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Resolution of sex chromosome constitution by GISH and telomere-FISH in some species of Lepidoptera

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Abstract

We have developed a simple method to resolve the sex chromosome constitution in females of Lepidoptera by using combination of genomic in situ hybridization (GISH) and fluorescence in situ hybridization with $(TTAGG)_n$ telomeric probe (telomere-FISH). In pachytene configurations of sex chromosomes, GISH differentiated W heterochromatin and telomere-FISH detected the chromosome ends. With this method we showed that *Antheraea yamamai* has a standard system with a fully differentiated W-Z sex chromosome pair. In *Orgyia antiqua*, we confirmed the presence of neo-W and neo-Z chromosomes, which most probably originated by fusion of the ancestral W and Z with an autosome pair. In contrast to earlier data, *O. thyellina* females displayed a neo-ZW_1W_2 sex chromosome constitution. A neo-WZ_1Z_2 trivalent was found in females of *Samia cynthia* ssp. indet., originating from a population in Nagano, Japan. Whereas another subspecies collected in Sapporo, Japan, and determined as *S. c. walkeri*, showed a neo-W/neo-Z bivalent similar to *O. antiqua*, and the subspecies *S. c. ricini* showed a Z univalent (a Z/ZZ system). The combination of GISH and telomere-FISH enabled us to acquire not only reliable information about sex chromosome constitution but also an insight into sex chromosome evolution in Lepidoptera.

Introduction

Sex chromosomes are designated X and Y in systems with male heterogamety (e.g., mammals, flies, etc.) and W and Z in those with female heterogamety (e.g., birds, reptiles, moths and butterflies). In both systems, derived sex chromosome constitutions occur, which either lack the Y or W, or possess multiple sex chromosomes. Multiple sex chromosomes could arise when an ancestral sex chromosome fused with (or was translocated onto) an autosome, forming a neo-sex chromosome (e.g., neo-Y), while the other sex chromosome did not. Then the remaining sex chromosome and the autosome homologue became multiple sex chromosomes (e.g., X_1 and X_2, respectively). This scenario of the origin of multiple sex chromosomes appears common in the animal kingdom (e.g., Toder et al. 1997; Schmid et al. 2003; Dobigny et al. 2004; Jacobs 2004). Nevertheless, comparative studies of sex chromosome constitution in related species may uncover other scenarios and thus, extend our knowledge on the evolution of sex chromosomes (Graves 1998; Ayling and Griffin 2002).

In species with heteromorphic sex chromosomes, X and Y or W and Z can be distinguished by the chromosome size or specific banding/staining pattern. However, in species with multiple sex chromosomes, it is sometimes difficult to disclose the actual sex chromosome constitution. Fluorescence in situ hybridization (FISH) can help to identify individual sex chromosomes, if suitable molecular probes are available. Chromosome painting probes prepared by flow sorting or microdissection (Guan et al. 1994; Chowdhary et al. 1998) represent a very useful tool for the analysis of sex chromosome constitution (Toder et al. 1997; Shibata et al. 1999; Rens et al. 2004; Grützner et al. 2004). However, if adequate molecular markers of sex chromosomes are not available, one must conventionally compare the number of chromosomes between males and females, and analyze chromosome pairing,

the synaptonemal complex formation and/or chiasma formation (Wahrman et al. 1983; Jacobs 2004).

Moths and butterflies (Lepidoptera) possess a WZ/ZZ (female/male) sex chromosome system or its numerical variations such as Z/ZZ, W_1W_2Z/ZZ, and $WZ_1Z_2/Z_1Z_1Z_2Z_2$ (Suomalainen 1969a; Robinson 1971; Nilsson et al. 1988; Traut and Marec 1996, 1997; Rishi et al. 1999; Traut 1999). In some species, sex chromosome differentiation can be studied by using a pachytene mapping technique (Traut and Marec 1997). In preparations of pachytene oocytes, this simple method makes possible identification of sex chromosome bivalent (or trivalent) according to the W chromosome heterochromatin but fails if the W heterochromatin is not seen as, for example, in the carob moth, *Ectomyelois ceratoniae* (Mediouni et al. 2004). In addition, lepidopteran sex chromosomes pair during female meiosis and, though largely non-homologous, form a synaptonemal complex (SC), which is often not discernable from autosome SCs due to a complete synaptic adjustment of chromosomes involved in the SC (Marec and Traut 1994). In some species such as the ruby tiger moth, *Phragmatobia fuliginosa*, the formation of the sex chromosome SC makes it difficult to distinguish the W_1W_2Z trivalent from a bivalent (Traut and Marec 1997).

Identification of chromosomes in Lepidoptera had been problematic due to numerous chromosomes, which are uniform in size and shape, and lack primary constrictions (review: Wolf 1996), and due to the absence of convenient banding techniques. Recently, BAC-FISH resolved this difficulty in the silkworm (Sahara et al. 2003b; Yoshido et al. 2005). With respect to the sex chromosome, comparative genomic hybridization (CGH) was alternatively employed to study differentiation of sex chromosomes (Traut et al. 1999; Sahara et al. 2003a). CGH can identify the Y or W chromosomes even in species with homomorphic sex chromosomes, if there are sufficient differences in their gross molecular composition. A similar but simpler genomic in situ hybridization (GISH) can also be used to detect the Y or

W sex chromosomes (Traut et al. 1999; Sahara et al. 2003b). In contrast to FISH with chromosome-specific painting probes, CGH and GISH use genomic DNA probes and thus, are universally applicable for sex chromosome identification.

In more complicated meiotic configurations of multiple sex chromosomes, the location of chromosome ends, telomeres, can help to resolve the sex chromosome constitution. The telomeres of lepidopteran chromosomes consists of a long array of short tandem repeats, $(TTAGG)_n$ (Okazaki et al. 1993; Sahara et al. 1999). This telomere structure is conserved in most insect orders with some exceptions (Frydrychová et al. 2004). Telomeric probes can be easily generated by a non-template PCR method (Ijdo et al. 1991) and used for FISH (Sahara et al. 1999; Frydrychová et al. 2004; Weiss-Schneeweiss et al. 2004).

