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ORIGINAL ARTICLE

Step-by-step evolution of neo-sex chromosomes in geographical populations of wild silkmoths, *Samia cynthia* ssp.

A Yoshido[1], K Sahara[1], F Marec[2] and Y Matsuda[3]

[1]*Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Japan;* [2]*Institute of Entomology, Biology Centre ASCR, České Budějovice, Czech Republic and* [3]*Laboratory of Animal Genetics, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan*

Geographical subspecies of wild silkmoths, *Samia cynthia* ssp. (Lepidoptera: Saturniidae), differ considerably in sex chromosome constitution owing to sex chromosome fusions with autosomes, which leads to variation in chromosome numbers. We cloned *S. cynthia* orthologues of 16 *Bombyx mori* genes and mapped them to chromosome spreads of *S. cynthia* subspecies by fluorescence *in situ* hybridization (FISH) to determine the origin of *S. cynthia* neo-sex chromosomes. FISH mapping revealed that the Z chromosome and chromosome 12 of *B. mori* correspond to the Z chromosome and an autosome (A_1) of *S. c. ricini* (Vietnam population, $2n=27$, Z0 in female moths), respectively. *B. mori* chromosome 11 corresponds partly to another autosome (A_2) and partly to a chromosome carrying nucleolar organizer region (NOR) of this subspecies. The NOR chromosome of *S. c. ricini* is also partly homologous to *B. mori* chromosome 24. Furthermore, our results revealed that two A_1 homologues each fused with the W and Z chromosomes in a common ancestor of both Japanese subspecies *S. c. walkeri* (Sapporo population, $2n=26$, neo-Wneo-Z) and *S. cynthia* subsp. indet. (Nagano population, $2n=25$, neo-WZ_1Z_2). One homologue, corresponding to the A_2 autosome in *S. c. ricini* and *S. c. walkeri*, fused with the W chromosome in *S. cynthia* subsp. indet. Consequently, the other homologue became a Z_2 chromosome. These results clearly showed a step-by-step evolution of the neo-sex chromosomes by repeated autosome–sex chromosome fusions. We suggest that the rearrangements of sex chromosomes may facilitate divergence of *S. cynthia* subspecies towards speciation.

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Introduction

Moths and butterflies (Lepidoptera) have sex chromosome systems with female heterogamety, which is also characteristic of their closest relatives, caddis flies (Trichoptera). The majority of lepidopteran species show a WZ/ZZ (female/male) pair of sex chromosomes (reviewed by Traut *et al*. (2007)). Besides the common WZ/ZZ system, variants without the W chromosome (Z/ZZ) and with multiple sex chromosomes, such as W_1W_2Z/ZZ and $WZ_1Z_2/Z_1Z_1Z_2Z_2$, have been found (for example, Nilsson *et al*., 1988; Traut and Marec, 1997; Yoshido *et al*., 2005b). The multiple sex chromosome systems may have originated from either fission or sex chromosome–autosome fusions (Marec *et al*., 2010). The latter scenario, giving rise to neo-sex chromosomes, seems to be common not only in vertebrates (Toder *et al*., 1997; Schmid *et al*., 2003), but also in some insects, such as flies of the genus *Drosophila* (Flores *et al*., 2008) and true bugs of the genus *Dysdercus* (Bressa *et al*., 2009). This scenario is also more likely the source of multiple sex chromosomes in Lepidoptera (Yoshido *et al*., 2005b; reviewed by Marec *et al*. (2010)).

Wild silkmoths of the genus *Samia* (Lepidoptera: Saturniidae) represent a complex of closely related species native to Asia (Peigler and Naumann, 2003). In the literature, they are mostly treated as geographical subspecies of *Samia cynthia* (Drury), the type species of the genus, obviously because of largely allopatric occurrence, morphological similarity and unclear taxonomic relationships. Recently, Peigler and Naumann (2003) reconstructed the genus and identified 19 different *Samia* species. Nevertheless, here we prefer the former classification of subspecies, which is consistent with that used in our previous cytogenetic study (see Yoshido *et al*., 2005b). In a previous study, we showed that three different geographic populations of *S. cynthia* exhibit a unique polymorphism in chromosome number, resulting from variations in the sex chromosome systems. Three different sex chromosome constitutions have been identified: Z/ZZ in a Vietnam population (the Eri silkworm, *S. c. ricini*) with $2n=27/28$, neo-Wneo-Z/neo-Zneo-Z in a Sapporo population (the ailanthus silkworm, *S. c. walkeri*) with $2n=26/26$ and neo-WZ_1Z_2/$Z_1Z_1Z_2Z_2$ in a Nagano population (the Shinju silkworm,

Correspondence: Dr A Yoshido, Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Hokkaido University, N9, W9, Kita-ku, Sapporo 060-8589, Japan.
E-mail: yoshido@abs.agr.hokudai.ac.jp
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S. cynthia subsp. indet.) with $2n = 25/26$. On the basis of these findings, it has been proposed that a common ancestor of *S. cynthia* subspecies had $2n = 28$ chromosomes and a WZ/ZZ sex chromosome constitution (Yoshido *et al*., 2005b). The ancestral karyotype could be still preserved in some extant subspecies of *S. cynthia*, as Kawaguchi (1937) described populations (Kyoto and Fukuoka, Japan) of the Shinju silkworm, *S. c. pryeri*, with 14 meiotic bivalents in male silkworm, and Song *et al*. (1996) found 28 chromosomes in mitotic metaphase complements from a specimen collected in China. According to the proposed scheme of the sex chromosome evolution in *S. cynthia* (Yoshido *et al*., 2005b), a neo-Wneo-Z sex chromosome pair in *S. c. walkeri* resulted from a fusion of an autosomal pair with the original W and Z chromosomes. The sex chromosome constitution of *S. cynthia* subsp. indet. may have derived from the subsequent fusion event of the neo-W chromosome with another autosome; then, the homologue of this autosome became a Z_2 chromosome in this subspecies. However, this hypothesis has not yet been proven, mainly because of the lack of suitable cytogenetic and/or molecular markers to elucidate the origin of individual parts of the neo-sex chromosomes.

Gene-based chromosome maps have been constructed only in four lepidopteran species: the silkworm (*Bombyx mori*), the tobacco hornworm (*Manduca sexta*) and two butterflies, *Heliconius melpomene* and *Bicyclus anynana*. Gene mapping in these species was carried out either by genetic linkage analysis (*B. mori*, *H. melpomene* and *B. anynana*) or by physical localization of genes using bacterial artificial chromosomes-fluorescence *in situ* hybridization (FISH with BAC of *B. mori* and *M. sexta* as probes) (Jiggins *et al*., 2005; Yoshido *et al*., 2005a; Yasukochi *et al*., 2006, 2009; Pringle *et al*., 2007; Beldade *et al*., 2009). Linkage analysis is a laborious and time-consuming procedure, especially in genetically uncharacterized species, and BAC-FISH mapping requires a BAC library, which is not available in *S. cynthia*. Here we present an alternative strategy of gene mapping that is based on chromosomal localization of *S. cynthia* orthologues of known *B. mori* genes by conventional FISH with PCR-generated probes. We cloned *S. cynthia* orthologous DNA fragments of both the sex-linked and autosomal *B. mori* genes. By FISH mapping, we then successfully identified autosomes involved in the evolution of neo-sex chromosomes in geographical populations of this species. This is the first application of FISH for the identification of neo-sex chromosomes by direct gene mapping in Lepidoptera, and most probably in all invertebrates.

