

# Cytogenetic Studies on *Gonatodes* (Reptilia, Squamata, Sphaerodactylidae)

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## Key Words

Banding analyses · FISH · Geckos · *Gonatodes* · Karyotype evolution · Meiotic chromosomes · Mitotic chromosomes

## Abstract

Mitotic and meiotic chromosomes of 5 species of the reptile genus *Gonatodes* are described by means of conventional staining, banding analyses and in situ hybridization using a synthetic telomeric DNA probe. The amount, location and fluorochrome affinities of constitutive heterochromatin, the number and positions of nucleolus organizer regions, and the patterns of telomeric DNA sequences were determined for most of the species. The karyotypes of *G. falconensis* and *G. taniae* from northern Venezuela are distinguished by their extraordinarily reduced diploid chromosome number of  $2n = 16$ , which is the lowest value found so far in reptiles. In contrast to most other reptiles, both species have exclusively large biarmed (meta- and submetacentric) chromosomes. Comparison of the karyotypes of *G. falconensis* and *G. taniae* with those of other *Gonatodes* species indicates that the exceptional  $2n = 16$  karyotype originated by a series of 8 centric fusions. The karyotypes of *G. falconensis* and *G. taniae* are further characterized by the presence of considerable amounts of  $(TTAGGG)_n$  telomeric sequences in the centro-

meric regions of all chromosomes. These are probably not only relics of the centric fusion events, but a component of the highly repetitive DNA in the constitutive heterochromatin of the chromosomes. The genome sizes of 4 *Gonatodes* species were determined using flow cytometry. For comparative purposes, all previously published cytogenetic data on *Gonatodes* and other sphaerodactylids are included and discussed.

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The reptilian genus *Gonatodes* is a diverse clade of monophyletic neotropical geckos comprising 30 described species with a distribution in Central America, tropical South America, and most of the major Caribbean Islands [Rivero-Blanco, 1979; Gamble et al., 2008; Schargel, 2008; Schargel et al., 2010; Uetz and Hošek, 2014]. These small-bodied lizards (28–65 mm) are mostly diurnal, forest dwelling and arboreal, with larger species frequently found closer to tree bases. Many *Gonatodes* are abundant in forested areas and are important components of Amazonian lizard communities [Rivero-Blanco, 1979; Vitt et al., 2000]. Some species inhabit human-modified environments and even highly urbanized areas. Females lay a single, large oval egg at a time, each female laying

several eggs annually. Communal egg-laying has been registered for a number of *Gonatodes* species. Most species of *Gonatodes* exhibit strong sexual dichromatism: females are cryptic, whereas males usually have bright-colored markings (green, orange, red, yellow) on the head, body sides, and the underside of the tail [Rivero-Blanco, 1979]. In several species, the males are characterized by striking intra-populational coloration differences (male polychromatism), with some species having up to 4 different color morphs in the same locality [Schargel, 2008; Schargel et al., 2010; Rivero-Blanco and Schargel, 2012]. Most divergences in the genus *Gonatodes* occurred in the last half of the Cenozoic era (~35 Mya), especially in the Oligocene and Miocene [Gamble et al., 2008].

There are only a limited number of cytogenetic studies on geckos, with approximately 5% of the recognized species examined [Olmo, 1986]. Up to date, the karyotypes of 9 species belonging to the genus *Gonatodes* are known (table 1). Their diploid chromosome numbers range between  $2n = 16$  and  $2n = 40$  and the fundamental numbers between  $FN = 31$  and  $FN = 46$ . In *G. taniae*, a species known from only 2 localities in the Central Coastal Mountain range in northern Venezuela [Schargel, 2008], the lowest diploid chromosome number known for reptiles ( $2n = 16$ ) was discovered [Schmid et al., 1994]. This completely biarmed karyotype was interpreted to be the final result of a repeated series of centric fusions starting from an ancestral karyotype with  $2n = 32$  telocentric chromosomes found in other *Gonatodes* species.

The present study examines the chromosomes of 5 further *Gonatodes* species from Venezuela, Trinidad and Cuba and provides results for several banding techniques and fluorescence in situ hybridization (FISH) experiments. The amount, chromosomal location and composition of the constitutive heterochromatin, the number and location of nucleolus organizer regions (NORs) as well as the patterns of repetitive telomeric DNA sequences were determined in *G. falconensis* from Venezuela. This species, which is closely related to *G. taniae* from a phylogenetic point of view [Schargel et al., 2010], also possesses an extremely reduced, completely biarmed karyotype with only  $2n = 16$  chromosomes. Although the karyotypes of *G. falconensis* and *G. taniae* are very similar, there are discrete differences which clearly allow differentiating between both species. The genome sizes of 4 *Gonatodes* species were determined using DNA flow cytometry. For completeness and for comparative purposes, all previously published cytogenetic data on the genus *Gonatodes* and other species of the family Sphaerodactylidae are reviewed.

## Materials and Methods

### Animals

Individuals representing 5 *Gonatodes* species (*G. albobularis*, *G. annularis*, *G. ceciliae*, *G. falconensis*, and *G. vittatus*) were collected during various expeditions to Venezuela, Trinidad and Cuba (table 1). The chromosomes of the Venezuelan species were prepared in a temporary cytogenetic laboratory in the biological field station 'Rancho Grande' located in the Henri Pittier National Park, Aragua State. The Cuban specimen was prepared in a temporary cytogenetic laboratory in the Departamento de Biología Animal y Humana, University of La Habana. Tissues obtained for chromosome preparations and determination of the nuclear DNA contents were transferred to 1.8-ml plastic tubes (Nunc), stored at 4°C or -20°C and transported to the laboratories in Würzburg (Germany), either packed in dry ice or at room temperature. The specimens collected on Trinidad were brought back from the field alive and processed in a conventional laboratory, University of Maryland. All procedures with the living animals strictly conformed to the guidelines established by the Animal Care Committees of the respective countries.

### Chromosome Preparation and Banding Analyses

Mitotic metaphases were prepared directly from the bone marrow and intestines of all animals after *in vivo* colchicine treatment. Male meiotic chromosomes were obtained from testes. The techniques used for the preparation of cell suspensions, hypotonic treatment and fixation of the cells have been described previously [Schmid et al., 2010]. C-banding of constitutive heterochromatin, AgNO<sub>3</sub> labeling of NORs, fluorescence stainings of chromosomes with the fluorochromes quinacrine mustard and DAPI, and fluorescence counterstainings with distamycin A/DAPI and distamycin A/mithramycin were also performed according to Schmid et al. [2010].

### Localization of (TTAGGG)<sub>n</sub> Telomeric Repeats

The repetitive telomeric DNA sequences were detected using a fluorescein-conjugated peptide nucleic acid (PNA) probe (Telomere PNA FISH kit/FITC: K5325; Dako Cytomation, Denmark). The detailed procedure of *in situ* hybridization and signal detection is provided by the manufacturer.

### Photography and Analysis of Banding Patterns

All the microscopic analyses were performed with Zeiss photomicroscopes III and Zeiss Axiophot microscopes equipped with incident HBO 50W mercury lamp illumination. The filter combinations necessary for the analyses of metaphases stained with the various fluorochromes or for FISH were described by Schmid et al. [2010]. All photographs were taken on Agfaortho 25 films. At least 5 karyotypes for each of the staining techniques applied were prepared from each of the 17 animals. C-banded karyotypes of *G. taniae* published by Schmid et al. [1994] were used for a detailed comparison with a C-banded karyotype of *G. falconensis*.

