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Chromosome aneupolyploidy in an endemic Malagasy gecko (Gekkonidae: *Geckolepis*)

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Abstract. Polyploidy is exceptional in mammals and rare in reptiles. Unscheduled polyploidy often leads to cell disorders and cancer through genomic rearrangements that can be difficult to trace and is particularly poorly studied in non-model vertebrates. In the present paper, using a combination of banding and Fluorescence In Situ Hybridization (FISH) techniques, we reconstruct and characterize the origin and significance of aneupolyploid karyotypes found in the bone marrow of an individual of the Malagasy gecko *Geckolepis typica*. Our results demonstrate that these anomalous karyotypes correspond to a proliferative cell disorder characterized by a succession of distinct structural events, from a preliminary duplication of the whole genome to the instability of loci of NORs and subsequent chromosome rearrangements that will produce aneuploid cells. Our comparison between the newly described normal, i.e., diploid, karyotype of the species (2n = 40) and aneutetraploid plates (4n = 77–80) revealed that translocations of NOR-bearing chromosomes are involved in generating aneuploidy. Furthermore, simultaneous and multiple translocations of the NOR-bearing chromosomes occurred with relatively high frequency (27% and 19%, respectively) on distinct chromosome pairs. In addition, in about 9% of 90 scored metaphase plates, structural instability of the NOR regions also produced detachments of rDNA arrays and generated an accessory, fully heterochromatic B-chromosome. Our data suggest that the duplication of the whole genome can be considered a preliminary stage in proliferative cell disorders, inducing chromosomal instability and structural rearrangements.

Key words. Squamata, aneuploidy, chromosome rearrangements, NORs, genetic disorder, B-chromosome, Madagascar.

Introduction

Polyploidy is both a major driving force of evolution and a pathogenetic condition. Polyploidisation, i.e., the inheritance of additional sets of chromosomes, can originate from two main mechanisms (Madlung 2013): allopolyploidy (additional sets originate from hybridisation of two different species), or autopolyploidy (additional sets are derived from the same or a closely related individual). In addition, polyploidy can be part of the developmental program of organisms, organs or tissues (scheduled allo- and autopolyploidy) or unexpected (unscheduled autopolyploidy), the latter generally representing a pathogenetic condition (Storchova & Kuffer 2008).

In general, scheduled polyploidy is a successful diversification mechanism in plant evolution, and multiple independent polyploid lineages occur in several distinct taxonomic groups (Gregory & Mable 2005, Jiao et al. 2011).

Even if less common than in plants, polyploidy also occurs in vertebrates, especially in fishes and amphibians (Gregory & Mable 2005, Schmid et al. 2015). In mammals and birds, true polyploidy (related to the whole organism) is generally fatal, with embryonic development being arrested during the early stages or embryos dying soon after birth (Storchova & Kuffer 2008). Exceptional cases concern the tetraploidy in two octodontid species, which to date are the only known tetraploid species of endothermic vertebrates (Gallardo et al. 2004, 2006). In reptiles, polyploidy is a rare condition (e.g., Mezzasalma et al. 2016), but it has been documented in independent evolutionary lineages, always concerning triploid, parthenogenetic species (Olmo & Signorino 2017).

In recent years, renewed interest has been paid to an increasing extent on unscheduled polyploidy, because of its link to cancer research (Storchova & Pellman 2004, Storchova & Kuffer 2008, Coward & Harding 2014). In general, unscheduled polyploidy is rarely tolerated and often associated with chromosomal instability, leading to alterations to both chromosome structure and number (Storchova & Pellman 2004, Otto 2007). In particu-

lar, chromosomal instability following duplication of the whole genome can promote deleterious rearrangements, genetic abnormalities, and tumorigenesis (e.g., Margolis 2005, Olaharski et al. 2006, Storchova & Kuffer 2008, Coward & Harding 2014).

In poikilothermic (="ectothermic") vertebrates, there are relatively few data about cancer, and less than 100 cases have been reported from reptiles, mostly concerning captive-kept specimens and mostly regarding the haematopoietic system, skin, liver and reproductive organs (Hernadez-Divers & Garner 2003, Garner et al. 2004, Robert 2010).

