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The origin and differentiation process of X and Y chromosomes of the black marsh turtle (*Siebenrockiella crassicollis*, Geoemydidae, Testudines)

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Abstract The black marsh turtle (*Siebenrockiella crassicollis*) has morphologically differentiated X and Y sex chromosomes. To elucidate the origin and evolutionary process of *S. crassicollis* X and Y chromosomes, we performed cross-species chromosome painting with chromosome-specific DNA from Chinese soft-shelled turtle (*Pelodiscus sinensis*) and chromosome mapping of the sex-linked genes of *S. crassicollis* using FISH. The X and Y chromosomes of *S. crassicollis* were hybridized with DNA probe of *P. sinensis* chromosome 5, which is homologous to chicken chromosome 5. *S. crassicollis* homologues of 14 chicken chromosome 5-linked genes were all localized to the X long arm, whereas two genes were mapped to the Y short arm and the other 12 genes were localized to the Y long arm in the same order as the X chromosome. This result suggests that extensive linkage homology has been retained between chicken chromosome 5 and *S. crassicollis* X and Y chromosomes and that *S. crassicollis* X and Y chromosomes are at an early stage of sex chromosome differentiation. Comparison of the locations of two site-specific repetitive DNA sequences on the X and Y chromosomes demonstrated that the centromere shift was the result of centromere repositioning, not of pericentric inversion.

Keywords Testudines · sex chromosomes · comparative mapping · chromosome homology · gene order · repetitive sequence · centromere repositioning

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Abbreviations

AMI	*Alligator mississippiensis*
ASI	*Alligator sinensis*
BCO	*Boa constrictor*
BrdU	5-bromo-2′-deoxyuridine
BSG	Barium hydroxide/saline/Giemsa
CCD	Charge-coupled device
CCR	*Caiman crocodilus*
cDNA	Complementary DNA
CLA	*Caiman latirostris*
CSI	*Crocodylus siamensis*
DDBJ	DNA Data Bank of Japan
Dig	Digoxigenin

dNTP	Deoxynucleotide triphosphate
dUTP	2′-deoxyuridine 5′-triphosphate
EQU	*Elaphe quadrivirgata*
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
GGA	*Gallus gallus*
GGA	*Gavialis gangeticus*
GHO	*Gekko hokouensis*
GSD	Genotypic sex determination
kb	Kilo base pairs
MYA	Million years ago
PCR	Polymerase chain reaction
PFL	*Protobothrops flavoviridis*
PMB	*Python molurus bivittatus*
PI	Propidium iodide
PSI	*Pelodiscus sinensis*
SCA	*Struthio camelus*
SCR	*Siebenrockiella crassicollis*
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
STR	*Staurotypus triporcatus*
TSC	*Tomistoma schlegelii*
TSD	Temperature-dependent sex determination
UV	Ultraviolet

Introduction

Testudines exhibits two different types of sex-determining systems: temperature-dependent sex determination (TSD) in which sex ratio is determined by the incubation temperature of eggs and genotypic sex determination (GSD) with both male heterogamety (XY/XX) and female heterogamety (ZZ/ZW) (Head et al. 1987; Sarre et al. 2004; Valenzuela and Lance 2004; Olmo and Signorino 2005). Most of testudinian species exhibit TSD, and heteromorphic sex chromosomes have been known in only eight GSD species: the XY/XX system in six species, Brazilian radiolated swamp turtle (*Acanthochelys radiolata*, formerly *Platemys radiolata*, Chelidae) (McBee et al. 1985), the eastern long-necked turtle (*Chelodina longicollis*, Chelidae) (Ezaz et al. 2006) and Murray river turtle (*Emydura macquarii*, Chelidae) (Martinez et al. 2008), Chiapas giant musk turtle (*Staurotypus salvinii*, Kinosternidae), Mexican giant musk turtle (*Staurotypus triporcatus*, Kinosternidae) (Bull et al. 1974), the black marsh turtle (*Siebenrockiella crassicollis*, Geoemydidae) (Carr and Bickham 1981); and the ZZ/ZW system in two species, the brown-roofed turtle (*Pangshura* (*Kachuga*) *smithii*, Geoemydidae) (Sharma et al. 1975) and Chinese soft-shelled turtle (*Pelodiscus sinensis*, Trionychidae) (Kawai et al. 2007). Chromosome numbers of turtles are highly variable, ranging from $2n=22$ to 66 (Ayres et al. 1969; Bickham and Baker 1976; Bickham et al. 1983). Their karyotypes are generally composed of two major chromosomal components, macrochromosomes and microchromosomes, according to their physical sizes; the most common diploid chromosome number is around 50–52, consisting of 12–14 pairs of macrochromosomes and 12–14 pairs of microchromosomes. The sizes of sex chromosomes are varied between species. *C. longicollis* and *P. sinensis* have the XY- and ZW-types of micro-sex chromosomes, respectively, and the other six species have large- or middle-sized sex chromosomes. The sex chromosomes of *E. macquarii* are considered to have been established by a translocation of an ancestral Y microchromosome as found in the species of a sister clade, *C. longicollis*, onto the tip of the fourth largest autosome (Martinez et al. 2008); however, the origins of the sex chromosomes of these species and their homologies have been still unknown except for the ZW micro-sex chromosomes of *P. sinensis*. Comparative gene mapping between *P. sinensis* and chicken revealed that the six largest chromosomes were almost equivalent between two species (Matsuda et al. 2005); however, *P. sinensis* Z chromosome was homologous to chicken chromosome 15 but not to the chicken Z chromosome (Kawagoshi et al. 2009). This result suggests that the ZW-type sex chromosomes of birds and turtles differentiated independently in each lineage from different autosome pairs of the common ancestor of Arcosauromorpha, which diverged around 250 million years ago (MYA) (Kumazawa and Nishida 1999; Janke et al. 2001; Rest et al. 2003; Iwabe et al. 2005).

The black marsh turtle (*S. crassicollis*, Geoemydidae) is abundant throughout much of the southeast Asian rainforest (Thailand, Sumatra, Borneo, Java and peninsular Malaysia) and has heteromorphic sex chromosomes of the XY/XX-type (Carr and Bickham 1981, 1986). We revealed that the ZW sex chromosomes of *P. sinensis* correspond to chicken chromosome 15 (Kawagoshi et al. 2009); however, the origins of the testudinian XY sex chromosomes are still unknown. In this study, to elucidate the origin and differentiation process of *S. crassicollis* XY chromosomes, we first conducted chromosome painting with chromosome-specific DNA probes from *P.*

sinensis, and then performed chromosome mapping of *S. crassicollis* homologues of chicken genes located on the chromosome that is homologous to *S. crassicollis* XY chromosomes. We also cloned site-specific repetitive DNA sequences from *S. crassicollis* and used them as cytogenetic markers to determine the process of chromosomal rearrangements that occurred between the X and Y chromosomes. This is the first report on the origin and differentiation process of the XY-type sex chromosomes in Testudines, which was examined by comparative gene mapping.

Material and methods

Cell culture and chromosome preparation

One captive bred male of *S. crassicollis* was purchased and used for this study. After intraperitoneal injection of pentobarbital, the heart, lung, and the mesentery were removed and used for cell culture. All experimental procedures with the animal conformed to the guideline established by the Animal Care Committee, Nagoya University, Japan. The tissues were minced and cultured in Dulbecco's modified Eagle's medium (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Invitrogen-GIBCO), 100 μg/ml kanamycin, and 1% antibiotic-antimycotic (PSA) (Invitrogen-GIBCO). The 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 chromosome preparation, the fibroblast cells at log phase were harvested after colcemid treatment (120 ng/ml) for 45 min, suspended in 0.075 M KCl at room temperature for 20 min and fixed with methanol/acetic acid (3:1) three times. The cell suspension was dropped on cleaned glass slides and air-dried. For gene mapping by FISH, replication R-banding was performed to identify each chromosome as described previously (Matsuda and Chapman 1995). The fibroblast cell cultures were treated with BrdU (12 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) at late replication stage for 12 h including 45 min colcemid treatment, and chromosome preparations were made as mentioned above. The slides were dried at room temperature for 2–3 days. After staining the slides with Hoechst 33258 (1 μg/ml) for 10 min, R bands were obtained by heating them at 65°C for 3 min and exposing them to UV light at 65°C for an additional 6.5 min. The slides were kept at −80°C until use.

