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Chromosome banding in Amphibia

XXX. Karyotype aberrations in cultured fibroblast cells

M. Schmid,[a] C. Steinlein,[a] and T. Haaf[b]

[a] Department of Human Genetics, Biocenter, University of Würzburg, Würzburg;
[b] Department of Human Genetics, University of Mainz, Mainz (Germany)

Abstract. The present study reports for the first time on the numerical and structural chromosome anomalies that spontaneously arise in aging cultured fibroblast cells of Amphibia. The analyses were conducted on kidney fibroblasts of three anuran species with extremely divergent genome sizes *(Bufo rubropunctatus*, *Scaphiopus holbrooki*, *Gastrotheca riobambae)*, in the sixth up to the 14th culture passage. The chromosomal rearrangements were identified by means of the 5-bromodeoxyuridine/deoxythymidine (BrdU/dT) replication banding technique. The aberrations can be either confined to a single chromosome, or else involve all chromosomes of the karyotype. The most frequent structural aberrations in the cell cultures of *S. holbrooki* and *G. riobambae* are tandem fusions between two or more chromosomes. These tandem fusions originating in vitro in long-termed cell cultures reflect the chromosome mutations which also took place during amphibian phylogenesis.

The manifold studies on chromosome repatterning that occurred during the phylogeny of the Amphibia have been reviewed repeatedly (Morescalchi, 1973; Schmid, 1980; Schmid and Haaf, 1989; King, 1990). These comparative cytogenetic data permit close insights into the operant laws of karyological evolution in amphibians. Thus, the classical compilation of Morescalchi (1973) impressively demonstrates that in all three amphibian orders (Anura, Urodela, and Apoda) the number of microchromosomes and telocentric chromosomes was reduced in favor of large meta- or submetacentric chromosomes during the evolution from primitive to highly specialized genera. The evolution of karyotypes from the primitive genera, which still possess microchromosomes and telocentric chromosomes alongside a few meta- or submetacentric chromosomes, to the more highly evolved genera with exclusively biarmed chromosomes is explained by assuming a series of tandem fusions, centric (Robertsonian) fusions, and non-reciprocal translocations taking place between the chromosomes.

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Request reprints from M. Schmid, Department of Human Genetics
University of Würzburg, Biozentrum, Am Hubland
D–97074 Würzburg (Germany); telephone: +49-931-888-4077
fax: +49-931-888-4058; e-mail: m.schmid@biozentrum.uni-wuerzburg.de

The wealth of results obtained from these comparative cytogenetic studies has been facilitated mainly by the relatively easy and fast techniques of chromosome preparation. These methods consist in using either short-term cultures of lymphocytes or embryonic cells, or else directly the bone marrow, spleen, gut, cornea, or gonads of animals treated previously in vivo with colchicine. All these techniques reveal the constitutional karyotype of the individuals examined and, with some very few exceptions of occasionally found chromosome mutations, do not offer the possibility to detect systematically all the spontaneously occurring, age-related chromosomal changes.

In contrast, there is no single report in the literature describing the chromosome aberrations arising in long-term amphibian fibroblast cultures. The two major reasons for this are: (1) Amphibian fibroblasts possess only a limited growth efficiency under a variety of different culture conditions, the cells usually giving up mitotic divisions before reaching the fifth culture passage, and (2) the induction of multiple banding patterns with the classical G-, R- and Q-banding procedures for identification of the individual chromosomes is not possible in metaphase chromosomes of amphibians.

The results presented in this article describe the different categories of numerical and structural chromosome aberrations that spontaneously occur in kidney fibroblasts of the anuran species *Bufo rubropunctatus* (Bufonidae), *Scaphiopus holbrooki* (Pelobatidae) and *Gastrotheca riobambae* (Hylidae). The chro-

