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Cytogenetic analysis on geographically distant parthenogenetic populations of *Tityus trivittatus* Kraepelin, 1898 (Scorpiones, Buthidae): karyotype, constitutive heterochromatin and rDNA localization

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Abstract

Tityus trivittatus Kraepelin, 1898 is the most medically important scorpion species of Argentina, and parthenogenetic populations are present in the major cities of this country. We performed a detailed cytogenetic analysis of specimens of three synanthropic parthenogenetic populations, all distant about 900 km from each other, using Ag-NOR, C-banding, DAPI/CMA_3 staining and FISH with autologous 28S rDNA probes. The karyotype of females and embryos from the three populations showed 2n=6, with two large and four middle-sized holokinetic chromosomes. Constitutive heterochromatin was found in terminal and interstitial location and its pattern allowed the identification of three chromosome pairs. NORs were found on the terminal heterochromatic region of one pair of middle-sized chromosomes. The use of fluorochromes to characterize heterochromatin showed the absence of GC-rich heterochromatin and a low and variable number of AT-rich heterochromatic regions. We propose that a possible explanation for the lack of karyotypic variation between these geographically distant populations could be a recent colonization of urban areas by human means of synanthropic specimens from a single lineage of northeastern Argentina.

Keywords
Scorpion, holokinetic chromosomes, parthenogenesis, karyotype, FISH, NOR

Introduction

Tityus C. L. Koch, 1836 (Buthidae) is the most diversified genus of the order Scorpiones, with about 200 described species. It occurs from Central America to southern South America, in tropical and temperate areas. Several species of this genus are medically important, and most of the dangerous scorpion species in South America belong to the genus *Tityus* (Salomón and de Roodt 2001, de Roodt et al. 2003). This genus presents holokinetic chromosomes, as well as other genera of the family Buthidae, and a great intra- and interspecific variation of chromosome number, ranging from 2n=5 to 2n=27 (Schneider et al. 2009).

Tityus trivittatus Kraepelin, 1898 is the most medically important scorpion species of Argentina and it is responsible for several casualties (Maury 1997, de Roodt et al. 2010). It occurs in southern Brazil, Paraguay, and northern and central Argentina. It reaches Buenos Aires and La Plata cities, being the southernmost species of the genus. *Tityus trivittatus* became a synanthropic species in many areas, being present in most of the major cities of Argentina and Paraguay. *Tityus trivittatus*, as many species of the genus, is facultatively parthenogenetic; sexual populations were reported in Paraguay, southern Brazil and northern Argentina, however, Argentinean populations to the south of latitude 28° S are formed exclusively by parthenogenetic females (Maury 1997, Ojanguren Affilastro 2005).

Parthenogenesis in *Tityus* is quite common and besides *T. trivittatus* it has been mentioned to occur in several species, i.e. *T. columbianus* (Thorell, 1876), *T. confluens* Borelli, 1899, *T. metuendus* Pocock, 1897, *T. serrulatus* Lutz & Mello, 1922, *T. stigmurus* (Thorell, 1876) and *T. uruguayensis* Borelli, 1901 (Matthiesen 1962, Zolessi 1985, Toscano-Gadea 2005, Lourenço 2008, Ross 2010, Seiter 2012). Parthenogenesis was confirmed in *T. trivittatus* based on the observation of virgin females which could produce offspring after lifetime isolation in captivity (Toscano-Gadea 2005). Thelytokous parthenogenesis seems to be the principal mode of asexual reproduction in scorpions, except for the claim of arrhenotokous parthenogenesis in *T. metuendus*, which was severely disputed (Lourenço and Cuellar 1999, Francke 2008). Among these species, cytogenetic studies have been performed in parthenogenetic populations of *T. serrulatus* (2n=12) and *T. stigmurus* (2n=16), and in sexual populations of *T. confluens* (2n=13 in males), *T. metuendus* (2n=15 in males and females and 2n=16 in males) and *T. trivittatus* (2n=14 male), all from Brazil (Piza 1948, 1950, 1952, Schneider and Cella 2010, Mattos et al. 2013). However, we consider that the identity of the specimens of *T. trivittatus* and *T. confluens* from central Brazil analyzed in those studies is doubtful and should be confirmed with a deep taxonomic study of the group, since they have all been collected in areas placed far from the confirmed distribution of these species (Maury 1970, 1974, 1997, Murua et al. 2002, Fernández Campón and Lagos

Silnik 2009). Records of *T. confluens* from central Brazil mentioned in Bertani et al. (2005) could probably belong to other closely related species.

In this contribution, we have cytogenetically studied specimens from three synanthropic parthenogenetic Argentinean populations of *T. trivittatus*, from Buenos Aires, Posadas, and Catamarca cities, all distant about 900 km from each other. The karyotype, constitutive heterochromatin distribution and composition, and ribosomal DNA localization were characterized.

Materials and methods

We have studied females and embryos of *T. trivittatus* collected from urban populations at the cities of Buenos Aires (34°35.66'S, 58°24.68'W) and Posadas (Misiones province) (27°24.99'S, 55°55.96'W), both in Argentina. Seven females and eleven embryos (of four of these females), were collected by the authors in old subterranean tunnels below the children's Hospital "Dr Ricardo Gutiérrez", placed in a highly urbanized area of Buenos Aires city. Nine females and seven embryos (of one of these females) were collected by the authors in a backyard of a house in the periphery of Posadas city, Misiones province. Also, two adult females (one of them with six embryos) were provided by the Department of Zoonoses of Catamarca province, Argentina. The exact locality of the specimens of Catamarca is unknown, but these specimens are most likely to have been collected in the city of San Fernando del Valle de Catamarca (28°28.14'S, 65°46.77'W), the biggest city of the province, where the Department of Zoonoses is placed.

All the specimens were carried alive to laboratory and killed by cooling down to -20°C. Their ovaries and embryos were dissected in saline solution (0.154 M NaCl), incubated in hypotonic solution (1:1 saline solution:distilled water) for 30 min, then fixed for 30 min in a freshly prepared Carnoy fixative (ethanol:chloroform:acetic acid, 6:3:1) and stored in fresh fixative. Pieces of ovaries or embryos were placed on slides and dissociated in a drop of 60% acetic acid with tungsten needles. Preparations with a drop of suspension were placed on a heating histological plate at 40–45°C; suspension was spread on the slides using a tungsten needle.

Conventional staining was made with 5% Giemsa solution in distilled water for 12–15 minutes. The C-banding was performed according to the protocol described by Sumner (1972) and stained with Giemsa or DAPI (4'-6-diamidino-2-phenylindole). The study of the nucleolar organizer regions (NORs) was made by silver-staining technique according to Howell and Black (1980). Fluorescent staining with DAPI and CMA_3 (chromomycin A_3) was carried out according to Rebagliati et al. (2003).

Ribosomal genes were detected by Fluorescence in situ hybridization (FISH) technique with 28S rDNA probe. Total genomic DNA of *T. trivittatus* was extracted using a DNeasy Tissue Kit (QIAGEN, Hilden, Germany). Unlabelled 28S rDNA probes were generated by PCR using primers 28Sa (5′-GACCCGTCTTGAAACACGGA-3′) and 28Sb (5′-TCGGAAGGAACCAGCTACTA-3′) (Whiting et al. 1997). The sequence of the 331bp fragment of the 28S rDNA gene was deposited in the

NCBI database under the accession number KF723293. The probes were labelled by random primed labeling with DIG-11-dUTP using a DIG-High Prime labeling kit. FISH was performed as described by González et al. (2004) with slight modifications, and the probes were detected with Anti-digoxigenin-fluorescein Fab fragments (Roche Applied Science, Mannheim, Germany). The preparations were counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, CA, USA).

To determine the karyotype, chromosome measurements of well-spread prometaphase cells from specimens of each population were made using Micro-Measure software, version 3.3 (Reeves and Tear 2000). The relative length of each chromosome was calculated as a percentage of total complement length (%TCL). This analysis was based on one female from Buenos Aires (10 cells), four embryos of one female from Posadas (10 cells), and one female (7 cells) and two embryos (10 cells) of another female from Catamarca. These data allowed us to prepare an idiogram.

Results

The study of females and embryos of *Tityus trivittatus* from the parthenogenetic populations of Buenos Aires, Posadas, and Catamarca showed the same chromosome number of 2n=6, with two large and four middle-sized holokinetic chromosomes (Fig. 1a). Each large-sized chromosome presented an average value of 20.72% of the TCL, and the average value of the similar sized medium chromosomes was 14.64% of the TCL (Table 1) (Fig. 2c). The very few cells observed at early anaphase showed parallel arrangement of the sister chromatids, which is characteristic of holokinetic chromosomes (Fig. 1b).

The study of specimens from the three localities revealed a complex pattern of C-bands with terminal, subterminal and interstitial localization, which made it possible to identify three chromosome pairs. This pattern was observed both with Giemsa and DAPI staining, although DAPI allowed a better resolution of smaller C-bands. The two large-sized chromosomes (pair 1) presented terminal and subterminal C-bands at each terminal region and one submedial band. The heterochromatic bands at one of the terminal regions are closer and the submedial band is located near of these bands. A pair of middle-sized chromosomes (pair 2) carried a C-band in one terminal region, a medial C-band and a conspicuous terminal and a subterminal C-band at the other terminal region. The other middle-sized chromosomes (pair 3) carried C-bands at each terminal region and a subterminal C-band (Figs 1c, d, 2c). In the specimens from Buenos Aires one of the terminal bands of pair 3 is more conspicuous and the subterminal band is closer to it.

Silver staining visualized active NORs at a terminal region of two middle-sized chromosomes (Fig. 1e). Cells with sequential C-banding and silver staining showed that NORs are located at the double-banded terminal region of pair 2 (Fig. 1f).

DAPI/CMA_3 sequential staining revealed no bright CMA_3 bands. Most cells showed chromosomes homogeneously stained with DAPI. Other cells showed some bright DAPI bands that were coincident with C-bands (Fig. 1g). The number of bright

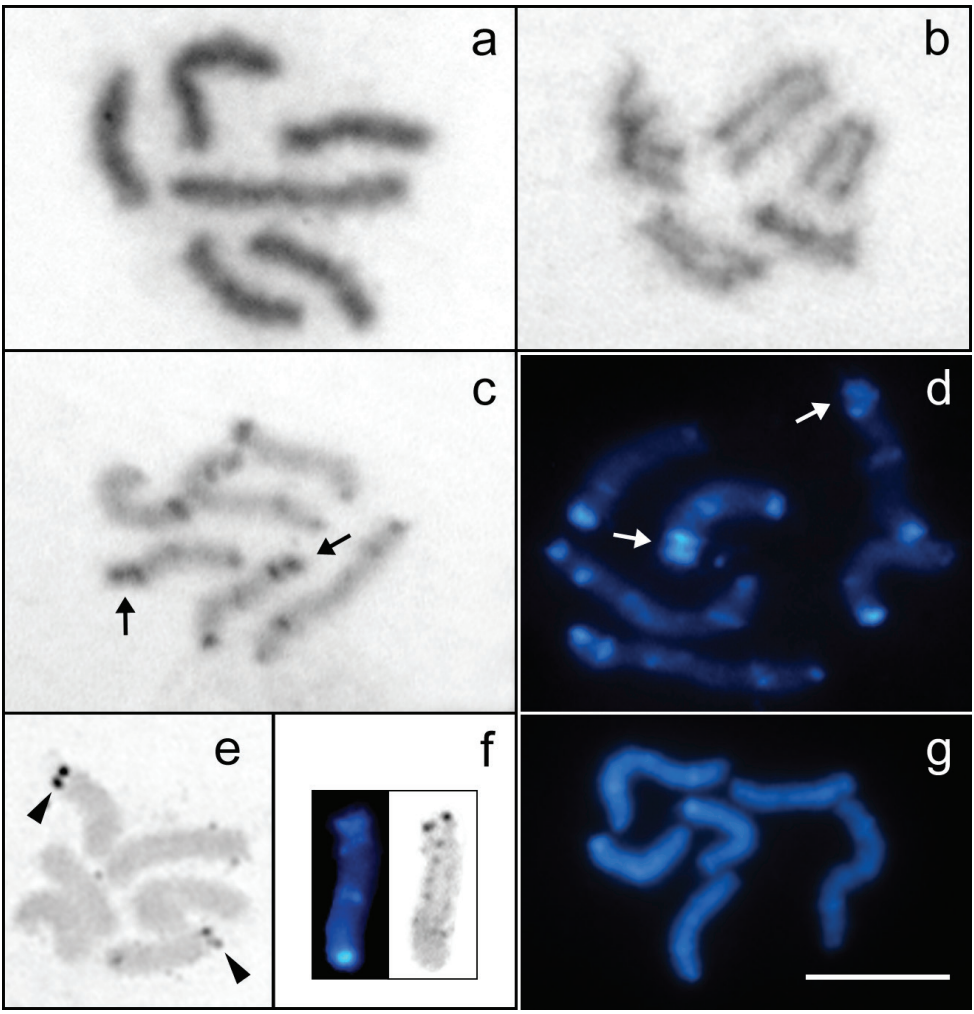

Figure 1. Mitotic cells of *Tityus trivittatus* (2n=6). **a** Giemsa-stained prometaphase **b** Early anaphase **c** C-banded prometaphase stained with Giemsa (Buenos Aires city) **d** C-banded prometaphase stained with DAPI (Buenos Aires city) **e** Silver-stained metaphase **f** Sequential C-banding and silver staining on chromosome 2 **g** DAPI-banded prometaphase after DAPI/CMA_3 staining. The arrows point to the double-banded terminal region of pair 2. Arrowheads point to the NORs. Scale bar= 10 μm.

DAPI bands was less than the number of C-bands, and the smaller C-bands were not detected. This technique did not provide reliable results, since the number of DAPI bands was variable between cells with the same degree of chromosome condensation.

DAPI counterstaining in FISH technique revealed a similar pattern of bright bands as C-banding, which allowed for identification of each chromosome pair. Hybridization signals with the autologous 28S rDNA probes were located at the double-banded terminal region of pair 2 (Fig. 2a, c). Late mitotic prophase chromosomes revealed that the rDNA cluster is embedded in the conspicuous terminal C-band of pair 2 (Fig. 2b).

Table 1. Chromosome measurements of the studied populations of *Tityus trivittatus*. Relative lengths expressed as percentage of total chromosome length (%TCL). Mean values and their standard deviations (SD) are given.

Chromosome number	Buenos Aires	Catamarca	Posadas
	%TCL ± SD	%TCL ± SD	%TCL ± SD
1	21.53 ± 0.99	21.54 ± 0.60	20.89 ± 0.79
2	19.96 ± 0.34	20.45 ± 0.65	19.92 ± 0.93
3	15.37 ± 0.57	15.44 ± 0.51	15.87 ± 0.55
4	14.93 ± 0.34	14.82 ± 0.45	15.07 ± 0.49
5	14.35 ± 0.33	14.28 ± 0.48	14.41 ± 0.63
6	13.86 ± 0.40	13.47 ± 0.55	13.84 ± 0.48

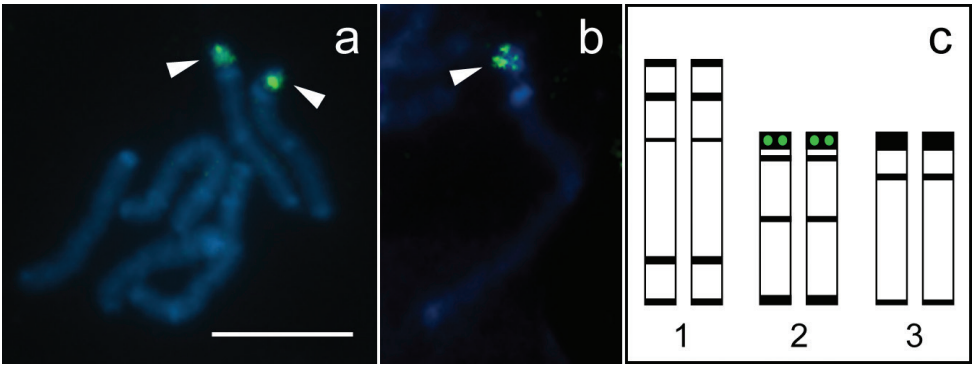

Figure 2. Fluorescence *in situ* hybridization with 28S rDNA probe and idiogram of the karyotype of *Tityus trivittatus*. **a** Mitotic prometaphase with hybridization signals **b** Chromosome 2 at late prophase with hybridization signal; **c.** Idiogram showing distribution of constitutive heterochromatin (black bands) and 28S rDNA clusters (green circles). Chromosomes are counterstained with DAPI (blue). Arrowheads point to hybridization signals (green). Scale bar= 10 μm.

Discussion

The chromosome number found in the specimens of the Argentinean populations of *Tityus trivittatus* herein studied is one of the lowest in Buthidae, and it is also present in *Tityus martinpaechi* Lourenço, 2001 and some individuals of *Tityus bahiensis* (Perty, 1834) (Piza 1939, Schneider et al. 2009, Mattos et al. 2013).

In other species of *Tityus*, Mattos et al. (2013) described two different patterns of heterochromatin distribution: species with small blocks of constitutive heterochromatin in the terminal regions of some chromosomes and species with more conspicuous blocks of constitutive heterochromatin in the terminal regions of all chromosomes and in the interstitial regions of some or all chromosomes. The specimens of *T. trivittatus* herein studied share the latter pattern of constitutive heterochromatin distribution.

The use of DAPI and CMA_3 fluorochromes to characterize heterochromatin of *T. trivittatus* showed the absence of GC-rich heterochromatin and a low and variable number of AT-rich heterochromatic regions, which were coincident with some of the

bands revealed by C-banding. In other Buthidae species the number of heterochromatic regions revealed by DAPI/CMA_3 staining was also lower than that visualized by C-banding and these regions were almost exclusively AT-rich (only *T. martinpaechi* and *Rhopalurus agamemnon* (C. L. Koch, 1839) show GC-rich terminal regions in one chromosome pair) (Schneider and Cella 2010, Mattos et al. 2013). This difference could be related to the protocol of each technique: C-banding method implies a differential extraction of DNA that leads to a greater contrast between euchromatin and heterochromatin, whereas during direct DAPI/CMA_3 staining there is no DNA extraction and the number of heterochromatic regions observed could be less (Sumner 2003, Barros-e-Silva and Guerra 2010). The low number of heterochromatic regions revealed with the latter technique could be also related to the holokinetic nature of the chromosomes, since it has been suggested that this type of chromosomes could be more rigid (Mandrioli and Manicardi 2012). A structural difference of the chromatin condensation of buthid mitotic chromosomes could hinder the specific fluorochrome binding to DNA.

The number and terminal location of NORs, as well as their association with constitutive heterochromatin found in the specimens of *T. trivittatus*, are all common features reported in other species of *Tityus* (Schneider et al. 2009, Schneider and Cella 2010, Mattos et al. 2013). Moreover, the terminal location of NORs is found in many other species of invertebrates and plants with holokinetic chromosomes (e.g.:Hemiptera, Lepidoptera, Odonata, Nematoda, Juncaceae, Cyperaceae and *Cuscuta* Linnaeus) (Albertson 1984, Rebagliati et al. 2003, Guerra and García 2004, Mola and Papeschi 2006, Criniti et al. 2009, Nguyen et al. 2010, Heckmann et al. 2011, Sousa et al. 2011, Poggio et al. 2011, Maryańska-Nadachowska et al. 2013), and this location of NORs could be a functional requirement to ensure chromosome stability in this type of chromosomes (Heckmann et al. 2011).

Tityus trivittatus is an invasive synanthropic species that easily colonizes urban areas due to its great adaptability, ubiquity and parthenogenetic reproduction. This species was probably introduced into Buenos Aires city during the first half of the twentieth century by anthropogenic means (Maury 1970). Parthenogenetic reproduction may allow the establishment of different karyotypes in isolated synanthropic populations. Nevertheless, all specimens of the three populations herein analyzed show the same karyotype in spite of the fact that the populations are about 900 km apart. The lack of variation between the studied populations could be due to a recent colonization of all these urban areas by specimens from a wild sexual (or even parthenogenetic) population with three pairs of homologous chromosomes. Another possible explanation is that all specimens from these cities belong to a single lineage that originally colonized cities from north-eastern Argentina, where its presence has been recorded long time ago (Werner 1902, Mello-Leitão 1934), and once it became synanthropic, specimens from these populations were easily transported from one city to another by human means. The last hypothesis seems more plausible, and is supported by the recent and fast colonization of all the cities of western Argentina (Murua et al. 2002, Fernández Campón and Lagos Silnik 2009), in areas that are far from the "Wet Chaco", the original habitat of *T. trivittatus* (Ojanguren-Affilastro 2005). In two distant parthenogenetic popula-

tions of *T. serrulatus* with conserved karyotype, a particular combination of genes was proposed to have been selectively advantageous (Schneider and Cella 2010). This fact could also be related to the establishment of a particular karyotype in *T. trivittatus*.

Taking into account the high incidence of intra- and interpopulation chromosome rearrangements reported in other species of *Tityus*, further cytogenetic studies of unequivocally identified sexual and parthenogenetic populations of *T. trivittatus* are needed to reveal potential chromosome variation within this species.

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RESEARCH ARTICLE

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Karyotypes of six spider species belonging to the families Gnaphosidae, Salticidae, Thomisidae, and Zodariidae (Araneae) from Turkey

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Abstract

In this study, the karyotypes of six spider species from Turkey belonging to the families Gnaphosidae, Salticidae, Thomisidae, and Zodariidae were analyzed. Male chromosomal features including diploid chromosome numbers and sex chromosome systems were determined as 2n=22, X_1X_20 in *Drassyllus sur* Tuneva & Esyunin, 2003, *Nomisia exornata* (C. L. Koch, 1839), and *Nomisia orientalis* Dalmas, 1921; 2n=28, X_1X_20 in *Sitticus caricis* (Westring, 1861); 2n=23, X0 in *Xysticus gallicus* Simon, 1875 and 2n=42, X_1X_20 in *Pax islamita* (Simon, 1873), respectively. The chromosome morphology of all species was acrocentric. Data obtained contribute to knowledge of the karyotype evolution of araneomorphs.

Keywords

Araneae, diploid number, sex chromosome system

Introduction

Spiders are one of the most important animal groups, and contain approximately 44 500 species all around the world (Platnick 2014) and consists of three primary clades, namely Mesothelae, Mygalomorphae and Araneaomorphae, the last one being phylogenetically most derived and the largest group (Coddington and Levi 1991). Infraorder

Araneaomorphae contains more than 41 000 species (Platnick 2014). Despite this diversity, only 771 species of spiders have been karyotyped so far (Araújo et al. 2014).

