Genetica (2008) 133:119–127
DOI 10.1007/s10709-007-9189-7

Cytogenetic analysis of four species of *Pseudis* (Anura, Hylidae), with the description of ZZ/ZW sex chromosomes in *P. tocantins*

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Received: 21 October 2006 / Accepted: 30 July 2007 / Published online: 23 August 2007

Abstract *Pseudis paradoxa paradoxa*, *P. p. platensis*, *P. bolbodactyla*, *P. fusca* and *P. tocantins* were analyzed cytogenetically by conventional chromosomal staining, C-banding, silver staining and fluorescent in situ hybridization with an rDNA probe. *Pseudis tocantins* chromosomes were also stained with distamycin A/DAPI. All of the species had a diploid number of 2n = 24 chromosomes and the nucleolar organizer region (NOR) was located on pair 7. However, the karyotypes could be differentiated based on the morphology of chromosomal pairs 2 and 8, the region that the NORs occupied on the long arms of the homologous of pair 7, and the pattern of heterochromatin distribution. The subspecies *P. p. paradoxa* and *P. p. platensis* had identical karyotypes. Heteromorphism in NOR size was seen in *P. p. paradoxa*, *P. p. platensis*, *P. bolbodactyla* and *P. fusca*. Heteromorphic sex chromosomes (ZZ/ZW) were identified in *P. tocantins*. The W chromosome was subtelocentric and larger than the metacentric Z chromosomes. The differences observed in the C-banding pattern and in the position of the NOR on the sex chromosomes suggested that inversions and heterochromatinization were responsible for the morphological differentiation of these chromosomes.

Keywords Amphibia · Heterochromatin · Karyotype · NOR · *Pseudis* · Sex chromosomes

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Introduction

The taxonomic position and phylogenetic relationships of the genus *Pseudis* have always been a matter of extensive discussion. Savage and Carvalho (1953) grouped *Pseudis* together with the genus *Lysapsus* in the family Pseudidae, whereas Duellman (2001) included both genera in the subfamily Pseudinae as a sister group of the Hylinae. However, based on molecular studies, Darst and Canatella (2004) included the two genera in the subfamily Hylinae, a relationship recently confirmed by an extensive reassessment of the subfamily Hylinae based on the analysis of mitochondrial gene sequences (Faivovich et al. 2005). Until the 1990s, the genus *Pseudis* consisted only of the species *P. minuta* and *P. paradoxa*, the latter being subdivided into seven subspecies: *P. p. paradoxa*,

P. p. platensis, *P. p. bolbodactyla*, *P. p. fusca*, *P. p. occidentalis*, *P. p. caribensis* and *P. p. nicefori* (Gallardo 1961). Caramaschi and Cruz (1998) considered *P. p. bolbodactyla* and *P. p. fusca* to be full species (*P. bolbodactyla* and *P. fusca*, respectively), and described *P. tocantins* as another species in this genus. Kwet (2000) subsequently described *P. cardosoi*, a species morphologically similar to *P. minuta.*

Barrio and Rubel (1970) used conventional chromosomal staining to analyze *P. p. platensis* from Argentina, and Busin et al. (2001) analyzed *P. minuta* and *Pseudis* sp. (aff. *minuta*), two species from the southern Brazilian state of Rio Grande do Sul (RS), by conventional and differential chromosomal staining. These studies revealed karyotypes of 2n = 24 for *P. p. platensis* and *P. minuta* and 2n = 28 for *Pseudis* sp. (aff. *minuta*), with the latter species having four pairs of telocentrics in addition to bi-armed chromosomes. According to Busin et al. (manuscript in preparation), *P. cardosoi* specimens from the type-locality (Centro de Pesquisa e Conservação—Pró-Mata, municipality of São Francisco de Paula—RS) and *Pseudis* sp. (aff. *minuta*) from Tainhas—RS, previously analyzed by Busin et al. (2001), have identical karyotypes, suggesting that *P. cardosoi* and *Pseudis* sp. (*aff. minuta*) are the same species.

In amphibians, cytogenetically recognizable sex chromosomes are rare and there is generally little heteromorphism, suggesting that the chromosomes are still in the initial stage of morphological differentiation. In many species, sex chromosomes can be identified in mitotic metaphases only with the use of differential staining techniques. For example, the homomorphic Z and W chromosomes in *Buergeria buergeri* are distinguished by the nucleolar organizer region (NOR) that is located on the Z but not on the W chromosome (Ohta 1986; Schmid et al. 1993). In contrast, in some species, the sex chromosomes may show sufficient morphological differentiation to allow their recognition by conventional staining, as in *Eleutherodactylus euphronides* and *E. shrevei*, in which the W chromosome is 3–4 times larger than the Z chromosome (Schmid et al. 2002a). The variation seen in the degree of heteromorphism among amphibian sex chromosomes could provide useful clues for understanding the evolution of sex chromosomes in vertebrates.

