

Chromosome Evolution in Dendropsophini (Amphibia, Anura, Hylinae)

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Key Words

B chromosomes · Dendropsophini · Hylidae ·
Interstitial telomeric sequence · Karyotype diversity

Abstract

Dendropsophini is the most species-rich tribe within Hylidae with 234 described species. Although cytogenetic information is sparse, chromosome numbers and morphology have been considered as an important character system for systematic inferences in this group. Using a diversity of standard and molecular techniques, we describe the previously unknown karyotypes of the genera *Xenohyla*, *Scarthyia* and *Sphaenorhynchus* and provide new information on *Dendropsophus* and *Lysapsus*. Our results reveal significant karyotype diversity among Dendropsophini, with diploid chromosome numbers ranging from $2n = 22$ in *S. goinorum*, $2n = 24$ in *Lysapsus*, *Scinax*, *Xenohyla*, and almost all species of *Sphaenorhynchus* and *Pseudis*, $2n = 26$ in *S. carneus*, $2n = 28$ in *P. cardosoi*, to $2n = 30$ in all known *Dendropsophus* species. Although nucleolar organizer regions (NORs) and C-banding patterns show a high degree of variability, NOR positions in

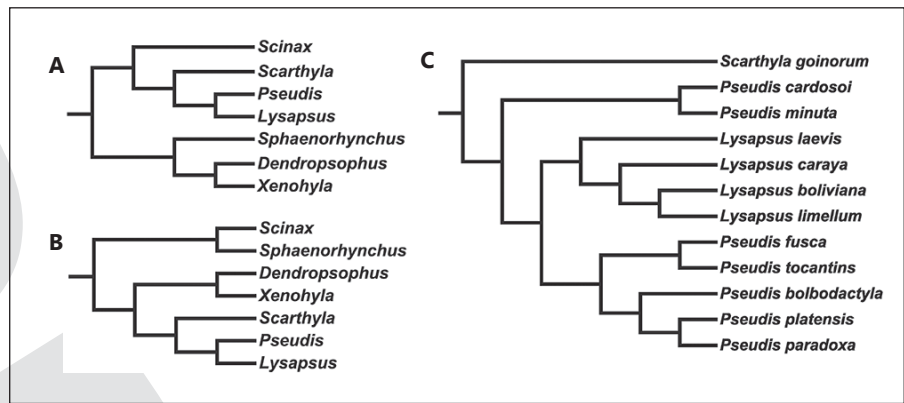
$2n = 22, 24$ and 28 karyotypes and C-banding patterns in *Lysapsus* and *Pseudis* are informative cytological markers. Interstitial telomeric sequences reveal a diploid number reduction from 24 to 22 by a chromosome fusion event. The diploid number of *X. truncata* corroborates the character state of $2n = 30$ as a synapomorphy of *Dendropsophus*.

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The treefrog family Hylidae is the most species-rich family of anurans, with 926 recognized species (Amphibian Species of the World: an Online Reference, version 5.6, <http://research.amnh.org/vz/herpetology/amphibia/>). It comprises 3 subfamilies: Hylinae, Pelodyadinae and Phyllomedusinae. Hylinae is divided in 4 tribes: Cophomantini, Dendropsophini, Hylini, and Lophiohylini [Faivovich et al., 2005], with Dendropsophini being the most species-rich with 234 described species (Amphibian Species of the World: an Online Reference, version 5.6; Faivovich et al. [2005]). Furthermore, Dendropsophini is the most problematic in terms of the low support for its monophyly [Faivovich et al., 2005; Wiens et al., 2006

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Fig. 1. Alternative phylogenetic hypotheses of Dendropsophini, showing the 4 groups that are consistently obtained: *Scinax*, *Sphaenorhynchus*, the clade *Scarthylya* + *Pseudis* + *Lysapsus*, and the clade *Xenohyla* + *Dendropsophus* by Faivovich et al. [2005] (A), by Wiens et al. [2010] (B) and phylogenetic relationship hypothesis for *Lysapsus* and *Pseudis* [Aguiar et al., 2007; Garda and Cannatella, 2007] (C).



suppl. data, 2010]. Although always poorly supported, it has been repeatedly recovered as monophyletic [see discussion in Wiens et al., 2010] or polyphyletic in recent analyses [Pyron and Wiens, 2011].

Currently, 7 genera are included in Dendropsophini, *Dendropsophus* (94 species), *Lysapsus* (4 species), *Pseudis* (7 species), *Scarthylya* (2 species), *Scinax* (111 species), *Sphaenorhynchus* (14 species), and *Xenohyla* (2 species) (Amphibian Species of the World: an Online Reference, version 5.6). The individual monophyly of most of these genera is well supported by molecular data, but relationships among them – particularly the positions of *Scinax* and *Sphaenorhynchus* – are still unstable (fig. 1A, B) [Faivovich et al., 2005; Wiens et al., 2010]. Two phylogenetic analyses of *Lysapsus* and *Pseudis* were recently published (fig. 1C) [Aguiar et al., 2007; Garda and Cannatella, 2007]. The monophyly of *Scarthylya* and *Xenohyla* has not been tested. While it does not seem controversial in *Xenohyla* [Faivovich et al., 2005], additional research is necessary in *Scarthylya*.

Knowledge on chromosome number and morphology in hylids is still sparse, and in Dendropsophini, only 74 of the 234 assigned species have known karyotypes [Catroli and Kasahara, 2009; Cardozo et al., 2011], with most information limited to chromosome numbers. These are available for 1 of the 14 species of *Sphaenorhynchus*, 3 of the 4 species of *Lysapsus*, all species of *Pseudis*, 24 of the 94 species of *Dendropsophus*, and 39 of the 111 species of *Scinax* [Catroli and Kasahara, 2009; Cardozo et al., 2011]. No cytogenetic information is available for *Scarthylya* and *Xenohyla*.

Even though cytogenetic information is available for 31% of the species of Dendropsophini, possibly, the best example of how chromosome number and karyotype diversity has been used as evidence of relationship in the

tribe is the genus *Dendropsophus* [Duellman and Cole, 1965; Duellman, 1967, 1970; Bogart, 1973; Kaiser et al., 1996; Gruber et al., 2005]. This genus includes all Neotropical species formerly placed in *Hyla* that are known or suspected to have 30 chromosomes [Faivovich et al., 2005]. The species of *Dendropsophus* have been suspected to be related based on chromosome number since the 1960s [Duellman and Cole, 1965; Duellman, 1967]. Recently, the chromosome number of $2n = 30$ has been considered a putative synapomorphy of the genus, with the caveat that the karyotype in *Xenohyla*, the sister group of *Dendropsophus* [Faivovich et al., 2005; Wiens et al., 2010], remains unknown. *Dendropsophus* also has 6 known different fundamental numbers (FN), and some species present B chromosomes [Gruber et al., 2005].

Within the tribe, apart from *Dendropsophus*, only *P. cardosoi* with somatic chromosome number of $2n = 28$ [Busin et al., 2001] is known to have a number of chromosomes different from the putatively plesiomorphic hylid number, $2n = 24$. The $2n = 28$ karyotype is so far an autapomorphy of *P. cardosoi* [Busin et al., 2008].

In this contribution, using a diversity of standard and molecular techniques, we describe the previously unknown karyotypes of *Xenohyla*, *Scarthylya* and *Sphaenorhynchus* and also present more information on 2 species of *Dendropsophus* and 1 of *Lysapsus*. With these data, we discuss chromosome evolution in this speciose and complex treefrog group.