C banding

To examine the chromosomal distribution of constitutive heterochromatin in *S. crassicollis* chromosomes, C banding was performed by the standard BSG method (Sumner 1972) with slight modification; chromosome slides were treated with 0.2 N HCl at room temperature for 5 min and then 5% $Ba(OH)_2$ at 50°C for 2 min.

Chromosome painting

Chromosome-specific DNA probes of *P. sinensis* were used for comparative chromosome painting of *S. crassicollis* chromosomes. The *P. sinensis* chromosome paints were prepared and provided by Fengtang Yang and Patricia O'Brien, Department of Veterinary Medicine, Cambridge University, UK. Chromosome painting was performed as described in Matsubara et al. (2003) with slight modification. DNA probe (1 μg) was labeled with biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland) using a nick translation kit (Roche Diagnostics). After pre-hybridization for 15 min at 37°C, hybridization was carried out at 37°C for 5 days. After hybridization, the slide was washed, incubated with fluorescein-conjugated avidin (FITC-avidin) (Roche Diagnostics), and stained with 0.75 μg/ml propidium iodide (PI).

Molecular cloning of repetitive DNA sequences

High molecular weight genomic DNA was extracted from liver tissue. Genomic DNA was digested with 18 restriction endonucleases, *Apa*I, *Alu*I, *Bam*HI, *Bgl*I, *Bgl*II, *Csp*45I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Pvu*II, *Sac*I, *Sal*I, *Spe*I, *Xba*I, and *Xho*I, size-fractionated with 2% agarose gel electrophoresis, and then stained with ethidium bromide. The prominent DNA bands of repetitive sequences were eluted from the gel using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into pGEM®-7Zf(+) vector (Promega, Tokyo, Japan), and transformed into competent cells of *Escherichia coli* strain DH5α (TOYOBO, Osaka, Japan). The sizes of DNA fragments inserted into the vector were confirmed by electrophoresis of PCR products that were amplified

using T7 and Sp6 primers, and the clones were used for FISH.

Molecular cloning of *S. crassicollis* homologues of chicken genes

Testes of *S. crassicollis* were homogenized and lysed with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted following the manufacturer's instruction. Reverse transcription-PCR and molecular cloning of cDNA fragments were performed as described previously (Kawai et al. 2009). Nucleotide sequences were determined using an ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Foster, CA, USA) after sequencing reactions with a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

FISH mapping of genes

FISH was performed for chromosomal localization of repetitive DNA sequences and cDNA fragments of functional genes following Matsuda and Chapman (1995). Repetitive DNA fragment (250 ng) was labeled with biotin-16-dUTP by nick translation and hybridized to chromosome slides. After hybridization, the slides were incubated with FITC-avidin and stained with 0.75 μg/ml PI. For chromosome mapping of cDNA fragments, 250 ng cDNA probe was labeled with biotin-16-dUTP (Roche Diagnostics) by nick translation. After hybridization, the probe DNA was reacted with goat anti-biotin antibody (Vector Laboratories), and then stained with Alexa Fluor 488 rabbit anti-goat IgG (H + L) conjugate (Invitrogen-Molecular Probes) and subsequently counter-stained with 0.75 μg/ml PI.

Southern blot hybridization

Genomic DNA of *S. crassicollis* was digested with nine restriction endonucleases (*Alu*I, *Apa*I, *Bam*HI, *Hae*III, *Hin*dIII, *Hin*fI, *Rsa*I, *Hpa*II, *Msp*I). The digested DNA was fractionated in 2% agarose gel and transferred onto nylon membranes (Roche Diagnostics). DNA fragments of repetitive sequences were labeled with digoxigenin (DIG)-11-dUTP using PCR DIG Labeling Mix (Roche Diagnostics) and hybridized to the membranes. Hybridization was carried out at 45°C overnight in DIG Easy Hyb solution (Roche Diagnostics). After hybridization, the membranes were washed at 45°C in 0.1% SDS/2× SSC, 0.1% SDS/1× SSC, 0.1% SDS/0.5× SSC and 0.1% SDS/0.1× SSC for 15 min each. Chemiluminescent signals were detected using anti-digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics) and exposed to BioMax MS autoradiography film (Kodak, Rochester, NY, USA).

Slot-blot hybridization

To examine the conservation of repetitive sequences among different species, slot-blot hybridization was conducted with DIG-11-dUTP-labeled repetitive DNA fragments. Genomic DNA was collected from three species of Testudines, the black marsh turtle (*S. crassicollis*, Geoemydidae), Mexican giant musk turtle (*S. triporcatus*, Kinosternidae), and Chinese soft-shelled turtle (*P. sinensis*, Trionychidae); seven species of Crocodilia, Siamese crocodile (*Crocodylus siamensis*, Crocodylidae), the false gharial (*Tomistoma schlegelii*, Crocodylidae), Indian gharial (*Gavialis gangeticus*, Gavialidae), American alligator (*Alligator mississippiensis*, Alligatoridae), Chinese alligator (*Alligator sinensis*, Alligatoridae), the common caiman (*Caiman crocodilus*, Alligatoridae), and the broad-nosed caiman (*Caiman latirostris*, Alligatoridae) (Kawagoshi et al. 2008); two species of Aves, chicken (*Gallus gallus*, Phasianidae, Galliformes) and ostrich (*Struthio camelus*, Struthionidae, Struthioniformes); and four species of Squamata, Burmese python (*Python molurus bivittatus*, Pythonidae), Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae), habu (*Protobothrops flavoviridis*, formerly *Trimeresurus flavoviridis*, Viperidae), and Hokou gecko (*Gekko hokouensis*, Gekkonidae). Genomic DNA (200 ng) was denatured with NaOH and transferred onto nylon membranes using BIO-DOT SF blotting equipment (Bio-Rad, Hercules, CA, USA). Hybridization and detection of signals were performed as described in the section of Southern blot hybridization.

Results

Karyotype of *S. crassicollis*

Chromosome numbers of $2n=50$ and $2n=52$ have been reported for *S. crassicollis* (Killebrew 1977;

Carr and Bickham 1981, 1986). In the present study, we examined 17 Giemsa-stained metaphase spreads of one male *S. crassicollis*. Chromosome number was $2n=50$ for all metaphase spreads, and the karyotype consisted of four pairs of large chromosomes, five and two pairs of middle- and small-sized chromosomes, respectively, and 14 pairs of indistinguishable microchromosomes (Fig. 1). Sex chromosomes were the third or fourth largest heteromorphic chromosomes; the X and Y were submetacentric and metacentric, respectively.

C bands of *S. crassicollis* chromosomes

C-positive heterochromatin blocks were observed in the centromeric regions of almost all autosomes, and the centromeric heterochromatin was large in several pairs of chromosomes (Fig. 2). The short arm of the X chromosome and a distal half of the Y short arm were heterochromatic and an intense heterochromatin block was located in the pericentromeric region of the X long arm.

Chromosome painting probed with chromosome-specific DNA of *P. sinensis*

FITC-labeled chromosome 5-specific DNA probe of *P. sinensis* painted the X and Y chromosomes of *S. crassicollis* but not any other chromosomes (Fig. 3), indicating that *S. crassicollis* X and Y sex chromosomes are a counterpart of *P. sinensis* chromosome 5, which is homologous to chicken chromosome 5 (Matsuda et al. 2005). The painted signal was not detected in the pericentromeric region of the X long arm, which corresponded to C-positive heterochromatin.

