Evidence for different origin of sex chromosomes in snakes, birds and mammals, and stepwise differentiation of snake sex chromosomes

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Abstract

All snake species exhibit genetic sex determination with the ZZ/ZW type of sex chromosomes. To investigate the origin and evolution of snake sex chromosomes, we constructed by FISH a cytogenetic map of the Japanese four-striped rat snake (*Elaphe quadrivirgata*) with 109 cDNA clones. Eleven of the 109 clones were localized to the Z chromosome. All human and chicken homologues of the snake Z-linked genes were located on autosomes, suggesting that the sex chromosomes of snakes, mammals and birds were all derived from different autosomal pairs of the common ancestor. We mapped the 11 Z-linked genes of *E. quadrivirgata* to chromosomes of two other species, the Burmese python (*Python molurus bivittatus*) and the habu (*Trimeresurus flavoviridis*), to investigate the process of W chromosome differentiation. All and three of the 11 clones were localized to both the Z and W chromosomes in *P. molurus* and *E. quadrivirgata*, respectively, whereas no cDNA clones were mapped to the W chromosome in *T. flavoviridis*. Comparative mapping revealed that the sex chromosomes are only slightly differentiated in *P. molurus* whereas they are fully differentiated in *T. flavoviridis*, and *E. quadrivirgata* is at a transitional stage of sex chromosome differentiation. The differentiation of sex chromosomes was probably initiated from the distal region on the short arm of the proto-sex chromosome of the common ancestor, and then deletion and heterochromatization progressed on the sex-specific chromosome from the phylogenetically primitive boids to the more advanced viperids.

Introduction

All snake species are subject to genetic sex determination with sex chromosomes, as are mammals and birds, and they have female heterogamety (ZZ males and ZW females). Comparative gene mapping between human and chicken revealed that human XX/XY and chicken ZZ/ZW sex chromosomes have no homologies (1, 2), suggesting that the sex chromosomes of mammals and birds were derived from different pairs of autosomes of the common ancestor. Beçak *et al*. (3) found that there is close karyological similarity between snakes and birds, such as distinct differentiation of macro- and microchromosomes and constant occurrence of ZW-type sex chromosomes. This leads us to predict the presence of homology between ophidian and avian sex chromosomes. However, no attempts have been yet made to investigate the conservation of the linkage homologies of snake chromosomes to human and chicken chromosomes by comparative gene mapping, although this approach would provide fundamental information on the genome evolution and the origin of sex chromosome differentiation in amniotes. In our previous study (4), we constructed a preliminary cytogenetic map of the Japanese four-striped rat snake (*Elaphe quadrivirgata*) with 52 EST (expressed sequence tagged) clones, which were isolated from the cDNA library of the brain tissue and were identified as snake homologues of human and chicken orthologous genes by a search of the DNA database. Out of 52 EST clones, two genes, *TAX1BP1* and *WAC*, whose human homologues are located on human chromosome 7 and 10 respectively, were localized to the Z chromosome. In addition, snake homologues of three chicken Z-linked genes, *DMRT1*, *ACO1/IREBP* and *CHD1*, were molecularly cloned by RT-PCR, and were

subjected to chromosome mapping. All three homologues were mapped to the short arm of the snake chromosome 2, suggesting that the sex chromosomes of snakes, mammals and birds were differentiated independently from different autosomes of the common ancestor. However, only a few genes were mapped on the snake Z chromosome, and the homology of the snake Z chromosome to human and chicken chromosomes has not been investigated in detail.

It is speculated from the observations of differently evolved sex chromosome pairs that heteromorphic sex chromosomes have developed from a pair of homologous chromosomes (5). In this scenario, a gene mutation that conferred a sexual advantage firstly occurred on one of the homologues, and a partially heterozygous chromosomal region was consequently formed. Meiotic recombination between the proto-sex chromosomes was suppressed around the heterologous region to preserve the linkage of sex-linked genes. The suppression of recombination favored the accumulation of gene mutations on the sex-specific chromosome, leading to numerous deletions of the functionally inactivated genes and accumulation of repetitive DNA sequences (6, 7). The mammalian Y chromosome and avian W chromosome became highly degenerated and extensively heteochromatized, with the exception of monotremes and palaeognathous birds, which have less differentiated sex chromosomes (8-13). The human Y chromosome still contains 27 homologues of X-linked single copy genes and pseudogenes (14), and chicken has also the Z and W forms of six "gametologous" genes, which arose by the cessation of recombination due to sex chromosome differentiation, *ATP5A1, CHD, HINTZ, PKCI, SPIN* and *UBA2* (15-17). The degeneration status of the snake W chromosomes varies among species (3, 18, 19). The Z and W chromosomes are

homomorphic in the boid species. In contrast, the W chromosomes are highly degenerated and heterochromatic in the poisonous snakes belonging to the Elapidae and the Viperidae. The colubrid species, which have moderately differentiated sex chromosomes, are at an intermediate stage of sex chromosome differentiation between the Boidae and the poisonous snakes. Thus, snakes are a good animal model for studying the evolutionary process of sex chromosome differentiation in vertebrates.

Here we report a high resolution cytogenetic map of the Japanese four-striped rat snake constructed with 105 EST clones. We demonstrate the conservation of the linkage homologies of snake chromosomes with human and chicken chromosomes, and discuss the genome evolution and the origins of sex chromosomes in amniotes. Furthermore, we compare the structures of sex chromosomes among three snake species, the Japanese four-striped rat snake (Colubridae), the Burmese python (*Python molurus bivittatus*, Pythonidae) and the habu (*Trimeresurus flavoviridis*, Viperidae) to track the process of sex chromosome differentiation during the evolution of snakes. Firstly, the morphologies and G- and C-banded patterns of sex chromosomes were compared. Secondly, the cDNA clones localized to the sex chromosomes of the Japanese four-striped rat snake were comparatively mapped to the chromosomes of two other species. In addition, we cloned a novel sex chromosome-specific repetitive DNA sequence from the Japanese four-striped rat snake, which is also conserved in both the python and the habu, and used it as a cytogenetic marker for comparative mapping of sex chromosomes. We also cloned two sexual differentiation genes, *DMRT1* and *SOX9*, from the habu, and determined their chromosomal locations in the three

snake species to search for candidate genes of sex determination in snakes. Finally we discuss the origin and the process of differentiation of snake sex chromosomes.

