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ORIGINAL ARTICLE

The fate of W chromosomes in hybrids between wild silkmoths, *Samia cynthia* ssp.: no role in sex determination and reproduction

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Moths and butterflies (Lepidoptera) have sex chromosome systems with female heterogamety (WZ/ZZ or derived variants). The maternally inherited W chromosome is known to determine female sex in the silkworm, *Bombyx mori*. However, little is known about the role of W chromosome in other lepidopteran species. Here we describe two forms of the W chromosome, W and neo-W, that are transmitted to both sexes in offspring of hybrids from reciprocal crosses between subspecies of wild silkmoths, *Samia cynthia*. We performed crosses between *S. c. pryeri* ($2n=28$, WZ/ZZ) and *S. c. walkeri* ($2n=26$, neo-Wneo-Z/neo-Zneo-Z) and examined fitness and sex chromosome constitution in their hybrids. The F_1 hybrids of both reciprocal crosses had reduced fertility. Fluorescence *in situ* hybridization revealed not only the expected sex chromosome constitutions in the backcross and F_2 hybrids of both sexes but also females without the W (or neo-W) chromosome and males carrying the W (or neo-W) chromosome. Furthermore, crosses between the F_2 hybrids revealed no association between the presence or absence of W (or neo-W) chromosome and variations in the hatchability of their eggs. Our results clearly suggest that the W (or neo-W) chromosome of *S. cynthia* ssp. plays no role in sex determination and reproduction, and thus does not contribute to the formation of reproductive barriers between different subspecies.
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INTRODUCTION

Sex chromosomes consist of two types: those appearing only in one sex (termed Y or W) and those appearing in both sexes in one or two copies (X or Z). It is believed that the sex chromosomes (XY or WZ) have evolved from a pair of originally recombining homologous chromosomes, which differentiated through the restriction of recombination after one member of the pair acquired a sex-determining function (Charlesworth *et al.*, 2005). This model of sex chromosome evolution primarily concerns taxa where recombination is present in both sexes. To what extent is this model applicable to taxa where recombination is fully suppressed in the heterogametic sex, such as in males of flies and females of moths and butterflies (reviewed in Marec, 1996), remains to be clarified.

Sex-limited chromosomes (Y in males or W in females) genetically degenerate due to the lack of recombination and, therefore, their DNA compositions usually differ greatly from the other genomic regions. Although there are many mysteries about the function of these chromosomes, their importance for sex-specific trais has been revealed in various organisms. For example, in some vertebrates the Y or W chromosomes provide primary sex determination signals (Bachtrog *et al.*, 2014). In contrast, the Y chromosome of *Drosophila melanogaster* has no effect on the sex determination cascade, which is controlled by the ratio between the number of X chromosomes and the number of sets of autosomes (Salz and Erickson, 2010). However, the *Drosophila* Y chromosome also plays an important role as it contributes significantly to fertility and male fitness (Brosseau, 1960; Lemos *et al.*, 2008). In chicken, the *DMRT1* gene located on the Z chromosome is considered a key player in the sex determination pathway (Smith *et al.*, 2009), while the role of W chromosome is still unclear; at least recent data suggest that the W chromosome affects female fertility traits (Moghadam *et al.*, 2012).

The sex chromosomes X or Z play an important role in fundamental evolutionary processes, such as speciation or adaptation (Qvarnström and Bailey, 2009). A large contribution of the X chromosome to reproductive isolation (the so-called large X-effect) has been well established in models with male heterogamety, especially in species of the *Drosophila* genus (Presgraves, 2008). A growing body of evidence suggests that also the Y chromosomes may play a role in the evolution of reproductive barriers (e.g., Sweigart, 2010; Campbell *et al.*, 2012), in addition to their role in sex determination or sex-specific functions and traits (Dean and Mank, 2014).

Moths and butterflies (Lepidoptera) have holokinetic chromosomes and sex chromosome systems with female heterogamety. Besides the most common WZ/ZZ (female/male) system, numerical variations of the sex chromosomes such as Z0/ZZ, W_1W_2Z/ZZ and $WZ_1Z_2/Z_1Z_1Z_2Z_2$ occur in different branches of the lepidopteran phylogenetic tree (Marec *et al.*, 2010). Basal groups (non-Ditrysia) share a Z0/ZZ system with Trichoptera (caddis-flies), the sister order of Lepidoptera, and available data suggest that the W chromosome arose later in the evolution of Lepidoptera in a common ancestor of the non-ditrysian Tischeriina plus advanced Lepidoptera, the Ditrysia (Lukhtanov, 2000).

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Although it seems clear that the sex in Lepidoptera is determined by the sex chromosome constitution, the actual role of the W and Z chromosomes remains unknown except for the silkworm, *Bombyx mori* (see below). It has been proposed that the sexual development depends either on the presence of W chromosome encoding a female-determining signal (dominant W) or on the dosage of Z chromosomes carrying male-promoting genes and acting against female-promoting genes located on autosomes, the so-called Z-counting mechanism (Traut *et al.*, 2007; Sahara *et al.*, 2012). The 'dominant W' sex determination mechanism has been well established in *B. mori*. In this species, the W chromosome carries a dominant female-determining factor (*Fem*) that promotes femaleness, irrespective the number of Z chromosomes present in the genome (Fujii and Shimada, 2007). Recently Kiuchi *et al.* (2014) made the surprising discovery that the feminizing factor in *B. mori* is not a protein-coding gene but a W-encoded small RNA named *Fem* piRNA. The authors also showed that the *Fem* piRNA downregulates the expression of a Z-linked gene, *Masculinizer* (*Masc*), which promotes male development in the absence of the W chromosome. However, it is not yet known (i) whether the *Fem* piRNA-*Masc* sex-determining pathway is conserved in other lepidopteran species with the W chromosome and (ii) whether *Masc* plays a role in species with a Z0/ZZ sex chromosome system that are thought to have the Z-counting mechanism of sex determination (Traut *et al.*, 2007).

Based on the disproportionate association of sex-linked traits that distinguish closely related species, the lepidopteran Z chromosome is expected to have a large effect on species divergence (Sperling, 1994). Indeed, an increasing number of reports point to a major role of sex chromosomes in postzygotic and/or prezygotic reproductive barrier in some lepidopteran species (e.g., Naisbit *et al.*, 2002; Dopman *et al.*, 2005). Moreover, recent results suggest that structural rearrangements of sex chromosomes which involve autosomes, the so-called neo-sex chromosomes, can also contribute to reproductive isolation and speciation in Lepidoptera (Yoshido *et al.*, 2011a; Nguyen *et al.*, 2013). However, little is known about the actual impact of sex chromosomes and their meiotic behaviour on fitness of hybrids between distinct populations or closely related species.

