Hereditas 147: 43–52 (2010)

Chromosome analysis in *Pseudopaludicola* (Anura, Leiuperidae), with description of sex chromosomes XX/XY in *P. saltica*

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Taxonomic changes have frequently occurred in the anuran genus *Pseudopaludicola* as a consequence of high morphological similarity among its species. The present work reports karyotypic analysis of three *Pseudopaludicola* species sampled in their type locality and four *Pseudopaludicola* populations from distinct localities, aiming at contributing to the systematics of this genus. Chromosomes were stained with Giemsa or submitted to the silver staining (Ag-NOR) and C-banding techniques. The karyotype was 2n=22 in *P. mineira*, *Pseudopaludicola* sp. and two populations of *P. saltica.* The chromosome pair 8 was heteromorphic in *P. saltica*, characterizing a XX/XY sex-determination system with telocentric X and submetacentric Y. Highly similar karyotypes with 2n=18 chromosomes were observed in *P. canga, P.* aff. *canga* from Barreirinhas, State of Maranhão, Uberlândia, State of Minas Gerais and Icém, State São Paulo. The high similarity among the karyotypes 2n=18 suggested that the populations of *P.* aff. *canga* belong to the group 'pusilla', the same group of *P. canga.* The data demonstrated also that *P.* aff. *canga* from Barreirinhas (northeast region) is cytogenetically identical to *P. canga* with regarding the NOR site position in pair 3 and the presence of a heterochromatic block in the pair 2, whereas *P.* aff. *canga* from Uberlândia and Icém (southeast) had the NOR in the pair 9. Moreover, the cytogenetic data discriminated *P. mineira* and *Pseudopaludicola* sp. from the previously analyzed species with 22 chromosomes, and suggested that *Pseudopaludicola* sp. is an undescribed species. Sexual heteromorphic chromosomes are firstly reported in *Pseudopaludicola* and the data indicated the need of an extensive taxonomic review in this genus.

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The genus *Pseudopaludicola* Miranda Ribeiro, 1926, currently in the family Leiuperidae (Grant et al. 2006), consists of 12 species (Frost 2009). This genus is widely distributed throughout South America, from northern Colombia to Argentina. In Brazil, nine *Pseudopaludicola* species have been recognized (Lobo 1992; Frost 2009). These species are smaller than 20 mm, morphologically similar, and some of them exist in sympatry (Lobo 1992).

The *Pseudopaludicola* genus has recently gone through several taxonomic changes, mainly based on molecular analyses. Frost et al. (2006) allocated *Pseudopaludicola* in the family Leptodactylidae, together with the genera *Pleurodema, Edalorhina, Physalaemus, Paratelmatobius, Scythrophrys, Adenomera, Lithodytes, Leptodactylus* and *Vanzolinius.* However, Grant et al. (2006) proposed a new arrangement for the family Leptodactylidae, presently consisting of four genera, *Hydrolaetare*, *Leptodactylus*, *Paratelmatobius* and *Scythrophrys.* The remaining genera, including *Pseudopaludicola*, were allocated in the family Leiuperidae.

The species in the genus *Pseudopaludicola* are currently separated into the groups 'pusilla' and 'falcipes', according to their morphological traits (Lynch 1989). Their behav-

DOI: 10.1111/j.1601-5223.2009.02153.x

ioral, bioacoustic and morphological characteristics have been intensively studied for taxonomic purpose (Haddad and Cardoso 1987; Lynch 1971, 1989; Lobo 1992, 1994, 1995, 1996). However, several aspects of intra and intergeneric phylogenetic relatedness and validation of new species remain unclear. According to several reports, a broad revision in this genus is still needed for the better understanding of its systematics (Lynch 1989; Lobo 1996).

Cytogenetic analyses in the genus *Pseudopaludicola* are scarce in the literature and, thus far, the karyotypes described for *P. falcipes* and *P. ameghini* (*sensu* Cope 1887), in the group 'falcipes', were analyzed only by conventional staining with Giemsa (Saez and Brum 1960; Brum and Saez 1968; Beçak 1968; Batistic et al. 1969; Batistic 1970). These techniques revealed uncommon variation in the number of chromosomes, from 2n=16 to 2n=22, in morphologically similar populations identified as *P. falcipes* (Beçak 1968; Batistic et al. 1969; Batistic 1970). However, a recent cytogenetic study on *P. falcipes* (2n=22) from the type locality revealed that the previously described chromosome number variation (2n=16 to 2n=22) attributed to the species *P. falcipes* (Beçak 1968; Batistic 1970), actually represents distinct species (Fávero et al. unpubl.). These karyotypes are related to diverse species, such as *P. mystacalis* (2n=16), *P. ameghini* (*sensu* Cope 1887) and *P. ternetzi* (2n=20). Cytogenetic studies have been proven helpful as a tool to assist in the characterization and systematics of cryptic species, such as the ones existing in the *Pseudopaludicola* genus.

The objective of the present work was to analyze the karyotypes of several Brazilian *Pseudopaludicola* species and populations, aiming at contributing to better understand the systematics of this genus. Cytogenetic data are described for the species *P. mineira* and *P. saltica* ('falcipes' group) and *P. canga* ('pusilla' group) from their type localities, and four populations, some of which are suspected of being new species.

