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Molecular phylogeny and karyotype differentiation in *Paratelmatobius* and *Scythrophrys* (Anura, Leptodactylidae)

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Abstract *Paratelmatobius* and *Scythrophrys* are leptodactylid frogs endemic to the Brazilian Atlantic forest and their close phylogenetic relationship was recently inferred in an analysis that included *Paratelmatobius* sp. and *S. sawayae*. To investigate the interspecific relationships among *Paratelmatobius* and *Scythrophrys* species, we analyzed a mitochondrial region (approximately 2.4 kb) that included the ribosomal genes 12S and 16S and the tRNAval in representatives of all known localities of these genera and in 54 other species. Maximum parsimony inferences were done using PAUP* and support for the clades was evaluated by bootstrapping. A cytogenetic analysis using Giemsa staining, C-banding and silver staining was also done for those populations of *Paratelmatobius* not included in previous cytogenetic studies of this genus in order to assess their karyotype differentiation. Our results suggested *Paratelmatobius* and *Scythrophrys* formed a clade strongly supported by bootstrapping, which corroborated their very close phylogenetic relationship. Among the *Paratelmatobius* species, two clades were identified and corroborated the groups *P. mantiqueira* and *P. cardosoi* previously proposed based on morphological characters. The karyotypes of *Paratelmatobius* sp. 2 and *Paratelmatobius* sp. 3 described here had diploid chromosome number 2n = 24 and showed many similarities with karyotypes of other *Paratelmatobius* representatives. The cytogenetic data and the phylogenetic analysis allowed the proposal/corroboration of several hypotheses for the karyotype differentiation within *Paratelmatobius* and *Scythrophrys*. Namely the telocentric pair No. 4 represented a synapomorphy of *P. cardosoi* and *Paratelmatobius* sp. 2, while chromosome pair No. 5 with interstitial C-bands could be interpreted as a synapomorphy of the *P. cardosoi* group. The NOR-bearing chromosome No. 10 in the karyotype of *P. poecilogaster* was considered homeologous to chromosome No. 10 in the karyotype of *Scythrophrys* sp., chromosome No. 9 in the karyotype of *Paratelmatobius* sp. 1, chromosome No. 8 in the karyotypes of *Paratelmatobius* sp. 2 and of *Paratelmatobius* sp. 3, and chromosome No. 7 in the karyotype of *P. cardosoi*. A hypothesis for the evolutionary divergence of these NOR-bearing chromosomes, which probably involved events like gain in heteochromatin, was proposed.

Keywords Chromosome evolution · Nucleolar organizing region · Heterochromatin

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Introduction

Paratelmatobius B. Lutz and Carvalho, 1958, and *Scythrophrys* Lynch, 1971, are genera of leptodactylid frogs endemic to the Brazilian Atlantic forest. Currently, five species are recognized in the genus *Paratelmatobius* and are separated into two groups based on morphological characteristics (Pombal Jr. and Haddad 1999). The *P. cardosoi* group contains *P. cardosoi* Pombal Jr. and

Haddad, 1999, and *P. mantiqueira* Pombal Jr. and Haddad, 1999, whereas *P. gaigeae* (Cochran 1938), *P. lutzii* B. Lutz and Carvalho, 1958, and *P. poecilogaster* Giaretta and Castanho, 1990, form the *P. lutzii* group. In a recent study, an undescribed species of the *P. cardosoi* group was found at Piraquara, in the Serra do Mar of Paraná State, Brazil (Lourenço et al. 2003a). All known *Paratelmatobius* species have a limited distribution and occur in small, specific microhabitats in the Atlantic forest in the Serra do Mar and Serra da Mantiqueira (reviewed by Pombal Jr. and Haddad 1999). The genus *Scythrophrys* is currently monotypic and specimens of *S. sawayae* have been found in the Serra do Mar in the states of Paraná and Santa Catarina (see Frost 2006). An undescribed species of this genus was also reported based on chromosome data analysis (Lourenço et al. 2003b).

Similarities between *Paratelmatobius* and *Scythrophrys* have been mentioned by some authors (Lynch 1971; Heyer 1975; Garcia 1996), but only recently the close phylogenetic relationship between them was proposed by Frost et al. (2006). In this phylogenetic study, morphological and specially molecular data were used and the genera *Paratelmatobius* and *Scythrophrys* were represented by one specimen of *Paratelmatobius* sp. and one of *S. sawayae*.

