Chromosomal studies in four species of genus *Chaunus* (Bufonidae, Anura): localization of telomeric and ribosomal sequences after fluorescence in situ hybridization (FISH)

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Abstract Fluorescence in situ hybridization (FISH) using telomeric and ribosomal sequences was performed in four species of toad genus *Chaunus*: *C. ictericus*, *C. jimi*, *C. rubescens* and *C. schneideri*. Analyses based on conventional, C-banding and Ag-NOR staining were also carried out. The four species present a 2n = 22 karyotype, composed by metacentric and submetacentric chromosomes, which were indistinguishable either after conventional staining or banding techniques. Constitutive heterochromatin was predominantly located at pericentromeric regions, and telomeric sequences $(TTAGGG)_n$ were restricted to the end of all chromosomes. Silver staining revealed Ag-NORs located at the short arm of pair 7, and heteromorphism in size of NOR signals was also observed. By contrast, FISH with ribosomal probes clearly demonstrated absence of any heteromorphism in size of rDNA sequences, suggesting that the difference observed after Ag-staining should be attributed to differences in chromosomal condensation and/or gene activity rather than to the number of ribosomal cistrons.

Keywords Ag-NORs · Amphibian cytogenetics · C-banding · *Chaunus* · FISH with ribosomal and telomeric probes · Karyotypes

Abbreviations
ITS   Interstitial telomeric sites
FISH   Fluorescence in situ hybridization
FN   Fundamental number
NORs   Nucleolar organizer regions

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Introduction

Substantial modifications on the classification of living amphibians were recently proposed by Frost et al. (2006) following an extensive study based on molecular and morphological characters. In order to recover historical relationships several taxonomic changes were implemented, rearranging many traditionally old standing groups like the former toad genus *Bufo*. Previously, *Bufo* was considered widely distributed in all continents except for Artic region, Australia, New Guinea and adjacent islands, and included about 258 species (Pramuk 2006). Frost et al. (2006) recovered *Bufo* as a paraphyletic taxon and then split it up into 17 formal generic entities. Presently, South American species are currently assembled in four genera: *Chaunus*, *Rhaebo* (*guttatus* group), *Rhinella* (*margaritifer* group) and *Nannophryne* (*variegatus* group). *Chaunus* encompasses 45 species that are widespread from Lower Rio Grande Valley region of southern Texas (USA) and southern Sonora (Mexico) south through tropical Mexico and Central America to southern South America (Frost 2007).

Karyotypes constituted of 22 biarmed chromosomes were reported for *Chaunus achalensis*, *C. arenarum*, *C. arequipensis*, *C. arunco*, *C. atacamensis*, *C. crucifer*, *C. dorbignyi*, *C. fernandezae*, *C. granulosus*, *C. marinus*, *C. poeppigii*, *C. pygmaeus*, *C. rubescens*, *C. rubropunctatus*, *C. schneideri* and *C. spinulosus* (King 1990; Kuramoto 1990; Piazze 1995; Kasahara et al. 1996; Baldissera et al. 1999; Azevedo et al. 2003).

Other bufonid genera as *Anaxyrus*, *Atelopus*, *Bufo*, *Capensibufo*, *Melanophryniscus*, *Nannophryne*, *Nectophrynoides*, *Ollotis*, *Rhinella*, *Schismaderma* and *Werneria* also have karyotypes with 22 biarmed chromosomes (King 1990; Kuramoto 1990). Exceptions to this pattern are found in some species of *Amietophrynus* that present three different diploid numbers: 2n = 20 and FN = 40 probably due to events of chromosomal fusions (Beckert and Doyle 1968; Bogart 1968; Bogart and Perret 1977), and 2n = 40 and 2n = 44, which were considered as polyploids (Bogart and Tandy 1976; King 1990; Kuramoto 1990). Bogart (1972) and Formas (1978) highlighted the chromosomal uniformity of *Chaunus* (*Bufo* at that time) and other bufonids and suggested that the first members of the genus that arrived in South America had a 2n = 22 karyotype and a secondary constriction at pair 7.

Despite a reasonable number of cytogenetic studies in amphibians, including *Chaunus*, the majority of them were exclusively based on description of diploid number and chromosomal morphology. Only at the end of the 1970's and during the 1980's, differential staining (e.g. localization of nucleolar organizer regions after silver staining–Ag-NORs-, and distribution of constitutive heterochromatin (mainly by C-banding) in amphibian chromosomes were initially employed.

In *Chaunus*, a single Ag-NOR-bearing pair was detected: pair 7 in *C. arenarum*, *C. crucifer*, *C. ictericus*, *C. marinus*, *C. rubescens* and *C. schneideri*, and pair 5 in *C. granulosus* and *C. pygmaeus* (Schmid 1978, 1980, 1982; Kasahara et al. 1996; Baldissera et al. 1999; Azevedo et al. 2003).