In this paper, we present a new tool for the study of sex chromosome constitution in females of Lepidoptera, which is based on the combination of GISH and FISH with the $(TTAGG)_n$ telomeric probe (telomere-FISH). We demonstrate the usefulness of this combined method for the study of sex chromosomes in the Japanese oak silkworm (*Antheraea yamamai*), three subspecies of cynthia silkmoths (the eri-silkworm, *Samia cynthia ricini*, the Shinju-silkworm, *S. c.* ssp. indet., and the ailanthus silkworm, *S. c. walkeri*), and two *Orgyia* species, the vapourer moth (*O. antiqua*) and the white-spotted tussock moth (*O. thyellina*). Results achieved suggest that the sex chromosome constitution in Lepidoptera is more variable than previously assumed and provide evidence of sex chromosome divergence in geographical populations and between related species.

Materials and methods

Insects

Antheraea yamamai (Guérin-Méneville) (Saturniidae) larvae were from a culture kept in the laboratory of K.S. The culture was provided to the Hokkaido University by the Sericultural Institute of Nagano prefecture some 25 years ago, where it was established more than 200 years ago from a sample of natural population collected in Nagano, Japan. In *Samia cynthia* (Drury) (Saturniidae), we used larvae originating from three different subspecies/populations. (i) A laboratory culture *Samia cynthia* ssp. was obtained from specimens collected by H. Saito (Kyoto, Japan) in Nagano in the 1990s. The specimens were originally identified as *S. c. pryeri* (Butler) but here we avoid to classify them in subspecies due to their chromosome number, which differed from *S. c. pryeri* (see Discussion), and further refer to *S. c.* ssp. indet. (ii) A population collected in Sapporo, Japan, in 2003, which was identified as *Samia cynthia walkeri* (Felder & Felder). (iii) A laboratory culture of *Samia cynthia ricini* (Donovan), originating from a Vietnam population, was kindly provided by T. Shimada (Tokyo, Japan). Specimens of *Orgyia thyellina* Butler (Lymantriidae) were collected in Naganuma, Hokkaido, Japan, in 2000. *Orgyia antiqua* (L.) specimens were from a culture kept in the laboratory of F.M. (for its origin, see Rego and Marec 2003).

Chromosome preparations

Spread preparations of pachytene oocytes were made as described in Sahara et al. (1999). Briefly, ovaries of last instar larvae were dissected in a saline solution, swollen for 10 min in a hypotonic solution (83mM KCl and 17mM NaCl; Marec and Traut 1994) and fixed for 15-30 min in Carnoy's (ethanol, chloroform, acetic acid, 6:3:1). Cells were dissociated in 60% acetic

acid and spread on a heating plate at 50°C. Then preparations were passed through a graded ethanol series (70%, 80%, 98%) and stored in the freezer until use.

Fluorescence in situ hybridization (FISH)

In each species, the whole genomic DNA was extracted separately from females and males according to the standard procedure (Blin and Stafford 1976). The female DNA was labelled by nick translation using a Nick Translation System (Invitrogen, Tokyo, Japan) either with Cy3-dCTP (Amersham, Tokyo, Japan) or Fluorescein-12-dCTP (PerkinElmer, Boston, MA). $(TTAGG)_n$ telomeric probes were generated by non-template polymerase chain reaction (PCR) method of Ijdo et al. (1991), following the protocol of Sahara et al. (1999) with one modification: the probes were directly labelled with Cy3-dCTP during PCR amplification.

Genomic in situ hybridization (GISH) was carried out using the procedure given in Sahara et al (2003b). After removal from the freezer, chromosome preparations were passed through an ethanol series and air-dried. Denaturation was done at 72°C for 3 to 4 min in 70% formamide, 2 x SSC. For one preparation, the probe cocktail for GISH alone contained 500 ng of Cy3-labelled female DNA (red), 25 μg of sonicated salmon sperm (Sigma-Aldrich, Tokyo, Japan) DNA and 3 μg of unlabelled sonicated male genomic DNA in 10 μl of hybridization solution (50% formamide, 10% dextran sulfate, 2 x SSC). The probe cocktail for the combination of telomere-FISH and GISH contained 500 ng of fluorescein-labelled female DNA (green), 100 ng of Cy3-labelled telomeric probe (red), 25 μg of sonicated salmon sperm DNA and 3 μg of unlabelled sonicated male genomic DNA in 10 μl of hybridization solution. After hybridization in moist chamber at 37°C for 3 days, slides were washed at 62°C in 0.1 x SSC containing 1% Triton X-100. The slides were then counterstained and mounted in antifade based on DABCO (for composition, see Traut et al. 1999) containing 0.5 μg/ml of DAPI (Sigma-Aldrich, Tokyo, Japan). Preparations were observed in a Leica DMRE HC

fluorescence microscope. Digital images were acquired and processed as described in Sahara et al. (2003b).

Results

Sex chromosome constitution in *Antheraea yamamai*

In A. *yamamai*, we observed 31 bivalents in female pachytene complements (Fig. 1a) and 62 chromosomes in mitotic metaphases (not shown). The numbers are consistent with the diploid chromosome number of 2n=62 given in Kawaguchi (1934). The WZ bivalent was easily distinguished by deep staining of the W-chromosome thread with the Cy3-labelled female genomic probe (Fig. 1a). Besides the WZ, two bivalents carrying the nucleolar organizer region (NOR) were identified according to spherical nucleoli weakly stained with DAPI (Fig. 1a). In addition to the W chromatin, the female genomic probe highlighted an interstitial heterochromatic segment in one of the NOR bivalents (Fig. 1a, arrowhead). With the combination of GISH and telomere-FISH, the genomic probe painted the entire W chromosome and telomeric probe detected exclusively both ends of the WZ bivalent (Fig. 1b). Therefore, we concluded that *A. yamamai* has a standard sex chromosome system with fully differentiated W and Z sex chromosomes.

Sex chromosome constitution in *Orgyia thyellina* and *O. antiqua*

Orgyia thyellina showed 11 bivalents in each pachytene oocyte. GISH signals were observed in the middle part of a long bivalent, which also carried an NOR (Fig. 1c). The strong hybridization signals suggested that the labelled chromosome represents the W. Up to here, our observation was consistent with the earlier reports (Traut and Clarke 1996; Traut and Marec 1997). However, the position of hybridization signals of the telomeric probe indicated that a very long neo-Z chromosome pairs with two W chromosomes, W_1 (Fig. 1d, arrow) and W_2 (Fig. 1d, arrowhead). The W_1 chromosome appeared shorter than W_2, but contained most of the W heterochromatin. Whereas the longer W_2 chromosome carried a shorter piece of the

W heterochromatin and also the NOR, like the corresponding region of the Z chromosome. Mitotic oogonia showed an odd chromosome number (2n=23). In accordance with our finding in the pachytene stage, two chromosomes with GISH signals were observed, the W_1 strongly highlighted with the female genomic probe (Fig. 1e, arrow) and the W_2 with a small cluster of GISH signals at one end (Fig. 1e, arrowhead). Based on the above results we concluded that *O. thyellina* has a neo-ZW_1W_2 sex chromosome system.

