherto unknown retrotransposons. The results show that our approach is feasible and that the W-chromosome composition of *C. pomonella* is not principally different from that of *Bombyx mori* or from that of Y chromosomes of several species with an XY sex-determining mechanism. The W chromosome has attracted repetitive sequences during evolution but also contains unique sequences.

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Introduction

The W chromosome is an equivalent of the Y chromosome in sex chromosome systems with heterogametic females such as WZ/ZZ (female/male) and derived variants. Female heterogamety is common in vertebrates, as it is conserved in birds (Pigozzi 1999; Ellegren 2000) and snakes (Jones and Singh 1985) and also occurs in some lizards, amphibians, and fish (Wallace et al. 1999; Graves and Shetty 2001; Schmid and Steinlein 2001; Schartl 2004). In invertebrates, however, it is known only in two sister orders of insects, the Trichoptera (caddis flies) and Lepidoptera (moths and butterflies; Traut 1999), and in parasitic fluke worms of the family Schistosomatidae (Špakulová and Casanova 2004). Heterogametic females were also reported from a few plant species, but this remains to be confirmed (Vyskot and Hobza 2004).

In Lepidoptera, the W chromosome is found in the advanced clade that combines Tischeriina and Ditrysia and contains the vast majority of moth and butterfly species, whereas primitive Lepidoptera clades share the ancestral Z/ZZ (female/male) sex chromosome system with related caddis flies (Marec and Novák 1998; Traut and Marec

1997; Lukhtanov 2000). The lepidopteran W chromosome, like W chromosomes in birds and many Y chromosomes in XY systems, is heterochromatin-rich and gene-poor. In somatic interphase nuclei, it forms a heterochromatin body (or bodies), the so-called 'W chromatin' or 'sex chromatin', which is easily discernable in polyploid cells, where it consists of multiple copies of the W chromosome (Traut and Marec 1996). In the flour moth, *Ephestia kuehniella* Zeller, the W body comprises more than 1,000 W chromosomes in some polyploid tissues (Traut and Scholz 1978). In contrast to Y chromosomes in flies and mammals, lepidopteran W chromosomes pair completely during meiotic prophase I and form regular synaptonemal complexes with the Z chromosome in spite of the obvious lack of homology (Weith and Traut 1986; reviewed by Marec 1996). In this respect, it resembles the W chromosomes in birds, which also show heterosynapsis with the Z chromosomes (Pigozzi 1999). Unlike generally small Y chromosomes in many animals, such as beetles, flies, and mammals (Virkki et al. 1991; Graves 2005; Steinemann and Steinemann 2005), and small W chromosomes in carinate birds (Pigozzi 1999), the lepidopteran W is more or less comparable in size with the Z chromosome, and the WZ pair often belongs to the largest chromosomes in the genome (Traut and Marec 1997; Traut et al. 1999; Mediouni et al. 2004; Fuková et al. 2005; Yoshido et al. 2005). The females of Lepidoptera lack crossing-over, and their meiosis is achiasmatic (Marec 1996 and references therein). Accordingly, the whole W chromosome does not recombine and is thought to be subject to degeneration similar to the *Drosophila* Y chromosome (Steinemann and Steinemann 2005). As it apparently has no pseudoautosomal region, whole-length degeneration can be expected in contrast to the avian W chromosome (Pigozzi 1999) or mammalian Y chromosome (Pask and Graves 1999; Graves 2006).

So far, only few genes have been proposed to be present on the lepidopteran W chromosome. The W-linked egg-size-determining gene, *Esd*, was discovered in the silkworm, *Bombyx mori* (L.) (Kawamura 1988, 1990), using two types of triploid females, ZZW and ZWW. In the tiger swallowtail butterfly, *Papilio glaucus* L., the female-limited dark wing colour was thought to be controlled by a W-linked locus, but observed exceptions in matrilinear inheritance (Scriber and Evans 1987; Scriber et al. 1996) and the lack of association between the maternally transmitted mitochondrial DNA haplotypes and the W-limited phenotype (Andolfatto et al. 2003) make W-linkage questionable. Gotter et al. (1999) showed that the period gene (*per*) in the silkmoth, *Antheraea pernyi* (Guérin-Méneville), is located on the Z chromosome (*perZ*), but several copies of the *per* sequence are found on the W chromosome (*perW*). At least one of the *perW* copies is a functional gene that encodes a truncated protein, whereas another copy (*per* antisense) encodes an antisense RNA. Finally, numerous experimental data suggest that sex in *B. mori* is controlled by a female-promoting epistatic factor located on the W chromosome. However, this putative feminizing gene has not been identified yet (Ohbayashi et al. 2002). Results obtained in sex chromosome mutants of *E. kuehniella* suggest that the W chromosome carries a male-killing factor, which might serve as a feedback control of sexual development rather than as a female determinant (Marec et al. 2001).

Within the past few years, some progress has been achieved in molecular characterization of the lepidopteran W chromosome. Using comparative genomic hybridization (CGH) with male-derived and female-derived genomic DNA probes, Traut et al. (1999) showed that W chromosomes in *B. mori*, *E. kuehniella*, and the wax moth, *Galleria mellonella* (L.), are molecularly well differentiated from the Z chromosomes. A comparison of hybridization signals of the two probes suggested that the W chromosomes consisted largely of DNA sequences of two types: (1) common interspersed repetitive sequences that occur elsewhere in the respective genome but are accumulated in the W chromosomes and (2) W-specific repetitive sequences. In some species, such as *E. kuehniella* and *G. mellonella*, the W-specific sequences predominate, whereas this fraction is barely noticeable in the W chromatin of *B. mori* (Sahara et al. 2003a). The latter finding is in agreement with the difficulty of isolating DNA fragments from the W chromosome of *B. mori*; the few that were recovered contained transposons (Abe et al. 1998, 2000; Ohbayashi et al. 1998). Recently, Sahara et al. (2003b) demonstrated the identification of the W chromosome in *B. mori* using fluorescence in situ hybridization (FISH) with W-chromosome-derived bacterial artificial chromosome (BAC) probes. The W-BAC probes stained the whole W chromosome, although they were derived from W sequences of only about 170 kb. This suggested that the W-BAC clones contain sequences that are spread over the whole W chromosome. Most probably, these regions consist of retrotransposable elements, which are the main structural component of the W chromosome (Abe et al. 2005). Thus, the *B. mori* W chromosome appears to be molecularly rather homogeneous like the chicken W chromosome (Berlin and Ellegren 2004).

A detailed molecular analysis of the lepidopteran W chromosome faces difficulties due to the absence of functional genes and the accumulation of repetitive sequences and transposons. In *B. mori*, two approaches have been used to analyse the W chromosome: (1) identification and subsequent analysis of W-specific randomly amplified polymorphic DNA (RAPD) markers and (2) shotgun sequencing of W-specific BAC clones selected from BAC

libraries using the W-specific RAPD markers. Both approaches revealed preferentially various transposable elements (reviewed by Abe et al. 2005). In this study, we present an alternative approach for obtaining W-chromosome sequences that is based on laser microdissection of W-chromatin bodies from highly polyploid cells of the female Malpighian tubules. W-chromosome DNA was amplified from the W-chromatin samples by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). The DOP-PCR products proved suitable for W-chromosome painting by FISH and for investigating W-chromosome DNA sequences by cloning and sequencing.

