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Chromosomal analysis of three Brazilian "eleutherodactyline" frogs (Anura: Terrarana), with suggestion of a new species

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Abstract

The karyotypes of four Brazilian "eleutherodactyline" samples were analyzed aiming to provide additional cytogenetic data for future understanding of the evolutionary and systematic relationships of this large anuran group. The populations consisted of *Pristimantis dundeei* (Chapada dos Guimarães and Rondonópolis, Mato Grosso), *Pristimantis* aff. *dundeei* (Aripuanã, Mato Grosso) and *Ischnocnema paulodutrai* (Ilhéus, Bahia). The data revealed that *P. dundeei* and *P.* aff. *dundeei* have 2n=28 chromosomes, whereas *I. paulodutrai* has 2n=30. All pairs of chromosomes were telocentric, except for the subtelocentric pair 4 in *I. paulodutrai*. Differences in Ag-NOR pattern and interstitial heterochromatin positions clearly distinguished *P.* aff. *dundeei* from *P. dundeei*, and differentiated them from *I. paulodutrai*. The specimens of *I. paulodutrai* showed two distinct color patterns, but they did not differ in their cytogenetic characteristics. Karyotypes with 2n=28 and 2n=30 chromosomes have not been previously described for Brazilian "eleutherodactylines" which, to date, had been characterized as 2n=20, 2n=22 and 2n=34. The NOR position differences identified between *P. dundeei* and *P.* aff. *dundeei*, allied to their known distinct behavior and ecological data, suggested that the *P.* aff. *dundeei* from the Aripuanã sampling location is a new species. Similarities between *I. paulodutrai* and species currently assigned to *Pristimantis* are herein discussed on the basis of chromosome number and morphological characteristics.

Key words: *Pristimantis*, *Ischnocnema*, cytogenetics, karyotype, NOR, C-banding

Introduction

Eleutherodactylus was, for a long time, the largest vertebrate genus and had been considered to encompass a high degree of speciation (Bogart & Hedges 1995). However, following recent comprehensive studies (Frost *et al.* 2006; Heinicke *et al.* 2007; Hedges *et al.* 2008), this taxon has undergone a radical restructuring, including partitions into new taxa. Approximately 700 species, formerly included in the subfamily Eleutherodactylinae (Leptodacylidae), were recently split into the new genera *Euhyas* (94 species), *Craugastor* (116 species), *Pelorius* (6 species), *Syrrhopus* (24 species), and *Eleutherodactylus* (492 species), according to Frost *et al.* (2006). They were assigned to the family Brachycephalidae, which contain more than 800 species grouped in 19 genera (Frost *et al.* 2006). In a further reorganization, Heinicke *et al.* (2007) recognized two additional genera, *Pristimantis* (393 species), and *Limnophys* (15 species) and considered *Euhyas*, *Pelorius* and *Syrrho-*

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pus as subgenera of the genus *Eleutherodactylus*. In addition, the former *Eleutherodactylus* species natives to southeastern Brazil were allocated in the genus *Ischnocnema*.

Hedges *et al.* (2008) placed 882 described "eleutherodactyline" species in a new taxon, Terrarana, based on the analysis of 344 species through molecular characters. Nearly 85 species are reported in Brazil, assigned to the families: Strabomantidae (*Strabomantis*, *Euparkerella*, *Holoaden*, *Noblella*, *Oreobates* and *Pristimantis*), Craugastoridae (*Haddadus*), Eleutherodactylidae (*Adelophryne*, *Phyzelaphryne*) and Brachycephalidae (*Brachycephalus* and *Ischnocnema*).

A high degree of diploid number (2n) variation (2n=18 to 2n=36) and complex sexual chromosome systems have been identified among the more than 100 species of *Eleutherodactylus* (*sensu lato*) thus far analyzed (see Kuramoto 1991, Bogart & Hedges, 1995, Kaiser *et al.* 1995, Schmid *et al.* 2002, Schmid *et al.* 2003, Siqueira *et al.* 2004; Campos *et al.* 2006). Structural rearrangements have been postulated as determinant in their conspicuous chromosome variation (Bogart 1991; Schmid *et al.* 2003).