Entelegyne spiders form a very diversified clade of araneomorphs. Their karyotypes are characterized by a predominance of acrocentric chromosomes, $X_1X_2 0$ sex chromosome system (Araújo et al. 2005), relatively low diploid chromosome numbers (ranges from 10 to 49, Kořínková and Král 2013), and chiasmatic meiosis (Kumbıçak 2010). Acrocentric karyotypes of entelegynes with lower chromosome numbers could be derived from ancestral acrocentric karyotypes by tandem fusions (Suzuki 1954) or by cycles of centric fusions and subsequent pericentric inversions (Kořínková and Král 2013). The latter hypothesis is supported by the fact that centric fusions are the most frequent source of chromosome polymorphism found in populations of entelegyne spiders (Kořínková and Král 2013).

In spiders, the $X_1X_2 0$ system could be the ancestral sex chromosome determination as inferred from its presence in the most primitive recent spiders, namely the suborder Mesothelae and basal families of the infraorder Mygalomorphae (Suzuki 1954).

Salticidae, Thomisidae, Gnaphosidae, and Zodariidae are some of the largest families in the order Araneae (Platnick 2014). Despite the high diversity of these spider groups, cytogenetic data have been collected only in 223 species belonging to these clades (Araújo et al. 2014).

This study presents karyotypes of six species belonging to the genera *Drassyllus* Chamberlin, 1922 and *Nomisia* Dalmas, 1921 (Gnaphosidae), *Sitticus* Simon, 1901 (Salticidae), *Xysticus* C. L. Koch, 1835 (Thomisidae), and *Pax* Levy, 1990 (Zodariidae). Our study brings new data and fills some gaps in cytogenetics of these families.

Material and methods

Material: Spiders were collected in Mediterranean, Southeast and Central Anatolia (Turkey) during the year 2012. Collection data of particular species (localities including their coordinates, dates of collection, number of individuals studied) are listed in Table 1. Voucher specimens were deposited in the collection of Department of Molecular Biology and Genetics, Art and Science Faculty, Nevşehir Hacı Bektaş Veli University (Nevşehir, Turkey). The identification of spiders was made by O. Seyyar (Department of Biology, Art and Science Faculty, Niğde University, Niğde, Turkey).

Chromosome preparations and observation: Slides for chromosome observations were made by the spreading technique of Traut (1976), with some modifications. This method consisted of three basic steps. First, the gonads were hypotonized in 0.075 M KCl for 12-15 min in room temperature (RT). Second, gonads were fixed in two batches of freshly prepared Carnoy fixative (ethanol: chloroform: glacial acetic acid; 6:3:1), first batch for 10 min and second one for 20 min (RT). Finally, a cell suspension was prepared from a piece of tissue in a drop of 60% acetic acid on a slide using a pair of tungsten needles. The slide was placed on a histological plate at 42 °C and the drop was evaporated by mov-

Table 1. Material used for chromosome analysis.

Family	Species	Locality	Coordinates	Date of Collection	Number of Individuals Studied
Gnaphosidae	*Drassyllus sur* Tuneva & Esyunin, 2003	Gaziantep, Sakçagözü	37°10'18"N, 36°55'39"E	04.04.2012	7♂
	Nomisia exornata (C. L. Koch, 1839)	Antalya, Aksu	36°55'30"N, 30°48'29"E	24.03.2012	11♂
	Nomisia orientalis Dalmas, 1921	Antalya, Gazipaşa	36°16'23"N, 32°17'33"E	24.03.2012	2♂
		Osmaniye, Düziçi	37°15'02"N, 36°26'36"E	21.05.2012	5♂
		Adıyaman, Kahta	37°48'46"N, 38°38'20"E	11.03.2012	4♂
		Gaziantep, Islahiye	37°01'21"N, 36°37'24"E	06.04.2012	9♂
Salticidae	*Sitticus caricis* (Westring, 1861)	Nevşehir, Göreme	38°38'44"N, 34°50'06"E	10.05.2012	8♂
		Nevşehir, Zelve	38°40'16"N, 34°51'43"E	27.06.2012	3♂
Thomisidae	*Xysticus gallicus* Simon, 1875	Adana, Çamalan	37°19'12"N, 34°36'28"E	12.04.2012	6♂
		Mersin, Bozyazı	36°06'04"N, 32°58'38"E	15.04.2012	2♂
		Mersin, Aydıncık	36°08'36"N, 33°22'59"E	15.04.2012	3♂
Zodariidae	*Pax islamita* (Simon, 1873)	Osmaniye, Toprakkale	37°04'24"N, 36°08'42"E	09.06.2012	5♂

ing it with a tungsten needle. Slides were stained with 5% Giemsa in Sørensen phosphate buffer (pH=6.8) for 27 min (RT). Chromosome spreads were investigated an Olympus BX53 microscope and photographed using a DP26 digital camera (Olympus) using CELLSENS software (Olympus). Relative chromosome lengths (RCL) including standard deviations were calculated as a percentage of the total chromosome length of the diploid set including sex chromosomes (%TCL) from 10 mitotic metaphase plates for each species by CELLSENS software. Classification of chromosome morphology was based on the arm ratio (Levan et al. 1964).

Results

Gnaphosidae

The chromosomes of *Drassyllus sur* Tuneva & Esyunin, 2003 (2n♂=22) were acrocentric. The sex chromosome system was formed by chromosomes X_1 and X_2 which were medium-sized elements (Fig. 1A). The autosome pairs decreased gradually in size. Length of autosome pairs decreased from 9.74±0.29% to 6.89±0.12% of TCL. Relative length of X_1 and X_2 was 8.45±0.06% and 7.57±0.17% of the diploid set, respectively.

There were 10 autosomal bivalents and two sex chromosomes at diplotene (Fig. 2A). Sex chromosomes were positively heteropycnotic from leptotene to metaphase II (Fig. 2B).

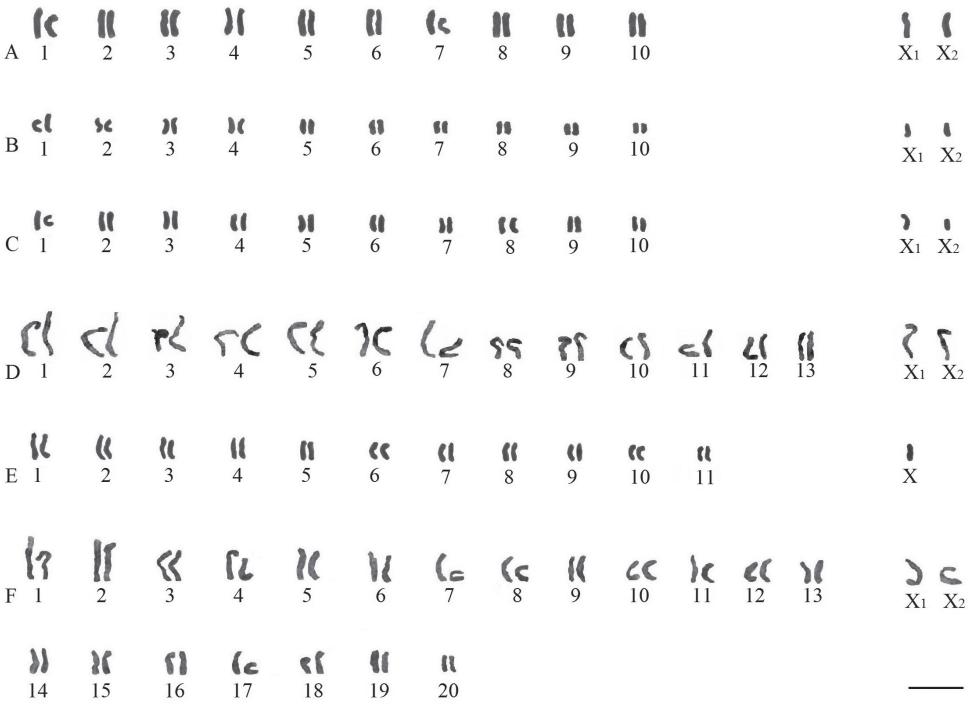

Figure 1. Karyotypes of species based on spermatogonial metaphases. **A** *Drassyllus sur*, 2n♂=22, X_1X_20 **B** *Nomisia exornata*, 2n♂=22, X_1X_20 **C** *Nomisia orientalis*, 2n♂=22, X_1X_20 **D** *Sitticus caricis*, 2n♂=28, X_1X_20 **E** *Xysticus gallicus*, 2n♂=23, X0 **F** *Pax islamita* 2n♂=42, X_1X_20. Bar=10 μm.

The karyotype of *N. exornata* (C. L. Koch, 1839) (Fig. 1B) (2n♂=22, X_1X_20) was acrocentric. Autosome pairs decreased gradually in size from 10.3±0.21% to 5.85±0.17% of TCL. Relative length of X_1 and X_2 were 7.46±0.13% and 6.65±0.08% of TCL, respectively.

The autosomes of *N. orientalis* Dalmas, 1921 (Fig. 1C) (2n♂=22, X_1X_20) was acrocentric. RCL of autosome pairs were decreased gradually from 10.61±0.24% to 6.62±0.19% of TCL. The gonosomes X_1 (7.91±0.12% of TCL) and X_2 (6.10±0.07% of TCL) showed acrocentric morphology.

The sex chromosomes were positively heteropycnotic from leptotene to diakinesis in both *Nomisia* species studied. Plates consisted of 10 autosomal bivalents and two univalent sex chromosomes from pachytene to metaphase I (Fig. 2C–E). At meiotic anaphases, 10 chromosomes segregated to one pole and 12 chromosomes to another pole (Fig. 2F).

Salticidae

The autosomes of *Sitticus caricis* (Westring, 1861) (2n♂=28, X_1X_20) were acrocentric. RCL decreased gradually from 8.47±0.42% to 5.04±0.16% of TCL (Fig. 1D). The sex

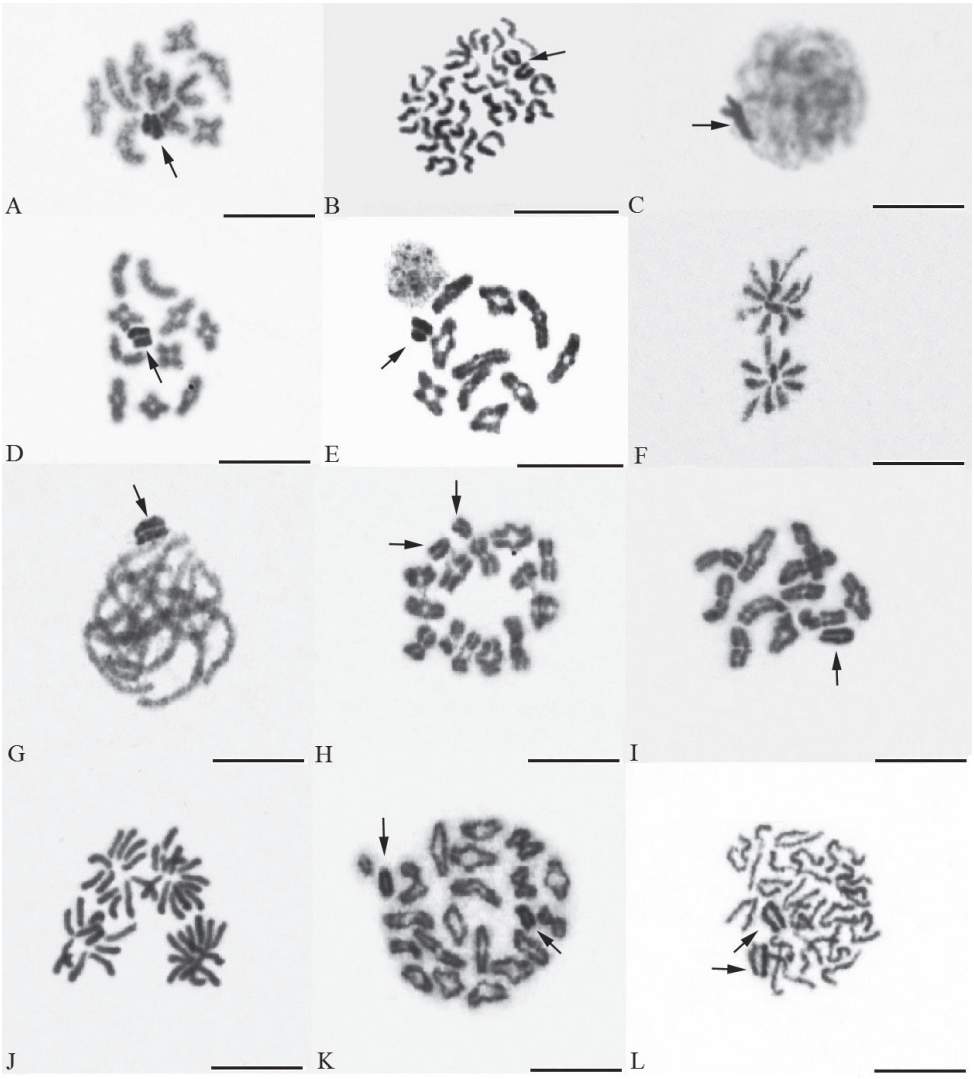

Figure 2. Meiosis of gnaphosid, salticid, thomisid and zodariid males. *Drassyllus sur* **A** diplotene **B** metaphase II, *Nomisia exornata* **C** early pachytene **D** diakinesis, *Nomisia orientalis* **E** diakinesis **F** part of anaphase II showing one plate with 10 chromosomes and another plate with 12 chromosomes, *Sitticus caricis* **G** pachytene **H** diplotene, *Xysticus gallicus* **I** diakinesis **J** anaphase II, *Pax islamita* **K** diakinesis **L** half of metaphase II (arrows indicate sex chromosomes). Bar=10 μm.

chromosomes X_1 (7.33 ±0.51% of TCL) and X_2 (6.72±0.38% of TCL) were medium sized in comparison with the autosomes.

Leptotene, zygotene, and pachytene nuclei included a positively heteropycnotic sex chromosome body that was located at the periphery of the nucleus (Fig. 2G). At late prophase I (i.e. diplotene and diakinesis), 13 autosomal bivalents and two univalent sex chromosomes were determined (Fig. 2H).

Thomisidae

The chromosome set of *Xysticus gallicus* Simon, 1875 (2n♂=23, X0) contained 11 acrocentric pairs and a small X chromosome (Fig. 1E). Autosome pairs decreased gradually in size from 10.28±0.62% to 6.46±0.39% of TCL. Relative length of X chromosome was 6.77±0.46% of TCL. This chromosome was longer than the smallest autosome pair.

From leptotene to diakinesis, X chromosome was formed by an intensively stained material. Diakinetic plates exhibit 11 autosomal bivalents (Fig. 2I). At metaphase II and anaphase II, X chromosome was isopycnotic with autosomes (Fig. 2J).

Zodariidae

The karyotype of *Pax islamita* (Simon, 1873) consisted of acrocentric chromosomes; the diploid number was 42 (Fig. 1F). Autosome pairs exhibited a gradual decrease of relative lengths from 6.42±0.58 to 3.31±0.24% of TCL. This species showed X_1X_20 sex chromosome system. The acrocentric gonosomes showed similar size. Their relative lengths were 5.92±0.66% and 5.37±0.18% of TCL, respectively.

From beginning of pachytene to metaphase I, plates consisted of 20 autosomal bivalents and two not associated sex chromosomes on the periphery of nucleus (Fig. 2K). Sex chromosomes were positively heteropycnotic during prophase and metaphase II. Metaphases II consisted of 20 or 22 chromosomes, respectively. Metaphases II with 22 chromosomes contained two X chromosomes (Fig. 2L).

Discussion

Karyotypes of 771 spider species from 277 genera are known at present (Araújo et al. 2014). Diploid chromosome numbers of spiders range from 7 (Suzuki 1954) to 128 (Král et al. 2013). Entelegynae araneomorphs exhibit lower diploid numbers and mostly monoarmed chromosomes when compared with the predominantly high chromosome numbers and biarmed chromosomes of mygalomorphs (Kořínková and Král 2013). The sex chromosome system of most entelegynes is X_1X_20 type. This system is supposed to be the ancestral form in spiders. It was found in more than 77% of spiders (Araújo et al. 2005).

So far, chromosome numbers have been established for 38 species of gnaphosid spiders. The majority of species (33 in a total) have 2n♂=22 including X_1X_20 sex chromosome system (Araújo et al. 2014). Cytogenetics of *Drassyllus* is still not adequately explored. However, two karyotypes of *Drassyllus* have been published: *D. pumilus* (C.L. Koch, 1839) (2n♂=22, X_1X_20) (Kumbıçak et al. 2009) and *D. praeficus* (L. Koch, 1866) (2n♂=22, X_1X_20) (Kumbıçak et al. 2013). The same karyotype was found in *D. sur* (this study). Karyotypes of the *Nomisia* species analyzed show also the same karyotype with 2n♂=22 and X_1X_20 sex chromosome system (Gorlova et al. 1997,

Kumbıçak et al. 2011, this study). *Drassyllus* and *Nomisia* belong to different subfamilies (Zelotinae and Gnaphosinae, respectively) (Ubick 2005, Seyyar et al. 2009). With one exception (*Urozelotes rusticus*, (L. Koch, 1872) Srivastava and Shukla 1986), all members of these subfamilies karyotyped so far presents 2n♂=22, X_1X_20 (Araújo et al. 2014), confirming the homogeneity of chromosome numbers, morphology and sex chromosomes systems in the family Gnaphosidae.

Male diploid numbers in salticids vary from 2n=14 in *Menemerus illigeri* (Audouin, 1826) (Gorlova et al. 1997) to 2n=28 in most salticids (109 in a total, Araújo et al. 2014). According to the previous studies, salticids exhibit considerable diversity of the sex chromosome systems (X0, X_1X_20, $X_1X_2X_30$, and $X_1X_2X_3Y$). 12 male karyotypes has been found in salticids, namely: 29, $X_1X_2X_30$; 28, X_1X_20; 27, X0; 27, X_1X_2Y; 26, $X_1X_2X_3Y$; 26, X_1X_20; 25, X_1X_2Y; 25, X0; 23, X0; 22, X_1X_20; 21, X0 and 14, X_1X_20 (Araújo et al. 2014). Ancestral karyotype of salticids is probably formed by 28 chromosomes including X_1X_20 system (Maddison and Leduc-Robert 2013). Our results showed 2n♂=28, X_1X_20 in *Sitticus caricis*. This chromosome number as well as the acrocentric chromosome morphology is the same as found in *Sitticus* species studied so far, namely *S. littoralis* (Hahn, 1832) (Suzuki 1954) and *S. terebratus* (Clerck, 1757) (Hackman 1948). *Sitticus* is the only genus belonging to Amycoida clade (Maddison and Hedin 2003) karyotyped so far. So, the current knowledge is not sufficient to explain the karyotype evolution of this clade, therefore new studies on the other amycoids are needed.

The male karyotype of *Xysticus gallicus* displays the general pattern described for most Thomisidae: a diploid chromosome number 23 and X0 sex chromosome system including acrocentric sex chromosome. All *Xysticus* species analyzed so far present this karyotype with exception of *X. triguttatus* Keyserling 1880 (Painter 1914). According to the phylogeny of Benjamin et al. (2008), *Xysticus* is sister group to *Coriarachne* Thorell, 1870, that also presents 2n=23, X0 (*C. fulvipes* Karsch, 1879; Suzuki 1952). There are two hypotheses explaining the origin of acrocentric X0 sex chromosome system in spiders. According to Datta and Chatterjee (1989, 1992) the acrocentric X chromosome can be derived by centric fusion of the X_1 and X_2 chromosomes, followed by pericentric inversions. Also, the acrocentric X chromosome could have originated from tandem fusion between acrocentric X_1 and X_2 chromosomes (Pekár and Král 2001).

Our study represented a diploid number of 42 acrocentric chromosomes and X_1X_20 system in *Pax islamita*. This finding is compatible with the results reported by Král et al. (2011). However, these authors have also found heterozygotes for autosomal centric fusion (2n♂=41, X_1X_20) in addition to the standard individuals. Furthermore, they revealed different pattern of sex chromosome behaviour at male germline of this species. According to their results, the gonosomes were recognised as early as spermatogonial prophase and prometaphase due to their precocious condensation, positive heteropycnosis, and association. However, our data showed the sex chromosomes were indistinguishable at mitotic prophase and prometaphase from autosomes. It was possible to recognise them from autosomes at the beginning of meiotic prophase only due to positive heteropycnosis.

In conclusion, our study described the karyotype features of five araneomorph spiders for the first time and confirms some findings of Král et al. 2011 for *P. islamita*.

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Comparative cytogenetics of *Physalaemus albifrons* and *Physalaemus cuvieri* species groups (Anura, Leptodactylidae)

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Abstract

Recently, *Physalaemus albifrons* (Spix, 1824) was relocated from the *P. cuvieri* group to the same group as *P. biligonigerus* (Cope, 1861), *P. marmoratus* (Reinhardt & Lütken, 1862) and *P. santafecinus* Barrio, 1965. To contribute to the analysis of this proposition, we studied the karyotypes of *P. albifrons*, *P. santafecinus* and three species of the *P. cuvieri* group. The karyotype of *P. santafecinus* was found to be very similar to those of *P. biligonigerus* and *P. marmoratus*, which were previously described. A remarkable characteristic that these three species share is a conspicuous C-band that extends from the pericentromeric region almost to the telomere in the short arm of chromosome 3. This characteristic is not present in the *P. albifrons* karyotype and could be a synapomorphy of *P. biligonigerus*, *P. marmoratus* and *P. santafecinus*. The karyotype of *P. santafecinus* is also similar to those of *P. marmoratus* and *P. biligonigerus* owing to the presence of several terminal C-bands and the distal localization of the NOR in a small metacentric chromosome. In contrast, the *P. albifrons* karyotype has no terminal C-bands and its NOR is located intersti-

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tially in the long arm of submetacentric chromosome 8. The NOR-bearing chromosome of *P. albifrons* very closely resembles those found in *P. albonotatus* (Steindachner, 1864), *P. cuqui* Lobo, 1993 and some populations of *P. cuvieri* Fitzinger, 1826. Additionally, the *P. albifrons* karyotype has an interstitial C-band in chromosome 5 that has been exclusively observed in species of the *P. cuvieri* group. Therefore, we were not able to identify any chromosomal feature that supports the reallocation of *P. albifrons*.

