Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes

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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 3 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 protosex 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.

comparative map | chromosome homology | FISH | sex-determining gene | reptile

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 finding leads us to predict the presence of homology between ophidian and avian sex chromosomes. However, no attempts have yet been 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 another study (4), we constructed a preliminary cytogenetic map of the Japanese four-striped rat snake (*Elaphe quadrivirgata*) with 52 EST 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. Of 52 EST clones, two genes, *TAX1BP1* and *WAC*, whose human homologues are located on human chromosomes 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 first occurred on one of the homologues, and a partially heterozygous chromosomal region was consequently formed. Meiotic recombination between the protosex 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 because of sex-chromosome differentiation, *ATP5A1*, *CHD1*, *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

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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. First, the morphologies and G- and C-banded patterns of sex chromosomes were compared. Second, 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 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*, below) were newly mapped to the *E. quadrivirgata* (EQU) chromosomes by the direct R-banding FISH method (Fig. 1). A preliminary cytogenetic map of this species was constructed with 52 EST clones and 3 cDNA clones isolated by RT-PCR in our study (4), and the 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 Table 2, which is published as supporting information on the PNAS web site. 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 had 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 of 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), ≈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.

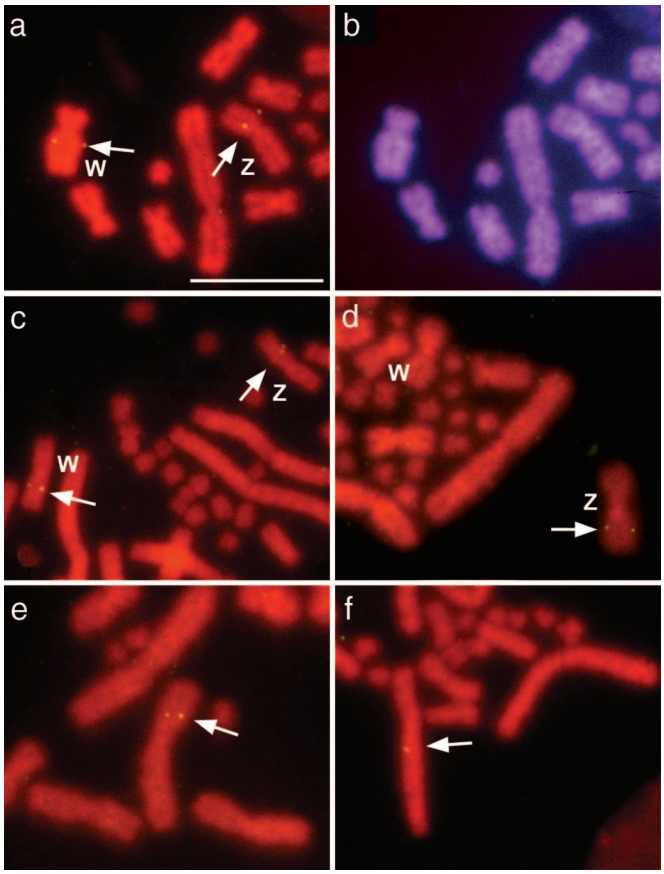

Fig. 1. FISH mapping of *RAB5A* (*a–d*), *DMRT1* (e), and *SOX9* (*f*) to snake chromosomes. Arrows indicate the hybridization signals. *RAB5A* is mapped on both the Z and W chromosomes of *E. quadrivirgata* (*a*) and *P. molurus* (*c*) and only on the Z chromosome of *T. flavoviridis* (*d*). Hoechst-stained 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 (*f*), respectively, in *T. flavoviridis*. (Scale bar, 10 μm.)

Eleven of 105 EST clones were localized to the Z chromosome of the Japanese four-striped rat snake (Figs. 1*a* and 2). Three of the 11 genes were also mapped to the W chromosome, and 8 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