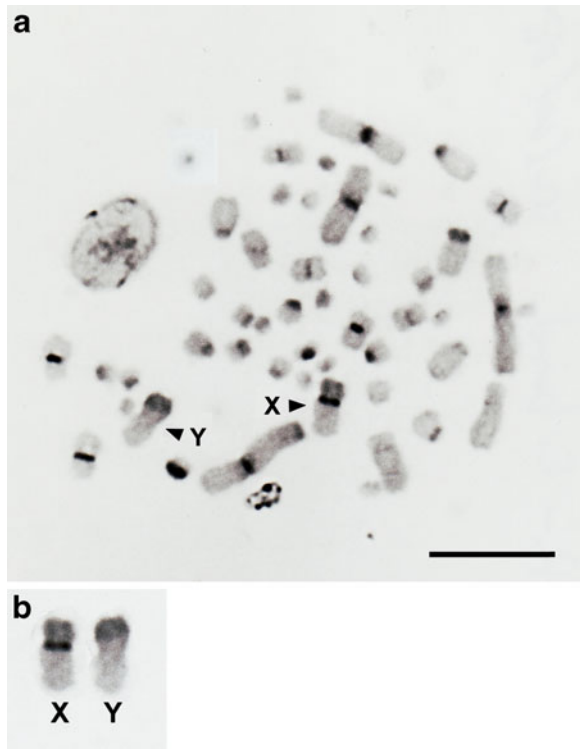

Fig. 2 C-banded metaphase spread of male *S. crassicollis* (**a**) and enlarged photograph of the X and Y chromosomes (**b**). *Arrowheads* indicate the X and Y chromosomes. *Scale bar* represents 10 μm

Molecular cloning of *S. crassicollis* homologues of chicken chromosome 5-linked genes

S. crassicollis homologues of the following 14 chicken chromosome 5-linked genes were cloned using a total cDNA pool derived from *S. crassicloiis* testes: *BUB1B*, *CPT1A*, *CRY2*, *CTR9*, *DDB1*, *DEAF1*, *DYNC1H1*, *EIF4G2*, *GARNL1*, *PAPOLA*, *PSMC1*, *PYGL*, *ST5*, and *WT1*. PCR primers of 14 genes are shown in Table 1. Nucleotide sequences of the cDNA fragments of 14 genes

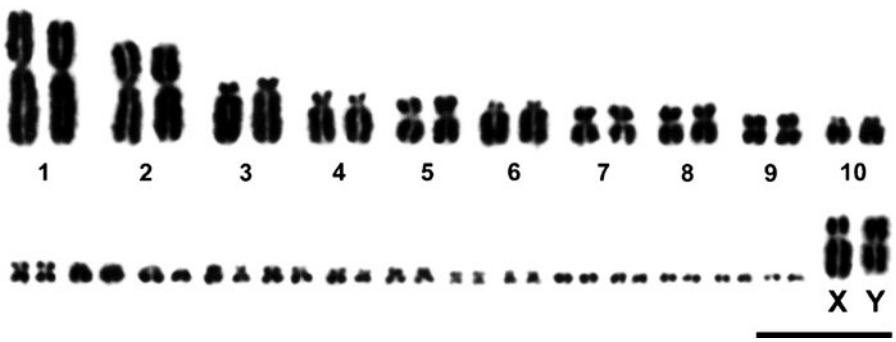

Fig. 1 Giemsa-stained karyotype of male *Siebenrockiella crassicollis*. The X and Y chromosomes are submetacentric and metacentric, respectively. *Scale bar* represents 10 μm

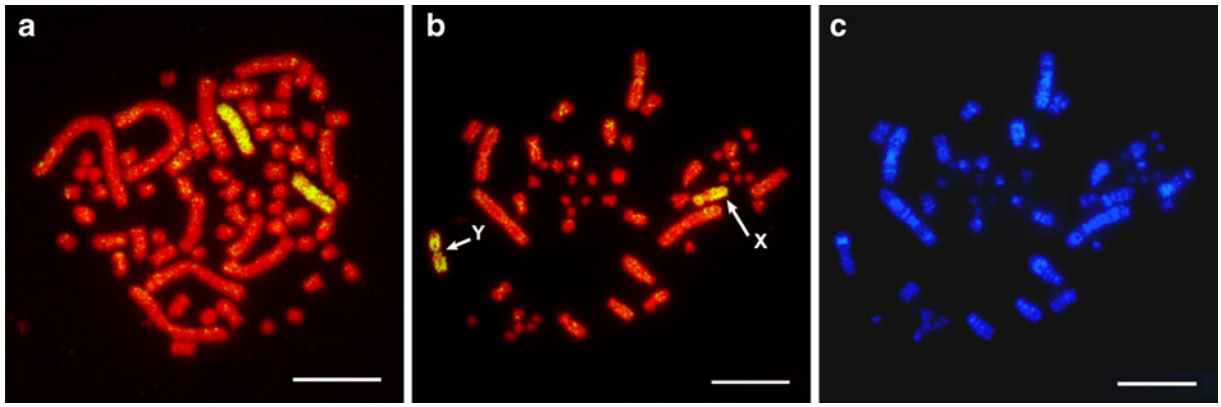

Fig. 3 Chromosome painting with chromosome 5-specific DNA probe of *P. sinensis* to PI-stained metaphase spreads of female *Pelodiscus sinensis* (**a**) and male *S. crassicollis* (**b**). (**c**) Hoechst-stained pattern of the same metaphase spread (**b**). The probe painted chromosome 5 of *P. sinensis* (**a**) and the X and Y chromosomes of *S. crassicollis* (**b**). *Arrows* indicate the X and Y chromosomes. *Scale bar* represents 10 μm

were deposited in DDBJ (http://www.ddbj.nig.ac.jp/). Nucleotide sequence identities of the equivalent regions of the cDNA fragments between chicken and *S. crassicollis* ranged from 65.4% to 96.4% (Table 2). The *CPT1A* cDNA fragment showed the lowest identity (65.4%) with the chicken homologue.

FISH mapping of *S. crassicollis* homologues of chicken chromosome 5-linked genes

Hoechst-stained bands obtained by the replication banding method, which correspond to G bands, made it possible to identify each chromosome precisely and

Table 1 Degenerate oligonucleotide primers used for molecular cloning of *S. crassicollis* homologues of 14 chicken chromosome 5-linked genes

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
BUB1B	CAGATTGAAGAGATGGAGAAGAAGC	GACTCAAGTCACCATGGACTATTTCTGC
CPT1A	CATGTTGTACAGYTTCCAGACNTC	GTKGAYARTCTCCAAGGCTCAG
CRY2	CGCTTYCAGGCCATCATHAGCC	GGTCTTCCACACANGAAGGGAC
CTR9	CCAGATGACTTGCTTGGATACATTGG	CTGYTCTGTGACCTTCTTCAAATGGC
DDB1	TCAACCTSAAGGARGTGAACTACATG	ATGCCRTTGACNGTGCCGAAGAG
DEAF1	AACTGGGACCCNTCNGTGTAYGAC	GRCAGAANGTGGAGCAGTAGTTGAC
DYNC1H1	RGTCAGCGAGTTCAAGGARG	CATGCTGCCTGARTGGCCAT
EIF4G2	CTATGCCTTGAGCTCCTCAATGTGG	GAGGTGGCATATCCTTCGACTGTC
GARNL1	CTTTYCTCAATGCACCAAGAGTTGAAGC	GCATTCAGGRCTWGTNACACCTAAATACTG
PAPOLA	GCTATGCTAGTAGCAAGAACTTGCC	GTTGTGTGGCAGTTTGAGGAATGC
PSMC1	GACCAGATGCTGCNAGCAAACTG	CCATCAGACCAGCTTCTGTACAG
PYGL	TCAACACNATGCGCCTNTGGTC	CRAACATGGAGGANGGGTTGATCTTC
ST5	CTTTGCACAGGATGTGGASTCC	CACAGGCAGCTCCTTCAGYTTG
WT1	AACCACTCYTTCAARCAYGARGACCCCAT	CAYTGGAAKGGTTTCACACCTGTGTGTC
	AACCACTCYTTCAARCAYGARGACCCCAT	CAGCTGGAGTTTGGTCATGT

BUB1B budding uninhibited by benzimidazoles 1 homolog beta (yeast), *CPT1A* carnitine palmitoyltransferase 1A (liver), *CRY2* cryptochrome 2 (photolyase-like), *CTR9* Paf1/RNA polymerase II complex component, homolog (*S. cerevisiae*), *DDB1* damage-specific DNA binding protein 1, 127 kDa, *DEAF1* deformed epidermal autoregulatory factor 1 (*Drosophila*), *DYNC1H1* dynein, cytoplasmic 1, heavy chain 1, *EIF4G2* eukaryotic translation initiation factor 4 gamma, 2, *GARNL1* GTPase activating Rap/RanGAP domain-like 1, *PAPOLA* poly(A) polymerase alpha, *PSMC1* proteasome (prosome, macropain) 26S subunit, ATPase, 1, *PYGL* phosphorylase, glycogen, liver, *ST5* suppression of tumorigenicity 5, *WT1* Wilms tumor 1

Table 2 The cDNA fragments of *S. crassicollis* homologues of chicken chromosome 5-linked genes and nucleotide sequence identities between chicken and *S. crassicollis* cDNA fragments

