

# The Y chromosome of the Okinawa spiny rat, *Tokudaia muenninki*, was rescued through fusion with an autosome

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**Abstract** The genus *Tokudaia* comprises three species, two of which have lost their Y chromosome and have an XO/XO sex chromosome constitution. Although *Tokudaia muenninki* (Okinawa spiny rat) retains the Y chromosome, both sex chromosomes are unusually large. We

conducted a molecular cytogenetic analysis to characterize the sex chromosomes of *T. muenninki*. Using cross-species fluorescence in situ hybridization (Zoo-FISH), we found that both short arms of the *T. muenninki* sex chromosomes were painted by probes from mouse chromosomes 11 and 16. Comparative genomic hybridization analysis was unable to detect sex-specific regions in the sex chromosomes because both sex probes highlighted the large heterochromatic blocks on the Y chromosome as well as five autosomal pairs. We then performed comparative FISH mapping using 29 mouse complementary DNA (cDNA) clones of the 22 X-linked genes and the seven genes linked to mouse chromosome 11 (whose homologue had fused to the sex chromosomes), and FISH mapping using two *T. muenninki* cDNA clones of the Y-linked genes. This analysis revealed that the ancestral gene order on the long arm of the X chromosome and the centromeric region of the short arm of the Y chromosome were conserved. Whereas six of the mouse chromosome 11 genes were also mapped to Xp and Yp, in addition, one gene, *CBX2*, was also mapped to Xp, Yp, and chromosome 14 in *T. muenninki*. *CBX2* is the candidate gene for the novel sex determination system in the two other species of *Tokudaia*, which lack a Y chromosome and *SRY* gene. Overall, these results indicated that the Y chromosome of *T. muenninki* avoided a loss event, which occurred in an ancestral lineage of *T. osimensis* and *T. tokunoshimensis*, through fusion with an autosome. Despite retaining the Y chromosome, sex determination

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in *T. muenninki* might not follow the usual mammalian pattern and deserves further investigation.

**Keywords** X chromosome · evolution · Zoo-FISH · FISH · *CBX2* · Spiny rat · *Tokudaia*

### Abbreviations

ASA	American Standards Association
BSA	Bovine serum albumin
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
dUTP	Deoxyuridine 5-triphosphate
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
HMG	High-mobility group
IHB	Intercalary heterochromatin block
kb	Kilo base pairs
MMU	<i>Mus musculus</i> chromosome
MYA	Million years ago
ORF	Open reading frame
PAR	Pseudoautosomal region
qRT-PCR	Quantitative real-time PCR
<i>RBMY1A1</i>	RNA binding motif protein, Y chromosome, family 1, member A1
SSC	Saline sodium citrate
TMU	<i>Tokudaia muenninki</i> chromosome
TTO	<i>Tokudaia tokunoshimensis</i> chromosome
UV	Ultraviolet
Zoo-FISH	Cross-species fluorescence in situ hybridization

### Introduction

The genus *Tokudaia* belongs to the subfamily Murinae (Muridae, Rodentia) and consists of three species: *Tokudaia muenninki* (Okinawa spiny rat), *Tokudaia osimensis* (Amami spiny rat), and *Tokudaia tokunoshimensis* (Tokunoshima spiny rat). Each species is endemic to an island in the southernmost part of Japan and is named after the island on which it is found. The sex chromosomes in this genus are unusual among mammals. The diploid chromosome numbers of *T. osimensis* and *T. tokunoshimensis* are an odd number, 25 and 45, respectively. Neither *T. osimensis* nor *T. tokunoshimensis* has a Y chromosome and sex is determined with an XO/XO sex chromosome constitution in both species (Honda

et al. 1977, 1978; Kobayashi et al. 2007). Although the diploid chromosome number of *T. muenninki* is 44 with XX/XY sex chromosome constitution, both sex chromosomes are unusually large (Tsuchiya et al. 1989; Murata et al. 2010). The euchromatic regions of the X and Y chromosomes occupy 8% and 4% of the haploid genome, respectively, which suggests that an autosomal addition has occurred (Murata et al. 2010). On the basis of molecular phylogenetic analysis, *T. muenninki* was the first species in the genus to diverge, which suggests that the Y chromosome was lost in the common ancestor of *T. osimensis* and *T. tokunoshimensis* after this event (Murata et al. 2010). Three stepwise events that led to the disappearance of the Y chromosome have been suggested; gene transpositions from the Y chromosome to autosomes, superseding Y-linked genes by new genes on the X chromosome or autosomes, and Y-to-X translocation containing Y-linked genes (Arakawa et al. 2002; Kuroiwa et al. 2010).

The *SRY* gene, which is the master gene for male sex determination in placental mammals (Sinclair et al. 1990; Koopman et al. 1991) and is located on the Y chromosome, has been lost completely in *T. osimensis* and *T. tokunoshimensis* (Soullier et al. 1998; Sutou et al. 2001). Other Y chromosome genes have also been lost in *T. osimensis*, including *RBMY1A1* (RNA binding motif protein, Y chromosome, family 1, member A1) (Kuroiwa et al. 2010), which plays a role in spermatogenesis (Mazeyrat et al. 1999; Elliott 2004). However, most proto-Y-linked genes have been conserved in both sexes of *T. osimensis* and *T. tokunoshimensis* through translocation from the Y chromosome to the distal region of the long arm of the X chromosome. The absence of *SRY* indicates that *T. osimensis* and *T. tokunoshimensis* must have a novel sex-determining mechanism. Recently, *CBX2* (chromobox homolog 2, synonym is *M33*) has emerged as a candidate gene for sex determination in these species (Kuroiwa et al. 2011). *CBX2* is a single copy gene that is located on an autosome in mice and humans. However, quantification of the copy number of *CBX2* in *T. osimensis* and *T. tokunoshimensis* by quantitative real-time PCR showed that there were two or three more copies of *CBX2* per haploid genome in males than in females, which suggests that *CBX2* might be involved in a novel sex-determining mechanism through gene dosage (Kuroiwa et al. 2011).

Although *T. muenninki* possesses a Y chromosome, several aspects of sex determination in this species are

unusual (Tsuchiya et al. 1989; Murata et al. 2010). Multiple copies of the *SRY* gene (>70) are found in *T. muenninki*, and these are distributed along the entire long arm of the Y chromosome (Murata et al. 2010). Most copies are not functional; however, an open reading frame (ORF) is conserved in three copies (Murata et al. 2010). A single amino acid substitution that is specific to *T. muenninki* was detected in the DNA binding surface domain in the high-mobility group (HMG)-box of all *SRY* copies (Murata et al. 2010). This region is important for the binding of *SRY* to target DNA sequences, which suggests that the DNA-binding ability of all *SRY* proteins produced in *T. muenninki* is weakened by this mutation.

To reveal the composition of the X and Y chromosomes in *T. muenninki*, we performed cross-species fluorescence in situ hybridization (Zoo-FISH) with chromosome-specific DNA probes constructed from the laboratory mouse (*Mus musculus*). To describe the composition in more detail and identify sex-specific regions in the sex chromosomes, we performed comparative genomic hybridization (CGH) analysis. When this provided limited information, we then conducted comparative FISH analysis using 29 mouse cDNA clones of functional genes linked to the X chromosome and chromosome 11 and FISH mapping using two *T. muenninki* cDNA clones of the Y-linked genes.

