Comparison of the Z and W sex chromosomal architectures in elegant crested tinamou (*Eudromia elegans*) and ostrich (*Struthio camelus*) and the process of sex chromosome differentiation in palaeognathous birds

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Abstract

To clarify the process of avian sex chromosome differentiation in palaeognathous birds, we performed molecular and cytogenetic characterization of W chromosome-specific repetitive DNA sequences for elegant crested tinamou (*Eudromia elegans*, Tinamiformes), and constructed comparative cytogenetic maps of the Z and W chromosomes with nine chicken Z-linked gene homologues for *E. elegans* and ostrich (*Struthio camelus*, Struthioniformes). A novel family of W-specific repetitive sequences isolated from *E. elegans* was found to be composed of GC-rich 293-bp elements that were tandemly arrayed in the genome as satellite DNA. No nucleotide sequence homologies were found for the Struthioniformes and neognathous birds. The comparative cytogenetic maps of the Z and W chromosomes of *E. elegans* and *S. camelus* revealed that there are partial deletions in the proximal regions of the W chromosomes in the two species, and the W chromosome is more differentiated in *E. elegans* than in *S. camelus*. These results suggest that a deletion firstly occurred in the proximal region close to the centromere of the acrocentric proto-W chromosome and advanced toward the distal region. In *E. elegans* the W-specific repeated sequence elements were amplified site-specifically after deletion of a large part of the W chromosome occurred.

Introduction

Avian species are categorized into two large groups: the Palaeognathae (ratites and palaeognathous carinates (tinamous)) and the Neognathae (all other carinates). They are classified morphologically based on the palatal form, and this classification has been confirmed at the molecular level by DNA-DNA hybridization and nucleotide sequencing of the nuclear and mitochondrial ribosomal RNA genes (Sibley and Ahlquist 1990; van Tuinen et al. 1998, 2000). There are remarkable differences in the constitution of sex chromosomes between the two groups. Neognathous birds have highly differentiated W chromosomes that are comparatively smaller than the Z chromosome, highly heterochromatized and late replicating (Takagi 1972; Takagi and Sasaki 1974; Schmid et al. 1989). In contrast, the palaeognathous ratites (the Struthioniformes) retain the most primitive forms of avian sex chromosomes, which are largely homomorphic between the Z and W chromosomes (Takagi et al. 1972; de Boer 1980; Ansari et al. 1988) since palaeognathous birds and neognathous birds diverged about 120 million years ago (van Tuinen and Hedges 2001). Comparative chromosome painting with the chicken Z chromosome-specific DNA revealed that the extensive homology between the Z and W chromosomes is also preserved on a molecular basis in emu (*Dromaius novaehollandiae*) (Shetty et al. 1999). The homology was also confirmed by comparative mapping of the sex chromosomes of emu, ostrich and double-wattled cassowary, which demonstrated that the Z and W chromosomes are homomorphic except for some marginally differentiated regions (Ogawa et al. 1998; Nishida-Umehara et al. 1999; Shetty et al. 2002). These data suggest that the W chromosomes of the Struthioniformes are hardly differentiated molecularly and still retain much Z homology.

Tinamous are classified as palaeognathous carinates and are phylogenetically

positioned as a sister group to the ratites (van Tuinen et al. 1998, 2000; Cracraft 2001). One half to two-thirds of the W chromosomes consist of heterochromatin in elegant crested tinamou (*Eudromia elegans*), red-winged tinamou (*Rhynchotus rufescens*) and spotted tinamou (*Nothura maculosa*) (Sasaki et al. 1980; Pigozzi and Solari, 1999, 2005). Therefore, the W chromosomes of the Tinamiformes are considered to be at an intermediate stage in heterochromatization between the largely euchromatic W chromosomes of the palaeognathous ratites and the highly heterochromatic W chromosomes of neognathous birds. This has been confirmed by cytogenetic studies of meiotic chromosome pairing. In *R. rufescens* and *N. maculosa* the recombination nodules on the Z and W chromosomal pair are distributed in much longer regions than in neognathous birds but are restricted to shorter segments than those of the two rhea species, *P. pennata* and *R. americana* (Pigozzi and Solari 1997, 1999, 2005). However, W-heterochromatin has not been molecularly cloned from any Tinamiformes species, and there is little information about comparative chromosome mapping in these taxa either.

Several female-specific repetitive DNA sequences have been cloned for some neognathous birds: the *XhoI*-family, *EcoRI*-family and *SspI*-family of chicken (*Gallus gallus*) (Tone et al. 1982, 1984; Kodama et al. 1987; Saitoh et al. 1991; Saitoh and Mizuno 1992; Itoh and Mizuno 2002), the *PstI*-family of turkey (*Meleagris gallopavo*), the *Taq*I-family of Japanese common pheasant (*Phasianus versicolor*)(Saitoh et al. 1989) and LfW-1 of lesser black-backed gull (*Larus fuscus*)(Griffiths and Holland 1990). Some of them are major components of the W-heterochromatin, and their nucleotide sequences are highly diverged between different species as rapidly evolved molecules. We recently cloned a novel family of repetitive sequences from Galliformes

species, which is an interspersed-type repetitive sequence amplified site-specifically on the W chromosome (Yamada et al. 2006). This family of repetitive sequences is highly conserved in neognathous birds but not in palaeognathous birds. All these results collectively suggest that the W-heterochromatin of *E. elegans* is composed of other types of repetitive sequences whose origins are different from those of the known W-specific repetitive sequences.

To define the sex chromosomal architecture of palaeognathous birds and to elucidate the process of avian sex chromosome differentiation, we first molecularly cloned a novel family of W-specific repetitive sequences from *E. elegans*, and characterized them by nucleotide sequencing and chromosomal and filter hybridization. Secondly, we cloned eight and nine homologues of chicken Z-linked genes from *S. camelus* and *E. elegans*, respectively, and localized them to the Z and W chromosomes of these two species. Finally we discuss the process of sex chromosome differentiation that occurred in palaeognathous birds.

Materials and methods

Specimens, cell cultures and chromosome preparation

The lymphocyte cells prepared from the blood of one male and female each of *Eudromia elegans* were cultured in RPMI 1640 medium supplemented with 18% fetal bovine serum, 3 μg/ml concanavalin A (Sigma), 10 μg/ml lipopolysaccharide (Sigma), 90 μg/ml phytohaemagglutinin (HA15, Murex) and 5×10^{-5} M mercaptoethanol. Cell

cultures were incubated at 39°C in a humidified atmosphere of 5% CO_2 in air. BrdU (25 μg/ml) was added to the culture 48 h later, and cell culturing was continued for an additional 5 h including 1 h of colcemid treatment (0.025 μg/ml) before harvesting. The cells were collected, suspended in 0.075 M KCl, and fixed with 3:1 methanol: glacial acetic acid following a standard protocol. The cell suspension was dropped on glass slides and air-dried. After staining of the chromosome slides with Hoechst 33258 (1 μg/ml) for 5 min, R-bands were obtained by heating the slides for 5 min at 65°C and exposing them to UV light at 65°C for an additional 3 min (Matsuda and Chapman 1995). The fibroblast cells prepared from skin tissues of one male and female each of *E. elegans* and one female of *Struthio camelus* were cultured in 199 medium supplemented with 18% fetal bovine serum under the same conditions used for the lymphocyte cell cultures. BrdU (25 μg/ml) was added at log phase and the cell culturing was continued for an additional 5 h. The cells were harvested after 30 min of colcemid treatment (0.025 μg/ml) and fixed in the same way as in the case of lymphocyte cell cultures, and chromosome preparations were made. For C-banding analysis, the chromosome slides were prepared from fibroblast cells cultured without BrdU treatment.

C-banding

To examine the chromosomal distribution of constitutive heterochromatin in male and female *E. elegans,* chromosome C-banding was made with the BSG (barium hydroxide/saline/Giemsa) method (Sumner 1972) with slight modification.

Molecular cloning of female-specific repetitive DNA sequences

High molecular weight genomic DNA was extracted from the blood of one male and female each of *E. elegans.* The genomic DNA was digested with 23 restriction endonucleases: *Alu*I, *Apa*I, *Bgl*II, *Bam*HI, *Bst*XI, *Dde*I, *Eco*RI, *Eco*RV, *Hae*III, *Hap*II, *Hin*dIII *Hin*fI, *Mbo*I, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Taq*I, *Xba*I and *Xho*I, and fractionated by electrophoresis with 1% and 3% agarose gels, and stained with ethidium bromide. Female-specific DNA bands were isolated from the gel, and the DNA fragments were eluted using a QIAquick Gel Extraction Kit (Qiagen) and ligated into pBluescript II SK(+), and transformed into competent *Escherichia coli* JM109 cells (Takara Bio). The sizes of the DNA fragments inserted in the vector were confirmed by electrophoresis of the PCR products that were amplified with T3 and T7 primers, and the clones were used for fluorescence in situ hybridization (FISH).

Nucleotide sequencing

Nucleotide sequences were determined using an ABI PRISM3100 DNA Analyzer (Applied Biosystems) after the sequencing reaction with a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

Southern blot hybridization

The genomic DNA digested with restriction endonucleases was fractionated on a 1%

agarose gel by electrophoresis and transferred onto a Hybond N+ nylon membrane (Amersham Biosciences). The DNA fragment cloned from the female-specific DNA band was labeled with digoxigenin-11-dUTP using a PCR DIG Labeling Mix (Roche Diagnostics), and was hybridized to the membrane as a probe overnight at 42°C using DIG Easy Hyb (Roche Diagnostics). The membrane was washed sequentially at 42°C in 2 × SSC, 1 × SSC, 0.5 × SSC and 0.1 × SSC for 15 min each. The chemiluminescent signals were detected with anti-Digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics), and exposed to BioMax MS Autoradiography Film (Kodak).