Chaunus species also share similar C-banding patterns either obtained after treatment with barium hydroxide or using fluorochromes. Constitutive heterochromatin was found at pericentromeric regions and on the telomeres (Schmid 1980; Piazze 1995; Kasahara et al. 1996; Azevedo et al. 2003).

In this paper, to further investigate karyotypic similarity in the genus, in addition to the conventional analyses with Giemsa, silver staining and C-banding in four species of *Chaunus* (*C. ictericus*, *C. jimi*, *C. rubescens* and *C. schneideri*), we also localized ribosomal and telomeric sequences by fluorescence in situ hybridization (FISH). The karyotype and banding patterns of *C. jimi* have never been previously reported, as well as the localization of telomeric and ribosomal sequences by FISH.

Material and methods

Cytogenetic analyses were carried out on eight individuals of four species of *Chaunus* collected from different Brazilian regions (Table 1).

Metaphases were obtained from blood cultures of *C. ictericus*, *C. rubescens*, *C. schneideri* and from one specimen of *C. jimi*, according to Schmid (1978) with modifications. Blood samples were obtained from the ventricule and 0.1 ml of blood was transferred to a flask containing 4 ml of RPMI medium, 1 ml of fetal calf serum, 250 μl of phytohemagglutinin and 50 μl of heparin. Cells were cultivated for 3–5 days at 26°C and then harvested after a treatment with 50 μl of colchicine 0.01% during one hour. Chromosomes were also obtained from bone marrow, liver and spleen of a single individual of *C. jimi* (Schmid 1978) and from testis (Schmid 1978; Bogart 1973).

Chromosome analyses were done after Giemsa staining, C-banding (Sumner 1972), Ag-NOR staining (Howell and Black 1980) and for localization of telomeric sequences after FISH; digoxigenin-labeled (TTAGGG)$_n$ was used as a probe following the Oncor's protocol (catalog number P5097-DG.5). Hybridization signals were detected by incubation with fluorescein isothiocyanate (FITC)-labeled anti-digoxigenin and the slides were counterstained with propidium iodide in antifade solution. The ribosomal probe HM 456 was biotin-labeled by nick-translation according to the manufacturer's (Life Technologies). Chromosome signals were visualized using a Zeiss Axiophot microscope equipped with a FITC filter and photographed using Ektachrome 400 (Kodak) color slide film.

Table 1 Species, number of individuals, sex and localities of *Chaunus* studied in this work

| Species	Sex	Locality
Chaunus ictericus	3 males	Carapicuíba, São Paulo state (23°31′S, 46°50′W)
Chaunus jimi	2 females	Ibiraba, Bahia state (10°47′ S, 42°49′W)
Chaunus rubescens	2 males	Santa Bárbara, Minas Gerais state (19°57′S, 43°24′W)
Chaunus schneideri	1 female	Lagoa Santa, Minas Gerais state (19°37′S, 43°53′W)

Results

Conventional staining

Chaunus ictericus, *C. jimi*, *C. rubescens* and *C. schneideri* share similar karyotypes with 2n = 22 and FN = 44. Pairs 2, 3, 4 and 6 are submetacentrics and the remaining pairs are metacentrics (Fig. 1).

The four species exhibited a secondary constriction at the short arm of one or both homologues of pair 7 (108 and 260 metaphases, respectively) (Table 2). Furthermore, the secondary constriction exhibited heteromorphism in size in 133 out of 432 metaphases analyzed (Table 2, Fig. 2).

Meioses of *C. rubescens* and *C. ictericus* revealed 11 bivalents with terminal chiasmata in metaphase I, and 11
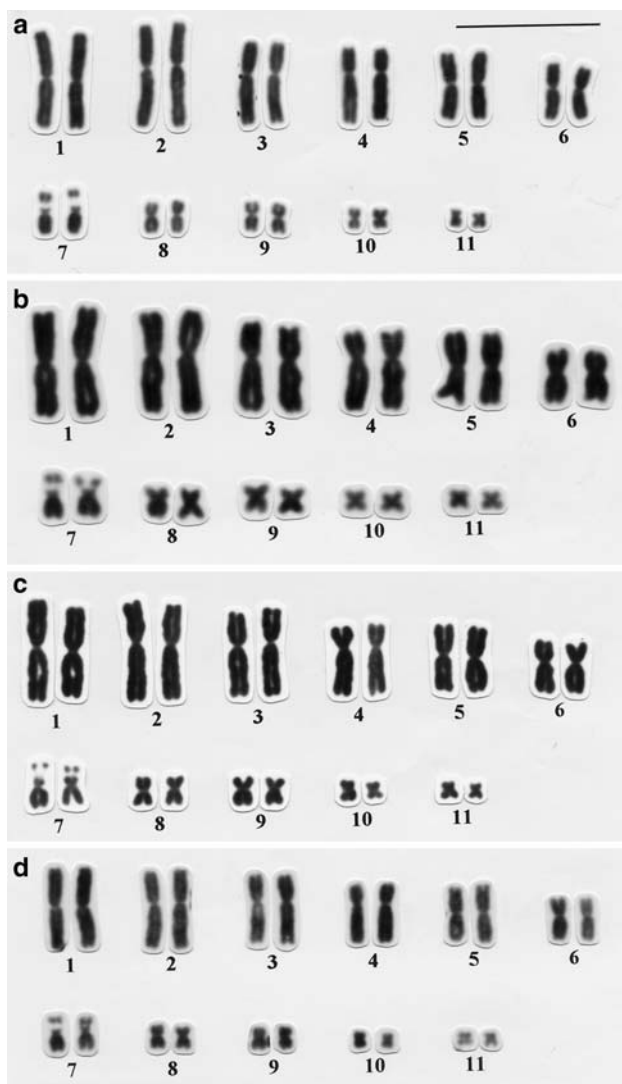
Table 2 Number of metaphases analyzed after conventional staining and occurrence of secondary constriction in *Chaunus*