MATERIAL AND METHODS

The analyzed specimens were sampled in eight Brazilian locations (Table 1). The voucher specimens were deposited in the Coleção Zoológica at the Federal University of Mato Grosso (UFMT), Mato Grosso State, Brazil, in Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC) at the State University of Campinas (UNICAMP) and in Museu de Zoologia of the State University of São Paulo, São José do Rio Preto (DZSJRP), in São Paulo State, Brazil. A map of sampling locations in which the studied specimens were surveyed is shown in Fig. 1.

Mitotic metaphases were obtained from cell suspensions of intestinal epithelium and testis of animals previously

Fig. 1. Map of sampling locations in which the studied specimens of *Pseudopaludicola* species were surveyed in Brazil, as listed in Table 1.

treated with 2% colchicine solution for five h, according to King and Rofe (1976) and Schmid (1978), with few modifications. Chromosomes were stained with Giemsa 10% or submitted to the (Ag-NOR) silver impregnation (Howell and Black 1980) and C-banding (Sumner 1972) techniques, with modification: samples were pretreated with 50% acetic acid, according to Siqueira et al. (2008). The samples were examined under a photomicroscope Olympus BX60, and images were captured using QCapture™ 2.81.0 and Image Pro-plus™ 4.5. The chromosomes were measured and classified according to Green and Sessions (1991), as shown in Table 2.

RESULTS

The karyotypes of the *Pseudopaludicola* specimens studied in the present work consisted of 2n=22 or 2n=18 chromosomes.

Karyotypes 2n=22

The species *P. mineira* and *P. saltica*, and the population of *Pseudopaludicola* sp. showed 2n=22 chromosomes. The karyotypes of these species were observed to differ only in the morphology of the pairs 6, 7, 8 and 11 (Table 2, 3, Fig. 2).

In most populations, the nucleolar organizing region (NOR) was detected in the telomeric region of the long arm of the pair 8 (Fig. 3). However, in *Pseudopaludicola*

Table 1. *Number of specimens of the analyzed Pseudopaludicola species and their respective sampling localities in Brazil. (ZUEC= Zoology Museum of the State University of Campinas, SP, Brazil; DZSJRP = Zoology Museum of the State University of São Paulo, São José do Rio Preto, SP, Brazil; UFMT = Zoology Collection of the Federal University of Mato Grosso, Brazil).*

Species	Locality	Number of specimens	Accession number
P. mineira	Serra do Cipó, Minas Gerais (type locality) (19°14'S; 43°33'W)	8 ♂ ; 1♀	ZUEC 14319 – 14323, 14326, 14329 e 14330
Pseudopaludicola sp.	Andaraí, Bahia (12°48'S; 41°19'W)	10 ♂ ; 3 ♀	ZUEC 14255, 14256, 14257, 14260, 14261, 14264 – 14271
P. saltica	Chapada dos Guimarães, Mato Grosso (type locality) (15°27'S; 55° 44'W)	13 ♂ ; 1♀	ZUEC 14227, 14228, 14232, 14234, 14236, 14237, 14240, 14242 –14247 UFMT 8542
P. saltica	Uberlândia, Minas Gerais (18°55'S; 48°16'W)	8 ♂; 2♀	ZUEC 14291, 14292, 14295, 14296, 14301, 14302, 14304, 14305, 14307, 14310
P. canga	Serra dos Carajás, Pará (type locality) (6º03'S; 50°28'W)	3 ♂; 12♀	ZUEC 14343, 14353, 14363, 14364, 14366, 14369 – 14374, 14376, 14378, 14379, 14380
Pseudopaludicola aff. *canga*	Barreirinhas, Maranhão (2°44'S; 42°49'W)	2♂	ZUEC 13860 e 13867
Pseudopaludicola aff. *canga*	Uberlândia, Minas Gerais (18°55'S; 48°16'W)	8 ♂; 3♀	ZUEC 14181, 14185, 14186, 14187, 14209, 14212, 14214, 14216, 14217, 14222 e 14223
Pseudopaludicola aff. *canga*	Icém, São Paulo (20°20'S; 49°11'W)	5 ♂; 1♀	DZSJRP 8727, 8728, 8747, 8749, 8750, 8752

sp. the NOR was interstitially located near the telomere of the long arm of the pair 8 (Fig. 3B). With the exception of *P. mineira* and of two *Pseudopaludicola* sp. specimens (ZUEC 14255 and 14266), the NOR was heteromorphic in all of the analyzed individuals (Fig. 3B–D). The NOR heteromorphism comprised a difference in size among the homologous chromosomes of the pair 8, especially in the population of *Pseudopaludicola* sp. (Fig. 2B), and were identified as 8 and 8'.

The C-banding technique revealed heterochromatic blocks in the centromeric region of all chromosomes. In *Pseudopaludicola mineira*, pericentromeric bands were observed in the long and short arms of the pair 1, in the short arm of pairs 2 and 7, and in the long arm of the pairs 9 and 10 (Fig. 4A). Interstitial blocks were present in both arms of pair 8 (Fig. 4A), and the one in the long arm was associated to the secondary constriction and coincident to the NOR (Fig. 3A). In *Pseudopaludicola* sp., heterochromatic interstitial blocks were observed in the short and long arms of the pair 1 and in the long arms of the pairs 8 and 9 (Fig. 4B). In the pair 8, this block was adjacent to a NOR positive secondary constriction.

