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Highly conserved linkage homology between birds and turtles: Bird and turtle chromosomes are precise counterparts of each other

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Abstract

The karyotypes of birds, turtles and snakes are characterized by two distinct chromosomal components, macrochromosomes and microchromosomes. This close karyological relationship between birds and reptiles has long been a topic of speculation among cytogeneticists and evolutionary biologists; however, there is scarcely any evidence for orthology at the molecular level. To define the conserved chromosome synteny among humans, chickens and reptiles and the process of genome evolution in the amniotes, we constructed comparative cytogenetic maps of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and the Japanese four-striped rat snake (*Elaphe quadrivirgata*) using cDNA clones of reptile functional genes. Homology between the turtle and chicken chromosomes is highly conserved, with the six largest chromosomes being almost equivalent to each other. On the other hand, homology to chicken chromosomes is lower in the snake than in the turtle. Turtle chromosome 6q and snake chromosome 2p represent conserved synteny with the chicken Z chromosome. These results suggest that the avian and turtle genomes have been well conserved during the evolution of the Arcosauria. The avian and snake sex Z chromosomes were derived from different autosomes in a common ancestor, indicating that the causative genes of sex determination may be different between birds and snakes.

Introduction

The karyotypes of birds, turtles and snakes are principally composed of two major chromosomal components, namely macrochromosomes and microchromosomes, which differ with respect to physical size, though the boundary between the two is not necessarily defined. Turtles have variable numbers of

chromosomes, ranging from 2n = 26 to 68 (Ayres *et al*. 1969, Bickham & Baker 1976, Bickham *et al*. 1983). The most common diploid number is around 50–52 in Emydidae, including 12–14 pairs of macrochromosomes and 12–14 pairs of microchromosomes, and 66 in Trionycidae, including 8–9 pairs of macrochromosomes and 24–25 pairs of microchromosomes (Bickham & Baker 1976, Bickham *et al*. 1983). Similar karyotypes are also observed in birds. The bird karyotypes are remarkably uniform, and the modal number is around 80, which consists of 7–10 pairs of macrochromosomes, including ZW sex chromosomes, and a large number of microchromosomes, though diploid chromosome numbers range from 50 in the Falconidae to over 100 in the Rallidae and Ramphastidae (Takagi & Sasaki 1974, de Boer 1984, Belterman & de Boer 1984, Sasaki *et al*. 1984). The first three pairs of macrochromosomes are outstandingly large, and the morphological similarities of the largest three chromosomes are shared by many of the species in diverse avian orders (Takagi & Sasaki 1974). Based on the comparison of G-banding patterns between bird and turtle chromosomes, Takagi & Sasaki (1974) suggested that the largest three pairs might have been transmitted without many structural changes from a common ancestor of birds and turtles. The range of karyotypic variation is very narrow in snakes. The most common diploid number of snakes is 2n = 36, which consists of eight pairs of macrochromosomes and ten pairs of microchromosomes (Beçak *et al*. 1964, Beçak & Beçak 1969, Singh 1972). The close karyological relationship between birds and reptiles has long been a topic of speculation among cytogeneticists and evolutionary biologists; however, there is hardly any evidence to confirm this similarity at the molecular level. Graves & Shetty (2000) demonstrated by comparative chromosome painting of the turtle (*Chelodina longicollis*) that chicken chromosome 4 painted the fourth largest pair of autosomes and the short arm of chromosome 7/8 in the turtle. The chicken Z chromosome was equivalent to the fifth-largest autosomal pair of the turtle (Graves & Shetty 2001). These results suggest that chromosome homology might have been preserved between turtles and birds; however, gene-based conserved synteny between the two genera has not been verified by comparative gene mapping.

As detailed physical and genetic linkage maps of the chicken have been constructed, extensive chromosome homology between the chicken and human genomes (about 100 conserved syntenic segments) has been revealed (Groenen *et al*. 2000, Schmid *et al*. 2000). The comparative maps of functional genes between chicken and mammalian species provide new insights into the evolution of vertebrate genomes (Burt *et al*. 1995, 1999, Nanda *et al*. 1999, 2000, Groenen *et al*. 2000, Schmid *et al*. 2000, Burt 2002). This approach makes it possible to compare chromosomes between species belonging to different classes or phyla, but reptiles have not been the subject of comparative mapping because there are almost no DNA probes for functional genes in reptiles. Comparative mapping between birds and reptiles would provide more detailed information about the evolution of the amniotes, which has not been studied yet. Partial sequencing of a large number of cDNAs to develop expressed sequence tags (ESTs) facilitates gene discovery using the EST database (dbEST), and ESTs provide a ready source of DNA probes for comparative gene mapping between any species. Orthologues are homologous genes from different species that evolved from a common ancestral gene and normally retain the same function during evolution. The identification of orthologous genes from reptile EST clones facilitates the direct comparison of human, avian and reptilian genomes by comparative gene mapping.

In this study we constructed cDNA libraries from the brain tissue and the 14-day-old whole embryos of the Chinese soft-shelled turtle and from the brain tissue of the Japanese four-striped rat snake. We isolated a large number of cDNA clones at random from the turtle and snake cDNA libraries, determined their partial sequences, and then searched for orthologues from the reptilian EST clones for comparative gene mapping. Here we address the relationships of genome organization between chicken and two reptilian species by constructing their comparative cytogenetic maps with the EST clones.

Materials and methods

Specimen

Adult females and embryos of the Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae, Testudinata) were purchased from a breeding farm in Japan, and used for constructing cDNA libraries and chromosome preparations. Wild individuals of

the Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae, Ophidia) were captured in the field in Japan and used for the experiments.

Construction of cDNA libraries, DNA sequencing and database analysis

The sources of RNA used for constructing cDNA libraries were the brain tissue of an adult female and whole 14-day embryos for the turtle, and the brain tissues of eighteen adult male and female individuals for the snake. Poly (A) mRNAs were isolated from the fresh tissues, and cloned into the λ uni-ZAP vector (Stratagene) using standard protocols. Lambda uni-ZAP clones were converted into pBluescript SK (+) clones, and transformed into XL1-Blue bacterial cells (Stratagene). Colonies were randomly picked and transferred into 96-well plates using the 'Q' Pix (GENETIX). The clones were grown overnight, and the plasmid DNAs were prepared using MultiScreen-NA and FB plates (Millipore, Bedford, MA). Sequencing reactions were performed with dideoxy dye-labelled terminator using SK primer according to the manufacturer's protocol (Applied Biosystems), and the nucleotide sequences were determined using an ABI PRISM3700 DNA Analyzer (Applied Biosystems). The nucleotide sequence comparisons versus the National Center for Biotechnology Information (NCBI) database were performed using the Blast X program. Individual ESTs were translated in all reading frames and compared against the NCBI 'non-redundant' nucleotide and/or peptide sequence database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?). All the EST clones mapped to turtle and snake chromosomes were deposited in DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome.html).

