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Evolution of sex chromosomes ZW of *Schistosoma mansoni* inferred from
chromosome paint and BAC mapping analyses

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Running title: Sex chromosome evolution of *S. mansoni*

Abstract

Chromosomes of schistosome parasites among digenetic flukes have a unique evolution because they exhibit the sex chromosomes ZW, which are not found in the other groups of flukes that are hermaphrodites. We conducted molecular cytogenetic analyses for investigating the sex chromosome evolution using chromosome paint analysis and BAC clones mapping. To carry this out, we developed a technique for making paint probes of genomic DNA from a single scraped chromosome segment using a chromosome microdissection system, and a FISH mapping technique for BAC clones. Paint probes clearly identified each of the 8 pairs of chromosomes by a different fluorochrome color.

Combination analysis of chromosome paint analysis with Z/W probes and chromosome mapping with 93 BAC clones revealed that the W chromosome of *Schistosoma mansoni* has evolved by at least four inversion events and heterochromatinization. Nine of 93 BAC clones hybridized with both the Z and W chromosomes, but the locations were different between Z and W chromosomes. The homologous regions were estimated to have moved from the original Z chromosome to the differentiated W chromosome by three inversion events that occurred before W heterochromatinization. An inversion that was observed in the heterochromatic region of the W chromosome likely occurred after W heterochromatinization. These inversions and heterochromatinization are hypothesized to be the key factors that promoted the evolution of the W chromosome of *S. mansoni*.

Key word: *Schistosoma mansoni*, chromosome paint, BAC mapping, FISH, sex chromosome

Introduction

Chromosomes are the central structures that contain the genome (or genetic information) of organisms. They are the mechanism by which genetic information is transmitted from cell to cell and generation to generation. Studies of chromosomal changes are useful for understanding alterations of gene order, genetic isolation, speciation and evolution (e.g., White 1978; Wagner et al. 1993; Strachan and Read 2011). Therefore, detailed karyotypes of each species and/or the mechanism of chromosome differentiation offers opportunities to investigate the biology of the species. Molecular cytogenetic techniques are very useful for analyzing chromosome evolution and genome biology of organisms (e.g., Ijido et al. 1991; Scherthan et al. 1994). In the case of schistosomes, molecular cytogenetic techniques are the best way to analyze chromosome structure and/or differentiation, because their chromosomes exhibit C-banding but not other multiple banding patterns such as G- and R-bands found in mammals, which are useful for detecting chromosome differentiation and evolution. (e.g., Yunis and Prakash, 1982).

Schistosomiasis, a human infection by blood flukes in the genus *Schistosoma*, is a major neglected tropical parasitic disease that is a source of morbidity affecting about 210 million people in 76 countries of the world (e.g., Chitsdulo et al. 2000). *S. mansoni* infects about 90 million people in Africa, the Middle East, the Caribbean and Brazil. The genome of *S. mansoni*, one etiologic agent of human schistosomiasis, was sequenced (363 megabase) (Berriman et al. 2009; Protasio et al., 2012). The genome data will be useful for drug discovery and new approaches for the control of schistosomiasis. Schistosome parasites have a unique sex determining mechanism (ZW sex chromosomes) among trematodes as other trematode groups which are hermaphroditic do not exhibit sex chromosomes. The male exhibits ZZ and the female ZW chromosomes (Short 1983). Evidence indicates that sex chromosomes have differentiated from homologous autosomes (Ohno 1967). In the case of *Schistosoma*, the W chromosome has a large constitutive heterochromatin block (e.g., Short 1983; Hirai et al. 2001; Lawton et al. 2011). It has been suggested that heterochromatinization and inversions of the W chromosome have been the main mechanism to prevent crossing over between the Z and W chromosomes (e.g., John 1988; Solari 1994; Charlesworth et al. 2005). Subsequently, such regions can harbor sex determination genes due to prevention of chiasma formation (genetic recombination) (Hirai et al., 1989, 1993). Actually, genetic linkage map analysis by crossing a female of one *S. mansoni* strain to a male of another strain coupled with chromosome mapping with BAC (bacteria artificial

chromosome) clones identified a non-recombination site between the Z- and W-specific genes (Criscione et al. 2009). Lepasant et al. (2012) gave suggested chromatin structural changes around the W-specific repeats are responsible for the initial event (non-recombination) in sex chromosome development. However, whether structural changes caused by inversions are part of the mechanism that resulted in the differentiation of the ZW remains to be determined and is the main subject of the present study. We conducted analyses of BAC clone mapping and employed chromosome paint methods to examine sex chromosome differentiation in *S. mansoni*.

