A novel family of repetitive DNA sequences amplified site-specifically on the W chromosomes in Neognathous birds

Kazuhiko Yamada[1], Chizuko Nishida-Umehara[1, 2], Junko Ishijima[1], Takahiro Murakami[1], Mami Shibusawa[1], Kimiyuki Tsuchiya[3], Masaoki Tsudzuki[4] and Yoichi Matsuda[1, 2] *

[1] *Laboratory of Animal Cytogenetics, Division of Genome Dynamics, Creative Research Initiative "Sousei", Hokkaido University, North 10 West 8, Kita-ku, Sapporo 060-0810, Japan; Fax:81-11-736-6304; E-mail:yoimatsu@ees.hokudai.ac.jp;* [2] *Division of Biological Sciences, Graduate School of Science, Hokkaido University, North 10 West 8, Kita-ku, Sapporo 060-0810, Japan;* [3] *Laboratory of Wild Animals, Department of Zootechnical Sciences, Faculty of Agriculture, Tokyo University of Agriculture, Atsugi 234-0034, Japan;* [4] *Laboratory of Animal Breeding and Genetics, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Hiroshima, Japan*

**Correspondence*

Key words: W chromosome, repetitive DNA sequence, Japanese quail, guinea fowl, Neognathous birds

Abstract

A novel family of repetitive DNA sequences were molecularly cloned from *Apa*I-digested genomic DNA of two Galliformes species, Japanese quail (*Coturnix japonica*) and guinea fowl (*Numida meleagris*), and characterized by chromosome *in-situ* hybridization and filter hybridization. Both the repeated sequence elements produced intensely painted signals on the W chromosomes, whereas they weakly hybridized to whole chromosomal regions as interspersed-type repetitive sequences. The repeated elements of the two species had high similarity of nucleotide sequences, and cross-hybridized to chromosomes of two other Galliformes species, chicken (*Gallus gallus*) and blue-breasted quail (*Coturnix chinensis*). The nucleotide sequences were conserved in three other orders of Neognathous birds, the Strigiformes, Gruiformes and Falconiformes, but not in Palaeognathous birds, the Struthioniformes and Tinamiformes, indicating that the repeated sequence elements were amplified on the W chromosomes in the lineage of Neognathous birds after the common ancestor diverged into the Palaeognathae and Neognathae. They are components of the W heterochromatin in Neognathous birds, and a good molecular cytogenetic marker for estimating the phylogenetic relationships and for clarifying the origin of the sex chromosome heterochromatin and the process of sex chromosome differentiation in birds.

Introduction

The avian species are phylogenetically classified into two primary clades based on the palatal form, Palaeognathous birds (the Palaeognathae) and Neognathous birds (the Neognathae), which diverged at the first stage in the evolution of modern birds. Palaeognathous birds, the ratites and tinamous, are monophyletic, and all the remaining carinate species belong to Neognathous birds. This phylogenetic relationship of the two lineages has also been confirmed at the molecular level by DNA-DNA hybridization and analyses of nuclear and mitochondrial ribosomal RNA gene sequences (Sibley & Ahlquist 1990, van Tuinen *et al*. 1998, 2000). Birds have the ZZ male and ZW female type of sex chromosomes. In most of the carinates, Z chromosomes are the fourth to the sixth largest chromosomes, accounting for about 7% of the genome (Ohno 1967, Takagi & Sasaki 1974), whereas the W chromosomes are usually small, heterochromatic and late replicating (Takagi 1972, Schmid *et al.* 1989). By contrast, the morphological differentiation of the W chromosomes is less distinct in the ratites, and the heterochromatization does not occur either (Takagi *et al.* 1972, de Boer 1980, Ansari *et al.* 1988, Nishida-Umehara *et al.* 1999). Large homologies between the Z and W chromosomes have been revealed in the ratites by comparative chromosome mapping, comparative chromosome painting (ZOO-FISH) and cytogenetic studies on meiotic chromosome pairing of the Z and W chromosomes; however, there are partially non-homologous regions between the Z and W chromosomes (Pigozzi & Solari 1997, 1999, Ogawa *et al.* 1998, Nishida-Umehara *et al.* 1999, Shetty *et al.* 1999). Tinamous are positioned as the sister group of the ratites in the Palaeognathae (van Tuinen *et al.* 1998, 2000, van Tuinen & Hedges 2001, Cracraft 2001). Heterochromatization of W chromosomes has occurred in tinamous, elegant crested tinamou (*Eudromia elegans*), red-winged tinamou (*Rynchotus rufescens*) and

spotted tinamou (*Nothura maculosa*), in which half to two-thirds of the W chromosomes are heterochromatic, although there is little morphological difference between the Z and W chromosomes (Sasaki *et al.* 1980, Pigozzi & Solari 1999, 2005).

Isolation of W chromosome-specific repetitive sequences and their molecular and cytogenetic characterization provides important information on the process and mechanism of avian sex chromosome differentiation. In chicken (*Gallus gallus*), three W-specific repeated sequence families have been isolated as components of the W-heterochromatin and molecularly characterized, i.e., the *Xho*I-family (Tone *et al.* 1982, 1984, Kodama *et al.* 1987), *Eco*RI-family (Saitoh *et al*. 1991, Saitoh & Mizuno 1992) and *Ssp*I-family sequences (Saitoh & Mizuno 1992, Itoh & Mizuno 2002). The copy numbers of the three repeated sequence families are large, and these sequences amount to 70% of the total DNA of the chicken W chromosome (Itoh & Mizuno 2002). Repeated sequence families that are similar to the *Xho*I-family sequence of *G. gallus* have also been isolated from turkey (*Meleagris gallopavo*) and Japanese common pheasant (*Phasianus versicolor*), and are called *Pst*I- and *Taq*I-family sequences, respectively (Saitoh *et al*. 1989). The W-specific repetitive sequences are highly diverged between different species as rapidly evolved molecules, and therefore, they are good molecular cytogenetic markers for estimating phylogenetic relationships in birds. However, molecular cloning of repetitive sequences on the W chromosome is limited to the Phasianidae species, and more extensive studies are necessary to clarify their origins and the process of nucleotide sequence divergence.

Here we report a novel family of repetitive sequences isolated from guinea fowl (*Numida meleagris*) and Japanese quail (*Coturnix japonica*). Nucleotide sequence analysis and chromosome *in-situ* hybridization revealed that they are novel interspersed-type repetitive sequences and amplified site-specifically on the W

chromosomes. The novel repetitive sequence family is categorized as a new type of W-heterochromatin sequences: they are highly conserved through the different orders of Neognathous birds, while they do not cross-hybridize to the genomic DNA of Palaeognathous birds.

Materials and methods

Specimens, cell culture and chromosome preparation

The fibroblast cells collected from skin tissue of *C. japonica* were cultured in 199 medium supplemented with 15% fetal bovine serum at 39˚C in 5% CO_2. The peripheral lymphocytes of *N. meleagris* were cultured for 45 h in RPMI 1640 medium containing 15% fetal bovine serum, 1% phytohemagglutinin (HA15, Murex), 3 μg/ml concanavalin A (Sigma) and 10 μg/ml lipopolysaccharide (Sigma) (Suzuki *et al*. 1999). The cells were harvested after colcemid treatment for 1 h. The cells were suspended in 0.075 M KCl, fixed in 3:1 methanol : acetic acid, then dropped on glass slides and air-dried. Slides were kept at -80˚C until use.

Molecular cloning

Genomic DNA was extracted from the cultured fibroblast cells of *C. japonica* and the whole blood cells of *N. meleagris* using standard techniques (Sambrook *et al.* 1989). The genomic DNA was digested with 26 restriction endonucleases, *Apa*I, *Ase*I, *Bam*HI, *Bgl*I, *Bgl*II, *Bst*XI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hpa*II, *Hin*dIII, *Hin*fI, *Mlu*I, *Msp*I, *Nsi*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Sal*I, *Sau*3AI, *Sma*I, *Taq*I, *Xba*I and *Xho*I, size fractionated by 1% and 3% agarose gel electrophoresis, and stained with ethidium bromide. The prominent DNA bands detected thereby were eluted from the gel using a

SUPREC™-01 (Takara), cloned into pBluescript II vector (Stratagene), and transferred into TOP10 *Escherichia coli* competent cells (Invitrogen). The sizes of genomic DNA fragments inserted in the clones were confirmed by electrophoresis of the PCR products that were amplified with T3 and T7 primers, and the clones were used to perform fluorescence *in-situ* hybridization (FISH).

FISH

FISH was performed as described by Matsuda & Chapman (1995). DNA clones 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 FITC-labeled streptavidin (Amersham), and chromosomes were stained with propidium iodide. The FISH images were captured with the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK) using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope.