Cell culture, chromosome preparation and FISH

Preparation of R-banded chromosomes and fluorescence *in-situ* hybridization (FISH) were performed as described previously (Matsuda & Chapman 1995, Suzuki *et al*. 1999). The fibroblast cells collected from the embryos of the turtle and the heart tissues of the female snakes were cultured in 199 medium supplemented with 15% fetal bovine serum at 26°C in 5% CO_2 5-Bromodeoxyuridine (BrdU) was incorporated during the late replication stage for differential staining. The cells were harvested after colcemid treatment for 1 h, suspended in 0.075 mol/L KCl, fixed in 3:1 methanol:acetic acid three times, then dropped on glass slides and air-dried. R-banded chromosomes were obtained by exposure of chromosome slides to UV light after staining with Hoechst 33258. Slides were kept at −80°C until use.

The cDNA fragments amplified from the EST clones by PCR were used as probes for FISH mapping. The inserts were amplified using Insert Check Ready Blue (Toyobo) which included universal M13 P7 and M13 P8 primers. DNA amplification was performed in a total reaction volume of 100 μl containing 100 ng of plasmid as a template. PCR products were electrophoresed on a 1% agarose gel, recovered using Sprec-DNA Recovery Filter Tubes (Takara Biomedical), and purified according to the manufacturer's instructions.

The DNA probes were labelled by nick translation with biotin-16-dUTP (Roche Diagnostics) using a standard protocol. The hybridized cDNA probes were reacted with goat anti-biotin antibodies (Vector Laboratories), and then stained with fluoresceinated donkey anti-goat IgG (Nordic Immunology). The slides were stained with 0.50 μg/ml propidium iodide for observation. FISH images were observed under a Nikon fluorescence microscope using Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA100 films were used for microphotography.

Molecular cloning of reptilian homologues of chicken Z-linked genes

The turtle and snake homologues of the chicken Z-linked genes, DMRT1, ACO1 and CHD1, were molecularly cloned by RT-PCR. Total RNAs were extracted from testes of the turtle and the snake using Trizol (Invitrogen). The cDNAs were synthesized using SuperScript II Rnase H(−) Reverse Transcriptase (Invitrogen).Various sets of PCR primers were synthesized based on the conserved regions of the three genes. The degenerate primer pairs used in the RT-PCR reactions were as follows: Primers for DMRT1: F1, 5′-GCA GCG GGT GAT GGC NGC NCA GGT-3′; R1, 5′-GCC AGA ATC TTG ACT GCT GGG YGG YGA-3′. Primers for ACO1:F1, 5′-GAC AGY TTR CAR AAG AAT CAR GAY-3′; R1, 5′-CCY TTR AAT CCT TGC TTN GYT CC-3′; F2, 5′-GTG CTC ACY RTN ACN AAG CAC CT-3′; R2, 5′-AGG TCT CCC TGN GTD ATN GCY TC-3′. Primers for CHD1:F1, 5′-CTC CAG AAG ATG

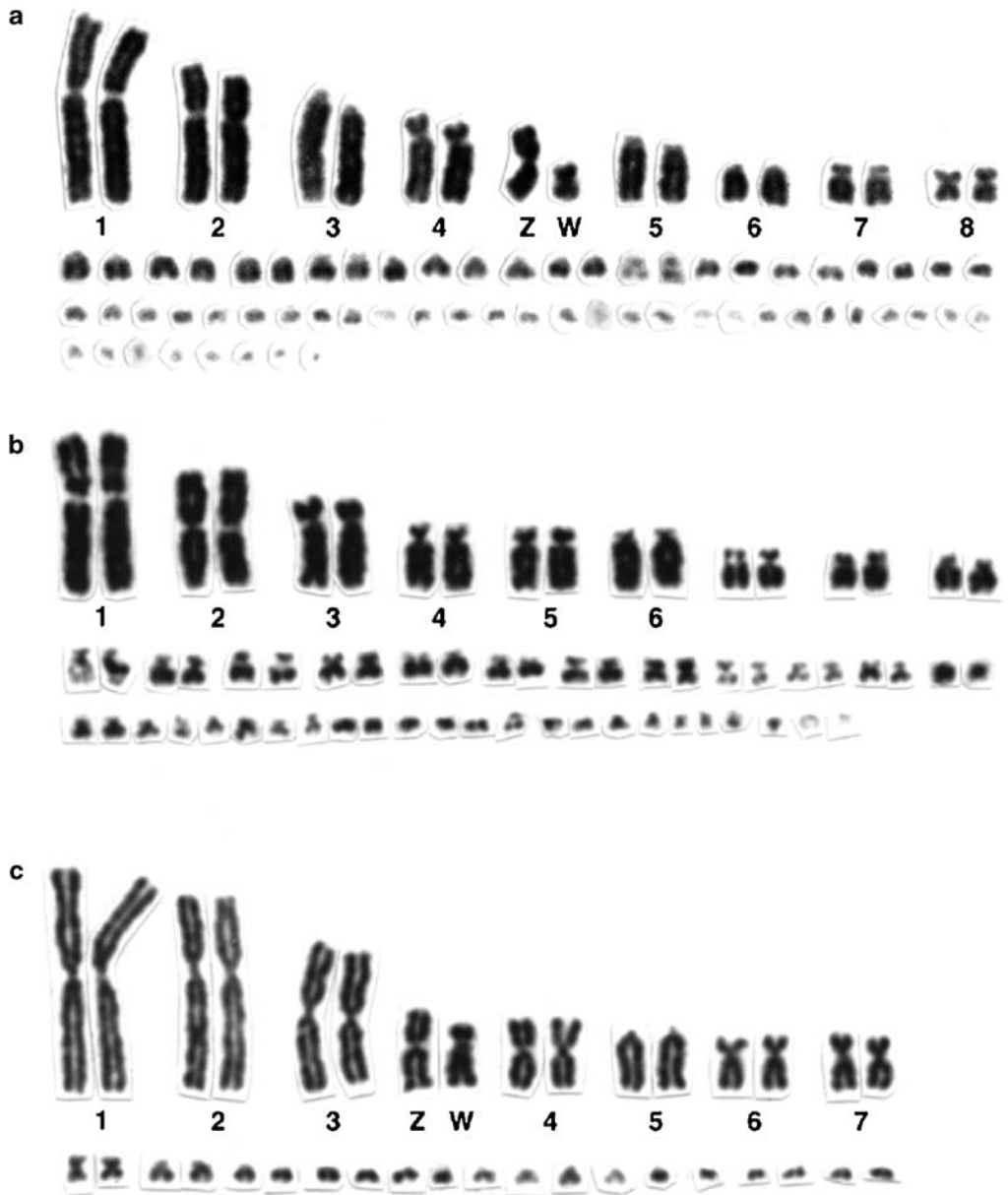

Figure 1. Giemsa-stained karyotypes of chicken (2n = 78) (**a**), the Chinese soft-shelled turtle (2n = 66) (**b**) and the Japanese four-striped rat snake (2n = 36) (**c**).

TGG AAT ATT ATA AYT GC-3′; R1, 5′-TAT TGT TTT NCC NAG NCC CAT TTC A-3′; F2, 5′-TGG TGC AAA GGN AAT AGT TGY ATH C-3′; R2, 5′-AGY TCY TTG TGN AGR CTT GCA TAA CC-3′; F3, 5′-TGT AAC CAT TGC TAC CTC ATT AAR CC-3′; R3, 5′-AGA TCA TTY TGT GGA TTC CAR TCN GAA TCR-3′. Amplification of the fragments was achieved using the Ex Taq system (Takara Biomedical). The PCR conditions were an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; and finally 72°C for 5 min. The PCR products with more than one band were separately isolated and subcloned using the pGEM-T Easy Vector System (Promega).