In view of the taxonomic uncertainties surrounding various *Pseudis* species and subspecies, in this study, we used conventional chromosomal staining, C-banding, AgNOR staining, FISH with rDNA probes and fluorescence staining with distamycin A/DAPI to examine the relationships among two subspecies of *P. paradoxa* (*P. p. paradoxa* and *P. p. platensis*), *P. bolbodactyla*, *P. fusca* and *P. tocantins* collected at different localities in Brazil. We also describe a new case of ZZ/ZW sex chromosome heteromorphism in *P. tocantins*.

Materials and methods

The following specimens were analyzed: three males and four females of *P. p. paradoxa* from Bacabal-MA (04°12′00″ S 44°48′00″ W), eight males and five females of *P. p. platensis* from Corumbá-MS (18°54′00″ S 56°48′00″ W), 15 males and 10 females of *P. bolbodactyla* from Quirinópolis-GO (18°30′00″ S 50°24′00″ W), six males of *P. fusca* from Coronel Murta-MG (16°36′00″ S 42°12′00″ W), one male and one female of *P. fusca* from Salinas-MG (16°06′00″ S 42°12′00″ W), and four males and seven females of *P. tocantins* from Porto Nacional-TO (10°42′00″ S 48°24′00″ W), all located in Brazil.

The frogs were collected with the permission of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, process no. 02001008875/01-11, license no. 039/03) and are deposited in the "Prof. Adão José Cardoso" Natural History Museum of the State University of Campinas, under the ZUEC accession numbers 11792–11799, 11802–11805 and 12840 (*P. p. platensis*—Corumbá, MS), 13235 to 13239 (*P. fusca*—Coronel Murta, MG) and 13227 to 13234 (*P. tocantins*—Porto Nacional, TO), and in the Museu Nacional, Rio de Janeiro, under the MNRJ accession numbers 33857–33863 (*P. p. paradoxa*—Bacabal, MA), 34031–34055 (*P. bolbodactyla*—Quirinópolis, GO) and 35456–35458 (*P. tocantins*—Porto Nacional, TO). The two specimens of *P. fusca* collected in Salinas—MG (BC198.09 and 198.10—laboratory register numbers) have not yet been registered.

Chromosomal preparations were obtained from intestinal and testicular cell suspensions of frogs previously treated with colchicine for 4–6 h according to the method of Schmid (1978). The material was stained with 10% Giemsa and also processed for C-banding by the method of Sumner (1972) with a slight alteration in the duration of treatment with barium hydroxide. AgNOR staining was done as described by Howell and Black (1980) and fluorescent in situ hybridization (FISH) was done according to Viégas-Péquignot (1992) using a recombinant plasmid (HM123) containing fragments of *Xenopus laevis* rDNA (Meunier-Rotival et al. 1979) to detect ribosomal genes. The probe was labeled with biotin by nick translation according to the manufacturer's instructions (Gibco). C-banded metaphases of male and female specimens of *P. tocantins* were submitted sequentially to fluorescence staining with distamycin A/DAPI (Schweizer 1980). The chromosomes were classified using the criteria proposed by Green and Sessions (1991).

Results

The *P. paradoxa* subspecies, *P. bolbodactyla*, *P. fusca* and *P. tocantins* had a diploid number of 2n = 24 chromosomes, with their karyotypes consisting of metacentric, submetacentric and subtelocentric chromosomes (Figs. 1, 4A, D, and 6).

Pseudis p. paradoxa and P. p. platensis

The two subspecies had identical karyotypes, with the same chromosomal morphology, C-banding pattern and number and location of NORs. Chromosomal pairs 1 and 9–12 were metacentric, pairs 2, 4, 5 and 8 were submetacentric and pairs 3 and 6 were subtelocentric. Chromosomal pair 7 was of the metacentric or submetacentric type, the former (morph 7a) being large and the latter (morph 7b), small. The proximal long arms of these chromosomes showed a secondary constriction that was relatively small or even absent in morph 7a and very large in morph 7b. The only combinations seen in males and females were 7a7a (in 80% of the *P. p. paradoxa* and 60% of the *P. p. platensis* specimens that were karyotyped) and 7a7b (in 20% of the *P. p. paradoxa* and 40% of the *P. p. platensis* specimens that

