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**The molecular basis of chromosome orthologies and sex chromosomal differentiation in palaeognathous birds**

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**Abstract**

Palaeognathous birds (Struthioniformes and Tinamiformes) have morphologically conserved karyotypes and less differentiated ZW sex chromosomes. To delineate interspecific chromosome orthologies in palaeognathous birds we conducted comparative chromosome painting with chicken (*Gallus gallus*, GGA) chromosome 1-9 and Z chromosome paints (GGA1-9 and GGAZ) for emu, double-wattled cassowary, ostrich, greater rhea, lesser rhea and elegant crested tinamou. All six species showed the same painting patterns: each probe was hybridized to a single pair of chromosomes with the exception that the GGA4 was hybridized to the fourth largest chromosome and a single pair of microchromosomes. The GGAZ was also hybridized to the entire region of the W chromosome, indicating that extensive homology remains between the Z and W chromosomes on the molecular level. Comparative FISH mapping of four Z- and/or W-linked markers, the *ACO1/IREBP*, *ZOV3* and *CHD1* genes and the EE0.6 sequence, revealed the presence of a small deletion in the proximal region of the long arm of the W chromosome in greater rhea and lesser rhea. These results suggest that the karyotypes and sex chromosomes of palaeognathous birds are highly conserved not only morphologically, but also at the molecular level; moreover, palaeognathous birds appear to retain the ancestral lineage of avian karyotypes.

## Introduction

Avian karyotypes are generally characterized by the high diploid number of chromosomes, ranging from 74 to 86 in about two thirds of species. Karyotypes can be subdivided into large macrochromosomes including ZW-type sex chromosomes (7-10 pairs) and a large number of almost indistinguishable microchromosomes (Takagi & Sasaki 1974, Belterman & de Boer 1984) or into groups A-D (Masabanda *et al*. 2004). The karyotypic similarities and differences between bird species have been studied morphologically by conventional Giemsa staining and chromosome banding, and, for the past 10 years, molecular cytogenetically by comparative FISH mapping with chromosome-specific paints, cDNA and genomic DNA clones, mostly developed in chicken (*Gallus gallus*) (Griffin *et al.* 1999, Suzuki *et al.* 1999, Schmid *et al.* 2000, Shibusawa *et al.* 2001, 2002). Cross-species chromosome hybridization (termed Zoo-FISH) and subsequent comparative gene mapping delineates accurately the chromosomal orthologies between distantly related species and the chromosome rearrangements that have occurred during evolution. Comparative chromosome painting in birds with chicken probes has been performed for over 30 species in at least nine orders (Shetty *et al.* 1999, Schmid *et al.* 2000, Raudsepp *et al.* 2002, Guttenbach *et al.* 2003, Kasai *et al.* 2003, Derjusheva *et al.* 2004, Shibusawa *et al.* 2004a, b, Itoh & Arnold 2005, de Oliveria *et al.* 2005, Nanda *et al.* 2006, reviewed in Griffin *et al*. 2007). These studies collectively have revealed that the avian karyotypes are highly conserved at the molecular level with rare exceptions including the Falconiformes and the Psittaciformes which display both the fragmentation of macrochromosomes and the

decrease in microchromosomal number through fusions (de Oliveira *et al.* 2005). Reciprocal chromosome translocations have not yet been reported.

Avian species are categorized into two large clades based on the palatal form: the Palaeognathae and the Neognathae. The Palaeognathae consist of the Struthioniformes (Ratites) and the sister group Tinamiformes. This classification is also confirmed molecular phylogenetically (Sibley & Ahlquist 1990, van Tuinen & Hedges 1998, 2000, Cracraft 2001). G-banding suggests that palaeognathous birds have morphologically the typical avian karyotype (Takagi *et al.* 1972, de Boer 1980, Ansari *et al.* 1988), which is also shared by most neognathous birds (Takagi & Sasaki 1974, Belterman & de Boer 1984); there is however a remarkable difference in the sex chromosome differentiation between the two taxa. The W chromosomes of neognathous birds are highly differentiated: they are smaller than the Z chromosomes, conspicuously heterochromatin-rich and late replicating (Takagi *et al.* 1972, Schmid *et al.* 1989). In contrast, the sex chromosomes of Struthioniformes species remain the most primitive stage of avian sex chromosome differentiation: the Z and W chromosomes are extensively homomorphic and euchromatic (Takagi *et al.* 1972, de Boer 1980, Ansari *et al.* 1988).

Chromosome numbers of Tinamiformes species have been reported to be around 2n=80, and they also appear to have the typical avian karyotypes (Sasaki *et al.* 1984, Belterman & de Boer 1990, Pigozzi & Solari 1999, 2005), whereas the W chromosomes with the large heterochromatin blocks are in the intermediate state between the euchromatic W chromosomes of the Ratites and the highly heterochomatized W

chromosomes of neognathous birds (Sasaki *et al.* 1980, Pigozzi & Solari 1999, 2005, Tsuda *et al.* 2007). In palaeognathous birds, cross-species chromosome hybridization has been performed only for emu (*Dromaius novaehollandiae*) (Shetty *et al.* 1999) and for greater rhea *(Rhea americana)* (Guttenbach *et al*. 2003), with chicken chromosome 1-9 paints demonstrating that their karyotypes have complete orthology with the chicken macrochromosomes except that the chicken chromosome 4 paint hybridizes to the fourth largest chromosome and an additional pair of microchromosomes. Chromosome painting with the chicken Z probe showed that the homology between the Z and W chromosomes is highly conserved at the molecular level in emu, however, the sex chromosomes of other Ratites and Tinamous have only been morphologically studied by conventional Giemsa staining and/or G- and C-banding (Takagi *et al.* 1972, Sasaki *et al.* 1980, Ansari *et al.* 1988, Belterman & de Boer 1990, Pigozzi & Solari, 1999, 2005). Comparative mapping with a few Z- and/or W-linked molecular makers has been also performed only for emu, ostrich and double-wattled cassowary (Ogawa *et al.* 1998, Nishida-Umehara *et al.* 1999).

Here we have conducted comparative chromosome painting with chicken chromosome-specific DNA probes for six species belonging to two Palaeognathae orders, and delineated chromosome homologies and interchromosomal rearrangements among the palaeognathous bird species. The state of sex chromosome differentiation was also examined by comparative chromosome mapping of Z- and/or W-linked markers and morphological comparison of banding patterns between the Z and W chromosomes. Karyotypic evolution in the context of sex chromosome differentiation in

palaeognathous birds is discussed.

## Materials and methods

### *Specimens*

Five species of the Struthioniformes and one species of the Tinamiformes were used for chromosomal analysis: emu (*Dromaius novaehollandiae*), double-wattled cassowary (*Casuarius casuarius*), ostrich (*Struthio camelus*), greater rhea (*Rhea americana*) and lesser rhea (*Pterocnemia pennata*) of the Struthioniformes and elegant crested tinamou (*Eudromia elegans*) of the Tinamiformes. Small pieces of skin tissues were taken by biopsy and used for cell culture.