Species	Specimen	Number of constrictions			
		None	One	Two	
				Homomorphic	Heteromorphic
C. ictericus	AF 270	22	5	88	–
	AF 271	10	7	5	–
	AF 311	–	3	12	29
C. jimi	AF 84	17	1	–	–
	AF 85	10	48	–	49
C. rubescens	AF 387	–	3	10	6
	AF 388	5	5	4	1
C. schneideri	AF 320	–	36	8	48
	Total	64	108	127	133

chromosomes in metaphase II (Fig. 3). Heteromorphic chromosomes were not found neither in mitotic nor in meiotic analyses.

Localization of Ag-NORs and rDNA after FISH

All species presented the secondary constriction at the short arm of pair 7 which corresponds to the Ag-NOR sites (Figs. 2 and 4). Although Ag-NORs were always present in pair 7, one or both homologues were positively stained in all species (Table 3); in the last case, heteromorphism in Ag-NORs size was also detected in a variable frequency (Table 3). In some metaphases the size of one Ag-NOR was clearly duplicated (Fig. 4d). None of the 458 metaphases exhibited increasing in size of Ag-NORs of both homologues simultaneously (Table 3).
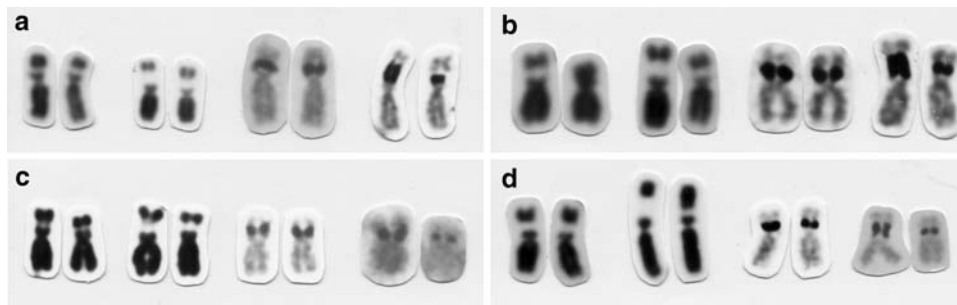
FISH with HM 456 ribosomal probe evidenced signals at the short arm of each homologue of pair 7 and no heteromorphism in size was detected, even when heteromorphic constrictions were observed in DAPI (Fig. 5).

C-banding

Chaunus ictericus, *C. jimi*, *C. rubescens* and *C. schneideri* showed similar pattern of constitutive heterochromatin at the pericentromeric regions of all pairs. Faintly stained interstitial C-bands were also detected in some chromosomes (Fig. 6).

FISH with telomeric probe

The sequence $(TTAGGG)_n$ was detected on the telomeric regions of all chromosomes of the four species (Fig. 7). The telomeric sequences are relatively short and in some cases the signals visualization was tenuous.

Fig. 1 Conventional stained karyotypes of *Chaunus* species with 2n = 22. (**a**) *C. ictericus*. (**b**) *C. jimi*. (**c**) *C. rubescens*. (**d**) *C. schneideri*. Note the presence of an interstitial secondary constriction at the short arm of pair 7 in all metaphases. Bar = 10 μm

Fig. 2 Localization of secondary constriction and NORs in short arm of pair 7 in four metaphases of each *Chaunus* species. (**a**) *C. ictericus.* (**b**) *C. jimi.* (**c**) *C. rubescens.* (**d**) *C. schneideri.* Homomorphic constriction, heteromorphic constriction, homomorphic NORs and heteromorphic NORs in **a, c** and **d**; constriction in a single homologue, heteromorphic constriction, homomorphic and heteomorphic NORs in **b**