The karyotypes were identical in the two analyzed populations of *P. saltica*, from Chapada dos Guimarães, Mato Grosso, Brazil (type locality) and from Uberlândia, Minas Gerais, Brazil (Table 2, 3, Fig. 2C–D). They consisted of metacentric (pairs 1, 2, 5, 7, 9, 10 and 11) and submetacentric chromosomes (pairs 3, 4 and 6). In males, the NOR-bearing pair 8 was heteromorphic (XY) with telocentric and submetacentric homologous (Fig. 2C–D, 3C–D). In females, both chromosomes in the pair 8 were telocentric (XX) (Fig. 2C–D, 3D). In *P. saltica*, besides the centromeric heterochromatic blocks, positive C-bands were detected in the interstitial regions of the long arms of the pairs 1, 2, 3, 4 and 6, and in the short arms of the pairs 1 and 2 (Fig. 4C–D). A heterochromatic block coincident to the NOR was observed in the long arm of the pair 8, which in females showed a size heteromorphism (Fig. 4C–D). However, the Ag-NOR reaction was weaker and bluish compared to the intensely stained magenta heterochromatic blocks.

The homologous of the pair 8 were polymorphic in the only analyzed *P. saltica* female from the type locality, due to the size heteromorphism of the secondary constriction, positive to C-banding, localized in the long arm coincident with the NOR (Fig. 5A). In males, the relative size of the submetacentric chromosomeY was a little larger than the telocentric X chromosome (Fig. 5B, Table 2). However,

in the specimen UFMT 8542, the X chromosome was larger than the Y, with increased staining in the NOR site and in the heterochromatin block (Fig. 5C).

Aiming at verifying the meiotic behavior of the sex chromosomes in *P. saltica,* they were analyzed at diakinesis in testis cells stained with Giemsa (Fig. 6A) and submitted to the C-banding technique (Fig. 6B). On the diakinesis, eleven bivalents were observed, which followed the expected behavior of homologous in this meiotic phase.

Karyotypes with 2n=18

The karyotypes of *P. canga* from Serra dos Carajás and *P.* aff. *canga* from Barreirinhas, Uberlândia and Icém consisted of 2n=18, with no differences` in the chromosome classification. The pairs 1, 2, 5 and 6 were metacentric, the pairs 3, 4 and 9 submetacentric and the pairs 7 and 8 telocentric (Table 2, Fig. 7). In all analyzed populations, the long arm of one of the homologous of the pair 3 had a slight size heteromorphism, which did not alter the classification of this pair (Table 2).

The NOR site was localized in the pericentromeric region of the short arm of the pair 3 both in *P. canga* and *P.* aff. *canga* - Barreirinhas (Fig. 8A–B), near the telomere of the long arm of the pair 9 in *P.* aff. *canga* - Uberlândia (Fig. 8C) and at the telomeric region of the long arm of the pair 9 in *P.* aff. *canga* - Icém (Fig. 8D).

The C-banding pattern was similar among the four *Pseudopaludicola* populations and comprised few non-centromeric heterochromatic blocks. In all analyzed populations, a C-band was detected in the short arm of the pair 1. Only *P. canga* and *P.* aff. *canga* - Barreirinhas had the heterochromatic block in the pericentromeric region of the long arm of the pair 2 (Fig. 9).

Table 2. *Morphometric analysis of the chromosomes in the studied Pseudopaludicola. Classification according to Green and Sessions (1991): M=metacentric; SM=submetacentric; T=telocentric; †chromosome X; ◆ chromosome Y.*

	Number of chromosomes											
	1	2	3	4	5	6	7	8	8'	9	10	11
	P. mineira (Serra do Cipó, Minas Gerais state)											
Relative size	15.66	13.47	11.29	10.33	9.20	8.49	7.92	6.71	-	6.11	5.72	5.03
Arm ratio	1.16	1.19	1.85	1.81	1.14	1.15	1.71	1.34	-	1.16	1.17	1.18
Classification	M	M	SM	SM	M	M	SM	M	-	M	M	M
	Pseudopaludicola sp. (Andaraí, Bahia state)											
Relative size	14.71	12.53	11.06	10.28	8.90	8.07	7.45	6.91	5.79	5.35	5.01	3.93
Arm ratio	1.15	1.37	1.99	1.87	1.19	1.31	1.39	2.31	1.85	1.25	1.25	1.93
Classification	M	M	SM	SM	M	M	M	SM	SM	M	M	SM
	P. saltica (Chapada dos Guimarães, Mato Grosso state)											
Relative size	14.65	12.50	11.80	10.90	8.23	7.77	7.33	5.72	6.10	5.37	4.95	4.64
Arm ratio	1.19	1.43	2.46	2.47	1.24	1.94	1.09	8.30	2.27	1.16	1.21	1.17
Classification	M	M	SM	SM	M	SM	M	T†	SM ◆	M	M	M
	P. saltica (Uberlândia, Minas Gerais state)											
Relative size	14.75	12.60	11.60	10.80	9.23	8.31	7.67	5.68	6.26	4.94	4.87	4.53
Arm ratio	1.18	1.46	2.59	2.60	1.20	1.87	1.12	8.98	2.13	1.18	1.19	1.21
Classification	M	M	SM	SM	M	SM	M	T†	SM ◆	M	M	M
	P. canga (Serra dos Carajás, Pará state)											
Relative size	14.89	12.31	11.49	10.55	9.68	8.88	7.46	6.64	-	6.57	-	-
Arm ratio	1.15	1.31	2.41	2.01	1.31	1.19	10.0	9.30	-	1.72	-	-
Classification	M	M	SM	SM	M	M	T	T	-	SM	-	-
	Pseudopaludicola aff. *canga* (Barreirinhas, Maranhão state)											
Relative size	14.73	13.13	12.16	9.63	9.15	8.61	7.56	6.86	-	5.97	-	-
Arm ratio	1.10	1.26	2.38	1.84	1.34	1.25	10.0	11.0	-	2.02	-	-
Classification	M	M	SM	SM	M	M	T	T	-	SM	-	-
	Pseudopaludicola aff. *canga* (Uberlândia, Minas Gerais state)											
Relative size	14.85	13.05	10.37	10.54	9.52	9.23	8.17	6.56	-	7.30	-	-
Arm ratio	1.27	1.39	2.59	2.08	1.28	1.41	13.6	10.72	-	2.01	-	-
Classification	M	M	SM	SM	M	M	T	T	-	SM	-	-
	Pseudopaludicola aff. *canga* (Icém, São Paulo state)											
Relative size	15.29	12.17	10.98	10.25	9.51	8.83	8.42	6.65	-	6.87	-	-
Arm ratio	1.18	1.47	2.09	2.68	1.19	1.39	14.8	12.0	-	1.87	-	-
Classification	M	M	SM	SM	M	M	T	T	-	SM	-	-