Materials and methods

Insects

We used larvae of three *S. cynthia* (Drury) subspecies originating from distant geographical regions: *S. c. walkeri* (Felder and Felder) from Sapporo (Hokkaido, Japan), *S. cynthia* subsp. indet. from Nagano (Honshu, Japan) and *S. c. ricini* (Donovan) from a Vietnam population (for details, see Yoshido *et al*., 2005b). The larvae were reared on *Ailanthus altissima* trees at the Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan.

Cloning and sequencing

Genomic DNA was extracted separately from female and male larvae of *S. c. walkeri* by a standard phenol–chloroform procedure and used as a template for PCR amplification of *S. cynthia* orthologues of *B. mori* genes with primers listed in Table 1. The sequence-tagged site primers were designed for genes encoding 3-hydroxy-3-methylglutaryl-CoA reductase, DOPA decarboxylase, elongation factor 1α subunit (EF-1α), attacin, chitinase, lysozyme, hemolin and storage protein 1 according to *S. c. ricini* sequences available in a public database. To design further sequence-tagged site primers, sequence information of *S. c. ricini* gene orthologues for Y-box protein (BYB), ribosomal protein L4 (RpL4), ribosomal protein L18 (RpL18), cytoplasmic actin (A4) and translation elongation factor 2 (eEF-2*)* was obtained from SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). For the orthologues of *kettin*, *topoisomerase II* (*Topo II*) and *xanthine dehydrogenase I* (*XDH I*), degenerate oligonucleotide primers were designed from the nucleotide sequences in the conserved regions of *B. mori* and *Drosophila melanogaster*. PCR amplifications were carried out with an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 52–60 °C and 2 min extension at 72 °C, and a final extension step at 72 °C for 5 min. Amplified gene fragments were cloned into pGEM T-easy vector (Promega KK, Tokyo, Japan), and their nucleotide sequences were determined with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan), following the sequencing protocol supplied by the manufacturer.

Chromosome preparations

Spread preparations were made from pachytene oocytes, which were obtained from ovaries of last instar larvae as described by Yoshido *et al*. (2005b). Briefly, the ovaries were dissected in a saline solution and fixed for 10–15 min in Carnoy's fixative (ethanol, chloroform, acetic acid, 6:3:1). Cells were dissociated in 60% acetic acid and spread on the slide using a heating plate at 50 °C. The preparations were then passed through a graded ethanol series (70, 80 and 98%) and stored in a −30 °C freezer until further use.

Fluorescence *in situ* hybridization

Genomic *in situ* hybridization (GISH) was carried out according to the methods described by Sahara *et al*. (2003). Female genomic DNA was labelled by nick translation using a Nick Translation Mix (Roche Diagnostics, Mannheim, Germany) with Green-dUTP (Abbott Molecular Inc., Des Plaines, IL, USA) and used as a probe. For FISH mapping of orthologous genes, the cloned fragments were labelled with Cy3-dCTP (GE Healthcare, Piscataway, NJ, USA) by PCR under conditions described above, except for a 4 min extension. We purified the probes using a Wizard SV Gel and PCR Clean-Up System (Promega KK) as per the manufacturer's protocol.

Chromosome preparations were removed from the freezer, passed through the graded ethanol series and air-dried. They were denatured at 72 °C for 3.5 min in 70% formamide, 2 × standard sodium citrate. FISH mapping and GISH were simultaneously performed for one

Table 1 Primers used in this study and the size of amplified fragments of *Samia cyntia walkeri* orthologues of *Bombyx mori* genes, and corresponding orthologous genes and/or ESTs of *S. cynthia*

PCR amplification of S. c. walkeri *orthologues*			S. cynthia *orthologues and ESTs*		Bombyx mori *genes*			
Forward primer sequence	*Reverse primer sequence*	*Product size (bp)*	*Accession no.*	*EST (SilkBase code)*[a]	*Accession no.*	*Putative function*	*Chr. no.*	*Location in KAIKObase*[b]
GGNCCNGGNGAYGARGGNGARTAYAC	GGNGGRTANACNCGRTCYTCYTCNCC	1820	AB543309[c]	Not found	AB079865	Kettin	1 (Z)	6 505 696–6 533 895
TAAAACAGTTAAACAAAAGCAGGTCAT	CTCATCTCCYTGTGCACCCCCTTGGTT	1663	AB543308	S06A01NCLL0010_M07	AB098537	BYB	1 (Z)	10 855 943–10 857 680
GTTTGGGGAGCACATGGAGAAT	CATTTCCGTCTGCGTTCGCTTGG	ca. 2500	DQ465407	S06A01NCLL0005_L15	AB274990	3-Hydroxy-3-methylglutaryl-CoA reductase	2	916 956–919 459
ACCGAATTAGAAGTGGTGATG	GCTTTCTTCACTCATACGTGA	ca. 2300	AF015065	Not found	AF372836	DDC	4	12 504 927–12 521 471
GGTATCGACAAACGTACCATCG	ATGGATTTAGGGTTGTCCTCTG	1085	AF015084	I09A02NGRL0007_G07	D13338	EF-1α	5	17 106 041–17 107 432
GTTCGCTAAGTTGTTCCTAGTG	GTGCTGGGTTCCCACGAGGACTT	921	AB059394	I09A02NGRL0003_D23	D76418	Attacin	6	18 601 317–18 602 129
CATATTCCGCACGATGCGGG	GTTACCTAAGCCCATGTTCAT	ca. 2000	AB201280	Not found	AB104488	Chitinase	7	14 117 862–14 119 517
GTCCAGGAGATCAACAAGACCAAG	CAGCTTCAGGAGGTTCAGCTTGTT	ca. 1800	AB543313, AB543314	S13A01NGRL0010_F15	AY769271	RpL4	11	2 852 029–2 862 665
GGNGCNAARCTNTGYAAYATHTTYTC	TGRAARTAYTCYTTNGCYTCYTTNGA	1391	AB543310[c]	Not found	AF013277	Topo II	11	8 589 427–8 602 332
CATAAACACGACAGGAAAGTTCGGC	CTTGGTGCGCACGTATGGTTTAGT	ca. 2000	AB543311, AB543312	S06A01NCLL0020_P12	AY769287	RpL18	11	9 399 580–9 401 164
GCTTTGCATTGCGATGCGAAACGT	ACAGTCGCTAATATCTGGCAGTCC	ca. 3000	AB048258	I10A02NGRL0003_P23	L37416	Lysozyme	12	3 312 565–3 309 259
GCNCAYGGNTCNCARTGYGGNTTYTGYAC	TARAARTGYTCYTGNCCNCCCATNCGRCA	1985	AB543315[c]	Not found	D38159	XDH I	12	8 502 165–8 493 509
GTTGCCGCGTTGGTAGTAGACAA	CCAGACTCGTCGTACTCCTGTTT	1169	AB543316	I10A02NGRL0006_L21	U49644	Cytoplasmic actin A4	17	2 936 956–2 938 173
ATTCAGCGTACAATTCTTATGATGGG	CCAATGATCAAATACGCATTGTGGGAA	1113	AB543317	S06A01NCLL0007_L10	DQ443396	eEF-2	19	12 802 871–12 811 573
AAAATGGGCATGCTACCGCGTGG	GCTCATCAACATGACGTCTTTGTA	ca. 2300	AB288051	S13A02NGRL0010_N13	X12978	SP1	23	16 906 448–16 911 216
GGGTAAACAATGGCGTCTAAG	GCTCTGCAGCCGTAGTATCCC	ca. 3000	AB220992	I09A02NGRL0003_K24	AY515321	Hemolin	24	13 879 059–13 884 127