## Materials and methods

### Sample collection, cell culture, and DNA and RNA extraction

The three species of *Tokudaia* are all endangered (The IUCN Red List of Threatened Species; <http://www.iucnredlist.org/> 15/9/2011) and have been protected by the Japanese government as natural treasures since 1972. With permission from the Agency for Cultural Affairs and the Ministry of the Environment in Japan, we trapped the Okinawa spiny rat, *T. muenninki*, on Okinawa-jima Island in March 2008 and February 2009 (Yamada et al. 2010). We collected tail tissue from four males and three females for use in the study. Cell culture and DNA extraction were performed as described by Murata et al. (2010). Total RNA was extracted from fibroblasts derived

from tail tissue from a *T. muenninki* male using TRIZOL Reagent (Invitrogen) in accordance with the manufacturer's protocol. The cDNAs were synthesized using SuperScript II Reverse Transcriptase (Invitrogen).

### DNA probes

Seven mouse cDNA clones of functional genes linked to chromosome 11 and 22 linked to the X chromosome were used in the study. Their gene names, symbols, accession numbers, sizes, and chromosomal location in mouse (*M. musculus*), rat (*Rattus norvegicus*), and three *Tokudaia* species are listed in Table 1. A 6.6-kilo base pairs (kb) mouse genomic DNA fragment was used for chromosomal localization of the 18S–28S rRNA gene (Kominami et al. 1982).

Clones of *T. muenninki* *DDX3Y* (AB672502; 1.8 kb) and *UTY* (AB672501; 2.0 kb), which are both linked to the Y chromosome, were also used for FISH mapping. The mouse homologues referred to here are *Ddx3y* (NM\_012008) and *Uty* (NM\_009484), and the rat genes are *DDX3Y* (FJ775727) and *UTY* (FJ775728). The *T. muenninki* homologues of these genes were cloned by degenerate PCR. The primer pairs used in the PCR were as follows: *DDX3Y*, 5'-TGA TCG TGG AAG TGG ATC C-3' and 5'-CAC AGC TGG TAA TGT ACT GCA-3'; *UTY*, 5'-GAC GCT GTT GAA CAA GGC AA-3' and 5'-GGA GTG GGT GGA TTC AAC TTC-3'. The PCR for *DDX3Y* was carried out in 10 µl of 1× Ex *Taq* buffer that contained 0.5 µl of template cDNA, 2 mM MgCl<sub>2</sub>, 0.5 µM of each primer, 0.2 mM each of the four dNTPs, and 0.25 U of Ex *Taq* (TaKaRa Bio Inc.). The reaction profile was denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60 °C, and extension for 2 min at 72°C, and then a final extension at 72°C for 7 min. The PCR for *UTY* was carried out in 20 µl of 1× PrimeSTAR GXL buffer (Mg<sup>2+</sup> plus) that contained 1.0 µl of template cDNA, 0.3 µM of each primer, 0.2 mM each of the four dNTPs, and 0.25 U of PrimeSTAR GXL DNA Polymerase (TaKaRa Bio Inc.). The reaction profile was denaturation for 3 min at 94°C, followed by 35 cycles of denaturation for 10 s at 98°C, annealing for 15 sec at 60°C, and extension for 1.5 min at 68°C, and then a final extension at 72°C for 5 min.

**Table 1** The list of mouse cDNA clones used for FISH mapping and their chromosomal locations

Gene symbol	Gene name	cDNA clone Size (kb)	Chromosome location				
			Mouse <sup>a</sup>	Rat <sup>a</sup>	<i>T. muenninki</i> <sup>d</sup>	<i>T. osimensis</i> <sup>e</sup>	<i>T. tokunoshimensis</i> <sup>e</sup>
STK10	Serin/threonine kinase 10	3.1	11 A4 <sup>b</sup>	10q 12.3	Xp, Yp	–	–
ADAM19	A disintegrin and metalloproteinase domain 19 (meltrin beta)	1.4	11 A5-B1.1 <sup>c</sup>	10q 21.3-q 22	Xp, Yp	–	–
DOC2 β	Double C2 beta	–	11 B3-B5	10q	Xp, Yp	–	–
COASY	Coenzyme A synthase	2.0	11 D	10q 32.1	Xp, Yp	–	–
GAA	Glucosidase, alpha, acid	2.0	11 D-E	10	Xp, Yp	–	–
CBX2	Chromobox homolog 2 ( <i>Drosophila</i> Pc class)	1.5	11 E2	10q 32.3	Xp, Yp, 14	3q 24, 6p 11.2 <sup>f</sup>	10q 25-26, 14q 12-13.1 <sup>f</sup>
ARHGDI A	Rho GDP dissociation inhibitor (GDI) alpha	–	11 E2	10q 32.3	Xp, Yp	–	–
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	1.2	X A2-A3.1	Xq 11.1	Xq	Xp 11.3	Xq 11
ELK1	ELK1, member of ETS oncogene family	1.2	X A2-A3.1	Xq 11.1	Xq	Xp 11.3	Xq 11
PDHA1	Pyruvate dehydrogenase E1 alpha 1	0.9	X A4	Xq 11.1	Xq	Xp 11.3	Xq 11
TSPAN7	tetraspanin 7	2.0	X A1.3-A2	Xq 11.2-12	Xq	Xp 11.2-11.3	Xq 11-12
MAOA	Monoamine oxidase A	1.4	X A2	Xq 12	Xq	Xp 11.12-11.2	Xq 12
PLP2	Proteolipid protein 2	1.2	X A2-A3.1	Xq 12	Xq	Xp 11.12-11.2	Xq 12
GATA1	GATA binding protein 1	1.0	X A2	Xq 12	Xq	Xp 11.12-11.2	Xq 12
PIGA	Phosphatidylinositol glycan anchor biosynthesis, class A	1.1	X F5	Xq 12-13	Xq	Xp 11.12	Xq 12
SAT1	Spermidine/spermine N1-acetyl transferase 1	1.1	X F3-F4	Xq 13-14	Xq	Xp 11.11-11.12	Xq 12-13.1
PHKA2	Phosphorylase kinase alpha 2	2.3	X F3-F4	Xq 13-14	Xq	Xp 11.11-11.12	Xq 12-13.1
DXHXS423	DNA segment, Chr X, human DXS423	0.8	X C3	Xq 21.3	Xq	Xq 11	Xq 13.3
EFNB1	Ephrin B1	1.0	X D	Xq 22.1	Xq	Xq 12	Xq 21
PGK1	Phosphoglycerate kinase 1	1.5	X D	Xq 22.3-31.1	Xq	Xq 12-21	Xq 21-22
PLP1	Proteolipid protein (myelin) 1	1.5	X F1-F2	Xq 32	Xq	Xq 21-22	Xq 22-23
HTR2C	5-Hydroxytryptamine (serotonin) receptor 2 C	1.4	X F1	Xq 34-35.1	Xq	Xq 23.1	Xq 24.1
SLC25A5	Solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5	1.4	X A4	Xq 36	Xq	Xq 23.1	Xq 24.1
FHL1	Four and half LIM domains 1	2.0	X A6-A7.1	Xq 36	Xq	Xq 23.2	Xq 24.2
IDH3G	Isocitrate dehydrogenase 3 (NAD+) gamma	1.3	X A7.3-B	Xq 37.1-37.2	Xq	Xq 23.3	Xq 24.3
PLXNA3	Plexin A3	1.3	X B-C1	Xq 37.1-37.2	Xq	Xq 23.3	Xq 24.3
CETN2	Centrin 2	0.8	X B	Xq 37.1-37.2	Xq	Xq 23.3	Xq 24.3
GDI1	Guanosine diphosphate (GDP) dissociation inhibitor 1	1.1	X B-C1	Xq 37.1-37.2	Xq	Xq 23.3	Xq 24.3
BGN	biglycan	1.7	X B	Xq 37.2	Xq	Xq 23.3	Xq 24.3

<sup>a</sup>Kuroiwa et al. (1998)<sup>b</sup>Kuramochi et al. (1999)<sup>c</sup>Kurohara et al. (2000)<sup>d</sup>Present study<sup>e</sup>Kobayashi et al. (2008)<sup>f</sup>Kuroiwa et al. (2011)

## Chromosome preparation and Zoo-FISH analysis

The preparation of R-banded chromosomes was performed as described by Matsuda et al. (1992a), Matsuda and Chapman (1995), and Kobayashi et al. (2008).