In Brazil, only seven "eleutherodactyline" species have been cytogenetically analyzed: *E. binotatus* (currently *Haddadus binotatus*) (Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008), *E. guentheri* (currently *Ischnocnema guentheri*) (Beçak 1968; Brum-Zorrilla & Saez 1968; Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008), *E. holti* (currently *I. holti*) (De Lucca *et al.* 1974), *E. juipoca* (currently *I. juipoca*) (Campos *et al.* 2008), *E. lacteus* (currently *I. lactea*) (De Lucca & Jim 1974), *E. parvus* (currently *I. parva*) (Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008) and *E. fenestratus* (currently *Pristimantis fenestratus*) (Siqueira *et al.* unpublished data). The karyotyped species can be discriminated in two groups of distinct diploid chromosome number and morphology. One group is characterized by 2n=20 and 2n=22 chromosomes, most or all of them metacentric and submetacentric, represented by species from the southern and southeastern Brazil (*I. holti*, *I. lactea*, *I. guentheri*, *I. juipoca*, *I. parva* and *Haddadus binotatus*). The second group is characterized by 2n=34 chromosomes, all of them telocentric, represented by *E. fenestratus* from the northern and northeastern Brazil.

In the present work, the karyotypes of *Pristimantis dundeei*, *Ischnocnema paulodutrai*, and a third taxon here nominated *Pristimantis* aff. *dundeei* (based on ecological and behavioral differences) were analyzed aiming at increasing the number of karyotyped Brazilian "eleutherodactyline" species and improving the understanding of their evolutionary relatedness and taxonomic status.

Material and Methods

The Brazilian "eleutherodactyline" frogs analyzed in this work were sampled in four locations, at the central western and northeastern regions, under a permit issued by the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (License nº 206/2005 - CGFAU/LIC). *Pristimantis dundeei* was sampled in Mato Grosso State, in the type-locality Chapada dos Guimarães (15° 40' 00" S, 55° 84' 00" W, ten females and two males) and in Rondonópolis (16° 40' 00" S, 54° 44' 00" W, five females and one male). *Pristimantis* aff. *dundeei* were sampled in Aripuanã, Mato Grosso State (10º 15' 00" S, 59º 23' 00" W, two females and three males). *Ischnocnema paulodutrai* was sampled in Bahia State, at the campus of the Universidade Estadual de Santa Cruz (UESC), Ilhéus (14º 47' 00" S 39º 10' 00" W, five males and five females). Two distinct dorsal morphological patterns were identified among the *I. paulodutrai* specimens, characterized by typical dorsal specked staining or brown dorsal stripes. Although it has been observed that *Pristimantis* aff. *dundeei* also exhibit distinct morphological patterns, with or without a median dorsal stripe (C. Strüssmann, personal information), the specimens analyzed in the present work had no stripes.

All voucher specimens were deposited in the Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC), at the Universidade Estadual de Campinas (UNICAMP), and in the Coleção "Célio F. B. Haddad" at the Universidade Estadual Paulista (UNESP) Rio Claro, São Paulo, Brazil, under the accession numbers: ZUEC 14.061

to 14.073 (*P. dundeei*, from Chapada dos Guimarães, MT), ZUEC 14.044 to 14.049 (*P. dundeei*, from Rondonópolis, MT), ZUEC 14.052 to 14.054 (*P.* aff. *dundeei*, from Aripuanã, MT) and CFBH 11964 to 11973 (*I. paulodutrai*, from Ilhéus, BA). The specimens CFBH 11963 (female), 11972 and 11973 (males) had median dorsal stripe**.**

Mitotic chromosomes were obtained from a suspension of intestinal epithelium and testicular cells of animals pre-treated with 2% colchicine for at least 4h, as described by King & Rofe (1976) and Schmid (1978). Conventional staining with 10% Giemsa solution, Ag-NOR labelling (Howell & Black 1980) and C-banding (Sumner 1972) were used in the chromosome analyses. The C-banding technique was modified by applying (1) a pre-treatment in 50% acetic acid, for 30 minutes, previously to the 0.2N HCl hydrolysis for 30 to 60 minutes, and (2) by incubation at 60ºC for both treatments with 5% $Ba(OH)_2$, for 15 to 20 seconds, and the saline solution (2xSSC), for 15 to 20 minutes.