Slot-blot hybridization

To examine the nucleotide sequence divergence of the repetitive DNA sequences, slot-blot hybridization probed with a repeated sequence element was performed. Genomic DNA was extracted from blood samples collected from one male and female each of the following 11 species of five orders and used for slot-blot hybridization: elegant crested tinamou (*Eudromia elegans*) of the Tinamiformes, emu (*Dromaius novaehollandiae*), double-wattled cassowary (*Casuarius casuarius*), greater rhea (*Rhea americana*), lesser rhea (*Pterocnemia pennata*) and ostrich (*Struthio camelus*) of the Struthioniformes, chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*) and guinea fowl (*Numida meleagris*) of the Galliformes, Siberian crane (*Grus leucogeranus*) of the Gruiformes and Blakiston's fish owl (*Ketupa blakistoni*) of the Strigiformes. The DNA was denatured with NaOH and blotted onto a Hybond N+ nylon membrane using BIO-DOT SF blotting equipment (Bio-Rad Laboratories). The probe DNA was labeled

with digoxigenin-11-dUTP using a PCR DIG Labeling Mix (Roche Diagnostics), and hybridized to the membrane overnight at 42°C using DIG Easy Hyb (Roche Diagnostics). Then the membrane was washed, and the chemiluminescent signals were detected using the same procedure as for Southern blot hybridization.

To estimate the amount of the repetitive DNA sequences in the genome, eight different concentrations of female genomic DNA and the repetitive sequences were prepared. Slot-blot analysis was performed using a DNA fragment of the repetitive sequence labeled with digoxigenin-11-dUTP as probe. The luminescent hybridization signals were measured using BIO-PROFILE Image Analysis Software (VILBER LOURMAT), and the intensity of the signals was compared between the genomic DNA and the repetitive sequence.

Molecular cloning of chicken Z-linked gene homologues

For comparative FISH mapping of the Z and W chromosomes, we molecularly cloned homologues of the following chicken Z-linked genes from *E. elegans* and *S. camelus*: ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle (*ATP5A1*), chromodomain helicase DNA binding protein 1 (*CHD1*), growth hormone receptor (*GHR*), neurotrophic tyrosine kinase receptor, type 2 (*NTRK2*), protein kinase C inhibitor (*PKCI*), ribosomal protein S6 (*RPS6*), spindlin (*SPIN*) and tropomodulin 1 (*TMOD*) for *S. camelus* and *E. elegans,* and soluble aconitase 1/iron-responsive element binding protein (*ACO1/IREBP*) for *E. elegans*. The fibroblast cells of *E. elegans* and *S. camelus* were lysed with TRIzol Reagent (Invitrogen), and

total RNA was extracted following the manufacturer's instructions. The cDNA was obtained by RT-PCR using Oligo $(dT)_{12-18}$ Primer (Invitrogen) and SuperScript II RNase H^- Reverse Transcriptase (Invitrogen), and was used as the PCR template to amplify the homologues of the chicken Z-linked genes. Genomic DNA of male and female *E. elegans* was extracted from blood cells and used as the PCR template to amplify genomic DNA fragments of the *CHD1* gene. Twenty nanograms of DNA were incubated in 20 μl of 1 × ExTaq Buffer containing 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 μM degenerate primers or 1.0 μM primers, and 0.25 U of TaKaRa Ex Taq (Takara Bio). The PCR conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 48-58°C for 30 s and 72°C for 35 s; and finally 72°C for 5 min for a final extension. The cDNA fragments of *PKCI* and *RPS6* of *S. camelus* and *E. elegans* were extended using the 3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen). The nucleotide sequences of the primers and temperatures at the annealing step in the PCR reaction used for cDNA cloning of the genes are listed in Table 1. The PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The target DNA bands were isolated from the gels and extracted using a QIAquick Gel Extraction Kit (Qiagen). The DNA fragments were cloned using pGEM-T Easy Vector System I (Promega), and then transformed into competent *E. coli* JM109 cells.

Fluorescence in situ hybridization (FISH)

FISH analysis was performed for chromosomal localization of the repetitive DNA

sequences and the functional genes as described previously by Matsuda and Chapman (1995). The DNA fragments of the repetitive DNA sequences were labeled with biotin-16-dUTP using a nick translation kit (Roche Diagnostics) and ethanol-precipitated with salmon sperm DNA and *E. coli* tRNA. After hybridization, the slides were incubated with avidin-fluorescein (Roche Diagnostics) and stained with 0.75 μg/ml propidium iodide (PI).

For chromosome mapping of the chicken Z-linked gene homologues, multiple cDNA fragments isolated for each gene were mixed and used as probes (Table 1). The cDNA fragments of *GHR* and *ATP5A1* of *S. camelus* and *ATP5A1* of *E. elegans* were labeled with CyDye 3-dUTP (Amersham Biosciences) using a nick translation kit, and other cDNA fragments were labeled with biotin-16-dUTP. After hybridization with the cDNA fragments labeled with biotin-16-dUTP, the probes were reacted with goat anti-biotin antibody (Vector Laboratories) and then stained with Alexa Fluor 488 rabbit anti-goat IgG (H+L) conjugate (Molecular Probes). The chromosome slides were counterstained with 0.75 μg/ml PI. The FITC signals of the repetitive sequences and Cy3 fluorescence signals of cDNA fragments were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope, and were analyzed with the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK). The Alexa signals were observed under a Nikon fluorescence microscope using Nikon filter sets B-2A and UV-2A. DYNA HG ASA100 films (Kodak) were used for microphotography.

Results

C-banded karyotype of *E. elegans*

The C-banded patterns of chromosomes were examined for female and male *E. elegans* (Fig. 1). A large C-positive heterochromatin block was observed in the proximal region of the W chromosome, which accounted for two-thirds of its long arm (Fig. 1a), whereas there were no large C-positive bands on the Z chromosome (Fig. 1a, b). There were C-positive bands in the centromeric regions of all autosomes, and large centromeric C-bands were observed in two pairs of macrochromosomes and about half of the microchromosomes.

Molecular cloning of female-specific repetitive DNA sequences

Genomic DNA of male and female *E. elegans* was digested with 23 restriction endonucleases, fractionated by agarose gel electrophoresis and stained with ethidium bromide. Female-specific bands around 0.3 kb in size were detected in the *Bam*HI-, *Bgl*II- and *Bst*XI-digests (Fig. 2). The female-specific *Bam*HI band was isolated from the gel, and the DNA fragments were eluted and cloned into plasmid vectors. Twenty-four clones were obtained and used for FISH analysis.

Chromosomal location of female-specific repetitive DNA sequences

The chromosomal location of the DNA fragments isolated from the female-specific DNA band was examined by FISH. The hybridization signals were detected in the centromeric region of the W chromosome for 19 out of 24 clones, all of which painted about one-third of the long arm of the W chromosome (Fig. 3a), whereas no hybridization signals were detected on the metaphase spreads of two male individuals (Fig. 3b). No signals were detected for the remaining five clones. The painted signal of the W-specific repetitive sequences covered about two-thirds of the C-positive heterochromatin region on the W chromosome (see Fig. 1a).

Nucleotide sequences of W-specific repeated sequence elements

Nucleotide sequences were determined for 19 clones of the W-specific repetitive DNA sequences (accession numbers AB254044-AB254062) isolated from the 0.3-kb *Bam*HI band (Fig. 4). All the DNA fragments were grouped into the same family of repetitive DNA sequences. No internal repeats were detected by dot-matrix analysis (data not shown). The W-specific repeated sequence family was composed of 291-293-bp elements. Almost all of the cloned DNA fragments contained *Bgl*II and *Bst*XI restriction sites, indicating that the female-specific bands detected in the *Bam*HI-, *Bgl*II- and *Bst*XI-digests were composed of the same repeated sequence family. The nucleotide sequences were compared between the clones for all combinations of the 19 clones, and

the results showed that the nucleotide sequence homologies between the clones ranged from 89.8% to 99.0%. The G + C contents of the fragments were 61.1% on average, ranging from 59.5% to 62.8%, indicating that the repeated sequence family is GC-rich. The consensus sequence of the novel W-specific repeated sequence elements was searched for nucleotide sequence homology to other DNA sequences using the DDBJ database (http://www.ddb.nig.ac.jp/), but no sequences with significant homology were detected.

Genomic organization of W-specific repetitive sequences

To examine the organization of the W-specific repetitive sequences in the genome of *E. elegans*, the genomic DNA of female *E. elegans* digested with *Bam*HI, *Bgl*II, *Bst*XI, *Hae*III, *Hinf*I, *Hpa*II or *Msp*I was subjected to Southern blot hybridization probed with the EEL-*Bam*HI 21 fragment. The hybridization resulted in polymeric ladder signals of tandem repeats of the 293-bp basic monomer unit in *Bam*HI-, *Bgl*II-, *Bst*XI- and *Hinf*I-digested genomic DNA (Fig. 5). In the *Bam*HI-, *Bgl*II- and *Bst*XI-digests, the same-sized monomer units were present in the greatest abundance, with progressively decreasing copy numbers of each higher order of repeat unit, indicating that the restriction sites of the three endonucleases are very highly conserved in the repetitive sequences. The number of *Hin*fI cleavage sites was much smaller than those of *Bam*HI, *Bgl*II and *Bst*XI in the tandem arrays of the 293-bp monomer units, and hence more-intense hybridization bands were observed at higher molecular weight in the *Hin*fI-digest. DNA bands with molecular weight lower than 293 bp were observed in the

*Hae*III- and *Msp*I-digests, and were derived from internal restriction sites of the two endonucleases conserved in the repeated sequence elements (see Fig. 4). *Hpa*II and *Msp*I endonucleases were used to examine the methylation status of the repetitive sequences in the genome. Their restriction sites are both 'CCGG', and *Hpa*II does not cleave when the second cytosine is methylated, whereas *Msp*I does. There was a remarkable difference in hybridization pattern between the *Hpa*II- and *Msp*I-digests; there were very few *Msp*I-bands at higher molecular weight than the 293-bp monomer unit, while ladder bands were observed from low to high molecular weight in the *Hpa*II-digest. In addition, there were intermediate-sized bands between each ladder band of multiple 293-bp monomer units, which were derived from internal cleavage sites in the repeated sequence elements (see Fig. 4). The difference in hybridization pattern between the *Hpa*II- and *Msp*I-digests indicates that the repeated sequences are highly methylated in the genome.