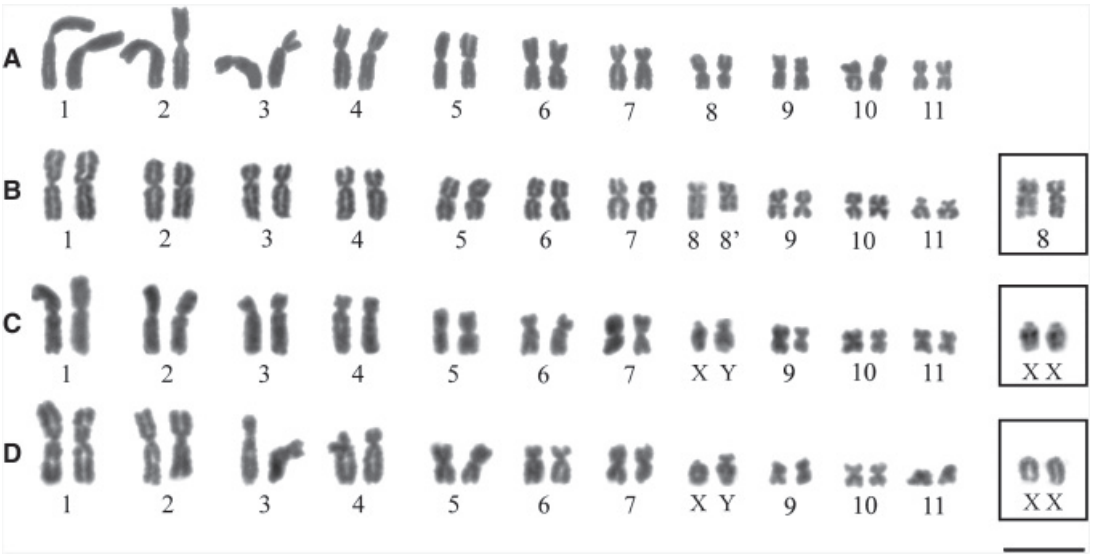

Fig. 2A–D. Karyotypes with 2n=22 chromosomes stained with Giemsa. (**A**) *P. mineira*, (**B**) *Pseudopaludicola* sp., (**C**) male specimen of *P. saltica* from Chapada dos Guimarães, (**D**) male specimen of *P. saltica* from Uberlândia. In the standout: (**B**) homomorphic pair 8 of *Pseudopaludicola* sp.; (**C**, **D**) the sex chromosomes XX of *P. saltica*. Bar = 5 μm.

DISCUSSION

The data presented herein revealed cytogenetic characteristics previously unknown in the *Pseudopaludicola* genus, which are of fundamental importance for the systematics of this anuran group and for the understanding of its evolutionary biology. Karyotype polymorphisms were suitable to separate the analyzed *Pseudopaludicola* species and populations into the 'falcipes' and 'pusilla' groups.

The karyotypes (2n=22) were conservative between the species *P. mineira* and *P. saltica*, sampled in their type localities. However, the *P. saltica* from Uberlândia, and the population of *Pseudopaludicola* sp. showed morphological polymorphisms in the chromosomes 7, 8 and 11, which distinguished *P. mineira* from *Pseudopaludicola* sp. Moreover, these two species were differentiated by the NOR site in the pair 8, which was terminal in *P. mineira* and subterminal in *Pseudopaludicola* sp., and by polymorphisms in the heterochromatin distribution as well. Therefore, the karyotypic analysis indicated that *Pseudopaludicola* sp. is a distinct taxon, possibly a new, undescribed species.

The fact that the *P. saltica* specimens from Uberlândia did not differ from the specimens sampled in the type locality (Chapada do Guimarães) confirmed that they are the same species. In these populations, the morphological differentiation of the pair 8 between males and females is an evidence of XX/XY sex-determination system, which was previously unknown in the genus *Pseudopaludicola.* The differentiation of the pair 8 might be directly involved in speciation within *Pseudopaludicola*, since the NOR is also localized in this same chromosome pair in other species (2n=22) of the genus, as for instance *P. mineira* and *Pseudopaludicola* sp., herein described, and *P. falcipes* from the type locality (Porto Alegre, Rio Grande do Sul state, Brazil) and *Pseudopaludicola* sp. from Poconé, Mato Grosso state, Brazil (Fávero et al. unpubl.).

