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Evidence for independent instances of chromosome number reduction in the genus *Pseudopaludicola* (Anura: Leptodactylidae)

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Abstract. We describe the chromosome morphology, C-bands, NORs position, and DAPI/CMA3 staining of five species of *Pseudopaludicola* (*P. boliviana*, *P. canga*, *P. falcipes*, *P. mystacalis*, and *P. ternetzi*) from several populations from Argentina, Brazil, Paraguay, and Uruguay. In addition, the telomeric sequence locations of *P. mystacalis* and *P. canga* are evaluated using FISH. We re-analyse the progressive reduction of chromosome numbers recently proposed for the genus by optimisation of character basic chromosome numbers (x) and fundamental chromosome numbers (FN) on current phylogenetic hypotheses. Our evidence supports a progressive reduction of chromosome numbers in *Pseudopaludicola*, and multiple origins of the karyotype 2n = 20. Additionally, we discuss the interpretation of the current *Pseudopaludicola* phylogenetic hypothesis and a series of inconsistencies in the chromosome literature for the genus.

Key words. C-bands, Chromosome reduction, CMA3, Character Optimisation, DAPI, NORs.

Introduction

The genus *Pseudopaludicola* currently comprises 18 recognized species with a vast distribution in northern and central South America, throughout Argentina, Brazil, Bolivia, Colombia, Guiana, Paraguay, Peru, Suriname, Uruguay, and Venezuela (Frost 2014). The latest phylogenetic hypothesis proposed by Veiga-Menoncello et al. (2014) based on mitochondrial genes recovered the genus as a natural group under parsimony and Bayesian analyses, supporting the previous proposals by Lynch (1989) and results obtained by Lobo (1995) that were based on morphological characters.

The first cytogenetic studies in *Pseudopaludicola* were performed using histological sections of gonads, which allowed quantifying the chromosome number (2n = 2x = 22) in *P. falcipes* (Sáez & Brum 1960, Brum-Zorrilla & Sáez 1968). Beçak (1968) carried out the first detailed analysis of chromosome morphology in a population from São Jose do Rio Preto, state of São Paulo (Brazil), assigned to *P. falcipes*, with 2n = 2x = 18 chromosomes. The specimens studied by Beçak (1968) likely correspond to the recently described species *P. atragula* Pansonato, Mudrek, Veiga-Menoncello, Rossa-Feres, Martins & Strüssmann, 2014a, given their chromosome morphology and geographical location close to São Jose do Rio Preto. Batistic et al. (1969) and Batistic (1970) described the chromosome morphologies of several Brazilian species/populations, all of them assigned to *P. falcipes*, indicating the existence of four different diploid numbers (2n = 2x = 16, 18, 20, and 22 chromosomes, respectively). Additionally, the chromosome morphology of *P. ameghini* was reported as 2n = 2x = 20 chromosomes by Beçak (1968) and Batistic (1970). More than four decades later, the knowledge about the chromosomal characteristics of *Pseudopaludicola* has increased markedly. More recently, Duarte et al. (2010) carried out a detailed analysis of the chromosome morphology, C-bands and Ag-NORs in *P. canga*, *P. facureae*, *P. mineira*, *P. saltica*, *P.* sp. (from Andaraí, Bahia, Brazil), and two populations from different Brazilian localities referred to as *P.* aff. *canga* (one of them currently *P. atragula*). These authors described the presence of heteromorphic sex

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chromosomes of the type XX/XY in *P. saltica*. Besides, Toledo et al. (2010), in the description of *P. murundu*, reported on the karyotype of this species. The available information on *Pseudopaludicola* is complemented with the work by Fávero et al. (2011) who described the chromosome morphology, C-banding pattern and Ag-NORs in *P. ameghini*, *P. falcipes*, *P. mystacalis*, *P. ternetzi*, and eight populations from different regions of Brazil, which were named by them as *P.* sp. (aff. *falcipes*), *P.* sp (aff. *mystacalis* I), *P.* sp. (aff. *mystacalis* II), *P.* sp. 1, *P.* sp. 2 (now *P. atragula*), and *P.* sp. 3. Based on the extensive data previously published and their phylogenetic hypotheses, Veiga-Menoncello et al. (2014) proposed a progressive reduction of the chromosome number in the genus *Pseudopaludicola* from a plesiomorphic karyotype of 2n = 2x = 22 chromosomes to a karyotype with 2n = 2x = 16 as was observed in *P. mystacalis*.

In the present work we describe the chromosome morphology, distribution, and composition of heterochromatin with C-banding and DAPI/CMA3 staining, as well as the position of NORs, in five species of *Pseudopaludicola* form several populations distributed in Argentina, Brazil, Paraguay, and Uruguay. The telomeric sequence locations of *P. mystacalis* and *P. canga* were also studied using Fluorescent in situ Hybridisation (FISH). Finally, the character state's basic and fundamental chromosome numbers were optimised on the basis of the phylogenetic hypothesis proposed by Veiga-Menoncello et al. (2014), in order to assess the significance of chromosome rearrangements for the karyotypic evolution of this genus.

Materials and methods

One hundred and one specimens belonging to five species of *Pseudopaludicola* (*P. boliviana*, *P. canga*, *P. falcipes*, *P. mystacalis*, and *P. ternetzi*) from Argentina, Brazil, Paraguay, and Uruguay were analysed cytogenetically (Fig. 1). Specimens were euthanised by administration of lidocaine, fixed in 10% formalin, and stored in 70% ethanol. Vouchers are housed in the herpetological collections of Instituto de Investigación Biológica del Paraguay, Asunción, Paraguay (IIBP-H), Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical, Posadas, Misiones, Argentina (LGE), Museu Paraense Emílio Goeldi, Pará State, Brazil (under acronyms BOC, PCS), and Museo Nacional de Historia Natural, Montevideo, Uruguay (MNHN). Detailed voucher information, locality data and sex of each studied specimen is provided in the appendix.

Chromosome spreads were prepared from intestinal epithelium and testes (Schmid et al. 2010) and stained with a Giemsa-PBS solution (pH 6.8). The silver-staining of nucleolar organizer regions (Ag-NORs) and C-banding techniques were performed according to Howell & Black (1980) and Sumner (1972), respectively.

Staining with DAPI/CMA3 was done according to Barros-e-Silva & Guerra (2010), with modifications. Figures with the results of these techniques are shown only for chromosome pairs bearing NORs, while the other chromosomes of the diploid complement are summarized in ideograms. The FISH technique was applied to specimens of *P. mystacalis* and *P. canga* following Pinkel et al. (1986) with a telomeric DNA sequence $(TTAGGG)_n$ as a probe. This telomeric probe was generated by PCR according Ijdo et al. (1991) and labelled by nick translation with biotin-14-dATP following the manufacturer's specifications (Bionick labelling system, Invitrogen).