Results and discussion

Cytogenetic map of the Japanese four-striped rat snake

Fifty-three EST clones and one cDNA clone of the *SOX9* gene (see "Chromosome mapping of *DMRT1* and *SOX9*" section) were newly mapped to *Elaphe quadrivirgata* (EQU) chromosomes by the direct R-banding FISH method (Fig. 1). The first preliminary cytogenetic map of this species was constructed with 52 EST clones and three cDNA clones isolated by RT-PCR in our previous study (4), and the second cytogenetic map constructed in this study consequently defines the locations of a total of 109 cDNA clones (Fig. 2 and Table 1). The 105 EST clones mapped on the snake chromosomes and their accession numbers and chromosomal locations in the snake, human (*Homo sapiens*: HSA) and chicken (*Gallus gallus*: GGA) are listed in Supplementary Table 1. The chromosome homologies were investigated among the three species, and the numbers of homologous chromosome segments were found to be 25 and 49 for chromosomes 1-7 and the Z chromosomes between the snake and chicken and between the snake and human, respectively. We previously constructed a cytogenetic map of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) with 92 cDNA clones (4, 20), which revealed that the chromosomes have been highly conserved between the chicken and the turtle, with the six largest chromosomes being almost equivalent to

each other. All the data collectively suggest that the number of chromosome rearrangements that occurred between the snake and chicken was much more than that between the turtle and chicken. The primitive reptiles diverged into two major lineages, Lepidosauria (lizards and snakes) and Archosauromorpha (turtles, crocodilians and birds), around 260 million years ago (21, 22). The large differences of chromosome numbers between the rat snake (2n=36) and chicken (2n=78) probably resulted from two independent events of chromosome rearrangements: the accumulation of fusions between macro- and microchromosomes in the lineage of snakes leading to the increase in chromosome size and the decrease of microchromosomes; and the fission of macrochromosomes that occurred in the lineage of birds, which caused the increase of macro- and microchromosomes. Several types of cytogenetic evidence of the fission and/or fusion events that occurred in the two lineages were found in this study. For instance, the large chromosome segments of the long arm of EQU2 corresponded to three chicken microchromosomes, GGA12, GGA13, and GGA18, and the long arm of EQU3 corresponded to GGA8, GGA20 and GGA26 (Fig. 2). In like manner, the chromosomal segments homologous to GGA19, GGA12 and GGA27 were localized to EQU1p, EQU6p and EQUZq, respectively. More comparative mapping data for the snake, chicken and other amniote species will be needed to decide between the alternatives.

Eleven of 105 EST clones were localized to the Z chromosome of the Japanese four-striped rat snake (Figs. 1a and 2). Three of the 11 genes were also mapped to the W chromosome, and eight other genes were localized only to the Z chromosome, indicating that certain homologous regions remain between the Z and W chromosomes. No human and chicken homologues of the 11

snake Z-linked genes were located on their sex chromosomes (Fig. 2). In humans, *GAD2*, *WAC* and *KLF6* were located on HSA10p. *LOC90693*, *TAX1BP1* and *AMPH* were localized to HSA7p, *CTNNB1* and *RAB5A* to HSA3p, and *TUBG1*, *GH1* and *MYST2* to HSA17q. In chicken, *GAD2*, *WAC*, *KLF6*, *AMPH*, *CTNNB1* and *RAB5A* were located on GGA2p, and *TUBG1* and *GH1* were located on a pair of microchromosomes, GGA27. On the other hand, the snake homologues of human X-linked genes, *EIF2S3*, *SYAP1* and *ATRX*, were localized to EQU4, EQU4 and a microchromosome, respectively, and the snake homologues of six chicken Z-linked genes, *ZFR*, *PHAX*, *C9orf72*, *UBQLN1*, *KIAA0368* and *TOPORS,* were all mapped to EQU2p. These results confirm our previous finding that the sex chromosomes of snakes, mammals and birds were derived from different autosomal pairs of the common ancestor and differentiated independently in each lineage.

Comparison of karyotypes among three snake species by chromosome banding

The G- and C-banded karyotypes of *Python molurus*, *Elaphe quadrivirgata* and *Trimeresurus flavoviridis* are shown in Figure 3. The snake karyotypes are highly conserved, and the most common diploid number is 2n = 36, consisting of eight pairs of macrochromosomes and 10 pairs of microchromosomes (3, 23, 24). The Z chromosomes were the fourth or fifth largest metacentric chromosomes for all three species, while the G-banded patterns were different among the species. The sex chromosomes of *P. molurus* were morphologically homomorphic, and the G-banded patterns of the Z and W chromosomes were the same (Fig. 3a). In *E. quadrivirgata*, the W

chromosome was submetacentric, and its size was about four-fifths that of the metacentric Z chromosome (Fig. 3c). The submetacentric W chromosome of *T. flavoviridis* was about two-thirds the size of the metacentric Z chromosome (Fig. 3e). In *P. molurus,* C-positive heterochromatin was localized to the telomeric and centromeric regions on both the Z and W chromosomes (Fig. 3b), and it was found that heterochromatization of the sex-specific W chromosome has not occurred. In contrast, the deletion of euchromatic regions and chromosomal heterochromatization is far advanced on the W chromosomes of *E. quadrivirgata* and *T. flavoviridis*. The short arms of the W chromosomes were found to be degenerated in the two species. A large amount of C-positive heterochromatin was distributed on the interstitial region of the long arm of the *E. quadrivirgata* W chromosome (Fig. 3d). In *T. flavoviridis*, a large amount of heterochromatin was distributed over the entire long arm and the centromeric region of the W chromosome (Fig. 3f).

