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Rigorózní práce

# Molecular cytogenetic analysis of the W sex chromosome in Lepidoptera

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**Annotation**:
In the presented study, we developed a straightforward method to isolate W sex chromosome-specific DNA sequences in the codling moth, *Cydia pomonella*. We utilized the presence of a female specific sex chromatin consisting solely from the W chromosome and cut it from polyploid nuclei by the means of laser microdissection. The material obtained was used for the painting probe development and for the generation of the W chromosome-specific plasmid library. The occurrence of predominantly highly repetitive sequences in the W chromosome-specific library confirmed the hypothesis on the W chromosome composition based on results from comparative genomic hybridization experiments in *C. pomonella* and other lepidopteran species.

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**Declaration**: This work was done in collaboration with the co-authors (Walther Traut, Magda Vítková, Petr Nguyen, Svatava Kubíčková and František Marec).

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

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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.

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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.

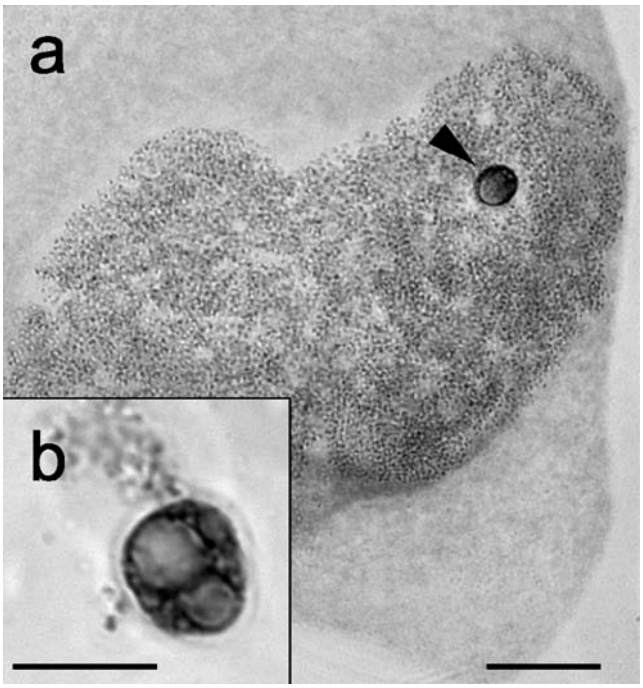


**Fig. 1** **a** A highly polyploid nucleus of the Malpighian tubule cell from a codling moth female larva, showing a spherical W-chromatin body deeply stained with orcein. **b** A detail of the W-chromatin body. *Bar* indicates 20 μm (**a**) and 10 μm (**b**)

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-

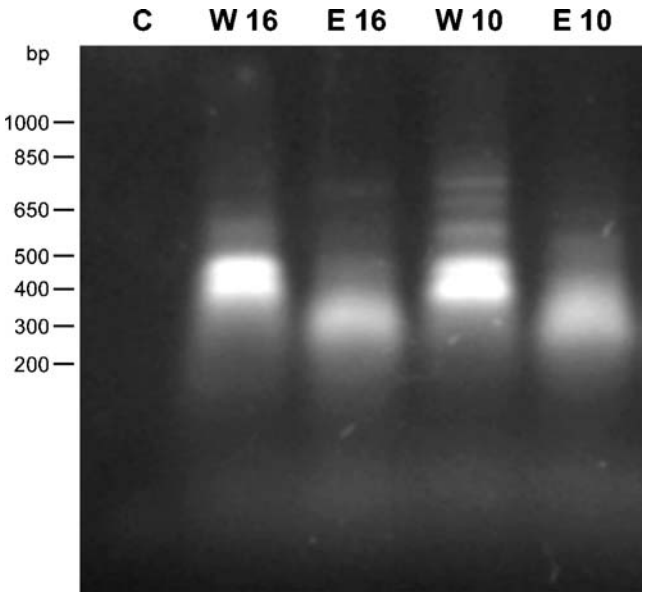


**Fig. 2** Electrophoresis of the microdissected samples of DNA amplified by DOP-PCR. Amplification products were separated in a 1% agarose gel and stained with ethidium bromide. Size specification in bp was done with 1 Kb Plus DNA Ladder (Invitrogen Life Technologies). *Lanes*: *C*, blank control; *W 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