Nucleotide sequencing

The cloned fragments whose fluorescence signals were detected on the W chromosomes were used for nucleotide sequencing. Nucleotide sequences were determined using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham) with Texas Red-labeled T3 and T7 primers and a SQ-5500 DNA sequencer (Hitachi). The nucleotide sequence analysis was performed with the computer software of GeneWorks (Intelligenetics).

Southern blot hybridization

The genomic DNA extracted from four Galliformes species, *C. japonica*, *N. meleagris*, *G. gallus* and blue-crested quail (*Coturnix chinensis*), was digested with *Apa*I. The restriction-digested genomic DNA was fractionated on 1% agarose gels by horizontal gel electrophoresis, and the DNA fragments were transferred onto Hybond N^+ nylon membranes (Amersham). The probes were labeled with digoxigenin-11-dUTP using a PCR DIG Labeling Mix (Roche Diagnostics) and hybridized to the membranes overnight at 42°C in DIG Easy Hyb solution (Roche Diagnostics). The membranes were 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 Anit-Digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics), and exposed to BioMax MS Autoradiography Film (Kodak). To examine the genomic organization of the repetitive sequences in detail, the genomic DNA of *C. japonica* and *N. meleagris* was digested with seven endonucleases, *Bam*HI, *Bgl*II, *Eco*RI, *Hae*III, *Hin*fI, *Hpa*II and *Msp*I, and subjected to Southern blot hybridization.

Slot-blot hybridization

Slot-blot hybridization was used for examining the nucleotide sequence conservation of the repetitive sequences among different species and for estimating the amount of the repetitive sequences in the genomes of *C. japonica* and *N. meleagris*. Genomic DNA was collected from 13 species of six orders; emu (*Dromaius novaehollandiae*) and lesser rhea (*Pterocnemia pennata*) of the Struthioniformes, elegant crested tinamou (*E. elegans*) of the Tinamiformes, *C. chinensis*, *C. japonica*, *G. gallus* and *N. meleagris* of the Galliformes, Blakiston's fish-owl (*Ketupa blakistoni*) and Eurasian eagle owl (*Bubo bubo*) of the Strigiformes, Siberian white crane (*Grus leucogeranus*) and hooded

crane (*Grus monacha*) of the Gruiformes, and Japanese mountain hawk eagle (*Spizaetus nipalensis*) and crested serpent eagle (*Spilornis cheela*) of the Falconiformes. Genomic DNA was extracted from the whole blood cells except in the cases of *B. bubo*, *S. nipalensis* and *S. cheela*, in which DNA was extracted from the cultured fibroblast cells. The genomic DNA was denatured with NaOH, and transferred onto a Hybond N^+ nylon membrane using a BIO-DOT SF blotting equipment (Bio-Rad). The DNA probe was labeled with digoxigenin-11-dUTP using a PCR DIG Labeling Mix, and hybridized to the membrane at 42˚C in DIG Easy Hyb solution. The chemiluminescent signals were detected using the same procedure used in the case of Southern blot hybridization.

For estimating the amount of the repetitive sequences in the genome, five different concentrations of female genomic DNA, male genomic DNA and PCR products of the repeated sequence element were prepared for *C. japonica* and *N. meleagris*, and used for slot-blot hybridization. The intensities of the hybridization bands on the membrane were estimated and compared using a public domain image processing and analysis program, NIH image (http://rsbweb.nih.gov/nih-image/).

Results

Molecular cloning of repetitive sequences

Genomic DNA of female *C. japonica* and *N. meleagris* was digested with 26 restriction endonucleases, and the prominent DNA bands with the sizes of about 1.1 kb and 2.3 kb in the *Apa*I-digest were isolated for *C. japonica* and *N. meleagris*, respectively (Figure 1). Nine and ten clones were obtained from *C. japonica* and *N. meleagris*, respectively, and used for FISH analysis.

Chromosomal distribution

Painted fluorescence signals were detected on the W chromosomes for two clones of *C. japonica* (CJA-*Apa*I 1 and CJA-*Apa*I 4) and two clones of *N. meleagris* (NME-*Apa*I 9 and NME-*Apa*I 10), while faint signals were also distributed in the whole genome (Figures 2a, b). These results revealed that the DNA fragments were interspersed-type repetitive sequences and amplified site-specifically on the W chromosomes. Two clones of *N. meleagris* hybridized to the interstitial heterochromatic region on the long arm of chromosome 4 (data not shown). No fluorescence signals were detected for the remaining seven clones of *C. japonica* and six clones of *N. meleagris*. In *N. meleagris*, a fluorescence signal was additionally observed on one microchromosome besides the W chromosome (Figure 2b). The CJA-*Apa*I 1 and NME-*Apa*I 9 fragments interspecifically cross-hybridized to the W chromosomes of *N. meleagris* and *C. japonica*, respectively (data not shown). Fluorescence signals were also observed on the W chromosomes of *G. gallus* and *C. chinensis* using NME-*Apa*I 9 as a probe (Figures 2c, d).

Variations of the chromosomal distribution of the NME-*Apa*I element were examined for an additional two females and three males of *N. meleagris* collected from the same breeding colony. Signals on one microchromosome and two microchromosomes were detected in one male and female each and in one female, respectively, while there were no signals on microchromosomes in one male and one female (Figure 3). The repeated sequence element was also amplified on microchromosomes besides the W chromosome in *N. meleagris*; however, the site-specific amplification on microchromosomes varied between individuals.

Nucleotide sequences

The sizes and G+C contents were 1054 bp and 50.2% for CJA-*Apa*I 1 (Accession No. AB189143), 1048 bp and 49.7% for CJA-*Apa*I 4 (AB189144), 2335 bp and 50.8% for NME-*Apa*I 9 (AB189146), and 2335 bp and 50.4% for NME-*Apa*I 10 (AB189147) (Figure 4). The nucleotide sequence similarities were 96.5% between CJA-*Apa*I 1 and CJA-*Apa*I 4, and 97.6% between NME-*Apa*I 9 and NME-*Apa*I 10. There were also high interspecific similarities of the nucleotide sequences between the CJA elements (CJA-*Apa*I 1 and CJA-*Apa*I 4) and the NME elements (NME-*Apa*I 9 and NME-*Apa*I 10) in the overlapped regions, ranging from 81.5% to 82.3%. The nucleotide sequences at positions 1049-1054 of NME-*Apa*I 9 and at positions 1048-1053 of NME-*Apa*I 10 were 'GGGCCT', and the nucleotide sequences at the same position of CJA-*Apa*I 1 and CJA-*Apa*I 4 were 'GGGCCC', which was the sequence of the *Apa*I restriction site. The difference in the size between the CJA-*Apa*I and NME-*Apa*I elements was caused by the *Apa*I restriction sites that were newly produced by base substitutions in the repeated sequence elements of *C. japonica*. The two CJA clones isolated in this study were therefore partial fragments of the elements that were equivalent to NME-*Apa*I 9 and NME-*Apa*I 10 in *N. meleagris*.

The nucleotide sequences of CJA-*Apa*I 1 and NME-*Apa*I 9 were searched for homology with the DDBJ database (http://www.ddbj.nig.ac.jp). Significant homology was found between CJA-*Apa*I 1 and an anonymous genomic DNA clone of *G. gallus* (AC145927), with 82.6% identity in a 1056-bp overlap. There were no nucleotide sequences with significant homology beyond this clone.

Genomic organization of the repeated sequence elements

The NME-*Apa*I 9 fragment was used as a probe for Southern blot hybridization

with *Apa*I-digested genomic DNA of female individuals of four Galliformes species, *G. gallus*, *C. japonica*, *C. chinensis* and *N. meleagris* (Figure 5). The genomic DNA of *N. meleagris* was obtained from the individual with the hybridization signal on one microchromosome. Intensely hybridizing bands were observed at 2.3 kb in all the species, and the molecular size was the same as that for the NME-*Apa*I 9 fragment. The intense bands at about 1050 bp corresponded to the CJA-*Apa*I 1 and CJA-*Apa*I 4 fragments, which were the partial fragments produced by the internal *Apa*I sites in the repeated sequence elements. This type of the hybridization bands was commonly observed in three of the four species (the exception was *G. gallus*). These results indicated that the genomic organization of the repeated elements was highly conserved through the Galliformes, and that the internal *Apa*I sites did not exist in the elements of *G. gallus*. The hybridization bands with the intermediate size between 2.3 kb and 1050 bp and the bands smaller than 1050 bp might be derived from other *Apa*I restriction sites that existed internally in the elements. However, no hybridization bands other than 2.3-kb were observed in *G. gallus*, indicating that the 2.3-kb fragments were basal units of the repetitive sequences and were highly conserved in the Galliformes. The copy number of the NME-*Apa*I elements was lower in *G. gallus* than in the other three species.

