Karyotype evolution in monitor lizards: cross-species chromosome mapping of cDNA reveals highly conserved synteny and gene order in the Toxicofera clade

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Abstract The water monitor lizard (*Varanus salvator macromaculatus* (VSA), Platynota) has a chromosome number of $2n=40$: its karyotype consists of 16 macrochromosomes and 24 microchromosomes. To delineate the process of karyotype evolution in *V. salvator macromaculatus*, we constructed a cytogenetic map with 86 functional genes and compared it with those of the butterfly lizard (*Leiolepis reevesii rubritaeniata* (LRE); $2n=36$) and Japanese four-striped rat snake (*Elaphe quadrivirgata* (EQU); $2n=36$), members of the Toxicofera clade. The syntenies and gene orders of macrochromosomes were highly conserved between these species except for several chromosomal rearrangements: eight pairs of VSA macrochromosomes and/or chromosome arms exhibited homology with six pairs of LRE macrochromosomes and eight pairs of EQU macrochromosomes. Furthermore, the genes mapped to microchromosomes of three species were all located on chicken microchromosomes or chromosome 4p. No reciprocal translocations were found in the species, and their karyotypic differences were caused by: low frequencies of interchromosomal rearrangements, such as tandem fusions, or centric fissions/fusions between macrochromosomes and between macro- and microchromosomes; and intrachromosomal rearrangements, such as paracentric inversions or centromere repositioning. The chromosomal rearrangements that occurred in macrochromosomes of the *Varanus* lineage were also identified through comparative cytogenetic mapping of *V. salvator macromaculatus* and *V. exanthematicus*.

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Morphologic differences in chromosomes 6–8 between the two species could have resulted from pericentric inversion or centromere repositioning.

Keywords Monitor lizard · Toxicofera · Squamata · Synteny and gene order · Karyotype evolution · Cytogenetic map

Abbreviations

BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
EQU	*Elaphe quadrivirgata*
FISH	Fluorescence in situ hybridization
GGA	*Gallus gallus*
LRE	*Leiolepis reevesii rubritaeniata*
MYA	Million years ago
rRNA	Ribosomal RNA
VSA	*Varanus salvator macromaculatus*

Introduction

The genome sequences of the domestic chicken (*Gallus gallus*; International Chicken Genome Sequencing Consortium ICGSC 2004) precipitated advances in comparative genomics between Reptilia and Aves that facilitated comparison of genomic structures between two taxa at the molecular level. Comparison of the chromosome maps of the Chinese soft-shelled turtle (*Pelodiscus sinensis*), Siamese crocodile (*Crocodylus siamensis*), Japanese four-striped rat snake (*Elaphe quadrivirgata*), butterfly lizard (*Leiolepis reevesii rubritaeniata*), and Australian central bearded dragon (*Pogona vitticeps*) with *G. gallus* revealed that syntenies and gene orders have been highly conserved between Aves, Testudines, Crocodylia, and Squamata (Matsuda et al. 2005; Matsubara et al. 2006, 2012; Srikulnath et al. 2009b; Uno et al. 2012; Young et al. 2013) since the Sauropsida (all existing reptiles and birds) diverged from the Synapsida around 320 MYA (Shedlock and Edwards 2009). The draft genome assembly of the green anole (*Anolis carolinensis*) confirmed that the sytenies and gene orders of the green anole are also highly conserved in the chicken genome (Alföldi et al. 2011).

Squamate reptiles, the most diverse reptilian order, are traditionally classified into three suborders: Serpentes (snakes), Amphisbaenia (worm lizards), and Lacertilia (lizards). The most common chromosome number in snakes is $2n=36$: their karyotypes consist of 16 macro- and 20 microchromosomes. There is relatively little karyotypic variation ($2n=30$–42) in snakes, whereas worm lizards show a large variation in chromosome number ($2n=30$–50; Olmo and Signorino 2005). The extant lizards can be categorized into six infraorders—Iguania, Gekkota, Scincomorpha, Diploglossa, Dibamia, and Platynota (Uetz 2013)—and they also exhibit a large variation in both chromosome number ($2n=24$–46) and chromosome morphology (Olmo and Signorino 2005). Karyotypes with few or no microchromosomes are found in the Lacertidae family of Scincomorpha and in the Gekkota, whereas karyotypes containing many microchromosomes are found in the Iguania, Platynota, Dibamia, Diploglossa, and Scincomorpha excluding the Lacertidae.

Molecular phylogenetic studies suggest that iguanians (Iguania) and anguimorphs (Platynota and Diploglossa) are closely related to snakes, and all are members of the so-called Toxicofera clade, which bear oral toxin-secreting glands (Vidal and Hedges 2005). However, the close genetic relationship of iguanians with snakes and anguimorphs remains controversial (Townsend et al. 2004; Vidal and Hedges 2005; Srikulnath et al. 2010; Wiens et al. 2012). The common chromosome number of Iguania is $2n=36$: these karyotypes consist of 12 macro- and 24 microchromosomes. This differs from the most common karyotype of Serpentes ($2n=36$), which comprises 16 macro- and 20 microchromosomes (Olmo and Signorino 2005). Our previous chromosome mapping study (Srikulnath et al. 2009b), which compared chromosomal locations of 54 functional genes between the butterfly lizard (Iguania) and the Japanese four-striped rat snake, revealed that most macrochromosomes were homologous to each other, whereas several chromosomal rearrangements—such as centric fission/fusion of macrochromosomes, telomere-to-telomere tandem fusion between macrochromosomes and between macro- and microchromosomes, and centromere repositioning—had occurred between the species. By contrast, anguimorphs show a large variation in the numbers of both macro- and microchromosomes ($2n=30$–44) within the same taxon (Olmo and Signorino 2005). However, the degree of conservation of synteny and gene order of anguimorph chromosomes relative to snake and iguanian chromosomes, and the pattern of karyotype evolution in anguimorphs, remain unknown. Comparative gene mapping in anguimorphs, snakes, and iguanians is necessary

to clarify the process of karyotype evolution in the Toxicofera clade.

The monitor lizards (varanid lizards, *Varanus* sp., Varanidae), an ancient group of anguimorph lizards, inhabit Afro-Arabia, Western to Southeast Asia, the Indonesian Archipelago, Papua New Guinea, and Australia (Ast 2001; Townsend et al. 2004; Amer and Kumazawa 2008). Currently, the Varanidae family comprises 73 extant species (Uetz 2013). According to King and King (1975), the chromosome number of all monitor lizards is $2n=40$: these karyotypes comprise 16 macro- and 24 microchromosomes. The only variation in their karyotypes is in the morphology of the macrochromosomes. The karyotype of the water monitor lizard (*Varanus salvator macromaculatus*) comprises two pairs of large metacentrics (1st and 2nd), two pairs of medium-sized metacentrics (3rd and 4th), one pair of small acrocentrics (5th), three pairs of small submetacentrics (6th–8th), and 12 pairs of microchromosomes. This is considered the primitive form of varanid lizard karyotypes (King and King 1975; Chaiprasertsri et al. 2013). However, the karyotype of the Savannah monitor lizard (*Varanus exanthematicus*) differs in several pairs of small macrochromosomes from that of *V. salvator macromaculatus* (King and King 1975). This suggests that the two monitor lizards might be good models to elucidate the process of chromosomal changes in the lineage of *Varanus*. Here, we construct a comparative cytogenetic map of functional genes for *V. salvator macromaculatus* using fluorescence in situ hybridization (FISH), and examine its conserved synteny with *L. reevesii rubritaeniata* and *E. quadrivirgata* chromosomes. Using comparative mapping data from these species, we delineate the process of karyotype evolution in the Toxicofera clade. Additionally, we also construct a cytogenetic map of *V. exanthematicus* chromosomes 6–8, to identify the chromosomal rearrangements that occurred between the two monitor lizard species.