Our previous studies showed that different subspecies of wild silkmoths, *Samia cynthia* ssp. (Lepidoptera; Saturniidae), exhibit a unique sex chromosome polymorphism. Geographical populations of *S. cynthia* ssp. with allopatric distributions differ in chromosome numbers and have at least four different sex chromosome constitutions, predominant WZ/ZZ in *S. c. pryeri* (west Japan) with $2n=28$♀/28♂, Z0/ZZ in *S. c. ricini* (Vietnam) and sporadically in *S. c. pryeri* (a part of Kagoshima population, Kyushu, Japan) with $2n=27$♀/28♂, neo-WZ_1Z_2/$Z_1Z_1Z_2Z_2$ in *S. cynthia* subsp. indet. (Nagano, Japan) with $2n=25$♀/26♂, and neo-Wneo-Z/neo-Zneo-Z in *S. c. walkeri* (Sapporo, Japan) with $2n=26$♀/26♂ (Yoshido *et al.*, 2005, 2011a, 2013). The variations in the constitution of sex chromosomes were derived from the loss of W chromosome and fusion between the sex chromosomes and autosomes, resulting in different chromosome numbers (Yoshido *et al.*, 2011a, b; Sahara *et al.*, 2012). For example, the neo-Z chromosome in *S. c. walkeri* arose by fusion between the Z chromosome and chromosome 13 of *S. c. pryeri* (Figure 1). In addition, *S. cynthia* ssp. also exhibit unusual differences between W chromosomes. The W chromosome in *S. c. pryeri* consists of two parts, the highly heterochromatic part (black in Figure 1) and less heterochromatic part (striped grey in Figure 1). The former part was found only in *S. c. pryeri* while the latter part corresponds to the ancestral segment (striped grey in Figure 1) of the neo-W chromosome in *S. c. walkeri* (Yoshido *et al.*, 2013). Hence *S. cynthia* ssp. provide a unique opportunity to address fundamental biological questions, such as the evolution of neo-sex chromosomes, their role in the formation of reproductive barriers between populations and closely related species and the role of multiple sex chromosome systems in sex determination.

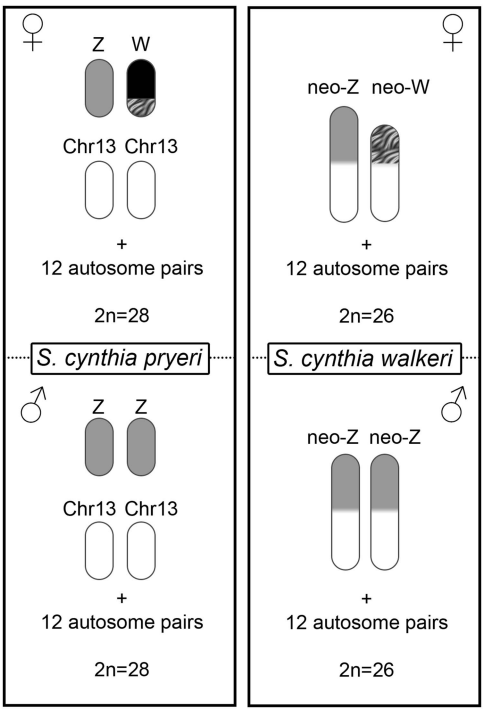


Figure 1 Schematic illustrations of sex chromosome constitutions in both sexes of *Samia cynthia pryeri* and *Samia cynthia walkeri*. *S. c. pryeri* has a WZ/ZZ sex chromosome system with $2n=28$♀/28♂ and *S. c. walkeri* a neo-Wneo-Z/neo-Zneo-Z system with $2n=26$♀/26♂. The neo-Z chromosome of *S. c. walkeri* arose by fusion of an ancestral Z chromosome (grey) and an autosome corresponding to chromosome 13 (Chr13) of *S. c. pryeri*. The W chromosome in *S. c. pryeri* consists of a highly heterochromatic part (black) and euchromatin-like part (striped grey), the latter corresponding to a part of the neo-W chromosome in *S. c. walkeri*.

Here we performed reciprocal crosses between *S. cynthia* ssp. (*S. c. pryeri* and *S. c. walkeri*) with distinct sex chromosome constitutions and different W chromosomes and examined egg hatch rates in F_1, F_2 and backcross hybrids. We also examined the constitution of sex chromosomes and their behaviour in the F_2 and backcross hybrids using molecular cytogenetic markers. This detailed analysis of hybrids allowed us to test the role of different W chromosomes in sex determination of *S. cynthia* ssp. and whether they have any impact on fitness and contribute to the formation of reproductive barriers between the subspecies.

MATERIALS AND METHODS

Insects

We used specimens of two *S. cynthia* subspecies (Saturniidae), *S. c. walkeri* (Sapporo population, Hokkaido) and *S. c. pryeri* (Toyota population, Honshu). Two subspecies were collected at respective sites in 2005–2011 and reared on *Alianthus altissima* at the Field Science Center for Northern Biosphere (Hokkaido University, Sapporo, Japan; Yoshido *et al.*, 2005, 2013).

Crosses

Mating experiments were carried out during the period from 2010 to 2012 in the Field Science Center for Northern Biosphere, Hokkaido University. After eclosion, virgin females and males were mated individually in mesh cages ($24\times80\times25$–50 cm) for 1–3 days. Behaviour of moths in cages was

monitored at intervals of 6–10 h to check for mating. Mated females were kept separately in cages, where they laid eggs for 1–3 days. Eggs from individual females were collected and placed in plastic containers. About 10–15 days after oviposition, hatchability of the eggs was recorded. Scatterplots of fecundity (i.e., the number of eggs laid) and the egg hatchability were created using Excel templates for independent data, as shown in Weissgerber *et al.* (2015). In addition, the data obtained were statistically compared by one-way analysis of variance followed by Tukey's multiple comparisons test, using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). Bodies of the parents used for these crosses were stored at − 30 °C to verify their genotype, if needed. Hatched larvae were reared on *A. altissima*.

Chromosome preparations

Mitotic chromosomes were obtained from wing discs of the last instar larvae as described in Yoshido *et al.* (2014). Spread preparations of pachytene complements were made from gonads of both sexes as given in Yoshido *et al.* (2013). Tissues of the last larvae were dissected in a saline solution, swollen for 10–15 min in a hypotonic solution (75 mM KCl) and then fixed for 10–15 min in Carnoy fixative (ethanol, chloroform, acetic acid, 6:3:1). Ovaries were fixed immediately after dissection, that is without hypotonic treatment. Cells were dissociated in 60% acetic acid and spread on a heating plate at 50 °C. Preparations were then passed through a graded ethanol series (70, 80, 98%, 30 s each) and stored in the freezer at − 20 °C until use.

Fluorescence in situ hybridization (FISH)

For FISH with *S. cynthia walkeri* fosmid clones, we selected a Z chromosome marker, clone 45A6 and a chromosome 13 marker, clone 32B23 (for details, see Yoshido *et al.*, 2011b). Fosmid probes were labelled using a Nick Translation Kit (Abbott Molecular Inc., Des Plaines, IL, USA) with Green-dUTP (Abbott Molecular Inc.) and Cy3-dUTP (GE Healthcare, Buckinghamshire, UK), respectively. For the detection of W chromosomes, we used FISH with W chromosome painting probes (the so-called type-2 W-probes), which highlighted the whole W chromosome in *S. c. pryeri* and a part of the neo-W chromosome in *S. c. walkeri* (Yoshido *et al.*, 2013). The probes were prepared and then labelled with Cy3-dUTP (GE Healthcare) as described in Yoshido *et al.* (2013). FISH with W chromosome painting probes were carried out according to the reprobing protocol for Lepidoptera (Yoshido *et al.*, 2014) after FISH with fosmid probes.