Materials and method

Chromosome microdissection

We established an improved simple microdissection technique for obtaining paint probes from chromosomes and/or chromosome segments based on our previous trials (Taguchi et al. 2007). Basically, we followed a Micro-FISH technique, HeadStart microdissection, to make paint probes from schistosome chromosomes (Christian et al., 1999). Briefly, chromosomes spread on a cover slip (24 x 60 mm²) were scraped one-by-one as individual chromosomes or segments using a glass needle of about 2 µm diameter made using a glass capillary puller (Narishige Tokyo, Japan). In our hands, the glass capillary (GC100-10, Clark Electromedical Instruments, England) was the best quality for making the needle. The cover slip chromosome spread preparations were

made as previously described (Hirai and Hirai, 2004). The preparation was stained for 2 min with 4% Giemsa in pH 6.8 Sörensen's phosphate buffer, and after drying was directly scraped or kept in a humid chamber at 4°C until used. The stained preparation was set on a horizontal turntable of an inverted microscope (Zeiss Axiovert 135) equipped with a micromanipulator (Eppendorf 5171). Chromosomes were scraped under conditions of high humidity established by surrounding the chromosome preparation with wet (distilled water soaked) tissue papers and maintaining the room at more than 50% humidity. A tube (200 µl) for collecting chromosome segments was set close to the work plate of the module unit for rapid transfer. We scraped each individual autosome and the ZW sex chromosomes of *S. mansoni*. Chromosome preparations were made with *S. mansoni* (NMRI strain) sporocyst stages from *Biomphalaria glabrata*-infected snails as previously described (Hirai and Hirai, 2004).

DNA collection

PCR to collect genomic DNA of a chromosome fragment was performed as previously described (e.g., Bohlander et al., 1992; Guan et al., 1993; Christian et al., 1999). Briefly, a scraped chromosome segment was put into a 200-µl tube under humid conditions described above. After centrifugation at 14,000 rpm for 10 min, 3 µl of degenerate oligonucleotide-primed (DOP)-PCR (Telenus et al. 1992) reaction solution (0.5 µl Thermo Sequenase reaction buffer, 0.24 µl 5 mM dNTP, 0.12 µl 100 µM DOP primer, 2.04 µl distilled water, 0.3 µl

Thermo Sequenase DNA Polymerase (GE Healthcare, E79000Y) was added to the tube containing the scraped chromosome fragments. The primer was as previously described (5'-CCG ACT CGA GNN NNN NAT GTG G-3') (Telenius et al. 1992).

The conditions of the DNA amplification were slightly modified from those previously described (Christian et al., 1999). Only a small volume PCR reaction solution was used and three rounds of PCR were used for amplification and labeling. The DOP-PCR reaction was performed as follows. (1) The first PCR with 3 μ l reaction solution mentioned above: 1 cycle at 94°C for 10 min, 12 cycles at 94°C for 1 min-30°C for 2 min-a ramp of 0.1 °C/s up to 65°C for 3 min. (2) The second PCR with total 10 μ l reaction solution [3 μ l of 1st PCR product + 7 μ l (1 μ l 10x Thermo Sequenase reaction buffer, 0.8 μ l 2.5 mM dNTP, 0.33 μ l 100 μ M DOP primer, 4.77 μ l distilled water, 0.1 μ l Thermo Sequenase DNA polymerase): 1 cycle at 95°C for 5 min, 25 cycles at 94°C for 1 min-56°C for 1 min-72°C for 3 min, 1 cycle at 72°C, held at 4°C]. After the 2nd PCR, DNA amplification was checked with a Nano Drop Spectrophotometer (Scrum, ND-1000) instead of electrophoresis. (3) The third PCR for labeling with a total 10 μ l reaction solution [1.5 μ l 2nd PCR product + 8.5 μ l (1 μ l 10x Thermo Sequenase reaction buffer, 0.8 μ l 2.5 mM dNTP, 0.33 μ l 100 μ M DOP primer, 5.87 μ l distilled water, 0.1 Thermo Sequenase DNA polymerase, 0.4 μ l digoxigenin-11-dUTP or biotin-16-dUTP)]: PCR run conditions were the same as for the 2nd PCR.