Materials and methods

Specimens, cell culture, and chromosome preparation

Fibroblasts from a male water monitor lizard (*V. salvator macromaculatus*) used in the work of Chaiprasertsri et al. (2013) were recovered from liquid nitrogen and cultured. A male Savannah monitor lizard (*V. exanthematicus*) was purchased from a pet shop in Nagoya, Japan. Morphologic identification of the species was performed as previously described (Bennett and Thakoordyal 2003; and data not shown). After intraperitoneal injection of pentobarbital, the heart, lungs, and mesenteries were removed and used for cell culture. The animal's gender was identified morphologically, and confirmed by its internal anatomy. All experimental procedures using animals conformed to guidelines established by the Animal Care and Use Committee, Nagoya University, Japan. The tissues were minced, and cultured in Gibco® Dulbecco's modified Eagle's medium (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 15 % Gibco® Fetal Bovine Serum (Life Technologies Corporation), 100 µg/ml kanamycin, and 1 % Gibco® Antibiotic-Antimycotic (Life Technologies Corporation). The cultures were incubated at 26 °C in a humidified atmosphere of 5 % CO_2 in air. Primary cultured fibroblasts were harvested using trypsin and subcultured. For replication R-banding, fibroblast cultures from the two species were incubated with 5-bromo-2′-deoxyuridine (12 µg/ml; Sigma-Aldrich Corporation, St. Louis, MO, USA) for 12 h, including 45 min of colcemid treatment (120 ng/ml) before harvesting, and chromosome preparations were made following a standard air-drying method. After staining the chromosome slides with Hoechst 33258 (1 µg/ml) for 8 min, the slides were heated at 65 °C for 3 min, and exposed to ultraviolet light at 65 °C for an additional 6 min (Matsuda and Chapman 1995). The slides were kept at −80 °C until use.

Molecular identification of *V. exanthematicus*

For the molecular identification of *V. exanthematicus*, partial DNA fragments of the mitochondrial *ND2* gene were cloned, and their nucleotide sequences determined. Whole genomic DNA was extracted from the liver of a male *V. exanthematicus* following a standard salting-out protocol, and used as a template for polymerase chain reaction (PCR). Primers and PCR conditions were previously described (Kumazawa and Endo 2004). The PCR products were cloned using the pGEM®-T-Easy Vector System I (Promega Corporation, Madison, WI, USA), and the nucleotide sequences of the DNA fragments were determined using an ABI 3130 Automated Capillary DNA Sequencer (Applied Biosystems, Life Technologies Corporation). The nucleotide sequences were used to search the National Center for Biotechnology Information (NCBI) database using the

BLASTx and BLASTn programs (http://blast.ncbi.nlm. nih.gov/Blast.cgi), and were deposited in the DNA Data Bank of Japan (AB795272; http://www.ddbj.nig.ac.jp/ index-e.html). The nucleotide sequences of the *V. exanthematicus* mitochondrial *ND2* gene were aligned with those of other platynotan lizards and outgroups, comprising the banded gecko (*Coleonyx variegatus*), American alligator (*Alligator mississippiensis*), Siamese crocodile (*C. siamensis*), and domestic chicken (*G. gallus*) taken from the NCBI database (Supplementary Table S1). All unalignable and gap-containing sites were carefully removed, and sequence divergence between the species was estimated using the default parameters of Molecular Evolutionary Genetics Analysis 4 software (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA; Kumar et al. 2004), using uncorrected pairwise distances.

Cross-species chromosome mapping

The chromosomal locations of the 18S–28S and 5S ribosomal RNA (rRNA) genes, telomeric (TTAGGG)*n* sequences, and 86 functional genes were determined by FISH, as previously described (Matsuda and Chapman 1995; Srikulnath et al. 2009a). cDNA fragments cloned from *L. reevesii rubritaeniata* (54 genes; Srikulnath et al. 2009a, b), *Gekko hokouensis* (one gene), *Lacerta agilis* (30 genes; Srikulnath et al., unpublished data), and *E. quadrivirgata* (one gene; Matsubara et al., unpublished data; Table 1; Supplementary Table S2), and a 99-base pair (bp) fragment (pCSI5S; EU723235) of the *C. siamensis* 5S rRNA genes were used for chromosome mapping. Nucleotide sequence homologies of all reptilian cDNA fragments with those of the chicken were examined and the cDNA fragments were confirmed to be homologs of chicken genes. We labeled 250 ng of cDNA or 5S rDNA probe with biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland) by nick translation following the manufacturer's protocol. After hybridization, the probes were detected with goat anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA), and stained with Alexa Fluor® 488 rabbit anti-goat IgG (H+L) conjugate (Molecular Probes®, Life Technologies Corporation). Slides were subsequently counterstained with 0.75 μg/ml propidium iodide. Hybridization signals were captured using a cooled charge-coupled device (CCD) camera mounted on a Nikon fluorescence microscope (Nikon Corporation,

Tokyo, Japan), and processed using software by Nikon Microsystems Imaging Solutions Ltd. (Tokyo, Japan).

Dual-color FISH was performed to compare the chromosomal locations of the 18S–28S rRNA genes and telomeric (TTAGGG)*n* sequences in *V. salvator macromaculatus* and *V. exanthematicus*. A partial 1.8-kb fragment (pCSI1) of the 8.2-kb fragment (EU727190) of the *C. siamensis* 18S–28S rRNA genes, and a biotin-labeled 42-bp oligonucleotide complementary to (TTAGGG)*n* sequences (Sigma-Aldrich Corporation), were used as probes. We labeled 250 ng of the 18S–28S rDNA probe with digoxigenin-11-dUTP (Roche Diagnostics) and hybridized to *V. salvator macromaculatus* and *V. exanthematicus* chromosomes with the biotin-labeled TTAGGG repeats. After hybridization, the digoxigenin- and biotin-labeled probes were stained with anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics) and avidin labeled with fluorescein isothiocyanate (avidin-FITC; Vector Laboratories), respectively. Fluorescence hybridization signals were captured using a cooled CCD camera mounted on a Leica DMRA microscope (Leica Microsystems, Wetzlar, Germany), and processed using 550CW-QFISH software by Leica Microsystems Imaging Solutions Ltd. (Cambridge, UK).

Results

Molecular identification of *V. exanthematicus*

The data set of aligned *ND2* fragments showed 761 nucleotides, comprising 63 variable sites and 472 parsimony informative sites. The degree of sequence divergence between the *V. exanthematicus ND2* fragment obtained in this study (AB795272) and that previously deposited in the database (AF407496) was 1 % (data not shown). This result confirmed that the animal used in this study was *V. exanthematicus*, considering that intraspecific variation of *ND2* sequences was shown to be about 10 % in lizards (Glor and Laport 2012).

Karyotypes of *V. salvator macromaculatus* and *V. exanthematicus*

We examined over 20 Hoechst-stained metaphase spreads for one male *V. salvator macromaculatus* and one male *V. exanthematicus*. Diploid chromosome numbers were 40 in both species, and morphologic

Table 1 List of 86 cDNA clones mapped to *Varanus salvator macromaculatus* chromosomes and their chromosomal locations in the butterfly lizard (*Leiolepis reevesii rubritaeniata*), the Japanese four-striped rat snake (*Elaphe quadrivirgata*), and chicken (*Gallus gallus*)