Gene[a]	Length of cDNA fragment (bp)	Length of nucleotide sequence in the overlapped region of chicken gene	Identity (%) between SCR and GGA[b]	Accession number
BUB1B	1,570	1,245	75.0 (937/1249)	AB674487
CPT1A	1,566	1,566	65.4 (1030/1575)	AB674488
CRY2	1,074	1,069	87.0 (935/1075)	AB674489
CTR9	979	977	89.7 (876/977)[c]	AB674490
DDB1	1,095	1,095	88.7 (972/1096)	AB674491
DEAF1	1,192	1,012	84.3 (853/1012)[c]	AB674492
DYNC1H1	1,046	1,046	86.4 (904/1046)[c]	AB674493
EIF4G2	1,093	1,093	96.4 (1054/1093)[c]	AB674494
GARNL1	1,409	1,409	81.0 (1155/1425)	AB674495
PAPOLA	1,057	1,057	95.2 (1006/1057)[c]	AB674496
PSMC1	1,092	1,092	87.2 (952/1092)[c]	AB674497
PYGL	977	974	81.1 (790/974)[c]	AB674498
ST5	1,316	1,316	88.8 (1168/1316)[c]	AB674499
WT1	781	780	89.7 (700/780)[c,d]	AB674500, B674501

[a] *BUB1B* budding uninhibited by benzimidazoles 1 homolog beta (yeast), *CPT1A* carnitine palmitoyltransferase 1A (liver), *CRY2* cryptochrome 2 (photolyase-like), *CTR9* Paf1/RNA polymerase II complex component, homolog (*S. cerevisiae*), *DDB1* damage-specific DNA binding protein 1, 127kDa, *DEAF1* deformed epidermal autoregulatory factor 1 (*Drosophila*), *DYNC1H1* dynein, cytoplasmic 1, heavy chain 1, *EIF4G2* eukaryotic translation initiation factor 4 gamma, 2, *GARNL1* GTPase activating Rap/RanGAP domain-like 1, *PAPOLA* poly(A) polymerase alpha, *PSMC1* proteasome (prosome, macropain) 26S subunit, ATPase, 1, *PYGL* phosphorylase, glycogen, liver, *ST5* suppression of tumorigenicity 5, *WT1* Wilms tumor 1

[b] The number in parenthesis indicates the number of identical bases/the number of bases including gaps in the overlapped region between cDNA fragments of two species. *SCR S. crassicollis*, *GGA Gallus gallus*

[c] There are no gaps in nucleotide sequences between *S. crassicollis* and chicken cDNA fragments

[d] The total cDNA length of *WT1* fragment concatenated with multiple PCR products

to determine the subchromosomal locations of the genes. Fourteen genes were all localized to the X long arm, whereas two of them (*DDB1* and *DEAF1*) were mapped to the Y short arm, and the other 12 genes were all localized at the same positions on the Y long arm as those of the X chromosome (Figs. 4, 5). For FISH mapping, more than 25–30 metaphase spreads were observed for each gene, and the hybridization efficiency on the X and/or Y chromosomes ranged from 20% to 32% of metaphase spreads.

Molecular cloning of repetitive sequences and their chromosomal distribution

Genomic DNA of *S. crassicollis* was digested with 18 restriction endonucleases, and the prominent DNA bands at approximately 300 bp in *Alu*I digest were isolated (data not shown). Thirteen clones were obtained and used for FISH analysis. FISH signals were detected for two of 13 DNA clones. The SCR-*Alu*I-01 sequence was distributed predominantly in the centromeric heterochromatin regions of three pairs of macrochromosomes, six pairs of middle- and/or small-sized chromosomes and nine pairs of microchromosomes (Fig. 6). Small signals were also detected in the telomeric regions of several pairs of large- and middle-sized chromosomes. In the sex chromosomes, the signals were observed in the telomeric regions of both the X and Y chromosomes, the pericentromeric region of the X long arm, and an interstitial region of the Y short arm. The SCR-*Alu*I-09 sequence was localized to the centromeric heterochromatin of chromosome 1 and 2 and two pairs of middle-sized chromosomes and to the pericentromeric region of the X long arm and an interstitial region of the Y short arm (Fig. 6). The SCR-*Alu*I-01 and SCR-*Alu*I-09 sequences were not overlapped with the intense C-positive heterochromatin of the X long arm.

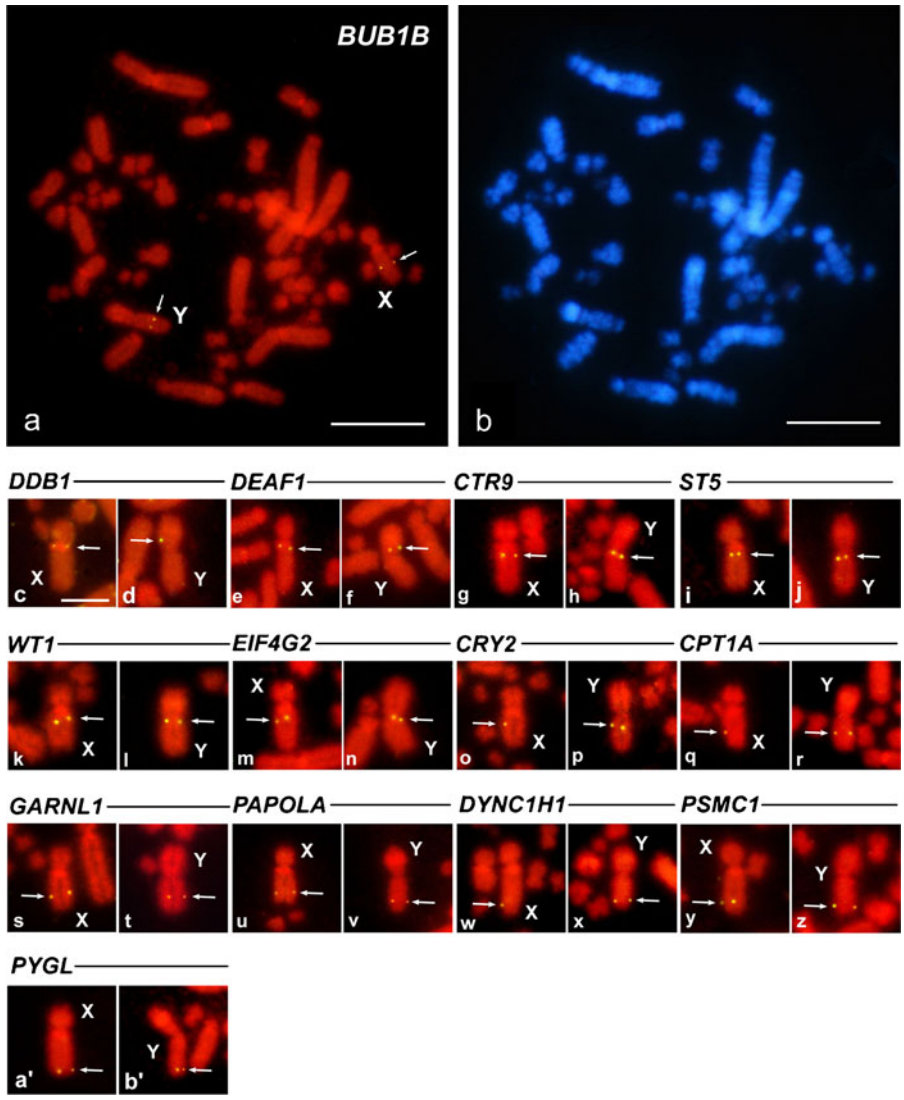

Fig. 4 Chromosomal localization of *S. crassicollis* homologues of 14 chicken chromosome 5-linked genes, *DDB1*, *DEAF1*, *CTR9, ST5, WT1*, *EIF4G2*, *CRY2*, *BUB1B*, *CPT1A*, *GARNL1*, *PAPOLA*, *DYNC1H1*, *PSMC1*, and *PYGL*, to chromosomes of male *S. crassicollis*. **a**, **b** FISH pattern of *BUB1B* on PI-stained metaphase spread (**a**) and Hoechst-stained pattern of the same metaphase spread (**b**). **c**–**z**, **a′**, **b′** FISH patterns of *DDB1* (**c**), *DEAF1* (**e**), *CTR9* (**g**), *ST5* (**i**), *WT1* (**k**), *EIF4G2* (**m**),*CRY2*(**o**), *CPT1A*(**q**), *GARNL1*(**s**), *PAPOLA*(**u**), *DYNC1H1*(**w**), *PSMC1*(**y**) and *PYGL*(**a′**) on PI-stained X chromosomes, and FISH patterns of *DDB1* (**d**), *DEAF1* (**f**), *CTR9* (**h**), *ST5* (**j**), *WT1* (**l**), *EIF4G2* (**n**), *CRY2* (**p**), *CPT1A* (**r**), *GARNL1* (**t**), *PAPOLA* (**v**), *DYNC1H1* (**x**), *PSMC1* (**z**) and *PYGL* (**b′**) on PI-stained Y chromosomes. *Arrows* indicate the hybridization signals of the genes. *Scale bars* represent 10 μm (**a**, **b**) and 2.5 μm (**c**–**z**, **a′**, **b′**)