Ethanol precipitation was performed with the following composition: 5 μ l 3rd PCR product, 5 μ l Cot-1 DNA, 0.5 μ l salmon sperm DNA, 1.65 μ l 3M sodium acetate, 0.5 μ l Ethachinmate (Nippon Gene, Japan), 40 μ l distilled water, 100 μ l 99.5% ethanol. After standing at 4 °C for 1 h, the reaction was centrifuged at 15,000 rpm for 30 min. After centrifugation the supernatant was discarded and the pellet was dissolved with 50 μ l formamide.

Fluorescent in situ hybridization (FISH) and microscopy

Paint FISH was done with 3 μ l labeled DNA formamide solution (30 μ l 30% dextran sulfate, 10 μ l 20xSSC and 5 μ l genomic DNA). Hybridization followed the steps previously described (Hirai and Hirai 2004). Briefly, chromosome DNA was denatured by alkaline treatment (pH 12.5 2 x SSC solution for 4 min). After denaturation of the probe DNA at 70°C for 5 min, the probe mixture was placed onto a slide. After 16 h hybridization and post-hybridization washing, the hybridizations were detected using a mixture of 1/100 μ l anti-Dig-antibody-FITC Fab fragments (Roche) for digoxigenin-labeled probes (green color paint) and of 0.5/100 μ l Ultra Avidin Rhodamine (Leinco Technologies Inc.) for biotin-labeled probes (red color paint). In the case of three color FISH detection, a mixture of these two detection solutions was applied to a probe mixture labeled by digoxigenin and biotin (usually yellow paint color). BAC clone mapping on chromosomes followed a previously described technique (Hirai and Hirai 2004).

Result and discussion

Paint analyses

We produced paint probes for each of the chromosomes of *S. mansoni* using a microdissection technique. Figure 1 shows the data of chromosomes 1-7. Although chromosomes 5-7 have been reported in a previous study (Taguchi et al. 2007), we added probes for autosomes 1-4 in the present study.

Chromosome identification becomes easier by using the probes, which can detect each autosome, especially in gene and genome mapping. In addition, we made paint probes for the Z and W chromosomes (Figure 2). DAPI stained chromosomes clearly identified the W chromosome with the large heterochromatin block (Figure 2a, bright region). A paint probe of the W chromosome hybridized only with an inner region (Figure 2b, bright green color) of the large heterochromatic block between the short and long arms of the W (compare between Figure 2a and 2b). Although the signal of the short arm was weak, almost the entire length of the heterochromatic region was identified with the W paint probe (Figure 2b, bracket). Double FISH with the Z and W paint probes shows the Z and W chromosomes more clearly (Figure 2c and 2d). The Z probe hybridized along the entire length of the Z chromosome and two parts (green color) of the short and long arms of the W chromosome (Figure 2d). The two positive parts (green) on the W stained by the Z probe are located on both sides of the W probe signal (pink color) (Figure 2d).

Chromosome paint analysis is useful for detecting karyotypes with unidentifiable characteristics due to similar morphology as well as for elucidating phylogenetic homology of chromosomes. In the present study, we identified and validated paint probes for chromosomes 1, 2, 3, 4, 5, 6, 7, Z, and W. As the DNA probes allow us to identify each chromosome, we were now in a position to reconstruct chromosome rearrangements in the genus *Schistosoma*. As shown in the Z and W, the paint probes visually show homologous and differentiated regions between the sex chromosomes. The data supports our contention that an ancestor homologous pair differentiated into the two sex chromosomes, W with and Z without heterochromatin block around the centromeric region. Although this has also been shown by C-band analysis (Short 1983), DNA paint probes demonstrate more clearly that heterochromatinization apparently occurred in a Z homologue, as other regions except the heterochromatin are completely homologous between the Z and W (Figure 3). The genes related to sex accumulate on potential sex chromosomes, due to heterochromatinization that prevents gene exchange (recombination) between the former homologues. As shown in Figure 4, prevention of recombination in the heterochromatic region was actually observed in female meiotic cell divisions of *S. mansoni* (Hirai *et al.* 1989; Spotila *et al.* 1989; Criscione *et al.* 2009). Three chiasmata only in euchromatic regions were usually observed in meiotic bivalent chromosome of the Z-W pair, but crossing over never occurred in the heterochromatic region (Figure 4). A linkage map analysis indicated two interesting events, that the 2nd