To examine the genomic organization of the repeated sequence elements in detail, the CJA-*Apa*I 1 and NME-*Apa*I 9 fragments were hybridized to the genomic DNAs of *C. japonica* and *N. meleagris* digested with seven endonucleases, respectively (Figure 6). The *Bgl*II sites were conserved in *C. japonica*, and three intensely hybridizing bands were observed at molecular weight of 2-3 kb (Figure 6a). The multiple bands observed at lower molecular weight in the *Hae*III- and *Hin*fI-digests might correspond to the DNA fragments derived from many internal *Hae*III and *Hin*fI restriction sites

contained in the elements (see Figure 4). Many intense hybridization bands with size smaller than 3 kb were observed in the *Msp*I-digest, while no hybridization bands were found at lower molecular weight in the *Hpa*II-digest. Nucleotide sequence analysis revealed that several restriction sites of the two isoschizomers were contained in the CJA-*Apa*I elements, and thus the lower molecular weight bands corresponded to the DNA fragments derived from the internal *Msp*I sites. *Msp*I cleaves when the CG sequence is methylated, whereas *Hpa*II does not cleave this sequence when the cytosine is methylated. The difference in the hybridization patterns between the *Hpa*II- and *Msp*I-digests therefore indicated that the elements were hypermethylated in fibroblast cells. Similar patterns were observed in the hybridization with NME-*Apa*I 9 in *N. meleagris* (Figure 6b). *Bam*HI and *Bgl*II sites were conserved in *N. meleagris* as well as *Bgl*II sites in *C. japonica*; however, the restriction sites were more conserved in *N. meleagris* than *C. japonica.* The extensive hypermethylation was also found in peripheral whole blood cells of *N. meleagris.*

The amount of the repeated sequence elements in the genome of *N. meleagris* and *C. japonica* was estimated by slot-blot hybridization (Figure 7). The intensities of the chemiluminescence hybridization signals probed with NME-*Apa*I 9 and CJA-*Apa*I were compared among the probe DNA and male and female genomic DNA of *N. meleagris* and *C. japonica*. The NME-*Apa*I element represented 0.21% and 0.15% of the female and male genomes of *N. meleagris*, respectively, and the CJA-*Apa*I element accounted for 0.47 % and 0.28 % of the female and male genomes of *C. japonica*, respectively. The total copy numbers were larger in females than males, indicating that the elements were abundant on the W chromosomes.

Nucleotide sequence conservation in birds

To examine the nucleotide sequence conservation of the repeated sequence elements in avian species, slot-blot hybridization probed with NME-*Apa*I 9 was performed for 13 species of six orders. The NME-*Apa*I 9 probe cross-hybridized to the genome of the species of the Strigiformes, Gruiformes and Falconiformes as well as the Galliformes (Figure 8). The hybridization signals were also found in the male genome of all six species, and the intensities of hybridization bands were not much different between males and females. By contrast, no hybridization signals were observed for *S. camelus* and *P. pennata* of the Struthioniformes and *E. elegans* of the Tinamiformes.

The chromosomal distribution of the repeated sequence elements was examined for the six species of the three orders, *K. blakistoni*, *B. bubo*, *G. leucogeranus*, *G. monacha*, *S. nipalensis* and *S. cheela*, which produced hybridization signals by slot-blot hybridization. The painted signals probed with NME-*Apa*I 9 were located on the W chromosomes of the Gruiformes and Falconiformes, and weak signals were also detected on the W chromosomes of the Strigiformes (Figure 9).

Discussion

We cloned a novel family of interspersed-type repetitive DNA sequences from *C. japonica* and *N. meleagris*, which were site-specifically amplified on the W chromosomes. The repeated sequence elements had high similarities of nucleotide sequences (81-82%) between the two species, and were also conserved in other Galliformes species, *G. gallus* and *C. chinensis*. The similarity of Southern hybridization patterns of four species indicates that the genomic organization of the

repeated sequence elements is highly conserved, as are their chromosomal locations on the W chromosomes in the Galliformes. Three different types of W-specific repetitive sequences, the *Xho*I-family (Tone *et al*. 1982, 1984; Kodama *et al*. 1987), *Eco*RI-family (Saitoh *et al*. 1991, Saitoh & Mizuno 1992) and *Ssp*I-family sequences (Itoh & Mizuno 2002) have been isolated from *G. gallus*. The *Xho*I- and *Eco*RI-family sequences have about 68% homology, and are characterized by arrays of about 21-bp tandem repeat, which contain $(A)_{3\text{-}5}$ and $(T)_{3\text{-}5}$ clusters separated by 6-7 or 6-8 GC-rich base pairs (Kodama *et al*. 1987, Saitoh *et al*. 1991). The *Ssp*I-family sequence is composed of about 0.5-kb monomer units that are tandemly repeated in the genome of *G. gallus*. The *Xho*I-, *Eco*RI- and *Ssp*I-family sequences cover separate parts of the W chromosome. The nucleotide sequences of the three families show distinct genus-specificity, being limited to the genus *Gallus* and not found in other genera. The novel family of repetitive sequences cloned in this study had no homologies with any W-specific repeated sequence families of the Galliformes, and showed several different features. The repeated sequence elements of *C. japonica* and *N. meleagris* were not W-specific but rather interspersed-type repetitive sequences that were distributed throughout all chromosomal regions. No internal repetitions were found, and they were amplified site-specifically on the W chromosomes and distributed in almost the whole heterochromatic regions of the W chromosomes in four Galliformes species (*N. meleagris, C. japonica, G. gallus* and *C. chinensis*). The repeated sequences were remarkably hypermethylated as condensed chromatin in the genome, indicating that they may have a role in chromatin organization in interphase nuclei and in the chromosomal architecture of the W chromosome at metaphase. The fluorescence signals were also found on microchromosomes in *N. meleagris.* The additional signals might be caused by site-specific amplification of the repeated sequence on autosomes

besides the W chromosome, which occurred in the individuals of the breeding colony used in this study. These results indicate that the origin of the novel repeated sequence family is distinctly different from the already known W-specific repetitive sequences. The repeated sequence element of *N. meleagris* cross-hybridized to genomic DNA of six species in three other orders of Neognathous birds (the Strigiformes, Gruiformes and Falconiformes), but did not cross-hybridize to genomic DNA of Palaeognathous birds (the Struthioniformes and Tinamiformes). The repeated sequence family is a basal component of the W chromosome, which is highly conserved through Neognathous birds, and is a good molecular cytogenetic marker for phylogenetic relationships of birds.

In the early stage of avian speciation, the ancient types of sex chromosomes had not been differentiated morphologically. The primitive types of sex chromosomes have been retained in Palaeognathous birds since the common ancestor diverged into the Palaeognathae and Neognathae (de Boer 1980, Ansari *et al.* 1988, Ogawa *et al*. 1998, Nishida-Umehara *et al*. 1999, Shetty *et al*. 1999). In the lineage of Neognathous birds, the W-specific repetitive sequences were amplified with the degeneration of the W chromosome (Charlesworth 1991, Charlesworth & Charlesworth 2000), and the divergence of their nucleotide sequences was accelerated as rapidly evolved molecules. By contrast, the repeated sequence family cloned in this study were a non-W-specific interspersed-type of repetitive sequences and are commonly shared in Neognathous birds but not in Palaeognathous birds. These results suggest that the repeated sequence family occurred in the genome of Neognathous birds after the Neognathae and Palaeognathae diverged from the common ancestor 110 - 130 million years ago (MYA) and were amplified site-specifically on the W chromosomes with sex chromosome differentiation (García-Moreno & Mindell DP 2000, Haddrath & Baker 2001, van

Tuinen and Hedges 2001).