Relative lengths (RL) and braquial relations (BR) were scored using the software Micromeasure v3.3 (Reeves & Tear 2000). Karyotypes were arranged according to decreases in chromosome size, following the nomenclature of Levan et al. (1964), as modified by Green & Sessions (1991), and preserving the apparent homology with data available from the literature. We used 'x' (basic chromosome number), 2n (somatic chromosome number), and

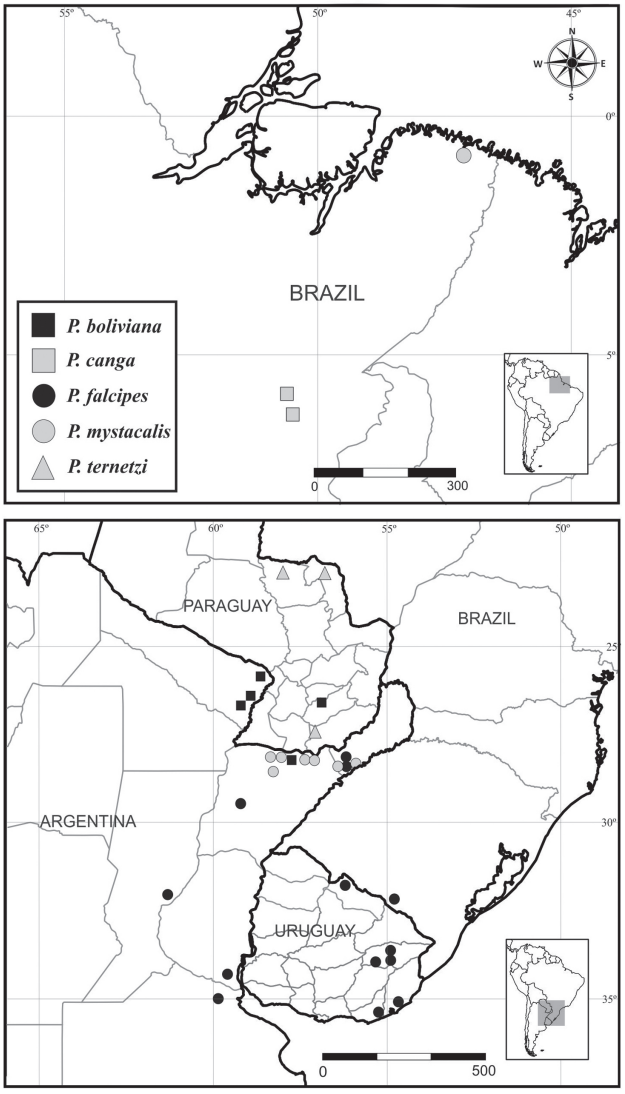

Figure 1. Localities where the specimens analysed in the present work were collected.

Table 1. Morphometric data of the karyotypes of species included in the present study. Relative length (RL), braquial relation (BR), standard deviation (SD), chromosome type (CT): m – metacentric; sm – submetacentric; st – subtelocentric; t – telocentric.

Chromosome pair	*Pseudopaludicola falcipes*			*Pseudopaludicola bolviana*			*Pseudopaludicola ternetzi*			*Pseudopaludicola canga*			*Pseudopaludicola mystacalis*		
	RL	BR±SD	CT	RL	BR±SD	CT	RL	BR±SD	CT	RL	BR±SD	CT	RL	BR±SD	CT
1	16.00	1.09±0.08	m	14.74	1.33±0.01	m	16.08	1.21±0.05	m	16.38	1.10±0.01	m	22.12	1.03±0.02	m
2	13.46	1.40±0.01	m	12.2	1.14±0.03	m	12.94	1.14±0.08	m	12.93	1.25±0.32	m	17.23	1.18±0.08	m
3	11.08	1.13±0.13	m	13.37	1.57±0.03	m	12.03	2.47±0.27	sm	13.09	1.99±0.32	sm	12.86	1.11±0.14	m
4	11.53	2.05±0.01	sm	10.43	1.66±0.03	m	12.19	2.03±0.25	sm	12.28	1.76±0.25	sm	11.94	2.32±0.45	sm
5	9.73	1.07±0.08	m	9.48	1.29±0.08	m	9.28	1.56±0.78	m	12.11	1.25±0.19	m	10.67	1.82±0.28	sm
6	7.98	1.13±0.18	m	8.97	1.15±0.06	m	10.8	1.35±0.10	m	11.19	1.45±0.46	m	9.89	1.54±0.05	m
7	7.62	1.91±0.10	sm	8.35	1.35±0.03	m	7.34	1.06±0.02	m	8.21	1.87±0.17	sm	9.05	2.18±0.10	sm
8	5.98	4.37±1.60	st	7.95	1.07±0.01	m	7.33	1.73±0.02	sm	7.5	7.31±0.47	t	6.24	2.68±0.28	sm
9	5.82	1.11±0.01	m	7.3	1.34±0.12	m	6.63	1.81±0.27	sm	6.31	9.23±0.21	t			
10	5.47	1.25±0.64	m	7.21	2.16±0.48	sm	5.38	1.21±0.27	m						
11	5.33	1.39±0.05	m												

'FN' (fundamental number of chromosome arms) as suggested by White (1954). Other abbreviations used are: 'NORs' (nucleolar organizer regions), 'p' (short arm), 'q' (long arm), and 'SC' (secondary constrictions).

To evaluate the karyological diversification of *Pseudopaludicola* we considered two characters: (1) basic chromosome number (x), with character states 11, 10, 9, and 8; and (2) fundamental number (FN) with states 44, 42, 40, and 32. We considered these states as both ordered and unordered and optimised them as per the phylogenetic hypothesis of Veiga-Menoncello et al. (2014), whose ingroup relationships did not differ under maximum parsimony or Bayesian methods. This analysis was conducted with using TNT 1.1 (Goloboff et al. 2008).

Results

Four different chromosome numbers were found in karyotypes of *Pseudopaludicola*, 22 (*P. falcipes*), 20 (*P. boliviana* and *P. ternetzi*), 18 (*P. canga*), and 16 (*P. mystacalis*). No heteromorphic sex chromosomes were identified with differential staining techniques in any of these species.

All populations of *Pseudopaludicola falcipes* examined presented 2n = 2x = 22 (FN = 44), with pairs 1–3, 5–6, 9–11 metacentric, 4 and 7 submetacentric, and 8 subtelocentric (Fig. 2 A, Tab. 1). The C-positive bands were observed across the pericentromeric region in 3p, 4q, 8q, and 10q (Fig. 3 A). The NORs were located in 8q in the pericentromeric region, coinciding with the usually evident secondary constrictions (Fig. 4A), with such regions being DAPI-negative/CMA3-positive (Figs 4B, C).