chiasma located at the boundary region between heterochromatin and euchromatin of the long arm showed a hot spot for recombination and that the Z chromosome region corresponding to the W heterochromatin block has Z specific genes (Criscione *et al.* 2009). Whether or not heterochromatinization occurred as a single event remains to be determined. In the present study, however, we were able to estimate the inversions that probably occurred before heterochromatinization by mapping BAC clones (below).

Chromosome mapping of BAC clones

To date 93 BACs were localized on the Z and W chromosomes (Figure 5a). Out of them, 33 clones were localized to common regions of the Z and W (red letters). Thirty-five clones were observed only on the Z (blue letters). Eleven clones were specific for the W (purple letters). Nine clones were observed on both chromosomes, but the locations were different between the Z and W (black letters). Three clones were also observed on both sex chromosomes, but the locations were slightly different between the Z and W (orange letters). Green letter clones (29F12 and 29F20) showed hybridization (Figure 5 inset) at different locations between the Z and W and partial transposition in the W (29F20).

These results allow us to estimate the existence of inversions that occurred during evolution of the Z and W (Figure 5b). Minimum estimation is the occurrence of 4 inversions, 3 in the Z chromosome and 1 in the W

chromosome. The first postulated inversion is shown by light green bars, in which 5 clones (black letters in Figure 5a) moved from the mid region of the Z to the border between euchromatin and heterochromatin of the short arm of the W (= pericentric inversion: inversion 1). The second may be a paracentric inversion shown by the yellow lines (15C3, 29F2) (inversion 2). The third may be a paracentric inversion in the short arm of the Z (83-1F, 83-9A, 29D5) (inversion 3). Finally, the fourth may be a pericentric inversion on the W, movement from the border of euchromatin and heterochromatin to the proximal region of the long arm (29F20) (inversion 4), identified by a black bracket (Figure 5b). The clone 29F20 was also located at the mid region of the Z chromosome, and moved to the border region of heterochromatin and euchromatin of the W. It allows us to postulate that the clone might have moved by a pericentric inversion (dark green line) or heterochromatinization in the diverged W chromosome.

Conventional experiments also postulated existence of inversions in evolution of the W (Short 1983; Lawton et al. 2011), but the locations of the inversions were not defined. In the present study, BAC chromosome mapping indicated approximate positions of inversions, although the exact breakage points and the order of occurrence are not clear. Localization of the BAC clones between the Z and W chromosomes allowed us to estimate the positions of the inverted areas that occurred in the ancestral sex chromosomes. The order in which these inversions occurred will require more intensive analysis. Paint analysis and BAC mapping reconfirmed heterochromatinization in the W

previously shown by C-band analysis (Short 1983; Hirai et al. 1989), and detected homologous regions of the Z and W. From these findings uncovered by molecular cytogenetic techniques, three inversions on the Z and one on the W of *S. mansoni* were for the first time defined. This coupled with the study by Lepesant et al. (2012) provides further insights into ZW sex chromosome evolution.

Based on relationships between inversions and heterochromatin, assuming these mapping data are correct, a hypothesis of the ZW evolution can be proposed. (1) We postulate that inversion 1 occurred on one of the ancestral homologous chromosomes of the ZW before heterochromatinization, because BACs transposed (Figure 5b, green lines) from the mid region of the Z to a corresponding region with the heterochromatin and euchromatin border after differentiation of the W. (2) Inversion 2 occurred at the middle portion of the long arm of the ancestral chromosome of the W. Although the order in which inversion 1 and 2 occurred cannot be determined at present, this inversion area may be the starting point of heterochromatinization of the W. As heterozygous inversions act as crossing-over suppressors (White 1973), these inversions likely reduced genetic recombination between the ZW precursors. This is postulated to have resulted in the separation of the Z and W, followed by heterochromatinization based on repetitive sequences and sex-specific gene differentiation in this region. Therefore, most of the BACs that mapped to the region of the Z showed specificity for the Z (Figure 5a, blue letters). (3)

Inversion 3 occurred in the short arm of the Z after separation of the Z and W, because the inversion is observed only on the Z. (4) Inversion 4 occurred only in the W after evolution of the sex chromosome, because mapping data showed movement of part of the clone (29F20) only on the W. Although BAC mapping suggests movement of two clones (29F12 and 29F20) from outside of the inversion area (Figure 5, dark green line), we should perform further analyses to determine this in the future, as the transposition of BACs cannot be explained by simple inversion, assumed heterochromatinization as well.