The slides were examined under a BX60 Olympus microscope, and the images were captured using the software Image Pro-Plus 4.5.1 version. Mitotic chromosomes were measured and classified according to Green & Sessions (1991).

Results

A diploid number (2n) of 28 telocentric chromosomes and a fundamental number (FN) of 28 characterized the *P. dundeei* and *P.* aff. *dundeei* localities. In the *P. dundeei* specimens, the Ag-NOR sites were adjacent to the centromere in the pair 1, coincident with a secondary constriction (Fig. 1a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes. C-bands were faint and adjacent to the centromere of the pair 1, and interstitially positioned in the pairs 2, 4 and 11. In pair 1, the heterochromatin was adjacent to the NOR (Fig. 1a, b).

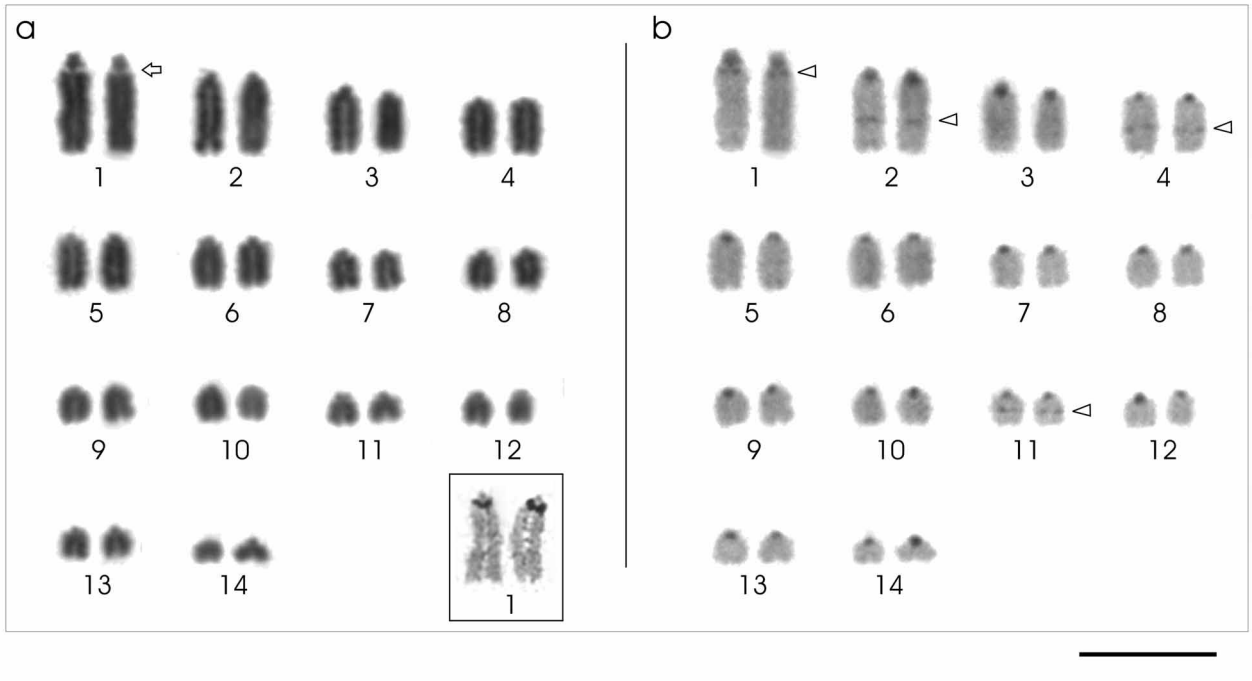

FIGURE 1. Karyotype of *Pristimantis dundeei*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions, which are coincident with the NOR position (inset). The arrowheads indicate interstitial heterochromatin. Bar = 10 μm.

In *P.* aff. *dundeei*, the Ag-NOR sites were adjacent to the centromere of the pair 10, coincident with a sec-

ondary constriction (Fig. 2a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes, and faint C-bands were observed interstitially in pair 4 as well as adjacent to the centromere of pair 10, coincident to the NOR (Fig. 2a, b).