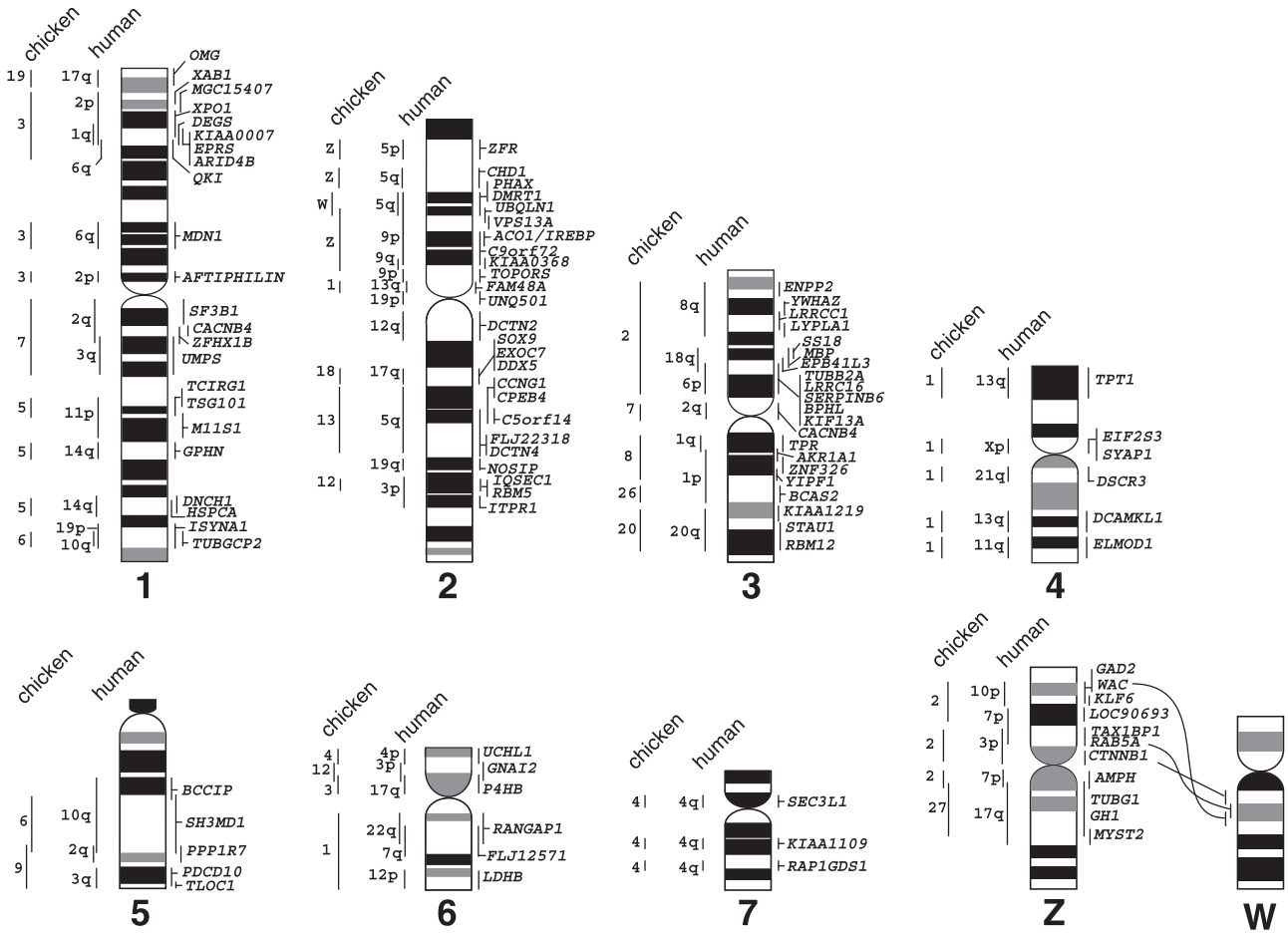

Fig. 2. A comparative cytogenetic map of chromosomes 1–7 and the Z and W chromosomes of *E. quadrivirgata*. For chromosome mapping of *CHD1*, *DMRT1*, *ACO1*, and *SOX9*, cDNA fragments isolated by RT-PCR were used, and all other genes were mapped by using EST clones. The chromosomal locations of the 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 of the snake chromosomes. Gene symbols are according to the human nomenclature.

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 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 *P. molurus*, *E. quadrivirgata*, and *T. flavoviridis* are shown in Fig. 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, whereas 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. 3*a*). In *E. quadrivirgata*, the W chromosome was submetacentric, and its size was ≈4/5 that of the metacentric Z chromosome (Fig. 3*c*). The submetacentric W chromosome of *T. flavoviridis* was ≈2/3 the size of the metacentric Z chromosome (Fig. 3*e*). In *P. molurus*, C-positive heterochromatin was localized to the telomeric and centromeric regions on both the Z and W chromosomes (Fig. 3*b*), 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. 3*d*). In *T. flavoviridis*, a large amount of heterochromatin was distributed over the entire long arm and the centromeric region of the W chromosome (Fig. 3*f*).

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 BamHI digest of *E. quadrivirgata* genomic DNA, and one clone containing sex chromosome-specific repetitive DNA sequence was identified. The BamHI B4 fragment (accession no. AB254800) was localized to the distal regions on

Table 1. The list of the genes mapped to microchromosomes of *E. 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

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.

