University of South Bohemia in České Budějovice
Faculty of Science

Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp.

RNDr. Thesis

Mgr. Jindra Šíchová

České Budějovice

2016

Yoshido A., Šíchová J., Kubíčková S., Marec F., Sahara K. (2013) Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Chromosome Research* **21**: 149-164. DOI: 10.1007/s10577-013-9344-1

IF = 2.478

Annotation

Geographical subspecies of wild silkmoths, *Samia cynthia* ssp. (Lepidoptera: Saturniidae) exhibit a unique polymorphism in chromosome numbers, resulting from variations in the sex chromosome systems. Three different sex chromosome constitutions were identified: Z0/ZZ in *S. c. ricini* (2n=27/28), neo-Wneo-Z/neo-Zneo-Z in *S. c. walkeri* (2n=26/26), and neo-$WZ_1Z_2/Z_1Z_1Z_2Z_2$ in *S. cynthia* subsp. indet. (2n=25/26). It has been proposed, that the common ancestor of *S. cynthia* subspecies had a classical WZ/ZZ constitution with diploid chromosome number of 2n=28/28 and the neo-sex chromosomes in *S. c. walkeri* and *S. cynthia* subsp. indet. arose by repeated sex chromosome-autosome fusions. Our sampling effort enabled us to examine five populations of *S. cynthia* with an ancestral-like karyotype with the aim to verify a hypothesis about sex chromosome evolution in this species complex. Obtained results suggest that the curious WZ system of *S. cynthia pryeri* may represent an ancestral state of the *Samia* species complex. However, they do not exclude an alternative hypothesis of its derived origine.

Declaration [in Czech]

Prohlašuji, že svoji rigorózní práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 01.03.2016 .....

Jindra Šíchová

Prohlášení o autorském podílu Jindry Šíchové na publikaci:

Yoshido A., Šíchová J., Kubíčková S., Marec F., Sahara K. (2013) Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp. *Chromosome Research* **21**: 149-164. DOI: 10.1007/s10577-013-9344-1

Prohlašuji, že Jindra Šíchová se významně podílela na výše zmíněné publikaci. Podílela se na laserové mikrodisekci pohlavního chromozomu W martináče *Samia cynthia pryeri*, na amlifikaci a následné reamplifikaci vyřezané DNA a na přípravě sond pro fluorescenční in situ hybridizace (FISH).

Atsuo Yoshido
(první autor)

František Marec
(korespondující autor)

Chromosome Res (2013) 21:149–164
DOI 10.1007/s10577-013-9344-1

Rapid turnover of the W chromosome in geographical populations of wild silkmoths, *Samia cynthia* ssp.

Atsuo Yoshido · Jindra Šíchová · Svatava Kubíčková · František Marec · Ken Sahara

Received: 22 January 2013 / Revised: 25 February 2013 / Accepted: 26 February 2013 / Published online: 21 March 2013

Abstract Our previous studies revealed a considerably high level of chromosomal polymorphism in wild silkmoths, *Samia cynthia* ssp. (Lepidoptera: Saturniidae). Geographical populations of this species complex differ in chromosome numbers and show derived sex chromosome systems including Z0/ZZ in *S. cynthia ricini* (2*n*=27/28; Vietnam), neo-Wneo-Z/neo-Zneo-Z in *S. cynthia walkeri* (2*n*=26/26; Sapporo, Hokkaido) and neo-$WZ_1Z_2/Z_1Z_1Z_2Z_2$ in *S. cynthia* subsp. indet. (2*n*=25/26; Nagano, Honshu). In this study, we collected specimens of *S. cynthia pryeri* in Japanese islands Kyushu, Shikoku and Honshu, with an ancestral-like karyotype of 2*n*=28 in both sexes and a WZ/ZZ sex chromosome system, except for one population, in which females have lost the W chromosome. However, the *S. cynthia pryeri* W chromosome showed a very unusual morphology: It was composed of a highly heterochromatic body, which remained condensed throughout the whole cell cycle and of a euchromatin-like "tail." We examined molecular composition of the W and neo-W chromosomes in *S. cynthia* subspecies by comparative genomic hybridisation and fluorescence in situ hybridisation with W chromosome painting probes prepared from laser-microdissected W chromatin of *S. cynthia pryeri*. These methods revealed that the molecular composition of highly heterochromatic part of the *S. cynthia pryeri* W chromosome is very different and lacks homology in the genomes of other subspecies, whereas the euchromatin-like part of the W chromosome corresponds to a heterochromatic part of the neo-W chromosomes in *S. cynthia walkeri* and *S. cynthia* subsp. indet. Our findings suggest that the curious WZ system of *S. cynthia pryeri* may represent an ancestral state of the *Samia* species complex but do not exclude an alternative hypothesis of its derived origin.

Keywords Fluorescence in situ hybridisation · heterochromatin · insects · Lepidoptera · sex chromosome evolution

Abbreviations

FISH	Fluorescence in situ hybridisation
CGH	Comparative genomic hybridisation
GISH	Genomic in situ hybridisation

Responsible Editor: Walther Traut.

A. Yoshido · K. Sahara
Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Hokkaido University, N9, W9, Kita-ku, Sapporo 060-8589, Japan

J. Šíchová · F. Marec
Institute of Entomology, Biology Centre ASCR, České Budějovice, Czech Republic

J. Šíchová · F. Marec
Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

S. Kubíčková
Veterinary Research Institute, Brno, Czech Republic

Present Address:
A. Yoshido (✉)
Division of Biological Sciences and Center for Genome Dynamics, Faculty of Science, Hokkaido University, North 10, West 8, Kita-ku, Sapporo 060-0810, Japan
e-mail: yoshido@mail.sci.hokudai.ac.jp

Present Address:
K. Sahara
Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, 3-18-8, Ueda, Morioka 020-8550, Japan

DAPI	4′,6-Diamidino-2-phenylindole
SSC	Saline sodium citrate
COI-COII	Cytochrome c oxidase subunits I, II

Introduction

It is generally believed that heteromorphic sex chromosomes have evolved from a homologous pair of autosomes, independently in diverse lineages of eukaryotes (Ohno 1967). This has been well demonstrated in vertebrates, where the sex chromosomes of mammals, birds and snakes were derived from different pairs of autosomes of the common ancestor (Nanda et al. 1999; Matsubara et al. 2006). However, recent studies on the origin and evolution of Y chromosomes in species of the *Drosophila* genus, based on the whole genome sequences, revealed that the *Drosophila melanogaster* Y chromosome does not share any genes with the X chromosome, except for *Ste-Su*(*Ste*) and rDNA gene clusters (Carvalho 2002). The lack of homology suggests that the Y chromosome has not originated from the X chromosome partner but may have been derived from a supernumerary (B) chromosome (Hackstein et al. 1996; Graves 2005; Carvalho et al. 2009). The Y chromosome is entirely heterochromatic and consists mainly of transposable elements and repetitive sequences; it contains only about 20 single-copy genes (Carvalho et al. 2009; Kaiser and Bachtrog 2010). However, the Y chromosome in the *Drosophila pseudoobscura* lineage is not homologous to the *D. melanogaster* Y. It is a neo-Y chromosome that has probably arisen through a number of sex chromosome–autosome fusions (Carvalho and Clark 2005). Due to the absence of recombination in males, the neo-Y chromosome shows a typical signs of molecular degradation as found in *Drosophila miranda*, a close relative of *D. pseudoobscura* (Bachtrog 2005; Bachtrog et al. 2008). The findings in mammals and *Drosophila* allow us to understand the origin and evolutionary processes of the male-limited Y chromosomes in XY systems.

Moths and butterflies (Lepidoptera) have sex chromosome systems with female heterogamety, referred to as WZ and Z0 depending on the presence or absence of a W chromosome. Primitive Lepidoptera share the Z0 system with their sister group, caddis flies (Trichoptera), and both the groups form the only clade with heterogametic females among insects (Traut et al. 2007). The W chromosome occurs in the vast majority of Lepidoptera, specifically in the clades Ditrysia plus Tischeriina (Traut and Marec 1996; 1997; Lukhtanov 2000; Marec et al. 2010). In many species, the W chromosome can be identified using comparative genomic hybridisation (CGH) or genomic in situ hybridisation (GISH) provided that its DNA content is sufficiently differentiated from the Z chromosome (Traut et al. 1999; Sahara et al. 2003b). Furthermore, results of CGH and GISH suggested that lepidopteran W chromosomes are predominantly composed of accumulated repetitive DNA sequences, also occurring but scattered in the other chromosomes (Traut 1999; Traut et al. 1999; Sahara et al. 2003a; Mediouni et al. 2004; Fuková et al. 2005; Yoshido et al. 2006). In addition, the complete absence of meiotic recombination in lepidopteran females resulted in the accelerated molecular divergence of the maternally inherited W chromosomes, and hence, some sequence homologies were found only between W chromosomes of very closely related species (Abe et al. 2002; Vítková et al. 2007; Yoshido et al. 2007). Thus, a little is known about the origin and evolutionary processes of the heterochromatin-rich and gene-poor W chromosomes of Lepidoptera.