Nucleotide sequences of repetitive sequences

The length and G + C content were 308 bp and 70.8% for SCR-*Alu*I-01 (AB674502) (Fig. 7a) and 287 bp and 69.3% for SCR-*Alu*I-09 (AB674503) (Fig. 7b). SCR-*Alu*I-09 was composed of seven 36 bp-unit and one 34 bp-unit elements (Fig. 7c). Nucleotide sequence identities and G + C content among the internal units of SCR-*Alu*I-09 ranged from 66.7% between unit 5 and 6 to 97.2% between unit 1 and 3, and their G + C content was 68.8% on average, ranging from 61.1% (unit 6) to 75.0% (unit 1 and 3). There was no sequence homology between SCR-*Alu*I-01 and SCR-*Alu*I-09. These nucleotide sequences were compared with all the DNA sequences in the nonredundant database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequence of SCR-*Alu*I-01 had the homology with partial sequences of two BAC clones (CHY3-80H12 and CHY3-29H12) derived from the painted turtle (*Chrysemys picta*) genome. Homologies of SCR-*Alu*I-01 with CHY3-29H12 and CHY3-80H12 were both 87% in the equivalent regions of 302 bp. No sequences with significant homology were detected for SCR-*Alu*I-09.

Genomic organization of repetitive sequences

In hybridization probed with SCR-*Alu*I-01, polymeric hybridization bands were found at 308, 616, and 924 bp in *Alu*I digest, indicating that this sequence can be

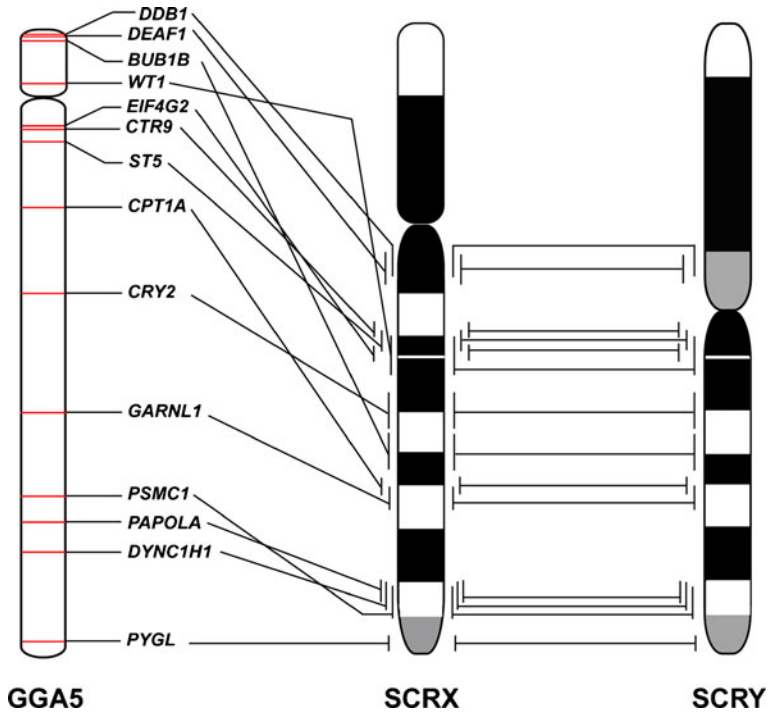

Fig. 5 Comparative maps of 14 functional genes, *DDB1*, *DEAF1*, *CTR9*, *ST5*, *WT1*, *EIF4G2*, *CRY2*, *BUB1B*, *CPT1A*, *GARNL1*, *PAPOLA*, *DYNC1H1*, *PSMC1*, and *PYGL* on chicken chromosome 5 (GGA5) and *S. crassicollis* X (SCRX) and Y (SCRY) chromosomes. The gene order of 14 genes on GGA5 was taken from the Ensemble Chicken Web Server (http://uswest.ensembl.org/Gallus_gallus/Info/Index) and Chicken Genome Resources (http://www.ncbi.nlm.nih.gov/genome/guide/chicken/)

categorized as satellite DNA; however, one another repeated unit of about 400 bp with *Alu*I site, which was not molecularly cloned in the present study, was contained in this family (Fig. 8a). In *Apa*I digest, hybridization bands comprising two units of the SCR-*Alu*I-01 element and their dimeric bands were found at about 600 and 1,200 bp, respectively. The same sizes of hybridization bands were also observed in *Alu*I digest. These results suggest that *Alu*I and *Apa*I sites of SCR-*Alu*I-01 are highly conserved in this sequence family and *Apa*I site is located in every other unit. *Hin*fI and *Rsa*I sites were also highly conserved; however, the unit sizes of the SCR-*Alu*I-01 elements with *Hin*fI and *Rsa*I sites were different from those with *Alu*I and *Apa*I sites, suggesting that the SCR-*Alu*I-01 sequences consist of various unit sizes of elements with different restriction sites. The hybridization bands detected at less than 308 bp in *Hae*III and *Msp*I digests were derived from multiple internal restriction sites contained in the elements. CCGG sequence is cleaved by *Hpa*II and *Msp*I, and *Hpa*II does not cleave when the CG sequence is methylated. In digests cleaved with two isoschizomers, all hybridization bands were observed at less than 600 bp in *Msp*I digest, while no low molecular weight bands were found in *Hpa*II digest. This remarkable difference indicates that the SCR-*Alu*I-01 sequences are hypermethylated in the genome.

Smears of DNA in the range from 300 bp to 2 kb were observed when digested with *Alu*I and hybridized with SCR-*Alu*I-09 probe (Fig. 8b). Hybridization patterns also appeared as broad smear in *Bam*HI, *Hin*dIII, *Hin*fI, *Rsa*I and *Hpa*II digests, suggesting that these restriction sites are less conserved in the SCR-*Alu*I-09 sequences. Intense single bands were observed in *Hae*III and *Msp*I digests, which might be derived from conserved internal restriction sites in the SCR-*Alu*I-09 sequence elements. The *Hpa*II sites were highly methylated in the SCR-*Alu*I-09 sequences as well as the SCR-*Alu*I-01 sequences.

Nucleotide sequence conservation of repetitive sequences

Nucleotide sequence conservation of the SCR-*Alu*I-01 and SCR-*Alu*I-09 sequences was examined by slot-blot hybridization for 16 species from three reptilian orders and two avian orders (Fig. 9). In SCR-*Alu*I-01, intense hybridization signals were observed in three testudinian species, whereas weak signals were observed for the other all species (Fig. 9a). In contrast, the hybridization signal for