The divergence time of avian sex chromosomes were estimated by comparative analysis of the nucleotide sequences of five known gametologous genes (*ATP5A1*, *UBAP2*, *SPIN*, *HINT* and *CHD*) on avian Z and W chromosomes, which arose via the cessation of recombination and differentiation of sex chromosomes (García-Moreno & Mindell 2000, de Kloet & de Kloet 2003, Handley *et al.* 2004). Handley *et al.* (2004) proposed the presence of at least two evolutionary strata in the process of avian sex chromosome differentiation: Z-W recombination ceased in the oldest stratum 102-170 MYA before the split of Neoaves and Eoaves. The disruption of chromosomal recombination in the second stratum occurred independently in the different lineages between 58 and 85 MYA when the major radiation of the existing Neognathous birds occurred. The sex chromosomes of the ratites are largely homomorphic except for subtle sex chromosome differentiation (Ansari *et al.* 1988, Ogawa et al. 1998, Nishida-Umehara et al. 1999), and no sex-specific forms have been found for the five gametologs. In tinamous, the differentiation of the Z and W genes only occurred in *SPIN* (de Kloet & de Kloet 2003), revealing that the cessation of recombination between the Z and W chromosomes in the lineage of the Tinamiformes occurred independently from Neognathous species after the ancestors of this order diverged from the ancestors of the ratites approximately 90 MYA (Haddrath & Baker 2001, van Tuinen & Hedges 2001,). The cessation of recombination, degeneration and heterochromatization in the W chromosome of the Tinamiformes has proceeded farther than that in the ratites. The intermediate type of sex chromosome differentiation in the Tinamiformes between those in the ratites and Neognathous birds is confirmed by cytogenetic observation of meiotic chromosome paring; in *R. rufescens* and *N. maculosa* the recombination nodules in the Z and W chromosomal pair are distributed

in much longer segments than in Neognathous birds but restricted to shorter segments than in the lesser rhea (*P. pennata*) (Pigozzi and Solari 1999, 2005). In *E. elegans* the W chromosome is heterochromatic in half to two-thirds of the whole chromosomal region (Sasaki *et al*. 1980). No nucleotide sequence similarities were found by slot-blot hybridization between the repeated sequence elements cloned in this study and W-heterochromatin of *E. elegans*, indicating that the W-heterochromatin of *E. elegans* is composed of other types of repetitive sequences whose origins are different from them. The present data on the novel family of repetitive sequences are well correlated with the phylogenetic relationships between the Palaeognathae and Neognathae species. The W-heterochromatin might have appeared independently in the lineage of the Tinamiformes after the divergence of the Palaeognathe and Neognathae, and therefore the nucleotide sequences of the W-heterochromatin of the Tinamiformes must be different from those of Neognathous birds. The molecular cloning of the W-specific repetitive sequences of the Tinamiformes and their characterization gives us a clue for clarifying the processes of W chromosome differentiation and heterochromatization in birds.

Acknowledgements

We express our appreciation to Yokohama Zoological Gardens, Yokohama, for providing the specimens of emu and elegant crested tinamou, Kanazawa Zoological Gardens, Yokohama, for lesser rhea, Ueno Zoological Gardens, Tokyo, for Eurasian eagle owl, Tama Zoological Park, Tokyo, for Siberian white crane, and Inokashira Park Zoo, Tokyo, for hooded crane. We used the specimens of Blakiston's fish owl with the co-operation of the Blakiston’s fish owl conservation programs organized by the

Ministry of the Environment, Japan, and the specimens of Japanese mountain hawk-eagle were provided from The Project Team for Research and Conservation of the Japanese Mountain Hawk-Eagle. The specimens of crested serpent eagle, which were collected from wounded individuals taken lawfully to the Okinawa Zoo, Okinawa Kids Discovery Kingdom Foundation, Okinawa, were provided by Teruo Kinjo, Okinawa Zoo. 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.

References

Ansari HA, Takagi N, Sasaki M (1988) Morphological differentiation of sex chromosomes in three species of ratite birds. *Cytogenet Cell Genet* **47**: 185-188.

Charlesworth B (1991) The evolution of sex chromosomes. *Science* **251**: 1030-1033.

Charlesworth B, Charlesworth D (2000) The degeneration of Y chromosomes. *Phil Trans R Soc Lond B* **355**: 1563-1572.

Cracraft J (2001) Avian evolution, Gondwana biogeography and the Cretaceous-Tertiary mass extinction event. *Proc R Soc Lond B* **268**: 459-469.

de Boer LEM (1980) Do the chromosomes of the kiwi provide evidence for a monophyletic origin of the ratites? *Nature* **287**: 84-85.

de Kloet RS, de Kloet SR (2003) Evolution of the spindlin gene in birds: independent cessation of the recombination of sex chromosomes at the spindlin locus in neognathous birds and tinamous, a palaeognathous avian family. *Genetica* **119**: 333-342.

García-Moreno J, Mindell DP (2000) Rooting a phylogeny with homologous genes on opposite sex chromosomes (gametologs): a case study using avian CHD. *Mol Biol Evol* **17**: 1826-1832.

Haddrath O, Baker AJ (2001) Complete mitochondrial DNA genome sequences of extinct birds: ratite phylogenetics and the vicariance biogeography hypothesis. *Proc R Soc Lond B* **268**: 939-945.

Handley L-JL, Ceplitis H, Ellegren H (2004) Evolutionary strata on the chicken Z chromosome: implications for sex chromosome evolution. *Genetics* **167**: 367-376.

Itoh Y, Mizuno S (2002) Molecular and cytological characterization of *Ssp*I-family repetitive sequence on the chicken W chromosome. *Chromsome Res* **10**: 499-511.

Kodama H, Saitoh H, Tone M, Kuhara S, Sakaki Y, Mizuno S (1987) Nucleotide sequences and unusual electrophoretic behavior of the W chromosome-specific repeating DNA units of the domestic fowl, *Gallus gallus domestics*. *Chromosoma* **96**: 18-25.

Matsuda Y, Chapman VM (1995) Application of fluorescence *in situ* hybridization in genome analysis of the mouse. *Electrophoresis* **16**: 261-272.

Nishida-Umehara C, Fujiwara A, Ogawa A, Mizuno S, Abe S, Yoshida MC (1999) Differentiation of Z and W chromosomes revealed by replication banding and FISH mapping of sex-chromosome-linked DNA markers in the cassowary (Aves, Ratitae). *Chromosome Res* **7**: 635-640.

Ogawa A, Murata K, Mizuno S (1998) The location of Z- and W-linked marker genes and sequence on the homomorphic sex chromosomes of the ostrich and the emu. *Proc Natl Acad Sci USA* **95**: 4415-4418.

Ohno S (1967) *Sex Chromosomes and Sex-linked Genes.* Berlin-Heiderberg-New York: Springer-Verlag.

Pigozzi MI, Solari AJ (1997) Extreme axial equalization and wide distribution of recombination nodules in the primitive ZW pair of *Rhea americana* (Aves, Ratitae). *Chromosome Res* **5**: 421-428.

Pigozzi MI, Solari AJ (1999) The ZW pairs of two paleognath birds from two orders show transitional stage of sex chromosome differentiation. *Chromosome Res* **7**: 541-551.

Pigozzi MI, Solari AJ (2005) Meiotic recombination in the ZW pair of a tinamid birds shows a differential pattern compared with neognaths. *Genome* **48**: 286-290.

Saitoh H, Harata M, Mizuno S (1989) Presence of female-specific bent-repetitive DNA sequences in the genomes of turkey and pheasant and their interactions with W-protein of chicken. *Chromosoma* **98**: 250-258.

Saitoh Y, Saitoh H, Ohtomo K, Mizuno S (1991) Occupancy of the majority of DNA in the chicken W chromosome by bent-repetitive sequences. *Chromosoma* **101**: 32-40.

Saitoh Y, Mizuno S (1992) Distribtion of XhoI and EcoRI family repetitive DNA sequences into separate domains in the chicken W chromosome. *Chromosoma* **101**: 474-477.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Sasaki M, Nishida C, Takagi N, Hori H (1980) Sex-chromosomes of the elegant crested tinamou, *Eudromia elegans* (Aves: Tinamiformes: Tinamidae). *Chromosome Info Serv* **29**: 19-21.

Schmid M, Enderle E, Schindler D, Schempp W (1989) Chromosome banding and

DNA replication patterns in bird karyotypes. *Cytogenet Cell Genet* **52**: 139-146.

Shetty S, Griffin DK, Graves JAM (1999) Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Res* **7**: 289-295.

Sibley CG, Ahlquist JE (1990) *Phylogeny and Classification of Birds: A study in Molecular Evolution.* New Haven: Yale University Press.

Suzuki T, Kurosaki, T, Shimada K *et al.* (1999) Cytogenetic mapping of 31 functional genes on chicken chromosomes by direct R-banding FISH. *Cytogenet Cell Genet* **87**: 32-40.

Takagi N (1972) A comparative study of the chromosome replication in 6 species of birds. *Jpn J Genet* **47**: 115-123.

Takagi N, Sasaki M (1974) A phylogenetic study of bird karyotypes. *Chromosoma* **46**: 91-120.

Takagi N, Itoh M, Sasaki M (1972) Chromosome studies in four species of Ratitae (Aves). *Chromosoma* **36**: 281-291.