Abbreviations: BYB, Y-box protein; DDC, DOPA decarboxylase; DOP-PCR, degenerate oligonucleotide-primed PCR; EF-1α, elongation factor 1α; eEF-2, translation elongation factor 2; EST, expressed sequence tag; RpL4, ribosomal protein L4; RpL18, ribosomal protein L18; SP1, sex-specific storage-protein 1; Topo II, topoisomerase II; XDH I, xanthine dehydrogenase I.

[a] ESTs showing the highest score were taken from http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi.

[b] Inferred from http://sgp.dna.affrc.go.jp/KAIKObase/.

[c] An orthlogous sequence was obtained using DOP-PCR.

preparation with a probe cocktail containing 500 ng Green-labelled female DNA (green), 100 ng Cy3-labelled gene fragment (red), 3 μg unlabelled sonicated male genomic DNA and 25 μg sonicated salmon sperm DNA (Sigma-Aldrich, Tokyo, Japan) in 10 μl hybridization solution (50% formamide, 10% dextran sulphate, 2 × standard sodium citrate). After hybridization in a moist chamber at 37 °C for 3 days, the slides were washed at 62 °C in 0.1 × standard sodium citrate containing 1% Triton X-100, and then counterstained and mounted in antifade solution containing 1,4-diazabicyclo[2,2,2]octan (DABCO; for composition, see Traut *et al*., 1999) and 0.5 $\mu g\,ml^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Preparations were observed in a Leica DMRE HC fluorescence microscope. Digital images were acquired and processed as described in Sahara *et al*. (2003).

Construction of molecular phylogenetic trees

To examine relationships between *S. cynthia* subspecies, a molecular phylogenetic analysis was carried out using (i) a fragment of mitochondrial DNA (*COI–COII*) containing complete sequences of genes encoding cytochrome oxidase subunit I, $tRNA^{Leu}$, and cytochrome oxidase subunit II, $tRNA^{Lys}$, and (ii) a partial coding sequence of the nuclear gene *EF-1α*. In each subspecies, the *COI–COII* fragment was amplified from the respective mitochondrial DNA by degenerate oligonucleotide-primed PCR using two primers, 5′-CGAAAATGACTTT AYTCNACTAATC-3′ (forward) and 5′-TCATTAGAAGT ANTTGNTAATTTAC-3′ (reverse). The *EF-1α* sequence was amplified from the respective genomic DNA using primers given in Table 1. PCR conditions were as described above. PCR products (2355 bp *COI–COII* and 1085 bp *EF-1α* fragments) were sequenced with an ABI PRISM 3100 Genetic Analyzer following the sequencing protocol supplied by the manufacturer.

Neighbour-joining trees of *S. cynthia* subspecies were constructed using the Molecular Evolutionary Genetics Analysis 4 software (Tamura *et al*., 2007; http://www.megasoftware.net/). Bootstrap values were estimated with 1000 replicates. The Chinese oak silkmoth, *Antheraea pernyi* (Saturniidae), and *B. mori* were used as outgroups. The corresponding *COI–COII* and *EF-1α* sequences of these two species were obtained from a public database. GenBank accession numbers of all sequences used are given in Supplementary Table S1.

Results

S. cynthia orthologues of *B. mori* genes

We cloned *S. c. walkeri* (Sapporo population) orthologues of 16 *B. mori* and/or *S. c. ricini* genes (Table 1). BlastN searches of the coding region in *S. c. walkeri* orthologues of 13 genes (except for *kettin*, *Topo II* and *XDH I*) against expressed sequence tag libraries of *S. c. ricini* in SilkBase and public databases indicated very high sequence identities (at least 96%). TblastX searches of *S. c. walkeri* orthologues of three *B. mori* genes, *Topo II*, *kettin* and *XDH I*, showed 91, 89 and 76% identities, respectively.

FISH mapping of *S. cynthia* orthologues of *B. mori* Z-linked genes

We carried out FISH mapping of the *S. cynthia* orthologues of *B. mori* Z-linked genes (*BYB* and *kettin*) to female pachytene complements in three *S. cynthia* subspecies/populations. In these complements, the Z chromosome was easily identified as the only univalent among autosome bivalents of *S. c. ricini* (Figure 1a), whereas the neo-Wneo-Z bivalent of *S. c. walkeri* (Figure 1b) and the neo-WZ_1Z_2 trivalent of *S. cynthia* subsp. indet. (Figure 1d) were recognized according to green hybridization signals of the female genomic probe, which bound to the ancestral W-heterochromatin segment of the neo-W chromosome. In *S. c. ricini*, a probe made from the orthologue of *B. mori BYB* hybridized to the Z-chromosome univalent (Figure 1a). A clear hybridization signal of the probe was found on the neo-Z chromosome in the neo-Wneo-Z bivalent of *S. c. walkeri* (Figures 1b and c) and on the Z_1 chromosome in the neo-WZ_1Z_2 trivalent of *S. cynthia* subsp. indet. (Figures 1d and e). The *kettin* orthologous probe also hybridized to the Z univalent in *S. c. ricini* (Figure 1f, Supplementary Figure S1a), the original Z part of the neo-Z chromosome in *S. c. walkeri* (Figure 1g, Supplementary Figure S1b) and the Z_1 chromosome in *S. cynthia* subsp. indet. (Figure 1h, Supplementary Figure S1c). In the latter two subspecies, hybridization signals of the *kettin* orthologous probe were observed on unpaired regions of the sex chromosomes.

