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Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes

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Abstract. Recent progress of chicken genome projects has revealed that bird ZW and mammalian XY sex chromosomes were derived from different autosomal pairs of the common ancestor; however, the evolutionary relationship between bird and reptilian sex chromosomes is still unclear. The Chinese soft-shelled turtle *(Pelodiscus sinensis)* exhibits genetic sex determination, but no distinguishable (heteromorphic) sex chromosomes have been identified. In order to investigate this further, we performed molecular cytogenetic analyses of this species, and thereby identified ZZ/ZW-type micro-sex chromosomes. In addition, we cloned reptile homologues of chicken Z-linked genes from three reptilian species, the Chinese soft-shelled turtle and the Japanese four-striped rat snake *(Elaphe quadrivirgata)*, which have heteromorphic sex chromosomes, and the Siam crocodile *(Crocodylus siamensis)*, which exhibits temperature-dependent sex determination and lacks sex chromosomes. We then mapped them to chromosomes of each species using FISH. The linkage of the genes has been highly conserved in all species: the chicken Z chromosome corresponded to the turtle chromosome 6q, snake chromosome 2p and crocodile chromosome 3. The order of the genes was identical among the three species. The absence of homology between the bird Z chromosome and the snake and turtle Z sex chromosomes suggests that the origin of the sex chromosomes and the causative genes of sex determination are different between birds and reptiles.

Amniotes have a great diversity of sex chromosome constitutions and sex determination systems. Mammals exhibit male heterogamety (XX female, XY male) with exceptional cases of XO/XO systems in some rodents (*Ellobius* and *Tokudaia*, Murinae) (Vorontsov et al., 1980; Kolomiets et al., 1991; Sutou et al., 2001; Arakawa et al., 2002), whereas birds invariably have female heterogamety (ZZ male, ZW female) (Takagi and Sasaki, 1974; Belterman and de Boer, 1984; Sasaki et al., 1984). Reptiles exhibit extraordinary variability. All crocodilians, the tuatara, most turtles and some lizards have temperature-dependent sex determination (TSD) systems (Ferguson and Joanen, 1982; Head et al., 1987; Lang and Andrews, 1994; Cree et al., 1995; Ciofi and Swingland, 1997; Valenzuela and Lance, 2004). All snakes and other reptilian species (most lizards and a small number of turtles) possess genetic sex determination (GSD) systems. All snakes have female heterogamety (Beçak et al., 1964; Beçak and Beçak, 1969; Singh, 1972), whereas both XX/XY- and ZZ/ZW-type sex chromosomes have been re-

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ported in lizards and turtles (Olmo and Signorino, 2005). However, the sex chromosomes have been identified only for a few species of the lizards and turtles that exhibit GSD. Recently sex-specific microchromosomes were identified in several reptilian species using molecular cytogenetic techniques (Ezaz et al., 2005, 2006), and these results lead us to predict that the number of reptilian species having differentiated sex chromosomes that are hardly distinguishable by conventional morphological analysis is more than previously estimated.

With the recent progress of chicken genome research, detailed physical and genetic linkage maps of chicken have been constructed, and extensive homology between chicken and human chromosomes has been revealed (Burt et al., 1999; Groenen et al., 2000; Schmid et al., 2000). Comparative mapping of functional genes between chicken and human now provides compelling evidence that the human XY and chicken ZW sex chromosomes have no homology, suggesting that the sex chromosomes of mammals and birds were derived from different autosomes of the common ancestor (Nanda et al., 1999, 2000, 2002). There is a close karyotype similarity between birds and snakes, such as the existence of macro- and microchromosomes, which are divided broadly into two categories based on the physical size, and constant occurrence of female-heterogametic ZW-type sex chromosomes (Beçak et al., 1964; Beçak and Beçak, 1969; Singh, 1972). Therefore, it has been speculated that the Z chromosomes of snakes and birds have a common origin (Ohno, 1967). However, very few attempts have been made to compare sex chromosomes between birds and reptiles by gene mapping, although this approach will provide essential information on genome evolution and the origin of sex chromosomes in amniotes. We recently constructed comparative cytogenetic maps of the Chinese soft-shelled turtle *(Pelodiscus sinensis)* with 92 cDNA clones and of the Japanese four-striped rat snake *(Elaphe quadrivirgata)* with 55 cDNA clones using fluorescence in situ hybridization (FISH) (Matsuda et al., 2005; Kuraku et al., 2006). We also molecularly cloned homologues of three chicken Z-linked genes, *DMRT1, ACO1/IREBP* and *CHD1*, for the turtle and the snake, and mapped them to chromosomes of these two reptiles (Matsuda et al., 2005). All three homologues were mapped to the long arm of the turtle chromosome 6 and the short arm of the snake chromosome 2. All these data collectively suggest that the sex chromosomes of snakes, mammals and birds were differentiated independently from different autosomes of the common ancestor. The Chinese soft-shelled turtle is a species that exhibits GSD (Ji et al., 2003). Nevertheless, no heteromorphic sex chromosomes have been identified in its karyotype (Bickham et al., 1983; Sato and Ota, 2001). With respect to the Japanese four-striped rat snake, the Z and W chromosomes have already been described (Itoh et al., 1970; Matsuda et al., 2005), but only a few genes have been mapped on the Z chromosome. Thus, the origin of the reptilian sex chromosomes and their homologies to human and chicken chromosomes are still unclear. The karyotypes of birds, turtles and snakes are principally composed of two major chromosomal components, macrochromosomes and microchromosomes, which differ in physical size. The karyotypes of the Crocodilia contrast with the typical karyotypes of birds and other reptiles in almost or completely lacking microchromosomes, although they have 7–12 pairs of small chromosomes (Cohen and Gans, 1970). In all crocodilian species so far studied in this regard, TSD has been reported (Ferguson and Joanen, 1982; Lang and Andrews, 1994; Valenzuela and Lance, 2004), and this confirms the absence of heteromorphic sex chromosomes in this group of reptiles, as suggested by Cohen and Gans (1970).

Here we report the identification of the micro-sex chromosomes for the Chinese soft-shelled turtle. We also report the comparative mapping of homologues of chicken Z-linked genes in three reptilian species, the Chinese soft-shelled turtle, the Japanese four-striped rat snake and the Siam crocodile *(Crocodylus siamensis)*. C-banding, comparative genomic hybridization, Ag-NOR staining and FISH with the 18S-28S ribosomal RNA gene were applied to the turtle chromosomes, and the female-specific heteromorphic microchromosomes were identified. We also cloned homologues of chicken Z-linked genes from those three reptilian species, mapped them to chromosomes of each species, and examined the conserved linkage homologies of the chicken Z sex chromosome and the reptilian chromosomes. Finally, we discuss the origin and the process of differentiation of bird and reptilian sex chromosomes according to our findings.