Gene symbol	Sequenced length of cDNA fragment (bp)	Chromosomal location				Accession number
		V. salvator macromaculatus	*L. reevesii rubritaeniata*	*E. quadrivirgata*	Chicken	
ATP5A1[a]	987	1p	2p23.1–p23.3	2p	Zp	AB480291 and AB480292
GHR[b]	771	1p	2p22–p23.3	2p	Zp	AB480290
CHD1[b]	961	1p	2p21	2p	Zq	AB480289
DMRT1[b]	628	1p	2p12–p21	2p	Zp	AB480288
RPS6[b]	521	1p	2p11.3–p12	2p	Zp	AB480287
ACO1/IREBP[a]	1,122	1p	2p11.2–p11.4	2p	Zq	AB480285 and AB480286
SOX9[b,f]	603, 717	1q	2q11.2–q11.4	2q	18	AB490350 and AB490351
TOB1[c]	950	1q	_[g]	_[h]	18	AB794087
RUFY1[b]	545	1q	2q12.2–q21.1	2q	13	AB490352
TKT[b]	943	1q	2q11.1	2q	12	AB490349
ALAS1[c]	1,060	1q	_[g]	_[h]	12	AB794074
BRD2[b]	691	1q	2q22.2	_[h]	16	AB480353
XAB1[b]	489	2p	1p22.4	1p	3	AB490344
FBXW11[d]	926	2p	_[g]	1p	13	AB792691
ESR1[b]	951	2p	1p21.2	1p	3	AB490345
ACVR1[c]	845	2p	_[g]	_[h]	7	AB794073
CACNB4[b]	882	2q	1q12.2–q13.1	1q	7	AB490346
WT1[b]	542	2q	1q21.1–q22.1	1q	5	AB490347
DYNC1H1[b]	997	2q	1q32.1–q32.3	1q	5	AB490348
CYP2C21-like[c]	1,331	2q	_[g]	_[h]	_[i]	AB794068
SOX5[b,f]	851, 705	3p	5q22.1–q22.4	_[h]	1p	AB490376 and AB490377
TTC26[b]	772	3p	5q21.3–q22.3	6q	1p	AB490375
RANGAP1[b]	1,024	3p	5q21.2–q22.1	6q	1p	AB490374
DYRK2[b]	1,086	3p	5q12–q21.2	_[h]	1p	AB490373
UCHL1[b]	595	3p	5p11.1–p12.1	6p	4q	AB490372
EXOC1[b]	1,177	3q	5p11.2–p12.2	7p	4q	AB490371
ACSL1[b]	748	3q	5p12.1–p12.2	7q	4q	AB490370
DCLK2[b]	688	3q	5p12.1–p21	_[h]	4q	AB490369
RAP1GDS1[b]	991	3q	5p21–p22.2	7q	4q	AB490368
SMAD1[c]	944	3q	_[g]	_[h]	4q	AB794085
MYST2[b]	1,261	4p	6p21.1–p22.2	Zq	27	AB490378
STAT3[c]	1,654	4p	_[g]	_[h]	27	AB793734
TOP2A[c,f]	882, 477	4p	_[g]	_[h]	27	AB793735 and AB793736
TOP2B[c]	1,639	4q	_[g]	_[h]	2p	AB793737
CTNNB1[b]	1,201	4q	6q11	Zp	2p	AB490379
WAC[b]	1,122	4q	6q21–q23	Zp	2p	AB490381
GAD2[b]	672	4q	6q21–q23	Zp	2p	AB490380
TPT1[b]	438	5	3p11.1–q11	4p	1q	AB490359
IPO5[c,f]	703, 492	5	_[g]	_[h]	1q	AB793729 and AB793730
EIF2S3[b]	733	5	3q12.3–q21.1	4p	1q	AB490361
OCA2[b]	782	5	3q12.1–q12.2	_[h]	1q	AB490360
ELMOD1[b]	682	5	3q22.1–q22.3	4q	1q	AB490362

Table 1 (continued)

Gene symbol	Sequenced length of cDNA fragment (bp)	Chromosomal location				Accession number
		V. salvator macromaculatus	*L. reevesii rubritaeniata*	*E. quadrivirgata*	Chicken	
ADAM12[c]	933	6q	_[g]	_[h]	6	AB794067
PSAP[b]	1,325	6q	3p11.2–p12.2	5q	6	AB490358
BTRC[b]	889	6q	3p21.1–p21.2	_[h]	6	AB490357
SH3PXD2A[b]	1,210	6q	3p22.1	5q	6	AB490356
SLIT[e]	995	6q	_[g]	_[h]	6	AB794071
EPHA4[c]	888	6q	_[g]	_[h]	9	AB794079
SKIL[c]	1,686	6q	_[g]	_[h]	9	AB794070
TLOC1[b]	782	6q	3p22.3	5q	9	AB490355
NCL[c]	1,780	6q	_[g]	_[h]	9	AB794069
HDAC3[b]	929	7p	4p11.1–p11.2	3p	13	AB490365
SS18[b,f]	614, 508	7q	4p12.2–p21.2	3p	2q	AB490364 and AB490397
ENPP2[b]	961	7q	4p22.2–p22.4	3p	2q	AB490363
ZNF326[b]	892	8p	4q12.1–q12.3	3q	8	AB490366
RPE65[c]	1,130	8p	_[g]	_[h]	8	AB793733
CNTN2[c]	922	8q	_[g]	_[h]	26	AB793728
USP49[c]	1,210	8q	_[g]	_[h]	26	AB794088
RBM12[b]	943	8q	4q21.2–q22.1	3q	20	AB490367
RPN2[c]	1,229	8q	_[g]	_[h]	20	AB794084
CUL4B[b]	723	Micro	Micro	_[h]	4p	AB490387
ATRX[b]	804	Micro	Micro	Micro	4p	AB490386
AR[b]	941	Micro	Micro	Micro	4p	AB490385
CHD2[b,f]	654, 692	Micro	Micro	_[h]	10	AB490388 and AB490389
POLG[c]	1,565	Micro	_[g]	Micro	10	AB794083
BRD7[b]	784	Micro	Micro	Micro	11	AB490390
EEF2K[c]	970	Micro	_[g]	Micro	14	AB794077
UBN1[c]	1,153	Micro	_[g]	_[h]	14	AB794072
PDXDC1[c]	1,619	Micro	_[g]	_[h]	14	AB794082
NF2[b]	940	Micro	Micro	_[h]	15	AB490393
SF3A1[b]	937	Micro	Micro	_[h]	15	AB490394
ATP2A2[b]	972	Micro	Micro	Micro	15	AB490391
SBNO1[b]	1,345	Micro	Micro	_[h]	15	AB490392
DNM1[c]	1,014	Micro	_[g]	Micro	17	AB794076
GRIN1[c]	893	Micro	_[g]	Micro	17	AB794080
TRIM37[b,f]	631, 577	Micro	Micro	_[h]	19	AB490382 and AB490383
AMH[e,f]	712, 709	Micro	_[g]	_[h]	19	AB794387 and AB794388
ENO1[c]	917	Micro	_[g]	Micro	21	AB794078
TMEM57[c]	800	Micro	_[g]	_[h]	23	AB794086
RNF19B[c]	942	Micro	_[g]	_[h]	23	AB793732
HSPA8[b]	906	Micro	Micro	Micro	24	AB490395
ARNT[c]	1,034	Micro	_[g]	_[h]	25	AB794075
EEF2[b]	975	Micro	Micro	1q	28	AB490384
KRT8[c]	835	Micro	_[g]	_[h]	_[i]	AB794081

Table 1 (continued)

Gene symbol	Sequenced length of cDNA fragment (bp)	Chromosomal location				Accession number
		V. salvator macromaculatus	*L. reevesii rubritaeniata*	*E. quadrivirgata*	Chicken	
ACTN4[b]	1,069	Micro	Micro	_[h]	_[i]	AB490396
PPP2R1A[c]	1,169	Micro	_[g]	_[h]	_[i]	AB793731

[a] Nucleotide sequences of cDNA fragments of *L. reevesii rubritaeniata* were taken from Srikulnath et al. (2009a). For mapping of *ATP5A1*, and *ACO1/IREBP*, two different fragments of the gene were simultaneously hybridized to chromosomes

[b] The cDNA fragments were obtained from of *L. reevesii rubritaeniata* in Srikulnath et al. (2009a, b)

[c] The cDNA fragments were obtained from *Lacerta agilis* in Srikulnath et al. (unpublished data)

[d] The cDNA fragments were obtained from of *Gekko hokouensis* in Srikulnath et al. (unpublished data)

[e] The cDNA fragments were obtained from of *E. quadrivirgata* in Matsubara et al. (unpublished data)

[f] Nucleotide sequences of two accession numbers were determined separately by forward and reverse primers in one clone

[g] No data in the *L. reevesii rubritaeniata*

[h] No data in the *E. quadrivirgata*

[i] No data in chicken

differences were evident in three pairs of chromosomes (6th–8th) between the two species (Fig. 1). The eight pairs of macrochromosomes of *V. salvator macromaculatus* comprised two pairs of large metacentrics (1st and 2nd), two pairs of metacentrics (3rd and 4th), one pair of acrocentrics (5th), and three pairs of submetacentrics (6th–8th; King and King 1975; Chaiprasertsri et al. 2013), whereas the 6th and 8th chromosomes were acrocentric and the 7th submetacentric in *V. exanthematicus* (Fig. 1). Our data on the *V. exanthematicus* karyotype were inconsistent with a previous report (King and King 1975), which described chromosomes 6–8 as acrocentric.