Identification of autosomal parts of the neo-sex chromosomes

We searched for *S. cynthia* orthologues that would map to autosomal parts of the neo-sex chromosomes in *S. c. walkeri* and *S. cynthia* subsp. indet. These autosomal parts correspond to A_1 and A_2 autosomes of *S. c. ricini*, as shown in the previously proposed scheme of the sex chromosome evolution in this species (see Figure 4 in Yoshido *et al*., 2005b). Among randomly selected orthologues of *B. mori* autosomal genes, a probe prepared from the *S. cynthia* orthologue of the *XDH I* gene, which is located on *B. mori* chromosome 12, hybridized to an autosome bivalent in *S. c. ricini* (Figure 2a). The probe mapped to the putative A_1 part of the neo-W and neo-Z sex chromosomes in *S. c. walkeri* (Figure 2b, Supplementary Figure S1d) and to the neo-W and Z_1 sex chromosomes in *S. cynthia* subsp. indet. (Figure 2c, Supplementary Figure S1e). *Lysozyme* is another representative of the gene located on the *B. mori* chromosome 12. The *S. cynthia* orthologous probe of this gene also hybridized to the A_1 segments of neo-sex chromosomes in *S. c. walkeri* (Figure 2d, Supplementary Figure S1f) and *S. cynthia* subsp. indet. (data not shown). These results indicated that at least a part of the A_1 segment of the two subspecies with neo-sex chromosomes corresponds to a part of the *B. mori* chromosome 12.

In *B. mori*, the *Topo II* and *RpL18* genes are located on chromosome 11. A probe prepared from the *S. cynthia* orthologue of the *Topo II* gene mapped to an autosome in *S. c. walkeri* (Figure 3a), whereas in *S. cynthia* subsp. indet., hybridization signals of the probe were observed on the neo-W and Z_2 sex chromosomes (Figure 3b, Supplementary Figure S1g). Similarly, the *RpL18* orthologous probe hybridized to autosomal segments of both the neo-W and Z_2 sex chromosomes in *S. cynthia* subsp. indet. (Figure 3c, Supplementary Figure S1h), whereas in *S. c. walkeri*, the probe hybridized to a pair of autosomes (data not shown). Hence, a part of the *B. mori* chromo-

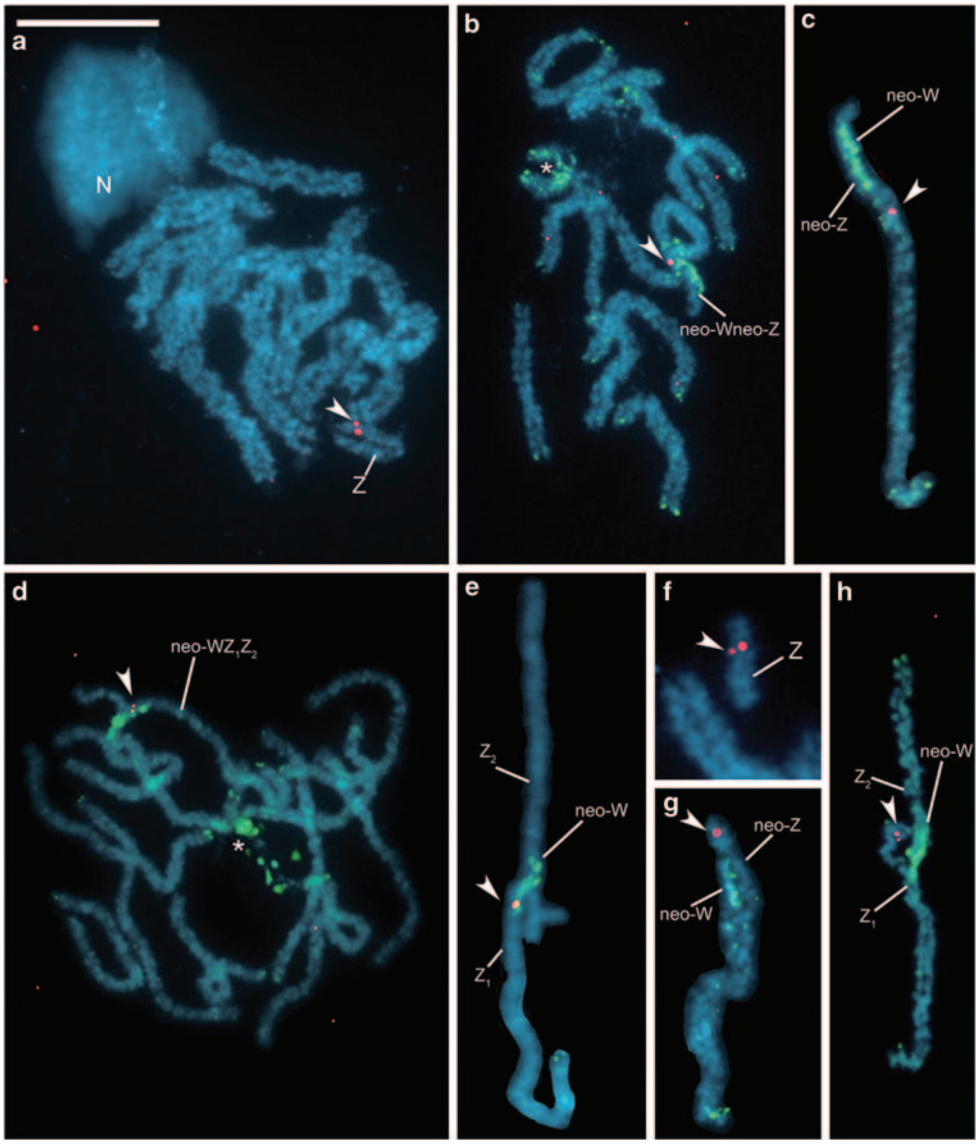

Figure 1 FISH mapping of *S. cynthia* orthologues of *B. mori* Z-linked genes on female pachytene chromosomes of three subspecies of *S. cynthia*. Red signals (arrowheads) are Cy3-labelled probes of *BYB* (**a**–**e**) and *kettin* (**f**–**h**) orthologous genes. GISH with Green-labelled female genomic probe (green signals) identified the original W compartment composed of heterochromatin (**b**–**e**, **g**, **h**) and also highlighted a block of heterochromatin in the NOR autosome (asterisk in **b**, **d**). Chromosomes were counterstained with DAPI (light blue). *S. c. ricini* (**a**, **f**): the U-shaped, apparently self-paired univalent of the Z chromosome with *BYB* orthologue signals on both chromatids is seen in the pachytene complement besides 13 autosome bivalents; also note a conspicuous nucleolus (N) associated with one end of the NOR autosome (**a**); and a Z-chromosome univalent with *kettin* orthologue signals on both chromatids (**f**). *S. c. walkeri* (**b**, **c**, **g**): a pachytene complement composed of a neo-Wneo-Z sex chromosome bivalent and 12 autosome bivalents (**b**); signals of the *BYB* (**b**, **c**) and *kettin* (**g**) orthologous probes are located on the neo-Z chromosome. *S. cynthia* subsp. indet. (**d**, **e**, **h**): a pachytene complement consists of a neo-sex chromosome trivalent (neo-WZ_1Z_2) and 11 autosome bivalents (**d**); signals of the *BYB* (**d**, **e**) and *kettin* (**h**) orthologous probes are located on the Z_1 chromosome. Bar represents 10 μm (**a**–**e**, **h**), 7.5 μm (**g**) and 5 μm (**f**).