All examined specimens of *Pseudopaludicola boliviana* presented 2n = 2x= 20 (FN = 40), with pairs 1–9 being metacentric and 10 submetacentric (Fig. 2 B, Tab. 1). The C-bands were distributed in the centromeric regions in all chromosomes, pericentromeric in 2p, 3q, 6p, and interstitial in 5q (Fig. 3 B). The NORs were located in the distal region of the long arm of pair 2 (Fig. 3 D), with such region being DAPI-negative/CMA3-positive (Figs 4E, F).

The specimens of *Pseudopaludicola ternetzi* presented 2n = 2x = 20 (FN = 40). The pairs 1–2, 5–7, and 10 were metacentric, whereas pairs 3–4, 8–9 were submetacentric (Fig. 2C, Tab. 1). The heterochromatin revealed by C-banding was distributed in the centromeric and pericentromeric regions in almost all chromosomes (Fig. 3C). The NORs were located terminally at 8q (Fig. 4G), being the pair bearing the DAPI-negative/CMA3-positive NORs (Figs 4H, I).

Pseudopaludicola canga presented 2n = 2x = 18 (FN = 32) with pairs 1–2, 5–6 being metacentric, 3–4, 9 submetacentric, and pairs 7–8 telocentric (Fig. 2D, Tab. 1). The C-bands were distributed in the centromeric and pericentromeric regions of all chromosomes of the complement, and small bands were observed in the telomeres of both telocentric pairs (Fig. 3D). The NORs were located across the pericentromeric region of the pair 4 (Fig. 4J), with that region being DAPI-negative/CMA3-positive (Figs 4K, L).

The studied populations of *Pseudopaludicola mystacalis* shared a diploid number of 16 chromosomes (2n = 2x = 16; FN = 32). The pairs 1–3 and 6 were metacentric, whereas pairs 4–5, 7–8 were submetacentric (Fig. 2E, Tab. 1). The C-banding pattern was distributed over all centromeres of the chromosomes of the complement, at the interstitial region of 1p and the pericentromeric region of 2q (Fig. 3E). The NORs were located pericentromerically on 4p, coinciding with the usually evident secondary constrictions (Fig. 4M). The DAPI/CMA3-staining technique revealed the NORs region as being DAPI-negative/CMA3-positive (Figs 4N, O).

In *Pseudopaludicola falcipes* and *P. ternetzi*, DAPI/CMA3-staining provided evidence of centromeric and pericentromeric CMA3-positive bands. In *P. ternetzi*, in addition to the NORs region, another DAPI-negative/CMA3-positive band was observed in the subtelomeric region of 4q. On the other hand, *P. boliviana* presented multiple DAPI-positive bands in both arms of chromosome

pairs 1, 5, 7, 9, an extensive block at pair 6p, a small band at pair 10p, and a CMA3-positive band at pair 6p. *Pseudopaludicola canga* presented the pericentromeric and centromeric regions of all chromosomes of the complement and an DAPI-positive interstitial band at pair 2q, while in *P. mystacalis* the telomeres of pairs 2–4, 6, and the interstitial region of the pair 7 were CMA3-positive (Fig. 5).

In situ hybridisation experiments using telomeric probes $(TTAGGG)_n$ revealed only small fluorescence signals distally, across the telomeres of all chromosomes, both in *Pseudopaludicola canga* and *P. mystacalis* (Fig. 6).

Optimisation of the character state basic chromosome number as per the phylogenetic hypothesis of Veiga-Menoncello et al. (2014) evidenced the character state x = 11 as the plesiomorphic condition for *Pseudopaludicola*, while x = 10 as an autapomorphy of *P. boliviana* and an ambiguous synapomorphy for *P. ternetzi* + *P. ameghini*, x = 8 autapomorphic for *P. mystacalis* and x = 9 as an ambiguous synapomorphy for the clade ([*P. facureae* + *P. atragula*] + [*P. canga* + *P.* sp. 3]). When the character states are considered as non-additive, an ambiguity arises at the nodes for all species with chromosome number different from 22, except for *P. boliviana* (Fig. 7A). When states are considered as ordered, a progressive reduction from 2n = 22 to 20 and subsequently to 18 and 16 is observed (Fig. 7B).

Optimisation of the states assigned to the character fundamental number (FN) presented an ambiguity at the nodes for the species with FN = 40, except *Pseudopaludicola boliviana* (that is, *P. ameghini* and *P. ternetzi*), FN = 32 (*P. canga*, *P. facureae*, *P. mystacalis, P. atragula*, and *P.* sp. 3), and another in the species with FN = 42 (*P. saltica* and *P. murundu*) (Fig. 7). If the FN character states are considered as additive, the FN changes from 44 to 40 and finally

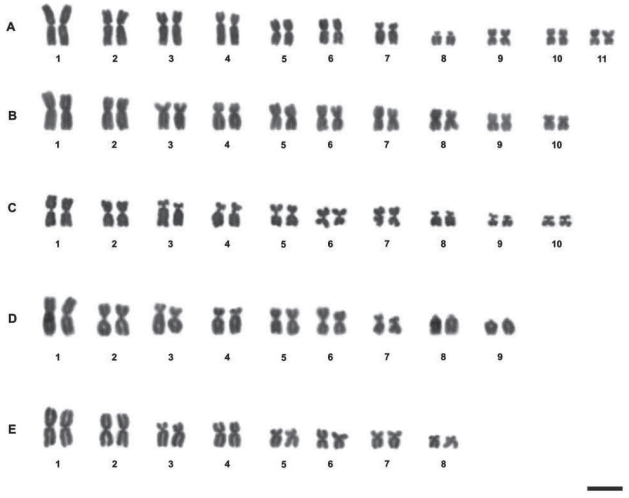

Figure 2. Giemsa-stained karyotypes: A) *Pseudopaludicola falcipes*; B) *P. boliviana*; C) *P. ternetzi*; D) *P. canga*; E) *P. mystacalis*. Scale bar = 10 μm.

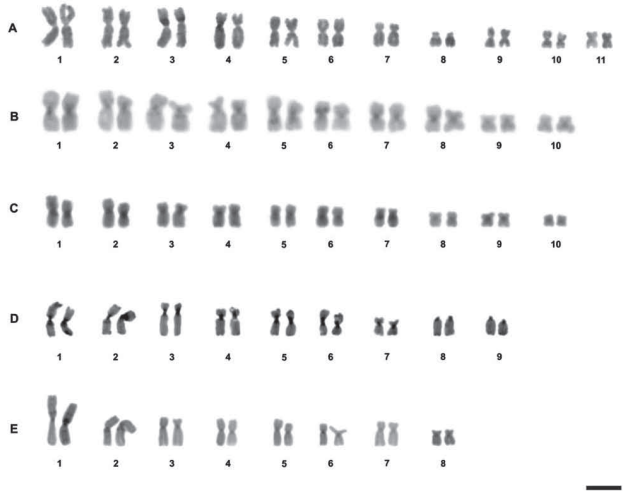

Figure 3. C-banded karyotype patterns: A) *Pseudopaludicola falcipes*; B) *P. boliviana*; C) *P. ternetzi*; D) *P. canga*; E) *P. mystacalis*. Scale bar = 10 μm.