In this study, we report on an unusual chromosome condition found in the bone marrow cells of a female specimen of the endemic Malagasy gecko, *Geckolepis typica* Grandidier, 1867. This is a medium-sized nocturnal gecko species, occurring in spiny thickets and deciduous forests across southern and western Madagascar (Köhler et al. 2009), but its taxonomy is not yet well resolved and different genetic lineages are known to exist (Lemme et al. 2013). We analyse the abnormal polyploid condition found in the bone marrow cells of a specimen of *G. typica*, comparing it with the newly described normal karyotype of the species and reconstructing a particular pathway of genomic events that led to chromosomal instability, distinct chromosome rearrangements, and the generation of B-chromosomes.

Materials and methods

We examined two females (field numbers GA 409 and GG 8) and two males (GA 420 and GA 411) of *Geckolepis typica* originating from the Toliara region. All the samples used in this study were collected during previous fieldwork in Madagascar between 2003 and 2004, and conserved as tissue samples and cell suspensions at the Department of Biology of the Università degli Studi di Napoli Federico II, Naples, Italy.

To provide an unambiguous taxonomic allocation of the examined samples, they were also genotyped. Genomic DNA was extracted from tissue samples according to the standard method by Sambrook et al. (1989) and a fragment of the mitochondrial NADH dehydrogenase subunit 4 (ND4) was amplified by means of standard Polymerase Chain Reaction (PCR). PCRs were performed using the primer pair ND4fi1 5'- GCAAATACAAACTAYGAACG -3' (Jackman et al. 2008) and LeuR1 5'-CATTACTTTTTACTTGGATTTGCACCA -3' (Arevalo et al. 1994). PCRs were conducted in a volume of 25 µl and parameters were set as follows: an initial denaturation step at 94°C for 5 min, followed by 36 cycles at 94°C for 30 s, 52°C for 35 s, 72°C for 1 min; final extension at 72°C for 7 min.

Amplicons were sequenced in both directions on an automated sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA) using the BIGDYE TERMINATOR v. 3.1 (ABI) kit. Sequences were blasted in GenBank and chromatograms were checked, edited using CHROMAS LITE 2.1.1, and aligned in CLUSTALW with BIOEDIT 7.0.5.3 (Hall 1999). Chromosomes were obtained from intestinal epithelium and femur and humerus marrow cells of four individuals, previously injected with a dose (0.1 ml/10 g body weight) of colchicine (1 mg/ml). Metaphase plates were obtained from the intestinal epithelium according to the scraping + air-drying method (King & Rofe 1976) and from bone marrow cells as described by Schmid (1978).

Besides standard staining (with a 0.5% Giemsa solution at pH 7), we performed a C-banding procedure following Sumner (1972) and a sequential C-banding + CMA + DAPI as described by Mezzasalma et al. (2015, 2017a). In addition, NOR-FISH was performed as described by Petraccioli et al. (2015), but using as a probe the PCR-amplified and biotinylated 18S rRNA gene of the gekkonid *Tarentola mauritanica* (Linnaeus, 1758). Metaphase plates were detected and recorded using an epifluorescence microscope (Axioscope Zeiss) equipped with a digital camera.

Results

The four studied samples exhibited the same ND4 haplotype (accession number: LT838333), and a comparison between the newly determined sequences and the homologous fragment retrieved from GenBank allowed us to assign the four examined gecko samples to *G. typica* (OTU J according to Lemme et al. 2013).

The two male specimens (GA 410, GA 411), a female (GG8), and the intestine cells of the other female specimen (GA 409) exhibited a karyotype of 2n = 40 chromosomes, with pairs 3, 6 and 9 shaped as metacentric, pairs 2 and 4 as submetacentric, and the remaining pairs as telocentric elements that gradually decreased in length (Fig. 1). NOR-FISH evidenced the presence of loci of NORs on the centromeric tip of telocentric chromosome pair 10 (Fig. 1). C-banding revealed a marked C-band, positive to both CMA and DAPI, on the tip of chromosome pair 10, corresponding to the NOR-associated heterochromatin. Other, but more feeble C-bands, positive only to DAPI, were present in pericentromeric regions of various biarmed chromosomes (Figs 1 B and C).