The Zoo-FISH analysis was performed using biotin- and Cy3-labeled chromosome-specific painting probes from the laboratory mouse (Cambio Ltd.). FISH was carried out as described by Nakamura et al. (2007) with slight modifications. The probes were denatured at 75°C for 10 min and pre-annealed by incubation at 37°C for 1 h. The chromosome slides were hybridized with the biotin- or Cy3-labeled probes at 37°C for 5 days. After hybridization with the biotin-labeled probes, the slides were washed in 50% formamide in 2× saline sodium citrate (SSC) at 37°C for 15 min, in 2× SSC for 15 min, and then in 4× SSC for 5 min at room temperature. To detect the hybridization signals of the biotin-labeled probes, the chromosome slides were incubated with avidin fluorescein isothiocyanate (FITC) (Vector Laboratories) or streptavidin-Cy5 (GE Healthcare UK Ltd.) diluted 1:500 in 1% BSA/4× SSC at 37°C for 1 h. The slides were washed sequentially on a shaker with 4× SSC, 0.1% Nonidet P-40/4× SSC, and then 4× SSC for 5 min each at room temperature. After hybridization with the Cy3-labeled probe, the slides were washed sequentially in 4× SSC, 0.1% Nonidet P-40/4× SSC, and then 4× SSC for 5 min each at room temperature.

## CGH analysis

The CGH analysis was conducted using metaphase spreads from one male and one female of *T. muenninki* as described by Kobayashi et al. (2007) with slight modifications. The genomic DNA for the probes was also extracted from one male and one female. The male and female genomic DNA probes were labeled differently, with Cy3-deoxyuridine 5-triphosphate (dUTP; GE Healthcare) and fluorescein-12-dUTP (Invitrogen), respectively, by nick translation at 15°C for 90 min. The labeled DNA probes were denatured at 75°C in formamide and dissolved in hybridization buffer (50% formamide, 2× SSC, 10% dextran sulfate, and 2 mg/ml BSA). The cocktail of probes for one slide contained 700 ng of labeled DNA from the female and 700 ng of labeled DNA from the male. Slides were hybridized at 37°C for 4 days. The slides were then washed sequentially in 4× SSC, 0.1%

Nonidet P-40 in 4× SSC, 4× SSC, and 2× SSC for 5 min each at room temperature, and then rinsed in 2× SSC.

## FISH analysis

The FISH analysis using cDNA clones as probes was performed as described by Kuroiwa et al. (2010). The exception was the FISH analysis using the 18S–28S rRNA gene clone as a probe. In this case, the chromosome slides were treated with RNase (1 µg/µl in 2× SSC) for 1 h, washed three times in 2× SSC for 3 min each, and dehydrated in 70% and 100% ethanol at 4°C for 5 min each before the chromosomes were denatured in 70% formamide in 2× SSC at 69°C for 2 min. After hybridization and washing, the slides were incubated under parafilm for 1 h at 37°C with fluoresceinated avidin (avidin-FITC; Vector Laboratories) diluted 1:500 in 1% bovine serum albumin (BSA)/4× SSC [instead of goat anti-biotin antibody (Vector Laboratories)]. The slides were washed sequentially on a shaker with 4× SSC, 0.1% Nonidet P-40 in 4× SSC, and 4× SSC for 10 min each, and then rinsed with 2× SSC and stained with 0.75 mg/ml propidium iodide for 3 min.

## Microphotography and image capture

The chromosomal slides were observed under a Nikon fluorescence microscope using Nikon filter sets B-2A (450–490 nm) and ultraviolet (UV)-2A (330–380 nm). Kodak Ektachrome American Standards Association (ASA) 100 films were used for microphotography. The digital FISH images were captured using the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope.

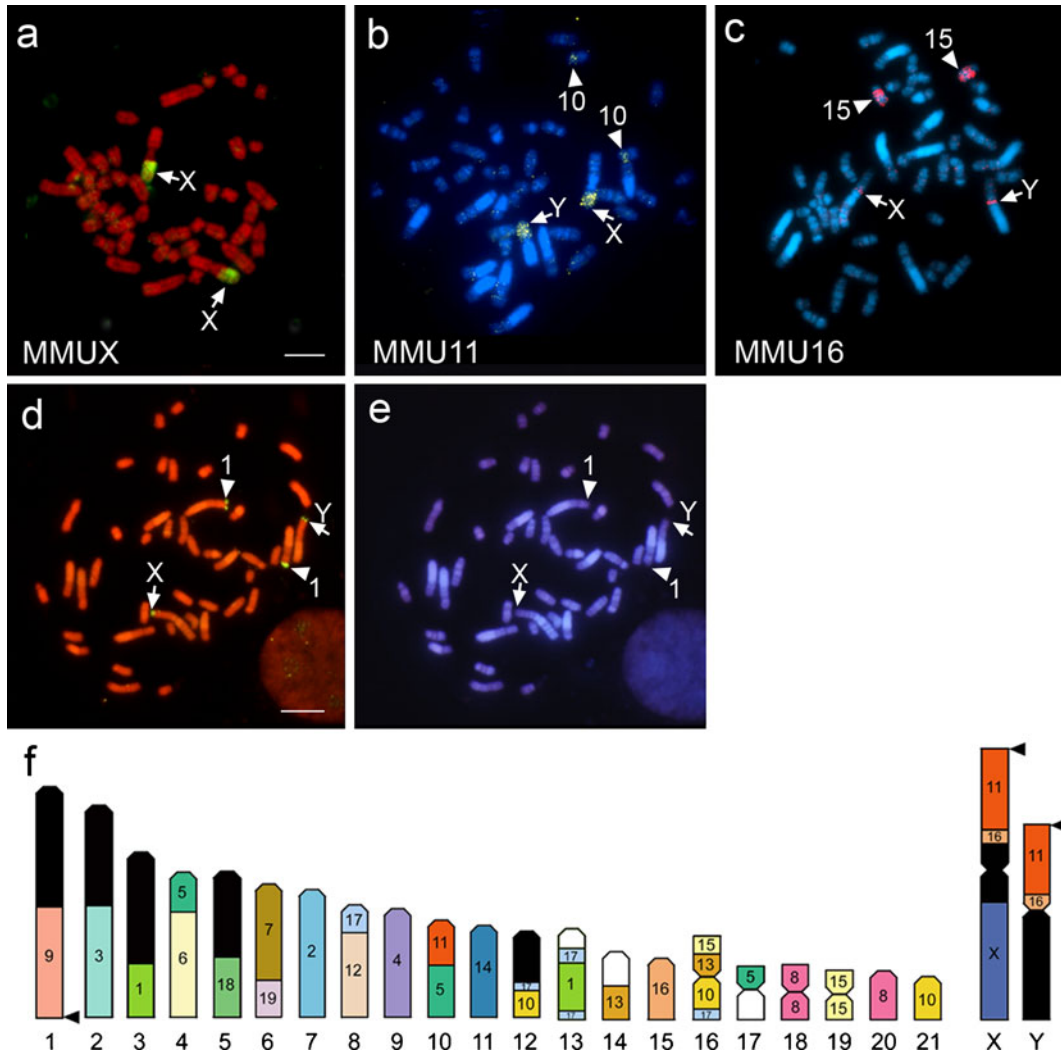
## Results

### Zoo-FISH analysis

We conducted the Zoo-FISH analysis on chromosomes from a male and a female specimen of *T. muenninki* using all *M. musculus* chromosome (MMU)-specific painting probes except for the Y chromosome probe. Each chromosome of *T. muenninki* could be identified on the basis of the Hoechst-stained bands that were obtained by the replication