Slot-blot hybridization was performed to estimate the amount of the W-specific repetitive sequences in the genome of female *E. elegans* (Fig. 6). Eight different concentrations of the female genomic DNA and the EEL-*Bam*HI 21 fragment were hybridized with digoxigenin-11-dUTP-labeled EEL-*Bam*HI 21, and the intensities of the signals were compared. The repetitive DNA sequence consequently accounted for about 0.2% of the genome.

Nucleotide sequence conservation of W-specific repetitive sequences

Nucleotide sequence conservation of the EEL-*Bam*HI repeated sequence family in

palaeognathous and neognathous birds was examined by slot-blot hybridization probed with digoxigenin-11-dUTP-labeled EEL-*Bam*HI 21 (Fig. 7). Genomic DNAs were collected from male and female individuals of 11 species that belonged to five different orders. An intense hybridization signal was detected in the genomic DNA of female *E. elegans*, but no signals were detected in male *E. elegans* or in males of any other species. No reliable hybridization bands were detected except in female *E. elegans* genomic DNA upon longer exposure of the membrane to X-ray-film either.

Chromosomal locations of chicken Z-linked gene homologues in *S. camelus* and *E. elegans*

The homologues of eight chicken Z-linked genes, *ATP5A1*, *CHD1*, *GHR*, *NTRK2*, *PKCI*, *RPS6*, *SPIN* and *TMOD,* were cloned from *S. camelus* and *E. elegans* (Table 2). The cDNA fragment of *ACO1/IREBP* was only cloned from *E. elegans* because the chromosome mapping of *ACO1/IREBP* has been reported for *S. camelus* (Ogawa et al. 1998). The chromosomal locations of these nine genes in *S. camelus* and *E. elegans* are shown in Figure 8 and Figure 9. The order of the Z-linked genes, *TMOD – ACO1/IREBP – RPS6, NTRK, PKCI – SPIN – CHD1 – GHR – ATP5A1*, in *S. camelus* was the same as that on the Z chromosomes of *E. elegans* (Fig. 9). The hybridization signals of *TMOD* and *ACO1/IREBP* were detected only on the Z chromosomes in two species, in which were localized near the centromere on the long arm. *RPS6, NTRK2* and *PKCI* were located proximal to the *SPIN* locus on the Z chromosomes in two species. In *S. camelus*, *SPIN* was located near the center of the W chromosome, and the

hybridization signals of *RPS6, NTRK2* and *PKCI* were also detected proximal to *SPIN* on the W chromosome. The locations of these four genes on the W chromosome were the same as those on the Z chromosome in this species. In *E. elegans*, no signals of *RPS6, NTRK2* or *PKCI* were observed on the W chromosome. *SPIN* was localized near the centromere on the W chromosome, and this locus was contained in the W-specific heterochromatin region. The chromosomal locations of *CHD1*, *GHR* and *ATP5A1* on the Z chromosome were the same as those on the W chromosomes in two species.

Nucleotide sequence similarities of Z-linked genes among chicken, *S. camelus* and *E. elegans*

The nucleotide sequence similarities of the eight Z-linked genes were compared in the equivalent regions of cDNA fragments among chicken (*G. gallus*), *S. camelus* and *E. elegans* (Table 3). The identities were the highest for seven genes (*ATP5A1*, *CHD1*, *GHR*, *NTRK2*, *PKCI*, *RPS6* and *SPIN*) between *S. camelus* and *E. elegans* (90.5 - 98.3%) and for *TMOD* between *G. gallus* and *S. camelus* (90.1%). The lowest identities were observed between *G. gallus* and *E. elegans* for seven genes (*ATP5A1*, *CHD1*, *GHR*, *NTRK2*, *RPS6, SPIN* and *TMOD*) (84.5 – 97.1%) and between *G. gallus* and *S. camelus* for *PKCI* (89.4%). The same identities were observed between *G. gallus* and *S. camelus* and between *G. gallus* and *E. elegans* for *CHD1* (92.4%) and *RPS6* (93.2%), respectively.

Comparison of nucleotide sequences between the Z and W forms of the *CHD1* gene in *E. elegans*

To examine the presence of the Z and W chromosomal forms of the *CHD1* gene in *E. elegans*, we designed PCR primers based on the nucleotide sequences of the cDNA fragments of the *CHD1* gene (AB254883 and AB254884) as follows: F, 5'-GGATCAGTAGAAGAAGATAT-3'; R, 5'-AAGCAGCTCATCTCCTACAG-3', and amplified the genomic DNA fragments using genomic DNA of one male and female each of *E. elegans*. About 610-bp DNA bands were obtained from both sexes, and their nucleotide sequences were determined by direct sequencing of the PCR products. Only the sex-specific W form of the *CHD1* gene was obtained from the female. The genomic DNA fragments obtained for the *CHD1W* gene (AB255123) and the *CHD1Z* gene (AB255124) covered the entire exon 7 and parts of exons 6 and 8, in which there were deletions of 3 bp and 4 bp in *CHD1W* and 2-bp deletion at two sites and a 1-bp deletion in *CHD1Z* (Fig. 10). The nucleotide sequence identities between the genomic DNA fragments of the *CHD1Z* and *CHD1W* genes were 85.8% (295/344) in the intron and 95.8% (252/263) in the coding region. These results suggest that meiotic recombination no longer occurs at the *CHD1* locus between the Z and the W chromosomes in *E. elegans*, and the nucleotide sequences in the intron are more differentiated than those in the exons.

Discussion

Here a novel *Bam*HI family of female-specific repetitive DNA sequences was molecularly cloned from *E. elegans*. The hybridization signals of the sequences were localized to the proximal region of the long arm of the W chromosome, which accounts for about two-thirds of the C-positive heterochromatin region, suggesting that the repeated sequence family is a major component of the W chromosome heterochromatin of *E. elegans*. The W-specific repetitive sequences are composed of GC-rich 291-293-bp elements and organized in tandem arrays as satellite DNA (stDNA). Internal restriction sites of *Bgl*II and *Bst*XI are present in almost all of the *Bam*HI repeated sequence fragments. Southern blot hybridization probed with the *Bam*HI repeated sequence element revealed that the sizes and the polymeric ladder patterns of hybridized bands in the *Bam*HI digest were completely the same as those in the *Bgl*II and *Bst*XI digests. These results clearly indicate that the *Bam*HI repeated sequence is the same as the *Bgl*II and *Bst*XI sequence families. The repeated sequence family was not found in the ZZ male of *E. elegans*, other palaeognathous ratites or neognathous birds. The novel stDNA sequences might have been amplified on the W chromosome independently in the Tinamiformes lineage after the Struthioniformes and the Tinamiformes diverged from the common ancestor of palaeognathous birds. The hypermethylation status of the W-specific stDNA suggests that the repetitive sequence has some roles in chromatin organization of the W chromosome in interphase nuclei, and replication timing at S phase and chromosome condensation at metaphase.

The Z- and W-chromosomal forms of the *CHD1* gene, *CHD1Z* and *CHD1W*, are present in neognathous birds, and the presence of the sex-specific forms facilitates the use of this gene as a molecular marker for sexing (Ellegren 1996; Griffiths et al. 1996, 1998; Fridolfsson and Ellegren 1999). The presence of the Z- and W-chromosomal

forms has been reported for five other "gametologous" genes (*ATP5A1*, *UBAP2*, *SPIN* and *HINT*) in neognathous birds: these are relic genes shared between homologous sex chromosomes as a result of the cessation of recombination (García-Moreno and Mindell 2000, de Kloet and de Kloet 2003, Handley et al. 2004). No W-specific forms of these genes have been reported in palaeognathous ratite birds having the extensively homomorphic Z and W chromosomes. We found sexual dimorphism of the *CHD1* gene in *E. elegans* in this study, indicating that recombination is suppressed around the *CHD1* locus between the Z and W chromosomes in *E. elegans,* leading to the nucleotide sequence divergence between the *CHD1Z* and *CHD1W* genes (Fig. 11). These data strongly suggest that the sex chromosome differentiation occurred around the *CHD1* locus independently in the Tinamiformes lineage after the divergence of the Palaeognathae and the Neognathae. The Z- and W-chromosomal forms have also been reported for the *SPIN* gene in four Tinamiformes species, including *E. elegans* (de Kloet 2002; de Kloet and de Kloet 2003). In *E. elegans*, the *SPIN* locus is contained in the W-specific heterochromatin region, and chromosomal recombination may be suppressed around this locus. However, no sex-specific products of the *SPIN* gene were obtained in this study.