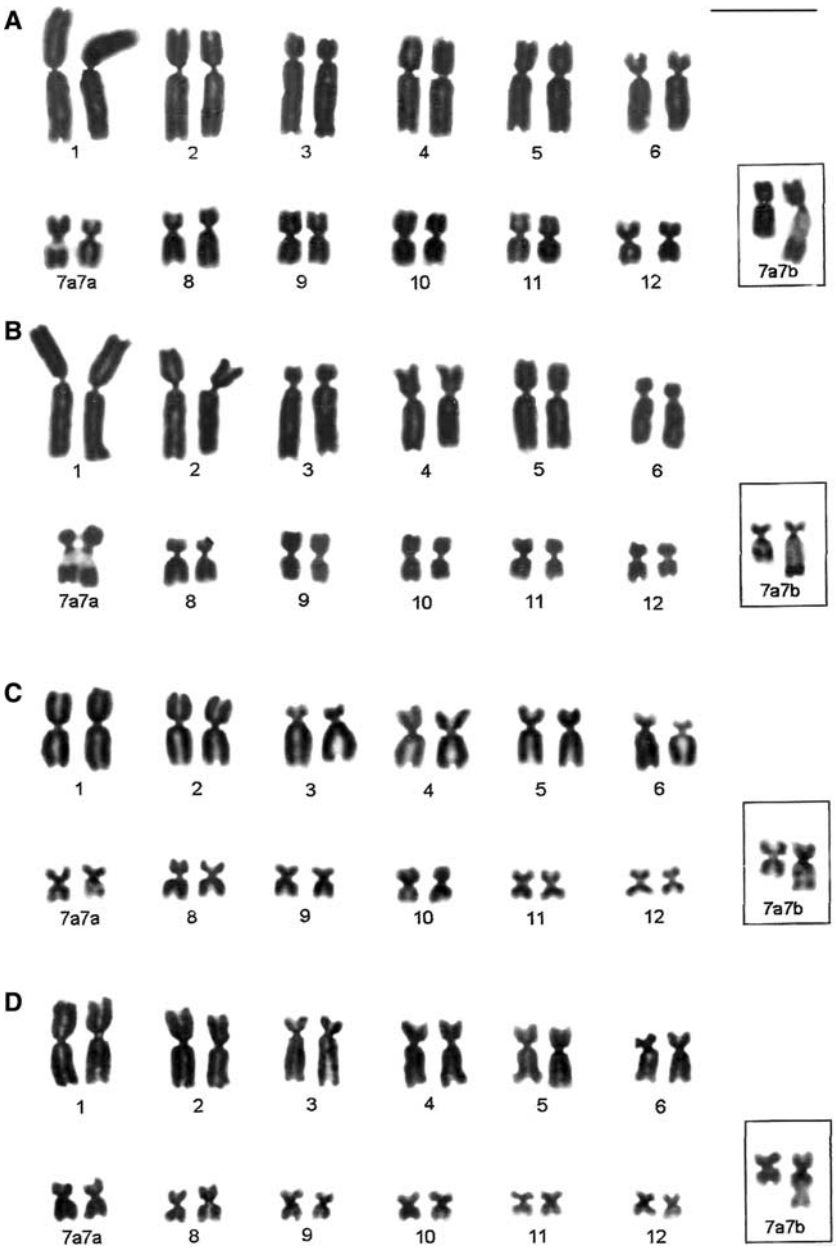

Fig. 1 Giemsa-stained karyotypes. (**A**) *P. p. paradoxa*. (**B**) *P. p. platensis*. (**C**) *P. bolbodactyla*. (**D**) *P. fusca*. Heteromorphic chromosomes 7a and 7b are highlighted. Bar = 5 μm

were karyotyped); the combination 7b7b was never seen (Figs. 1A, B and 6A, B). C-banding revealed heterochromatin in the centromeric regions of all chromosomes in both subspecies, in the telomeric region of the long arms of homologous 2 and 3, and in the pericentromeric region of the short arms of pair 5. Interstitial heterochromatin was detected on the long arms of chromosome 7. Heterochromatin was also detected from the pericentromeric to the subterminal region of the short arms of pairs 6 and 11 (Figs. 2A, B and 6A, B). NORs were detected in the pericentromeric region of the long arms of pair 7. The same specimens that showed heteromorphism in the size of the secondary constrictions also showed marked heteromorphism in NOR size with both Ag-staining and FISH with an rDNA probe (Fig. 3A, B).

P. bolbodactyla

The karyotype of *P. bolbodactyla* consisted of metacentric (pairs 1, 2, 9–12), submetacentric (pairs 4, 5 and 8) and subtelocentric (pairs 3 and 6) chromosomes. The long arms of chromosome 7 homologous also had a secondary constriction that was greater in one of the homologous in 40% of the specimens and altered the morphology to submetacentric (morph 7b), regardless of the specimeńs sex (Figs. 1C and 6C). C-banding revealed centromeric heterochromatin blocks in all chromosomes of the karyotype. Pericentromeric blocks were detected on the long arms of pairs 3 and 7 and on the short arms of pair 5. Telomeric ones were identified on the long arms of pairs 2 and 3. Heterochromatin blocks extending from the pericentromeric to the subterminal region were observed on the short