For our experiments, we selected a key pest of pome fruit in the temperate regions of the world, the codling moth, *Cydia pomonella* (L.). The codling moth is a member of the family Tortricidae, which together with such species-abundant groups as pyralid moths, noctuid moths, and butterflies, and also the genetic model, *B. mori*, belongs to the lepidopteran clade Ditrysia (Grimaldi and Engel 2004). The codling moth has a diploid chromosome number of $2n=56$ and a WZ/ZZ sex chromosome system. The WZ sex chromosomes represent the largest chromosome pair in the karyotype. The W chromosome is heterochromatic and comprises about 4% of the genome (Fuková et al. 2005). There is an additional motivation to study the W chromosome in this species. We set out to develop genetic sexing strains in the codling moth by inserting selectable transgenes into the W chromosome. For mapping these transgenes, there is a need for W-specific FISH probes and molecular markers of the W chromosome. The aim is to produce male-only progeny for the sterile insect technique (SIT) programmes to control this pest (Marec et al. 2005).

Materials and methods

Insects

We used a laboratory strain of the codling moth referred to as Krym-61. Details about its origin and rearing conditions are described in Fuková et al. (2005). Briefly, codling moth larvae were reared on artificial diet at a constant room temperature of 27±1°C and a non-diapausing light regime of 16L:8D, without humidity control.

Preparation of polyploid nuclei

For laser microdissection of W chromatin, Malpighian tubules were dissected out from the fifth instar female larvae in a physiological solution for *Ephestia* (Glaser 1917, cited by Lockwood 1961), swollen for 10 min in a hypotonic solution (75 mM KCl), and fixed in methanol/acetic acid (3:1) for 15 min. Then the tubules were transferred into a drop of 60% acetic acid, and the cells dissociated with tungsten needles. The resulting suspension of highly polyploid cells was dropped on a glass slide, coated with a polyethylene naphthalate membrane (P.A.L.M. GmbH, Bernried, Germany), and spread at 37°C using a heating plate. To avoid DNA contamination, the membrane was treated with ultraviolet light for 30 min before spreading. Preparations were passed through an ethanol series (70, 80, and 96%, 30 s each), air-dried, and stained with 4% Giemsa for 7 min.

Laser microdissection of W chromatin

W-chromatin bodies of polyploid nuclei of the female Malpighian tubule cells were identified in an inverted microscope and microdissected with the help of a laser microbeam system (P.A.L.M. MicroLaser System, P.A.L.M. GmbH) according to Kubickova et al. (2002). Only well-spread cells with a W-chromatin body close to the edge of the nucleus were selected for microdissection to minimize contamination with euchromatin. The W-chromatin body or, in the control series, a similarly sized piece of euchromatin was cut out with a laser microbeam (1.5–1.7 μJ/pulse, 1 μm in diameter) and catapulted by a single laser pulse (2 μJ/pulse) into a microtube cap containing 2 μl of PCR oil. The chromatin samples (ten chromatin pieces per microtube for cloning and sequencing and 16 pieces per microtube for the painting probe) were dispersed in 20 μl of 10 mM Tris–HCl, pH 8.8.

Preparation of W-chromosome painting probe

DNA of the microdissected samples was amplified by DOP-PCR in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) as described by Kubickova et al. (2002). DOP-PCR reactions were carried out in a total volume of 40 μl. The reaction mixture contained 60 mM Tris–HCl (pH 8.8), 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.6 μM degenerate primers (5′-CCGACTCGAGNNNNNNATGTGG-3′; VBC-Genomics GmbH, Vienna, Austria), 0.05% W-1 detergent, and 2 U *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR conditions were as follows. An initial denaturation step at 96°C for 3 min was followed by eight cycles of denaturation at 96°C for 1 min, annealing at 30°C for 1 min with 2 min transition from 30 to 72°C, and extension at 72°C for 2 min. The following 35 cycles were performed at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The amplification was accomplished by a final extension step at 72°C for 5 min. PCR products were analysed on a 1% agarose gel.

For FISH, the amplified DNA was labelled with SpectrumOrange-dUTP (Vysis, Richmond, UK) by DOP-PCR in a Mastercycler ep Gradient S (Eppendorf AG, Hamburg, Germany). As a template, 2 µl of the initial DOP-PCR reaction was used. The 20-µl labelling reaction contained: 50 mM Tris–HCl (pH 9.3), 15 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.2 mM each dNTP except 0.168 mM dTTP, 20 µM SpectrumOrange-dUTP, 1.6 µM DOP primers, 0.05% W-1, and 1 U LA polymerases Mix (Top-Bio, Prague, Czech Republic). An initial denaturation at 95°C for 3 min was followed by 30 cycles of 15 s at 94°C, 30 s at 56°C, and 2 min at 72°C. A final extension step was carried out at 72°C for 5 min.

Fluorescence in situ hybridization

Spread chromosome preparations were obtained from ovaries of fifth instar larvae as described in Mediouni et al. (2004). Briefly, the ovaries were dissected out, fixed in Carnoy fixative (ethanol, chloroform, acetic acid, 6:3:1), dissociated in 60% acetic acid, and spread on the slide at 45°C using a heating plate. After drying, the preparations were passed through a graded ethanol series (70, 80, and 100%) and stored at −20°C until further use.

FISH was carried out following the procedure of Traut et al. (1999) with slight modifications. The probe cocktail for one slide (10 µl; 50% deionized formamide and 10% dextran sulphate in 2× saline-sodium citrate [SSC] buffer) contained 300 ng of the labelled probe and 25 µg of sonicated salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA). In some experiments also, 3 µg of sonicated codling moth male genomic DNA was included in the probe cocktail as a species-specific competitor. The male competitor weakened hybridization signals, particularly in the euchromatin probe, but showed no obvious effect on the pattern of hybridization signals with W-chromosome probes. Therefore, its usage was omitted from subsequent experiments. The probe was denatured at 95°C for 5 min. Chromosome preparations were removed from the freezer, dehydrated in the graded ethanol series, and air-dried. Denaturation of chromosomes was done at 68°C for 3.5 min in 70% formamide in 2× SSC. The preparations were hybridized with the probe cocktail for 3 days at 37°C. Then the slides were washed for 5 min in 0.1× SSC containing 1% Triton X-100 at 62°C and for 2 min in 2× SSC containing 1% Triton X-100 at room temperature. The chromosomes were counterstained with 0.5 µg/ml of DAPI (4′,6-diamino-2-phenylindole; Sigma-Aldrich) in phosphate-buffered saline buffer and mounted in antifade based on DABCO (1,4-diazabicyclo(2.2.2)-octane; Sigma-Aldrich).

The preparations were inspected in a Zeiss Axioplan 2 microscope (Carl Zeiss Jena, Germany). Black and white images were recorded separately for each fluorescent dye with a cooled F-View CCD camera and captured with AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). Adobe Photoshop, version 6.0 was used to pseudocolour (light blue for DAPI and red for SpectrumOrange) and superimpose the fluorescent images.