Cross-species chromosome mapping of cDNA clones in *V. salvator macromaculatus*, and a cytogenetic map of *V. salvator macromaculatus*

We constructed a cytogenetic map of *V. salvator macromaculatus* with 86 genes (Figs. 2 and 3). This is the first reported cytogenetic map in anguimorph lizards. Over 30 metaphase spreads were observed for each gene, and hybridization efficiency ranged from approximately 20–80 %. Chromosome homology between *V. salvator macromaculatus* and the chicken was analyzed using the chicken genome database (http://www.ncbi.nlm.nih.gov/genome/guide/chicken/). Twelve genes mapped on *V. salvator macromaculatus*

(VSA) chromosome 1 localized to chicken (GGA) chromosomes Z, 12, 13, 16, and 18. Eight genes mapped on VSA2 localized to GGA3, 5, 7, and 13, and ten genes on VSA3 were located on GGA1p and 4q. Seven genes on VSA4 localized to GGA2p and 27, and five genes on VSA5 to GGA1q. Nine genes mapped on VSA6 localized to GGA6 and 9, three genes on VSA7 to GGA2q and 13, and six genes on VSA8 to GGA8, 20 and 26. Twenty-six genes mapped on VSA microchromosomes were located on GGA microchromosomes or GGA4p that was derived from a microchromosome of the ancestral avian karyotype (Nishida-Umehara et al. 2007).

Chromosomal locations of the 18S–28S ribosomal RNA genes and telomeric (TTAGGG)*n* sequences in *V. salvator macromaculatus* and *V. exanthematicus*

The 18S–28S rRNA genes localized to the secondary constriction in the proximal region of *V. salvator macromaculatus* and *V. exanthematicus* chromosome 1p. Hybridization signals for (TTAGGG)*n* sequences were observed at the telomeric ends of all chromosomes, and no interstitial signals were found (Fig. 4). To the best of our knowledge, this is the first study to report the chromosomal location of the 18S–28S rRNA genes and (TTAGGG)*n* sequences in varanid lizards.
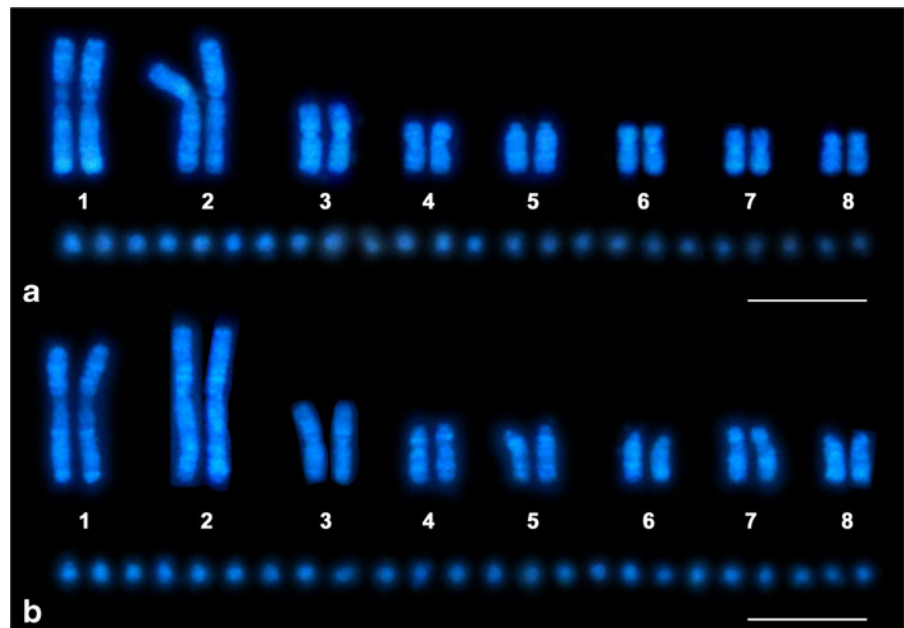
Comparative cytogenetic map between *V. salvator macromaculatus* and *V. exanthematicus*

Hoechst-stained karyotypes of *V. salvator macromaculatus* and *V. exanthematicus* revealed morphologic differences in chromosomes 6–8 (Fig. 1), indicating that rearrangements occurred in these chromosomes between the two species. To compare the chromosome structures and delineate the chromosomal rearrangements that occurred in chromosomes 6–8 between *V. salvator macromaculatus* and *V. exanthematicus* (VEX), we mapped nine genes to VEX6, three genes to VEX7, and five genes to VEX8, and constructed a comparative cytogenetic map of VSA and VEX (Figs. 5 and 6).

Discussion

Comparison of conserved synteny of *V. salvator macromaculatus* with other Toxicofera species and the chicken

Comparison of syntenies and gene orders of *V. salvator macromaculatus* with those of the chicken and two squamate reptiles (*L. reevesii rubritaeniata* and *E. quadrivirgata*) revealed that 11 syntenic groups of chicken macrochromosomes (GGA1p, 1q, 2p, 2q, 3, 4q, 5, 6, 7, 8, Z) were highly conserved in eight macrochromosomes and/or chromosome arms of *V.*

salvator macromaculatus (VSA1p, 2, 3, 4q, 5, 6q, 7q, and 8p), as well as *L. reevesii rubritaeniata* and *E. quadrivirgata* (Matsubara et al. 2006, 2012; Srikulnath et al. 2009b; Fig. 7). Genes that mapped to microchromosomes in *V. salvator macromaculatus* localized to chicken microchromosomes or GGA4p, whereas homologous segments of 19 chicken microchromosome-linked genes localized to VSA1q, 2p, 4p, 6q, 7p, and 8q (Table 1). Similarly, all genes that localized to *L. reevesii rubritaeniata* and *E. quadrivirgata* microchromosomes were also located on chicken microchromosomes (Matsubara et al. 2006, 2012, unpublished data; Srikulnath et al. 2009b). These results indicate that approximately half of chicken microchromosomes showed homology with macrochromosomes in the three Toxicofera species.