Materials and methods

Animals

Adult males and females of the Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae, Testudinata) were purchased from a breeding farm in Japan. A captive bred juvenile of the Siam crocodile (*Crocodylus siamensis*, Crocodylidae, Crocodilia), whose sex was unknown, was provided from the Okinawa Branch of the Takada Reptile Farm associated with the Okinawa Zoo, Okinawa Kids Discovery Kingdom Foundation, Okinawa. Females of the Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae, Ophidia) were captured in the field in Japan. All these animals were used for the experiments described below.

Cell culture and chromosome preparation

After anesthetization, the heart and the mesentery were collected from each animal, and used for cell culture. All experimental procedures with the animals conformed to the guidelines established by the Animal Care Committee, Hokkaido University. The tissues were minced, and the fibroblast cells of *P. sinensis* and *E. quadrivirgata* were cultured in MEM medium (Invitrogen) supplemented with 15% fetal bovine serum (Invitrogen), 42 μg/ml L-serine, 110 μg/ml sodium pyruvate, 30 μg/ml glycine, 100 μg/ml kanamycin and 1% Antibiotic-Antimycotic (PSA) (Invitrogen). For the cells from *C. siamensis,* Chang Medium C (Irvine Scientific) supplemented with 1% Antibiotic-Antimycotic (PSA) was used. The cell cultures were incubated at 26°C in a humidified atmosphere of 5% CO_2 in air. Primary cultured fibroblast cells were harvested using trypsin and then subcultured.

For conventional Giemsa staining, C-banding and comparative genomic hybridization, chromosome slides were prepared without 5-bromodeoxyuridine (BrdU) treatment. After colcemid treatment (120 ng/ml) for 45 min, the cells were harvested, suspended in 0.075 M KCl for 20 min at room temperature and fixed with 3:1 methanol:acetic acid

Table 1. Degenerate oligonucleotide primers used for cloning cDNA fragments of the chicken Z-linked gene homologues from *Pelodiscus sinensis*, *Elaphe quadrivirgata* and *Crocodylus siamensis*

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
ACO1/IREBP	GACAGYTTRCARAAGAATCARGAY[a] GTGCTCACYRTNACNAAGCACCT[a]	CCYTTRAATCCTTGCTTNGYTCC[a] AGGTCTCCCTGNGTDATNGCYTC[a]
ATP5A1	GAARACTGGCACHGCWGARRTRTCCTC[b] CGYCTKCTGGARAGAGCAGCBAARATG[b]	GGCAATBGADGTTTTSCCMGTCTGYCTGTC[b] CTGKTCWGAGATYTTSCCMTCAGWCCTG[b]
CHD1	TGTAACCATTGCTACCTCATTAARCC[a] CTCCAGAAGATGTGGAATATTATAAYTGC[a]	AGATCATTYTGTGGATTCCARTCNGAATCR[a] AGYTCYTTGTGNAGRCTTGCATAACC[a]
GHR	TGAGTTTATTGAGYTGGAYATWGAYGA[b, c] ATGGATCTTCGGCAKCTGYTGYTTA[b, c] GCCGGAGAAAACAGCTGTTAYTTYAA[d] AARGATGATGAYTCTGGACGWGCCAG[d]	GCTAHGGCAKGATTTTGTTCAGTTGG[b, c] ACTTCTTTGTACTGCAATTCATACTCCAG[b, c] GCYAAGATGGAGTTMACYTCRTCYARYTTTCC[d] TRTGRAYRGAVGTRTAGTCTGRGACAGG[d]
RPS6	CACTGGCTGCCAGAAGCTCAT[b]	GGCCTCCTTCATTCTCTTTG[b]

[a] Matsuda et al. (2005).
[b] Tsuda et al. (2007).
[c] Primers used for *C. siamensis* and *P. sinensis*.
[d] Primers used only for *E. quadrivirgata*.

following a standard protocol. The cell suspension was dropped on glass slides and air-dried. Replication R-banded chromosome preparation was performed for FISH mapping as described previously (Matsuda and Chapman, 1995). The cell cultures were incubated with BrdU (12 μg/ml) (Sigma) for 12 h during the late replication stage for differential staining. After BrdU treatment, including 45 min of colcemid treatment, the cells were harvested and fixed, and then chromosome slides were made by the air drying method. The chromosome slides were dried for 2 d at room temperature. After the chromosomes were stained with Hoechst 33258 (1 μg/ml) for 5 min, R-bands were obtained by heating the slides for 3 min at 65°C and exposing them to UV light at 65°C for an additional 5 min. The slides were kept at –80°C until use.

C-banding

To examine the distribution of constitutive heterochromatin in chromosomes of *P. sinensis*, chromosome C-banding was performed with the BSG (Barium hydroxide/Saline/Giemsa) method (Sumner, 1972) with slight modification.

Comparative genomic hybridization (CGH)

CGH was performed as described in Traut et al. (2001) with slight modification. Male and female genomic DNAs of *P. sinensis* were labeled with FITC-dUTP (Molecular Probes) and Texas Red-dUTP (Molecular Probes), respectively, using a nick translation kit (Roche Diagnostics) following the manufacturer's instructions, and ethanol-precipitated with salmon sperm DNA and *Escherichia coli* RNA. The chromosome slides were hardened at 65°C for 2 h, denatured at 70°C for 2 min in 70% formamide, 2× SSC, and dehydrated in 70% and 100% ethanol for 5 min each. A 20 μl mixture containing 250 ng of FITC-labeled male genomic DNA and 250 ng of Texas Red-labeled female genomic DNA, 50% formamide, 2× SSC, 10% dextran sulfate and 2 μg/μl BSA was hybridized to one slide by incubation for 3 d. The slides were washed in 4× SSC, 1% Nonidet P-40/4× SSC, 4× SSC and 2× SSC for 5 min each at room temperature. The fluorescence hybridization signals of FITC-labeled male DNA and Texas Red-labeled female DNA on metaphase chromosomes were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope, and were analyzed with the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK).

Ag-NOR staining

For visualization of the nucleolar organizer regions (NORs), metaphase chromosomes were stained with silver nitrate ($AgNO_3$) following Howell and Black (1980). Briefly, the solution mixture of 50% $AgNO_3$ and 2% gelatin containing 1% formic acid (2:1) were poured onto the chromosome slides, covered with cover slips, and left for about 45 s at 65°C. The slides were washed with water and dried. Ag-NOR staining was performed on the same slides after FISH analysis.