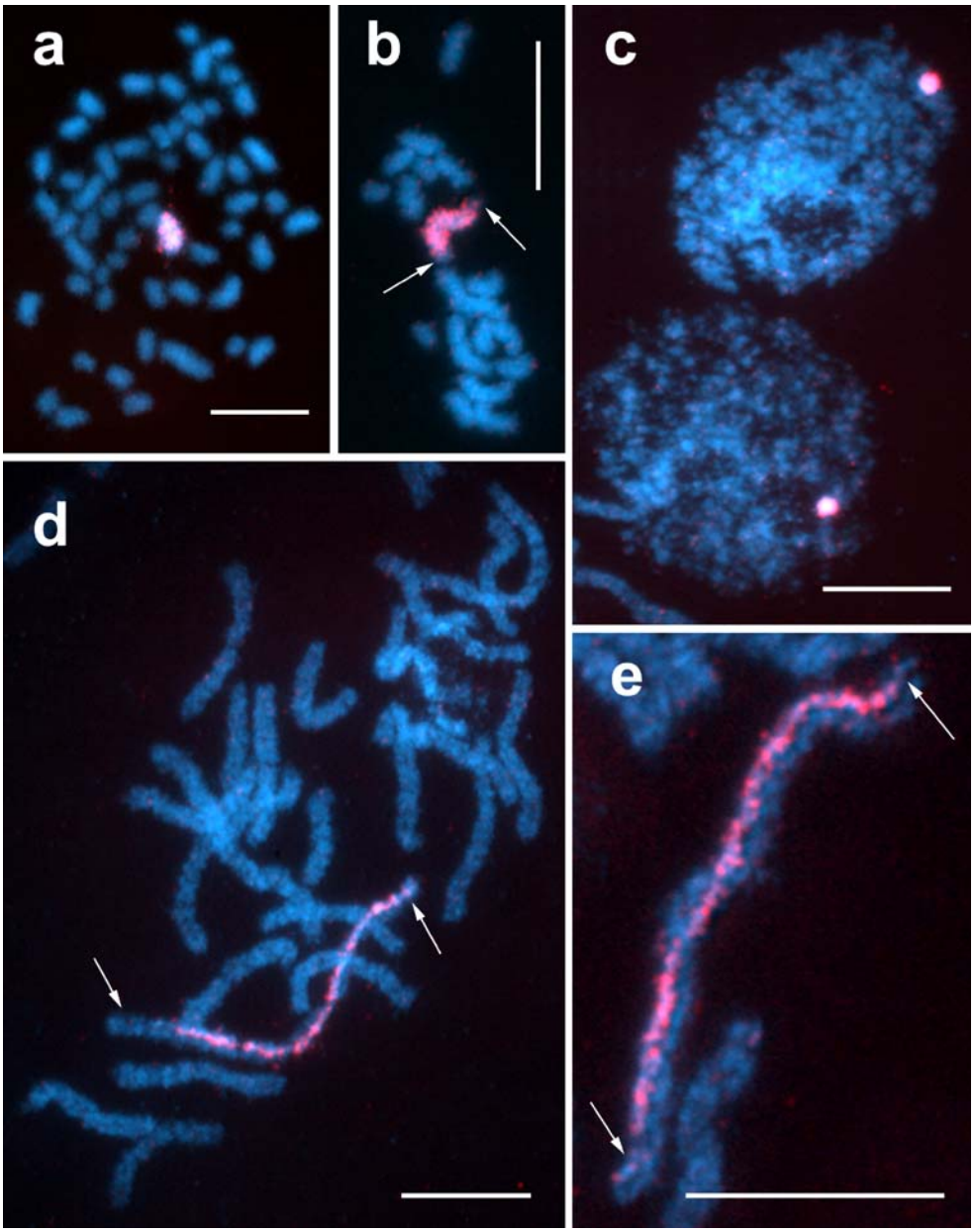


**Fig. 3** FISH with W-chromosome painting probes (*red hybridization signals*) in spread preparations of codling moth ovaries, counterstained with DAPI (*blue*). **a** Mitotic metaphase complement with a W chromosome strongly highlighted by the probe. **b** Incomplete set of late prophase chromosomes; 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-

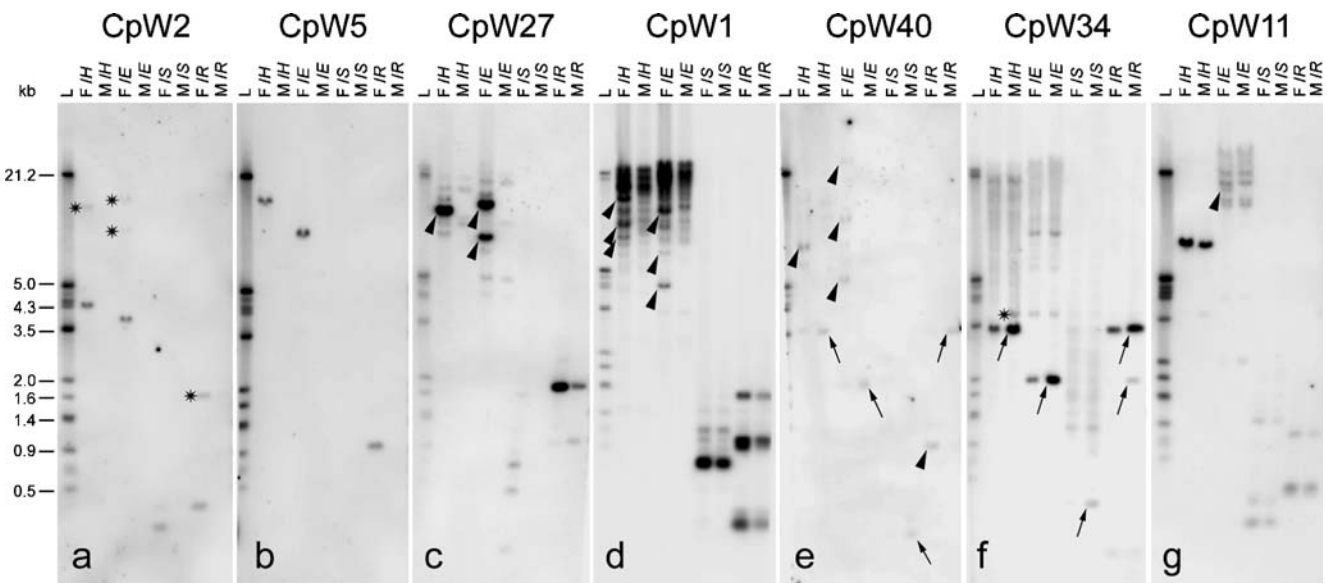


**Fig. 4** Southern hybridization of codling moth female and male genomic DNAs with CpW probes. **a** CpW2 motif showing a female-specific hybridization pattern; note a clear band plus upper barely visible band(s) (*asterisks*) in each female lane except the *S* lane. **b** CpW5 motif showing a conspicuous 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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