In advanced Lepidoptera, the Ditrysia, numerical variations of the standard WZ/ZZ (female/male) sex chromosome system were described, such as Z0/ZZ, W_1W_2Z/ZZ and WZ_1Z_2/$Z_1Z_1Z_2Z_2$ (Suomalainen 1969; Nilsson et al. 1988; Traut and Marec 1997; Rishi et al. 1999; Yoshido et al. 2005b). The derived systems are thought to originate either from the loss of the W chromosome (Z0/ZZ) or from sex chromosome–autosome fusion and fission (Marec et al. 2010; Sahara et al. 2012). These scenarios have also played a role in the evolution of unique variations of sex chromosome systems in wild silkmoths, *Samia cynthia* ssp. (Lepidoptera: Saturniidae) (Yoshido et al. 2011a). In this species complex, different geographical populations/subspecies exhibit different sex chromosome constitutions and chromosome numbers: Z0/ZZ ($2n$=27♀/28♂) in the Eri silkworm, *S. cynthia ricini* (Vietnam population); neo-Wneo-Z/neo-Zneo-Z ($2n$=26♀/26♂) in the ailanthus silkworm, *S. cynthia walkeri* (Sapporo population); and neo-WZ_1Z_2/$Z_1Z_1Z_2Z_2$ ($2n$=25♀/26♂) in the Shinju silkworm, *S. cynthia* subsp. indet. (Nagano population). The neo-sex chromosomes in the latter two subspecies have arisen by repeated sex chromosome–autosome fusions. In addition, originally Z parts and all the autosomal parts

of the neo-sex chromosomes correspond to the Z chromosome and particular autosomes in *S. cynthia ricini* (Yoshido et al. 2011b). These findings favour a hypothesis that a common ancestor of *S. cynthia* ssp. had a diploid chromosome number of $2n=28$ and a WZ/ZZ sex chromosome system (Yoshido et al. 2005b, 2011a). However, a stronger support of the hypothesis, i.e. obtained from cytogenetic analysis of a *S. cynthia* subspecies with ancestral karyotype characteristics, is still missing.

Kawaguchi (1937) described populations of the Shinju silkmoth, *S. cynthia pryeri*, from Fukuoka (Kyushu, Japan) and Kyoto (Honshu, Japan) with 14 meiotic bivalents and 28 mitotic chromosomes in males, but did not report any data from females. Nevertheless, this old report inspired us to carry out a more extensive survey of *S. cynthia* populations and search for specimens with an ancestral-like karyotype with the aim to verify our hypothesis on the sex chromosome evolution in *S. cynthia* subspecies/populations. In the present work, we collected samples of five populations of *S. cynthia pryeri* from localities in three Japanese islands, Kyushu, Shikoku and Honshu. We determined chromosome numbers in both sexes and performed a detailed analysis of the sex chromosome constitution using methods of molecular cytogenetics. Furthermore, we compared the molecular composition of the W chromosome of *S. cynthia pryeri* and neo-W chromosomes of the other *S. cynthia* subspecies by fluorescence in situ hybridisation (FISH) with W chromosome painting probes prepared by means of laser microdissection of the W chromatin from *S. cynthia pryeri* females.

Material and methods

Insects

We collected *S. cynthia* ssp. (Saturniidae) in five different localities in Japan, Toyota and Okayama (Honshu), Fukuoka and Kagoshima (Kyushu) and Takamatsu (Shikoku) in 2005–2011 (Fig. 1a). In addition, we used specimens of *S. cynthia walkeri* (Sapporo population, Hokkaido, Japan), *S. cynthia* subsp. indet. (Nagano population, Honshu, Japan) and *S. cynthia ricini* (Vietnam population) (for details, see Yoshido et al. 2005b). While Peigler and Naumann (2003) described the populations of *S. cynthia* collected in Honshu (central part), Kyushu and Ryukyu islands as *S. pryeri*, here we used the former classification of subspecies, i.e. *S. cynthia pryeri*, which is consistent with the manner used in our previous cytogenetic studies (Yoshido et al. 2005b, 2011a).

Construction of molecular phylogenetic tree

In samples of *S. cynthia* populations, a mitochondrial DNA region (2,355 bp) encompassing the *COI*, $tRNA^{Leu}$, *COII* and $tRNA^{Lys}$ genes was amplified by DOP-PCR using two degenerate oligonucleotide primers, 5′-CGAAAATGACTTTAYTCNACTAATC-3′ (forward) and 5′-TCATTAGAAGTANTTGNTAATTTAC-3′ (reverse), as described in Yoshido et al. (2011a). The PCR-generated fragments were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

A phylogenetic tree of *S. cynthia* geographic populations was constructed by the maximum likelihood method using the MEGA 5.0 software (Tamura et al. 2011), with the nucleotide substitution model GTR+G. The Chinese oak silkmoth, *Antheraea pernyi* (Saturniidae), and the silkworm, *Bombyx mori* (Bombycidae), were used as outgroups to construct the tree. GenBank accession numbers of all sequences used are AY048187 (*B. mori*), AY242996 (*A. pernyi*), AB558964 (*S. cynthia ricini*), AB558962 (*S. cynthia walkeri*), AB558963 (*S. cynthia* subsp. indet.), AB775192 (Toyota), AB775193 (Okayama), AB775194 (Takamatsu), AB775195 (Fukuoka), AB775196 and AB775197 (Kagoshima). Bootstrap values were estimated with 1,000 replicates.

Chromosome preparations

Spread chromosome preparations were made as described in Yoshido et al. (2005b), with one modification (see hypotonic treatment). Briefly, ovaries or testes of the last instar larvae were dissected in a saline solution and fixed for 10–15 min in Carnoy fixative (ethanol, chloroform, acetic acid, 6:3:1). Hypotonic treatment (75 mM KCl) for 10–15 min was done only for testes before fixation. 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 and 98 %, 30 s each) and stored in the freezer at −20 °C until use.

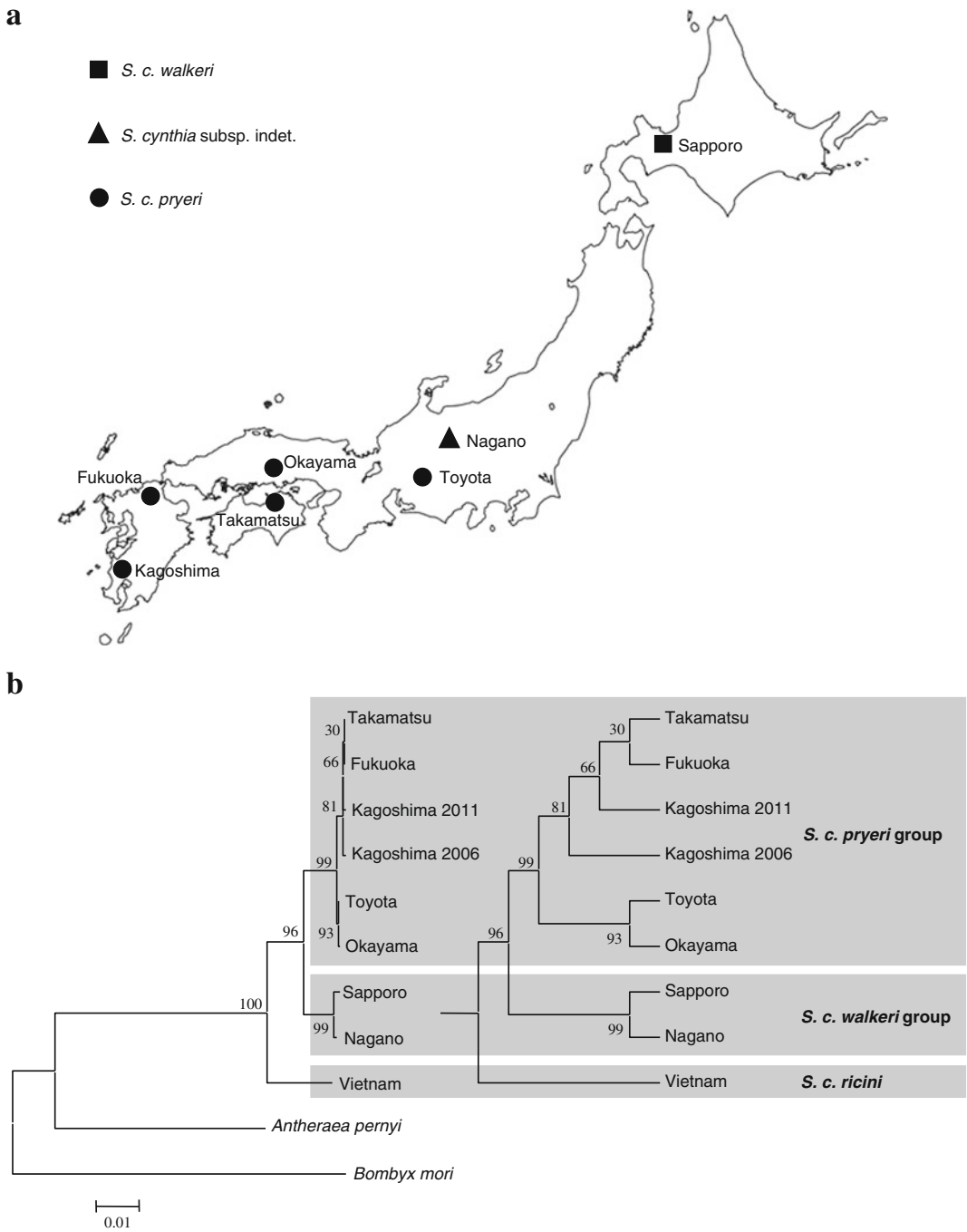


Fig. 1 **a** Distribution of *S. cynthia* subspecies in Japan. **b** Maximum likelihood tree showing phylogenetic relationships of eight geographic populations of *S. cynthia* ssp. (Saturniidae), constructed with the use of nucleotide sequences of a mitochondrial DNA fragment containing the *COI*, $tRNA^{Leu}$, *COII* and $tRNA^{Lys}$ genes. The domesticated silkworm, *B. mori* (Bombycidae), and the Chinese oak silkmoth, *A. pernyi* (Saturniidae), were used as outgroups. Scale indicates the number of base substitutions per site. The subtree on the right-hand side shows the *S. cynthia* ssp. clade separately (only topology is displayed). The bootstrap values (1,000 replicates) are shown next to the branches

Laser microdissection of the W chromosome and preparation of W chromosome painting probes

For laser microdissection of the *S. cynthia pryeri* W chromosome, we used preparations from ovaries of the Toyota populations. This was possible because the 'Toyota' W chromosome was easily identified by its small size and compact spherical form (see "Results"). Chromosome spreads were prepared following the protocol described in Fuková et al. (2007) for laser microdissection of W-chromatin bodies from polyploid nuclei, with some modifications. Briefly, the ovaries were dissected out from the fifth instar larvae, hypotonized for 10 min in 75 mM KCl, fixed for 15 min in methanol/acetic acid (3:1) and spread in 60 % acetic acid on a glass slide coated with a polyethylene naphthalate membrane at 50 °C. The preparation was stained with 4 % Giemsa. Microdissections were performed with the help of a PALM MicroLaser System (Carl Zeiss MicroImaging GmbH, Munich, Germany) as described in Kubickova et al. (2002). DNA of microdissected samples, each containing 10–20 W chromosomes, was used as a template for PCR amplification using a WGA4 GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR products were re-amplified using WGA3 GenomePlex WGA Reamplification Kit (Sigma-Aldrich) and then labelled with Cy3-dUTP (GE Healthcare UK, Buckinghamshire, UK) using the WGA3 Kit (for details, see Drosopoulou et al. 2012).