### *Cell culture and chromosome preparation*

The fibroblast cells prepared from the skin tissues were cultured in 199 medium (Invitrogen-GIBCO) supplemented with 18 % fetal bovine serum at 39°C in 5% $CO_2$ in air. After colcemid (0.025 μg/ml) treatment for 30 min, the chromosome preparation was made following a standard protocol. For karyotyping the chromosome slides were stained with 3% Giemsa solution for 10 min.

### *Chromosome banding*

To examine the distribution of constitutive heterochromatin on the Z and W chromosomes, C-banding was carried out with the BSG (Barium

hydroxide/Saline/Giemsa) method (Sumner 1972).

For morphological comparison of the Z and W chromosomes, replication G-banded chromosome preparations were made using the GBG (G-bands by BrdU using Giemsa) method as described in Nishida-Umehara *et al*. (1999). The replication R-banded chromosomes were prepared following Suzuki *et al.* (1999), and used for chromosome painting, Ag-NOR staining and chromosome mapping of DNA clones.

*DNA probes*

Chicken (*Gallus gallus*, GGA) chromosome-specific DNA probes of chromosome 1–9 and Z (GGA1-9 and GGAZ) were used for comparative chromosome painting (Griffin *et al.* 1999, Masabanda *et al*. 2004, www.farmachrom.net). Each probe was amplified by DOP-PCR following Carter *et al.* (1992) and hybridized to the metaphase spreads. The cosmid DNA clones of the *ACO1/IREBP* and *ZOV3* genes, the EE0.6 sequence derived from emu (Ogawa *et al*. 1998) and the genomic DNA fragment of the CHD1 gene cloned from lesser rhea in this study were used as probes for comparative mapping of the Z and W chromosomes. A 4.5 kb DNA fragment of the *CHD1* gene was amplified by polymerase chain reaction (PCR) with the genomic DNA of lesser rhea using the forward primer 2550(F): 5’-GTTACTGATTCGTCTACGAGA-3’ (Fridolfsson & Ellegren 1999) and the reverse primer P2(R): 5’-TCTGCATCGCTAAATCCTTT-3’ (Griffiths *et al*. 1996). For chromosome mapping of the 18S-28S ribosomal RNA genes, the 5.8-kb pHr21Ab and 7.3-kb pHr14E3 fragments of the human ribosomal RNA gene provided by the Japanese Cancer Research Resource Bank (JCRB), Tokyo, were used.

*Fluorescence* in-situ *hybridization (FISH) and Ag-NOR-staining*

FISH was performed as described in Matsuda & Chapman (1995) with slight modifications. For comparative chromosome painting, 1 microgram of chicken chromosome-specific DNA probe was labeled with biotin 16-dUTP using a nick translation kit (Roche Diagnostics), and hybridized to chromosome slides at 37˚C for four days. After hybridization, the slides were incubated with fluorosceinated avidin (FITC-avidin) (Roche Diagnostics), and stained with 0.50 μg/ml propidium iodide after washing. The FISH images were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope, and analysed with the 550CW-QFISH application program of Leica Microsystems Imaging Solutions Ltd. (Cambridge, UK).

For chromosome mapping of the *ACO1/IREBP, ZOV3* and *CHD1* genes, the EE0.6 sequence and the 18S-28S ribosomal RNA (rRNA) genes, 0.5 μg probe was labeled by nick translation with biotin 16-dUTP or digoxigenin (DIG) 11-dUTP (Roche Diagnostics). The genomic DNA clones of *ACO1/IREBP*, *ZOV3* and EE0.6 were hybridized to chromosomes of greater rhea, lesser rhea and elegant crested tinamou, and the DNA fragments of *CHD1* and the rRNA genes were hybridized to chromosomes of all six species. After hybridization overnight, the slides were washed, and then the hybridized probes labeled with biotin 16-dUTP and DIG 11-dUTP were detected with FITC-avidin and anti-DIG-rhodamine (Roche Diagnostics), respectively. The FISH signals were observed under a Nikon E800 fluorescence microscope using a B-2A filter

and microphotographed using Kodak Ektachrome ASA100 films.

The chromosomal distribution of the nucleolar organizer regions (NORs) was examined on the same metaphase spreads used for FISH analysis. After FISH, the slides were washed with distilled water, fixed with 3:1 methanol/glacial acetic acid for 5 min, rinsed with methanol and air-dried. Ag-NOR staining was performed as described in Howell & Black (1980).

## Results

*Karyotypes*

The diploid chromosome numbers of the six palaeognathous bird species were as follows: emu (2n=80), double-wattled cassowary (2n=92), ostrich (2n=80), greater rhea (2n=80), lesser rhea (2n=80) and elegant crested tinamou (2n=80), being identical with those reported by previous authors (Takagi *et al.* 1972, Ansari *et al*. 1988, Sasaki *et al*. 1980, 1984, Nishida-Umehara *et al*. 1999). Giemsa-stained preparations of the nine largest autosomes and ZW sex chromosomes of the six species are shown in Figure 1. The third largest chromosomes were acrocentric in ostrich, greater rhea, lesser rhea and elegant crested tinamou but subtelocentric in emu and double-wattled cassowary. The fourth largest chromosomes were all acrocentric except for the submetacentric chromosome 4 of elegant crested tinamou. The fifth largest autosomes were acrocentric except for the submetacentric chromosome 5 of greater rhea and lesser rhea.

The Z and W chromosomes were acrocentric in emu, double-wattled cassowary,

greater rhea, lesser rhea and elegant crested tinamou, whereas the Z and W chromosomes of ostrich were acrocentric and subtelocentric, respectively. The size differences between the Z and W chromosomes were small in the five species except elegant crested tinamou (Takagi *et al*. 1972, Sasaki *et al*. 1980, Ansari *et al*. 1988, Nishida-Umehara *et al.* 1999).

*Interspecific chromosomal homologies of macrochromosomes*

Chromosomal homologies were examined at the molecular level among the six palaeognathous bird species by comparative painting with chicken chromosome-specific DNA probes. All chicken painting probes (GGA1-9 and GGAZ) were efficiently cross-hybridized to chromosomes of all six species. The chromosome painting patterns with GGA1-9 in double-wattled cassowary and elegant crested tinamou were shown in Figures 2 and 3, respectively. Each chicken probe painted a single pair of chromosomes with the exception for GGA4 in all six species (Table 1), and chicken chromosome 1, 2, 3 and 5 corresponded to each chromosome 1, 2, 3 and 5, respectively. The GGA4 was hybridized to the fourth largest macrochromosome and additionally to a single pair of microchromosomes. The GGAZ was cross-hybridized to both the Z and W chromosomes in all six species, and the W chromosomes were also entirely painted like the Z chromosomes (Figure 4). The painting patterns of emu chromosomes with GGA1-9 and GGAZ and greater rhea chromosomes with GGA1-9 were consistent with the published data (Shetty *et al.* 1999, Guttenbach *et al*. 2003).

*Chromosomal location of the 18S-28S rRNA genes*

The 18S-28S rRNA genes were localized to a single pair of indistinguishable microchromosomes in emu, double-wattled cassowary, ostrich, greater rhea and lesser rhea, and to two pairs of microchromosomes in elegant crested tinamou (Figure 5). Ag-NORs were all localized to the chromosomal sites where the hybridization signals of the 18S-28S rRNA genes were located.