Chromosome preparations were removed from the freezer, passed through the graded ethanol series and air dried. Denaturation of chromosomes was carried out at 72 °C for 3.5 min in 70% formamide, 2× saline sodium citrate (SSC) buffer. For one preparation, the probe cocktail contained 500 ng of each labelled DNA probe, 3.0–5.0 μg of unlabelled sonicated male genomic DNA and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich, Tokyo, Japan) in 10 μl hybridization solution (50% formamide, 10% dextran sulphate, 2× SSC). The probe cocktail was denatured for 5 min at 90 °C and then spotted on denatured chromosomes. After incubation in a moist chamber at 37 °C for 3 days, slides were washed at 62 °C in 0.1× SSC containing 1% Triton X-100. The slides were then counterstained and mounted in antifade based on DABCO (1,4-diazabicyclo (2.2.2)-octane), containing 0.5 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) (both Sigma-Aldrich, Tokyo, Japan). Preparations were observed in a Leica DMRE HC fluorescence microscope (Leica Microsystems Inc., Tokyo, Japan) or a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Digital images were captured with a DFC350FX B&W CCD camera (Leica Microsystems Inc.) or an Olympus CCD monochrome camera XM10 equipped with cellSens 1.9 digital imaging software (Olympus Europa Holding, Hamburg, Germany) and processed with Adobe Photoshop CS4.

Cloning and Southern hybridization

DNA sequences derived from the W chromosome of *S. c. pryeri* (W-DNA) were obtained by laser microdissection (Yoshido *et al.*, 2013). PCR products amplified from W-DNA of microdissected samples by WGA4 and WGA3 Kit (Sigma-Aldrich, St. Louis, MO, USA) 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 the recipient. More than 300 clones were screened by FISH whether they are derived from the W chromosome or not. Cloned inserts derived from the W chromosome were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Probes for Southern hybridization were generated from the cloned inserts and labelled with thermostable alkaline phosphatase using AlkPhos Direct Labeling and Detection System (GE Healthcare). Genomic DNAs were extracted separately from females and males by standard phenol–chloroform procedure. Samples of the extracted DNAs were digested each with three restriction enzymes, *Hind*III, *Hae*III and *Alu*I (Takara, Kyoto, Japan), respectively. Then 3.0 μg of each sample were loaded on 1.5% agarose gel in TAE buffer and blotted onto nylon membrane, Hybond-N+ (GE Healthcare), by capillary transfer in 20× SSC. Hybridization and chemiluminescent detection with CDP-*Star* were carried out following the supplier's protocol. Membranes were exposed to Hyperfilm ECL (GE Healthcare).

Detection of W chromosomes in mated moths

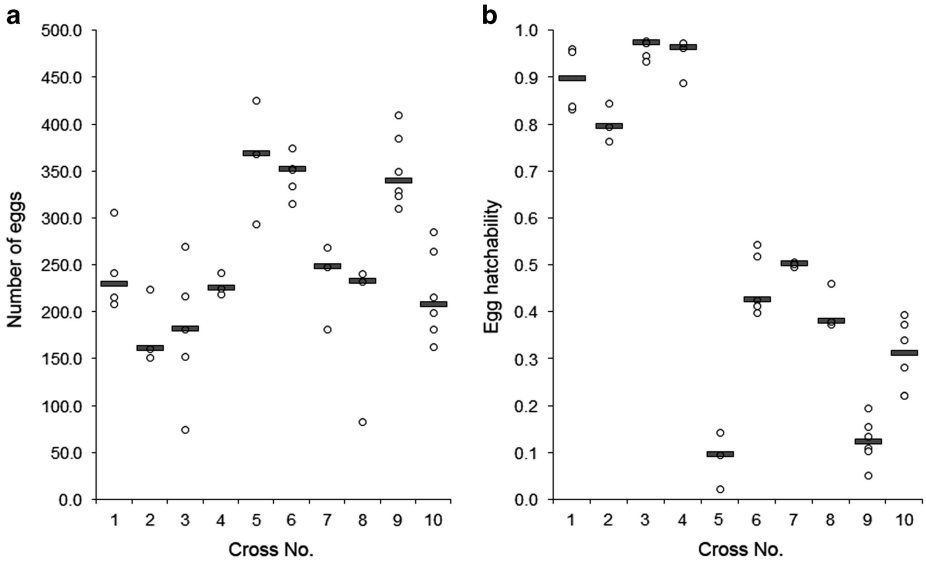
Genomic DNAs of mated moth parents were extracted separately from fathers and mothers by DNAzol reagent (Invitrogen, Tokyo, Japan). For the detection of *S. cynthia pryeri* W chromosomes, we designed primers 5′-CGAAATTTCGA TTACAACCC-3′ (forward; SCPW50-F) and 5′-TGTAGTTTTTCTGTATCC GG-3′ (reverse; SCPW50-R) derived from a sequence of the W chromosome, which was identified in this study (see Results). PCR reactions were performed using a reaction mixture composed of 5.0 ng of template genomic DNA, 10 pmol of each primers, 0.5 U of Ex-*Taq* polymerase and 1.0 μl of 10× Ex-*Taq* PCR buffer (Takara). PCR amplifications were conducted under the following conditions: 94 °C for 5 min, 18 cycles of 94 °C for 10 s, 54 °C for 10 s, 72 °C for 20 s and a final extension step at 72 °C for 1 min. Three microliters of PCR products were loaded on 1.5% agarose gel in TAE buffer. Gels were stained with ethidium bromide and photographed under UV light.

For the detection of neo-W chromosomes in *S. c. walkeri*, we used the sequences of *S. cynthia* ortholog of a gene coding for a glycine-rich protein (GRP2), which were amplified from genomic DNA of respective individuals by PCR using two primers, 5′-GGCGCTCCCATCGTACAGAA-3′ (forward; GRP2-F) and 5′-TCCGTAGGAGGTGCTGACAC-3′ (reverse; GRP2-R). The primer sequences were obtained from the *S. c. ricini* EST database (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). This primer set was designed to amplify the exonic sequences (576 or 588 bp, see Results). PCR conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 5 min. The PCR-generated fragments were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

RESULTS

Hatchability of eggs in crosses between *S. c. pryeri* and *S. c. walkeri*

Parental crosses between *S. c. pryeri* (SCP) and *S. c. walkeri* (SCW) revealed no significant differences in reproductive capacity from control crosses within respective populations (cross types No. 1–4 in Figure 2 and Supplementary Table S1). Both reciprocal crosses showed comparable fecundity (i.e., the number of eggs laid; Figure 2a) and fertility, with the egg hatchability as high as about 95% (Figure 2b and Supplementary Table S1, cross types No. 3 and 4). Also backcrosses and F_1 crosses showed no reduction of fecundity in comparison with both controls. Interestingly, females in all crosses of offspring derived from P crosses between SCP females and SCW males (cross type No. 3 in Figure 2 and Supplementary Table S1) laid a larger number of eggs than controls and all other crosses (Figure 2a and Supplementary Table S1, cross types No. 5, 6 and 9). However, the hatching rates in all four backcrosses and two reciprocal F_1 crosses were considerably reduced to about 10–50% (cross types No. 5–10 in Figure 2b and Supplementary Table S1). Particularly drastic reduction of fertility was observed in one type of F_1 females backcrossed to SCW males and in one type of crosses between F_1 hybrids, where the hatchability of eggs decreased to 8.8 and 12.5% on average, respectively (crosses No. 5 and 9 in Figure 2b and Supplementary Table S1).