Comparing the chromosome maps of *L. reevesii rubritaeniata* (LRE) and *E. quadrivirgata* (EQU) with that of *V. salvator macromaculatus*, we deduced the process of karyotype reorganization among these species by the most parsimonious explanation of chromosomal rearrangements. VSA5 was homologous to LRE3q and EQU4, and VSA6 to LRE3p and EQU5. The occurrence of VSA5 and VSA6 or EQU4 and EQU5 could be explained by centric fission of an ancestral bi-armed macrochromosome homologous to LRE3 (Figs. 7 and 8a). Alternatively, LRE3 resulted from centric fusion between the acrocentric proto-VSA6 and VSA5, or between the acrocentric proto-
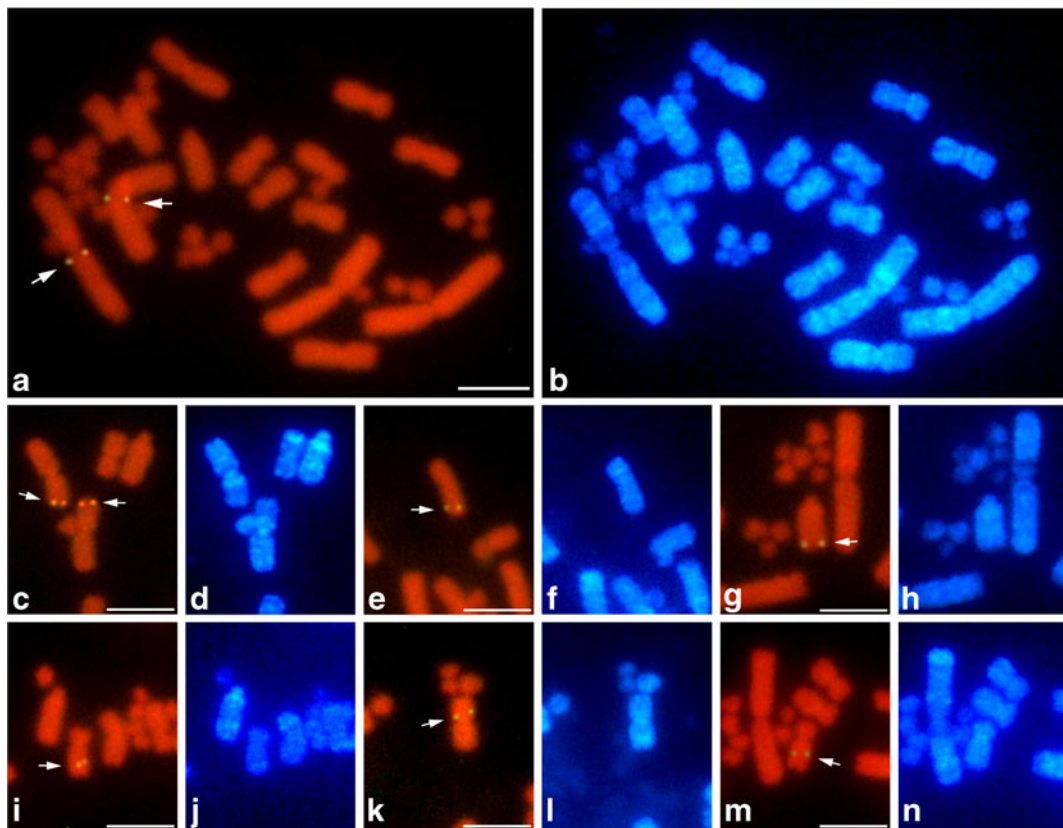
Fig. 2 Chromosomal locations of cDNA fragments of functional genes in *V. salvator macromaculatus*. The *SOX9* gene was located on chromosome 1q (**a**), *SOX5* on chromosome 3p (**c**), *WAC* on chromosome 4q (**e**), *ELMOD1* on chromosome 5 (**g**), *EPHA4* on chromosome 6q (**i**), *HDAC3* on chromosome 7p (**k**), and *USP49* on chromosome 8q (**m**). **b**, **d**, **f**, **h**, **j**, **l**, **n** Hoechst-stained patterns of the same metaphase spreads shown in (**a**), (**c**), (**e**), (**g**), (**i**), (**k**), and (**m**), respectively. *Arrows* indicate hybridization signals. *Scale bars* represent 5 μm

EQU4 and EQU5. VSA6 might have resulted from pericentric inversion or centromere repositioning in the acrocentric proto-VSA6, and EQU4 from centromere repositioning in the acrocentric proto-EQU4. Another plausible explanation is that LRE3 derived from the tandem fusion of two ancestral chromosomes, which remain as VSA5 and VSA6 and EQU4 and EQU5, followed by inactivation of one of the two centromeres on the derived dicentric chromosome (Fig. 8b).

EXOC1 and *UCHL1* near the centromere of LRE5p localized separately to VSA3q and VSA3p, respectively, and *EXOC1* to EQU7p, and *UCHL1* to EQU6p (Fig. 8c; Matsubara et al. 2006, 2012; Srikulnath et al. 2009b; this study). This implies that a small pericentric inversion occurred in VSA3, followed by centric fission in the lineage of *E. quadrivirgata*. The present forms of EQU6 and EQU7 might have resulted from pericentric inversion or centromere repositioning in the fission-derived acrocentric proto-EQU6 and proto-EQU7. Also plausible is that VSA3 resulted from centric fusion of the acrocentric proto-EQU6 and proto-EQU7, followed by pericentric inversion, leading to LRE5.

VSA7 corresponded to LRE4p and EQU3p, and VSA8 to LRE4q and EQU3q. VSA7 and VSA8 might have derived from centric fission of an ancestral bi-armed macrochromosome homologous to LRE4 and EQU3, followed by pericentric inversion or centromere repositioning in the acrocentric proto-VSA7 and proto-VSA8 (Fig. 9). Another plausible mechanism is centric fusion of the acrocentric proto-VSA7 and proto-VSA8, leading to LRE4 and EQU3.

VSA1 was homologous to LRE2 and EQU2, VSA2 to LRE1 and EQU1, and VSA4 to LRE6 and EQUZ (Fig. 7). However, microchromosome-linked genes in *V. salvator macromaculatus* and *L. reevesii rubritaeniata*, *TRIM37* and *AMH* (GGA19-linked gene homologs) and
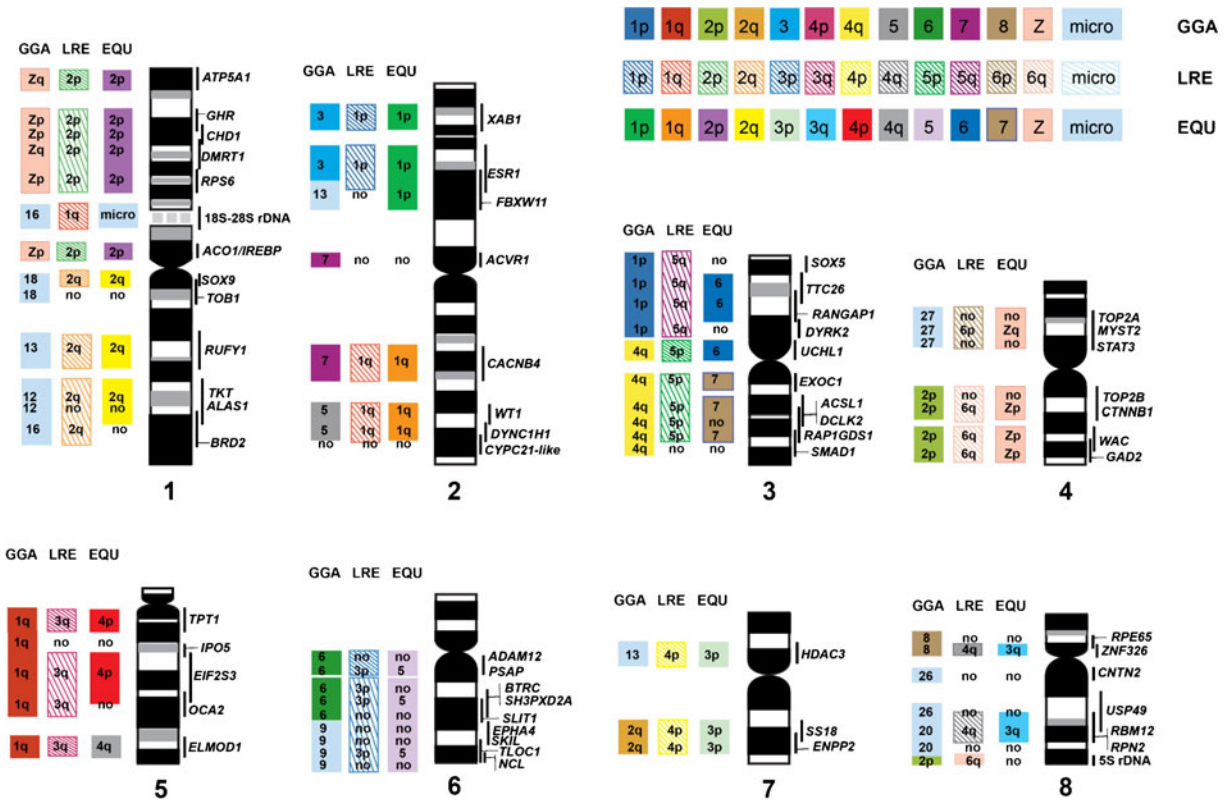
Fig. 3 Comparative cytogenetic map of *V. salvator macromaculatus* constructed with 60 functional genes and the 18S–28S and 5S ribosomal RNA genes. The ideograms of *V. salvator macromaculatus* macrochromosomes were constructed according to Hoechst-stained band patterns. Locations of the genes on *V. salvator macromaculatus* chromosomes are shown to the *right* of chromosomes. The chromosome numbers of the chicken (*G. gallus*, GGA), butterfly lizard (*L. reevesii rubritaeniata*, LRE), and Japanese four-striped rat snake (*E. quadrivirgata*, EQU), which show homologies with *V. salvator macromaculatus* chromosomes, are shown to the *left*. "no" indicates no data of chromosome homology, and "un" indicates a gene for which the chromosomal location has not been determined. The chromosomal locations of *L. reevesii rubritaeniata* genes were taken from Srikulnath et al. (2009b). The chromosomal locations of *E. quadrivirgata* genes were taken from Matsubara et al. (2006, 2012)