some 11 corresponds to the putative A_2 chromosome of *S. cynthia* (see Figure 4 in Yoshido *et al*., 2005b). Further FISH experiments showed that the orthologue of the *RpL4* gene located on *B. mori* chromosome 11 does not map to the neo-sex chromosomes, but to the nucleolar organizer region (NOR) bivalent in *S. cynthia* subsp. indet. (Figure 4a). This NOR bivalent was easily identified by a specific heterochromatin block highlighted by GISH with the *S. cynthia* female genomic probe (cf. Yoshido *et al*., 2005b). The *RpL4* orthologue also mapped to the NOR bivalents of *S. c. walkeri* and *S. c. ricini* (data not shown). This suggests that the NOR chromosome has the same origin in all three *S. cynthia* subspecies.

S. cynthia orthologous probes of genes located on *B. mori* chromosomes 2, 4, 5, 6, 7, 17, 19 and 23 (Table 1) hybridized to unspecified autosomes in two subspecies with the neo-sex chromosomes, *S. c. walkeri* and *S. cynthia* subsp. indet. (an example is shown in Figure 4b). Only the orthologue of the *hemolin* gene, a representative of *B. mori* chromosome 24, mapped to the NOR bivalents in all three *S. cynthia* subspecies (an example is shown in Figure 4c). These results suggest that the NOR chromosome of *S. cynthia* is composed of two segments, one

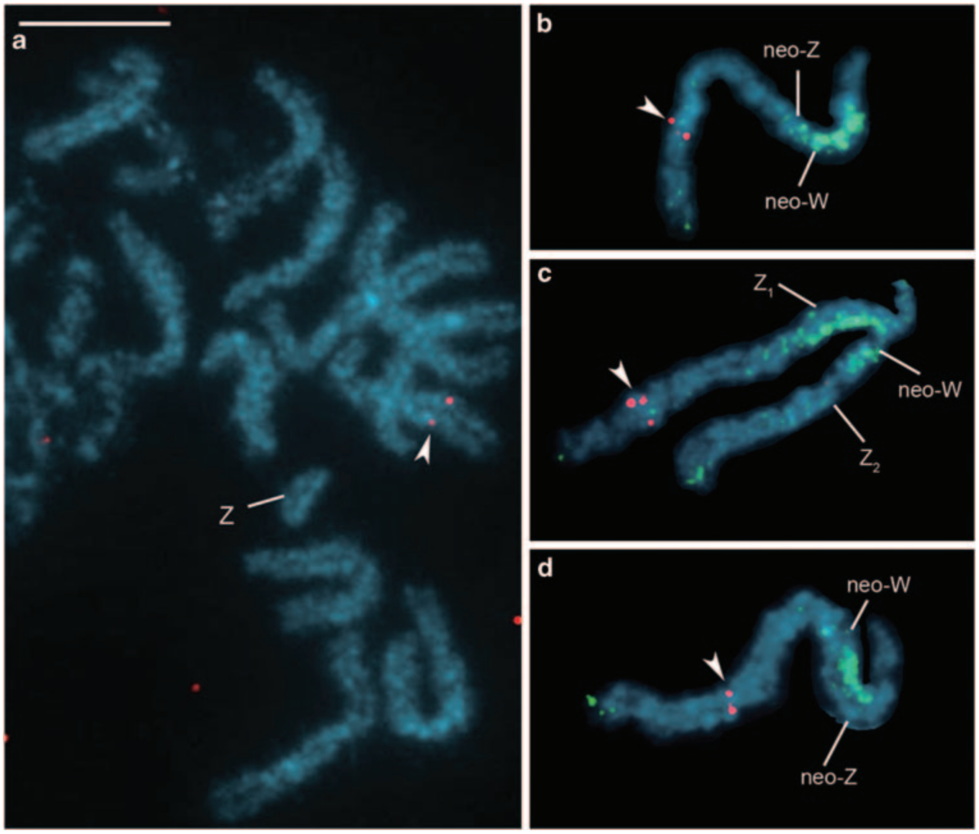

Figure 2 FISH identification of autosomal parts of the *S. c. walkeri* and *S. cynthia* subsp. indet. neo-sex chromosomes on female pachytene chromosomes of three subspecies of *S. cynthia*. The Cy3-labelled orthologous probe of the *B. mori XDH 1* gene (red signals, arrowheads) hybridized to an autosome bivalent, but not to the 'U-shaped' univalent of the Z chromosome in the female pachytene complement of *S. c. ricini* (**a**), whereas the *XDH 1* orthologous probe mapped to autosomal parts of both the neo-W and neo-Z chromosomes in *S. c. walkeri* (**b**) and to autosomal parts of both the neo-W and Z_1 chromosomes in *S. cynthia* subsp. indet. (**c**). The Cy3-labelled orthologous probe of the *B. mori lysozyme* gene (red signals, arrowhead) hybridized to the same autosomal segment of both the neo-W and neo-Z chromosomes in *S. c. walkeri* (**d**). GISH with Green-labelled female genomic probe (green signals) identified the original W-heterochromatin parts of the neo-W chromosome in *S. c. walkeri* (**b**, **d**) and *S. cynthia* subsp. indet. (**c**). Chromosomes were counterstained with DAPI (light blue). Bar represents 10 μm.

corresponding to a part of chromosome 11 and the other to a part of chromosome 24 of *B. mori*.

Evolutionary relationship between geographical subspecies of *S. cynthia*

The molecular phylogenetic analysis carried out using both the mitochondrial *COI–COII* and the nuclear *EF-1α* sequences confirmed the closer relationship of two Japanese subspecies, *S. c. walkeri* and *S. cynthia* subsp. indet., and separation of the Vietnam subspecies, *S. c. ricini*. A higher support for separation of the two Japanese subspecies was obtained with the *COI–COII* sequences, whereas the separation of *S. c. ricini* was obvious in both trees (Supplementary Figure S2a, b).