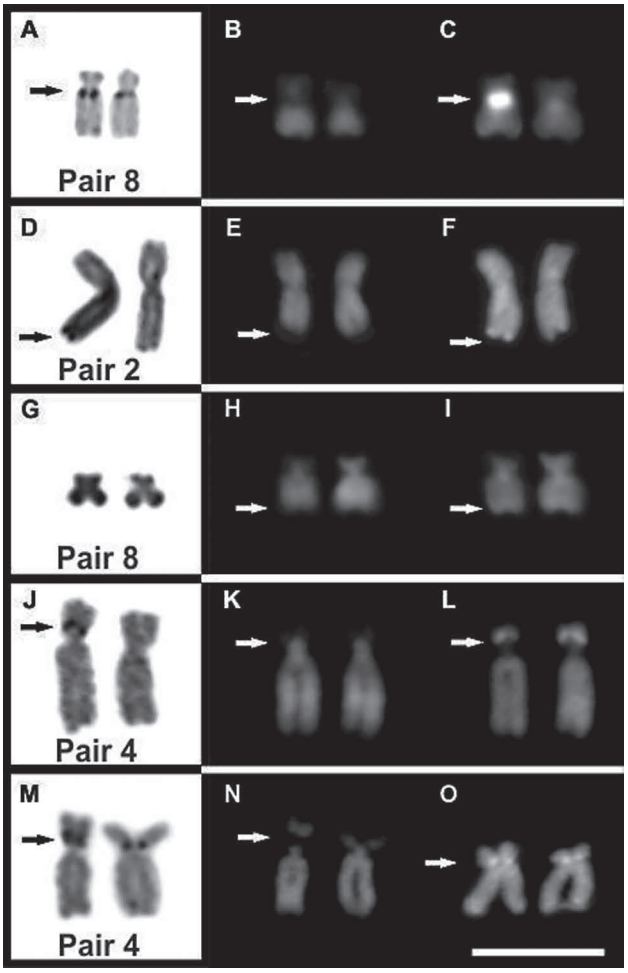

Figure 4. NOR-bearing chromosome pairs characterized by silver impregnation (left column), DAPI (middle), and CMA3 (right column): A–C) *Pseudopaludicola falcipes* pair 8; D–F) *P. boliviana* pair 2; G–I) *P. ternetzi* pair 8; J–L) *P. canga* pair 4; M–O) *P. mystacalis* pair 4. The arrows point out AT-or CG-rich regions. Scale bar = 10 μm.

to 32, while the node that supports the species with FN = 42 will remain ambiguous due to the lack of data for *P.* aff. *saltica* (Fig. 7).

Discussion

Karyotypic variation: independent instances of chromosome number reduction in *Pseudopaludicola*

The chromosome complement 2n = 22 (x = 11) is present in four nominal species of *Pseudopaludicola* (*P. falcipes*, *P. mineira*, *P. saltica*, and *P. murundu*) and two taxonomically unidentified forms (Sáez & Brum 1960, Brum-Zorrila & Sáez 1968, Batistic 1970, Duarte et al. 2010, Toledo et al. 2010, Fávero et al. 2011, this study), and optimise as the plesiomorphic condition for the genus. This character state is present in most genera of Leptodactylidae (i.e. *Edalorhina*, Bogart 1973, Lourenço et al. 2000a; *Physalaemus*, Kuramoto 1990, Silva et al. 1999, 2000, Amaral et al. 2000, Lourenço et al. 2006, Ananias et al. 2007, Quinderé et al. 2009, Tomatis et al. 2009, Vittorazzi et al. 2014; *Pleurodema*, Barrio & Rinaldi de Chieri 1970, Veloso

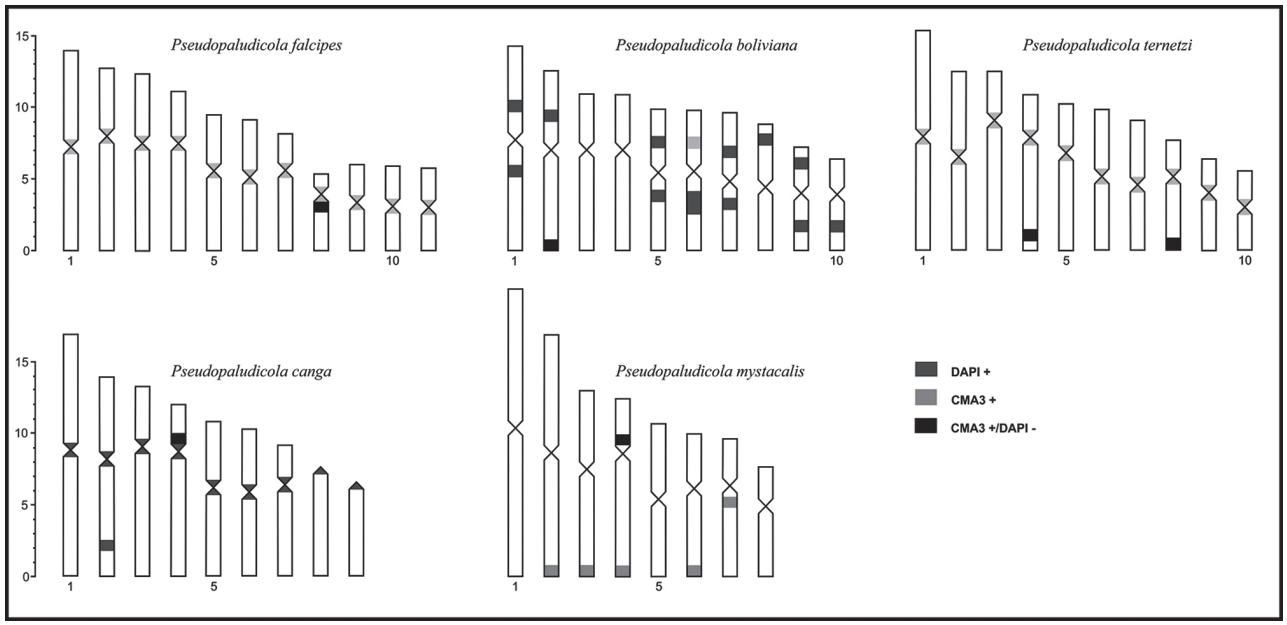

Figure 5. Ideograms with DAPI- or CMA3-bands present in the species analysed in this work: A) *Pseudopaludicola falcipes*; B) *P. boliviana*; C) *P. ternetzi*; D) *P. canga*; E) *P. mystacalis*. Scale indicates percentage relative size of chromosome pairs.