Fluorescence in situ hybridisation techniques

For the identification of W chromosomes, we used GISH, where labelled female genomic DNA (gDNA) probes were hybridised to female chromosome spreads at the excess of unlabelled male gDNA (see Sahara et al. 2003b). In some experiments, GISH was combined with FISH with telomeric $(TTAGG)_n$ probes that helped us to detect chromosome ends in pachytene oocytes (for further explanation and preparation of telomeric probes, see Yoshido et al. 2005b). We also used CGH with differently labelled female and male gDNA probes at a 1:1 ratio to examine molecular composition of the W chromosomes (see Sahara et al. 2003a). For both GISH and CGH, gDNAs were obtained separately from female and male larvae by standard phenol–chloroform extraction. For FISH with *S. cynthia walkeri* fosmid clones, we selected two Z chromosome markers, clones 19B8 and 45A6, and two chromosome 13 markers, clones 56J8 and 56J22 (for details, see Yoshido et al. 2011b). DNA probes were labelled using a Nick Translation Mix (Roche Diagnostics, Mannheim, Germany) with Green-dUTP, Orange-dUTP and Red-dUTP (Abbott Molecular Inc., Des Plaines, IL, USA) and Cy3-dCTP and Cy5-dUTP (GE Healthcare UK).

All FISH experiments were carried out following the procedure of Traut et al. (1999) for CGH with slight modifications. Briefly, after removal from the freezer, chromosome preparations were passed through the graded ethanol series and air-dried. Denaturation of chromosomes was done at 72 °C for 3.5 min in 70 % formamide, 2× saline sodium citrate (SSC). For one preparation, the probe cocktail contained 100–500 ng of each labelled DNA probe, 3 μg of unlabelled sonicated male gDNA and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich) in 10 μl hybridisation 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 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). Digital images were captured with a DFC350FX B&W CCD camera (Leica Microsystems Inc.) and processed with Adobe Photoshop CS4 as described in Yoshido et al. (2005a).

Results

Phylogenetic relationships between geographical populations of *S. cynthia* ssp.

We constructed a molecular phylogenetic tree using mitochondrial DNA sequences of the *COI* + $tRNA^{Leu}$ + *COII* + $tRNA^{Lys}$ region to examine the evolutionary relationship between five newly collected populations (Toyota, Okayama, Fukuoka, Kagoshima and Takamatsu; present study) and three previously examined populations (Sapporo, Nagano and Vietnam; Yoshido et

al. 2011a) of *S. cynthia* ssp. (Fig. 1b). In Kagoshima population, we used two female individuals collected in 2006 and 2011, respectively; since they differed in karyotype characteristics (see below), we treated them as representatives of two different populations. In the tree, the newly collected populations formed a common clade separated from the previously studied populations (cf. Figure S2 in Yoshido et al. 2011a). Consistently with Kawaguchi (1937), who described Fukuoka and Kyoto populations as *S. cynthia pryeri*, we classified the newly collected populations as *S. cynthia pryeri* group (Fig. 1a, b). Accordingly, the clade formed by Sapporo and Nagano populations was designated as *S. cynthia walkeri* group (Fig. 1b).

Sex chromosome constitution in the *S. cynthia pryeri* group

Cytogenetic analyses of *S. cynthia pryeri* populations revealed that specimens from Toyota and Fukuoka populations have identical chromosome numbers and a similar sex chromosome constitution (Fig. 2a–k). Males of both populations showed $n=14$ and $2n=28$ chromosomes in meiotic and mitotic complements (Fig. 2b, c). In females, a curious chromosome, largely composed of heterochromatin, was easily distinguished by strong DAPI staining not only in meiotic ($n=14$) and mitotic ($2n=28$) complements but also in interphase nuclei (Fig. 2d–f). Because no such chromosome was found in the Toyota and Fukuoka males (Fig. 2a–c), we concluded that this compact DAPI-highlighted element is a W chromosome. However, a detailed inspection of oogonial mitotic chromosomes revealed that the W chromosome is composed of two parts, a highly heterochromatic major part and a less condensed euchromatin-like part (Fig. 2g). W chromosome univalents, which were rarely observed in pachytene oocytes, showed a similar structure (Fig. 2h). The heterochromatic part of the W chromosome was strongly highlighted by GISH (Fig. 2i, *arrow*) and easily differentiated from the W pairing partner, the anticipated Z chromosome, in pachytene complements. Weaker GISH signals identified the telomeric and/or subtelomeric region of the less condensed part of the W chromosome (Fig. 2i, *arrowhead*). Similar to other *S. cynthia* subspecies (Yoshido et al. 2005b), GISH signals were also observed in an interstitial segment of a bivalent carrying the nucleolar organizer region (NOR) (Fig. 2i, *asterisk*) and telomeric regions of autosome bivalents.

In most pachytene oocytes of Toyota and Fukuoka populations, the W chromosome formed a curious bivalent with the anticipated Z chromosome that was wrapped around the highly heterochromatic W body. A similar WZ pairing was observed in the oriental tussock moth *Artaxa subflava* (Yoshido et al. 2006) and the butterfly *Bicyclus anynana* with abnormally small W chromosome (Van't Hof et al. 2008). FISH with the telomeric probe regularly identified three telomeres in the sex chromosome bivalent, two at the ends of the anticipated Z chromosome and one at the end of the less condensed part of the W chromosome that appeared paired with one end of the Z chromosome (Fig. 2j). FISH with two Z-derived fosmid clones, 45A6 and 19B8, identified the Z chromosome in the WZ bivalent (Fig. 2k) and confirmed thus that *S. cynthia pryeri* of Toyota and Fukuoka populations have a WZ/ZZ sex chromosome system.

In *S. cynthia pryeri* from Okayama and Takamatsu populations, we could not establish laboratory cultures because of discrepancy in eclosion timing between females and males. Therefore, we investigated sex chromosomes in these two populations using F_1 females from crosses between respective females and *S. cynthia walkeri* males (Sapporo population) (Fig. 3a–d). The *S. cynthia walkeri* males have $2n=26$ with a pair of neo-Z chromosomes that originated by fusion between the ancestral Z chromosome and an autosome, corresponding to chromosome 13 in *S. cynthia ricini* (see Yoshido et al. 2011b). Thus, if the mothers (females of Okayama and Takamatsu populations) had a sex chromosome constitution similar to that in the Fukuoka and Toyota populations, we expected to find in F_1 females a trivalent composed of *S. cynthia walkeri* neo-Z and *S. cynthia pryeri* W and chromosome 13. Indeed, pachytene complements in F_1 female progeny of an Okayama mother showed 12 autosome bivalents and a trivalent, easily identified by the curious heterochromatinized W body that was deeply stained with DAPI and strongly highlighted by GISH (Fig. 3a, *arrow*). GISH signals were also observed in the NOR-bivalent (Fig. 3a, *asterisk*). The trivalent configuration of the sex chromosomes and chromosome 13 was confirmed by telomere FISH (Fig. 3b). In rarely observed trivalents without the W ball-like structure, telomeric hybridisation signals were found in both ends of the extended W chromosome thread (Fig. 3c). Furthermore, fosmid-FISH mapping using two Z chromosome markers (45A6 and 19B8 clones) and two chromosome 13 markers (56J8 and

