RESEARCH ARTICLE

Comparative cytogenetic analysis of four species of *Dendropsophus* (Hylinae) from the Brazilian Atlantic forest

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Abstract

We conducted a cytogenetic study of four hyline frog species (*Dendropsophus elegans*, *D. microps*, *D. minutus* and *D. werneri*) from southern Brazil. All species had $2n = 30$ chromosomes, with interspecific and intraspecific variation in the numbers of metacentric, submetacentric, subtelocentric and telocentric chromosomes. C-banding and fluorochrome staining revealed conservative GC-rich heterochromatin localized in the pericentromeric regions of all species. The location of the nucleolus organizer regions, as confirmed by fluorescent *in situ* hybridization, differed between species. Telomeric probes detected sites that were restricted to the terminal regions of all chromosomes and no interstitial or centromeric signals were observed. Our study corroborates the generic synapomorphy of $2n = 30$ chromosomes for *Dendropsophus* and adds data that may become useful for future taxonomic revisions and a broader understanding of chromosomal evolution among hylids.

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Introduction

Genus *Dendropsophus* Fitzinger 1843 was long listed in the synonymy of the genus *Hyla*, based on similarities of morphology and life-history traits (Kellogg 1932). More than 80 years later, *Dendropsophus* was resurrected to distinguish species of *Hyla* with $2n = 30$ chromosomes (Faivovich *et al.* 2005), which had before simply been labelled the '30-chromosome *Hyla*' (e.g. Bogart 1973). Currently, genus *Hyla* is considered a member of the subfamily Hylinae (Hylidae), and its species fall into nine phylogenetic groups, with some species from throughout Central and South America, remaining unassigned to any species group (Faivovich *et al.* 2005; Frost 2014).

In addition to morphological similarities and chromosome number identity, molecular evidence suggests that *Dendropsophus* is a monophyletic genus (Faivovich *et al.* 2005; Wiens *et al.* 2010; Pyron and Wiens 2011). The chromosome number of *Xenohyla*, a sister group of *Dendropsophus* according to Faivovich *et al.* (2005) was recently determined to be $2n = 24$ (Suárez *et al.* 2013), supporting the hypothesis that $2n = 30$ is a synapomorphy of *Dendropsophus*. Although, the chromosome number is stable within *Dendropsophus*, variation in gross chromosome morphology (resulting in different fundamental numbers), the location of nucleolus organizer regions (NORs) and the presence of B-chromosomes have been described for some species (see Medeiros *et al.* 2003, 2006, 2013; Gruber *et al.* 2005; Suárez *et al.* 2013).

Comparative analysis of karyotypes is an important tool for taxonomy and for understanding chromosome evolution and geographic patterns (Schmid *et al.* 2010). In this paper, we describe the karyotypes of *D. elegans*, *D. microps*, *D. minutus* and *D. werneri*, allocated to four different species groups of *Dendropsophus*, while comparing them with data from the literature. These species have distinctive geographic distribution patterns, with range overlap in the southeastern

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and southern Brazilian Atlantic forest (IUCN 2013; Frost 2014). This study, along with our survey of the relevant literature provides interesting insights into the evolutionary and geographic diversifications of hylid karyotypes.

Material and methods

Species and collection localities

Specimens were collected from the municipality of São José dos Pinhais (25°47′ 41.97″S, 48°54′ 43.62″W), state of Paraná, Brazil. We collected six individuals of *D. werneri* (3♂ and 3♀), eight specimens of *D. elegans* (5♂ and 3♀), five specimens of *D. microps* (2♂ and 3♀) and nine specimens of *D. minutus* (5♂ and 4♀). Voucher specimens have been deposited at the Museu de Zoologia da Universidade Estadual de Campinas 'Adão José Cardoso', Campinas, São Paulo, Brazil (ZUEC 16983–17011).

Chromosome preparations, classical and molecular cytogenetic analyses

Mitotic chromosomes were obtained from femoral bone marrow suspensions following the protocol of Badissera *et al.* (1993). Conventional staining was achieved usinga 5% solution of Giemsa in a sodium phosphate buffer (pH 6.8) for 10 min. The AgNOR and C-banding techniques were performed according to the procedures of Howell and Black (1980) and Sumner (1972), respectively. Combined staining with 4′,6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3) was applied to obtain fluorescent bands (Schweizer 1981). Fluorescent *in situ* hybridization (FISH) was performed with an 18S rDNA probe obtained from *Prochilodus argenteus* (Hatanaka and Galetti 2004) that was labelled with biotin and highlighted by conjugated streptavidin–fluorescein isothiocyanate. In addition, we used a telomeric probe $(TTAGGG)_n$ according to Ijdo *et al.* (1991) that was labelled with digoxigenin and detected with antidigoxigenin–rhodamine conjugates. Both probes were amplified by polymerase chain reactions (PCR). Metaphases were examined on a Carl Zeiss Axiophot epifluorescence microscope equipped with epifluorescence filters and the chromosome images were captured using Case Data Manager software (Applied Spectral Imaging, MigdalHa'emek, Israel).