The more intensive and extensive determinations using chromosome paint probes that we established here coupled with BAC chromosome mapping will allow significant contributions to the evolution of schistosome sex chromosomes. Although multiple band analysis like G- or R-band found in mammals is not available in schistosomes, the molecular cytogenetic analyses introduced here could provide important clues for chromosome and genome evolution of these parasites. Chromosome paint analysis and BAC (or gene) chromosome mapping are relevant for investigations of chromosome evolution and determination of gene order. Recently, an intensive comparative analyses of the genomic sequence and the chromatin structure between male and female lead to a new hypothesis that sex differentiation in *S. mansoni* is based on developmental stage-dependent tagging of the W chromosome by non-coding RNA and a chromatin marking system (Lepessant et al. 2012). These new molecular and chromosomal (this study) techniques are important clues for

understanding not only sex chromosome evolution but also biological evolution of schistosome groups.

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Figure legends

Figures with Legends

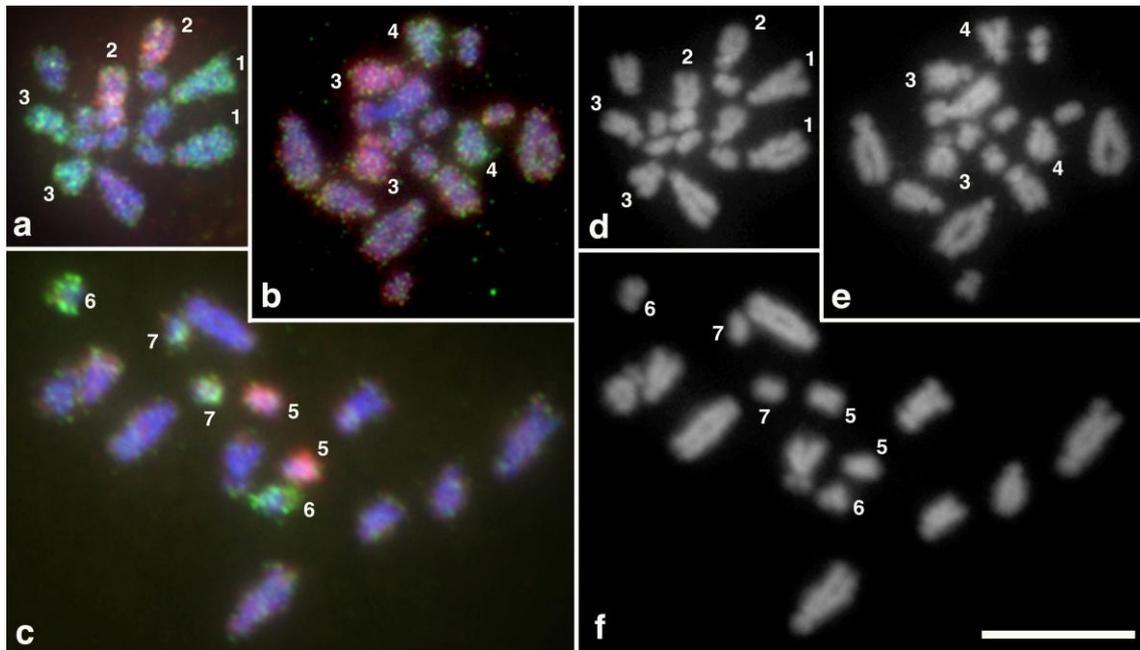


Figure 1. Chromosome paint with DNA probes of autosomes 1-7 of *S. mansoni*.

(a)-(c) Painted chromosomes. For detection color system see results section.

(d)-(f) DAPI (4',-6-Diamidine-2'-phenylnedole dihydrochloride) stained chromosomes corresponding to a, b, and c, respectively. Chromosomes are numbered. Scale is 10 μ m.

