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**Comparative chromosome painting map between two Ryukyu spiny rat species,
Tokudaia osimensis and *Tokudaia tokunoshimensis* (Muridae, Rodentia)**

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*Key Words: Tokudaia, comparative chromosome painting, chromosome homology,
ancestral karyotype, karyotype evolution*

Abstract

Ryukyu spiny rats (genus *Tokudaia*) are indigenous species that are confined to three islands of the Nansei Shoto archipelago, Amami-Oshima, Tokunoshima and Okinawa-jima, Japan. *Tokudaia tokunoshimensis* from Tokunoshima Island and *Tokudaia osimensis* from Amami-Oshima Island are closely related taxonomically, although their karyotypes are quite different: the diploid chromosome numbers and sex chromosome constitution are $2n=45$, X0/X0 for *T. tokunoshimensis* and $2n=25$, X0/X0 for *T. osimensis*. We conducted comparative chromosome painting with chromosome-specific DNA probes of the laboratory mouse (*Mus musculus*) to molecularly examine the chromosome homology between *T. tokunoshimensis* and *T. osimensis*, and deduced a possible ancestral karyotype of *Tokudaia* species and the process of evolutionary chromosome rearrangements. The proposed ancestral karyotype with the diploid number of $2n=48$, XX/XY was similar to the karyotype of *T. tokunoshimensis*, and the karyotype of *T. osimensis* would then have been established through at least 14 chromosomal changes, mainly centric fusion and tandem fusion, from the ancestral karyotype. The close karyological relationship between the ancestral karyotypes of *Tokudaia* and *Apodemus* also suggests that the chromosomal evolution in the *Tokudaia*-*Apodemus* lineage has been very slow and has accelerated only recently in the branch leading to *T. osimensis*.

Introduction

Ryukyu spiny rats (genus *Tokudaia*) are indigenous species that are confined to three islands, Amami-Oshima, Tokunoshima and Okinawa-jima, in the Nansei Shoto archipelago in the southeastern part of Japan. The genus *Tokudaia* belongs to the subfamily Murinae (Muridae; Rodentia), and consists of three extant species: *T. muenninki*, *T. osimensis* and *T. tokunoshimensis* (Endo & Tsuchiya 2006). *T. muenninki* from Okinawa-jima Island has been classified as a different subspecies from the other two *Tokudaia* species (Johnson 1946). *T. tokunoshimensis* from Tokunoshima Island was recently distinguished from *T. osimensis* from Amami-Oshima Island as a new species (Endo & Tsuchiya 2006). The genus *Tokudaia* has been molecular-phylogenetically determined to be a distinct lineage (Suzuki *et al.* 2000) that is positioned most closely to *Apodemus* based on the nucleotide sequences of several mitochondrial and nuclear genes (Michaux *et al.* 2002, Sato & Suzuki 2004). The divergence time of the *Tokudaia*-*Apodemus* clade was estimated to be approximately 6.5-8.0 million years ago (MYA) (Sato & Suzuki 2004). The genetic difference between *T. osimensis* and *T. tokunoshimensis* mitochondrial cytochrome *b* sequences was reported to be 8.8% (Suzuki *et al.* 1999), which roughly corresponds to a divergence time of 2 MYA on the basis of the divergence rate of this gene in murids (4.8% per million years; Suzuki *et al.* 2003). Although the phylogenetic position of *T. muenninki* is still unclear because there are no molecular phylogenetic data for this species, it is

speculated based on biogeographical considerations that first the *T. tokunoshimensis* – *T. osimensis* lineage diverged from the *T. muenninki* lineage; *T. tokunoshimensis* and *T. osimensis* were subsequently isolated on Tokunoshima Island and Amami-Oshima Island, respectively, and the three species consequently diverged independently from the common ancestor after they were confined to each of the three islands.