Cloning and sequencing

The DNA fragments obtained by DOP-PCR amplification were cloned into the pCR2.1-TOPO vector with the help of the TOPO TA Cloning Kit (Invitrogen Life Technologies, San Diego, CA, USA) using *Escherichia coli* DH5α competent cells as recipients. From the resulting transformant colonies, 54 clones were selected each from the W chromatin (CpW1-54) and the euchromatin sample (CpE1-54). Cloned inserts were sequenced from both sides using the SequiTherm ExcelII DNA sequencing kit (Epicentre Technologies, Madison, WI, USA) and the LI-COR 4200 Sequencer (LI-COR Biosciences GmbH, Bad Homburg, Germany). Sequencing primers were universal M13 forward (−21) (5′-TGT AAA ACG ACG GCC AGT-3′) and M13 reverse (−29) (5′-CAG GAA ACA GCT ATG ACC-3′).

CpE and CpW sequences

The insert sequences were stripped of the degenerate primers at both ends and collected in two libraries, CpW from W chromatin, and CpE from euchromatin. Two sequences of vector origin and one each of human and bacterial origin were discarded. Two more were removed from the CpW library because they did not recognize *C. pomonella* genomic DNA in Southern hybridization. Redundant sequences were detected with FASTA (Pearson and Lipman 1988) and removed from the libraries. The remaining non-redundant sequences were deposited under acc. nos. AM292089–AM292105 (for CpW) and AM292865–AM292906 (for CpE).

The two non-redundant libraries were used in databank searches. BLASTN searches were performed at National Center for Biotechnology Information (NCBI) against the nr database (all Genbank+EMBL+DDBJ+PDB sequences) and whole insect genomes (*Anopheles gambiae, Apis mellifera, B. mori, Drosophila melanogaster, and Tribolium castaneum).* BLASTX searches were done against the all non-redundant Genbank CDS translations+PDB+Swissprot+PIR+PRF database. To reveal any conspicuous structures such as direct or inverted repeats, the mreps program version 2.5 (Kolpakov et al. 2003) and ASSEMBLE version 3.15 (written by W. Traut) were used.

Southern hybridization

Probes for Southern hybridization were generated from insert containing plasmids. Plasmid DNA was labelled with digoxigenin 11-dUTP using DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) with M13-20 (forward, 5′-GTA AAA CGA CGG CCA GT-3′) and M13-26 (reverse, 5′-CAG GAA ACA GCT ATG AC-3′) primers made by Generi Biotech (Hradec Králové, Czech Republic).

Genomic DNAs were isolated separately from females and males by standard phenol–chloroform procedure (Blin and Stafford 1976). Samples of the extracted DNAs were digested each with four restriction enzymes, *Hin*dIII, *Eco*RI, *Sau*3AI, and *Rsa*I, separately. Then 4 μg of each sample were loaded on 1% agarose gel in Tris/acetate/ethylenediamine tetraacetic acid (TAE) buffer. DNA fragments were separated by horizontal electrophoresis at 5 V/cm and blotted onto positively charged nylon membrane (Hybond-N+; Amersham Biosciences, Buckinghamshire, UK) by capillary transfer in 20× SSC. Hybridization of genomic DNAs with DIG-labelled probes and subsequent chemiluminescent detection were carried out using the following reagents: DIG Easy Hyb Granules, DIG Wash and Block Buffer Set, Anti-Digoxigenin-AP, and CDP-*Star* ready-to-use (Roche Diagnostics GmbH), according to manufacturer's instructions with overnight hybridization at 45°C and stringent washes at 68°C. Membranes were exposed to Kodak X-Omat AR film, XAR-5 (Eastman Kodak, Rochester, NY). Alternatively, chemiluminescent images were recorded with a LAS-3000 Lumi-Imager (Fuji Photo Film Europe GmbH, Düsseldorf, Germany).

Results

Microdissection of W chromatin

Malpighian tubules from female larvae of the codling moth displayed large, oval, or gently lobed polyploid nuclei of a granular chromatin appearance with a conspicuous deeply stained W-chromatin body (Fig. 1a). In the majority of cells, the W body ranged from 7 to 9 μm in diameter (Fig. 1b). From the ratio between the W-body area in polyploid nuclei and half of the W-chromosome area in mitotic metaphases, we estimated that a medium-sized W body contains more than 250 W chromosomes (250 is certainly an underestimate, as the higher density of the W chromatin was not taken into account). Using a laser microbeam, we collected 10 or 16 W-chromatin bodies per sample, respectively. Thus, each W-chromatin sample represented several thousands of W chromosomes. Similarly, samples of euchromatin pieces were taken from the same nuclei.

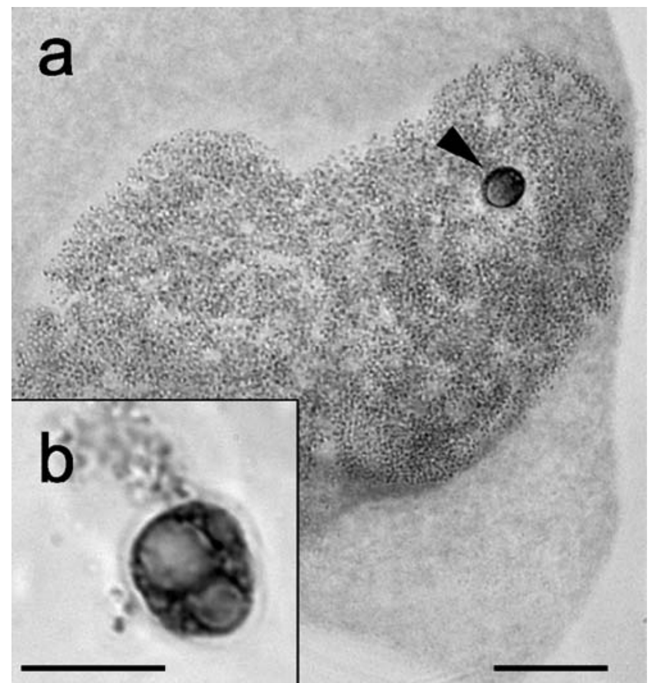

Fig. 1 **a** A highly polyploid nucleus of the Malpighian tubule cell from a codling moth female larva, showing a spherical W-chromatin body deeply stained with orcein. **b** A detail of the W-chromatin body. *Bar* indicates 20 μm (**a**) and 10 μm (**b**)

DNA of both W-chromatin and euchromatin samples was amplified by DOP-PCR and the products inspected by gel electrophoresis. The gel revealed a pattern of bands and smear (Fig. 2). DNA fragments ranged from less than 200 bp to more than 750 bp. This indicated that amplification of a multitude of DNA sequences had taken place. The bands indicated preferential representation of a few sequences, probably derived from repetitive elements.

W-chromosome painting by FISH

DOP-PCR products were labelled for FISH with Spectrum-Orange and hybridized to *C. pomonella* chromosomes. The euchromatin probe labelled all chromosomes although with stronger signals on the W chromosome (not shown). When the W-chromatin probe was hybridized, strong hybridization signals marked the W chromosome in mitotic plates and the W chromatin in interphase nuclei. We observed only very faint, scattered hybridization signals in the other chromosomes including the Z chromosome.