Tone M, Nakano N, Takao E, Narisawa S, Mizuno S (1982) Demonstration of W chromosome-specific repetitive DNA sequences in the domestic fowl, *Gallus g. domestics*. *Chromosoma* **86**: 551-569.

Tone M, Sakaki Y, Hashiguchi T, Mizuno S (1984) Genus specificity and extensive methylation of the W chromosome-specific repetitive DNA sequences from the domestic fowl, *Gallus gallus domestics*. *Chromosoma* **89**: 228-237.

van Tuinen M, Hedges SB (2001) Calibration of avian molecular clocks. *Mol Biol Evol* **18**: 206-213.

van Tuinen M, Sibley CG, Hedges SB (1998) Phylogeny and biogeography of ratite

birds inferred from DNA sequences of the mitochondrial ribosomal genes. *Mol Biol Evol* **15**: 370-376.

van Tuinen M, Sibley CG, Hedges SB (2000) The early history of modern birds inferred from DNA sequences of nuclear and mitochondrial ribosomal genes. *Mol Biol Evol* **17**: 451-457.

Figure legends

Figure 1. Ethidium bromide-stained gel of *Apa*I-digested genomic DNAs of *C. japonica* **(a)** and *N. meleagris* **(b)**. Arrows indicate the prominent DNA bands used for molecular cloning of repetitive DNA sequences. Molecular size markers are *Hin*dIII-digested λ DNA in the left lane and *Hin*cII-digested ϕX174 in the right lane.

Figure 2. FISH patterns probed with the repeated sequence elements. **(a, b)** Biotin-labeled CJA-*Apa*I 1 **(a)** and NME-*Apa*I 9 **(b)** were hybridized to metaphase spreads of *C. japonica* and *N. meleagris*, respectively. The arrow indicates a fluorescence signal on a microchromosome in *N. meleagris*. The arrowheads indicate Z chromosomes. **(c, d)** NME-*Apa*I 9 is hybridized to metaphase spreads of *G. gallus* **(c)** and *C. chinensis* **(d)**. Scale bar = 10 μm.

Figure 3. Variations on chromosomal distribution of the NME-*Apa*I 9 element among individuals in *N. meleagris.* **(a)** female No. 1, **(b)** female No. 2 **(c)** male No. 1, **(d)** male No. 2, **(e)** male No. 3. The fluorescence signals are located on no microchromosomes in **(a)** and **(d)**, one microchromosome in **(b)** and **(e)**, and two microchromosomes in **(c)**. Arrows indicate fluorescence signals on microchromosomes. Scale bar = 10 μm.

Figure 4. Nucleotide sequences of four repeated sequence fragments cloned from *N. meleagris* (NME-*Apa*I 9 and NME-*Apa*I 10) and *C. japonica* (CJA-*Apa*I 1 and CJA-*Apa*I 4). Dots indicate the bases that are the same as those of the NME-*Apa*I 9 fragment. Hyphens indicate gaps in the nucleotide sequences. One base nucleotide substitution is contained in *Apa*I restriction sites at positions 1049-1054 and 1048-1053

in NME-*Apa*I 9 and NME-*Apa*I 10, respectively, which are enclosed in a box. Underlines indicate internal restriction sites as follows: *Bam*HI (.....), *Bgl*II (.....), *Hae*III (.....), *Hin*fI (_____) and *Msp*I(*Hpa*II) (_____).

Figure 5. Southern blot hybridization patterns of *Apa*I-digested genomic DNAs of four Galliformes species probed with NME-*Apa*I 9. Lane 1; *G. gallus*, lane 2; *C. japonica*, lane 3; *C. chinensis*, lane 4; *N. meleagris*. Each lane contains 10 μg of genomic DNA. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested ϕX174 was used as a molecular size marker.

Figure 6. Southern blot hybridization patterns of genomic DNAs of *C. japonica* **(a)** and *N. meleagris* **(b)** probed with CJA-*Apa*I 1 and NME-*Apa*I 9, respectively. Lane 1; *Bam*HI, lane 2; *Bgl*II, lane 3; *Eco*RI, lane 4; *Hae*III, lane 5; *Hin*fI, lane 6; *Hpa*II, lane 7; *Msp*I. Each lane contains 10 μg genomic DNA. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested ϕX174 was used as a molecular size marker.

Figure 7. Estimation of copy numbers of the NME-*Apa*I 9 and CJA-*Apa*I 1 sequence elements in *N. meleagris* and *C. japonica*, respectively. Lane 1, NME-*Apa*I 9; Lane 2, genomic DNA of *N. meleagris* female; Lane 3, genomic DNA of *N. meleagris* male; Lane 4, CJA-*Apa*I 1; Lane 5, genomic DNA of *C. japonica* female; Lane 6, genomic DNA of *C. japonica* male. Hybridization was probed with NME-*Apa*I 9 for lanes 1-3, and with CJA-*Apa*I 1 for lanes 4-6.

Figure 8. Autoradiograph of slot-blot hybridization probed with NME-*Apa*I 9 to genomic DNAs of two Struthioniformes species, one Tinamiformes species, four

Galliformes species, two Strigiformes species, two Gruiformes species and two Falconiformes species. DNO; *D. novaehollandiae*, PPE; *P. pennata*, EEL *E. elegans* CCH; *C. chinensis*, CJA; *C. japonica*, GGA; *G. gallus*, NME; *N. meleagris*, KBL; *K. blakistoni*, BBU; *B. bubo*, GLE; *G. leucogeranus*, GMO; *G. monacha*, SNI; *S. nipalensis*, SCH; *S. cheela.*

Figure 9. FISH patterns probed with NME-*Apa*I 9 in six species of three orders. **(a)** *K. blakistoni*; **(b)** *B. bubo*; **(c)** *G. leucogeranus*; **(d)** *G. monacha*; **(e)** *S. nipalensis*; **(f)** *S. cheela*. Arrows indicate hybridization signals on the W chromosomes. Scale bar = 10 μm.

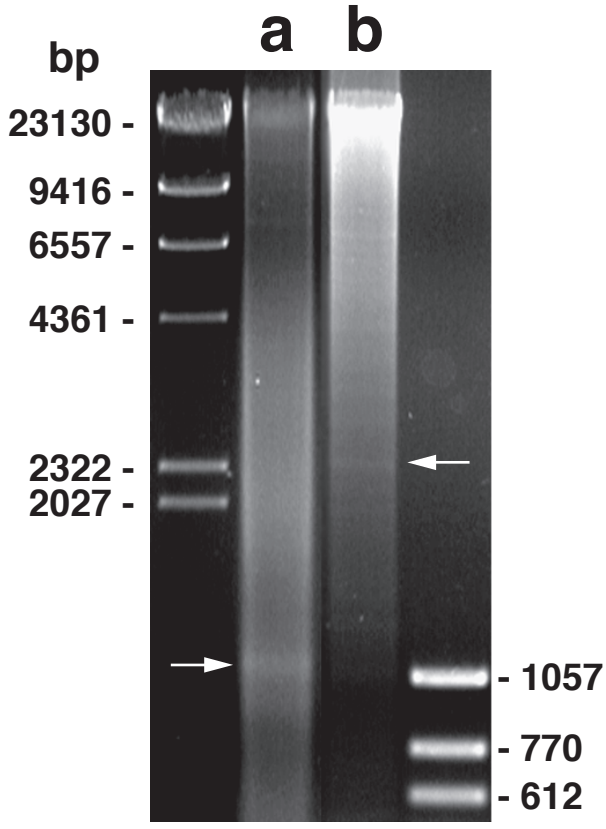

Figure 1 (Yamada *et al*.)