Keywords
Chromosome, NOR, C-banding, heterochromatin, *Physalaemus*

Introduction

Currently, the genus *Physalaemus* Fitzinger, 1826 is classified in the subfamily Leiuperinae Bonaparte, 1850 in the family Leptodatylidae Werner, 1896 (Pyron and Wiens 2011) and is composed of 46 species (Faivovich et al. 2012, Frost 2013). A detailed phylogenetic analysis of the species of *Physalaemus* is not yet available but some supraspecific groupings have been proposed. Lynch (1970) recognized four species groups: *P. pustulosus*, *P. biligonigerus*, *P. cuvieri* and *P. signifier*, which was followed until recently. Based on a phenetic analysis of morphometric characters, Nascimento et al. (2005) resurrected *Engystomops* Jiménez de la Espada, 1872 to include the species previously allocated to the *P. pustulosus* group (*sensu* Lynch, 1970), resurrected *Eupemphix* Steindachner,1863 for the single species *E. nattereri* Steindachner, 1863 (included in the *P. biligonigerus* group by Lynch, 1970) and recognized seven species groups of *Physalaemus*: *P. albifrons*, *P. cuvieri*, *P. deimaticus, P. gracilis, P. henselii*, *P. olfersii* and *P. signifer*. Because *Eupemphix* was paraphyletic with respect to *Physalaemus* in phylogenetic analyses that included eight (Pyron and Wiens 2011) and five (Faivovich et al. 2012) species of *Physalaemus*, Faivovich et al. (2012) proposed that *Eupemphix* is a junior synonym of *Physalaemus*, but did not allocate *P. nattereri* to any species group. The monophyly of each of the seven species groups of *Physalaemus* proposed by Nascimento et al. (2005) remains to be tested and possible synapomorphies of these groups are still to be recognized (see comments in Borteiro and Kolenc 2007, Tomatis et al. 2009, Vera Candioti et al. 2011).

According to the taxonomic proposal of Nascimento et al. (2005), *P. albifrons* (Spix, 1824) was removed from the *P. cuvieri* group (*sensu* Lynch 1970) and grouped together with *P. biligonigerus* (Cope, 1861), *P. marmoratus* (Reinhardt and Lütken, 1862) and *P. santafecinus* Barrio, 1965, three species that were previously allocated to the *P. biligonigerus* group proposed by Lynch (1970). Interestingly, Lobo (1996) indicated that the species of the *P. biligonigerus* group (*sensu* Lynch 1970; that included *P. marmoratus*, as *P. fuscomaculatus*) shared shovel-shaped metatarsal tubercles with *P. albifrons*. Otherwise, Vera Candioti et al. (2011) argued that larval oral morphology does not support the reallocation of *P. albifrons* proposed by Nascimento et al (2005), because the larval oral configuration of *P. albifrons* is almost identical to that of members of the *P. cuvieri* species group and differs from that of *P. biligonigerus*, *P. santafecinus* and probably *P. marmoratus*.

Detailed descriptions of the karyotypes of *P. biligonigerus* and *P. marmoratus* (as *P. fuscomaculatus*), which included the identification of the nucleolus organizer regions (NOR) and heterochromatic sites, were already provided (Amaral et al. 2000, Silva et al. 2000). On the other hand, only the chromosome number and morphology are known for *P. albifrons* (Denaro 1972), and no chromosomal data are available for *P. santafecinus*.

In the present work, we present a detailed characterization of the karyotype of *P. albifrons*, describe the karyotype of *P. santafecinus* and extend the cytogenetic analyses of the *P. cuvieri* group in order to better characterize the group from which *P. albifrons* was removed. Our aim is to provide additional evidence that could be used to compare the *P. albifrons* and P. *cuvieri* species groups.

Materials and methods

Specimens of *P. albifrons*, *P. santafecinus*, *P. albonotatus* (Steindachner, 1864), *P. centralis* Bokermann, 1962 and *P. cuqui* Lobo, 1993 from different localities in Brazil and Argentina were analyzed. For an unequivocal identification of the species, both morphological and acoustic characteristics were utilized. Each specimen's locality and voucher number in the scientific collection where it was deposited are provided in Table 1.

Metaphase chromosome spreads were obtained from cell suspensions of the intestine and testes of animals pre-treated with colchicine (2%) for at least 4 hours (according to Schmid et al. 2010, or adapted from King and Rofe 1976). Prior to the removal of the intestine and testes, the animals were deeply anesthetized with lidocaine gel 2%. Chromosomes were conventionally stained with 10% Giemsa and sequentially submitted to C-banding (King 1980) and silver staining by the Ag-NOR method (Howell and Black 1980) or to fluorescence *in situ* hybridization (FISH) (Viegas-Péquinot 1992) with the rDNA probe HM 123 (Meunier-Rotival et al. 1979). C-banded metaphases from *P. albifrons* were also stained with DAPI (0.5 mg/mL). For each species, at least 10 metaphases that were submitted to each technique were analyzed. Morphometric analyses were done using the MICROMEASURE v3.3 software (Reeves and Tear 2000). The chromosomes were classified according to the criteria proposed by Green and Sessions (1991).

Results

All of the analyzed individuals had a diploid complement of 22 chromosomes. By comparing all of the karyotypes of *Physalaemus* to each other, we noted a high interspecific similarity for the first seven chromosome pairs, and the homeology of these chromosomes could be inferred. Therefore, in each karyotype presented here, these chromosomes were ordered in such a way that their numbers could reflect these homology hypotheses even when their sizes suggested a different numbering. However, the smallest chromosomes (pairs 8–11) varied significantly among the species analyzed, and were numbered only

Table 1. Locality, voucher number and chromosome location of NORs and C-bands of the analyzed specimens. Abbreviations: CH – Chaco province; CT – Corrientes province; MA – Maranhão state; MT - Mato Grosso state; SA – Salta province; SP - São Paulo state; TO - Tocantins state; BC - Departamento de Biologia Celular da UNICAMP, Campinas, SP, Brazil; LGE - Laboratorio de Genética Evolutiva, Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Misiones, Argentina; MNRJ - Museu Nacional do Rio de Janeiro, RJ, Brazil; UFMT - Universidade Federal do Mato Grosso, MT, Brazil; ZUEC - Museu de História Natural, Universidade Estadual de Campinas, Campinas, SP, Brazil; p: short arm; q: long arm; int: interstitial; per: pericentromeric; 3cen-per: centromeric band that extend to the short arm. * only one chromosome of the pair. **In the ZUEC 13696 specimen, an additional terminal C-band is present in 2q (see text for details).

Species	Locality	Specimens	NOR locations	Principal non-centromeric C-bands
P. albifrons	Barreirinhas, MA, Brazil	**7** ♂ (MNRJ 24228, 24230, 24232, ZUEC 12361–3, 17925), **1**♀ (MNRJ 24227)	8q	3cen-per/5p int/8p per/ 9p per
P. albonotatus	Lambari D´Oeste, MT, Brazil (57.4°W, 16.4°S)	**6** ♂ (UFMT 4462, 4466, 4469–72), **1**♀ (UFMT 4465)	8q/9p/9q	2q int/3cen-per/5 int/
P. centralis	Palestina, SP, Brazil (49.2°W, 20.2°S)	**5** ♂ (ZUEC 13689–90, 13692, 3694, 13696)	9q per	2q int**/3cen-per/5p int/ 8q int/9q int/10p per
	Porto Nacional, TO, Brazil (48.6°W, 10.4°S)	**3** ♂ (ZUEC 13373, 13375, 13380)	9q per	2q int/3cen-per/5p int/ 8q int/9q int/10p per
P. cuqui	Near to Rio Piedras, Iruya, SA, Argentina (22°56'S; 64°39'W)	**1**♀ (LGE 6567)	3p*/8q/9p/9q	2q int/3cen-per/5p int
	Taco Pozo, CH, Argentina (25°34'S, 63°09'W)	**2** ♂ (LGE 1635–6)	8q/9p/9q	2q int/3cen-per/5p int
	Aguas Blancas, SA, Argentina (22°43'S, 64°22'W)	**1**♀ (LGE 6568)	8q/9p/9q	-
	Metán, SA, Argentina (25°06'S, 65°03'W)	**1**♀ (LGE 6569)	8q*/9p/9q	2q int/3cen-per/5p int
	Pichanal, SA, Argentina (25°24'S,64°09'W)	**1**♂ (LGE 6570)	8q/9p/9q	2q int/3cen-per/5p int
P. santafecinus	Ituzaingó, CT, Argentina (27°31'S, 56°40'W	**6** ♂ (LGE 077–8, 083–4, 087–8)	9q	1p per/1q int/2p per/3p/8p per/7q per/terminal in all chromosomes

by chromosome size. A detailed description of the karyotype of each species is presented below and the Appendix (Additional file 1) present all the karyotypes arranged together. Table 1 summarizes the data on NORs and non-centromeric C-bands.

Physalaemus albifrons

The *P. albifrons* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 9 and 11), submetacentric (pairs 4, 7, 8 and 10) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 1a; Table 2). C-banding followed by DAPI staining detected all of the centromeric regions and an

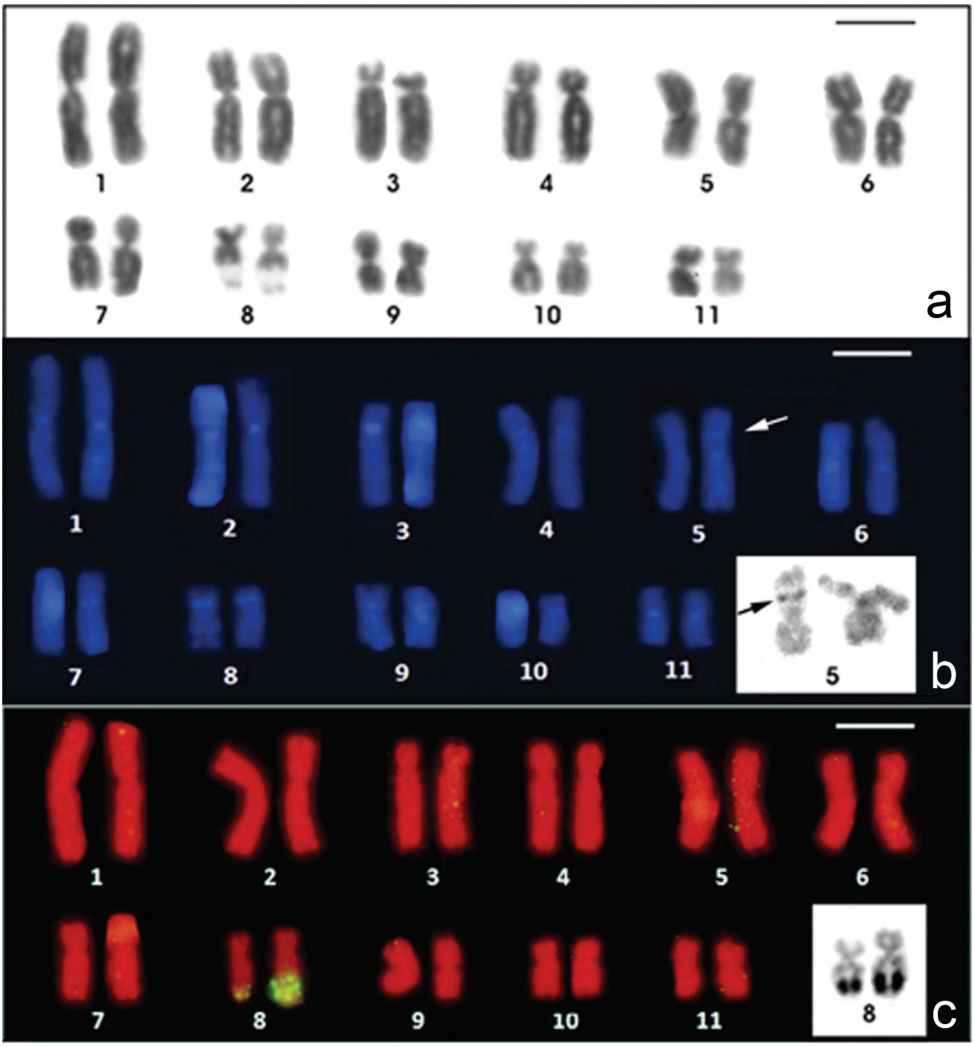

Figure 1. Karyotypes of *P. albifrons* after Giemsa-staining (**a**) C-banding followed by DAPI-staining (**b**) and in situ hybridization with a nucleolar rDNA probe (**c**). In **b** the inset shows the C-banded chromosome pair 5 stained with Giemsa; in **c** the inset shows the NOR-bearing chromosome pair 8 after silver staining. Arrows in **b** point the interstitial C-band in 5p. Bar=10mm.

interstitial heterochromatic band in the short arm of chromosome 5 as well as pericentromeric bands in the short arm of chromosomes 8 and 9 (Fig. 1b). The Giemsa stained C-banded metaphases showed this same pattern, but after DAPI staining, the bands could be more easily visualized. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 1b; Table 2).

The NORs were located distally in the long arm of chromosome 8 (Fig. 1c) and coincided with the secondary constrictions that were observed in Giemsa-stained meta-

Table 2. Morphometric parameters of the *P. albifrons*, *P. albonotatus*, *P. centralis*, *P. cuqui* and *P. santafecinus* karyotypes. The measurements were based on 10 metaphases of each species. CN: chromosome number; CI: centromeric index; SD: standard deviation; RL: relative length. CC: chromosome classification; m: metacentric; sm: submetacentric; st: subtelocentric. *[1]Chromosomes were numbered in order to reflect our hypotheses of homeology for the *Physalaemus* chromosomes even when their sizes suggested a different numbering. *[2]Value at the threshold between submetacentric and subtelocentric classifications.

P. albifrons											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.03	0.40 ± 0.04	0.24 ± 0.02	0.29 ± 0.02	0.46 ± 0.02	0.44 ± 0.04	0.36 ± 0.02	0.33 ± 0.04	0.43 ± 0.04	0.28 ± 0.04	0.45 ± 0.04
RL(%)	14.68	12.15	10.06*[1]	10.64*[1]	9.68	9.43	8.27	7.17	6.76	5.94	5.88
CC	m	m	st*	sm	m	m	sm	sm	m	sm	m
P. albonotatus											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.46 ± 0.03	0.45 ± 0.04	0.24 ± 0.02	0.33 ± 0.02	0.46 ± 0.03	0.43 ± 0.04	0.36 ± 0.07	0.39 ± 0.04	0.44 ± 0.03	0.42 ± 0.03	0.46 ± 0.03
RL(%)	13.87	12.18	10.00*[1]	10.42*[1]	9.61	9.48	8.31	7.32	7.05	5.98	5.78
CC	m	m	st*	sm	m	m	sm	m	m	m	m
P. centralis											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.01	0.39 ± 0.01	0.26 ± 0.02	0.30 ± 0.03	0.46 ± 0.03	0.43 ± 0.03	0.35 ± 0.03	0.42 ± 0.05	0.45 ± 0.01	0.40 ± 0.02	0.40 ± 0.04
RL(%)	13.82	12.24	10.07*[1]	10.26*[1]	10.03	9.36	7.99	7.27	7.12	6.31	5.52
CC	m	m	sm	sm	m	m	sm	m	m	m	m
P. cuqui											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.02	0.41 ± 0.03	0.24 ± 0.05	0.30 ± 0.03	0.44 ± 0.03	0.41 ± 0.02	0.34 ± 0.03	0.42 ± 0.01	0.42 ± 0.06	0.38 ± 0.01	0.43 ± 0.03
RL(%)	14.53	13.57	10.0*[1]	10.36*[1]	9.93	9.49	8.39	7.08	6.07	5.40	5.19
CC	m	m	st*	sm	m	m	sm	m	m	m	m
P. santafecinus											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.46 ± 0.02	0.40 ± 0.01	0.39 ± 0.02	0.27 ± 0.02	0.46 ± 0.02	0.43 ± 0.01	0.32 ± 0.02	0.39 ± 0.03	0.47 ± 0.01	0.43 ± 0.03	0.43 ± 0.04
RL(%)	14.11	13.21	12.34	10.88	10.27	9.67	8.60	5.68	5.41	5.35	4.47
CC	m	m	m	sm	m	m	sm	m	m	m	m

phases (Fig. 1a). In three specimens (ZUEC 17925, ZUEC 12363 and MNRJ 24224), a size heteromorphism was observed between the homologous NORs by FISH with an rDNA probe (Fig. 1c) and by silver staining (Fig. 1c - inset). In two specimens (MNRJ 24230 and 24232), the NOR-bearing homologous chromosomes 8 were homomorphic. For the remaining specimens, we were not able to determine if a NOR size heteromorphism was present.

Physalaemus albonotatus

The *P. albonotatus* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11), submetacentric (pairs 4 and 7) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 2a; Table 2). Curiously, chromosome 5 was larger than chromosomes 3 and 4 in some of the analyzed metaphases (as seen in Figure 2b). Heterochromatin was detected in the centromeres of all chromosomes and interstitially in the long arm of chromosome 2 and in the metacentric chromosome 5 (Fig. 2b). Only two C-banded chromosome pairs 5 were good enough to be measured. Therefore, we tentatively assigned the interstitial C-band of chromosome 5 to its short arm, but further analyses are necessary to test this hypothesis. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 2b - inset; Table 2).

Silver staining detected NORs distally in the long arm of chromosome 8 adjacent to a faint C-band and in both arms of chromosome 9 (Fig. 2d). The NOR in the long arm of chromosome 9 apparently coincided with a C-band (Fig. 2d). All of these NORs could be seen as secondary constrictions in Giemsa-stained metaphases (Figs. 2a and 2c).

Physalaemus centralis

The *P. centralis* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11) or submetacentric (pairs 3, 4, 7 and 8) (Fig. 3a; Table 2). A secondary constriction was detected in the pericentromeric region of the long arm of chromosome 9 and coincided with the NOR that was recognized by silver staining (Fig. 3a - inset). A NOR size heteromorphism was observed in all of the *P. centralis* specimens analyzed. C-bands were present interstitially in the long arm of chromosome 2, in the short arm of chromosome 5, in the long arms of chromosomes 8 and 9, in the pericentromeric region of the short arm of chromosome 10, and in all of the centromeres (Fig. 3b). Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 3b; Table 2).

In three specimens, a heteromorphic chromosome pair 8 composed of homologues with different morphologies and C-banding patterns was observed (Figs. 3a and 3b). While one chromosome 8 showed a conspicuous interstitial C-band that sometimes could be seen as two heterochromatic blocks (chromosome 8a in Fig. 3b), its homologue had no observable interstitial heterochromatic block (Fig. 3b). In the ZUEC 13696 specimen, the pericentromeric C-bands in the long arms of the homologous chromosomes 2 were heteromorphic in size. Additionally, the homologue that had the smaller pericentromeric C-band also had an additional and conspicuous terminal C-band in the long arm (Fig. 3b - inset).

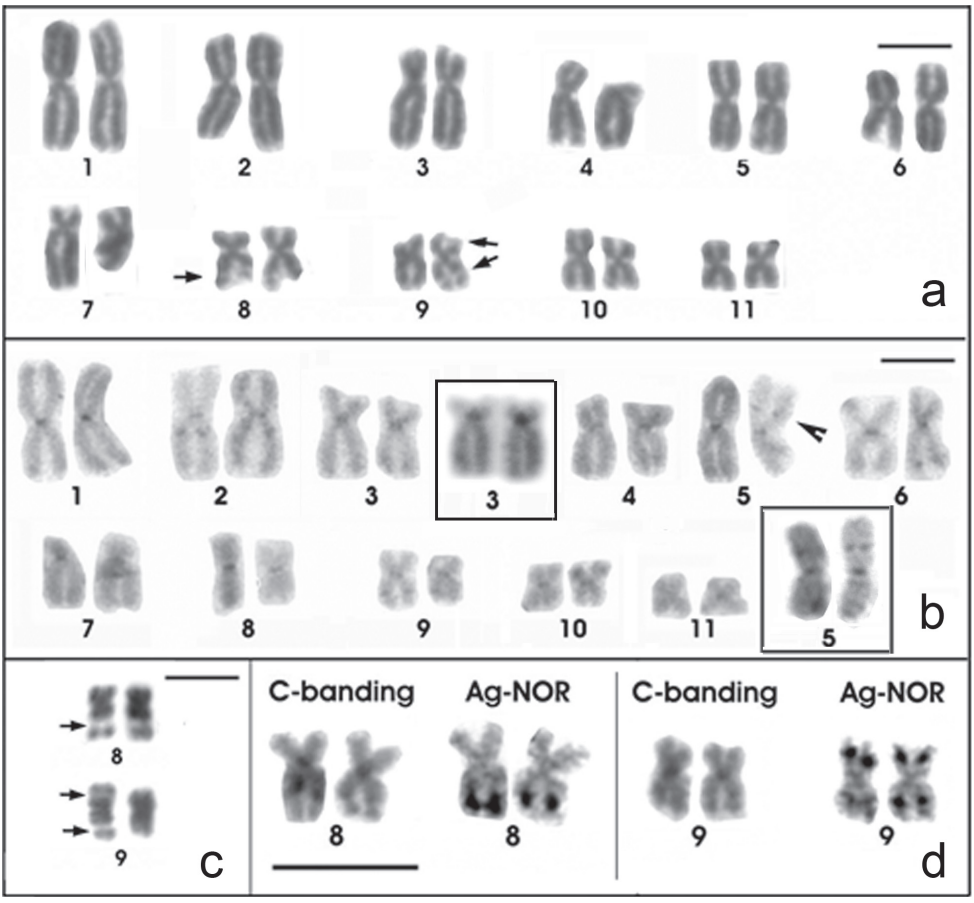

Figure 2. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. albonotatus*. In the insets in **b** C-banded chromosome pairs 3 and 5, showing evident pericentromeric and interstitial bands, respectively **c** NOR-bearing chromosome pairs of *P. albonotatus* stained with Giemsa. Arrows in **a** and **c** indicate secondary constrictions of the NORs. Arrowhead in **b** indicates the C-band in chromosome 5 **d** NOR-bearing chromosome pairs of one specimen of *P. albonotatus* sequentially submitted to the C-banding and the Ag-NOR methods. Note the NOR adjacent to an interstitial C-band in pair 8 and the NORs coincident with faint C-bands in pair 9. Bar=10mm.