Fig. 3 Meiotic cells in metaphase I (11 bivalents) (**a**) and (**c**) and metaphase II (n = 11) (**b**) and (**d**) in *Chaunus.* (**a**) and (**b**) *C. rubescens.* (**c**) and (**d**) *C. ictericus*
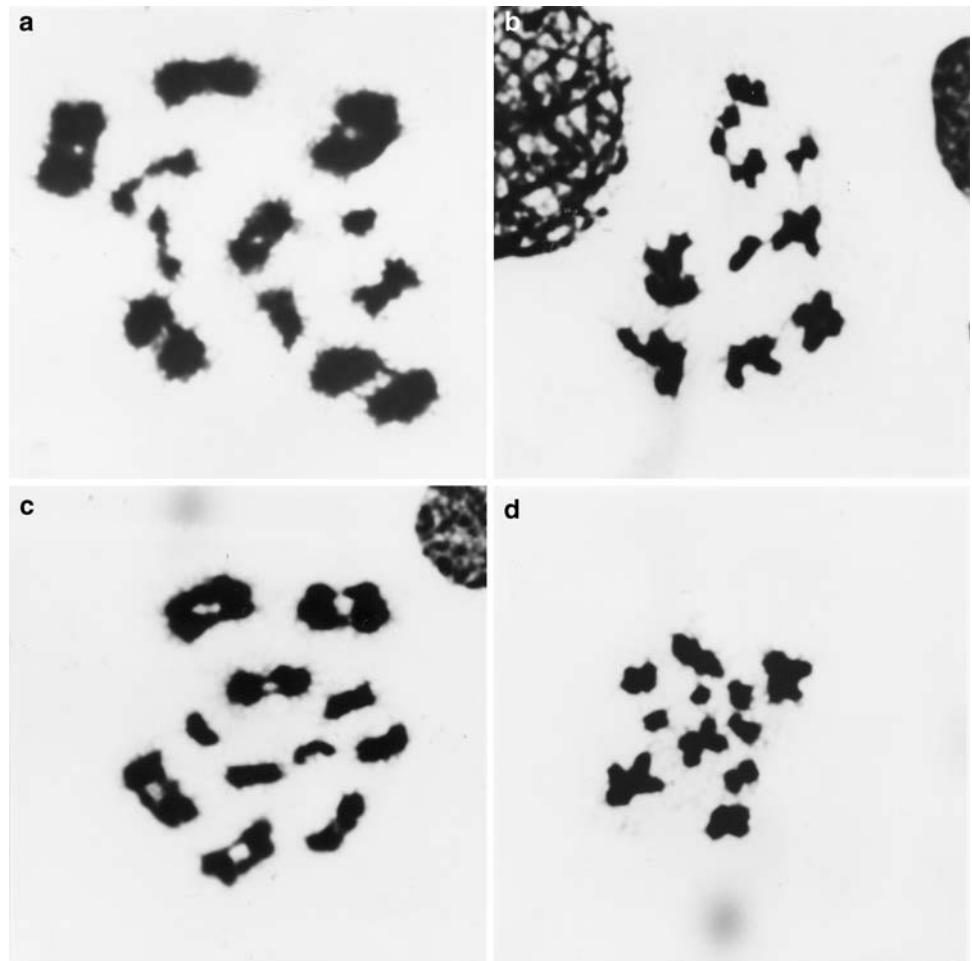
Discussion

Chaunus ictericus, *C. jimi*, *C. rubescens* and *C. schneideri* have indistinguishable karyotypes after conventional and differential staining (C-banding and Ag-NORs) similarly to those previously reported (Beçak 1968; Morescalchi and Gargiulo 1968; Bogart 1972; Brum-Zorilla and Saez 1971; Kasahara et al. 1996; Baldissera et al. 1999; Azevedo et al.

2003). Application of different staining techniques generally provides a better characterization of the anuran karyotypes, and the identification of species-specific patterns. For instance, Schmid (1978, 1980) recognized three distinct types of constitutive heterochromatin for 15 anuran species from eight genera, including some bufonids after quinacrine staining and C-banding: (i) positive C-bands, rich in AT; (ii) positive C-bands rich in GC and (iii) tenuous telomeric