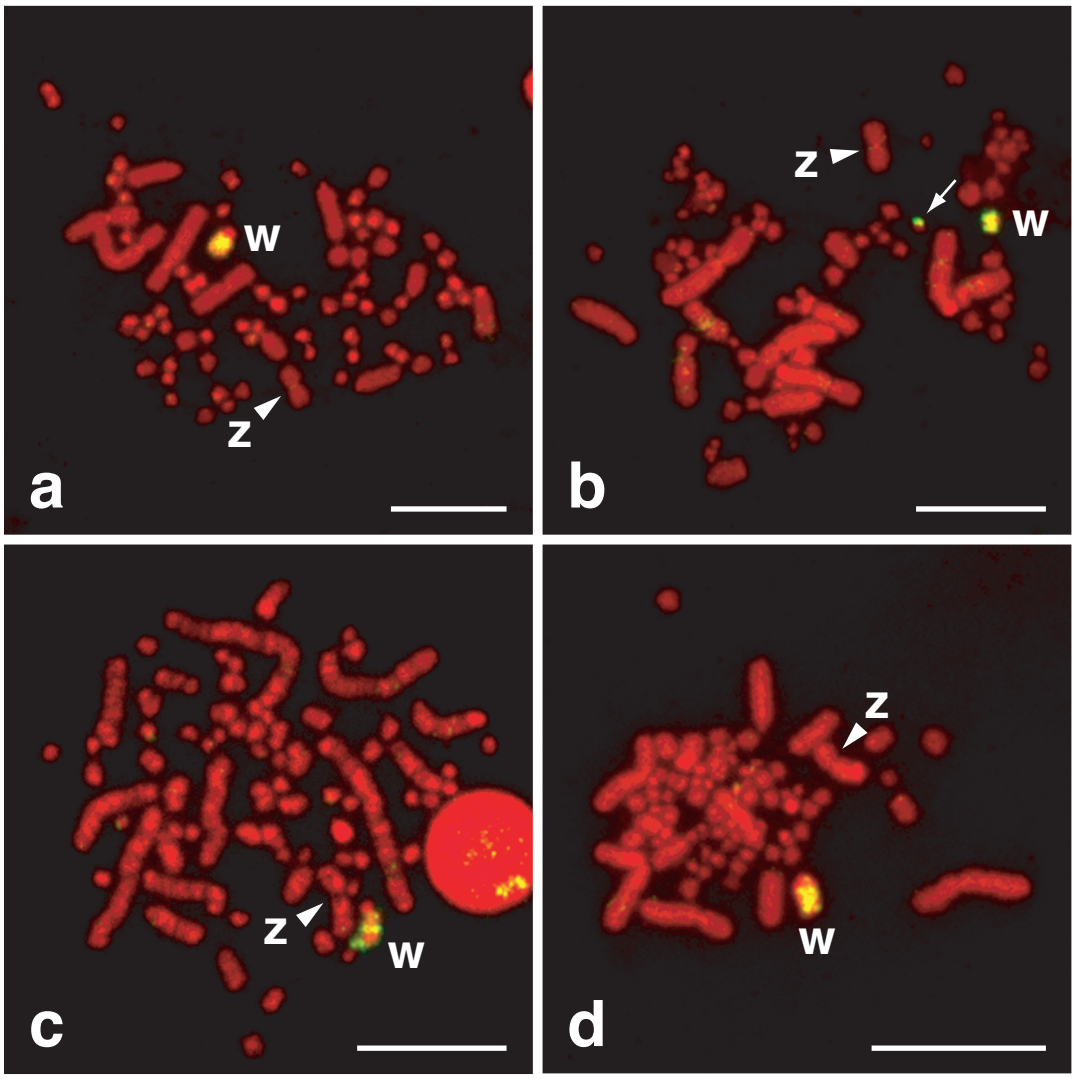

Figure 2 (Yamada *et al*.)

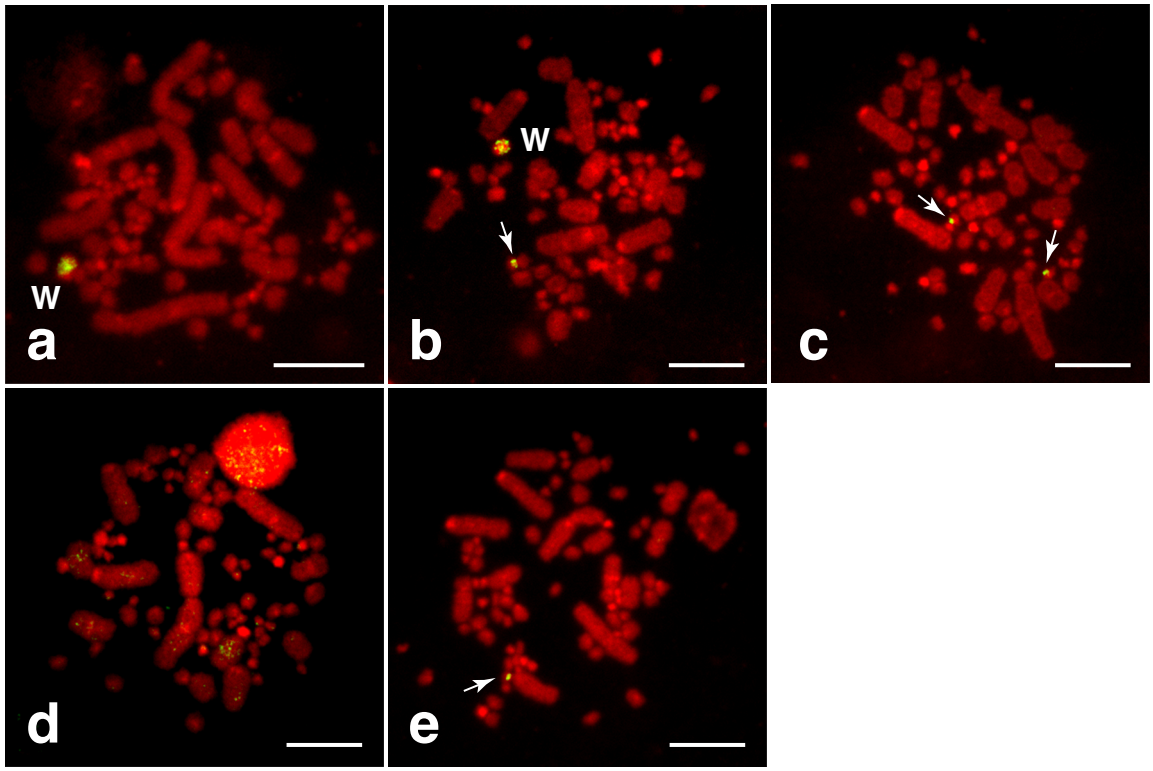

Figure 3 (Yamada *et al*.)

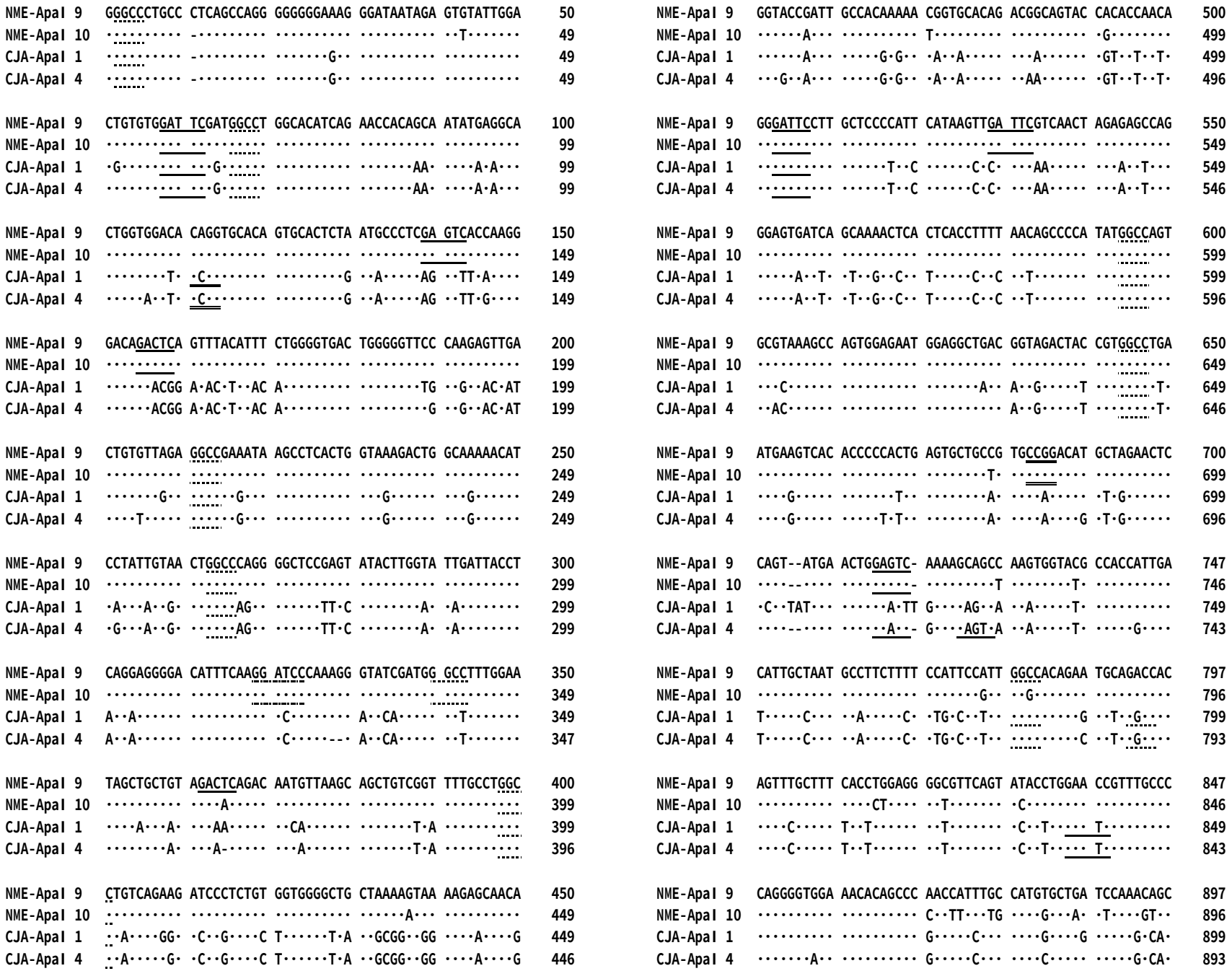

Figure 4 (1/2) (Yamada *et al.*)