In contrast, the relationships of these genera with other leptodactylids remain unclear. In 1986, Laurent formally recognized a group referred to as Cycloramphinae created to accommodate genera *Crossodactylus*, *Crossodactylodes*, *Cycloramphus*, *Hylodes*, *Megaelosia*, *Paratelmatobius*, *Rupirana*, *Scythrophrys*, *Thoropa* and *Zachaenus*. Heyer (1975) previously allocated this group to the informal taxonomic unit Grypiscine. However, the monophyly of this group was rejected by Frost et al. (2006) and Grant et al. (2006). According to Frost et al. (2006), this group consisted of three distantly related clades and *Paratelmatobius* + *Scythrophrys* was one of them. These authors also suggested a close relationship between the *Paratelmatobius* + *Scythrophrys* clade and *Leptodactylus*, although this hypothesis was only weakly supported by Bremer values of branch support. These results and the close relationship of these genera with *Pseudopaludicola* and *Pleurodema* + *Edalorhina* + *Physalaemus* supported a taxonomic reorganization that allowed the inclusion of *Paratelmatobius* and *Scythrophrys* in the family Leptodactylidae Werner, 1896 (1838) (Frost et al. 2006). This taxon corresponded to the previous subfamily Leptodactylinae (*sensu* Frost et al. 2004), with the exclusion of *Limnomedusa* and inclusion of *Paratelmatobius*, *Scythrophrys* and, provisionally, *Somuncuria*. Grant et al. (2006) also found a clade composed of *Paratelmatobius* + *Scythrophrys* + *Leptodactylus* that was distantly related to *Physalaemus* + *Edalorhina* + *Pleurodema* + *Pseudopaludicola*. Based on these data, *Paratelmatobius*, *Scythrophrys*, *Leptodactylus* and *Hydrolaetare* were grouped in the Leptodactylidae while the other genera, also considered as leptodactylids by Frost et al. (2006), were grouped in the Leiuperidae.

Cytogenetically, the species of *Paratelmatobius* and *Scythrophrys* show several similarities. All of them have a diploid number of 2n = 24 and the similarity among their karyotypes led to the suggestion of some interspecific homeologies (Lourenço et al. 2003a,b). An interstitial C-positive heterochromatic band in chromosome pair No. 1 is shared by all of the species studied to date. A nucleolar organizer region (NOR) situated on a small metacentric/submetacentric chromosome in *P. poecilogaster* and in karyotype II of *Scythrophrys* (*Scythrophrys* sp.) may be another homeology shared by species of these genera. This NOR is considered to be a plesiomorphic character relative to the other NOR locations present in the karyotypes of *Paratelmatobius* (in pairs Nos. 7 and 8).

In this study, we sampled all known localities of *Paratelmatobius* and *Scythrophrys* to assess their mutual interspecific relationships and with 54 species from other genera by analyzing a mitochondrial region (approximately 2.4 kb) that included the ribosomal genes 12S and 16S and the tRNAval. A cytogenetic analysis was also perfomed for those populations of *Paratelmatobius* not included in previous cytogenetic studies of this genus (Lourenço et al. 2000, 2003b) in order to assess their karyotype differentiation trends.

Material and methods

Phylogenetic analyses

Taxon sampling

All known localities of *Paratelmatobius* and *Scythrophrys* were sampled. Specimens of *Paratelmatobius* collected at Piraquara in Paraná State, Brazil, with the karyotype described by Lourenço et al. (2003b), were designated as *Paratelmatobius* sp. 1 (aff. *cardosoi*). Specimens of *Paratelmatobius* from Mogi das Cruzes, São Paulo State, Brazil and those from Parque Estadual Carlos Botelho, São Paulo State were referred to as *Paratelmatobius* sp. 2 and *Paratelmatobius* sp. 3, respectively. The localities and the number of specimens analyzed are shown in Table 1. The descriptions of the new species mentioned here are being prepared by one of the authors (CFBH) and other colleagues.