EEF2 (GGA28-linked gene homolog), localized to the distal ends of the short and long arms of EQU1, respectively (Fig. 7; Table 1). This result suggests that tandem fusion of microchromosomes to macrochromosomes occurred in both the p and q arms of EQU1 after this lineage diverged from the common ancestor of iguanian and anguimorph lizards. The chromosomal location of the GGA12-linked *TKT* gene in VSA1q differed from that in LRE2q and EQU2q: *TKT* localized to the proximal regions of LRE2q and EQU2q, although homology with GGA12 was found in the distal regions of chromosome 2q of *E. quadrivirgata* (Fig. 3) and *A. carolinensis* (Matsubara et al. 2006, 2012; Alföldi et al. 2011). The order of *SOX9* and *RUFY1* on LRE2q, EQU2q, and VSA1q did not differ between the three species,

suggesting that at least two paracentric inversions occurred sequentially in the ancestral chromosome of LRE2 and EQU2: a large paracentric inversion that first occurred at the breakpoints near the centromere and within the region homologous to GGA12 in the q arm of chromosome 2, followed by a paracentric inversion in the region containing *SOX9* and *RUFY1*. High-resolution gene mapping in this chromosomal region is necessary to confirm this possibility.

Comparison of chromosome structures in *V. salvator macromaculatus* and *V. exanthematicus*

The chromosome number of *V. salvator macromaculatus* and *V. exanthematicus* was shown to
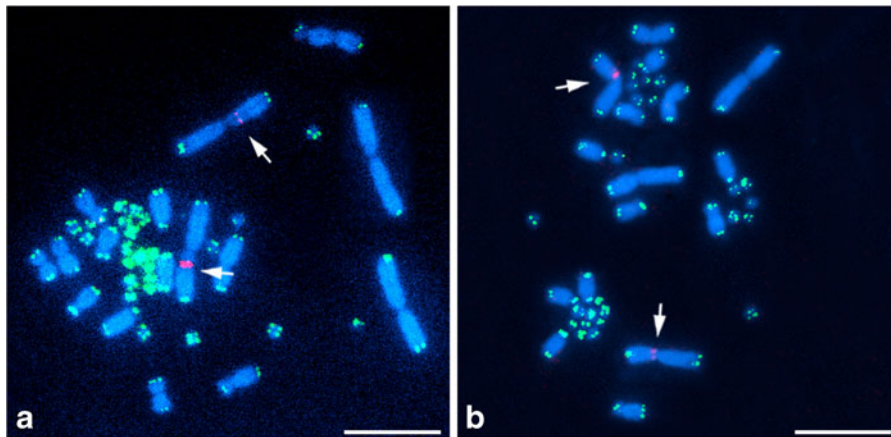
Fig. 4 Chromosomal locations of the 18S–28S ribosomal RNA genes and (TTAGGG)n sequences in a *V. salvator macromaculatus* male (**a**) and *V. exanthematicus* male (**b**). Hybridization patterns of rhodamine-labeled 18S–28S ribosomal RNA (rRNA) genes (*red*) and fluorescein isothiocyanate-labeled (TTAGGG)n sequences (*green*) on Hoechst-stained metaphase spreads. *Arrows* indicate the hybridization signals of the 18S–28S rRNA genes. *Scale bar* represents 10 μm

be 2n = 40, which comprises 8 pairs of macrochromosomes and 12 pairs of microchromosomes. This karyotypic feature is conserved throughout the genus *Varanus* (King and King 1975; Chaiprasertsri et al. 2013). The 18S–28S rRNA genes localized to the secondary constriction in the proximal region of *V. salvator macromaculatus* and *V. exanthematicus* chromosome 1p. These results were the same as that of *V. acanthurus*, in which the same chromosomal region was stained by Ag-NOR staining (King
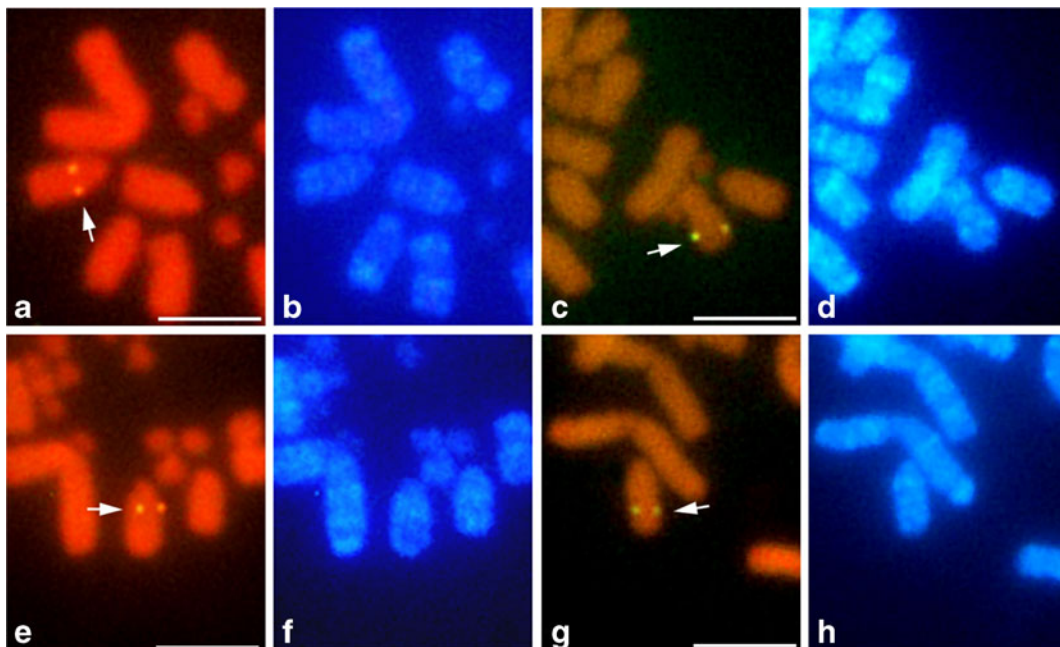