Remarkable karyotypic variations have been reported among the three *Tokudaia* species. The diploid chromosome number of *T. muenninki* is $2n=44$ with the XX/XY type of sex chromosomes (Tsuchiya *et al.* 1989), whereas the diploid numbers of *T. osimensis* and *T. tokunoshimensis* are $2n=25$ and $2n=45$, respectively, both of which have a unique X0/X0 sex determining system without a Y chromosome or a *Sry* gene (Honda *et al.* 1977, 1978, Soullier *et al.* 1998, Sutou *et al.* 2001, Arakawa *et al.* 2002, Kobayashi *et al.* 2007). The homology of the *T. osimensis* and *T. tokunoshimensis* X chromosomes with the mouse X chromosome has been revealed by comparative chromosome painting with a mouse X probe (Arakawa *et al.* 2002). The remarkable difference of the diploid chromosome number between *T. osimensis* and *T. tokunoshimensis* indicates that frequent chromosome rearrangements have occurred between the two species in less than 2 million years since they diverged from the common ancestor. Chromosome banding is a conventional method to compare the karyotypes between different species, and has been extensively used for studying the karyotypic evolution of vertebrates. Q-banding and G-banding analyses have been

performed for *T. osimensis* and *T. tokunoshimensis* (Arakawa *et al.* 2002, Kobayashi *et al.* 2007), and the G-banded ideograms of the two species have been established (Kobayashi *et al.* 2007). Chromosome banding is effective for the comparison of morphological similarities of chromosomes between relatively closely related species; however, it is difficult to accurately identify chromosome homology between different species using this method. Cross-species chromosome painting (termed ZOO-FISH) allows one to unambiguously identify homologous chromosome segments at the whole genome level and to delineate the chromosome rearrangements that have occurred in the lineage of each species since the two species diverged from the common ancestor. Comparative chromosome painting in the Muridae with mouse probes has been performed for over 13 species in at least six genera of two subfamilies (Murinae and Cricetinae): *Cricetulus griseus* (Yang *et al.* 2000), *Mus platythrix* (Matsubara *et al.* 2003), *Apodemus sylvaticus* and six other *Apodemus* species (Matsubara *et al.* 2004, Stanyon *et al.* 2004), *Rattus rattus* and *R. norvegicus* (Grützner *et al.* 1999, Guilly *et al.* 1999, Stanyon *et al.* 1999, Cavagna *et al.* 2002), *Rhabdomys pumilio* (Rambau & Robinson 2003) and *Otomys irroratus* (Engelbrecht *et al.* 2006). Based on these comparative painting data, the ancestral karyotypes of the Muridae have been proposed and discussed by several research groups (Stanyon *et al.* 2004, Engelbrecht *et al.* 2006).

Here we conducted comparative chromosome painting with mouse DNA probes for *T. osimensis* and *T. tokunoshimensis*, and molecularly identified the homologous

chromosome regions between the two *Tokudaia* species. Based on the chromosome homology data, we deduced a possible ancestral karyotype of *Tokudaia* species and the process of the chromosome rearrangements that have occurred between the two species. Finally we discuss the karyotype evolution in *Tokudaia* and the ancestral murid karyotype in conjunction with the comparative chromosome painting data of other Muridae species.

Materials and methods

Chromosome preparation

Wild individuals of *T. osimensis* were originally captured on Amami-Oshima Island in February 2004 with permission from the Agency for Cultural Affairs and the Ministry of the Environment in Japan. A small tip of the tail was taken from a live male animal and was used for fibroblast cell culture. Frozen fibroblast cells were used for *T. tokunoshimensis*. The cell line was established from the primary cultured fibroblast cells taken from the tail tissue of a wild male animal captured on Tokunoshima Island in 1977 (Honda *et al.* 1978), and has been stored in nitrogen liquid in our laboratory.

Preparation of R-banded chromosomes was performed as described by Matsuda *et al.* (1992) and Matsuda & Chapman (1995). The fibroblast cells were cultured in MEM medium supplemented with 15% fetal bovine serum at 37°C in 5% CO₂ in air.

Thymidine (300 µg/ml) was added to the cell cultures at log phase, and the cell culturing was continued for 14 h. The cells were washed with the serum-free culture medium three times, and then subsequently treated with BrdU (25 µg/ml) in the culture medium for an additional 5 h, including 30 min of colcemid treatment (0.02 µg/ml) before harvesting. Chromosome preparations were made following a standard protocol. After staining of the chromosome slides with Hoechst 33258 (1 µg/ml) for 5 min, R-banded chromosomes were obtained by heating the slides at 65°C for 3 min on a hot plate and subsequently exposing them to UV light for an additional 5 min at 65°C.