Our analyses also included many other species from the family Leptodactylidae, as well as species from the families Brachycephalidae, Ceratophryidae, Cycloramphidae and Leiuperidae, that were until recently included or

Table 1 Localities of the specimens and original source of the mtDNA sequences used in the phylogenetic analysis

Family/ subfamily	Species	Locality	Voucher Number	Original source of the sequences (GenBank accession number)
Leptodactylidae	*Paratelmatobius poecilogaster*	Brazil: Paranapiacaba, SP	CFBH3251, CFBH3253,**	This paper
	Paratelmatobius poecilogaster	Brazil: São Luiz do Paraitinga, SP	CFBH7731	This paper
	Paratelmatobius cardosoi	Brazil: Paranapiacaba, SP	CFBH3264-3265, CFBH3267	This paper
	Paratelmatobius gaigeae	Brazil: Município de Bananal, SP	CFBH7156	This paper
	Paratelmatobius sp. 1 (aff. *cardosoi*)	Brazil: Piraquara, PR	CFBH240	This paper
	Paratelmatobius sp. 2	Brazil: Mogi das Cruzes, SP	CFBH6469-6470	This paper
	Paratelmatobius sp. 3	Brazil: Pq. Estadual Carlos Botelho, São Miguel Arcanjo, SP	CFBH5448	This paper
	Scythrophrys sawayae	Brazil: Piraquara, PR	CFBH241, CFBH6072	This paper
	Scythrophrys sawayae	Brazil: São Bento do Sul, SC	CFBH3185	This paper
	Scythrophrys sp.	Brazil: Rancho Queimado, SC	BC4627, BC4628	This paper
	Leptodactylus (Lithodytes) sp.	Ecuador		Santos et al. (2003) (AY364538)
	Leptodactylus (Lithodytes) lineatus	Brazil: Apiacas		Darst and Cannatella (2004) (AY326012)
	Leptodactylus discodactylus	Ecuador		Frost et al. (2006) (DQ283433)
	Leptodactylus fuscus	Guyana		Frost et al. (2006) (DQ283404)
	Leptodactylus ocellatus	Argentina		Faivovich et al. (2005) (AY843688)
	Leptodactylus pentadactylus	Costa Rica: Río Pentencia, 2 mi N Tortuguero, Limón		Darst and Cannatella (2004) (AY326017)
Leiuperidae	*Edalorhina perezi*	Brazil: REAJ, AC	ZUEC9623	This paper
	Engystomops coloradorum	Ecuador: Pichincha		Ron et al. (2005) (AY834181 and AY834182)
	Engystomops guayaco	Ecuador: Guayas		Ron et al. (2005) (AY834172-AY834176)
	Engystomops montubio	Ecuador: Guayas		Ron et al. (2005) (AY834178)
	Engystomops pustulatus	Ecuador: Manabí		Ron et al. (2005) (AY834183)
	Engystomops pustulatus	Ecuador: Guayas		Ron et al. (2005) (AY834184)
	Engystomops randi	Ecuador: Guayas		Ron et al. (2005) (AY834179 and AY834180)
	Engystomops sp.	Brazil: Restauração, AC	ZUEC9652	This paper
	Engystomops sp.	Brazil: Foz do Rio Tejo, AC	ZUEC9641	This paper
	Eupemphix nattereri	Brazil: Luiz Antonio, SP		Darst and Cannatella (2004) (AY326020)
	Pleurodema brachyops	Guyana: Southern Rupununi Savanna, Aishalton		Faivovich et al. (2005) (AY843733)
	Physalaemus gracilis	No data		Pauly et al. (2004) (AY680272)
	Physalaemus riograndensis	Brazil: El Dourado, RS		Darst and Cannatella (2004) (AY326021)
	Pseudopaludicola falcipes	Argentina: Corrientes, Yapeyu		Faivovich et al. (2005) (AY843741)