Molecular cloning of reptile homologues of chicken Z-linked genes

We molecularly cloned reptile homologues of five chicken Z-linked genes: soluble aconitase 1/iron-responsive element binding protein *(ACO1/IREBP)*, ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle *(ATP5A1)*, chromodomain helicase DNA binding protein 1 *(CHD1)*, growth hormone receptor *(GHR)* and ribosomal protein S6 *(RPS6)*. Nucleotide sequences of the primers used for cDNA cloning of the genes are listed in Table 1. The fibroblast cells of the three reptilian species were lysed with TRIzol Reagent (Invitrogen), and total RNAs were extracted following the manufacturer's instructions. The cDNA was obtained by RT-PCR using Oligo $(dT)_{12–18}$ Primer (Invitrogen) and SuperScript II RNase H^- Reverse Transcriptase (Invitrogen), and was used as the PCR template to amplify the homologues of the chicken Z-linked genes. Twenty nanograms of cDNA was incubated in 20 μl of 1× ExTaq buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 μM degenerate primers and 0.25 U of TaKaRa Ex Taq (Takara Bio). The PCR conditions were as follows: an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 35 s; and finally 72°C for 5 min for a final extension. The PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. The target DNA bands were isolated from the gel and extracted using a QIAquick Gel Extraction Kit (Qiagen). The DNA fragments were cloned using pGEM-T Easy Vector System I (Promega), and were transformed into *E. coli* JM109 competent cells.

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

FISH mapping

FISH analysis was performed for chromosomal localization of the 18S-28S rRNA genes and the reptile homologues of chicken Z-linked genes as described previously (Matsuda and Chapman, 1995). The 5.8-

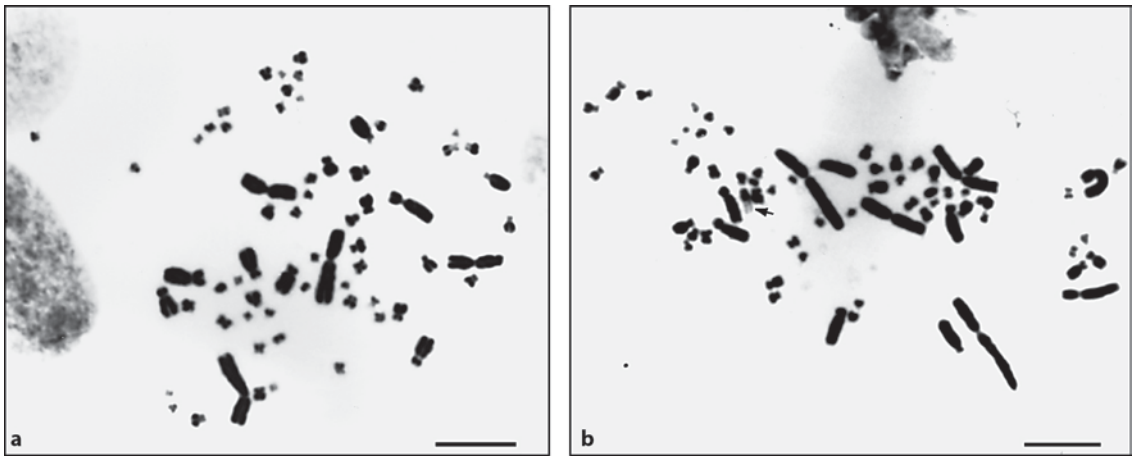

Fig. 1. Giemsa-stained metaphase chromosome spreads of male (**a**) and female (**b**) *Pelodiscus sinensis*. The arrow indicates a female-specific chromosome, half of which is only weakly stained by Giemsa. Scale bar = 10 μm.

Table 2. The length of cDNA fragments of the chicken Z-linked gene homologues cloned from *Pelodiscus sinensis, Elaphe quadrivirgata* and *Crocodylus siamensis*

Gene	Length of cDNA fragment (bp) and accession number		
	P. sinensis	*E. quadrivirgata*	*C. siamensis*
ACO1/IREBP	–	–	1122[a] (AB266722, AB266723)
ATP5A1	990[a] (AB266737, AB266738)	990[a] (AB266732, AB266733)	990[a] (AB266726, AB266730)
CHD1	–	–	1260[a] (AB266724, AB266725)
GHR	1310[a] (AB267378, AB267379)	1223[a] (AB266734, AB266735)	1310[a] (AB266727, AB266728)
RPS6	593 (AB266736)	593 (AB266731)	593 (AB266729)

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

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 as probes for chromosome mapping of the turtle 18S-28S rRNA genes. For chromosome mapping of reptile homologues of chicken Z-linked genes, two or more cDNA fragments isolated for each gene were mixed and used as probes to cover a wide region of the genes (Table 2). The DNA probes were labeled by nick translation with biotin-16-dUTP (Roche Diagnostics) following a standard protocol and ethanol-precipitated with salmon sperm DNA and *E. coli* tRNA. After hybridization probed with the 18S-28S rRNA genes, the slides were incubated with FITC-avidin (Vector Laboratories), and stained with 0.75 μg/ml propidium iodide. The slides hybridized with the cDNA fragments were reacted with goat anti-biotin antibody (Vector Laboratories) and then stained with Alexa Fluor 488 rabbit anti-goat IgG (H+L) conjugate (Molecular Probes). The hybridization signals were observed under a Nikon fluorescence microscope using Nikon filter sets B-2A and UV-2A, and the FISH images were microphotographed with DYNA HG ASA100 film (Kodak).

Results

Karyotypes of Pelodiscus sinensis

We closely examined the Giemsa-stained karyotypes of three males and three females. Ten metaphase spreads were examined for each individual. Comparison between male and female karyotypes revealed the possibility that there was a morphologically heteromorphic chromosome in all females (Fig. 1). This female-specific chromosome was a relatively large-sized subtelocentric microchromosome, about half of which was weakly stained by Giemsa.

C-bands in Pelodiscus sinensis chromosomes

The chromosomal distribution of C-bands was examined for a total of 29 and 26 metaphase spreads of female and male individuals, respectively. A large-sized heteromorphic microchromosome with a large C-positive band was observed in each female karyotype, but not in the male karyotypes (Fig. 2a, b). Further large C-positive bands were observed on two pairs of microchromosomes in both males and females. Small, clear C-bands were observed in the centromeric region of chromosomes 1 and 2, in the interstitial region of the long arm of the fifth-largest chromosome, and on about half of the microchromosomes. The same C-banded pattern was also observed in our previous study (Yamada et al., 2005).