R-banding method (Fig. 1). The MMU probes for all autosomes and the X chromosome were hybridized successfully to *T. muenninki* chromosomes (TMU). The images for the probes MMUX, MMU11, and

MMU16, are shown in Fig. 1a–c. The patterns for all the *T. muenninki* chromosomes are summarized in Fig. 1f. Although the long arm of the X chromosome of *T. muenninki* was covered with the MMUX probe



**Fig. 1** Karyotypic characterization in *T. muenninki*. **a–c** Zoo-FISH for *T. muenninki* with MMU-specific probes. Hybridization of FITC- (green) labeled mouse painting probes, MMUX, to PI-stained chromosomes of a *T. muenninki* female (**a**). Hybridization of Cy3- (yellow) and Cy5- (pink) labeled mouse painting probes, MMU11 (**b**) and MMU 16 (**c**), respectively, to Hoechst-stained chromosomes of a *T. muenninki* male. Arrows and arrowheads indicate the hybridization signals on the sex chromosomes and autosomes [TMU10 (**b**) and TMU15 (**c**)], respectively. The scale bar indicates 10  $\mu$ m. **(d, e)** Mapping of *T. muenninki* chromosomes by FISH using mouse genomic DNA clones of the 18S–28S rRNA genes. Hybridization signals of the 18S–28S rRNA genes were visualized by avidin-FITC on PI-stained chromosomes

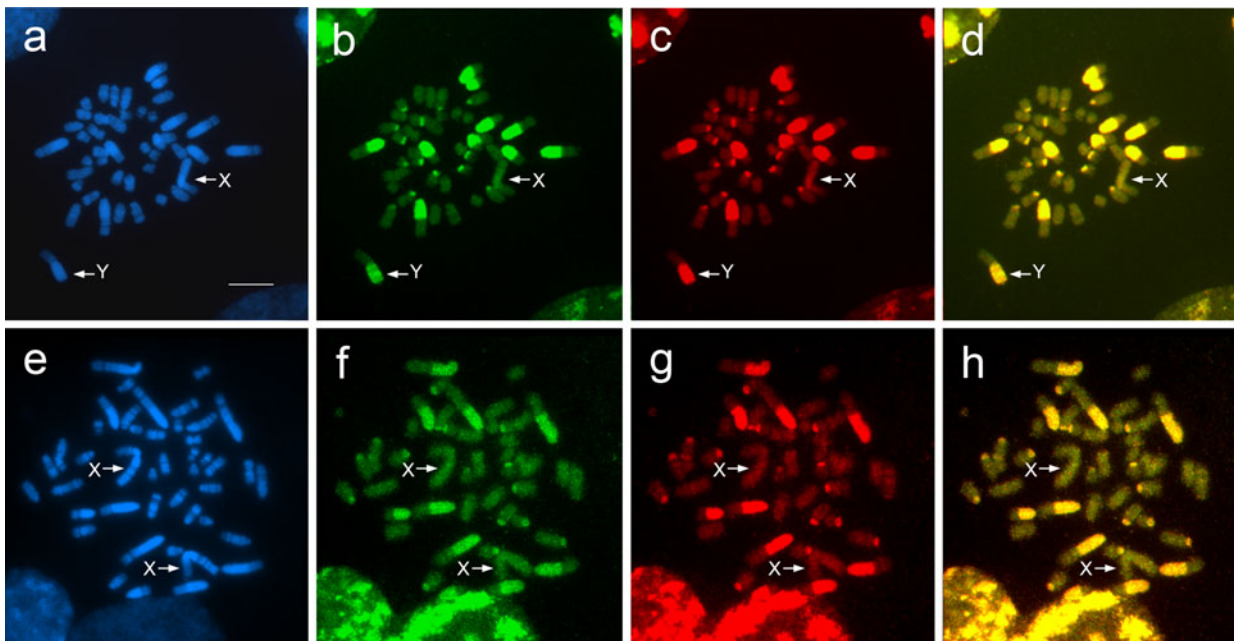
**(d)**. Hoechst G-banding patterns are shown in **e**. Arrows and arrowheads indicate the hybridization signals on the sex chromosomes and a pair of autosomes (TMU1), respectively. The scale bar indicates 10  $\mu$ m. **f** Comparative cytogenetic maps of *T. muenninki*. The comparative cytogenetic maps showing chromosome homologies between mouse and *T. muenninki* were constructed by Zoo-FISH analysis with mouse probes. The numbers under the chromosomes of *T. muenninki* indicate the chromosome numbers for this species. The numbers inside the chromosomes indicate the chromosome numbers for mouse that correspond to the chromosomal segments of *T. muenninki* indicated. Arrowheads indicate the locations of the 18S–28S rRNA genes. Large heterochromatic regions are shown in black

(Fig. 1a), the short arms of the X and Y chromosomes both hybridized with the mouse probes MMU11 and MMU16 (Fig. 1b and c, respectively). The 18S–28S ribosomal RNA genes were localized to the distal ends of chromosome 1 and the short arms of the sex chromosomes (Fig. 1d, e), which corresponded to MMU9 and MMU11, respectively (Fig. 1f).

Fourteen chromosomes of *T. muenninki* (TMU1, 2, 3, 5, 7, 9, 11, 14, 15, 17, 18, 19, 20, and 21) each were hybridized with a single mouse probe; six chromosomes (TMU4, 6, 8, 10, 12, and 13) each hybridized with two probes; and one chromosome (TMU16) was hybridized with four probes (Fig. 1f). Thirty-four conserved segments were detected between the mouse and *T. muenninki* chromosomes. The hybridization patterns between *T. muenninki* and the mouse probes could be grouped into three categories: (1) 11 mouse probes (MMU2, 3, 4, 6, 7, 9, 12, 14, 18, 19, and X) each hybridized to a single chromosome or chromosomal segment of *T. muenninki*; (2) six mouse probes (MMU1, 8, 11, 13, 15, and 16) each hybridized to two chromosomes; and (3) three mouse probes (MMU5, 10, and 17) each hybridized to more than three chromosomes (Fig. 1f).

## CGH analysis

We performed CGH to identify the male-specific region in the Y chromosome of *T. muenninki*. CGH signals with differently labeled genomic DNAs from a female and a male were highlighted in the long arm of the Y chromosome, as well as in the heterochromatic regions of five autosomal pairs in both sexes (Fig. 2). The long arm of the Y chromosome, Yq, was labeled intensely not only by the male probe, which contained Y chromosomal DNA, but also by the female probe, although the intensity of Hoechst fluorescence of Yq was not higher than that of any of the other chromosomes (Fig. 2a–c). The short arm of the Y chromosome, Yp, which is a proto-autosomal segment that has been translocated to the Y chromosome, as shown in Fig. 1, was stained equally by the female- and male-derived probes (Fig. 2d). Therefore, the loss or gain of a male-specific chromosomal region was not detected in the *T. muenninki* male by CGH analysis. Furthermore, when unlabeled female DNA and mouse C0t-1 DNA were used in the CGH analysis as competitors, no male-specific region was detected in the *T. muenninki* male (data not shown). In addition, the sex-specific region was not



**Fig. 2** CGH images of a male (a–d) and a female (e–h) metaphase spread for *T. muenninki*. The Hoechst G-banding patterns are shown in a and e. The CGH images (d, h) were obtained by merging the images of metaphase spreads hybridized with

female genomic DNA (b, f) and male genomic DNA (c, g). Arrows indicate the sex chromosomes. Scale bar represents 10  $\mu$ m

detected in diploid mitotic complements of the *T. muenninki* female (Fig. 2e–h).