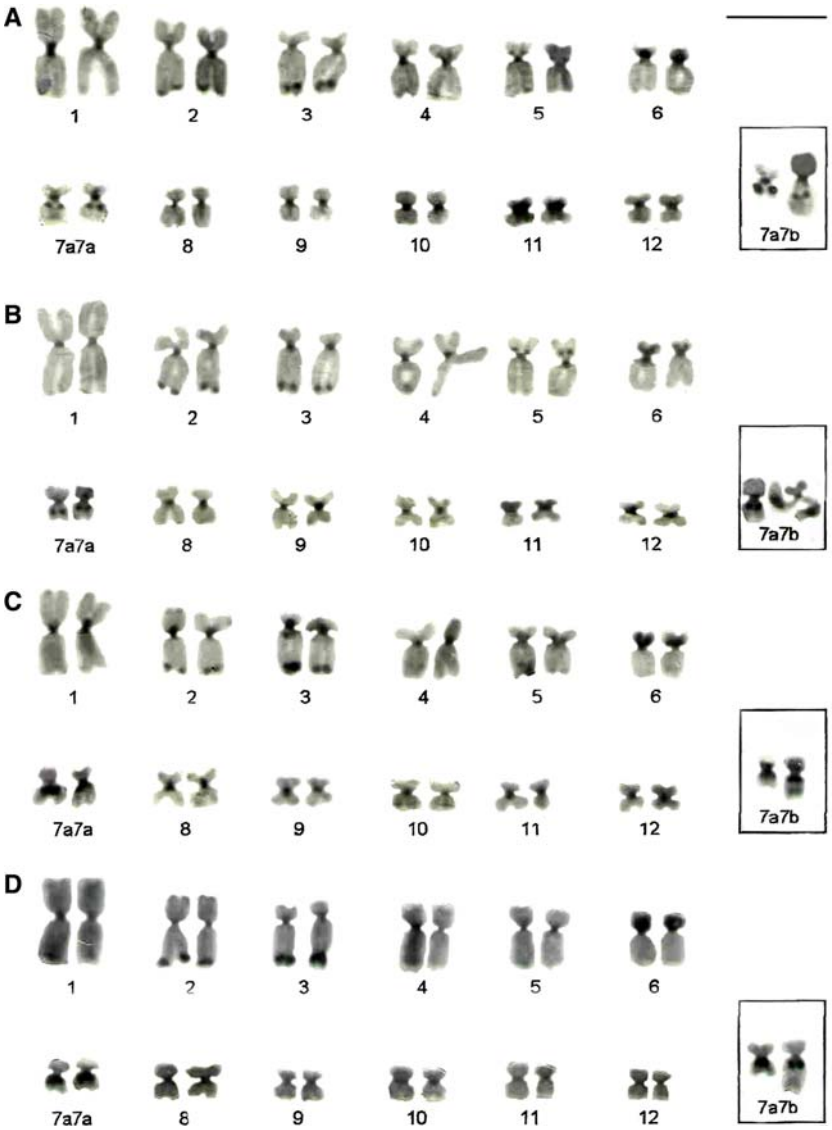

Fig. 2 C-banding patterns. (**A**) *P. p. paradoxa.* (**B**) *P. p. platensis*. (**C**) *P. bolbodactyla*. (**D**) *P. fusca*. Heteromorphic chromosomes 7a and 7b are highlighted. Bar = 5 μm

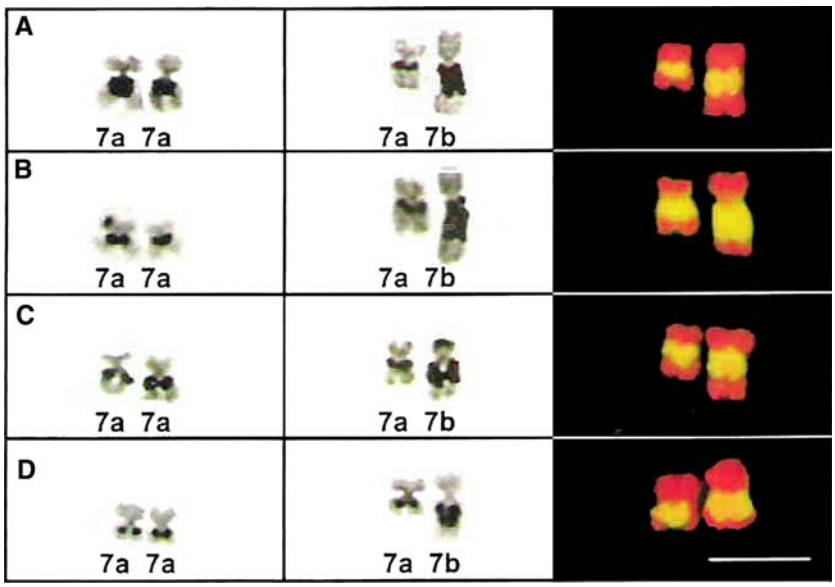

Fig. 3 Chromosomal pair 7 after silver staining and in situ hybridization with the HM123 probe. First column, homomorphic NOR (7a7a); second column, heteromorphic NOR (morphs 7a and 7b); third column, fluorescent in situ hybridization. Lane A, *P. p. paradoxa*; lane B, *P. p. platensis*; lane C, *P. bolbodactyla*; lane D, *P. fusca*. Bar = 5 μm

arms of homologous 6. A NOR was also detected on the long arms of pair 7 in the interstitial region immediately after the heterochromatin block (Figs. 3C and 6C). Heteromorphism in NOR size was observed in 40% of the specimens that also showed heteromorphism in the size of the secondary constrictions (Figs. 3C and 6C). FISH using the HM123 probe confirmed the results obtained by silver staining (Fig. 3C).