### DNA Flow Cytometry

Blood samples were obtained by cardiac puncture with a heparinized syringe and fixed immediately in 70% ethanol. The samples were centrifuged (10 min, 200 g), and the erythrocyte pellets resuspended and incubated in 1 ml pepsin solution (0.5% in 0.1 N HCl) at room temperature for 15 min. After addition of 5 ml staining

**Table 1.** Cytogenetic data available for the genus *Gonatodes*

Species <sup>a</sup>	Country	Specimens <sup>b</sup>	Locality of sampling	2n	FN <sup>c</sup>	Sex chromosomes <sup>d</sup>	Tech-niques <sup>e</sup>	References
<i>G. albogularis</i>	Cuba	1♂	Vedado, Ciudad de La Habana	32	32		1–4	present study
<i>G. annularis</i>	Venezuela	1♀	Campamento Río Grande, Bolivar State	32	32		1	present study
<i>G. ceciliae</i>								
cytotype A	Trinidad	1♂	Simla Research Station, 6.4 km N Arima, St. Georges County	26	33	XY	1	McBee et al., 1987
cytotype B	Trinidad	1♂	Simla Research Station, 6.4 km N Arima, St. Georges County	22	31	XY	1	McBee et al., 1987
cytotype C	Trinidad	1♂	Simla Research Station, 6.4 km N Arima, St. Georges County	24	32	XY	1	present study
<i>G. falconensis</i>	Venezuela	2♂, 1♀	Cueva de San Luis, Curimagua, Sierra de San Luis, Falcón State	16	32		1–3, 5, 7	present study
<i>G. hasemani</i>	Brazil	1♂, 1♀	Aripuaña, Mato Grosso State, 10°10'S/59°27'W	32	32		1	dos Santos et al., 2003
<i>G. humeralis</i>								
	Venezuela	1♂	28 km E El Palmar at Río Grande, Bolivar State	32	32		1	McBee et al., 1987
	Brazil	1♂	Tucuruí, Pará State, 03°45'S/49°40'W	32	32		1–3, 6	dos Santos et al., 2003
	Brazil	1e	Vai Quem Quer State, Pará State, 13°30'S/55°50'W	32	32		1–3, 6	dos Santos et al., 2003
	Brazil	2♂, 1♀	São João da Baliza, Roraima State, 00°57'S/59°54'W	32	32		1–3, 6	dos Santos et al., 2003
	Brazil	1♀	Aripuaña, Mato Grosso State, 10°10'S/59°27'W	32	32		1–3, 6	dos Santos et al., 2003
<i>G. rozei</i>								
cytotype A	Venezuela	2♂	32 km N Altigracia de Orituco, Guatopo National Park, Miranda State	32	32		1	McBee et al., 1984
cytotype B	Venezuela	1♂	32 km N Altigracia de Orituco, Guatopo National Park, Miranda State	40	46		1	McBee et al., 1984
<i>G. taniae</i>	Venezuela	4♂, 2♀	Henri Pittier National Park, biological field station 'Rancho Grande', Aragua State, 10°21'N/67°41'W	16	32		1, 2, 4, 5, 7	Schmid et al., 1994; present study
<i>G. vittatus</i>								
	Trinidad	1♂	Simla Research Station, 6.4 km N Arima, St. Georges County	32	32		1	McBee et al., 1987
	Trinidad	1♀	Milepost 44, Mayaro road, Cocos Bay, Narivo County	32	32		1	McBee et al., 1987
	Trinidad	1♂	Simla Research Station, 6.4 km N Arima, St. Georges County	32	32		1	present study
	Venezuela	n.i.	Caracas, Federal District	32	32		1	Rada de Martínez, 1980
	Venezuela	3♂, 1♀	El Limón, Aragua State	32	32		1, 2	Schmid et al., 1994; present study

<sup>a</sup> The *G. ceciliae* from Trinidad are deposited in the Texas Cooperative Wildlife Collection (TCWC), Texas A&M University, as Nos. 61816 (cytotype A) and 61817 (cytotype B) and in the Smithsonian Institution, Washington, DC (USNM) as No. 306161 (cytotype C). The *G. rozei* from Venezuela are deposited in the TCWC as Nos. 59302 and 59303 (cytotype A) and 59304 (cytotype B). The latter 3 specimens were reported as an undescribed species by McBee et al. [1984]. <sup>b</sup> e = Embryo; n.i. = sex not identified. <sup>c</sup> Because some of the chromosome pairs of *G. rozei* cytotype B (2n = 40,

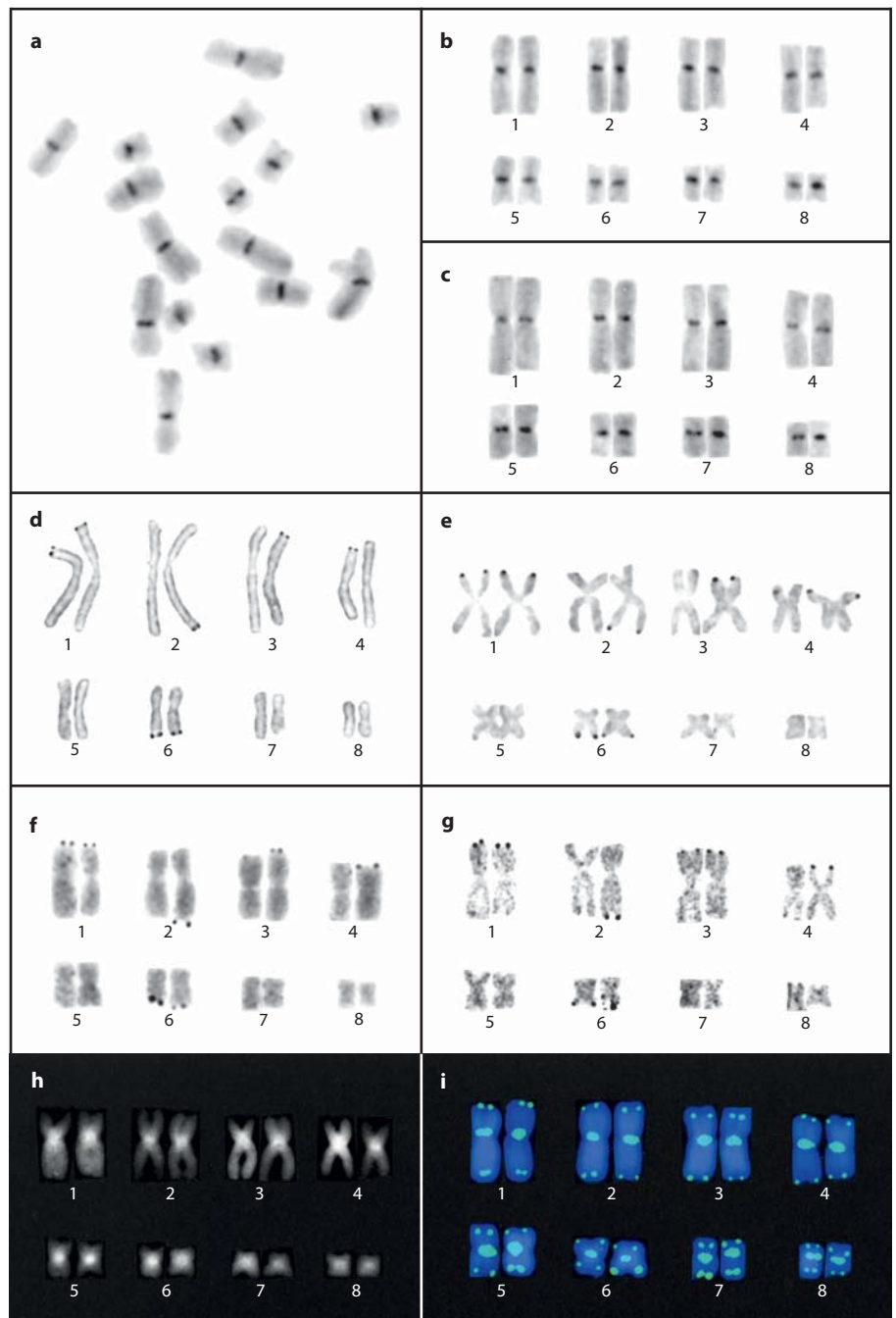
FN = 46) depicted in the published figure are very small without visible centromeric constrictions, the fundamental number is uncertain. <sup>d</sup> Since only male individuals of *G. ceciliae* were examined, the observation of heteromorphic XY sex chromosomes is preliminary. <sup>e</sup> 1 = Conventional staining; 2 = C-banding of constitutive heterochromatin; 3 = Ag-staining of NORs; 4 = quinacrine fluorescence; 5 = mithramycin fluorescence; 6 = BrdU-replication banding patterns; 7 = FISH with telomeric DNA probes.

solution (2 µg/ml DAPI in 0.2 M sodium citrate), the erythrocyte suspensions were kept at room temperature for 3 h [Otto, 1994] and were then mixed with chicken erythrocytes. Flow cytometric analyses were carried out with an epi-illumination flow system of conventional design (Partec Cell Analyzer CA II) at 365 nm (filter combination KG1/BG38/UG1 for excitation, TK420 as dichroic mirror, and GG435 as barrier filter). The nuclear DNA content was calibrated against the known genome size of chicken erythrocytes (2.33 µg DNA/nucleus) used as internal standards.