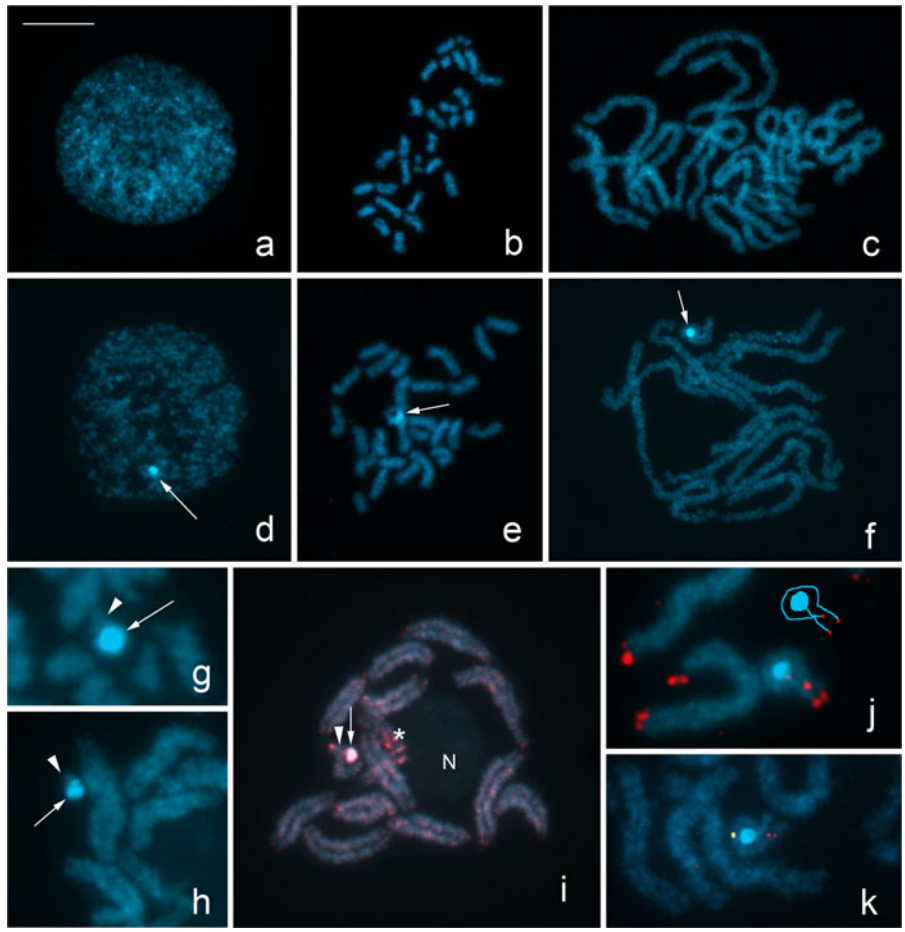
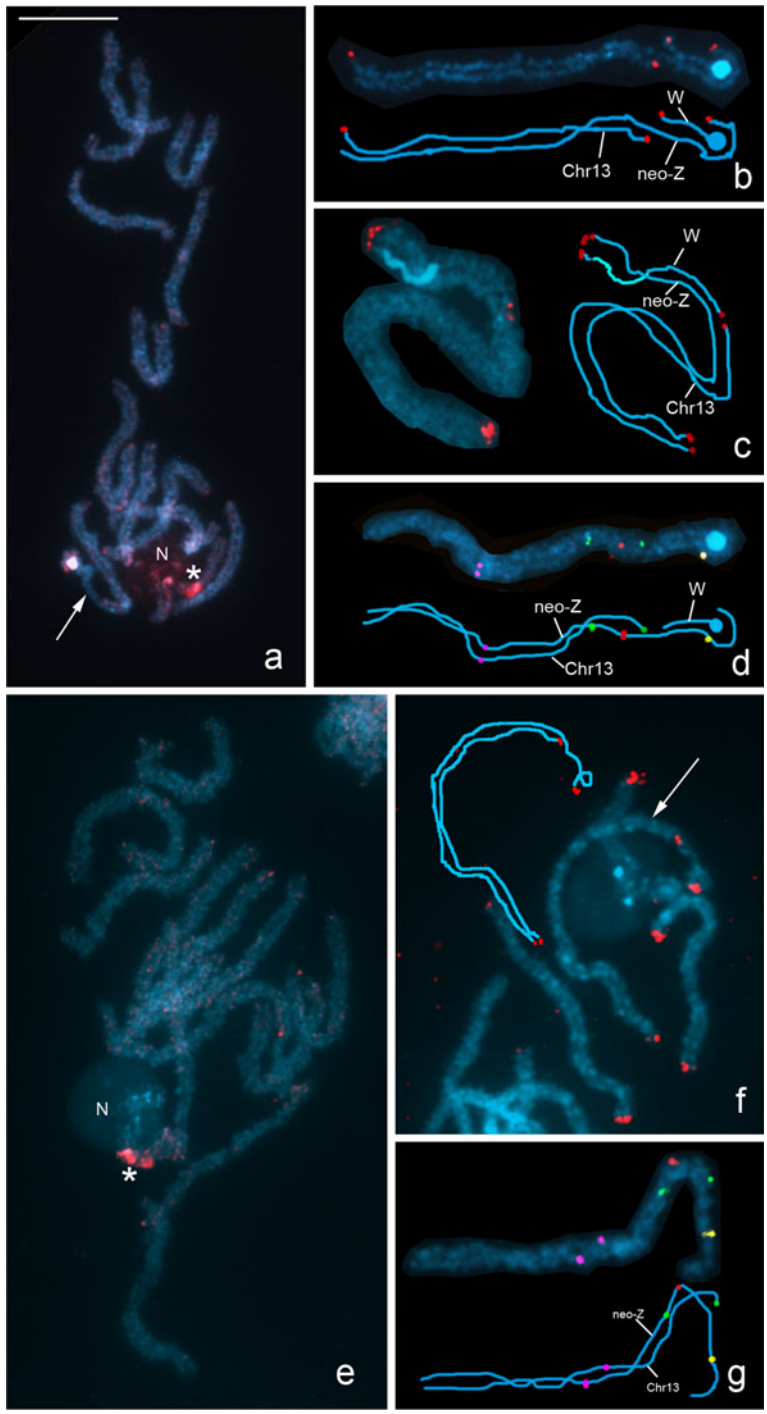


Fig. 2 Sex chromosome constitution in males (**a**–**c**) and females (**d**–**k**) of *S. cynthia pryeri* (Toyota and Fukuoka populations). Preparations were stained with DAPI (*light blue*). **a**, **d** Interphase nucleus; **b**, **e** mitotic metaphase complement; **c**, **f** pachytene complement; **g** a detail of mitotic metaphase W chromosome; **h** a detail of pachytene W univalent. *Arrow* in **d**–**f** indicates a curious heterochromatinized W chromosome. *Arrow* in **g** and **h** points to the highly heterochromatic part of the W chromosome; *arrowhead* points to the euchromatin-like part. **i** Pachytene complement stained with GISH. Cy3-labelled female genomic probe (*red signals*) strongly highlighted the heterochromatic part (*arrow*) and also identified the euchromatin-like part (*arrowhead*) of the W chromosome; the probe also highlighted a segment of the NOR-bivalent (*asterisk*) associated with the nucleolus (*N*). **j** A part of pachytene nucleus showing a curious WZ bivalent with chromosome ends identified by FISH with a Cy3-labelled $(TTAGG)_n$ telomeric probe (*red signals*). Drawing shows a schematic interpretation of the sex chromosome configuration. **k** A part of pachytene nucleus showing a WZ bivalent after FISH with two fosmid probes that mapped to the Z chromosome, Orange-labelled probe of the 19B8 clone (*red signals*) and Red-labelled probe of the 45A6 clone (*yellow signals*). *Bar* represents 10 μm (**a**–**f**, **i**), 5 μm (**h**, **j**, **k**) and 2.5 μm (**g**)

56J22 clones) confirmed that the trivalent is formed by the father-derived neo-Z chromosome and the mother-derived W chromosome plus chromosome 13 (Fig. 3d). Similar results were obtained in F_1 females from the cross between a Takamatsu female and a *S. cynthia walkeri* male (not shown). Both F_1 male progenies showed $2n=27$ chromosomes without any W heterochromatin (not shown). Hence, we concluded that wild silkmoths of the Okayama and Takamatsu populations have a $2n=28$ karyotype and a WZ/ZZ sex chromosome constitution with a highly heterochromatic W chromosome similar to that found in Fukuoka and Toyota populations.

Wild silkmoths of Kagoshima population seemed to be exceptional among *S. cynthia pryeri* group as F_1 females from the cross between a Kagoshima female collected in 2006 and *S. cynthia walkeri* male did not have any heterochromatinized chromosome in pachytene complements. We observed 13 elements in the complements, and GISH highlighted only an interstitial segment of the NOR-bivalent and weakly labelled



telomeric regions of some chromosomes (Fig. 3e). In addition, using telomere FISH, we identified an uneven bivalent (Fig. 3f, *arrow*). Then we confirmed by fosmid-FISH mapping that this uneven bivalent is formed by a father-derived neo-Z chromosome and a mother derived chromosome 13 (Fig. 3g). Similar results were obtained in F_1 females obtained from another female of Kagoshima population collected in 2006. The results together with $2n=27$ chromosomes found in F_1 males (not shown) suggested a Z0/ZZ sex