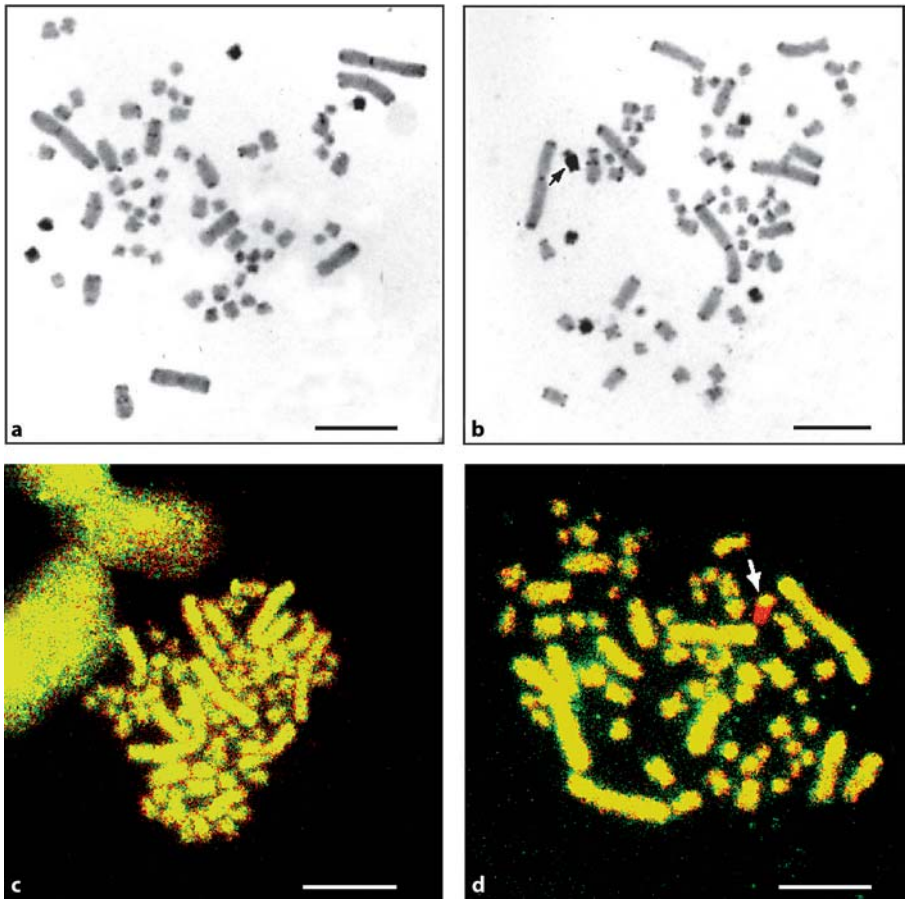

Fig. 2. C-banded metaphase chromosome spreads of male (**a**) and female (**b**) *Pelodiscus sinensis* and the patterns of comparative genomic hybridization with FITC-labeled male genomic DNA and Texas Red-labeled female genomic DNA to chromosomes of male (**c**) and female (**d**) *Pelodiscus sinensis*. (**b**) The arrow indicates the female-specific chromosome with a large and intense C-positive band. (**d**) The arrow indicates the chromosome with the female-specific region painted with Texas Red-labeled female-driven DNA. Scale bar = 10 μm.

Comparison of CGH patterns between male and female chromosomes of Pelodiscus sinensis

We performed CGH for three males and three females. The hybridization patterns of FITC-labeled male-derived DNA and Texas Red-labeled female-derived DNA were compared between the male and female metaphase spreads. In males, all chromosomes were equally stained with the male- and female-derived probes, whereas in females a female-specific heteromorphic chromosome was intensely stained with the Texas Red-labeled female-derived probe (Fig. 2c, d). The female-specific region painted red by CGH corresponded to the large C-positive heterochromatin block of the female-specific chromosome.

Location of the 18S-28S ribosomal RNA genes on Pelodiscus sinensis chromosomes

The fluorescence hybridization signals of the 18S-28S ribosomal RNA genes were localized to two microchromosomes in both males and females (Fig. 3). However, a distinct difference was observed in the size of the hybridization signals between the microchromosomes forming the heteromorphic pair in all three females (Fig. 3a): a much larger number of copies of the rRNA genes were clustered on the larger-sized chromosome, which corresponded to the large-sized C-band on the same chromosome. The copy number of the rRNA genes was much lower on the other microchromosome (Fig. 3a), which seemingly corresponded to the homologous pair with the hybridization signal in all three males (Fig. 3d). The difference in the size of the euchromatic chromosomal region was also found between the heteromorphic microchromosome pair in females. The euchromatic region of the female-specific chromosome was much smaller than that of the other chromosome, indicating that the deletion of the euchromatic chromosome region occurred in this chromosome with the amplification of the rRNA genes and the repetitive DNA sequences composing C-positive heterochromatin.

Ag-NOR in Pelodiscus sinensis chromosomes

Ag-NOR staining was performed for the same metaphase spreads used for FISH mapping of the 18S-28S rRNA genes. All male individuals had the same size of Ag-stained NORs on the short arms of the two microchromosomes that seemingly corresponded to the smaller components of the heteromorphic pair in females (see above) (Fig. 3f). In contrast, all female individuals showed ladder-like Ag-NOR bands on the female-specific microchromosome (Fig. 3c), and no Ag-NOR bands were observed on the other microchromosome with a small copy number of the 18S-28S rRNA genes.