Material and Methods

Individuals of 9 species of the genera *Dendropsophus*, *Lysapsus*, *Scarthylya*, *Sphaenorhynchus*, and *Xenohyla* were cytogenetically analyzed. Specimens were sacrificed with lidocaine 5%, fixed in formalin 10%, stored in ethanol 70%, and housed in the herpeto-

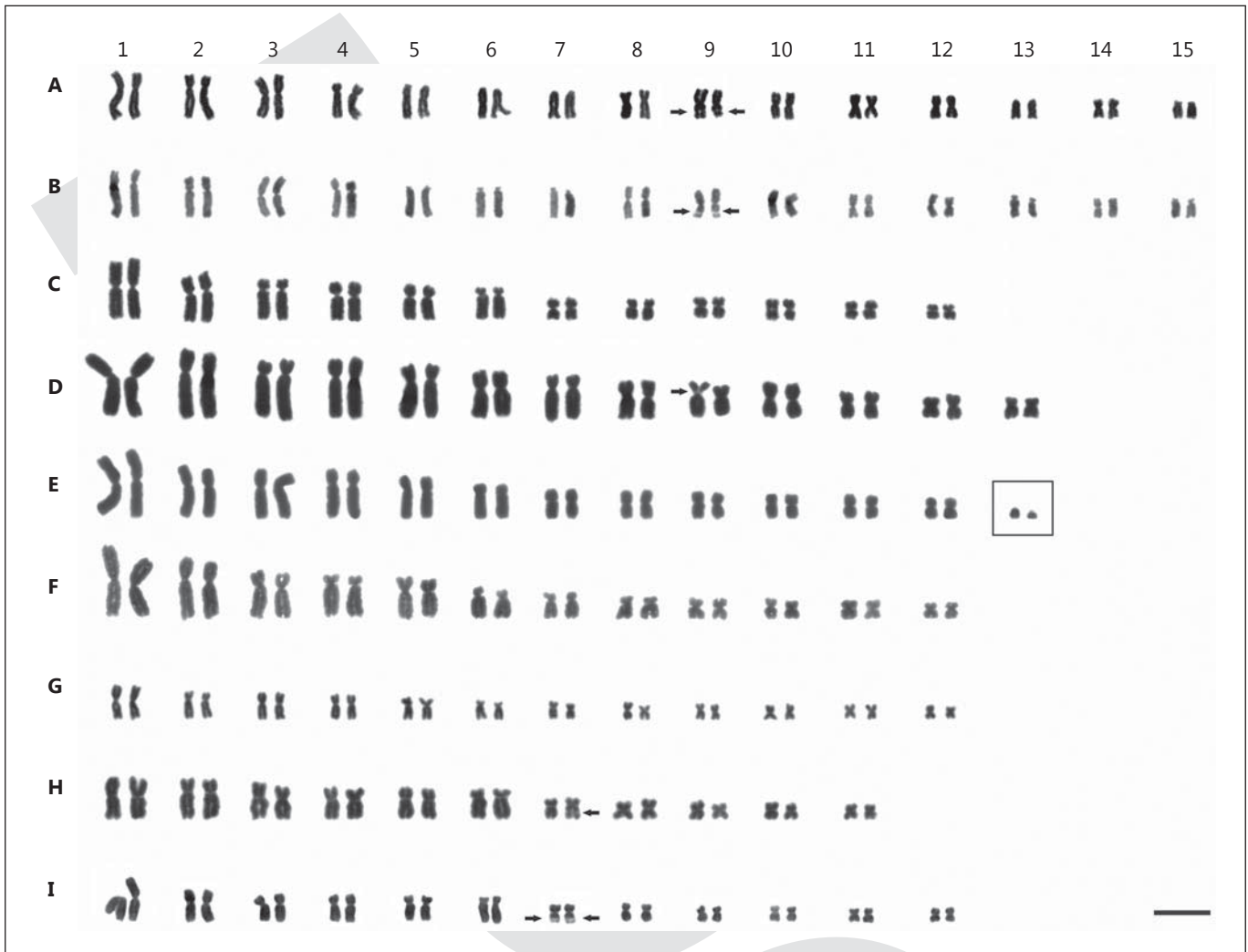


Fig. 2. Giemsa stained karyotypes. **A** *D. marmoratus*; **B** *D. melanargyreus*; **C** *S. caramaschii*; **D** *S. carneus*; **E** *S. dorisae*; **F** *S. lacteus*; **G** *L. laevis*; **H** *S. goinorum*; **I** *X. truncata*. Arrows indicate evident nucleolar constrictions. B chromosomes of *S. dorisae* are framed. Scale bar = 10 μ m.

logical collection of the Museu Paranaense Emílio Goeldi, Belém, Pará, Brazil, and in the Célio F. B. Haddad Amphibian collection, Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil (online suppl. appendix 1 for voucher information, locality data and sex of each analyzed specimen; see www.karger.com/doi/10.1159/000354997 for all online suppl. material).

Chromosome Preparations

Preparations of mitotic chromosomes were obtained from intestine and bone marrow suspensions following the protocol of Schmid et al. [2010]. Fixed cell suspensions were dropped or spread on slides and stained with a 5%-Giemsa solution with phosphate buffer. Metaphasic chromosomes were treated with different techniques: AgNO₃ impregnation [Howell and Black, 1980], C-

banding [Sumner, 1972], fluorochromes CMA₃/DAPI [Schweizer and Ambros, 1994], and FISH with 18S rDNA probes [Viegas-Péquignot, 1992]. Telomeric regions were revealed using human telomeric probes (ONCOR P4097-DG5) following the protocol of the manufacturer.

Staining with CMA₃/DAPI fluorochromes was done on untreated cells and on cells treated with C-banding or FISH procedures [Pieczarka et al., 2006]. Figures with results of all these techniques are shown only for *S. lacteus*, while results for the other species are summarized in ideograms in the results sections.

Analysis of Karyotypes and Banding Patterns

Karyotypes were constructed using Corel X3 software and chromosomes were classified following conventions of Levan et al. [1964], with modifications of Green and Sessions [1991]. To pre-

serve the chromosome homeology suggested by previous authors, the orders of some chromosome pairs were altered in *Dendropsophus* and *Lysapsus* species. The telocentric chromosomes of *Dendropsophus* were ordered following the proposal of Bogart [1973], whereas the second and third pairs of *L. laevis* were altered following Busin et al. [2006]. The short and long arms of chromosomes are termed p and q, respectively. The terms x (basic chromosome number), n (gametic chromosome number), 2n, and FN were used as suggested by White [1954]. Measurements and centromeric ratio of each chromosome pair were calculated on 10 metaphase plates with MicroMeasure v3.3 software (MicroMeasure for Windows, version 3.3, <http://www.colostate.edu/Depts/Biology/MicroMeasure>).

Taxonomic Considerations about Some Analyzed Taxa

The nomenclature of *Pseudis* and *Lysapsus* has recently generated some controversy (summarized in Amphibian Species of the World: an Online Reference, version 5.6). Its origin lay in the non-monophyly of *Pseudis*, as previously defined by Klappenbach [1985], in the analyses of Aguiar et al. [2007] and Garda and Cannatella [2007] and in the proposed solutions to deal with the situation. More recently, Wiens et al. [2010] and Pyron and Wiens [2011] recovered *Pseudis* as monophyletic. However, the position of the problematic clade composed of *P. cardosoi* and *P. minuta*, in the analyses of Aguiar et al. [2007] and Garda and Cannatella [2007], is not particularly well-supported by the most recent hypotheses. For the time being, we recognize the genera *Lysapsus* and *Pseudis*, but the issue will not be settled until more evidence for the monophyly of *Pseudis* is advanced.

Results

We present cytogenetic data of 9 *Dendropsophini* species from 5 genera: *Lysapsus* (*L. laevis*), *Scarthyla* (*S. goinorum*), *Sphaenorhynchus* (*S. lacteus*, *S. carneus*, *S. caramaschii*, and *S. dorisae*), *Xenohyla* (*X. truncata*), and *Dendropsophus* (*D. marmoratus* and *D. melanargyreus*), 8 of them for the first time. Nucleolar organizer region (NOR) locations, C-banding pattern, fluorochrome banding pattern, and locations of telomeric sequences also constitute original data for all karyological descriptions.