*Comparative mapping of Z- and W-linked DNA markers*

Comparative mapping of the *ACO1/IREBP* and *ZOV3* genes and the EE0.6 sequence was performed for greater rhea, lesser rhea and elegant crested tinamou. The hybridization signals of the clones were all detected for lesser rhea (Figure 6) and greater rhea (data not shown) but not for elegant crested tinamou. The 4.5 kb genomic DNA fragment of the *CHD1* gene isolated from lesser rhea was applied to chromosomes of all six species, but the hybridization signals were obtained only for two rhea species. The hybridization signal of *ACO1/IREBP* was detected near the centromere on the Z chromosome but not on the W chromosome in two rhea species (Figure 6a). The *CHD1*, *ZOV3* and EE0.6 were localized to both the Z and W chromosomes in the two species (Figure 6b, c). *ZOV3* was closely localized proximal to EE0.6, and the *CHD1* was localized proximal to *ZOV3* on the Z chromosomes. The locations of the genes and the order of *CHD1* - *ZOV3* - EE0.6 from the proximal on the Z chromosome was the same as those on the W chromosome. The locations of the four markers on the Z and W chromosomes in the two species were the same as those in ostrich reported previously

(Ogawa *et al.* 1998, Tsuda *et al*. 2007) (Figure 7).

*C-banded and replication G-banded patterns of the Z and W chromosomes*

The structural differences between the Z and W chromosomes were examined by C-banding and replication G-banding. The results are summarized in Figure 7 with the data of chromosome painting with the chicken Z probe and chromosome mapping of Z- and/or W-linked DNA markers. In ostrich, distinct C-positive bands were observed in the interstitial region of both the Z and W chromosomes and in the centromeric region of the W chromosome. In emu, double-wattled cassowary, greater rhea and lesser rhea, weak C-positive bands were observed in the centromeric regions of the W chromosomes. The GBG method produced high resolution G-bands, which were effective for morphological comparison between the Z and W chromosomes. The replication G-banded patterns in the distal half (approximately) of the Z chromosome (indicated by arrows in Figure 7) were identical between the Z and W chromosomes in five Struthioniformes species, whereas there were differences in banding patterns between the proximal half of the Z chromosomes and the corresponding regions of the W chromosomes. In elegant crested tinamou, the replication G-banded pattern was similar between the Z and W chromosomes in the distal quarter (roughly) of the Z chromosome. Two thirds of the W chromosome were composed of C-positive heterochromatin in this species, whereas no C-positive band was observed on the Z chromosome. These results strongly suggest that structural changes have occurred in the proximal regions of the W chromosomes of all six palaeognathous bird species.

**Discussion**

The morphological comparison of macrochromosomes and ZW sex chromosomes among five Struthioniformes species and one Tinamiformes species confirmed that the karyotypes have been highly conserved among palaeognathous birds, although there are a few morphological differences between the macrochromosomes (Takagi *et al.* 1972, Sasaki *et al.* 1980, 1984, Ansari *et al*. 1988, Nishida-Umehara *et al.* 1999, Guttenbach *et al.* 2003, present study). The karyotypes of palaeognathous birds, which are composed of several macrochromosomes and a large number of almost indistinguishable microchromosomes, are quite similar to those of the earliest diverged Carinates, the Galliformes and the Anseriformes (Takagi & Sasaki 1974, Belterman & de Boer 1984). Cross-species chromosome hybridization with chicken chromosome paints confirmed the karyotypic orthologies between the Palaeognathae and the Galloanserae at the molecular level. The painting probes of chicken chromosome 1-9 and Z were each hybridized to a single pair of chromosomes in all six palaeognathous bird species except that the GGA4 was hybridized to the fourth largest chromosome and an additional pair of microchromosomes. This result suggests that the higher diploid chromosome number of double-wattled cassowary (2n=92) than five other palaeognathous bird species (2n=80) was attributed to the fissions of chromosomes smaller than number 9. Chromosome painting with the GGA4 probe confirmed that the submetacentric chicken chromosome 4 resulted from a centric fusion between the ancestral type of acrocentric

chromosome 4 (GGA4q) and a smaller (GGA4p) (Shetty *et al.* 1999, Shibusawa *et al*. 2004b). The short arm of GGA4 is GC-rich, preserving the feature of GC-rich bird microchromosome (McQueen *et al.* 1996, 1998, International Chicken Genome Sequencing Consortium 2004); Griffin and colleagues have suggested that this smaller chromosome is orthologous to turkey chromosome 9 which has been since designated ancestral chromosome 10 (International Chicken Genome Sequencing Consortium 2004; Griffin *et al*. 2007). We previously delineated the process of karyotypic evolution of the Galliformes by comparing the chromosome painting data of 13 Galliformes species with their molecular phylogenetic tree constructed with the mitochondrial DNA sequences, and consequently proposed that the karyotype of emu is identical with the ancestral karyotype of the Galliformes (at least for the largest chromosome pairs) (Shibusawa *et al.* 2004b). The 18S-28S rRNA genes were localized to a pair of undistinguishable microchromosomes in all the five Struthioniformes species like chicken with the rRNA genes located on chromosome 16, suggesting that the rRNA genes might have been located on a single pair of microchromosomes in the ancestral avian karyotype. These data support that palaeognathous birds retain the ancestral karyotypes of the Galloanserae (Sibley & Ahlquist 1990), and that the ancient types of avian karyotypes have been independently conserved in both the lineages of the Palaeognathae and the Neognathae (including the Galloanserae and Neoaves) since they diverged about 120 million years ago (van Tuinen & Hedges 2001, Paton *et al.* 2002).

We recently constructed a comparative cytogenetic map between chicken and Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae) with a large number of

cDNA clones (Matsuda *et al.* 2005). The chicken-turtle comparative map revealed that the GGA4q and GGA4p correspond to the subtelocentric chromosome 4 and a pair of microchromosomes of the turtle, respectively. The acrocentric chromosome 4 (GGA4q) and the GGA4p microchromosome are an ancient feature of the reptile-avian lineage; indeed GGA4q appears almost intact in mammals, being represented as human chromosome 4 (Chowdhary & Raudsepp 2000). Homologies between the turtle and chicken chromosomes have been highly conserved, with the six largest chromosomes being almost equivalent to one another. These results suggest that the Archosauromprha (reptiles and birds) have shared the similar ancestral types of chromosomal organization for more than 210 million years (Janke & Arnason 1997, Kumar & Hedges 1998, Hedges & Poling 1999, Kumazawa & Nishida 1999, Mannen & Li 1999).