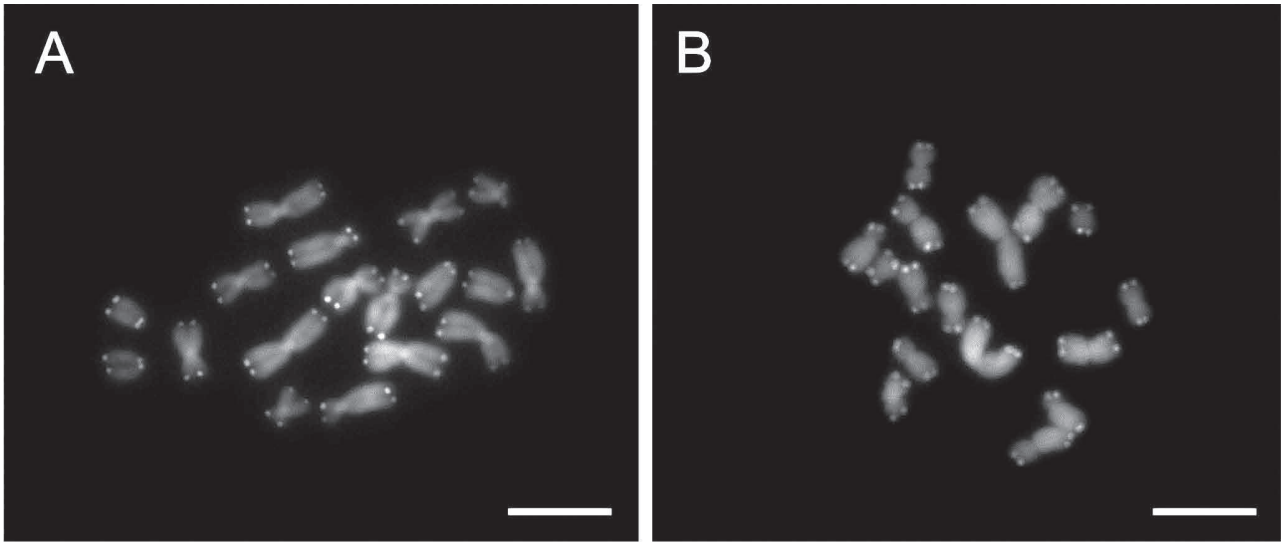

Figure 6. FISH-treated metaphase plates with telomeric probes: A) *P. canga*; B) *P. mystacalis*. Scale bar = 10 μm.

et al. 1973, Schmid et al., 1993, Lourenço et al. 2006, Valetti et al. 2009; *Engystomops*, Lourenço et al. 1998, 1999, Targueta et al. 2010; and *Leptodactylus*, Kuramoto 1990, Kasahara et al. 1998, Silva et al. 2000, 2004, 2006, Amaro-Ghilardi et al. 2004, 2006, Gazoni et al. 2012). Other chromosome numbers reported for this family are 2n = 26 in most *Adenomera* species (Kuramoto 1990, Campos et al. 2009, Zaracho & Hernando 2011); 2n = 24 in *Scythrophrys* (Lourenço et al. 2003a, 2008), *Paratelmatobius* (De Lucca et al. 1974, Lourenço et al. 2000b, 2003b, 2008), *Leptodactylus silvanimbus* (Amaro-Ghilardi et al. 2006), and *Adenomera marmorata* (Bogart 1974, Gazoni et al. 2012); 2n = 20 in *Leptodactylus* aff. *podicipinus* (Gazoni et al. 2012), *Engystomops pustulatus*, and *E. puyango* (Ron et al. 2010, Targueta et al. 2011); and 2n = 18 in *Lithodytes lineatus* (Bogart 1970). The karyotypes of *Rupirana cardosoi* and *Crossodactylodes* spp., both of which were included in the Leptodactylidae (subfamily Paratelmatobiinae) by Fouquet et al. (2013), remain unknown.

The plesiomorphic condition 2n = 22 previously proposed for *Pseudopaludicola* (Veiga-Menoncello et al. 2014) is supported by the available data, but karyological information in the putative monophyletic *P. pusilla* group is still incomplete. From the four nominal species included in this group (Cardozo & Suarez 2012; Veiga-Menoncello et al. 2014), only *P. boliviana* (this study) and an unnamed taxon (as *P.* sp., Veiga-Menoncello et al. 2014, or *P.* sp. 1, Fávero et al. 2011) have their karyotypes described.