O. antiqua has the diploid chromosome number of 2n=28 (Traut and Clarke 1997; Traut and Marec 1997). Accordingly, pachytene oocytes showed 13 autosome bivalents and a neo-W/neo-Z sex chromosome bivalent (Fig. 1f). In the sex chromosome bivalent, GISH identified a distal segment of the neo-W chromosome, representing about one third of the chromosome length (Fig. 1f, g). In contrast to *O. thyellina*, the neo-W/neo-Z was a regular bivalent with twin telomeric signals exclusively at the ends and did not involve the NOR (Fig. 1g). In mitotic chromosomes of oogonial cells, GISH also differentiated a distal segment of the neo-W chromosome (Fig. 1h).

Sex chromosome constitution in *Samia cynthia*

No W chromosome was found in preparations of *S. c. ricini* females (Fig. 2a, b). In oogonia, 2n=27 mitotic chromosomes were regularly observed (Fig. 2b). Post-pachytene oocyte complements consisted of 13 autosomal bivalents and a Z univalent (Fig. 2a, arrow). Stronger hybridization signals of the female genomic probe were observed in telomeric regions of most chromosomes and in the periphery of NOR in the bivalent associated with nucleolus (Fig. 2a, arrowheads). Accordingly, two NOR-carrying autosomes displayed strong GISH signals (Fig. 2b, arrowheads). The results suggest a Z/ZZ sex chromosome system in *S. c. ricini*.

In female pachytenes of *S. c*. ssp. indet., 12 bivalents were observed. GISH signals highlighted a short interstitial segment of one chromosome of the longest bivalent, which

formed a loop-like structure in the middle part (Fig. 2c). This unclear structure indicated that the bivalent might be, in fact, a sex chromosome trivalent and GISH detected the W chromatin. Similar to *S. c. ricini*, strong GISH signals were observed in the NOR bivalent (Fig. 2c, arrowhead) and also in telomeric segments of most bivalents. In pachytene oocytes of *S. c.* ssp. indet. stained with GISH plus telomere-FISH, the telomeric probe detected six ends of the presumable sex chromosomes and thus, confirmed the presence of a sex chromosome trivalent, in which a long neo-W chromosome was paired with two Z chromosomes, Z_1 and Z_2 (Fig. 2d). One end of the Z_1 chromosome remained unpaired in the central part of the trivalent. This free end was obviously responsible for the unclear loop-like structure in some pachytene nuclei (Fig. 2c, d). Based on the results of GISH plus telomere-FISH we concluded that the sex chromosome constitution of *S. c.* ssp. indet. is neo-WZ_1Z_2. Accordingly, mitotic oogonia showed an odd chromosome number of 2n=25. The longest chromosome was identified as the W according to a strong interstitial band of GISH signals (Fig. 2e). Two other but small chromosomes with clusters of GISH signals represented probably two homologous NOR chromosomes (Fig. 2e, arrowhead). The distribution of GISH hybridization signals in mitotic metaphases was consistent with that in tetraploid nuclei of the nurse cells, where the GISH-marked chromosomes were duplicated. In these tetraploid nuclei, two W chromosomes, each consisting of two chromatids, were arranged in parallel (Fig. 2f). Interestingly, autosomes formed quartets with homologues often arranged side-by-side. Two remaining couples of middle-sized chromosomes, located close to the W chromosomes, represented most probably the duplicated Z_1 and Z_2 chromosomes. From the length comparison of both Z chromosomes in pachytene nuclei we deduced that the longer couple represented two Z_1 chromosomes (Fig. 2f, long arrow) and the shorter two Z_2 chromosomes (Fig. 2f, short arrow). The grouping of both the autosome homologues and the sex chromosomes in the tetraploid nuclei most probably results from previous pairing of the

chromosomes in pachytene oocytes, from which the nurse cells originated (Rasmussen and Holm 1982).

S. c. walkeri differed from *S. c.* ssp. indet. in both the chromosome number and sex chromosome constitution. Pachytene nuclei of *S. c. walkeri* females showed 12 pairs of autosomes plus a neo-W/neo-Z sex chromosome bivalent, in which GISH identified a distal segment of the neo-W chromosome (Fig. 2g). GISH signals were also observed in the NOR bivalent (Fig. 2g, arrowhead) and in telomeric segments of most bivalents. The bivalent structure of sex chromosomes was confirmed by the presence of four hybridization signals of the telomeric probe, two labelling the W-ends and two the Z-ends (Fig. 2h). Thus, the diploid chromosome number was 2n=26 (Fig. 2i). A telomeric signal at the distal end of GISH-labelled segment clearly showed that this segment is, in fact, a terminal part of the neo-W chromosome. Then the corresponding end of the neo-W/neo-Z bivalent is formed by an unpaired terminal part of the neo-Z chromosome which is longer than the neo-W chromosome (Fig. 2h). The terminal location of GISH signals was confirmed in mitotic chromosomes of oogonial cells (Fig. 2i) and in chromosomes of tetraploid nurse cells (Fig. 2j). Two chromosomes located close to neo-W chromosomes of tetraploid nurse cells represented most probably the neo-Z chromosomes (Fig. 2j, arrow).