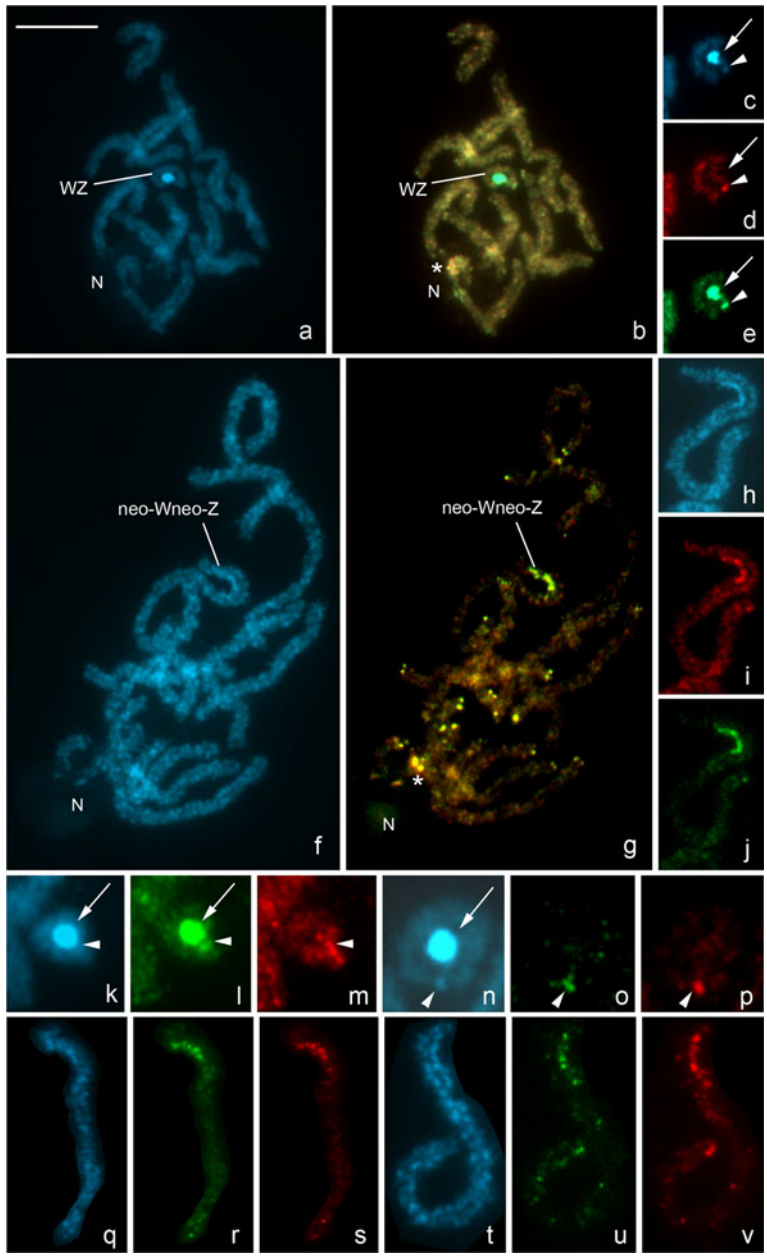
◀ **Fig. 3** Sex chromosome constitution in F_1 females from crosses between *S. cynthia pryeri* females and *S. cynthia walkeri* males. Okayama × Sapporo populations (**a**–**d**), Kagoshima × Sapporo populations (**e**–**g**). Chromosomes were stained with DAPI (*light blue*). **a** Pachytene complement stained with GISH; Cy3-labelled female genomic probe (*red signals*) identified the highly heterochromatinized part of the W chromosome (*arrow*) and strongly highlighted a part of the NOR-autosome bivalent (*asterisk*); *N* nucleolus. **b**, **c** A sex chromosome trivalent after FISH with the Cy3-labelled $(TTAGG)_n$ telomeric probe (*red signals*); drawing shows a schematic interpretation of the sex chromosome trivalent. **d** A sex chromosome trivalent after FISH with two fosmid probes that mapped to the ancestral part of the neo-Z chromosome, Orange-labelled probe of the 19B8 clone (*red signal*) and Red-labelled probe of the 45A6 clone (*yellow signal*), and two fosmid probes that mapped to the autosomal part of the neo-Z chromosome and to chromosome 13, Green-labelled probe of the 56J8 clone (*green signals*) and Cy5-labelled probe of the 56J22 clone (*magenta signals*). **e** Pachytene complement stained with GISH; Cy3-labelled female genomic probe (*red signals*) strongly highlighted a part of the NOR-autosome bivalent (*asterisk*) but did not detect a W chromosome; *N* nucleolus. **f** A part of pachytene nucleus after FISH with the Cy3-labelled $(TTAGG)_n$ telomeric probe (*red signals*), showing an uneven bivalent (*arrow*) with an unpaired terminal segment; drawing shows a schematic interpretation of the uneven bivalent. **g** A part of pachytene nucleus, showing an uneven bivalent after FISH with two fosmid probes that mapped to the ancestral part of the neo-Z chromosome, Orange-labelled probe of the 19B8 clone (*red signal*) and Red-labelled probe of the 45A6 clone (*yellow signal*), and two fosmid probes that mapped to the autosomal part of the neo-Z chromosome and to chromosome 13, Green-labelled probe of the 56J8 clone (*green signals*) and Cy5-labelled probe of the 56J22 clone (*magenta signals*). *Bar* represents 10 μm (**a**, **e**) and 5.0 μm (**b**–**d**, **f**–**g**)

chromosome system in the Kagoshima population. However, the karyotype and sex chromosome constitution of Kagoshima specimens collected in 2011 was different from those collected in 2006. In this case, we established a culture and performed a standard cytogenetic analysis in both sexes. We found $2n$=28♀/28♂ karyotypes and a WZ/ZZ sex chromosome system with a highly heterochromatic W chromosome in females, similar to that observed in *S. cynthia pryeri* females of Toyota and Fukuoka populations (see Fig. 2d–k). Thus, we concluded that both the WZ and Z0 sex chromosome constitutions occur in Kagoshima females.

Molecular differentiation of W chromosomes in *S. cynthia* subspecies by CGH

We carried out CGH using genomic DNA of females and males in specimens representing the *S. cynthia pryeri* group (Fukuoka population) and in *S. cynthia walkeri* (Sapporo population) (Fig. 4a–j). In *S. cynthia pryeri*, both the female (green) and male (red) genomic probes hybridised to all chromosomes, resulting in yellowish colouration of chromatin, with a few exceptions. Both probes highlighted a part of the NOR-bivalent at very similar intensities (Fig. 4b, *asterisk*). Stronger hybridisation signals of both probes at similar intensities were also observed in a part of the euchromatin-like part of the W chromosome (Fig. 4d, e, *arrowhead*), whereas the highly heterochromatic W body was preferentially and strongly differentiated only with the female genomic probe (Fig. 4b, d, e, *arrows*). Similar distribution and intensities of hybridisation signals were observed in the W chromosomes from other populations (Toyota, Okayama, Takamatsu and Kagoshima) of the *S. cynthia pryeri* group (not shown). In *S. cynthia walkeri* (Sapporo population), both probes strongly highlighted the ancestral W-part in the neo-W chromosome (Fig. 4f–j). Both probes also highlighted a part of the NOR-bivalent and telomeric regions of autosomes (Fig. 4g). Results of CGH in *S. cynthia* subsp. indet. females (Nagano population) were similar to those obtained in *S. cynthia walkeri* (not shown).

We also compared gross molecular composition of the W chromosomes between *S. cynthia* subspecies by cross-hybridisation of female genomic probes (Fig. 4k–v). The highly heterochromatic part of the conspicuous W chromosomes in *S. cynthia pryeri* group was highlighted only by *S. cynthia pryeri* female genomic probes prepared from female specimens carrying the W chromosome (Fig. 4k–p). Similar results were obtained with female genomic probes from all four populations of *S. cynthia pryeri* group (not shown), which suggests a common origin of the curious W chromosomes in these populations. Female genomic probes from other subspecies did not hybridise to the highly heterochromatic part of the conspicuous W chromosomes (Fig. 4k–p) though they highlighted either the whole euchromatin-like part of the W chromosome (*S. cynthia walkeri* probe, Fig. 4m, *arrowhead*) or the telomeric region of the latter part (*S. cynthia* subsp. indet. and *S. cynthia ricini* probes, Fig. 4n–p, *arrowhead*). By contrast, the heterochromatic part of the neo-W chromosome in *S. cynthia walkeri* was differentiated by female genomic probes from all subspecies (Fig. 4q–v).



Identification of shared parts of the neo-W and W chromosomes

For a detailed comparison of the *S. cynthia walkeri* neo-W chromosome and the *S. cynthia pryeri* W chromosome, we used W-chromosome painting probes prepared by laser microdissection of the W chromosome from females of *S. cynthia pryeri* (Toyota population) (Fig. 5a–n). In this experiment, we obtained two types of W-painting probes. The type-1 W-probe was highly specific for the heterochromatic part of the conspicuous W chromosome in *S. cynthia pryeri* (Fig. 5a–d, *arrows*) but did not hybridise to the euchromatin-like part of the W chromosome (Fig. 5b–d, *arrowhead*). However, the type-2 W-probe painted the whole W chromosome (i.e. including the euchromatin-like part) in *S. cynthia pryeri* females from Toyota population (Fig. 5e–h).

◀ **Fig. 4** Comparative genomic hybridisation (CGH) between respective sexes in females of *S. cynthia pryeri* (**a**–**e**) and *S. cynthia walkeri* (**f**–**j**) and between subspecies in females of *S. cynthia pryeri* (**k**–**p**) and *S. cynthia walkeri* (**q**–**v**). Pachytene chromosomes were counterstained with DAPI (*light blue*), female-derived genomic DNA probes were labelled with Green-dUTP (*green*), male-derived genomic DNA probes with Cy3-dCTP (*red*). *N* nucleolus. **a** (DAPI image) and **b** (merged image of both probes), Pachytene complement of *S. cynthia pryeri* female. The female genomic probe strongly highlighted the compact heterochromatin body of the W chromosome and both probes highlighted a part of the NOR-bivalent (*asterisk*). **c**–**e** A detail of the WZ bivalent: **c** DAPI image; **d** male genomic probe; **e** female genomic probe. The female probe differentiated both the heterochromatin body (*arrow*) and the euchromatin-like part (*arrowhead*) of the W chromosome, whereas the male genomic probe only the latter part (*arrowhead*). **f** (DAPI image) and **g** (merged image of both probes), Pachytene complement of *S. cynthia walkeri* female. CGH highlighted the ancestral heterochromatic part of the neo-W chromosome and an interstitial segment of the NOR-bivalent (*asterisk*). **h**–**j** A detail of the neo-Wneo-Z bivalent: **h** DAPI image; **i** male genomic probe; **j** female genomic probe. Both probes strongly bound to the heterochromatic segment of the neo-W chromosome. **k**–**p** A detail of the *S. cynthia pryeri* WZ bivalent stained with between-subspecies CGH. **q**–**v** A detail of the *S. cynthia walkeri* neo-Wneo-Z bivalent stained with between-subspecies CGH. **k**, **n**, **q**, **t** DAPI images; **l**, **r** *S. cynthia pryeri* female genomic probe; **m**, **s** *S. cynthia walkeri* female genomic probe; **o**, **u** *S. cynthia* subsp. indet. female genomic probe; **p**, **v** *S. cynthia ricini* female genomic probe. **k**–**p** The *S. cynthia pryeri* female genomic probe differentiated both parts of the W chromosome (*arrow* and *arrowhead*) in *S. cynthia pryeri*, whereas other *S. cynthia* subspecies probes only the euchromatin-like part (*arrowhead*). **q**–**v** All female genomic probes differentiated the ancestral heterochromatic part of the neo-W chromosome in *S. cynthia walkeri*. *Bar* represents 10 μm (**a**–**j**, **q**–**v**) and 5 μm (**k**-**p**)