The GGAZ probe was intensely cross-hybridized to the entire W chromosomes of five Struthioniformes species, indicating that their W chromosomes have been hardly differentiated molecularly and still retain most of their genes. Cytogenetic studies on meiotic chromosome pairing also showed the presence of high homologies between the Z and W chromosomes in two rhea species (Pigozzi & Solari 1997, 1999). The comparative cytogenetic mapping of four Z- and/or W-linked molecular markers showed that the proximal regions including the *ACO1/IREBP* gene of the acrocentric Z chromosomes have been deleted in the W chromosomes of greater rhea and lesser rhea as previously reported in ostrich and double-wattled cassowary; however, *ACO1/IREBP* is still located on the emu W chromosome (Ogawa *et al.* 1998, Nishida-Umehara *et al.* 1999). In addition to the chromosome deletion, GBG-banding demonstrated that some

chromosomal rearrangements have occurred in the W chromosomal regions corresponding to the proximal half of the Z chromosomes. It seems more likely that the structural differentiation in the proximal region of the W chromosome was caused by the cessation of meiotic recombination between the Z and W chromosomes (Nishida-Umehara *et al.* 1999, García-Moreno & Mindell 2000, de Kloet & de Kloet 2003, Tsuda *et al.* 2007). In contrast to the Struthioniformes, there are large heterochromatic regions on the W chromosomes of Tinamiformes species (Sasaki *et al.* 1980, Pigozzi & Solari 1999, 2005). The chromosomal deletion in the proximal region of the W chromosome was much larger in elegant crested tinamou than ostrich (Tsuda *et al.* 2007), and the C-banding and GBG-banding also showed the presence of the large deletion of the euchromatic regions in the W chromosome of this species. The sexual dimorphism has been found for the *CHD1* and spindlin (*SPIN*) genes of elegant crested tinamou, which are located in the proximal half of the W chromosome (de Kloet 2002, de Kloet & de Kloet 2003, Tsuda *et al*. 2007), suggesting that the Z and W copies of *CHD1* and *SPIN* have been diverged by the cessation of meiotic recombination in the region containing the two genes between the Z and W chromosomes. Recently we molecularly cloned the *Bam*HI-family of repetitive DNA sequences that consist of the large W-heterochromatin of elegant crested tinamou (Tsuda *et al.* 2007); however, this heterochromatin region was also intensely painted with chicken Z probe. This result suggests that a single copy or a few number of copies of the unique sequences on the Z chromosome, which are shared by both the Palaeognathae and the Neognathae, were site-specifically amplified on the W chromosome in this species. The W chromosomes

of Tinamiformes species, therefore, are considered to be at a transitional stage between the largely euchromatic W chromosomes of the Struthioniformes and the highly condensed heterochromatic W chromosomes of neognathous birds.

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**Figure legends**

*Figure 1.* Nine largest autosomal pairs and ZW sex chromosomes of six palaeognathous bird species represented by conventional Giemsa-staining. (**a**) emu (*Dromaius novaehollandiae*). (**b**) double-wattled cassowary (*Casuarius casuarius*). (**c**) ostrich (*Struthio camelus*). (**d**) greater rhea (*Rhea americana*). (**e**) lesser rhea (*Pterocnemia pennata*). (**f**) elegant crested tinamou (*Eudromia elegans*).

*Figure 2.* Chromosome painting with chicken chromosome 1-9 probes to PI-stained R-banded metaphase spreads of double-wattled cassowary (*Casuarius casuarius*) female. Scale bar indicates 10 μm.

*Figure 3.* Chromosome painting with chicken chromosome 1-9 probes to PI-stained R-banded metaphase spreads of elegant crested tinamou (*Eudromia elegans*) female. Scale bar indicates 10 μm.

*Figure 4.* Chromosome painting with chicken chromosome Z probe to metaphase spreads of the ZW females of five Struthioniformes species and one Tinamiformes species. (**a**, **b**) emu (*Dromaius novaehollandiae*, DNO). (**c**) double-wattled cassowary (*Casuarius casuarius*, CCA). (**d**, **e**) ostrich (*Struthio camelus*, SCA). (**f**) greater rhea (*Rhea americana*, RAM). (**g**, **h**) lesser rhea (*Pterocnemia pennata*, PPE). (**i**) elegant crested tinamou (*Eudromia elegans*, EEL). (**b**), (**e**) and (**h**) are Hoechst-stained patterns of the PI-stained R-banded metaphase spreads shown in (**a**), (**d**) and (**g**), respectively,

which show the same banding patterns as G-banding. Scale bar indicates 10 μm.

*Figure 5.* Chromosomal distribution of the 18S-28S ribosomal RNA genes and the nucleolar organizer regions (NORs) on metaphase spreads of the ZW females of five Struthioniformes species and one Tinamiformes species. (**a**-**c**) lesser rhea (*Pterocnemia pennata*, PPE). (**d**) emu (*Dromaius novaehollandiae*, DNO). (**e**) double-wattled cassowary (*Casuarius casuarius*, CCA). (**f**) ostrich (*Struthio camelus*, SCA). (**g**) greater rhea (*Rhea americana*, RAM). (**h**, **i**) elegant crested tinamou (*Eudromia elegans*, EEL). (**a**, **d**, **e**, **f**, **g**, **h**) FISH patterns of the 18S-28S ribosomal RNA genes on PI-stained R-banded metaphase spreads. (**c**) and (**i**) are Ag-NOR-stained patterns of the metaphase spreads shown in (**a**, **b**) and (**h**), respectively. (**b**) Hoechst-stained pattern of the metaphase spread shown in (**a**, **c**), which shows the same banding pattern as G-banding. Scale bar indicates 10 μm.

*Figure 6.* FISH mapping of the *ACO1/IREBP*, *CHD1* and *ZOV3* genes and the EE0.6 sequence on PI-stained R-banded metaphase spreads of lesser rhea (*Pterocnemia pennata*) female. (**a**) FISH mapping of *ACO1/IREBP* gene using a cosmid clone as biotinylated probe on PI-stained metaphase spread. (**b**) Detection of the *CHD1* gene on PI-stained metaphase spread using a 4.5 kb genomic DNA fragment as biotinylated probe. (**c**) Simultaneous detection of the *ACO1/IREBP* and *ZOV3* genes and the EE0.6 sequence on the Z and W chromosomes. The FITC signals of biotin-labeled *ACO1/IRFBP* and EE0.6 (greenish-yellow) and the rhodamin signals of DIG-labeled

*ZOV3* (red) are localized to the Z and W sex chromosomes. The order of the clones is *ACO1/IREBP* - *ZOV3* - EE0.6 from the proximal on the Z chromosome, whereas *ACO1/IREBP* is deleted on the W chromosome. Scale bar indicates 10 μm.

*Figure 7.* Summary of Giemsa-stained, C-banded and GBG-banded patterns and chromosome painting patterns with the chicken Z probe of the Z and W chromosomes, and the locations of four DNA markers on the Z and W chromosomes in emu (*Dromaius novaehollandiae*, DNO), double-wattled cassowary (*Casuarius casuarius*, CCA), ostrich (*Struthio camelus*, SCA), greater rhea (*Rhea americana*, RAM) and lesser rhea (*Pterocnemia pennata*, PPE) of the Struthioniformes, elegant crested tinamou (*Eudromia elegans*, EEL) of the Tinamiformes and chicken (*Gallus gallus*, GGA). The chromosomal locations of *ACO1/IREBP*, *ZOV3* and EE0.6 on the Z and W chromosomes of emu and ostrich, and double-wattled cassowary were taken from Ogawa *et al.* (1998) and Nishida-Umehara *et al.* (1999), respectively. The chromosomal location of *CHD1* in ostrich and the location of *ACO1/IREBP* and *CHD1* in elegant crested tinamou were taken from Tsuda *et al.* (2007).