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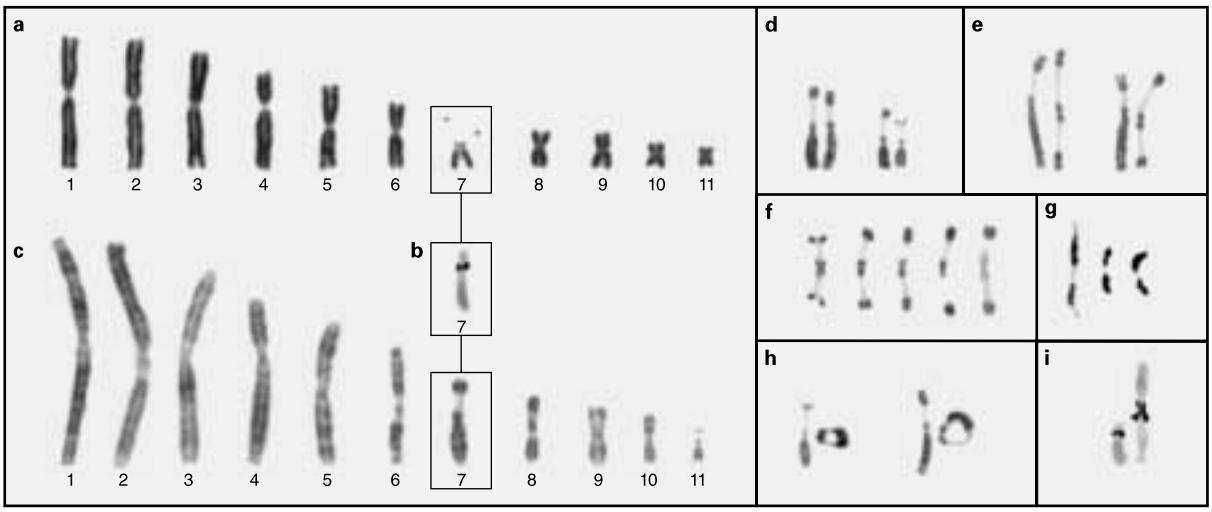

Fig. 1. (**a**) Giemsa-stained haploid karyotype of a male of *Bufo rubropunctatus*. (**b**) Silver-stained nucleolus organizer region (NOR) in the chromosome 7 short arm. (**c**) Haploid karyotype showing BrdU/dT replication banding patterns. (**d**) Two pairs of Giemsa-stained, normal structured chromosomes 7. (**e–f**) Aberrations of chromosomes 7 found in kidney fibroblasts from the sixth to the tenth culture passage. (**e**) BrdU/dT-banded chromosomes 7 consisting of one normal (left) and an aberrant isochromosome 7p (right). (**f**) Selected Giemsa-stained isochromosomes 7p from five different metaphases depicting symmetrical arrangement of nuclear constrictions and terminal satellites on both chromosome arms. (**g**) Selected silver-stained isochromosomes 7p from three metaphases showing distinct NOR labeling in both chromosome arms. (**h**) Two pairs of BrdU/dT-banded chromosomes 7 consisting of one normal homolog (left) and one ring chromosome (right). (**i**) Normal chromosome 7 (left) and two tandemly fused chromosomes 7 (right). Note that both chromosomes 7 are fused at their long arm telomeric regions with their NORs inserted at the fusion site.

mosomes were prepared from cells in the sixth up to the 14th culture passage, and the chromosome aberrations were identified by means of the 5-bromodeoxyuridine/deoxythymidine (BrdU/dT) replication banding technique.

Materials and methods

Animals and cell lines

One mature male individual each of *Bufo rubropunctatus*, *Scaphiopus holbrooki*, and *Gastrotheca riobambae* was supplied by a specialized animal dealer. The animals were kept in aquaterraria at 18–20 °C and fed with worms and flies. After sacrificing the animals with diethyl-ether, small kidney pieces were removed to set up cell cultures. All procedures with the animals conformed to the guidelines established by the Animal Care Committees.

Cell cultures and BrdU treatment

The kidney samples were minced and cultured in MEM (Gibco) supplemented with 13 % fetal calf serum (Boehringer) and 0.9 % penicillin-streptomycin (stock solution: 10,000 U/ml, Gibco). The cell cultures were maintained for 6 to 14 weeks as monolayer cultures in 25-cm^2 flasks (Nunc), and incubated at 26 °C under ordinary atmospheric conditions. Cells were harvested for chromosome analysis from subcultures using trypsin. Colcemid (Gibco) was added 2 h prior to harvest at a final concentration of 0.15 µg/ml of culture medium. Hypotonic treatment lasted 35 min at 37 °C in 0.027 M sodium citrate. The technique used for fixation of the cells in acetic acid:methanol (1:3) has been described previously (Schmid, 1978).