Physalaemus cuqui

The *P. cuqui* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11), submetacentric (pairs 4 and 7) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 4a; Table 2). Heterochromatic bands were observed interstitially in the long arm of chromosome pair 2, in the metacentric chromosome pair 5 and in the centromeric regions of all of the chromosomes (Fig. 4b). Only one C-banded chromosome pair 5 could be measured. Therefore, as well as for *P. albonotatus*, we tentatively assigned the interstitial C-band of chromosome 5 of *P. cuqui* to its short arm, but further analyses are necessary to test

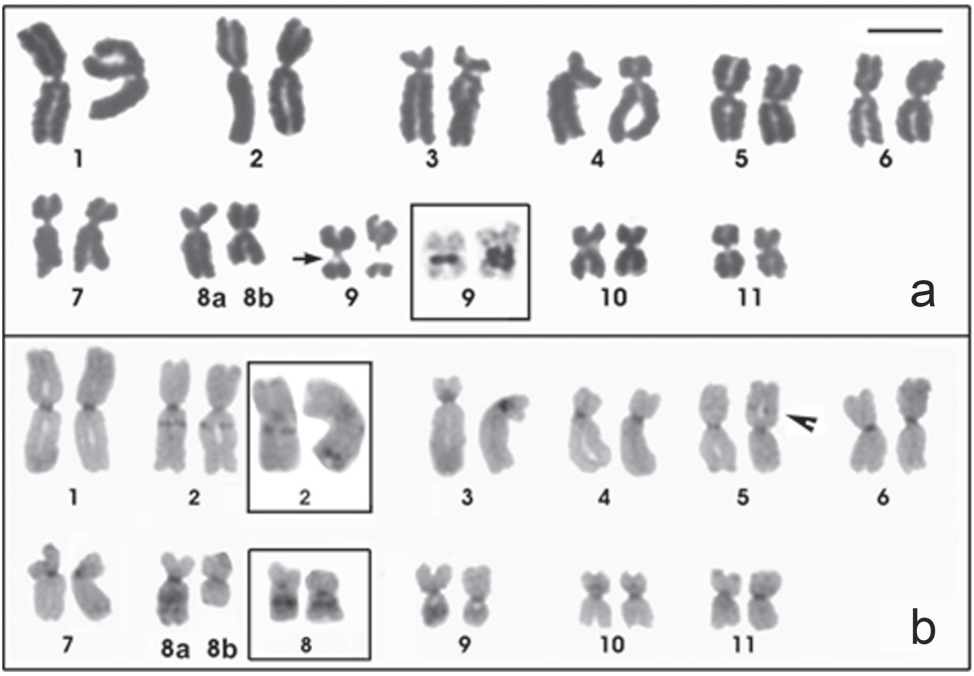

Figure 3. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. centralis*. In **a** an arrow indicates the secondary constriction of the NOR and the inset shows the NOR-bearing chromosome 9 after silver staining. Note the NOR size heteromorphism. In **b** the arrowhead indicates the C-band in 5p and the insets show the heteromorphic pair 2 and the homomorphic pair 8 of the ZUEC 13696 specimen. Note the conspicuous interstitial heterochromatin in the long arm of chromosome pair 8. Bar=10mm.

this hypothesis. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 4b; Table 2).

In three specimens, the Ag-NORs were located in the long arm of chromosome pair 8 and in the short and long arms of chromosome pair 9 (LGE 1635-1636, MLP DB 4973) (Fig. 4c – left), but only one chromosome 9 was silver-stained in the MLP DB 5560 specimen (Fig. 4c – middle). Additionally, one specimen (MLP DB 6480) showed an additional Ag-NOR in the short arm of one chromosome 4 (Fig. 4c – right). These Ag-NORs were coincident with the secondary constrictions visualized in Giemsa-stained metaphases (Fig. 4a).

Physalaemus santafecinus

The *P. santafecinus* chromosomes were classified as metacentric (pairs 1, 2, 3, 5, 6, 8, 9, 10 and 11) or submetacentric (pairs 4 and 7) (Fig. 5a; Table 2). The NORs were located distally in the long arm of chromosome 9 (Fig. 5a - inset). C-bands were detected in all the centromeric regions. Additionally, pericentromeric C-bands were present in the short arms

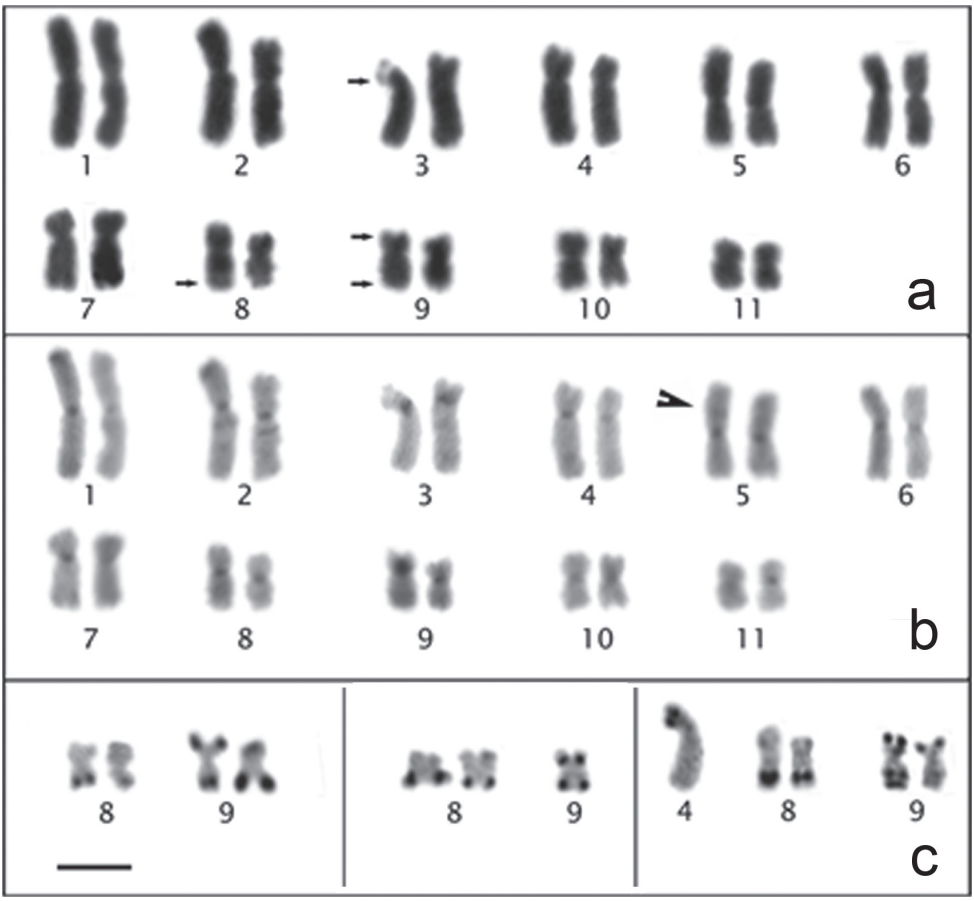

Figure 4. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. cuqui*. In **a** the arrows indicate the secondary constriction of the NOR. In **b** arrowhead indicates the C-band in 5p **c** Variability in the NOR-bearing chromosome pairs of three specimens of *P. cuqui.* Bar=10mm.

of chromosomes 1 and 2 and in the short arm of chromosome 8. Small C-bands were also detected proximally in the long arms of chromosomes 4 and 7 and distally in the long arm of chromosome 1. A conspicuous C-band was observed in the short arm of chromosome 3, which was almost entirely heterochromatic. Terminal faint C-bands could be seen in all of the chromosomes (Fig. 5b). When the Ag-NOR method was performed on C-banded metaphases, we could undoubtedly recognize the chromosome 9 as the NOR-bearing chromosome while chromosomes 8 had strong pericentromeric C-bands (data not shown).

Discussion

To date, 23 of the 46 species of *Physalaemus* were karyotyped and all of them have 2n=22 (Beçak 1968, Beçak et al. 1970, Denaro 1972, De Lucca et al. 1974, Silva

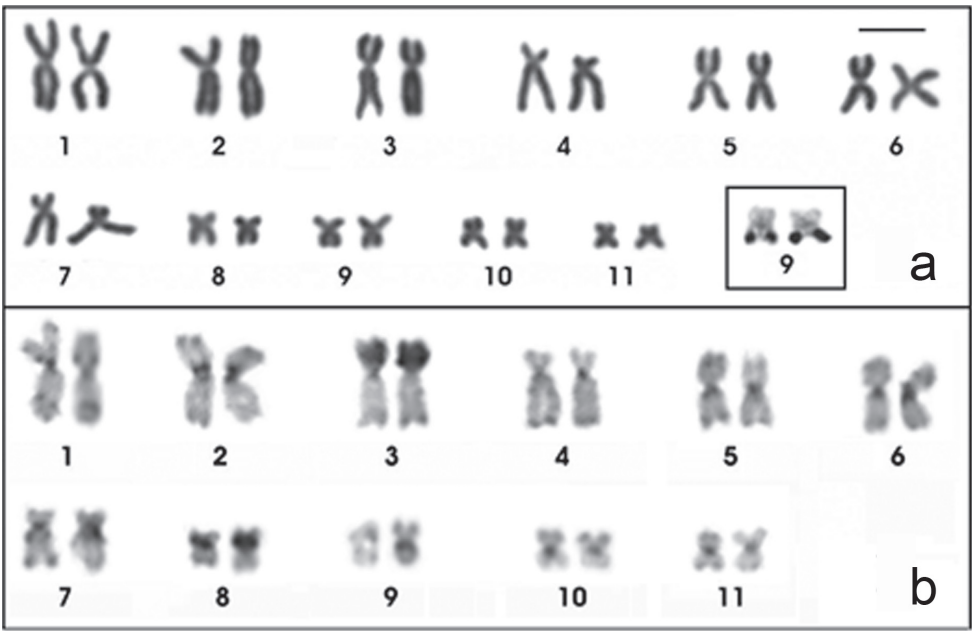

Figure 5. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. santafecinus*. In **a**, the inset shows the NOR-bearing chromosome 9 after silver staining. Bar=10mm.

et al. 1999, Silva et al. 2000, Amaral et al. 2000, Lourenço et al. 2006, Ananias et al. 2007, Tomatis et al. 2009, Milani et al. 2010 – included *P. feioi* Cassini et al., 2010 as *P. olfersii* (Lichtenstein & Martens, 1856), Nascimento et al. 2010, Provete et al. 2012). Interestingly, two distinct fundamental numbers (FN) can be recognized among the karyotypes of *Physalaemus* species. The five species of the *P. signifer* group already karyotyped have FN=42 and a telocentric chromosome 11 [see karyotype of *P. signifer* (Girard, 1853) in De Lucca et al. (1974), *P. crombiei* Heyer & Wolf, 1989 and *P. spiniger* (Miranda-Ribeiro, 1926) karyotypes in Silva et al. (2000), and a reference to the *P. atlanticus* Haddad and Sazima, 2004 and *P. moreirae* (Miranda-Ribeiro, 1937) karyotypes in the discussion of Ananias et al. (2007)], as does *P. nattereri* (Beçak 1968, Lourenço et al. 2006, Ananias et al. 2007) and *P. fernandezae* (Müller, 1926) (Tomatis et al. 2009). The karyotypes of the remaining species of *Physalaemus*, including the species of the *P. cuvieri* and the *P. albifrons* groups that we focused on in our present investigation, have FN=44 and a biarmed chromosome 11. Considering the close phylogenetic relationship inferred for *P. nattereri* and *P. signifer* (Pyron and Wiens 2011, Faivovich et al. 2012, Fouquet et al. 2013), which was the only species of the *P. signifer* group already included in phylogenetic analyses, it is possible to suppose that the telocentric chromosomes 11 of *P. nattereri* and *P. signifer* have the same origin. On the contrary, the similar chromosomes 11 of *P. fernandezae* and the *P. signifer* group probably result from a homoplasy (Tomatis et al. 2009).

The karyotype of *P. santafecinus* described here is very similar in chromosomal size and morphology to those of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* (Amaral et al. 2000, Silva et al. 2000). The chromosomes classified by Amaral et al. (2000) as 4 and 5 probably correspond to chromosomes 5 and 4, respectively, of the karyotype of *P. biligonigerus* described by Silva et al. (2000) and of the *P. santafecinus* karyotype. Such a discrepancy emerges, however, from the use of different criteria for the numeric classification of the chromosomes rather than from a real divergence between the karyotypes.

A remarkable characteristic of the *P. santafecinus* karyotype that is shared with the karyotypes of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* is a conspicuous C-block on the short arm of chromosome 3 (3p) (Table 3). This large heterochromatic C-block is not detected in *P. albifrons* or in any species of *P. cuvieri* group. Instead, a small C-band pericentromerically located on 3p was already detected in the karyotypes of the species currently allocated to the *P. cuvieri* group that were already studied by C-banding [i.e., *P. albifrons*, *P. albonotatus*, *P. centralis*, *P. cuqui* (present work), *P. ephippifer* (Steindachner, 1864) (Nascimento et al. 2010) and one of the populations of *P. cuvieri* Fitzinger, 1826 that was studied cytogenetically by Quinderé et al. (2009)]. Although the pericentromeric C-band in 3p of *P. ephippifer* could be easily observed, it was also much smaller than those observed in *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus.* In the latter four species, the larger size of this C-band probably explains the larger size of 3p in these karyotypes. A small pericentromeric C-band that extend from the centromere to the short arm of chromosome 3 was also present in *P. barrioi* Bokermann, 1967 (Provete et al., 2012), *P. olfersii* and *P. feioi* (as *P. olfersii*; Milani et al. 2010), which are the species of *P. gracilis* group (*P. barrioi*) and *P. olfersii* group (*P. olfersii* and *P. feioi*) already studied by C-banding.

Interestingly, a large 3p showing a large C-band was also observed in *P. nattereri* (Lourenço et al. 2006, Ananias et al. 2007), a species previously allocated to the *P. biligonigerus* group by Lynch (1970). Although a rigorous phylogenetic analysis of the *Physalaemus* genus is not yet available, in recent phylogenetic inferences *P. nattereri* was recovered as the sister species of *P. signifer* and was not closely related to *P. biligonigerus* (Pyron and Wiens 2011, Faivovich et al. 2012, Fouquet et al. 2013). In this phylogenetic context the most parsimonious hypothesis is to consider the large heterochromatic region in chromosome 3 of *P. nattereri* to be homoplastic with respect to the large heterochromatic region in chromosome 3 of the *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* karyotypes. This hypothesis is particularly plausible if we consider the evolutionary dynamics of satellite DNAs, which are the principal components of heterochromatin (reviewed in Charlesworth et al. 1994). The copy number of satellite DNA repeats can vary dramatically, as they are frequently involved in unequal crossing over and other events as rolling circle replication and conversion-like mechanisms (reviewed in Charlesworth et al. 1994, and in Ugarkovic and Plohl 2002).

On the other hand, the available data do not prevent the large C-band found on 3p of *P. santafecinus*, *P. biligonigerus* and *P. marmoratus* from being a synapomorphy of this group of species, which could have arisen from the amplification of a small C-band.

Table 3. Comparison of chromosome 3 of species of *P. cuvieri* (left column) and P. *albifrons* (right column) groups. Black areas in the ideograms represent C-bands. *[1]Based on Silva et al. (1999) and Quinderé et al. (2009). *[2]Nascimento et al. (2010). *[3]Based on Amaral et al. (2000).

***P. cuvieri* group (sensu Nascimento et al. 2005)**	**Chromosome 3**		***P. albifrons* group (sensu Nascimento et al. 2005)**
P. albonotatus			*P. albifrons*
P. centralis		 *3	*P. biligonigerus*
P. cicada	No C-banding data	 *3	*P. marmoratus (=P. fuscomaculatus)*
P. cuqui			*P. santafecinus*
P. cuvieri	 *1		
P. ephippifer	 *2		
P. erikae	No C-banding data		
P. fischeri	No C-banding data		
P. kroyeri	No C-banding data		

Despite the proposals of Lynch (1970) and Nascimento et al. (2005) disagree with regard to the relationships of these three species with other *Physalaemus* species, the close relationships of *P. santafecinus*, *P. biligonigerus* and *P. marmoratus* was considered in both studies. A phylogenetic analysis designed to study the relationships in the genus *Physalaemus*, however, is crucial to test this hypothesis. Also, further molecular characterization of the heterochromatic bands on 3p could help to provide additional evidence of the inferred heterochromatin amplification process.

In addition to the large C-band in 3p, the karyotype of *P. santafecinus* is also similar to those of *P. biligonigerus P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* (Amaral et al. 2000, Silva et al. 2000) based on the presence of several telomeric C-bands and a pericentromeric C-band in the short arm of chromosome 8 as well as the NOR location. In all of these karyotypes, the NOR-bearing chromosome is small and metacentric, and it was classified as chromosome 9 in the karyotype of *P. santafecinus* (described here) and in the karyotypes of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* described by Amaral et al. (2000). However, in the karyotype of *P. biligonigerus* described by Silva et al. (2000), the NOR-bearing chromosome was considered to be chromosome 8, which has a conspicuous pericentromeric C-band. Because Silva et al. (2000) apparently did not perform sequential C-banding and Ag-NOR in order to properly identify the NOR-bearing chromosome in C-banded metaphases, it is likely that the NOR-bearing chromosome is chromosome 9 in the C-banded karyotype shown by those authors.

In contrast to *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus*, the telomeric C-bands could not be detected in the karyotype of *P. albifrons*. Additionally, the NOR in *P. albifrons* was detected interstitially in the long arm of the submetacentric chromosome 8. This NOR-bearing chromosome very closely resembles the NOR-bearing chromosome found in some populations of *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009) as well as in *P. albonotatus* and *P. cuqui* (present work). The *P. albifrons* karyotype presented here is very similar to the Giemsa-stained karyotype described for this species by Denaro (1972). However, the chromosome classified by Denaro (1972) as No. 11 is probably the one we classified as No. 8, and the secondary constriction observed by Denaro (1972) is likely to be the site recognized as NOR by silver impregnation in the present work.

Despite the similarity between the NOR-bearing chromosome of *P. albifrons* and those of some species of the *P. cuvieri* group, it would be premature to consider this a synapomorphy of *P. albifrons* and species of the *P. cuvieri* group because the evolutionary divergence of this character (i.e., NOR location) has not yet been elucidated. We cannot discard the possibility that the NOR found in *P. albifrons* and in some *P. cuvieri* species is plesiomorphic with respect to the other NOR sites found in *Physalaemus* species. This interpretation derives from the fact that the NOR-bearing chromosome 8 found in other leiuperines, as *Pleurodema diplolister* (Peters, 1870) (Lourenço et al., 2006), resembles that of *P. albifrons* and some *P. cuvieri* species group and could constitute the same state of character.

Another chromosome feature found in *P. albifrons* that was also detected in species of the *P. cuvieri* group was the interstitial C-band in chromosome 5 (Table 4). This C-

Table 4. Comparison of chromosome 5 of species of *P. cuvieri* (left column) and *P. albifrons* (right column) groups. Black areas in the ideograms represent C-bands. *[1]C-band was tentatively assigned to the short arm (see text for details). *[2]Based on Silva et al. (1999) and Quinderé et al. (2009). *[3]Nascimento et al. (2010). *[4]Based on chromosomes described as No. 3 by Amaral et al. (2000).

***P. cuvieri* group (sensu Nascimento et al. 2005)**	**Chromosome 5**		***P. albifrons* group (sensu Nascimento et al. 2005)**
P. albonotatus	 *1		*P. albifrons*
P. centralis		 *4	*P. biligonigerus*
P. cicada	No C-banding data	 *4	*P. marmoratus (=P. fuscomaculatus)*
P. cuqui			*P. santafecinus*
P. cuvieri	 *2		
P. ephippifer	 *3		
P. erikae	No C-banding data		
P. fischeri	No C-banding data		
P. kroyeri	No C-banding data		

band was observed in all of the species of the *P. cuvieri* group already analyzed by the C-banding technique, including *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009), *P. ephippifer* (Nascimento et al. 2010), *P. albonotatus* (present work), *P. centralis* (present work) and *P. cuqui* (present work). However, this band was not detected in the C-banded karyotypes of the other three species currently allocated in the *P. albifrons* group (Amaral et al. 2000, Silva et al. 2000, present work) or in species of the *P. henselii* group (Tomatis et al. 2009), the *P. olfersii* group (Milani et al. 2010) and the *P. gracilis* group (Provete et al. 2012). Based on these data, the interstitial C-band in the medium-sized chromosome classified as No. 5 is a putative synapomorphy of *P. albifrons* and the species of the *P. cuvieri* group. However, because of the small size of this C-band, which could make its detection by the C-banding technique particularly difficult, and because of the dynamics of the satellite DNA sequences, which are subject to recurrent amplification/deletion events, this hypothesis must be taken with caution. A comprehensive phylogenetic study of the genus *Physalaemus* and a molecular characterization of this interstitial C-band would allow this hypothesis to be properly evaluated.

In conclusion, we were not able to recognize any chromosomal character that would support the reallocation of *P. albifrons* from the *P. cuvieri* group to the *P. albifrons* group together with *P. biligonigerus*, P. *marmoratus* and *P. santafecinus*.

Interestingly, in addition to the data regarding chromosomal characteristics, larval morphology also does not seem to support the composition of the *P. albifrons* group. *Physalaemus biligonigerus*, *P. santafecinus* and *P. marmoratus* have a similar larval oral disc configuration (LTRF 2/2, with a dorsal gap in the marginal papillae) that differs considerably from that of *P. albifrons*, whose oral disc is almost identical to that of the tadpoles of the *P. cuvieri* group and is thus characterized by an LTRF 2/3 with dorsal, ventrolateral and ventral gaps in the marginal papillae (Vera Candioti et al. 2011). During embryogenesis of the oral disc of *Physalaemus*, ventrolateral gaps appear in the marginal papillae, apparently in all species of the genus (see Vera Candioti et al. 2011). The ventrolateral gaps persist only in the tadpoles of *P. cuvieri* species group [except *P. fischeri* (Boulenger, 1890) and *P. cicada* Bokermann, 1966], in *P. riograndensis* Milstead, 1960 (*P. henselii* group) and in *P. albifrons* (see Vera Candioti et al. 2011). On the other hand, ventral gaps develop only in tadpoles of *P. albifrons*, in species of *P. cuvieri* group (except *P. fischeri*) and in two species of the *P. henselii* group [*P. henselii* (Peters, 1872) and *P. fernandezae* (Müller, 1926)]. Among the leiuperines, the ventrolateral gaps were only observed in some species of *Pseudopaludicola* (see Vera Candioti et al. 2011), and although its presence during development appears to be plesiomorphic for *Physalaemus*, its persistence in larval stages is a putative synapomorphy of the *P. cuvieri* group (including *P. albifrons*). Finally, the internal oral morphology of tadpoles of *P. albifrons* differs from that of *P. biligonigerus*, *P. marmoratus* and *P. santafecinus* based on the presence of three lingual papillae, which is a characteristic shared with some species of the *P. cuvieri* group (Oliveira et al. 2010).

