Genomic Structures of the kW1 Loci on the Z and W Chromosomes in Ratite Birds: Structural Changes at an Early Stage of W Chromosome Differentiation

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Key Words

kW1 sequence · Neognathous birds · Paleognathous birds · Ratite · Repetitive sequence · Reptiles · Sex chromosome differentiation

Abstract

The W chromosome of ratite birds shows minimal morphological differentiation and retains homology of genetic linkage and gene order with a substantial stretch of the Z chromosome; however, the molecular structure in the differentiated region is still not well known. The kW1 sequence was isolated from the kiwi as a W-specific DNA marker for PCR-based molecular sexing of ratite birds. In ratite W chromosomes, this sequence commonly contains a ~200-bp deletion. To characterize the very early event of avian sex chromosome differentiation, we performed molecular cytogenetic analyses of kW1 and its flanking sequences in paleognathous and neognathous birds and reptiles. Female-specific repeats were found in the kW1-flanking sequence of the cassowary (*Casuarius casuarius*), and the repeats have been amplified in the pericentromeric region of the W chromosomes of ratites, which may have resulted from the cessation of meiotic recombination between the Z and W chromo-

somes at an early stage of sex chromosome differentiation. The presence of the kW1 sequence in neognathous birds and a crocodilian species suggests that the kW1 sequence was present in the ancestral genome of Archosauria; however, it disappeared in other reptilian taxa and several lineages of neognathous birds.

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Sex chromosomes initially evolved from an autosomal pair after a master sex-determining gene or region appeared on one of the homologous autosomes [Ohno, 1967]. Suppression of meiotic recombination between proto-sex chromosomes is proposed to extend in a stepwise fashion that includes intrachromosomal rearrangement events, such as inversions [Lahn and Page, 1999; Handley et al., 2004; Hughes et al., 2012; Vicoso et al., 2013b]. This would be followed by genetic degeneration of the sex-specific chromosome and the accumulation of repetitive sequences, often leading to morphologically heteromorphic sex chromosomes [Charlesworth, 1991].

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However, the molecular events at the very early stage of the differentiation of sex chromosomes from autosomes remain elusive. Several species with morphologically homomorphic sex chromosomes at an initial stage of differentiation have been reported whose sex-determining genes are identified, i.e. the African clawed frog (*Xenopus laevis*) [Yoshimoto et al., 2008], a medaka fish (*Oryzias latipes*) [Myosho et al., 2012] and the tiger pufferfish (*Takifugu rubripes*) [Kamiya et al., 2012]. In medaka (*Oryzias latipes*) [Matsuda et al., 2002; Nanda et al., 2002] and in the papaya plant (*Carica papaya*) [Liu et al., 2004], the sex chromosomes are homomorphic except for a small sex-specific region that contains sex-determining gene(s) and multiple copies of simple repeated sequences and/or transposable elements.

Unlike mammals with male heterogamety, birds exhibit a sex chromosome system with ZZ/ZW-type female heterogamety. Extant bird species show a wide range of sex chromosome differentiation patterns. Ratites (kiwi, emu, cassowary, ostrich, and rhea) represent the most primitive stage of sex chromosome differentiation. The Z and W sex chromosomes of ratites are distinguishable only by their slight differences in size; the Z chromosome is slightly larger than the W [Takagi et al., 1972; de Boer, 1980], and C-positive heterochromatin is barely detectable in the W chromosomes [Ansari et al., 1988; Nishida-Umehara et al., 2007]. Comparative chromosome painting and gene mapping revealed extensive homology between the Z and W chromosomes of ratites, with recombination occurring along most of the length of the gametologs [Shetty et al., 1999; Nishida-Umehara et al., 2007; Janes et al., 2008], and structural differentiation has been found only for a relatively small deletion near the centromere of the acrocentric W chromosome [Nishida-Umehara et al., 2007; Tsuda et al., 2007]. *DMRT1* (double-sex and *mab-3*-related transcription factor 1) is a strong candidate for the sex-determining gene in birds, and its paralogs determine sex in birds, medaka fish (*O. latipes*) and *X. laevis* [Matsuda et al., 2002; Nanda et al., 2002; Scharl, 2004; Yoshimoto et al., 2008]. *DMRT1* is deleted in the chicken W chromosome and considered to be involved in testis determination by 2-fold gene dosage in ZZ males [Nanda et al., 2000; Smith et al., 2009]. *DMRT1* is also deleted in the W chromosome of a paleognathous bird species, the emu [Shetty et al., 2002]; however, it is still unknown whether it is present in other ratite species. Therefore, the ratite sex chromosome is a good model to understand the molecular basis of the initial and subsequent steps of sex chromosome differentiation. By contrast, neognathous birds have highly differentiated Z and

W chromosomes, and the W chromosome is heterochromatic. Tinamiformes (tinamous), which form the monophyletic group Palaeognathae with the Struthioniformes [Hackett et al., 2008; Harshman et al., 2008], show an intermediate type of ZW differentiation pattern: their Z and W chromosomes show a lower level of similarity, and the W degeneration that has occurred could have been aided by the accumulation of repetitive DNA sequences [Tsuda et al., 2007], although the differentiation of the sex chromosomes is not as extensive as in neognathous birds [Pigozzi and Solari, 1999; Mank and Ellegren, 2007; Tsuda et al., 2007].

Genomic analysis of sex chromosome pairs at an early stage of differentiation sheds light on their evolutionary processes. Several random amplified polymorphic DNA (RAPD) markers have been developed to detect sex-specific sequences in the ostrich [Bello and Sánchez, 1999] and emu [de Kloet, 2001]. The kW1 (kiwi W-specific DNA fragment) sequence is a sex-specific genomic sequence isolated from the kiwi [Huynen et al., 2002], which is a good molecular marker for obtaining a better understanding of the structural difference between the Z and W chromosomes at a very early stage of sex chromosome differentiation. A W-specific ~200-bp deletion of this non-coding sequence is common in ratites and facilitates PCR-based molecular sexing of all living ratites and an extinct ratite species, the moa [Bunce et al., 2003]. In the present study, we cloned the kW1 sequence from paleognathous and neognathous birds and reptiles to clarify the origin of this sequence. Then, to examine the genomic structural changes at an early stage of ratite W chromosome differentiation, we sequenced flanking regions of the kW1 locus from the Z and W chromosomes of the cassowary. Our findings suggest that the kW1 sequence was present in the genome of the common ancestor of Archosauria, and female-specific repeats in the flanking region of the kW1 sequence were amplified in the pericentromeric region of ratite W chromosomes due to the cessation of meiotic recombination.