Discussion

Neo-sex chromosomes that originated by autosome–sex chromosome fusions have been reported in various animals with male heterogamety (for example, Schmid *et al*., 2003; Král, 2007; Zhou *et al*., 2008; Bressa *et al*., 2009). However, reports on neo-sex chromosomes in animals with female heterogamety are limited to a few species of fish (Ueno *et al*., 2001) and moths (reviewed by Marec *et al*. (2010)). *S. cynthia* silkmoths represent a special case. Their sex chromosome constitution was altered by a series of chromosomal rearrangements in geographical subspecies after they differentiated from a putative ancestor with the diploid karyotype of $2n = 28$ and a WZ sex chromosome pair. Thus, a loss of the W chromosome resulted in a Z0 system as found in *S. c. ricini* (Vietnam population), whereas fusion of sex chromosomes with autosomes resulted in the neo-Wneo-Z and neo-WZ_1Z_2 systems as found in *S. c. walkeri* (Sapporo population) and *S. cynthia* subsp. indet. (Nagano population), respectively (Yoshido *et al*., 2005b).

In this study, we identified autosomes involved in the evolution of the complex sex chromosome systems in *S. cynthia* by comparative mapping of *S. cynthia* orthologues of *B. mori* genes. Figure 5 shows a schematic representation of the process of chromosomal rearrangements that occurred between *B. mori* and three geographical subspecies of *S. cynthia*. Our findings support a hypothetical scenario of the sex chromosome evolution in *S. cynthia* mentioned previously (see Figure 4 in Yoshido *et al*., 2005b). In addition, the findings point towards conserved synteny of genes between Z chromosomes of *B. mori* and *S. c. ricini* and also to homology of the *S. c. ricini* Z chromosome with parts of the neo-Z chromosome of *S. c. walkeri* and the Z_1 chromosome of

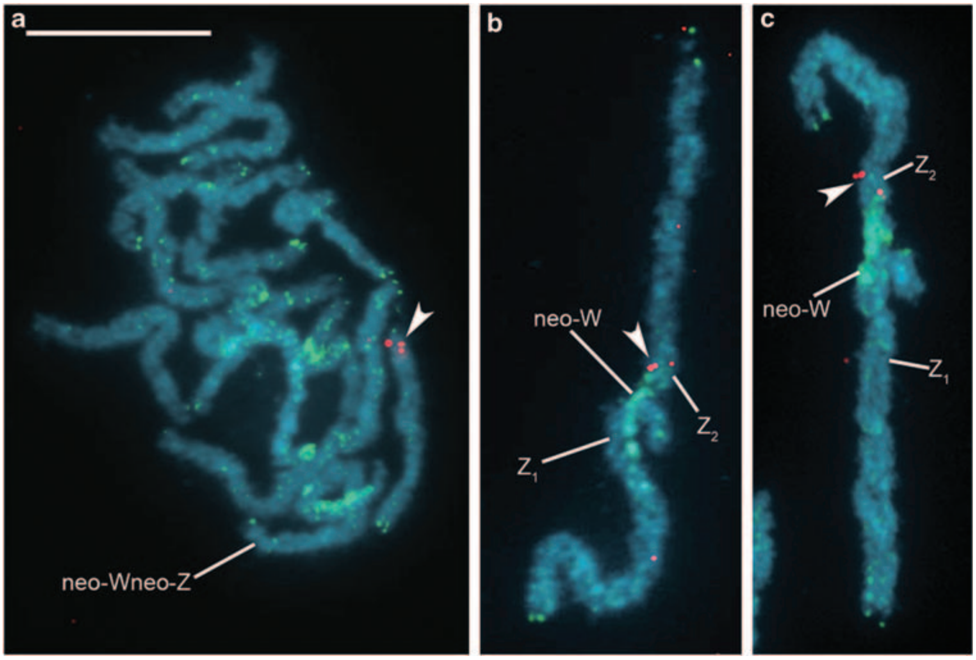

Figure 3 FISH identification of the autosomal segment of the neo-W chromosome homologous to the Z_2 chromosome in *S. cynthia* subsp. indet. on female pachytene chromosomes of *S. c. walkeri* (**a**) and *S. cynthia* subsp. indet. (**b**, **c**). The Cy3-labelled orthologous probe of the *B. mori Topo II* gene (red signals, arrowheads) hybridized to an autosomal bivalent but not to the neo-Wneo-Z bivalent in a female pachytene complement of *S. c. walkeri* (**a**), whereas in *S. cynthia* subsp. indet., the probe mapped to the Z_2 chromosome and the neo-W chromosome of the neo-WZ_1Z_2 trivalent (**b**). The Cy3-labelled orthologous probe of the *B. mori RpL18* gene (red signal, arrowhead) also hybridized to the Z_2 chromosome and the neo-W chromosome of the neo-WZ_1Z_2 trivalent in *S. cynthia* subsp. indet. (**c**). GISH with Green-labelled female genomic probe (green signals) identified the original W-heterochromatin parts of the neo-W chromosome in *S. c. walkeri* (**a**) and *S. cynthia* subsp. indet. (**b**, **c**). Chromosomes were counterstained with DAPI (light blue). Bar represents 10 μm.

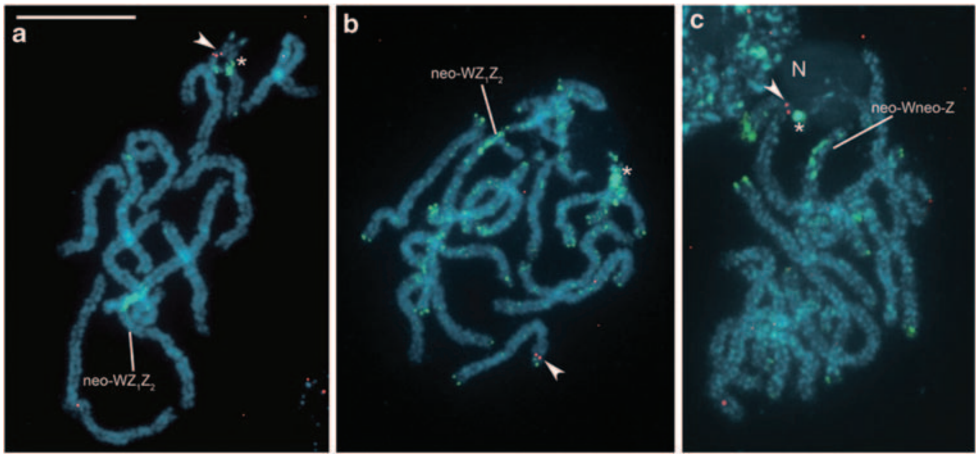

Figure 4 FISH mapping of *S. cynthia* orthologues of *B. mori* autosomal genes on female pachytene chromosome complements of *S. cynthia* subspecies. Red signals (arrowheads) are Cy3-labelled orthologous probes of the *B. mori* genes *RpL4* (**a**), *eEF-2* (**b**) and *hemolin* (**c**). GISH with Green-labelled female genomic probe (green signals) identified the original W-heterochromatin compartment of the neo-W chromosome and also highlighted a heterochromatin block on the NOR bivalent (asterisk) in *S. cynthia* subsp. indet. (**a**, **b**) and *S. c. walkeri* (**c**). Chromosomes were counterstained with DAPI (light blue). In *S. cynthia* subsp. indet., the *RpL4* orthologue mapped to the NOR bivalent (**a**), whereas the *eEF-2* orthologue mapped to an autosome bivalent (**b**). The *hemolin* orthologue mapped to the NOR bivalent in *S. c. walkeri* (**c**); note a conspicuous nucleolus (N) associated with the NOR bivalent. Bar represents 10 μm.