In metaphase complements, the compact W chromosome was deeply and continuously painted with the probe (Fig. 3a). In late prophase chromosomes, the hybridization pattern was uneven with noticeable weak labelling of the chromosome ends (Fig. 3b). Particularly, strong hybridiza-

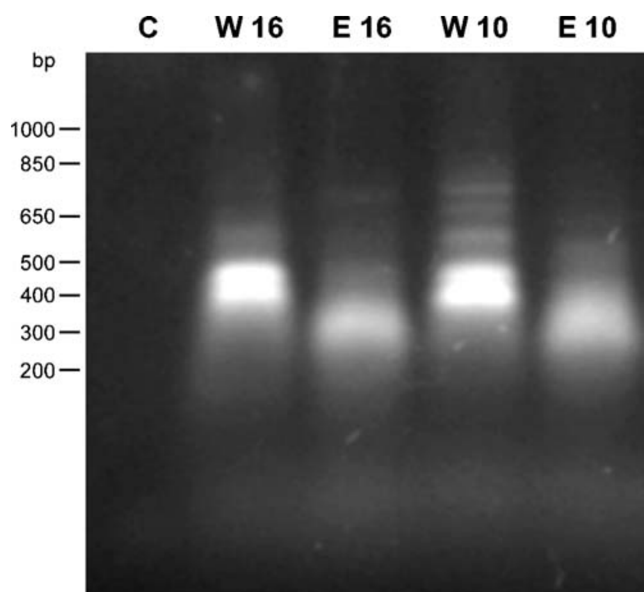

Fig. 2 Electrophoresis of the microdissected samples of DNA amplified by DOP-PCR. Amplification products were separated in a 1% agarose gel and stained with ethidium bromide. Size specification in bp was done with 1 Kb Plus DNA Ladder (Invitrogen Life Technologies). *Lanes*: *C*, blank control; *W 16* and *W 10*, W-chromatin samples obtained by amplification of 16 or 10 W bodies, respectively; *E 16* and *E 10*, euchromatin samples obtained from 16 or 10 non-W-chromatin nuclear areas

tion signals were observed in interphase nuclei in which the probe deeply stained the W body (Fig. 3c). To obtain higher resolution of the labelling pattern, we used pachytene chromosomes for FISH. In these, the probe highlighted almost the entire W-chromosome thread of the WZ bivalent, whereas the Z-chromosome thread remained unlabelled (Fig. 3d). However, hybridization signals were distributed unevenly along the W-chromosome thread (Fig. 3e). Especially remarkable was the weak signal at both chromosome ends comprising 5 and 10% of the chromosome length, respectively (see arrows in Fig. 3d,e). Notwithstanding this restriction, the W-chromatin probe proved to be a suitable W-painting probe. The results show that the probe preferentially contains W-chromosome sequences.

W-chromosome sequences

DOP-PCR-amplified DNA from microdissected chromatin samples was cloned and sequenced. From the W-chromatin sample, 53 sequences were collected. A sequence of human origin and one of bacterial origin were discarded, as were two more sequences that did not hybridize to genomic *C. pomonella* DNA in Southern blots. The sizes of the remaining 49 sequences varied from 70 to 1,088 bp. The collection proved to be highly redundant. After the removal of redundant sequences, our *C. pomonella* W-chromatin (CpW) library consisted of 17 non-redundant sequences (Table 1).

No conspicuous structures such as long direct or inverted repeats were detected in the collected sequences. Databank searches revealed sequence homology to retrotransposons in three sequences, CpW11 (acc. no. AM292093), CpW19 (acc. no. AM292096), and CpW27 (acc. no. AM292099), whereas no convincing homology has been found in the remaining sequences. CpW11 showed homology to non-long terminal repeat (LTR) retrotransposons of the TRAS family of *B. mori* and TRASSc of *Samia cynthia* (Kubo et al. 2001). CpW19 was similar to a segment of the gag–pol polyprotein of *D. melanogaster* LTR retrotransposons roo (Scherer et al. 1982) and BEL (Davis and Judd 1995). CpW27 displayed homology not only to the gag–rt region of the LTR retrotransposon MAX (Marsano et al. 2004) but also to the *B. mori* retrotransposon Kamikaze (Abe et al. 2001). Thus, at least 3 of 17 W-chromatin sequences were derived from transposons.

For comparison, 52 sequences from the euchromatin samples were collected in the same way as those from W chromatin. Two, containing vector sequence, were discarded. Among the remaining 50 sequences, sizes ranged from 63 to 412 bp. Sequence redundancy was considerably lower than in the W-chromatin sample. The *C. pomonella* euchromatin (CpE) library finally consisted of 42 non-redundant sequences (acc. nos. AM292865–AM292906). Five of the 42 sequences (CpE18, acc. no. AM292881; CpE21, acc. no. AM292884; CpE22, acc. no. AM292885; CpE38, acc. no. AM292898; CpE43, acc. no. AM292900) displayed homology to retrotransposon polyprotein and, hence, were probably of retrotransposon origin. Three sequences (CpE3, acc. no. AM292867; CpE20, acc. no. AM292883; CpE25, acc. no. AM292887) were obviously from coding regions of *C. pomonella* genes. CpE3 is part of a gene encoding a DEAD box protein. CpE20 is part of a gene-encoding cell-division cycle 27, and CpE25 is from a gene-encoding UDP-glucose pyrophosphorylase 2.

W-specificity and multiplicity of W-chromatin-derived sequences

To study W-specificity and multiplicity, the CpW sequences were hybridized to Southern blots of female and male genomic DNAs, digested separately with four different restriction enzymes. Two of them, *Sau*3AI and *Rsa*I, were four-base-pair cutters, two others, *Hin*dIII and *Eco*RI, were six-base-pair cutters, selected to obtain short and long restriction fragments. Results are summarized in Table 1, examples are shown in Fig. 4.

Two sequences (CpW2, CpW5) hybridized exclusively with female DNAs (Fig. 4a,b) and, hence, are W-specific sequences. CpW5 is considered a single-copy sequence, as