Using the two types of W-probes with different painting specificity, we carried out FISH in other *S. cynthia* subspecies. The type-1 W-probe did not hybridise to any part of the neo-W chromosome and did not highlighted any other chromosome region in *S. cynthia walkeri*, except weak hybridisation signals in several telomeric regions (Fig. 5i). On the contrary, the type-2 W-probe highlighted the heterochromatic segment of the neo-W chromosome in *S. cynthia walkeri* (Fig. 5j). Hybridisation signals of the type-2 W-probe colocalized with GISH signals, confirming thus that the type-2 W-probe identified the ancestral part of the neo-W chromosome (Fig. 5k–n). These results clearly show that the highly heterochromatic part of the W chromosome is specific to the *S. cynthia pryeri* and it is absent in the genome of other *S. cynthia* subspecies, whereas the euchromatin-like part of the *S. cynthia pryeri* W chromosome shares significant sequence homology with the ancestral heterochromatic segment of the neo-W chromosome in *S. cynthia walkeri*.

Discussion

Previous studies showed that neo-sex chromosomes in geographical populations of wild silkmoths, *S. cynthia* ssp., evolved by repeated autosome-sex chromosome fusions resulting in reduced chromosome numbers. These studies also proposed a hypothesis that an ancestral karyotype had a diploid chromosome number of $2n=28$ and a standard WZ/ZZ sex chromosome system (Yoshido et al. 2005b, 2011a, b). Our sampling effort in western part of Japan (Fig. 1a) enabled us to examine five populations of *S. cynthia*, all showing ancestral-like karyotypes and a WZ sex chromosome pair in females with the only exception: In Kagoshima population, we found another female karyotype ($2n=27$, Z0) in addition to that of $2n=28$, WZ. However, the W chromosome in females from these populations differs considerably from W chromosomes in other lepidopteran species (reviewed in Marec et al. 2010). It is composed of two parts, a conspicuous heterochromatic body and a euchromatin-like 'tail'. Moreover, it shows a remarkable morphological stability throughout cell cycles. A reconstructed molecular phylogenetic tree of all so-far examined populations of *S. cynthia* placed the five Western Japan populations into a common clade, designated as *S. cynthia pryeri* group, clearly separated from two other clades, one formed by central plus northern Japan populations, *S. cynthia walkeri* group, and the other by the Vietnam population, *S. cynthia ricini* group (Fig. 1b).

Results of previous studies on sex chromosome constitution in Lepidoptera and Trichoptera, the two sister orders with female heterogamety, suggested that the lepidopteran W chromosome arose in Tischeriina plus Ditrysia as an evolutionary novelty from the Z0 sex chromosome system (Traut and Marec 1996; Marec and Novák 1998; Lukhtanov 2000). However, secondary losses of the W chromosome have occurred independently and rather frequently in species of different clades of Ditrysia (see Fig. 3.4 in Marec et al. 2010). The W chromosome has also been lost in *S. cynthia* ssp. This happened at least twice in *S. cynthia ricini* (Yoshido et al. 2005b) and within Kagoshima population (this study), which belongs to the *S. cynthia pryeri* group (see Figs. 1 and 6). In the latter case,

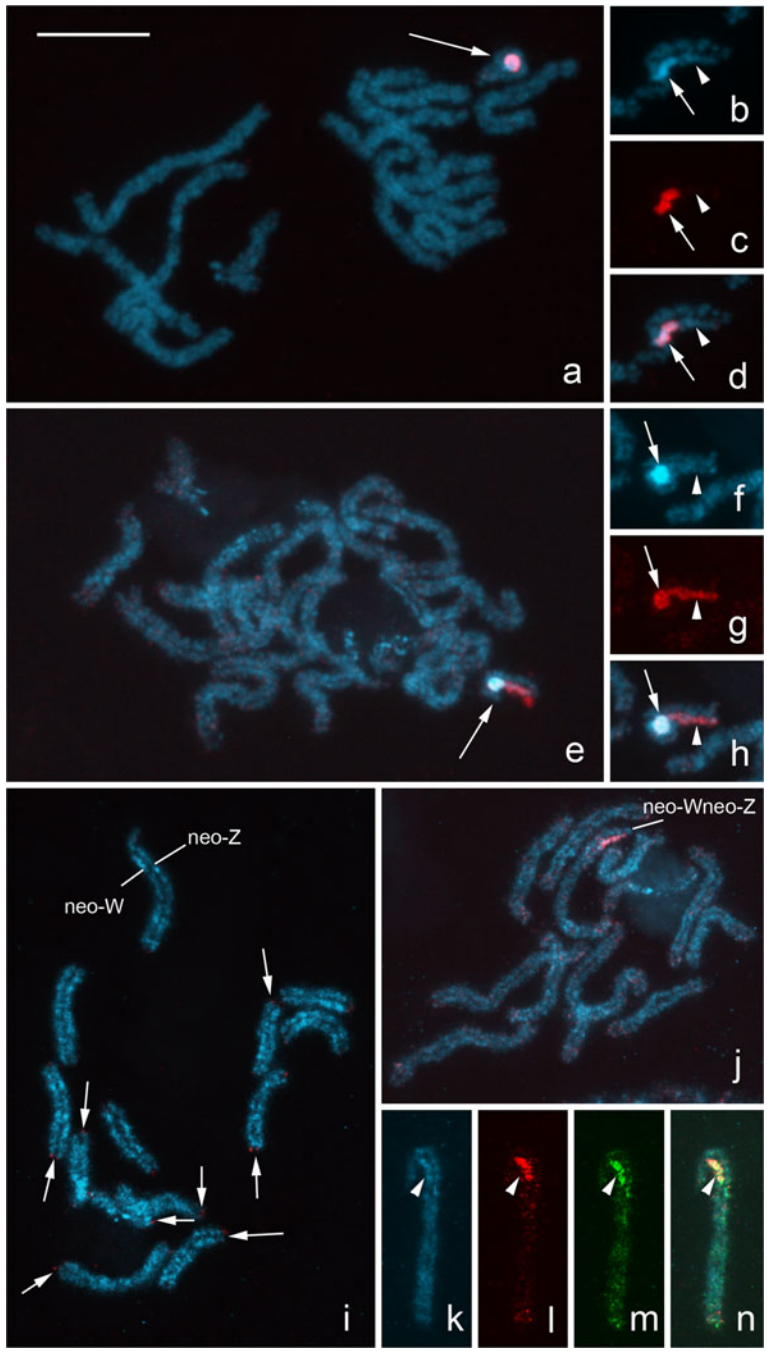


Fig. 5 FISH with W-chromosome painting probes in females of two *S. cynthia* subspecies, *S. cynthia pryeri* (**a**–**h**) and *S. cynthia walkeri* (**i**–**n**). Cy3-labelled W-probes (*red*) were prepared from *S. cynthia pryeri*. Pachytene chromosomes were counterstained with DAPI (*light blue*). **a**–**d** Type-1 W-probe hybridised only to the highly heterochromatic part of the W chromosome in *S. cynthia pryeri*. **a** Pachytene complement. **b**–**d** A detail of the WZ bivalent: **b** DAPI image; **c** W-probe; **d** merged image. **e**–**h** Type-2 W-probe hybridised to the whole W chromosome in *S. cynthia pryeri*. **e** Pachytene complement. **f**–**h** A detail of the WZ bivalent: **f** DAPI image; **g** W-probe; **h** merged image. **i** Type-1 W-probe hybridised only to telomeric regions of some bivalents in *S. cynthia walkeri*. **j** Type-2 W-probe hybridised to the heterochromatic segment of the neo-W chromosome in pachytene complements of *S. cynthia walkeri*. **k**–**n** A detail of the neo-Wneo-Z bivalent in *S. cynthia walkeri*: **k** DAPI image; **l** type-2 W-probe; **m** GISH with Green-labelled *S. cynthia walkeri* female genomic probe (*green*); **n** merged image. Note that GISH and W-probe signals largely colocalize and both identified the heterochromatic segment of the neo-W chromosome. *Bar* represents 10 μm (**a**–**h**) and 15 μm (**i**–**n**)

the W loss could result from asynaptic behaviour of the sex chromosomes, rarely observed in pachytene oocytes of *S. cynthia pryeri* (see Fig. 2h) that was followed by random segregation of the W and Z chromosomes. The W absence in *S. cynthia ricini* suggests a W-independent mechanism of sex determination in *S. cynthia*. Thus, dispensability of the W chromosome together with infrequent asynapsis of the sex chromosomes seems to be a plausible reason for repeated losses of the W chromosome in *S. cynthia* ssp. and possibly also for the relatively frequent W losses in the Ditrysia.