Fig. 4 Silver stained metaphases of *Chaunus* showing the localization of NORs (arrows). (**a**) *C. ictericus.* (**b**) *C. jimi.* (**c**) *C. rubescens.* (**d**) *C. schneideri.* Note the presence of heteromorphic NORs in **d**
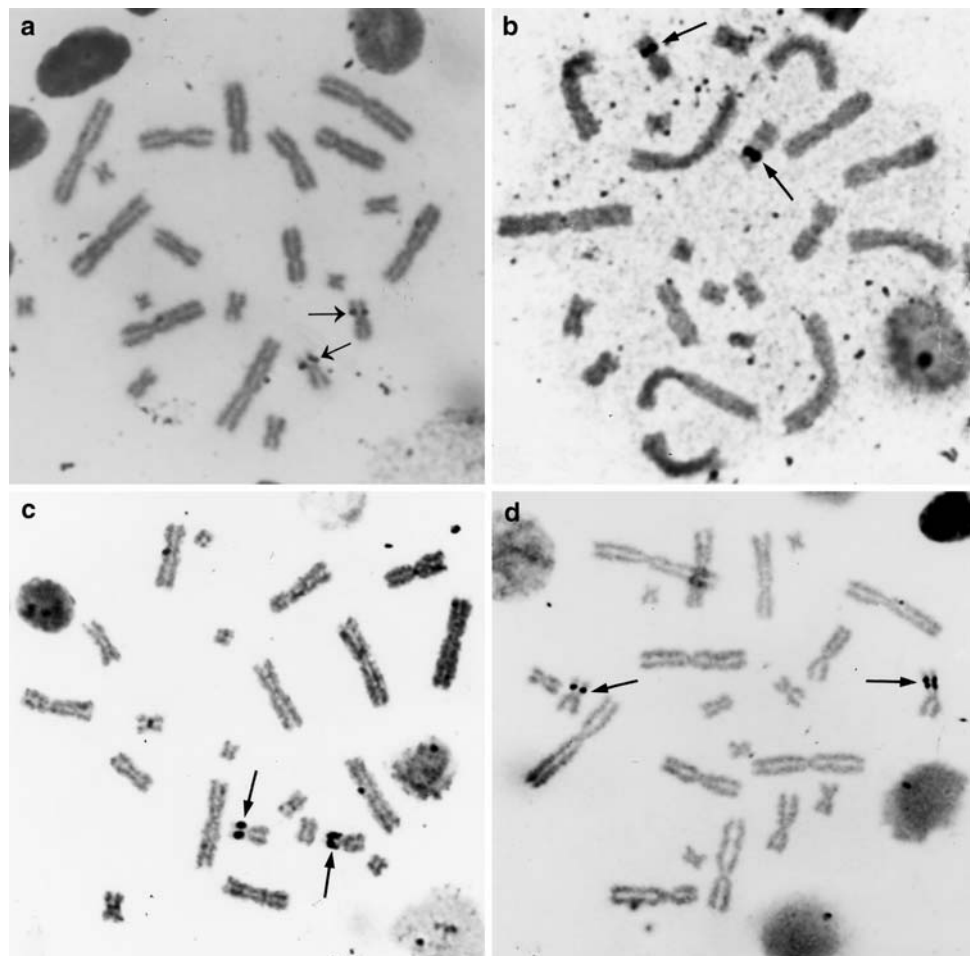
Table 3 Number and size of NORs detected on the short arm of pair 7 in *C. ictericus, C. jimi, C. rubescens* and *C. schneideri*

| Species	Specimen	r (%)	rr (%)	Rr (%)	Total
C. ictericus	AF 270	–	46 (93.9)	3 (6.1)	49
	AF 271	3 (17.6)	6 (35.3)	8 (47.1)	17
	AF 311	–	9 (25)	27 (75)	36
C. jimi	AF 84	23 (28.1)	7 (8.5)	52 (63.4)	82
	AF 85	–	40 (28.0)	103 (72.0)	143
C. rubescens	AF 387	–	18 (69.2)	8 (30.8)	26
	AF 388	–	18 (66.7)	9 (33.3)	27
C. schneideri	AF 320	–	13 (16.7)	65 (83.3)	78

r: a single NOR; rr: homomorphic NORs; Rr: heteromorphic NORs

C-bands. All species analyzed showed association between heterochromatin and Ag-NORs, and in some cases, patterns of distribution of heterochromatin were species-specific. Matsui et al. (1985) recognized six karyotypes of *Bufo bufo* species complex based on pattern of distribution of constitutive heterochromatin and Miura (1995) distinguished two distinctive karyotypes in *Bufo japonicus japonicus* also using C-banding patterns, despite the uniformity of those karyotypes after conventional staining.

C. ictericus, C. jimi, C. rubescens, and *C. schneideri* have C-bands distributed at the pericentromeric region of all chromosomes, exactly as observed by Kasahara et al. (1996) and Azevedo et al. (2003) for individuals of *C. ictericus* and *C. schneideri* from Botucatu, Jundiaí and Rio Claro (São Paulo state, Brazil). However the region of the short arm of pair 7 that corresponds to the Ag-NORs was not evidenced as positive C-band, in opposition to the observation of Kasahara, Silva and Haddad (1996) and Azevedo et al. (2003). In this case, the pattern of distribution of constitutive heterochromatin was quite conservative and none of the species here analyzed showed a species-specific pattern of C-bands.