Discussion

Neo-sex chromosomes, originating by autosome-sex chromosome fusion were reported in a number of animals, for example, in a marsupial, the swamp wallaby *Wallabia bicolor* (Toder et al. 1997), a frog, *Eleutherodactylus riveroi* (Schmid et al. 2003), a lizardfish, *Trachinocephalus myops* (Ueno et al. 2001), a fly, *Drosophila americana* (McAllister, 2003), a true bug, *Dundocoris nodulicarinus* (Jacobs 2004), etc. In moths and butterflies of the insect order Lepidoptera, four variants of sex chromosome systems have been described to date: (i) a WZ/ZZ (female/male) system (e.g., *Bombyx mori*; Tanaka, 1916), (ii) a W_1W_2Z/ZZ system (e.g., *Phragmatobia fuliginosa*; Seiler 1925; Traut and Marec 1997), (iii) a $WZ_1Z_2/Z_1Z_1Z_2Z_2$ system (e.g., *Yponomeuta* spp.; Nilsson et al. 1988), and (iv) a Z/ZZ system (e.g., *Talaeporia tubulosa*; Seiler 1921). In lepidopteran species with multiple sex chromosomes, it is believed that the Z chromosome in W_1W_2Z and the W in WZ_1Z_2 systems were rearranged by fusion (or translocation) between the ancestral Z or W sex chromosomes and an autosome (Suomalainen, 1969b; Traut, 1999). Consequently, the fused chromosomes are designated neo-Z and/or neo-W sex chromosomes, respectively. In some species, both the ancestral W and Z chromosomes fused each with one member of an autosome pair, resulting in a neo-W/neo-Z sex chromosome pair. This obviously happened in *Orgyia antiqua* (Traut and Marec 1997; Rego and Marec 2003; this study). On the other hand, multiple sex chromosomes, such as W_1 and W_2 or Z_1 and Z_2, could also arise from a sex-chromosome fission event (Suomalainen 1969a).

A question remains how lepidopteran species cope with segregation of multiple sex chromosomes during female meiosis I. As far as we know there are no reports on sex-ratio distortion that would indicate random segregation. Obviously, non-random segregation maintains the 1:1 sex ratio and retains the sex chromosome constitution. In the achiasmatic meiosis of females, chromosome segregation including sex chromosomes is ensured by

modified synaptonemal complexes, which mediate bivalent pairing until metaphase I and thus substitute the function of chiasmata beyond the pachytene stage (reviews: Rasmussen and Holm 1982; Marec 1996). Recent data suggest that also telomeric associations between postpachytene oocyte bivalents participate in ensuring the proper chromosome segregation (Rego and Marec 2003). Based on given information, we presume that the pachytene configuration of sex chromosome trivalents, WZ_1Z_2 or W_1W_2Z, in which W pairs with Z_1 plus Z_2 and Z pairs with W_1 plus W_2 (this study), is preserved till metaphase I resulting thus in co-segregation of Z_1Z_2 or W_1W_2, respectively. Similar co-segregation was observed in sex chromosome trivalents of radiation-induced mutants in *Ephestia kuehniella* Zeller (Marec et al. 2001).

Two species in our study, *Antheraea yamamai* and *O. antiqua*, showed a W-Z sex chromosome pair. However, the overall pattern of their sex chromosomes considerably differed. The W and Z in *A. yamamai* were fully differentiated by GISH, indicating gross differences in the DNA composition, which most probably reflects an accumulation of repetitive sequences and transposons in the W chromosome (cf. Sahara et al. 2003a). Taking into account the chromosome number of n=31, which is common in Lepidoptera (Suomalainen 1969b; Robinson 1971), it appears that *A. yamamai* has an ancestral type of sex chromosomes, typical for most species of the 'advanced' clade of Lepidoptera, the Ditrysia (see Traut and Marec 1996, 1997). Whereas the neo-W and neo-Z chromosomes of *O. antiqua* were each composed of an ancestral part and a part of autosomal origin (Traut and Marec 1997). The autosomal part of the neo-W chromosome was not composed of heterochromatin and not detected by GISH. This implies that these autosomal parts exhibit not only structural homology but also significant homology in the DNA composition. Obviously, they represent evolutionarily young parts of the sex chromosomes, which did not have sufficient time for differentiation. This evidence of young W chromosome parts maintaining homology and old

W chromosome parts being more differentiated from their former counterparts is the first comparative evidence of W chromosome degeneration in Lepidoptera, most likely due to the accumulation of transposable and repetitive elements. Thus, the non-recombining W chromosomes appear to degenerate in a similar manner as the Y chromosomes of flies and mammals, probably acting as a 'trap' (Steinemann et al. 1993) or 'graveyard' (Kjellman et al. 1995) for transposable elements (see also Traut et al. 1999). This finding is crucial for the application of theoretical models of sexual selection, reproductive isolation, and speciation (Prowell 1998; Presgraves 2002; Kirkpatrick and Hall 2004). In addition, the identification of the neo-W chromosome in *O. antiqua* demonstrates the robustness of GISH in identifying sex chromosomes, enabling insight to the evolution of sex chromosomes in Lepidoptera not before possible.

In *Orgyia thyellina*, previous studies also reported a neo-W/neo-Z sex chromosome pair (Traut and Clarke 1996, 1997; Traut and Marec 1997). Indeed, in figures of pachytene oocyte preparations presented in those studies, a very long sex chromosome bivalent is seen. Also the even number of chromosomes observed in female mitosis (2n=22) supported the bivalent constitution of sex chromosomes (Traut and Clarke 1996). However, the use of GISH plus telomere-FISH in our study revealed that the putative bivalent is, in fact, a neo-ZW_1W_2 trivalent, which forms a precise synaptonemal complex indiscernible from a bivalent with conventional staining. Accordingly, an odd chromosome number of 2n=23 was found in mitotic oogonia. Since the previous authors examined another population of *O. thyellina* (from Hirosaki, Aomori, Japan), the difference between our and previous results may reflect the actual between-population difference in the sex chromosome constitution.

Results of Traut and Clarke (1997) and Rego and Marec (2003) suggest that the low-number karyotypes of *O. antiqua* (n=14) and *O. thyellina* (n=11) evolved from high-chromosome-number karyotypes like n=30 in related species, *O. recens* and *O. ericae*

(Robinson 1971), by multiple chromosome fusions. This also regards their sex chromosomes. From the comparison of the structure of the neo-W/neo-Z bivalent in *O. antiqua* and the neo-ZW_1W_2 trivalent in *O. thyellina*, one can deduce three steps of the sex chromosome evolution (Fig. 3). At first, neo-W and neo-Z sex chromosomes, such as found in *O. antiqua*, arose from an ancestral WZ pair by fusion with a single pair of autosomes (Fig. 3b). In the next step, the neo-W and neo- Z fused each with one member of another autosome pair, carrying the NOR (Fig. 3c). We speculate that *O. thyellina* (Hirosaki population, Aomori, Japan) used in Traut and Clarke (1996) constitutes sex chromosome system indicated in Fig. 3c. In the third step, the neo-W chromosome-NOR autosome fusion was broken into two parts, but the break point was within the ancestral W-chromatin part, resulting in the W_1 and W_2 chromosomes, each carrying a piece of the original W chromatin and the W_2 carrying the NOR. This resulted in the neo-ZW_1W_2 trivalent found in this study in *O. thyellina* (Naganuma population, Hokkaido, Japan). A question remains how the broken ends acquired the telomeres. Possibly, the new telomeres could be generated from interstitial telomeric sites (ITS), representing remnants of karyotype evolution through chromosome fusion. Recently, such ITS were observed in most chromosomes of *O. antiqua* (Rego and Marec 2003).