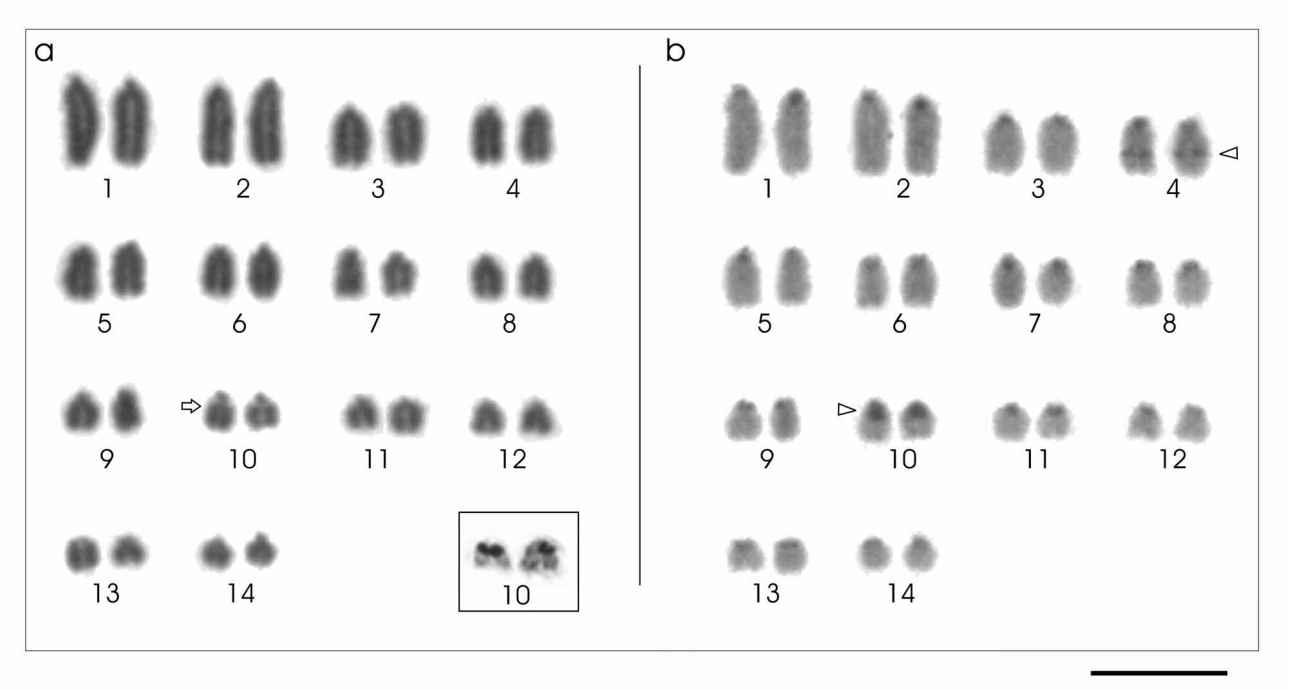

FIGURE 2. Karyotype of *Pristimantis* aff. *dundeei*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions, which are coincident with the NOR position (inset). The arrowheads indicate the interstitial heterochromatin. Bar = 10 µm.