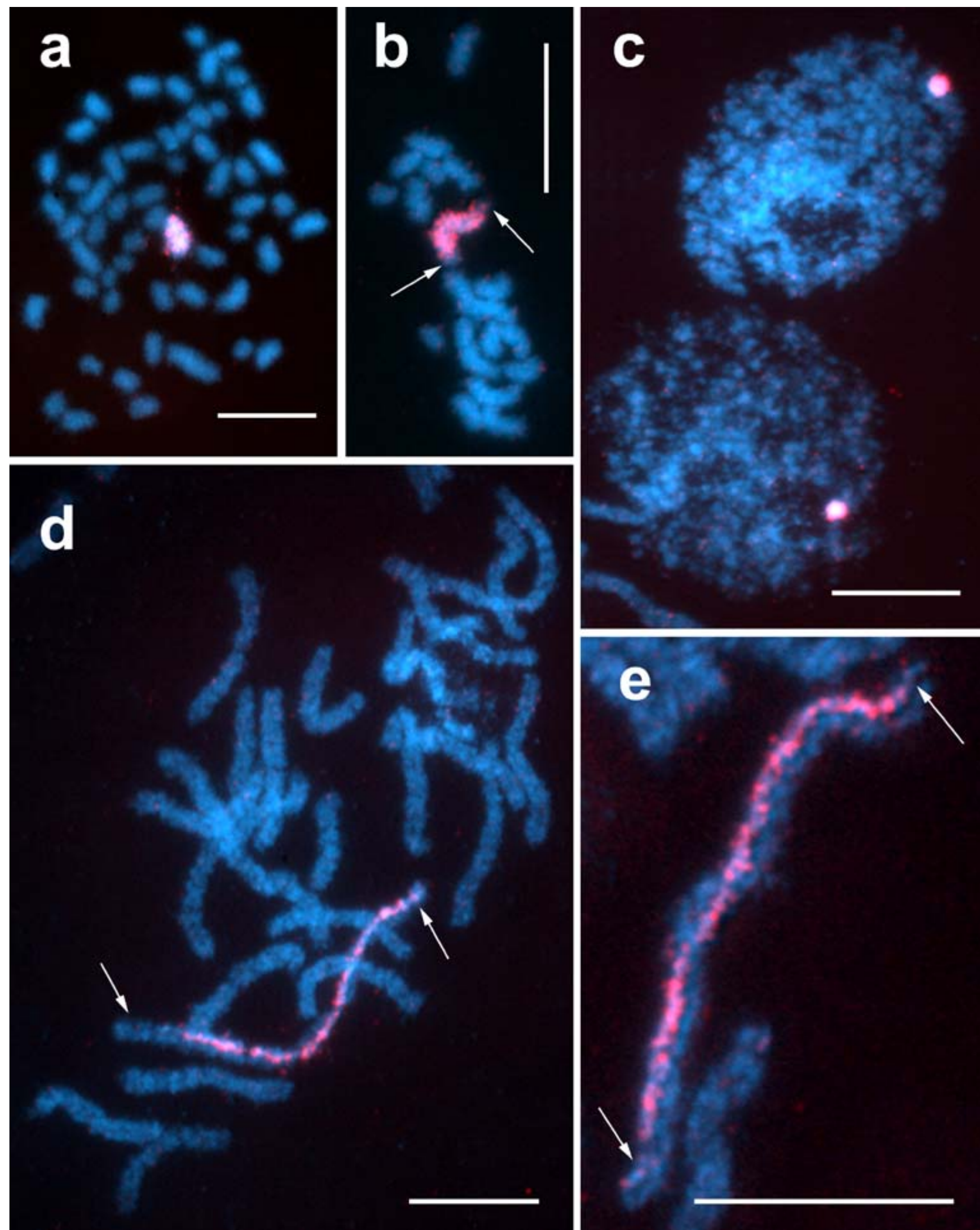

Fig. 3 FISH with W-chromosome painting probes (*red hybridization signals*) in spread preparations of codling moth ovaries, counterstained with DAPI (*blue*). **a** Mitotic metaphase complement with a W chromosome strongly highlighted by the probe. **b** Incomplete set of late prophase chromosomes; the W chromosome is strongly labelled with the probe except both telomeric regions, which are almost free of hybridization signals (*arrows*). **c** Two interphase nuclei, each showing a W-chromatin body deeply stained with the probe. **d** Pachytene complement; the WZ bivalent is identified by the binding of the probe to the W-chromosome thread; note weakly stained ends of the W chromosome (*arrows*). **e** A detail of pachytene WZ bivalent; note strong hybridization signals along the whole of W-chromosome thread with exception of both ends, which are almost free of signals (*arrows*). *Bars*=10 μm

it recognizes only a single band in *Hin*dIII, *Eco*RI, and *Rsa*I digests of female DNA (none in *Sau*3AI, the fragments are too small due to an internal restriction site). CpW2 may be present in the W chromosome in one or two more although incomplete copies, as the blots show one or two additional faint bands (Fig. 4a).

One sequence (CpW40) not only appears to be present in a few copies in the W chromosome but also shows a single copy in the Z chromosome (Fig. 4e). There are one or three bands in each female DNA lane with no counterpart in the male lanes and another band present in female and male lanes with stronger signals in the male lanes compatible with the presence of one copy of the Z chromosome in females and two copies in males.

Seven sequences (CpW1, CpW16, CpW17, CpW19, CpW21, CpW27, CpW33), among them are two with similarity to LTR retrotransposons, are multi-copy sequences with a preferential location in the W chromosome, as indicated by the strong signals in the female lanes (Fig. 4c,d). In CpW33 sequence, one band in each male lane shows a stronger signal than in corresponding female lanes, indicating Z-chromosomal copies (not shown).

Seven other multi-copy sequences, among them is one with similarity to *B. mori* TRAS retrotransposon family, are preferentially distributed either on the autosomes (CpW11, CpW25, CpW36, CpW42, CpW43, CpW54) according to the even strength of signals in female and male lanes

Table 1 Microdissected W-chromatin sequences

Sequence	Accession no.	Size (bp)	Copy number	Location	Similarity
CpW1	AM292089	378	Multi-copy	Mostly W-chromosomal	–
CpW2	AM292090	264	Single-copy or low-copy	W-specific	–
CpW5	AM292091	93	Single-copy	W-specific	–
CpW11	AM292093	377	Multi-copy	Mostly autosomal	Non-LTR retrotransposons TRAS
CpW16	AM292094	136	Multi-copy	Mostly W-chromosomal	–
CpW17	AM292095	625	Multi-copy	Mostly W-chromosomal	–
CpW19	AM292096	316	Multi-copy	Mostly W-chromosomal	LTR retrotransposons roo, BEL
CpW21	AM292097	262	Multi-copy	Mostly W-chromosomal	–
CpW25	AM292098	1,088	Multi-copy	Mostly autosomal	–
CpW27	AM292099	389	Multi-copy	Mostly W-chromosomal	LTR retrotransposons MAX, Kamikaze
CpW33	AM292100	70	Multi-copy	Mostly W- and Z-chromosomal	–
CpW34	AM292101	157	Multi-copy	Mostly Z-chromosomal	–
CpW36	AM292102	163	Multi-copy	Mostly autosomal	–
CpW40	AM292103	231	Low-copy	W- and Z-chromosomal	–
CpW42	AM292104	203	Multi-copy	Mostly autosomal	–
CpW43	AM292105	759	Multi-copy	Mostly autosomal	–
CpW54	AM292092	202	Multi-copy	Mostly autosomal	–

Distribution in the codling moth genome based on Southern hybridization to genomic DNAs from females and males, DNAs digested with *Hin*dIII, *Eco*RI, *Sau*3AI, and *Rsa*I restriction enzymes.

(Fig. 4g) or in the Z chromosome (CpW34), as the stronger signals in male lanes indicate (Fig. 4f).