Replication banding patterns

All experiments were performed on non-synchronized cells of the sixth to the 14th passage. 24 h before cell harvest, 100 µg/ml BrdU (Sigma) was added to the cultures. After 16 h, the cells were washed twice with conventional culture medium and fed with medium containing 48 µg/ml deoxythymidine (Sigma). Cultures were kept in this medium for the last 8 h. Differential staining of replication bands was achieved with a modified fluorescence-plus-Giemsa (FPG) technique (Perry and Wolf, 1974). Slides were aged for 3 days, then kept for 30 min in buffered 0.03 µg/ml eosin Y solution (Hazen et al., 1985). Eosin Y (standard yellow, Fluka) was employed in preference to Hoechst 33258 commonly used for the FPG technique because of the better resolution of the replication bands. After rinsing the slides in distilled water, the cells were UV irradiated for 30 min in buffer at a distance of 10 cm from the UV lamp (254 nm) and 1 cm below the buffer level. The components of the buffer used are: 0.03 M KCl, 0.15 M NaCl, 0.83 mM KH_2PO_4, adjusted to pH 5.5. Subsequently, preparations were rinsed in fresh buffer, and incubated in 2× SSC for 90 min (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate). Finally, the slides were stained for 6 min in 5 % Giemsa solution (pH 6.8), washed twice in distilled water and air-dried.

Regional banding of chromosomes

Demonstration of constitutive heterochromatin by C-banding and staining of nucleolus organizer regions with $AgNO_3$ were performed according to Schmid et al. (1983).

Photography and analysis of banding patterns

Microscopic analyses were conducted on Zeiss photomicroscopes III. All photographs were taken on Agfaortho 25 ASA film. Each metaphase containing one or more chromosome aberrations was photographed, the chromosomes cut out from the prints, and affixed to double-sided tape in parallel rows. This record system permits an easy identification of all chromosome rearrangements and aneuploidies.

Results

Bufo rubropunctatus

In accordance with the results obtained by Formas (1978), the South American toad *B. rubropunctatus* has 2n = 22 chromosomes. As in most other *Bufo* species (Bogart, 1972), the karyotype consists of six larger and five distinctly smaller meta- or submetacentric chromosomes (Fig. 1a). A prominent nucleo-

lar constriction is located in the chromosome 7 short arm (Fig. 1a) that proves to be the nucleolus organizer region (NOR) as shown by specific silver staining (Fig. 1b). By application of the BrdU-replication banding technique a large number of dark- and pale-stained replication bands are induced in the chromosomes during the first half of the S-phase, most of them being evenly distributed along the metaphase chromosomes (Fig. 1c).

Among 50 metaphases of the sixth to tenth cell culture passage analyzed, 15 (30%) show a normal diploid karyotype with two unaltered chromosomes 7 (Fig. 1d). In 27 metaphases (54%), one of the chromosomes 7 is missing, and an isochromosome 7p is present instead (Fig. 1e). This metacentric isochromosome 7p exhibits a perfect symmetry, with equal lengths of the nucleolar constrictions and sizes of the terminal satellites (Fig. 1f), as well as equal amounts of silver deposits (Fig. 1g) on both sides of the centromeric region. In six metaphases (12%), one of the chromosomes 7 is replaced by a ring chromosome (Fig. 1h). The ring chromosome 7 shows two nucleolar constrictions located in opposition to each other. It cannot be decided whether the ring chromosome 7 derived from the isochromosome 7p by telomeric fusion, or vice versa, if the isochromosome 7p is the product of a broken ring chromosome 7, although the first alternative seems to be the more likely event. Finally, in two of the cells (4%), a complex isoform of one chromosome 7 is seen in addition to a normal chromosome 7 (Fig. 1i). In this particular rearrangement the two chromosomes 7 are fused in tandem at their long arm telomeric regions with their NORs inserted at the fusion site.

Scaphiopus holbrooki

The conventional karyotype of the North American spadefoot toad *S. holbrooki* has been reported by Wasserman and Bogart (1968) and Morescalchi et al. (1977), and C-banding, mithramycin fluorescence and BrdU/dT replication banding were studied by Schmid et al. (2003). This species has 2n = 26 chromosomes, the pairs 1–6 being distinctly larger than pairs 8–13 (Fig. 2a). Pairs 3–5 have an acrocentric morphology, all the remaining pairs are meta- or submetacentric. The NOR is located in the chromosome 7 short arm. Constitutive heterochromatin is present at the centromeric and telomeric regions of all chromosomes, in the entire short arms of the acrocentric chromosomes 3 and 5, proximally and distally to the NOR in the chromosome 7 short arm, as well as interstitially in the pericentromeric regions of chromosomes 1–5 (Fig. 2a).