## Results

### *Gonatodes falconensis*

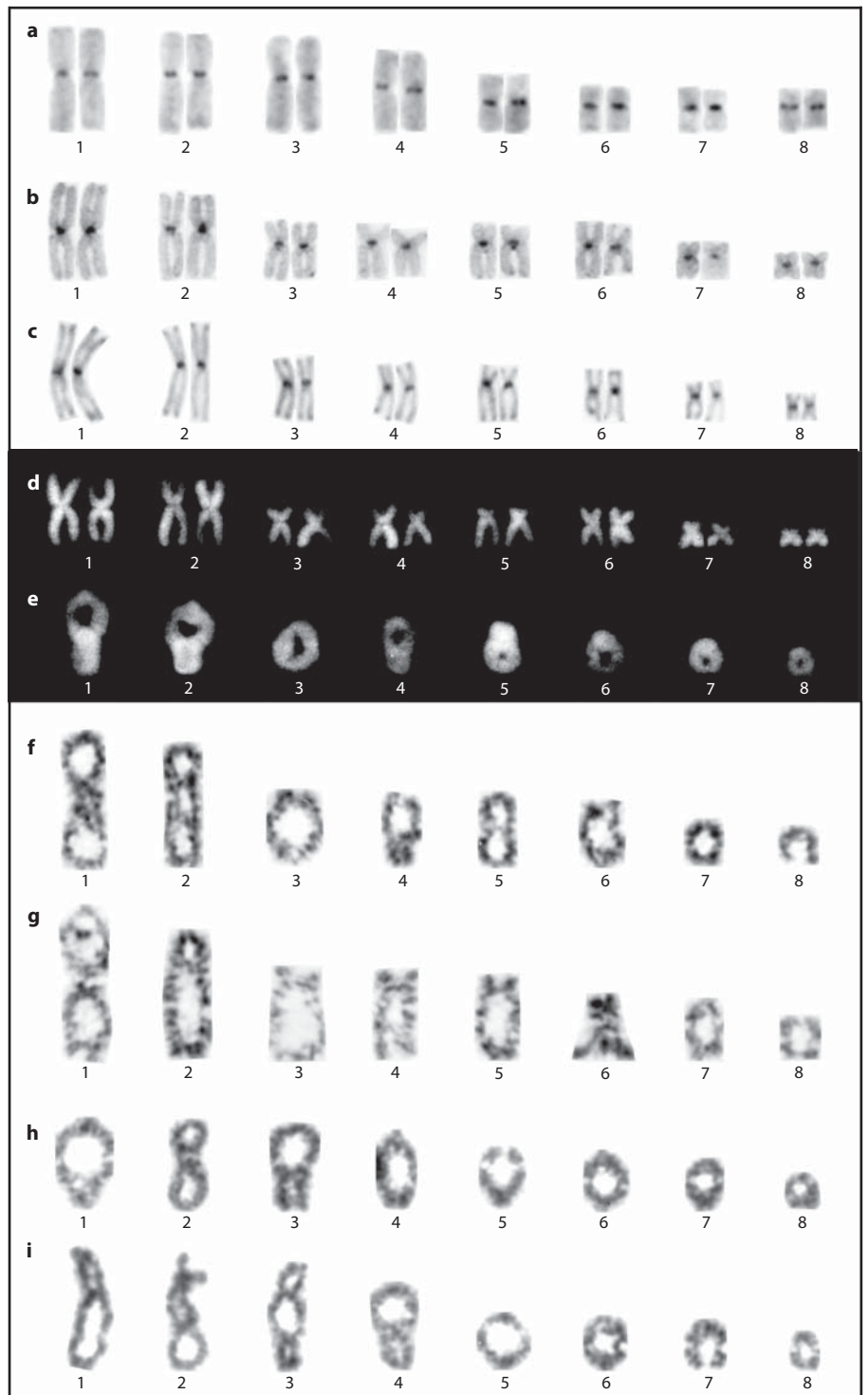
A C-banded metaphase and 2 C-banded karyotypes of *G. falconensis* are shown in figure 1a–c. As in *G. taniae* [Schmid et al., 1994], in all 3 individuals of *G. falconensis* examined, the diploid chromosome number is only 2n = 16, and the fundamental number is FN = 32. All chromo-



**Fig. 1.** Metaphase chromosomes of a *G. falconensis* male. **a–c** Metaphase (**a**) and 2 karyotypes (**b**, **c**) showing C-bands. **d–g** Four karyotypes exhibiting Ag-staining. Note the intra-individual variability of the number and location of the NORs. **h** Karyotype obtained after counterstaining chromosomes with distamycin A/mithramycin. The centromeric heterochromatin shows bright mithramycin fluorescence. **i** Karyotype showing the FISH pattern of the telomeric (TTAGGG)<sub>n</sub> repeats. Note the very large het-ITSs in the centromeric regions of all 8 chromosome pairs.

somes are metacentric or nearly metacentric. In the male as well as female karyotypes, the 16 chromosomes can be arranged in 8 homologous pairs. Chromosome pairs 1–4 decrease slightly in their lengths, with only small size differences between adjacent pairs, whereas pairs 5–8 are distinctly smaller (fig. 1b, c). There is no evidence for the presence of heteromorphic sex chromosomes, and no secondary constrictions can be detected in the karyotypes.

All chromosomes of *G. falconensis* exhibit distinct constitutive heterochromatin in their centromeric regions. Additionally, very faintly stained heterochromatin is located in all telomeric regions. Interstitial or sex-specific heterochromatic bands cannot be detected in the C-banded karyotypes (fig. 1a–c). After staining the chromosomes with the GC base pair-specific mithramycin, the centromeric heterochromatin of all chromosomes is character-



**Fig. 2. a–c** Comparison of C-banded karyotypes of *G. falconensis* (**a**) and *G. taniae* (**b**, **c**). Note distinct species-specific differences in the lengths of chromosomes 1–4. **d**, **e** Mitotic chromosomes (**d**) and male meiotic bivalents (**e**) of *G. taniae* from diakinesis after fluorescence counterstaining with distamycin A/mithramycin. Note absence of mithramycin-bright centromeric heterochromatin in the chromosomes. **f–i** Male meiotic bivalents of *G. taniae* (**f**, **g**) and *G. falconensis* (**h**, **i**) from diakinesis. Note species-specific differences of bivalent lengths 1–4.

ized by a very bright fluorescence (fig. 1h). The latter is in clear contrast to *G. taniae*, where all chromosome pairs show a uniform mithramycin fluorescence (fig. 2d, e) [Schmid et al., 1994]. Following staining with the AT base

pair-specific fluorochromes quinacrine mustard and DAPI or counterstaining with distamycin A/DAPI, no specific fluorescence is produced in the centromeric heterochromatin of the *G. falconensis* chromosomes (not shown).

**Table 2.** Relative lengths of the chromosomes in mitotic metaphases of *G. falconensis* and *G. taniae*

Species	Relative length of chromosomes, % <sup>a</sup>							
	1	2	3	4	5	6	7	8
<i>G. falconensis</i>	19.0	17.6	16.9	14.8	9.9	8.5	7.0	6.3
<i>G. taniae</i>	21.4	17.9	12.1	12.1	11.4	10.7	7.9	6.4

<sup>a</sup>The chromosomes of 2 C-banded metaphases each of *G. falconensis* and *G. taniae* were used for the measurements.