Cross No.	Cross type	No. of pairs	Crosses	
			female	male
1	C	4	SCW	SCW
2	C	3	SCP	SCP
3	P	5	SCP	SCW
4	P	3	SCW	SCP
5	B_1	3	SCP♀×SCW♂	SCW
6	B_1	5	SCW	SCP♀×SCW♂
7	B_1	3	SCW♀×SCP♂	SCW
8	B_1	3	SCW	SCW♀×SCP♂
9	F_1	6	SCP♀×SCW♂	SCP♀×SCW♂
10	F_1	6	SCW♀×SCP♂	SCW♀×SCP♂

Figure 2 Scatterplots of fecundity (**a**) and egg hatchability (**b**) in the control (C), parental (P) and F_1 crosses, and backcrosses (B_1) between *Samia cynthia pryeri* (SCP) and *Samia cynthia walkeri* (SCW). Grey bars represent median. Open circles show the number of eggs laid (**a**) and hatchability of eggs (**b**) for each mating.

Sex chromosome constitution in F_1 and F_2 hybrids of crosses between *S. c. pryeri* and *S. c. walkeri*

To determine the sex chromosome constitution in F_1 and F_2 hybrids of crosses between SCP females and SCW males (see Figure 1 for their sex chromosome constitution), we used the type-2 W-probe which painted the whole W chromosome of *S. cynthia pryeri* (Yoshido *et al.*, 2013) and two fosmid probes, one derived from the Z chromosome (45A6 clone) and the other from chromosome 13 (32B23 clone) of *S. cynthia* ssp. (Yoshido *et al.*, 2011b). FISH with these probes identified the Z chromosome, the W chromosome and two chromosome 13 in mitotic complements of SCP females with $2n=28$ (Figure 3a) and two neo-Z chromosomes in mitotic complements of SCW males with $2n=26$ (Figure 3b). With FISH we also confirmed that all studied F_1 hybrids of crosses between SCP females and SCW males have the expected sex chromosome constitution, that is, W, neo-Z plus a chromosome 13 in F_1 females with $2n=27$ (Figure 3c) and Z, neo-Z plus a chromosome 13 in F_1 males with $2n=27$ (Figure 3d). However, in mitotic complements of F_2 females, FISH mapping with the above probes showed not only two expected sex chromosome constitutions, that is, W, neo-Z plus a chromosome 13 with $2n=27$ (Figure 3e) and W, Z plus a chromosome 13 pair with $2n=28$ (Figure 3f), but also revealed two types of females without the W chromosome, having either neo-Z plus a chromosome 13 with $2n=26$ (Figure 3i) or Z plus a chromosome 13 pair with $2n=27$ (Figure 3j). Similarly in F_2 males, we found two expected sex chromosome constitutions, that is, neo-Z, Z plus a chromosome 13 with $2n=27$ (Figure 3g) and a neo-Z pair with $2n=26$ (Figure 3h), and two unexpected types of males with the W chromosome, having either W, neo-Z, Z plus a chromosome 13 with $2n=28$ (Figure 3k) or W plus a neo-Z pair with $2n=27$ (Figure 3l). All sex chromosome constitutions identified by FISH mapping in F_1 and F_2 hybrids of crosses between SCP females and SCW males are schematically illustrated in Figure 3.

Because the type-2 W-probe can identify the original parts of the neo-W chromosome in *S. c. walkeri* (see Yoshido *et al.*, 2013; Figure 4a in the present study), we also used this probe to determine the sex chromosome constitution by FISH in F_1 and F_2 hybrids originating from the reciprocal crosses between SCW females and SCP males (Figures 4c–l). FISH with the W-probe and fosmid probes (see above) confirmed that parents and F_1 hybrids used in this cross have the expected sex chromosome constitution, that is, neo-W plus neo-Z in SCW females with $2n=26$ (Figure 4a), a Z pair plus a chromosome 13 pair in SCP males with $2n=28$ (Figure 4b), neo-W, a Z chromosome plus a chromosome 13 in F_1 females with $2n=27$ (Figure 4c) and neo-Z, a Z chromosome plus a chromosome 13 in F_1 males with $2n=27$ (Figure 4d). Also in this case, F_2 females showed two expected sex chromosome constitutions, that is, neo-W plus neo-Z with $2n=26$ (Figure 4e) and neo-W, Z plus a chromosome 13 with $2n=27$ (Figure 4f), and two unexpected without neo-W, that is, neo-Z plus a chromosome 13 with $2n=26$ (Figure 4i) and Z plus a chromosome 13 pair with $2n=27$ (Figure 4j). In F_2 males, FISH mapping also revealed two expected sex chromosome constitutions, neo-Z, Z plus a chromosome 13 with $2n=27$ (Figure 4g) and a Z pair plus a chromosome 13 pair with $2n=28$ (Figure 4h), and two unexpected with the neo-W chromosome, that is, neo-Z, Z plus