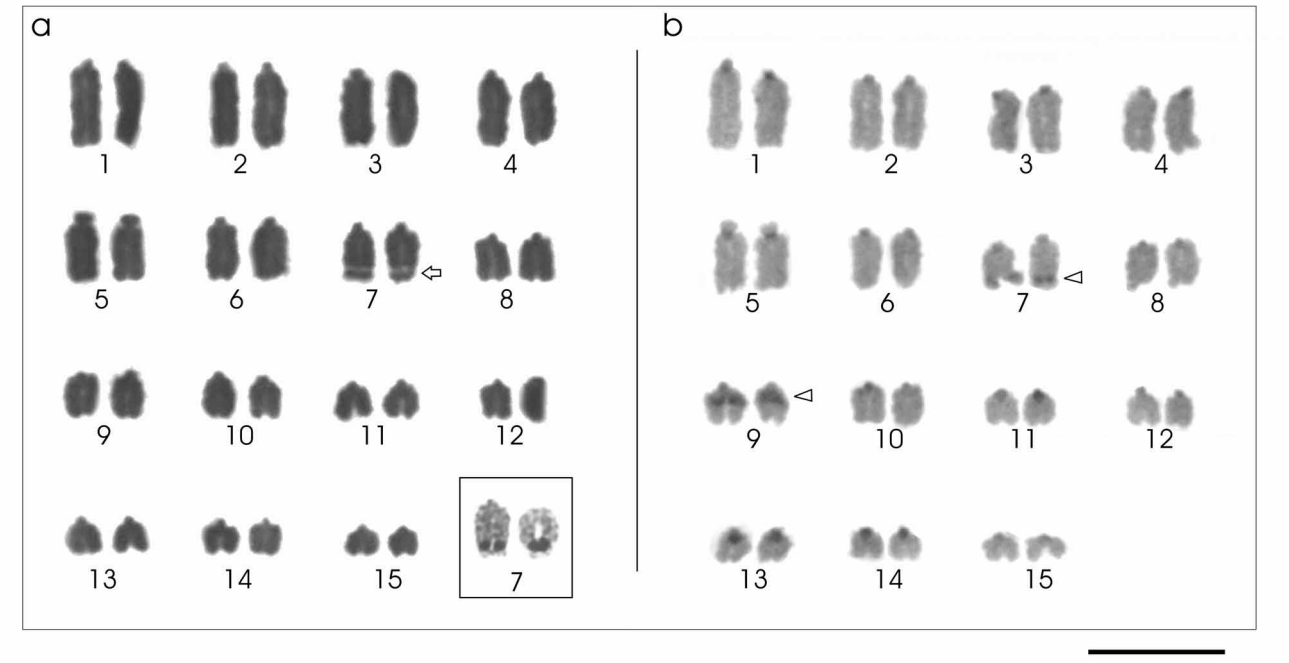

FIGURE 3. Karyotype of *Ischnocnema paulodutrai*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions. The arrowheads indicate interstitial heterochromatin. Inset: the Ag-NOR bearing chromosomes. Bar = 10 µm.

The *I. paulodutrai* karyotype consisted of 2n=30 chromosomes and FN 32, with all pairs classified as telocentrics, except for the subtelocentric pair 5 (Fig. 3a, b). The Ag-NOR sites were located at the subtelomeric regions of pair 7, coincident with a secondary constriction (Fig. 3a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes. Large blocks of heterochromatin were found interstitially in pair 9, and at the subtelomeric region of pair 7, coincident with the secondary constriction and NOR (Fig. 3a, b). Heterochromatic blocks, similar to the latter ones were also identified in meiotic cells of male specimens, in which 15 bivalents were found (Fig. 4a, b). In the analyzed samples, there were no detectable karyotypical differences between the two distinct morphological types of *I. paulodutrai* specimens. Metaphases with chromosomes associated by thin chromatin bridges, mainly between their telomeric and centromeric regions (Fig. 5a-d), were observed in the specimens of all taxa.