Molecular cloning and characterization of sex chromosome-specific repetitive sequences

A sex chromosome-specific repetitive DNA sequence was isolated from *E. quadrivirgata*. The chromosomal distribution was examined for 16 clones isolated from the 1.3-kb DNA band of the *Bam*HI digest of *E. quadrivirgata* genomic DNA, and one clone containing sex chromosome-specific repetitive DNA sequence was identified. The *Bam*HI B4 fragment (Accession No. AB254800) was localized to the distal regions on the long arm of the Z chromosome and the short arm of the W chromosome (Fig. 4a). The size of the fragment was 1261 bp, and its G+C content was 40.0 %, indicating that it was AT-rich.

To examine the genomic organization of the sex chromosome-specific *Bam*HI repeated sequence, the genomic DNA digested with six restriction endonucleases was subjected to Southern blot hybridization with the *Bam*HI B4 fragment as probe (Fig. 4d). A weakly hybridized band corresponding to the monomer unit was observed at 1.3 kb in the *Bam*HI digest. Ladder bands, some of which did not correspond to the sizes of polymeric bands of the *Bam*HI repeated sequence element, were detected around 2.5 to 10 kb, and intense hybridization signals were observed at higher molecular weight than 10 kb. This result indicates that the *Bam*HI sites are conserved in the repetitive DNA sequences but are not frequent in the genome. Many intensely hybridized bands were detected around 1.5 - 23 kb in the *Msp*I-digest but not in the *Hpa*II digest. The restriction sites of *Hpa*II and *Msp*I are both 'CCGG', and *Hpa*II does not cleave when the second cytosine is methylated, whereas *Msp*I cleaves when the CG sequence is methylated. The difference in hybridization patterns between the *Msp*I and *Hpa*II digests suggests that the *Bam*HI repeated sequence undergoes extensive methylation in the genome.

The *Bam*HI repeated sequence was conserved in the genome of *P. molurus* and *T. flavoviridis*, and cross-hybridized to the chromosomes of the two species (Figs. 4b and c). The hybridization signals were localized to the distal regions of the short arms of the Z and W chromosomes in the two species. Thus, the nucleotide sequence and chromosomal location of the *Bam*HI repeated sequence is highly conserved in Henophidia and Caenophidia.

Chromosome mapping of DMRT1 *and* SOX9

DMRT1 and *SOX9* are highly conserved in vertebrates as sexual differentiation genes with important roles in testis differentiation (25-27). We molecularly cloned *DMRT1* (Accession No. AB254801) and *SOX9* (Accession No. AB254802) from the adult testis of *T. flavoviridis* by RT-PCR. The primer sets for the *DMRT1* and *SOX9* genes amplified 1168-bp and 1390-bp products, respectively, and their chromosomal locations of the *DMRT1* and *SOX9* genes were determined for the three species by FISH. In our previous study (4) *DMRT1* was mapped to the short arm of *E. quadrivirgata* chromosome 2, which was found here to be homologous to the chicken Z chromosome (Fig. 2). *DMRT1* was also localized to the short arm of chromosome 2 in both *T. flavoviridis* (Fig. 1e) and *P. molurus* (data not shown) in this study. *SOX9* is located on the long arm of chromosome 17 in humans, which contains a segment homologous to the snake Z chromosome (Fig. 2). However, *SOX9* was localized to the long arm of chromosome 2 in *T. flavoviridis* (Fig. 1f) and two other species (data not shown). These results suggest that *DMRT1* and *SOX9* are not the candidate genes of sex determination situated the furthest upstream in the sex differentiation pathway of snakes.

Comparative cytogenetic maps of sex chromosome-linked genes

The *E. quadrivirgata* cDNA clones of 11 Z-linked genes were successfully localized to the chromosomes of *P. molurus* and *T. flavoviridis* (Figs. 1c and d). Figure 5 shows the comparative cytogenetic maps of sex chromosomes in the three species. The order of the Z-linked genes was

identical among the three species except that the location of *AMPH* was different between *E. quadrivirgata* and the two other species. In *P. molurus* and *T. flavoviridis*, *MYST2*, *GH1* and *TUBG* were all localized to the short arm of the Z chromosome, and *AMPH* was localized to the long arm, whereas all four genes were located on the long arm of the *E. quadrivirgata* Z chromosome. These results suggest that the order of the four genes on the Z chromosome of the common ancestor has been conserved in *P. molurus* and *T. flavoviridis* and that a small pericentric inversion occurred in the region containing *AMPH* on the *E. quadrivirgata* Z chromosome.

All 11 cDNA clones were mapped to both the Z and W chromosomes in *P. molurus*, and the order of the genes was identical between the Z and W chromosome. In *E. quadrivirgata*, the hybridization signals on the W chromosome were observed for only three clones, *CTNNB1*, *RAB5A* and *WAC*, and the genes were localized to the proximal C-negative euchromatic region on the long arm (Fig. 3d). No cDNA clones were mapped to the W chromosome of *T. flavoviridis*. The chromosome segments that contained the W homologues of the Z-linked genes were probably deleted during the process of W chromosome differentiation in *E. quadrivirgata* and *T. flavoviridis*, and were subsequently heterochromatized with the amplification of the repetitive sequences. The other possibility is a decrease of the hybridization efficiency due to the divergence in nucleotide sequence between the Z- and W-linked genes by the cessation of meiotic recombination.