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

†The chromosomal locations of chicken homologues were defined by using the BLASTN program of Ensembl (www.ensembl.org/index.html) and/or the tblastx program of NCBI.

the long arm of the Z chromosome and the short arm of the W chromosome (Fig. 4*a*). The size of the fragment was 1,261 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 BamHI repeated sequence, the genomic DNA digested with six restriction endonucleases was subjected to Southern blot hybridization with the BamHI B4 fragment as probe (Fig. 4*d*). A weakly hybridized band corresponding to the monomer unit was observed at 1.3 kb in the BamHI digest. Ladder bands, some of which did not correspond to the sizes of polymeric bands of the

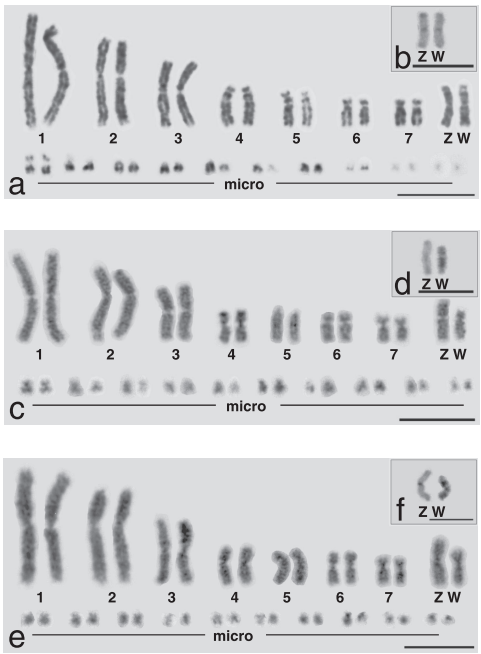

Fig. 3. G-banded karyotypes and C-banded sex chromosomes of three snake species, *P. molurus* (*a* and *b*), *E. quadrivirgata* (*c* and *d*), and *T. flavoviridis* (*e* and *f*). Macrochromosomes other than sex chromosomes are numbered according to size in each species.

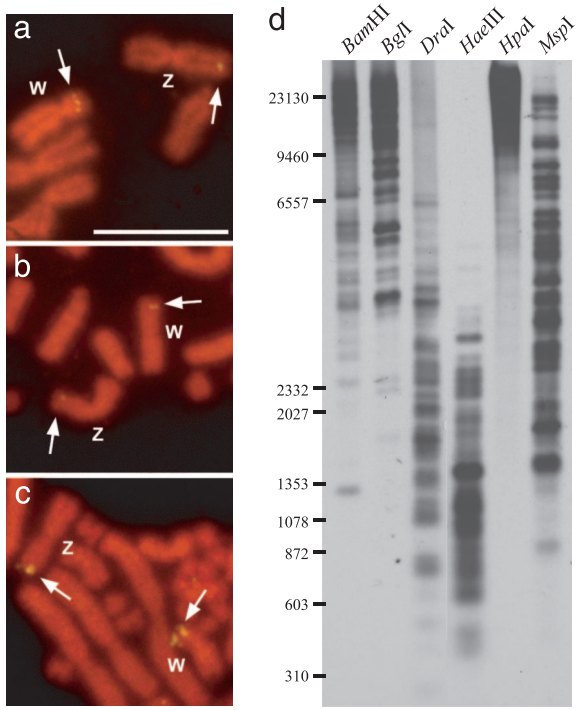

Fig. 4. Cytogenetic and molecular characterization of a sex chromosome-specific repetitive sequence. (*a–c*) Chromosomal localization of the BamHI repeat sequence to chromosomes of *E. quadrivirgata* (*a*), *P. molurus* (*b*), and *T. flavoviridis* (*c*). Arrows indicate the hybridization signals. (*d*) Southern blot hybridization of *E. quadrivirgata* genomic DNA probed with the BamHI H4 fragment. Each lane contained 5 μg of genomic DNA. A mixture of lambda DNA digested with HindIII and phiX174 phage DNA digested with HaeIII was used as a molecular size marker.

BamHI repeated sequence element, were detected ≈2.5–10 kb, and intense hybridization signals were observed at higher molecular weight than 10 kb. This result indicates that the BamHI sites are conserved in the repetitive DNA sequences but are not frequent in the genome. Many intensely hybridized bands were detected ≈1.5–23 kb in the MspI digest but not in the HpaII digest. The restriction sites of HpaII and MspI are both "CCGG", and HpaII does not cleave when the second cytosine is methylated, whereas MspI cleaves when the CG sequence is methylated. The difference in hybridization patterns between the MspI and HpaII digests suggests that the BamHI repeated sequence undergoes extensive methylation in the genome.