Using two methods, CGH and FISH with the W-painting probes of two types (Figs. 4 and 5), we showed that there is a considerable molecular divergence but also

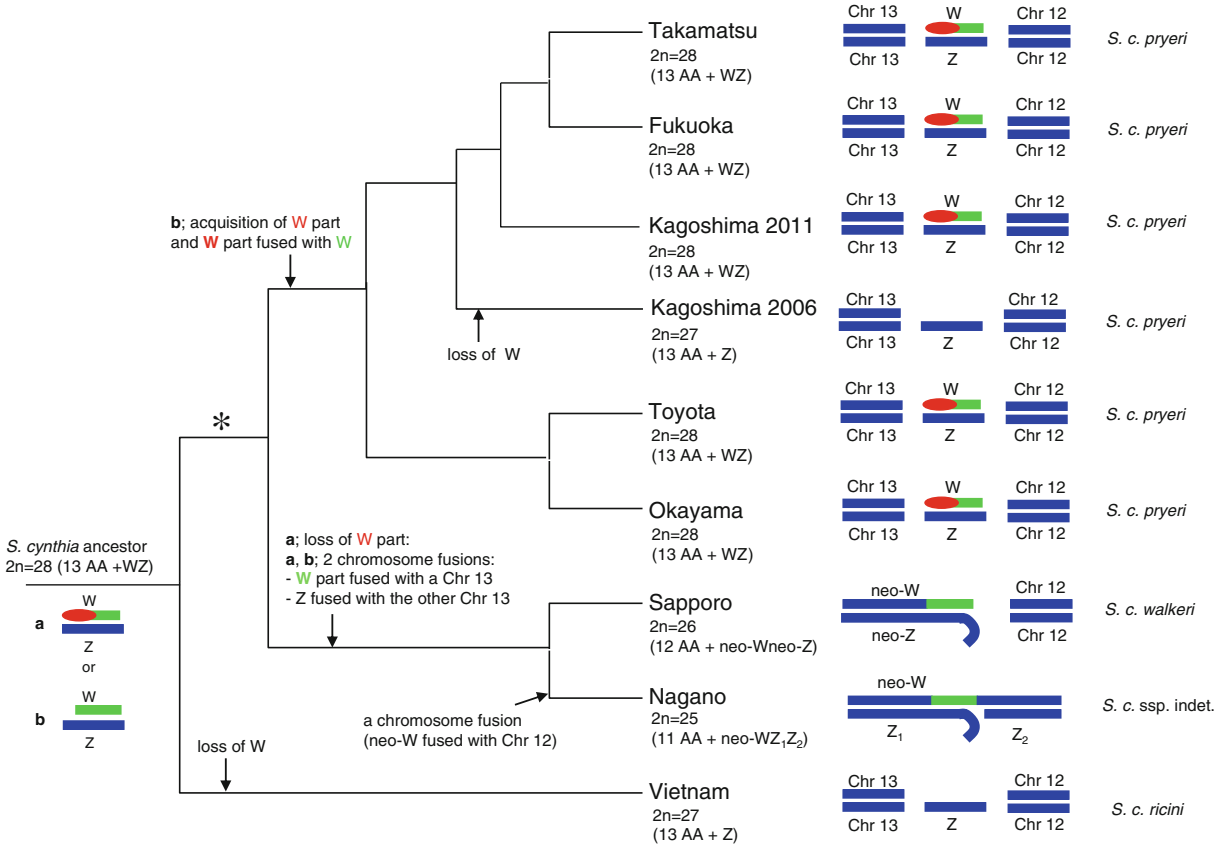


Fig. 6 Hypothetical scenarios of the sex chromosome evolution in eight geographical populations of *S. cynthia*. Chr12 and Chr13 indicate autosomes corresponding to chromosome 12 and chromosome 13 in *S. cynthia ricini*, respectively (see Yoshido et al. 2011b); *a* and *b* indicate two alternative forms of the ancestral W chromosome; *asterisk* indicates another scenario with an ancestral Z0/ZZ sex chromosome system in all *S. cynthia* ssp. and acquisition of the W chromosome of either type in a common ancestor of the Japanese populations

a region of conserved homology between the W chromosomes of *S. cynthia pryeri* group and the neo-W chromosomes of *S. cynthia walkeri* group. The results of CGH suggest that the highly heterochromatic part of the W chromosome is composed of DNA repeats occurring exclusively in females of the *S. cynthia pryeri* group. A large block of female-specific DNA was also found in the W chromosomes of two pyralid moths, *Plodia interpunctella* and *Garellia mellonella* (Vítková et al. 2007), but it did not reach the extent of preferential binding of the female genomic probe to the highly heterochromatic W part of *S. cynthia pryeri* (see Fig. 4a–e). CGH results also suggest that the euchromatin-like W part in *S. cynthia pryeri* group and the ancestral part of the neo-W chromosomes in *S. cynthia walkeri* group share homologous DNA sequences that are common in the other chromosomes of both sexes in all *S. cynthia* ssp. (Fig. 4f-p). A similar composition of the W chromosome was reported in several lepidopteran species including *B. mori*, where the W chromosome is largely composed of transposable elements dispersed in the whole genome (Sahara et al. 2003b; Abe et al. 2005; reviewed in Marec et al. 2010). Additionally, FISH with the W-painting probes of two-types (Fig. 5) further corroborated results of CGH. This method clearly showed that the highly heterochromatic part of the *S. cynthia pryeri* W chromosome is composed of sequences absent or underrepresented in the genomes of other subspecies and that the euchromatin-like part of the *S. cynthia pryeri* W chromosome corresponds to the ancestral W part of the neo-W chromosome in *S. cynthia walkeri* group.

On the basis of conserved homology and similarity between W chromosomes and in accord with the molecular phylogenetic tree (see Fig. 1b), we propose

three hypothetical scenarios for the sex chromosome evolution in *S. cynthia* subspecies and/or populations (Fig. 6). According to the first scenario, the W chromosome in *S. cynthia pryeri* group represents an ancestral state (Fig. 6a). This scenario is supported by the diploid chromosome number ($2n=28$) and the standard sex chromosome constitution (WZ) in *S. cynthia pryeri* females. Then the karyotypes of *S. cynthia ricini* and *S. cynthia pryeri* (some individuals of Kagoshima population) females could be easily explained by the secondary loss of the W chromosome. Further, the sex chromosome–autosome fusion in the *S. cynthia walkeri* group would be accompanied with deletion of the highly heterochromatic part of the *S. cynthia pryeri* W chromosome. The second scenario is based on the assumption that the W chromosome of a common ancestor of *S. cynthia* subspecies was composed only of a part corresponding to the euchromatin-like part of the W chromosome in the *S. cynthia pryeri* group (Fig. 6b). In this case, the ancestral W chromosome (a) would fuse with an autosome(s) resulting in neo-W chromosomes as found in the *S. cynthia walkeri* group or (b) would acquire the highly heterochromatic part as found in the *S. cynthia pryeri* group. Although the considerable sequence homology of the ancestral W part with the corresponding parts of the W chromosomes in all *S. cynthia* ssp. seems to favour this scenario, the unclear origin of the highly heterochromatic W part in the *S. cynthia pryeri* group represents a weak point. Theoretically, this part could have arisen by accumulation of repetitive elements occurring scattered in the genome or could have originated by fusion with a B chromosome. Thirdly, there is another possible scenario that a common ancestor of *S. cynthia* ssp. had a Z0/ZZ sex chromosome system. In this case, the W chromosome of either a or b type in Fig. 6 would arise in a common ancestor of the Japanese populations of *S. cynthia* (see branch marked with *asterisk* in Fig. 6). Then a common ancestor of all *S. cynthia* ssp. would have a karyotype of $2n=27$ as *S. cynthia ricini*, and the Japanese populations would further evolve following either the first or second scenarios. However, since we lack supporting arguments for any of these scenarios, the ancestral state of the karyotype in *S. cynthia* ssp. remains to be clarified.

Theories on the evolution of sex chromosomes are largely based on results obtained in organisms with male heterogamety (e.g. Charlesworth et al. 2005; Bachtrog 2006), but a little is known how these theories apply to organisms with female heterogamety such as birds (Ellegren 2011) or moths and butterflies (Sahara et al. 2012). In this work, we showed dynamic changes of the sex chromosome constitution, including the W chromosome loss on the one hand and the formation of neo-sex chromosomes on the other hand, within one species complex, *S. cynthia* ssp. Similar changes in the sex chromosome constitution were found also in organisms with male heterogamety, but within the whole genus such as *Drosophila* (Kaiser and Bachtrog 2010) or spiny rats, *Tokudaia* (Murata et al. 2012). Thus, geographical populations/subspecies of *S. cynthia* represent a unique model for the study of sex chromosome evolution. A comparative sequence analysis of sex chromosomes in *S. cynthia* ssp. could significantly contribute to understanding the early steps of molecular differentiation between the neo-W and neo-Z chromosomes or to clarifying the role of the highly differentiated W chromosomes. In addition, sex chromosomes appear to play a disproportionally large role in speciation (Presgraves 2008; Qvarnström and Bailey 2009). Recently, Kitano et al. (2009) showed that neo-sex chromosomes contribute to reproductive isolation between closely related species of the stickleback fishes. Moreover, there is growing evidence that not only X chromosomes but also Y chromosomes can contribute to the formation of reproductive barriers as found, for example, in crosses between mice species (Tucker et al. 1992; Campbell et al. 2012), European rabbit subspecies (Geraldes et al. 2008) and *Drosophila* species (Llopart et al. 2005; Ferree and Barbash 2009; Sweigart 2010). In view of these findings, further research on sex chromosomes in *S. cynthia* ssp. could help us to elucidate the role of sex chromosome constitution in reproductive isolation and speciation of organisms with female heterogamety.

Acknowledgments We thank Y. Yamada (Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan) for enabling us to rear *S. cynthia* subspecies on *Ailanthus altissima* trees. Our thanks also go to Y. Banno, Y. Sakamaki, T. Mano, K. Yamagishi, I. Miura and H. Nagata for providing the specimens of *S. cynthia* populations. This work was funded by grants 19–1114 (A.Y.), 23380030 (K.S.) and a research fellowship 21–7147 (K.S.) from the Japan Society for the Promotion of Science. Preparation of W chromosome painting probes by laser microdissection was financed by Grant Agency of the Academy of Sciences of the Czech Republic, grant IAA600960925 (J.Š. and F.M.). S.K. acknowledges support from Ministry of Agriculture of the Czech Republic (project MZE 0002716202). J.Š. received additional support from the grant GAJU 137/2010/P of the Grant Agency of the University of South Bohemia, České Budějovice, Czech Republic.