Evolution of sex chromosomes in snakes

Morphologically undifferentiated sex chromosomes have been described in several organisms,

such as the medaka fish (28-30) and papaya (31). The Y chromosome of the medaka is completely homologous to its counterpart except for a 250-kb male-specific chromosomal region containing the male-determining *DMY/DMRT1Yb* gene (28-30). The male-specific region of the papaya Y chromosome accounts for about 10 % of the chromosome (31). These instances lead us to suppose that the differentiated region between the Z and W chromosome of *P. molurus*, which possibly contains sex-determining gene(s), is too small to be detected by banding techniques and comparative FISH mapping. In *E. quadrivirgata* and *T. flavoviridis*, the short arm of the W chromosome is extensively degenerated, and almost no homology between the Z and W chromosomes remains except for the telomeric regions, where the *Bam*HI repeat element is localized. Homology to the Z chromosome is partially preserved in the region near the centromere on the long arm of the heterochromatic W chromosome in *E. quadrivirgata*, whereas no homology on the long arm was detected between Z and W chromosomes in *T. flavoviridis*. These results suggest that the differentiation of sex chromosomes was initiated from a distal region on the short arm of the proto-sex chromosome in the common ancestor through the occurrence of a sex differentiator on only one of an autosomal pair. The cessation of meiotic recombination due to chromosome rearrangements occurring in the sex-specific region is considered to favor the accumulation of gene mutations. This should lead to the partial deletion of euchromatic regions and heterochromatization with the accumulation of repetitive DNA sequences on the sex-specific chromosome, such as extended from the short arm to the long arm of the W chromosome in the *E. quadrivirgata* and *T. flavoviridis* lineages. After the divergence of the two lineages, the

degeneration might have become more advanced independently in the *T. flavoviridis* lineage.

Materials and methods

Animals

One adult female of the Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae) was captured in the field in Japan and used for chromosome banding, FISH mapping and Southern hybridization. The same individual was also used in our previous study (4). One adult female each of the Burmese python (*Python molurus bivittatus*, Pythonidae) and the habu (*Trimeresurus flavoviridis*, Viperidae), which were bred at the Japan Snake Institute, Japan, was used for chromosome banding and FISH. The original collection locality of the individual of *P. molurus bivittatus* is unknown. The individual of *T. flavoviridis* was originally captured in Tokunoshima Island in Japan. A testis of one male *T. flavoviridis* originally captured on Okinawa Island, Japan, was used for molecular cloning of the *DMRT1* and *SOX9* genes.

DNA probes

A large number of EST clones of *E. quadrivirgata* were obtained from the brain cDNA library in our previous study (4). We selected 53 additional EST clones of snake homologues of human genes with high E-value ($< 2e^{-35}$), and used them for chromosome mapping. The *T. flavoviridis* homologues of the *DMRT1* and *SOX9* genes were molecularly cloned as described in

our previous study (4). The primer sets for *DMRT1* were synthesized based on the sequence of *E. quadrivirgata* (Accession No. AB179698). The degenerate primer sets for *SOX9* were newly designed based on the conserved regions among *Eublepharis macularius*, *Calotes versicolor*, *Alligator mississippiensis* and *Gallus gallus* (Accession Nos. AF217252, AF061784, AF106572 and AB012236, respectively). The following primer pairs were used in the PCR reactions: Primers for *DMRT1*: F, 5'-AGT GAC GAG GTG GGC TGC TA-3'; R, 5'-ATC TTG ACT GCT GGG TGG TG-3'. Primers for *SOX9*: F, 5'-CCC AGC CNC ACN ATG TCG GA-3'; R, 5'-GTG AGC TGN GTG TAG ACN GG-3'. The PCR conditions were as follows: an initial denaturation at 94 ºC for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °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 3% agarose gels, and bands of the expected size were isolated and subcloned using a pGEM-T Easy Vector System (Promega). The nucleotide sequences of the cDNA fragments were determined using an ABI PRISM3100 DNA Analyzer (Applied Biosystems) after the sequencing reaction with dideoxy dye-labelled terminator using T7 and Sp6 primers according to the manufacturer's protocol (Applied Biosystems).

DNA extraction and cloning of repetitive DNA

Genomic DNA was extracted from liver tissue of the female *E. quadrivirgata*. The genomic DNA was digested with 18 restriction endonucleases, *Apa*I, *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Nsi*I, *Pvu*II, *Rsa*I, *Sac*I, *Sau*3AI, *Sma*I, *Taq*I, *Xba*I and *Xho*I, size

fractionated by electrophoresis on 1% and 3% agarose gels. The prominent DNA bands of repetitive sequences detected thereby were isolated from the gel, and the DNA fragments were eluted and subcloned into pBluescript II SK(+) (Stratagene), and then transferred into *Escherichia coli* TOP10 competent cells (Invitrogen). The nucleotide sequences of the clones that produced fluorescence hybridization signals were sequenced.

Southern blot hybridization

The genomic DNA of *E. quadrivirgata* was digested with six restriction endonucleases, *Bam*HI, *Bgl*I, *Dra*I, *Hae*III, *Hpa*II and *Msp*I. The DNAs were fractionated by electrophoresis on 1% agarose gel, and the DNA fragments were transferred onto a nylon membrane (Roche Diagnostics). The repeated sequence element of *E. quadrivirgata* was labeled with digoxigenin-dUTP using a PCR DIG Labeling Mix (Roche Diagnostics) and hybridized to the membrane in DIG Easy Hyb (Roche Diagnostics) overnight at 42°C. After hybridization, the membrane was washed sequentially at 42˚C in 2 X SSC with 0.1% SDS, 1 X SSC with 0.1% SDS, 0.5 X SSC with 0.1% SDS and 0.1 X SSC with 0.1% SDS for 15 min each, and was reacted with Anti-Digoxigenin-AP, Fab fragments (Roche Diagnostics). Then the membrane was reacted with CDP-Star (Roche Diagnostics) and exposed to BioMax MS Autoradiography Film (Kodak).

Chromosome preparation and FISH

Chromosome preparation and FISH were performed according to our previous studies (4, 32).

Chromosome preparations were made from blood lymphocytes and/or fibroblast cells taken from heart tissue. The cultured cells were treated with BrdU during late S phase for differential replication banding. R-banded chromosomes were obtained by exposure of chromosome slides to UV light after staining with Hoechst 33258. For G- and C-banding analyses, chromosome preparations were made from the cells cultured without BrdU-treatment. The G- and C-banded chromosomes were obtained with the GTG (G-bands by trypsin using Giemsa) method (33) and the CBG (C-bands by Barium hydroxide using Giemsa) method (34), respectively.