Analysis of karyotypes

Karyotypes were constructed and measured in Adobe® Photoshop® 9.0.2 software and chromosomes were classified following the conventions of Green and Sessions (1991). Measurements and centromeric ratio for each chromosome pair were calculated on 10 metaphase plates using a software tool.

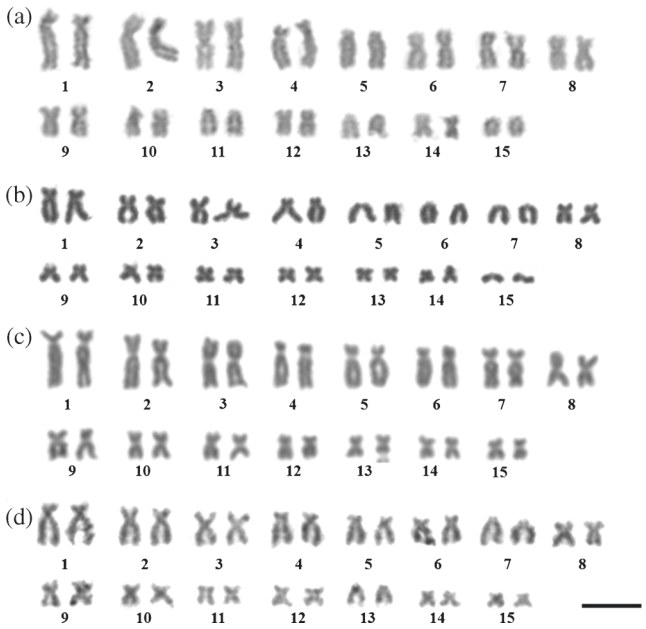

Figure 1. Giemsa-stained karyotypes of (a) *D. elegans*, (b) *D. microps*, (c) *D. minutus* and (d) *D. werneri* from the Brazilian Atlantic forest. All four species show a consistent karyotype of $2n = 30$. Bar = 10 μm.

Results

Diploid number and chromosomal formula

All analysed species had karyotypes consisting of $2n = 30$ chromosomes (figure 1) but each species had distinctive numbers of visible chromosome arms per karyotype (see table 1 for morphometric data from karyotypes).

Karyotype of *D. elegans* (*D. leucophyllatus* group) was composed of eight pairs of metacentric chromosomes (pairs 3, 6–10, 12, 13), three pairs of submetacentric chromosomes (pairs 1, 2 and 4), two pairs of subtelocentric chromosomes (pairs 5 and 14) and telocentric pairs 11 and 15, with a fundamental number (FN) equal to 56 (figure 1a). *Dendropsophus microps* (*D. parviceps* group) has a FN of 52 with eight pairs of metacentric chromosomes (pairs 3, 8–14), two pairs of submetacentric chromosomes (pairs 1 and 2), subtelocentric pair 4, and four pairs of telocentric chromosomes (pairs 5–7, 15) (figure 1b). *D. minutus* (*D. minutus* group) exhibited seven pairs of metacentric chromosomes (pairs 3, 8, 10–13, 15), seven pairs of submetacentric chromosomes (pairs 1, 2, 5–7, 9, 14) and subtelocentric pair 4 (FN = 60). In this species, secondary constrictions were usually observed in the terminal region of the long arm in one of the homologs of pair 13 (figure 1c). Finally, *D. werneri* (*D. microcephalus* group) had a FN of 60 with eight pairs of metacentric chromosomes (pairs 3, 8–10, 12–15), two pairs of submetacentric chromosomes (pairs 1 and 2) and five pairs of subtelocentric chromosomes (pairs 4–7 and 11) (figure 1d). Heteromorphic sex chromosomes were not detected in any sample of the analysed species.

Table 1. Morphometric data of the *Dendropsophus* species included in the present study.