P. fusca

The karyotype of *P. fusca* consisted of metacentric (pairs 1, 2, 8–12), submetacentric (pairs 4 and 5) and subtelocentric (pairs 3 and 6) chromosomes. A secondary constriction was detected by conventional Giemsa staining in the interstitial region of the long arms of chromosome 7 homologous. This constriction altered the chromosomal morphology to submetacentric in 30% of the specimens analyzed (two males from Coronel Murta, MG, and one female from Salinas, MG), with this condition being also independent of the specimen's sex (Figs. 1D and 6D). C-banding revealed heterochromatin blocks in the centromeric region of all chromosomes and in the telomeric region of the long arm of pairs 2 and 3. A heterochromatin block was also detected on the short arms of pair 6, where it extended to the subterminal region of the chromosomes, and a pericentromeric

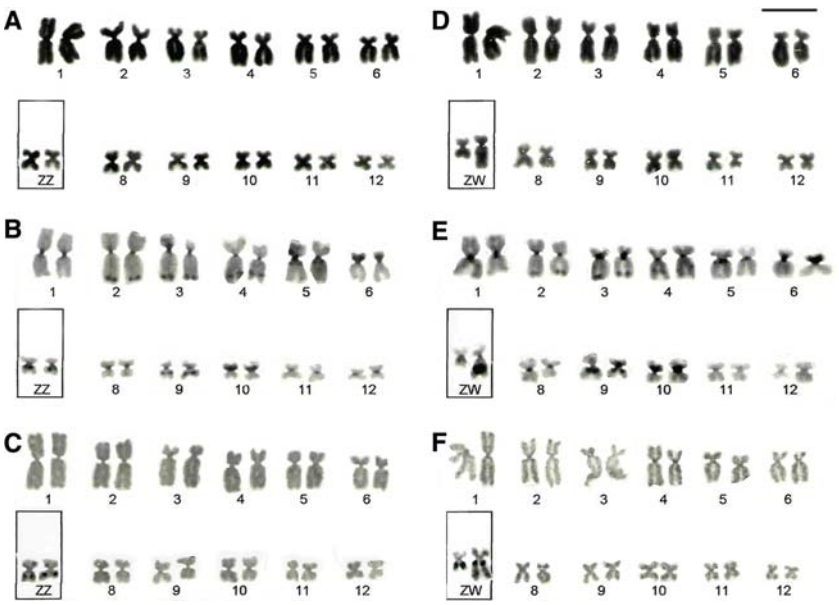

Fig. 4 Karyotypes of male and female *P. tocantins*. (**A**) Giemsa-stained male karyotype. (**B**) C-banding pattern of a male karyotype. (**C**) Silver-stained male karyotype. (**D**) Giemsa-stained female karyotype. (**E**) C-banding pattern of a female karyotype. (**F**) Silver-stained female karyotype. Bar = 5 μm

heterochromatin block was observed on the long arms of pair 7 (Figs. 2D and 6D). NORs were located in the interstitial region of the long arms of chromosome 7 posterior to the heterochromatin block, as seen in *P. bolbodactyla*. The specimens with heteromorphic secondary constrictions on pair 7 also showed marked heteromorphism in NOR size. FISH using the HM123 probe confirmed the results obtained by silver staining (Figs. 3D and 6D).