The probe DNAs were labeled by nick translation with biotin-16-dUTP (Roche Diagnostics). The hybridization was carried out at 37˚C for 1 or 2 days. The slides hybridized with genomic DNA clones were stained with fluoresceinated avidin (Roche Diagnostics), and then stained with 0.25 μg/ml propidium iodide. For cDNA mapping, the slides were reacted with goat anti-biotin antibody (Vector Laboratories), and then stained with fluoresceinated anti-goat IgG (Nordic Immunology). FISH images were observed under a Nikon fluorescence microscope using B-2A and UV-2A filter sets. Kodak Ektachrome ASA 100 films were used for microphotography.

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Figure legends

Fig. 1. FISH mapping of *RAB5A* (a, c and d), *DMRT1* (e) and *SOX9* (f) to snake chromosomes. Arrows indicate the hybridization signals. *RAB5A* is mapped on both the Z and W chromosomes of *Elaphe quadrivirgata* (a) and *Python molurus* (c), and only on the Z chromosome of *Trimeresurus flavoviridis* (d). Hoechst-stained G-banded pattern of the same metaphase as in (a) is shown in (b). *DMRT1* and *SOX9* are mapped on the short arm of chromosome 2 (e) and on the long arm of chromosome 2 (d), respectively, in *T. flavoviridis*. A scale bar indicates 10 μm.

Fig. 2. A comparative cytogenetic map of chromosomes 1-7 and the Z and W chromosomes of *Elaphe quadrivirgata*. For chromosome mapping of *CHD1, DMRT1, ACO1* and *SOX9,* cDNA fragments isolated by RT-PCR were used, and all other genes were mapped using EST clones. The chromosomal locations of the genes are shown to the right of *E. quadrivirgata* chromosomes. The ideogram of G-banded chromosomes constructed in our previous study (4) was used here. The genes mapped to microchromosomes of *E. quadrivirgata* and the chromosomal locations of their human and chicken homologues are given in Table 1. The human and chicken chromosome segments with homology to the snake chromosomes and their chromosome numbers are indicated to the left side of the snake chromosomes. Gene symbols are according to the human nomenclature.

Fig. 3. G-banded karyotypes and C-banded sex chromosomes of three snake species, *Python*

molurus (a and b), *Elaphe quadrivirgata* (c and d) and *Trimeresurus flavoviridis* (e and f). Macrochromosomes other than sex chromosomes are numbered according to size in each species.

Fig. 4. (a-c) Chromosomal localization of the *Bam*HI repeat sequence to chromosomes of *Elaphe quadrivirgata* (a), *Python molurus* (b) and *Trimeresurus flavoviridis* (c). Arrows indicate the hybridization signals. (d) Southern blot hybridization of *E. quadrivirgata* genomic DNA probed with the *Bam*HI H4 fragment. Each lane contained 5 μg of genomic DNA. A mixture of lambda DNA digested with *Hin*dIII and phiX174 phage DNA digested with *Hae*III was used as a molecular size marker.

Fig. 5. Comparative cytogenetic maps of sex chromosomes of *Python molurus*, *Elaphe quadrivirgata* and *Trimeresurus flavoviridis*. The ideograms of the Z and W chromosomes are made according to the G-banded patterns. The Z chromosome of *E. quadrivirgata* is depicted upside down to make the gene order on the Z chromosome correspond to those of the other two species. The locations of the 11 genes and the *Bam*HI repeat sequence are shown to the side of each chromosome.

Table 1. The list of the genes mapped to microchromosomes of *Elaphe quadrivirgata* and their chromosomal locations in human and chicken.

Gene symbol	Chromosome location		
	snake	human*	chicken†
NEF3	micro	8p	22
ASB6	micro	9q	-
RPL12	micro	9q	17
FLJ25530	micro	11q	-
HSPA8	micro	11q	24
GLCE	micro	15q	un
POLG	micro	15q	10
LOC283820	micro	16p	14
PARN	micro	16p	14
ATRX	micro	Xq	4

*The chromosomal locations were referred from the UniGene database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

† The chromosomal locations of chicken homologues were defined using the BLASTN program of Ensembl (http://www.ensembl.org/index.html) and/or the tblastx program of NCBI. un: the nucleotide sequence of the gene was annotated in the chicken genome sequence but its chromosomal location has not been yet identified. -: no significant homology was found.

Supplementary table 1. The list of cDNA clones including the clones previously mapped (Matsuda et al., 2005).