Samia cynthia ricini displayed a Z/ZZ sex chromosome system, which occurs in 'primitive' Lepidoptera such as Micropterigidae (Traut and Marec 1997) and in the entire order Trichoptera (caddis-flies) (Marec and Novák 1998), a sister group of the Lepidoptera. Therefore, the Z/ZZ system has been suggested as an ancestral character for the entire clade Amphiesmenoptera, consisting of the two orders, Lepidoptera and Trichoptera (Traut 1999). The W chromosome probably evolved in a common ancestor of 'primitive' Tischeriina plus 'advanced' Ditrysia (Traut and Marec 1996; Lukhtanov 2000), the latter comprising majority of extant Lepidoptera including Saturniidae. Following this, the Z/ZZ in *S. c. ricini* is obviously a derived system, which arose from a WZ/ZZ system by a loss of the W

chromosome. On the other hand, the sex chromosome constitution in two other subspecies, *S. c.* ssp. indet. (Nagano population) and *S. c. walkeri* (Sapporo population), differed considerably from that in *S. c. ricini*, namely because both included a neo-W chromosome. *S. c.* ssp. indet. and *S. c. walkeri* differed in the structure of neo-W chromosomes. In *S. c.* ssp. indet., the neo-W consisted of three parts, an interstitially located heterochromatin segment, derived from the original W chromosome, and two autosome-derived terminal segments, occupying opposite ends of the neo-W. In *S. c. walkeri*, the neo-W was composed of an original W heterochromatin plus an autosome.

Based on the comparison of sex chromosome constitution and chromosome numbers in the subspecies examined here, we propose a hypothetical scheme of the sex chromosome evolution in *S. cynthia* (Fig. 4). A common ancestor had probably 2n=28 chromosomes and a WZ/ZZ sex chromosome constitution (Fig. 4a). The karyotype of *S. c. ricini* females with 2n=27 can be easily explained by the loss of W chromosome (Fig. 4b). We suppose that the neo-W/neo-Z pair in *S. c. walkeri* with 2n=26 arose by fusion of the ancestral WZ chromosomes with a pair of autosome (A_1A_1) (Fig. 4c). The neo-WZ_1Z_2 trivalent in *S. c.* ssp. indet. with 2n=25 (Fig. 4d) represents the next evolutionary step, in which the neo-W chromosome fused with another autosome (A_2). Since Kawaguchi (1937) described populations (Kyoto and Fukuoka, Japan) of *S. c. pryeri* with n=14 determined by the number of meiotic bivalents in males, the karyotype of our hypothetical ancestor could be still preserved in some extant subspecies. Moreover, Dedderer (1928) described a laboratory population of *S. cynthia* with 12 meiotic and 24 mitotic chromosomes. Owing to large variation in chromosome number and sex chromosome constitution in different subspecies and/or populations (Robinson 1971; Song et al. 1996; this study), *S. cynthia* represents an excellent model for the study of karyotype and sex chromosome evolution. Furthermore, sex chromosomes may be involved in population and species divergence (Charlesworth et al.

1987). Recently, Kirkpatrick and Hall (2004) showed using a theoretical model that sex linkage of genes can have a substantial effect on the outcome of sexual selection, which is involved in species divergence. It is also know that the lepidopteran Z chromosome plays a disproportionately large role in species divergence (Prowell 1998, but see Reinhold 1998). From this point of view, the high polymorphism of sex chromosomes in *S. cynthia* makes this species a favourable model for evolutionary studies dealing with sexual selection, population genetics, hybridization, and speciation in Lepidoptera and other organisms with WZ sex chromosome systems.

The present study demonstrates that the combination of GISH and telomere-FISH is a simple, fast and reliable method for sex chromosome identification in the heterogametic sex. The method is particularly useful for the study of sex chromosome constitution in species with multiple sex chromosomes. In addition, it provides an insight into sex chromosome evolution without laborious analysis of the synaptonemal complex formation and/or chiasma formation or preparation of specific sex chromosome painting probes. The method can be used in both the systems with female heterogamety (WZ and derived systems) and male heterogamety (XY and derived systems) providing that organisms examined have molecularly differentiated sex chromosomes and a known telomere sequence.