Table 1. List of 59 EST clones mapped to Chinese soft-shelled turtle chromosomes. Closed boxes indicate conserved syntenies between chicken and the Chinese soft-shelled turtle, which are equivalent between the two species.

Gene symbol[a]	Insert length (kb)	Sequence length (bp)	E-value	Chromosome location in human	Chromosome location in the turtle	Chromosome location in chicken	Accession Number
PECI	2.0	809	e-105	6p24.3	1p		AU312267
NAV3	1.2	1033	e-123	12q14.3	1p	1	AU312263
NAPILI	2.0	811	2e-90	12q21.1	1p	1	AU312281
TRA1	2.5	531	2e-84	12q24.2-q24.3	1p	1	AU312248
RPL3	1.6	498	1e-95	22q13	1p	1	AU312265
MAP3K7IP1	1.0	561	3e-78	22q13.1	1p	1	AU312271
DPT	1.2	680	1e-79	1q12-q23	1q		AU312278
RPL8	1.0	824	e-148	8q24.3	1q		AU312288
USP5	1.2	800	e-106	12p13	1q	1	AU312276
PPP1CC	2.5	519	3e-64	12q24.1-q24.2	1q	15	AU312244
ZNF294	2.0	543	4e-71	21q22.11	1q	1	AU312299
C21orf33	2.5	655	e-113	21q22.3	1q	1	AU312295
EIF2S3	2.0	658	e-134	Xp22.2-p22.1	1q	1	AU312268
ARF1	2.0	649	2e-96	1q42	2p	2	AU312289
LAMR1	1.0	829	e-129	3p21.3	2p		AU312259
GARS	2.0	802	e-162	7p15	2p	2	AU312286
BAZIB	2.0	756	3e-97	7q11.23	2q	19	AU312277
NSMAF	4.0	521	5e-97	8q12-q13	2q	2	AU312241
EIF3S6	1.6	548	e-110	8q22-q23	2q	2	AU312274
KIAA0153	1.8	809	7e-49	22q13.31	2q		AU312266
NVL	2.0	794	e-101	1q41-q42.2	3p	3	AU312294
EPHX1	1.7	737	4e-79	1q42.1	3p	3	AU312282
XPO1	2.3	573	e-124	2p16	3p	3	AU312293
RNASEH1	3.0	520	5e-70	2p25	3q	3	AU312243
ARG1	1.2	760	5e-87	6q23	3q		AU312300
UCHL1	3.0	532	4e-77	4p14	4q	4	AU312247
PAPSS1	1.5	810	e-166	4q24	4q	4	AU312290
HMGB2	1.8	901	2e-90	4q31	4q	4	AU312262
FAT	2.1	527	e-100	4q34-q35	4q	4	AU312273
C14orf166	1.0	687	4e-95	14q22.1	5q	5	AU312301
EIF2S1	1.5	461	6e-73	14q24.1	5q	5	AU312298
COQ6	1.0	925	9e-66	14q24.2	5q	5	AU312260
EIF2B2	2.1	839	e-108	14q24.3	5q	5	AU312297
ACTC	1.5	739	e-148	15q11-q14	5q	5	AU312292
CLTA	1.0	799	3e-96	9p13	6p	Z	AU312285
CHD1	2.0	798	5e-92	5q15-q21	6q		AU312270
ALDH7A1	1.7	616	2e-84	5q31	6q	Unknown	AU312269
FBP1	1.5	732	e-134	9q22.3	6q	Z	AU312291
CDK9	1.5	275	5e-45	9q34.1	6q		AU312239
SIAT8C	1.4	420	1e-86	18q21.31	6q		AU312252
SLC20A1	3.0	740	2e-48	2q11-q14	micro		AU312245
SCG2	2.3	636	2e-53	2q35-q36	micro	9	AU312275
RASA2	3.0	561	3e-67	3q22-q23	micro	9	AU312254
PLD1	3.0	517	e-102	3q26	micro	9	AU312251
HNRPD	1.5	504	2e-87	4q21.1-q21.2	micro		AU312284
CTNNA1	4.5	520	5e-88	5q31	micro	13	AU312240
SKP1A	1.8	815	8e-95	5q31	micro		AU312280
SPARC	2.0	513	2e-65	5q31.3-q32	micro	13	AU312255
CSNK1A1	2.0	449	5e-73	5q32	micro	13	AU312296
GTF2I	2.0	761	1e-90	7q11.23	micro	19	AU312279
PTN	2.2	516	4e-71	7q33-q34	micro	1	AU312250
LHX2	1.5	710	e-129	9q33-34.1	micro	17	AU312297
COX15	1.2	519	3e-81	10q24	micro	6	AU312249
KARS	1.5	516	e-106	16q23-q24	micro	11	AU312242

Table 1. (Continued.)

Gene symbol[a]	Insert length (kb)	Sequence length (bp)	E-value	Chromosome location in human	Chromosome location in the turtle	Chromosome location in chicken	Accession Number
EEF2	1.2	526	2e-98	19pter-q12	micro	28	AU312258
EEF1A2	2.0	513	3e-86	20q13.3	micro	20	AU312256
TOP3B	1.8	804	e-118	22q11.2	micro	15	AU312272
COL4A5	2.1	795	e-112	Xq22	micro	4	AU312283
DCX	1.7	1125	e-107	Xq22.3-q23	micro	4	AU312264

[a]Human gene symbol.
Unkown: The nucleotide sequence of the gene is annotated in the chicken genome sequence but its chromosomal location is not yet identified.

The 5′-UTR of the DMRT1 gene was amplified using the 5′ RACE system version 2.0 (Invitrogen). The two pairs of primers for the ACO1 gene, F1/R1 and F2/R2, amplified 794 bp and 797 bp products, respectively. The three pairs of primers for the CHD1 gene, F1/R1, F2/R2, and F3/R3, amplified 443 bp, 584 bp and 401 bp products, respectively. The nucleotide sequences of the cDNA clones were determined using an ABI PRISM3100 DNA Analyzer (Applied Biosystems) after performing the sequencing reaction with dideoxy dye-labelled terminator using SK primer according to the manufacturer's protocol.

Results

The chromosome number of the Chinese soft-shelled turtle was 66 with nine pairs of macrochromosomes and 24 pairs of microchromosomes, which was quite similar to that of the chicken (Figure 1a, b). The present study confirmed the previous data reported by Sato & Ota (2001). The Japanese four-striped rat snake had 2n = 36, with eight pairs of macrochromosomes, including differentiated Z and W chromosomes, and 10 pairs of microchromosomes (Figure 1c). The submetacentric W chromosomes might have resulted from a pericentric inversion of the metacentric Z chromosome followed by partial deletion.

We isolated 382 and 1150 non-redundant EST clones from the cDNA libraries constructed from the adult brain and the 14-day-old embryos of the Chinese soft-shelled turtle, respectively. Two thousand and ninety-seven non-redundant ESTs were also isolated from the brain cDNA library of the Japanese four-striped rat snake. EST clones with Blast X scores less than $1e^{-45}$ were classified as putative reptile homologues of human genes in this study. Fifty-nine turtle and 52 snake homologues of human orthologous genes were carefully selected by eliminating family genes (Tables 1 and 2), and cytogenetically localized to chromosomes by FISH (Figure 2).