Materials and Methods

Specimens

The species used in this study were emu (*Dromaius novaehollandiae*), double-wattled cassowary (*Casuarus casuaris*), ostrich (*Struthio camelus*), and lesser rhea (*Pterocnemia pennata*; synonym *Rhea pennata*) of the Struthioniformes; elegant crested tinamou (*Eudromia elegans*, Tinamiformes), chicken (red junglefowl) (*Gallus gallus*, Galliformes), whooper swan (*Cygnus cygnus*, Anseriformes), Blakiston's fish-owl (*Bubo blakistoni*, synonym *Ketupa blakistoni*, Strigiformes), Japanese mountain hawk-eagle (*Ni-*

saetus nipalensis orientalis, synonym *Spizaetus nipalensis orientalis*, Falconiformes); and 3 reptilian species, Chinese soft-shelled turtle (*Pelodiscus sinensis*, Testudines), Siamese crocodile (*Crocodylus siamensis*, Crocodilia), and red-tailed boa (*Boa constrictor*, Squamata). One male and 1 female of each species were used excepting *E. elegans* (5 males and 6 females), *B. blakistoni* (3 males and 4 females) and *N. nipalensis orientalis* (4 males and 4 females). Fibroblast cells were obtained by skin biopsies from male and female individuals of all avian species, cultured, and then used for chromosome preparation and DNA extraction. Genomic DNA of *P. sinensis* and *C. siamensis* was extracted from liver tissues which were obtained in our previous study [Uno et al., 2012] and had been kept at -80°C . Genomic DNA of *B. constrictor* was extracted from peripheral blood obtained from live individuals maintained at the Japan Snake Institute, Gunma, Japan. All experimental procedures using animals conformed to the guidelines established by the Animal Care Committees of Hokkaido University and Nagoya University.

PCR and Nucleotide Sequence Analysis

The *CHD1* (chromodomain helicase DNA binding 1) gene was used as a control for the detection of sex-specific DNA fragments in neognathous birds, in which the size-different Z- and W-specific fragments are amplified by PCR [Fridolfsson and Ellegren, 1999]. PCR amplification was carried out in a 15- μl mixture containing 1.5 mM MgCl_2 , 0.2 mM each of dNTPs, 0.2 μM of each primer, 20 ng of genomic DNA, 0.25 U Takara Ex Taq polymerase (Takara Bio), and 1.5 μl of $10\times$ Ex Taq buffer. DNA fragments of the *CHD1* gene were amplified using the primers 2550F (5'-GT-TACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAAT-GATCCAGTGCTTG-3'), and PCR was performed by heating samples to 94°C for 2 min, then 30 cycles at 94°C for 30 s, 46°C for 30 s, and 72°C for 35 s, followed by heating to 72°C for 5 min. The kW1 sequence was amplified with the primers w1 (5'-CCT-TTAAACAAGCTGTAAAGCA-3') and k7 (5'-TCTCTTTT-GTTCTAGACACCCT-3') [Huynen et al., 2002] as described above, except that the annealing temperature was 48°C . PCR fragments were cloned into the pGEMT-easy vector (Promega), and their nucleotide sequences were determined. DNA sequences were aligned using ClustalW implemented in MEGA5 [Tamura et al., 2011], the alignments were refined with our eyes, and their phylogenetic tree was constructed with a neighbor-joining method. All sequences used for phylogenetic analysis were deposited in GenBank.

Genomic DNA Walking

The Z- and W-specific kW1-flanking sequences were amplified from genomic DNA of 1 male and 1 female *C. casuarius*, respectively, using a DNA Walking SpeedUp kit (Seegene). On the basis of the nucleotide sequences of the CcZ-(w1, k7) and CcW-(w1, k7) fragments, which were amplified from male and female genomic DNA of *C. casuarius*, respectively, Z- and W-specific genomic sequences were extended from the kW1 locus in k7 and w1 directions. The W-specific sequences were amplified by PCR using target-specific primers, TSP1 (5'-TGAGGGGACATCTTGTTT-3'), TSP2 (5'-TCTACAATGGAGGCTTTCAC-3'), TSP4 (5'-GTGAAAG-CCTCCATTGTAGA-3'), and TSP5 (5'-AAAACCAAGATGTC-CCCTCA-3'). Walking primers for the *C. casuarius* W sequences (TSP1 and TSP5) were designed across the female-specific deleted region of the CcW-(w1, k7) fragment. For amplification of Z-spe-

cific sequences, w1, k7, TSP10 (5'-ATAAAGAGCTACAACCA-CAG-3'), and TSP11 (5'-CTGTGGTTGTAGCTCTTTAT-3') were used as target-specific primers.

Chromosome Preparation and Fluorescence in situ Hybridization

Cultured fibroblast cells of *C. casuarius*, *D. novaehollandiae* and *S. camelus* were harvested after 6 h of treatment with BrdU (25 $\mu\text{g}/\text{ml}$), suspended in 0.075 M KCl, fixed in methanol/acetic acid (3:1), and spread onto clean glass slides using a standard protocol. Replication banding of chromosomes and fluorescence in situ hybridization (FISH) analysis was performed as described previously [Matsuda and Chapman, 1995].

Hybridization of DNA Blots

For Southern blot hybridization, 4 μg genomic DNA of female and male cassowaries was digested with *EcoRI* and *TaqI*. The DNA was fractionated in 1% agarose gel and transferred onto nylon membranes (Roche Diagnostics). DNA probes were labeled with DIG-11-dUTP using the PCR DIG Labeling Mix (Roche Diagnostics), followed by hybridization to the membranes at 45°C overnight in DIG Easy Hyb solution (Roche Diagnostics), and then the membranes were washed at 45°C in 0.1% SDS/ $2\times$ SSC, 0.1% SDS/ $1\times$ SSC, 0.1% SDS/ $0.5\times$ SSC, and 0.1% SDS/ $0.1\times$ SSC for 15 min each. The luminescent signals were generated with anti-digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics) and detected by exposing the membrane to Biomax MS-1 autoradiography film (Carestream Health).