Fig. 5 FISH with ribosomal probe HM456 (**a**, **c** and **e**) and DAPI staining (**b**, **d** and **f**). (**a** and **b**) *C. ictericus.* (**c** and **d**) *C. jimi.* (**e** and **f**) *C. schneideri.* Homomorphic NORs in **a**, **c** and **e** and homomorphic constrictions in **b** and heteromorphic constriction in **d** and **f**
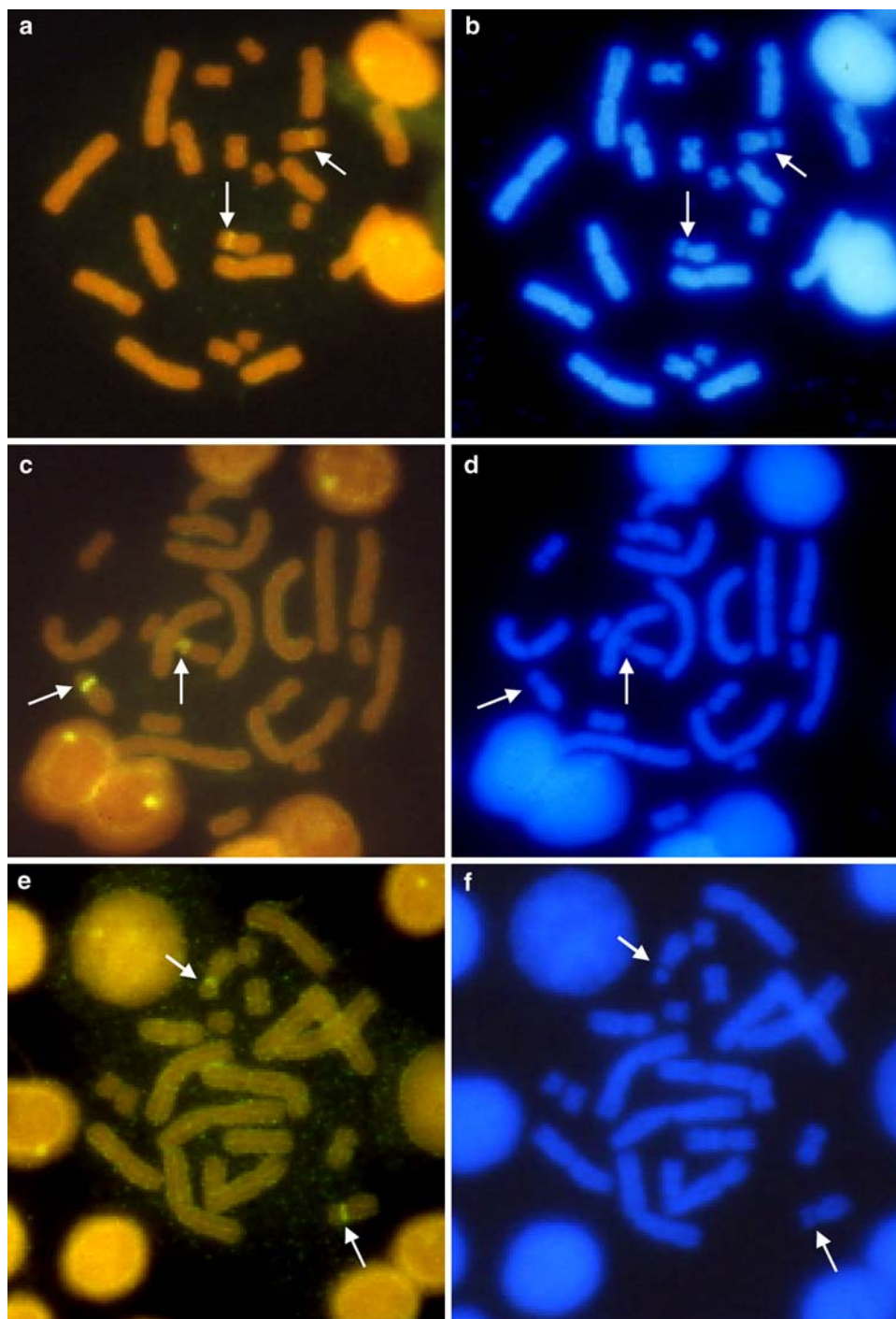
The telomeric sequence $(TTAGGG)_n$ is widely conservative among vertebrates (Meyne et al. 1989). Besides the telomeric pattern, many vertebrate species showed interstitial telomeric sites (ITS), and in many cases the presence of these sequences was correlated with events of chromosomal rearrangements (Meyne et al. 1990). Thereby, the localization of telomeric sequences has been used in studies of karyotype evolution in several species groups (Scherthan 1990; Lee et al. 1993; Schmid et al. 1994;

Vermeesch et al. 1996; Svartman and Vianna-Morgante 1998; Qumsiyeh et al. 1997; Garagna et al. 1997; Fagundes et al. 1997; Fagundes et al. 1997; Silva and Yonenaga-Yassuda 1997, 1998a, b).