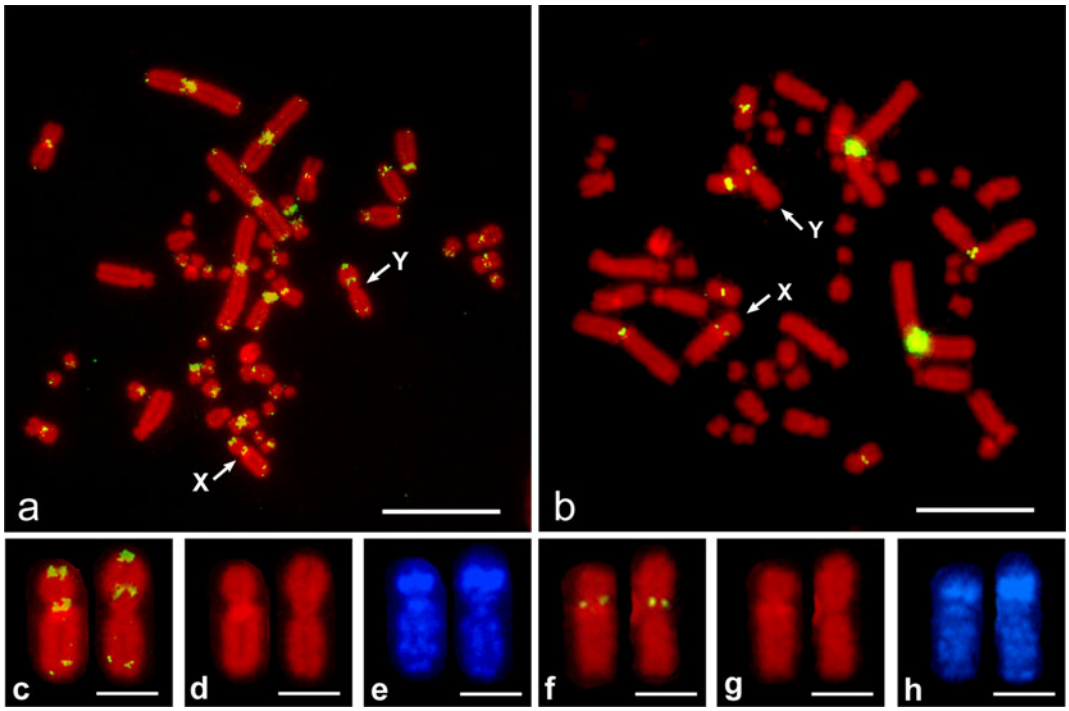

Fig. 6 Chromosomal distribution of the SCR-*Alu*I-01 and SCR-*Alu*I-09 sequences on male *S. crassicollis* chromosomes. **a**, **b** Distribution pattern of SCR-*Alu*I-01 (**a**) and SCR-*Alu*I-09 (**b**) on PI-stained metaphase spreads. *Arrows* indicate the X and Y chromosomes. **c**–**h** Enlarged photographs of the X and Y chromosomes. (**c**) FISH pattern of SCR-*Alu*I-01 on PI-stained X and Y chromosomes. **d**, **e** PI-stained (**d**) and Hoechst-stained (**e**) X and Y chromosomes. (**f**) FISH pattern of SCR-*Alu*I-09 on PI-stained X and Y chromosomes. **g**, **h** PI-stained (**g**) and Hoechst-stained (**h**) X and Y chromosomes. *Scale bars* represent 10 μm in (**a**, **b**) and 2.5 μm in (**c**–**h**)

SCR-*Alu*I-09 was observed for only *S. crassicollis*, and there were no signals in the other two testudinian species and all species of Crocodilia, Aves, and Squamata (Fig. 9b).

Discussion

In this study, we investigated the origin and evolutionary process of the X and Y sex chromosomes of *S. crassicollis* using cross-species chromosome painting with chromosome-specific DNA from Chinese soft-shelled turtle (*P. sinensis*) and chromosome mapping of the sex-linked genes of *S. crassicollis*. The X and Y chromosomes of *S. crassicollis* was homologous to chicken chromosome 5, and *S. crassicollis* homologues of 14 chicken chromosome 5-linked genes were all localized to the X and Y chromosomes in the same order, although the centromeric position was different between two chromosomes. Comparison of the locations of two site-specific repetitive DNA sequences on the X and Y chromosomes suggested that the centromere shift on the Y chromosome was the result of centromere repositioning that occurred on the proto-X chromosome, not of pericentric inversion.

Homology of sex chromosomes: chromosome painting and candidate gene mapping

Cross-species chromosome painting with *P. sinensis* chromosome-specific DNA revealed the homology of *S. crassicollis* X and Y sex chromosomes with *P. sinensis* chromosome 5, which correspond to chicken chromosome 5 (Matsuda et al. 2005). *S. crassicollis* homologues of 14 chicken chromosome 5-linked genes were all localized to the X and Y chromosomes of *S. crassicollis*, indicating that this genetic linkage has been retained in Testudines and Aves since Archosauromorpha first appeared about 250 MYA (Kumazawa and Nishida 1999; Janke et al. 2001;

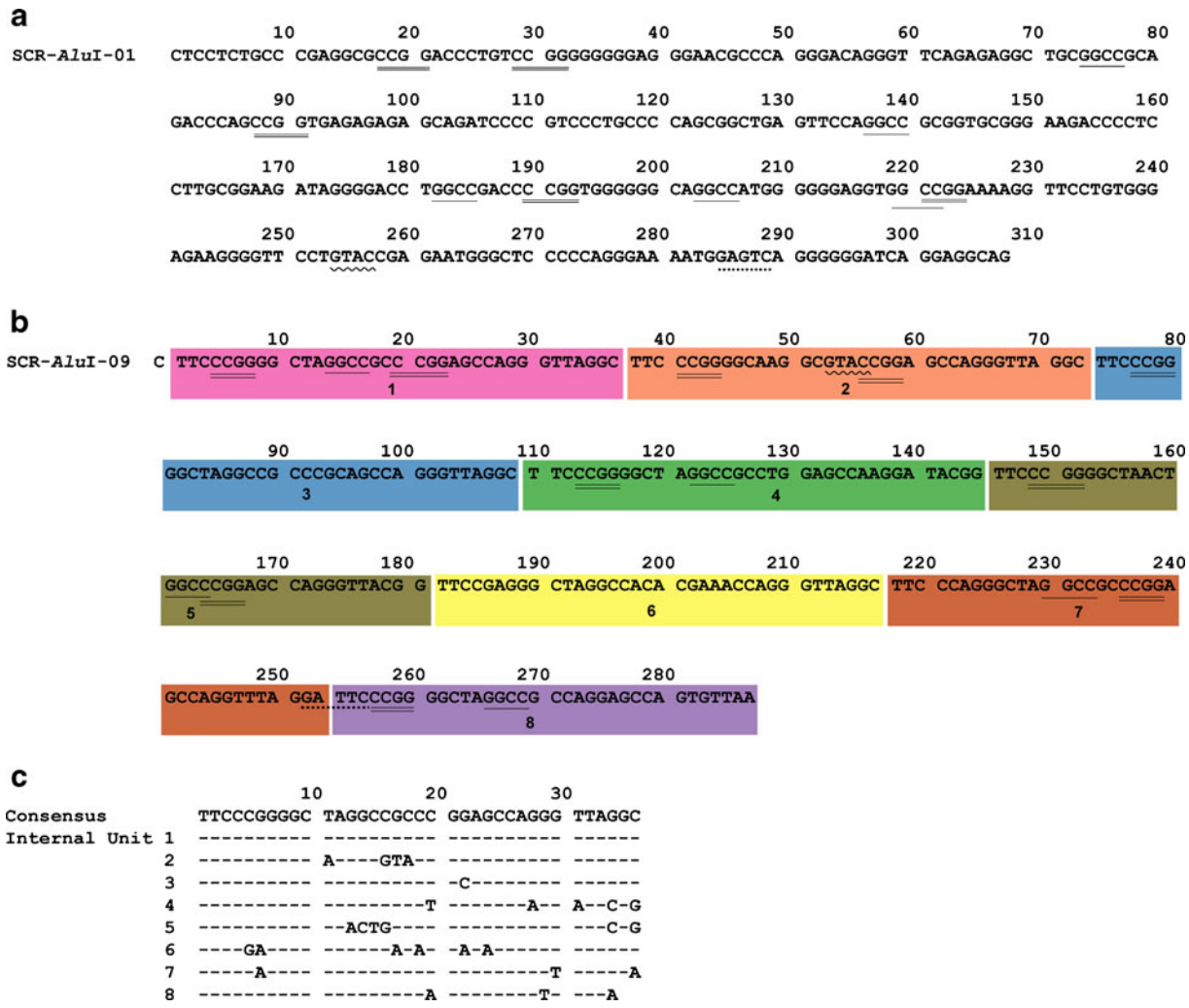

Fig. 7 Nucleotide sequences of the SCR-*Alu*I-01 and SCR-*Alu*I-09 fragments. **a** Nucleotide sequence of the SCR-*Alu*I-01 fragment. Internal restriction sites of four restriction endonucleases are represented as follows: *Hae*III (*straight line*), *Rsa*I (*crooked line*), *Hin*fI (*dotted line*), and *Msp*I (*double line*). **b** Nucleotide sequence of the SCR-*Alu*I-09 fragment. Each internal unit of SCR-*Alu*I-09 is shown by different *color box* and *number*. Internal restriction sites of four restriction endonucleases are represented as follows: *Hae*III (*straight line*), *Rsa*I (*crooked line*), *Hin*fI (*dotted line*), and *Msp*I (*double line*). **c** Nucleotide sequences of eight internal units of SCR-*Alu*I-09 and comparison of their nucleotide sequences. *Hyphens* indicate the same nucleotides as those of the consensus sequence of eight internal units shown at the *top*