Forty turtle homologues were specifically localized to the six largest pairs of macrochromosomes, and the remaining 19 homologue clones were localized to chromosomes smaller than chromosome 6 (the microchromosomes) (Table 1). Ten conserved segments, to which two or more genes were mapped, were identified between the human chromosomes and turtle chromosomes. Chromosome homologies between chicken and the turtle were examined using the current information on the human–chicken comparative map (Schmid *et al*. 2000, Burt 2002) (Figure 3). Twelve out of 13 clones located on the Chinese soft-shelled turtle chromosome 1 (*Pelodiscus sinensis* chromosome: PSI) were localized to seven regions homologous to human chromosomes (*Homo sapiens* chromosome: HSA) 1q, 6p, 12p, 12q, 21q, 22q and Xp, where conserved synteny has been also identified in chicken chromosome 1 (*Gallus gallus* chromosome: GGA). Seven genes on PSI2 were localized to regions homologous to HSA1q, 3p, 7p, 7q, 8q and 22q, which are orthologous to GGA2. Five genes on PSI3 were localized to the conserved regions of GGA3 homologous to HSA1q, 2p and 6q. Four genes on PSI4 and five genes on PSI5 were localized to regions conserved between GGA4 and HSA4p and 4q, and between GGA5 and HSA14q and 15q, respectively. The locations of the turtle homologues on chicken chromosomes were searched using the annotation database of the first draft chicken genome assembly, Ensembl Chicken Web Server (URL: http://www.ensembl.org/Gallus_gallus/) (International Chicken Genome Sequencing Consortium

Table 2. List of 52 EST clones mapped to Japanese four-striped rat-snake chromosomes.

Gene symbol[a]	Insert length (kb)	Sequence length (bp)	E-value	Chromosome location in human	Chromosome location in the snake	Chromosome location in chicken	Accession Number
EPRS	1.8	749	e-112	1q41-q42	1p	3	AU312324
ARID4B	1.6	690	3e-54	1q42.1-q43	1p		AU312346
DEGS	1.4	694	e-101	1q42.12	1p	Unknown	AU312341
AFTIPHILIN	1.8	514	5e-70	2p15	1p		AU312311
XPO1	2.0	775	e-155	2p16	1p	3	AU312325
MGC15407	1.5	716	2e-70	2p16.2	1p	3	AU312344
KIAA0007	2.3	418	8e-72	2p23.3	1p		AU312332
XAB1	1.2	767	8e-67	2p23.3	1p		AU312353
MDN1	2.4	679	2e-92	6q15	1p	3	AU312339
QKI	1.8	772	1e-50	6q26-q27	1p	3	AU312356
SF3B1	1.2	651	e-115	2q33.1	1q		AU312337
UMPS	2.4	566	1e-87	3q13	1q		AU312331
TUBGCP2	1.6	702	e-101	10q26.3	1q		AU312343
M11S1	1.8	592	1e-53	11p13	1q		AU312350
TSG101	1.8	727	7e-60	11p15	1q	Unknown	AU312316
GPHN	1.2	729	7e-82	14q23.3-q24.1	1q	5	AU312327
DNCH1	1.2	745	1e-85	14q32.3-qter	1q	5	AU312310
ISYNA1	1.8	731	1e-54	19p13.11	1q	5	AU312338
ZFR	1.8	738	e-100	5p13.3	2p		AU312309
PHAX	2.2	760	4e-71	5q23.3	2p		AU312322
C9orf72	3.0	727	e-143	9p21.1	2p	Z	AU312326
UNQ501	1.3	746	1e-56	19p13.2	2cen		AU312305
CPEB4	1.8	655	2e-74	5q21	2q		AU312333
DCTN4	2.3	671	1e-91	5q31-q32	2q	13	AU312349
C5orf14	1.8	730	2e-57	5q31.2	2q	Unknown	AU312304
CCNG1	3.0	734	5e-74	5q32-q34	2q	13	AU312308
FLJ22318	1.8	780	9e-98	5q35.3	2q		AU312329
DCTN2	1.8	765	e-117	12q13.2-q13.3	2q		AU312317
NOSIP	1.5	690	1e-60	19q13.33	2q		AU312303
SS18	2.5	599	9e-92	18q11.2	3p	2	AU312302
MBP	2.1	757	1e-44	18q23	3p	2	AU312318
BCAS2	1.3	839	3e-87	1p21-p13.3	3q	26	AU312354
EIF2S3	1.8	735	e-157	Xp22.2-p22.1	4p	1	AU312306
SYAP1	1.5	761	1e-98	Xp22.22	4p	1	AU312328
DSCR3	1.8	590	2e-98	21q22.2	4q	1	AU312319
PDCD10	1.3	791	7e-62	3q26.2	5q	9	AU312342
TLOC1	1.8	615	7e-70	3q26.2-q27	5q	9	AU312335
SH3MD1	2.3	764	4e-54	10q25.1	5q	6	AU312347
BCCIP	1.8	601	5e-56	10q26.1	5q	Unknown	AU312307
FLJ12571	2.3	738	3e-66	7q34	6q		AU312352
RANGAP1	1.5	756	9e-70	22q13	6q	1	AU312313
SEC3L1	2.0	711	1e-63	4q12	7p		AU312345
RAP1GDS1	1.3	702	e-118	4q23-q25	7q	4	AU312351
KIAA1109	2.0	782	1e-73	4q28.1	7q	4	AU312348
ASB6	1.0	675	1e-53	9	micro	17	AU312340
FLJ25530	1.1	786	5e-86	11q24.2	micro		AU312336
GLCE	2.5	431	6e-87	15q22.31	micro	10	AU312330
POLG	2.3	740	e-107	15q25	micro	10	AU312315
PARN	2.4	742	e-102	16p13	micro	14	AU312312
LOC283820	2.3	721	5e-93	16p13.12	micro		AU312323
TAX1BP1	1.8	752	1e-66	7pl5	Zp	2	AU312320
WAC	1.3	727	2e-79	10	Zp	2	AU312355

[a]Human gene symbol.

Unknown: The nucleotide sequence of the gene is annotated in the chicken genome sequence but its chromosomal location is not yet identified.