	Chromosome														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D. elegans															
RL%	10.85	10.11	9.20	8.20	7.37	7.04	6.96	6.71	5.55	5.55	5.30	5.14	4.72	4.56	3.73
CI	0.26	0.35	0.46	0.28	0.17	0.38	0.41	0.41	0.40	0.40	0.10	0.41	0.14	0.40	0.12
CT	sm	sm	m	sm	st	m	m	m	m	m	t	m	st*	m	t
D. microps															
RL%	10.72	9.04	8.78	8.27	7.49	7.47	6.46	6.45	5.81	6.07	5.30	5.04	4.13	4.11	3.49
CI	0.25	0.31	0.47	0.24	0.05	0.12	0.03	0.39	0.36	0.32	0.40	0.42	0.43	0.38	0.03
CT	sm	sm	m	st	t	t	t	m	m	m*	m	m	m	m	t
D. minutus															
RL%	10.14	9.58	9.02	8.23	7.99	7.51	7.35	6.31	5.02	5.11	4.92	4.81	4.64	3.98	3.51
CI	0.25	0.34	0.47	0.23	0.26	0.27	0.26	0.46	0.30	0.43	0.44	0.43	0.42	0.35	0.38
CT	sm	sm	m	st	sm	sm	sm	m	sm	m	m	m	m*	sm	m
D. werneri															
RL%	10.67	9.54	8.87	7.74	7.52	7.41	6.96	6.51	6.17	5.27	4.73	4.71	4.60	4.04	3.70
CI	0.25	0.34	0.40	0.22	0.24	0.23	0.17	0.37	0.38	0.45	0.43	0.44	0.21	0.41	0.41
CT	sm	sm	m	st	st	st	st	m	m	m	m	m	st*	m	m

RL, relative length; CI, centromeric index; CT, chromosome type; m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric (according to Green and Sessions 1991); *NOR-bearing chromosome pair.

C-banding, fluorochrome staining and NOR detection

D. werneri, *D. elegans* and *D. minutus* showed C-banding only at the centromeric region of most chromosome pairs and associated with NORs, which were identified at terminal regions on the long arms of pair 13 (figure 2). C-banding in *D. microps* was not successful to establish a pattern. After CMA_3/DAPI double-staining, most of the positive C-bands were CMA_3^+/$DAPI^-$ (i.e. GC-rich), including the NOR sites (figure 3), with the exception of *D. microps*, which showed neutral signals for both fluorochromes (CMA_3^0/$DAPI^0$) along its chromosomes (figure 3c). With the exception of *D. microps*, which presents an AgNOR-positive signal in the telomeric region of the short arm of pair 10, the other species had their NORs in the telomeric region of the long arm of pair 13 (figures 4a–d). In *D. minutus*, the NORs on chromosome 13 coincided with secondary constrictions in Giemsa-stained metaphases. NOR locations were confirmed by FISH with an 18S rDNA probe in all four species (figure 4).

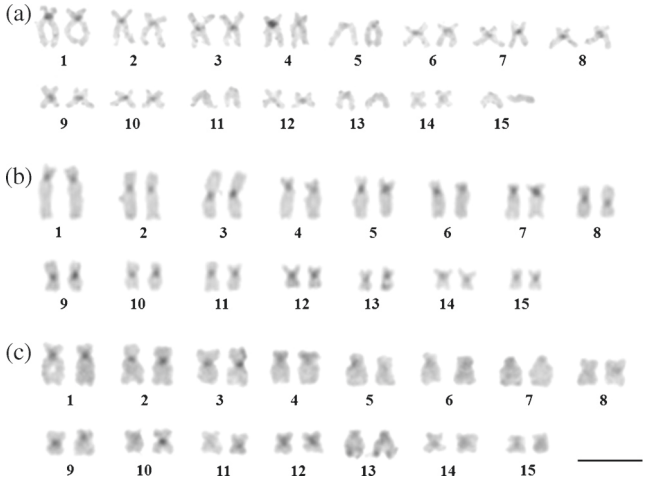

Figure 2. C-banded karyotypes of: (a) *D. elegans*, (b) *D. minutus* and (c) *D. werneri* from the Brazilian Atlantic forest. Bar = 10 μm.