### Comparative FISH and FISH analysis

We mapped mouse cDNA clones of 22 X-linked genes on chromosomes derived from a *T. muenninki* female by direct R-banding FISH. All 22 genes were mapped on the long arm of the X chromosome in *T. muenninki*. The gene order was identical to that of the other two *Tokudaia* species and the laboratory rat, *R. norvegicus* (Fig. 3, Kobayashi et al. 2008). The hybridization patterns of four genes on the X chromosome, *ELK1* (Fig. 4a, b), *SAT1* (Fig. 4c, d), *SLC25A5* (Fig. 4e, f), and *PLXNA3* (Fig. 4g, h), are shown in Fig. 4.

Mapping analysis of the *SRY* gene could not be used to identify the original Y chromosome region because multiple copies of *SRY* are distributed over the entire Yq and centromere region of the Y chromosome in *T. muenninki* (Murata et al. 2010). In light of this, we used nine mouse cDNA clones (unpublished data) to screen for single copy genes on the Y chromosome by Southern blot analysis. The *DDX3Y* and *UTY* genes appeared to occur as a single copy in the male *T. muenninki* genome. Thus, we cloned the *T. muenninki* cDNA for both *DDX3Y* and *UTY* and used them as probes for FISH.

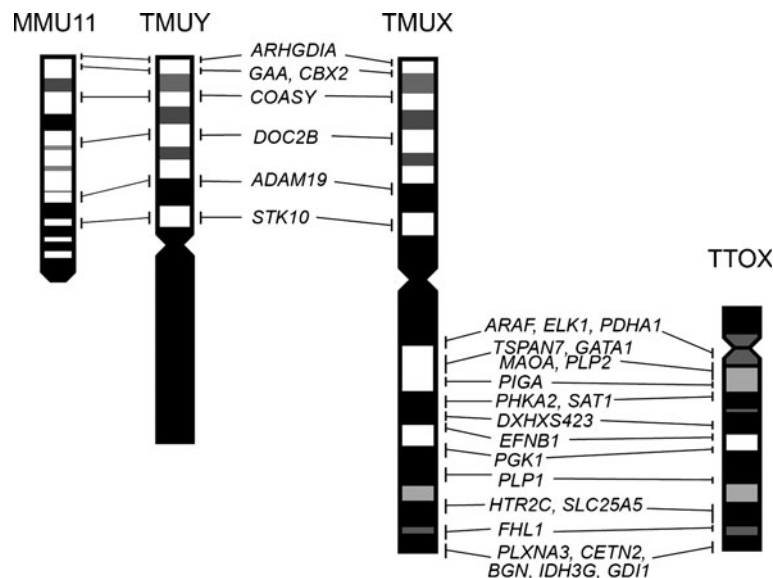
The sequence identity of the *DDX3Y* and *UTY* genes between mouse and *T. muenninki* was 95%. Both the *DDX3Y* and *UTY* genes were localized in *T. muenninki* to the proximal region of the centromere on Yp (Fig. 4i–l).

Given that the MMU11 painting probe hybridized to both Xp and Yp of *T. muenninki*, we performed comparative FISH mapping using probes from mouse cDNA clones of seven genes located on chromosome 11. All seven genes mapped to both Xp and Yp in *T. muenninki*. The hybridization patterns of four genes, *STK10* (Fig. 5a–d), *DOC2B* (Fig. 5e–h), *COASY* (Fig. 5i–l), and *CBX2* (Fig. 5m–o), are shown in Fig. 5. The gene orders on both Xp and Yp were identical to that on chromosome 11 of mouse (Fig. 3). However, *CBX2* was also localized to chromosome 14 in both sexes of *T. muenninki* (Fig. 5m–o).

### Discussion

#### Ancestral karyotype of the genus *Tokudaia*

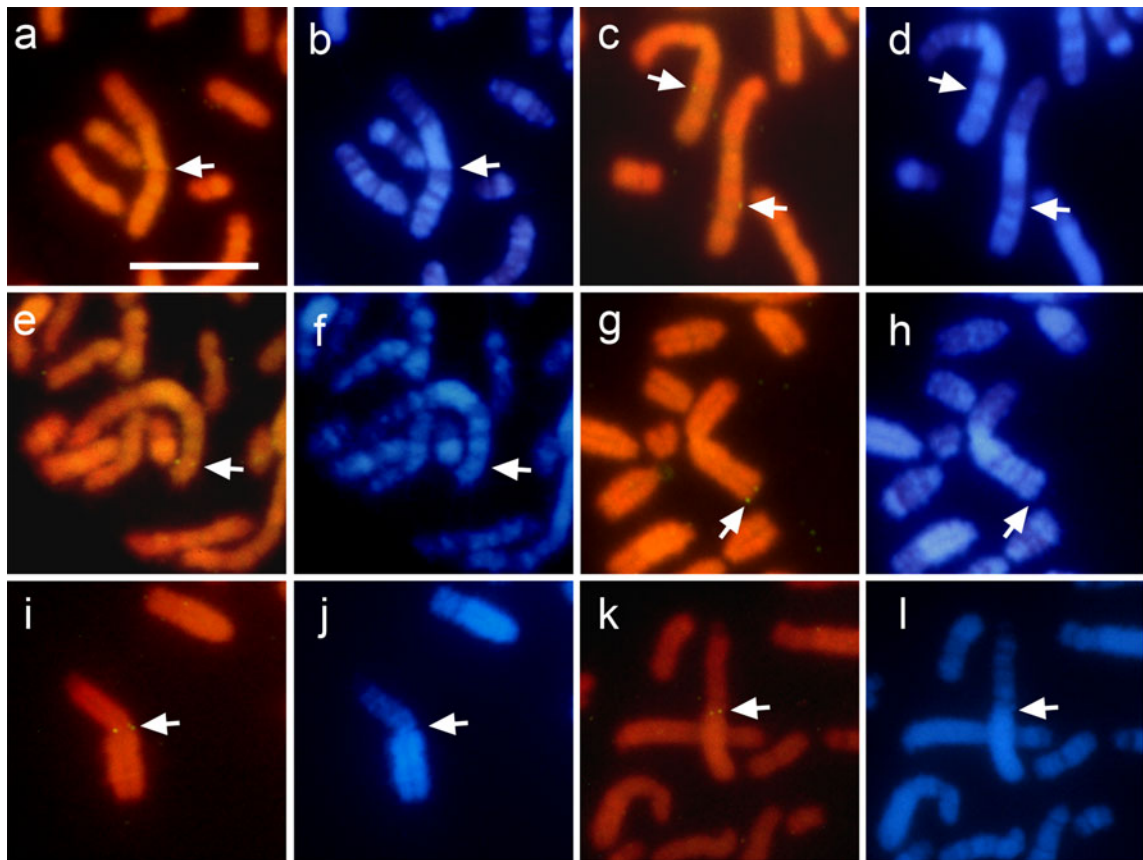
With the exception of several chromosome rearrangements described below, the karyotype of *T. muenninki* was largely identical to the one reported previously, which was predicted from maps of *T. osimensis* and *T.*