Bone marrow cells of the studied female (GA 409) exhibited metaphase plates (90 examined) with variable chromosome numbers, namely 77 (19%), 78 (27%), 79 (45%), and 80 (9%). For karyotype reconstruction and the detection of chromosome rearrangements, the recorded metaphase plates stained with both sequential NOR-FISH and C-banding + CMA+DAPI (66 in total) provided useful information. Both staining methods allowed us to follow rearranged loci of NORs, because they were the only genomic regions that responded positive to both fluorochromes as well as to NOR-FISH staining (Figs 2 and 3). In this manner, it was possible to detect that the variable and high chromosome numbers exhibited by bone marrow cells resulted from a duplication of the whole genome, followed by chromosome rearrangements involving the NOR-bearing elements. In all the examined bone marrow metaphase plates of the studied female specimen GA 409, the chro-

mosomes are grouped in 20 quartets with their morphological and chromatinic characteristics being very similar to those observed in the 20 chromosome pairs of normal diploid karyotypes found in intestinal cells of the same female and the other studied specimens (Figs 2 and 3). In addition, the NOR-bearing quartets were lacking one, two or three elements in all supernumerary plates, wheras NOR-FISH signals and C-bands positive to CMA and DAPI were evident on telocentric elements of the first quartet and, less frequently, also on elements of the second or fourth quartet (Figs 2 and 3). This proved that these rearrangements involved translocations of entire chromosomes, namely Robertsonian fusions between the elements of the quartet 10 and those of the quartet 1 or 2 (Fig. 3). After translocations on one or two of the relative chromosomes often occurred also the amplification of loci of NORs (Figs 2 and 3). It also should be stressed that all karyotypes with 80 elements (Fig. 2D) do not originate from a simple duplication of the whole genome. In fact, in all the observed metaphase plates with 80 chromosomes, one of the NOR-bearing chromosomes of quartet 10 had moved to an element of quartet 1, while an accessory, unpaired and fully heterochromatic chromosome was present. This supernumerary

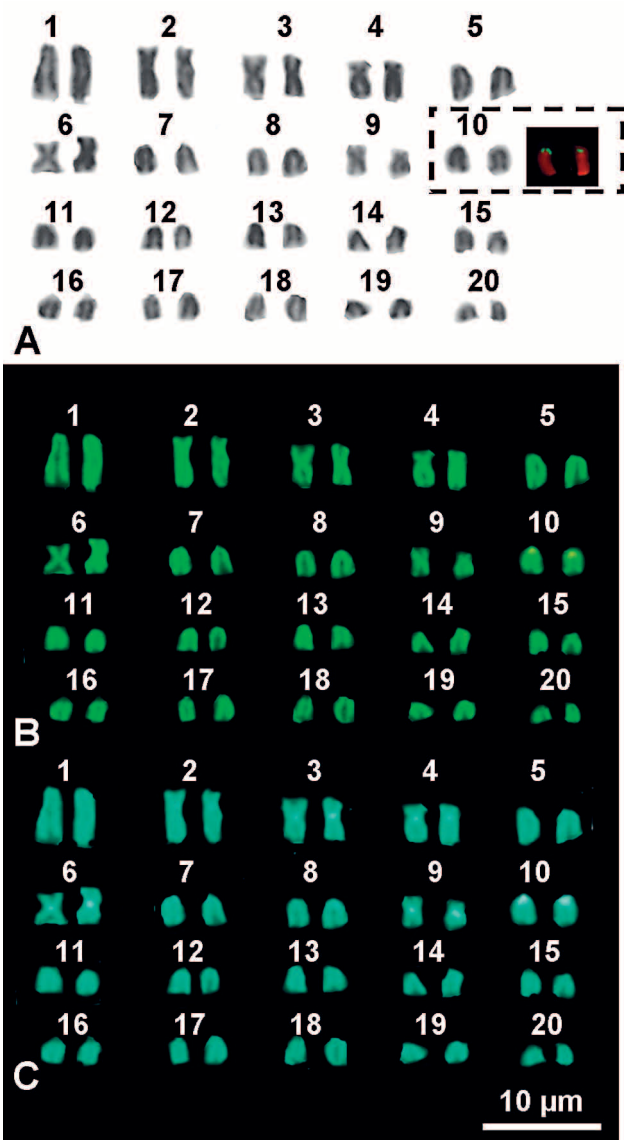

Figure 1. Normal karyotype from the intestine of a female *Geckolepis typica* (GA 409) stained with Giemsa (A) and sequentially stained with C-banding + CMA (B) + DAPI (C). The box includes the NOR-bearing pair stained with C-banding (left) and NOR-FISH (right).