S. cynthia subsp. indet. Our data suggest that the first step of neo-sex chromosome evolution was a fusion between an autosome pair (A_1), which corresponds to *B. mori* chromosome 12, and the original W and Z chromosomes in a common ancestor of both Japanese subspecies, *S. c. walkeri* and *S. cynthia* subsp. indet., whereas no such event occurred in *S. c. ricini* (Figure 5). In the next step, the neo-W chromosome of *S. cynthia* subsp. indet. was formed by a fusion of the neo-W of *S. c. walkeri* with another autosome (A_2) corresponding to *B. mori* chromosome 11, and therefore the other A_2 corresponds to the Z_2 sex chromosome in *S. cynthia* subsp. indet. Phylogenetic trees constructed with nucleotide se-

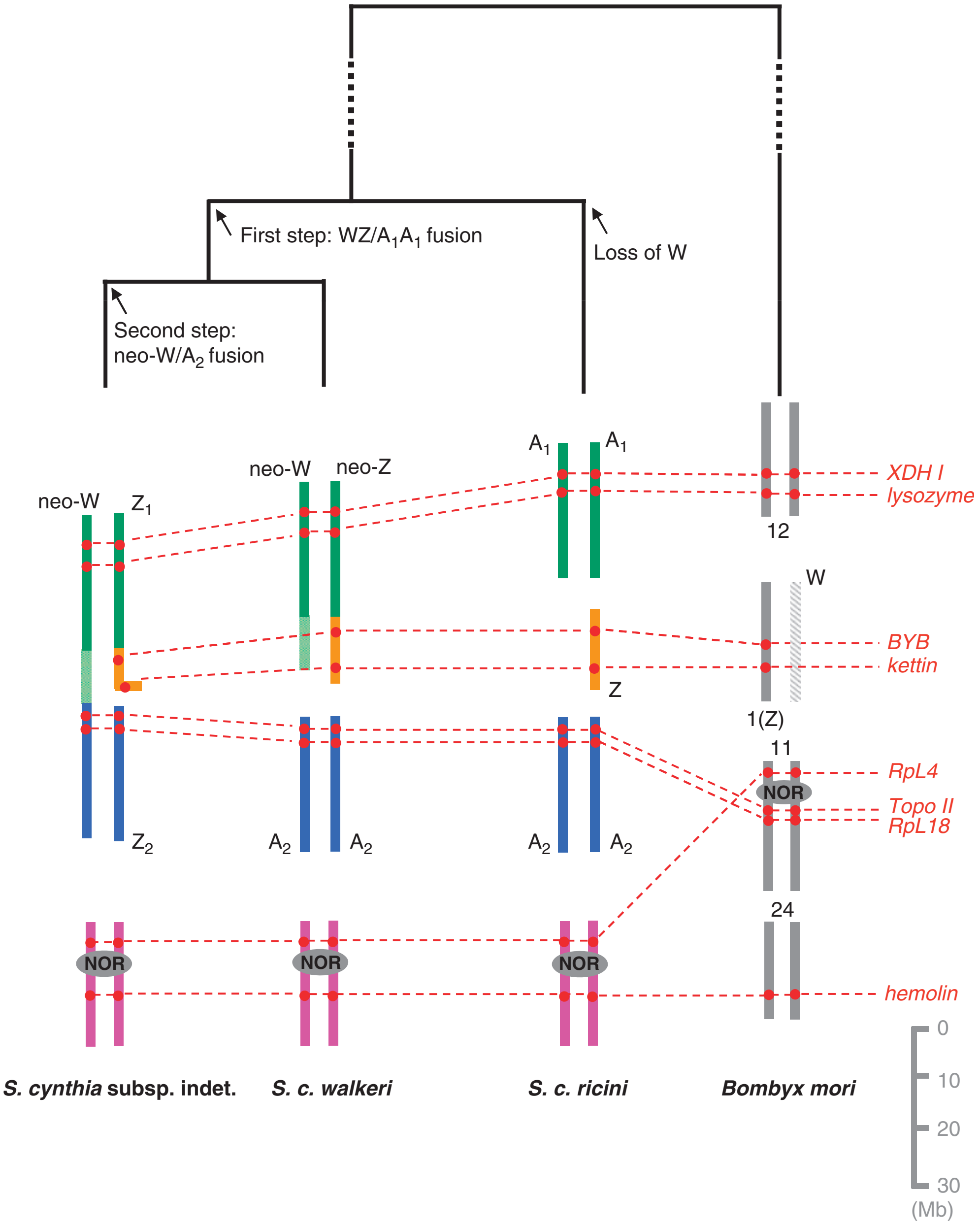

Figure 5 Schematic drawing of step-by-step evolution of the sex chromosome constitution in female silkmoth of *Samia cynthia* subspecies, based on comparative mapping of orthologues of *Bombyx mori* genes. In two Japanese subspecies, the neo-sex chromosomes evolved in two steps by fusions of the ancestral sex chromosomes with autosomes. In the first step, the W and Z chromosomes each fused with an autosome of the A_1A_1 pair, corresponding to chromosome 12 of *B. mori*; this resulted in the neo-Wneo-Z constitution of *S. c. walkeri* female silkmoth. In the second step, only the neo-W chromosome fused with an autosome (A_2), partly corresponding to chromosome 11 of *B. mori*; this resulted in a neo-WZ_1Z_2 constitution of *S. cynthia* subsp. indet. female silkmoth. On the contrary, loss of the W chromosome in the putative *S. cynthia* ancestor generated a Z0 constitution of *S. c. ricini*. A segment corresponding to another part of the *B. mori* chromosome 11 containing the NOR plus a segment corresponding to a part of *B. mori* chromosome 24 formed the only NOR chromosome in all three *S. cynthia* subspecies. *B. mori* chromosomes are represented by grey vertical bars drawn to relative scale in Mb taken from KAIKObase.

quences of mitochondrial and nuclear genes (Supplementary Figure S2a, b) also support the scenario of karyotype evolution in the geographic populations of *S. cynthia*.