Chromosome painting

The biotin- and Cy3-labeled chromosome-specific painting probes of the laboratory mouse were purchased from Cambio Ltd., Cambridge, UK. Chromosome *in-situ* hybridization with the painting probes was carried out as described by Matsubara *et al.* (2003) with slight modifications. The chromosome slides were aged at 65°C for 2 h, and denatured in 70% formamide, 2 X SSC at 70°C for 2 min, and dehydrated in 70% and 100% ethanol at 4°C for 5 min each. The probes were denatured at 75°C for 10 min and pre-annealed by incubation at 37°C for 45 min. The chromosome slides were hybridized with the probes at 37°C for 3 days. After hybridization, the slides were washed in 50% formamide, 2 X SSC at 37°C for 15 min, and in 2 X SSC for 15 min at room temperature. For detection of the hybridization signals of the biotin-labeled probes, the

chromosome slides were incubated with FITC-avidin (Roche Diagnostics) at 1:500 dilution in 1% BSA, 4 X SSC at 37°C for 1 h. The slides were washed on a shaker with 4 X SSC, 0.1% Nonidet P-40/4 X SSC, and 4 X SSC for 5 min each at room temperature, and stained with propidium iodide (PI).

Image capture

The FISH images were captured using a cooled CCD camera (Micro MAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope, and were analyzed with the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK).

Results

Chromosome painting

All mouse (*Mus musculus*, MMU) chromosome-specific paint probes except for the Y probe were successfully cross-hybridized to chromosomes of the two *Tokudaia* species (Figure 1). The painting patterns of all chromosomes in the two species are summarized in Figure 2.

Nineteen autosomal paints and the X paint detected 33 conserved segments between mouse and *Tokudaia osimensis* (TOS) chromosomes (Figure 2a). The

hybridization patterns of *T. osimensis* chromosomes with the mouse probes were grouped into three categories: (1) 11 mouse probes (MMU2, 3, 4, 6, 7, 9, 12, 15, 18, 19 and X) each hybridized to a single chromosome, chromosome arm or chromosome segment of *T. osimensis*; (2) six mouse probes (MMU1, 8, 11, 13, 14 and 16) each produced two painted signals; and (3) three mouse probes (MMU5, 10 and 17) each produced three or four painted signals. Only two *T. osimensis* chromosomes (TOS11 and X) were each hybridized by a single mouse probe, five chromosomes (TOS1, 2, 9, 10 and 12) were each hybridized by two probes, and four chromosomes (TOS3, 4, 5, 6, 7 and 8) were hybridized by three or four probes. Twelve inter-chromosomal associations with fourteen mouse chromosomes and/or chromosome segments were observed: MMU1/17, 11/16, 12/17, 5/11, 7/19, 1/13, 5/6, 10/17 (twice), 10/13, 8/14 and 8/15.

Thirty-two conserved chromosome segments were observed between mouse and *Tokudaia tokunoshimensis* (TTO) chromosomes (Figure 2b). Eleven mouse probes (MMU2, 3, 4, 6, 7, 9, 12, 15, 18, 19 and X) each hybridized to a single chromosome or chromosome segment of *T. tokunoshimensis*, seven mouse probes (MMU1, 8, 10, 11, 13, 14 and 16) each produced two painted signals, and MMU5 and MMU17 produced three and four painted signals, respectively. Fourteen *T. tokunoshimensis* chromosomes (TTO3, 4, 5, 7, 8, 11, 13, 15, 17-20, 22 and X) were each painted with a single mouse probe. Nine other chromosomes (TTO1, 2, 6, 9, 10, 12, 14, 16 and 21) were each

painted with two different probes. Nine inter-chromosomal associations with 14 mouse chromosomes and/or chromosome segments were observed: MMU7/19, 5/6, 10/17 (twice), 13/15, 11/16, 12/17, 5/11 and 1/17.

The chromosome associations MMU1/17, 5/6, 5/11, 7/19, 10/17 (twice), 11/16 and 12/17 were observed in both *T. osimensis* and *T. tokunoshimensis*, suggesting that these associations had probably been contained in the karyotype of their common ancestor. The chromosome associations MMU1/13, 8/14, 8/15 and 10/13 in *T. osimensis* were not observed in *T. tokunoshimensis*, while MMU13/15 in *T. tokunoshimensis* was not found in *T. osimensis*. The 18S-28S ribosomal RNA genes have been localized to the distal ends of the long arms of chromosomes 2, 3 and 9 in *T. osimensis* and the distal ends of chromosomes 8 and 10 in *T. tokunoshimensis* (Arakawa *et al.* 2002). The distal regions of the TOS2 long arm and TTO8 corresponded to MMU9, and the distal regions of the TOS3 long arm and TTO10 corresponded to MMU11 (Figure 2), indicating that the chromosomal locations of the 18S-28S rRNA genes were derived from the same origin, except for the 18S-28S rRNA genes in the distal region of the TOS9 long arm corresponding to MMU14.