Fifty BrdU/dT-banded metaphases from the sixth to eighth cell culture passage were analyzed. Thirty cells (60%) have a normal karyotype, whereas in the remaining 20 metaphases (40%) at least one numerical or structural chromosome aberration can be detected (Fig. 2). The prevalent anomalies are monosomies (34.3%) and trisomies (31.3%). Examples of such monosomic and trisomic cells are depicted in Fig. 2a, b, e–h). Tetrasomies occur at a frequency of 6.5% (Fig. 2e, g). The chromosomes most frequently involved in aneuploidies are 5, 7, 11 and 13. It is remarkable that the only structural aberrations found consist of tandem fusions between two chromosomes, like those shown in Fig. 2c–h. They amount to 27.9% of all aberrations. The chromosome most frequently involved in these tandem fusions is no. 1 (Fig. 2c–h). It is fused either with a non-homologous chromosome (Fig. 2c, d, f, g), or else with its own homolog (Fig. 2e, h). In most of the tandemly fused chromosomes two centromeric constrictions can be discerned (Fig. 2c–g). Both the long as well as the short arm of the various chromosomes can participate in the tandem fusions. It should be pointed out that in the 50 metaphases analyzed all the 13 chromosomes of *S. holbrooki* are involved in numerical and/or structural aberrations. The lowest number of aberrations per metaphase are simple trisomies (Fig. 2a), the highest number consist of one to three trisomies, one tetrasomy, two to three monosomies, and two tandem fusions (Fig. 2e–h).

Gastrotheca riobambae

The karyotype of this South American marsupial frog is one of the best studied among the Anura (Schmid et al., 1983, 1986; Schmid and de Almeida, 1988; Schmid and Klett, 1994). It is distinguished by highly heteromorphic XY sex chromosomes. The Y chromosome is considerably larger than the X chromosome and composed almost completely of constitutive heterochromatin. The sole NOR in the karyotype is located in the X chromosome short arm. This causes a sex-specific difference in the number of ribosomal RNA genes of about 2(♀):1(♂). No cytogenetic indications were found for a possible inactivation of one of the two X chromosomes in female cells. The banded karyotype of *G. riobambae* has been described in detail by Schmid et al. (1983), restriction endonuclease banding was reported by Schmid and de Almeida (1988), and the BrdU/dT replication banding patterns in metaphase chromosomes were first presented by Schmid and Klett (1994).

Thirty-six metaphases (72%) out of 50 metaphases from the tenth to the 14th cell culture passage yield a normal karyotype. In each of the remaining 14 cells (28%) at least three chromosome aberrations are present (Fig. 3). As in the cell cultures of *S. holbrooki*, the most frequent anomalies are monosomies (28.3%), trisomies (9.5%), and partial trisomies (9.5%). It should be emphasized that in all 14 aberrant karyotypes the Y chromosome is missing (Fig. 3). Apparently the loss of the complete Y chromosome has no deleterious effects on cell growth and function. This is understandable because the huge Y chromosome mainly consists of constitutive heterochromatin with its non-transcribed repetitive DNA sequences. In all normal and aberrant karyotypes examined, the X chromosome (i.e. chromosome no. 4) shows a normal structure (Fig. 3). Tetrasomies of autosomes occur with a frequency of 2% (Fig. 3e).

Concerning the structural chromosome rearrangements, again tandem fusions between two chromosomes represent the most frequent category (25.7%). They involve autosomes 1, 2, 5, 7 and 8 (Fig. 3a, c–e). In two metaphases (6.7%) two pairs of extremely large chromosomes are present (Fig. 3e) that are interpreted to be the result of multiple tandem fusions between more than two autosomes. However, the BrdU/dT replication banding patterns in these huge chromosomes are too complex to reveal their origin. Two cells (2.7%) contain a non-reciprocal translocation between autosomes 3 and 11 (Fig. 3a). A centric fusion of the acrocentric autosomes 9 and 13 is found in five cells (6.7%) (Fig. 3e). A further centric fusion between the two homologous acrocentric autosomes 11 is counted in another