There is a significant diversity of diploid chromosome numbers in the studied species, ranging from $2n = 22$ (FN = 44) in *S. goinorum*, $2n = 24$ (FN = 48) in *L. laevis*, *S. caramaschii*, *S. dorisae*, *S. lacteus*, and *X. truncata*, $2n = 26$ (FN = 52) in *S. carneus*, to $2n = 30$ (FN = 50) in *D. marmoratus* and *D. melanargyreus* (fig. 2). No heteromorphic sex chromosomes were observed in the analyzed species. Additionally, in 3 specimens of *S. dorisae*, there are 1 (PS 894) or 2 (PS 891, 899) supernumerary chromosomes ($2n = 24 + 1-2 B$) (see table 1 for chromosome morphometric data from karyotypes).

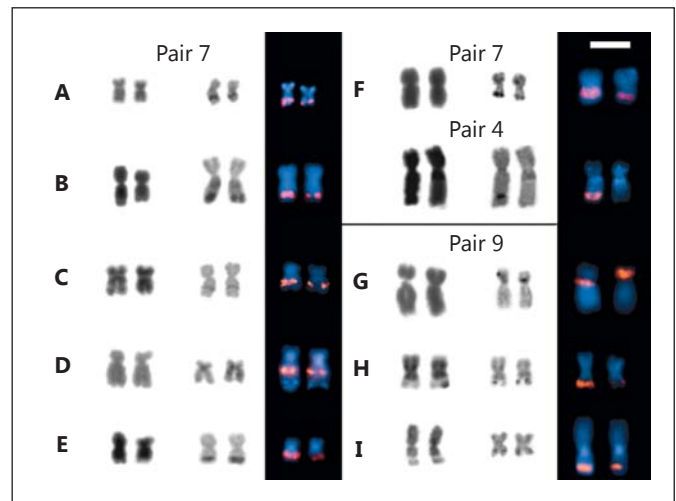


Fig. 3. NOR-bearing chromosome pairs characterized by presence/absence of nucleolar constrictions in conventionally stained cells (left column), silver impregnation (second column) and FISH with 18S rDNA probe (right column). **A** *L. laevis*; **B** *S. caramaschii*; **C** *S. dorisae*; **D** *S. lacteus*; **E** *X. truncata*; **F** *S. goinorum*, main NOR chromosome pair 7q and additional NOR on one chromosome of pair 4 in one specimen; **G** *S. carneus*; **H** *D. marmoratus*; **I** *D. melanargyreus*. Arrows indicate evident nucleolar constrictions. Scale bar = 10 μ m.

All karyotypes with 22 or 24 chromosomes have interstitial nucleolar constrictions on pair 7q (fig. 3A–F). In *S. carneus*, they occur on the pericentromeric region of pair 9p (fig. 3G); in species of *Dendropsophus* (30 chromosomes), they occur in the interstitial region of pair 9q (fig. 3H, I). In most cases, nucleolar constrictions are CMA₃⁺ (GC-rich regions, fig. 6), coincident with Ag-NOR marks and the location of 18S rDNA probes in FISH; additional signals of this gene are observed on one chromosome of pair 4q in a single male of *S. goinorum* (PS 892), and thus it does not seem to be sex-linked (fig. 3F).

In most analyzed species, positive C-bands are distributed predominantly throughout the centromeric region of most chromosome pairs, with the exception of *S. caramaschii*, which only shows a single heterochromatic band on pair 7q associated to NORs (figs. 4–6). Additionally, in the species of *Dendropsophus*, *S. carneus*, *S. dorisae*, and *X. truncata*, some interstitial and/or distal C-bands are also present, while in *L. laevis*, *S. goinorum* and *S. lacteus*, they show a more complex pattern with pericentromeric interstitial and distal C-positive bands (figs. 4–6). CMA₃/DAPI staining, performed after FISH or C-banding treatment, provides similar rich and varied banding

Table 1. Morphometric data of the karyotypes from the species included in the present study

| Chromosome pair | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|-------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| <i>D. marmoratus</i> | | | | | | | | | | | | | | | | |
| % Set | 10.97 | 10.01 | 9.59 | 7.99 | 7.01 | 6.58 | 6.47 | 6.47 | 6.85 | 6.27 | 6.22 | 5.36 | 4.95 | 4.39 | 4.30 | 3.48 |
| CR | 0.26±0.04 | 0.38±0.02 | 0.26±0.04 | 0.06±0.01 | 0.07±0.03 | 0.12±0.03 | 0.12±0.03 | 0.41±0.03 | 0.42±0.03 | 0.46±0.03 | 0.40±0.06 | 0.45±0.05 | 0.45±0.03 | 0.13±0.09 | 0.40±0.05 | 0.12±0.03 |
| Type | sm | m | sm | t | t | t | t | m* | m | m* | m | m | m | t | m | t |
| <i>D. melanargyreus</i> | | | | | | | | | | | | | | | | |
| % Set | 10.77 | 9.31 | 9.20 | 8.32 | 7.33 | 6.94 | 6.47 | 6.47 | 7.13 | 6.49 | 6.26 | 5.20 | 5.01 | 5.16 | 4.73 | 4.54 |
| CR | 0.30±0.03 | 0.33±0.04 | 0.44±0.04 | 0.33±0.05 | 0.13±0.04 | 0.12±0.03 | 0.12±0.03 | 0.12±0.03 | 0.42±0.04 | 0.46±0.03 | 0.45±0.04 | 0.44±0.02 | 0.45±0.03 | 0.18±0.03 | 0.44±0.03 | 0.21±0.05 |
| Type | sm | m | m | sm | t | t | t | t | m | m* | m | m | m | st | m | st |
| <i>P. laevis</i> | | | | | | | | | | | | | | | | |
| % Set | 14.00 | 10.88 | 11.45 | 10.10 | 9.51 | 8.09 | 7.10 | 6.88 | 6.70 | 6.57 | 6.59 | 6.57 | 6.13 | 6.13 | 6.13 | 6.13 |
| CR | 0.46±0.02 | 0.30±0.02 | 0.41±0.02 | 0.37±0.02 | 0.39±0.02 | 0.26±0.03 | 0.38±0.07 | 0.44±0.05 | 0.43±0.04 | 0.45±0.04 | 0.45±0.04 | 0.42±0.07 | 0.42±0.05 | 0.42±0.05 | 0.42±0.05 | 0.42±0.05 |
| Type | m | sm | m | sm | m | sm | sm* | m | m | m | m | m | m | m | m | m |
| <i>S. goinorum</i> | | | | | | | | | | | | | | | | |
| % Set | 14.21 | 12.71 | 11.56 | 11.10 | 11.10 | 10.09 | 6.98 | 6.31 | 5.88 | 5.88 | 5.48 | 5.06 | 4.60 | 4.60 | 4.60 | 4.60 |
| CR | 0.44±0.04 | 0.33±0.04 | 0.39±0.04 | 0.36±0.04 | 0.30±0.04 | 0.35±0.05 | 0.41±0.04 | 0.42±0.03 | 0.40±0.04 | 0.43±0.05 | 0.43±0.05 | 0.42±0.04 | 0.42±0.03 | 0.37±0.03 | 0.42±0.03 | 0.37±0.03 |
| Type | m | sm | m | sm | sm | sm | m* | m | m | m | m | m | m | sm | m | sm |
| <i>S. caramaschii</i> | | | | | | | | | | | | | | | | |
| % Set | 14.50 | 12.01 | 11.03 | 10.88 | 9.79 | 8.20 | 6.57 | 6.25 | 5.89 | 5.89 | 5.51 | 5.28 | 4.60 | 4.60 | 4.60 | 4.60 |
| CR | 0.45±0.02 | 0.39±0.01 | 0.34±0.03 | 0.27±0.03 | 0.34±0.02 | 0.22±0.02 | 0.42±0.04 | 0.37±0.03 | 0.48±0.02 | 0.48±0.02 | 0.42±0.04 | 0.42±0.03 | 0.37±0.03 | 0.42±0.03 | 0.42±0.03 | 0.37±0.03 |
| Type | m | sm | sm | sm | sm | st | m* | sm | m | m | m | m | m | m | m | sm |
| <i>S. dorisae</i> | | | | | | | | | | | | | | | | |
| % Set | 15.23 | 11.90 | 10.88 | 10.38 | 9.90 | 8.07 | 6.52 | 6.31 | 6.04 | 6.04 | 5.63 | 5.43 | 4.72 | 1.44# | 4.72 | 1.44# |
| CR | 0.45±0.02 | 0.39±0.03 | 0.34±0.04 | 0.33±0.04 | 0.38±0.03 | 0.28±0.03 | 0.45±0.03 | 0.43±0.04 | 0.44±0.03 | 0.44±0.03 | 0.43±0.05 | 0.44±0.03 | 0.41±0.04 | 0.00# | 0.41±0.04 | 0.00# |
| Type | m | sm | sm | sm | m | sm | m* | m | m | m | m | m | m | t# | m | t# |
| <i>S. lacteus</i> | | | | | | | | | | | | | | | | |
| % Set | 15.80 | 13.37 | 11.23 | 10.11 | 9.95 | 7.74 | 6.60 | 5.93 | 5.57 | 5.57 | 5.16 | 5.02 | 4.21 | 4.21 | 4.21 | 4.21 |
| CR | 0.47±0.03 | 0.35±0.02 | 0.33±0.03 | 0.24±0.06 | 0.34±0.04 | 0.23±0.05 | 0.32±0.03 | 0.31±0.05 | 0.42±0.03 | 0.42±0.03 | 0.42±0.04 | 0.41±0.04 | 0.37±0.03 | 0.37±0.03 | 0.37±0.03 | 0.37±0.03 |
| Type | m | sm | sm | st | sm | st | sm* | m | m | m | m | m | sm | sm | m | sm |
| <i>S. carneus</i> | | | | | | | | | | | | | | | | |
| % Set | 12.52 | 12.04 | 10.56 | 9.79 | 9.01 | 8.05 | 7.34 | 6.34 | 6.33 | 6.33 | 5.95 | 4.97 | 4.52 | 3.72 | 4.52 | 3.72 |
| CR | 0.39±0.02 | 0.37±0.03 | 0.27±0.05 | 0.33±0.03 | 0.32±0.04 | 0.35±0.04 | 0.30±0.04 | 0.40±0.04 | 0.36±0.04 | 0.36±0.04 | 0.46±0.03 | 0.44±0.03 | 0.44±0.03 | 0.44±0.03 | 0.44±0.03 | 0.37±0.03 |
| Type | m | sm | sm | sm | sm | sm | sm | m | sm* | sm* | m | m | m | m | m | sm |
| <i>X. truncata</i> | | | | | | | | | | | | | | | | |
| % Set | 16.29 | 12.05 | 10.47 | 9.61 | 9.13 | 8.60 | 6.52 | 6.11 | 5.74 | 5.74 | 5.35 | 5.29 | 4.83 | 4.83 | 4.83 | 4.83 |
| CR | 0.48±0.02 | 0.39±0.03 | 0.38±0.03 | 0.32±0.05 | 0.36±0.03 | 0.30±0.04 | 0.40±0.04 | 0.43±0.05 | 0.42±0.05 | 0.42±0.05 | 0.40±0.03 | 0.40±0.05 | 0.41±0.04 | 0.41±0.04 | 0.41±0.04 | 0.41±0.04 |
| Type | m | m | m | sm | sm | sm | m* | m | m | m | m | m | m | m | m | m |