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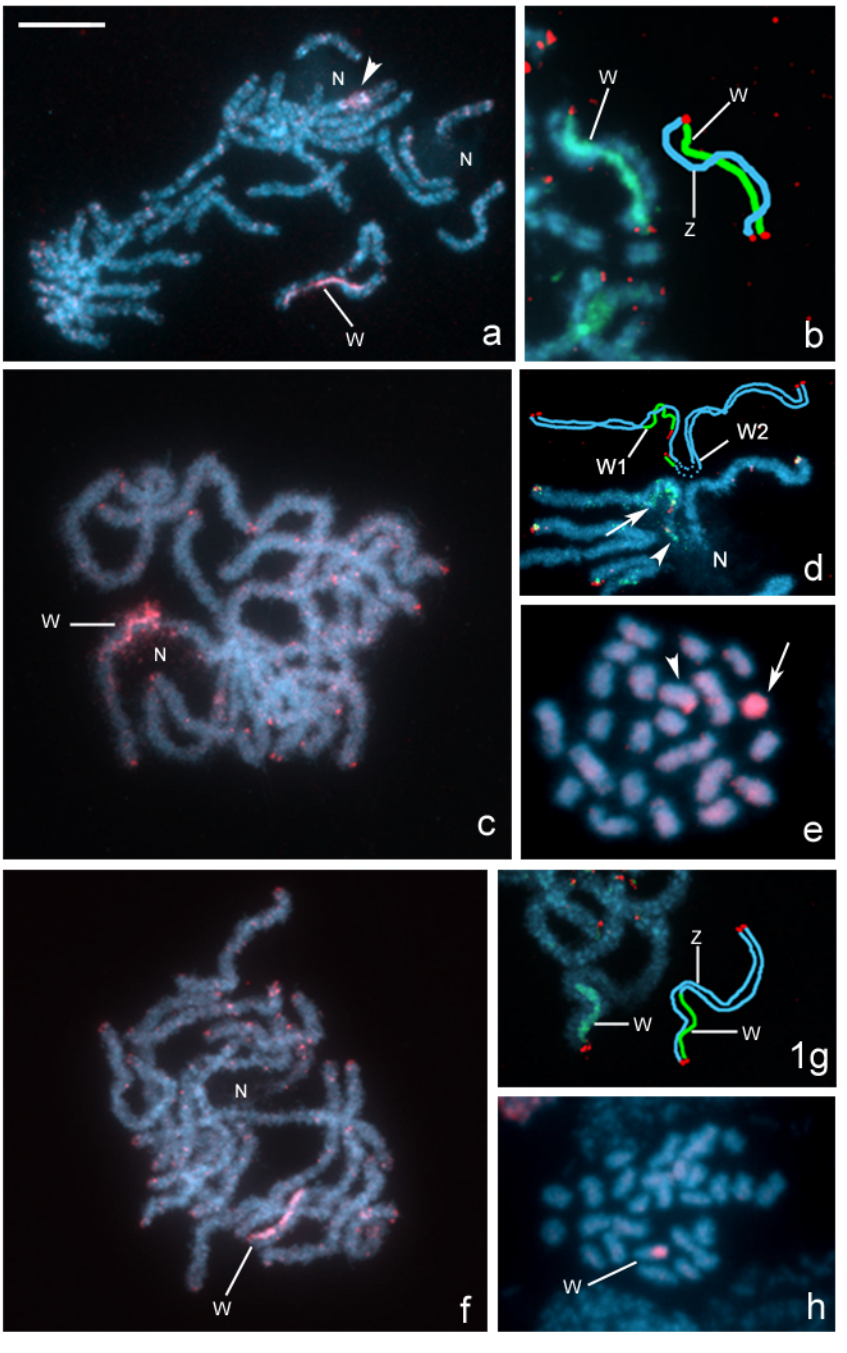
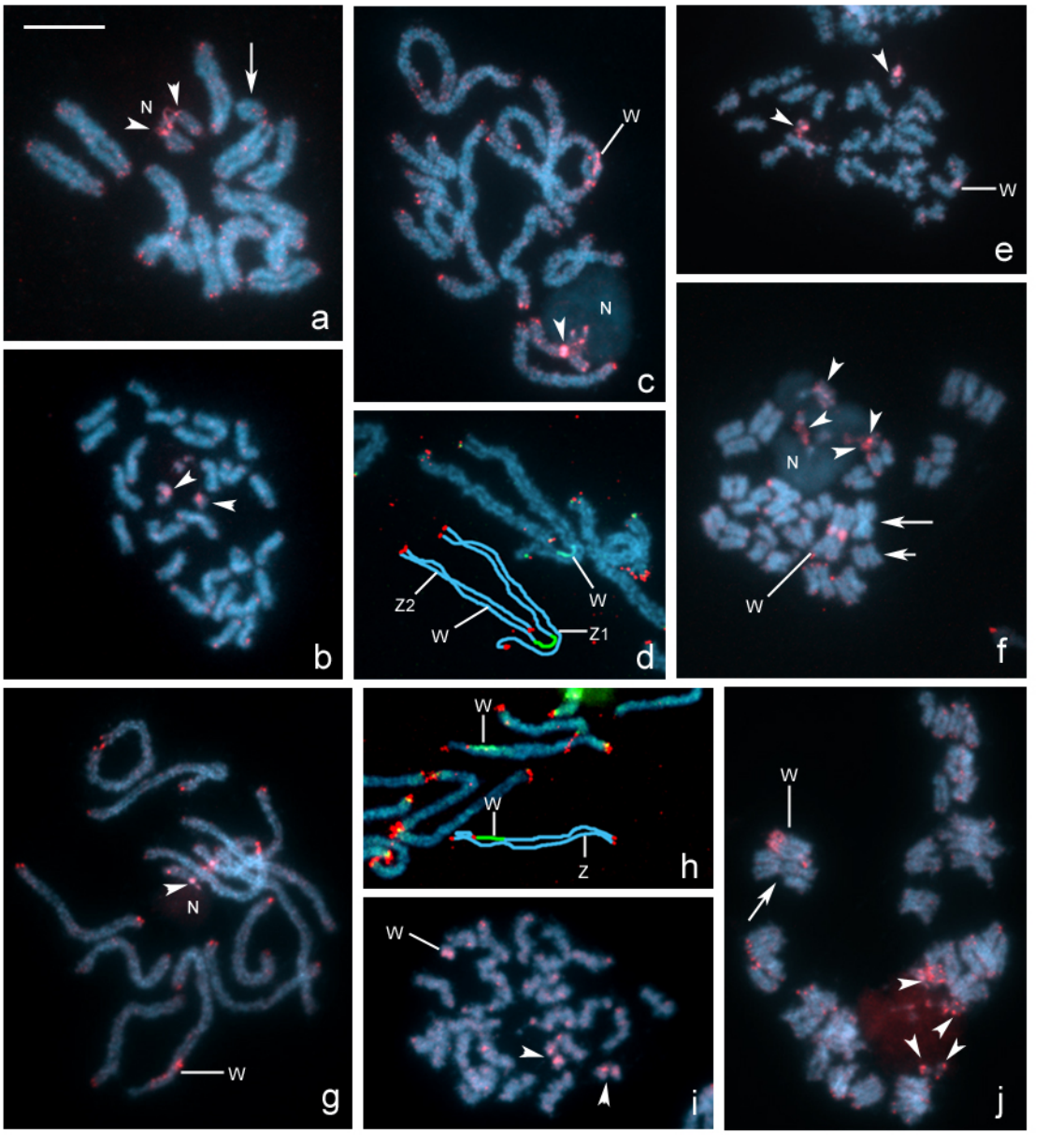