Silver staining was successfully performed in the chromosomes of 1 male *G. falconensis* and showed multiple NORs with an apparent intra-individual variability. In most metaphases analyzed, NORs are present in the short arm telomeric regions of both chromosomes 1 and 3, as well as in the long arm telomeric regions of both chromosomes 6. Additionally, NORs can be discerned in the short arm telomeric regions of 1 homologue 4 and in the long arm telomeric region of 1 homologue 2 (fig. 1d–g).

As expected, FISH shows the presence of (TTAGGG)<sub>n</sub> repeats in the telomeric regions of all chromosomes of *G. falconensis* (fig. 1i). Furthermore, very bright hybridization signals are present in the centromeric regions of all 8 chromosome pairs. These hybridization signals colocalize with the centromeric heterochromatin of the chromosomes (compare fig. 1b, c and i). The same chromosomal distribution of (TTAGGG)<sub>n</sub> repeats is present in the karyotype of *G. taniae* [Schmid et al., 1994].

Although the karyotypes of the closely related *G. falconensis* and *G. taniae* are extremely similar, a closer examination shows a clear difference (fig. 2a–c). Whereas in *G. falconensis* the chromosome pairs 1–4 decrease slightly in their lengths with only small size differences between adjacent pairs (fig. 2a), in *G. taniae* the pairs 1 and 2 are distinctly larger than the remaining pairs 3–8 (fig. 2b, c). This is quantitatively confirmed by length measurements of chromosomes at the mid-stage of metaphase (table 2). This difference between the mitotic karyotypes of *G. falconensis* and *G. taniae* is also evident in the meiotic chromosomes of male diakinesis (fig. 2f–i).

In male meiosis of *G. falconensis* and *G. taniae*, the majority of diakinetically bivalents possess terminally located chiasmata which give them a ring-like appearance (fig. 2f–i). Subterminal, interstitial and pericentromeric chiasmata are frequent. Achiasmatic bivalents are not found.

#### *Gonatodes vittatus*, *G. annularis* and *G. albogularis*

All 3 species have a diploid chromosome number of  $2n = 32$  and a fundamental number of  $FN = 32$ . The

karyotypes consist of 16 pairs of telocentric chromosomes in a graded series from large to small (fig. 3). In the specimens examined, no heteromorphic sex chromosomes are detectable. This is also in accordance with previous results obtained for *G. vittatus* from different localities in Venezuela and Trinidad (table 1).

All chromosomes of *G. vittatus* and *G. albogularis* exhibit distinct constitutive heterochromatin in their centromeric regions (fig. 3b, e, f). The same results, although with less quality, were obtained for *G. annularis* (not shown). Staining with the AT base pair-specific fluorochromes quinacrine mustard and DAPI or counterstaining with distamycin A/DAPI does not produce specific fluorescing signals in the centromeric heterochromatin of the *G. albogularis* chromosomes (fig. 3j, k). Silver staining was successfully performed in the chromosomes of the male *G. albogularis* and showed a single NOR pair in the tiny short arms of chromosome pair 2 in all metaphases analyzed (fig. 3g–i). No intra-individual variability in the number or location of the NORs exists in this specimen.

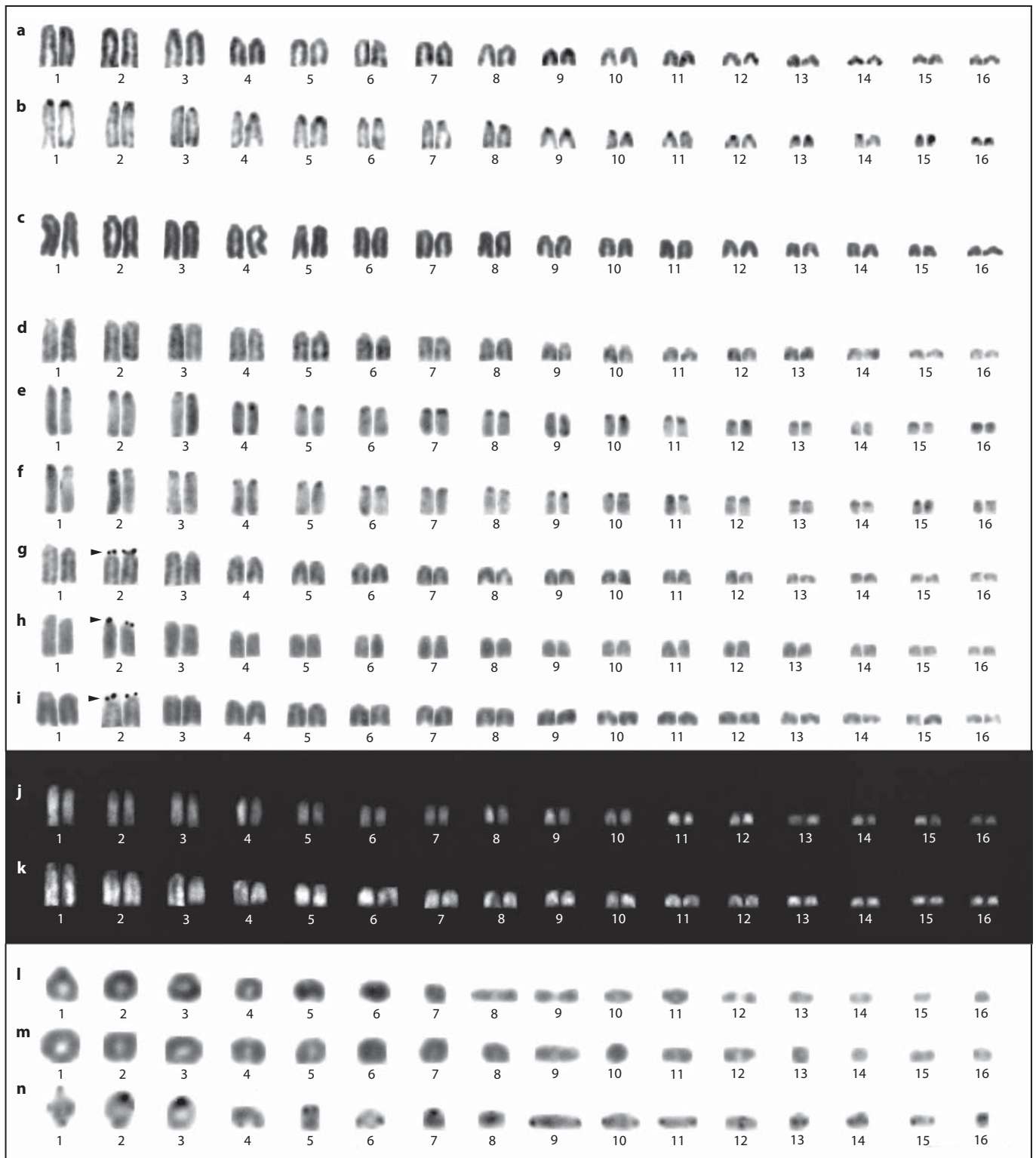
As in the male meiosis of *G. falconensis* and *G. taniae*, the majority of diakinetically bivalents of *G. vittatus*, *G. albogularis* and *G. annularis* show terminally located chiasmata and a ring-like appearance. When one of the terminal chiasmata in these bivalents opens, large rod-like or V-like elements develop (fig. 3l–n). Subterminal, interstitial and pericentromeric chiasmata are less frequent.

#### *Gonatodes ceciliae*

In the present study, an unknown karyological variant of *G. ceciliae* (cytotype C, table 1) with a diploid chromosome number of  $2n = 24$  and a fundamental number of  $FN = 32$  was found. This male individual, like the male of cytotype A described by McBee et al. [1987], has 2 pairs of large metacentric chromosomes and 1 pair of small metacentric chromosomes (fig. 4a). However, one each of the telocentric chromosomes 5 and 6 are centrally fused with one each of the telocentric chromosomes 7 and 8 (5;7 and 6;8 in fig. 4a). Accordingly, in the diakinesis stage of meiosis, 2 distinct trivalents are present in this individual (fig. 4b–d). As a further characteristic, the heteromorphic XY sex chromosomes, consisting of 1 large and 1 small telocentric element (fig. 4a), do not form a distinct sex bivalent at meiosis (fig. 4b–d).