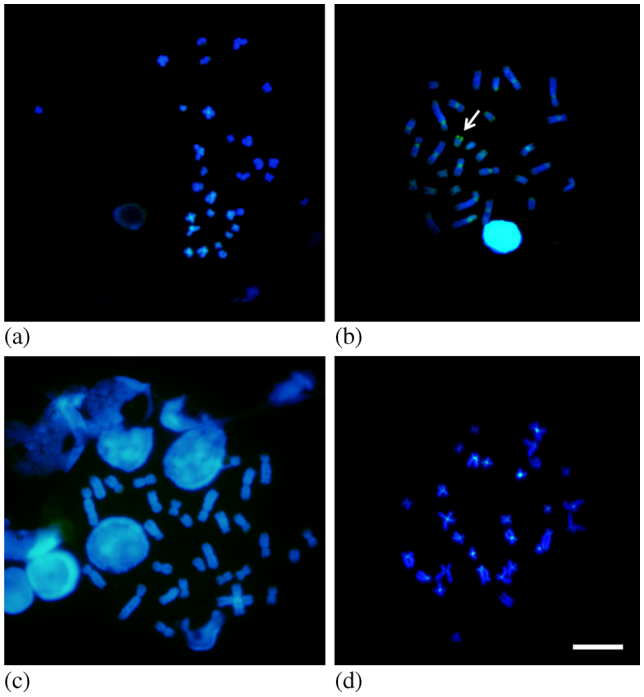

Figure 3. Sequential staining of karyotypes with CMA_3/DAPI. (a) *D. werneri*, (b) *D. elegans*, (c) *D. microps* and (d) *D. minutus*. The arrow in (b) indicates the NOR-bearing chromosome. There is no evidence of GC-rich regions in (c). Bar = 10 μm.

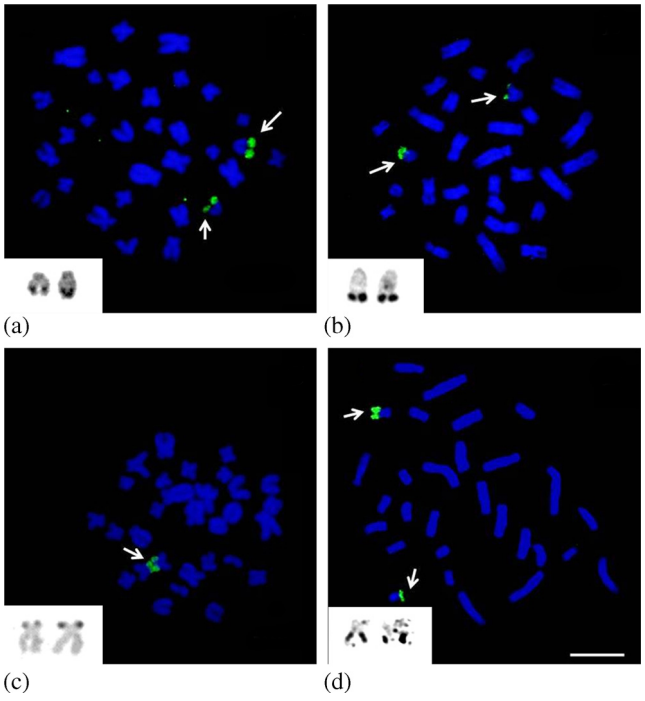

Figure 4. FISH with an 18S rDNA probe (arrows) and equivalent Ag-NOR sites (boxes). (a) Subtelocentric pair 13 of *D. werneri*, (b) subtelocentric pair 13 in *D. elegans*, (c) metacentric pair 10 in *D. microps* and (d) metacentric pair 13 in *D. minutus*. Bar = 10 μm.

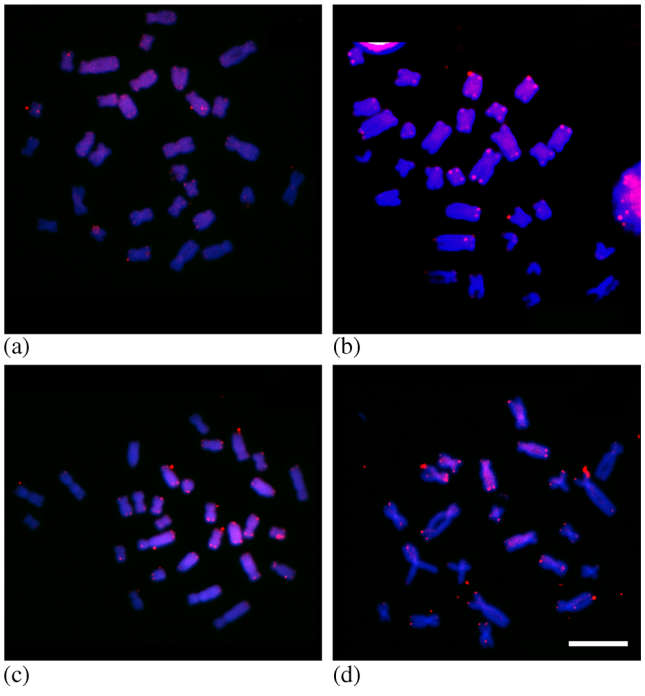

Figure 5. Telomeric hybridization using $(TTAGGG)_n$ to the karyotypes of: (a) *D. werneri*, (b) *D. elegans*, (c) *D. microps* and (d) *D. minutus*. Bar = 10 μm.