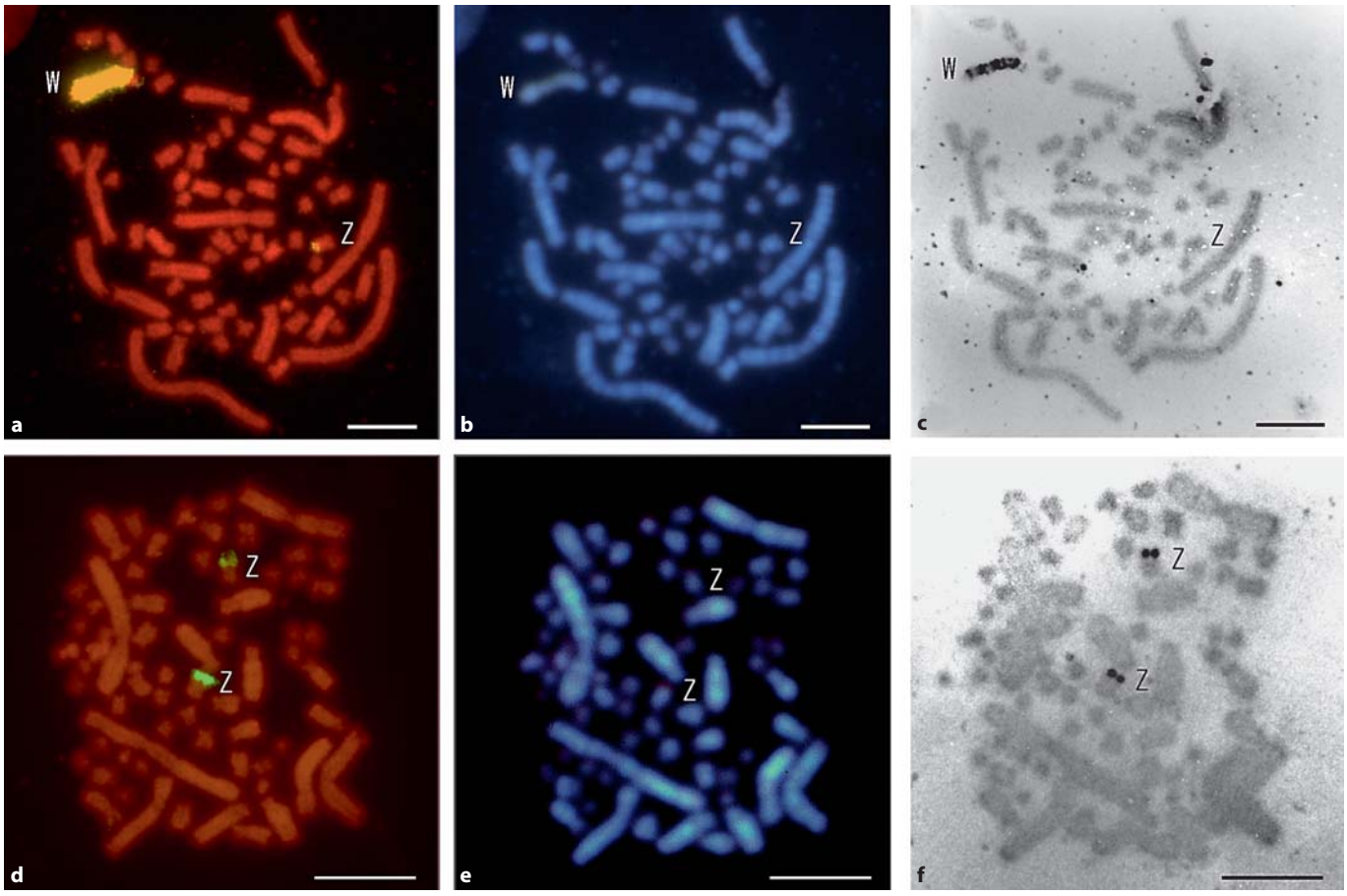

Fig. 3. Chromosomal distribution of the 18S-28S rRNA genes and NORs on metaphase chromosome spreads in female (**a–c**) and male (**d–f**) *Pelodiscus sinensis*. FISH signals of the 18S-28S rRNA genes are located on a pair of heteromorphic chromosomes in female (**a**) and on a pair of the same-sized microchromosomes in male (**d**). (**c**) Ag-stained NOR is distributed in the large C-positive heterochromatin region as ladder bands but no NOR band is detected on the other microchromosome in female. (**f**) The same-sized Ag-NOR bands are observed on two microchromosomes forming a pair in male. (**b**) and (**e**) are Hoechst-stained patterns of the metaphase spreads in female and male, respectively. Scale bar = 10 μm.

Molecular cloning and nucleotide sequences of reptile homologues of chicken Z-linked genes

The homologues of three chicken Z-linked genes, *ATP5A1, GHR* and *RPS6,* were newly cloned from all three reptilian species (Table 2). For cloning cDNA fragments of the *E. quadrivirgata GHR* gene, primer sets that were different from those used for *P. sinensis* and *C. siamensis* were used. The cDNA fragments of *ACO1/IREBP* and *CHD1* were only cloned from *C. siamensis* because the gene homologues of *P. sinensis* and *E. quadrivirgata* had already been cloned in our previous study (Matsuda et al., 2005). The sequence data of *ACO1/IREBP, CHD1* and *DMRT1* of *P. sinensis* and *E. quadrivirgata* were also taken from that previous study. The *DMRT1* homologue was not cloned from *C. siamensis* because of the absence of a testis sample. For each species, the nucleotide sequences were determined for three to five cDNA fragments amplified with corresponding primer sets, and their consensus sequences were deposited in DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome.html).

The total length of the cDNA fragments of the five genes were estimated by concatenating the sequences of multiple PCR products amplified with two to four pairs of primer sets (Table 2). The nucleotide sequence identities were compared in the equivalent regions of the cDNA fragments between the chicken and three reptilian species and among the three reptilian species (Table 3). The identities were the highest for three genes *(ATP5A1*, *CHD1* and *RPS6)* (87.4–89.7%) between chicken and *P. sinensis*, and for two genes *(ACO1/IREBP* and *GHR)* (81.6–84.0%) between chicken and *C. siamensis*. The lowest identities were observed between chicken and *E. quadrivirgata* for all five genes (67.5–84.4%). Comparison among the three reptilian species revealed that the highest identities were observed for all five genes (83.9–92.5%) between *P. sinensis* and *C. siamensis* and the lowest identities for four genes *(ACO1/IREBP, ATP5A1*, *CHD1* and *RPS6)* (80.3–84.5%) between *E. quadrivirgata* and *C. siamensis* and for *GHR* (67.3%) between *E. quadrivirgata* and *P. sinensis*.