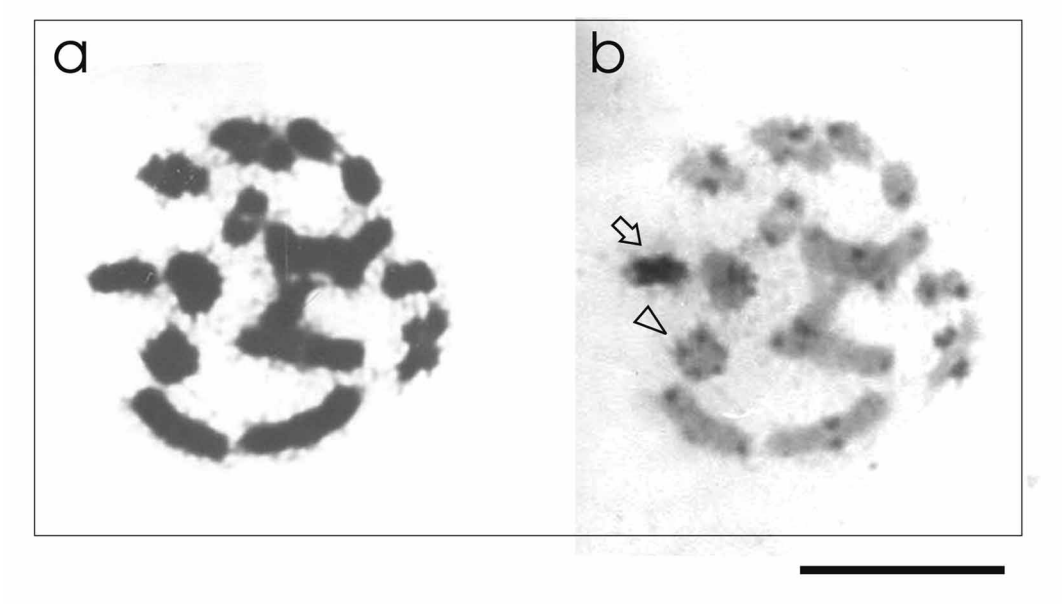

FIGURE 4. Diplotene of *I. paulodutrai*: (**a**) Giemsa- staining; (**b**) C-banding showing bivalents and centromeric heterochromatin in all pairs. The arrow indicates interstitial heterochromatin in the pair 9. The arrowhead indicates the NOR-bearing chromosome pair. Bar = 10 µm.

Discussion

The diploid number of 28 chromosomes, as described for *P. dundeei* and *P.* aff. *dundeei*, has been previously reported in other "eleutherodactylines", comprising ten species from Central America and one from the United States. Of those eleven species, *Eleutherodactylus* (*Euhyas*) *bakeri*, *E.* (*Eleutherodactylus*) *amplinympha*, *E.* (*Eleutherodactylus*) *martinicensis* (Kaiser *et al.* 1994), and *E.* (*Eleutherodactylus*) *johnstonei* (Kaiser *et al.* 1994; Bogart & Hedges 1995) remain in the *Eleutherodactylus* genus (Heinicke *et al.* 2007; Frost, 2007; Hedges *et al.* 2008). The chromosome number 2n=30, found in *I. paulodutrai*, has been described in twelve Central America species of "eleutherodactyline", including one *Pristimantis* species, *P. urichi* (Schmid *et al.* 2002). In Brazilian species, however, this is the first description of 2n=28 and 2n=30 chromosomes.

Despite the coincident chromosome numbers (2n=28 and 2n=30), as well as high number of telocentric ones, the Brazilian and Central America species are clearly differentiated by their chromosome fundamental number (FN). The Brazilian species were characterized by FN=28 in P. dundeei (2n=28) and P. aff. dundeei, and FN=32 in *I. paulodutrai* (2n=30), whereas the Central America *Eleutherodactylus* (either sensu lato or stricto) have been reported as FN=32 in 2n=28 species, and FN=36, 40 or 58 in 2n=30 species (León 1970, Bogart 1970a, 1970b, 1970c, 1981, 1991, DeWeese 1975, Savage & DeWeese 1979, 1980, Miyamoto 1983,

1984, Bogart & Hedges 1995, Kaiser *et al.* 1994, 1995, Schmid *et al.* 2002). Therefore, the data suggest that distinct mechanisms of chromosomal differentiation have originated the 2n=28 and 2n=30 karyotypes of the analyzed Brazilian species. High diploid chromosome numbers and similar chromosome morphology suggested that the *Eleutherodactylus* species in Central America are more closely related to the 2n=34 species native to northern Brazil, than to the species with 2n=20 and 2n=22, of the southern and southeastern Brazilian regions. The karyotypes of *Ischnocnema* species are in agreement with this hypothesis. In addition, the hypothesis is strongly reinforced by the recent assignment of former *Eleutherodactylus* species into the genus *Ischnocnema*, on basis of DNA sequence analyses (Heinicke *et al.* 2007), which included *I. guentheri*, *I. parva* and *I. juipoca* (all characterized by small chromosome number). Therefore, the karyotypes clearly distinguished these three southern *Ischnocnema* species from the *Pristimantis* species found in the Brazilian northern region.