Discussion

Stanyon *et al.* (2004) first proposed a working hypothesis about the ancestral murid karyotype based on the chromosome painting data of four Murinae species and one Cricetinae species: *Mus platythrix* (Matsubara *et al.* 2003), *Apodemus sylvaticus* (Matsubara *et al.* 2004, Stanyon *et al.* 2004), which has been positioned the most closely to *Tokudaia* (Sato & Suzuki 2004), *Rattus norvegicus* (Grützner *et al.* 1999, Guilly *et al.* 1999, Stanyon *et al.* 1999, Cavagna *et al.* 2002), *Rhabdomys pumilio* (Rambau & Robinson 2003) and *Cricetulus griseus* (Yang *et al.* 2000). The ancestral murid karyotype probably had the diploid number of $2n=54$ and contained the following chromosomes that are homologous to *Mus musculus*: MMU1a, 1b /17a, 2a, 2b/13a, 3, 4, 5a, 5b/11, 6a, 6b, 7/19, 8a, 8b, 9, 10a, 10b/17b, 10c/17c, 11a, 11b/16a, 12a, 12/17d, 13b/15, 14, 15b, 16b, 18, X and Y. The Giemsa-stained karyotype of the other *Tokudaia* species, *T. muenninki*, which is a rare species that is found on Okinawa-jima Island and may now be extinct, has been reported to be $2n=44$, XX/XY (Tsuchiya *et al.* 1989). This karyotype is composed of 17 acrocentric, three small submetacentric and one small metacentric autosomal pairs and the submetacentric X and Y chromosomes, and is similar to the *T. tokunoshimensis* karyotype except for the X chromosome, which is subtelocentric in this species and monosomic in both males and females. Based on the present chromosome painting data of *T. osimensis* and *T. tokunoshimensis* and the published data of the Giemsa-stained karyotype of *T. muenninki*, we deduced a possible ancestral karyotype of *Tokudaia* species and the process of chromosome rearrangements

based on the most parsimonious events of chromosome rearrangements and translocations. Although reciprocal painting data would be necessary to determine the true homology of chromosome segments of the mouse chromosomes, we concluded that the ancestral karyotype of *Tokudaia* species was $2n=48$, with 21 acrocentric, one submetacentric and one metacentric autosomal pairs, and a subtelocentric or submetacentric X chromosome and a Y chromosome, which was described using the nomenclature described in Stanyon *et al.* (2004) as follows: MMU2, 14a, 9, 16b, 11b/16a, 1b/17a, 5a, 12/17d, 7/19, 5b/11a, 13a, 1a, 5c/6, 3, 10b/17b, 10a, 4, 13b/15, 8a, 8b, 14b, 18, 10c/17c, X and Y (Figure 3).

According to our scheme shown in Figure 3, three pericentric inversions probably occurred in TTO3, 18 and 20. The position of the centromere on TTO12 might have been changed by centromere repositioning, as reported in several mammalian species (Ventura *et al.* 2001, Eder *et al.* 2003, Ferreri *et al.* 2005, Carbone *et al.* 2006). TTO6 might have resulted from tandem fusion between MMU10b/17b and 10a. In comparison to *T. tokunoshimensis*, at least eight centric fusions (TOS1-8), one fission, four tandem fusions (TOS9, TOS10, the short arms of TOS6 and TOS8) and one centromere repositioning (TOS12) appear to have occurred in the lineage of *T. osimensis*. The schematic ancestral karyotype of the genus *Tokudaia* was quite similar to the ancestral karyotype of the genus *Apodemus*, which we inferred from comparative painting among seven *Apodemus* species (*Apodemus peninsulae*, *A. agrarius*, *A. semotus*, *A. speciosus*,

A. argenteus, *A. gurka* and *A. sylvaticus*): the ancestral karyotype of *Apodemus* probably had a diploid chromosome number of $2n=48$, and shared 20 of the same chromosome segments and associations, 2, 9, 16b, 1b/17a, 5a, 12/17d, 7/19, 5b/11a, 1a, 5c/6, 3, 10b/17b, 10a, 4, 13b/15, 8a, 8b, 10c/17c, X and Y, with the ancestral karyotype of *Tokudaia* (Matsubara *et al.* 2004, Matsubara *et al.* unpublished data). In the morphological comparison of the G-banded chromosomes by Kobayashi *et al.* (2007), it was suggested that at least 10 centric fusions have occurred in the lineage of *T. osimensis*; however, it was difficult to accurately identify the homology of each chromosome between the two species, and other chromosome rearrangements could not be detected. The comparative chromosome painting with mouse probes made it possible to identify the chromosome homology at the molecular level and to delineate the process of the chromosome rearrangements that have occurred in the two *Tokudaia* species.