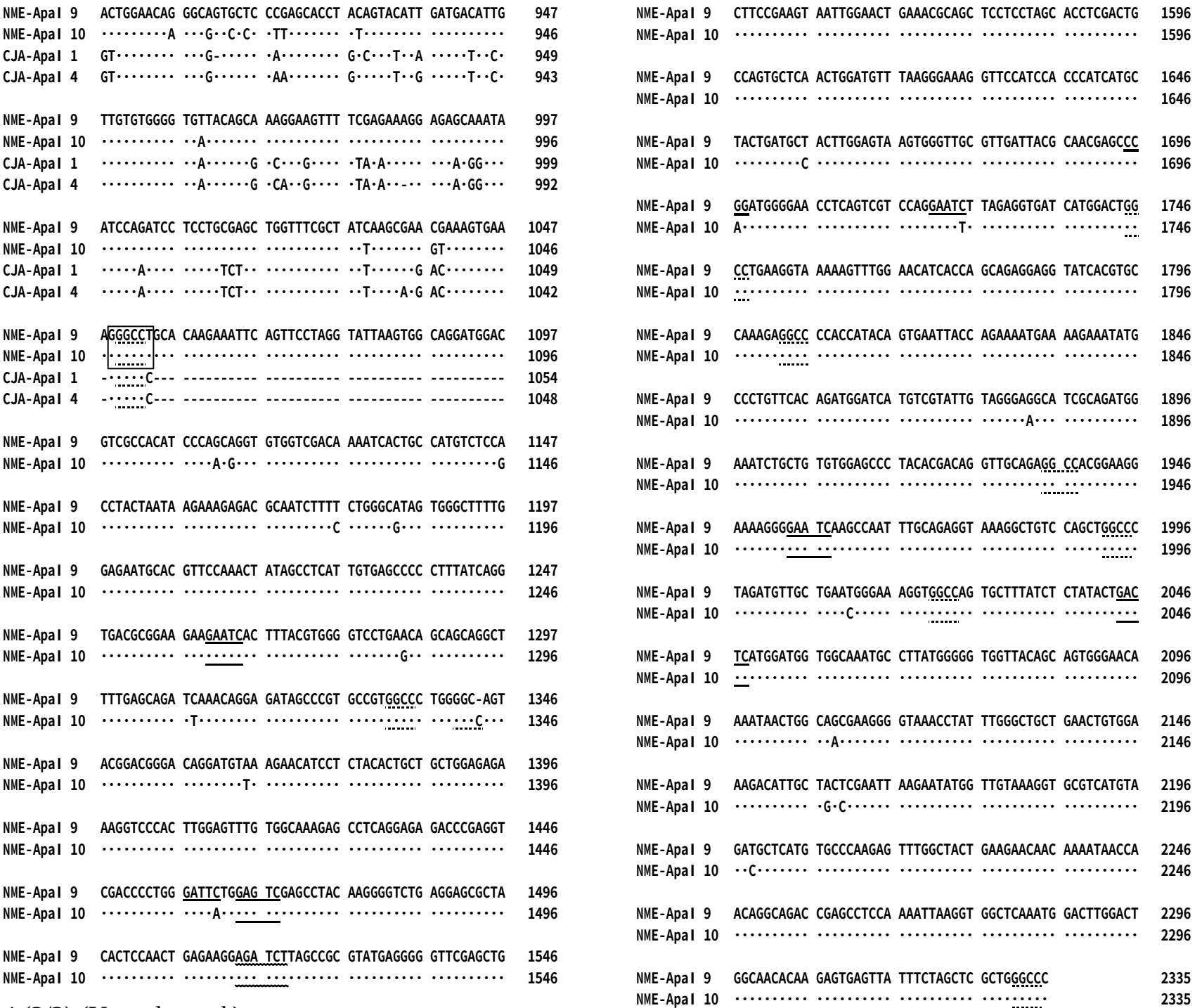

Figure 4 (2/2) (Yamada *et al*.)

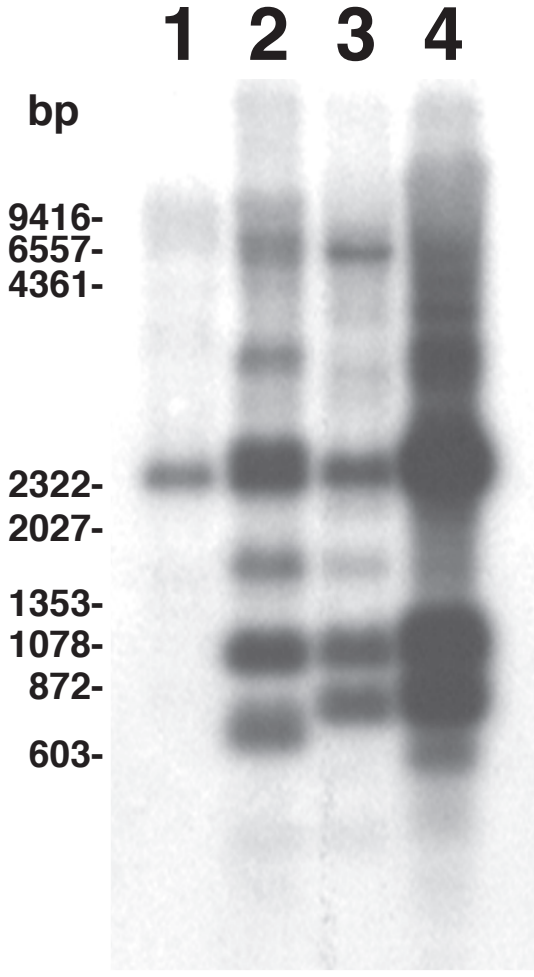

Figure 5 (Yamada *et al*.)