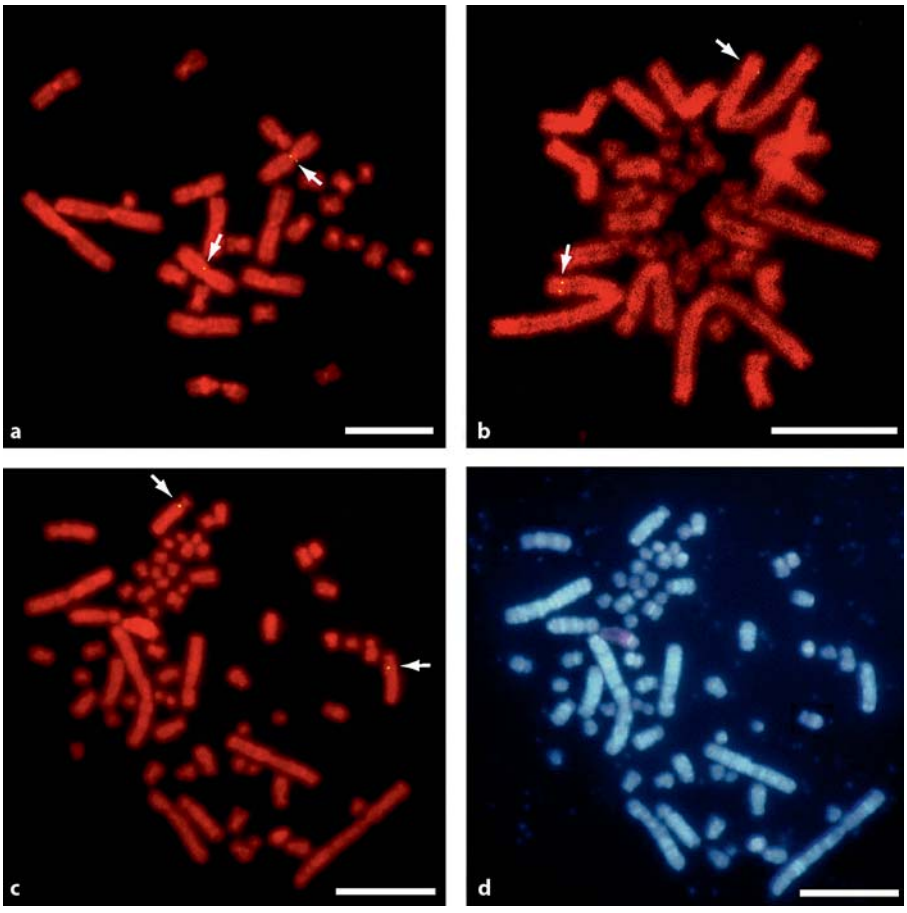

Fig. 4. FISH mapping of *ACO1/IREBP* in *Crocodylus siamensis* (**a**), *GHR* in *Elaphe quadrivirgata* (**b**) and *RPS6* in *Pelodiscus sinensis* (**c**) with cDNA fragments used as biotinylated probes. In all these, FISH patterns are shown on PI-stained chromosomes. Hoechst-stained pattern of the same metaphase spread as in **c** is shown in **d**. Arrows indicate hybridization signals. Scale bars = 10 μm.

Table 3. Nucleotide sequence identities of cDNA fragments of five genes among *Gallus gallus* (GGA), *Pelodiscus sinensis* (PSI), *Elaphe quadrivirgata* (EQU) and *Crocodylus siamensis* (CSI)

Gene	Identity (%)[a]					
	GGA-PSI	GGA-EQU	GGA-CSI	PSI-EQU	PSI-CSI	EQU-CSI
ACO1/IREBP[b]	81.7 (917/1122)	79.0 (886/1122)	84.0 (942/1122)	82.5 (926/1122)	86.5 (971/1122)	80.3 (901/1122)
ATP5A1	87.4 (865/990)	81.6 (808/990)	86.9 (860/990)	83.4 (826/990)	90.2 (893/990)	83.1 (823/990)
CHD1[c]	89.7 (1130/1260)	84.4 (1086/1286)	89.1 (1123/1260)	85.4 (1076/1260)	92.5 (1165/1260)	84.5 (1065/1260)
GHR	79.1 (1037/1310)	67.5 (825/1223)	81.6 (1069/1310)	67.3 (569/845)	83.9 (1099/1310)	67.6 (571/845)
RPS6	87.7 (520/593)	82.8 (491/593)	87.2 (517/593)	86.0 (510/593)	89.7 (532/593)	84.3 (500/593)

[a] The number in parentheses indicates the number of identical bases/the number of bases in overlapped region between cDNA fragments of two species.
[b] Nucleotide sequences of AB185397 and AB185398 (Matsuda et al., 2005) were used for *P. sinensis* and *E. quadrivirgata*, respectively.
[c] Nucleotide sequences of AB185401 and AB185402; and AB185399 and AB185400 (Matsuda et al., 2005) were used for *P. sinensis* and *E. quadrivirgata*, respectively.

Comparative mapping of reptile homologues of chicken Z-linked genes

The chromosomal locations of the reptile homologues of the five chicken Z-linked genes were determined for the three reptilian species (Figs. 4 and 5). The chromosomal locations of *ACO1/IREBP, CHD1* and *DMRT1* of *P. sinensis* and *E. quadrivirgata* were taken from Matsuda et al. (2005). All the genes were precisely localized to the long arm of chromosome 6 of *P. sinensis*, the short arm of chromosome 2 of *E. quadrivirgata* and chromosome 3 of *C. siamensis*. The order of six genes, *ACO1/IREBP–RPS6–DMRT1–CHD1–GHR–ATP5A1*, from the centromere to the distal