In amphibians, different patterns of distributions of telomeric sequences were reported. In *Anaxyrus woodhousei* and *A. terrestris* (Bufonidae), *Eleutherodactylus euphronides* and *Craugastor maussi* (Brachycephalidae), *Gastrotheca espeletia* (Amphignathodontidae), *Leptodactylus ocellatus,*
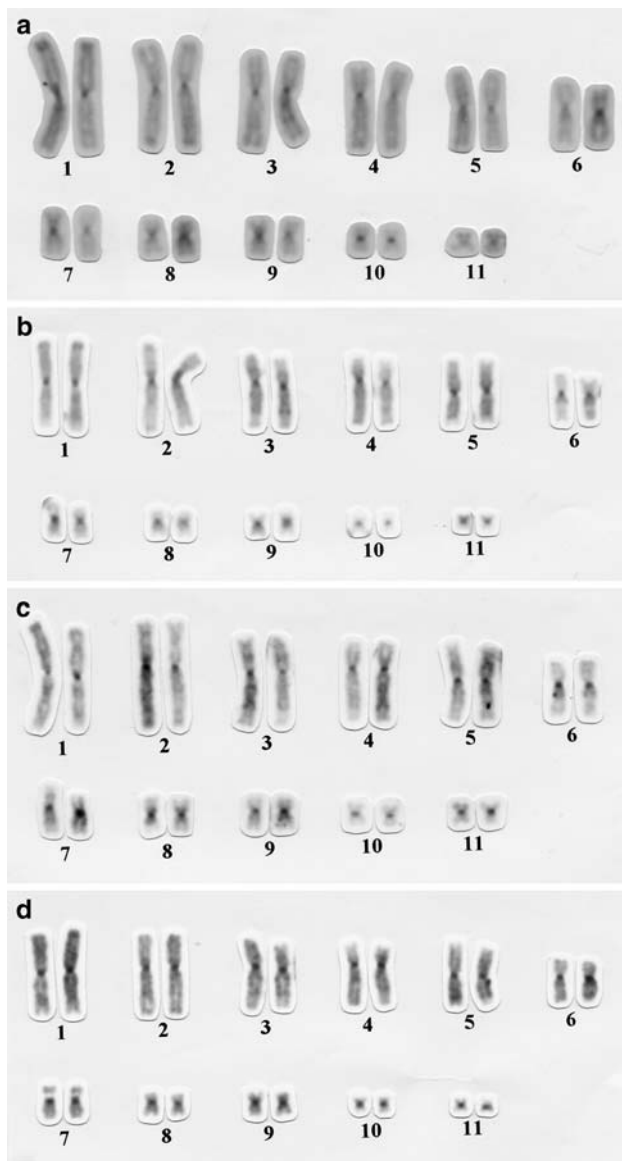
Fig. 6 Pattern of distribution of C-bands in *Chaunus*. (**a**) *C. ictericus*. (**b**) *C. jimi*. (**c**) *C. rubescens*. (**d**) *C. schneideri*

L. knudseni and *L. pentadactylus* (Leptodactylidae) exclusively telomeric signals were observed (Meyne et al. 1990; Foote et al. 1991; Schmid et al. 2002a, b, c; Amaro-Ghilardi et al. 2004). In *Xenopus laevis* (Pipidae) a telomeric pattern besides a ITS in a single chromosome pair was detected and in *Eleutherodactylus riveroi* (Brachycephalidae) two chromosomes with ITS were observed in addition to the telomeric signals (Meyne et al. 1990; Schmid et al. 2003). In five species of *Hyla* (*H. versicolor*, *H. chrysoscelis*, *H. femoralis*, *H. squirella*, *H. cinerea*), *Pseudacris nigrita* and *P. brimleyi* (Hylidae) many ITS in several chromosomes were observed, despite the conservative diploid number (2n = 24) of all species (Meyne et al. 1990; Wiley et al. 1992).

In opposition to the ITS patterns observed in hylids, the species of genus *Chaunus* herein reported showed only a telomeric localization of the (TTAGGG)$_n$ sequence. In fact, if the presence of interstitial telomeric sequences is indeed an evidence of chromosomal rearrangements during the differentiation of karyotypes, ITS were not supposed to be expected in the species of *Chaunus*, because these toads are characterized by a highly conservative diploid number and chromosomal morphology.

Chaunus ictericus, *C. jimi*, *C. rubescens* and *C. schneideri* showed a single Ag-NOR bearing pair 7, with positive signals on its short arms despite the variation in the number and size of Ag-NORs observed. An identical pattern of localization of NORs were previously described for individuals of *Chaunus ictericus*, *C. rubescens* and *C. schneideri* from Botucatu, Corumbataí, Jundiaí, Ribeirão Branco, Rio Claro (São Paulo state) and Brasília, Brazil (Kasahara et al. 1996; Baldissera et al. 1999; Azevedo et al. 2003)

Only one pair of NORs was detected in many species of bufonids and hylids after silver staining (Schmid 1978). Furthermore, high frequency of heteromorphism in size of Ag-NORs was observed, due to probable *in tandem* duplications of ribosomal sequence. Schmid (1982), after studying 260 individuals from 23 anuran genera, observed that Ag-NORs were present in a single pair of chromosomes in the majority of species, regardless their phylogenetic position. He also noticed variation of Ag-NOR size in 67% of the individuals, which was argued as a result of probable *in tandem* duplications or triplications. According to the author, duplications were always observed in only one homologue, and the heteromorphism was related to unequal meiotic crossovers, sister chromatid exchanges or errors during DNA duplication.