Fig. 5 Chromosomal locations of cDNA fragments of functional genes in a *V. exanthematicus* male. The *ADAM12* gene was located on chromosome 6 (**a**), *ENPP2* on chromosome 7q (**c**), *ZNF326* on chromosome 8q (**e**), and *RPN2* on chromosome 8q (**g**). **b, d, f, h** Hoechst-stained patterns of propidium iodide-stained metaphase spreads are shown in (**a**), (**c**), (**e**), and (**g**), respectively. *Arrows* indicate hybridization signals. *Scale bars* represent 5 μm
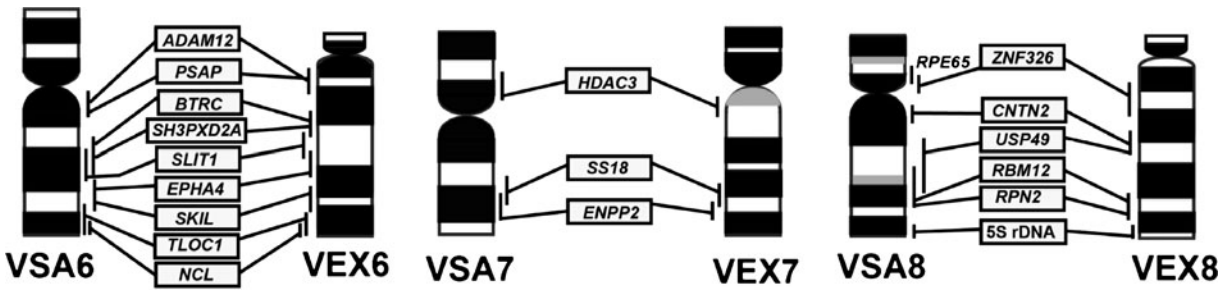
Fig. 6 Comparative cytogenetic maps of chromosomes 6–8 in *V. salvator macromaculatus* and *V. exanthematicus*, which were constructed with 17 functional genes and the 5S rRNA genes

et al. 1982). According to King and King (1975), the karyotypes of varanid lizards can be classified into six groups, based on the morphology of macrochromosomes. The *salvator* group, containing *V. salvator macromaculatus*, was located at the basal position of the karyotypic phylogeny of varanid lizards,

and *V. exanthematicus* (*gouldii* group) was positioned at the terminus of the tree. However, this karyological relationship was not in accordance with previously reported molecular phylogeny (Ast 2001), which indicated that *V. exanthematicus* was located at the basal position of the tree. King and King (1975) suggested that
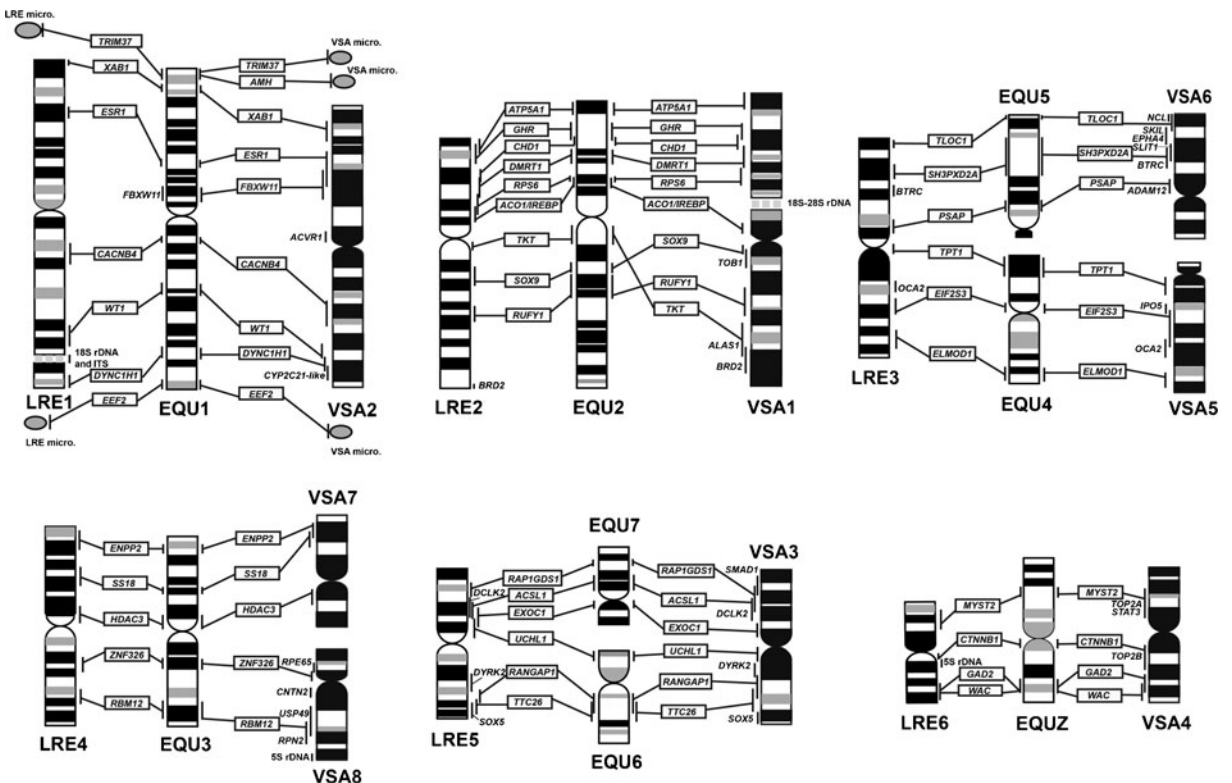

Fig. 7 Comparative cytogenetic maps of macrochromosomes among *V. salvator macromaculatus*, *L. reevesii rubritaeniata*, and *E. quadrivirgata*, constructed with 63 functional genes. The chromosome map of *L. reevesii rubritaeniata* (LRE) was taken from Srikulnath et al. (2009b). The ideograms of *E. quadrivirgata* (EQU) macrochromosomes were taken from Matsuda et al. (2005), and chromosomal locations of the genes from Matsubara et al. (2006, 2012). *V. salvator macromaculatus* (VSA) 3, VSA6, VSA7, EQU5, EQU7, and EQUZ are inverted to facilitate comparison
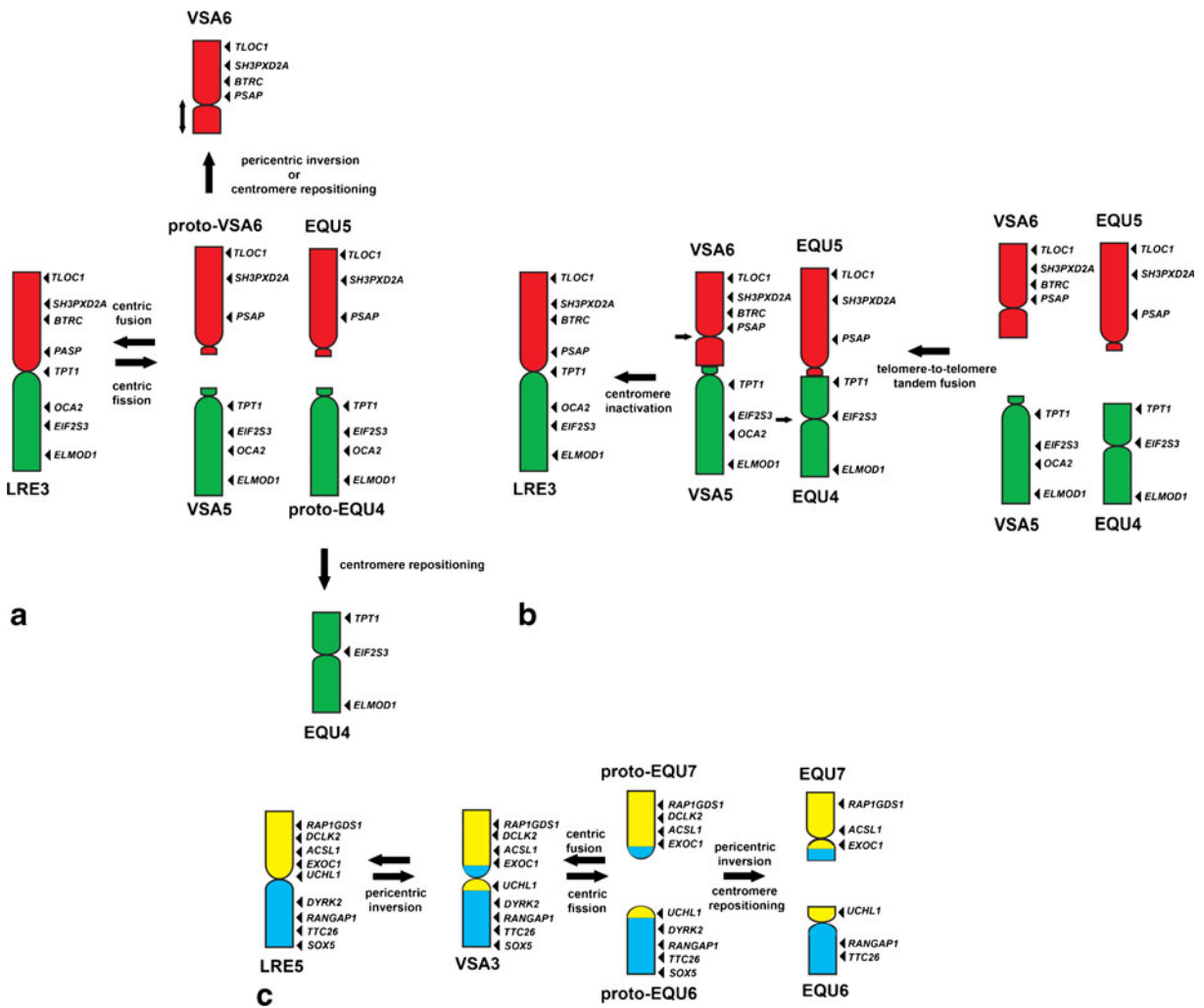
Fig. 8 Schematic representation of the process of chromosomal rearrangements that occurred in *V. salvator macromaculatus* chromosomes 3, 5, and 6 (VSA3, VSA5, and VSA6), *L. reevesii rubritaeniata* chromosomes 3 and 5 (LRE3 and LRE5), and *E. quadrivirgata* chromosomes 4–7 (EQU4–EQU7). VSA3, VSA6, EQU5, and EQU7 are inverted to facilitate comparison. Chromosomal locations of the genes are shown to the *right* of chromosomes by *arrowheads*. The syntenies and gene orders of LRE3p, LRE3q, LRE5p, and LRE5q, their homologous chromosomes, and chromosome arms and/or segments are shown in *red*, *green*, *yellow*, and *blue*, respectively. **a** The diagram schematically summarizes the occurrence of LRE3 and VSA5, VSA6, EQU4, and EQU5 by two different processes of chromosomal rearrangements. One is a centric fission event of the bi-armed, followed by subsequent centromere repositioning in acrocentric proto-EQU4, and pericentric inversion or centromere repositioning in acrocentric proto-VSA6. The alternative process is that LRE3 was derived from centric fusion between VSA5 and acrocentric proto-VSA6, or between acrocentric proto-EQU4 and EQU5. The