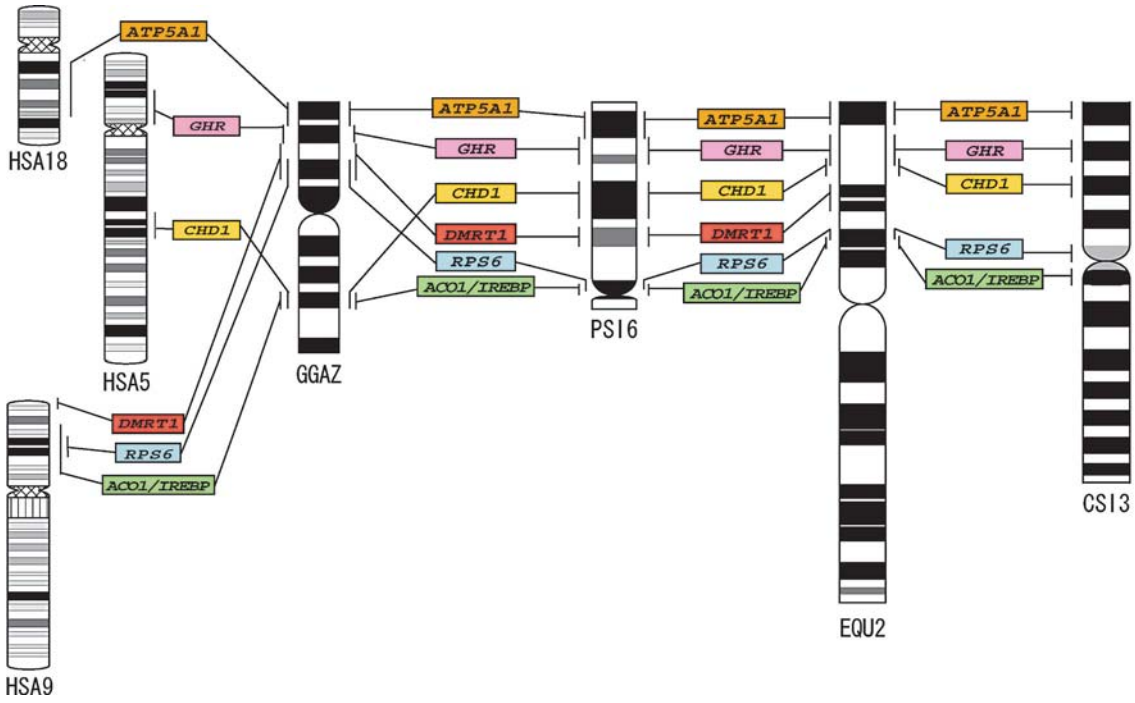

Fig. 5. Comparative chromosome maps of the homologues of chicken Z-linked genes in humans and three reptilian species, *Pelodiscus sinensis* (PSI), *Elaphe quadrivirgata* (EQU) and *Crocodylus siamensis* (CSI). The ideogram of the G-banded chicken (*Gallus gallus*; GGA) Z chromosome is taken from the ARKdb (http://www.thearkdb.org/), and human chromosomes 5, 9 and 18 from the human (*Homo sapiens*; HSA) chromosome ideograms at the standard ISCN (1981) 850-band level (Francke, 1994). *P. sinensis* chromosome 6 is arranged upside down to make the gene order correspond to that on *E. quadrivirgata* chromosome 2p and *C. siamensis* chromosome 3p. The chromosomal locations of the *ACO1/IREBP, CHD1* and *DMRT1* genes in *P. sinensis* and *E. quadrivirgata* are taken from our previous study (Matsuda et al., 2005).

end on *P. sinensis* chromosome 6q was identical with that on *E. quadrivirgata* chromosome 2p. The order of two closely linked genes, *ACO1/IREBP* and *RPS6*, of *P. sinensis* and *E. quadrivirgata* was confirmed by two-color FISH (data not shown). In *C. siamensis* the order of *RPS6–CHD–GHR–ATP5A1* from the centromere to the distal end on the short arm of chromosome 3 was the same as those on *P. sinensis* chromosome 6q and *E. quadrivirgata* chromosome 2p; however, *ACO1/IREBP* and *RPS6* were located on different arms across the centromere on chromosome 3. The hybridization signal of *RPS6* was located near the centromere on the short arm of chromosome 3, whereas *ACO1/IREBP* was localized near the centromere on the long arm.

Discussion

ZZ/ZW-type sex chromosomes in Pelodiscus sinensis

Previous authors reporting the standard karyotypes of *P. sinensis* did not mention the presence of a heteromorphic pair (Bickham et al., 1983; Sato and Ota, 2001). This may have been due to their failure to notice the heteromorphism in the microchromosome pair. Sex chromosomes bearing NORs have been reported in some species of mammals (Goodpasture and Bloom, 1975; Yonenaga-Yassuda et al., 1983; Stitou et al., 1997), birds (Sasaki et al., 1994), amphibians (Schmid et al., 1983; Wiley, 2003) and fish (Born and Bertollo, 2000; Artoni and Bertollo, 2002). In this study, large FISH signals of the 18S-28S ribosomal RNA gene cluster were obtained on the female-specific microchromosome in *P. sinensis*. The other microchromosome with the rRNA genes was much smaller. In males the rRNA genes were localized to a pair of microchromosomes, which were considered to be identical with the smaller of the heteromorphic pair of microchromosomes in females. These results strongly suggest that the female-specific microchromosome and its smaller counterpart are the W and Z sex chromosomes, respectively. Micro-sex chromosomes have been reported for a ZZ/ZW lizard, *Pogona vitticeps* (Ezaz et al., 2005), and an XX/XY turtle, *Chelodina longicollis* (Ezaz et al., 2006); however, *P. sinensis* is the first example of ZZ/ZW-type micro-sex chromosomes in turtles. These findings suggest that many GSD reptiles with cryptic sex chromosomes may also have micro-sex chromosomes.

Species with karyotypes whose NORs are located only on the sex chromosomes are limited to a few mammals and amphibians, such as the rat kangaroo *Potorous tridactylus* and the fruit bats *Carollia perspicillata* and *Carollia castanea* (Goodpasture and Bloom, 1975), the marsupial frog *Gastrotheca riobambae* (Schmid et al., 1983) and the pine

woods treefrog *Hyla femoralis* (Wiley, 2003), and *P. sinensis* is the first case for reptiles. The 18S-28S rRNA genes were co-localized with the female-specific C-positive heterochromatin that can be discriminated from the heterochromatin of the Z chromosome and autosomes by comparative genomic hybridization. The ladder Ag-NOR bands in the W heterochromatin suggest that the rRNA genes have been amplified with the repetitive DNA sequences composing the C-positive W heterochromatin. FISH signals of the rRNA genes were observed on the two sex chromosomes in both sexes; however, Ag-NORs were detected only on the large-sized W chromosome in all females, whereas all males had two Ag-NORs on a pair of the Z microchromosomes. The sex-specific difference in the copy number of the rRNA genes between the ZZ males and ZW females appears to be compensated by sex-specific regulation of the gene expression leading to the occurrence of inactivation of the NOR on the Z chromosome in females. It is probable that the expression of the old gene on the Z chromosome is inhibited, owing to the presence of the 18S-28S rRNA genes newly amplified on the W chromosome.

Ohno's law (1967) asserts that the heteromorphic sex chromosomes originated from a homologous autosomal pair through the occurrence of dominant sex determinant on only one of the pair. By such modification, GSD should be founded with the birth of the proto sex-specific chromosome. The cessation of meiotic recombination due to chromosome rearrangements, e.g., inversions or translocations, and/or constitutional changes, e.g., accumulation of heterochromatin occurring in the sex-specific region, is considered to favor the accumulation of gene mutations, and this should lead to the functional inactivation of the genes followed by the partial deletion of the sex-specific chromosomes (Charlesworth, 1991; Charlesworth and Charlesworth, 2000). The absence of chromosome recombination would also foster the accumulation of repetitive DNA sequences. Thus, the sex chromosome differentiation proceeds by deletion of euchromatic chromosomal region(s) followed by extensive accumulation of heterochromatin. Cytogenetic evidence that the initiation of sex chromosome differentiation can be caused by stochastic effects such as subtle expansion of heterochromatin on one homologue has been reported in the tilapia *(Oreochromis niloticus)* (Griffin et al., 2002). The differentiation of a pair of microchromosomes, not the sixth largest chromosome, to the sex chromosomes might also have incidentally occurred in *P. sinensis*, and the sex chromosome pair of *P. sinensis* may represent an intermediate situation between the initial stage of sex chromosome differentiation and the fully differentiated sex chromosomes. The 18S-28S rRNA genes on the W chromosomes seem to have been co-amplified with the repetitive sequences in this species; however, they are not inactivated, and maintain their function.