% Set = Relative size; CR = centromeric ratio; Type: m = metacentric; sm = submetacentric; st = subtelocentric; t = telocentric; * = NOR-bearing chromosome pair; # = B chromosome.

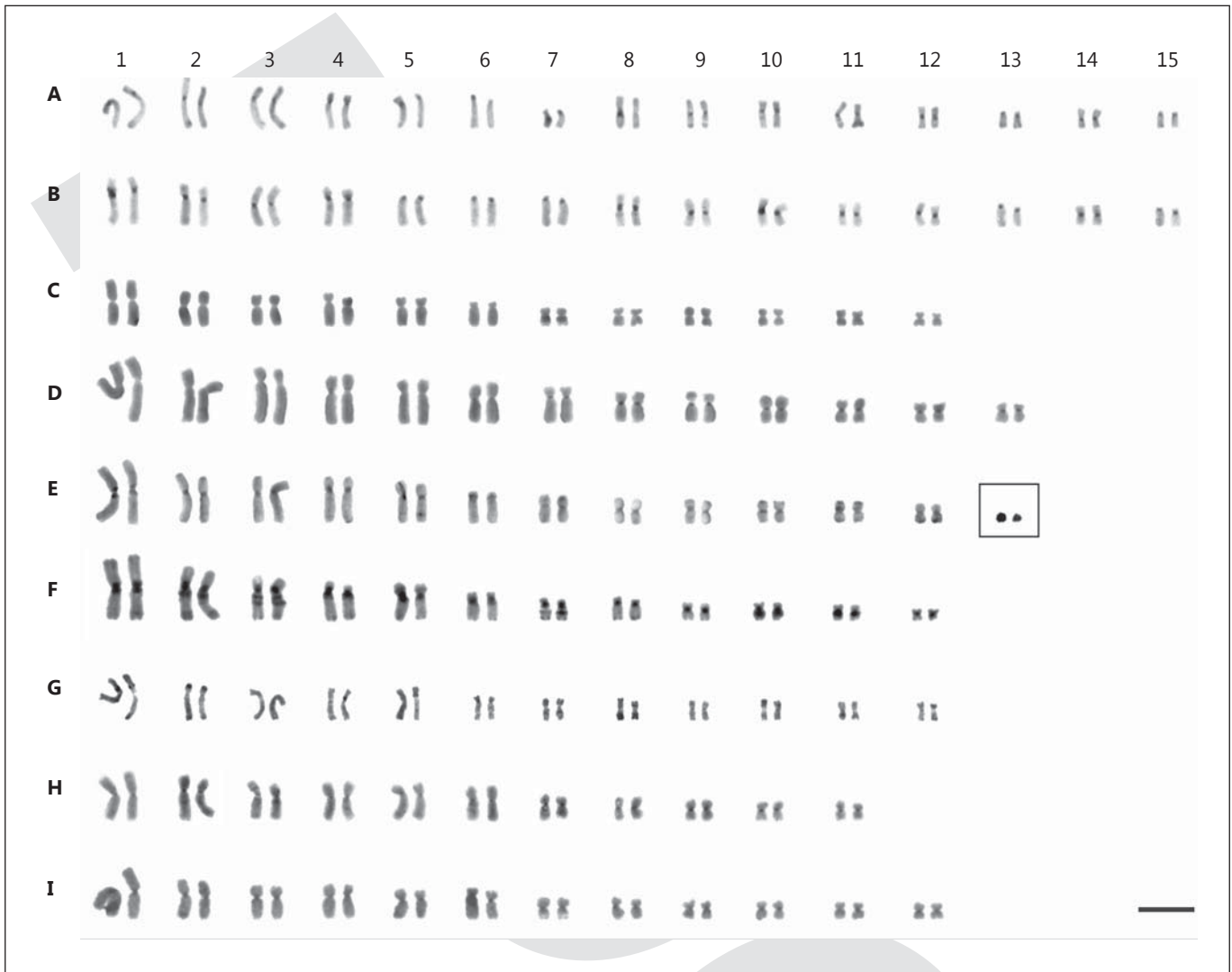


Fig. 4. C-banded karyotype patterns. **A** *D. marmoratus*; **B** *D. melanargyreus*; **C** *S. caramaschii*; **D** *S. carneus*; **E** *S. dorisae*; **F** *S. lacteus*; **G** *L. laevis*; **H** *S. goinorum*; **I** *X. truncata*. B chromosomes of *S. dorisae* are framed. Scale bar = 10 μ m.

patterns, improving the brightness and contrast of C-bands. Conversely, the pattern observed in cells without pretreatment shows in most cases only CMA₃ bands associated to NOR locations (fig. 5). In almost all analyzed species, C-banded cells show enhanced fluorescence of centromeric heterochromatin (CMA₃⁺/DAPI⁺ bands), while in *L. laevis*, pairs 1–3 and 7–11 had DAPI⁺ centromeric heterochromatin (fig. 5) and CMA₃⁺ in *S. goinorum* and *X. truncata*. Interstitial and distal C-bands were mainly CMA₃⁺/DAPI⁺ in most species and DAPI⁺ in *S. lacteus*. Figures 5 and 6 summarize the results of these techniques.