References

Abe H, Sugasaki T, Terada T et al (2002) Nested retrotransposons on the W chromosome of the wild silkworm *Bombyx mandarina*. Insect Mol Biol 11:307–314

Abe H, Mita K, Yasukochi Y, Oshiki T, Shimada T (2005) Retrotransposable elements on the W chromosome of the silkworm, *Bombyx mori*. Cytogenet Genome Res 110:144–151

Bachtrog D (2005) Sex chromosome evolution: molecular aspects of Y-chromosome degeneration in *Drosophila*. Genome Res 15:1393–1401

Bachtrog D (2006) A dynamic view of sex chromosome evolution. Curr Opin Genet Dev 16:578–585

Bachtrog D, Hom E, Wong KM, Maside X, de Jong P (2008) Genomic degradation of a young Y chromosome in *Drosophila miranda*. Genome Biol 9:R30

Campbell P, Good JM, Dean MD, Tucker PK, Nachman MW (2012) The contribution of the Y chromosome to hybrid male sterility in house mice. Genetics 191:1271–1281

Carvalho AB (2002) Origin and evolution of the *Drosophila* Y chromosome. Curr Opin Genet Dev 12:664–668

Carvalho AB, Clark AG (2005) Y chromosome of *D. pseudoobscura* is not homologous to the ancestral *Drosophila* Y. Science 307:108–110

Carvalho AB, Koerich LB, Clark AG (2009) Origin and evolution of Y chromosomes: *Drosophila* tales. Trends Genet 25:270–277

Charlesworth D, Charlesworth B, Marais G (2005) Steps in the evolution of heteromorphic sex chromosomes. Heredity 95:118–128

Drosopoulou E, Nakou I, Šíchová J, Kubíčková S, Marec F, Mavragani-Tsipidou P (2012) Sex chromosomes and associated rDNA form a heterochromatic network in the polytene nuclei of *Bactrocera oleae* (Diptera: Tephritidae). Genetica 140:169–180

Ellegren H (2011) Sex-chromosome evolution: recent progress and the influence of male and female heterogamety. Nat Rev Genet 12:157–166

Ferree PM, Barbash DA (2009) Species-specific heterochromatin prevents mitotic chromosome segregation to cause hybrid lethality in *Drosophila*. PLoS Biol 7:e1000234

Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. Genome 48:1083–1092

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

Geraldes A, Basset P, Gibson B et al (2008) Inferring the history of speciation in house mice from autosomal, X-linked, Y-linked and mitochondrial genes. Mol Ecol 17:5349–5363

Graves JAM (2005) Recycling the Y chromosome. Science 307:50–51

Hackstein JHP, Hochstenbach R, Hauschteck-Jungen E, Beukeboom LW (1996) Is the Y chromosome of *Drosophila* an evolved supernumerary chromosome? Bioessays 18:317–323

Kaiser VB, Bachtrog D (2010) Evolution of sex chromosomes in insects. Annu Rev Genet 44:91–112

Kawaguchi E (1937) Chromosomenketten beim Rassenbastarde des Seidenspinners *Philosamia cynthia*. (Zytologische Untersuchungen am Seidenspinner und seinen Verwandten. III.) Cytologia Fujii II (8):1023–1033

Kitano J, Ross JA, Mori S et al (2009) A role for a neo-sex chromosome in stickleback speciation. Nature 461:1079–1083

Kubickova S, Cernohorska H, Musilova P, Rubes J (2002) The use of laser microdissection for the preparation of chromosome-specific painting probes in farm animals. Chromosome Res 10:571–577

Llopart A, Lachaise D, Coyne JA (2005) Multilocus analysis of introgression between two sympatric sister species of *Drosophila*: *Drosophila yakuba* and *D. santomea*. Genetics 171:197–210

Lukhtanov VA (2000) Sex chromatin and sex chromosome systems in nonditrysian Lepidoptera (Insecta). J Zool Syst Evol Res 38:73–79

Marec F, Novák K (1998) Absence of sex chromatin corresponds with a sex-chromosome univalent in females of Trichoptera. Eur J Entomol 95:197–209

Marec F, Sahara K, Traut W (2010) Rise and fall of the W chromosome in Lepidoptera. In: Goldsmith MR, Marec F (eds) Molecular biology and genetics of the Lepidoptera. CRC, Boca Raton, pp 49–63

Matsubara K, Tarui H, Toriba M et al (2006) Evidence for different origin of sex chromosomes in snakes, bird, and mammals and step-wise differentiation of snake sex chromosomes. Proc Natl Acad Sci USA 103:18190–18195

Mediouni J, Fuková I, Frydrychová R, Dhouibi MH, Marec F (2004) Karyotype, sex chromatin and sex chromosome differentiation in the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). Caryologia 57:184–194

Murata C, Yamada F, Kawauchi N, Matsuda Y, Kuroiwa A (2012) The Y chromosome of the Okinawa spiny rat, *Tokudaia muenninki*, was rescued through fusion with an autosome. Chromosome Res 20:111–125

Nanda I, Shan Z, Schartl M et al (1999) 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet 21:258–259

Nilsson N-O, Löfstedt C, Dävring L (1988) Unusual sex chromosome inheritance in six species of small ermine moths (*Yponomeuta*, Yponomeutidae, Lepidoptera). Hereditas 108:259–265

Ohno S (1967) Sex chromosomes and sex-linked genes. Springer, Berlin

Peigler RS, Naumann S (2003) A revision of the silkmoth genus *Samia*. University of the Incarnate Word, San Antonio

Presgraves DC (2008) Sex chromosomes and speciation in *Drosophila*. Trends Genet 24:336–343

Qvarnström A, Bailey RI (2009) Speciation through evolution of sex-linked genes. Heredity 102:4–15

Rishi S, Sahni G, Rishi KK (1999) Inheritance of unusual sex chromosome evidenced by $AA^{W}Z$ trivalent in *Trabala vishnu* (Lasiocampidae, Lepidoptera). Cytobios 100:85–94

Sahara K, Marec F, Eickhoff U, Traut W (2003a) Moth sex chromatin probed by comparative genomic hybridization (CGH). Genome 46:339–342

Sahara K, Yoshido A, Kawamura N et al (2003b) W-derived BAC probes as a new tool for identification of the W

chromosome and its aberrations in *Bombyx mori*. Chromosoma 112:48–55

Sahara K, Yoshido A, Traut W (2012) Sex chromosome evolution in moths and butterflies. Chromosome Res 20:83–94

Suomalainen E (1969) On the sex chromosome trivalent in some Lepidoptera females. Chromosoma 28:298–308

Sweigart AL (2010) Simple *Y*-autosomal incompatibilities cause hybrid male sterility in reciprocal cross between *Drosophila virilis* and *D. americana*. Genetics 184:779–787

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Traut W (1999) The evolution of sex chromosomes in insects: differentiation of sex chromosomes in flies and moths. Eur J Entomol 96:227–235

Traut W, Marec F (1996) Sex chromatin in Lepidoptera. Q Rev Biol 71:239–256

Traut W, Marec F (1997) Sex chromosome differentiation in some species of Lepidoptera (Insecta). Chromosome Res 5:283–291

Traut W, Sahara K, Otto TD, Marec F (1999) Molecular differentiation of sex chromosome probed by comparative genomic hybridization. Chromosoma 108:173–180

Traut W, Sahara K, Marec F (2007) Sex chromosomes and sex determination in Lepidoptera. Sex Dev 1:332–346

Tucker PK, Sage RD, Warner J, Wilson AG, Eicher EM (1992) Abrupt cline for sex-chromosomes in a hybrid zone between 2 species of mice. Evolution 46:1146–1163

Van't Hof AE, Marec F, Saccheri IJ, Brakefield PM, Zwaan BJ (2008) Cytogenetic characterization and AFLP-based genetic linkage mapping for the butterfly *Bicyclus anynana*, covering all 28 karyotyped chromosomes. PLoS One 3:e3882

Vítková M, Fuková I, Kubíčková S, Marec F (2007) Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). Chromosome Res 15:917–930

Yoshido A, Bando H, Yasukochi Y, Sahara K (2005a) The *Bombyx mori* karyotype and the assignment of linkage groups. Genetics 170:675–685

Yoshido A, Marec F, Sahara K (2005b) Resolution of sex chromosome constitution by genomic in situ hybridization and fluorescence in situ hybridization with $(TTAGG)_n$ telomeric probe in some species of Lepidoptera. Chromosoma 114:193–202

Yoshido A, Yamada Y, Sahara K (2006) The W chromosome detection in several lepidopteran species by genomic *in situ* hybridization (GISH). J Insect Biotech Sericol 75:147–151

Yoshido A, Yasukochi Y, Marec F, Abe H, Sahara K (2007) FISH analysis of the W chromosome in *Bombyx mandarina* and several other species of Lepidoptera by means of *B. mori* W-BAC probes. J Insect Biotech Sericol 76:1–7

Yoshido A, Sahara K, Marec F, Matsuda Y (2011a) Step-by-step evolution of neo-sex chromosomes in geographical populations of wild silkmoths, *Samia cynthia* ssp. Heredity 106:614–624

Yoshido A, Yasukochi Y, Sahara K (2011b) *Samia cynthia* versus *Bombyx mori*: comparative gene mapping between a species with a low-number karyotype and the model species of Lepidoptera. Insect Biochem Mol Biol 41:370–377