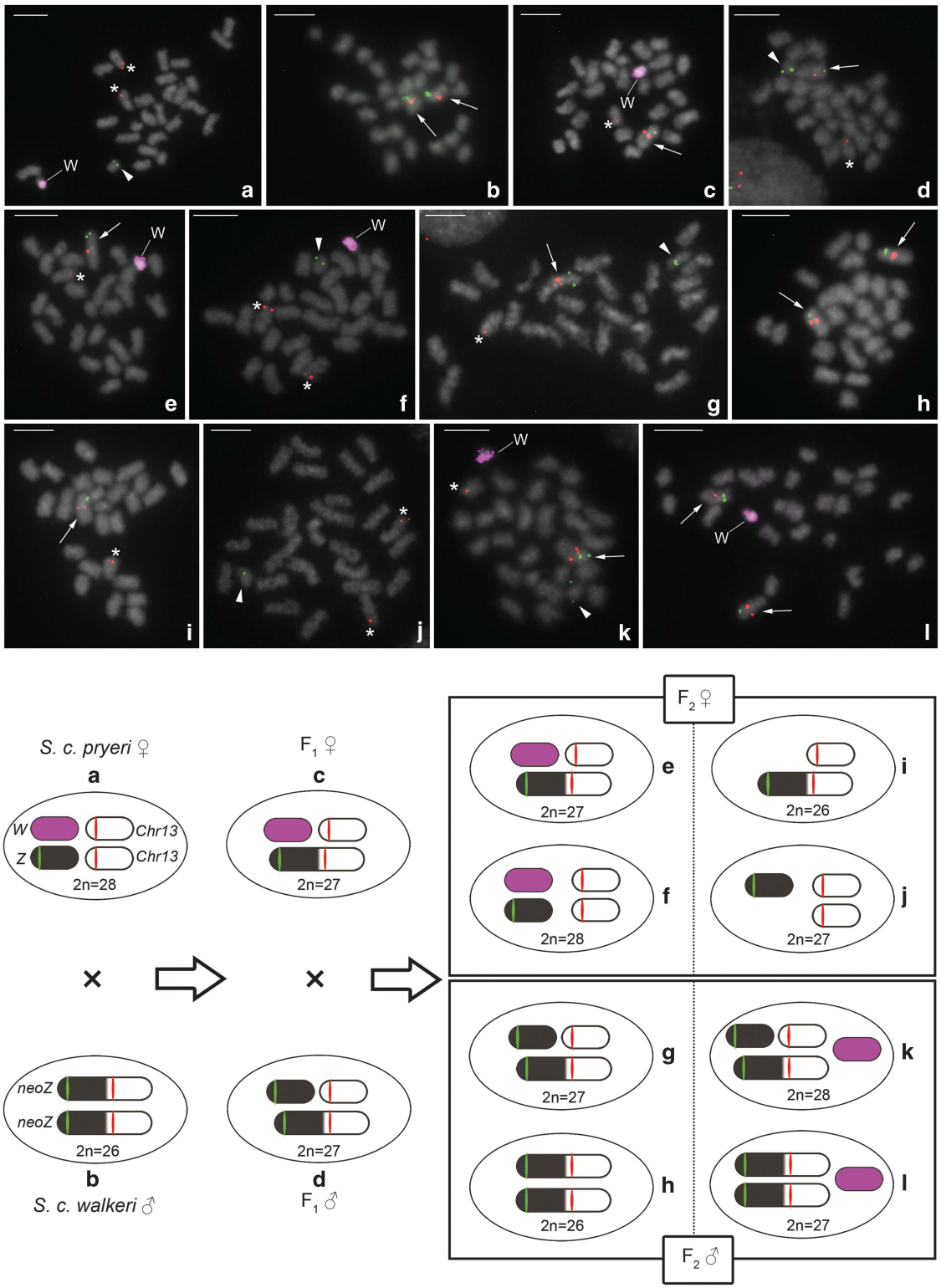


Figure 3 Upper panel: FISH with sex chromosome derived probes in mitotic metaphase complements of parents (**a**, **b**) and F_1 (**c**, **d**) and F_2 (**e**–**l**) offspring of crosses between *Samia cynthia pryeri* females and *Samia cynthia walkeri* males. Chromosomes were stained with DAPI (grey). Cy3-labelled probe of the 32B23 fosmid clone (red signals) mapped to chromosome 13 or the corresponding autosomal part of the neo-Z chromosome and Green-labelled probe of the 45A6 fosmid clone (green signals) to the Z chromosome or the ancestral part of the neo-Z chromosome. Cy3-labelled W-painting probe (magenta signals) highlighted the *S. c. pryeri* W chromosome. Bar = 5.0 μm. Arrows, arrowheads and asterisks indicate the neo-Z chromosome, Z chromosome, and chromosome 13, respectively. (**a**) *S. c. pryeri* female, (**b**) *S. c. walkeri* male, (**c**) F_1 female, (**d**) F_1 male, (**e**, **f**, **i**, **j**) F_2 females and (**g**, **h**, **k**, **l**) F_2 males. Mitotic metaphase complements of F_2 hybrids were examined in eight males and eight females (Supplementary Table S2). Lower panel: schematic illustrations of sex chromosome constitutions in parents and F_1 and F_2 hybrids from matings between *S. c. pryeri* females and *S. c. walkeri* males, based on the FISH results.

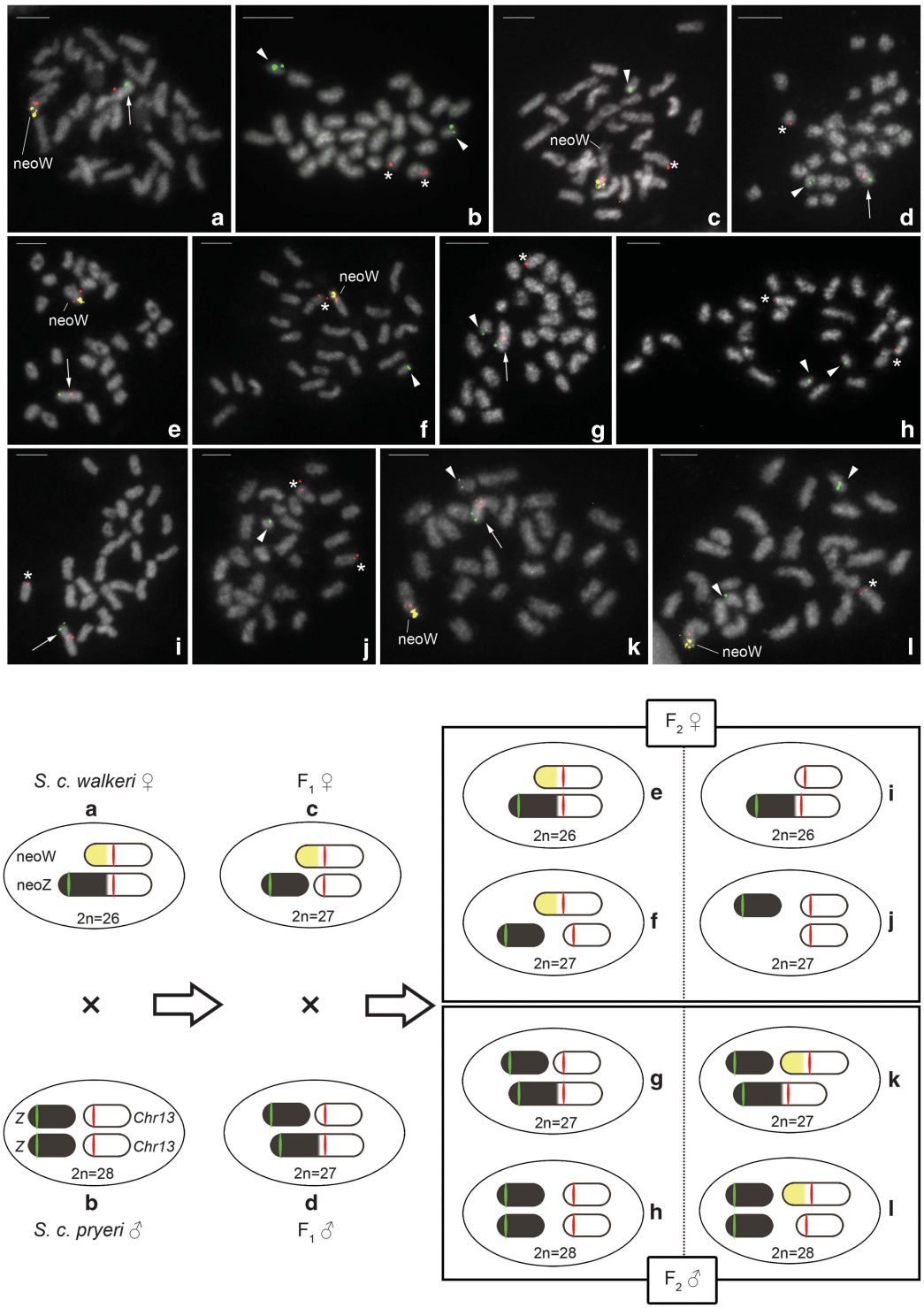


Figure 4 Upper panel: FISH with sex chromosome-derived probes in mitotic metaphase complements of parents (**a**, **b**) and F_1 (**c**, **d**) and F_2 (**e**-**l**) offspring of crosses between *Samia cynthia walkeri* females and *Samia cynthia pryeri* males. Chromosomes were stained with DAPI (grey). Cy3-labelled probe of the 32B23 fosmid clone (red signals) mapped to chromosome 13 or the corresponding autosomal part of the neo-Z and neo-W chromosomes, and Green-labelled probe of the 45A6 fosmid clone (green signals) to the Z chromosome or the ancestral part of the neo-Z chromosome. Cy3-labelled W-painting probe (yellow signals) highlighted the ancestral part of the neo-W chromosome of *S. c. walkeri*. Bar = 5.0 μm. Arrows, arrowheads, and asterisks indicate the neo-Z chromosome, Z chromosome and chromosome 13, respectively. (**a**) *S. c. walkeri* female, (**b**) *S. c. pryeri* male, (**c**) F_1 female, (**d**) F_1 male, (**e**, **f**, **i**, **j**) F_2 females and (**g**, **h**, **k**, **l**) F_2 males. Mitotic metaphase complements of F_2 hybrids were examined in eight males and eight females (Supplementary Table S2). Lower panel: schematic illustrations of sex chromosome constitutions in parents and F_1 and F_2 hybrids from matings between *S. c. walkeri* females and *S. c. pryeri* males, based on the FISH results.

a neo-W with $2n = 27$ (Figure 4k) and a Z pair, a chromosome 13 plus a neo-W with $2n = 28$ (Figure 4l). The sex chromosome constitutions identified in F_1 and F_2 hybrids of crosses between SCW females and SCP males are schematically illustrated in Figure 4.