The B chromosomes in *S. dorisae* are telocentric and mostly heterochromatic, with a fluorochrome pattern CMA₃⁺/DAPI⁺ (figs. 4, 7). Meiotic analyses in specimens with 2 B chromosomes (PS 891, 899) revealed them as univalents (51.25%) or associated as a bivalent (48.75%) in diakinesis (fig. 7A, B). Furthermore, metaphase II cells show none (14.47%), 1 (75%) or 2 (10.53%) B chromosomes (fig. 7C–E), indicating meiotic instability of these extra chromosomes.

FISH using telomeric probes (TTAGGG)_n reveals small distal fluorescence in the telomeres of all chromosomes in the studied species (fig. 8). Additionally, a

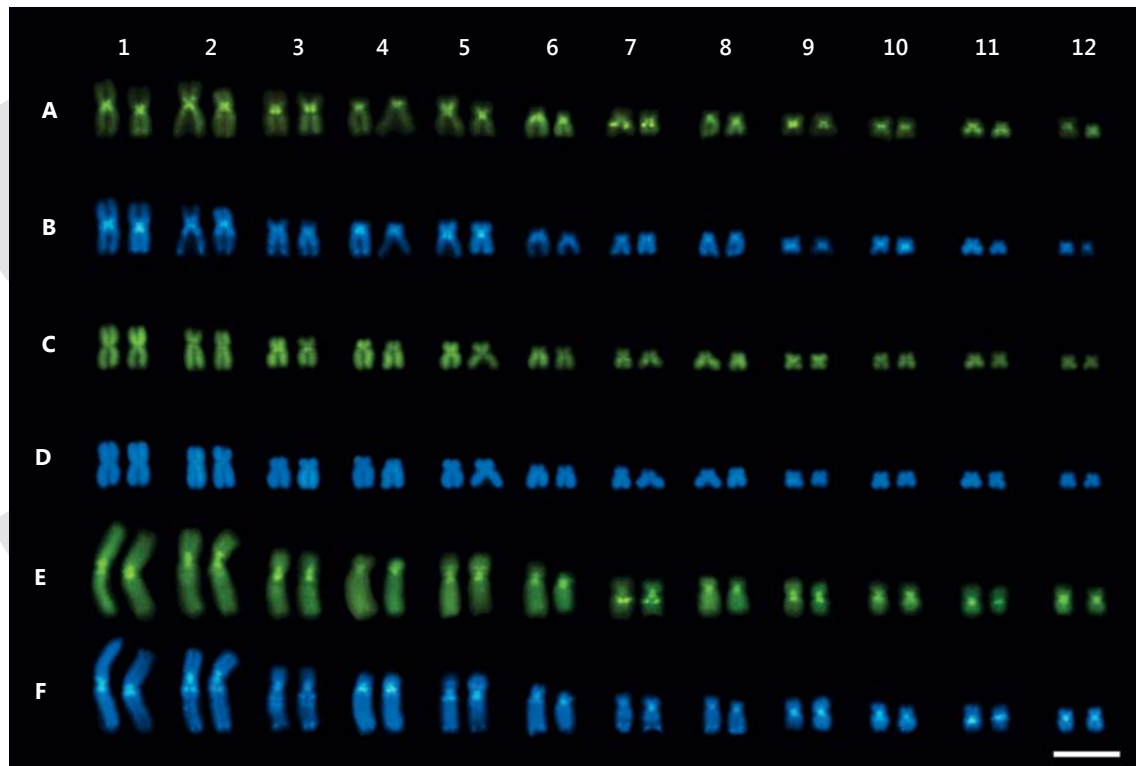


Fig. 5. Differential CMA₃ (A, C, E)/DAPI (B, D, F) banding patterns of *S. lacteus*, after different pretreatment procedures. A, B C-banded cells; C, D cells without pretreatment; E, F cells after formamide denaturation in FISH experiments. Scale bar = 10 μ m.

brighter fluorescent signal covers almost half of pair 2p of *S. lacteus* (CMA₃⁻/DAPI⁻) and most of the B chromosomes in *S. dorisae* (CMA₃⁺/DAPI⁺) (fig. 8E, F, respectively). A CMA₃⁺ interstitial telomere sequence (ITS) occurs on centromere of pair 3 in *S. goinorum*.

Discussion

The cytogenetic data available for at least 82 species of Dendropsophini indicate a great diversity of chromosome and fundamental numbers, NOR positions and heterochromatin patterns. Diploid numbers range from $2n = 2x = 22$ in *S. goinorum* to $2n = 2x = 30$ in all *Dendropsophus* species studied so far [Catroli and Kasahara, 2009; Cardozo et al., 2011; present work].

The karyotype with $x = 12$ is the more prevalent in Dendropsophini; it has been observed in all studied species of *Scinax* and *Lysapsus*, most *Pseudis* and *Sphaenorhynchus* species (except *P. cardosoi* and *S. carneus*) and *X. truncata*. This character state is shared by most mem-

bers of Cophomantini, Hylini and Lophiohylini and has been suggested as a putative synapomorphy of Hylineae [Duellman, 2001]. However, as previously indicated by Faivovich et al. [2005], its corroboration depends on a better understanding of the taxonomic distribution of this character state in the more basal members of most tribes.

The monophyly of Dendropsophini has been corroborated in some studies with low support [Faivovich et al., 2005; Wiens et al., 2005, 2010] or rejected [Wiens et al., 2006 suppl. data, 2010 suppl. data; Pyron and Wiens, 2011]. The 4 groups that are consistently referred to in all analyses are *Scinax*, *Sphaenorhynchus*, the clade composed of *Scarthyla*, *Lysapsus* and *Pseudis*, and the clade that includes *Xenohyla* and *Dendropsophus*. Although described as nonmonophyletic, the most recent analysis [Pyron and Wiens, 2011] indicates with no support that *Scinax* and *Sphaenorhynchus* are sister taxa and with low support that the clade that includes *Lysapsus*, *Pseudis* and *Scarthyla* is the sister group of the clade composed of *Xenohyla* and *Dendropsophus*.