We used slot-blot hybridization to estimate the copy numbers of repeated sequences. Four different concentrations of RNase-treated whole genomic DNA were denatured with 0.4 N NaOH for 10 min and transferred to nylon membranes (Roche Diagnostics) using BIO-DOT SF blotting equipment (Bio-Rad). DNA probes were labeled with DIG-11-dUTP using the PCR DIG Labeling Mix, and hybridized with the membranes at 45°C in DIG Easy Hyb solution. The luminescent signals were detected as described above, and densitometric analysis of the signals was performed using NIH Image 1.63 software (<http://rsb.info.nih.gov/nih-image/>).

Molecular Cloning and Chromosome Mapping of the DMRT1 Gene

Total RNA was extracted from the testis of *S. camelus* using TRIzol reagent (Invitrogen). Three micrograms of total RNA were mixed with 0.5 μg of Oligo (dT)₁₂₋₁₈ primers (Invitrogen) and incubated for 10 min at 70°C . After cooling on ice, the solution was modified to contain $1\times$ first-strand buffer, 0.1 M DTT, 10 mM dNTP, and 200 U SuperScript II RNase H-reverse transcriptase (Invitrogen). Reactions were performed for 50 min at 42°C and then terminated by incubation for 15 min at 70°C . Amplification of cDNA was carried out in 20 μl of $1\times$ Ex Taq buffer containing 1.5 mM MgCl_2 , 0.2 mM dNTPs, 5.0 μM degenerate primers, and 0.25 U TaKaRa Ex Taq (Takara Bio) with the forward primer 5'-GCAGCGGGTGATGGCNGCNCAGGT-3' and the reverse primer 5'-GGCCACGGCCATCTGGTAYTGNGAGTA-3'. PCR was performed under the conditions described above except that the annealing temperature was 55°C . The resultant *DMRT1* cDNA fragment (379 bp) (accession No. AB536737) was cloned and used as a probe for screening a testis cDNA library of *S. camelus*. Five micrograms of poly(A)⁺ RNA was cloned into the lambda ZAP vector to construct a cDNA library using a ZAP-cDNA synthesis

Fig. 1. PCR amplification of kW1 sequence fragments and molecular sexing with the *CHD1* gene in avian and reptilian species. The analysis involved 4 ratite bird species (*D. novaehollandiae*, *C. casuarius*, *S. camelus*, and *P. pennata*), a tinamid bird (*E. elegans*),

4 neognathous bird species (*G. gallus*, *C. cygnus*, *B. blakistoni*, and *N. nipalensis orientalis*), and 3 reptilian species (*P. sinensis*, *C. siamensis* and *B. constrictor*). F = female; M = male; - = sex not determined; Ma = DNA size marker (*HaeIII*-digested ϕ X174 DNA).

Table 1. The w1-k7 fragments isolated from 7 avian and 1 crocodilian species and the kW1-flanking sequences of *C. casuarius*

DNA fragment Species	Chromosome	Length of DNA fragment, bp	Accession No.	
w1-k7 fragment				
<i>Casuarius casuarius</i>	Z	286	AB536723	
	W	103	AB536724	
<i>Dromaius novaehollandiae</i>	Z	295	AB841267	
	W	103	AB841268	
<i>Pterocnemia pennata</i>	Z	289	AB536725	
	W	103	AB536726	
<i>Struthio camelus</i>	Z	294	AB564694	
	W	110	AB564693	
<i>Eudromia elegans</i>	W	271	AB536727	
<i>Bubo blakistoni</i>	Z	280	AB536728	
	W	295	AB536729	
<i>Nisaetus nipalensis orientalis</i>	Z	288	AB536730	
	W	295	AB536731	
<i>Crocodylus siamensis</i>		285	AB536732	
kW1-flanking sequence				
<i>Casuarius</i>	CcZ-w1	Z	888	AB536735
<i>casuarius</i>	CcZ-k7	Z	763	AB536736
	CcW-w1	W	967	AB536733
	CcW-k7	W	1,786	AB536734

kit (Stratagene) and Gigapack III Gold packaging extracts (Stratagene). Hybridization signals were detected using a digoxigenin luminescent detection system (Roche Diagnostics), as suggested by the supplier. The selected clone contained about 1.3 kb of the homologous sequence of *DMRT1* (AB536738). Given that the clone had a 3' UTR region that is rich in repeat sequences, a subclone (668 bp) without the 3' UTR region was used for FISH analysis to eliminate nonspecific hybridization signals. A mixture (1:1) of the 668-bp cDNA clone and the 379-bp PCR product, which spans exons 2 through 5 (831 bp) of the *DMRT1* sequence, was used as a FISH probe.

Results

kW1 Sequences in Birds and Reptiles

We performed PCR amplification of the kW1 sequences from the genomic DNA of 9 avian species and 3 reptilian species that represent the orders Testudines, Crocodilia and Squamata. This involved the use of k7 and w1 primers and determination of their nucleotide sequences (figs. 1, 2; table 1). The 286- to 295-bp w1-k7 fragments were detected in both sexes in 4 ratite species, whereas additional 103- or 110-bp fragments were amplified only in females. By contrast, the w1-k7 fragment was

Fig. 2. Alignment of nucleotide sequences of the w1-k7 fragments of 8 avian species (*C. casuarius*, *D. novaehollandiae*, *P. pennata*, *S. camelus*, *A. australis manteui*, *E. elegans*, *B. blakistoni*, and *N. nipalensis orientalis*). The nucleotide sequence of the kW1 sequence of *A. australis manteui* (AF308932) was taken from Huynen et al. [2002].