#### Comparative Cytogenetic Aspects

Chromosomal repatterning occurring during evolution can be elucidated, at least partially, either with the



**Fig. 3.** a–k Metaphase chromosomes of *G. vittatus* (a, b), *G. annularis* (c) and *G. albogularis* (d–k), showing conventional Giemsa staining (a, c, d), C-banding of constitutive heterochromatin (b, e, f), Ag-staining of NORs (arrowheads) (g–i), and quinacrine mustard staining (j, k). l–n Male meiotic bivalents of *G. albogularis* after conventional Giemsa staining (l, m) and C-banding (n).

help of multiple G- and R-banding procedures or modern molecular cytogenetic methods such as cross-species chromosome painting using chromosome-specific DNA probes generated from flow-sorted or microdissected chromosomes that are used as templates for DNA amplification by DOP-PCR. The direct induction of G- and R-bands in the chromosomes of reptiles and other poikilothermic vertebrates, either by enzymatic treatment or by heating in specific buffer solutions, is not possible or yields unsatisfactory results [for reviews, see Schmid and Guttenbach, 1988; Schmid et al., 2010]. Although the induction of R-bands with the 5-bromodeoxyuridine (BrdU) replication banding technique is possible in the chromosomes of *Gonatodes* [dos Santos et al., 2003] and other reptiles, the small size of most reptile chromosomes and the low number of induced R-bands inhibit the identification of the corresponding homeologous chromosome regions. On the other hand, chromosome-specific DNA probes have not yet been generated for *Gonatodes* or other sphaerodactylids. Therefore, and like in the report of Schmid et al. [1994], the following formal approach was chosen to test whether the 8 biarmed (meta- and submetacentric) chromosomes of *G. falconensis* and *G. taniae* could be considered homeologous to the 16 single-armed (telocentric) chromosomes of *G. vittatus*, *G. albogularis* and *G. annularis*. The 8 chromosome pairs of *G. taniae* were arranged in a column (fig. 5, GTA(a)). These chromosomes were cut apart at their centromeric regions, and the 32 resulting telocentric chromosomes were arranged in a second column (fig. 5, GTA(b)). Finally, the telocentric chromosomes of *G. vittatus*, *G. annularis* and *G. albogularis* were arranged in 3 further parallel columns (fig. 5, GVI, GAN, GAL) in such a way that a maximum correspondence of chromosome lengths was obtained. The comparison shows nearly identical lengths of biarmed and single-armed chromosomes. It seems obvious that in the genus *Gonatodes* the differences between the individual karyotypes are best explained by multiple centric fusions. Nevertheless, it is obligatory to confirm this oversimplified approach by chromosome painting [e.g. Trifonov et al., 2011].

#### Genome Sizes

The genome sizes of *G. albogularis* (1 ♂), *G. taniae* (1 ♀), *G. falconensis* (1 ♂), and *G. vittatus* (1 ♂) were determined using DNA flow cytometry of DAPI-stained erythrocytes. As an example of these measurements, the histogram obtained for *G. falconensis* is shown in figure 6. *G. albogularis* has the lowest genome size (1.6 pg DNA/nucleus), whereas those of *G. taniae*, *G. falconensis* and

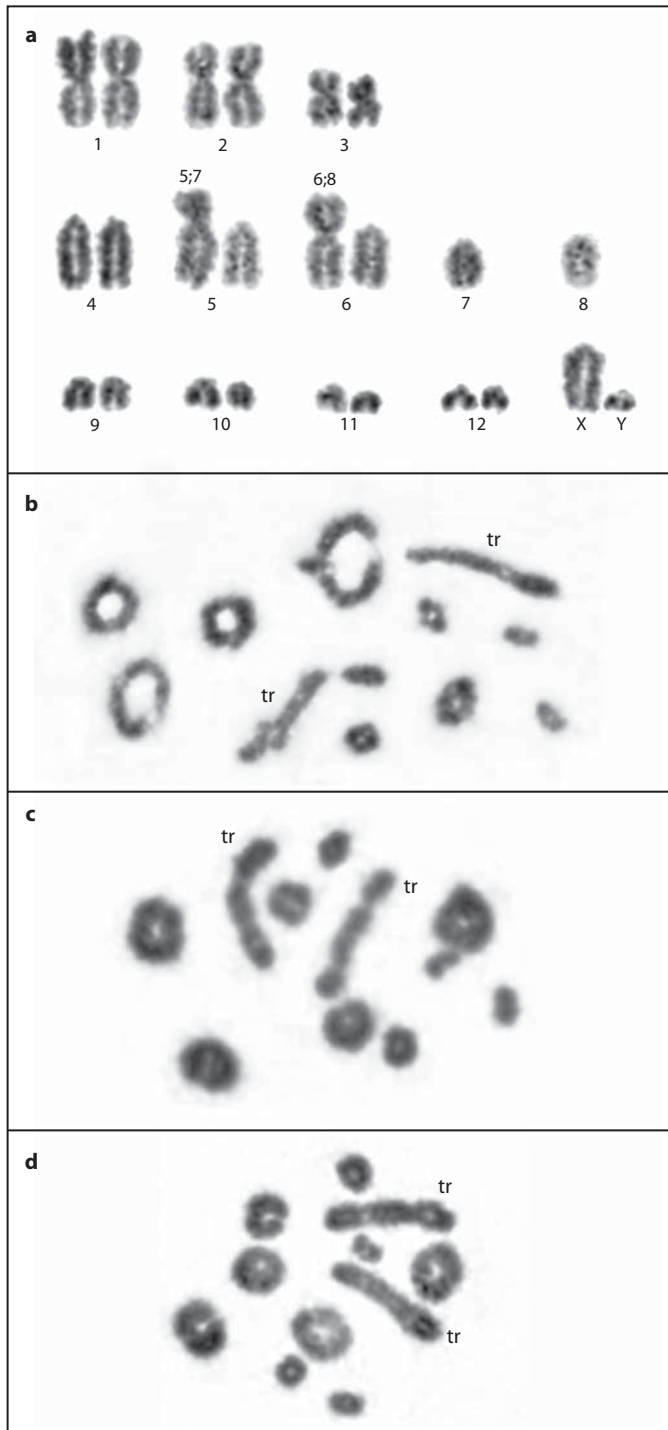
*G. vittatus* are distinctly higher (3.1, 4.5 and 7.8 pg DNA/nucleus, respectively). However, it is necessary to add the caveat that the quality of the fixed erythrocytes is influenced by the conditions of isolation (field or laboratory), transport to the laboratory (temperatures of  $-20^{\circ}\text{C}$  to  $40^{\circ}\text{C}$ ), storage time of the fixed samples in the laboratory, and the staining efficiency of the fixed erythrocytes. Furthermore, it should be noted that the erythrocytes were stained with the AT base pair-specific DAPI, and not with ethidium bromide which anneals to double-stranded DNA regardless of its base pair composition. Therefore, erythrocyte nuclei of those species containing high amounts of AT-rich repetitive DNA will be more intensively labeled than nuclei of those depauperate in such sequences or which have enriched amounts of GC-repetitive DNA. This can lead to biased DNA values. In spite of these difficulties, the results obtained by DNA flow cytometry provide the first genome size values for the genus *Gonatodes*.