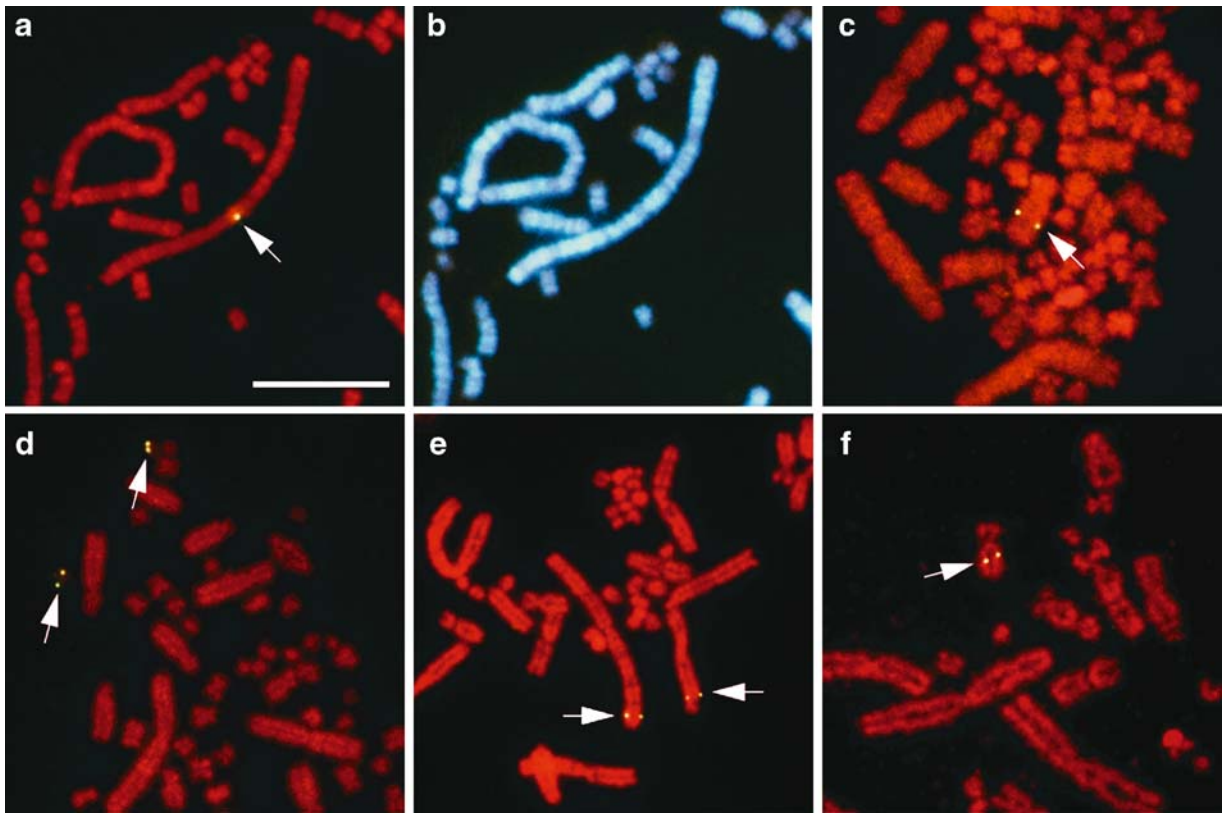

Figure 2. Comparative FISH mapping of EST clones in the Chinese soft-shelled turtle and the Japanese four-striped rat snake. PPP1CC (**a**), ACTC (**c**) and SLC20A1 (**d**) genes are localized to chromosome 1, chromosome 5 and a pair of microchromosomes in the Chinese soft-shelled turtles, respectively. TUBGCP2 (**e**) and FLJ12571 (**f**) genes are localized to chromosome 1 and chromosome 6 in the Japanese four-striped rat snake, respectively. (**b**) Hoechst-stained chromosomes of the metaphase spread shown in (**a**), which show the same banding patterns as G-banding. Scale bar indicates 10 μm. Arrows indicate the fluorescence signals.

2004, Wallis *et al*. 2004). Forty-six (78.0%) out of 59 mapped genes were physically localized to chicken chromosomes based on the genome sequences. Chromosomal locations were directly compared between chicken and the turtle for 28 (82.4%) of 34 genes mapped in turtle chromosomes 1–5. The five largest chromosomes were equivalent between chicken and the turtle for 26 of these 28 genes (the exceptions were PPP1CC on GGA15 and BAZ1B on GGA19). It has been revealed that the biarmed GGA4 was derived from a centric fusion between the ancestral types of acrocentric chromosome 4 and a microchromosome (Shetty *et al*. 1999, Nishida-Umehara *et al*. unpublished). The homology to HSAXq, which corresponds to the p arm of GGA4, was observed in a microchromosome in *P. sinensis*, indicating that that PSI4 retained the ancestral type of chromosome 4 (Table 1).

Forty-six snake homologues of human genes were localized to the eight largest pairs of macrochromosomes, including the sex Z chromosome, the remaining six homologues being localized to chromosomes smaller than chromosome 7 (Table 2). Eleven conserved segments were identified between the human chromosomes and snake chromosomes (Figure 4). Twenty-five (54.3%) out of the 46 genes mapped onto the snake chromosomes 1–7 and Z were localized directly to chicken chromosomes by the search with Ensembl. Five snake homologues, EPRS (HSA1q41-q42), XPO1 (HSA2p16), MGC15407 (HSA2p16.2), MDN1 (HSA6q15) and QKI (HSA6q26-q27), mapped onto the short arm of the Japanese four-striped rat snake chromosome (*Elaphe quadrivirgata* chromosome: EQU) 1 were identified on GGA3, which also has conserved synteny with HSA1q, 2p and 6q. Three genes on EQU1q, GPHN