*Table 1* . Comparative chromosome painting in six Palaeognathae species with chicken chromosome paints 1 - 9 and Z

| Species | 2n | Chromosome | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| chicken<br>*Gallus domesticus* | 78 | GGA1 | GGA2 | GGA3 | GGA4 | GGA5 | GGA6 | GGA7 | GGA8 | GGA9 |
| emu[a]<br>*Dromaius novaehollandiae* | 80 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |
| cassowary<br>*Casuarius casuarius* | 92 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |
| ostrich<br>*Struthio camelus* | 80 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |
| greater rhea[b]<br>*Rhea americana* | 80 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |
| lesser rhea<br>*Pterocnemia pennata* | 80 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |
| tinamou<br>*Eudromia elegans* | 80 | 1 | 2 | 3 | 4+m | 5 | x | x | x | x |

x : the corresponding chromosome detected by chicken probe

m : microchromosome

[a] Shetty *et al.* 1999

[b] The data of chromosome painting with GGA1-9 were taken from Guttenbach *et al.* 2003

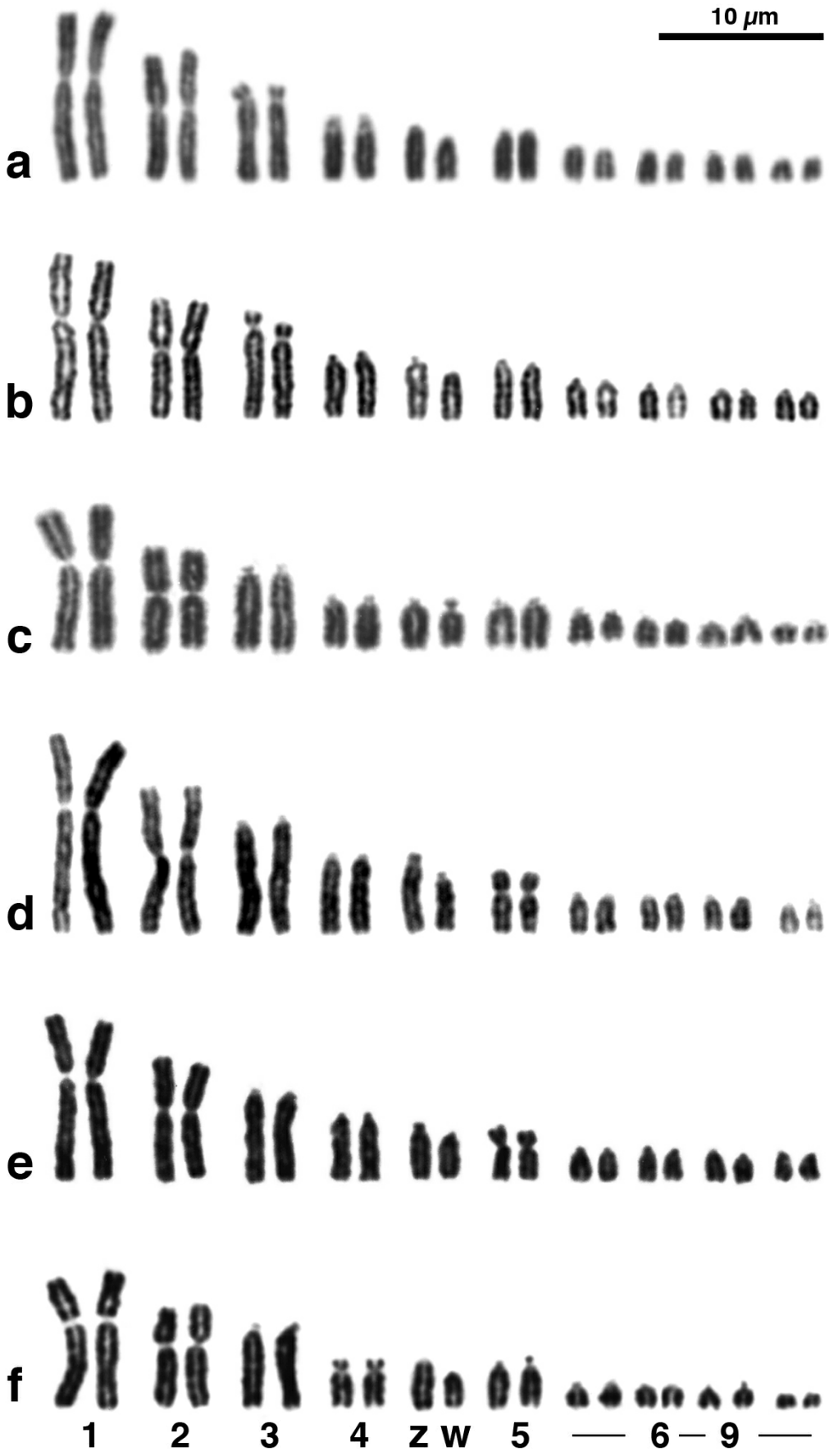



**Figure 1 (Nishida-Umehara *et al.*)**

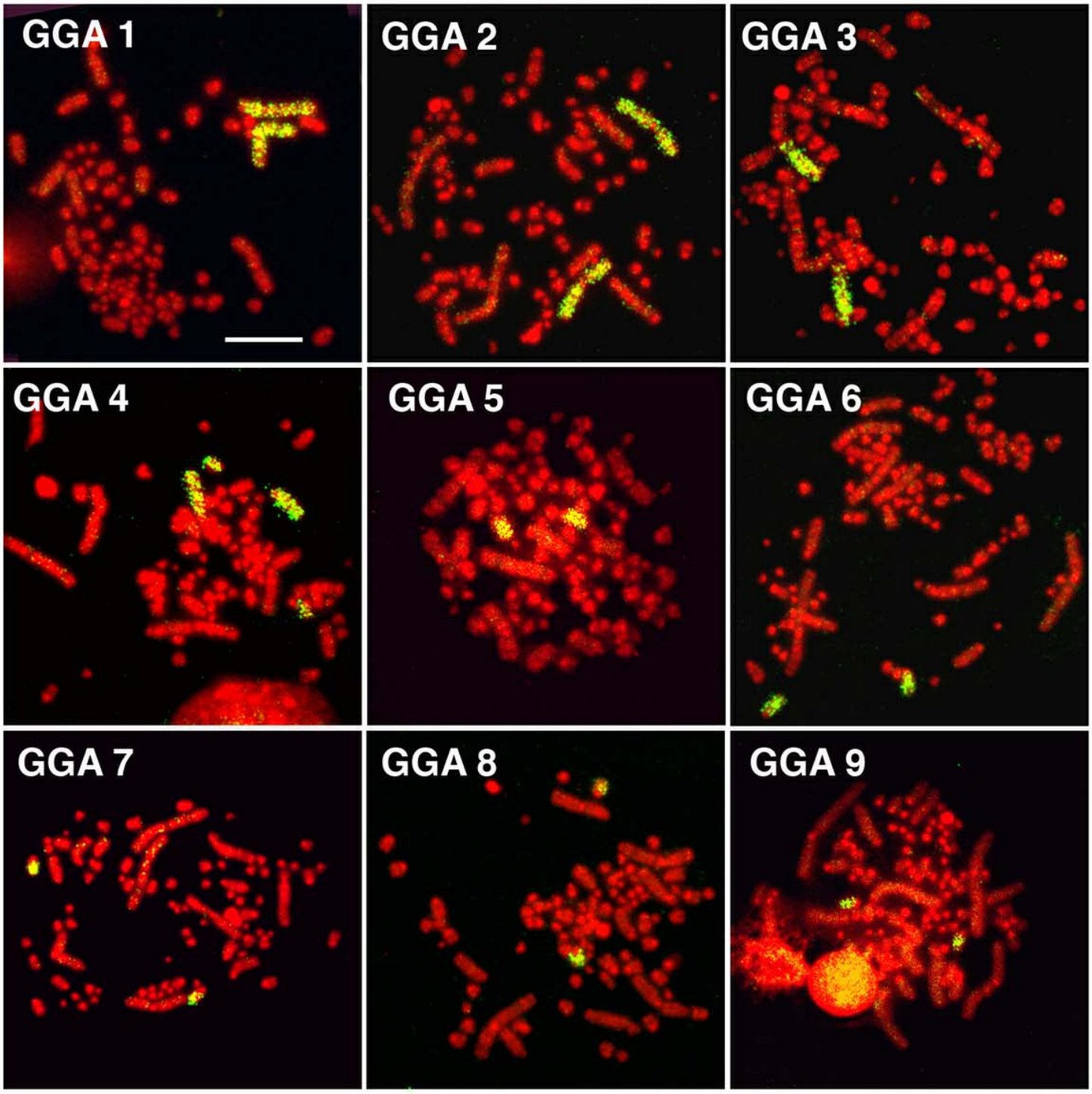



**Figure 2 (Nishida-Umehara *et al.*)**

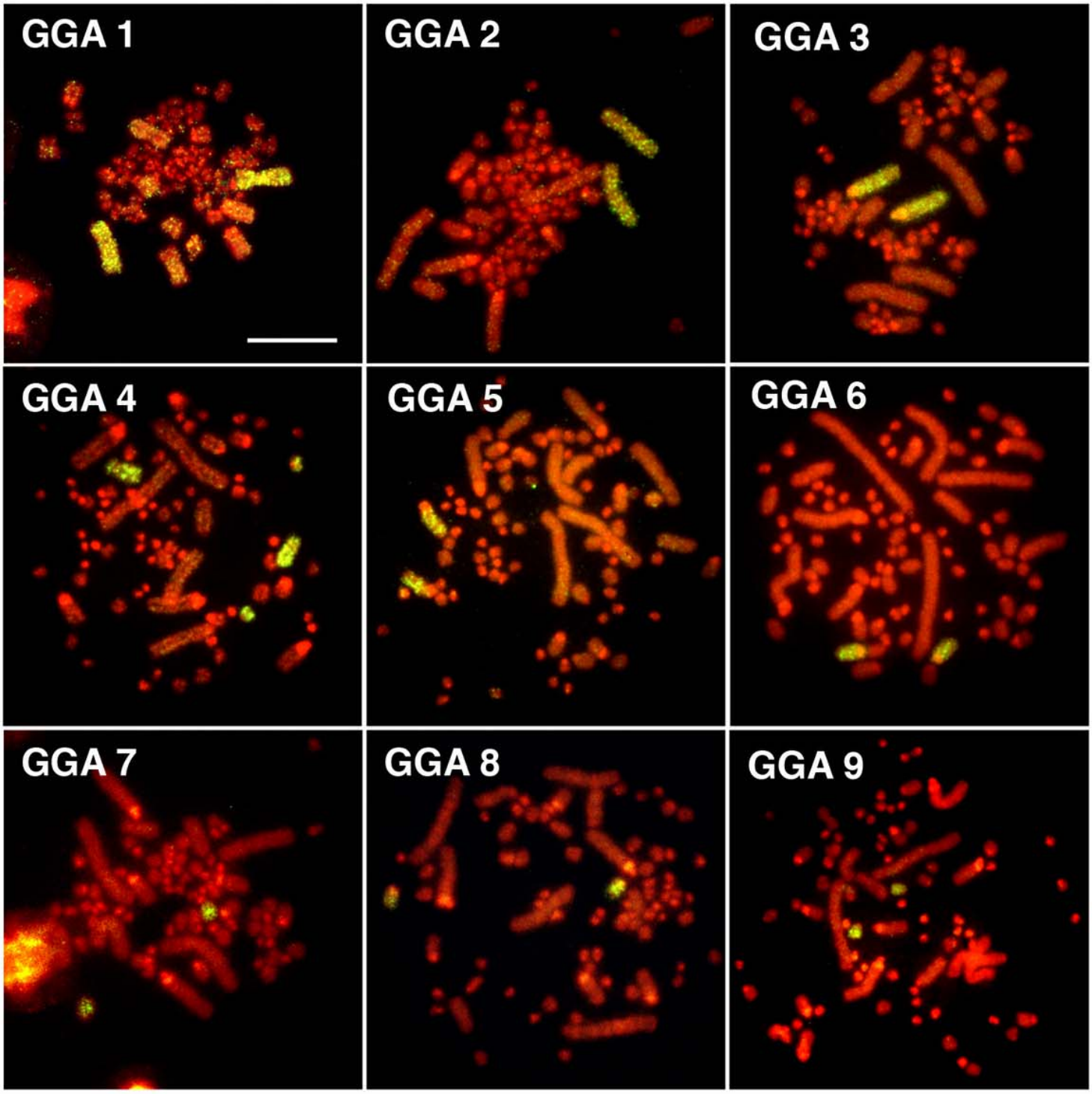