Discussion

Although molecular data on Y chromosomes are rapidly growing (e.g. Charlesworth and Charlesworth 2000; Hellborg and Ellegren 2004; Vyskot and Hobza 2004; Gvozdev et al. 2005; Graves 2006), information about W chromosomes is still scarce and limited to the silkworm (Abe et al. 2005) and chicken (Berlin and Ellegren 2004). In this paper, we present a new and feasible approach that enabled us to obtain both the W-chromosome-specific painting probe and first sequence data from the W chromosome in the codling moth, *C. pomonella*. The probe represents the first painting probe in insects prepared by laser microdissection, although manual microdissection was already used for the development of X and Y probes in the fruit fly, *Ceratitis capitata* (Willhoeft et al. 1998).

With the help of laser microbeam, we sampled W-chromatin bodies from highly polyploid nuclei of codling moth females and thus obtained roughly several thousand copies of the W-chromosome DNA molecule per sample. The copy number exceeds by two orders the number of microdissected chromosomes used for preparation of chromosome-painting probes in mammals (ten was used by Kubickova et al. 2002), and it is higher than the number of sorted chromosomes usually used for the same purpose (400–500 chromosomes; e.g. Chaves et al. 2004; Stanyon et al. 2004). The high copy number of W chromosomes sampled may have helped us to recover a few unique DNA sequences, in addition to multi-copy ones (see below).

The W-chromosome painting probe exhibited a highly specific binding to the codling moth W chromosome and only scattered and very faint signals in the other chromosomes including the Z chromosome. A detailed analysis of hybridization signals in pachytene oocytes revealed that the probe stained most of the W chromosome with the exception of both ends. A similar labelling pattern was obtained after genomic in situ hybridization (GISH) with a female-derived genomic probe (Fuková et al. 2005). The absence of terminal signals with both types of probes indicates that the subtelomeric regions are composed of sequences, differing from the bulk of the W chromosome. On the other hand, the lack of hybridization signals in the Z chromosome supports our previous results of GISH and CGH showing a high level of molecular differentiation of the W chromosome from the Z chromosome (Fuková et al. 2005). Some homology may, however, be left over, as the presence of the W/Z-specific sequence (CpW40) tells. Thus, the codling moth belongs to the lepidopteran species displaying an advanced stage of sex chromosome evolution (Traut et al. 1999).

By cloning and sequencing of W-chromatin-derived DNA fragments, we identified 17 non-redundant sequences that represent single-copy and multi-copy sequence motifs of the codling moth W chromosome. Three sequences are obviously derived from transposable elements, as they exhibit homology to retrotransposons described in *D. melanogaster* and *B. mori*. The remaining motifs do not bear sufficient similarity to known sequences in the data-

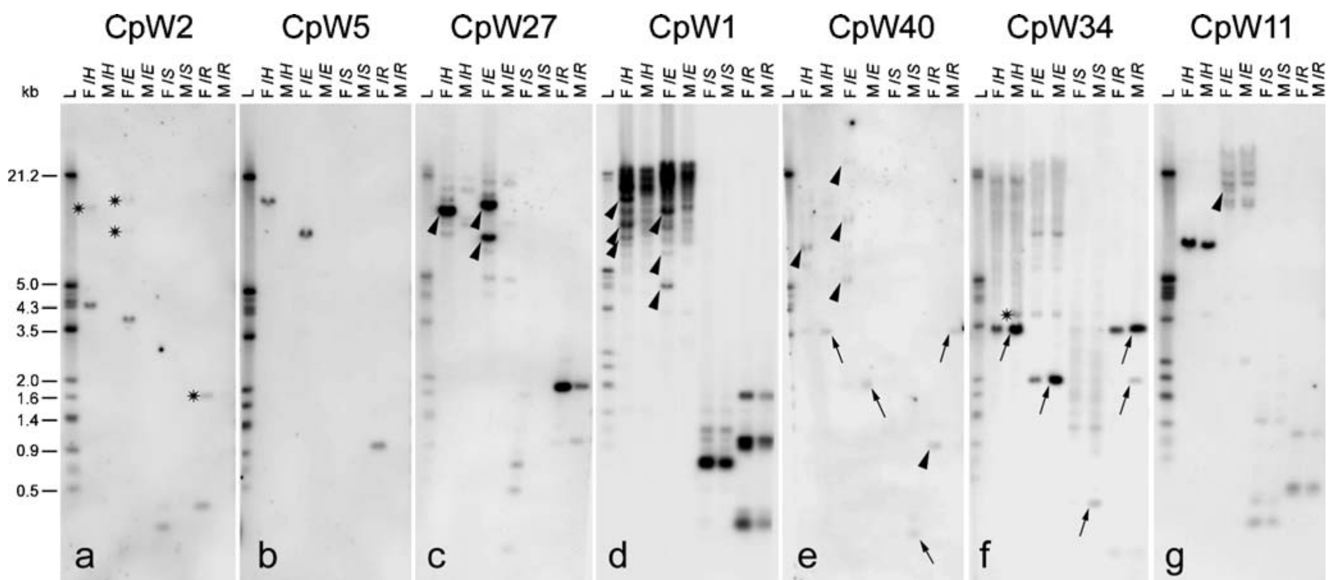

Fig. 4 Southern hybridization of codling moth female and male genomic DNAs with CpW probes. **a** CpW2 motif showing a female-specific hybridization pattern; note a clear band plus upper barely visible band(s) (*asterisks*) in each female lane except the *S* lane. **b** CpW5 motif showing a conspicuous band in each female lane except the *S* lane but none in male lanes. **c** CpW27 motif displaying 1–2 strong female-specific bands in *H* and *E* lanes (*arrowheads*), in addition to multiple weak bands in both sexes, and 3–4 weaker female-specific bands in *S* lane (1 inner restriction site); also note a much stronger band in F/*R* lane then the corresponding band in M/*R* lane. **d** CpW1 motif showing a similar multiple bands in both sexes with a few additional bands in *H* and *E* female lanes (*arrowheads*); multiple bands in *S* and *R* lanes of both sexes reflect two inner restriction sites. **e** CpW40 motif showing 1–3 weak bands in each female lane except *S* (*arrowheads*) and a weak band in each male lane (*arrows*) with a very weak counterpart in female lanes. **f** CpW34 motif showing a similar hybridization pattern in both sexes but with 1–2 stronger bands in male lanes (*arrows*), indicating sequences located in the Z chromosome; two bands in *R* lane reflect one inner restriction site; *asterisk* indicates a male-specific band in *H* lane. **g** CpW11 motif showing a similar hybridization pattern and similar abundance in both sexes except the female-specific band in *E* lane (*arrowhead*); three and two bands in *S* and *R* lanes of both sexes reflect two and one inner restriction sites, respectively. *L*, DNA ladder (digoxigenin-labelled DNA molecular-weight marker III; Boehringer Mannheim GmbH, Germany) with size specifications in kb. The abbreviations above lanes, F /*H*, M /*H*, F /*E*, M /*E*, F /*S*, M /*S*, F /*R*, and M /*R* denote female and male genomic DNAs, digested with four restriction enzymes, *Hin*dIII, *Eco*RI, *Sau*3AI, and *Rsa*I, respectively

banks. The 17 sequence motifs can be classified into four groups (see Table 1): (1) female-specific single-copy and low-copy sequences, i.e. sequences localized exclusively in the W chromosome; (2) a low-copy sequence occurring in both sexes but located only in the W and Z chromosomes; (3) multi-copy sequences that are highly enriched in the W chromosome but occur also elsewhere; (4) ubiquitous multi-copy sequences not only predominantly occurring in autosomes but also present in the sex chromosomes. The latter two groups of interspersed repetitive sequences were expected to occur in the codling moth W chromosome, because with CGH, the W chromosome was equally differentiated by both the female and male genomic DNA probes. This was explained by accumulation of repetitive sequences occurring scattered in the respective genome (Fuková et al. 2005). In this paper, we show that the codling moth W chromosome also contains W-specific sequences (CpW2 and CpW5).