	10	20	30	40	50	60
<i>C. casuarius</i> Z	GTATAATGTT	CACCATTTTG	TTACTAATAA	TGAAGTAAAA	GCCTCTGTCA	CAGAGCA--T
<i>D. novaehollandiae</i> Z	.A.....	.....	.....	.....G.....	.....T.....	.....
<i>P. pennata</i> Z	.....	.....C.....	.....	.....A...T.C.....	.....	.....
<i>S. camelus</i> Z	.....	.....	.....	.....CT.....	.....	.....
<i>A. a. manteui</i>	.....C.....	.....C.....	.....G.....	CA.....	AT...C.....	T.....CTG
<i>B. blakistoni</i> Z	..G.C.C.....	.....	.....	.....	A.....	T.A.....
<i>N. n. orientalis</i> Z	-G.....	.....G..A.....	.....	.....	A.....	T..GA.C--
<i>C. siamensis</i>	.....G..A.....	.....T.....	.....G..A..G.....	.....G.....	TGG.G.CGG	T...AG--G
<i>C. casuarius</i> W	A....GCA.....	.....	.....G.....	.....C.....	.....CA.TG	T.....
<i>D. novaehollandiae</i> W	A....GCA.....	.....	.....G.....	.....C.....	.....CA.TG	T.....
<i>P. pennata</i> W	A....CA.....	.....	.....G.....	.....	A.A..CA.TG	T.....
<i>S. camelus</i> W	A....CA.....	.....	.....G.....	.....	.....CA.TG	T.....
<i>E. elegans</i> W	..GG.....	.....A.....	.....	.....CT.....	.....AA.....	T...ATG--
<i>B. blakistoni</i> W	..G.....	.....	.....G.....	.....	.....	T...CTG--G
<i>N. n. orientalis</i> W	..G.G.....	.....	.....T.....	.....	.....	T...TG--G
	70	80	90	100	110	120
<i>C. casuarius</i> Z	TTTAACAGGA	TGCTCCAAAC	CAAAATG--G	TGATATTTTA	TAGCACCCAA	CACTTTCCGC
<i>D. novaehollandiae</i> Z	.....	.....AC.....	.....G...TG..A.....	.....G.....	.....	.....T.A.
<i>P. pennata</i> Z	.....	.....A.....	.....TG..A.....	.....	.....TT..	.....T.T..TT..
<i>S. camelus</i> Z	.....	.....A.....	.....TG..A.....	.....	.....TG.A.....	.....TTT..
<i>A. a. manteui</i>	.....G.....	.....A.....	.....C.C.TG..AT.....	.....	C...G.G.....	TG...T.AT
<i>B. blakistoni</i> Z	.....	CA.C.A.....	.....G...TG..A.....	.....C.....	.....	.....
<i>N. n. orientalis</i> Z	.....	.....A.....	.....GG..TG..A.....	.....	C.A..TG...	.....ATT..
<i>C. siamensis</i>	.....	CAT.AA..T..A..G...TG..A..G..A.....	.....	.....	.....CTG...	.....C..T..
<i>C. casuarius</i> W	.....	CA..A.....	.....G...T--	.....	.....	.....
<i>D. novaehollandiae</i> W	.....	C...A.....	.....G...T--	.....	.....	.....
<i>P. pennata</i> W	.....	A.....	.....G...T--	.....	.....	.....
<i>S. camelus</i> W	.....	C...A.....	.....G...T--	.....	.....	.....
<i>E. elegans</i> W	.....C--	.....	..G...TG..G.....	.....	C...TG...	..AC..TT..
<i>B. blakistoni</i> W	.....	.....A.....	.....G...TG..A.....	.....	C.CTGTG...	..GTG...T..
<i>N. n. orientalis</i> W	.....	.....A.....	.....GGC.TG..A.....	.....	C.CTGTG...	..TG...T..
	130	140	150	160	170	180
<i>C. casuarius</i> Z	TACATTGATG	TATTGTTG-G	AGTTTATAAA	GAGTACAAC	CACAGCTTCC	ACAGA-ACTA
<i>D. novaehollandiae</i> Z	.....	.....G...CA--	.....C.....	.....T.....	.....	.....G.....
<i>P. pennata</i> Z	.....	.....	.....	--T..TTGG	AGT.TA.AAA	..G..CT...
<i>S. camelus</i> Z	.....	.....G.....	.....	.....GT.....	..T.C.....	.....G..
<i>A. a. manteui</i>	G.....	..G..A.CAA.....	.....	.....	.....	.....G--
<i>B. blakistoni</i> Z	-T.C.....	..G...CA--	.....	.....T.....	..T.....T	G.....C
<i>N. n. orientalis</i> Z	..TG...A	..G...CA-A	.....C..G.....	.....T.....	..T.A.....	..CA..G..
<i>C. siamensis</i>	.....	..G..AC.A--	.....	.....	.....T..T..	..G...T...
<i>C. casuarius</i> W	.....	.....	.....	.....	.....	.....
<i>D. novaehollandiae</i> W	.....	.....	.....	.....	.....	.....
<i>P. pennata</i> W	.....	.....	.....	.....	.....	.....
<i>S. camelus</i> W	.....	.....	.....	.....	.....	.....
<i>E. elegans</i> W	A.TT.CA...	..G..A...-	.....	CA.A.T...	.....AT..--	.....
<i>B. blakistoni</i> W	.....	..G...CA--	T.....	.....T.....	.....	.....
<i>N. n. orientalis</i> W	.....	..G...CA--	.....	.....T.....	.....	.....T.C
	190	200	210	220	230	240
<i>C. casuarius</i> Z	TCATAATCAG	GCCTGCAGAC	ATGGACCCTT	TAAATCTGCG	GAGGGTCTGG	-AAAAACGTA
<i>D. novaehollandiae</i> Z	..A.GC...	..T..TG..T	C..A.A...	C...C...C	A.....T...	....A.A..
<i>P. pennata</i> Z	.....C.C.	AT...TG...	.....	.....T...T	..A.....	-C.CG..A..
<i>S. camelus</i> Z	..G..C...A...	..TG...T	.....	.....C...C	.....A..A..	-G...T...A..
<i>A. a. manteui</i>	..G.GC...	..TG...T	G..A..A...	.....C...T	.....T...T	.....A..
<i>B. blakistoni</i> Z	CT.G..CTG.	..T..TG..T	G..A..A...	C...T.G..C	AG..A.T...	G.....A..T
<i>N. n. orientalis</i> Z	..G.GC...	..ATG...	..A.TAT.G	.....C..C..C	A.....T...	.....A..
<i>C. siamensis</i>	..CGC.C...	..T..TT...	G..A..TTC	C...C.T.CC	ACTA..G...	....G..A.G
<i>C. casuarius</i> W	.....	.....	.....	.....	.....	.....
<i>D. novaehollandiae</i> W	.....	.....	.....	.....	.....	.....
<i>P. pennata</i> W	.....	.....	.....	.....	.....	.....
<i>S. camelus</i> W	.....	.....	.....	.....	.....	.....
<i>E. elegans</i> W	..G.GC...	.....TG..T	G..AA.....	.....GC...C	A.....T..A	....TA.A.G
<i>B. blakistoni</i> W	..G.GC...	.....TG..T	G..A.....	.....T.C...C	A.....T...	....T...T..
<i>N. n. orientalis</i> W	..G.GC...	.....TG...	..A...A..	.....C...C	A.....T...	....T...T..
	250	260	270	280	290	300
<i>C. casuarius</i> Z	TTT-GGGATG	TGTTTCAAAA	TAAAGATCT	GTGC-----	TCAAAAAGG	GGACGATGTC
<i>D. novaehollandiae</i> Z	..A.G.....	.....GA...T	.....G.....	CA.CTCTGC	.....G.G..	A...C.C..T
<i>P. pennata</i> Z	.....	.....CA...T	.....	.....CTCTCC	.....G.....	.....A.....
<i>S. camelus</i> Z	..A.A.....	.....A...T	.....	.....CTGTCC	.....G.....	A...C.....
<i>A. a. manteui</i>	..A.G.....	.....GA...T	.....G.....	..C..CTCTGC	.....G.G..	A..GC.....
<i>B. blakistoni</i> Z	..A.G...G.A	.....A...T	.....T.....	CAACTCTGC	..T.....GCA	A...C.....
<i>N. n. orientalis</i> Z	..A.G...--	---G.GTT	.....T.....	..C..CTCTGC	..T.....G	A...CG.A..
<i>C. siamensis</i>	..ACG...CT	AT..CA...T	A...CC.A.A	-----	..G.G...G	A...C.CA.G
<i>C. casuarius</i> W	.....	.....	.....	-----CCCC	.....G..	A...CG.A..
<i>D. novaehollandiae</i> W	.....	.....	.....	-----CCCC	.....G..	A...CG.A..
<i>P. pennata</i> W	.....	.....	.....	-----CCCC	.....G..	A...C.....
<i>S. camelus</i> W	.....	.....	.....	-----CCCC	.....G..	A...C.....
<i>E. elegans</i> W	..G.G.....	.....A...T	.....T.....	A...CTCTAC	..G...G..	A...T.CA.G
<i>B. blakistoni</i> W	..G.G...GG.	..C.A...T	G.....	CT.CTCTGC	..G...G..	A...C.....
<i>N. n. orientalis</i> W	..G.G.A.GG.	.....A...T	G.....	CT.CTCTGC	..G...G..	A...C.CT..