Handley et al. (2004) proposed that there were at least two strata in the process of avian sex chromosome differentiation: recombination between Z and W chromosomes initially ceased around the small region partially differentiated from the ancestral homomorphic sex chromosome in the oldest stratum 102-170 MYA, before the split of Neoaves and Eoaves. The disruption of chromosome recombination in the second stratum occurred independently in the different lineages 58-85 MYA, at the time of the major radiation of the existing neognathous birds. Comparative FISH mapping of

chicken Z-linked genes to the Z and W chromosomes of *S. camelus* and *E. elegans* showed two chromosomal hybridization patterns of the genes: 1) hybridization signals were located on both the Z and W chromosomes, and 2) hybridization signals were detected on the Z chromosome but not on the W chromosome. Two possible evolutionary events might be responsible for the absence of hybridization signals on the W chromosome: 1) deletion of the chromosomal segment that contains the W homologues of the Z-linked genes; and 2) a decrease of hybridization efficiency due to divergence in the nucleotide sequence between the Z- and W-linked genes due to the suppression of recombination. Hybridization signals of *TMOD* and *ACO1/IREBP*, which are located near the centromere on the long arm of the Z chromosome, were not detected on the W chromosomes in these two species. In *S. camelus*, six other genes were localized to both the Z and W chromosomes, suggesting that differentiation due to a small deletion occurred in the proximal region of the W chromosome, as reported by Ogawa et al. (1998). In *E. elegans*, hybridization signals of *RPS6*, *NTRK2* and *PKCI* were detected on the Z chromosome but not on the W chromosome. This is indicative of a difference in the state of sex chromosome differentiation between *E. elegans* and *S. camelus*, whose lineages diverged around 83 MYA (van Tuinen and Hedges, 2001). Considering that the proximal half of the W chromosome of *E. elegans* is composed of heterochromatin and that the W chromosome is morphologically shorter than that of *S. camelus*, the absence of hybridization signals of *RPS6, NTRK2* and *PKCI* may be due to the deletion of about the proximal half of the euchromatic long arm of the ancestral W chromosome, and *E. elegans* is at an advanced stage of sex chromosome differentiation in comparison with *S. camelus*.

Multiple copies of the Z-linked *PKCI* gene are located on the W chromosome of *G.*

gallus, and their nucleotide sequences are divergent from that of the Z homologue. The *WPKCI/ASW* gene is highly expressed in the undifferentiated gonads of female chicken embryos (O'Neill et al. 2000, Hori et al. 2000), and it is consequently supposed that this gene has an important role in sex determination followed by gonadal differentiation. In palaeognathous ratites, no W-specific forms of the *PKCI* gene have been found, and this leads us to predict that the female-specific function of this gene was acquired in the lineage of neognathous birds along with the differentiation of sex chromosomes. The *PKCI* gene was localized to the same location between the Z and W chromosomes of *S. camelus* with the same hybridization efficiency in this study, suggesting that the genetic divergence might not have occurred between the Z- and W-linked *PKCI* in *S. camelus* (O'Neill et al. 2000). The presence of the W homologue of *PKCI* was not confirmed in *E. elegans*, either.

The hybridization signals of *SPIN* were detected on both the Z and W chromosomes in *S. camelus* and *E. elegans*, but the locations of the genes on the W chromosomes were different between the two species. The W homologue of *SPIN* was located around the center of the long arm in *S. camelus,* whereas it was mapped to the heterochromatin region near the centromere of the W chromosome in *E. elegans*. The *CHD1* gene of *E. elegans* was located just distal to the *SPIN* locus on the Z chromosome, while it was localized far from the *SPIN* locus, located near the centromere, on the W chromosome. The difference in the chromosomal location of *SPIN* and *CHD1* between the Z and W chromosomes in *E. elegans* suggests two possibilities: One is that the *SPIN* gene on the W chromosome translocated near the centromere via a paracentric inversion. The other is that the *SPIN* and *CHD1* loci on the W chromosome were kept apart by the *Bam*HI family of W-specific repetitive

sequences amplified between the two loci.

An early stage of W chromosome differentiation from the proto-sex chromosomes was clearly demonstrated in *S. camelus* in this study. This species retains a partially differentiated type of W chromosome in which the chromosomal deletion occurred from a region proximal to the centromere to a locus proximal to *RPS6-NTRK2-PKCI*. In *E. elegans*, the deletion occurred in a wider chromosomal region than in *S. camelus*. Chromosomal deletion advanced from a region near the centromere toward a distal region in *E. elegans*, and the deleted region consequently extended from the centromere to a locus proximal to *SPIN*. The absence of recombination in the deleted chromosome region accelerated the site-specific amplification of the W-specific EEL-*Bam*HI repeated sequence due to the absence of recombination subsequently occurring between the *SPIN* locus and the *CHD1* locus. However, the euchromatic region has been preserved between the *CHD1* locus and the distal end, and therefore the W chromosome differentiation in *E. elegans* is at a transitional stage between that in *S. camelus,* which has a partially deleted W chromosome, and neognathous birds, which have highly degenerated and heterochromatic W chromosomes. The number of genes localized to the sex chromosomes in the two species is still small to confirm this scenario of avian sex chromosome differentiation, and there is no information on the divergence of nucleotide sequences between Z- and W-linked genes except for the *SPIN* and *CHD1* genes of *E. elegans*. Further investigations will be needed to fully define the process of avian sex chromosome differentiation.

Acknowledgements

We express our appreciation to Yokohama Zoological Gardens, Yokohama, for providing skin and blood samples of elegant crested tinamou, and Kimiyuki Tsuchiya for skin samples of ostrich. This work was supported by Grants-in-Aid for Scientific Research (No.15370001 and No.16086201) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Figure legends

Figure 1

C-banded metaphase spreads of female (a) and male (b) *Eudromia elegans*. Scale bar = 10 μm.

Figure 2

Ethidium bromide-stained gel of female and male genomic DNA of *Eudromia elegans* digested with three endonucleases, *Bam*HI, *Bgl*II and *Bst*XI. Arrowheads indicate female-specific DNA bands. The female-specific DNA band in the *Bam*HI-digest was used for molecular cloning of repetitive DNA sequences. ϕX174 phage DNA digested with *Hae*III was used as a molecular size marker in the left lane.

Figure 3

Chromosomal distribution of the *Bam*HI repeated sequence on PI-stained metaphase spreads of female (a) and male (b) *Eudromia elegans.* The EEL-*Bam*HI 21 fragment was used as biotinylated probe. FISH signal of the repeated sequence is located on the proximal region of the W chromosome. Scale bar =10 μm.

Figure 4

Nucleotide sequences of the *Bam*HI repeated sequence elements isolated from *Eudromia elegans*. The consensus sequence of 19 fragments is indicated at the bottom. Internal restriction sites of seven endonucleases are indicated as follows: *Bam*HI (_____), *Hin*fI (_ _ _ _ _ _), *Bgl*II (.....), *Bst*XI (_____), *Hae*III (□) and *Hpa*II/*Msp*I (_ _ _ _ _).

Figure 5

Southern blot hybridization patterns of female *Eudromia elegans* genomic DNA digested with *Bam*HI, *Bgl*II, *Bst*XI, *Hae*III, *Hin*fI, *Hpa*II and *Msp*I. EEL-*Bam*HI 21 was labeled with digoxigenin-11-dUTP and used as probe. ϕ X174 phage DNA digested with *Hae*III was used as a molecular size marker.

Figure 6

Quantitative slot-blot hybridization with eight different concentrations of female *Eudromia elegans* genomic DNA and the EEL-*Bam*HI 21 fragment. EEL-*Bam*HI 21 was labeled with digoxigenin-11-dUTP and used for hybridization.

Figure 7

Slot-blot hybridization probed with digoxigenin-11-dUTP-labeled EEL-*Bam*HI 21 fragment. Genomic DNAs of 11 species that belong to five orders were used: EEL (*Eudromia elegans*) of the Tinamiformes, and DNO (*Dromaius novaehollandiae*), CCA (*Casuarius casuarius*), RAM (*Rhea americana*), PPE (*Pterocnemia pennata*) and SCA (*Struthio camelus*) of the Struthioniformes as palaeognathous birds. GGA (*Gallus gallus*), CJA (*Coturnix japonica*) and NME (*Numida meleagris*) of the Galliformes, GLE (*Grus leucogeranus*) of the Gruiformes and KBL (*Ketupa blakistoni*) of the Strigiformes as neognathous birds.

Figure 8

Chromosomal localization of Z- and W-linked genes in *Eudromia elegans* and *Struthio*

camelus by FISH. A. FISH mapping of *GHR* to *S. camelus* chromosomes (a) and *ACO1/IREBP* to *E. elegans* chromosomes (b, c). (a) The cDNA fragment of the *S. camelus GHR* gene was labeled with CyDye 3-dUTP and used for hybridization. The hybridization signals of *GHR* were located on both the Z and W chromosomes on Hoechst-stained metaphase spreads of *S. camelus*. (b, c) The cDNA fragment of *E. elegans ACO1/IREBP* was labeled with biotin-16-dUTP. The Alexa fluorescence signals of *ACO1/IREBP* were localized to the Z chromosome but not to the W chromosome on PI-stained metaphase spreads of *E. elegans* (b). Hoechst-stained pattern of the same metaphase spread is shown in (c). Arrows indicate hybridization signals. Scale bars =10 μm. B. Location of chicken Z-linked gene homologues on the Z and W chromosomes of *S. camelus* (upper row) and *E. elegans* (lower row). Alexa fluorescence signals were visualized on PI-stained chromosomes for *TMOD*, *ACO1/IREBP*, *RPS6, NTK2*, *PKCI, SPIN* and *CHD1* in *S. camelus* and *E. elegans*. Alexa signals of *GHR* were localized to PI-stained chromosomes of *E. elegans*, and Cy3 fluorescence signals of *GHR* and *ATP5A1* were localized to Hoechst-stained chromosomes of *S. camelus* and of *S. camelus* and *E. elegans*, respectively. Seven genes (*TMOD* is an exception) were localized to both the Z and W chromosomes in *S. camelus*. In *E. elegans*, FISH signals were detected on both the Z and W chromosomes for four genes, *SPIN*, *CHD1*, *GHR* and *ATP5A1*. The Z and W chromosomes in each photograph were sorted from the same metaphase spread.