In F_2 hybrids of both reciprocal crosses, we did not observe any major bias in the sex ratio or proportion of individual types of sex chromosome constitutions. Both the expected and unexpected types (see Figures 3e–l and 4e–l) occurred in F_2 hybrids in similar frequencies (Supplementary Table S2). To clarify this observation, we examined meiotic pairing of sex chromosomes in pachytene complements of F_1 hybrids from both reciprocal crosses. Pachytene oocytes of F_1 females (SCP females × SCW males; Figure 3c) mostly showed a sex chromosome trivalent, which consisted of the neo-Z and W sex chromosomes and a chromosome 13 (Supplementary Figure S1a and b; Yoshido *et al.*, 2013). In the trivalent, the chromosomes were either fully synapsed (Supplementary Figure S1a) or only partially paired (Supplementary Figure S1b), presumably depending on the pachytene substage. While the chromosome 13 was always found either well synapsed or at least paired with the corresponding part of the neo-Z chromosome, in a few pachytene complements the W chromosome remained unpaired and formed a univalent (Supplementary Figure S1c). In F_1 females (Figure 4c) from the reciprocal crosses (SCW females × SCP males), pachytene oocytes mostly showed a bivalent composed of the neo-W chromosome and a chromosome 13 and a Z chromosome univalent (Supplementary Figure S1e). Exceptionally, we observed a curious trivalent composed of the fully synapsed neo-W chromosome with a chromosome 13 and with a terminal segment of the Z chromosome (Supplementary Figure S1d). The analysis of meiotic pairing thus revealed considerable differences in the configuration of sex chromosomes between F_1 females from both reciprocal crosses (Table 1). In F_1 males from both crosses, all pachytene nuclei showed a sex chromosome trivalent, which consisted of the neo-Z and Z chromosomes and a chromosome 13 (not shown).

Development of a molecular marker for the identification of the *S. c. pryeri* W chromosome

To identify candidates for molecular markers, we cloned, sequenced and analysed DNA fragments derived from the microdissected samples of *S. c. pryeri* W chromosomes, amplified by PCR (Yoshido *et al.*, 2013). From more than 300 sequences cloned, we obtained eight sequences, which were specific for the highly heterochromatic part of the W chromosome in *S. c. pryeri*, as proven by FISH (not shown). A sequence analysis showed that seven of eight clones contain homologous sequences. In total, we identified two different repetitive sequences, further referred to as major and minor W-repeats (Supplementary Table S3). Then one of the seven clones, SCPW50 containing 356 bp of the major W-repeats, was used in this study as a molecular marker of the *S. c. pryeri* W chromosome. Using FISH we confirmed that the SCPW50 probe highlights the whole highly heterochromatic part of the W chromosome in *S. c. pryeri* (Supplementary Figure S2a–d, arrows). In contrast, the SCPW67 clone (885 bp) of the minor W-repeats mapped to the end of the *S. c. pryeri* W chromosome (Supplementary Figure S2e and f, arrowheads).

We hybridized the SCPW50 and SCPW67 sequences to Southern blots of female and male genomic DNAs, digested separately with three different restriction enzymes. Southern hybridization clearly showed that SCPW50 sequences are a part of clustered tandem repeats, almost exclusively present in the female genomic DNA (Supplementary Figure S2g, left) and that SCPW67 sequences are also a part of clustered tandem repeats, preferentially present in the female genomic DNA but also occurring in the male genomic DNA (Supplementary Figure S2g, right). Based on the results of Southern hybridization and sequence analysis, we concluded that the major W-repeats and minor W-repeats are organized in tandem arrays of a 206 bp and a 490 bp sequence, respectively.

In the next step, we designed a pair of PCR primers (SCPW50-F/SCPW50-R; see Materials and methods), specific for the major W-repeats. Using the primers, two distinct DNA fragments (approx. 200 and 400 bp) were amplified exclusively from genomic DNAs of female progeny of *S. c. pryeri* mothers (Supplementary Figure S2h). The results showed that the *S. c. pryeri* W chromosome is specifically detected with the designed PCR primers.

Table 1 Pairing configurations of sex chromosomes observed in pachytene nuclei of F_1 females from reciprocal crosses between *Samia cynthia pryeri* (SCP) and *Samia cynthia walkeri* (SCW)

P crosses	*Total of nuclei (no.)*	*Trivalent (no.)*	*Bivalent+univalent (no.)*	*Incomplete pairing (%)*
SCP♀ × SCW♂	150	144[a]	6[b]	4.0
SCW♀ × SCP♂	177	11[c]	166[d]	93.8

[a]Trivalent composed of neo-Z and W sex chromosomes and a chromosome 13 (see Supplementary Figure S1a and b).
[b]Neo-Z chromosome and chromosome 13 formed a bivalent, W chromosome was a univalent (Supplementary Figure S1c).
[c]Trivalent composed of the fully synapsed neo-W chromosome with chromosome 13 and a partially synapsed Z chromosome (Supplementary Figure S1d).
[d]Neo-W chromosome and chromosome 13 formed a bivalent, Z chromosome was a univalent (Supplementary Figure S1e).

Development of a molecular marker for the identification of the *S. c. walkeri* neo-W chromosome

We could not identify any sequence specific for part of the neo-W chromosome in *S. c. walkeri* that was highlighted by the type-2 W-probe used in cytogenetic experiments. Thus a molecular marker for the neo-W chromosome identification has been developed with the help of sequence polymorphism of the *GRP2* (glycine-rich protein 2) gene (Supplementary Table S3), which is located in the autosomal parts of the neo-sex chromosomes in *S. c. walkeri* and in chromosome 13 of *S. c. pryeri* (Supplementary Figure S3; see also Figure 1 in Yoshido *et al.*, 2011b). We amplified DNA fragments of *S. cynthia* ortholog of *GRP2* by PCR using specific primers (GRP2-F/GRP2-R; see Materials and methods) from four females and four males in *S. c. walkeri* and *S. c. pryeri*, respectively. The fragments generated from *S. c. walkeri* DNA were 588 bp long and those from *S. c. pryeri* 576 bp long (Supplementary Table S3). A sequence analysis of the *GRP2* fragments revealed several synonymous substitutions and an insertion/deletion in the exonic region between the subspecies. In addition, a non-synonymous substitution was found only in all *S. c. wakeri* females but not in males and in all *S. c. pryeri* specimens examined, indicating that the non-synonymous substitution is fixed only in *S. c. walkeri* females and can be used as a neo-W chromosome marker. Overall, we distinguished the neo-W, neo-Z and chromosome 13 according to different amino acids, derived from the nucleotide sequences of respective fragments of *S. cynthia GRP2* orthologs (Supplementary Figure S3).