Fig. 3. Phylogenetic tree constructed with nucleotide sequences of 15 kW1 sequence fragments isolated from 8 avian species and 1 reptilian species. The nucleotide sequence of the w1-k7 fragment of *A. australis manteui* (AF308932) was taken from Huynen et al. [2002]. Bootstrap values (1,000 replicates) are indicated at the branch nodes. The tree was rooted with the kW1 sequence of *C. siamensis* and constructed from the aligned sequences. Z = Z-linked sequence derived from males; W = W-linked sequence derived from females.

amplified only in females of *E. elegans* (fig. 1). PCR amplification was attempted for further 4 males and 5 females, and consequently, the PCR products of the w1-k7 fragments were only obtained from females (online suppl. fig. 1; see www.karger.com/doi/10.1159/000362479 for all online suppl. material). The same result has also been reported in a previous study [Ogata et al., 2006]. The nucleotide sequences of the 271-bp female-specific w1-k7 fragments amplified from all 6 females were identical. In neognathous birds, no bands were obtained for *G. gallus* and *C. cygnus* of Galloanserae. However, w1-k7 fragments were amplified in 2 species of Neoaves, *B. blakistoni* and *N. nipalensis orientalis*, in which only 1 DNA band was observed at ~290 bp in both sexes (3 males and 4 females in *B. blakistoni* and 4 males and 4 females in *N. nipalensis orientalis*) (fig. 1; online suppl. fig. 2). In *B. blakistoni*, direct sequencing of the PCR products revealed that only 280-bp fragments were amplified from 3 males and 280- and 295-bp fragments from 2 females (4 clones of 280 bp and 5 clones of 295 bp were obtained from the DNA band) (fig. 2). In *N. nipalensis orientalis*, 288- bp and 295-bp fragments were cloned from 2 females (5 clones of 288 bp and 4 clones of 295 bp); however, only 288-bp fragments were obtained from 4 males

(fig. 2). These results suggest that the female-specific DNA fragments were larger than the Z-derived bands of males owing to a 15-bp nucleotide deletion in the *B. blakistoni* Z-homolog and a 7-bp deletion in the *N. nipalensis orientalis* Z-homolog. Nucleotide sequence identities of the w1-k7 fragments between *B. blakistoni* and *N. nipalensis orientalis* were 71.9% for Z-homologs and 93.2% for W-homologs in overlapping regions. The identity between the W-derived female-specific fragments of these 2 species (93.2%) was also much higher than between the Z- and W-derived fragments of the same species (77.7% identity in *B. blakistoni* and 78.6% in *N. nipalensis orientalis*). Among the 3 reptilian species, the kW1 sequence was amplified only from *C. siamensis*. Nucleotide sequence identities of the w1-k7 fragments of 6 avian species and 1 crocodilian species with the *C. casuarius* Z-derived w1-k7 fragment (AB536723) were calculated, including gaps but excluding insertions or deletions of 2 or more bases; the identities were 81.3% for *D. novae-hollandiae* (AB841267), 78.6% for *P. pennata* (AB536725), 85.0% for *S. camelus* (AB564694), 77.8% for the North Island brown kiwi (*Apteryx australis manteui*) (AF308932), whose nucleotide sequence of the w1-k7 fragment was taken from Huynen et al. [2002], 69.9% for *B. blakistoni* (AB536728), 72.8% for *N. nipalensis orientalis* (AB536730), and 66.7% for *C. siamensis* (AB536732).

