The W chromosome Detection in Several Lepidopteran Species by Genomic *in situ* Hybridization (GISH)

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(Abstract)

We newly identified the W chromosome in eight lepidopteran species and confirmed the absence of W chromosome in *Caligula japonica* females with the Z0 sex chromosome constitution by means of genomic *in situ* hybridization (GISH). In pachytene oocytes of species with the WZ/ZZ sex chromosome system, female-derived genomic probes of the respective species highlighted the whole W chromosome thread in the WZ bivalent and also stained specific autosomal region(s) with telomeric and/or subtelomeric signals. On the basis of GISH results obtained in this study and earlier published, we classified karyotypes of lepidopteran species into three types.

Key words: FISH, heterochromatin, Lepidoptera, nucleolar organizer region, pachytene chromosome, sex chromosome

INTRODUCTION

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, 1969; Nilsson *et al.*, 1988; Traut and Marec, 1996, 1997; Rishi *et al.*, 1999; Traut, 1999; Yoshido *et al.*, 2005b). Pachytene mapping in Lepidoptera can detect the W chromosome in pachytene oocytes (Traut and Marec, 1997). However, this method fails in species which the W does not form a heterochromatin thread. The problem of W chromosome identification can be overcome with the help of comparative genomic hybridization (CGH) or genomic *in situ* hybridization (GISH) providing that there is a sufficient level of molecular differentiation between the W and Z sex chromosomes (Traut *et al.*, 1999; Mediouni *et al.*, 2004; Sahara *et al.*, 2003b; Fuková *et al.*, 2005; Yoshido *et al.*, 2005b). With CGH, it is also possible to estimate gross sequence composition of the W chromosome (Sahara *et al.*, 2003a).

In Lepidoptera, CGH and GISH helped so far to detect W chromosome in 11 species (belonging to 5 families) out of 12 species examined using these methods. In this paper, we carried out GISH in nine other lepidopteran species using respective female-derived genomic probes and in eight of them identified the W chromosome. Our results confirmed the easiness of W chromosome identification in Lepidoptera by GISH and extended the practically applicable range to 21 species from 10 families.

MATERIALS AND METHODS

Insects

Specimens of *Spilarctia seriatopunctata* (Arctiidae), *Papilio xuthus* (Papilionidae), *Inachus io geisha* (Nymphalidae), and *Pieris brassicae* (Pieridae) were collected in Sapporo in 2003. *Caligula japonica* (Saturniidae), *Orgyia recens* (Lymantriidae), and *Artaxa subflava* (Lymantriidae) were collected in Sunagawa, Naganuma, and Ishikari, Hokkaido, respectively, in 2005. *Mamestra brassicae* (Noctuidae) was provided by Dr. Hayakawa (Saga University). *Antheraea pernyi* (Saturniidae) was obtained from Dr. Kajiura (Shinsyu University) through National Bioresource Project (NBRP).

Chromosome preparation

Spread chromosome preparations were done as described in Yoshido *et al.* (2005a) with a slight modification of the procedure developed by Traut (1976). Briefly, ovaries of the matured last instar larvae or young pupae were dissected in *Ephestia*'s saline solution (Marec and Traut, 1993) and fixed with Carnoy's fluid (ethanol, chloroform, acetic acid, 6:3:1). In some species, the ovaries were incubated in a hypotonic solution (83 mM KCl and 17 mM NaCl) before fixing. Chromosomes were spread in 60% acetic acid at 50°C using a heating plate. Preparations were passed through a graded ethanol series (70%, 80%, and 98%) and stored at -20°C until use.