Gene symbol*	Insert size (kb)	Sequence length (bp)	E-value†	Accession no.	Chromosome location		
					Snake	Human‡	Chicken§
OMG	1.7	634	6e-56	BW999947	1p	17q11.2	19
XAB1	1.2	767	8e-67	AU312353	1p	2p23.3	3
MGC15407	1.5	716	2e-70	AU312344	1p	2p16.2	3
XPO1	2.0	775	1e-155	AU312325	1p	2p16	3
DEGS	1.4	694	1e-101	AU312341	1p	1q42.12	3
KIAA0007	2.3	418	8e-72	AU312332	1p	2p23.3	3
EPRS	1.8	749	1e-112	AU312324	1p	1q41-q42	3
ARID4B	1.6	690	3e-54	AU312346	1p	1q42.1-q43	un
QKI	1.8	772	1e-50	AU312356	1p	6q26-27	3
MDN1	2.4	679	2e-92	AU312339	1p	6q15	3
AFTIPHILIN	1.8	514	5e-70	AU312311	1p	2p15	3
SF3B1	1.2	651	1e-115	AU312337	1q	2q33.1	7
CACNB4	1.7	449	4e-39	BW999948	1q, 3cen	2q22-q23	7
ZFHX1B	1.8	588	1e-84	BW999949	1q	2q22	7
UMPS	2.4	566	1e-87	AU312331	1q	3q13	7
TCIRG1	3.2	552	5e-48	BW999950	1q	11q13.4-q13.5	5
TSG101	1.8	727	7e-60	AU312316	1q	11p15	5
M11S1	1.8	592	1e-53	AU312350	1q	11p13	un
GPHN	1.2	729	7e-82	AU312327	1q	14q23.3-q24.1	5
DNCH1	1.2	745	1e-85	AU312310	1q	14q32.3-qter	5
HSPCA	2.4	792	1e-97	BW999951	1q	14q32-q33	un
ISYNA1	1.8	731	1e-54	AU312338	1q	19p13.11	un
TUBGCP2	1.6	702	1e-101	AU312343	1q	10q26.3	6
ZFR	1.8	738	1e-100	AU312309	2p	5p13.2	Z
PHAX	2.2	760	4e-71	AU312322	2p	5q23.2	W
VPS13A	2.6	596	1e-71	BW999952	2p	9q21	un
UBQLN1	2.0	724	1e-91	BW999953	2p	9q21.2-q21.3	Z
C9orf72	3.0	727	1e-143	AU312326	2p	9p21.1	Z
KIAA0368	4.5	456	9e-72	BW999954	2p	9q31.3	Z
TOPORS	2.9	722	6e-50	BW999955	2p	9p21	Z
FAM48A	1.8	442	2e-45	BW999956	2cen	13q13.3	1
UNQ501	1.3	746	1e-56	AU312305	2cen	19p13.2	-
DCTN2	1.8	765	1e-117	AU312317	2q	12q13.2-q13.3	un
EXOC7	1.3	664	2e-104	BW999957	2q	17q25.1	18
DDX5	1.9	740	4e-122	BW999958	2q	17q21	18
CCNG1	3.0	734	5e-74	AU312308	2q	5q32-q34	13
CPEB4	1.8	650		AU312333	2q	5q21	13
FLJ22318	1.8	775	2e-75	AU312329	2q	5q35.3	-
DCTN4	2.3	671	1e-91	AU312349	2q	5q31-q32	13
C5orf14	1.8	730	2e-57	AU312304	2q	5q31.2	13
NOSIP	1.5	690	1e-60	AU312303	2q	19q13.33	-
IQSEC1	2.6	774	4e-71	BW999959	2q	3p25.2	-
RBM5	2.2	778	1e-65	BW999960	2q	3p21.3	12
ITPR1	1.9	336	2e-56	BW999961	2q	3p26-p25	-
ENPP2	3.3	744	3e-113	BW999962	3p	8q24.1	2
YWHAZ	1.4	595	6e-68	BW999963	3p	8q23.1	un
LRRCC1	1.6	620	4e-55	BW999964	3p	8q21.2	2
LYPLA1	3.5	769	1e-50	BW999965	3p	8q11.23	-
SS18	2.5	599	9e-92	AU312302	3p	18q11.2	2
MBP	2.1	757	1e-44	AU312318	3p	18q23	2
EPB41L3	1.5	758	1e-86	BW999966	3p	18p11.32	2
TUBB2A	3.2	670	2e-74	BW999967	3p	6p25	2
LRRC16	2.9	707	7e-107	BW999968	3p	6p22.2	2
SERPINB6	2.4	782	5e-64	BW999969	3p	6p25	2
BPHL	1.3	606	4e-84	BW999970	3p	6p25	2
KIF13A	1.8	741	3e-108	BW999971	3p	6p23	2

TPR	2.2	702	1e-88	BW999972	3q	1q25	8
AKR1A1	1.6	679	6e-63	BW999973	3q	1p33-p32	8
ZNF326	1.2	768	5e-94	BW999974	3q	1p22.2	8
YIPF1	1.6	679	2e-50	BW999975	3q	1p33-p32.1	8
BCAS2	1.3	839	3e-87	AU312354	3q	1p21-p13.3	26
KIAA1219	0.9	699	5e-77	BW999976	3q	20q11.23	20
STAU1	2.2	749	4e-99	BW999977	3q	20q13.1	20
RBM12	3.2	763	3e-77	BW999978	3q	20q11.21	20
TPT1	1.5	762	2e-84	BW999979	4p	13q12-q14	1
EIF2S3	1.8	735	e-157	AU312306	4p	Xp22.2-p22.1	1
SYAP1	1.5	761	1e-98	AU312328	4p	Xp22.22	1
DSCR3	1.8	590	2e-98	AU312319	4q	21q22.2	1
DCAMKL1	2.9	405	5e-38	BW999980	4q	13q13	1
ELMOD1	2.5	582	6e-65	BW999981	4q	11q22.3	1
BCCIP	1.8	601	5e-56	AU312307	5q	10q26.1	un
SH3MD1	2.3	764	4e-54	AU312347	5q	10q25.1	6
PPP1R7	1.5	778	5e-66	BW999982	5q	2q37.3	9
PDCD10	1.3	791	7e-62	AU312342	5q	3q26.2	9
TLOC1	1.8	615	7e-70	AU312335	5q	3q26.2-q27	9
UCHL1	0.7	723	2e-35	BW999983	6p	4p14	-
GNAI2	3.5	744	4e-101	BW999984	6p	3p21	12
P4HB	2.7	723	1e-65	BW999985	6p	17q25	3
FLJ12571	2.3	738	3e-66	AU312352	6q	7q34	1
RANGAP1	1.5	756	9e-70	AU312313	6q	22q13	1
LDHB	1.3	547	6e-82	BW999986	6q	12p12.2-p12.1	1
SEC3L1	2.0	711	1e-63	AU312345	7p	4q12	4
KIAA1109	2.0	782	1e-73	AU312348	7q	4q28.1	4
RAP1GDS1	1.3	702	1e-118	AU312351	7q	4q23-q25	4
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RPL12	0.8	757	2e-86	BW999988	micro	9q34	17
FLJ25530	1.1	786	5e-86	AU312336	micro	11q24.2	-
HSPA8	1.9	750	3e-99	BW999989	micro	11q24.1	24
GLCE	2.5	431	6e-87	AU312330	micro	15q22.31	un
POLG	2.3	740	1e-107	AU312315	micro	15q25	10
LOC283820	2.3	721	5e-93	AU312323	micro	16p13.12	14
PARN	2.4	742	1e-102	AU312312	micro	16p13	14
ATRX	3.3	654	9e-68	BW999990	micro	Xq13.1-q21.1	4
GAD2	3.8	708	7e-133	BW999991	Zp	10p11.23	2
WAC	1.3	727	2e-79	AU312355	Zp, Wq	10p12.1	2
KLF6	2.2	781	2e-56	BW999992	Zp	10p15	2
LOC90693	3.5	762	1e-47	BW999993	Zp	7p15.3	2
TAX1BP1	1.8	752	1e-66	AU312320	Zp	7p15	-
RAB5A	2.2	742	2e-87	BW999994	Zp, Wq	3p24-p22	2
CTNNB1	2.6	747	8e-133	BW999995	Zcen, Wcen	3p21	2
AMPH	2.6	776	2e-37	BW999996	Zcen	7p14-p13	2
TUBG1	0.9	669	8e-81	BW999997	Zq	17q21	27
GH1	0.9	768	6e-59	BW999998	Zq	17q24.2	27
MYST2	1.2	734	3e-77	BW999999	Zq	17q21.32	un