The BamHI repeated sequence was conserved in the genome of *P. molurus* and *T. flavoviridis* and cross-hybridized to the chromosomes of the two species (Fig. 4 *b* 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 BamHI 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 1,168-bp and 1,390-bp products, respectively, and their chromosomal locations of the *DMRT1* and *SOX9* genes were determined for the three species by FISH. In our study (4), *DMRT1* was mapped to the short arm of *E. quadrivirgata*

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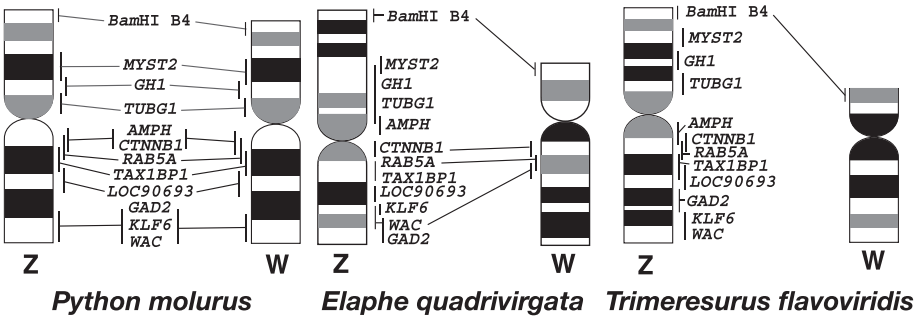

Fig. 5. Comparative cytogenetic maps of sex chromosomes of *P. molurus*, *E. quadrivirgata*, and *T. flavoviridis*. The ideograms of the Z and W chromosomes are made according to the G-banded patterns. The Z chromosome of *E. quadrivirgata* is depicted upside down to make the gene order on the Z chromosome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

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. 1*e*) 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. 1*f*) 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* (Fig. 1 *c* and *d*). Fig. 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 *TUBG1* 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. 3*d*). 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 ≈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 BamHI 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 the 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 protosex chromosome in the common ancestor through the occurrence of a sex differentiator on only one of an autosomal pair. The cessation of meiotic recombination because of chromosome rearrangements occurring in the sex-specific region is considered to favor the accumulation of gene mutations. This accumulation 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 (*E. quadrivirgata*, Colubridae) was captured in the field in Japan and used for chromosome banding, FISH mapping, and Southern blot hybridization. The same individual was also used in our previous study (4). One adult female each of the Burmese python(*P. molurus bivittatus*, Pythonidae) and the habu (*T. flavoviridis*, Viperidae), which were bred at the Japan Snake Institute, 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 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 (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 *G. gallus* (accession nos. AF217252, AF061784, AF106572, and AB012236, respectively). The following primer pairs were used in the PCRs: Primers for *DMRT1*: forward, 5′-AGT GAC GAG GTG GGC TGC TA-3′; reverse, 5′-ATC TTG ACT GCT GGG TGG TG-3′. Primers for *SOX9*: forward, 5′-CCC AGC CNC ACN ATG TCG GA-3′; reverse, 5′-GTG AGC TGN GTG TAG ACN GG-3′. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 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 by using a pGEM-T Easy Vector System (Promega, Madison, WI). The nucleotide sequences of the cDNA fragments were determined by using an ABI PRISM3100 DNA Analyzer (Applied Biosystems, Foster City, CA) after the sequencing reaction with dideoxy dye-labeled 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, ApaI, BamHI, BglI, BglII, EcoRI, EcoRV, HaeIII, HindIII, HinfI, NsiI, PvuII, RsaI, SacI, Sau3AI, SmaI, TaqI, XbaI, and XhoI, 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, La Jolla, CA) and then transferred into *Escherichia coli* TOP10 competent cells (Invitrogen, Carlsbad, CA). 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, BamHI, BglI, DraI, HaeIII, HpaII, and MspI. The DNAs were fractionated by electrophoresis on 1% agarose gel, and the DNA fragments were transferred onto a nylon membrane (Roche Diagnostics, Basel, Switzerland). The repeated sequence element of *E. quadrivirgata* was labeled with digoxigenin-dUTP by 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× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, 0.5× SSC with 0.1% SDS, and 0.1× 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, Rochester, NY).

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, Burlingame, CA) and then stained with fluoresceinated anti-goat IgG (Nordic Immunology, Tilburg, The Netherlands). FISH images were observed under a fluorescence microscope (Nikon, Tokyo, Japan) using B-2A and UV-2A filter sets. Kodak Ektachrome ASA 100 films were used for microphotography.

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