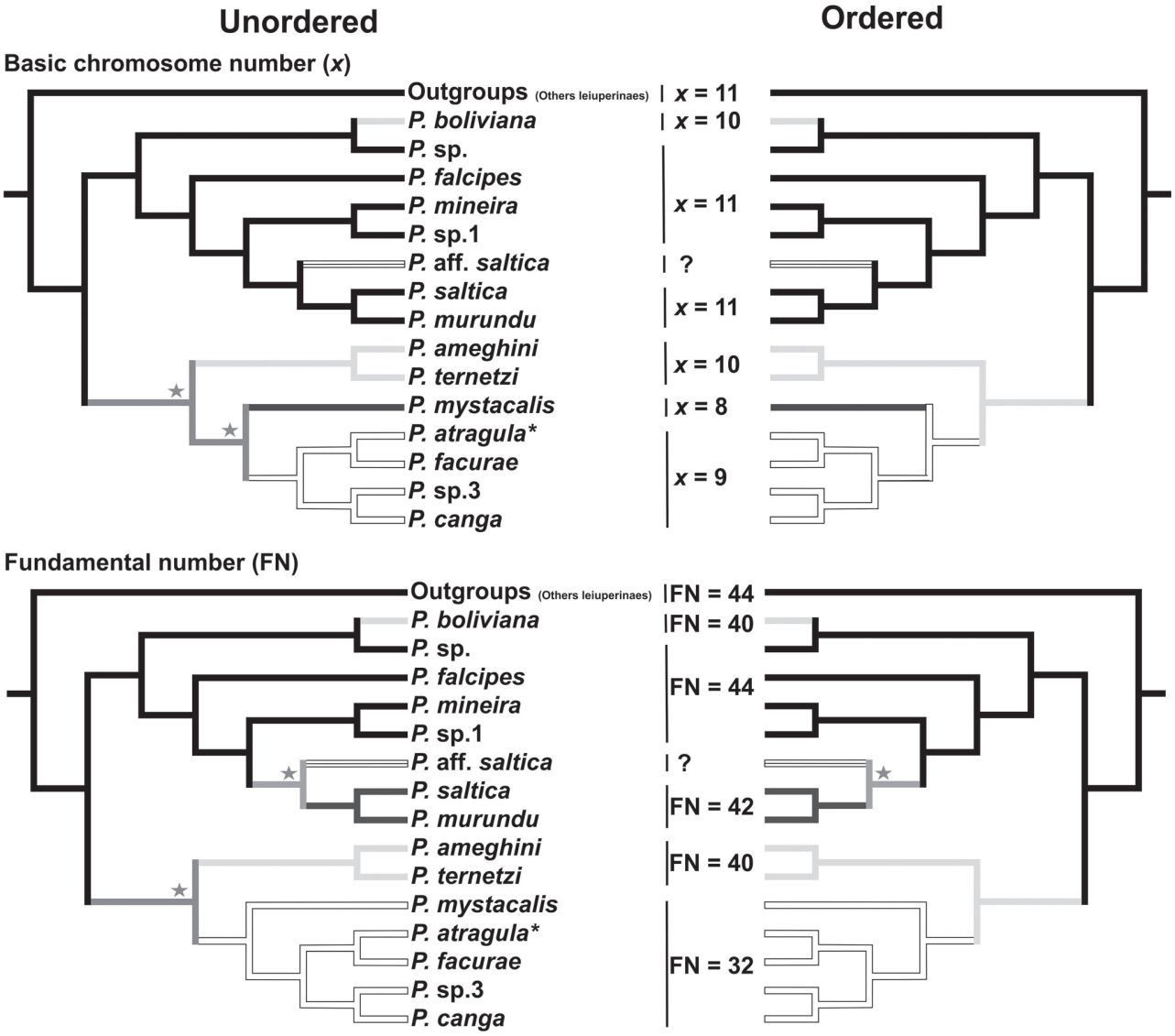

Figure 7. Direct optimisation of basic (right) character states and fundamental chromosome numbers (left) of *Pseudopaludicola* species in the phylogenetic hypothesis proposed by Veiga-Menoncello et al. (2014). The right column considers the states ordered, the left unordered. Stars indicate ambiguous nodes. **Pseudopaludicola atragula* corresponds to the terminal named *P.* sp. 2 in Veiga-Menoncello et al. (2014).

Amongst the species with karyotypes of x = 11 (2n = 22), two different karyotypic conditions were described: plesiomorphic (FN = 44) in *P. falcipes*, *P. mineira*, and two taxonomically unidentified forms, all with bi-armed chromosomes (Sáez & Brum 1960, Brum-Zorrila & Sáez 1968, Batistic 1970, Duarte et al. 2010, Toledo et al. 2010, Fávero et al. 2011, this study), and with a telocentric pair of chromosomes (FN = 42), as is present in *P. saltica* and *P. murundu* (Duarte et al. 2010, Toledo et al. 2010). The plesiomorphic state of *Pseudopaludicola* (2n = 22; FN = 44) is present also in most leiuperine frogs, except for *Physalaemus fernandezae*, *P. nattereri*, and the *P. signifer* species group with 2n = 22; FN = 42 (Silva et al. 2000, Lourenço et al. 2006, Ananias et al. 2007, Tomatis et al. 2009). The karyotypes with a telocentric pair present in *P. saltica* and *P. murundu* suggest a derived condition. This character state cannot be correctly optimised, as it is not known from the terminal named *P.* aff. *saltica* by Veiga-Menoncello et al. (2014). In this regard, the presence of a telocentric pair may be a synapomorphy of the clade named "*P. saltica* group" (Veiga-Menoncello et al. 2014), or of the less inclusive clade involving the sister species *P. murundu* and *P. saltica*.

Within *Pseudopaludicola*, three other basic chromosome numbers were identified: x = 10 in *P. ameghini*, *P. ternetzi*, and *P. boliviana* (Fávero et al. 2011, this study), x = 9 in *P. atragula*, *P. canga*, *P. facureae*, and one nameless taxon (Duarte et al. 2010; this study), and x = 8 in *P. mystacalis* (Fávero et al. 2011; this study). Those taxa with x = 10, *P. ameghini* and *P. ternetzi,* are sister species and phylogenetically distant from *P. boliviana*, which is included in the *P. pusilla* group. In this sense, the 2n = 20 observed in *P. boliviana* provides evidence of an independent instance of reduction of the chromosome number in the genus. Furthermore, this karyotype could not be considered homologous of those described for *P. ternetzi* and *P. ameghini*, because of different chromosome morphologies and very different banding patterns (see Fávero et al. 2011, this study). All taxa with x = 9 are nested within a single clade, for which the presence of 2n = 18 represents an ambiguous synapomorphy.

Finally, the karyotype of *Pseudopaludicola mystacalis* with 2n = 16 is an autapomorphy of this taxon and one of the smallest diploid numbers known in Anura. Furthermore, 2n = 16 is only shared with two phylogenetically distant taxa: *Chiromantis doriae* (Rhacophoridae; Tan, 1987) and *Cardioglossa gracilis* (Arthroleptidae; Bogart & Tandy, 1981), while *Arthroleptis poecilonotus*, *A. stenodactylus*, and *A.* sp. present 2n = 14 (Arthroleptidae; Green & Session 2007; Schmid et al. 2010).

Basic chromosome number and fundamental number optimisation

Veiga-Menoncello et al. (2014) proposed a scenario of progressive reduction in the chromosome number in the evolution of *Pseudopaludicola* from the plesiomorphic 2n = 22 to 2n = 16, based on the phylogenetic hypothesis obtained and data available from the literature. The topology of the tree obtained by the authors suggests multiple events of reduction of the basic chromosome number at several internal nodes. As was mentioned above, x = 10 is present in two independent lineages, taxa with x = 9 comprise a single clade, and x = 8 is an autapomorphy of *P. mystacalis*. In this sense, when a character state optimisation is performed considering them unordered, x and FN do not corroborate a progressive reduction in the basic chromosome number from x = 11 to 10, 9, and subsequently to 8 (see ambiguities in Fig. 6). Conversely, when the character states are considered ordered, the chromosome number is progressively reduced (Fig. 6). This gradual and progressive chromosome reduction is the most logical and parsimonious scenario for the chromosome evolution in *Pseudopaludicola*, since it involves the same number of state changes but fewer chromosomal rearrangements.

In other anuran taxa with noticeable variation in their chromosome numbers, scenarios without involving a gradual chromosome change can be observed. In *Aplastodiscus* (Hylidae), the plesiomorphic 2n = 24 suffered two independent instances of reduction, from 2n = 24 to 22, and another one from 2n = 24 to 20, and subsequently to 18 (Gruber et al. 2012). In the karyotype evolution of *Alsodes* (Alsodidae), three independent abrupt transformations occurred from the plesiomorphic condition of 2n = 26, involving increments or reductions of several pairs of haploid chromosomes, to a condition of 2n = 22, 30, and 34 (Blotto et al. 2013).

Differential staining and the significance in karyotypic diversification in *Pseudopaludicola*

Chromosomal studies conducted in *Pseudopaludicola* have not identified sex chromosomes in most species. However, Duarte et al. (2010) described a chromosomal sex-determination system of XX/XY in *P. saltica*, and such condition is to date regarded as an autapomorphy of this taxon.