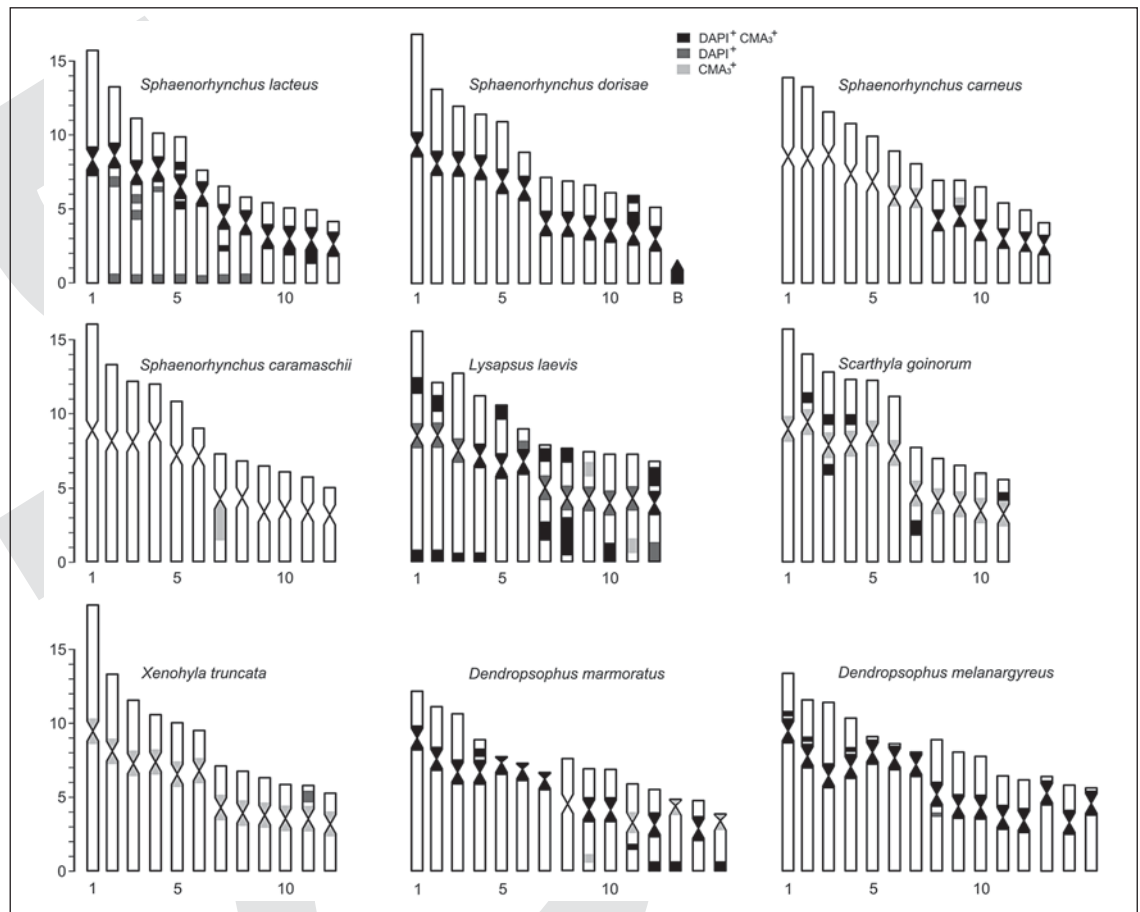


Fig. 6. Idiograms with marked heterochromatin fluorescence of C-banded karyotypes of the species studied in this paper. Scale indicates percentage relative size of chromosome pairs.

Scinax: Diversity within the Plesiomorphic 24-Chromosome Complement

Several species of *Scinax* (39 of 111 species) have been included in cytogenetic analyses [recently reviewed by Cardozo et al., 2011]. All studied species have $2n = 2x = 24$ biarmed chromosomes, and pairs 1, 2 and 6 have sizes and morphologies typical of species of both major clades recognized [Cardozo et al., 2011]. Species of the *S. ruber* clade, as many other hylids, have NORs on pair 11q, except for *S. alter*, which has terminal NORs on pair 3q. Most species of the *S. catharinae* clade have NORs on pair 6p, and this character state has been proposed as a putative synapomorphy of the clade [Cardozo et al., 2011]. Finally, the C-banding pattern is predominantly centromeric in *Scinax*, but in the *S. catharinae* clade, there is a greater amount of pericentromeric heterochromatin than in the *S. ruber* clade [Cardozo et al., 2011].

Lysapsus, *Pseudis* and *Scarthyla*: Homeology, Robertsonian Rearrangements and Centromeric ITS

The species of *Lysapsus* and *Pseudis* share similar chromosome morphology, with $2n = 24$ (FN = 48) chromosomes, and the *P. cardosoi* karyotype ($2n = 28$; FN = 48) is an autapomorphic condition due to Robertsonian rearrangements [Busin et al., 2000, 2008]. *L. caraya* differs from other taxa having all metacentric chromosomes and NORs on 6p [Busin et al., 2006], whereas the remaining species with $2n = 24$ have nuclear constrictions and NORs on pair 7q [Busin et al., 2000; present work]. Although *P. cardosoi* bears the NORs on 5q [Busin et al., 2000, 2008], these chromosomes should be considered homeologous to 7q chromosomes of other *Lysapsus* and *Pseudis* karyotypes, as the different location in the karyotype would be a consequence of the fissions of pairs 1 and 4 that originated the 28-chromosome karyotype [Busin et al., 2000].

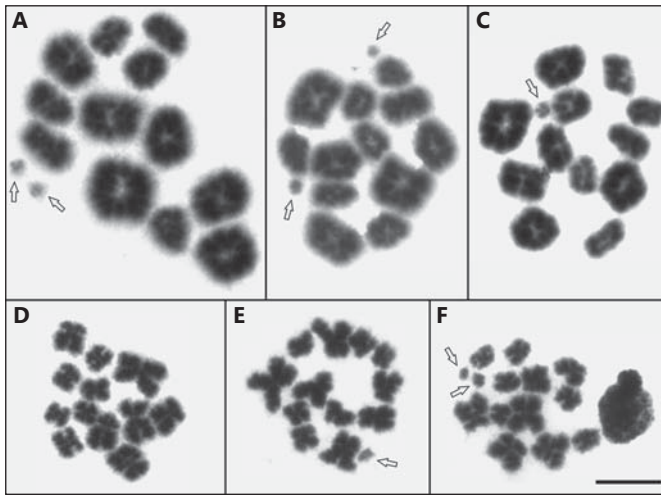


Fig. 7. Meiotic behavior of B chromosomes in *S. dorisae* with 2 supernumerary chromosomes (arrows). Diakinesis cells with 2 B chromosomes as univalents (**A, B**) or associated as a bivalent (**C**); metaphase II with none (**D**), 1 (**E**) or 2 (**F**) B chromosomes. Scale bar = 10 μ m.

Additional NORs were detected on 1q and 8q pairs in *L. bolivianus* and on 7q in *L. caraya* [Busin et al., 2006].

The C-banding patterns in species of *Lysapsus* and *Pseudis* include centromeric bands on most chromosome pairs [Busin et al., 2000, 2006, 2008; present work]. Additionally, interstitial and distal C-bands are present on chromosome pairs 1–3 and 6–8 [Busin et al., 2000, 2006, 2008; present work]. These character states are putative synapomorphies of this group. In *Pseudis*, the clade composed of *P. minuta* and *P. cardosoi* is characterized by an interstitial heterochromatic block on pair 2p (or its homeologue, see Busin et al., [2000]). The clade containing all other species (*P. fusca*, *P. tocantins*, *P. bolbodactyla*, *P. platensis*, and *P. paradoxa*) shows distal bands on 2q, 3q and proximal on 6p [Busin et al., 2008], and these represent putative synapomorphies of this clade. The presence of a ZZ/ZW chromosome system of sexual determination in *P. tocantins* [Busin et al., 2008], involving chromosome pair 7, is an autapomorphy of this taxon, and the origin of such system remains unknown. The species of *Lysapsus* share a C-banding pattern with several interstitial and distal bands; the conspicuous interstitial bands on 1p and distal on 2p, only present in these species [Busin et al., 2006; present work], are putative synapomorphies of this clade.

The cytogenetic data presented here show that *S. goinorum*, with $2n = 22$ biarmed chromosomes, shares the

position of NORs on 7q with most species of *Lysapsus* and *Pseudis*. The C-banding pattern is characterized by the occurrence of centromeric and interstitial bands on pairs 2p, 3p, 3q, 4p, 7q, and 11p (fig. 6).

The presence of a centromeric ITS on pair 3 in *S. goinorum* could be interpreted as an evidence of chromosomal rearrangements that led to the reduction of chromosome number in this species from 24 to 22. However, there are diverse mechanisms that explain the origin of ITS sequences other than chromosomal rearrangements and need to be briefly considered here. These include the amplification of the oligonucleotide (TTTAGGG)_n associated to heterochromatin extension, mutations, transposition or unequal crossing-over, or the acquisition of ITS related to transcriptional processes associated to the DNA repair machinery [Tsipouri et al., 2008; Carvalho et al., 2009 and references therein; Ventura et al., 2009; Gaspin et al., 2010].