Hatchability of eggs in crosses between F_2 hybrids with and without the W/neo-W chromosome

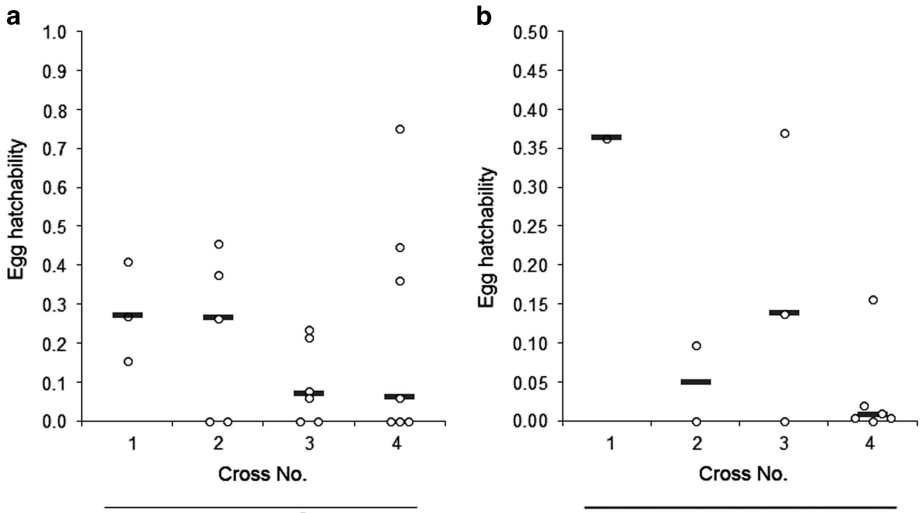
We did not find any remarkable phenotype differences between F_2 males with and without the W (or neo-W) chromosome. Similarly, no obvious differences between F_2 females with different sex chromosome

constitutions were found. Almost every F_2 hybrid irrespective of the presence or absence of the W (or neo-W) chromosome could mate with the opposite sex.

To find out whether the presence or absence of the W (or neo-W) chromosome affects fertility of F_2 hybrid, we performed crosses between each type of F_2 hybrids and examined the relationship between fertility of these crosses and the presence or absence of the W (or neo-W) chromosome (Figure 5). In crosses between F_2 hybrids from the SCP female × SCW male, individual F_2 females laid varying proportions of fertile (hatched) and sterile eggs in respective pairs (Figure 5a and Supplementary Table S4). After egg laying, we extracted genomic DNAs from respective parents and performed PCR tests for the presence or absence of the SCP W chromosome using the W-specific molecular marker. In 21 pairs of F_2 hybrids, the PCR tests revealed crosses between females (W−) and males (W−), females (W+) and males (W+), females (W−) and males (W+), and females (W+) and males (W−) in the numbers of 3, 5, 6, and 7 pairs, respectively (Supplementary Figure S4 and Table S4). The analysis of egg hatchability in crosses between F_2 hybrids showed no apparent association between the presence or absence of the W chromosome and the level of their fertility (Figure 5a). Crosses between F_2 hybrids from the SCW female × SCP male showed lower egg hatchability than those from the reciprocal crosses, with a high proportion of sterile eggs (Figure 5b). In 12 pairs of F_2 hybrids, sequencing for the detection of the neo-W molecular marker (Supplementary Figure S3) showed all combinations of F_2 parents with respect to the presence (neo-W+) or absence (neo-W−) of the neo-W chromosome in numbers of 1, 2, 3 and 6 pairs, respectively (Supplementary Table S4). Similar to F_2 hybrids originating from the reciprocal crosses, there was no clear association between the presence or absence of the neo-W chromosome and sterility/fertility of pairs established (Figure 5b). Overall, the results suggest that neither the W nor the neo-W chromosomes, by themselves, affect the level of fertility in *S. cynthia* ssp.

DISCUSSION

In this work, we clearly showed that the female-specific W chromosome (or neo-W) is transmitted not only to females but also to males in the progenies of hybrids from crosses between geographical subspecies of *S. cynthia* with distinct sex chromosome constitutions. We observed this phenomenon also in the progenies of backcrosses (data not shown). In *S. cynthia* ssp., the W (neo-W) chromosome is generally inherited only by females of the respective geographical populations with the exception of two cases, a part of the *S. c. pryeri* Kagoshima population that lost the W chromosome (Yoshido *et al.*, 2013) and *S. c. ricini*, which has a Z0/ZZ sex chromosome system (Yoshido *et al.*, 2011a). To our knowledge, transmission of the W (or neo-W) chromosomes to males is limited to hybrids produced by F_1 females of crosses between *S. cynthia* ssp. How could it happen that the F_1 females transmitted the W (or neo-W) chromosomes to the male progeny? In F_1 females from crosses between *S. c. pryeri* females and *S. c. walkeri* males, we observed only very few pachytene oocytes with a W chromosome univalent which would result in random segregation of the chromosome (Supplementary Figure S1c and Table 1). In the vast majority of pachytene oocytes, the W chromosome formed a sex chromosome trivalent with the neo-Z chromosome derived from father and a chromosome 13 derived from mother (see Supplementary Figure S1a and b in this study and Figure 3c in Yoshido *et al.*, 2013). Since in this trivalent, the W plus chromosome 13 were both paired with the neo-Z chromosome, regular segregation of the W chromosome to female offspring was expected. Thus, the event that enabled the W chromosome transmission to both sexes had to occur at later stages of meiosis, that is, after pachytene. In the achiasmatic meiosis of lepidopteran females, paired chromosomes remain associated through a modified



Cross No.	No. of pairs	Crosses	
		F_2 female	F_2 male
1	3	W-	W-
2	5	W+	W+
3	6	W-	W+
4	7	W+	W-

Cross No.	No. of pairs	Crosses	
		F_2 female	F_2 male
1	1	neoW-	neoW-
2	2	neoW+	neoW+
3	3	neoW-	neoW+
4	6	neoW+	neoW-

Figure 5 Scatterplots of the egg hatchability in crosses between F_2 hybrids of *Samia cynthia* ssp. (**a**) Hatchability of eggs in 21 pairs of F_2 hybrids from crosses between *S. c. pryeri* females and *S. c. walkeri* males. W+ and W− indicate F_2 hybrids with and without the W chromosome, respectively. (**b**) Hatchability of eggs in 12 pairs of F_2 hybrids from crosses between *S. c. walkeri* females and *S. c. pryeri* males. neo-W+ and neo-W− indicate F_2 hybrids with and without the neo-W chromosome, respectively. Grey bars represent median. Open circles show hatchability of eggs for each mating.

synaptonemal complex (SC) beyond the pachytene stage until metaphase I (reviewed in Marec, 1996). Since the short W chromosome showed a weaker affinity to the neo-Z chromosome than chromosome 13 due to the absence of homology (Supplementary Figure S1b), it could be often released from the trivalent, even when the trivalent is still held together by the modified SC. On the contrary, in F_1 females originating from the reciprocal crosses, the neo-W chromosome mostly formed a pachytene bivalent with a chromosome 13 inherited from the father (Supplementary Figure S1e and Table 1), while a trivalent was observed rarely (Supplementary Figure S1d and Table 1). Thus, the unpaired Z chromosome inherited from the father could randomly segregate either with the neo-W chromosome or with chromosome 13. The observed pairing patterns in F_1 females of oth reciprocal crosses suggest that a weaker synapsis of the W chromosome with the ancestral part of the neo-Z chromosome and the absence of pairing of the Z chromosome with the ancestral part of the neo-W chromosome result in a partially random segregation of the W and Z chromosomes, respectively. Thus, the failure of meiotic pairing seems to be responsible for the occurrence of unexpected sex chromosome constitutions in both sexes of F_2 hybrids.