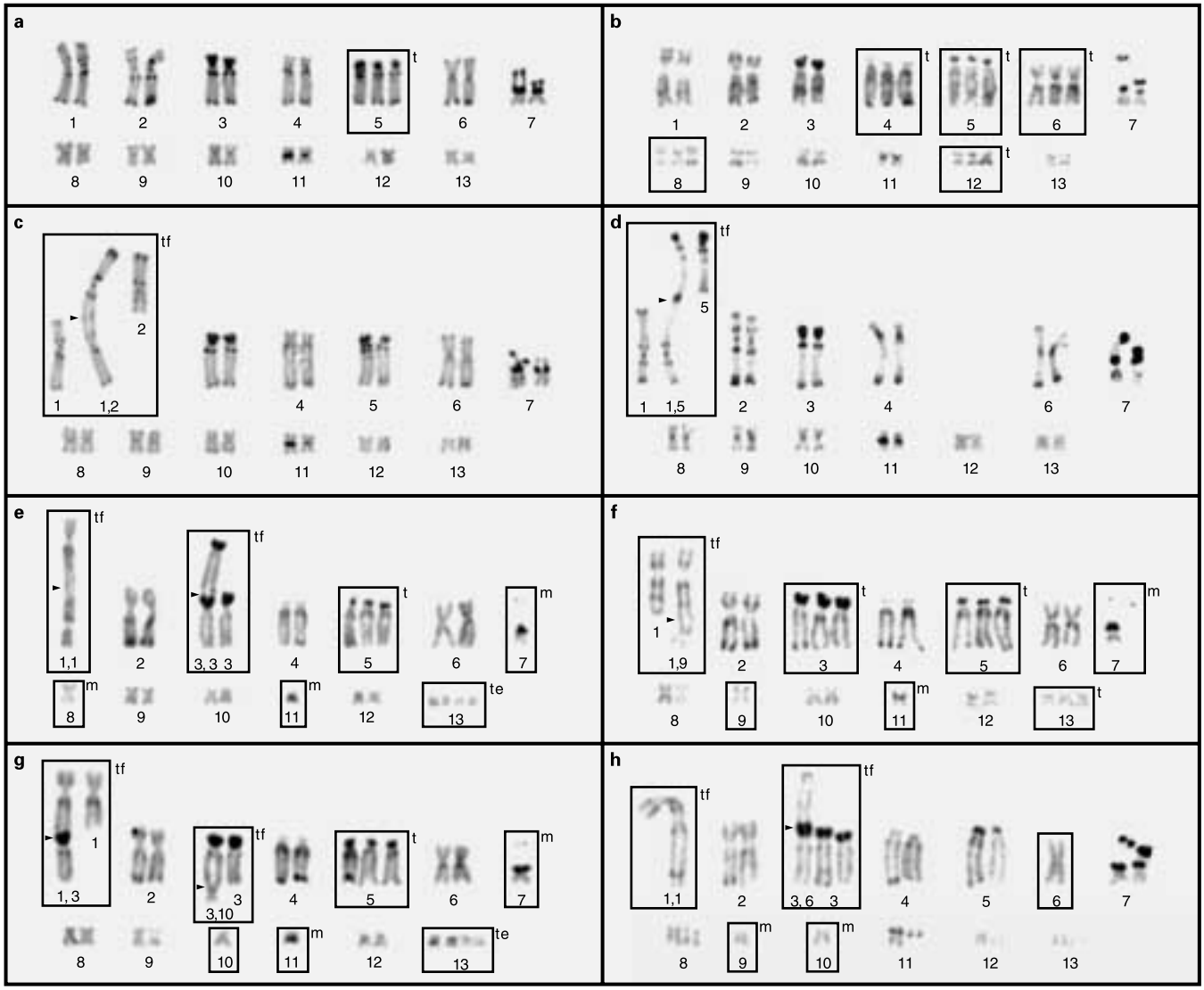

Fig. 2. (**a–h**) BrdU/dT-banded karyotypes of a male of *Scaphiopus holbrooki* prepared from kidney fibroblasts in the sixth to the eighth culture passage. The chromosomes showing numerical and/or structural aberrations are framed. The abbreviations used are as follows: (m) monosomy, (t) trisomy, (te) tetrasomy, (tf) tandem fusion. The black arrowheads beside the tandemly fused chromosomes mark the fusion site.

five cells (6.7%) (Fig. 3d, e). However, this condition can also be explained assuming an isochromosome formation by one homolog 11 plus a simultaneous monosomy of the other homolog, which of course, is a more complex chromosomal mutation event. Finally, in a single metaphase (1.5%), an allocyclic or prematurely condensed chromosome no. 1 is detectable (Fig. 3f).