Interspecific comparison in the *P. cuvieri* group

Some of the species in the *P. cuvieri* group are sibling species with important intraspecific morphological variation. Therefore, the identification of these species that is based exclusively on their morphology is sometimes very difficult. Occasionally, species misidentification has occurred, for example, among *P. cuvieri*, *P. albonotatus*, *P. cuqui* and *P. centralis* (Barrio, 1965). Our results revealed conspicuous cytogenetic differences among most species of the *P. cuvieri* group. The exception is the great similarity between the karyotypes of *P. albonotatus* and *P. cuqui*. Additionally, the karyotypes of the species analyzed here were distinguished from the previously analyzed karyotype of *P. cuvieri.* The interspecific variation described in this work regarding heterochromatin and NOR distribution is of fundamental importance for the comparative analysis of the *P. cuvieri* species group.

An interstitial C-band was observed near the centromere in the long arm of chromosome 2 of *P. albonotatus*, *P. centralis* and *P. cuqui*; whereas in the karyotype of *P. ephippifer* (Nascimento et al. 2010) there is an interstitial C-band in the short arm of chromosome 2. A corresponding interstitial C-band in the short arm of chromosome 2 was reported in *P. cuvieri* populations from Rio Claro (Silva et al. 1999) and Palmeiras (Quinderé et al. 2009). If these heterochromatic bands were homeologous, it is conceivable that rearrangements (mainly pericentric inversions) involving chromosome 2 might have occurred during the divergence of these species. Interestingly, the present work reports evidence of a rearrangement involving chromosome 2 in *P. centralis*. In the ZUEC 13696 specimen of *P. centralis*, heteromorphism for the intrachromosomal location of heterochromatic regions in the chromosome pair 2 suggested that paracentric inversion might have been involved in this chromosomal rearrangement.

Despite the overall similarity in chromosomal morphology among the species currently allocated to the *P. cuvieri* group, chromosome pairs 8 and 9 differ greatly. The differences in these chromosomes probably arose from the distinct locations of the NOR in these karyotypes, as these rDNA genes occupy different sites in pairs 8 and/or 9 of these species. The observed pattern of NOR occurrence can be helpful in distinguishing the analyzed species of the *P. cuvieri* group. Noticeably, a pericentromeric NOR site was found exclusively in the *P. centralis* karyotype. However, the NOR-bearing chromosomes (chromosome pairs 8) from the species *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009), *P. albonotatus*, *P. cuqui* and *P. albifrons* are quite similar and their homeology could be possible. Otherwise, the evolutionary relationship of this chromosome with the other NOR-bearing chromosomes found in species of *P. cuvieri*, *P. albifrons* and other species groups remains unclear, and further studies are necessary to elucidate the rearrangements that give rise to the great diversification of the NOR-bearing chromosomes in this genus.

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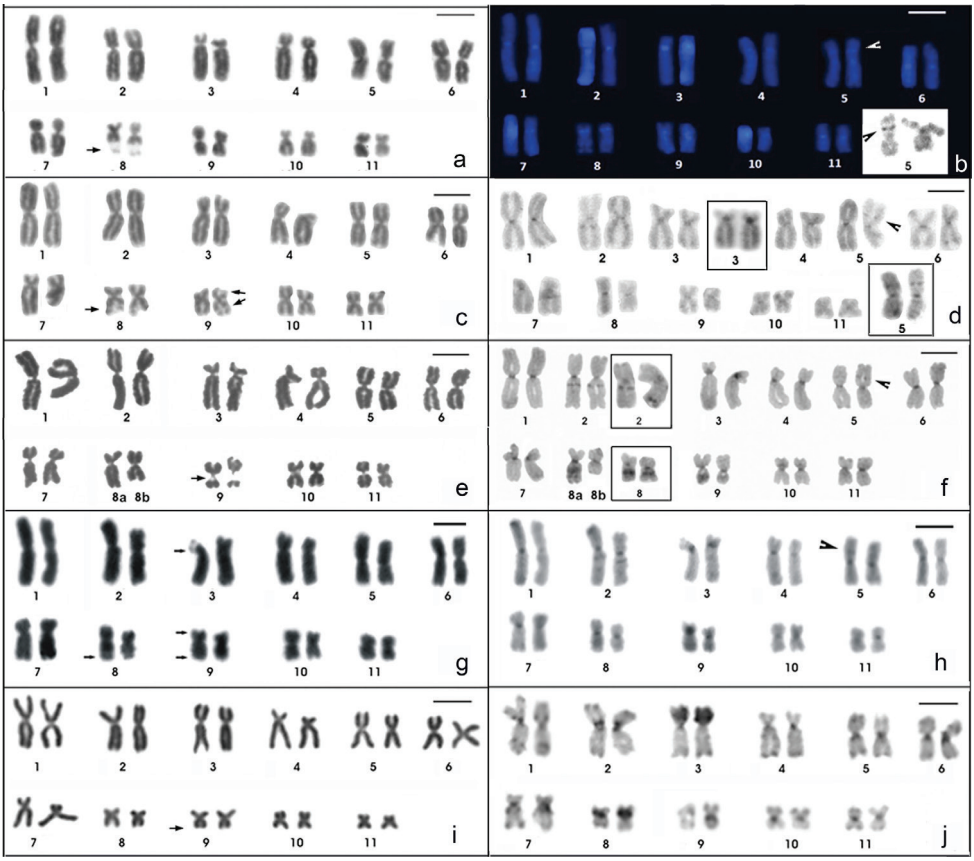
Appendix



Additional file 1. Giemsa-stained (**a, c, e, g, i**) and C-banded karyotypes of *P. albifrons* (**a, b**) *P. albonotatus* (**c, d**) *P. centralis* (**e, f**) *P. cuqui* (**g, h**) and *P. santafecinus* (**i, j**). In **b** the chromosomes were stained with DAPI after C-banding, except those in the inset, which were stained with Giemsa. In the insets in **d** C-banded chromosome pairs 3 and 5, showing evident pericentromeric and interstitial bands, respectively. The insets in **f** show the heteromorphic pair 2 and the homomorphic pair 8 of the ZUEC 13696 specimen. Arrows point NORs. Arrowheads point the C-band in 5p. Bar=10mm.

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Cytotaxonomy of two species of genus *Chrysolaena* H. Robinson, 1988 (Vernonieae, Asteraceae) from Northeast Paraguay

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Abstract

Chromosome counts and karyotypes of two species of *Chrysolaena* H. Robinson 1988 are presented in this paper. Mitotic analysis revealed that both taxa have x=10, a basic chromosome number considered characteristic of the genus. The chromosome number and the karyotype of *C. cristobaliana* are reported for the first time, as well as a new cytotype and the karyotype of *C. sceptrum*. *Chrysolaena cristobaliana* showed heptaploid cytotype with $2n$=7x=70 and a karyotype composed of 46 *m* + 24 *sm* chromosomes. On the other hand, *C. sceptrum* presented tetraploid cytotype with $2n$=4x=40 and a karyotype with 30 *m* + 10 *sm* chromosomes. Accessory chromosomes were observed in cells of both species. The chromosome analysis showed that these species differ in the chromosome number and the total chromosome length, although they showed similar chromosome morphology and asymmetry indexes. The results support the use of chromosome data in taxonomic treatments of the American members of the tribe Vernonieae.

Keywords

B chromosomes, chromosome numbers, karyotype, Lepidaploinae, polyploidy

Introduction

The genus *Chrysolaena* (Vernonieae, Asteraceae) includes 18 species mainly concentrated in southern Brazil and northeast of Argentina. From this area, the genus extends to north Peru and the Amazon region of Brazil, and southward, to the center of the

province of Buenos Aires in Argentina. Most of the species of *Chrysolaena* occur in Brazil (15 spp.), Paraguay (11 spp.) and Argentina (6 spp.). However, a small number of species are found in Bolivia, Peru and Uruguay. Species of the genus are characterized by sericeous or velutinous indumentum, glandular anthers appendages, style without a basal node and glandular cypselas (Robinson 1988). Another important distinguishing feature that separates it from the other American genera of the tribe is the morphology of the pollen grains (Type C) which is tricolporate, echinolophate, with presence of polar lacuna but lacking of equatorial lacuna (Keeley and Jones 1979, Via do Pico and Dematteis 2013). However, the more distinguishable feature of *Chrysolaena* is probably the base chromosome number, because this is the single American genus of the tribe with x=10 (Dematteis 1997, Via do Pico and Dematteis 2012a, 2012b).

Since the taxonomic treatment realized by Robinson (1988), where the author segregates *Chrysolaena* from *Vernonia* Schreb., 1791, most of the studies have been focused in nomenclature, anatomy, cytology and palynology (Martins and Oliveira 2007, Oliveira et al. 2007b, Mendonça et al. 2007, Dematteis 2009, Galastri et al. 2010, Via do Pico and Dematteis 2012a, 2012b, 2013, Appezzato-da-Glória et al. 2012). Despite these contributions, the chromosome information is still scarce. Chromosome studies carried out in the genus, reported basic number x=10 and different ploidy levels or cytotypes in nine species of *Chrysolaena*: both diploid and tetraploid populations have been found in *C. flexuosa* (Sims) H. Robinson, 1988, *C. propinqua* (Hieron.) H. Robinson, 1988, *C. lithospermifolia* (Hieron.) H. Robinson, 1988, and *C. obovata* (Less.) Dematt., 2009, whereas only diploid populations are known for *C. verbascifolia* (Less.) H. Rob., 1988. In *Chrysolaena simplex* (Less.) Dematt., 2007, have been found tetraploid cytotypes and in *C. sceptrum* (Chodat) Dematt., 2009, octoploid. Both *Chrysolaena cognata* (Less.) Dematt., 2009, and *C. platensis* (Spreng.) H. Robinson, 1988, show a greater cytological variation with diploid, tetraploid, hexaploid, and octoploid populations, and even odd polyploids in *C. cognata* (Galiano and Hunziker 1987, Dematteis 1997a, 2002, 2009, Angulo and Dematteis 2009b). Despite these studies, only the karyotypes of *C. flexuosa*, *C. simplex*, *C. platensis*, *C. cognata*, *C. verbascifolia*, *C. propinqua* and *C. lithospermifolia* have been analyzed (Ruas et al. 1991, Dematteis 1997a, Angulo and Dematteis 2009a, 2009b, Via do Pico and Dematteis 2012b) and these analyses did not include all the cytotypes of the species.

Chrysolaena cristobaliana Dematt., 2009, and *C. sceptrum* are erect shrubs with well-developed xylopodia and its distribution is mostly restricted regarding to the other species of the genus. Both taxa grow on high fields and "Cerrados" from northeast of Paraguay and southeastern Mato Grosso and Mato Grosso do Sul in Brazil (Dematteis 2009). Cytological information of these two species is very scarce and only the chromosome number of a single population of *C. sceptrum* has been reported (Dematteis 2002).

In the present study, *C. cristobaliana* and *C. sceptrum* were cytologically examined in order to extend the cytogenetic knowledge and provide information taxonomically useful. The chromosome number and the karyotype of *C. cristobaliana* are reported for the first time, as well as a new cytotype and the karyotype of *C. sceptrum.*

Materials and methods

The specimens were obtained from natural populations from department of Amambay, northeast of Paraguay. Voucher specimens are kept at the herbarium of the Instituto de Botánica del Nordeste (CTES). Location and Voucher specimens: *Chrysolaena cristobaliana*: Paraguay, Dpto. Amambay: Chirigüelo, 2 km W Pedro Juan Caballero. Cerrado degraded, near neighborhood. *Dematteis and Vega 4283,* (CTES). *C. sceptrum*: Paraguay, Dpto. Amambay: Chirigüelo, 2 km W Pedro Juan Caballero. Cerrado degraded, near neighborhood. *Dematteis and Vega*, *4289* (CTES).

Mitotic chromosome preparations were made from root meristems obtained from germinating seeds. The roots were pretreated for about 5 h in 0.002 M 8-hydroxyquinoline solution at room temperature, fixed in 3:1 absolute alcohol/acetic acid, and then stained using Feulgen's technique. Permanent microscope slides were prepared by mounting in Euparal.

At least 10 metaphases were drawn for each population using a Zeiss camera lucida (Carl Zeiss, Germany), selecting the best for measurements. The nomenclature used to describe the chromosome morphology was the one proposed by Levan et al. (1964). The morphology of the chromosomes was determined using the centromeric index (i=short arm x 100/total length of the chromosome). Accordingly, the chromosomes were classified as metacentrics (*m*): 50–37.5, submetacentrics (*sm*): 37.5–25, and subtelocentrics (*st*): 25–12.5. Ideograms were drawn based on the average centromeric index and arranged in order of decreasing size. Because the polyploid nature of the species is unknown and taking into account the concept of ideogram (diagrammatic representation of the gametic chromosome set (n) of a species), the chromosomes were grouped in pairs.

The following karyological parameters were evaluated: total karyotype length (TKL), centromeric index (i), chromosome length (c), arm ratio (ar), and their averages (I, C and AR, respectively); in addition, the ratio between the smallest and the largest chromosome (R</>) was calculated. The karyotype asymmetry was estimated using intrachromosomal (A_1) and interchromosomal (A_2) indexes suggested by Romero Zarco (1986) and the symmetry classes of Stebbins (SC) (Stebbins 1971).

Results

The species analyzed, the somatic chromosome numbers, the ploidy level, and the karyotypic parameters calculated are detailed in Table 1.

Both species analyzed presented base chromosome number x=10. *Chrysolaena sceptrum* showed a tetraploid cytotype with 2n=4x=40 (Fig. 1a, b), with a karyotype composed of 30 metacentric (*m*) and 10 submetacentric (*sm*) chromosomes (Figure 2a). In a few cells from 0-2 accessory or B chromosomes were observed. These elements were metacentric and showed an average size of 1.47 µm. *Chrysolaena cristobaliana* presented a heptaploid cytotype with 2n=7x=70, 0-7 accessory chromosomes per cell (Fig. 1c, d) and a karyotype formed by 46 (*m*) and 24 (*sm*) chromosomes (Fig. 2b).

Table 1. Chromosomal number, ploidy level, karyotype formula, total karyotype length (TKL), average chromosome length (C), average centromeric index (I), average arm ratio (AR), ratio between the smallest and the largest chromosome (R</>), intrachromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2), symmetry classes of Stebbins (SC) of the *Chrysolaena* species analyzed. SE: standard error.

Species and voucher	*C. cristobaliana* 4283	*C. sceptrum* 4289
2n	70	40
Ploidy	7x	4x
Karyotype formula	2n=46m+24sm+0-7Bs	2n=30m+10sm+0-2Bs
TKL ± SE (µm)	92.42 ± 0.10	41.56 ± 0.09
C (µm)	2.64	2.08
I ± SE	39.71 ± 0.65	40.61 ± 0.85
AR ± SE	0.66±0.019	0.69±0.024
R </>	0.43	0.48
A_1	0.33	0.31
A_2	0.23	0.21
SC	1B	1B

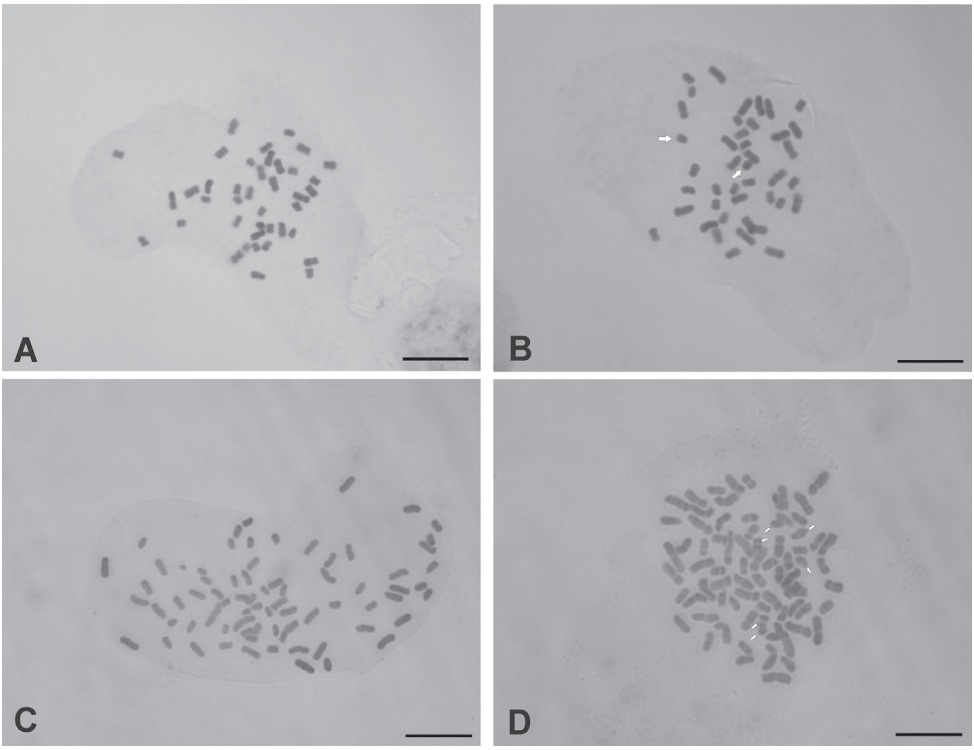

Figure 1. A–D Somatic chromosomes of *Chrysolaena*. **A–B** *C. sceptrum*: **A** 2*n*=4*x*=40 **B** 2*n*=4*x*=40+2 Bs **C–D** *C. cristobaliana*: **C** 2*n*=7*x*=70 **D** 2*n*=7*x*=70+6 Bs. Bar= 5 µm. White arrows denote B-chromosomes.

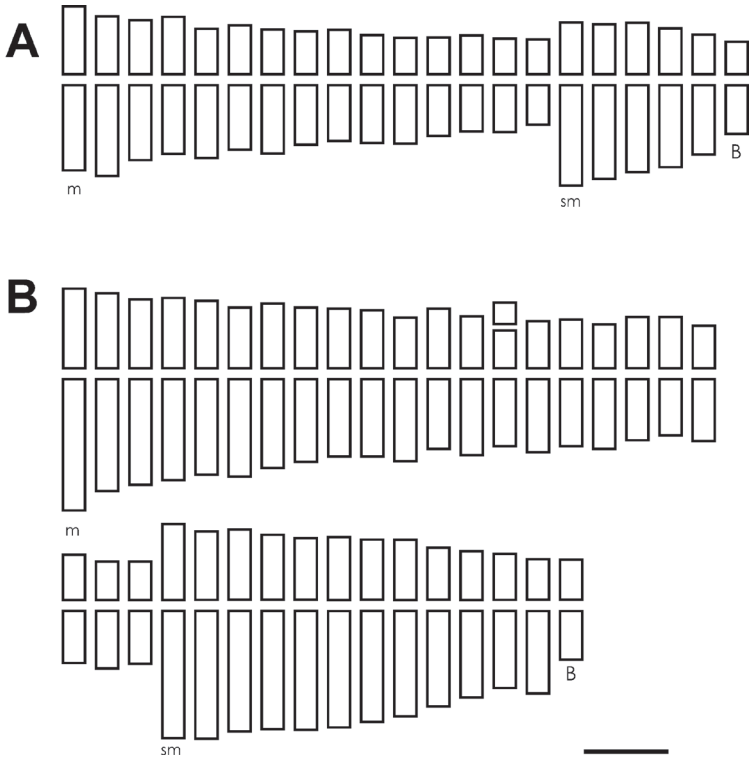

Figure 2. A–B Ideograms. **A** *Chrysolaena sceptrum*: 2*n*=30*m*+10*sm*+0-2 Bs **B** *C. cristobaliana*: 2*n*=46*m*+24*sm*+0-7 Bs. Bar= 1.5 µm.

The accessory chromosomes displayed a metacentric morphology, and an average size of 1.61 µm. A single secondary constriction was observed in pair N° 25.

Both taxa showed moderately symmetrical karyotypes. The majority of chromosomes were metacentric, with fewer submetacentric pairs. Gradual differences in chromosome size were observed. *Chrysolaena cristobaliana* showed an average centromeric index I=39.71, an asymmetry index A_1=0.33 and A_2=0.23; while *C. sceptrum* presented values of I= 40.61, A_1= 0.31 and A_2= 0.21. The average chromosome length (C) in *C. cristobaliana* was 2.64 µm, while in *C. sceptrum* it was 2.08 µm (see Table 1). According to the classification of Stebbins (1971) both species presented symmetry type 1B.

Discussion

Chromosome numbers

In this study we reported for the first time the chromosome number and the karyotype of *C. cristobaliana.* Besides, the karyotype of *C. sceptrum* was recorded for the first time, as well as a new cytotype.

The base chromosome number x=10 is considered characteristic of *Chrysolaena* and clearly distinguishes this genus from the remaining American groups of the tribe. This number also has been found in the two species here analyzed, which is consistent with previous studies carried out in others *Chrysolaena* species (Dematteis 1997a, 1997b, 1998, 2002, 2009, Dematteis et al. 2007, Angulo and Dematteis 2009a, 2009b, Via do Pico and Dematteis 2012a, 2012b). The only cytological record available for *C. sceptrum* was reported by Dematteis (2002) for a population from Paraguay, which showed an octoploid cytotype with $2n$=8x=80. In our study we report a new cytotype (tetraploid, $2n$=4x=40) and the first karyotype analysis of the species. *Chrysolaena sceptrum* has been considered by some authors as a synonym of *C. cognata* (Jones 1981, Robinson 1988), or a variety of this species (Cabrera 1944). However, there are many differences between these two entities, such as the shape and width of the leaves, and the number of florets per head, among the most conspicuous features (Dematteis 2009). Dematteis (2009) refers to the number of chromosomes as another distinguishing characteristic between these two species. *Chrysolaena cognata* presents $2n$=20, 40, and 60, and *C. sceptrum* presents octoploid cytotype ($2n$=80). Nevertheless, further counts reported also the octoploid cytotype ($2n$=80) for *C. cognata* (Via do Pico and Dematteis 2012b), and in this study we report the tetraploid cytotype ($2n$=40) for *C. sceptrum*. For this reason, the number of chromosomes already would not be a useful character to distinguish the two species each other. Although *C. sceptrum* has been cited for Argentina (Cabrera 1944), it has not been found to date in that country. The specimens cited by Cabrera (1944) clearly belong to *C. cognata,* which is widely distributed in Argentina. The geographic distribution of *C. sceptrum* is exclusive of Mato Grosso and Mato Grosso do Sul in Brazil and east of Paraguay. Morphological differences listed above and geographical distribution would be the most conspicuous features to distinguish between *C. cognata* and *C. sceptrum*.