**Fig. 3** Comparative cytogenetic maps of seven genes from mouse chromosome 11 on the sex chromosomes of *T. muenninki* (TMUX, TMUY) and mouse chromosome 11 (MMU11) and 22 X-linked genes in *T. muenninki* and *T. tokunoshimensis*. The locations and order of the genes are shown on the side of each

chromosome. The gene order and ideogram of MMU11 was taken from the NCBI database (<http://www.ncbi.nlm.nih.gov/21/9/2011>). The gene order and ideograms of *T. tokunoshimensis* X chromosomes were taken from Kobayashi et al. (2008). TTO, *T. tokunoshimensis* chromosome





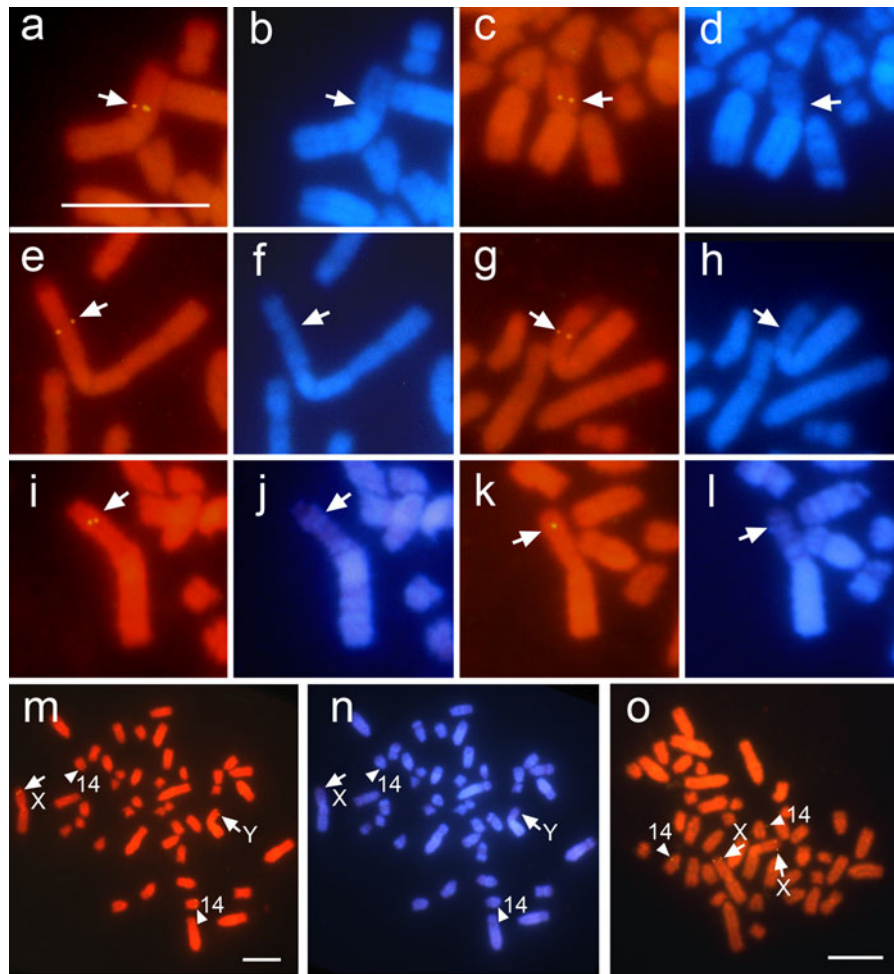
**Fig. 4** Chromosome mapping of four X-linked genes and two Y-linked genes on *T. muenninki* chromosomes. The hybridization signals are indicated by arrows. Four X-linked genes, *ELK1* (a, b), *SAT1* (b, c), *SLC25A5* (e, f), and *PLXNA3* (g, h), were mapped from the central to the distal part of *T. muenninki* Xq in the order shown.

Two Y-linked genes, *DDX3Y* (i, j) and *UTY* (k, l) were localized to the proximal region of the centromere in *T. muenninki* Yp. R- and Hoechst G-banding patterns are shown in (a, c, e, g, i, k) and (b, d, f, h, j, l), respectively. The scale bar indicates 10  $\mu$ m

*tokunoshimensis* generated by chromosome painting (Nakamura et al. 2007): MMU1b/17a/17e, a single segment of MMU14, and the two distinct chromosomal segments of MMU15 occurred in *T. muenninki*, whereas MMU1b/17a, two distinct chromosomal segments of MMU14, and a single segment of MMU15 occurred in *T. osimensis* and *T. tokunoshimensis*. The former segments found in *T. muenninki* are also conserved in the ancestral karyotype of the genus *Apodemus* (Matsubara et al. 2004), which is the most closely related group to the genus *Tokudaia* in Murinae (Michaux et al. 2002; Sato and Suzuki 2004; Rowe et al. 2008), suggesting that the ancestor of genus *Tokudaia* might have the same segments as *T. muenninki*, MMU1b/17a/17e, two distinct chromosomal segments of MMU14 and a single segment of MMU15. Therefore, we have demonstrated that the ancestral karyotype of the genus *Tokudaia* had a diploid

chromosome number of 48 and contained the following chromosomes that are homologous to mouse: 1a, 1b/17a/17e, 2, 3, 4, 5a, 5b/11a, 5c/6, 7/19, 8a, 8b, 9, 10a, 10b/17b, 10c/17c, 11b/16a, 12/17d, 13a, 13b/15a, 14, 15b, 16b, 18, X, and Y (Fig. 6). Lineage-specific chromosome rearrangements in *T. muenninki*, inferred from the ancestral karyotype, were as follows: (1) fusion between segments homologous to MMU11b/16a and the ancestral Y in TMUY; (2) centric fusions between segments homologous to MMU11b/16a and MMUX in TMUX and between segments homologous to MMU10b/17b and MMU13b/15a in TMU16; and (3) two pericentric inversions in TMU17 and TMU19 (Fig. 6).

Furthermore, the localization of the 18S–28S ribosomal RNA genes to two chromosomal segments homologous to MMU9 and MMU11 in *T. muenninki* (Fig. 1d–f) was also conserved in the other two *Tokudaia*



**Fig. 5** Chromosome mapping of the *STK10* (a–d), *DOC2B* (e–h), *COASY* (i–l), and *CBX2* (m–o) genes on *T. muenninki* chromosomes. The *STK10*, *DOC2B*, and *COASY* genes were localized to both Xp (a, e, i) and Yp (c, g, k) in *T. muenninki*. *CBX2* was mapped to Xp, Yp, and a pair of autosomes (TMU14) in the male