Table 3. *Summary of differences observed among the karyotypes of Pseudopaludicola species with 2n=22 chromosomes. M = metacentric; SM = submetacentric; T = telocentric.*

Species	Chromosome pairs				NOR in pair 8	C-band
	6	7	8	11		
P. mineira	M/ M	SM/ SM	M/ M	M/ M	telomeric	1, 2, 7, 8, 9, 10
Pseudopaludicola sp.	M/ M	M/ M	SM/SM	SM/ SM	sub-telomeric	1, 8, 9
	SM/SM	M/ M	T/SM	M/ M	telomeric	1, 2, 3, 4, 6, 8
P. saltica	SM/SM	M/M	T/T	M/M	telomeric	1, 2, 3, 4, 6, 8

Fig. 3A–D. Localization of the NOR site in the species with karyotype 2n=22, by the silver impregnation technique. (**A**) *P. mineira*, (**B**) *Pseudopaludicola.* sp. (**C**) *P. saltica* from Chapada dos Guimarães, (**D**) *P. saltica* from Uberlândia. In the outstand: (**B**) heteromorphic pair 8; (**C, D**) homomorphic pair 8. Bar = 5 μm.

The morphology of the pairs 3 and 8 of these two last populations, however, was significantly different compared with the ones 2n=22 described herein. In all of these analyzed species, the pair 8 bears the NOR in the telomeric region and has a heterochromatic block associated to this region, while in *P. falcipes* the NOR is localized in the long arm of the subtelocentric chromosome 8, near the centromere. The data suggested that modifications in the nucleolar organizing region and, possibly, in the heterochromatin, can be related to changes in the structure of this chromosome pair and have contributed to the karyotypic differentiation of these species. Batistic (1970) observed a 2n=22 karyotype in meiotic metaphases of a population identified as *P. falcipes*, from Feira de Santana, Bahia state, Brazil. As the study was restricted to meiotic metaphases and limited to the number of chromosomes detection, additional comparisons with this population were not possible.

Pseudopaludicola canga is the first species within the group 'pusilla' to have its karyotype studied. The *P. canga* and the three populations of *P.* aff. *canga* – Barreirinhas, Uberlândia and Icém have 2n=18 karyotypes. The two former ones could be distinguished from the others only by the NOR site and by the presence of a C-band in chromosome 2. Considering this high karyotypic similarity, it is conceivable that these *Pseudopaludicola* populations are closely related to *P. canga* and possibly belong to the the same group 'pusilla'. This hypothesis could be tested in a phylogenetic study comprising species of the groups 'falcipes' and 'pusilla'.

According to Giaretta and Kokubum (2003), *P. canga* is known only from its type locality, Serra dos Carajás, Pará, Brazil. However, *P. canga* and *P.* aff. *canga* - Barreirinhas were not distinguished in the cytogenetic analysis. Hence, the possibility that they belong to the same species cannot be discarded, which would expand the known *P. canga* geographic distribution. This hypothesis must be tested in additional studies on their morphology and bioacoustics, since they have been considered distinct taxa (G. A. Vasconcelos pers. comm.).

Furthermore, no differences were detected in the karyotypes of *P.* aff. *canga* - Uberlândia and *P.* aff. *canga* - Icém, which also suggests that these two populations belong to the same taxon. The taxonomic identification of the population *P.* aff. *canga* (2n=18) from Icém (São Paulo state, Brazil) was based on morphological similarities with *P. falcipes* (2n=22). However, the chromosome analysis showed that this population is indeed closely related to the *P. canga* species. Correspondingly, the karyotype 2n=18 described by Beçak (1968) and Batistic (1970) for specimens from the region of São José do Rio Preto (São Paulo

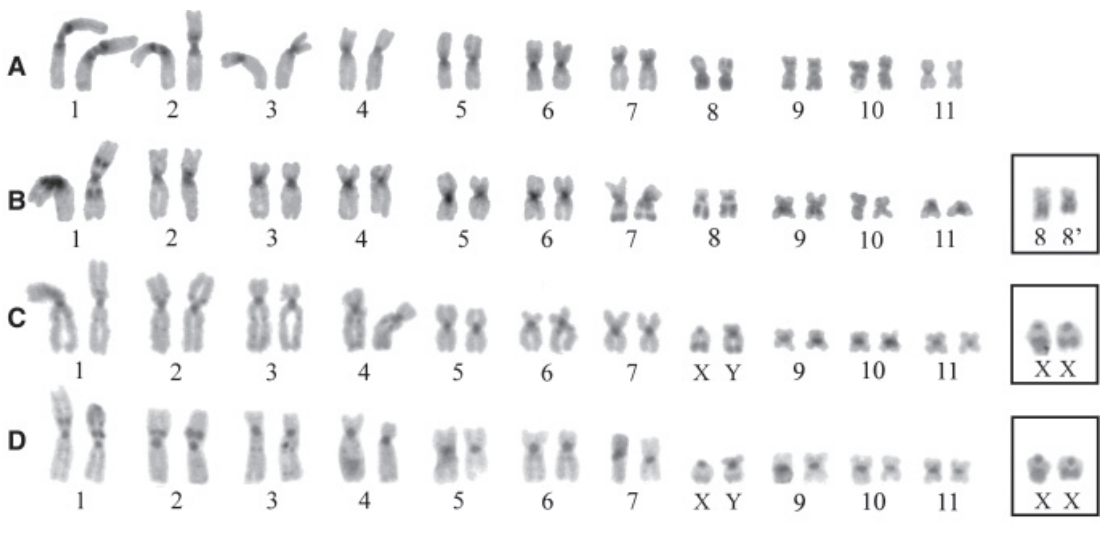

Fig. 4A–D. Karyotypes with 2n=22 chromosomes submitted to the C-banding technique. (**A**) *P. mineira* (**B**) *Pseudopaludicola* sp. (**C**) male of *P. saltica* from Chapada dos Guimarães, (**D**) male of *P. saltica* from Uberlândia. In standout: (**B**) heteromorphic pair 8; (**C, D**) sex chromosomes XX of *P. saltica*. Bar = 5 μm.