A neighbor-joining tree was constructed for the Z- and W-linked kW1 sequences from 8 avian species that included *A. australis manteui*, which was rooted with the sequence of *C. siamensis* (fig. 3). The ratite W-linked sequences were grouped as a cluster with high bootstrap values, although bootstrap values at the cluster roots of Neognathae and Palaeognathae were lower. However, the W-linked sequences of Neoaves (*B. blakistoni* and *N. nipalensis orientalis*) were grouped as a cluster with the Z-linked sequences of *B. blakistoni* and *N. nipalensis orientalis* and also 5 ratite species.

Nucleotide Sequences of Genomic Regions Flanking the kW1 Sequence

We obtained DNA fragments of unknown regions on either side of the 286-bp CcZ-(w1, k7) and 103-bp CcW-(w1, k7) sequences from *C. casuarius* by genomic walking with PCR amplification (fig. 4A). The female-specific sequences (CcW-k7 and CcW-w1) spanned a 2.8-kb genomic DNA fragment (CcW) (fig. 4B). DNA fragments obtained from a male (CcZ-k7 and CcZ-w1) covered about 2.0 kb and contained a region highly homologous to the CcW sequence. Dot matrix analysis of the CcW

Fig. 4. Genomic structure of the kW1 sequence and its flanking region in *C. casuarius*. **A** Maps of the primers used for PCR-based genome walking. CcZ-(w1, k7) and CcW-(w1, k7) indicate the fragments of the kW1 sequence amplified from male and female genomic DNA, respectively, using w1 and k7 primers. Dashed line indicates deletion in the kW1 sequence of the W chromosome. **B** Schematic representation of the genomic structure of the kW1 sequence and its flanking regions. Z- and W-derived sequences (CcZ and CcW) are illustrated as horizontal bars. Orange boxes

(703 bp) and yellow boxes (274 bp) show highly homologous regions (sequence identity: 77 and 78%, respectively), and green boxes (300 bp) show regions with low homology (22%) between the CcZ and CcW sequences. Striped boxes in blue show repeat units in the CcW sequence. The fragments used for FISH analysis are shown as colored bars above the CcZ sequence and below the CcW sequence. **C** Alignment of the nucleotide sequences of 2 repeat units (unit 1 and unit 2) detected in the CcW sequence.

fragment revealed the presence of ~143-bp repeats in the flanking region of the kW1 sequence (data not shown); nucleotide sequence identity between unit 1 (142 bp) and unit 2 (143 bp) repeats was 89.5% (128 bp/143 bp) (fig. 4C). BLAST searches revealed that the genomic contig of the Z sex chromosome of the zebra finch (*Taeniopygia guttata*, Passeriformes) (NW_002234465) showed 63.6% nucleotide sequence identity (763/1,199 bp) in the highly homologous region of the CcZ sequence. A search with RepeatMasker showed significant nucleotide sequence similarity of the Z-linked CcZ-(w1, k7) sequence (71.3%) with the coding region of 379-aa integrase of a *Polinton* DNA

transposon of the American alligator (*Alligator mississippiensis*), which was positioned at 12,805 to 13,941 bp in the consensus sequence called the *Polinton-1_AMi* sequence (online suppl. table 1). Similarities were also found for the Z-linked w1-k7 sequences of the other 4 ratite species (*D. novaehollandiae*, *S. camelus*, *P. pennata*, and *A. australis manteui*; 62.6–74.2%), the W-linked w1-k7 sequences of a tinamid species (*E. elegans*; 62.0%), and the Z- and W-linked w1-k7 sequences of 2 neognathous bird species (*B. blakistoni* and *N. nipalensis orientalis*; 65.0–73.6%). The w1-k7 fragment of *C. siamensis* showed the highest identity (90.5%) to the *Polinton-1_AMi* sequence.

Fig. 5. Chromosomal localization of kW1 and its flanking sequences by FISH in females of 3 ratite species (*C. casuarius*, *D. novaehollandiae* and *S. camelus*). **A, B** Hybridization patterns of biotin-labeled CcW-w1 (**A**) and CcW-k7 (**B**) on Hoechst-stained metaphase spreads of *C. casuarius*. The hybridization signals (green) were localized in the pericentromeric regions of the W chromosomes. **C** Hybridization pattern of biotin-labeled CcW-(w1, k7) on *C. casuarius* chromosomes. No hybridization signal was detected. **D, E** Hybridization patterns of biotin-labeled CcW-w1 (**D**) and CcW-k7 (**E**) on metaphase spreads of *D. novaehollandiae*. **F** Hybridization pattern of CcW-w1 unit 1 on *C. casuarius* chromosomes. **G** Hybridization pattern of biotin-labeled CcW-w1(-) unit 1 (green) and DIG-labeled EE0.6 (red) on a DAPI-stained meta-

phase spread of *C. casuarius*. The *D. novaehollandiae* homolog of EE0.6 (pGE EE0.6) [Ogawa et al., 1998] was used as a FISH probe to identify the Z and W sex chromosomes. A weak hybridization signal of CcW-w1(-) unit 1 was detected in the pericentromeric region of the W chromosome. The EE0.6 sequences were localized to the distal regions of the Z and W chromosomes. **H, I** Two-color FISH of biotin-labeled *DMRT1* cDNA (green) and DIG-labeled mixed probes of 3 CcW fragments [CcW-(w1, k7), CcW-w1, and CcW-k7] (red) on metaphase spreads of *C. casuarius* (**H**) and *S. camelus* (**I**). Arrows and arrowheads indicate hybridization signals of CcW probes and *DMRT1* cDNA, respectively. Scale bars = 10 μm.

Fig. 6. Genomic organization of kW1 and its flanking sequences in *C. casuarius*. **A** Southern blot hybridization patterns of *EcoRI*- and *TaqI*-digested genomic DNA of *C. casuarius* probed with CcW-w1 unit 1 (245 bp) and CcZ-(w1, k7) (337 bp) fragments. F = Female; M = male. **B** Slot-blot hybridization patterns of *C. casuarius* genomic DNA probed with the CcW-w1 sequence. The amounts of female and male genomic DNA and probe DNA (CcW-w1) are shown on the right and left of the picture, respectively.