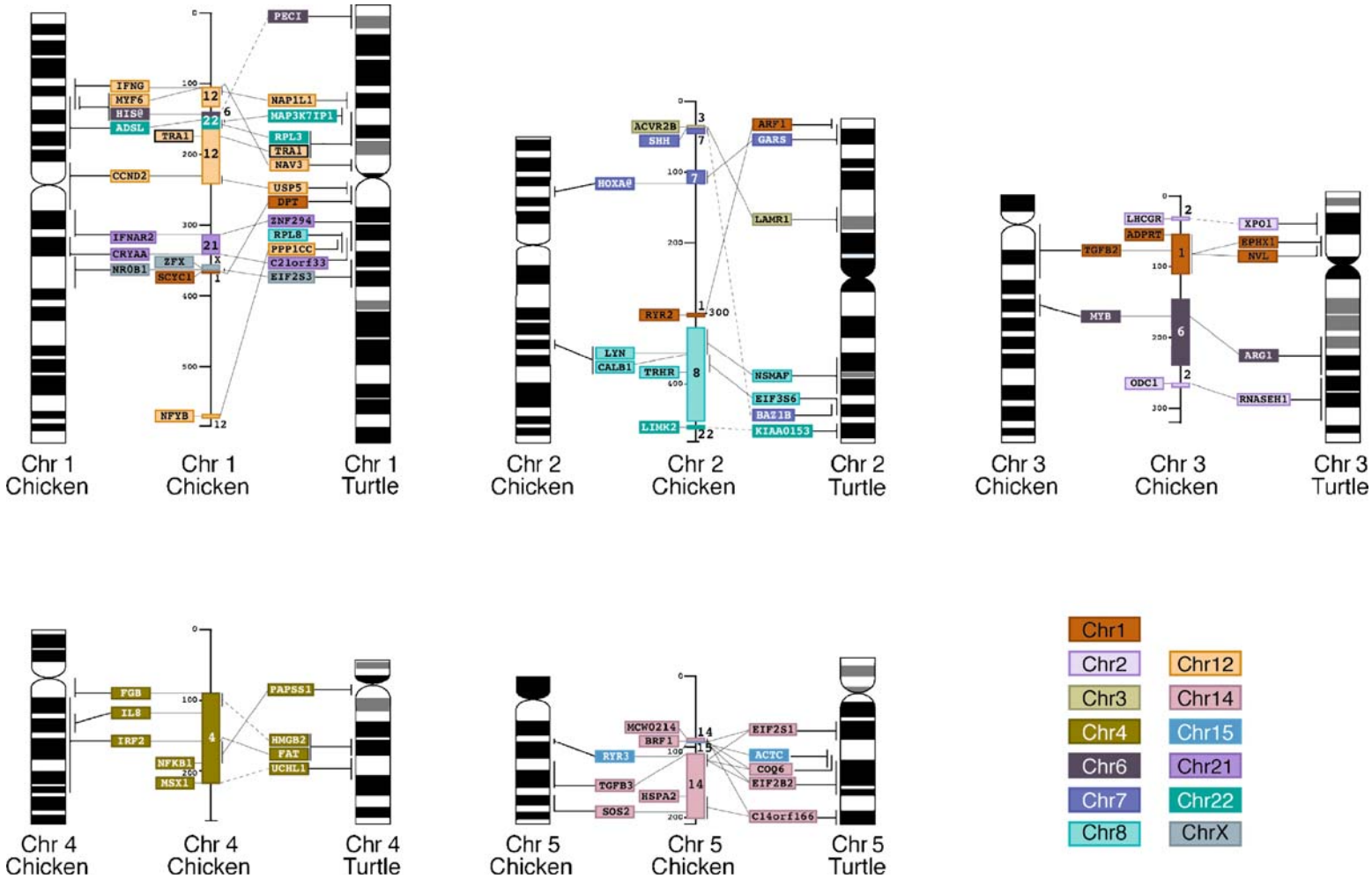

Figure 3. Comparative chromosome map between chicken and the Chinese soft-shelled turtle. The gene symbols are described following human gene nomenclature. The G-band ideogram of chicken chromosomes was obtained from ARKdb-chick Roslin Institute (http://www.thearkdb.org/browser?species=chicken). The G-band ideogram of the turtle chromosomes was made with Hoechst 33258-stained band patterns obtained by our replication R-banding method. The chromosome homologies between these species were indirectly compared according to the current information on the chicken–human comparative map (Schmid *et al*. 2000, Burt 2002). The chicken linkage maps are shown between the cytogenetic chromosome maps of chicken and the turtle. The conserved regions that are homologous to human chromosomes are represented by the coloured boxes on the linkage map. The numeral value on the linkage map indicates map position (cM). The solid lines indicate the locations of the genes finely localized to the conserved subchromosomal regions of human chromosomes. The dotted lines indicate the locations of the turtle genes that are localized to the vicinity of the subchromosomal regions conserved between human and chicken. TRA1 on PSI1 is mapped to both turtle and chicken chromosomes. Chromosome homology to HSA8q where human RPL8 is located has not been identified in GGA1.

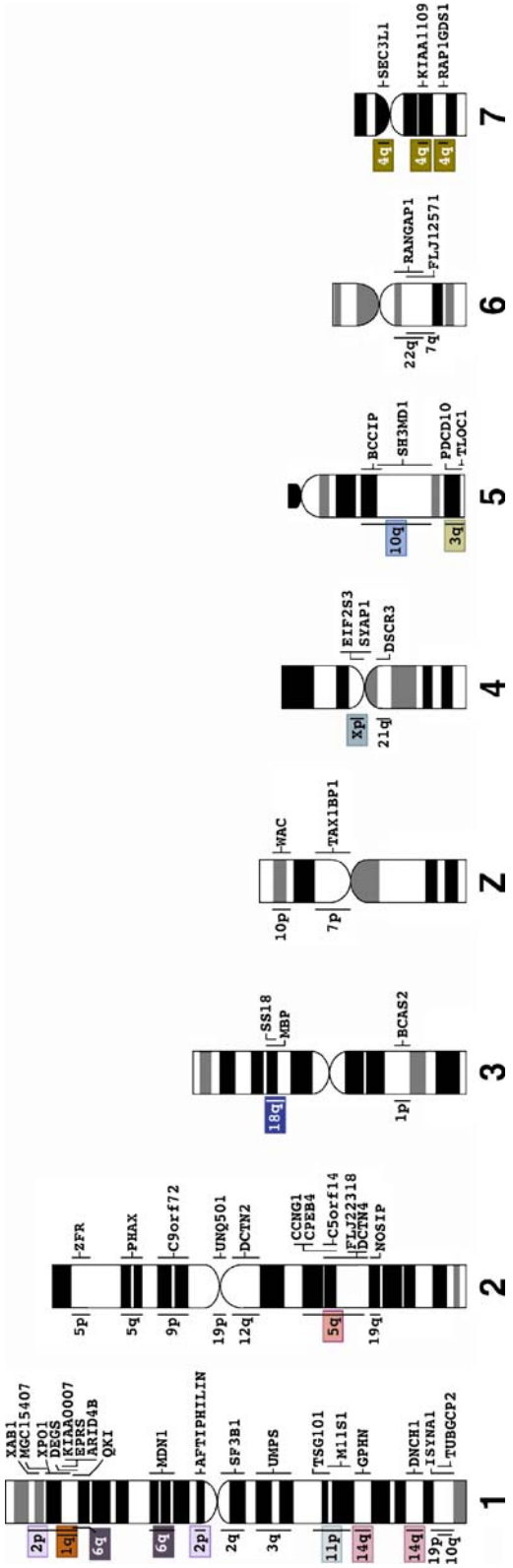

Figure 4. Comparative cytogenetic map of the Japanese four-striped rat snake. The G-band ideogram of the snake chromosomes was made according to Hoechst 33258-stained band patterns. The locations of the genes are represented to the right of the snake chromosomes. The human chromosomes with linkage homology to the snake chromosomes are shown to the left of chromosomes. The conserved segments of human chromosomes, to which two or more genes on the same chromosome arm are localized, are shown in colour. Bars demonstrate the extents of the conserved chromosome segments.

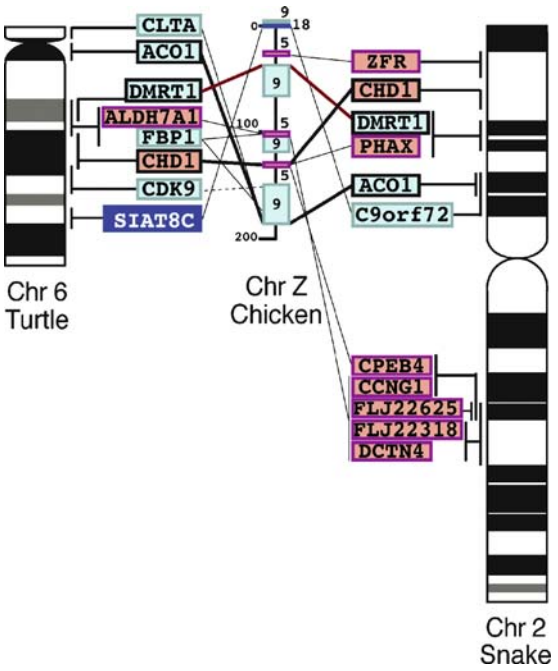

Figure 5. Comparison of the chicken Z chromosome with the Chinese soft-shelled turtle chromosome 6 and the Japanese four-striped rat snake chromosome 2. The linkage map of the chicken Z chromosome is shown between the cytogenetic chromosome maps of chicken and the turtle. The coloured boxes indicate the homology to the respective human chromosomes. The turtle and snake CHD1, ACO1 and DMRT1 genes were cloned as cDNA fragments by RT-PCR. The turtle CHD1 gene was also mapped using its EST clone.