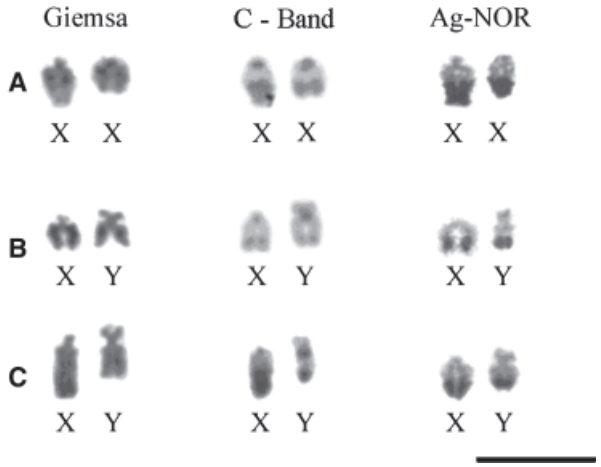

Fig. 5A–C. Sex chromosomes of *P. saltica* from the type locality submitted to the Giemsa conventional staining, C-banding and Ag-NOR. (**A**) chromosomes X of the female voucher specimen ZUEC 14234, (**B**) chromosomes XY of the male voucher specimen ZUEC 14172 (**C**), chromosomes XY of the specimen UFMT 8542. Bar = 5 μm.

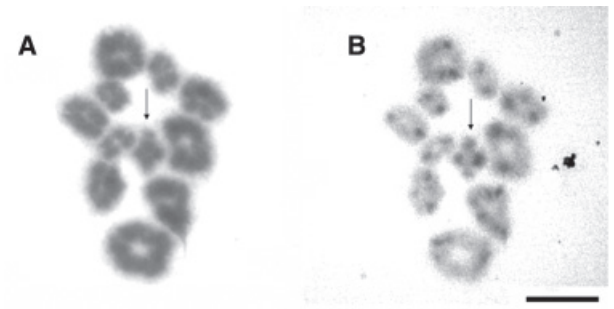

Fig. 6A–B. Diakinesis in the meiotic prophase I of germinative cells of male *P. saltica* from the Chapada dos Guimarães. Chromosomes were stained with Giemsa (**A**) and submitted to the C-banding technique (**B**). Arrows indicate the pair of sex chromosomes. Bar = 5 μm.

state) and identified as *P. falcipes,* is similar to the *P. canga* (2n=18) karyotype. Therefore, our data suggest that these populations are actually related to *P. canga,* despite having morphological similarities to *P. falcipes* and morphologically different of *P. canga* (D. C. Rossa-Feres unpubl.). In addition to the *Pseudopaludicola* karyotype 2n=18 in Icém, two other distinct karyotypes are found in sympatry, consisting of 2n=16 and 2n=20. These specimens are cytogenetically related, respectively, to *P. mystacalis* (2n=16) and to *P. ameghini* (*sensu* Cope 1887) and *P. ternetzi,* both with 2n=20 (Fávero et al. unpubl.). The cytogenetic studies have indicated that the high morphological similarity among *Pseudopaludicola* species has frequently misled taxonomic identifications, and reinforces the need of an extensive review in the genus.

The cytogenetic data presented herein suggested that the karyotypes 2n=18 chromosomes, probably belong to the group 'pusilla' and are evolutionary conservative, since they have rather similar chromosome morphology and heterochromatin pattern. However, they are variable in relation to the NOR-bearing pair and this characteristic clearly separated the 2n=18 karyotypes in two groups: one with the NOR site in the pair 3 (*P. canga* and *P.* aff. *canga* - Barreirinhas) and the other with the NOR in the pair 9 (*P.* aff. *canga* from Uberlândia and Icém).

Chromosome number variation among species in the same genus, as demonstrated in *Pseudopaludicola,* is quite rare in the order Anura. Various chromosomic rearrangement mechanisms, involving centric fusion and/or fission events, were used to explain the origin of numerically different karyotypes, as well as possible evolutionary stages that led to this variation (Beçak 1968; Bogart 1973, 1991; King 1990). Batistic (1970) proposed that fusion mechanisms may have originated numerically distinct karyotypes in *Pseudopaludicola,* besides other chromosomic rearrangements. In addition, the author suggested that these karyotypes were originated from an ancestral karyotype 2n=22, with a progressive decrease toward the karyotype 2n=16.

The present work describes karyotypes 2n=22 in *P. mineira*, *Pseudopaludicola* sp. and *P. saltica* and 2n=18 in *P. canga* and *P.* aff. *canga* – Barreirinhas, Uberlândia and Icém, but the cytogenetic data did not allow inference on the possible mechanisms associated to the speciation in this anuran group of species.