#### Discussion

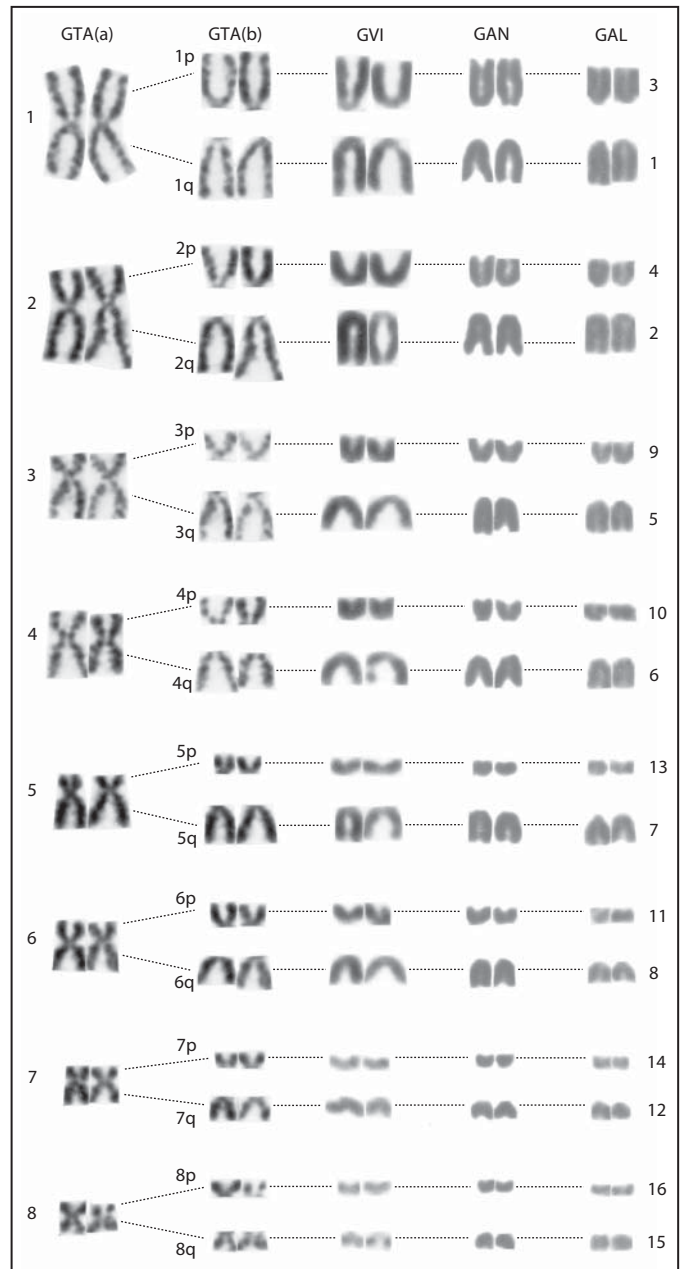
Up to date, cytogenetic data for 9 *Gonatodes* species are available (table 1). Six species (*G. albogularis*, *G. annularis*, *G. hasemani*, *G. humeralis*, *G. rozei* cytotype A, and *G. vittatus*) possess a diploid chromosome number of  $2n = 32$  and a fundamental number of  $FN = 32$ . These monotonous, completely unarmed karyotypes with 32 telocentric chromosomes of continuously decreasing lengths are considered to represent the ancestral condition in the genus. Most of the other *Gonatodes* karyotypes can be theoretically derived by a series of centric (and tandem) fusion events which reduced the unarmed karyotypes into more diversified biarmed karyotypes presenting meta- and submetacentric chromosome pairs with different morphologies.

In the 2 males of *G. ceciliae* analyzed by McBee et al. [1987], collected at the Simla Research Station in Trinidad, 2 different karyotypes were detected. One male (cytotype A, table 1) had  $2n = 26$  and  $FN = 33$ , with 2 pairs of large metacentric chromosomes, 1 pair of small metacentric chromosomes, and 9 pairs of telocentric chromosomes gradually decreasing in size. Additionally, it had a distinctly heteromorphic chromosome pair consisting of a large metacentric and a small telocentric element which were interpreted as XY sex chromosomes [McBee et al., 1987]. This karyotype can be derived from the presumed ancestral  $2n = 32/FN = 32$  karyotype by 3 centric fusions between 6 pairs of large telocentric autosomes and a con-

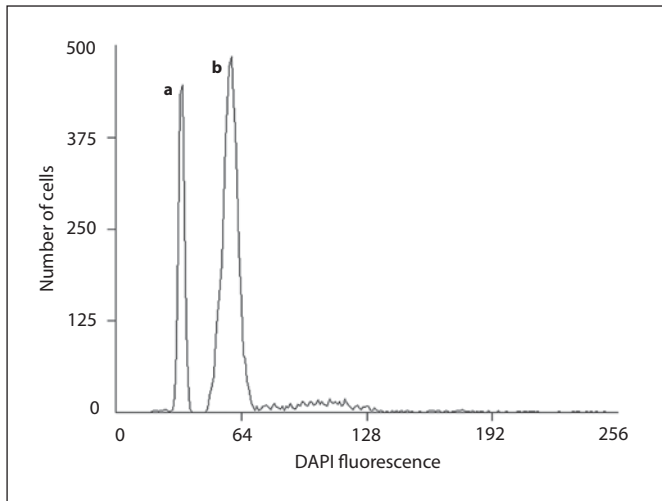




**Fig. 4.** **a** Conventionally stained karyotype of *G. ceciliae* cytotype C (male) with 2 centric fusion chromosomes, 5;7 and 6;8, as well as heteromorphic telocentric XY sex chromosomes. **b–d** In meiosis of this male individual, the diakinesis show 2 distinct trivalents (tr) each. The heteromorphic XY sex chromosomes do not form a conspicuous sex bivalent.



**Fig. 5.** Formal comparison of the supposedly homeologous chromosomes of *G. taniae* (GTA), *G. vittatus* (GVI), *G. annularis* (GAN), and *G. albogularis* (GAL). The 16 biarmed chromosomes of *G. taniae* shown in the first column GTA(a) were cut apart exactly at their centromeric region, and the resulting 32 telocentric chromosomes were arranged to an artificial karyotype in the second column GTA(b). The 32 telocentric chromosomes of the other 3 *Gonatodes* species are arranged in parallel columns to obtain maximum correspondence of chromosome lengths. Dotted lines connect homeologous chromosomes.



**Fig. 6.** Histogram obtained by DNA flow cytometry of a mixture of DAPI-stained erythrocytes of chicken (a) and *G. falconensis* (b). The nuclear DNA content is directly proportional to the DAPI fluorescence intensity, expressed as channel numbers on the x-axis. Chicken erythrocytes were used as internal standard with a known DNA content of 2.33 pg DNA/nucleus. The nuclear DNA content of the *G. falconensis* erythrocytes is calculated from the ratio of the peak channel number of the *G. falconensis* erythrocytes (b) and chicken (a) multiplied by the known nuclear DNA amount of the chicken.

siderable size increase of the X chromosome. The latter event could have originated by addition or amplification of repetitive DNA (constitutive heterochromatin), possibly followed by a pericentric inversion in the originally telocentric X chromosome. Thereby, the fundamental number was increased from FN = 32 to FN = 33. The second male specimen identified as *G. ceciliae* (cytotype B, table 1) shared the same heteromorphic XY sex chromosomes, but had  $2n = 22$  and FN = 31, with an additional pair of large metacentric chromosomes. This karyotype was explained by the occurrence of a further centric fusion between 2 pairs of large telocentric autosomes plus an independent tandem fusion between 2 pairs of small telocentric autosomes [McBee et al., 1987]. The 2 male specimens of *G. ceciliae* used by McBee et al. [1987] to produce the karyotypes they reported are deposited at the Texas Cooperative Wildlife Collection (TCWC) and were examined by us to ascertain their identity. The 2 specimens do fall within the variation described for *G. ceciliae*. However, the 2 specimens are different color morphs. One of the specimens (TCWC 61816) lacks reticulations on the head, whereas in the other specimen (TCWC 61817) reticulations are present (table 1).

It becomes obvious that *G. ceciliae* is either in a very dynamic stage of karyological differentiation or, alternatively, the high karyotype variability may indicate the existence of various cryptic species. Before a final conclusion can be made, the 3 *G. ceciliae* cytotypes A, B and C must be re-examined by C-banding and chromosome painting using chromosome-specific DNA probes. Furthermore, it is necessary to study the karyotypes of much more specimens of *G. ceciliae* from Trinidad and north-eastern Venezuela. Finally, the chromosomes of the sister species *G. ocellatus* (endemic to Tobago) must be included in the chromosome analysis.