FISH Analysis of the kW1 and kW1-Flanking Sequences and DMRT1

We examined the chromosomal locations of the kW1 and kW1-flanking sequences in female ratites (*C. casuarius*, *D. novaehollandiae* and *S. camelus*) by FISH probing with 3 CcW sequence fragments [CcW-(w1, k7), CcW-w1 and CcW-k7] and 3 CcZ sequence fragments [CcZ-(w1, k7), CcZ-w1 and CcZ-k7]. Both CcW-w1 and CcW-k7 fragments showed large hybridization signals on the *C. casuarius* W chromosome (fig. 5A, B), but no signal was found for CcW-(w1, k7) (fig. 5C) and the CcZ sequence fragments (data not shown) because of a single copy or few copies of the small nucleotide sequences. Large hybridization signals of CcW-w1 and CcW-k7 were also observed on the W chromosomes of *D. novaehollandiae* (fig. 5D, E) and *S. camelus* (data not shown). We then

isolated the repeat unit sequence (CcW-w1 unit 1) from the *PvuII*-digested CcW-w1 fragment and hybridized it to *C. casuarius* chromosomes. A strong hybridization signal derived from the 245-bp fragment was observed in the pericentromeric region of the W chromosomes of *C. casuarius* (fig. 5F), *D. novaehollandiae* and *S. camelus* (data not shown). A weak hybridization signal was also observed for the CcW-w1(-) unit 1 sequence (fig. 5G), which lacks repeat unit 1, in the pericentromeric region of the *C. casuarius* W chromosome; this suggests that the *C. casuarius* W chromosome also has a low copy number of multiple CcW-w1(-) unit 1 sequences. The *DMRT1* gene was mapped near the centromere on the long arm of the Z chromosome in *C. casuarius* and *S. camelus*, whereas no signals were found on the W chromosomes (fig. 5H, I).

Genomic Organization of the kW1 Sequence and the kW1-Flanking Repeat

Southern blot hybridization was performed to examine the organization of the kW1 sequence and kW1-flanking repeat in the *C. casuarius* genome (fig. 6A). The observation of primarily female-specific bands in the *EcoRI* digest probed with CcW-w1 unit 1 indicated that there are many copies of this repeat sequence in the W chromosome. Non-W-specific bands may be from the Z-linked homolog or autosomal. The CcZ-(w1, k7) probe showed a major band at around 7 kb in the *EcoRI* digest of both male and female genomes at almost the same intensity, except for other W-specific bands; this indicated no amplification of the CcZ-(w1, k7) sequence in the W chromosome. Hybridization signals of both probes did not show tandem arrays in the digests of *EcoRI* and *TaqI* (an internal cleavage site of CcW-w1 unit 1). The copy number of the repeat was determined by quantitative slot-blot hybridization of serially diluted genomic DNA probed with the CcW-w1 fragment (fig. 6B). The copy number of the CcW-w1 sequence was estimated to be ~50 copies on the W chromosome and ~20 copies on the Z chromosome and/or autosomes.

Nucleotide Sequence Conservation of the kW1 and kW1-Flanking Sequences

Nucleotide sequence conservation of the kW1 and kW1-flanking sequences was examined by Zoo blot hybridization. Figure 7 shows the hybridization patterns of the genomic DNA of 7 avian and 3 reptilian species probed with 6 different DNA fragments. Hybridization intensity was higher in females than in males for all probes except for CcZ-(w1, k7) in *D. novaehollandiae* and *C. ca-*

Fig. 7. Zoo blot hybridization probed with kW1 and its flanking sequence probes in 7 avian species and 3 reptilian species. 50 ng of female and male genomic DNA were blotted for each species. Two concentrations of probes (7.8 and 15.6 pg) were blotted on the same membrane as a control. F = Female; M = male.

suarius. The W-specific unit 1 repeat (CcW-w1 unit 1) contained in CcW-w1 seemed to be W-specific. Weak signals were detected in *B. blakistoni*, *N. nipalensis orientalis* and *C. siamensis* for the CcW-w1, CcW-w1(-) unit 1, and CcW-k7 fragments; however, no sex differences were found in their signal intensities. Hybridization signals were not found for the genomes of *E. elegans*, *G. gallus*, *C. cygnus*, *P. sinensis*, and *B. constrictor*.

Discussion

Analysis of kW1 sequences from both paleognathous and neognathous birds and reptiles suggested that the kW1 sequence was present in the common ancestor of Archosauria (diapsid amniotes whose living representatives consist of birds and crocodilians), i.e. 220–240 million years ago (MYA) [Kumar and Hedges, 1998; Kumazawa and Nishida, 1999; Benton and Donoghue, 2007], but not in early diverging lineages of sauropsids, Testudines and Squamata. The identification of Z- and W-specific kW1 sequences from the Neoaves, *B. blakistoni* and *N. nipalensis orientalis*, but not from the chicken and *C. cygnus*, suggests that the kW1 sequence was lost or entirely

differentiated in the lineage of Galloanserae. Whereas a 183–192-bp deletion was observed in the W-linked w1-k7 fragments in 4 ratite species (*D. novaehollandiae*, *C. casuarius*, *S. camelus*, and *P. pennata*), a similar deletion was not found in *B. blakistoni* or *N. nipalensis orientalis*. Instead, 15-bp and 7-bp deletions in the Z-homologs were observed in *B. blakistoni* and *N. nipalensis orientalis*, respectively. The molecular phylogenetic analysis of these kW1 sequences revealed parallel conservation within the Z-homologs and W-homologs in each ratite and neognathous bird lineage. The W chromosomes of ratites have evolved multiple copies of the kW1 sequence [Huynen et al., 2002; present study]. These results suggest that the initial differentiation at the kW1 locus between the protosex chromosomes occurred in the Palaeognathae and Neognathae independently after they diverged from their common ancestor ~120 MYA [van Tuinen and Hedges, 2001]. This parallel process of divergence was also found between ratites (Struthioniformes) and tinamous (Tinamiformes) in Palaeognathae. In *E. elegans*, the Z-linked homolog of the kW1 sequence was lost or entirely differentiated, and large nucleotide deletions as observed in ratite W-homologs were not found in the *E. elegans* W-homolog. This suggests that the initial event also occurred

independently in the daughter lineages (ratites and tinamous) after the ratite-tinamou separation from the common ancestor of Palaeognathae ~90 MYA [van Tuinen and Hedges, 2001].