Discussion

The only method that induces reliable multiple bands along amphibian metaphase chromosomes, and herewith allows their exact identification, is the 5-bromodeoxyuridine/deoxythymidine (BrdU/dT) technique (Schmid et al., 1990b). BrdU/dT replication bands were first obtained in bone marrow metaphase chromosomes of *Rana esculenta*, *R. temporaria* and *Pyxicephalus adspersus* (Schempp and Schmid, 1981), and later on in distinctly higher quality in chromosomes from cultured fibroblasts or lymphocytes of 19 anuran species belonging to the genera *Rana*, *Hyla*, *Bufo*, *Xenopus*, *Gastrotheca*, *Litoria*, *Odontophrynus*, and *Scaphiopus* (Schmid and Steinlein, 1991; Schmid and Klett, 1994; Miura, 1995; Miura et al., 1995; Wiley and Little, 2000; Schmid et al., 2002, 2003).

The present work is the very first report focusing on chromosome aberrations spontaneously originating in long-termed, aging amphibian fibroblast cultures. It shows that the rearrangements can be either confined to a single chromosome, like in *B. rubropunctatus*, or else involve all chromosomes of the karyotype, like in *S. holbrooki* and *G. riobambae*.

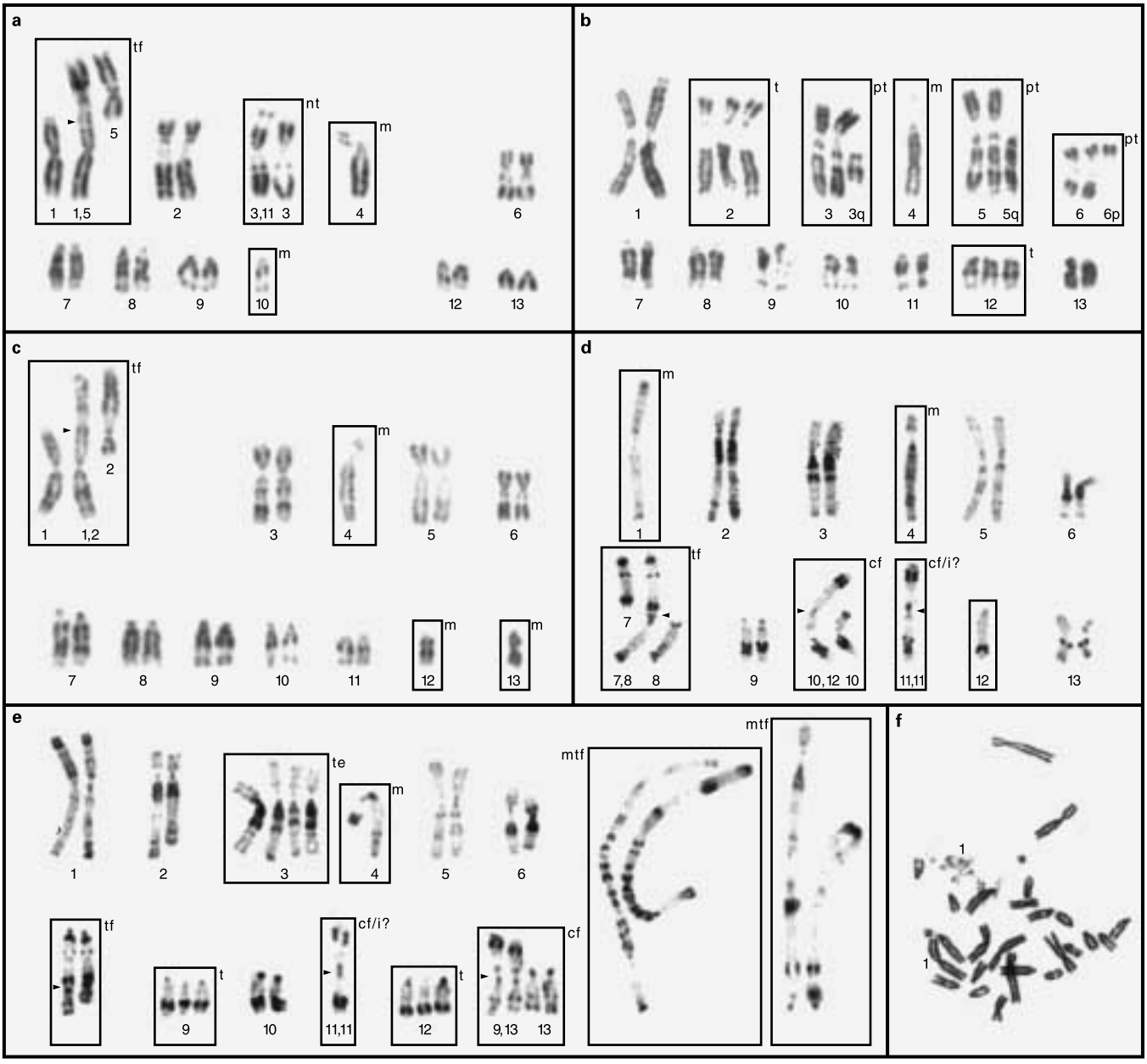

Fig. 3. (**a–e**) BrdU/dT-banded karyotypes of a male *Gastrotheca riobambae* prepared from kidney fibroblasts in the tenth to the 14th culture passage. The chromosomes showing numerical and/or structural aberrations are framed. The abbreviations used are as follows: (cf) centric fusion, (cf/i?) centric fusion or isochromosome, (m) monosomy, (mtf) multiple tandem fusion, (pt) partial trisomy, (t) trisomy, (te) tetrasomy, (tf) tandem fusion, (nt) non-reciprocal translocation. The black arrowheads beside the tandemly fused chromosomes mark the fusion sites. The single chromosome 4 is the X sex chromosome. Note that in all karyotypes the X chromosome is the only chromosome not involved in numerical or structural anomalies. The Y chromosome is missing in all cells. (**f**) Giemsa-stained metaphase with an allocyclic or prematurely condensed chromosome 1.

In the *B. rubropunctatus* fibroblasts all aberrations detected are restricted to one of the chromosomes 7. The most frequent anomaly is an isochromosome 7p which most probably originated by a singular event (clonal origin) and has spread throughout the cultures, rather than having arisen independently in different cells. Most probably the ring chromosome 7p was derived from this isochromosome 7p. However, there is no easy parsimonious explanation for the origin of the complex tandem fusion of two chromosomes 7 with their NORs inserted at their fusion site.