Table 1 continued

Family/ subfamily	Species	Locality	Voucher Number	Original source of the sequences (GenBank accession number)
Bufonidae	*Chaunus marinus*	Peru: Madre de Dios; Cusco Amazonico		Darst and Cannatella (2004) (AY325994)
	Dendrophryniscus minutus	Peru: Huanuco		Faivovich et al. (2005) (AY843582)
	Melanophryniscus klappenbachi	Argentina: Chaco		Faivovich et al. (2005) (AY843699)
	Melanophryniscus stelzneri	No data		Darst and Cannatella (2004) (AY325999)
Brachycephalidae	*Craugastor augusti*	Mexico: Jalisco		Darst and Cannatella (2004) (AY326011)
	Eleutherodactylus chloronotus	Ecuador: Napo		Darst and Cannatella (2004) (AY326007)
	Eleutherodactylus thymelensis	Ecuador: Carchi		Darst and Cannatella (2004) (AY326009)
	Euhyas cuneata	Cuba: Soledad, Cienfuegos Province		Feller and Hedges (1998) (Y10944)
	Phrynopus sp.	Ecuador: Carchi		Darst and Cannatella (2004) (AY326010)
Centrolenidae/	*Allophryne ruthveni*	Guyana: Kabocali Camp		Faivovich et al. (2005) (AY843564)
Centroleninae	*Cochranella* sp. 1	Ecuador: Carchi		Darst and Cannatella (2004) (AY326025)
	Cochranella sp. 2	No data		Darst and Cannatella (2004) (AY326023)
	Centrolene grandisonae	Ecuador		Santos et al. 2003 (AY364540)
	Centrolene prosoble pon	Panama: Cocle, El Cope, Parque Nacional 'Omar Torrijos'		Faivovich et al. (2005) (AY843574)
	Centrolene sp.	Ecuador: Napo		Darst and Cannatella (2004) (AY326022)
	Hyalinobatrachium sp.	No data		Darst and Cannatella (2004) (AY326024)
Ceratophryidae	*Ceratophrys cranwelli*	Argentina: Santa Fé, Vera		Faivovich et al. (2005) (AY843575)
	Ceratophrys ornata	No data		Darst and Cannatella (2004) (AY 326013)
	Telmatobius marmoratus	Bolivia: Bautista Saavedra, Canton Charazan, La Paz		Faivovich et al. (2005) (AY843769)
Cycloramphidae/ Cycloramphinae	*Alsodes gargola*	Argentina: Neuquen, Alumine		Faivovich et al. (2005) (AY843565)
	Alsodes monticola	Chile		Darst and Cannatella (2004) (AY326016)
	Cycloramphus boraceiensis	Brazil: Ubatuba, SP		This paper
	Eupsophus calcaratus	Argentina: Neuquen, Huiliches		Faivovich et al. (2005) (AY843587)
	Limnomedusa macroglossa	Argentina: Missiones, Aristobulo del Valle, Balneario Cunapiru		Faivovich et al. (2005) (AY843689)
	Odontophrynus achalensis	Argentina: Prov. Cordoba		Frost et al. (2006) (DQ283248)
	Odontophrynus americanus	Argentina: Buenos Aires, Escobar, Loma Verde		Faivovich et al. (2005) (AY843704)
	Proceratophrys avelinoi	Argentina: Misiones, Guarani, San Vicente		Frost et al. (2006) (DQ283038)
	Zachaenus parvulus	Brazil: Petrópolis, RJ	CFBH10120	This paper

Table 1 continued

Family/ subfamily	Species	Locality	Voucher Number	Original source of the sequences (GenBank accession number)
Cycloramphidae/ Hylodinae	*Crossodactylus caramaschii* *	Brazil: Parque Estadual Intervales, SP + CFBH 5415		Nuim, P.A.S. (unpublished) (AY143346) + Vences et al. (2003) (AY263235)
	Crossodactylus gaudichaudii	Brazil: Rio de Janeiro, RJ	ZUEC552	This paper
	Hylodes asper	Brazil: Paranapiacaba, SP	ZUEC11439	This paper
	Megaelosia goeldii	Brazil: Teresópolis, RJ, Rio Beija-Flor		Frost et al. (2006) (DQ283072)
Dendrobatidae	*Colostethus infraguttatus*	Ecuador: Manabi		Darst and Cannatella (2004) (AY326028)
	Dendrobates auratus	No data		Darst and Cannatella (2004) (AY326036)
	Epipedobates femoralis	Peru: Madre de Dios; Cusco Amazonico		Darst and Cannatella (2004) (AY326027)
	Phyllobates bicolor	No data		Darst and Cannatella (2004) (AY326031)
Thoropidae	*Thoropa miliaris*	Brazil: Ubatuba, SP	CFBH6233	This paper
	Thoropa miliaris	Brazil: Rio de Janeiro, RJ	BC164.02	This paper
	Thoropa miliaris	Brazil: Ubatuba, SP		Frost et al. (2006) (DQ 283331)