**Figure 3 (Nishida-Umehara *et al.*)**

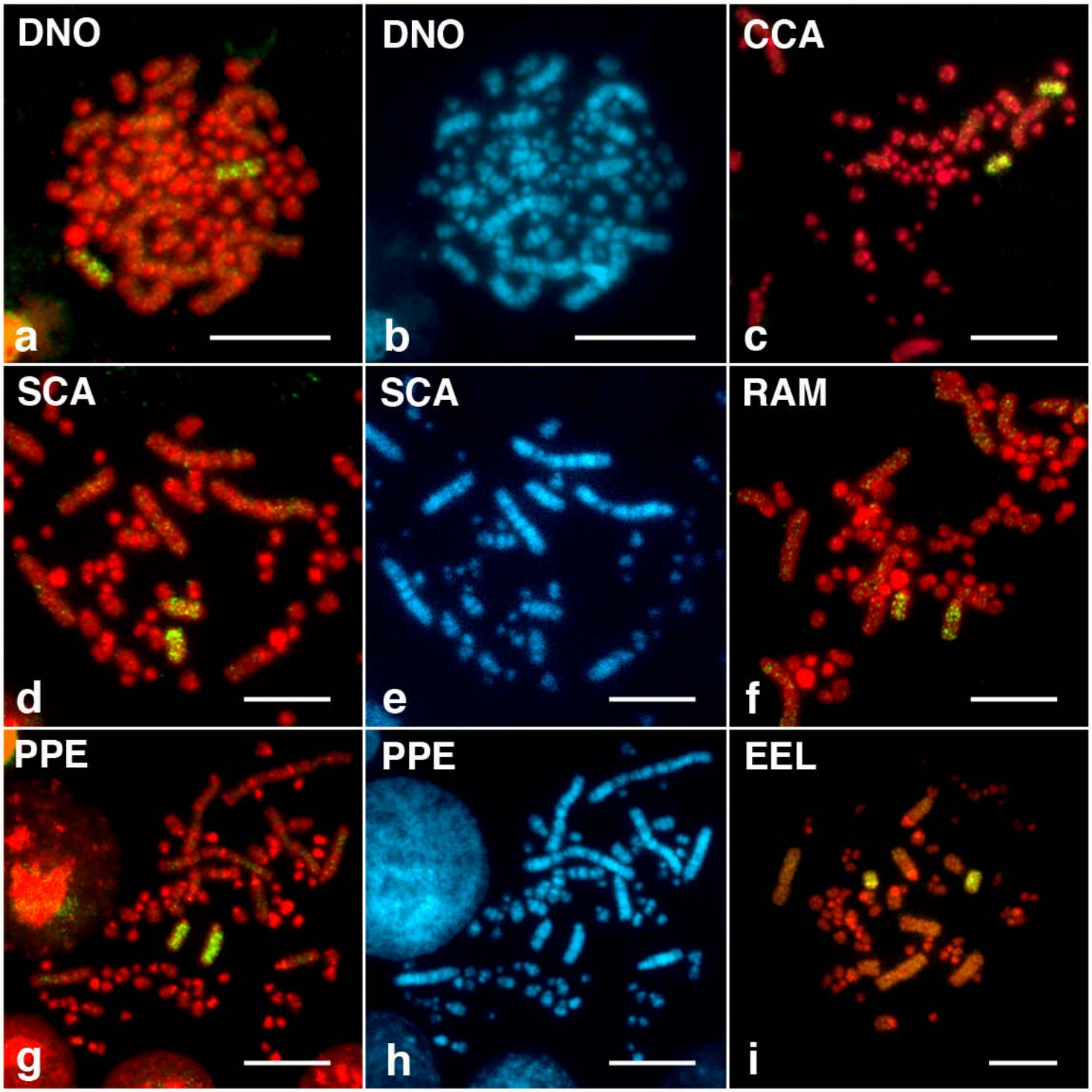



**Figure 4 (Nishida-Umehara *et al.*)**

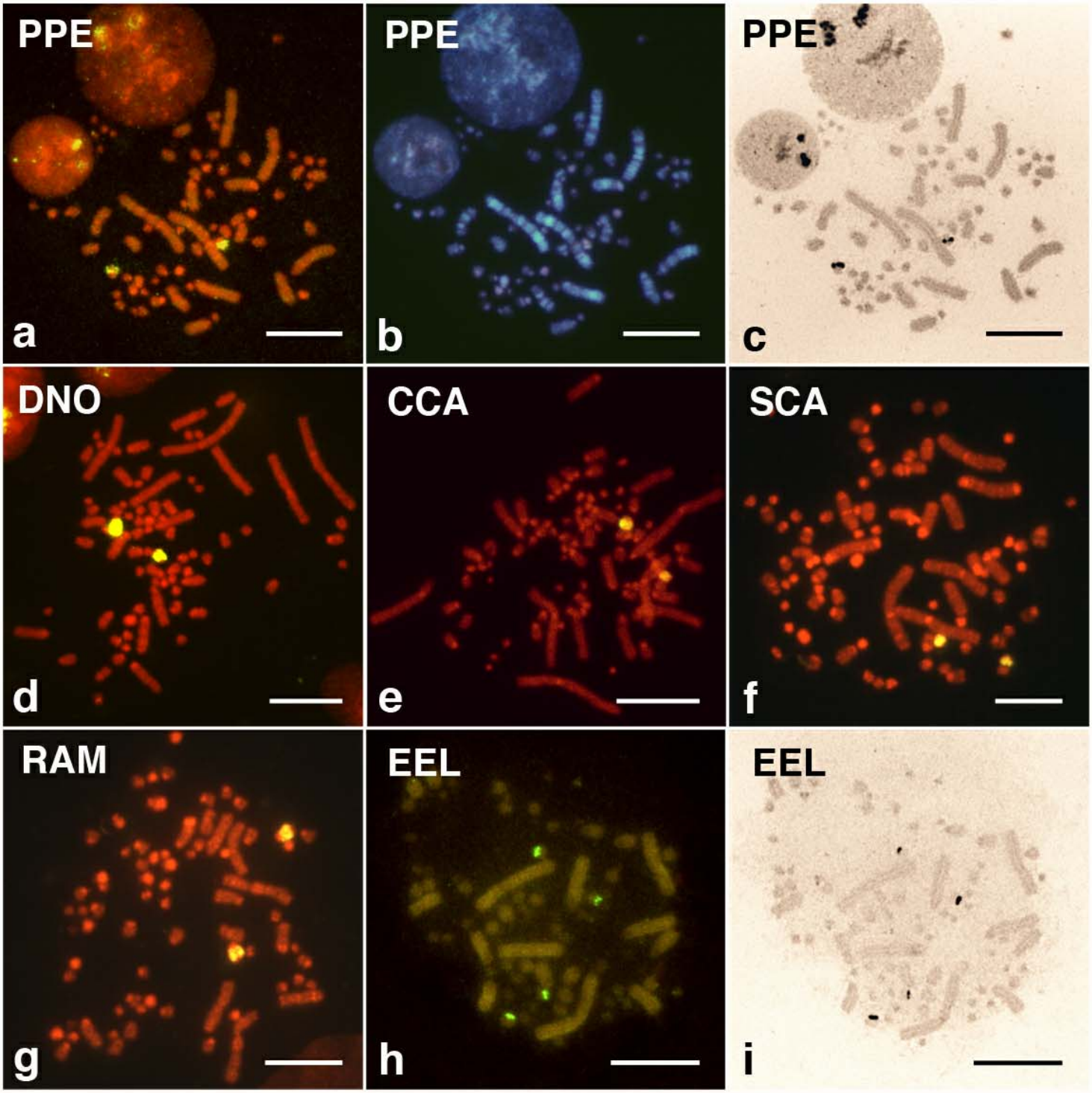



**Figure 5 (Nishida-Umehara *et al.*)**

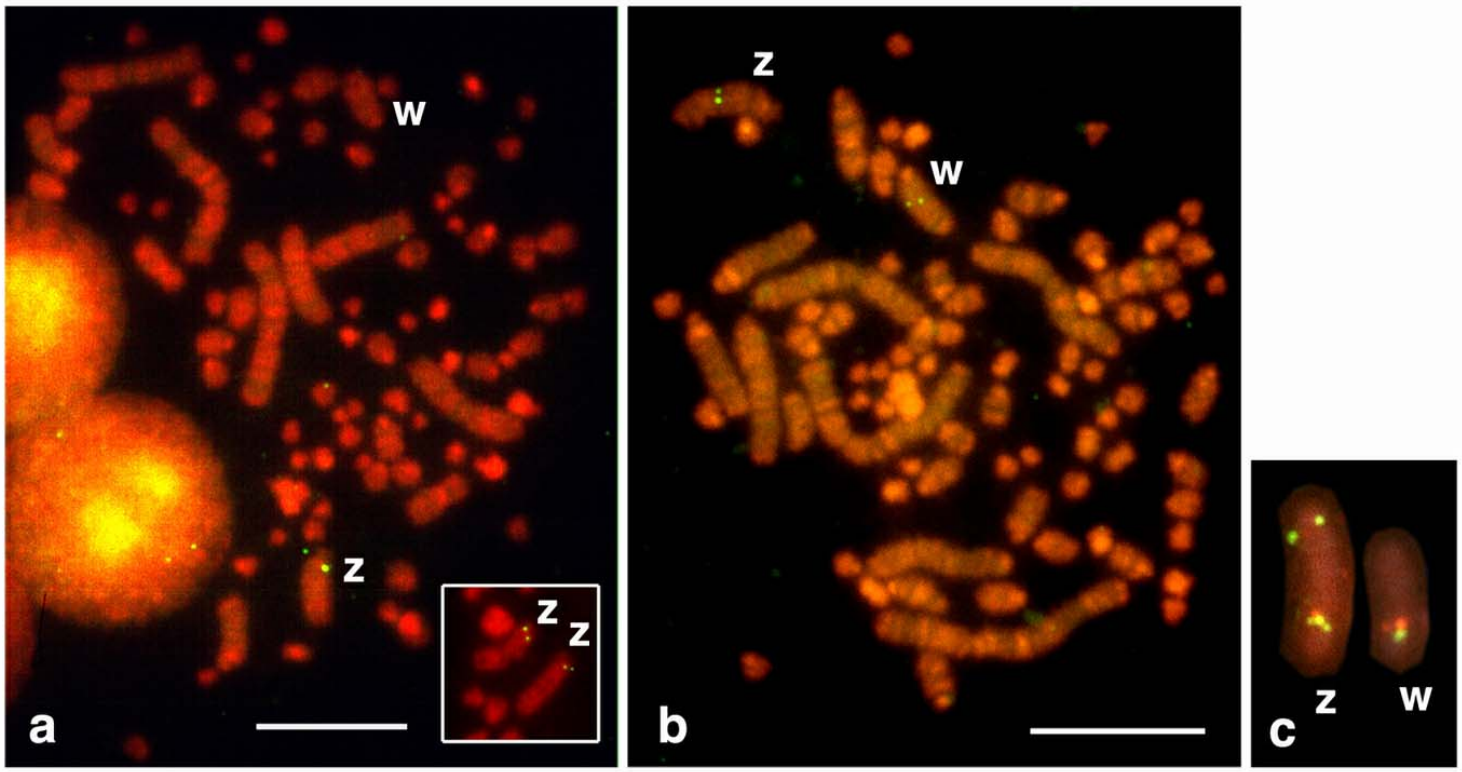



**Figure 6 (Nishida-Umehara *et al.*)**

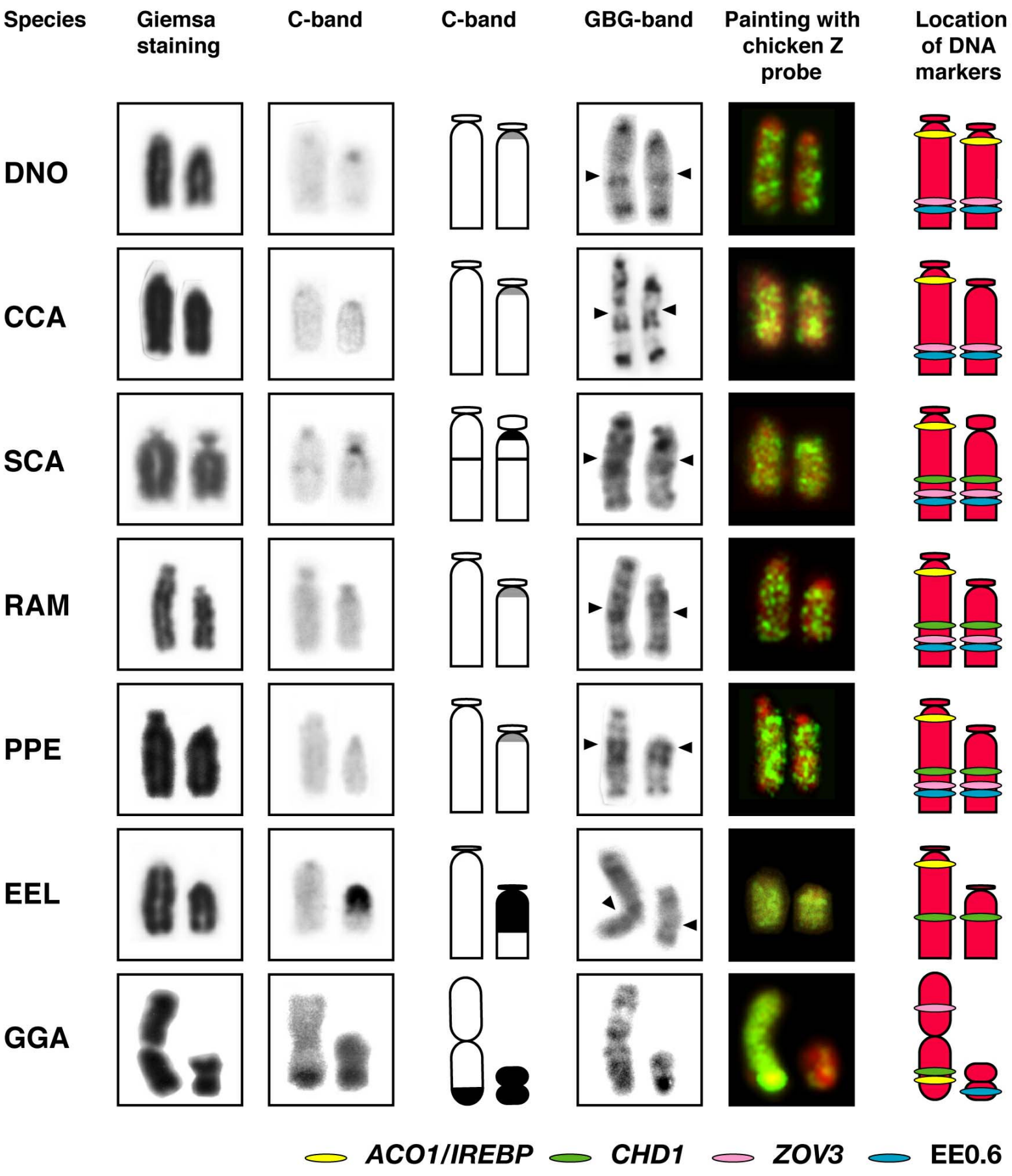



**Figure 7 (Nishida-Umehara *et al.*)**