The exceptional diploid chromosome number of  $2n = 16$  discovered in *G. falconensis* and *G. taniae* is the lowest found for reptiles. The karyotypes of both species are devoid of telocentric chromosomes, but only contain metacentric and submetacentric chromosomes. Furthermore, the biarmed karyotypes of *G. falconensis* and *G. taniae*, and the uniarmed karyotypes of *G. vittatus*, *G. albogularis* and *G. annularis* show a good agreement in the lengths of the supposedly homeologous chromosomes. Therefore, it is most probable that the chromosome complements of *G. falconensis* and *G. taniae* were derived from a presumed ancestral karyotype consisting of  $2n = 32$  telocentric chromosomes by a series of 8 subsequent fusion cycles. *G. falconensis*, *G. taniae* and *G. purpurogularis* are 3 very closely related montane species, restricted to the Coastal Mountain range and the Cordillera de Merida in northern and northwestern Venezuela (montane group 2 = *G. falconensis* complex of Schargel [2008]). All 3 species are unique among *Gonatotodes* in being only weakly sexually dimorphic in coloration relative to other species in this genus. Based on a phylogenetic maximum parsimony analysis of DNA sequences of the 12S mitochondrial gene and the *C-mos* nuclear gene, both *G. purpurogularis* and *G. taniae* were rejected as valid species [Schargel, 2008]. Consequently, *G. purpurogularis* and *G. taniae* were considered conspecific and junior synonyms of *G. falconensis*. However, further studies for a more rigorous delineation of species limits in this small *Gonatotodes* clade were recommended. Actually, as the present comparative analyses show, *G. falconensis* and *G. taniae* can be clearly differentiated by various cytogenetic characters, which suggest that these 2 species are not conspecific. Apparently, the karyotype evolution in these 2 species has proceeded faster than the molecular differentiation of their DNA in mitochondrial or some nuclear genes. This is a further case in which cytogenetic dissimilarities in closely related species are helpful with the unambiguous identification of species or with the discovery

of cryptic species. Similar results were obtained in the karyosystematics of some neotropical amphibians [Kaiser et al., 1995; Schmid et al., 2010, 2012; Díaz et al., 2012].

The evolutionary most parsimonious interpretation is that the common ancestor of *G. falconensis* and *G. taniae* already possessed the highly reduced  $2n = 16$  karyotype and that the observed differences between both karyotypes originated subsequently in both lineages by reciprocal translocations, inversions or other rearrangements. However, at least from a theoretical point of view, the interesting possibility that 8 centric fusions and fusion cycles were independent events which occurred in parallel after separation of the lineages leading to *G. falconensis* and *G. taniae* should also be considered. In this case the fusions in the 2 lineages could have involved different telocentric chromosomes (which is the most probable scenario), or exactly the same telocentric chromosomes (which seems highly improbable). The a priori probability that in both lineages exactly the same 8 fusion chromosomes randomly have evolved from a common ancestral karyotype with  $2n = 32$  telocentric chromosomes by 8 subsequent fusions and fusion cycles is calculated as follows:

Suppose that in the lineage leading to *G. falconensis* (lineage I), the first centric fusion occurs between the chromosomes A and B. The number of combinations of  $n$  different chromosomes in a karyotype consisting of  $m$  chromosomes is simply determined by the well-known binomial coefficient

$$C_n^m = \binom{m}{n} = \frac{m!}{(m-n)! \times n!} \quad (0 \leq n \leq m).$$

Therefore, the random probability that in the lineage leading to *G. taniae* (lineage II) exactly the same chromosomes A and B fuse is

$$p(A; B) = (C_n^m)^{-1} = \left( \frac{m!}{(m-n)! \times n!} \right)^{-1} = \left( \frac{16!}{(16-2)! \times 2!} \right)^{-1} = \frac{1}{120}.$$

Following the first centric fusion AB and its homozygous fixation in lineage I, a second fusion takes place between the chromosomes C and D. Accordingly, the probability that the same chromosomes C and D fuse in the lineage II is

$$p(C; D) = \left( \frac{14!}{(14-2)! \times 2!} \right)^{-1} = \frac{1}{91}.$$

If this calculation is carried out for all 8 subsequent fusions with  $m = 16, 14 \dots 6, 4, 2$ , the resulting random a

priori probability  $p(t)$  that in the lineage II exactly the same subsequent centric fusion chromosomes AB, CD, EF, GH, IJ, KL, MN, and OP originate as in the lineage I reads as follows

$$p(t) = (C_2^{16})^{-1} \times (C_2^{14})^{-1} \times (C_2^{12})^{-1} \dots \times (C_2^6)^{-1} \times (C_2^4)^{-1} \times (C_2^2)^{-1} = 1.22 \times 10^{-11}.$$

Thus, mathematically, the a priori probability for a random fixation of 8 identical centric fusions in the 2 independently evolving *Gonatodes* lineages I and II with an ancestral karyotype of  $2n = 32$  telocentric elements is as low as  $1.22 \times 10^{-11}$ . This practically excludes the possibility of identical fusions and fusion series during the chromosome evolution of the 2 *Gonatodes* species. Although such stochastic calculations deserve academic interest by their own right, the experimental way to prove if the karyotypes of *G. falconensis* and *G. taniae* contain the same or different fusion chromosomes is by cross-species chromosome painting.

Another point which should be investigated is if *G. purpurogularis* does also exhibit the exceptionally reduced  $2n = 16$  karyotype. This montane species, closely related to *G. falconensis* and *G. taniae*, was known only from the type locality in Calderas (Barinas State, Venezuela), but was later also sampled from the southern tip of the Cordillera de Merida in western Venezuela [Esqueda, 2004; Schargel, 2008]. If the conspicuous  $2n = 16$  karyotype is also present in *G. purpurogularis*, it would potentially represent an unambiguous synapomorphy characterizing this *Gonatodes* clade.

The only previously analyzed *Gonatodes* karyotype that does not fit in the model of a fusion continuum with the endpoints of  $2n = 32$  uniarmed chromosomes and  $2n = 16$  biarmed chromosomes is of *G. rozei* cytotype B (table 1). This male individual was sampled, together with 2 males of *G. rozei* cytotype A, on the same rock in the Guatopo National Park in Venezuela [McBee et al., 1984]. Its karyotype consisted of  $2n = 40$  and  $FN = 46$ , with only 2 distinctly biarmed chromosomes and several pairs of microchromosomes. It is not known whether these microchromosomes actually consist of euchromatin. Because of the significant karyotypic differences between cytotypes A and B, we decided to examine the specimens used by McBee et al. [1984]. The 3 male specimens (TCWC 59302, 59303, and 59304) are indeed conspecific and referable to the recently described *G. rozei*. Despite the fact that *G. rozei* is the most polychromatic species of *Gonatodes*, all 3 specimens exhibit the same color morph (morph I, see Rivero-Blanco and Schargel [2012]). The 3

**Table 3.** Cytogenetic data available for the family Sphaerodactylidae (excluding the genus *Gonatodes*)

Species	Country	2n	FN	Sex chromo- somes <sup>a</sup>	Techniques <sup>b</sup>	References
<i>Chatogekko amazonicus</i>	Brazil	36	36		1–4	dos Santos et al., 2003
<i>Euleptes europaea</i>	Mediterranean islands	42	44	XY/XX?	1, 5	Gornung et al., 2013
<i>Pristurus carteri</i>	Oman	36	36		1	Branch, 1980
<i>Teratoscincus scincus</i>	Central Asia	34	42		1	de Smet, 1981
	Central Asia	36	36		1	Manilo and Pisanets, 1984
	Central Asia	36	36		1	Manilo, 1993
	China	36	36		1	Zheng et al., 1998

<sup>a</sup> Only 1 male, 1 female and 1 juvenile from Sardinia were examined in the study of Gornung et al. [2013]. <sup>b</sup> 1 = Conventional staining; 2 = C-banding of constitutive heterochromatin; 3 = Ag-staining of NORs; 4 = BrdU-replication banding patterns; 5 = FISH with telomeric DNA probes.

specimens were visibly dissected in the leg for the purpose of obtaining bone marrow tissue for karyotyping. However, there is the possibility of mislabeled samples inasmuch as a different undescribed species of *Gonatodes* was sampled in the same area by the same researchers collecting specimens for the McBee et al. [1984] publication [Rivero-Blanco, pers. commun.].