The genetic content of the lepidopteran Z chromosomes is little known except for *B. mori*. Molecular linkage analyses revealed only a few orthologues of *B. mori* Z-linked genes that were also located on the Z chromosomes in other lepidopteran species, such as the European corn borer *Ostrinia nubilalis* (Dopman *et al.*, 2005), *Heliconius* butterflies (Jiggins *et al.*, 2005; Pringle *et al.*, 2007) and the butterfly *B. anynana* (Van't Hof *et al.*, 2008; Beldade *et al.*, 2009). Recently, BAC clones containing *M. sexta* orthologues of four *B. mori* Z-linked genes were mapped to the Z chromosomes of two species of the family Sphingidae, *M. sexta* and *Agrius convolvuli*, indicating the conserved synteny of the Z-linked genes between the three species (Yasukochi *et al.*, 2009). These results suggest that Z chromosomes are conserved in three large clades of Lepidoptera: the Pyraloidea

(*O. nubilalis*), Papilionoidea (*Heliconius* sp. and *B. anynana*) and Bombycoidea (*B. mori* and two sphingids). In this study, FISH mapping of two orthologues of the *B. mori* Z-linked genes (*kettin* and *BYB*; Table 1) in *S. cynthia* clearly showed that both genes are located on the Z chromosome of *S. c. ricini* and also on the original Z compartment in *S. c. walkeri* and *S. cynthia* subsp. indet (Figures 1a–h and 5). Our approach, which is based on the identification of conserved gene fragments and their comparative FISH mapping, is applicable to a wide range of species and thus it could facilitate studies on the evolution of sex chromosomes in Lepidoptera and their closest relatives, the Trichoptera. This method could also accelerate the construction of comparative maps of Lepidoptera, which has so far been performed for a limited number of species with BAC-FISH mapping (Sahara *et al*., 2007; Yasukochi *et al*., 2009).

Our study revealed that *S. cynthia* orthologues of two genes (*Topo II* and *RpL18*) of the *B. mori* chromosome 11 are located in the autosomal compartment of the neo-W chromosome in *S. cynthia* subsp. indet. (Nagano population) (Figure 3). However, another *S. cynthia* orthologue of the *B. mori* chromosome 11-linked gene, *RpL4*, did not map to the neo-W chromosome but to the NOR chromosome in all three *S. cynthia* subspecies (Figure 4). *B. mori* chromosome 11 can be easily recognized by an interstitially located nucleolus, which divides the chromosome into a shorter arm and a longer arm (Yoshido *et al*., 2005a). The *RpL4* gene is located on the shorter arm (approximately 2.86 Mb in KAIKObase), and the *Topo II* and *RpL18* genes are located on the longer arm (approximately 8.60 and 9.40 Mb, respectively). Besides the orthologue of the *RpL4* gene, an orthologue of the *hemolin* gene of *B. mori* chromosome 24 mapped to the NOR chromosome of *S. cynthia*, indicating homology of this chromosome with the shorter arm of chromosomes 11 and 24 of *B. mori* (Figure 5). These chromosomal rearrangements can be explained by two fission/fusion events involving two chromosomes of *S. cynthia* (A_2 and the NOR chromosome) and two chromosomes of *B. mori* (11 and 24).

In sex chromosome systems with male heterogamety, the Y chromosome is thought to originate from a homologous autosomal pair through the acquisition of a dominant, male-sex-determining role. The absence of meiotic recombination between X and Y chromosomes accelerates the accumulation of mutations on the Y chromosome, which leads to the functional inactivation of Y-linked genes followed by genetic erosion and accumulation of repetitive sequences (Charlesworth, 1996). However, the conception of the Y chromosome as a ‘graveyard’ (Steinemann *et al*., 1993; Kjellman *et al*., 1995) has been revised recently. In the highly evolved Y chromosomes of primates, the palindrome-driven sister chromatid and/or intrachromatid recombination prevents degradation and loss of Y-chromosome genes, and thus contributes to continuing evolution of the Y chromosome (Lange *et al*., 2009; Hughes *et al*., 2010). Moreover, recent findings in *Drosophila* suggest that the Y chromosome has an important role in male fitness and also contributes to adaptive phenotypic variation through a regulatory role of Y-linked polymorphic elements in gene expression (Lemos *et al*., 2008). In contrast, a gradual degeneration appears to be the primary driving force in evolutionarily young Y chromosomes as shown in the plant models *Carica papaya* and *Silene latifolia* (Jamilena *et al*., 2008). In addition, once the degenerated Y chromosome fuses or translocates to an autosome, a new cycle of its evolution starts. Hence, organisms carrying a primitive Y chromosome or an evolutionarily young neo-Y chromosome represent valuable models for the study of sex chromosome differentiation. Recent studies using genomic approaches provided supporting evidence for the hypothesis on early steps of sex chromosome evolution in several plants and animals with the XY system (Liu *et al*., 2004; Yamato *et al*., 2007; Bachtrog *et al*., 2008; Zhou *et al*., 2008). However, similar approaches have not been applied yet in the W chromosome of Lepidoptera. Geographic populations of *S. cynthia* examined in this study are the only representatives of lepidopteran species in which the autosomal origin of the neo-sex chromosomes has been molecularly determined. As *S. c. ricini* (Vietnam population) has a Z0 sex chromosome constitution with the original set of autosomes, comparative analyses between autosomal parts of the neo-W chromosomes in Japanese subspecies of *S. cynthia* and the original set of autosomes in *S. c. ricini* may significantly contribute to understanding the early steps of sex chromosome evolution in organisms with female heterogamety.

A question remains whether the evolutionary changes in sex chromosome constitution of *S. cynthia* populations have a role in speciation. A disproportionate association between traits that distinguish closely related species and the Z chromosome, found in several moths and butterflies, suggests that the lepidopteran Z chromosome has a large effect on species divergence (Sperling, 1994; Prowell 1998). Similarly, the Z-linkage of key components of reproductive isolation in flycatchers, such as species recognition, species-specific male traits and hybrid incompatibilities, suggests an important role of the avian Z chromosome in adaptive speciation (Saether *et al*., 2007). However, it seems that the so-called ‘Large Z-effect’ (or ‘Large X-effect’ in systems with male heterogamety) on speciation can often be attributed to the fact that the Z/X-linked traits evolve faster than autosomal traits leading to incompatibilities between the Z/X-linked and the interacting autosomal genes (Mank *et al*., 2007; Tang and Presgraves, 2009). Recent results suggest that the so-called ‘Faster-Z effect’ in a female heterogametic system is greater than the ‘Faster-X effect’ in organisms with male heterogamety (Mank *et al*., 2010). In *S. cynthia*, owing to the tendency to autosome–sex chromosome fusions, different sets of originally autosomal genes become sex-linked and subjected to a faster rate of evolution. These fusions also multiply the number of genes exposed to faster divergence, which may accelerate the accumulation of genetically based incompatibilities between populations. The above considerations are consistent with recent evidence on a significant role of neo-sex chromosomes in reproductive isolation of a vertebrate system, the three-spine stickleback fish (*Gasterosteus aculeatus*) (Kitano *et al*., 2009). Thus, we suggest that the step-by-step evolution of neo-sex chromosomes in geographical populations of *S. cynthia* by repeated autosome–sex chromosome fusions may facilitate population/species divergence and contribute to the formation of reproductive barriers.

Conflict of interest

The authors declare no conflict of interest.

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