Rest et al. 2003; Iwabe et al. 2005). This linkage has also been extensively conserved in human chromosome 11 and 14 for eight genes (*CPT1A, CRY2, CTR9, DDB1, DEAF1, EIF4G2, ST5, WT1*) and five genes (*DYNC1H1, GARNL1, PAPOLA, PSMC1, PYGL*), respectively, although only *BUB1B* is localized to human chromosomes 15 (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The genetic linkage of *S. crassicollis* X and Y chromosomes therefore have been highly conserved in amniotes; however, *S. crassicollis* XY sex chromosomes have no homology with the ZW sex chromosomes of *P. sinensis*, which is homologous to chicken chromosome 15 (Kawagoshi et al. 2009), suggesting that their sex chromosomes were differentiated independently within the same order from different autosomes of the common ancestor. The eastern long-necked turtle (*C. longicollis*) of Chelidae has micro-XY sex chromosomes, which are detectable by C banding, reverse fluorescent staining, or comparative genomic hybridization (Ezaz et al. 2006). In contrast, Murray river turtle (*E. macquarii*) and Brazilian radiolated swamp turtle (*A. radiolata*) belonging to the same

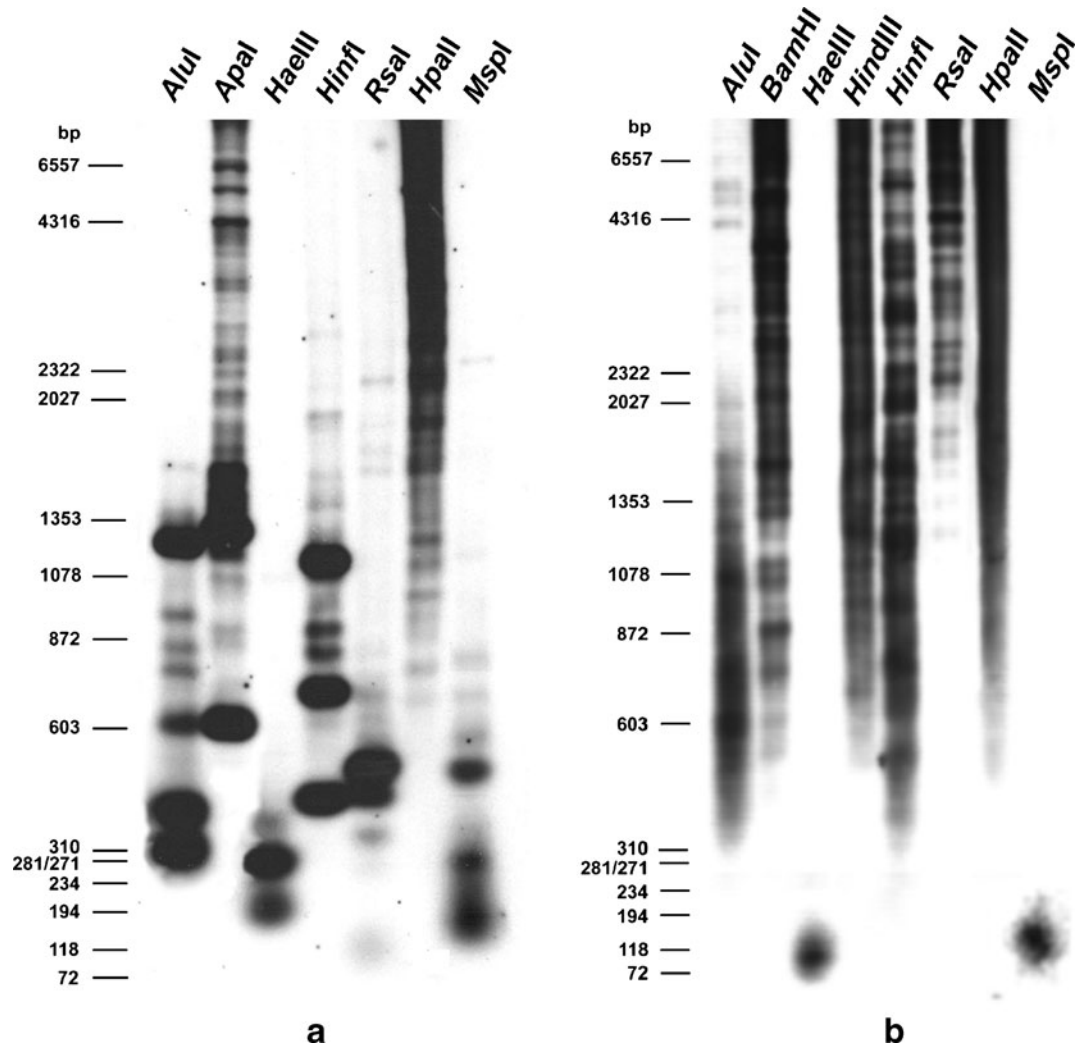

Fig. 8 Southern blot hybridization of *S. crassicollis* genomic DNA probed with the SCR-*Alu*I-01 (**a**) and SCR-*Alu*I-09 (**b**) fragments. A mixture of λDNA–*Hin*dIII and φX174 DNA–*Hae*III digests was used as a molecular weight marker

family have large-sized (submetaentric X and metacentric Y) and middle-sized (metacentric X and acrocentric Y) sex chromosomes, respectively (McBee et al. 1985; Martinez et al. 2008). This significant size differences among three species implied independent origins of sex chromosomes even within the same family. Comparative mapping of these three species with *S. crassicollis* is required to understand the diversity of sex chromosomal origins in XY-type turtles. Male heterogamety is also found in two species of Kinosternidae, Chiapas giant musk turtle (*S. salvinii*) and Mexican giant musk turtle (*S. triporcatus*) (Bull et al. 1974), which are the most closely related to *S. crassicollis* among XY-type turtles. *S. salvini* and *S. triporcatus* also have the fourth largest X chromosome, and the centromere and short arms of their X chromosomes were heterochromatized. These characteristics are similar to those of *S. crassicollis* X chromosome, and thus it would be considerable interest to investigate sex chromosomal homologies among three species.

The order of 14 chicken chromosome 5-linked genes on *S. crassicollis* Y chromosome was the same as that on the long arm of X chromosome, although two genes (*DDB1* and *DEAF1*) were localized to the proximal region of the Yp. The X and Y chromosomes of *S. crassicollis* therefore are at an early stage of sex

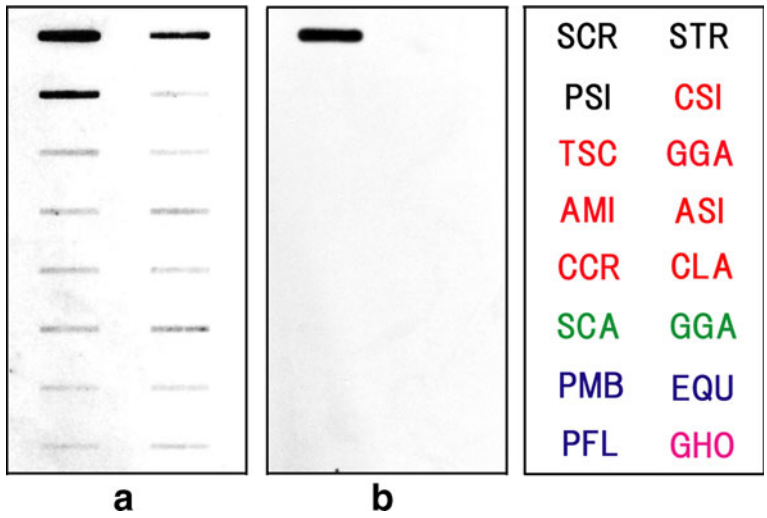

Fig. 9 Slot-blot hybridization probed with the SCR-*Alu*I-01 (**a**) and SCR-*Alu*I-09 (**b**) fragments. Genomic DNA used for this experiment were as follows: SCR (*Siebenrockiella crassicollis*), STR (*Staurotypus triporcatus*), and PSI (*Pelodiscus sinensis*) of Testudines written in *black*; CSI (*Crocodylus siamensis*), TSC (*Tomistoma schlegelii*), GGA (*Gavialis gangeticus*), AMI (*Alligator mississippiensis*), ASI (*Alligator sinensis*), CCR (*Caiman crocodilus*), and CLA (*Caiman latirostris*) of Crocodilia written in *red*; SCA (*Struthio camelus*) and GGA (*Gallus gallus*) of Aves written in *green*; PMB (*Python molurus bivittatus*), EQU (*Elaphe quadrivirgata*) and PFL (*Protobothrops flavoviridis*) of Serpentes written in blue; GHO (*Gekko hokouensis*) of Sauria written in purple

chromosome differentiation with no deletion of the Y homologues. The gene order of *S. crassicollis* X chromosome was largely different from that of chicken chromosome 5, indicating that intra-chromosomal rearrangements have occurred extensively between two lineages, although the gene order in the common ancestor is still unknown.