P. tocantins

The karyotypes of male and female *P. tocantins* consisted of metacentric (pairs 1, 2, 8–12), submetacentric (pairs 4 and 5) and subtelocentric (pair 6) chromosomes. The chromosomes of pair 7 were homomorphic and metacentric (ZZ) in males whereas in females one of the homologous was metacentric (Z) like the male chromosome and the other was subtelocentric (W), with its size being similar to that of the chromosomes of pair 4. No secondary constrictions were detected in any of the metaphase chromosomal pairs of the 11 specimens analyzed (Figs. 4A, D and 6E). The C-banding pattern obtained for male and female specimens indicated the presence of heterochromatin in the centromeric region of all chromosomes, in the telomeric region of the long arms of homologous 2 and 3, in the subterminal region of the long arms of homologous 4 and in the pericentromeric region of the short and long arms of homologous 5; heterochromatin was also seen along the short arms of pairs 6 and 10 and as a small block of in the interstitial region of the long arm of pair 9. The Z chromosomes had a strongly labeled heterochromatin block in the pericentromeric region of the long arms, and a strongly labeled, large interstitial heterochromatin block extending to the subterminal region was also seen on the long arms of the W chromosome (Figs. 4B, E, 5B and 6E). The same pattern was observed when metaphases previously stained for C-banding were stained with distamycin A/DAPI (Fig. 5C). NORs were detected on the long arms of the Z and W sex chromosomes (pair 7). In the Z chromosomes, NORs were located in the interstitial region posterior to the heterochromatin block, a location similar to that observed in *P. bolbodactyla* and *P. fusca*. In the W chromosome, the NOR was located pericentromerically anterior to the large heterochromatin block, as observed in subspecies of *P. paradoxa*. No heteromorphism in NOR size was seen in any of the 11 specimens analyzed (Figs. 4C, F and 6E). FISH using the HM123 probe confirmed the results obtained by AgNOR staining (Fig. 5E).

Discussion

Cytotaxonomic considerations

The diploid chromosome number (2n = 24) of the karyotypes obtained for the species analyzed here agrees with the number reported for *P. p. platensis* (Barrio and Rubel 1970) and *P. minuta* (Busin et al. 2001). The presence of NORs on the long arms of chromosomes 7 is also common to all karyotypes, except for *P. cardosoi*, which differs from the other species by having 2n = 28 chromosomes and an NOR located on the long arms of pair 5, which is probably homologous to chromosome 7 of the other species (see discussion in Busin et al. 2001). Although various authors have proposed a trend towards a reduction in chromosome number during the evolution of amphibian karyotypes (Beçak et al. 1970; Bogart 1973; Miura et al. 1995), the occurrence of karyotypes with 2n = 24 chromosomes in all species of the genus except for *P. cardosoi* supports the hypothesis raised by Busin et al. (2001) that karyotypes with 2n = 28 chromosomes represent an apomorphic condition in *Pseudis.*

The karyotypes of the species analyzed here had a highly conserved chromosomal morphology. Small differences among species were only seen in the morphology of chromosomes 2 and 8; however, based on the values proposed by Green and Sessions (1991), the centromeric indices were within the classification limit, indicating that these differences might not have been seen if a larger sample had been analyzed.

The pattern of heterochromatin distribution in the genomes of the *P. paradoxa* subspecies, *P. bolbodactyla*, *P. fusca* and *P. tocantins* revealed homologous heterochromatin bands in the telomeric region of the long arms of pairs 2 and 3 and on the short arms of the almost completely heterochromatic chromosomes 6. The long arms of pair 7 also contained a heterochromatin block, the position of which varied among *P. bolbodactyla*, *P. fusca* and *P. tocantins* and the *P. paradoxa* subspecies. *Pseudis tocantins* showed the greatest distribution of heterochromatin in the genome, with additional bands on chromosomes 4, 5, 9 and 10, in addition to a large heterochromatin block on the W chromosome of females. According to King (1991), most neobatrachian species analyzed by C-banding show interspecific diversity in the amount and distribution of heterochromatin in their genomes, suggesting that amplification of these regions is an evolutionary trend in Amphibia.

Comparison of the C-banding patterns obtained for *P. paradoxa (P. p. paradoxa* and *P. p. platensis)*, *P. bolbodactyla*, *P. fusca* and *P. tocantins* with those for *P. minuta* and *P. cardosoi* reported by Busin et al. (2001)

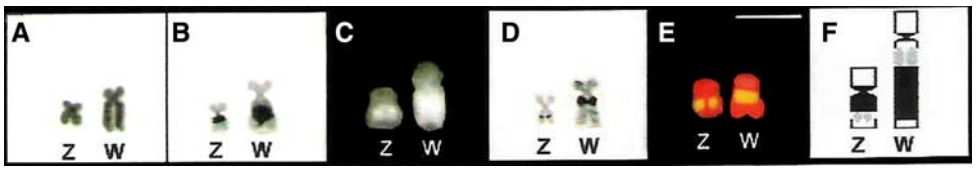

Fig. 5 Z and W sex chromosomes of *P. tocantins* after standard and differential staining. (**A**) Giemsa staining. (**B**) C-banding. Bar = 5 μm. (**C**) Distamycin A/DAPI staining. White areas indicate heterochromatin and black areas indicate euchromatic regions. (**D**) Silver staining (note that the chromosomes shown in **A** and **C** were first submitted to C-banding, followed by silver staining). (**E**) Fluorescent in situ hybridization. Bar = 5 μm. (**F**) Schematic representation of the Z and W sex chromosomes. Black areas indicate the heterochromatin region and gray areas indicate the NOR