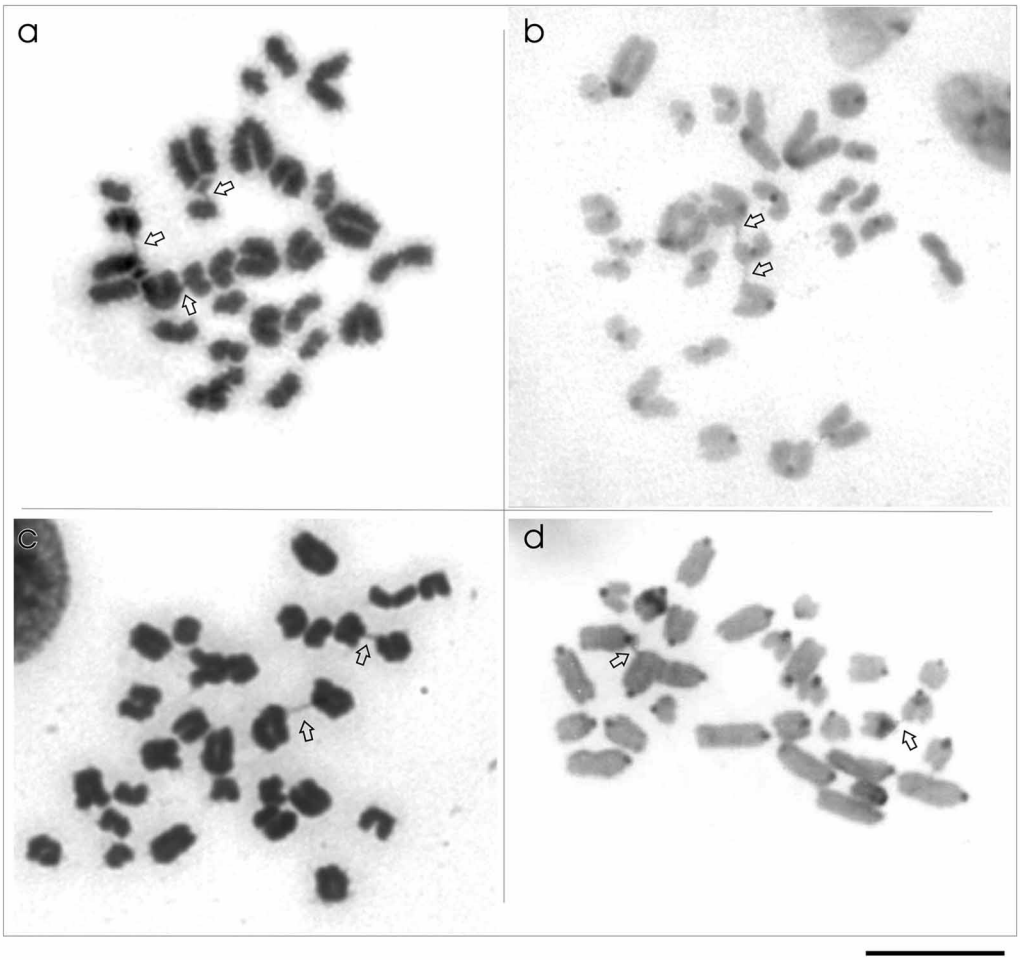

FIGURE 5. Giemsa-stained (**a** and **c**) and C-banded (**b** and **d**) metaphases showing chromosomes associated by thin filaments (arrows) in *P. dundeei* and *I. paulodutrai*, respectively. Bar = 10 µm.

However, in the midwestern region, at Mato Grosso State, *Pristimantis* species already analyzed were characterized by high and small chromosome numbers. The *P. dundeei* species has 2n=28, as described herein, whereas *P. crepitans* has 2n=22 (Siqueira *et al.*, unpublished data).

Ischnocnema paulodutrai is the first northeastern Brazilian species of this genus to be karyotyped. Although it was included in the southeastern group by Heinicke *et al.* (2007) and Hedges *et al.* (2008), its species series, *Ischnocnema ramagii*, occurs in the isolated remnants of Atlantic Coastal Forest in the states of Paraíba, Pernambuco and Bahia in northeastern Brazil.