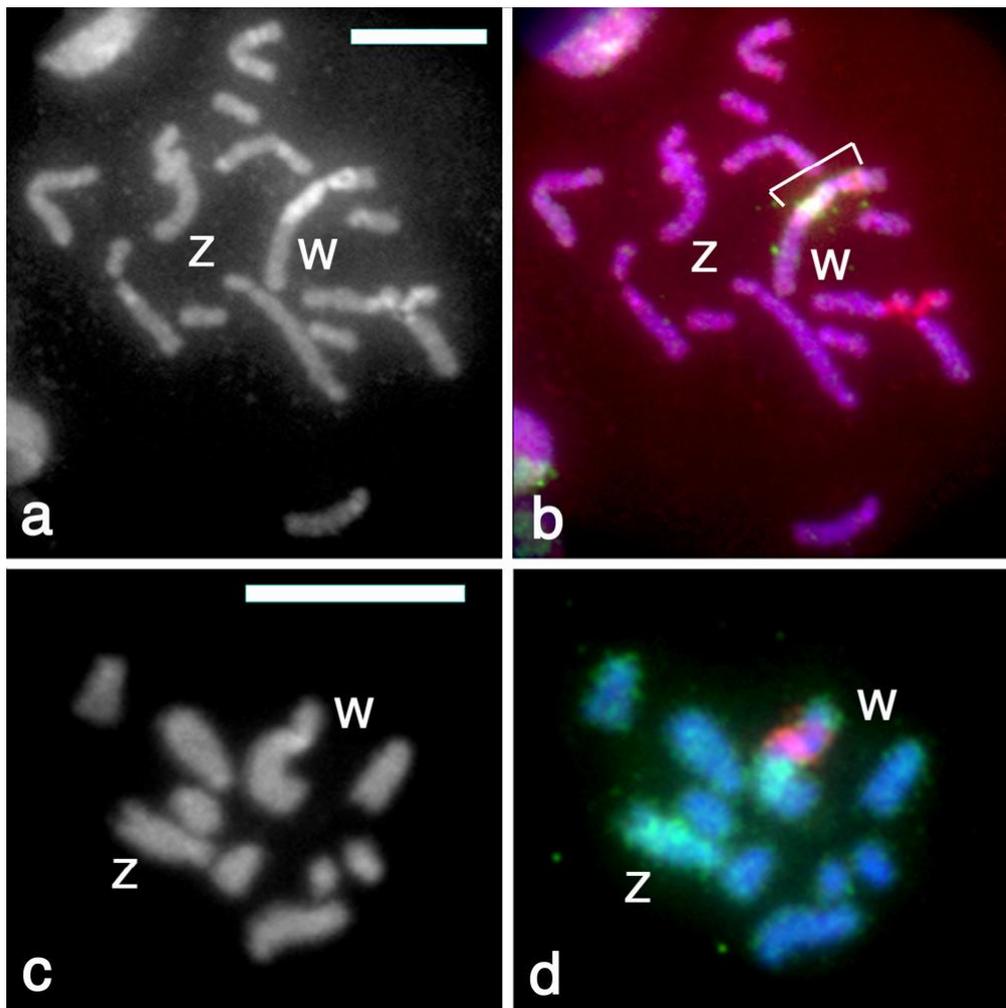


Figure 2. Chromosome paint analysis of the Z and W of *S. mansoni*. a) Conventional DAPI stained metaphase chromosomes. b) Chromosomes painted with the W probe. Bright green color (DIG-FITC system) is positive signal for W paint probe. White bracket shows a signal block on the W chromosome. c) Partial metaphase chromosomes stained by DAPI. d) Double FISH data analyzed with both of Z (labeled by DIG-FITC system: green color) and W (labeled by biotin-Rhodamine system: pink color) paint probes. Scale is 10 μ m.