Fávero et al. (2011), in an extensive cytogenetic survey of several Brazilian populations of *Pseudopaludicola*, described the diploid numbers, NORs position, and heterochromatin distributions of *P. ameghini*, *P. falcipes*, *P. mystacalis*, and *P. ternetzi*. They also included several taxonomically unassigned populations, which were named according to a confusing criterion: *Pseudopaludicola* sp. (aff. *mystacalis*) I, *Pseudopaludicola* sp. (aff. *mystacalis*) II, *Pseudopaludicola* sp. (aff. *falcipes*), *Pseudopaludicola* sp. 2, and *Pseudopaludicola* sp. 3. This confusion was partially resolved by Veiga-Menoncello et al. (2014), although multiple questions about the identity of several Brazilian specimens still remain. All the specimens analysed by Fávero et al. (2011) from São Paulo, Maranhão, and Mato Grosso states sharing 2n = 16 and NORs in pair 4p were not included in the phylogenetic analysis by Veiga-Menoncello et al. (2014), and were later identified as *P. mystacalis* based on acoustic parameters (Pansonato et al. 2014b).

Fávero et al. (2011) proposed differences in the morphology of chromosome pair 7 between *P. ameghini* and *P. ternetzi* (subtelocentric in *P. ameghini*; submetacentric in *P. ternetzi*). They quote a taxon as "*P.* cf. *ternetzi*" with pair 7 metacentric, specimen DZSJRP 6456, which appears to be nested in the *P. ternetzi* clade in the phylogenetic hypothesis by Veiga-Menoncello et al. (2014). Pair 7 of *P. ternetzi* also presents a metacentric morphology in our study (Tab. 1), which suggests that the chromosome morphology of this pair should be revised and regarded to not be a reliable character to distinguish between *P. ameghini* and *P. ternetzi.* The differences observed could be the consequence of differential condensation of the chromatids that render measuring of this small chromosome pair difficult.

It must be noted that the diploid number 2n = 16 of the specimen DZSJRP 8723 studied by Fávero et al. (2011) and identified as "*Pseudopaludicola* sp. (aff. *mystacalis*) I" was incorrectly quantified later rectified to 2n = 20 by Veiga-Menoncello et al. (2014) and reassigned as referring to *P. ternetzi*. However, this specimen presented a metacentric pair 3 and NORs on 4p (Fig. 3G in Fávero et al. 2011), whereas *P. ternetzi* and *P. ameghini* share a submetacentric pair 3 and NORs on 8q (9q of Fávero et al. 2011). This inconsistency was not taken into consideration by Veiga-Menoncello et al. (2014), who explained that the original error by Fávero et al. (2011) was based on the poor quality of the biological material used.

The results of the present work about the cytogenetics of *Pseudopaludicola canga*, *P. falcipes*, *P. mystacalis*, and *P. ternetzi* are consistent with those previously published for Brazilian populations (Duarte et al. 2010, Fávero et al. 2011). The metacentric pair 1 in *P. mystacalis* (2n = 16), which occurs in nearly 23% of the haploid chromosome set, is obviously much longer than in other *Pseudopaludicola* species with 2n = 18, 20, and 22 chromosomes (nearly 16% of the haploid set). Remarkably, the second chromosome pair in *P. mystacalis* shares a similar morphology and relative size with pair 1 of other *Pseudopaludicola* species, which suggest a homology between them. Therefore, pair 1 of *P. mystacalis* should have arisen from a major karyotype rearrangement. If we consider the karyotypes of *P. atragula, P. canga*, *P. facureae*, and one undetermined form reported by Duarte et al. (2010) and also *P. mystacalis*, all with FN = 32, chromosome homology may be inferred (based on relative length and chromosome morphology) between all pairs of the chromosome complement, except pairs 3, 8, and 9 of the taxa with 18 chromosomes. Pair 1 of *P. mystacalis* could be the consequence of at least two possible rearrangement events between pairs 3 and 8 of the karyotypes with 2n = 18. This assumption is based on apparent homology, as pairs 2–4, 5–7 of *P. mystacalis* are apparently homologous to pairs 1, 2, 4–7 of taxa with 2n = 18. Pairs 3 and 8 in the latter karyotypes could be affected by such rearrangement, leading to the large metacentric pair 1 of *P. mystacalis;* the telocentric pair 9, on the other hand, might have experienced an independent rearrangement event, for instance a paracentric inversion, as is suggested by the similar relative size shared with pair 8 of *P. mystacalis* (nearly 6% of the haploid set).

Although the event(s) involved in the rearrangements proposed could not be tested with the available data, the NORs position supports a homology between the pairs 4 in *P. canga* and *P. mystacalis*, while the similar morphology and relative size allow establishing of a putative homology between the remaining chromosome pairs.

Interstitial telomeric sequences (ITS) are generally interpreted as evidence of karyotypic rearrangements (see discussion in Suárez et al. 2013). Despite this, telomeric DNA loss may be an alternative mechanism that is frequently observed during chromosome fusions (Slijepcevic 1998). Schubert et al. (1992) and Nanda et al. (1995) reported the occurrence of numerous Robertsonian fusions in mice, but ITS cannot be detected by FISH with telomeric probes. Something similar appears to happen in anurans, as Schmid et al. (2010) observed spontaneous centric fusions in several species of Brachycephaloidea, but were able to enforce in situ hybridisation only in five of those species: *Eleutherodactylus adelus*, *E. pantone*, *Craugastor taurus*, *C. longirostris*, and *Pristimantis fenestratus*; nevertheless, they did not detect any positive ITS. In *Pseudopaludicola mystacalis* and *P. canga* (both with FN = 32), the absence of ITS does not reveal which chromosomes are implicated in the arrangement(s) that reduced the karyotypes from 18 to 16 chromosomes. A similar situation is present in *Aplastodiscus* (Hylidae), where *A. ehrhardti* (2n = 22) and *A. callipygius* (2n = 20) have no ITS, and the independent reduction events are supported by the analyses of cytological markers in a phylogenetic context (Carvalho et al. 2009, Gruber et al. 2012). In this sense, the absence of ITS in *P. canga* and *P. mystacalis* is not enough evidence to void the hypothesis of chromosome rearrangement proposed above.

In *Pseudopaludicola*, an important variation of C-positive bands has been reported, with pericentromeric, interstitial and telomeric heterochromatic bands being present in multiple regions of the chromosome complement in most species analysed (Duarte et al. 2010, Toledo et al. 2010, Fávero et al. 2011, this study). However, the presence of related species with different chromosome number does not allow a correct assessment of the homologies, which render the interpretation of C-banding difficult. The current lack of a reliable homology assessment between chromosomes of related species is reinforced by evidence stemming from fluorochrome staining; the centromeres of *P. canga* are CMA3-positive (predominance of CG-rich sequences) whereas *P. mystacalis* present neutral centromeres (uniform staining with DAPI and CMA3) and most telomeres with CG-rich sequences (CMA3-positive).

Although the value of the number and location of NORs for establishing homologies between chromosome segments has been questioned (Dobigny et al. 2004) in Leptodactylidae (sensu Fouquet et al. 2013), NORs have repeatedly been reported from a small chromosome pair with similar relative size assigned to pair 8 in several *Leptodactylus* spp. (Amaro-Ghilardi et al. 2006, Gazoni et al. 2012), in most *Pleurodema* species (Lourenço et al.