FISH and image processing

We carried out GISH according to the procedure of Sahara *et al.* (2003b). Briefly, female genomic DNAs were labeled by a Nick Translation System (Invitrogen, Tokyo, Japan) with Cy3-dCTP (Amersham, Tokyo, Japan). Chromosome preparations passed through an ethanol series and air-dried were denatured at 72°C for 3.5 min in 70% formamide, 2×SSC. Probe cocktail contained 500 ng of labeled respective female genomic DNA (Cy3; red), 25 g of sonicated salmon sperm DNA (Sigma-Aldrich, Tokyo, Japan), and 3 g of sonicated male genomic DNA in 10 l of hybridization solution (50% formamide, 10% dextran sulfate, 2×SSC). Hybridization in moist chamber was carried out at 37°C for 3 days. Afterwards, the slides were washed at 62°C in 0.1×SSC containing 1% TritonX-100. Mounting and counterstaining was done with antifade [0.233g 1,4-diazabicyclo(2.2.2)-octane, 1 ml 0.2 M Tris-HCl, pH 8.0, 9 ml glycerol] containing 0.5 g/ml of DAPI (4', 6-diamidino-2-phenylindole; Sigma-Aldrich, Tokyo, Japan). A Leica DMRE HC fluorescence microscope equipped with a Photometrics CoolSNAP CCD camera was used for observation and image capturing. Digital image processing and pseudocolouring was carried out with Adobe Photoshop, Version 7.0. Routinely, red coloring was used for Cy3 and light blue for DAPI images.

RESULTS AND DISCUSSION

CGH or GISH represents a powerful tool for sex chromosome identification in some organisms (Traut *et al.*, 1999; Traut and Winking, 2001). So far, these methods have been used to identify sex chromosomes in 12 lepidopteran species (Table 1). In this study, we extended the list in Table 1 by nine lepidopteran species. In eight species, GISH painted the whole chromosome thread of a bivalent (Figs. 2-9). The thread obviously represented the W chromosome forming the sex chromosome bivalent with its pairing partner, the Z chromosome. Thus, the species have a WZ/ZZ (female/male) sex chromosome system. On the other hand, the whole-chromosome painting was not observed in *C. japonica* (Fig. 1; Table 1). In this species, one of the longest chromosomes formed a univalent in female pachytene nuclei. As meiosis progressed, the univalent preferentially condensed and became deeply stained with DAPI (not shown). In accordance with the known haploid chromosome number of n=31 (Robinson, 1971), pachytene complements consisted of 31 elements, but 30 were bivalents and one univalent, obviously the Z chromosome. Accordingly, female mitotic complements consisted of 2n=61 chromosomes. Our results clearly showed that females of *C. japonica* have a ZO sex chromosome constitution, which was not known yet.

The Z chromosome surrounded the W in pachytene nuclei of *A. subflava* (Fig. 3). The characteristic nature of the sex chromosome bivalent in this species most probably results from the large difference between the size of sex chromosomes, the long Z chromosome and very short W chromosome. In *S. seriatopunctata* (Fig. 5), we determined for the first time the haploid chromosome number, which is n=31.

GISH signals were not restricted to the W chromosomes only but they were found also in autosomes. The autosomal signals stained specific segments in some species besides the predominant but fainter spots in telomeric and/or subtelomeric regions. After distribution of the autosomal signals we classified species examined into 3 groups: (i) without autosomal signals except for telomeric and/or subtermeric regions, (ii) with highlighted nucleolar organizer region (NOR) –chromosomes, and (iii) with strong signal only in non NOR-chromosome except for telomeric and/or subtermeric regions. The NOR consists of ribosomal gene cluster (Shaw and Doonan, 2005) and sometimes associates heterochromatic region near by depending on lepidopteran species (Yoshido *et al.*, 2005b). The first group involved *A. subflava* (Fig. 3), *O. recens* (Fig. 4), *Spilarctia seriatopunctata* (Fig. 5), *Papillio xuthus* (Fig. 7), *Cydia pomenella* (Fukova *et al.*, 2005), *O. antiqua*, and *O. thyellina* (Yoshido *et al.*, 2005b), and *Galleria mellonella* (Traut *et al.*, 1999). The second group involved *A. pernyi* (Fig. 2), *P. brassicae* (Fig. 8), *I. i. geisha* (Fig. 9), *A. yamamai*, *Samia cynthia walkeri*, *S. c. ricini*, and *S. c. indet.* ssp. (Yoshido *et al.*, 2005b), and *Ephestia kuehniella* (Traut *et al.*, 1999). Among them *A. pernyi*, *P. brassicae*, and *I. i. geisha* also have highlighted signals in the telomeric and/or subtelomeric regions of the bivalents. The signals appeared in most bivalents of *A. pernyi*, in two bivalents of *P. brassicae*, and in five bivalents of *I. i. geisha* (Figs. 2, 8, 9, respectively). It is notable that *I. i. geisha* displayed at least five nucleoli in a haploid genome, and three of them were strongly labeled by GISH. The last group included only *B. mori* (Sahara *et al.*, 2003b; Yoshido *et al.*, 2005a). Finally, we were not able to determine, whether the autosome with a GISH signal in *M. brassicae* (Fig. 6) is or is not the NOR-chromosome.