Figure 9

Comparative cytogenetic maps of the Z and W chromosomes of *Struthio camelus* and *Eudromia elegans*, which were constructed with homologues of nine chicken (*Gallus*

gallus) Z-linked genes. The ideogram of the G-banded chicken Z chromosome was taken from the ARKdb (http://www.thearkdb.org/), and is arranged upside down to make the gene orders on the distal region of the chicken Z chromosome correspond to those on the Z chromosomes of *S. camelus* and *E. elegans*. The genes whose hybridization signals were detected on the Z chromosomes but not on the W chromosomes are indicated in red. The genes indicated in black were localized to both the Z and W chromosomes. The chromosomal locations of the *ACO1/IREBP* and *ZOV3* genes of *S. camelus* were taken from Ogawa et al. (1998).

Figure 10

Nucleotide sequences of the genomic DNA fragments of the *CHD1W* and *CHD1Z* genes obtained from one female and male each of *Eudromia elegans*. The nucleotide sequences of a partial exon 6, exon 7 and a partial exon 8 contained in the genomic DNA fragments are shaded. Hyphens indicate gaps found between the two fragments.

Table 1 Degenerate oligonucleotide primers used for cloning cDNA fragments of the chicken Z-linked gene homologues from*S. camelus* and *E. elegans* and temperatures at annealing step in PCR reaction

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Temperature at annealing step (ºC)	Accession number of cDNA fragments
ATP5A1	GAARACTGGCACHGCWGARRTRTCCTC	GGCAATBGADGTTTTSCCMGTCTGYCTGTC		AB254864, AB254880
	CCATTGGYCGKGGYCAGCRTGAGCTSATYA	GCRGACACATCACCMGCCTGYGTTTC	58	AB254865, AB254881
	CGYCTKCTGGARAGAGCAGCBAARATG	CTGKTCWGAGATYTTSCCMTCAGWCCTG		AB254866, AB254882
CHD1	TCAYGARCATCARYTGTATGGVCCTT	CTGCTAGGATGTCCAGCATCC	55	AB254867, AB254883
	GTTACTGATTCGTCTACGAGA[a]	TCTGCATCGCTAAATCCTTT[b]		AB254868, AB254884
GHR	ATGGATCTTCGGCAKCTGYTGYTTA	ACTTCTTTGTACTGCAATTCATACTCCAG		AB254869, AB254885
	CCCCCTGTSCAYCTTAACTGGACTCTGC	AGATCTGGGTCAATCCCTTTAATCTTTGGA	58	AB254870, AB254886
	TGAGTTTATTGAGYTGGAYATWGAYGA[c]	GCTAHGGCAKGATTTTGTTCAGTTGG		AB254871
	AARGATGATGAYTCTGGACGWGCCAG[d]	GCTAHGGCAKGATTTTGTTCAGTTGG		AB254887
NTRK2	ATYCCWGTCATYGARAAYCCMCAGTA[c]	TGYTGKGANGCCAGGTAVACCATDCC		AB254872
	GABGAYTCWGCCAGYCCHCTSCAYCA[d]	TGYTGKGANGCCAGGTAVACCATDCC	58	AB254888
	GGBGAYCCMCTCATCATGGTYTTTGA	GGYTCYCKYTGCCARCANCCCAGCATST		AB254873, AB254889
PKCI	CACSATCTTYGGSAAGATYATCCGCA	CCAGGAGGCCARYBCAWYTGVCGACC	55	AB254874, AB254890
	CAAGCTCCAACACATTTCCTAG[e]	————————		AB254875, AB254891
RPS6	GAAGCTHAAYATCTCTTTCCCWGCCA[c]	GGCCTCCTTCATTCTCTTTG		AB254876
	CACTGGCTGCCAGAAGCTCAT[d]	GGCCTCCTTCATTCTCTTTG	55	AB254892
	CAGGACCAAGGCTCCTAAGA[e]	————————		AB254877, AB254893
SPIN	GCAGAATACAGCATGGATGG	TCTAGGATGTYTTCACCAARTCRTAGAC	55	AB254878, AB254894
TMOD	CTRGAGAAATAYCGDGACCTGGATG	CTCTTCCTCACWAGGTCRTTGTTRTTC		AB254879[f]
	CTRGAGAAATAYCGDGACCTGGATG	CATGCCMAGRATVGCWGCAATGTCACA[d]	58	AB254895
	GGCATGCACACSYTSATGAGYAACCAGC[d]	CTCTTCCTCACWAGGTCRTTGTTRTTC		AB254896
ACO1/IREBP	GACAGYTTRCARAAGAATCARGAY[d, g]	CCYTTRAATCCTTGCTTNGYTCC[d, g]	48	AB254897
	GTGCTCACYRTNACNAAGCACCT[d, g]	AGGTCTCCCTGNGTDATNGCYTC[d, g]		AB254898

[a]Fridolfsson and Ellegren (1999)
[b]Griffiths et al. (1996)
[c]Primers used only for *S. camelus*
[d]Primers used only for *E. elegans*
[e]Primers used for 3'RACE
[f]The PCR product was obtained only from *S.camelus*
[g]Matsuda et al. (2005)

Table 2 The length of cDNA fragments of the chicken Z-linked gene homologues cloned from *S. camelus* and *E. elegans*

Gene symbol	Gene name	Length of cDNA fragment (bp) and accession number	
		S. camelus	*E. elegans*
ATP5A1	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle	1419[a] (AB254864-254866)	1419[a] (AB254880-254882)
CHD1	chromodomain helicase DNA binding protein 1	1771[a] (AB254867-254868)	1727[a] (AB254883-254884)
GHR	growth hormone receptor	1643[a] (AB254869-254871)	1544[a] (AB254885-254887)
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	806[a] (AB254872-254873)	905[a] (AB254888-254889)
PKCI	protein kinase C inhibitor	498[a] (AB254874-254875)	492[a] (AB254890-254891)
RPS6	ribosomal protein S6	786[a] (AB254876-254877)	747[a] (AB254892-254893)
SPIN	spindlin	580 (AB254878)	580 (AB254894)
TMOD	tropomodulin 1	901 (AB254879)	903[a] (AB254895-254896)
ACO1/IREBP	soluble aconitase 1 /iron-responsive element binding protein	-	1145[a] (AB254897-254898)

[a]Total length of cDNA fragment concatenated by multiple PCR products

Table 3 Nucleotide sequence identities of cDNA fragments of nine Z-linked genes among *G. gallus* (GGA), *S. camelus* (SCA) and *E. elegans* (EEL)

	Identity (%)[a]		
Gene symbol	GGA-SCA	GGA-EEL	SCA-EEL
ATP5A1	92.7 (1316/1419)	91.5 (1298/1419)	94.9 (1347/1419)
CHD1	92.4 (1636/1771)	92.4 (1595/1727)	94.0 (1624/1727)
GHR	89.2 (1466/1643)	85.7 (1323/1544)	90.5 (1397/1544)
NTRK2	93.4 (753/806)	92.4 (836/905)	96.3 (776/806)
PKCI	89.4 (445/498)	91.9 (452/492)	95.9 (472/492)
RPS6	93.2 (717/769)	93.2 (696/747)	97.7 (730/747)
SPIN	97.8 (567/580)	97.1 (563/580)	98.3 (570/580)
TMOD	90.1 (812/901)	84.5 (763/903)	86.9 (738/849)
ACO1/IREBP	-	90.3 (1034/1145)	-

[a]The number in parenthesis indicates the number of identical bases / the number of bases in overlapped region between cDNA fragments of two species