The chromosome segments and chromosome associations contained in the ancestral karyotype of the genus *Tokudaia* deduced in this study, MMU1a, 1b/17a, 3, 4, 5a, 5b/11a, 7/19, 8a, 8b, 9, 10a, 10b/17b, 10c/17c, 11b/16a, 12/17d, 13b/15, 16b, 18, X and Y, are shared with the ancestral murid karyotype proposed by Stanyon *et al.* (2004), which account for 19 of the 26 autosomal pairs and one sex chromosome pair comprising the ancestral murid karyotype. Recent chromosome painting data of *Otomys irroratus* (Engelbrecht *et al.* 2006) showed that 13 autosomal and one sex chromosome

pairs of the ancestral *Tokudaia* karyotype, MMU1b/17a, 3, 4, 7/19, 8a, 8b, 10a, 10b/17b, 10c/17c, 11b/16a, 13b/15, 16b, 18, and X and Y, were also conserved in *Otomys*. These results suggest that the karyotype of the genus *Tokudaia* has remained quite similar to that of the ancestral murid chromosome constitution and provide important information for understanding the process of karyotype evolution in murids.

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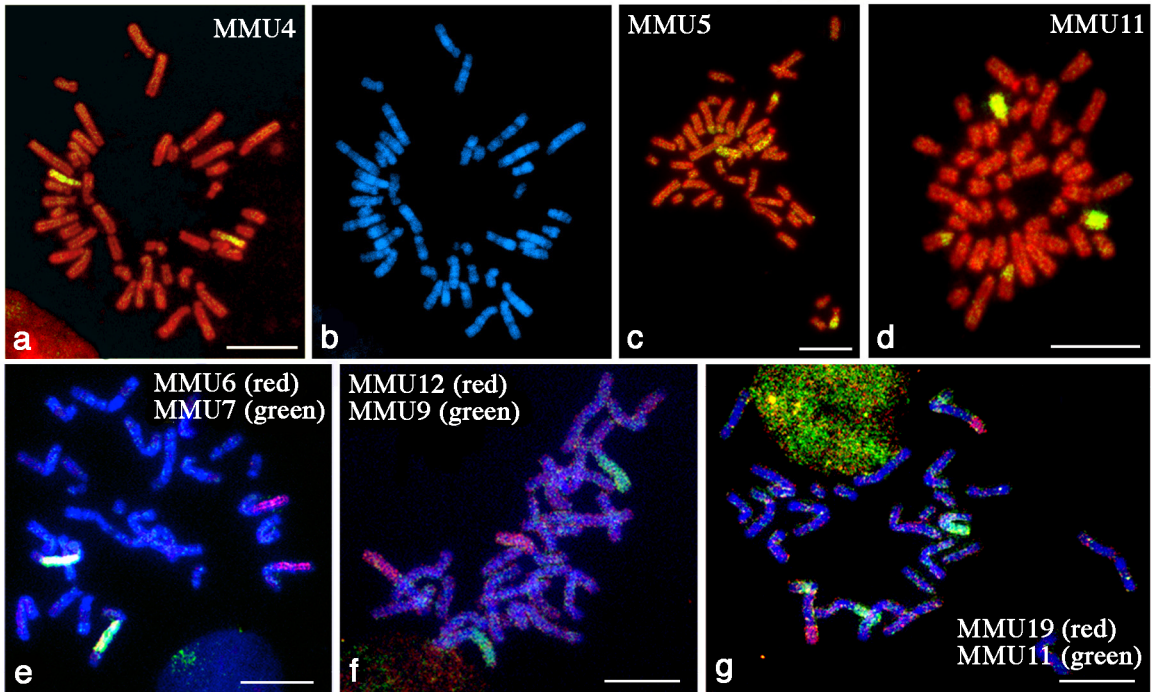
Figure legends