*Human gene symbol.

†The E-values versus human genes in the blastx probram of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) were represented.

‡The locations were referred from NCBI UniGene.

§ The locations of chicken homologues were defined using the BLASTN program of the Ensembl (http://www.ensembl.org/index.html) and/or the tblastx program of NCBI. un: The nucleotide sequence of the gene is annotated in the chicken genome sequence but its chromosomal location is not yet identified. -: no significant homology was found.

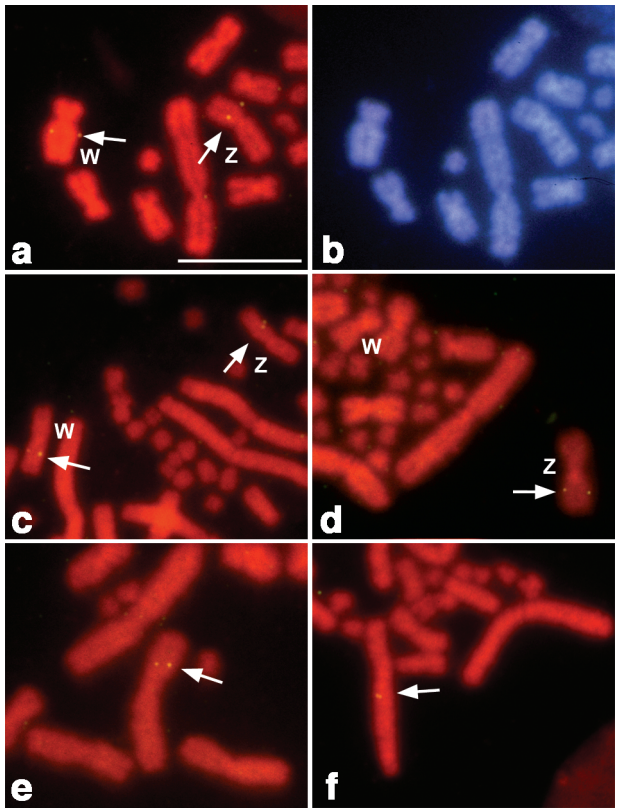

Figure 1

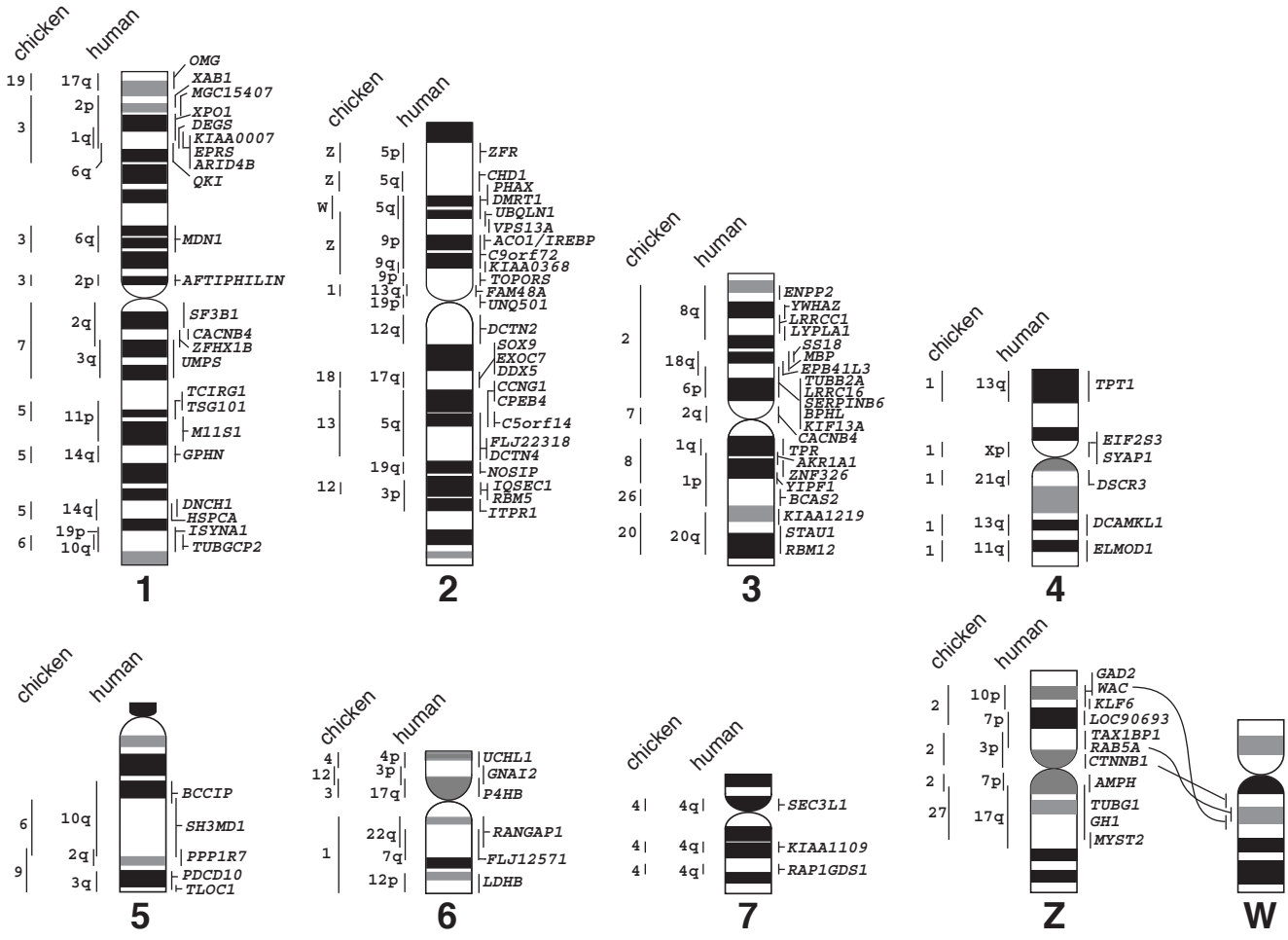
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Figure 2