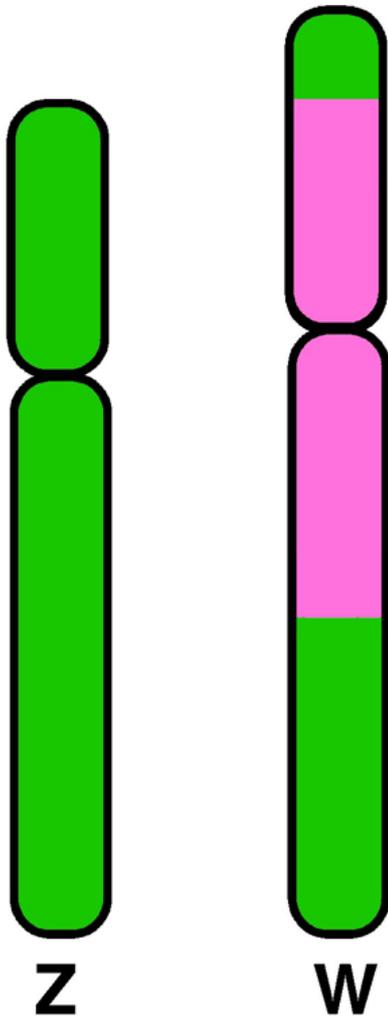


Figure 3. Schematic illustration of relationship of the Z and W chromosomes observed with C-band and chromosome paint analyses. Green color shows common regions between both stained by the Z paint probe. Pink color shows region specific for the W chromosome stained by the W paint probe and C-band treatment.

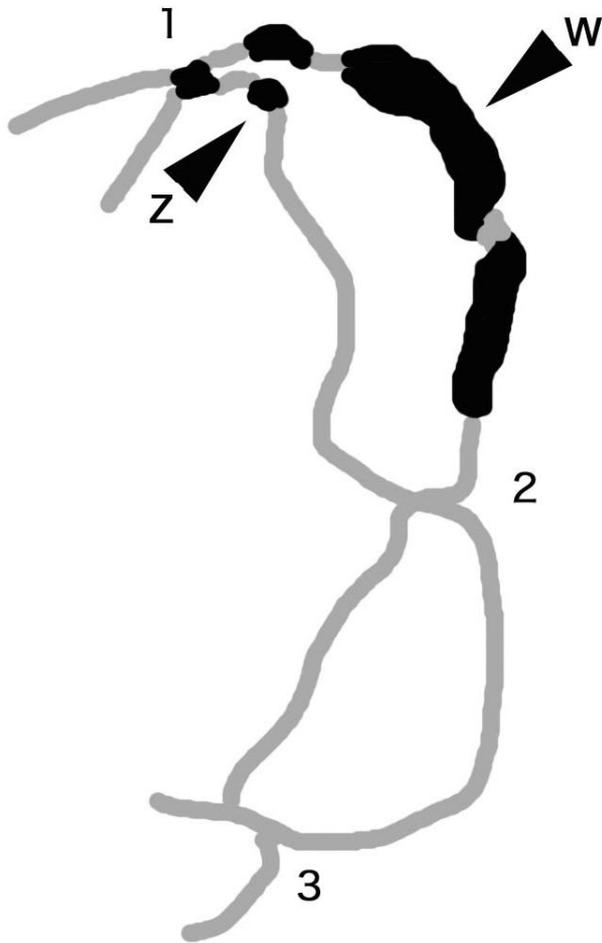


Figure 4. Illustration of Z and W chromosome pairing in diakinesis of male meiosis of *S. mansoni*. Black is heterochromatin and grey is euchromatin. Arrowhead is centromere region. Z, the Z chromosome. W, the W chromosome. Number shows location of chiasma.