(HSA14q23.3-q24.1), DNCH1 (HSA14q32.3-qter) and ISYNA1 (HSA19p13.11) were localized to GGA5, which has been found to be homologous to HSA14q but not to HSA19p. The short and long arms of EQU2 showed homology to GGAZ and GGA13, respectively. The homologies to HSA9p and HSA5q are shared between EQU2p and GGAZ and between EQU2q and GGA13. The short and long arms of EQU3 were homologous to GGA2 and GGA26, respectively. Conserved synteny to HSA18q and HSA1p was also found in GGA2 and GGA26, respectively. EQU4p and EQU4q showed homology to HSAXp and HSA21q, respectively, which are included in the syntenic region with GGA1. EQU5q, which corresponded to HSA3q and HSA10q, had homology to two chicken chromosomes, GGA6 and GGA9. The long arm of EQU6 was homologous to GGA1, which has conserved homology with HSA22q. Conserved synteny with HSA4q was found on GGA4 and EQU7q. Two genes, TAX1BP1 (HSA7p15) and WAC (HSA10), mapped onto EQUZ were not located on the chicken sex Z chromosome, and were instead localized to chicken autosome GGA2, which also has conserved synteny with HSA7p and HSA10. Twenty-four snake genes were localized to chicken chromosomes that show conserved synteny with the human chromosomes in which their human homologues are located; the one exceptional gene was ISYNA1 (HSA19p13.11). The highly conserved linkage homologies were also found between the snake and chicken chromosomes. Each of the arms of the largest five biarmed snake chromosomes corresponded to one chicken chromosome; however, the chromosomal correspondence identified between the turtle and chicken was not found between the snake and chicken.

No sex chromosomes were identified morphologically in the Chinese soft-shelled turtle (Figure 1). Six genes located on HSA5q, 9p, 9q and 18q, which are homologous to chicken Z chromosome, were localized to PSI6 (Figure 5). Three turtle homologues of chicken Z-linked genes, DMRT1, ACO1 and CHD1 (an EST clone of CHD1 was also identified in this study), which were cloned from adult testis of the turtle by RT-PCR, were also localized to PSI6, indicating that PSI6 is equivalent to the chicken Z chromosome. On the other hand, two genes located on HSA10p and 7p, in which no homology to the chicken Z chromosome has been found, were localized to the snake sex Z chromosome that is homologous to the short arm of GGA2. The snake EST clones of eight genes located on HSA 5p, 5q and 9p and three snake homologues of the chicken Z-linked genes DMRT1, ACO1 and CHD1 cloned by RT-PCR were localized to EQU2 (Figure 5), indicating that the short arm of EQU2 corresponds to the chicken Z chromosome.

The 1164-bp cDNA fragment of the turtle DMRT1 gene (Accession No. AB179697) and the 1235-bp cDNA fragment of the snake DMRT1 (AB179698) had 82.6% (683/827 bp) and 74.8% (624/834 bp) nucleotide identities with the equivalent region in the chicken DMRT1 cDNA fragment (AF211349), respectively (data not shown). The amino acid sequences of the turtle and snake DMRT1 genes showed 82.9% (228/275 AA) and 75.5% (209/277 AA) identities with the equivalent region in the chicken DMRT1 (Figure 6). In both cases, 100% identity was found in the DM domain with the chicken gene at the amino acid level. The nucleotide and

```
Gg          ------------------------------------------------
Ps      1   MPNDSSFNKPSASSDHHAQGGKMGSFGKAAVLMTAAAPAGGGG-AAGVLA    49
Eq      1   MPNETSFSKPSASSEHHAQGTKTGPFGKAAGLAAAGGGGNSGGLTQGPAG    50

Gg      1   --PGAGKKLPRLPKCARCRNHGYSSPLKGHKRFCMWRDCQCKKCSLIAER    48
Ps     50   PAAASGKKSPRLPKCARCRNHGYSSPLKGHKRFCMWRDCQCKKCSLIAER    99
Eq     51   TVTAAGKKSPRLPKCARCRNHGYSSPLKGHKRFCMWRDCQCKKCSLIAER   100

Gg     49   QRVMAAQVALRRQQAQEEELGISHPVPLPSAPEPVVKK-SSSSSSCLLQD    97
Ps    100   QRVMAAQVALRRQQAQEEELGISHPIPLPSAPELFVKKENNGGSSCLLLE   149
Eq    101   QRVMAAQVALRRQQAQEEELGISHPIPLPSATEMYVKKENNANSSC-LLE   149

Gg     98   SSSPAHSTSTVAAAAASAPPEGRMLIQDIPSIPSRGHLESTSDLVVDSTY   147
Ps    150   SSSPTHST-NTATTASSTPAEGRMLIQDIPSITSRGHLESTSDLVVDSTY   198
Eq    150   STSPPQST-TTATVVSTSSTEGRMLIQDIPSLASRGHLESTSDLVMDPTY   198

Gg    148   YSSFYQPSLYPYYNNLYNYSQYQMAVATESSSSETGGTFVGSAMKNSLRS   197
Ps    199   YSSFYQPSLYPYYNNLYNYSQYQMAVASESSSSDMGGTLVGSPVKNSLRS   248
Eq    199   YSSLYQPSLYPYYNNLYNYSQYQMAVATETTSGDMGSPLSGSPVKSSLRS   248

Gg    198   LPATYMSSQSGKQWQMKGMENRHAMSSQYRMCSYYPPTSYLGQGVGSPTC   247
Ps    249   LPATYMSSQSGNQWQMKSTESRHAMSSPYRMHSYYSPASYLGQSVSTPAC   298
Eq    249   LPTTYMSSQSGNQWQVKSPEGRHSLSSQYRMHSYYPSSSYLGQSVGAPAC   298

Gg    248   VTQILASEDTPSYSESKARVFSPPSSQDSGLGCLSSSESTKGDLECEPHQ   297
Ps    299   VPQIFTFEDSPSYSESKASVFSPPSSQ----------------------   325
Eq    299   VPQIFTFEENPSYSDTKANVFSPPSSQDSG-------------------   328

Gg    298   EPGAFAVSPVLEGE   311
Ps          --------------
Eq          --------------
```

Figure 6. Comparison of amino acid sequences of the DMRT1 genes of chicken (Gg), the Chinese soft-shelled turtle (Ps) and the Japanese four-striped rat snake (Eq). The amino acids that are identical between two or three genera are highlighted in green columns. The pink highlighting indicates the DM domain.

amino acid sequences of the turtle and snake DMRT1 genes were conserved between chicken and the two reptile species, and the identity of the turtle DMRT1 gene was higher than that of the snake. The concatenated 1122-bp (AB185397) and 1122-bp (AB185398) cDNA fragments of the turtle and snake ACO1 genes showed 81.7% (917/1122 bp) and 79.0% (886/1122 bp) nucleotide identities, and 88.8% (332/374 AA) and 88.0% (329/374 AA) identities at amino acid level with the chicken ACO1 fragment (D16150), respectively (data not shown). The nucleotide sequence identities of the CHD1 cDNA fragments of the turtle (915 bp, AB185401 and 345 bp, AB185402) and snake (941 bp, AB185399 and 345 bp, AB185400), which were separately cloned fragments of the full cDNA sequences, were 89.7%

(1130/1260 bp) (88.6%, 811/915 bp and 92.5%, 319/345 bp) and 84.4% (1086/1286 bp) (84.0%, 790/941 bp and 85.8%, 296/345 bp) with the equivalent region in the chicken CHD1Z fragment (AF004397), respectively (data not shown). The amino acid identities were 98.1% (411/419 AA) (97.4%, 297/305 AA and 100%, 114/114 AA) and 97.4% (417/428 AA) (97.5%, 306/314 AA and 97.4%, 111/114 AA) for the turtle–chicken and snake–chicken comparisons, respectively. The nucleotide sequence identities of the two reptile genes to their chicken homologues were higher in the turtle than the snake.