2006, Baldo et al. unpub. data), *Physalaemus* (Silva et al., 1999, 2000, Amaral et al., 2000, Tomatis et al., 2009), and *Pseudopaludicola* (Duarte et al., 2010, Toledo et al., 2010, Fávero et al., 2011, this study). Accordingly, in order to preserve chromosome homology, we consider that chromosome pairs 8 and 9 of *P. ternetzi* and *P. ameghini*, and pairs 3, 4 and 8, 9 of *P. canga* and *P. facureae* as described by Duarte et al. (2010) are inverted. Consequently, there are three different chromosome pairs bearing NORs in *Pseudopaludicola*: pair 2 in *P. boliviana*, pair 4 in *P. canga* and *P. mystacalis*, and pair 8 in *P. ameghini*, *P. facureae*, *P. falcipes*, *P. mineira*, *P. murundu*, *P. saltica*, and *P. ternetzi* (Duarte et al., 2010, Toledo et al., 2010, Fávero et al., 2011, this study). Based on the phylogenetic hypothesis of Veiga-Menoncello et al. (2014), the NORs are positioned on pair 8 in species with 2n = 20 and 22 (except in *P. boliviana*, present study), representing the plesiomorphic condition for the genus. The only known taxon with 2n = 16 has NORs on pair 4. Within the clade recovered by Veiga-Menoncello et al. (2014) with 2n = 18, the sister terminals *P. canga* and *P.* sp. 3 present NORs also on pair 4, and the sister terminals *P. atragula and P. facureae* across pair 8 (Duarte et al., 2010, this study).

Although our knowledge of the systematics, evolution and cytogenetics of species of *Pseudopaludicola* has increased markedly in recent years, some weak points still remain. Cytogenetic descriptions of *P. llanera*, *P. pusilla*, and *P. ceratophyes* and their inclusion in phylogenetic hypotheses are necessary for better understanding the chromosome evolution in the genus. Establishing a precise homology between the karyotypes of species with different chromosome numbers and those with the same diploid numbers acquired from independent origins would help us to understand how this genus has undergone its remarkable karyotypic diversification.

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Appendix

Specimens examined

Pseudopaludicola boliviana: Argentina: Corrientes Province: General Paz Department: Itá Ibaté City, LGE 2972 (male). Formosa Province: Formosa Department: Tatané, LGE 3029–30 (males), LGE 3031 (female), LGE 3032–4 (males), LGE 3035 (female), LGE 3069 (male); Tres Marías, LGE 3074–5 (males), LGE 3079–80 (males). Pilcomayo Deparment: Provincial Route 6, near Frontera, LGE 3037 (female), LGE 3086 (female). Paraguay: Caazapá Deparment: National Route 8, 12 km north from Caazapá Town, LGE 1677–80 (males), LGE 1681 (female), LGE 1682–5 (males).

Pseudopaludicola canga: Brazil: Pará State: Parauapebas Municipality: Carajás, PCS 236 (female), PCS 237 (male); Canaã dos Carajas Municipality, Serra da Bocaina, BOC 21 (male), BOC 22 (female), BOC 88 (male), BOC 89 (female).

Pseudopaludicola falcipes: Argentina: Buenos Aires Province: Luján Locality: Luján City, highway National Route 5, LGE 3098 (male), LGE 3100–1 (males). Corrientes Province: Curuzú Cuatiá Department: Perugorría, Estancia El Oscuro, LGE 3110 (male), LGE 3111 (female), LGE 3112 (male), LGE 3114 (female). Entre Ríos Province: Gualeguaychú Department: Arroyo Ramírez, LGE 3223–4 (two males), LGE 3226 (male), LGE 3227 (male). Misiones Province: Candelaria Deparment: Ñu Pyahú, Provincial Route 3, 3.5 km from National Route 12, LGE 1374 (female), LGE 1375 (male), LGE 1377 (female), LGE 1440 (female), LGE 1449 (male), LGE 2994 (male), LGE 2995 (male), LGE 2996 (female), LGE 2997 (female). Capital Department: right margin of Pindapoy Chico stream, LGE 6065–6 (males). Santa Fé: La Capital Deparment: Comuna de Leyes, Margins Leyes stream, LGE 3283 (female), LGE 3284 (male), LGE 3286 (male). Brazil: Rio Grande do Sul State: Br 153 km 654, 16 km from Aceguá Town, LGE 1499 (male), LGE 1500 (female). Uruguay: Rivera Department: Pueblo Madera (10 km south of Rivera City), MNHN 9521–2 (males), MNHN 9523 (male), MNHN 9524 (male), MNHN 9525–6 (males), MNHN 9527 (male), MNHN 9528 (female); Curticeras, MNHN 9529 (female), MNHN 9530 (male), MNHN 9531 (female), MNHN 9532–3 (males), 9534–5 (females). Rocha Department: Barra de la Laguna de Rocha, MNHN 9536 (female); Barra de Valizas, MNHN 9537 (female). Treinta y Tres Department: Arroyo Tigre, MNHN 9538 (male); Bañado de los Oliveras, MNHN 9539 (male); Quebrada de los Cuervos, MNHN 9540 (male).

Pseudopaludicola mystacalis. Argentina: Corrientes: General Paz Department: Provincial Route 13, 6 km. from National Route 12, LGE 1748 (male); Itá Ibaté City, LGE 1550 (male), LGE 1553 (female); Provincial Route 13, 7 km from Caá Catí City, LGE 1732 (female), LGE 1741 (male); Ituzaingó Department: Near Ituzaingó City, LGE 1554 (male), LGE 1556 (male), LGE 1559 (male); National Route 12 and Provincial Route 22; vicinity of Santa María natural reserve, LGE 2979 (male); Industrial Park, Ituzaingó City, LGE 2685 (male), near Villa Olivari Town, LGE 1549 (male); San Miguel Department: National Route 118, 17 km. from Loreto, LGE 1735 (female). Misiones: Capital Department: Right margin of Pindapoy Chico stream, LGE 6067 (male), LGE 6069 (male). Concepción Department: Itacaruaré town LGE 2691 (male), LGE 3014 (male), LGE 3017 (male). Brazil: Pará State: Bragança Municipality: Bacuriteua, LGE 8826 (male), LGE 8827–8 (females), LGE 8829 (male).

Pseudopaludicola ternetzi: Paraguay: Concepción Department: Distrito San Alfredo, Estancia Garay Kué, Reserva Natural Privada "Cerrados del Tagatiyá", IIBP-H 804–5 (males), IIBP-H 808 (female), IIBP 2528 (male), IIBP-H 2529 (female). Amambay Department: Estancia Pirá Potrero. Itapúa Department: National Route 1, 11 km west from General Delgado, IIBP-H 1244 (male).