Telomeric sequence mapping

Telomeric probe hybridized uniformly to the ends of all chromosomes and no additional (interstitial or centromeric) positive signals were detected in the analysed species (figure 5).

Discussion

D. werneri karyotype

D. werneri was formerly called *Hyla baileyi* and its karyotype was described under that name by Skuk and Langone (1992). The karyotype of *D. werneri* revealed by our study is similar to that described by Skuk and Langone (1992), including the absence of uniarmed chromosomes but with morphological differences in pairs 6–8, 12 and 13, featuring the highest fundamental number within the *D. microcephalus* group (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet). Bogart (1973) argued that reduced number of subtelocentric chromosomes in anurans may be a derived condition. Therefore, its karyotype suggests that *D. werneri* should be positioned in a derived branch of the *Dendropsophus* phylogeny. Subsequently, according to the most recent published topologies (Wiens *et al.* 2010; Pyron and Wiens 2011; Medeiros *et al.* 2013), other species of the *D. microcephalus* group, such as *D. berthalutzae*, *D. sanborni*, *D. nanus* and *D. walfordi* were placed on the terminal branches of this group. Given that there is no general rule that a gradual shift toward increasing FN may result in derived phylogenetic position, further investigation is needed to confirm the phylogenetic position of *D. werneri* within the *D. microcephalus* group.

Variability in chromosomal formula

Although, the studied species share a same diploid number, they present distinct chromosome morphologies, revealing intrinsic karyotype variation (Gruber *et al.* 2005). Because of these differences, we suggest that *Dendropsophus* karyotype might have evolved by non-Robertsonian rearrangements, such as pericentric inversions. However, the simple centromere repositioning, which alters the chromosome morphology without any accompanying chromosomal rearrangements (Rocchi *et al.* 2012), cannot be disregarded as a mechanism for change. Unfortunately, we were unable to ascertain this because of the limited resolution of the applied techniques. This may explain the existence of different karyotype formula reported for individuals of the same species of *Dendropsophus* from different locations, in view of its wide distribution. Even within a species group, it is not possible to establish a diagnostic feature based only on karyotype characters. This becomes clear when comparing the available information for the *D. microcephalus* group (see table 1 in electronic supplementary material). In fact, based on morphological features, Fouquet *et al.* (2011) argued that a more detailed investigation of the phylogenetic relationships of *Dendropsophus* was required. In addition, Medeiros *et al.* (2013) also suggested that further taxonomic investigations of *D. nanus* and *D. walfordi* from *D. microcephalus*

group was needed. Our data also corroborate such conclusions and suggests that *Dendropsophus* needs additional deeper evaluation.

NOR: correlation with heterochromatin and diversity

C-bands were distributed predominantly in the centromeric region of most chromosome pairs, an expected pattern in *Dendropsophus* (Kaiser *et al.* 1996; Gruber *et al.* 2005; Medeiros *et al.* 2013; Suárez *et al.* 2013). Double-staining by CMA_3/DAPI revealed that the heterochromatin is GC-rich in all species except in *D. microps*, which showed a neutral reaction with both fluorochromes. Suárez *et al.* (2013) also showed a similar CMA_3/DAPI banding pattern in *D. marmoratus* and *D. melanargyreus* (*D. marmoratus* group), revealing the absence of AT-rich or GC-rich repetitive DNA sequences in the heterochromatic regions (Schweizer 1981). The correlation of NOR sites with GC-rich sites is also relatively common among vertebrates (see Schmid *et al.* 2010 and its references), since such regions correspond to known isochores in eukaryotic genomes.