In the *C. casuarius* W chromosome, 2 repeated sequence elements (CcW-w1 unit 1 and unit 2) were found in the region flanking the kW1 locus. Hybridization to the pericentromeric region of W chromosomes of *C. casuarius*, *D. novaehollandiae* and *S. camelus* suggested that female-specific repeats had been amplified in the common ancestor of ratites, whose copy number of the CcW-w1 sequence was not more than 50. However, this amplification was not found in the tinamid acrocentric W chromosome, which is more morphologically differentiated than those of ratite birds and contains a large copy number of tandem arrayed repetitive DNA sequences in the proximal region [Tsuda et al., 2007]. The failure to detect homology with this satellite DNA in the W-linked kW1-flanking repeats of ratites suggests that rapid amplification of the satellite DNA occurred independently during the process of W chromosome differentiation in the lineage of tinamids.

Comparative cytogenetic maps of *P. sinensis* and the Japanese four-striped rat snake (*Elaphe quadrivirgata*) revealed that the gene order on the *S. camelus* Z chromosome is largely identical to that of turtle chromosome 6 and snake chromosome 2 [Matsuda et al., 2005; Matsubara et al., 2006, 2012; Kawai et al., 2007; Tsuda et al., 2007; Uno et al., 2012]. This finding strongly suggests that these reptilian Z sex chromosomes and the avian sex chromosomes derived independently from different autosomal pairs of their common ancestor, and the avian ancestral Z chromosome (= ratite Z) has been conserved in reptilian autosomes [Pokorná et al., 2012], although the Z chromosome of the Hokou gecko (*Gekko hokouensis*) exceptionally shares the homology with the chicken Z chromosome [Kawai et al., 2009], which is not found in other gekkotans [Trifonov et al., 2011; O'Meally et al., 2012]. This conserved homology has been also observed between the snake Z chromosomes and lacertilian autosomes in squamates [Srikulnath et al., 2009, 2013; Vicoso et al., 2013b]. However, the homolog of the avian sex-linked kW1 sequence was not found in Testudines and Squamata, whereas it has been conserved in Crocodylia, which exhibit a temperature-dependent sex determination system with no sex chromosomes. Homology analysis using RepeatMasker revealed that the kW1 sequence was highly similar to the nucleotide sequence that encodes a putative integrase derived from a novel type of DNA transposable element, called *Polinton*, which popu-

lates the genomes of protists, fungi and animals including vertebrates (fish, amphibians, reptilians, and birds) [Kapitonov and Jurka, 2006]. The similarities to the integrase coding region of the *Polinton* transposon from the American alligator ranged from 62.0 to 74.2% in 6 paleognathous bird species, 65.0 to 73.6% in 2 neognathous species, and 90.5% in *C. siamensis*. These results suggest that the kW1 sequences in Aves and Crocodylia share a common ancestor, which was acquired as a DNA transposable element in autosomes before the original divergence of the 2 lineages.

In the chicken, *DMRT1* has been identified as a master-switch sex-determining gene [Smith et al., 2009]; *DMRT1* is present on the Z chromosome but absent on the W chromosome, and is believed to trigger sexual development in a dose-dependent manner [Nanda et al., 2000]. The *DMRT1* gene was located near the centromere of the Z chromosome in *D. novaehollandiae*, but no copy was found in the W chromosome [Shetty et al., 2002]. The same result was shown for *C. casuarius* and *S. camelus* in the present study. The absence of *DMRT1* in the W chromosome of *D. novaehollandiae*, *C. casuarius* and *S. camelus* indicates the possibility that the dosage of Z-linked *DMRT1* mediates testis determination in ratites as well as in the chicken. Genomic sequencing and transcriptome analysis of *D. novaehollandiae* [Vicoso et al., 2013a] identified the Z-linked region with reduced coverage in females, called 'stratum 1', that occupied 5% of the *D. novaehollandiae* Z chromosome which was adjacent to the ancestral sex-determining region containing *DMRT1* (stratum 0). In this region, the recombination with the W chromosome has been suppressed, leading to the genetic divergence between the Z and W chromosomes. In the present study, *DMRT1* was localized near the centromere on the long arm of the Z chromosome in *C. casuarius* and *S. camelus*, where a deletion has occurred on the W chromosome of ratite species (*D. novaehollandiae*, *C. casuarius* and *S. camelus*) [Nishida-Umehara et al., 2007]. This result suggests that the kW1 sequence and its flanking region containing repeated sequences in *C. casuarius* and *S. camelus* may correspond to the 'stratum 1' region of *D. novaehollandiae*. A reduced frequency of meiotic recombination in the region adjacent to the centromere (known as the centromere effect) has been reported in many organisms [Rahn and Solari, 1986; Choo, 1998; Mahtani and Willard, 1998]. The kW1-flanking repeats therefore may have been amplified in this sex-specific non-recombining region near the centromere of the W chromosome and subsequently spread in the pericentromeric region.

Delineating the process of sex chromosome differentiation is difficult in neognathous birds because of their extensively heteromorphic sex chromosomes. The order of Z-linked genes also differs between species as a result of independent intra-chromosomal rearrangements in each lineage [International Chicken Genome Sequencing Consortium, 2004; Backström et al., 2006; Itoh et al., 2006; Modi et al., 2009]. By contrast, sex chromosome differentiation was slower in ratites than in neognathous birds. Detailed analysis of the small sex-specific genomic region of ratite sex chromosomes should provide essential information on the process and mechanism of sex chromosome differentiation in birds and vertebrates. However, the kW1-flanking sequence isolated in the present study is too short, and hence extensive nucleotide sequence data in the pericentromeric region of the ratite W chromosome are needed to clarify the molecular events at an early stage of sex chromosome differentiation. The kW1 and kW1-flanking sequences isolated in the present study will be a good anchor marker for such large-scale research.

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