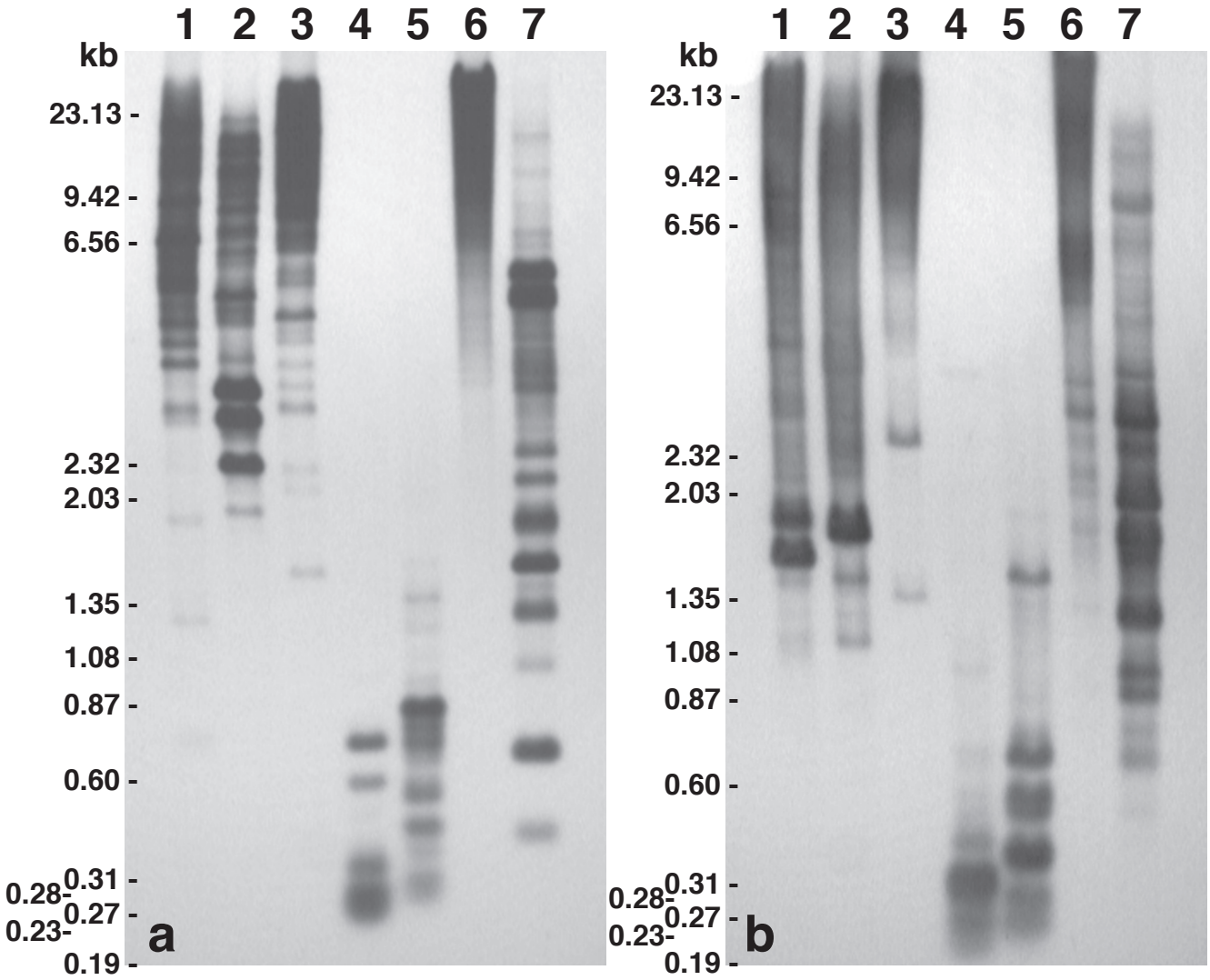

Figure 6 (Yamada *et al*.)

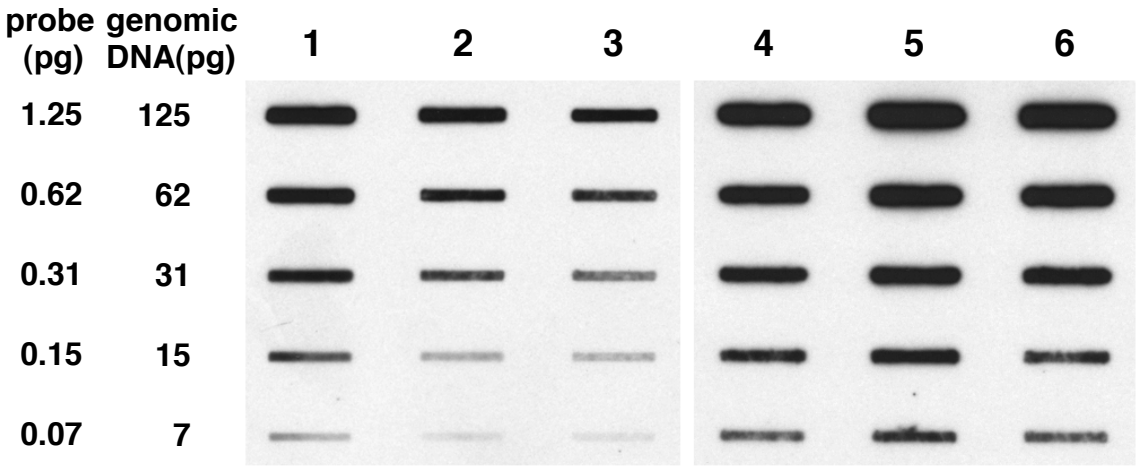

Figure 7 (Yamada *et al*.)

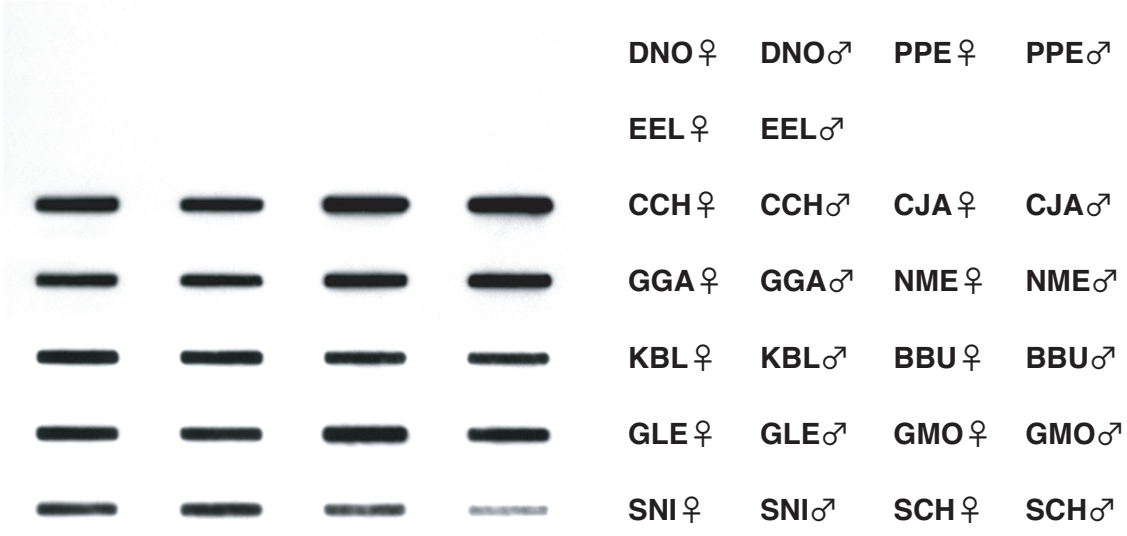

Figure 8 (Yamada *et al*.)

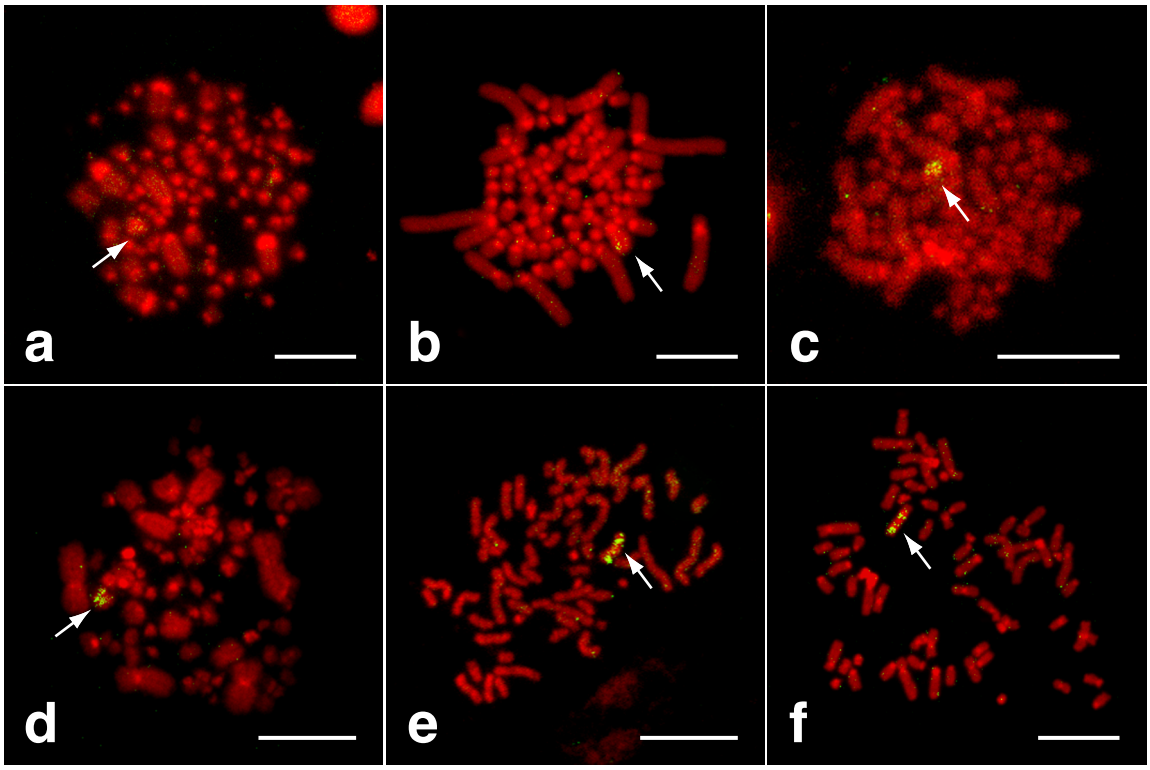

Figure 9 (Yamada *et al*.)