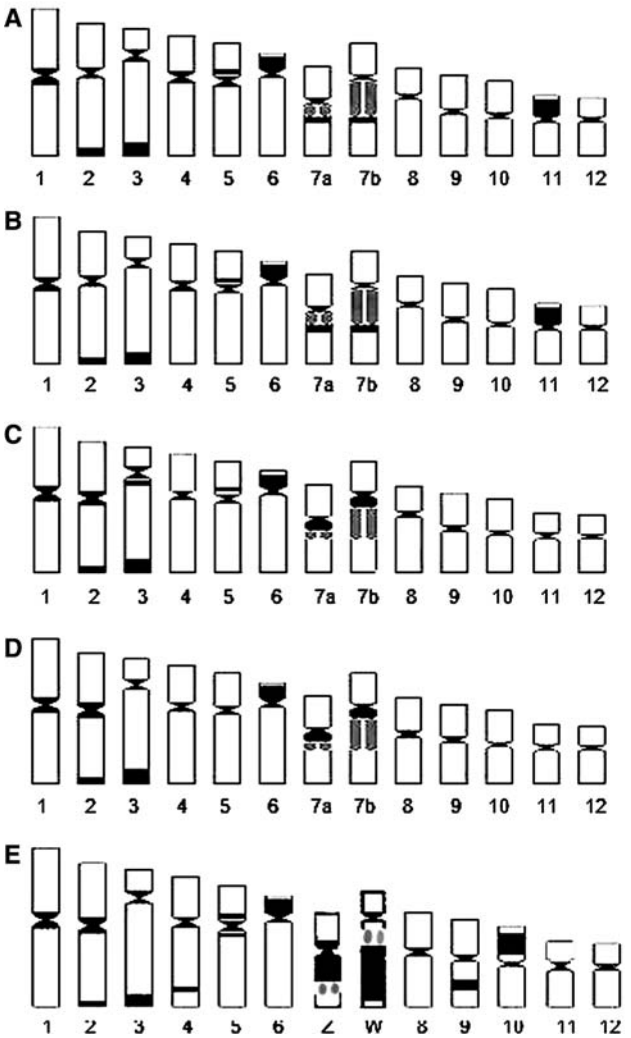

Fig. 6 Ideograms. (**A**) *P. p. paradoxa*. (**B**) *P. p. platensis*. (**C**) *P. bolbodactyla*. (**D**) *P. fusca*. (**E**) *P. tocantins*. Heterochromatin regions are indicated in black and NORs are in gray

revealed two groups of species. The two large and clearly visible interstitial heterochromatin blocks in pair 2 of *P. minuta* and in pair 1 of *P. cardosoi*, which are homologous and characteristic of the two species, were not detected in any of the species analyzed here. This finding agrees with the hypothesis by Busin et al. (2001) that the *P. cardosoi* karyotype with 2n = 28 arose by chromosomal rearrangements of the centric fission type from a 2n = 24 karyotype, similar to that of *P. minuta.*

Although the location of the NOR on the long arms of chromosomes 7 is a common condition in *Pseudis* species with 2n = 24 chromosomes, the position it occupies varies among species, indicating that inversion-type rearrangements occurred during the evolution of this chromosomal pair. NORs were observed in the pericentromeric region anterior to the heterochromatin block in the *P. paradoxa* subspecies and in the W chromosome of *P. tocantins*, whereas in *P. bolbodactyla*, *P. fusca* and in the Z chromosomes of *P. tocantins* the NORs were located in the interstitial region posterior to the heterochromatin block. According to Busin et al. (2001), *P. minuta* and *P. cardosoi* have NORs in the subtelomeric region and a heterochromatin block in the interstitial region of the arm, although in *P. cardosoi* the heterochromatin block was located on chromosome 5 (homologous to chromosome 7 of the other species).

Interestingly, species with NORs at the same position also share similar external morphological characteristics that bring them closer together. In their analysis of *Pseudis* species, Caramaschi and Cruz (1998) recognized two distinct groups among the species analyzed: the first involved *P. fusca*, *P. bolbodactyla* and *P. tocantins* which had no external carpal callus or only a vestigial one, and the second, consisting of *P. p. paradoxa*, *P. p. platensis*, *P. p. caribensis*, *P. p. occidentalis* and *P. p. nicefori*, was characterized by a well-developed round carpal callus. On the other hand, *Pseudis minuta* and *P. cardosoi* can be distinguished from the other two groups by a double and lateral vocal sac, whereas the other species have a simple, subgular vocal sac (Kwet 2000). Hence, the proposal that this genus can be classified into three groups based on their morphological characteristics is supported by the variable position that the NOR occupies on the long arms of chromosomal pair 7. In the special case of *P. tocantins* in which the NORs are located in different regions of the Z and W chromosomes, its inclusion in the group with the NOR located in the interstitial region posterior to the heterochromatin block, as observed for the Z chromosome, is

justified since it has been conventionally accepted that the chromosome present in only one of the sexes (W) is derived from homomorphic ancestral chromosomes (Ohno 1967; Singh et al. 1976; Jones and Singh 1981; Bull 1983; Jones 1984). However, additional studies on the location of NORs in *P. paradoxa* subspecies other than those analyzed here are necessary to confirm the proposed grouping of representatives of the genus *Pseudis* based on the position of the NOR.