In both *S. holbrooki* and *G. riobambae* a variety of chromosome anomalies appear during senescence of their fibroblast cultures, the most common ones being monosomies and trisomies that apparently are quite well tolerated by the cells. The most frequent structural chromosome aberrations in the cell cultures of both species are tandem fusions between two chro-

mosomes that result in unusually large, mostly dicentric chromosomes. The breaks preceding these tandem fusions are located in the telomeric regions of the chromosomes, which in all chromosomes of both species consist of constitutive heterochromatin (Schmid et al., 1983, 2003). With regard to this high rate of spontaneous in vitro tandem fusions it is important to note the comparative cytogenetic investigation of Morescalchi (1973) which demonstrates that tandem fusions played one of the principal roles during the evolution of amphibian karyotypes (see Introduction). Furthermore, from a detailed comparison between the late replication banding patterns induced in the chromosomes of frogs belonging to the genus *Rana* it was found that a tandem fusion between the two small chromosomes 11 and 13 in an ancestral 2n = 26 species has produced the larger chromosome no. 6 in the 2n = 24 species (Miura et al., 1995). It is conceivable that the structural chromosome aberrations observed in vitro reflect the chromosome mutations which also preferentially took place during amphibian phylogenesis. This implies that the telomeric regions of amphibian chromosomes are prone to be involved in tandem fusions and non-reciprocal translocations.

It must be emphasized that the various categories of chromosome anomalies accumulating in aging amphibian fibroblast cells are not related to the genome size of the species examined. Thus, although the various numerical and structural chromosome changes in the fibroblast cultures of *S. holbrooki* and *G. riobambae* are very similar, the genome sizes of the two species are extremely divergent. Whereas *S. holbrooki* has one of the smallest genomes found so far in vertebrates (2.0 pg DNA per nucleus) (Schmid et al., 2003), the nuclear DNA content of *G. riobambae* amounts to 8.5 pg (Schmid et al., 1990a).

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