Based on the results of FISH and silver-staining techniques, NORs were found to be adjacent to or embedded in C-banded heterochromatin. An association between constitutive heterochromatin and rDNA cistrons is frequently reported in anurans (e.g. Amaro-Ghilardi *et al.* 2008; Campos *et al.* 2009; Noleto *et al.* 2011). In three of the four analysed *Dendropsophus* species, pair 13 was identified as the NOR-bearing pair and the NOR was always positioned in the telomeric region of the long arm. A different situation was observed in *D. microps* which showed the NOR in the long arm of pair 10 as well as a chromosomal association of these regions (figure 4c). rDNA chromosomal sites represent very dynamic regions of the genome and their dispersion in anuran genomes could be due to interchromosomal rearrangements due to translocations or transpositions by mobile genetic elements which may move ribosomal gene loci to other chromosomes, mainly when there is constitutive heterochromatin associated with NORs. This situation has already been reported in majority of cases with NOR variability in the genome of several vertebrates (Woznicki *et al.* 2000; Datson and Murray 2006). Subsequently, *D. microps* and *D. minutus* presented NOR patterns that were similar to those described by Gruber *et al.* (2005). However, while *D. elegans* individuals showed that chromosome pair 13 was the NOR carrier, individuals from Ubatuba presented multiple NOR locations on pairs 10 and 14 (Gruber *et al.* 2005). This difference could represent an interpopulation NOR polymorphism which seems to be common in other anurans (Medeiros *et al.* 2003; Noleto *et al.* 2011). Multiple NOR-bearing chromosomal pairs are considered as a derived state in anurans (King *et al.* 1990). Therefore, a single switch in the position of the subtelocentric pair 14 to position 13 in the karyotype of *D. elegans* from Ubatuba makes pair 13, the primary NOR-bearing chromosome for *Dendropsophus* and thus, the ribosomal cistrons may be scattered to pair 10 that was what we observed in *D. microps* in the present study.

Telomeric sites in Dendropsophini karyotypes

The possible presence of interstitial telomeric sites (ITSs) might be useful in detecting chromosomal rearrangements (Robertsonian fusions), although other mechanisms may explain the origin of ITSs, such as the amplification of the oligonucleotide $(TTAGGG)_n$ associated with heterochromatin extension, mutations, transposition, or unequal crossing over (Tsipouri *et al.* 2008; Carvalho *et al.* 2009 and their references). The absence of interstitial telomeric repeats in the chromosomes of *Dendropsophus* species support the concept that the $2n = 30$ karyotype probably derived from a $2n = 24$ ancestor, but although how this has happened remains unknown. Centric fissions are one of the possibilities. The available information on Dendropsophini suggests two possible karyoevolutionary pathways from the plesiomorphic karyotype of 24 chromosomes: (i) signals of chromosomal fusion (centromeric ITS) leading to reduction in the chromosome number in *Scarthylagoinorum* ($2n = 22$; $FN = 44$); and (ii) increase of the diploid number in all known *Dendropsophus* species ($2n = 30$) (Suárez *et al.* 2013). In this respect, the use of telomeric probes in Dendropsophini is an attractive tool for comparative analysis and could provide further information regarding the identities of the chromosomes involved in the several transformations from the ancestral karyotype.

Conclusions

The diploid number of 30 chromosomes has been a convenient way to categorize the genus *Dendropsophus* (e.g. Kaiser *et al.* 1996; Gruber *et al.* 2005; Medeiros *et al.* 2006, 2013; Suárez *et al.* 2013). However, this feature was not considered a definitive synapomorphy of the genus by Faivovich *et al.* (2005). The recent description of the $2n = 24$ karyotype of *Xenohyla* (Suárez *et al.* 2013), a sister group of *Dendropsophus*, makes it clear that $2n = 30$ is a reliable delineator for the genus. Although, the karyotypes of only ~31% of *Dendropsophus* species have been studied, our data are consistent with the concept that a chromosome number of $2n = 30$ should be considered a synapomorphy of the genus (Gruber *et al.* 2005; Catroli and Kasahara 2009; Medeiros *et al.* 2013; Suárez *et al.* 2013).

Our study has revealed species-specific karyotypes with stories to tell. These data feature the details of chromosome morphology and allow us to trace their evolution with considerable confidence. Data such as these show unequivocally that karyotype investigations are a useful tool for evaluating relationships among lineages. Despite this utility, we acknowledge that it is not yet possible to establish detailed, reliable species-level phylogenies based on karyotypes since

the karyotypes of some two thirds of *Dendropsophus* species have not yet been described. Further studies with a focus on chromosome mapping of repetitive DNA sequences might confirm some chromosomal homeologies and contribute to the phylogenetic analyses of the cytotaxonomic relationships among neotropical frogs.

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