The heteromorphism in NOR size detected by silver staining among homologous of the *P. paradoxa* subspecies and in *P. bolbodactyla* and *P. fusca* also occurs in other anurans (Lourenço et al. 1998; Busin et al. 2001; Veiga-Menoncello et al. 2003) and probably reflects duplications and in tandem triplication of the NOR. According to Schmid (1982), NOR size may vary among individuals of the same species because of differences in the number of ribosomal gene copies present in each NOR.

In conclusion, although the *P. paradoxa* subspecies, *P. bolbodactyla*, *P. fusca* and *P. tocantins* had a karyotype with the same chromosomal number, the differences in the morphology of homologous 2 and 8, in the amount and location of some non-centromeric heterochromatin bands, and in the position of the NORs on the long arms of pair 7 differentiate these species. However, no cytogenetic differences were detected between the two *P. paradoxa* subspecies to justify their subspecies status.

Sex chromosomes

The karyotype of the seven *P. tocantins* females was characterized by heteromorphism in the size and morphology of the chromosomes of pair 7 that almost doubled the size of one of the homologous, a situation not observed in any of the male specimens. The heteromorphism in homologous of chromosome 7 in the female karyotype was more evident when the metaphases were submitted to C-banding and distamycin A/DAPI staining. These procedures revealed a large heterochromatin block on the long arms of one of the homologous that confirmed the presence of heteromorphic sex chromosomes in the $ZZ^{\male}/ZW^{\female}$ system of this species.

The heterochromatin pattern and NOR position also varied markedly between the Z and W chromosomes of *P. tocantins*. The metacentric Z chromosomes had a pericentromeric heterochromatin block and an interstitial NOR posterior to this block. In contrast, the reverse situation was observed for submetacentric W chromosomes, clearly indicating the occurrence of pericentric inversions in the differentiation of these chromosomes.

According to Ohno (1967), the evolution of sex chromosomes involves a series of events that allow the accumulation of differences between the homologous of a chromosomal pair in genes responsible for sex determination. The varying degrees of heteromorphism among amphibian sex chromosomes suggest that morphological differentiation first results from reduced crossing-over in one pair of the ancestral homomorphic chromosomes (Ohno 1967), followed by a gain of heterochromatin in the sex chromosome present in only one of the sexes (Y or W) (Singh et al. 1976; Jones and Singh 1981; Bull 1983) and, finally, morphological differentiation of the heterochromatic chromosome. However, Schmid et al. (2002b) demonstrated that the Y chromosomes of *Gastrotheca walkeri* and *G. ovifera* (Hylidae) contained a smaller amount of heterochromatin than expected, suggesting that the heteromorphism in this case may have resulted from a loss of heterochromatin in the Z and X chromosomes.

However, other authors have proposed that pericentric inversion may also be involved in the initial stages of sex chromosome differentiation. Iturra and Veloso (1989) described the presence of heteromorphic chromosomes in male (XY) specimens of *Eupsophus miguelli* and *E. roseus* (Leptodactylidae) in which all chromosomes of the complement had heterochromatin in the pericentromeric region, except for the Y chromosome. This finding suggested that X and Y differentiation had resulted from pericentric inversion and the loss of pericentromeric heterochromatin. A similar situation has also been demonstrated in the genus *Pyxicephalus* by Schmid (1980), who proposed that the difference between the W and Z chromosomes is due to inversion of the heterochromatin block in the W chromosome.

Pericentric inversion is clearly demonstrable in the sex chromosome of *P. tocantins* and may have been an important event for the differentiation of these chromosomes. Although pericentric inversions have been suggested as a possible mechanism for initiating the differentiation of sex chromosomes in vertebrates (John 1988), such structural rearrangements have not been implicated as an initial process in amphibians and thus may have occurred after the gain of heterochromatin. However, the possibility that inversion was the initial step in the differentiation of *P. tocantins* sex chromosomes cannot be ruled out since in the karyotypes of other species without differentiated sex chromosomes the NOR is located at different positions on the long arms of chromosomes 7, indicating the occurrence of inversions during the evolution of this chromosomal pair. Thus, sex chromosome heteromorphism may have started with an inversion followed by a gain in heterochromatin and subsequent morphological differentiation of the W chromosome.

Acknowledgements The authors thank Ulisses Caramaschi and Carlos Alberto Gonçalves da Cruz for identification of the species

used in this study, Odair de Aguiar Junior for valuable discussions, Sérgio Siqueira Júnior for collecting *P. tocantins* and *P. fusca*, Adrian Antônio Garda for collecting some *P. fusca*, and Klélia Aparecida de Carvalho for technical assistance.

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