On the basis of the results of our FISH and Southern hybridizations, we conclude that the codling moth W chromosome is not only mainly composed of interspersed repetitive sequences but also contains unique sequences. The majority of sequences occur scattered also in other chromosomes, whereas the female-specific DNA components are underrepresented. Thus, the sequence composition of the W chromosome is similar to that of *B. mori*, i.e. it consists mainly of retrotransposable elements (Sahara et al. 2003b; Abe et al. 2005). This image of lepidopteran W chromosomes fits well into evolutionary theories on the genetical degeneration of the non-recombining Y or W chromosomes through loss of functional genes and accumulation of repetitive sequences (Charlesworth and Charlesworth 2000; Gvozdev et al. 2005; Steinemann and Steinemann 2005; Graves 2006).

The present study demonstrates that laser microdissection of the female-specific sex chromatin followed by DOP-PCR is a straightforward way to obtain both W-chromosome painting probes for FISH and a glimpse into the DNA composition of the heterochromatic W chromosome. The technique is applicable to any lepidopteran species with a W chromosome that forms heterochromatic W-chromatin bodies, i.e. to the great majority of species investigated (Traut and Marec 1996). The sequences obtained here will significantly facilitate further molecular analysis of the codling moth W chromosome. We are presently isolating flanking regions of selected W-chromosome sequences like

CpW2 and CpW5 to generate FISH probes for mapping the W chromosome. Besides, the two W-specific sequences are being used as molecular markers of the female sex. Our progress in W-chromosome analysis will facilitate our goal to develop genetically engineered sexing strains in the codling moth (Marec et al. 2005), an essential step for the improvement of the SIT programmes and related strategies to control this pest.

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Synthesis and perspectives

Summary of results and discussion

In chapter one, I presented the karyotype analysis and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Pyralidae). The first result was that the sex chromosomes could not be detected by conventional cytogenetic methods. The carob moth karyotype did not show any outstanding chromosome pair. In pachytene preparations, all bivalents displayed alternating chromomere pattern without any conspicuous heterochromatin disabling thus the sex chromosome pair discrimination. A similar situation could be found in *Bombyx mori* where the sex chromosomes identification was enabled only by a large translocation of chromosome 2 to the W chromosome (Kawamura and Niino 1991). Recently, the WZ bivalent in *B. mori* was differentiated by comparative genomic hybridization (CGH; Traut et al. 1999). In our study I found that the sex chromosome pair in *E. ceratoniae* was morphologically indistinct but displayed a moderate molecular differentiation. Based on our CGH results, the co-authors and I concluded that the W chromosome of *E. ceratoniae* contained mainly repeats located elsewhere in the genome and hence W-specific sequences were under-represented. Traut et al. (1999), Sahara et al. (2003a) and Nagaraja et al. (2005) also found the presence of common repetitive elements in the *B. mori* W chromosome. The low differentiated *E. ceratoniae* and *B. mori* sex chromosomes represented rather an exception among other lepidopteran sex chromosome pairs which are highly heterochromatinized and differentiated from the Z chromosome as seen e.g. in a pyralid model species, *Ephestia kuehniella* (Marec and Traut 1994).

The codling moth (*Cydia pomonella*, Tortricidae) karyotype showed a similar pattern with chromosome sizes ranging from 0.5 μm to 3 μm. In the codling moth metaphase preparations, in contrast to the rather homogeneous carob moth karyotype, I could classify several chromosome groups according to the chromosome length. The largest chromosome pair of the codling moth karyotype represented the sex chromosomes. The Z and W chromosomes were very well differentiated. The two counterparts could be easily identified in meiotic prophase after staining with conventional cytogenetic dyes under light microscopy. The W chromosome was completely heterochromatinized in contrast to the fully euchromatic Z with alternating chromomere and interchromomere pattern similar to the autosomes. Recognition of the W in mitotic metaphase sets was, however, rather problematic due to the similar size of the sex chromosomes and the largest autosome pair. Also, the differential staining with DAPI, an AT-specific fluorescent dye, often failed, especially during the early metaphase in which the chromosomes were not fully condensed yet. I provided a highly reliable way to the W chromosome identification in various stages of the cell cycle by the means of genomic *in situ* hybridization (GISH). The principle of the method consisted in hybridizing labeled female genomic

DNA to a spread of female chromosomes in the presence of a competitor. In this case, I used unlabeled male genomic DNA. After hybridization the W chromosome was highlighted by the female probe because the sequences from the female-specific chromosome could not be competed by the male DNA. A similar method was adopted for disclosing the respective representation of female-specific sequences and sequences widely spread in the genome on the W. Unlike in GISH, the CGH technique utilized labeled both female and male genomic DNA probes as well as an excess of unlabeled male competitor DNA. The hybridization pattern showed that the codling moth W chromosome consisted predominantly of repeats occurring also elsewhere in the genome but accumulated in the W (chapter three).

In order to facilitate the field application of the Sterile Insect Technique (SIT) I present in chapter two an implementation of transgenesis for the development of genetic sexing strains in the codling moth, *C. pomonella*. The current state of genetic sexing in Lepidoptera is reviewed and benefits and possible drawbacks of the transgenic approach are discussed. The paper describes the principle of the transgenic sexing strains construction in *C. pomonella* and cytogenetic and molecular tools available so far for the accomplishment of the codling moth transgenesis.

The currently available genetic sexing techniques in Lepidoptera (Strunnikov 1975; Marec et al. 1999), which is based on balanced sex-linked recessive lethal mutations, cannot be easily applied for mass rearing due to their high requirements on handling and routine checking of the sexing strain genetic consistency. In addition, a big problem for an insect mass-rearing facility is the necessity to maintain and propagate two strains, the balanced lethal strain and a wild-type strain, which have to be cross-hybridized in order to produce male-only progeny. The introduction of a dominant conditional lethal gene into the female-specific W chromosome can bring an elegant solution to this situation (Marec et al. 2005). In chapter two, my co-authors and I suggest an adoption of the transgenic approach in order to deliver a conditional lethal (the cold sensitive *Notch*^{60g11} allele; Fryxell & Miller 1995) into the codling moth W chromosome. The transgenesis technique in this pest is already established in the laboratory of Lisa G. Neven (Ferguson et al. 2004). The transgene construct for the codling moth eggs injection contains the *piggyBac* transposon with *Notch*^{60g11} in tandem with the enhanced green fluorescent protein (EGFP) marker gene under the *B. mori Actin A3* promoter. In the first trials the *Notch*^{60g11} allele, originally identified in *D. melanogaster* (Fryxell & Miller 1995), proved to be lethal in the codling moth after two-day exposure to 12 °C (L.G. Neven, pers. comm.).