Genomic organization of sex chromosome repeats and XY differentiation

Two novel families of repetitive sequences were isolated, which served as good cytogenetic markers to delineate the differentiation process of *S. crassicollis* X and Y chromosomes. SCR-*Alu*I-09 was located in the pericentromeric region of the Xq and the interstitial region of the Yp. SCR-*Alu*I-01 was co-localized with SCR-*Alu*I-09 and also localized to the distal ends of both the short and long arms of the X and Y chromosomes. Centromere repositioning is most likely to explain this result. Centromere repositioning occurs without the disruption of gene order (Montefalcone et al. 1999) and is caused by the following phenomena: the activation of a neocentromere, acquisition and/or amplification of neocentromeric DNA, and inactivation and/or deletion of the old centromere (Wong and Choo 2001). According to our schematic representation, the SCR-*Alu*I-01 and -09 sequences and *DDB1* and *DEAF1* in the pericentromeric region of the proto-X long arm moved to the Y short arm with no change to the order of SCR-*Alu*I-01/SCR-*Alu*I-09–*DDB1*–*DEAF1* (Fig. 10). Meiotic recombination should be suppressed around the subcentromeric regions containing *DDB1* and *DEAF1* genes between the X and Y chromosomes, and then the X chromosome-specific repetitive sequences may have been amplified in the pericentromeric region of the X long arm, which are intensely stained with PI and with Giemsa by C banding. The alternative hypothesis is that a pericentric inversion occurred in the pericentromeric region; however, the order of SCR-*Alu*I-01/SCR-*Alu*I-09–*DDB1*–*DEAF1* on the Xq was the same as that on the Yp.

SCR-*Alu*I-01 was conserved through three testudinian families, Geoemydidae, Kinosternidae, and Trionychidae; however, weak signals were observed in Crocodilia, Aves, and Squamata. These results suggested that this sequence originated in the genome of the common ancestor of sauropsids and diverged and/or was amplified in Testudines. In contrast, the signal of SCR-*Alu*I-09 was only found in *S. crassicollis*, indicating that this sequence was amplified species-specifically as a

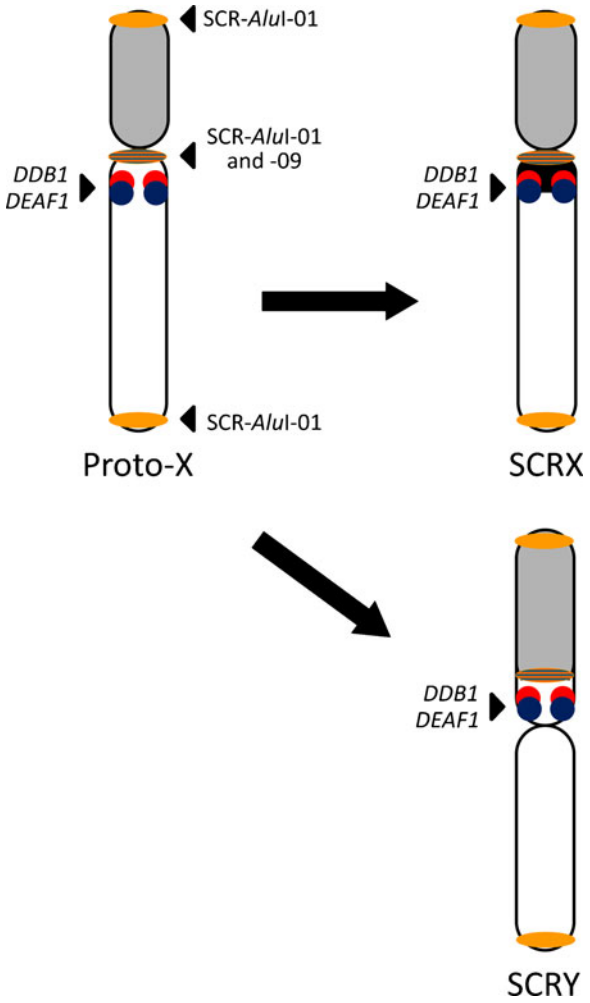

Fig. 10 Schematic representation for the differentiation process of the X and Y chromosomes in *S. crassicollis*. Chromosomal locations of *DDB1* and *DEAF1* are represented by *red* and *blue circles*, respectively. *Yellow ellipse* and *yellow*-and *blue*-striped *ellipse* indicate the chromosomal location of SCR-*Alu*I-01 and the co-location of SCR-*Alu*I-01 and SCR-*Alu*I-09, respectively. Two different types of heterochromatic regions are each *gray*- and *black-painted*. *Arrows* show the differentiation into the X (SCRX) and Y (SCRY) chromosomes from the proto-X chromosome, showing that X chromosome-specific repetitive sequences were amplified in the pericentromeric region of the X long arm and that the co-localized SCR-*Alu*I-01 and -09 sequences and *DDB1* and *DEAF1* genes in the pericentromeric region of the proto-X long arm moved to the Y short arm as a result of centromere repositioning

rapidly evolving molecule. The repetitive sequences comprising the heterochromatized regions of the X and Y short arms and the pericentromeric C-positive heterochromatin block of the X long arm have not been isolated yet. Molecular characterization of these sequences is important to understand the differentiation process of *S. crassicollis* sex chromosome.

The Y-specific region that is responsible for male sex determination has not been identified in *S. crassicollis*, but the sex-determining locus is more likely to be located near the pericentromeric region where it is structurally differentiated between the X and Y chromosomes. There were no known genes related to sexual differentiation in the chicken chromosome segment homologous to this region; however, *WT1*, which is a significant nuclear zinc-finger transcription factor that is required for early kidney and gonad development in mammals (Haber et al. 1991; Armstong et al. 1992; Kreidberg et al. 1993; Rackley et al. 1993; Roberts 2005), was localized to the subcentromeric region of the long arm of Y chromosome in this species. The three-spined stickleback (*Gasterosteus aculeatus*) and Nile tilapia (*Oreochromis niloticus*) have duplicated *WT1* genes, and one of them is located on the X and Y sex chromosomes in both species (Ross and Peichel 2008; Lee and Kocher 2007). Three inversions occurred in the chromosomal region containing the Y-linked *WT1* gene in the stickleback (Ross and Peichel 2008); however, the role of the Y-linked *WT1* gene for gonadal differentiation is unknown. In turtles, *WT1* expression has been examined in two TSD and one GSD species (Spotila et al. 1998; Valenzuela 2007). In the painted turtle (*C. picta*), sexual dimorphic expression of *WT1* was found in the developing adrenal-kidney-gonad (AKG) complex of embryos incubated at 25°C (male-producing temperature) and 30°C (female-producing temperature) during developmental stage 12 (Valenzuela 2007); the expression level was much higher at 25°C than 30°C. A GSD turtle, the smooth soft-shell turtle (*Apalone mutica*) also exhibited the thermal sensitive expression pattern (i.e., sexual dimorphic expression) of *WT1* between incubation temperatures of 25°C and 30°C during developmental stage 12 (Valenzuela 2007), indicating that this species retain the thermal-sensitivity to sexual differentiation after it acquired the GSD system. Neither expression stages nor expression levels of sexual differentiation-related genes during gonadal development has been examined in *S. crassicollis*. It is unclear whether the *WT1* clone isolated in the present study was derived from the X or Y chromosome; however, it will be of great interest to compare the expression of the X-linked *WT1* with that of the Y copy, which may have become functionally divergent from the X copy, as the sex chromosomes differentiated.

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