According to Uetz and Hošek [2014], the family Sphaerodactylidae currently consists of 209 extant species and the 12 genera *Aristelliger* (8 species), *Chatogekko* (1 species), *Coleodactylus* (5 species), *Euleptes* (1 species), *Gonatodes* (30 species), *Lepidoblepharis* (18 species), *Pristurus* (26 species), *Pseudogonatodes* (7 species), *Quedenfeldtia* (2 species), *Saurodactylus* (2 species), *Sphaerodactylus* (103 species), and *Teratoscincus* (6 species). In addition to the karyotypes of the 9 *Gonatodes* species reported in the present study, cytogenetic studies were performed in only 4 further species (table 3). Their karyotypes consist of a graded series of telocentric or subtelocentric chromosomes, only few submetacentric elements, and show no clear break between macro- and microchromosomes. Most species possess diploid chromosome numbers of  $2n = 36$ , but the Mediterranean *Euleptes europaea* is exceptional in having  $2n = 42$  chromosomes, which is the highest value so far detected in the family Sphaerodactylidae, as well as one of the highest among all lizards, excluding the triploid species [Gornung et al., 2013]. It is essential to obtain much more karyological data on further sphaerodactylids belonging to all 12 genera, before any conclusion about the ancestral karyotype in this family can be drawn.

Although there is an exceptional diversity of sex-determining mechanisms operating in lizards and sex chromosome morphologies [for recent reviews, see Ezaz et al., 2009; Gamble, 2010], almost nothing is known about this

in the family Sphaerodactylidae. There are no reports concerning the effects of incubation temperature on the sex of hatchlings in any sphaerodactylid species [Gamble, 2010]. Apart from *G. ceciliae*, heteromorphic XY sex chromosomes were tentatively hypothesized in *E. europaea* [Gornung et al., 2013] (table 3). In a single male examined of this species, there was a small but distinct heteromorphism in the length of the short arms in chromosome pair 1. This heteromorphism was not detected in the female specimen studied. However, since only 1 male, 1 female and 1 juvenile from Sardinia were examined, and no C-banding of the constitutive heterochromatin was applied to the chromosomes, it cannot be excluded that this heteromorphism is a sex-independent autosomal heteromorphism.

Intra-individual variations in the number and/or location of Ag-stained NORs, as demonstrated in *G. falconensis* in the present study, are not rare in poikilothermic vertebrates [for review, see Schmid et al., 2010, 2012]. These have also been detected in 2 specimens of *G. humeralis*, in which 1–8 NORs were present at the long arm telomeric regions of several telocentric chromosomes [dos Santos et al., 2003].

A conspicuous feature of the karyotypes of *G. falconensis* and *G. taniae* is the presence of large clusters of telomeric (TTAGGG)<sub>n</sub> repeats in the heterochromatic centromere regions of all 8 chromosome pairs. Heterochromatic intrachromosomal telomeric sequences (het-ITs) were demonstrated in the karyotypes of a variety of vertebrates [for review, see Schmid et al., 2010]. The het-ITs detected so far in lizard chromosomes are listed in table 4. Apart from *G. falconensis* and *G. taniae*, the sphaerodactylid *E. europaea* also shows large het-ITs in the centromeric regions of all chromosomes, and in the teids *Aspidoscelis sexlineata* and *A. gularis*, het-ITs are

**Table 4.** Lizard species with het-ITSs in the chromosomes

Family/species	Chromosome sites with het-ITS	References
Agamidae <i>Leiolepis reevesii rubritaeniata</i>	interstitial region of chromosome 1	Srikulnath et al., 2009
Gymnophthalmidae <i>Leposoma guianense</i> <i>Leposoma oswaldoi</i> <i>Leposoma scincoides</i>	centromeric region of chromosome 1 centromeric region of chromosomes 1, 3 and 7 centromeric region of chromosome 1	Pellegrino et al., 1999 Pellegrino et al., 1999 Pellegrino et al., 1999
Phrynosomatidae <i>Cophosaurus texanus</i> <i>Sceloporus olivaceus</i>	interstitial bands on at least 3 chromosomes interstitial bands on at least 3 chromosomes	Meyne et al., 1990 Meyne et al., 1990
Phyllodactylidae <i>Gymnodactylus amarali</i>	centromeric region of centric fusion chromosome 3;4	Pellegrino et al., 2009
Polychrotidae <i>Polychrus acutirostris</i>	centromeric region of chromosomes 1, 3, 4, X <sub>1</sub> , and Y	Bertolotto et al., 2001
Sphaerodactylidae <i>Euleptes europaea</i> <i>Gonatodes falconensis</i> <i>Gonatodes taniae</i>	centromeric region of all chromosomes centromeric region of all chromosomes centromeric region of all chromosomes	Gornung et al., 2013 present study Schmid et al., 1994
Teiidae <i>Aspidoscelis sexlineata</i> <i>Aspidoscelis gularis</i>	centromeric region of most chromosomes centromeric region of most chromosomes	Meyne et al., 1990 Meyne et al., 1990

located in the centromeric regions of most chromosomes. In contrast, only few centromeric regions possess het-ITSs in the gymnophthalmids *Leposoma guianense*, *L. oswaldoi* and *L. scincoides*, the phyllodactylid *Gymnodactylus amarali*, and the polychrotid *Polychrus acutirostris*. In the agamid *Leiolepis reevesii rubritaeniata* and the phrynosomatids *Cophosaurus texanus* and *Sceloporus olivaceus*, het-ITSs are located in interstitial positions of some chromosomes.

It is conceivable that het-ITSs are relics of chromosomal repatternings that occurred during karyotype evolution which shifted telomeric (TTAGGG)<sub>n</sub> repeats into internal positions. Chromosome rearrangements, capable to internalize telomeric (TTAGGG)<sub>n</sub> repeats, are mainly centric (Robertsonian) fusions, inversions, tandem translocations, and insertions. Actually, in *L. reevesii rubritaeniata*, the single het-ITS was traced back to an evolutionarily fixed tandem fusion between chromosome 1 and an 18S + 28S-carrying microchromosome. In *G. amarali*, the sole het-ITS is located exactly in the centromeric region of a centric fusion chromosome formed by the originally telocentric chromosomes 3 and 4, which is found in heterozygous and homozygous condition in wild populations. And, finally, in *G. falconensis* and *G. taniae*, whose chromosomes supposedly originated by a series of subsequent centric fusions, very large het-ITSs

are located in the centromeric regions of all chromosomes.

It seems obvious that in the meta- and submetacentric chromosomes of *G. falconensis* and *G. taniae*, het-ITSs with their (TTAGGG)<sub>n</sub> repeats are present at the centromeric regions (i.e. the fusion sites of telocentric chromosomes). However, it is very unlikely that so many (TTAGGG)<sub>n</sub> repeats, as estimated by the size and fluorescence intensity of the hybridization signals at the centromeric regions, could have originated through centric fusions alone (compare size of telomeric and centromeric hybridization signals in fig. 1i). In the most favorable event, that is in the case of a perfect telomeric fusion without terminal breaks or deletions, just a doubling of the telomeric hybridization signals, but not the observed remarkably large centromeric hybridization signals would be detected. An alternative explanation is that in the centromeric heterochromatin of the ancestral telocentric chromosomes enough of the (TTAGGG)<sub>n</sub> repeats were already present as a major component of the repetitive DNA. The (TTAGGG)<sub>n</sub> repeat is known to be a component of the repetitive satellite DNAs of some vertebrates [Southern, 1970; Fry and Salser, 1977; Arnason et al., 1988], and several authors suggested that telomeric-like sequences are components of the satellite DNA in some vertebrates [Wiley et al., 1992; Garagna et al., 1997; Garri-

do-Ramos et al., 1998; Pagnozzi et al., 2000; Metcalfe et al., 2004]. This may also account for the very large signals in the centromeric regions of all telocentric chromosomes in *E. europaea* obtained after in situ hybridization with a telomeric DNA probe [Gornung et al., 2013].

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