Figure 1. Comparative chromosome painting of two *Tokudaia* species with mouse (*Mus musculus*, MMU) chromosome-specific probes. **(a-d)** The chromosome hybridization with biotin-labeled mouse paint probes, MMU4 **(a)**, MMU5 **(c)** and MMU11 **(d)**, to PI-stained *T. tokunoshimensis* chromosomes. The Hoechst-stained pattern of the same metaphase chromosome spread **(a)** is shown in **(b)**. **(e-g)** The chromosome hybridization with biotin-labeled and Cy3-labeled mouse paint probes to Hoechst-stained *T. osimensis* chromosomes. **(e)** Cy3-labeled MMU6 (red) and biotin-labeled MMU7 (green). **(f)** MMU12 (red) and MMU9 (green). **(g)** MMU19 (red) and MMU11 (green). Scale bar =10 μm .

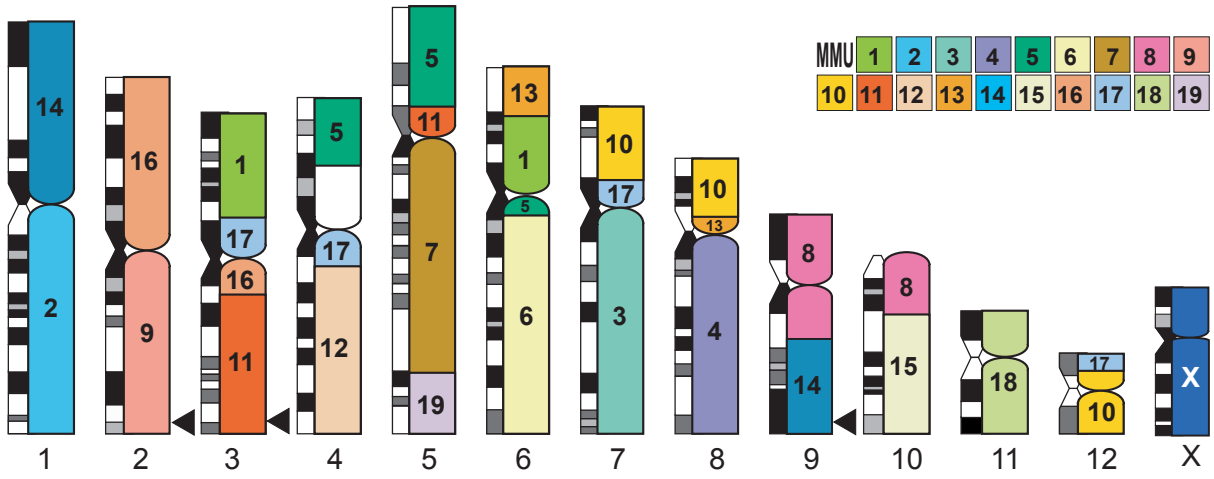
Figure 2. G-banded chromosome ideograms and chromosome painting patterns of *T. osimensis* **(a)** and *T. tokunoshimensis* **(b)**. G-banded chromosome ideograms of *T. osimensis* and *T. tokunoshimensis* were taken from Kobayashi *et al.* (2007). The comparative cytogenetic maps showing chromosome homologies between mouse and *T. osimensis* **(a)** and between mouse and *T. tokunoshimensis* **(b)** were constructed by comparative chromosome painting with mouse probes. The number below each chromosome indicates the chromosome number of the species. The numbers inside chromosomes indicate the chromosome numbers of mouse, which correspond to the chromosome segments of the *Tokudaia* species. Arrowheads indicate the locations of the

18S-28S rRNA genes, which were taken from Arakawa *et al.* (2002).

Figure 3. Schematic representation of the ancestral karyotype of the *Tokudaia* species and chromosome rearrangements that occurred in *T. osimensis* and *T. tokunoshimensis* after the divergence from the common ancestor. The numbers inside chromosomes indicate the mouse chromosome numbers, which correspond to the chromosome segments of the *Tokudaia* species. The numbers under chromosomes of *T. tokunoshimensis* and *T. osimensis* indicate the chromosome numbers of the two species. The numbers over chromosomes of the schematic ancestral karyotype indicate the chromosomes, chromosome segments or chromosome associations which are homologous to mouse chromosomes. They were numbered following the nomenclature of Stanyon *et al.* (2004). Arrows show the derivation of the chromosomes of *T. tokunoshimensis* and *T. osimensis* from the ancestral karyotype. Two arrows over one chromosome indicate the successive occurrence of a centric and a tandem fusion. Inv: pericentric inversion. CR: centromere repositioning.



(a)



(b)