The karyotype of *I. paulodutrai*, as described in the present work, suggests that it might be more closely related to the *P. dundeei* and *P.* aff. *dundeei* species, than to the remaining congeneric *Ischnocnema* species in Brazil, with low (2n=20 and 22) chromosome numbers. Such variability in "eleutherodactyline" is an elucidative example that high variation in chromosome number indicates polyphyletic groups that deserve a further taxonomic investigation.

The *P.* aff. *dundeei* karyotype showed diverse NOR-bearing and C-band pattern as compared to the *P. dundeei* populations, and constitute strong evidence that most likely they are two distinct species. The chromosome morphology, NOR locations and heterochromatin pattern has been useful to distinguish among different species, as already described for other anurans - e.g. *Physalaemus petersi* (Lourenço *et al.* 1999), *Pseudis minuta* and *Pseudis* aff. *minuta*, currently *Pseudis cardosoi* (Busin *et al.* 2000), *Dendropsophus nanus* and *D. sanborni* (Medeiros *et al.* 2003), *Scythrophrys* species (Lourenço *et al*. 2003a, 2008), *Paratelmatobius* (Lourenço *et al.* 2003b, 2008) and *Pristimantis fenestratus* and *Pristimantis* aff. *fenestratus* and *Barycholos ternetzi* and *Barycholos* aff. *ternetzi* (Siqueira *et al.* in preparation).

Such existence of a new species is also supported by the fact that *P.* aff. *dundeei* is out of the area of *P. dundeei* distribution, proposed by Heyer & Muñoz (1999), as well as by their differences in ecological, behavioral and acoustical characteristics (Christine Strüssmann, personal communication). On the other hand, both high similarity in chromosome morphology and undifferentiated interstitial C-band distribution in the pair 4 indicated that *P.* aff. *dundeei* and *P. dundeei* are closely related taxa.

In the analyzed *I. paulodutrai* specimens, the identical karyotypes between those distinguished by dorsal polymorphism reinforce that cytogenetically these two morphotypes belong to the same species. Vila Flor *et al.* (2004) described similar dorsal polymorphism in *Eleutherodactylus* (currently *Ischnocnema*) populations from Salvador, Bahia State, and from Maceió, Alagoas State, both in the northeastern Brazil. However, such polymorphism was not found in an *Ischnocnema* population sampled by those authors in Ilhéus, Bahia State. Based on those data, the authors concluded that the species of Ilhéus, Bahia state, which had only one color pattern, might be *I. paulodutrai* and the one from Salvador and Maceió presenting both patterns, might be *I. ramagii*. However, our specimens sample from Ilhéus (Bahia), the type-locality of the species, exhibited also two color patterns and all of them were here identified as *I. paulodutrai*. Considering these data, we suggest that the color polymorphism, which was previously described only in *I. ramagii*, is also present in *I. paulodutrai* being a case of homologous polymorphism among these *Ischnocnema* species (see De la Riva 1997).

Metaphases of *E.* (*Euhyas*) *glaucorieus, E.* (*Euhyas*) *bakeri*, and *E.* (*Pelorius*) *nortoni*, as reported by Bogart (1991), exhibited interchromosome thread connections in metaphase plates, although the authors did not mention this phenomenon. The observed associations seem very similar to those here described in metaphases of *I. paulodutrai* and *P. dundeei*. Furthermore, in *P. fenestratus* from Rio Branco, Acre state, Brazil, similar interchromosome threads between chromosomes and intra-individual variation in chromosome number have been found (Siqueira *et al.* unpublished data). Although intraspecific variation in chromosome number was not observed within the species *P. dundeei* and *I. paulodutrai*, such thread associations may also have an important role in the chromosome number alteration during the speciation in the "eleutherodactyline" taxa.

The karyotypic data on *P. dundeei*, *P.* aff. *dundeei* and *I. paulodutrai* populations, described in the present work, represent a forward step in the understanding of Brazilian "eleutherodactyline" species regarding their evolutionary relatedness and additional understanding of their taxonomic status. A further analysis of *P.* aff. *dundeei* as well as *I. paulodutrai* is needed to obtain a more solid taxonomic reassessment of these species.

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