Figure 1_ Yoshido et al



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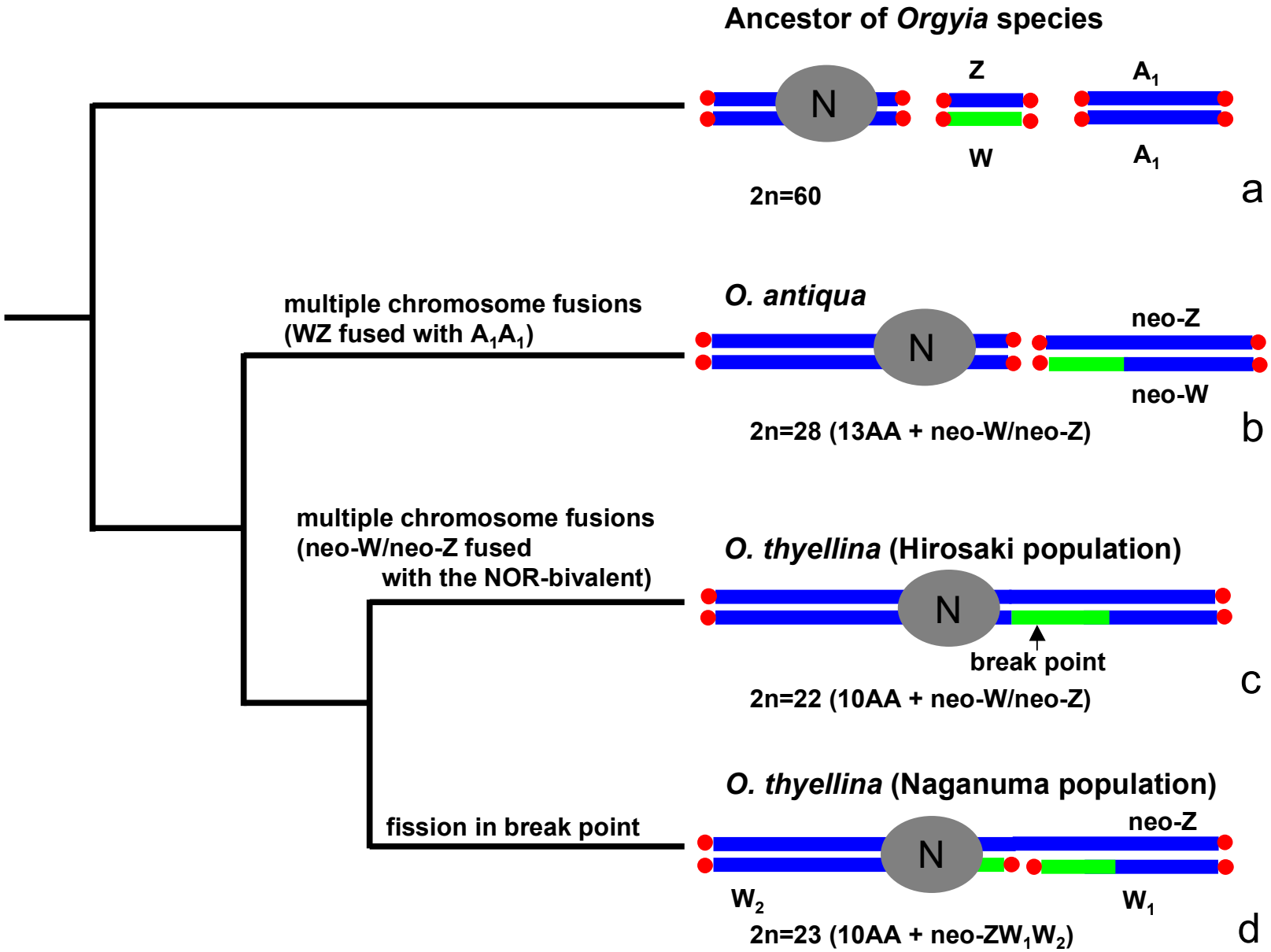