In Anura, the presence of noncentromeric ITS has been described for *Xenopus laevis* and *X. clivii* (Pipidae) [Meyne et al., 1990; Nanda et al., 2008], *Pristimantis fenestratus* and *P. riveroi* (Strabomantidae) [Schmid et al., 2010] and for 7 species of the Hyline tribe: 5 species of *Hyla* ($2n = 24, 48$) and 2 species of *Pseudacris* ($2n = 24$) [Meyne et al., 1990; Wiley et al., 1992]. Nevertheless, noncentromeric ITS were not observed in any of the 20 species analyzed with the telomeric probe of the other Hyline tribes, including 8 taxa with the ancestral $2n = 24$ karyotype [Anderson, 1996; Carvalho et al., 2009; Ferro et al., 2012; Gruber et al., 2012; present work]. At the same time, the presence of centromeric ITS has only been reported for some species of *Aplastodiscus* with $2n = 22$ (*A. albofrenatus*, *A. arildae* and *A. eugenioi*) and $2n = 18$ (*A. leucopygius*). However, other species with $2n = 22$ (*A. ehrhardti*) or $2n = 20$ (*A. callipygius*) have no ITS, although the transformations between $2n = 24$ and $2n = 22$ and those between $2n = 24$, $2n = 20$ and $2n = 18$ are well-supported by the analyses of diverse cytological markers in a phylogenetic context [Carvalho et al., 2009; Gruber et al., 2012]. The only other anuran for which centromeric ITS were reported is *P. cruentus* [Schmid et al., 2010].

Similar to subtelomeric repeats and unlike true telomeres that are euchromatic, a particular feature of the ITS is the acquisition of a heterochromatinized state [Vaquero-Sedas and Vega-Palas, 2011]. By analyses of 55 vertebrate karyotypes with the telomeric probe, Meyne et al. [1990] concluded that the majority of the ITS is located near or within the heterochromatic blocks revealed by C-banding and that most of the ITS and the heterochromatic blocks colocalize pericentromerically. Among anurans, this situ-

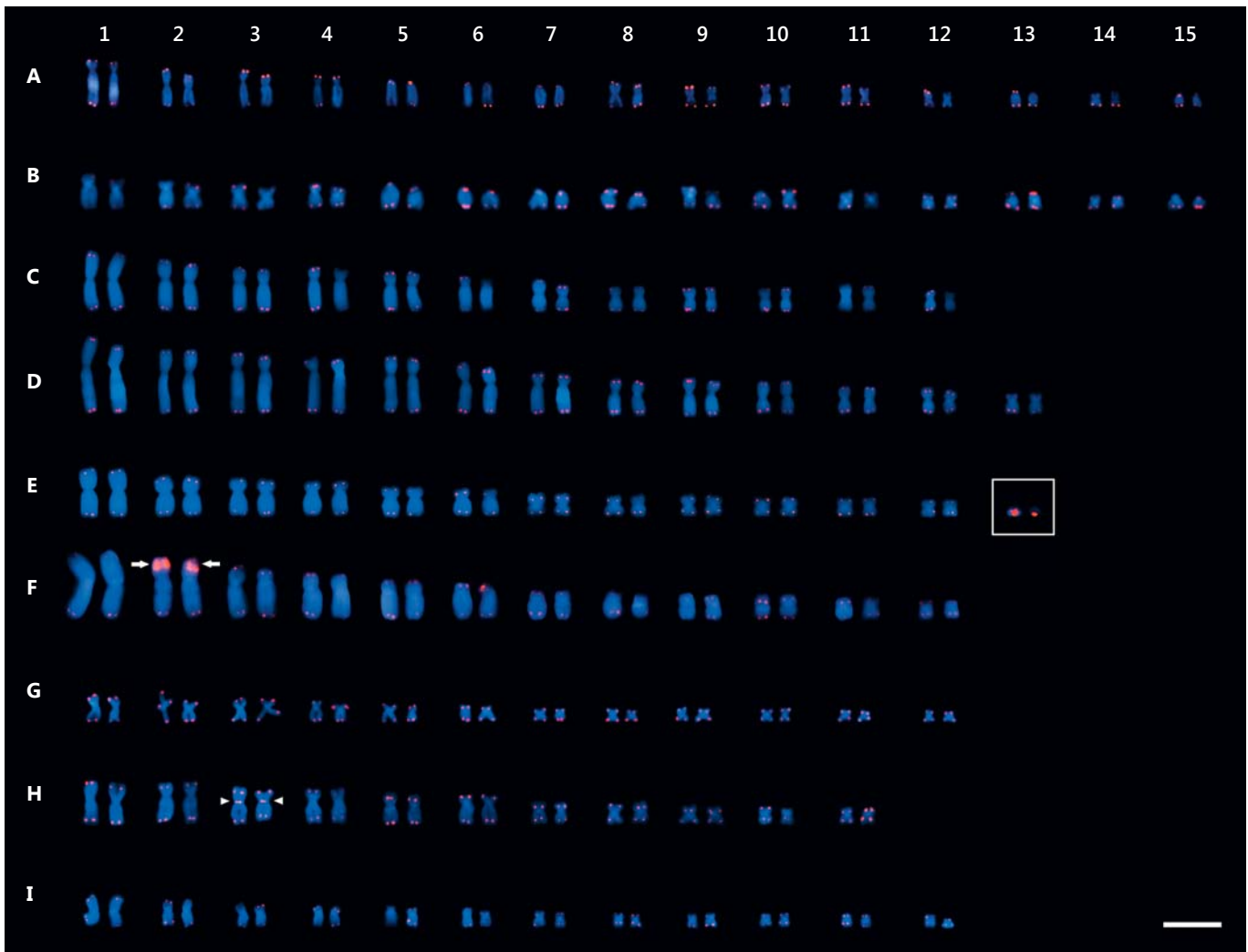


Fig. 8. FISH with telomere ONCOR probes (TTAGGG)_n (red) and counterstained with DAPI. **A** *D. marmoratus*; **B** *D. melanargyreus*; **C** *S. caramaschii*; **D** *S. carneus*; **E** *S. dorisae*; **F** *S. lacteus*; **G** *L. laevis*; **H** *S. goinorum*; **I** *X. truncata*. Intense telomeric signal on B chro-

mosomes of *S. dorisae* are framed. Arrows indicate block signals on pair 2p in *S. lacteus*. Arrowheads show centromeric signals on pair 3 in *S. goinorum*. Scale bar = 10 μm.

ation also occurs in *X. clivii*, which have heterochromatic ITS on 12 of their 18 chromosome pairs equilocally distributed between the heterologue chromosomes [Nanda et al., 2008] and in *Pristimantis cruentus* with very large heterochromatic ITS in the centromeric and pericentromeric heterochromatic regions in 14 of their 17 chromosome pairs [Schmid et al., 2010]. According to Greilhuber and Loidl [1983] and Schweizer and Loidl [1987], heterochromatic blocks can spread throughout the genome by unequal crossing-over, amplification or transposition events that take place during the interphase promoted by the chromosome vicinity and/or the Rabl orientation. In

hylids, the heterochromatic blocks are equilocally distributed on the set of heterologous chromosomes, as observed in the 5 genera with karyotypes described in the present contribution. In *Aplastodiscus*, many of these blocks also colocalize with ITS [Carvalho et al., 2009; Gruber et al., 2012]. As heterochromatic blocks, ITS are equilocalized [Wiley et al., 1992, figs. 1, 2; Carvalho et al., 2009, fig. 5; Gruber et al., 2012, fig. 5]. The equilocal distribution and colocalization of heterochromatic blocks and ITS support the idea that ITS originated from the same evolutionary processes described for heterochromatic blocks [Redi et al., 2001; Kagansky et al., 2009], i.e.

mechanisms of dispersion, contraction, extension, and chromatin modification. The latter are particularly evident in the 3 species of *Aplastodiscus* ($2n = 22$) with widespread centromeric ITS [Gruber et al., 2012] that according to our criteria mimic the true telomere resultant from the postulated chromosome fusion.