P. saltica sex chromosomes

Heteromorphic sex chromosomes are rare in anurans. To the present time, in the family Leiuperidae, only *Engystomops petersi* was known to have differentiated sex chromosomes (Lourenço et al. 1999), and herein they were firstly reported in the genus *Pseudopaludicola*. Interestingly, a pair of chromosomes in *P. saltica* was heteromorphic in males and homomorphic in females, characterizing a XX /XY♂ sex determination system. Both the telocentric X and the submetacentric Y chromosomes bear the NOR site, which is coincident with an interstitial heterochromatin block.

The sex determination systems XX/XY, ZZ/ZW and W0/00 are found in the leiuperid *Physalaemus petersi* (Lourenço et al. 1999), the hylid *Pseudis tocantins* (Busin et al. 2008), the leiopelmatid *Leiopelma hochstetteri* (Green 1988; Schmid et al. 1991; Green and Canatella 1993), and the cycloramphid *Proceratophrys boiei* (Ananias et al. 2007), among others. Multiple sex systems involving autosomes pairs, such as XXAA♀/ XYAA♂/ XAA^{Y}♂ were also described in the straboman-

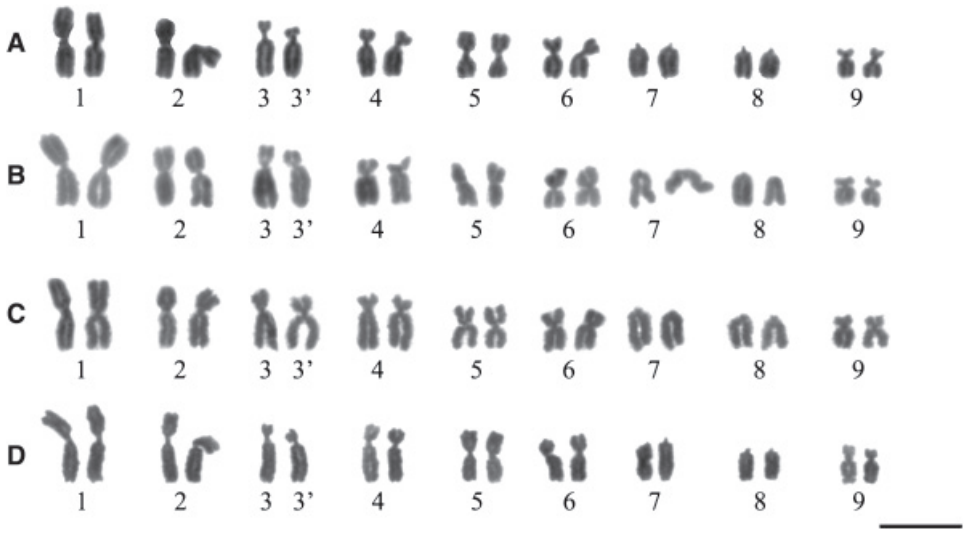

Fig. 7A–D. Karyotypes with 2n=18 chromosomes stained with Giemsa. (**A**) *P. canga*, (**B**) *P.* aff. *canga* - Barreirinhas, (**C**) *P.* aff. *canga* - Uberlândia, (**D**) *P.* aff. *canga* - Icém. Bar = 5 μm.

tids *Eleutherodactylus maussi* (currently *Strabomantis biporcatus*) and *E. riveroi* (currently *Pristimantis riveroi*) (Schmid et al. 2002a, 2003). In several anuran species, as for instance *Pseudepidalea viridis* (Bufonidae), the sex chromosomes could be differentiated only after chromosome banding (Odierna et al. 2007). The sex chromosomes are NOR-bearing in some of these species, as for instance *P. petersi* (Lourenço et al. 1998), *Pseudis tocantins* (Busin et al. 2008) and *Leiopelma archeyi* (Green 2002).

Iturra and Veloso (1989) and Schmid et al. (1991) suggested that sex chromosomes in amphibians, as well as in other vertebrates, had an autosomal ancestral origin. According to Ohno (1967), the differentiation of sex chromosomes might have gradually occurred by accumulating polymorphisms between the homologous of the pair of chromosomes responsible for genetic sex determination. Afterwards, crossing-over suppression and heterochromatin gain in only one chromosome of one of the sexes (Y or W) might have led to gradual differentiation of sex chromosomes (Singh et al. 1976; Jones and Singh 1981; Bull 1983). The heterochromatinization process has been suggested to explain the evolution of the chromosomes Y and W in mammals, birds and some reptiles (Schmid et al. 1988), as well as in salamanders of the genus *Triturus* (Schmid 1983; Schmid et al. 1979) and the marsupial anuran *Gastrotheca pseustes* (Schmid et al. 1990; Amphignathodontidae). However, Schmid et al. (1988, 2002b) demonstrated that the chromosome Y of *Gastrotheca walkeri* and *G. ovifera* had a lower quantity of heterochromatin than expected, suggesting that the heteromorphism, in this case, may have occurred by the loss of heterochromatin in the chromosome Y.