In Lepidoptera, little is known about the role of maternally inherited W chromosomes in sex determination except for the silkworm (*B. mori*). In this species, sex determination depends on the presence or absence of the W chromosome, which carries a dominant female-determining factor, a small PIWI-interacting RNA named *Fem* piRNA. The *Fem* piRNA controls female-specific splicing of the *B. mori doublesex* (*Bmdsx*) gene by downregulating expression of the Z-linked *Masculinizer* (*Masc*) gene (Kiuchi *et al.*, 2014). However, our study clearly showed that the W (or neo-W) chromosome in *S. cynthia* ssp. does not determine femaleness. Furthermore, our results suggest that the sex in *S. cynthia* ssp. could be determined by the number of Z chromosomes, that is, by a Z-counting mechanism (see Traut *et al.*, 2007), irrespective of the presence or absence of the W (neo-W) chromosome. What is then the molecular mechanism that controls the sexual development in *S. cynthia* ssp.? It is now generally accepted that the *doublesex* (*dsx*) acts as a double switch gene at the bottom of sex-determining pathway in insects (Suzuki, 2010; Gempe and Beye, 2011), and we can assume that the *dsx* gene plays this role also in *S. cynthia* ssp. However, upstream components of the sex-determining pathway in *S. cynthia* ssp. are not yet known. The Z-counting mechanism of sex determination assumes the existence of a male-promoting gene such as *Masc* on the Z chromosome acting against a female-promoting gene on an autosome (Traut *et al.*, 2007). The occurrence of *Masc* orthologs in several other lepidopteran species may indicate an evolutionarily conserved role for *Masc* in sex determination (Kiuchi *et al.*, 2014).

Data obtained in model lepidopteran species suggest that the W chromosome is important for fitness of females. In *B. mori*, W-linked mutations have been isolated that cause masculinization of the external genitalia in females and abnormalities in the ovaries (Fujii *et al.*, 2010; Hara *et al.*, 2012). In *Ephestia kuehniella*, inheritance of several radiation-induced Z-chromosome fragments containing a piece of the W chromosome exclusively by females along with female-biased sex ratio in the progeny suggested the presence of a male-killing factor on the W chromosome (Marec *et al.*, 2001). However, unlike the above findings, the W (neo-W) chromosome in *S. cynthia* ssp. appears to have no effect on fertility or viability. In both sexes of F_2 hybrids, we did not find any remarkable phenotype differences between specimens with and without the W (or neo-W) chromosome. Almost every F_2 hybrid regardless the presence or absence of the W (or neo-W) chromosome could mate with the opposite sex. Furthermore, in crosses between F_2 hybrids, no association between the presence or absence of the W (or neo-W) chromosome and variations in hatch rates was found (Figure 5). These findings suggest that the W (or neo-W) chromosome has no influence on fitness of females in *S. cynthia* ssp.

Hybrid sterility or inviability of the heterogametic sex has been demonstrated in many animals including a number of lepidopteran species (Qvarnström and Bailey, 2009). For example, hybridization experiments between species and/or populations of *Heliconius* butterflies showed sterility in female hybrids, following thus the Haldane's rule in the heterogametic sex (Naisbit *et al.*, 2002). Two *S. cynthia* ssp. used in our study, *S. c. walkeri* and *S. c. pryeri* with distinct sex chromosome constitutions, can be defined as allopatric clades (Yoshido *et al.*, 2013). It is unknown whether they hybridize in nature or not (Peigler and Naumann, 2003). Our hybridization experiments in laboratory conditions showed no evidence of prezygotic reproductive barriers, with the exception of their geographical distributions. Both reciprocal crosses were fully fertile (Figure 2). However, F_1 hybrids (both sexes) showed significantly reduced fertility (see the egg hatchability in backcrosses and F_1 crosses in Figure 2b and Supplementary Table S1). In addition, more than half of crosses between F_2 hybrids were almost sterile (Figure 5). These results suggest the existence of postzygotic reproductive barriers between the two geographically isolated *S. cynthia* ssp.

It is well known that hybrids produced by crosses between species or populations with distinct karyotypes, such as in the house mouse or *Drosophila* fruit flies, have heterozygous karyotypes and typically show reduced fertility to germ cell death for abnormalities of meiotic pairing, recombination failure and chromosome missegregation (Forejt, 1996; Kulathinal and Singh, 1998). However, our results of crosses between *S. cynthia* ssp. with distinct sex chromosome constitutions revealed that the sex chromosome (W or Z chromosome) missegregation has little effect on their hybrids and therefore may not necessarily contribute to the reproductive barrier between the two *S. cynthia* ssp.

Hybrid incompatibility can cause reproductive barriers between closely related species (Coyne and Orr, 2004). Genes that cause hybrid incompatibility within and between species have been identified in several model organisms (Presgraves, 2010). Among plants, recent results of cross-breeding analysis between two *Silene* species (*S. latifolia* and *S. diclinis*) with distinct sex chromosome constitutions suggest that aberrant phenotypes in the hybrids occur because of hybrid incompatibility rather than improper inheritance of neo-sex chromosomes (Weingartner and Delph, 2014). Our results also point to hybrid incompatibility rather than sex chromosome missegregation as a cause of the postzygotic reproductive barrier between *S. cynthia* subspecies. Although the mechanism of hybrid incompatibility cannot be inferred from our data, we can at least conclude that the turnover of sex chromosomes alone is not responsible for the remarkable sterility of F_2 hybrids between geographical subspecies of *S. cynthia*.

Sex-specific Y or W chromosomes are known to play an important role in sex determination, fertility, fitness or speciation. However, our results suggest that the W (neo-W) chromosome in *S. cynthia* ssp. has no significant effect on any of the above functions. The sex chromosome constitutions could thus become diverse in different populations or subspecies. It has been proposed that the lepidopteran W chromosome arose in a common ancestor of Tischeriina plus Ditrysia as an evolutionary novelty from a Z0 sex chromosome system and that it was lost occasionally in different groups of advanced Lepidoptera (Lukhtanov, 2000; see Figure 3.4 in Marec *et al.*, 2010). In this respect, geographical subspecies of *S. cynthia* represent unique

models for tracking the evolutionary process of rise and fall of the W chromosome within one species complex. Further research of *S. cynthia* ssp. will provide important insights not only about the evolution of sex chromosomes and their actual role in the formation of reproductive barriers, but also about the evolution of sex determination in lepidopteran species.

DATA ARCHIVING

Sequence data have been submitted to GenBank: accession numbers LC033561–LC033565. Data available from Dryad repository: http://dx.doi.org/10.5061/dryad.2752g.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)