On the basis of the above considerations, we hypothesize that the centromeric ITS found in *S. goinorum* with $2n = 22$ represents a true signal of a chromosomal fusion that led to the reduction of chromosome number in this species. The karyotype of *S. goinorum* differs from those of other Dendropsophini species with 24 chromosomes because it has an additional large chromosome (6 large chromosomes in *S. goinorum* vs. 5 in the remaining taxa), a single medium chromosome (2 in the remaining taxa) and one small chromosome less (4 vs. 5 in the remaining taxa). Thus, pair 3 of *S. goinorum*, which carries the centromeric ITS, seems to be the product of a chromosome fusion of a medium chromosome (sixth pair, commonly subtelocentric) and one of the smallest chromosomes (pairs 8–12, commonly metacentric). However, additional chromosome rearrangements are necessary to explain the extant shape, size and principally the centromeric ITS of pair 3, which are most parsimoniously explained by the fusion of 2 telocentric chromosomes. These additional rearrangements on both chromosomes could involve the loss of the short arm of a subtelocentric chromosome and/or the occurrence of a pericentric inversion, not involving telomeres, on one or both chromosomes leading to telocentric morphologies prior to the postulated fusion [reviewed in Schubert, 2007]. Studies in the other species of *Scarthyia*, *S. vigilans*, will determine if this reduction in chromosome number is a synapomorphy of *Scarthyia* or an autapomorphy of *S. goinorum* and could also be useful to identify the mechanisms involved in the reduction of the diploid number.

Sphaenorhynchus: Chromosome Number, Telomeric Amplification and B Chromosome

We had available only 4 of the 12 species of *Sphaenorhynchus*. The still unknown phylogenetic relationships in this genus do not allow to infer the polarity of the transformations in chromosome number. *S. carneus* ($2n = 26$) differs from the other studied species of *Sphaenorhynchus* ($2n = 24$). Similarly to the clade that includes *Lysapsus*, *Pseudis*, *Scarthyia*, and *Sphaenorhynchus*, species with $2n = 24$ have NORs and nucleolar constrictions on pair 7q. Nevertheless, in *S. carneus*, these regions are pericentromeric on pair 9p. The distribution and amount of heterochromatin show important variations in *Sphaeno-*

rhynchus. Thus, while *S. lacteus* comparatively has more heterochromatin distributed on all chromosomes, *S. caramaschii* has almost no heterochromatin, and the other 2 species have scarce centromeric heterochromatin.

B chromosomes are dispensable elements that do not recombine with the A complement from which they usually originate [Beukeboom, 1994]. In Anura, supernumerary chromosomes have been reported in only 2% of studied taxa, involving 16 species of 12 families [for a review, see Green, 2004; Schmid et al., 2010]. In Dendropsophini, the presence of supernumerary chromosomes so far has been reported in *D. nanus* [Medeiros et al., 2006], and here we report it in *S. dorisae*.

An apparent causal relationship can be inferred regarding the large telomeric band that covers almost half of pair 2p of *S. lacteus*, which is euchromatic, and B chromosomes of *S. dorisae*, which are entirely heterochromatic, as the former could be considered as a center to produce B chromosomes as previously reported for diverse tandem arrays from the A complement [Camacho et al., 2000]. According to Jones and Houben [2003 and references therein], spontaneous amplification of coding and noncoding tandem repeat sequences derived from A chromosomes seems to be strongly associated with the origin and evolution of B chromosomes. A telomerase-independent lengthening of the telomeres [Cheng et al., 2012; Lovejoy et al., 2012] may explain this particular phenomenon in *S. lacteus*, although the repetitive nature of the telomeric tandem array, which is a target of molecular alterations in the number of repetitions, is an appropriate explanation [Jones and Houben, 2003]. However, a more exhaustive analysis is necessary to prove its consistency as a good cytological landmark. On the other hand, the amplification of telomeres via evolutionary processes related to a heterochromatic state acquisition is an alternative explanation for the B chromosome of *S. dorisae* [Tsipouri et al., 2008; Carvalho et al., 2009 and references therein]. A more inclusive phylogenetic hypothesis of *Sphaenorhynchus* and a more resolute cytogenetic analysis including its remaining species is necessary to understand the origins of the supernumerary chromosomes and telomere amplification in the genus.

Xenohyla and *Dendropsophus*: Origin and Diversity of the 30-Chromosome Complement

X. truncata has a chromosome complement with 12 biarmed chromosome pairs. As observed in *Lysapsus*, *Pseudis* and *Sphaenorhynchus*, NORs are located on pair 7q, and the C-banding pattern is predominantly centromeric.

The NORs on 7q in *Sphaenorhynchus* (except *S. carneus*), *Pseudis*, *Scarthyla*, *Lysapsus*, and *Xenohyla* (all species with $2n = 24$, except *S. goinorum*) are the putative plesiomorphic condition in Dendropsophini. In members of Cophomantini and Lophiohylini, the NORs are always on the smaller chromosome pairs, while in Hylini, they are generally located on the smaller pairs or on other chromosome pairs, but never on pair 7 [Catroli and Kasahara, 2009]. Thus, the NORs on 7q optimize a putative synapomorphy of Dendropsophini in the phylogenetic hypotheses of Faivovich et al. [2005] and Wiens et al. [2010]. Additional polymorphic NORs, as detected in *S. goinorum* on one chromosome of pair 4q [present work], on pairs 1q and 8q in *L. bolivianus* and on 7q in *L. caraya* [Busin et al., 2006], have been reported for many amphibian species [for review see Schmid et al., 2010].

In *Dendropsophus*, only 28 of 94 described species have chromosome data available [Catroli and Kasahara, 2009; present work]. All of them share the diploid number of $2n = 30$, but there is extensive variation in the FN (online suppl. table 1). There are no chromosome data for the *D. garagoensis* and *D. minimus* groups, nor for the 6 species unassigned to any group. For the putatively basal *D. marmoratus* group [Faivovich et al., 2005], there are karyotypic data only for 3 species (*D. marmoratus*, *D. melanargyreus* and *D. nahdereri*), all characterized by an FN = 50 and 5 telocentric chromosome pairs [Bogart, 1973; Gruber et al., 2005; present work].

In most *Dendropsophus* species studied, the secondary constriction and NORs are present on only one homologous pair and show a high degree of variability between and within the groups. Multiple NOR sites were reported for *D. ebraccatus*, *D. elegans* [Gruber et al., 2005] and for *D. nanus* [Medeiros et al., 2003]. However, the uncertain homeology of chromosome pairs among taxa does not allow elucidating whether this variability is overestimated or real.

It has been hypothesized that the $2n = 30$ karyotype originated by centric fission events from a $2n = 24$ ancestral karyotype [Bogart, 1973]. Our report of this chromosome number in *X. truncata* supports this idea indicating that the $2n = 30$ karyotype is indeed a putative synapomorphy of *Dendropsophus*. While this has been suspected or assumed for a long time [Duellman and Cole, 1965; Duellman, 1967; Faivovich et al., 2005], the unknown situation in *Xenohyla* did not allow to identify the limits of the 30-chromosome clade and the point where the transformation from $2n = 24$ to $2n = 30$ took place. The use of whole chromosome hybridization-employing probes of *Xenohyla* could help to understand the mechanism im-

plied in this variation in chromosome number. *Dendropsophus* is an excellent model to study chromosome evolution, and the use of more resolutive analyses in more species is necessary to understand the origin of the diversity of 30-chromosome karyotypes and the mechanisms causing them.

The available information on Dendropsophini shows at least 4 independent transformations in chromosome numbers from the plesiomorphic karyotype of 24 chromosomes: a reduction (*S. goinorum*) and 3 independent increments (*S. carneus*, *Dendropsophus* and *P. cardosoi*). There are still many issues unsolved, and one of the most important is the establishment of the chromosomes involved in those changes. The presence of ITS in *S. goinorum* karyotypes provides clues which could involve chromosome pairs in rearrangements leading to reduction in the chromosome number. In this sense, the use of telomeric probes in Dendropsophini, as in other hylids with different chromosome numbers, could provide further information regarding the identities of the chromosomes involved in the several known transformations from the $2n = 24$ karyotype.

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