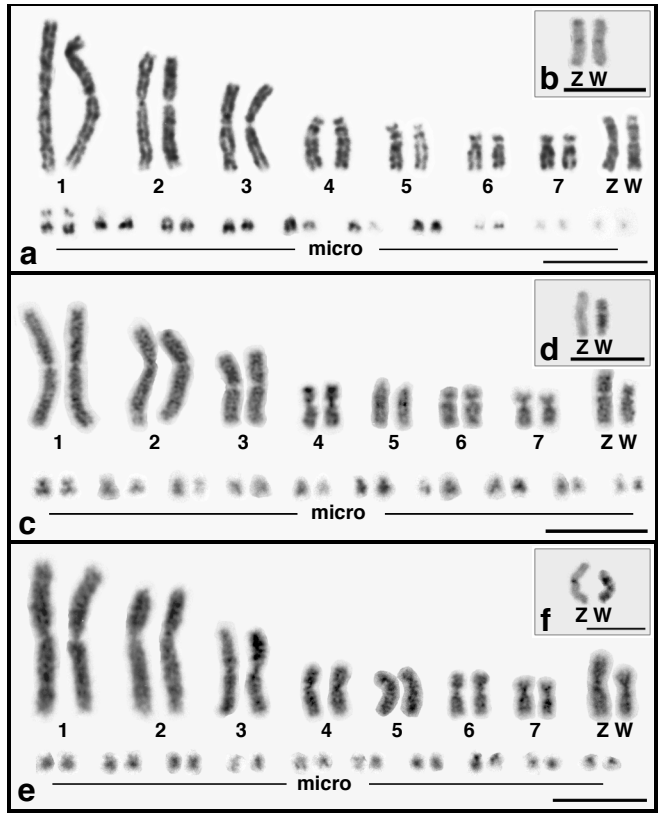
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Figure 3

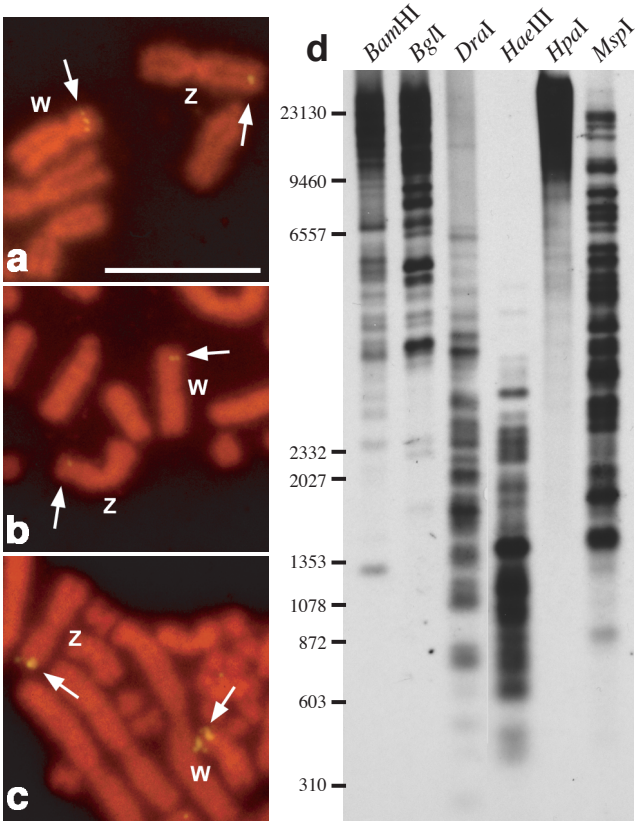
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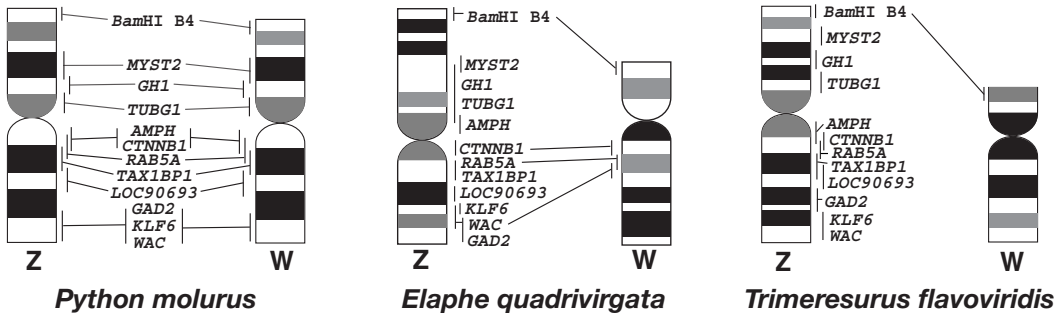
Figure 4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W
Trimeresurus flavoviridis

Figure 5