Once the transgenic strain is established, production of male-only offspring is straightforward and requires no additional technology implementation in the mass rearing facility. An important advantage of the method relies in the releases of non-transgenic males since the transgene is present in the female-specific W chromosome. Moreover, the males do not suffer any negative influence of the transgene as was shown e.g. in *Ceratitis capitata*. In this species, the trangene utilized for genetic

sexing had a negative impact on the fly fertility (Robinson 2002). Further, the transgenic sexing strains, as suggested in the chapter two allow for a specific genetic background to be introduced, e.g. the wild-type male one. Such a modification may highly improve the performance of sterile males in the field and allow the usage of the sexing strain constructed in any area to suppress local codling moth populations.

The last chapter was concerned with a genomic survey of the codling moth W chromosome using a cytogenetic approach. Based on CGH results in several lepidopteran species Sahara et al. (2003a) formulated the hypothesis that the W chromosome was rich in repetitive sequences. In the final chapter, I found an abundance of highly repetitive sequences in the W, which supports this hypothesis. The W chromosome consisted mainly from repeats scattered all over the genome which probably accumulated in the respective chromosome due to the lack of recombination and hence an effective mechanism to restrain their appearance in female Lepidoptera. However, even in the highly differentiated codling moth W chromosome there was a sequence located exclusively in the sex chromosome pair. This may represent the homologous region still remaining after extensive W degeneration. Also, W-specific sequences, though under-represented, were identified. Similarity to known sequences was revealed only in three from 17 unique W-located sequences. All of these three sequences were significantly similar to transposable elements from either *B. mori* or *Drosophila melanogaster*. The sequence data represent the second largest survey on the molecular composition of the W chromosome in Lepidoptera (after the silkworm, *B. mori*; Abe et al. 2005) and the first using the technique of laser microdissection. The single- and low-copy W-specific sequences were proposed for the development of W-specific DNA markers for codling moth sexing using PCR technique. The W-painting probes, prepared for the transgene location mapping in the codling moth genome during the transgenic sexing strain development, were the first chromosome-specific paints prepared by laser microdissection in insects.

Synthesis and future research

Sterile Insect Technique and codling moth transgenesis

Several improvements of the transgenesis methodology have been also suggested. The trial experiments showed that the fluorescent marker selected interferes with excessive auto-fluorescence of all developmental stages of the codling moth (L.G. Neven, pers. comm.). In the paper, we proposed to replace the enhanced EGFP for another fluorescent marker, DsRed2. Further improvement of the transgenesis procedure can be accomplished by utilization of the promoter with a more stable expression profile for driving of the transgene transcription in the codling moth. So far only the *B. mori Actin A3* promoter exhibits a chimeric expression pattern (L.G. Neven, pers. comm.; and also my

personal experience). The adoption of 3xP3 promoter would promote the transgene eye-specific expression during all developmental stages. The question on the conditional lethal effect of the *Notch*^{60g11} allele with restricted expression profile did not, however, rise in this thesis.

The transgenic procedures aimed for the genetic sexing strains construction require extensive screening for the transgene presence and its expression in progeny of putative transgenic females. In order to test the exhibition of the desired female lethality one has to identify the sex of insects after the induction of the transgene expression. The determination of the sex in embryos and first instar larvae is, therefore, of importance for checking the sex ratio without the requirement for raising the insects to the adulthood when the sex can be easily determined. I am currently testing PCR sexing methodologies for the early developmental stages of the codling moth. For this purpose, I am using the W chromosome-specific markers that were developed in chapter four.

In order to locate the transgene in the codling moth genome, I prepared a W-chromosome painting probe (see chapter four). Such a probe is, however, limited in its usage since it requires a new technically demanding laser microdissection procedure after its depletion. Recently, the codling moth BAC library was constructed and it is now available at L. G. Neven's laboratory (L.G. Neven, pers. comm.). The W-specific BAC clones can be selected by screening the BAC library with the W-microdissected sequences, which show exclusive or preferential W location and are single- or low-copy number. Such W-specific BAC clones may be used for development of the W chromosome painting probes. In the silkworm, *B. mori*, Sahara et al. (2003b) showed that the W-specific BAC clones, comprising only about one per cent of the whole W chromosome sequence (170 kb), painted the entire W. According to the authors this reflects the accumulation and distribution of transposable elements in the silkworm W chromosome.

Molecular cytogenetic methods for the sex chromosome evolution in Lepidoptera

Cytogenetics is a powerful approach to answer evolutionary questions, but, due to the biology of Lepidoptera or financial constraints, many cytogenetic methods adopted in humans and plants are unavailable in Lepidoptera. Lepidopteran cytogenetics needs, however, to keep up by utilizing and developing novel methodologies. For example, except the work presented here with sex chromosomes, there is currently no banding or other technique to identify other specific chromosomes. Identification of specific chromosomes, once not limited to those few distinguished due to size or AT richness, will extend the use of laser microdissection. Once such a technique is developed, one can build chromosome specific libraries for sequencing or to be used as chromosome-specific PCR templates. The methodology of FISH itself is far from perfected in Lepidoptera. The Fiber-FISH technique to

map individual EST or AFLP sequences or the utility of species-specific C_0t-1 DNA is still to be implemented. Indeed, a solution for a chromosome banding technique may evolve from the development of C_0t-1- or C_0t-3-specific DNA (Wang 1995).

Likewise, the survey of the W- chromosome presented in this thesis, is not optimal. The use of a BAC library to identify W-specific clones would facilitate surveys. Also, the small-insert libraries used here are known to have cloning issues with repetitive DNA. The generation of W-specific medium/large insert library and the usage of fiber-FISH with female EST sequences would enhance gene-hunting of rare genes. Identifying their number and their relative position would be of importance to further develop theories on the W evolution. By using laser microdissection of the Z chromosome a similar survey can be conducted. Ultimately, sequencing and comparative analyses will allow models of molecular evolution to be tested. These comparative analyses across lepidopteran families will ultimately provide evidence on the number and sequence conservation of W/Z. In addition, they will show if repeated additions from autosome pairs have occurred as is the situation in mammals. Likewise, one can look for sex-determining or sex-influencing genes and use comparative analyses to hypothesize how lepidopteran sex-determination has evolved.

The phylogenetic context due to the huge number of species in Lepidoptera and the diversity exhibited across families make Lepidoptera a perfect model system for the study of sex chromosome evolution. Also various examples of neo-sex chromosomes, multiple sex chromosomes and secondary losses of the W chromosome, which can be found in several groups of Lepidoptera (Suomalainen 1969; Ennis 1976; Nilsson et al. 1988; Rishi et al. 1999; Rego & Marec 2003; Yoshido et al. 2005b), document the usefulness of lepidopteran model system in sex chromosome evolutionary studies. In general, Lepidoptera display the full 'life cycle' of the W chromosome. It is important to study how its molecular and structural differentiation from the Z occurs, how and if it is influenced by rearrangements with the autosomes and ultimately if saturation with heterochromatic elements is the final step before it is completely lost from genome (Traut & Marec 1997).

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