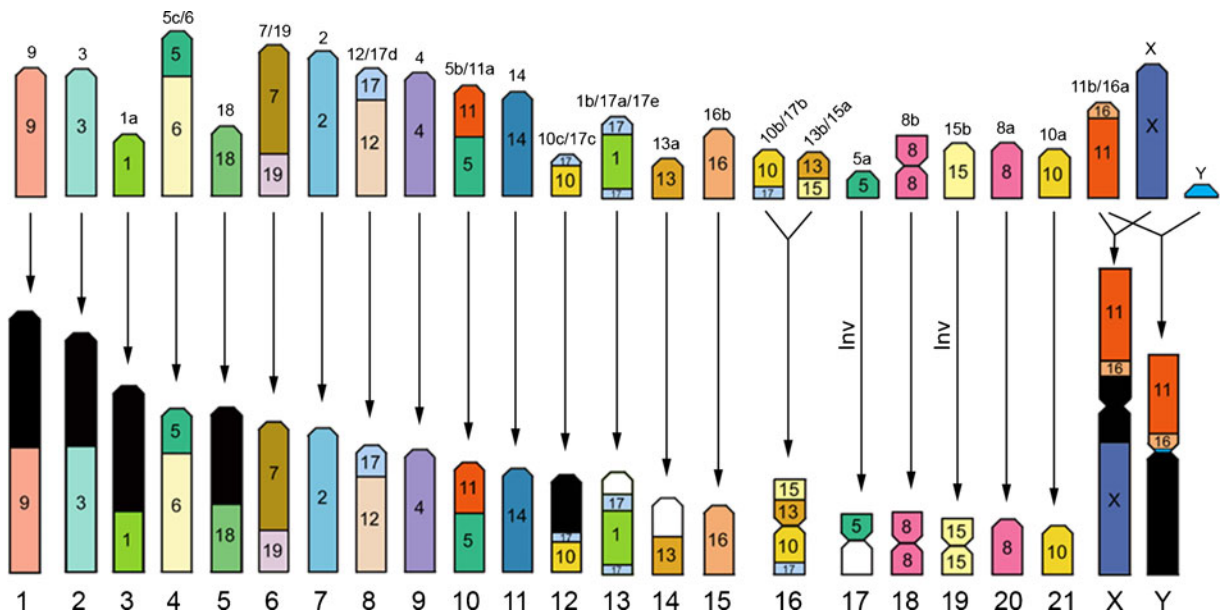
(m) and Xp and TMU14 in the female (o). The hybridization signals are indicated by *arrows* on the sex chromosomes and *arrowheads* on the autosomes. R- and Hoechst G-banding patterns are shown in (a, c, e, g, i, k, m, o) and (b, d, f, h, j, l, n), respectively. The scale bar represents 10  $\mu$ m

species (Arakawa et al. 2002; Nakamura et al. 2007) and six *Apodemus* species (Matsubara et al. 2004), which suggested that no chromosomal variation had occurred in *T. muenninki* in these regions.

#### Fusion of autosomes and the sex chromosomes

The short arms of the X and Y chromosomes of *T. muenninki* were painted by the MMU11 and MMU16 probes, which indicated that fusions had occurred between a pair of autosomes and the sex chromosomes. This was not observed in the other two *Tokudaia* species, where the chromosomal segments recognized by MMU11 and MMU16 corresponded to chromosome 3q

in *T. osimensis* and chromosome 10 in *T. tokunoshimensis*, respectively (Nakamura et al. 2007). The fusion of a pair of autosomes with both the X and Y chromosomes is a rare chromosomal rearrangement in mammals. The only other reported cases are in: the giant mole-rat (*Fukomys mechowii*), the African pygmy mouse (*Mus minutoides*), and three bovine species (*Boselaphus tragocamelus*, *Tragelaphus spekei*, and *Tragelaphus imberbis*) (Deuve et al. 2006; Veyrunes et al. 2004, 2010; Gallagher et al. 1998; Rubes et al. 2008). Species with X-autosome translocations all have intercalary heterochromatic blocks (IHBs) between the autosomal and the ancestral sexual segments, which probably enables the timing of



**Fig. 6** A schematic representation of the ancestral karyotype of the *Tokudaia* species and chromosome rearrangements that occurred in *T. muenninki* after its divergence from the common ancestor. The numbers inside the chromosomes indicate the mouse chromosome numbers that correspond to the indicated chromosomal segments of the *Tokudaia* species. The lower chromosomes correspond to the chromosomes of *T. muenninki*, and the numbers underneath are the chromosome numbers for this species. The numbers above the upper chromosomes, those of the schematic ancestral karyotype,

indicate the chromosomes, chromosomal segments, or chromosome associations that are homologous to mouse chromosomes. They were numbered following the nomenclature of Stanyon et al. (2004) and Nakamura et al. (2007). Arrows show how the chromosomes of *T. muenninki* were derived from the ancestral karyotype. Two-pronged arrows to a single chromosome indicate the successive occurrence of fusions. “Inv” indicates a pericentric inversion

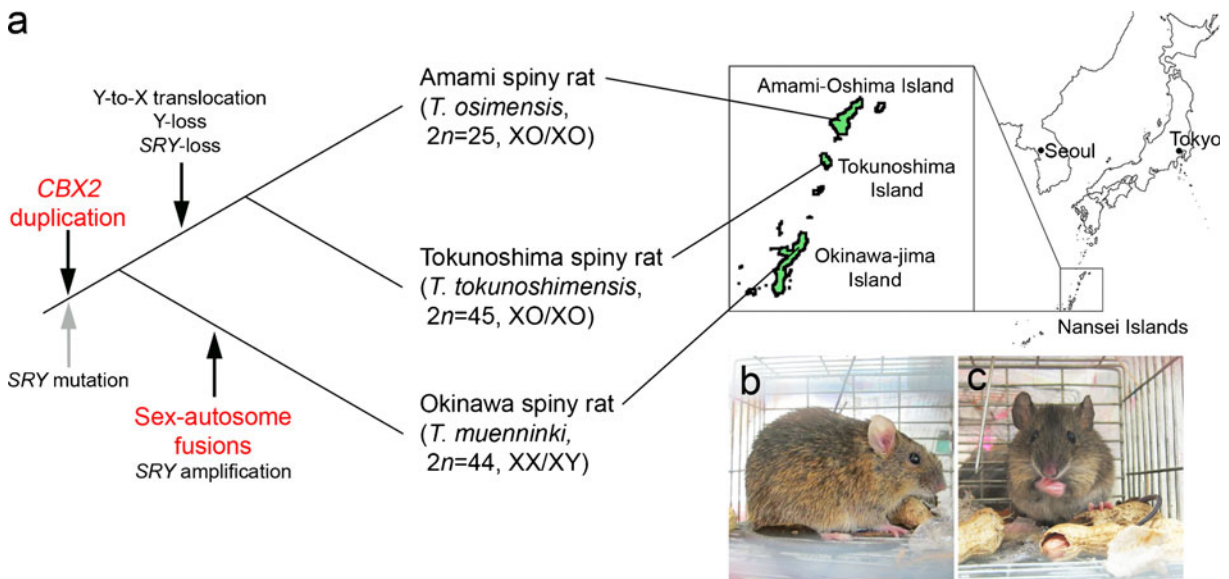
replication of the sex and autosomal segments to be regulated independently (Dobigny et al. 2004). The centromeric heterochromatin block of *T. muenninki* might function as an IHB and an X-autosomal boundary in this species (Murata et al. 2010).

#### Avoidance of Y chromosome loss through fusion with an autosome

*T. muenninki* is known to be the only species with a Y chromosome in the genus *Tokudaia*; as a consequence, it follows the general XY pattern of mammalian sex determination (Tsuchiya et al. 1989). However, our results indicated that the Y chromosome of *T. muenninki* has evolved in a unique manner. The Y chromosome might have had instability persisting in the common ancestral species of the genus *Tokudaia* (Fig. 7). In an ancestral lineage common to the two XO/XO spiny rats, *T. osimensis* and *T. tokunoshimensis*, most of the Y-linked genes (except *SRY* and *RBMY1A1*) escaped to the X chromosome, and the Y chromosome was lost subsequently (Kuroiwa et al. 2010). In contrast, in the

ancestral population of *T. muenninki*, the X and Y chromosomes fused with a pair of autosomes.