Chrysolaena cristobaliana has never been cytologically analyzed. This study reports as novelty the base chromosome number (x=10), the chromosome number ($2n$=7x=70, heptaploid) and the karyotype of the species. *Chrysolaena cristobaliana* and *C. sceptrum* are closely related species. They are distributed in the same geographic region, and even populations of both entities can be found living in the same area. The main morphological features that differentiate these two species are the branch of the stem and the leaf shape. *Chrysolaena cristobaliana* presents densely branched and elliptical, lanceolate to oblanceolate leaves, whilst *C. sceptrum* has single stems and narrowly lanceolate to linear leaves. The chromosome counts realized here show that these two entities can also be distinguished by the chromosome number.

Chrysolaena cristobaliana is also closely related to *C. cognata*, one of the most widely distributed species of the genus. However, both species differ in the leaf shape, the pubescence type, the florets number and the geographical distribution. The results of this study added the chromosome number as a feature to distinguish these two closely related species.

B chromosomes

Jones (1995) estimated that accessory chromosomes occur in about 10–15% of flowering plant species. Their distribution among angiosperm families is quite heterogeneous. They have been described in more than 150 species of Asteraceae and Poaceae. Generally, these chromosomes are heterochromatic, small, and very variable in number between individuals (Guerra 1988). The irregular distribution of B chromosomes among Angiosperm families suggests that species in certain groups are more likely to bear them than species in other families (Levin et al. 2005). Numerous cases of accessory chromosomes are known in the tribe Vernonieae (Angulo and Dematteis 2009b, Dematteis 1998, Galiano and Hunziker 1987, Oliveira et al. 2007a, Angulo and Dematteis 2012). The occurrence of accessory chromosomes in *C. cristobaliana* and *C. sceptrum* are new records for the genus. In both species, Bs presented a metacentric morphology and similar size. Previous studies reported accessory chromosomes in *C. flexuosa*, *C. cognata*, *C. propinqua*, and *C. verbascifolia* (Via do Pico and Dematteis 2012b). In most of this species, Bs are present in a low frequency (1-4), as reported here in *C. sceptrum.* However, in *C. verbascifolia* it has been observed between 0 and 7 B chromosomes per cell, as well as in *C. cristobaliana*. This variation was observed between individuals of the same population and even within the same individual. Another case in which it has been observed a high frequency of B chromosomes is *Lepidaploa canescens* (Kunth) H. Robinson, 1990, (sub nom=*Vernonia geminata*). In this species it has been observed from 0 to 6 accessory chromosomes amongst the cells of a given individual (Oliveira et al. 2007a). The B chromosomes of *C. cristobaliana* and *C. sceptrum* varied in cells of the same plant, which suggests non disjunction in the mitotic anaphase. Variation in chromosome number in the same plant is a rule used to discriminate B chromosomes from the normal chromosome complement (Jones and Rees 1982). Apparently, there is no difference in frequency between diploid and polyploids species (Jones and Rees 1982, Palestis et al. 2004, Trivers et al. 2004), but, there is a trend suggesting that Bs have a higher frequency in species with a large genome size (Trivers et al. 2004). So far, in the analyzed species of *Chrysolaena*, there is no differences in frequency between diploids and polyploids, since in *C. verbascifolia* (2*n*=20) and *C. cristobaliana* (2*n*=70) it has been observed the same frequency of Bs.

In species of plants and animals, that carries B chromosomes, those individuals from a given population with and without Bs, cannot generally be phenotypically distinguished from each other. However, in some species, there are instances in which B chromosomes change certain morphological characteristics (Jones and Rees 1982, Jones and Houben 2003) or cause some selectively advantageous effects (Teoh and Jones 1978, Jones and Rees 1982). Apparently, the B chromosomes found in *Chrysolaena* species have no effect on the phenotype or development of the individuals.

Karyotype

Chrysolaena cristobaliana and *C. sceptrum* were never been karyotypically characterized. Karyotype analysis is essential for the cytogenetic characterization of species and to examine the variation between its individuals and/or populations. The comparison of karyotypes of different species also allows the taxonomic and evolutionary analysis of a taxon, such as a genus. Besides, many times, differences in karyotype asymmetry can indicate how these chromosomes have diversified in size and morphology within a group (Guerra 1988). The karyotypes of the taxa here studied are formed by metacentric and submetacentric chromosomes, and are quite symmetrical due the predominance of metacentric chromosomes. This is a common feature in species of the tribe Vernonieae (Ruas et al. 1991, Dematteis 1997a, 1997b, 1998, Dematteis and Fernández 1998, 2000, Oliveira et al. 2007a, 2007b, Angulo and Dematteis 2009b, Via do Pico and Dematteis 2012b). *Chrysolaena sceptrum* presents the most symmetric karyotype, which is reflected in the highest average centromeric index and the lowest intrachromosomal asymmetry coefficient (A_1). *Chrysolaena cristobaliana* shows the highest interchromosomal asymmetry coefficient (A_2), which reflects the amplitude of its chromosome size. The asymmetry indexes, A_1 and A_2, calculated show similar values to other species of *Chrysolaena* previously analyzed (Angulo and Dematteis 2009b, Via do Pico and Dematteis 2012b). According to Stebbins (1971), both species fit into the symmetry category 1B, since the relation between the longest and shortest chromosome was between 2:1 - 4:1. This author suggested that classes 1B and 1C are absent in higher plants and only occur in animals (particularly in reptiles), which have karyotypes characterized by great differences in chromosome size, but with predominantly median or submedian centromeres (Stebbins, 1971). However, several subsequent works demonstrated that the class 1B is present among plants, e.g. in the genera *Helianthus* Linnaeus, 1753, *Crotalaria* Linnaeus, 1753, and *Onobrychis* Miller, 1754 (Gupta and Gupta 1978; Kulshreshtha and Gupta 1981; Almada et al. 2006; Hesamzadeh Hejazi and Mahdi Ziaei Nasab 2010).

Chrysolaena cristobaliana presents the longest karyotype (TKL), which is correlated with its ploidy level ($2n=7x=70$). Although the species differed in their chromosome number and total karyotype length, they had similar chromosomal morphology and asymmetry indices. Therefore, karyotype data do not seem to be of great use for group taxonomy, since the chromosomes are small and the karyotype differences cannot be detected rapidly by the analysis of one or a few cells of each species, but only by comparing average measurements. According to Ruas et al. (1991), Dematteis (1996, 1998), Dematteis and Fernández (1998, 2000) and Oliveira et al. (2007a,b), despite the occurrence of variation in chromosome number among species of *Vernonia sensu lato*, the karyotypes with conventional techniques does not discriminate well the species already studied, due to small variation in chromosome size and centromeric position.

Ploidy levels

The species of *Chrysolaen*a exhibit abundant polyploidy. The majority of species studied so far, include diploid and polyploid populations. There are some exceptions of species with a single known cytotype, such as the diploid *C. verbascifolia* (Dematteis 1997, Via do Pico and Dematteis 2012b). The review of chromosomal studies reveals that the tetraploid cytotype is the most common. From nine species in which chromosome number is known, seven had populations with tetraploid cytotypes (Dematteis 1997a, 1997b, 1998, 2002, 2009, Dematteis et al. 2007, Angulo and Dematteis 2009b, Via do Pico and Dematteis 2012a, b). Ploidy differences are not restricted to comparisons between species, but also occur frequently within species (Miller 1978, Burton and Husband 1999, Weiss et al. 2003). Available data on *Chrysolaena* and the results of this study show that the genus is cytologically complex, and polyploidy is an important mechanism in the differentiation and adaptation of species. The species, as well as, different populations of same species present numerous ploidy levels, even odd cytotypes. Some species would be polyploid series and would present more than one cytotype. Besides, there may be a species complex, with entities morphologically related but with different ploidy levels. In this group of species it could be occurring continuous processes of polyploidization and hybridization, which would lead to the different cytotypes observed. According to Jones (1979) and Ruas et al. (1991), the Vernonieae of the New World, in contrast to those of the Old World, show marked diversity in chromosome number and a high ratio of polyploid species. Polyploids may combine to give rise to a complex of polyploid species, which promotes morphological and ecological changes that hinder the taxonomic treatments (Galiano and Hunziker 1987, Dematteis 2002).

Moreover, the chromosome number found in *C. cirstobaliana* is the second report of impair ploidy level for the genus *Chrysolaena*. In *C. cognata* a mixed population with pentaploid ($2n=5x=50$) and hexaploid specimens ($2n=6x=60$) was found in Misiones, Argentina (Dematteis 2002). However, the population of *C. cristobaliana* is not mixed, and all individuals analyzed showed heptaploid ploidy level ($2n=7x=70$). Within the Vernonieae, another case of odd polyploidy was reported in *Lessingianthus macrocephalus* (Less.) H. Robinson, 1988, which presented $2n=11x=176$, one of the higher ploidy levels found within the Asteraceae (Angulo and Dematteis 2012). The high ploidy level with odd chromosome complement, suggests that an irregular meiosis behavior could lead to sterility. Generally, in plants of the Asteraceae family, with these features, is very common the apomictic reproduction. Apomixis is usually defined as a natural process that allows clonal reproduction through seeds, avoiding meiosis and fertilization, and resulting in offspring that are genetically identical to the maternal plant (Nogler 1984). Apomixis was recorded in 2.9 % of the genera of the Asteraceae family and is very common, e.g., in the tribe Eupatorieae (Farco et al. 2012). Considering this background, the population of *C. cristobaliana* analyzed in this study probably present apomictic reproduction. However, other studies should be conducted to test this issue.

Conclusions

There are many important characters of taxonomic weight to separate *Chrysolaen*a from other Vernonieae groups, such as the morphology of the pollen grains and the morphological characters. However, the chromosome number is considered one of the most important features, since *Chrysolaena* is the only American member of the tribe with the base number x=10, which is mainly present in the Old World Vernonieae. Historically, base chromosome numbers have been widely employed for the delimitation of generic and infrageneric taxa in the Compositae (Sundberg et al. 1986). Studies from pollen morphology previously carried out in *Chrysolaena* showed the occurrence of pollen type C (proposed as typical of the genus) in *C. cristobaliana* and *C. sceptrum* (Via do Pico and Dematteis 2013). In addition to this, the chromosome counts carried out in these two species confirm the base chromosome number of x=10 proposed for the genus, and support the taxonomic position of these entities. These results contribute to the knowledge regarding the cytology of the *Chrysolaena* genus and support the use of chromosome number for the taxonomy of the American Vernonieae.

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Genomic organization of repetitive DNAs and its implications for male karyotype and the neo-Y chromosome differentiation in *Erythrinus erythrinus* (Characiformes, Erythrinidae)

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Abstract

Studies have demonstrated the effective participation of repetitive DNA sequences in the origin and differentiation of the sex chromosomes in some biological groups. In this study several microsatellites and retrotranposable sequences were cytogenetically mapped in the *Erythrinus erythrinus* (Bloch & Schneider, 1801) male genome (karyomorph C), focusing on the distribution of these sequences in the sex chromosomes and in the evolutionary processes related to their differentiation. Males of *E. erythrinus* – karyomorph C – present 2n = 51 chromosomes (7m + 2sm + 6st + 36a), including the X_1X_2Y sex chromosomes. The C-positive heterochromatin has a predominant localization on the centromeric region of most chromosome pairs, but also in some telomeric regions. The 5S rDNA sites are located in the centromeric region of 27 chromosomes, including 26 acrocentric ones and the metacentric Y chromosome. The retrotransposons *Rex* 1 and *Rex* 6 show a dispersed pattern in the karyotype, contrasting with the *Rex* 3 distribution which is clearly co-localized with all the 27 5S rDNA sites. The microsatellite sequences show a differential distribution, some of them restricted to telomeric and/or interstitial regions and others with a scattered distribution on the chromosomes. However, no preferential accumulation of these elements were observed in the neo-Y chromosome, in contrast to what usually occurs in simple sex chromosome systems.

Keywords
FISH, microsatellites, retrotransposable sequences, sex chromosomes

Introduction

Sex chromosomes have been widely studied in several invertebrate, vertebrate and plant individuals, focusing on their origin and differentiation (Kubat et al. 2008, Ezaz et al. 2009, Kaiser and Bachtrog 2010, Cioffi et al. 2011, 2013), providing excellent opportunities to investigate the evolutionary processes acting on the genome (Bachtrog et al. 2011). Regarding fishes, different sex chromosome systems can occur, from simple to multiple ones (Devlin and Nagahama 2002), in which repetitive DNA sequences have been increasingly used in order to investigate the evolutionary processes of sex chromosome differentiation (Koga et al. 2002, Lippman et al. 2004, Gross et al. 2009, Cioffi et al. 2011, Martins et al. 2012). In fact, repetitive sequences can accumulate in the sex-specific chromosome due to the reduction of the recombination rate between the proto-sex pair, thus contributing to its differentiation (Vallender and Lahn 2004, Charlesworth et al. 2005).

Repetitive sequences include different classes of *in tandem* repeats, such as satellite DNAs, minisatellites and microsatellites, and interspersed repeats, like the transposable elements (TEs) (Jurka et al. 2005). Microsatellites are constituted by short sequences from 1 to 6 base pairs and, as such, classified as mono-, di-, tri-, tetra-, penta and hexanucleotides (Schlötterer and Harr 2001). Concerning the TEs, they can be grouped into two categories: the retrotransposons, which move into the genome via an intermediate RNA, and the transposons, which are directly transposed into the genome through a DNA copy (Charlesworth et al. 1994, Kazazian 2004).

Erythrinidae (Characiformes), are a small Neotropical fish family composed of three genera, *Erythrinus* Scopoli, 1777, *Hoplerythrinus* Gill, 1896 and *Hoplias* Gill, 1903 (Oyakawa 2003). Among the species of this group, *Hoplias malabaricus* (Bloch, 1794) and *Erythrinus erythrinus* (Bloch & Schneider, 1801) present a great diversity of karyomorphs and differentiated sex chromosome systems (Bertollo et al. 2000, Bertollo et al. 2004). In fact, four karyomorphs (A to D) were already described for *E. erythrinus*, and with exception of karyomorph A that not have differentiated sex chromosomes, the karyomorphs B, C and D share an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex system, but with different diploid numbers and chromosome morphology (Bertollo et al. 2004).

In this study several microsatellites and retrotransposable sequences were cytogenetically mapped in the *Erythrinus erythrinus* male genome (karyomorph C), focusing on their distribution in the sex chromosomes and in the evolutionary processes related to the differentiation of the neo-Y chromosome.

Methods

Material collection and classical cytogenetic analyses

Six male specimens of *E. erythrinus* (karyomorph C), from the Manaus region (3°13'41.4"S, 59°43'43.1"W – Amazon State, Brazil) were analyzed. The experiments followed ethical conducts, and anesthesia was used prior to sacrificing the animals. Mitotic chromosomes were obtained from the anterior portion of the kidney, according to Bertollo et al. (1978). In addition to the standard Giemsa staining, the C-banding method (Sumner 1972), was also employed to detect the distribution of the C-positive heterochromatin on the chromosomes.

Probe preparation

Oligonucleotide probes containing microsatellite sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(TA)_{15}$ were directly labeled with Cy5 during synthesis by Sigma (St. Louis, MO, USA), as described by Kubat et al. (2008). The retrotransposable elements *Rex* 1, *Rex* 3 and *Rex* 6 were obtained by PCR according to Volff et al. (1999). The 5S rDNA probe included 120 base pairs (bp) of the 5S rRNA encoding gene and 200 bp of the non-transcribed spacer (NTS) (Martins et al. 2006). All these probes were labeled with DIG-11-dUTP using DIG-Nick-translation Mix (Roche), and used for the fluorescence *in situ* hybridization (FISH) experiments.

Fluorescence *in situ* hybridization and signal detection

The FISH method was conducted as follows: slides with fixed chromosomes were maintained at 37 °C for 1 hour. Subsequently, they were incubated with RNAse (10 mg/ml) for 1 hour at 37 °C in a moist chamber. Next, it was performed a 5-minute wash with 1xPBS and 0.005% pepsin was applied to the slides (10 minutes at room temperature). The slides were then washed again with 1xPBS. The material was fixed with 1% formaldehyde at room temperature for 10 minutes. After further washing, the slides were dehydrated with 70%, 85% and 100% ethanol, 2 minutes in each bath. The chromosomal DNA was denatured in 70% formamide/2xSSC for 3 minutes at 72 °C. The slides were dehydrated again in a cold ethanol series (70%, 85% and 100%), 5 min each. The hybridization mixture, containing 100 ng of denatured probe, 10 mg/ml dextran sulfate, 2xSSC and 50% formamide (final volume of 30 µl) were heated to 95 °C for 10 minutes and then applied on the slides. Hybridization was performed for a period of 16-18 hours at 37 °C in a moist chamber. After hybridization, the slides were washed for 5 minutes with 2xSSC and then rinsed quickly in 1xPBS. The signal detection was performed using anti-digoxigenin rhodamine (Roche) for the 5S rDNA,

Rex 1, *Rex* 3 and *Rex* 6 probes. Subsequently, the slides were dehydrated again in an ethanol series (70%, 85% and 100%), 2 minutes each. After the complete drying of the slides, the chromosomes were counterstained with DAPI/antifade (1.2 mg/ml, Vector Laboratories).

Microscope analyses

Approximately 30 metaphase spreads were analyzed to confirm the diploid chromosome number, karyotype structure and FISH results. Images were captured by the CoolSNAP system software, Image Pro Plus, 4.1 (Media Cybernetics, Silver Spring, MD, USA), coupled to an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan). The chromosomes were classified as metacentric (m) , submetacentric (sm), subtelocentric (st) or acrocentic (a), according to their arm ratios (Levan et al. 1964).

Results

Males of *E. erythrinus* – karyomorph C – present 2n = 51 chromosomes (7m + 2sm + 6st + 36a), including the X_1X_2Y sex chromosomes. While the chromosomes X_1 and X_2 are acrocentric, the Y is the largest metacentric chromosome in the karyotype (Fig. 1a). The C-positive heterochromatin has a predominant localization on the centromeric region of most chromosome pairs, but also in some telomeric regions (Fig. 1b). The 5S rDNA sites are located in the centromeric region of 27 chromosomes, including 26 acrocentric ones and the metacentric Y-chromosome (Fig. 2). The retrotransposons *Rex* 1 and *Rex* 6 show a dispersed pattern in the karyotype, contrasting with the *Rex* 3 distribution which is clearly co-localized with all the 27 5S rDNA sites (Fig. 2).

The microsatellite sequences show a differential distribution, some of them restricted to telomeric and/or interstitial regions and others with a scattered distribution on the chromosomes. Microsatellites $(CA)_{15}$, $(GA)_{15}$, $(CAC)_{10}$ and $(CAG)_{10}$ are mainly accumulated in the telomeric regions of the chromosomes and in some interstitial sites, but with a different distribution, since some chromosomes present higher signals than other ones (Figs. 2 and 3). However, the $(CA)_{15}$ sequences present a greater distribution compared with the other three classes of microsatellites, including on the Y-chromosome. In fact, this chromosome show a greater accumulation for the $(CA)_{15}$ microsatellite, mainly in interstitial and telomeric regions of the long arms. In turn, the microsatellites $(CAA)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$ and $(TA)_{15}$ present a dispersed distribution among the autosomes and on the Y chromosomes (Fig. 3). In contrast, $(GAG)_{10}$ microsatellite is poorly represented in the genome of *E. erythrinus*, with only four chromosomes showing mapped sites in their centromeric region. The Y-chromosome shows no labeling for this microsatellite (Fig. 3). Figure 4 highlights the distribution of all repetitive sequences analyzed along the Y-chromosome of the species.

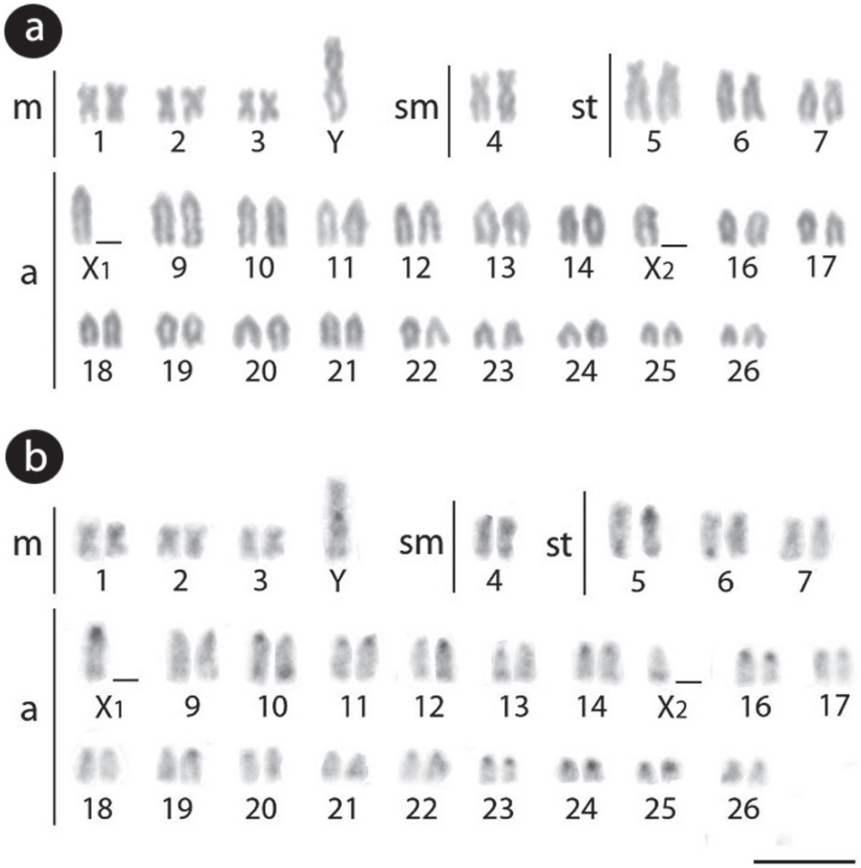

Figure 1. Male Karyotype of *Erythrinus erythrinus* arranged from Giemsa-stained (**a**) and C-banded chromosomes (**b**). Bar = 5 μm.