Our results confirmed the potential of GISH to identify the lepidopteran W chromosome as shown in representatives of 10 different families. GISH is also a very useful technique to resolve the sex chromosome constitution in females of Lepidoptera with multiple sex chromosomes (Yoshido *et al.*, 2005b). GISH enables us to detect the W chromosome even in a single female specimen using the female genomic DNA as a probe and simultaneously as a competitor (A. Yoshido, unpublished). This method is particularly well suited for obtaining the first and fast information on sex chromosomes in species with a small population size and/or in unidentified species.

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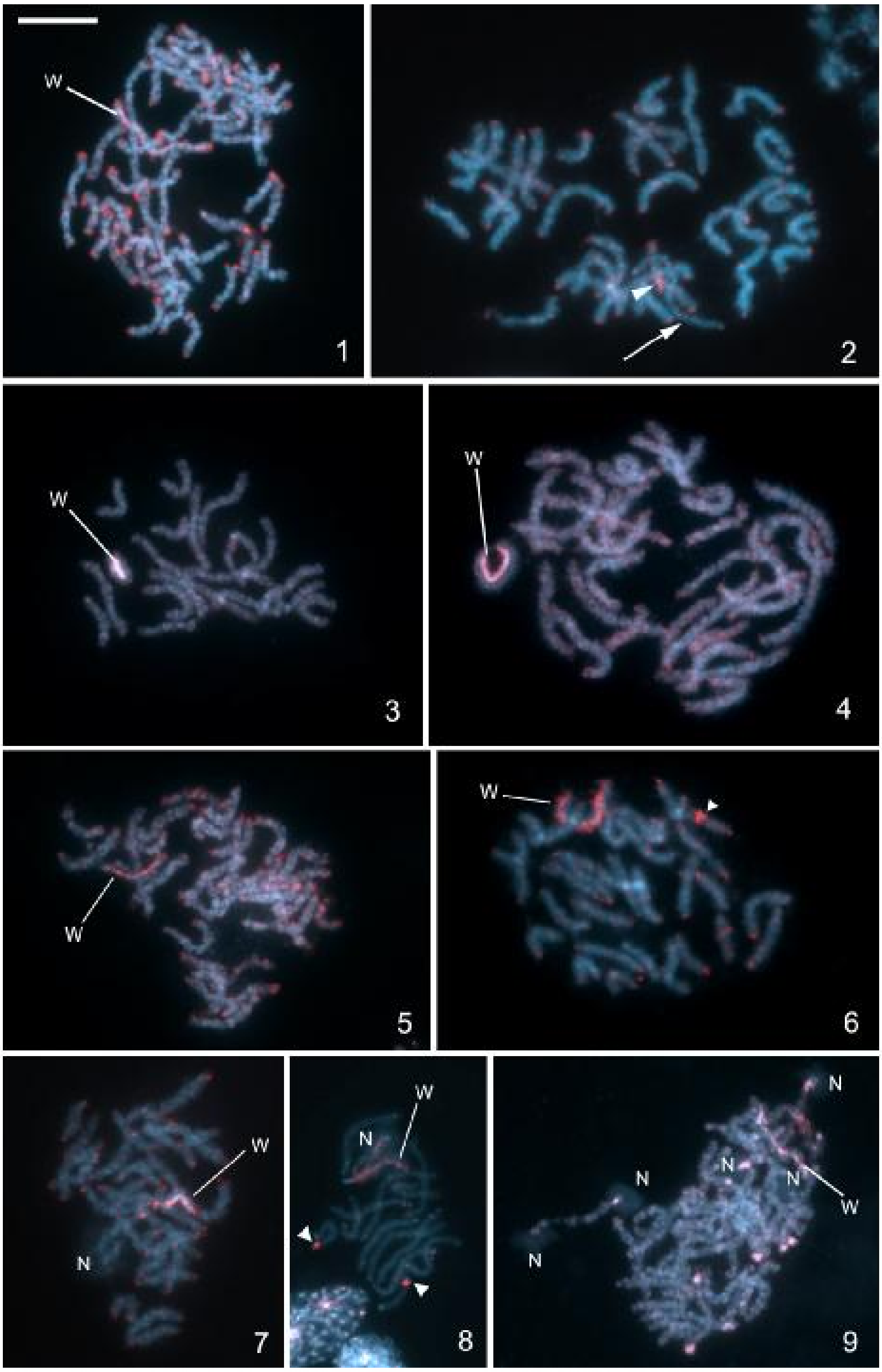
Figure legends