Origins and differentiation of sex chromosomes in reptiles

The homologues of six chicken Z-linked genes were localized to *P. sinensis* chromosome 6q and *E. quadrivirgata* chromosome 2p, and the order of the genes, *ACO1/IREBP–RPS6–DMRT1–CHD– GHR–ATP5A1* from the centromere, was identical between them. Only two Z-linked genes have been found in *E. quadrivirgata,* and their human and chicken homologues are located on human chromosome 10p and 7p and chicken chromosome 2p, respectively (Matsuda et al., 2005). These results indicate that the sex chromosomes of *P. sinensis* and *E. quadrivirgata* and the chicken Z sex chromosomes were derived from different autosomal pairs of the common ancestor. This order of the homologues of five chicken Z-linked genes has also been conserved in *C. siamensis* chromosome 3 with the exception that *RPS6* and *ACO1/IREBP* are located on different chromosome arms, chromosome 3p and 3q, respectively. Their chromosomal location on opposite sides of the centromere suggests the possibility that a centromere repositioning occurred between *ACO1/IREBP* and *RPS6* on the ancestral *C. siamensis* chromosome 3p after the centric fusion between the ancestral acrocentric *C. siamensis* chromosome 3q and chromosome 3p that is homologous to *P. sinensis* chromosome 6q and *E. quadrivirgata* chromosome 2p, as reported in primates (Montefalcone et al., 1999; Ventura et al., 2001; Eder et al., 2003), in nonprimate mammals (Ferreri et al., 2005; Carbone et al., 2006) and in birds (Kasai et al., 2003).

The molecular time scale of amniote evolution indicates that the latest common ancestors of mammals (synapsids) and reptiles and birds (diapsids) first appeared in the Carboniferous period around 310 million years ago (MYA) (Kumar and Hedges, 1998). Recent phylogenetic analyses of birds, and those three (crocodilians, turtles and snakes) and other extant reptile groups using mitochondrial genomes and many nuclear genes revealed that birds are most closely related to crocodilians, and that these two groups together constitute a clade (Archosauria) sister to turtles. These three groups compose the Archosauromorpha with a number of fossil taxa (Kumazawa and Nishida, 1999; Janke et al., 2001; Iwabe et al., 2005). On the other hand, squamates, including snakes, constitute a different clade, the Lepidosauria, which diverged earlier from the Archosauromorpha (Kumar and Hedges, 1998; Kumazawa and Nishida, 1999; Kumazawa, 2004; Dong and Kumazawa, 2005). The linkage of the chicken Z-linked genes and their order in the common ancestor of birds and reptiles seems to have been highly conserved in both the Archosauromorpha and the Lepidosauria since they diverged 250–290 MYA (Kumar and Hedges, 1998; Mannen and Li, 1999; Janke et al., 2001). The nucleotide sequence comparison of the cDNA fragments reveals higher nucleotide sequence identities between chicken and *C. siamensis* or *P. sinensis* and between *C. siamensis* and *P. sinensis*, and lower identities between chicken and *E. quadrivirgata* and between *E. quadrivirgata* and the other two reptiles. It is therefore safe to state that the genetic relationships as indicated by the nucleotide sequences of chicken Z-linked gene homologues further confirms the phylogenetic relationships of birds and three reptilian groups deduced from the nucleotide sequences of the mitochondrial genomes and other nuclear genes.

We recently constructed comparative cytogenetic maps of the Z sex chromosomes in the ostrich *(Struthio camelus)*

and the crested tinamou *(Eudromia elegans)* of the Palaeognathae (Tsuda et al., 2007), which diverged from the Neognathae around 120 MYA (van Tuinen and Hedges, 2001). *ACO1/IREBP, ATP5A1, CHD1, GHR* and *RPS6* were localized to the Z chromosome in both species, and the order of these genes was identical with those in *P. sinensis* and *E. quadrivirgata.* This suggests that palaeognathous birds share the same order of the genes on the acrocentric Z chromosomes with autosomes of both the Archosauromorpha and the Lepidosauria, and that the gene order on a pair of chromosomes of the common ancestor of birds and reptiles also have been conserved in palaeognathous birds. The acrocentric Z chromosome of palaeognathous birds is the ancestral type of bird sex chromosomes (de Boer, 1980; Ansari et al., 1988; Pigozzi and Solari, 1997; Ogawa et al., 1998; Nishida-Umehara et al., 1999; Shetty et al., 1999). The gene order on the metacentric chicken Z chromosome is different from that of the primitive form of the bird sex chromosome. This is considered to have resulted from multiple intrachromosomal rearrangements that have occurred independently in the lineage of the Phasianidae of the Galliformes (Shibusawa et al., 2004).

Reptiles exhibit different features of sex determination from birds and mammals. In all snakes, most lizards and some turtles, sex is determined by the genes carried on differentiated sex chromosomes. All snakes so far studied appropriately exhibit female heterogamety (e. g., Beçak et al., 1964; Beçak & Beçak, 1969; Singh, 1972), whereas there are both male (XX/XY) and female (ZZ/ZW) heterogamety in the lizards and turtles with GSD (Olmo and Signorino, 2005). Besides GSD or chromosomal sex determination (CSD), TSD is widespread in reptiles (Ciofi and Swingland, 1997). The mechanisms of sex determination and the primary factors of its complexity in birds and reptiles remain unknown (Sarre et al., 2004; Smith and Sinclair, 2004). The *DMRT1* gene, which encodes a putative transcription factor with a conserved DM *(dsx* and *mab-3)* domain, is located on the Z sex chromosome in both chicken, with the degenerated W chromosome, and emu, with extensively homomorphic sex chromosomes (Nanda et al., 2000; Shetty et al., 2002), suggesting the possibility that *DMRT1* is a strong candidate for an avian sex determining gene. In *E. quadrivirgata* and *P. sinensis*, the *DMRT1* homologue is mapped to autosomes, and the discordance between the chicken and reptilian Z sex chromosomes suggests that the critical sex-determining genes may be different between birds and reptiles, and thus the mechanisms of sex determination have evolved independently in the two taxa. In *P. sinensis*, no functional genes have been mapped on the Z chromosome, and hence the possible conservation of chicken and human chromosomes with the Z chromosome of *P. sinensis* is unknown. Further experiments will be required to examine what candidate genes of sex determination are located on the sex chromosomes. Characterization of reptilian sex chromosomes by molecular cytogenetic techniques and comparative gene mapping among reptilian lineages would be beneficial for understanding the evolution of sex chromosomes and sex determination mechanisms in vertebrates.

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Note added in proof

In our recent paper (Matsubara et al., 2006), eleven functional genes were localized to the Z sex chromosome of the Japanese four-striped rat snake *(Elaphe quadrivirgata)*, whose human and chicken homologues are located on human chromosome 3p, 7p, 10p and 17q, and chicken chromosome 2 and 27, respectively. This result also strongly suggests that the sex chromosomes of snakes, mammals and birds were all derived from different autosomal pairs of the common ancestor.