The specific localization of ribosomal DNA after FISH has been used in addition to the Ag-NORs in different groups of organisms. Porter et al. (1991) investigated the localization of rDNA in 16 species of lizards and four of snakes and they observed variation in the localization of these sequences even in cases in which karyotypes were totally similar. They concluded that throughout the evolutionary process of the squamates, the organization of rDNA in the genome has changed substantially although the morphology of the karyotypes had been kept. In salmonid fishes, Fujiwara et al. (1998) corroborate the occurrence of NOR heteromorphisms in *Oncorhynchus myskiss* and *Hucho perryi* after FISH with ribosomal probe. Lourenço et al. (1998) detected the occurrence of NOR polymorphism in *Physalaemus petersi* after silver staining and FISH with ribosomal probe.

Contrarily to the *in tandem* duplications firstly hypothesized by Schmid (1978, 1982), our results indicate that the heteromorphism of Ag-NORs-size in the short arm of pair

Fig. 7 Distribution of telomeric sequence (TTAGGG)$_n$ after FISH in *Chaunus*, showing an exclusively telomeric pattern. (**a**) *C. ictericus*. (**b**) *C. jimi*. (**c**) *C. rubescens*. (**d**) *C. schneideri*
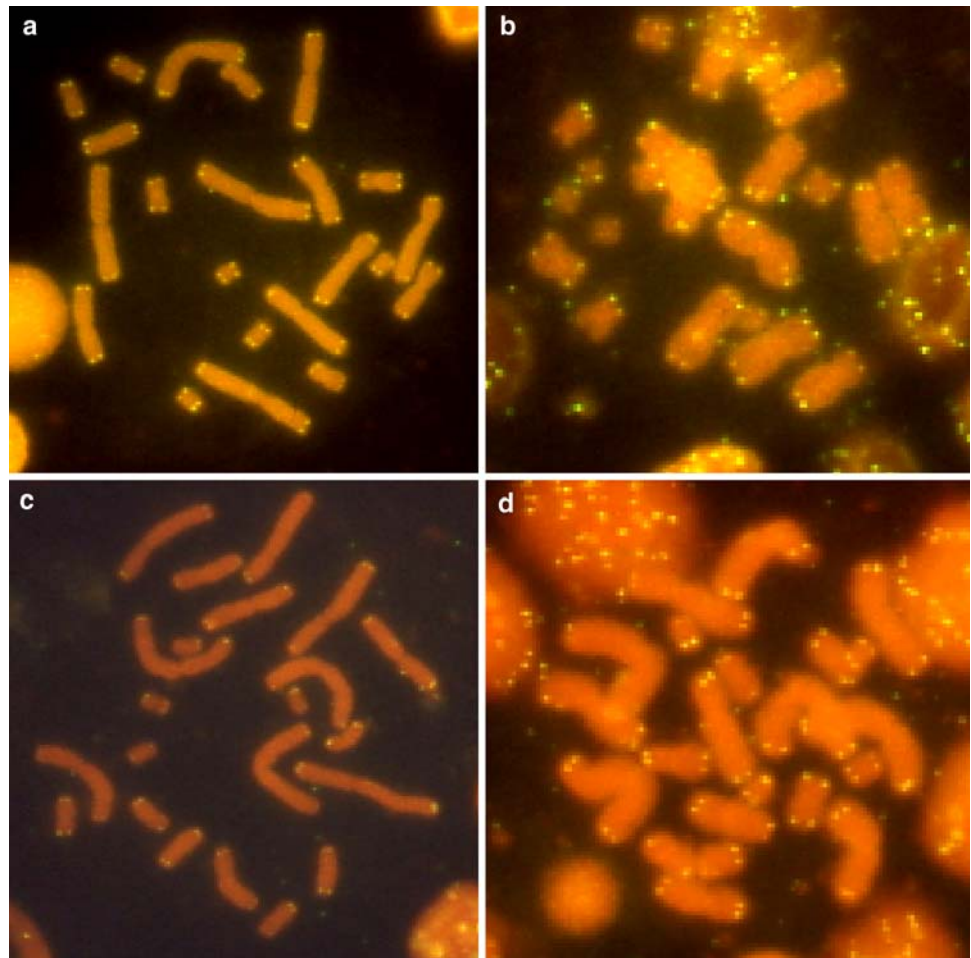
7 either in conventional or silver staining is probably due to a difference in activity of ribosomal cistrons and/or chromosomal condensation, since FISH with ribosomal probes evidenced signals with the same size. If duplications had been occurred, ribosomal regions of *Chaunus* should present differences in size of signals after FISH with ribosomal probe, as observed by Fujiwara et al. (1998).

Based on our results, we could emphasize that genus *Chaunus* is a good example of conservative karyotype among anuran amphibians. The morphological differentiation of these species does not seem to be related to the structural or morphological chromosome modifications on the basis of distribution of constitutive heterochromatin, localization of Ag-NORs and distribution of telomeric and ribosomal sequences in the karyotypes.

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