Discussion

The molecular time scale of vertebrate evolution indicates that the earliest ancestors of mammals (synapsids) and birds (diapsids) first appeared in the Carboniferous period around 310 million years ago (MYA) (Kumar & Hedges 1998). Mitochondrial DNA comparisons suggest that birds are most closely related to crocodilians, and the divergence between the two lineages is estimated to have occurred at 210–250 MYA (Janke & Arnason 1997, Kumar & Hedges 1998, Hedges & Poling 1999, Kumazawa & Nishida 1999, Mannen & Li 1999). The phylogenetic position of turtles relative to other amniotes has remained uncertain for some time. The traditional placement of turtles was separate from the diapsid reptiles, as the sole descendants of a presumably primitive anapsid reptilian group. A recently proposed molecular phylogeny, which was estimated from the nucleotide sequences of complete mitochondrial genomes and the nuclear genes, indicates that turtles should be grouped in the Archosauria with birds and crocodilians, and lizards and snakes can be classified into a different clade (the Lepidosauria) (Caspers *et al*. 1996, Zardoya & Meyer 1998, Hedges & Poling 1999, Kumazawa & Nishida 1999, Mannen & Li 1999, Mindell *et al*. 1999, Cao *et al*. 2000, Iwabe *et al*. 2005). Closely related species are generally expected to share more conserved segments than distantly related species; however, this rule is not always applicable between chicken and the Chinese soft-shelled turtle and between human and mouse. Based on the comparative mapping data of human, mouse and chicken, the organization of the human genome is suggested to be closer to that of the chicken, with 72 predicted chromosome rearrangements (ranging from 44 to 93), which is much less than the at-least 171 rearrangements between mouse and human (Burt *et al*. 1999). The present study reveals extensive chromosomal homologies between chicken and the Chinese soft-shelled turtle, although the two lineages diverged from the common ancestor more than 210 MYA. By contrast, chromosomal rearrangements have occurred more frequently between mouse and human than between chicken and the turtle (Carver & Stubbs 1997, Burt *et al*. 1999, Schmid *et al*. 2000, Burt 2002), although the lineage of human and mouse diverged more recently (80–90 MYA; Hasegawa *et al*. 2003). Our data suggest that the karyotypes of birds and turtles, consisting of few macrochromosomes and a large number of microchromosomes, have remained relatively stable and conserved, and thus inter- and intrachromosomal rearrangements have hardly occurred in the two lineages after divergence from the common ancestor. A higher frequency of interchromosomal rearrangements, which occurred between macrochromosomes and also between macro- and microchromosomes, led to the karyotype with several large-sized macrochromosomes and fewer microchromosomes in the lineage of snakes. The higher conserved synteny in the chicken–turtle comparison than the chicken–snake comparison supports the proposed molecular phylogenetic relationships among the three genera (Zardoya & Meyer 1998, Cao *et al*. 2000).

Reptiles exhibit different features of sex determination from birds and mammals. In all snakes, most lizards and a minority of turtles, sex is determined by genes carried on sex chromosomes, according to male (XX/XY) or female (ZZ/ZW) heterogamety. Only the ZZ/ZW mechanism exists in snakes (Beçak *et al*. 1964, Beçak & Beçak 1969, Singh 1972, Jones & Singh 1985), whereas both XX/XY and ZZ/ZW mechanisms have been found in lizards and turtles (Bull *et al*. 1974, King & Rofe 1976, Carr & Bickham 1981). In contrast to the genotypic (GSD) or chromosomal sex determination (CSD), temperature-dependent sex determination (TSD) is widespread in reptiles (Pieau *et al*. 1999, Pieau & Dorizzi 2004). The mechanisms of sex determination and the primary factors of its complexity in birds and reptiles remain unknown (Sarre *et al*. 2004, Smith & Sinclair 2004). In the Chinese soft-shelled turtle, which belongs to the Triionychidae, sex is genetically determined, not temperature-dependent (personal communication by H. Ota), although no heteromorphic

sex chromosomes have been identified. The extensive conservation of synteny between the chicken Z chromosome and human chromosomes 5 and 9 is demonstrated by comparative mapping of chicken homologues of human genes (Nanda *et al*. 1999, 2000, Schmid *et al*. 2000, Burt 2002). On the other hand, chicken homologues of the human X-linked genes have been localized to chicken chromosomes 1 and 4 (Schmid *et al*. 2000, Burt 2002, Kohn *et al*. 2004), indicating that the avian sex Z chromosome and the mammalian sex X chromosome have evolved independently from different autosomes of a common ancestor. Although sex chromosomes are not discriminated morphologically from other autosomes in the Chinese soft-shelled turtle, chromosome 6 has extensive conserved linkage homology to human chromosomes 5 and 9, and the three chicken Z-linked genes are localized to chromosome 6. These results suggest that the ancestral chromosomes of the avian sex Z chromosomes have been conserved as an autosome in the turtle genome for around 230–240 MY (Hedges & Poling 1999, Kumazawa & Nishida 1999).

The DMRT1 gene, which encodes a putative transcription factor with a conserved DM (*dsx* and *mab-3*) domain, is located in the minimal region of human chromosome 9p that is deleted in XY male-to-female sex reversal patients (Raymond *et al*. 1999). The structural homology between vertebrate and invertebrate DMRT1 genes and their remarkable capacity to complement their function across different phyla indicate that the DMRT1 genes are conserved forms of an ancient sexual regulator (Raymond *et al*. 1998). The chicken DMRT1 homologue is located on the Z chromosome, and therefore DMRT1 is thought to be a strong candidate for an avian sex determining gene (Nanda *et al*. 2000, Shetty *et al*. 2002). The chromosomal location of the DMRT1 homologue on the snake chromosome 2 and the discordance between the chicken and snake sex Z chromosomes suggest that the critical sex-determining genes may be different between birds and snakes, and thus the mechanisms of sex determination have evolved independently in the two genera.

The present approach of comparative mapping using an extensive number of ESTs is a new strategy to investigate chromosome homologies between reptiles and birds. The disposition of conserved chromosome segments among reptiles, birds and mammals provides clues for clarifying the phylogenetic hierarchy of genome evolution in vertebrates.

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