Figs. 1-9. GISH identification of the W chromosome in lepidopteran species. W-chromosome painting together with hybridization signals of various intensities in other chromosomes are obvious in all figures except for *Caligula japonica*, which has a ZO sex chromosome constitution. Fig. 1, *C. japonica*; Fig. 2, *Antheraea pernyi*; Fig. 3, *Artaxa subflava*; Fig. 4, *Orgyia recens*; Fig. 5, *Spilarctia seriatopunctata*; Fig. 6, *Mamestra brassicae*; Fig. 7, *Papilio xuthus*; Fig. 8, *Pieris brassicae*; Fig. 9, *Inachus io geisha.* Light blue, chromosomes counterstained with DAPI; red, GISH signals. N, nucleolus. Arrow indicates a Z chromosome univalent in *C. japonica*. Arrowheads indicate autosomal heterochromatic segments highlighted by GISH. Bar = 10 m.


W
1
2
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3
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4
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W
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9

Table 1. Summary of data on the identification of sex chromosome constitution by CGH or GISH in Lepidoptera

Family Species	Collection site	Collection year	Sex chromosome system	CGH or GISH done by/in
Arctiidae				
Spilarctia seriatopunctata	Sapporo	2003	WZ/ZZ	This study: Fig. 5
Bombycidae				
Bombyx mori			WZ/ZZ	Traut *et al*. (1999)
Bombyx mandarina	Sakado	1982	WZ/ZZ	Yoshido *et al*. (2006)
Lymantriidae				
Artaxa subflava	Ishikari	2005	WZ/ZZ	This study: Fig. 3
Orgyia thyellina	Naganuma	2000	W_1W_2 neo-Z /neo-Z neo-Z	Yoshido *et al*. (2005b)
Orgyia antiqua	Germany	1999	neo-W neo-Z/neo-Z neo-Z	Yoshido *et al*. (2005b)
Orgyia recens	Naganuma	2005	WZ/ZZ	This study: Fig. 4
Noctuidae				
Mamesta brassicae	Hirosaki and Shimonoseki	1990s	WZ/ZZ	This study: Fig. 6
Nymphalidae				
Inachus io geisha	Sapporo	2003	WZ/ZZ	This study: Fig. 9
Papilionidae				
Papilio xuthus	Sapporo	2003	WZ/ZZ	This study: Fig. 7
Pieridae				
Pieris brassicae	Sapporo	2003	WZ/ZZ	This study: Fig. 8
Pyralidae				
Ectomyelois eratoniae	Tunisia	1999	WZ/ZZ	Mediouni *et al*. (2004)
Ephestia kuehniella	Czech Republic		WZ/ZZ	Traut *et al*. (1999)
Galleria mellonella	Czech Republic		WZ/ZZ	Traut *et al*. (1999)
Saturniidae				
Antheraea peryni	Nagano		WZ/ZZ	This study: Fig. 2
Antheraea yamamai	Nagano	1880s	WZ/ZZ	Yoshido *et al*. (2005b)
Caligula japonica	Sunagawa	2005	Z/ZZ	This study: Fig. 1
Samia cynthia ricini	Vietnam	1990s	Z/ZZ	Yoshido *et al*. (2005b)
Samia cynthia ssp. indet	Nagano	1990s	neo-WZ_1Z_2/$Z_1Z_1Z_2Z_2$	Yoshido *et al*. (2005b)
Samia cynthia walkeri	Sapporo	2003	neo-W neo-Z/neo-Z neo-Z	Yoshido *et al*. (2005b)
Tortricidae				
Cydia pomonella	Russia	1961	WZ/ZZ	Fuková *et al*. (2005)