Figure 3 Yoshido et al

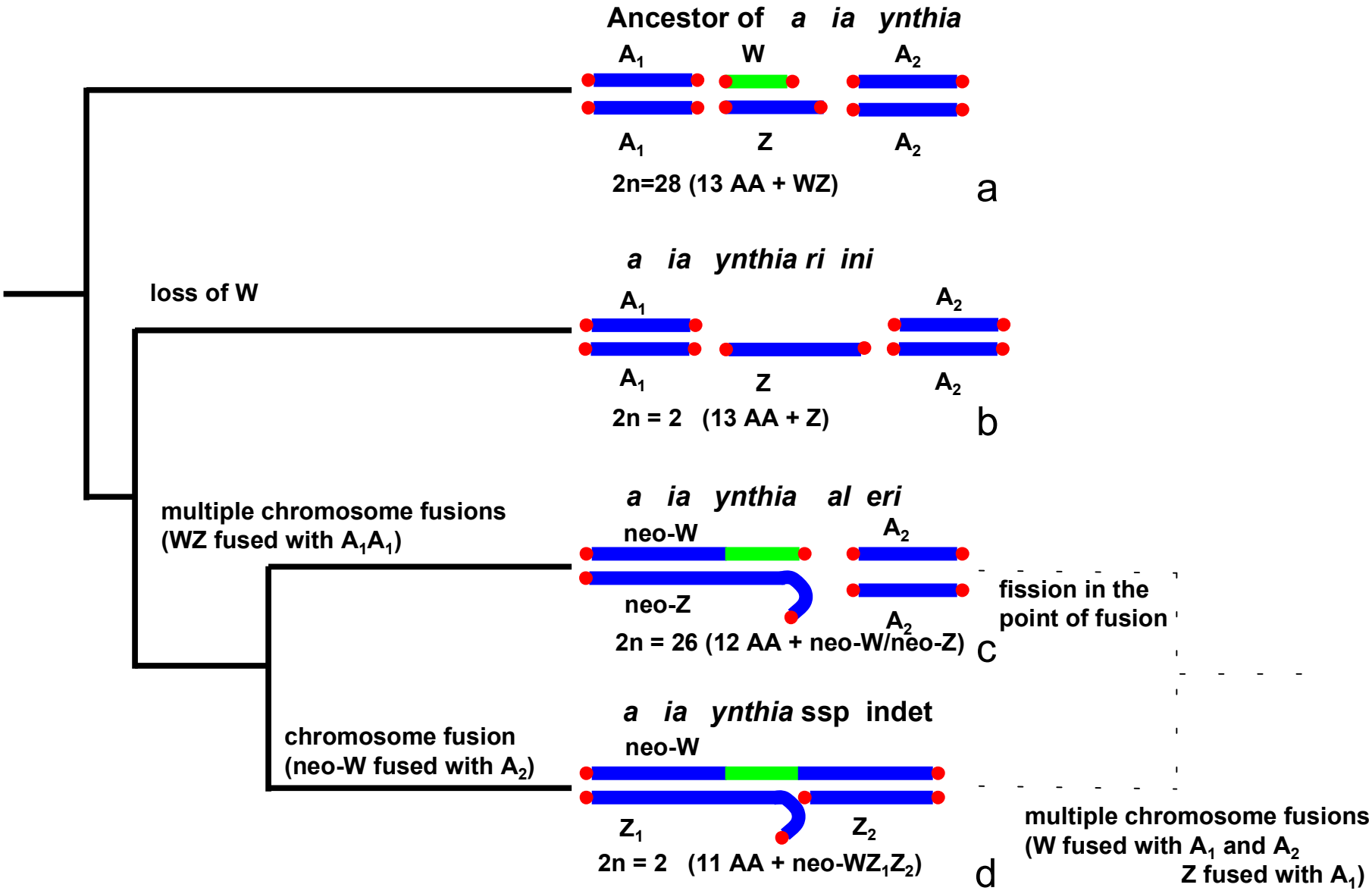

Figure 4 Yoshido et al

Figure legends

Fig. 1. Sex chromosome constitution in females of *Antheraea yamamai* (**a**, **b**), *Orgyia thyellina* (**c**, **d**, **e**), and *Orgyia antiqua* (**f**, **g**, **h**) examined by GISH (red signals, Cy3-labelled female genomic probes) in **a**, **c**, **e**, **f**, and **h** or by combination of GISH (green signals, fluorescein-labelled female genomic probes) and telomere-FISH (red signals, Cy3-labelled $(TTAGG)_n$ probes) in **b**, **d**, and **g**. Blue, chromosomes counterstained with DAPI; N, nucleolus. Drawings in **b**, **d**, and **g** show a schematic interpretation of sex chromosome configurations. **a**, pachytene complement showing a sex chromosome bivalent, identified by GISH-highlighted W chromosome thread; arrowhead indicates an autosomal heterochromatic segment of the NOR-bivalent. **b**, a WZ bivalent showing a fully differentiated W chromosome and telomeric signals at both ends. **c**, pachytene complement showing GISH-differentiated W chromatin. **d**, a part of pachytene nucleus with a neo-ZW_1W_2 trivalent, in which GISH-labelled W chromatin split into two chromosomes, W_1 (arrow) and W_2 (arrowhead), as results from the position of telomeric signals. **e**, GISH detection of W_1 (arrow) and W_2 (arrowhead) sex chromosomes in oogonial mitotic metaphase. **f**, pachytene complement showing GISH-differentiated W chromatin. **g**, a part of pachytene nucleus with a neo-W/neo-Z bivalent; GISH differentiated about one third of the neo-W chromosome; telomeric signals are located at the bivalent ends. **h**, GISH detection of the neo-W chromosome in oogonial mitotic prometaphase. Bar indicates 10 μm (**a**, **c**, **f**, **g**), 5 μm (**b, h**), and 7.7 μm (**d**, **e**).

Fig. 2. Sex chromosome constitution in females of *Samia cynthia* subspecies, *S. c. ricini* (**a**, **b**), *S. c*. ssp. indet. (**c**, **d**, **e**, **f**), and *S. c. walkeri* (**g**, **h**, **i**, **j**), examined by GISH (red signals, Cy3-labelled female genomic probes) in **a**, **b**, **c**, **e**, **f**, **g**, **i**, and **j** or by combination of GISH (green signals, fluorescein-labelled female genomic probes) and telomere-FISH (red signals, Cy3-labelled $(TTAGG)_n$ probes) in **d** and **h**. Blue, chromosomes counterstained with DAPI; N, nucleolus; arrowheads indicate autosomal heterochromatic segments in NOR chromosomes.

Drawings in **d** and **h** show a schematic interpretation of sex chromosome configurations. **a**, postpachytene complement showing 13 bivalents and a Z chromosome univalent (arrow). **b**, oogonial mitotic metaphase. **c**, pachytene complement, in which GISH identified W chromatin in an obscure bivalent forming a loop-like structure. **d**, a neo-WZ_1Z_2 pachytene trivalent; GISH differentiated a short central segment of the long neo-W chromosome; positions of telomeric signals indicate the ends of two Z chromosomes, Z_1 and Z_2. **e**, GISH detection of the neo-W chromosome in oogonial mitotic metaphase; **f**, GISH detection of two copies of the neo-W chromosome in tetraploid nurse cell chromosomes; two copies of putative Z_1 (long arrow) and Z_2 (short arrow) are seen near the neo-W chromosome pair. **g**, pachytene complement showing GISH-differentiated W chromatin. **h**, a part of pachytene nucleus with a neo-W/neo-Z bivalent; GISH differentiated a distal part of the neo-W chromosome; telomeric signals indicate the actual ends of the neo-W and neo-Z chromosomes. **i**, GISH detection of the neo-W chromosome in oogonial mitotic prometaphase; **j**, GISH detection of two copies of the neo-W chromosome in tetraploid nurse cell chromosomes; arrow indicates two copies of the putative neo-Z chromosome; Bar = 10 μm.

Fig. 3. Predicted phylogenetic relationship and hypothetical evolutionary process of sex chromosomes in *Orgyia* species. Fusion of the ancestral sex chromosome (**a**) with an autosome pair (A_1A_1) results in the neo-W/neo-Z constitution of *O. antiqua* females (**b**). Next fusion event of the neo-W/neo-Z sex chromosomes with the NOR chromosome pair generates the sex chromosome constitution of *O. thyellina* (Hirosaki population) females (**c**). Break point within the ancestral W chromatin part of the neo-W chromosome-NOR chromosome fusion generates two W chromosomes, resulting in a neo-ZW_1W_2 system in *O. thyellina* (Naganuma population) females (**d**).

Fig. 4. Predicted phylogenetic relationship and hypothetical evolutionary process of sex chromosomes in *Samia cynthia*. Loss of the W chromosome in the putative *Samia cynthia*

female ancestor (**a**) generates the karyotype and sex chromosome constitution of *S. c. ricini* females (**b**). Fusion of the ancestral sex chromosomes (**a**) with an autosome pair (A_1A_1) results in the neo-W/neo-Z constitution of *S. c. walkeri* females (**c**). Next neo-W chromosome-autosome fusion can explain a neo-WZ_1Z_2 system in *S. c.* ssp. indet. females (**d**). Alternatively, fission of the neo-W chromosome in *S. c.* ssp. indet. (**d**) leads to the sex chromosome system of *S. c. walkeri* females (**c**).