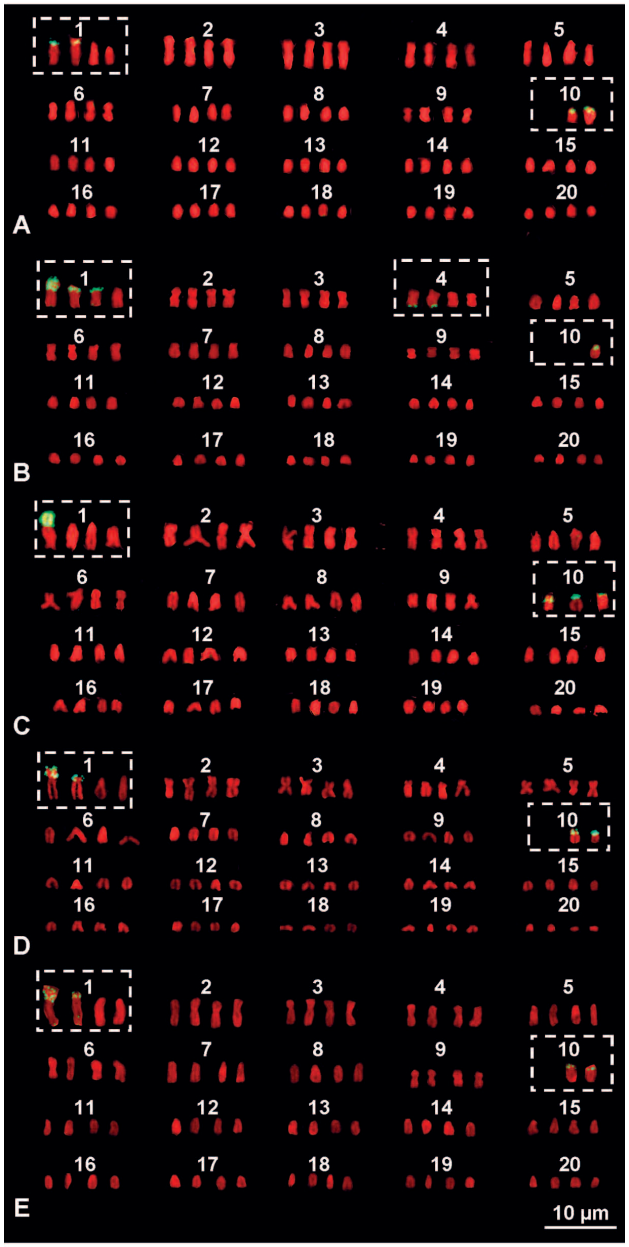

Figure 2. (A–E) Aneutetraploid karyotypes from bone marrow cells of *Geckolepis typica* female GA 409 stained with NOR-FISH. The boxes include elements showing hybridisation signals.

chromosome was always shaped as a telocentric element, highly positive to C-banding + CMA + DAPI (Fig. 3).

Discussion

Our results demonstrate that the karyotype of *Geckolepis typica* is composed of 2n = 40 chromosomes that are mostly shaped as telocentric elements and gradually decrease in length, and have NORs on the tips of chromosome pair 5. This chromosome formula is similar or differs slightly from those of congeneric species and phylogenetically more closely related genera of endemic Malagasy geckos (Mezzasalma et al. 2017b; unpublished data). Interspecific karyological similarities and differences as well as the chromosomal diversification of the genus *Geckolepis* will be discussed in detail elsewhere, whereas in the present paper, we reconstruct and characterize the origin and significance of the anomalous karyotypes of a studied female (GA 409) of this species.

All the examined metaphase plates found in the bone marrow of specimen GA 409 had nearly or exactly twice as many chromosomes as those of normal 2n = 40 karyotypes of other tissues and specimens. In all instances, these supernumerary chromosomes can be subdivided in homologous quartets, with the only exception being NOR-bearing elements that are never present as complete quartets. In addition, the missing NOR-bearing chromosomes were always located in other chromosome pairs. Therefore, the bone marrow of the examined female should be considered