The enlargement of the Y chromosome through fusion with an autosome extended the pseudoautosomal regions (PARs). PARs behave like an autosomal pair and recombine during male meiosis, which is thought to play a critical role in spermatogenesis (Kauppi et al. 2011; Matsuda et al. 1992b; Mohandas et al. 1992). The XY male mice with inverted X PAR, Y PAR flanked at the distal end by X PAR boundary together with adjacent X-specific material, or both of the variant PARs, are known to produce XO progeny by leading to the production of unusual X and Y products and consequent sex chromosome loss (Burgoyne and Evans 2000). If variant PAR was associated with the Y chromosome loss in an ancestral lineage common to the two XO/XO spiny rats, *T. osimensis* and *T. tokunoshimensis*, the Y chromosome would be stabilized by the acquisition of new PARs in the ancestral lineage of *T. muenninki*, and thus, loss of the Y chromosome was avoided. To test this hypothesis, future research should include the identification and sequencing of an ancestral PAR in three *Tokudaia* species.



**Fig. 7** The evolution of sex chromosomes, *SRY*, and *CBX2* in the genus *Tokudaia*. **a** The evolutionary events inferred from the present study (red) and previous studies (black) are shown in the phylogeny, together with the geographical distribution of *Tokudaia* species. *T. muenninki*, *T. osimensis*, and *T. tokunoshimensis*

Most human Y-linked genes are relics of a recent autosomal addition (Waters et al. 2001). The PAR of placental mammals was part of a larger autosomal addition to the X and Y chromosomes 130–80 million years ago (MYA) (Toder and Graves 1998), which suggests that the ancestral PAR of placental mammals was larger than the present human PAR (Waters et al. 2007). Enlargement of the Y chromosome by the addition of an autosome could be an important chromosomal phenomenon to stabilize X–Y pairing in the lineage of placental mammals.

#### Structure of the giant X and Y chromosomes

All mouse cDNA clones of the X-linked genes were mapped to Xq (Fig. 3). The gene order was consistent with that of rat and the two other *Tokudaia* species (Kobayashi et al 2008), which suggests that the proto-X chromosome was retained in Xq and had not undergone chromosomal rearrangement, e.g., inversion and deletion on a broad scale.

In our CGH analysis, probes derived from either males or females hybridized to Yq of *T. muenninki* (Fig. 2). This might have been caused by the accumulation of repetitive sequences on Yq that were common to both sexes. The accumulation of numerous

inhabit only Okinawa-jima, Amami-Oshima, and Tokunoshima Islands, respectively. **b**, **c** Adult female (**b**) and juvenile to sub-adult male (**c**) of *T. muenninki* trapped as part of conservation activities

repetitive sequences could be the reason why a male-specific region was not detected clearly by CGH, even though many *SRY* sequences are known to occur on Yq of *T. muenninki* (Murata et al. 2010).

Previous attempts to map the *SRY* gene did not detect an ancestral Y segment that was common to other mammalian Y chromosomes due to the large number of mainly nonfunctional *SRY* copies. In the present study, using FISH to map two single-copy Y-linked genes, *DDX3Y* and *UTY*, we identified the region of Yp close to the centromere as the euchromatic region of the proto-Y chromosome in the genus *Tokudaia* (Fig. 4). Now that this region has been identified, further characterization of the Y-linked genes in *T. muenninki* by Southern blotting and FISH analyses is needed to reveal the gene content of the original Y region. Sequence determination of the region using a bacterial artificial chromosome library is also required to determine the gene order and gene structure of the putative ancestral Y chromosome in *T. muenninki*.

Neo-X? Neo-Y?

Comparative FISH mapping of seven mouse cDNA clones linked to chromosome 11 showed that the order

of genes on *T. muenninki* Xp and Yp was identical to that of mouse chromosome 11. No obvious chromosome rearrangement, e.g., inversion, was detected between the X and Y chromosomes. However, it remains possible that some divergence in nucleotide sequence has occurred between the X and Y chromosomes if the proto-autosomal segments are in the early stages of differentiation. On the basis of mitochondrial sequence data for cytochrome *b*, divergence times between *T. muenninki* and the two other species are estimated to be around 2.5–2.7 MYA (Murata et al. 2010; on the basis of the substitution rate of this gene in murids, 4.8% per MY; Suzuki et al. 2003). In the black muntjac (*Muntiacus crinifrons*), some sequence divergence has occurred between neo-sex chromosomes within the past 0.5 MY (Zhou et al. 2008). Therefore, there has been sufficient time for mutations to accumulate between the sex chromosomes of *T. muenninki*.

#### Duplication of *CBX2* in the genus *Tokudaia*

Gene duplication has been proposed as a primary source of material for the acquisition of evolutionary novelties, e.g., new gene functions and new patterns of expression (Ohno 1970; Sidow 1996). Several mechanisms have been put forward to explain how paralogs diverge from one another. In one process, asymmetric evolution, one gene copy retains the ancestral function through purifying selection, whereas the other copy can undergo neofunctionalization or pseudogenization (Lynch and Conery 2000). Such a duplication event allowed the evolution of a sex-determining gene in a model fish species, medaka (*Oryzas latipes*, Schartl 2004).

Previously, we examined the copy numbers and chromosomal locations of ten genes, including *CBX2*, to identify candidate genes for sex determination in the two *Tokudaia* species that lack *SRY* (Kuroiwa et al. 2011). The *Cbx2/CBX2* gene is a single copy gene in mice and humans and is known to be involved in gonadal differentiation. The knockout of *Cbx2* in mouse causes male-to-female sex reversal, and that mouse with the karyotype 40, XY has normal female external genitalia, which indicates that *Cbx2/CBX2* represses ovarian development (Katoh-Fukui et al. 1998; Biason-Lauber et al. 2009). *CBX2* occurs at two loci in *T. osimensis* and *T. tokunoshimensis*, owing to the translocation of a duplicated copy of the gene to another autosome. Furthermore, two or three more copies of *CBX2* are present

in males of these species as compared with females. It is likely that these extra copies of *CBX2* repress ovarian development and hence cause the undifferentiated gonad to develop testes in these species. Therefore, the differences in gene dosage between sexes might be involved in a novel sex-determining mechanism (Kuroiwa et al. 2011). In the present study, we have shown that *T. muenninki* also possesses multiple copies of *CBX2*. This suggests that the duplication of *CBX2* occurred in a common ancestor of all three *Tokudaia* species, after which the autosomal segment that contained the original *CBX2* copy became linked to the *T. muenninki* sex chromosomes through chromosomal fusion. The comparison of the copy number of *CBX2* between *T. muenninki* male and female is needed to reveal when *Tokudaia* species acquired the extra copies of *CBX2* in male genome.

*T. muenninki* possesses multiple copies of the *SRY* gene on the Y chromosome (Murata et al. 2010). Most of the copies are pseudogenes, with a complete ORF conserved in only three copies (Murata et al. 2010). A mutation, which results in a single amino acid substitution in the DNA binding surface domain of the HMG box of all *SRY* copies, might have weakened the DNA binding ability of the *SRY* protein in *T. muenninki*. If the Y-linked *CBX2* in *T. muenninki* has acquired a male-specific function through early differentiation between Xp and Yp, *CBX2* might also have become an important gene for sex determination in *T. muenninki* in the presence of a weakened *SRY*. Future research should include the sequencing of each copy of *CBX2* and a functional analysis of the *SRY* genes in *T. muenninki* to test this hypothesis.

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