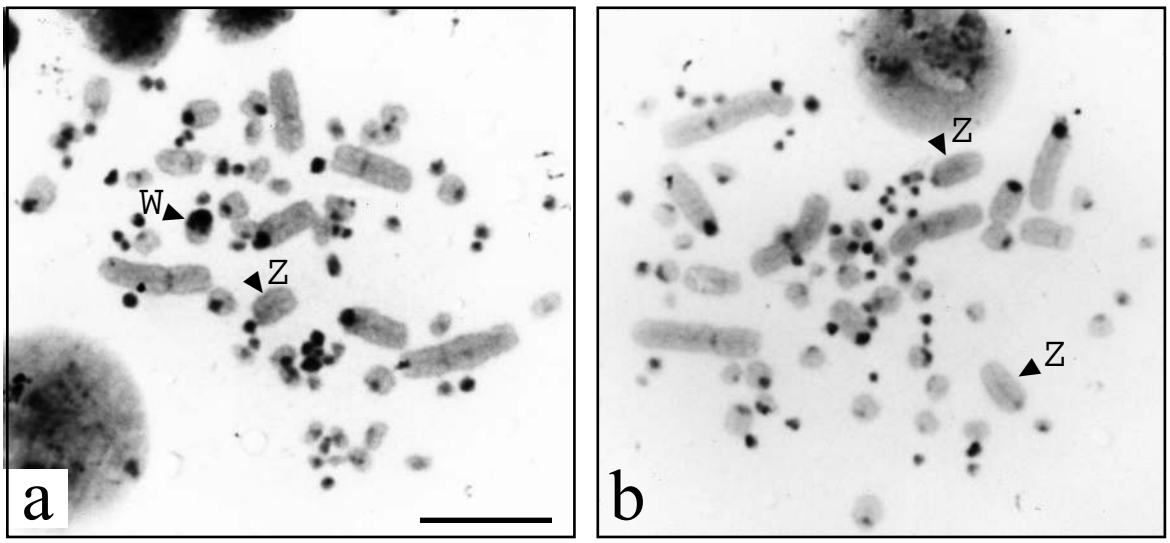

Figure 1

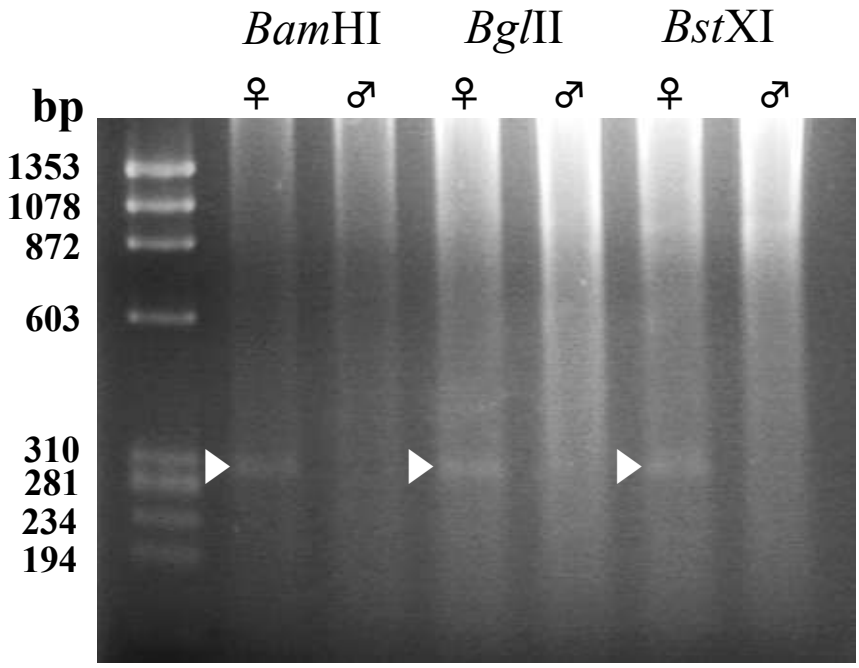

Figure 2

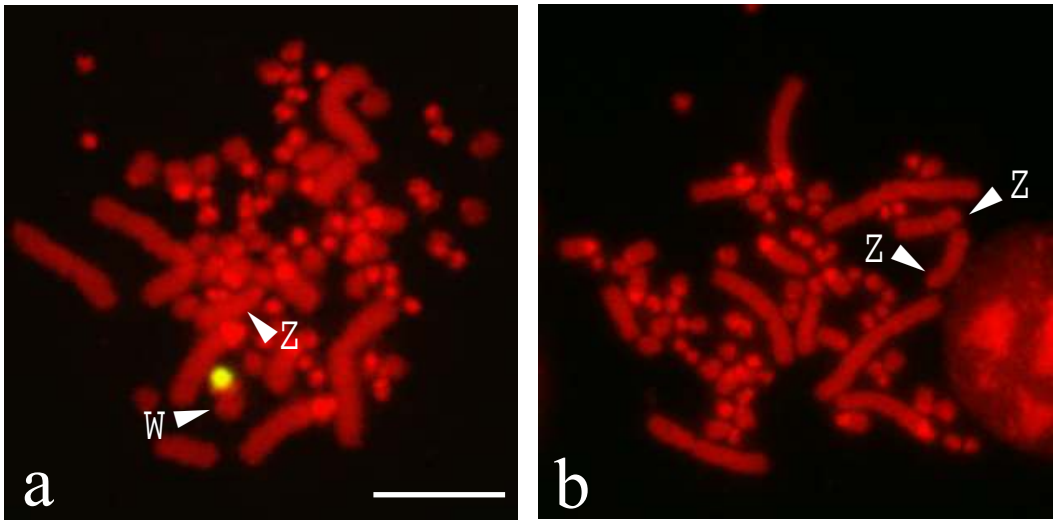

Figure 3

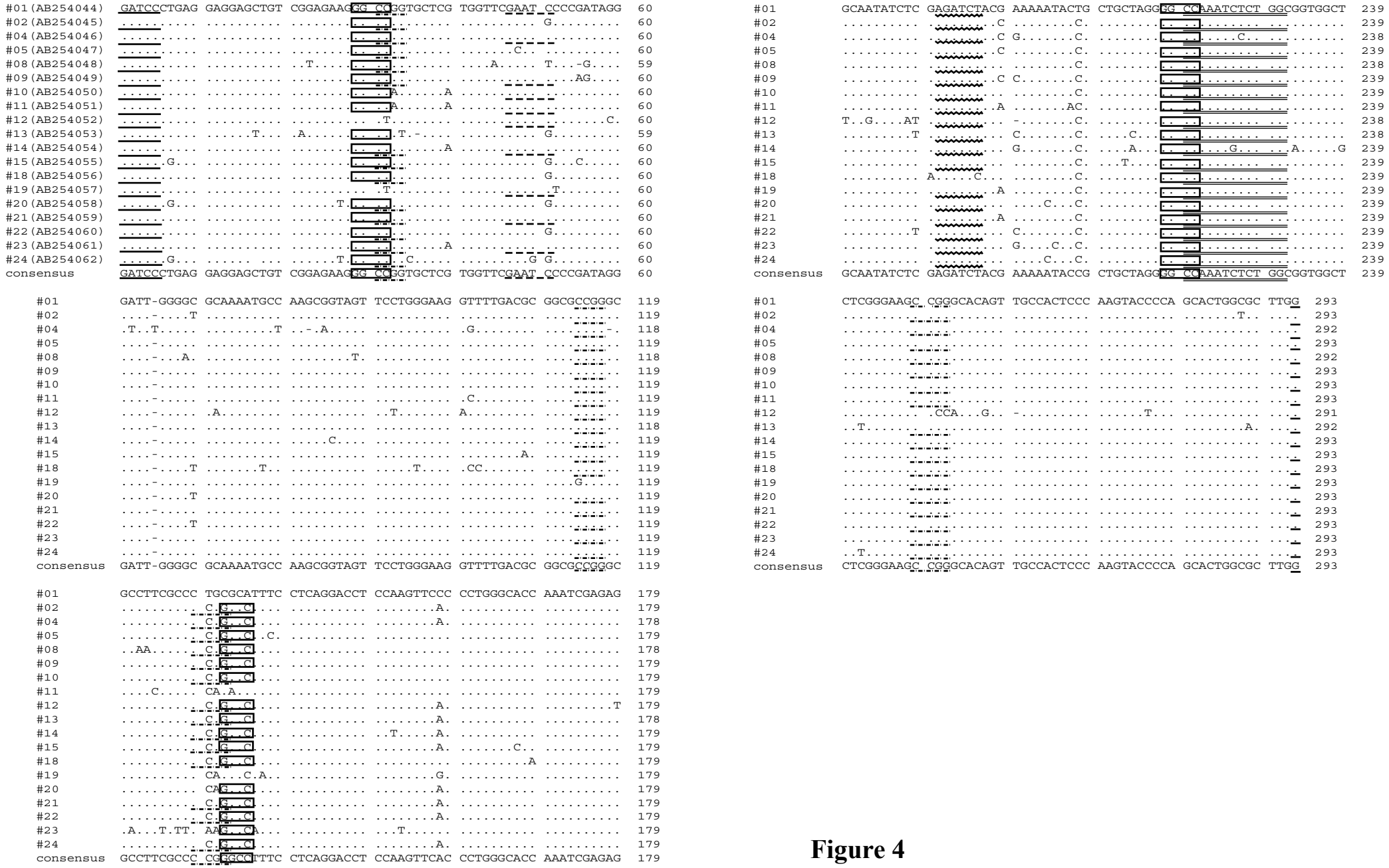

Figure 4

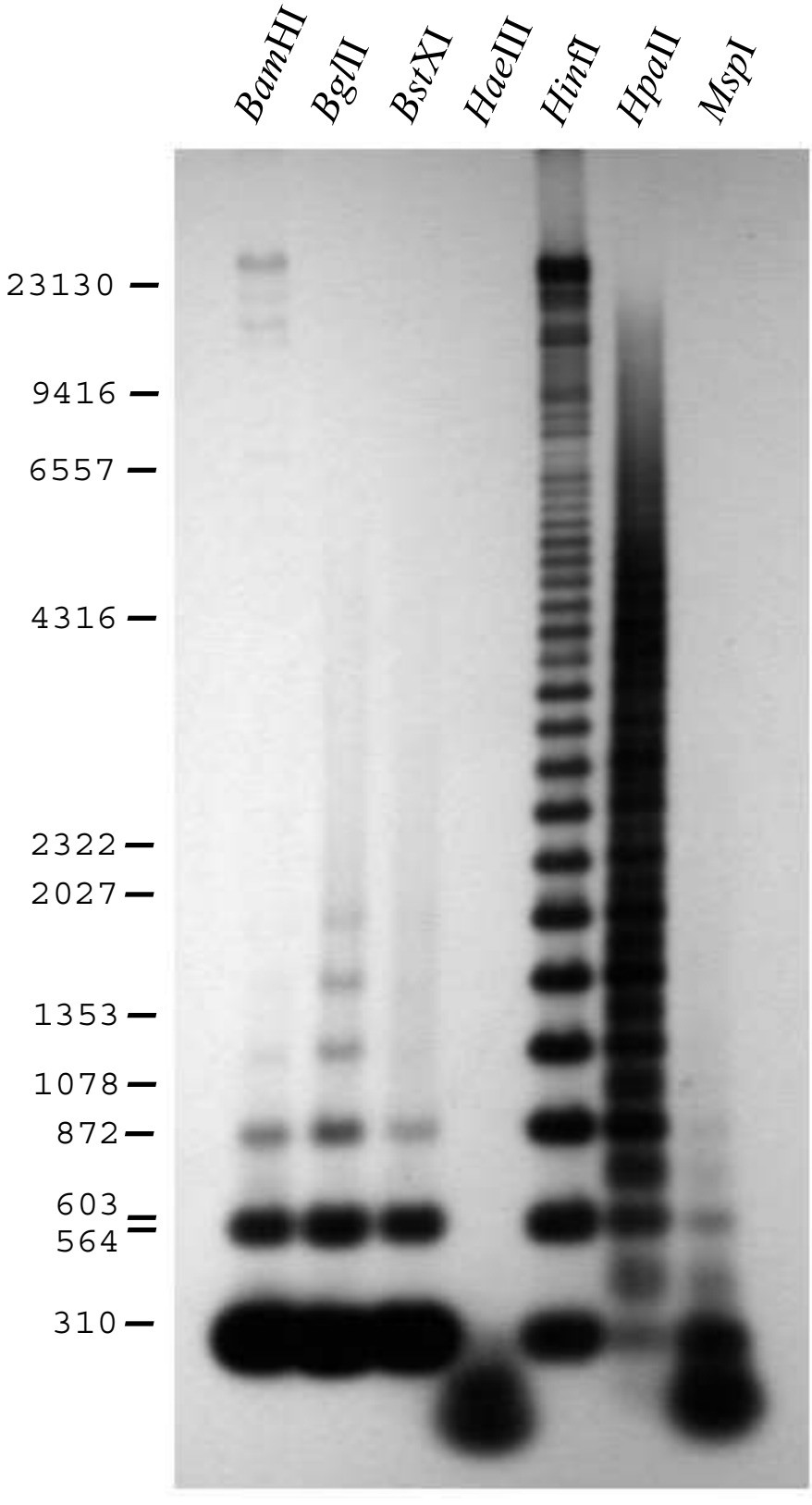
BamHI
BglII
BstXI
HaeIII
HinfI
HpaII
MspI
23130
9416
6557
4316
2322
2027
1353
1078
872
603
564
310