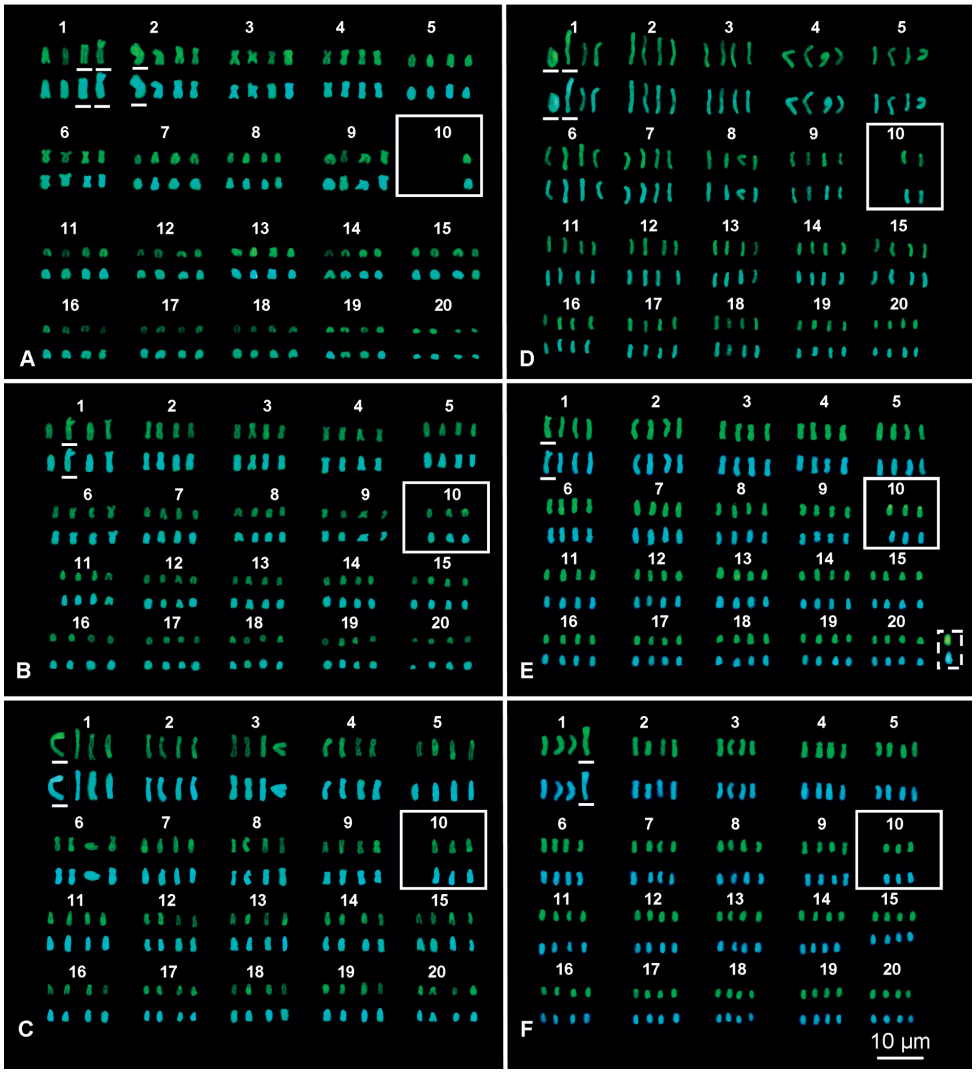

Figure 3. (A–F) Aneutetraploid karyotypes from bone marrow cells of *Geckolepis typica* female GA 409, sequentially stained with C-banding + CMA (upper rows) + DAPI (lower rows). The solid-line boxes include the tenth aneuploid quartets with elements showing bright centromeric heterochromatin, positive both to CMA and DAPI. The elements of the first or second quartets carrying large heterochromatic C-bands positive to CMA and DAPI are underlined. In (E), the box with a dashed outline includes the unpaired, accessory B-chromosome.

auto-tetraploid, with aneuploidy concerning the NOR-bearing chromosomes. Some animal tissues and organs, for example, the hepatocytes, osteoclasts, megakaryocytes or embryonic trophoblasts of mammals, usually do have a fractional content of polyploid cells (0.5–20% in humans) that occur as a part of the normal developmental program and usually result in the formation of differentiated cells in the end (Otto 2007, Storchova & Kuffer 2008). However, our results allow us to exclude the possibility of the auto-tetraploid bone marrow cells here observed representing a case of scheduled polyploidy, because bone marrow cells of the other examined samples, as well as intestine cells of the same female specimen GA 409, exhibited only normal, i.e., diploid, karyotypes.

In mammals, unscheduled tetraploid cell lines often represent an intermediate stage of various pathogenetic (cancer-related) structural changes, including chromosomal rearrangements such as duplications, deletions and/or nonreciprocal translocations (Storchova & Pellman 2004, Storchova & Kuffer 2008).