The initial stages of sex chromosome differentiation in vertebrates could be directly related to chromosome pericentric inversions (John 1988). Apparently, the origin of the sex chromosomes in the leptodactylids *Eupsophus miguelli* and *E. roseus* (Iturra and Veloso 1989), and *E. insularis* (Cuevas and Formas 1996) involved chromosome pericentric inversions. The differences between the chromosomes W and Z in *Pyxicephalus adspersus* (Ranidae) seem to comprise an inversion of a heterochromatin block in the chromosome W (Schmid 1980). Busin et al. (2008) suggested that the differentiation of sex chromosomes in *Pseudis tocantins* might have begun with an inversion followed by a heterochromatin gain, and subsequent morphological differentiation of the chromosome W. In *P. saltica*, however, the long arm of the chromosome Y is homologous to the chromosome X and both have a single heterochromatin block coincident to the NOR. An increased NOR was observed in the specimen UFMT 8542, in which the size of the chromosome X was larger than the Y. In the other species with 22 chromosomes, the NOR-bearing pair 8 also has a heterochromatin block in the long arm, which is totally or partially coincident with the NOR. Therefore, the

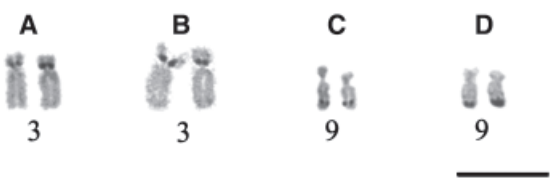

Fig. 8A–D. Localization of the NOR site in the species with karyotype 2n=18, by silver impregnation. (**A**) *P. canga*, (**B**) *P.* aff. *canga* - Barreirinhas, (**C**) *P.* aff. *canga* - Uberlândia (**D**) *P.* aff. *canga* - Icém. Bar = 5 μm.

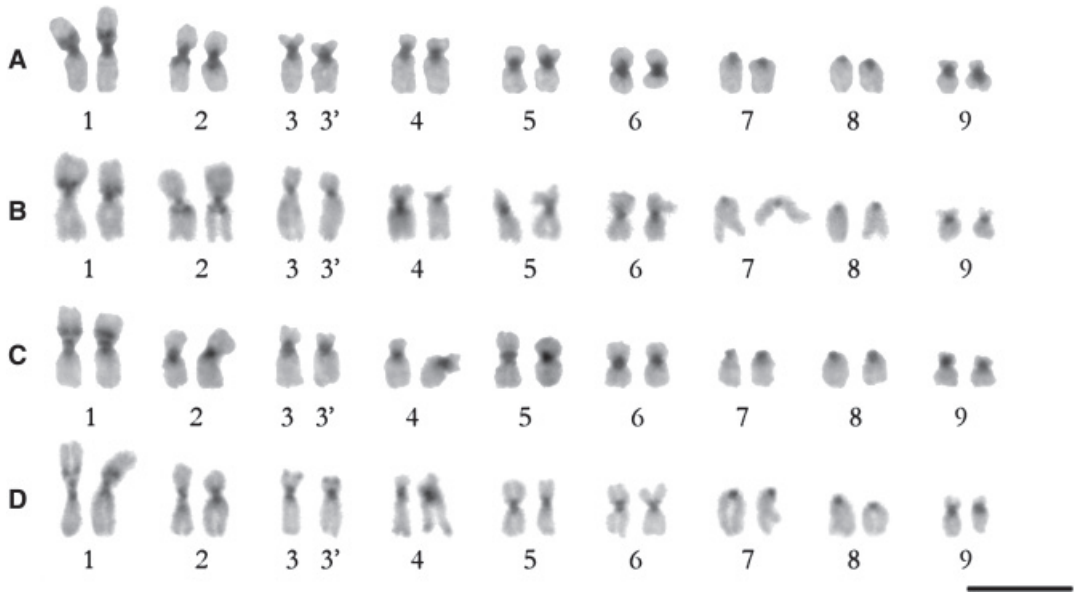

Fig. 9A–D. Karyotypes with 2n = 18 chromosomes submitted to the C-banding technique. (**A**) *P. canga*, (**B**) *P.* aff. *canga* - Barreirinhas, (**C**) *P.* aff. *canga* - Uberlândia, (**D**) *P.* aff. *canga* - Icém. In the outstand: the chromosome pair 2 of another *P. canga* metaphase, clearly showing the positive C-band in the pericentromeric region of the long arm. Bar = 5 μm.

pair 8 and the sex chromosomes may have a common origin, and the sex chromosomes are probably a derived condition. There is no evidence that the chromosome inversion mechanisms may have contributed to the differentiation of these chromosomes.

Therefore, based on these observations, two hypotheses could be raised to explain the mechanism of sex chromosome differentiation in *P. saltica*: (1) in an ancestral, a chromosome translocation, possibly involving the pair 8, originated the short arm of the chromosome Y, or (2) there was fission in the short arm of a submetacentric pair. However, analysis of meiotic cells in diakinesis showed no evidence that the short arm of the chromosome Y could have originated from a translocation process. Only bivalents were visualized in the meiosis, and this does not support the translocation hypothesis. The data presented herein indicated that the most probable hypothesis is that the chromosome X was originated by fission of the short arm of a submetacentric chromosome. The sex chromosomes in *P. saltica* could be in an initial stage of differentiation, as similarly proposed for other anuran species.

Acknowledgements – The authors are thankful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Proc. 6/60055-0) for financial support, Fundação de Ensino de São Paulo (FUNDESP) for the scollarship to T. C. D., Sérgio Siqueira Junior, Eduardo R. Fávero, Leandro Magrini, Gilda V. Andrade, Rodrigo A. Silva, Carolina P. Candeira e Fernando R. Silva for helping with frog samplings, and Célio F. B. Haddad for assisting with specimen taxonomic identification. The specimens were collected with permission of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA – process no. 02001.002003/2005–16).

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