bi-armed VSA6 and EQU4 probably resulted from the same process. *Arrows* indicate the directions of chromosomal rearrangement. The *bidirectional arrow* indicates the region where centromere repositioning or pericentric inversion might have occurred. **b** The diagram schematically summarizes the tandem fusion event between VSA5 and VSA6, or EQU4 and EQU5, followed by inactivation of one of two centromeres on the derived dicentric chromosomes, leading to LRE3. *Large arrows* indicate the direction of chromosomal rearrangements. *Small arrows* indicate centromeres that might have been inactivated in LRE3 after the tandem fusions. **c** Two different processes for the occurrence of LRE5, VSA3, EQU6, and EQU7. In the first, centric fission occurred in VSA3 after a small pericentric inversion, followed by pericentric inversion or centromere repositioning in the fission-derived acrocentric proto-EQU6 and proto-EQU7, leading to the bi-armed EQU6 and EQU7 (Srikulnath et al. 2009b). Alternatively, centric fusion occurred between acrocentric proto-EQU6 and proto-EQU7, followed by pericentric inversion in VSA3, leading to LRE5
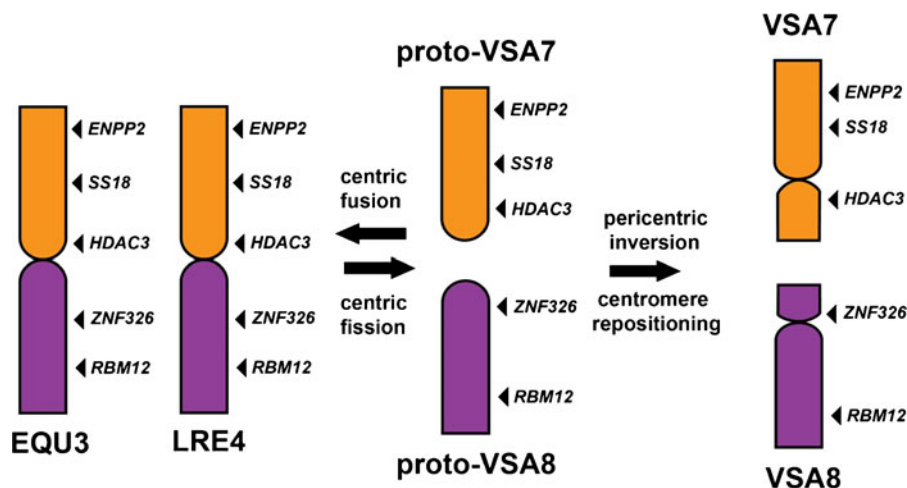
Fig. 9 Schematic representation of the process of chromosomal rearrangements that occurred in *V. salvator macromaculatus* chromosomes 7 and 8 (VSA7 and VSA8), *L. reevesii rubritaeniata* chromosome 4 (LRE4), and *E. quadrivirgata* chromosome 3 (EQU3). VSA7 is inverted to facilitate comparison. The syntenies and gene orders of LRE4p and LRE4q and their homologous chromosomes are shown in *orange* and *violet*, respectively. The

diagram schematically summarizes the centric fission event of EQU3 or LRE4, followed by pericentric inversion or centromere repositioning in acrocentric proto-VSA7 and proto-VSA8. Alternatively, centric fusion of acrocentric proto-VSA7 and proto-VSA8 occurred, leading to the bi-armed EQU3 or LRE4. The bi-armed VSA7 and VSA8 probably resulted from the same process. *Arrows* indicate the direction of chromosomal rearrangements

chromosomes tended to change from bi-armed to acrocentric by pericentric inversions in varanid lizards during their evolution. In this study, the hybridization signal of interstitial telomeric sites that appear to be remnants of chromosomal rearrangements such as fusion or inversion was not found in any chromosomes of the two varanid lizards, but Hoechst-stained karyotypes of *V. salvator macromaculatus* and *V. exanthematicus* revealed morphologic differences in chromosomes 6–8. The chromosomal rearrangements that occurred in chromosomes 6–8 between VSA and *V. exanthematicus* were then identified by comparative mapping with 17 functional genes. Although we could not determine the direction of chromosome reorganization, VEX6 and VEX8 seem to retain the acrocentric proto-VSA6 and proto-VSA8 (Figs. 8 and 9). The syntenies and gene orders of three chromosomes were highly conserved between the two species, whereas *HDAC3* on VSA7p localized to VEX7q and *ZNF326* on VSA8p to VEX8q, and all genes on the long arm of submetacentric VSA6 localized to acrocentric VEX6 (Fig. 6). The submetacentric VSA6 probably resulted from at least two pericentric

inversions: a large pericentric inversion in the region containing *ADAM12* and *PSAP* in VEX6, followed by an additional pericentric inversion. A pericentric inversion probably occurred between the submetacentric VSA7 and VEX7, and the morphologic difference between VSA8 and VEX8 could derive from a pericentric inversion or centromere repositioning. More comparative chromosome mapping data are required to resolve these issues.

Comparison of the cytogenetic maps of *V. salvator macromaculatus*, *L. reevesii rubritaeniata*, and *E. quadrivirgata*, and of *V. salvator macromaculatus* and *V. exanthematicus*, in this study revealed patterns of karyotype reorganization in the Toxicofera clade, and also in the lineage of varanid lizards. However, comparative gene mapping for more lacertilian species—especially the Gekkota and Lacertidae, which have karyotypes with few or no microchromosomes—is necessary for precise delineation of the process of karyotype evolution in Squamata. A greater understanding of the chromosome segments conserved among reptiles, and between reptiles and birds, will provide

insight into the phylogenetic hierarchy of genome evolution in amniotes.

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