Figure 5

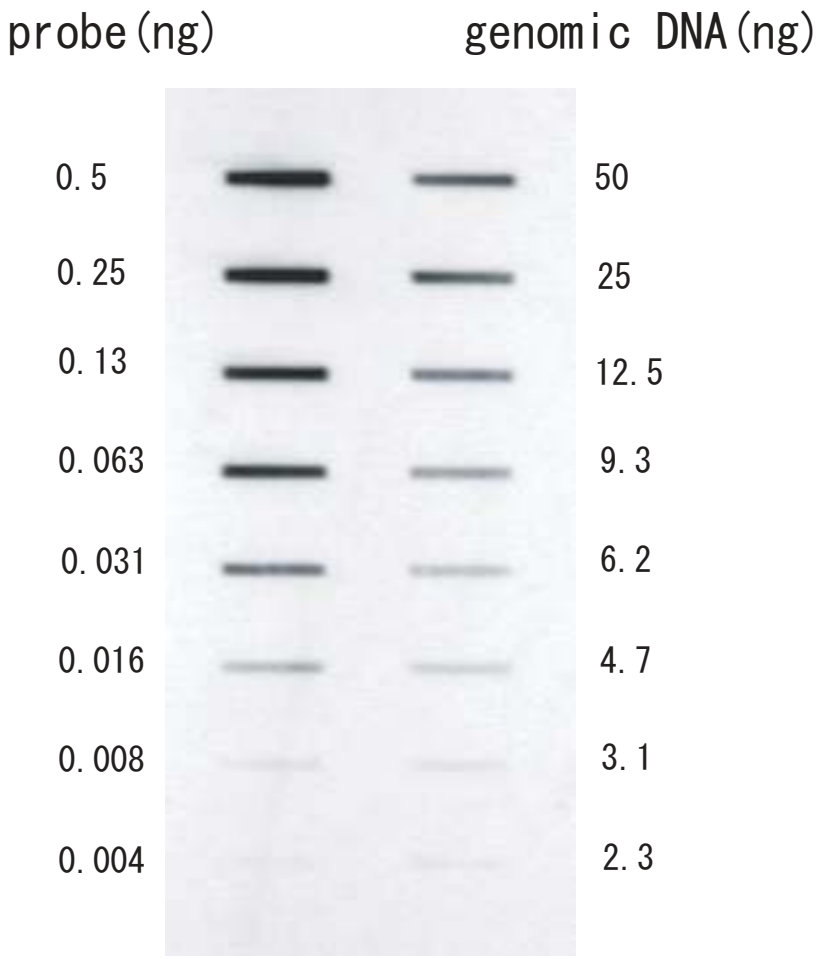
probe(ng)
genomic DNA(ng)
0.5
0.25
0.13
0.063
0.031
0.016
0.008
0.004
50
25
12.5
9.3
6.2
4.7
3.1
2.3

Figure 6

EEL♀ EEL♂

DNO♀ DNO♂ CCA♀ CCA♂

RAM♀ RAM♂ PPE♀ PPE♂ SCA♀ SCA♂

GGA♀ GGA♂ CJA♀ CJA♂ NME♀ NME♂

GLE♀ GLE♂ KBL♀ KBL♂

Figure 7

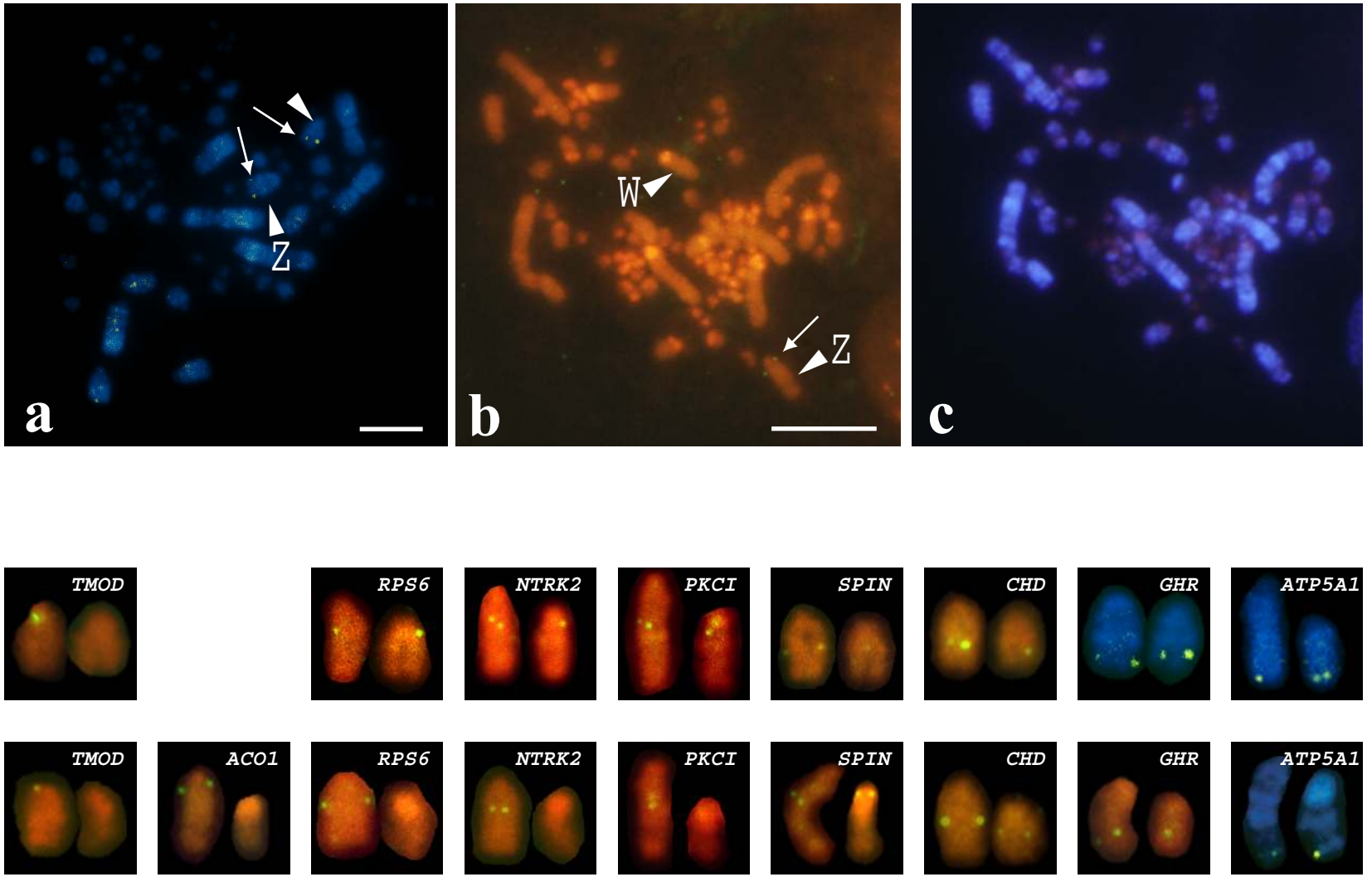
a
Z
b
W
Z
c
TMOD
RPS6
NTRK2
PKCI
SPIN
CHD
GHR
ATP5A1
TMOD
ACO1
RPS6
NTRK2
PKCI
SPIN
CHD
GHR
ATP5A1