Discussion

General distribution of repetitive sequences in the whole genome

The repetitive fraction of the genome can be a useful tool for the identification of recent genomic changes that occurred during the evolutionary process. Retrotransposons usually carry regulatory sequences and may attract methylation, thus influencing the gene expression (Martin et al. 2009). In addition, these sequences can also be a substrate for chromosomal rearrangements, including inversions and translocations (Ozouf-Costaz et al. 2004).

The *Rex* family seems to be abundant in different teleost species (Gross et al. 2009), with a varied distribution, from a scattered pattern to a preferential accumulation in some regions of the chromosomes (Gross et al. 2009, Ferreira et al. 2011). *Rex* 3 has been the most analyzed retrotransposon in fishes, showing different distributional

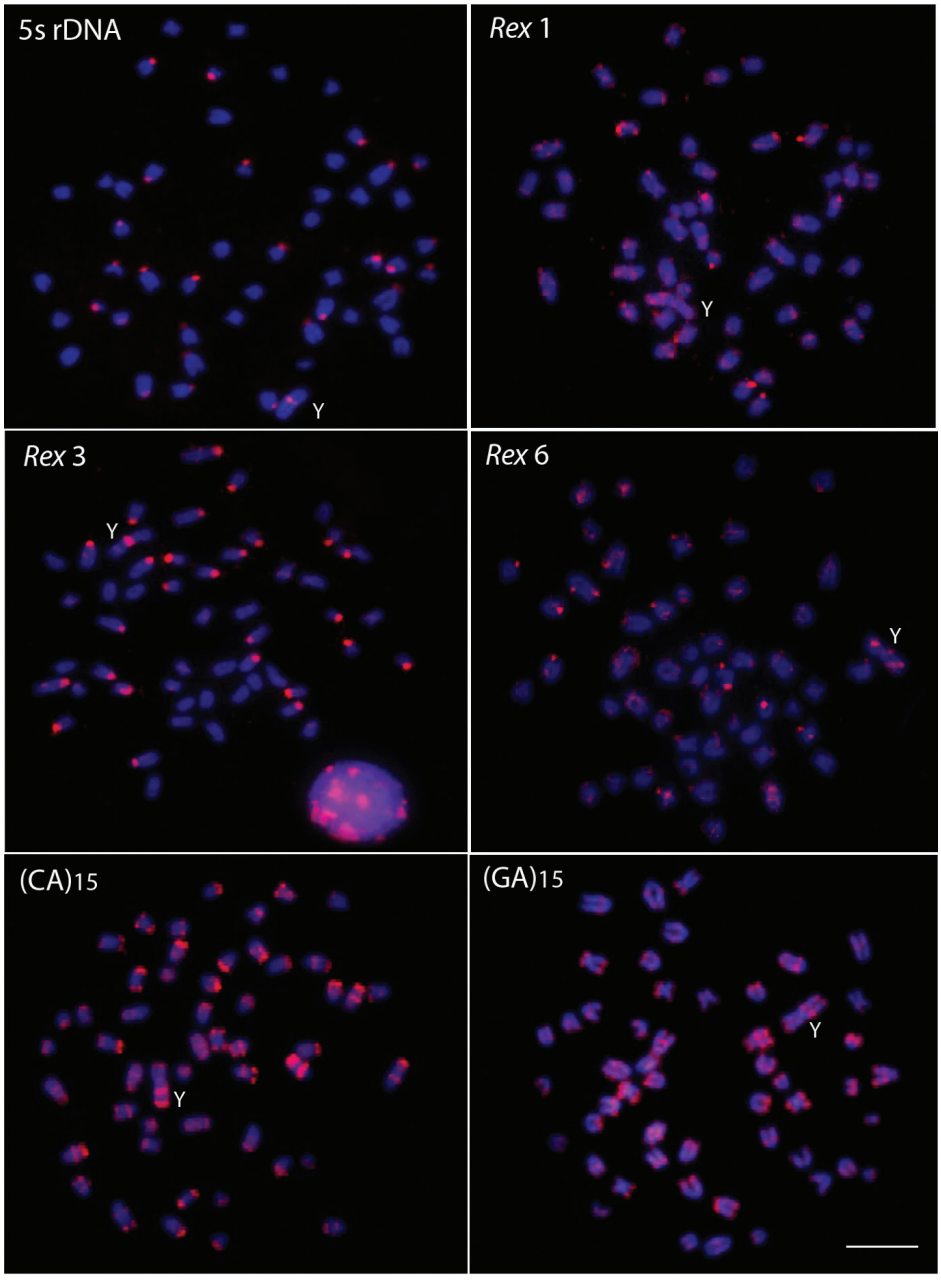

Figure 2. Male metaphase plates of *Erythrinus erythrinus* probed with 5S rDNA, *Rex* 1, *Rex* 3 and *Rex* 6 transposons and microsatellite sequences. Bar = 5 μm.

patterns in the genome of different species (Gross et al. 2009). In *E. erythrinus Rex* 3 showed a clear compartmentalized distribution in the centromeric region of the chromosomes, which was also observed in other fish species, such as *Notothenia coriiceps*

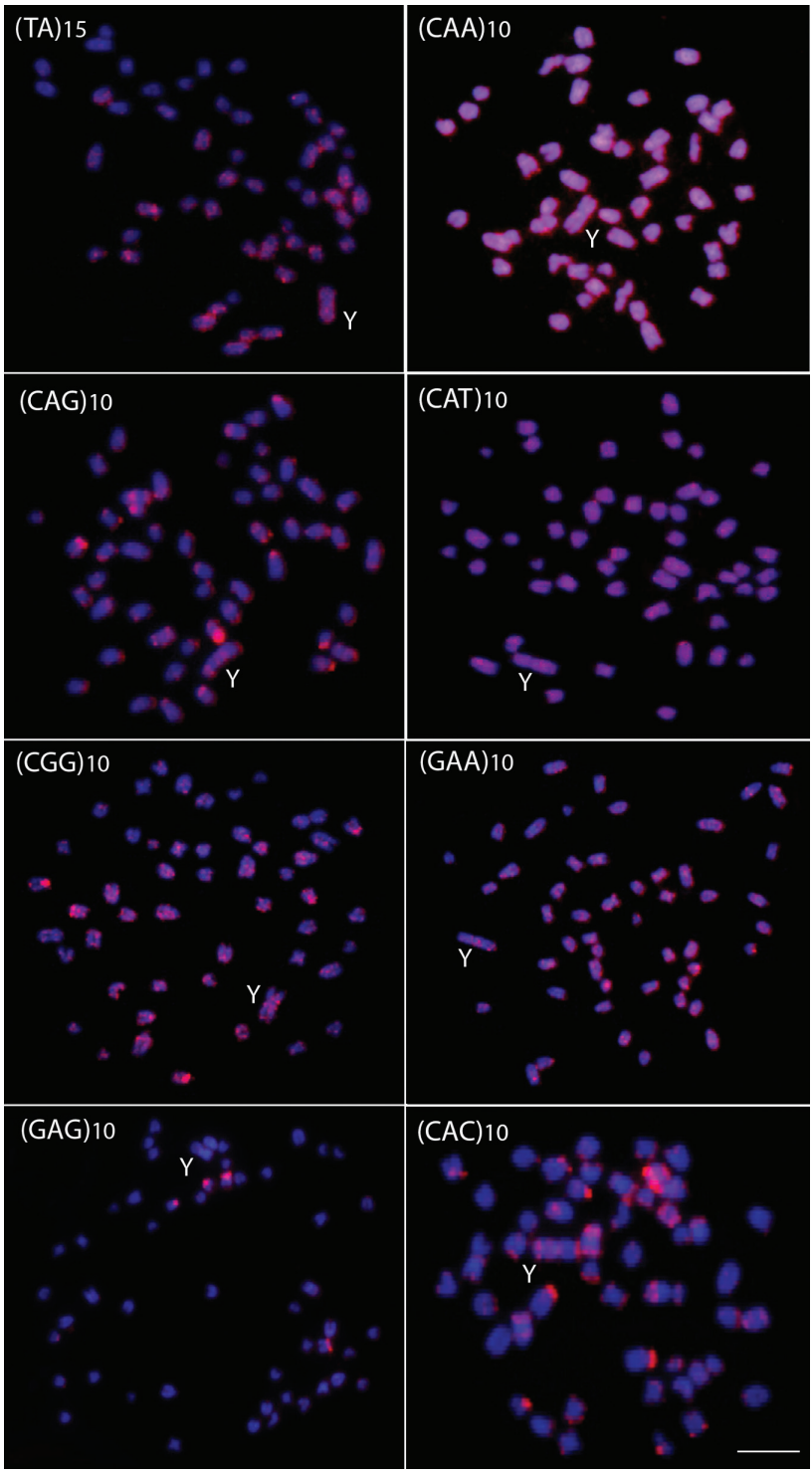

Figure 3. Male metaphase plates of *Erythrinus erythrinus* probed with microsatellite sequences. Bar = 5 μm.

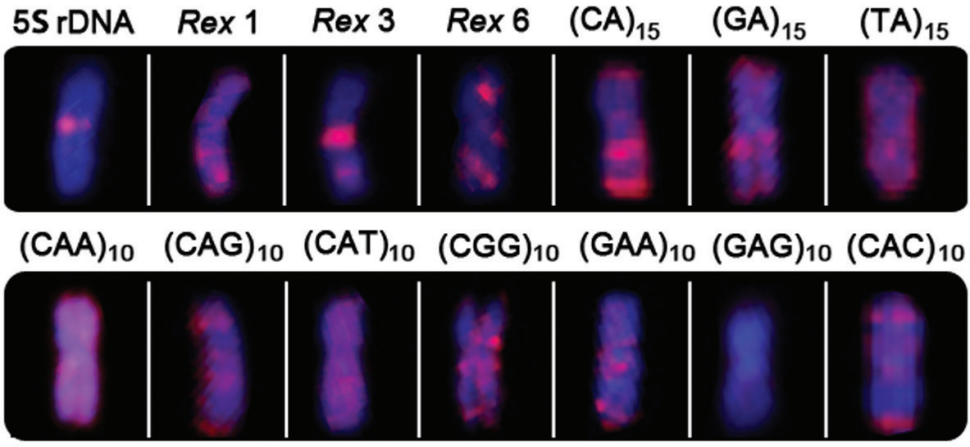

Figure 4. Distribution of repetitive DNA sequences in the Y chromosome of *Erythrinus erythrinus*.

Richardson, 1844 and *Chionodraco hamatus* (Lönnberg, 1905), with a compartmentalized distribution in the pericentromeric region (Ozouf-Costaz et al. 2004). As in the present study, scattered signals for the *Rex* 1 and *Rex* 6 retrotransposons were also found among the cichlid fishes, although many species also showed pericentromeric accumulation of these elements (Valente et al. 2011).

In *E. erythrinus* (karyomorph C), *Rex* 3 showed a clear colocalization with 5S rDNA sites in the centromeric region of several chromosomes. Our data agree with previous results achieved for this same karyomorph (Martins et al. 2012) and for karyomorph D (Cioffi et al. 2010), showing a surprising spreading of 5S rDNA/*Rex* 3 transposons in the genome of this fish, which contrasts with other karyomorphs of this species where the same event is not found. In this sense, in addition to classical cytogenetic rearrangements, these families of repetitive DNAs were useful to demonstrate the hidden biodiversity not detected by conventional morphological analyzes in this fish group. According to Volff et al. (1999), the *Rex* 3 retrotransposon can be associated with gene coding regions, as well as be inserted in introns and in the vicinity of promoter regions, thus probably allowing the dispersion of some genes with which they are associated. It is possible that such dispersion mediated by transposable elements is not a relatively rare event among fishes. In fact, a 5S rDNA dispersion was also recently found in the marine fish *Ctenogobius smaragdus* (Valenciennes, 1837), suggesting the mediation of repetitive elements (Lima-Filho et al. 2014). In addition, in *Rachycentron canadum* (Linnaeus, 1766) the Tol2 element, belonging to the family of hAT transposons, shows a huge colocalization with the 18S rDNA sites in the karyotype (Costa et al. 2013), indicating other TEs than those of the *Rex* family associated with ribosomal DNA families.

Microsatellites mapping has shown both similar as well as different distribution patterns between species (Kubat et al. 2008, Cioffi et al. 2010, Pokorná et al. 2011, Cioffi et al. 2012). This is also true for *E. erythrinus* where $(CA)_{15}$, $(GA)_{15}$, $(CAC)_{10}$ and

$(CAG)_{10}$ microsatellites are mainly compartmentalized in the telomeric and interstitial regions of the chromosomes, while $(TA)_{15}$, $(CAA)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$ and $(GAA)_{10}$ microsatellites show a more scattered distribution throughout the genome. In turn, the $(GAG)_{10}$ microsatellite is poorly represented in the genome of this species. Although $(CA)_{15}$ and $(GA)_{15}$ dinucleotides have a preferential accumulation in the telomeric regions of other fish species (Cioffi et al. 2011, Cioffi et al. 2012), they were also mapped in the interstitial region of several *E. erythrinus* chromosomes.

Distribution of the repetitive sequences in the sex chromosomes

The cytogenetic mapping of repetitive DNAs has improved the knowledge of the evolutionary origin of the neo-Y chromosome. In fact, the chromosomal mapping of repetitive DNA sequences has shown differential accumulations on the sex-specific chromosomes (Kubat et al. 2008, Cioffi et al. 2012, Xu et al. 2013).

In *E. erythrinus*, a centric fusion was proposed to be related with the origin of the big metacentric Y chromosome found in karyomorphs B, C and D and the differentiation of the $X_1X_1X_2X_2/X_1X_2Y$ multiple sex system in these karyomorphs (Bertollo et al. 2004). This proposal was strengthened by the colocalization of 5S rDNA/*Rex* 3 transposon in the centromeric region of several acrocentric chromosomes, and also of the metacentric Y-chromosome (Cioffi et al. 2010, Martins et al. 2012). Indeed, important role for DNA repetitive sequences, as the *Rex* family, has been assigned for chromosomal rearrangements and differentiation of the sex chromosome systems in fish species (Ozouf-Costaz et al. 2004, Cioffi and Bertollo 2012).

In turn, the mapping of microsatellites in the chromosomes has also been useful tools for analyzing the differentiation of sex chromosomes. In simple sex chromosomes, such as the ZZ/ZW system of *Leporinus reinhardti* Lütken, 1875 and *Triportheus auritus* (Valenciennes, 1850) (Cioffi et al. 2012), and the XX/XY system of *Hoplias malabaricus* – karyomorph B (Cioffi et al. 2010), there was a preferential accumulation of different microsatellites in the heterochromatic region of the sex-specific chromosome. However, in multiple sex chromosomes, such as the $X_1X_1X_2X_2/ X_1X_2Y$ system of *Hoplias malabaricus* – karyomorph D (Cioffi et al. 2011) and on the rock bream fish *Oplegnathus fasciatus* (Temminck & Schlegel, 1844) (Xu et al. 2013), although a preferential accumulation of some microsatellites was also found in the neo-Y chromosome, it was not so marked as in the simple sex chromosome systems.

Additionally, no preferential accumulation of microsatellites was found to occur in the sex chromosomes of *E. erythrinus*. In fact, there were no significant differences in the distribution of the microsatellites analyzed concerning to autosomes and sex chromosomes, based on the neo-Y chromosome which is easily identifiable in this species. It is well known that the suppression of the recombination is a crucial step in the differentiation of the sex pair, leading to the differentiation of the sex-specific chromosomes. Multiple sex chromosome systems originate from chromosomal rearrangements from simple systems and can itself reduce or eliminate the recombination near

breakpoints, reinforcing previous suggestions that other events, such as accumulation of repetitive DNAs, may not be necessary for this process (Moreira-Filho et al. 1993, Vieira et al. 2003).

Conclusion

The repetitive sequences used in this study did not show a differential accumulation in the neo-Y chromosome of *E. erythrinus*, showing a similar distribution to the other chromosomes of the complement. However, it is clear that different repetitive DNAs may exhibit differential distribution patterns in chromosomes, including the neo-Y one (Figure 4), probably reflecting differences in the time of chromosomal occupation, as well as of strategies for dispersal throughout the genome.

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Similarities and differences among the chromosomes of the wild guinea pig *Cavia tschudii* and the domestic guinea pig *Cavia porcellus* (Rodentia, Caviidae)

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Abstract

Cavia tschudii Fitzinger, 1867 is a wild guinea pig species living in South America that according to the analysis of mitochondrial genes is the closest wild form of the domestic guinea pig. To investigate the genetic divergence between the wild and domestic species of guinea pigs from a cytogenetic perspective, we characterized and compared the C, G and AgNOR banded karyotypes of molecularly identified *Cavia tschudii* and *Cavia porcellus* Linnaeus, 1758 specimens for the first time. Both species showed 64 chromosomes of similar morphology, although *C. tschudii* had four medium size submetacentric pairs that were not observed in the *C. porcellus* karyotype. Differences in the C bands size and the mean number of AgNOR bands between the karyotypes of the two species were detected. Most of the two species chromosomes showed total G band correspondence, suggesting that they probably represent large syntenic blocks conserved over time. Partial G band correspondence detected among the four submetacentric chromosomes present only in the *C. tschudii* karyotype and their subtelocentric homologues in *C. porcellus* may be explained by the occurrence of four pericentric inversions that probably emerged and were fixed in the *C. tschudii* populations under domestication. The role of the chromosomal and genomic differences in the divergence of these two *Cavia* species is discussed.

Keywords

Karyotype, G, C and AgNOR banding, active NORs, pericentric inversions, domestication

Introduction

Cavia tschudii Fitzinger, 1867 is a wild species of guinea pig (Rodentia, Caviidae) which inhabits northern Chile, southern Peru and Bolivia and northwestern Argentina (Weir 1974, Woods and Kilpatrick 2005). The domestic guinea pig *Cavia porcellus* Linnaeus, 1758 has a cosmopolitan distribution and is an experimental animal, pet, and even is consumed as food in countries of the Andean Altiplano (Tello 1972).

There is a consensus that *C. porcellus* is a domestic form derived from one of the five currently recognized wild species of guinea pigs that inhabit South America (Woods and Kilpatrick 2005). The crosses between *C. porcellus* and *C. fulgida* Wagler, 1831 yielded offspring which behaved according to the Haldane's rule (Haldane 1922), since females were fertile and males were sterile (Detletfsen 1914). By contrast, the crosses between *C. porcellus* and *C. aperea sensu* Erxleben, 1777 (Pictet and Ferrero 1951, Rood 1972) and between *C. porcellus* and *C. cutleri* Tschudi, 1844 (*sensu* Bennet, 1836) (Castle 1916) produced hybrids which were fertile in both sexes. *C. aperea* or *C. tschudii* have been repeatedly considered as the most probable ancestor of the domestic guinea pig. Later, molecular analyses of the mitochondrial cytochrome *b* and 12S RNA genes clearly showed that the closest species to *C. porcellus* is *C. tschudii* and not the genetically related *C. aperea* (Spotorno et al. 2004, Dunnum and Salazar-Bravo 2010). Based on these molecular results and on the analysis of mummified guinea pig remains found in archeological sites, Spotorno et al. (2007) suggested that the domestication of the wild guinea pig occurred in southern Peru-northern Chile.

Considering that the karyotype provides useful characters in taxonomic and systematic studies and that changes in the number and structure of chromosomes may contribute to speciation (King 1993, Searle 1993, Capanna and Redi 1994, Capanna and Castiglia 2004, Marques-Bonet and Navarro 2005, Faria and Navarro 2010), we describe and compare now for the first time the G, C and AgNOR banded karyotypes in molecularly identified specimens of the wild montane guinea pig *C. tschudii* and the domestic guinea pig *C. porcellus*. Our objective is to discover the chromosomal and genomic differences between these two species of *Cavia* in relation to the divergence associated with the domestication process.

Material and methods

Skulls, skins and liver samples for DNA analysis of all the studied animals were preserved in the collection of the Laboratorio de Citogénetica de Mamíferos, Facultad de Medicina, Universidad de Chile (LCM). We examined five *Cavia tschudii* specimens, four males (LCM 3199b, 3110, 3080, 3225) and one female (LCM 3232), collected in the locality of Molinos, Valle de Lluta, 18°23'S, 69°45'W, Arica, I Región, Chile, and four *Cavia porcellus* animals, two males (LCM 2454, 3192) from the laboratory Pirbright breed, Instituto de Salud Pública, Santiago, Chile, and two females (LCM 2479, 2489) from the Andean creole breed, Arica Agromarket, Arica, Chile.

Chromosomes were obtained from marrow cells using conventional in vivo colchicine, hypotonic method, preceded by yeast injection to improve the mitotic index (Lee and Elder 1980). Metaphase cells were G-banded and C-banded by the methods described by Chiarelli et al. (1972) and Sumner (1972), respectively. The active nucleolar organizing regions (NORs) were detected by the silver staining procedure (Sánchez-Rufas et al. 1982). At least 10 good-quality metaphases for each of the staining methods per taxon were selected under a light microscope and digitally captured and stored. Chromosomes were counted, cut out and ordered by size and form using ADOBE PHOTOSHOP version 6.0. The centromeric indexes calculated by measuring the chromosomal arms in 12 metaphases of each species, allowed the classification of the chromosomes as metacentric, submetacentric, subtelocentric and telocentric (Levan et al. 1964). Chromosomes of both species were ordered in the groups defined by Fernández and Spotorno (1968) for *C. porcellus* (groups A, B and C), adding a fourth group (group D) of submetacentric chromosomes for *C. tschudii* (Fig. 1). Male and female G-banded karyotypes from each species were compared and the chromosomes were classified as having totally corresponding, partially corresponding or unique G band patterns (Spotorno 1977, Walker et al. 1979). The size and distribution of the C and AgNOR bands were evaluated in six metaphases of *C. tschudii* and nine of *C. porcellus*. To determine the total number of active AgNOR in each of the species, $AgNOR^+$ sites were identified and counted in the chromosomes of 24 metaphases per species. The statistical significance of the differences was estimated using a Chi squared test.