This study reports for the first time that similar anomalies also may occur in squamate reptiles, i.e., as a cell disorder of the bone marrow, the main erythropoietic and granulopoietic tissue in Sauria (Glomsky & Pica 2006). Furthermore, this proliferative disorder followed a progressive succession of distinct structural events, from a preliminary duplication of the whole genome to the instability of loci of NORs and subsequent chromosome rearrangements that produce aneuploid cells. Rearrangements that were unambiguously involved in the generation of aneutetraploid karyotypes included nonreciprocal translocations of the NOR-bearing elements, preferentially by means of centric fusions, affecting the elements of first telocentric quartet or, less frequently, by tandem fusions, affecting those of the second metacentric quartet. Interestingly, in the scored plates, cases of simultaneous, multiple (two or three) translocations involving NOR-bearing chromosomes occurred with relatively high frequency (27 and 19%, respectively). Mutants, polymorphic populations, or varieties originating from simultaneous, multiple chromosome rearrangements are quite rare and their occurrence is relevant to both evolutionary and cancer-related studies, because they may often lead to further genomic instability, disclosing an intrinsic propensity to structural rearrangements of the chromosomes involved (see King 1982, Olmo et al. 2004).

In our case, other structural rearrangements occurred in addition to translocations of NOR-bearing chromosomes in aneutetraploid plates. In particular, the regions of translocated NORs underwent a significant amplification and in some cases (9% of scored metaphase plates), the high structural instability of amplified NOR regions resulted in their fragmentation and the generation of an accessory, unpaired chromosome, probably constituted by NOR-associated heterochromatin, that represents a newly generated B-chromosome.

B-chromosomes are accessory, dispensable elements found in many groups of plants and animals (Makunin et al. 2014), but their occurrence has rarely been documented from reptiles (Kupriyanova 1980, Castiglia 2004). While their origin, evolution, and functions are extensively debated (Camacho 2004, Makunin et al. 2014, Mezzasalma et al. 2015), they are usually thought to arise from ordinary A-chromosomes and subsequently follow their own evolutionary pathways (Camacho et al. 2000, Camacho 2004).

In general, B-chromosomes are smaller than regular A-chromosomes and mainly composed of constitutive heterochromatin (e.g., satellite sequences, transposable elements) and rDNA repeats (Beukeboom 1994, Camacho et al. 2000). Indeed, chromosome regions containing clusters of rDNA (as do the NOR-bearing regions) are known to exhibit high structural instability and represent recombination hotspots in cancer cells (see McStay 2016). These regions can be characterized by duplications, deletions and unequal chromosome exchanges and have the potential of generating B-chromosomes (Camacho et al. 2000).

According to this evidence, the instability of the NOR-bearing regions probably also triggered the duplication and subsequent fragmentation of rDNA arrays and their translocations to telomeric regions of elements of the fourth quartet in some metaphase plates.

It should also be stressed that the results provided here represent one of only a few cases where the origin of B-chromosomes can be unambiguously traced and linked to a series of progressive events, including the instability of the NOR-bearing regions, which, in turn, was probably caused by an unscheduled duplication of the whole genome and tetraploidy.

In general, unscheduled tetraploidy can be caused by one of three main mechanisms: cell fusion, mitotic errors, or failed cytokinesis (Storchova & Pellman 2004). Furthermore, the "tetraploid-intermediate model" presumes that particular genetic defects can lead to tetraploidy, aneuploidy and tumorigenesis (Castillo et al. 2007).

In the present case, it is not possible to ascertain the cell mechanisms that generated the observed aneutetraploid disorder, and more samples from the Tulear population of *G. typica* should be analysed to evaluate the incidental frequency of this cell disorder and its possible links to environmental factors.

In any case, the haemopoietic proliferative disorder here evidenced followed a progressive, multiple-step pathway that is generally concordant with the "tetraploid-intermediate model", including a duplication of the whole genome and high levels of chromosome instability that will lead to multiple, non-reciprocal translocations of NOR-bearing chromosomes and subsequent amplifications of NORs. Interestingly, in some instances, this mechanism also led to the genesis of accessory B-chromosomes.

We cannot exclude the possibility that other cryptic rearrangement also occurred in the observed aneutetraploid karyotypes, but the loss of chromosomes generating aneuploidy only involved the NOR-bearing pair, and no other variations in the chromosome number or morphology were observed. In conclusion, the observations here reported represent a rare instance of haematopoietic neo-

plasia in a wild reptile and demonstrate that unscheduled tetraploidy can be a preliminary stage in complex genome events, inducing chromosome rearrangements and promoting cancer, in poorly studied vertebrate taxa.

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