Figure 8

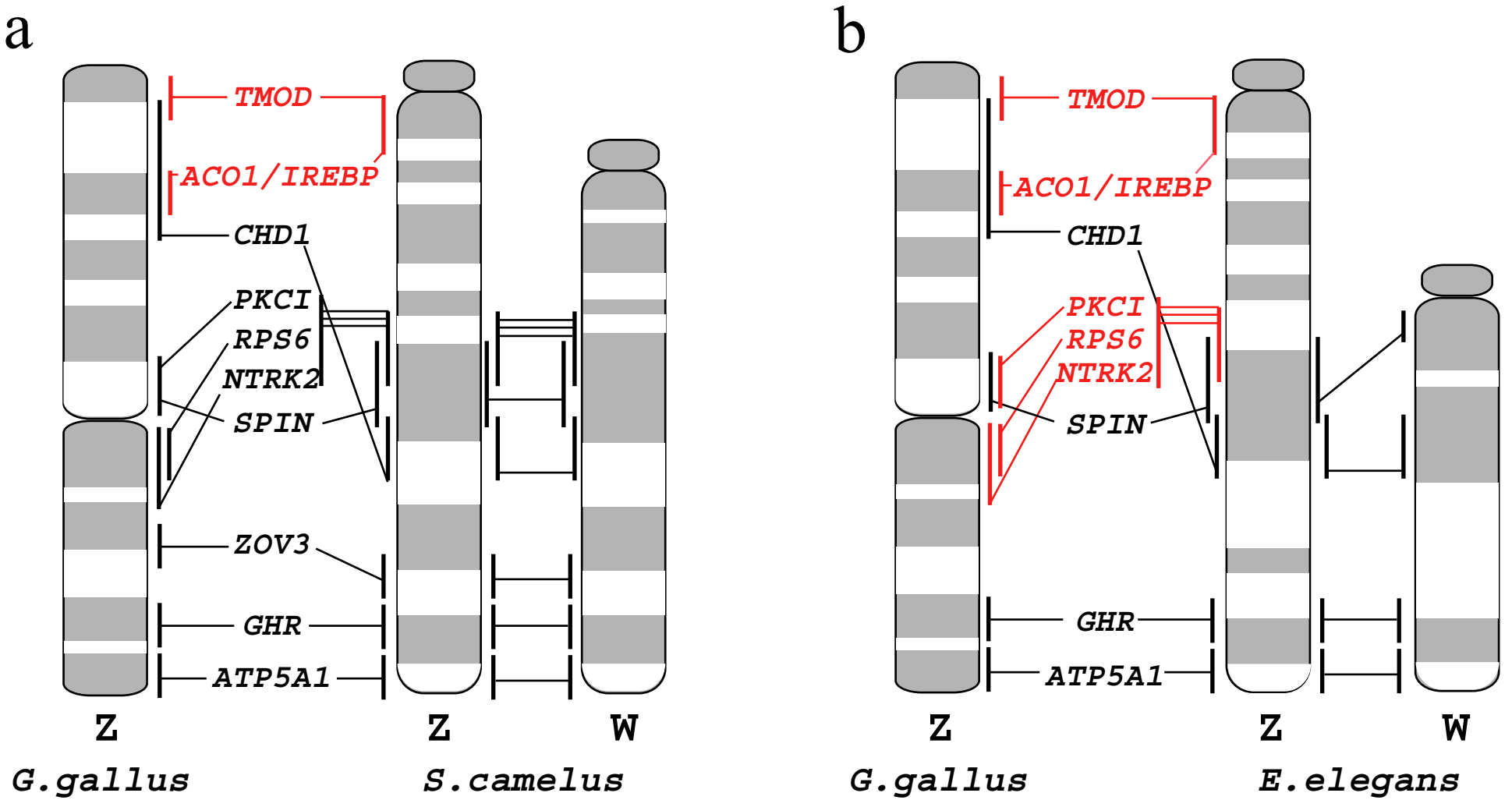
a
TMOD
ACO1/IREBP
CHD1
PKCI
RPS6
NTRK2
SPIN
ZOV3
GHR
ATP5A1
Z
Z
W
G.gallus
S.camelus
b
TMOD
ACO1/IREBP
CHD1
PKCI
RPS6
NTRK2
SPIN
GHR
ATP5A1
Z
Z
W
G.gallus
E.elegans

Figure 9

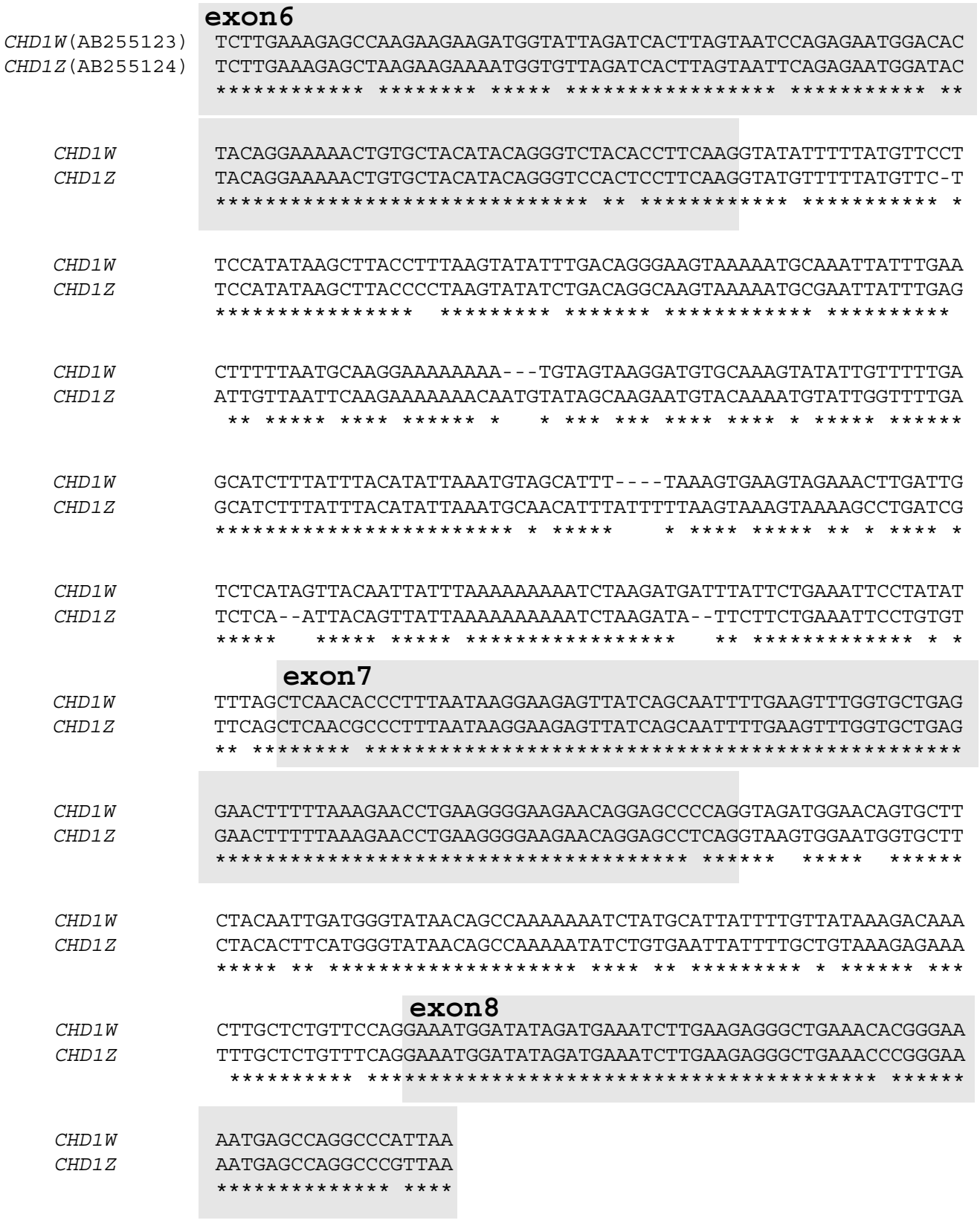
exon6
CHD1W(AB255123) TCTTGAAAGAGCCAAGAAGAAGATGGTATTAGATCACTTAGTAATCCAGAGAATGGACAC
CHD1Z(AB255124) TCTTGAAAGAGCTAAGAAGAAAATGGTGTTAGATCACTTAGTAATTCAGAGAATGGATAC
CHD1W TACAGGAAAAACTGTGCTACATACAGGGTCTACACCTTCAAGGTATATTTTTATGTTCCT
CHD1Z TACAGGAAAAACTGTGCTACATACAGGGTCCACTCCTTCAAGGTATGTTTTTATGTTC-T
CHD1W TCCATATAAGCTTACCTTTAAGTATATTTGACAGGGAAGTAAAAATGCAAATTATTTGAA
CHD1Z TCCATATAAGCTTACCCCTAAGTATATCTGACAGGCAAGTAAAAATGCGAATTATTTGAG
CHD1W CTTTTTAATGCAAGGAAAAAAAA---TGTAGTAAGGATGTGCAAAGTATATTGTTTTTGA
CHD1Z ATTGTTAATTCAAGAAAAAAACAATGTATAGCAAGAATGTACAAAATGTATTGGTTTTGA
CHD1W GCATCTTTATTTACATATTAAATGTAGCATTT----TAAAGTGAAGTAGAAACTTGATTG
CHD1Z GCATCTTTATTTACATATTAAATGCAACATTTATTTTTAAGTAAAGTAAAAGCCTGATCG
CHD1W TCTCATAGTTACAATTATTTAAAAAAAAATCTAAGATGATTTATTCTGAAATTCCTATAT
CHD1Z TCTCA--ATTACAGTTATTAAAAAAAAAATCTAAGATA--TTCTTCTGAAATTCCTGTGT
exon7
CHD1W TTTAGCTCAACACCCTTTAATAAGGAAGAGTTATCAGCAATTTTGAAGTTTGGTGCTGAG
CHD1Z TTCAGCTCAACGCCCTTTAATAAGGAAGAGTTATCAGCAATTTTGAAGTTTGGTGCTGAG
CHD1W GAACTTTTTAAAGAACCTGAAGGGGAAGAACAGGAGCCCCAGGTAGATGGAACAGTGCTT
CHD1Z GAACTTTTTAAAGAACCTGAAGGGGAAGAACAGGAGCCTCAGGTAAGTGGAATGGTGCTT
CHD1W CTACAATTGATGGGTATAACAGCCAAAAAAATCTATGCATTATTTTGTTATAAAGACAAA
CHD1Z CTACACTTCATGGGTATAACAGCCAAAAATATCTGTGAATTATTTTGCTGTAAAGAGAAA
exon8
CHD1W CTTGCTCTGTTCCAGGAAATGGATATAGATGAAATCTTGAAGAGGGCTGAAACACGGGAA
CHD1Z TTTGCTCTGTTTCAGGAAATGGATATAGATGAAATCTTGAAGAGGGCTGAAACCCGGGAA
CHD1W AATGAGCCAGGCCCATTAA
CHD1Z AATGAGCCAGGCCCGTTAA

Figure 10