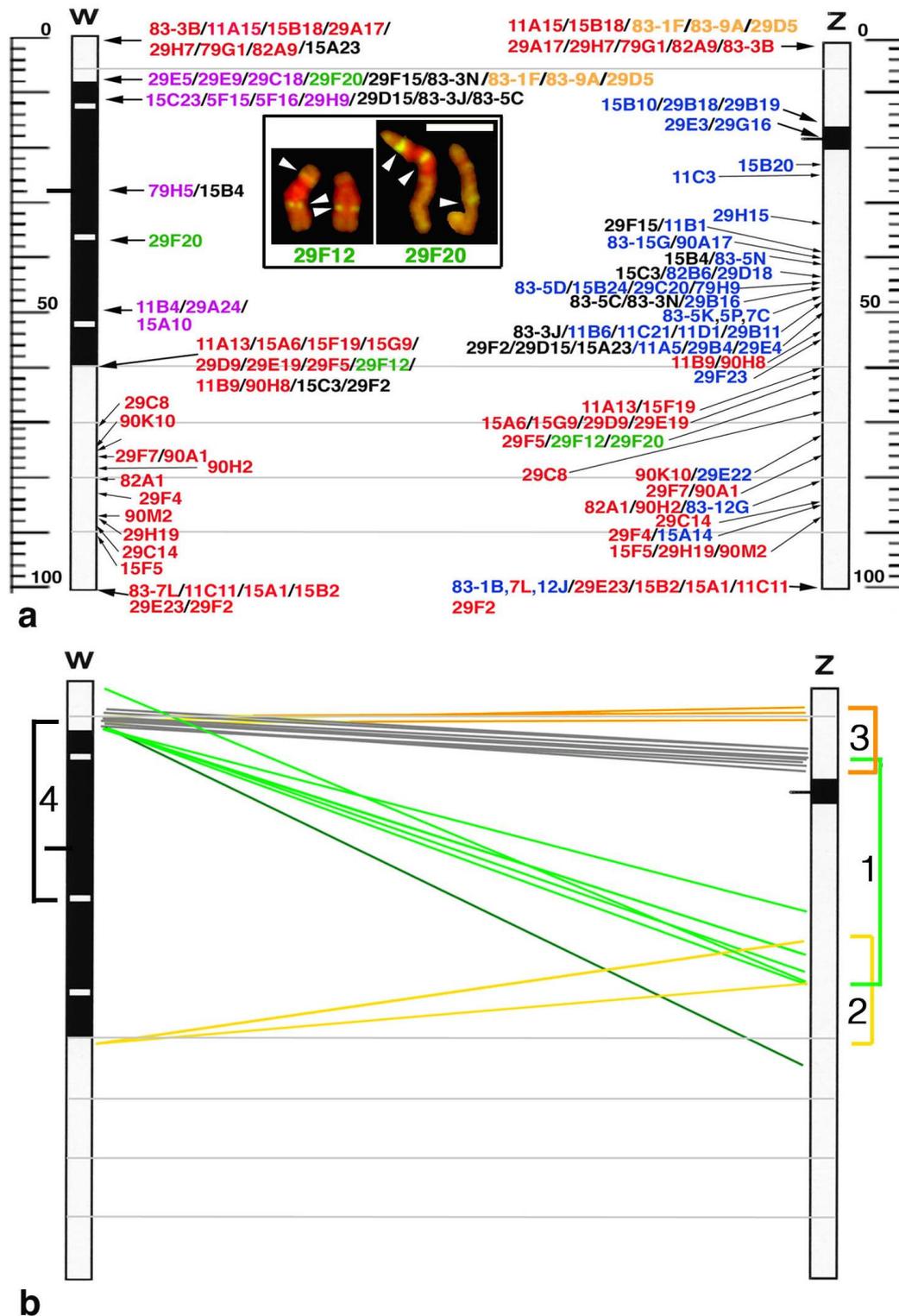


Figure 5. Localization of BAC clones (a) and chromosome structural changes (b) inferred from regions of BAC clones on the Z and W of *S. mansoni*. Blue letters

are clones specific for the Z. Purple letters are clones specific for the W. Red letters are clones located at common regions of the Z and W. Black letters are clones located at different regions between the Z and W. Orange letters are clones rearranged in the short arm of the Z. Green letters are clones shown by real picture of hybridization on the Z and W (inset, Scale is 10 μ m). Lines of (b) between the Z and W indicate locations of the same clones between both chromosomes. Light green lines indicate which 5 clones (black letters in Figure 5a) moved from the mid region of the Z to the border between euchromatin and heterochromatin of the short arm of the W (inversion 1). Yellow lines indicate movement of two clones (153C and 29F2) from the mid region of the Z to the border region between euchromatin and heterochromatin of the long arm of the W (inversion 2). Orange lines show movement of three clones (83-1F, 83-9A, 29D5) within the short arm of the Z chromosome (inversion 3). Black lines show locations that the clones that did not move to the same location between the Z and W chromosomes. Black bracket indicates movement of a clone (29F20) from the border of euchromatin and heterochromatin to the proximal region of the long arm (inversion 4). Clone 29F20 might have been located at the mid region of the ancestor Z chromosome, and moved to the border region of heterochromatin and euchromatin of the W by a pericentric inversion (dark green line) or heterochromatinization in the W. For details see text.