Results

Chromosome number, size and morphology

For *Cavia tschudii* and *Cavia porcellus* we consistently found a 2n=64, FNa=100-102; the variation in the FNa of both species was due to the polymorphism of chromosome 1 (Fig. 1). *Cavia tschudii* showed five pairs of submetacentric chromosomes (group D, Fig.1a) of which four pairs (numbers 27, 28, 29 and 30) were not present in the *C. porcellus* karyotype (Fig. 1b). The X chromosome of *C. tschudii* was a large submetacentric similar to that of *C. porcellus* and the Y chromosome was a subtelocentric larger than that of *C. porcellus* (Fig. 1).

G bands

The comparison of *C. tschudii* and *C. porcellus* G-banded karyotypes revealed total correspondence for 25 of the 31 autosomal pairs and for the X chromosomes of both species (Figs. 2, 3, Table 1). The four submetacentric chromosomes present only in the *C. tschudii* karyotype showed partial G band correspondence with four *C. porcel-*

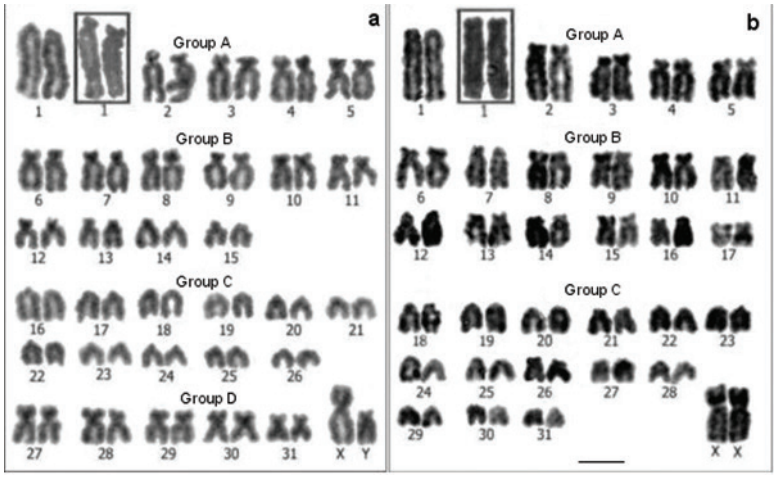

Figure 1. Conventional stained karyotypes: **a** *Cavia tschudii* male **b** *Cavia porcellus* female. Chromosomal pair Nº 1 shows subtelocentric morphology in other individuals of both species (**a** and **b** insets). Bar = 5 μm.

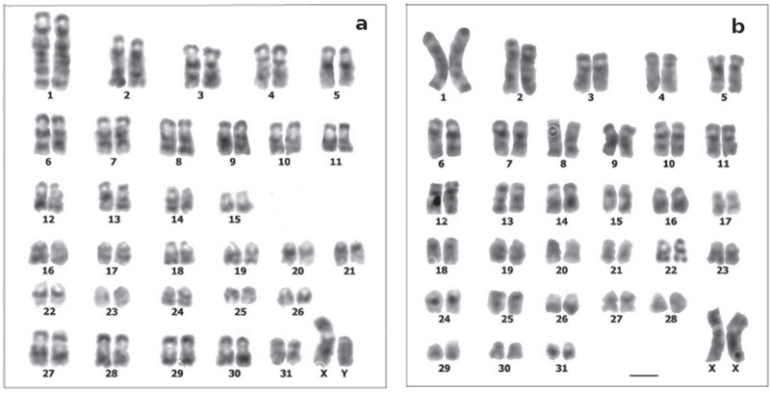

Figure 2. G-banded karyotypes : **a** *Cavia tschudii* male **b** *Cavia porcellus* female. Chromosomes numbered according to original karyotype descriptions (see Fig. 1). Bar = 5 μm.

lus subtelocentric chromosomes (Fig. 6, Table 1). Only the Y chromosomes and two autosomal pairs (*C. tschudii* chromosomes 12, 13 and *C. porcellus* chromosomes 14, 18) were unique of each species karyotype (Table 1).

C bands

The chromosomal distribution of the C bands was similar in the karyotypes of the two species, being located preferentially in the centromeres and the short arms of the chromosomes (Fig. 4). However, the amount of constitutive heterochromatin was ap-

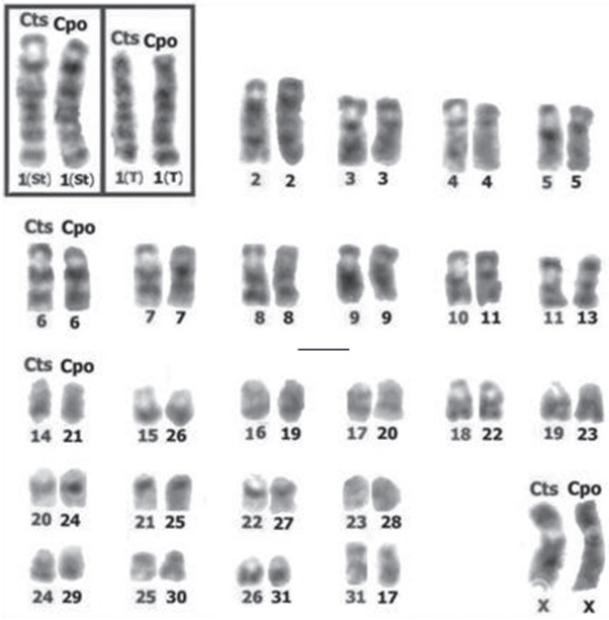

Figure 3. Chromosomes of *Cavia tschudii* (Cts) and *Cavia porcellus* (Cpo) with total G band correspondence. Cts chromosomes are at the left and Cpo at the right of each chromosomal group. Note that the long arms of subtelocentric (St) and telocentric (T) forms of pair 1 show total G band correspondence (inset). Chromosomes numbered according to original karyotype descriptions (see Fig. 1). Bar = 5 μm.

Table 1. Correspondence of *Cavia tschudii* and *Cavia porcellus* chromosomes according to their G band patterns[1].

Chromosomes with total G band correspondence																										
Cts	1	2	3	4	5	6	7	8	9	10	11	14	15	16	17	18	19	20	21	22	23	24	25	26	31	X
Cpo	1	2	3	4	5	6	7	8	9	11	13	21	26	19	20	22	23	24	25	27	28	29	30	31	17	X
Chromosomes with partial G band correspondence																										
Cts	27	28	29	30																						
Cpo	10	12	15	16																						
Unique species chromosomes																										
Cts	12	13			Y																					
Cpo			14	18		Y																				

[1]Chromosome numbers are the one of each species karyotype (see Fig. 1); in the same column chromosomes with total or partial G band correspondence. Cts = *Cavia tschudii*, Cpo = *Cavia porcellus*.

preciably greater in *C. tschudii* than in *C. porcellus*, spreading over most of the short arms in several subtelocentric chromosomes (Fig. 4a). The X chromosomes of both karyotypes, equal in size, morphology and G bands (Figs 1–3), showed a C^+ band in the paracentromeric region of the short arm (Fig. 4). Both Y chromosomes were completely heterochromatic, being larger the Y chromosome of *C. tschudii* than the *C. porcellus* one (Fig. 4).

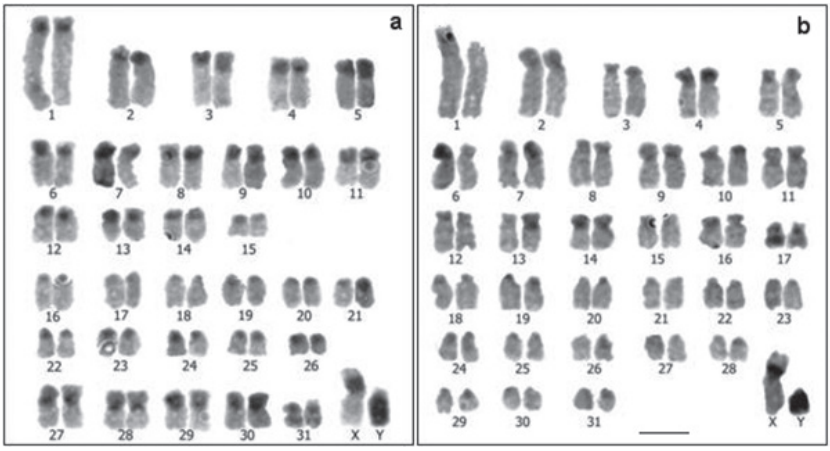

Figure 4. C-banded karyotypes: **a** *Cavia tschudii* male **b** *Cavia porcellus* male, showing heteromorphism for chromosome 1. Most of the chromosomes of both species were tentatively identified according size and morphology. Bar = 5 μm.

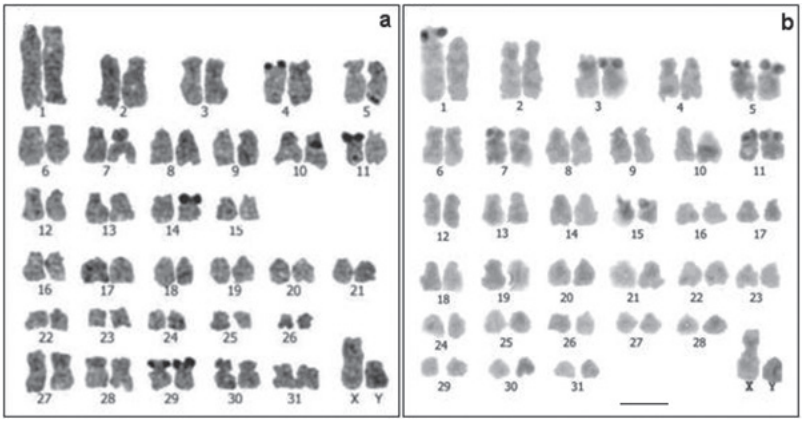

Figure 5. AgNOR-banded karyotypes: **a** *Cavia tschudii* male with four nucleolar chromosomal pairs (4, 11, 14 and 29) **b** *Cavia porcellus* male with five nucleolar chromosomal pairs (1, 3, 5, 7 and 11). The nucleolar chromosomes of both species were tentatively identified according to their size and morphology. Bar = 5 μm.

AgNOR bands

Multiple AgNOR bands were detected in the karyotypes of both species, consistently located in the telomeres of several chromosomal pairs (Fig. 5). The analysis of some AgNOR banded metaphases per species indicated that the number of AgNOR bands was different between the two species and also among the individuals. Thus, the results showed that in *C. porcellus* the mean and maximum numbers of chromosomes with active NORs (5.76 and 9.0, respectively) were higher than those of *C. tschudii* (4.13 and 7.0, respectively). Moreover, when we examined all the 3.072 chromosomes from 48 metaphases of both species, each of them having 64 chromosomes, we found a total

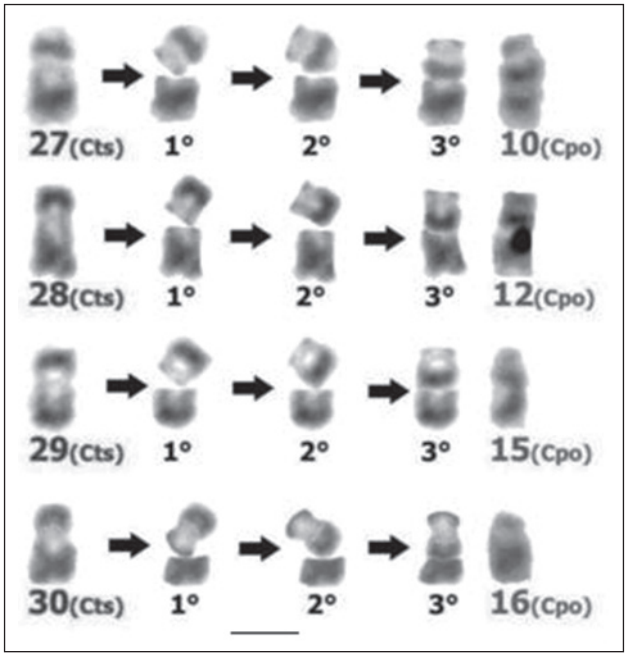

Figure 6. Rough simulation of the changes associated with the occurrence of pericentric inversions in *C. tschudii* chromosomes. Submetacentric *C. tschudii* chromosomes (Cts, first column at the left) that originate the subtelocentric *C. porcellus* chromosomes (Cpo, last column at the right): 1º) chromosomal break, 2º) rotation of the cleaved segment, 3º) rejoining and sealing with the original segment. Chromosomes numbered according to original karyotype descriptions (see Fig. 1). Bar = 5 μm.

of 237 AgNOR$^+$ sites, 138 of them located in *C. porcellus* chromosomes and 99 in *C. tschudii* chromosomes. Accordingly, the number of chromosomes bearing active NOR was significantly higher in the *C. porcellus* karyotype than in the *C. tschudii* one (χ^2 = 6.956; $p < 0.05$; df = 1).

Discussion

Cavia tschudii and *Cavia porcellus* diploid numbers (2n=64), previously described with basic cytogenetic techniques (Ohno et al. 1961, Fernández and Spotorno 1968, Dunnum and Salazar-Bravo 2006), were confirmed; nevertheless the fundamental number of autosomal arms (FNa = 100-102) were different to those reported before. For *C. porcellus*, Fernández and Spotorno (1968) described an FNa = 96, while for *C. tschudii*, Dunnum and Salazar-Bravo (2006) found an individual in the Bolivian Altiplano with an FNa which ranged from 104 to 108. The FNa variability of guinea pig species may be due to polymorphisms for the presence of short arms in the chromosomes described as subtelocentric or telocentric in these species. The polymorphism for chromosome 1 short arms detected previously for *C. cobaya* Pallas, 1766, a synonym of *C. porcellus,*

(Ohno et al. 1961, Schmid 1965, Zenzes et al. 1977) and for both species in this study, as well as the report of entirely heterochromatic short arms in the subtelocentric autosomes of *C. porcellus* (Bianchi and Ayres 1971), give support to such hypothesis.

The number and morphology of *C. tschudii* and *C. porcellus* chromosomes were similar to those reported for other subspecies and species of the genus *Cavia*. So, with the exception of *C. intermedia* Cherem, Olimpio, Ximenez, 1999, and a population of *C. magna* Ximenez, 1980, having 2n=62 (Gava et al. 1998, Cherem et al. 1999, Gava et al. 2012), for all of the other taxa of the genus the same 2n=64 diploid number has been described, although with different numbers of autosomal arms. An FNa=124 was recorded for *C. aperea pamparum* (George et al. 1972); FNa=116 for *C. aperea aperea* specimens from Pernambuco, Brasil (Maia 1984) and 114 for some individuals from the Bolivian lowlands (Dunnum and Salazar-Bravo 2006); FNa=124 for *C. magna* and *C. fulgida* (Pantaleão 1978) and 114 for *C. nana* Thomas, 1917 (*Cavia tschudii sodalis,* 1926) (Dunnum and Salazar-Bravo 2006).

The analysis of the C bands showed that although they had a similar distribution in the chromosomes of the two species, they were smaller in size in the autosomes and in the Y chromosome of *C. porcellus* than in the *C. tschudii* ones, suggesting that a loss of heterochromatin occurred during the domestication process. In accordance with this result, measurements of the genome sizes of 31 hystricognath rodent species (Gallardo et al. 2003) indicated that the genome of *C. tschudii* (9.1 pg) is larger than that of *C. porcellus* (8.2 ± 0.4 pg), having the first species the largest genome size among the 30 diploid species analyzed.

Five chromosomal pairs bearing NOR at the short arm telomeres were found by Zenzes et al. (1977) in the karyotype of the domestic *Cavia*, so being in agreement with our results. Using a double-staining procedure they could identify those chromosomes as numbers 1, 3, 9, 12 and 14 of the quinacrine banded stained karyotype. An accurate identification of the *C. porcellus* and *C. tschudii* nucleolar chromosomes described here would require the use of a similar double-staining procedure to allow the comparisons with other descriptions.

The differences in the number of AgNOR bands found between the two *Cavia* species analyzed here and among the individuals in each of them, confirmed the tendency to variability in NOR expression usually described for mammals. It has been proposed that this variability would depend mainly on the specific metabolic demands of cells and individuals (Mikelsaar et al. 1977, Mayr et al. 1987, Sánchez et al. 1989, Suzuki et al. 1990, Berríos et al. 1992, Zurita et al. 1997, Walker et al. 1999, Walker and Flores 2007). The greater number of AgNOR bands found in the *C. porcellus* karyotype than in the *C. tschudii* one would reveal a greater transcriptional activity of the ribosomal genes in the genome of the domestic form. One possible functional explanation of this result is that since *C. porcellus* has been selected for productive purposes, it would require higher rates of protein synthesis than the wild form. It should be mentioned that in a recent comparison of brain gene expression levels between four pairs of domestic/wild mammals, the largest differences were found between the domestic and wild guinea pigs, although *C. aperea*, genetically related to *C. tschudii*, was used as the wild guinea pig species (Albert et al. 2012).

Comparison of the G-banded karyotypes of the two *Cavia* species included in this study revealed that most of the autosomal pairs and the X chromosomes showed total G band correspondence, suggesting that these chromosomes constitute large syntenic blocks present in the common ancestor of both species and conserved over time. The differences in morphology and the partial G band correspondences detected between four chromosomal pairs of these two species, suggest that the four submetacentric chromosomes present only in the *C. tschudii* karyotype would have suffered pericentric inversions originating the four subtelocentric chromosomes of *C. porcellus* (Fig. 6, Table 1).

Cavia porcellus would be the domestic successor of *C. tschudii* from which it would have originated more than 4000 and possibly 7000 years ago (Wing 1986) by a process of domestication and artificial selection in the *C. tschudii* populations which inhabit southern Peru and northern Chile (Spotorno et al. 2007, Dunnum and Salazar-Bravo 2010). While being domesticated, those populations must have been small in size and with only a few individuals participating as parents in the reproductive process, which over many generations would have produced high levels of endogamy. These characteristics would have facilitated the fixation of the pericentric inversions which must have emerged spontaneously and frequently in the populations. Specifically, the heterozygotes for the pericentric inversions would have decreased their fertility since their gametes would be unbalanced as a result of crossing-over in the inverted segment (Coyne et al. 1993, King 1993). As a consequence, gene flow between the original homozygotes and the homozygotes for the inversion would have been reduced, originating genetic divergence between the two chromosomal forms.

Nevertheless, it has been demonstrated recently that the fertility of the inversion carriers is not always reduced (Muss and Schwanitz 2007). In some cases and depending on the size, genetic content, and chromosomal location of the inversion, the chromosomal inverted region pairs non-homologously with its normal partner forming a straight bivalent which does not present any loop, so causing crossing-over suppression (Torgasheva and Borodin 2010). If that is the case, the absence or reduced recombination between the inverted and non inverted genomic regions in the *Cavia* pericentric inversions, would be the cause of genetic divergence accumulation and reduction of gene flow between the two chromosomal forms, as it was proposed as a general model of speciation by several authors (Noor et al. 2001, Navarro and Barton 2003, Hoffman and Rieseberg 2008).

A critical assessment of reproductive isolation in crosses between *Cavia* species as previously reported, confronts the appropriate identification of specimens, the reliability of the taxonomy at the time, and the nature of the differences eventually found. For instance, crosses between *C. porcellus* and individuals from Arequipa, Perú identified as *C. cutleri* Bennett, 1836, which correspond to the original description of *C. cutleri* based on a single specimen from Ica, Peru (see Weir 1974), produced fertile offspring according to Castle (1916). By contrast, other wild specimens from Ica, Peru that also received the name *C. cutleri* by Tschudi in 1849, were finally renamed as *C. tschudii* by Fitzinger in 1867 (see Weir 1974); the latter is now the usually accepted name for the wild montane guinea pig (Woods and Kilpatrick 2005). Therefore, *C. cutleri* Bennet, 1838 is now considered a synonym of *C. porcellus* (Woods and Kilpatrick 2005). If

Castle in fact crossed *C. porcellus* laboratory animals with *C. cutleri* Bennet (= *C. porcellus*) specimens, in reality he might be doing intraspecific crosses, and the fertility of the descendants would be an expected result. In any case, the assignation of the individuals from Arequipa to *Cavia cutleri* Bennet was not well documented in that study, since it was based only on the smaller body size of those individuals with respect to domestic *C. porcellus* (Castle 1916). Moreover, he did not indicated the mating times taken by the crosses, neither the number of pairs in which crosses were attempted, reporting only that a large number of descendants were obtained (n=107), as many as those obtained in crosses within each form (n=108). In sum, if the chromosomal and nucleolar differences we are reporting here in molecularly identified specimens of *C. tschudii* and *C. porcellus* were also found in other populations, we predict that their eventual hybrids will show some degree of genomic incompatibility.

Reproduction of wild mammal species in captivity is a difficult and not always successful task. It is even more difficult to obtain descendants from crosses between different chromosomal races or species in the laboratory (Walker et al. 1984, 1999, Hauffe and Searle 1998, Castiglia and Capanna 2000, Franchini et al. 2008, Nunes et al. 2011). In crosses between phyllotine rodent species, we reported previously a decrease in the proportion of pairs with births and in the litter's size together with an increase in the time between mating and birth, compared to those registered for the intraspecific crosses (Walker et al. 1984, 1999). Although we repeatedly tried to cross our specimens of *C. tschudii* with *C. porcellus* in our laboratory, we have had no success yet.

If the analysis of the crosses realized between chromosomal races of *Mus* and *Sorex* rodents (Hauffe and Searle 1998, Castiglia and Capanna 2000, Franchini et al. 2008, Nunes et al. 2011) would have only considered the number of descendants obtained, it would not have been possible to reach conclusions about the fertility level of those hybrids. To estimate fertility, specific reproductive aspects must be studied, such as the success obtained in crosses between the parental forms and some hybrid characters, i.e.: the normality of their meiotic process, the histology of their gonads, the cell composition of their germinal line and the chromosomal constitution of the gametes that eventually they produce. In consequence, to evaluate the fertility level of eventual *C. tschudii* × *C. porcellus* hybrids, the reproductive characters just mentioned above must be analyzed in the descendants of crosses between individuals of the parental species taxonomically well identified. Specifically, the fertility of the heterozygotes for the pericentric inversions described here should be further investigated to evaluate the contribution of those chromosomal changes to the divergence of the two *Cavia* species.

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