The accession numbers of the sequences taken from GenBank and the collection number of the voucher specimens are shown. Abbreviations: CFBH: Célio Fernando Baptista Haddad collection (deposited in the Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, SP); BC: Departamento de Biologia Celular da Unicamp; REAJ: Reserva Extrativista do Alto Juruá; ZUEC: Museu de História Natural, Universidade Estadual de Campinas, Campinas, SP, Brazil

*Incomplete sequence of about 1100 bp obtained by joining two fragments deposited in GenBank

**One tadpole from this site was studied, but could not be preserved

partially included in the family Leptodactylidae (see Frost et al. 2006 and Grant et al. 2006). One member of the Cycloramphidae, *Zachaenus parvulus*, has already been suggested to be closely related to our group of interest (Heyer 1975, 1983), but was not included in the phylogenetic inference provided by Frost et al. (2006) and Grant et al. (2006). We also included species of the families Bufonidae, Centrolenidae and Dendrobatidae because some phylogenetic analyses have shown that these families, especially the Centrolenidae, are related to the Leptodactylidae (Darst and Cannatella 2004; Frost et al. 2006). The localities of all these specimens and the GenBank accession numbers of the sequences not generated in this work are shown in Table 1.

DNA extraction, amplification, purification and sequencing

Total DNA from liver and muscle samples preserved in 95% ethanol or that had been freshly collected from anesthetized animals was extracted using a commercial genomic DNA isolation kit (Amersham Biosciences). The mtDNA region containing the 12S rRNA, 16S rRNA and tRNAval genes was amplified by the polymerase chain reaction (PCR) using the primers MVZ59(L), MVZ 50(H), 12L13, Titus I(H), Hedges16L2a, Hedges16H10, 16Sar-L and 16Sbr-H (see the review by Goebel et al. 1999 for the primer sequences).

The PCR products were purified using GFX PCR DNA and gel band purification kits (Amersham Biosciences) or UltraClean PCR Clean-up kits (MO BIO Laboratories, Inc.) according to the manufacturer's specifications. The purified fragments were used in sequencing reactions with ABI Prism BigDye Terminator chemistry (version 2.0; Applied Biosystems, Inc.). After purification with isopropanol and ethanol, the sequenced products were read on an automated sequencer (ABI Prism; Applied Biosystems, Inc.), with both directions of each amplified fragment being sequenced. The sequences were edited using Bioedit (http://www.jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) and a continuous fragment of approximately 2.4 bp was generated for each specimen. Three specimens from each locality of *Paratelmatobius* and two from each site of *Scythrophrys* were used, except for *P. gaigeae* from Bananal and *P. poecilogaster* from São Luiz do Paraitinga, both in São Paulo State, and *S. sawayae* from São Bento do Sul, Santa Catarina State, Brazil, for which only one sample each was used. When interindividual variation was detected in the sequences, the different haplotypes were treated as distinct operational taxonomic units (OTUs) in the phylogenetic analysis.

Phylogenetic inferences

The sequences were aligned using the Clustal W option in the software Bioedit, with the default parameters for gap penalties. Manual adjustments to the alignment were made using the Bioedit editor and some ambiguously aligned regions were excluded (a total of 126 characters). The final product was a matrix with 2405 characters and 82 OTUs. Maximum parsimony analyses were done using PAUP* (version 4β10) (Swofford 2000). For the tree searches, the heuristic algorithm was used with tree-bisection-reconnection (TBR) branch swapping, and random addition of the sequences with 10 replicates. Clade support was assessed by bootstrapping (Felsenstein 1985) done with heuristic searching and 1,000 pseudoreplicates.

Two treatments were used to deal with indels, which were considered as missing data in some inferences and as a fifth state in others. In both cases, different weights (1 or 2) for the transversions were tested. The ACCTRAN option was used to optimize the characters. In all of the analyses, the cladograms were rooted in the species representative of Brachycephalidae (*sensu* Frost et al. 2006) since, according to Faivovich et al. (2005) and Frost et al. (2006), these species constitute a basal clade when compared with all of the other genera included in the present study.

Chromosomal analysis

One specimen of *Paratelmatobius* sp. 2 collected at Mogi das Cruzes and three specimens of *Paratelmatobius* sp. 3 from Parque Estadual Carlos Botelho, São Miguel Arcanjo, both located in São Paulo State, were used. After treatment *in vivo* with 2% colchicine solution for at least 5 hours, the specimens were anesthetized and their intestines and testes were processed according to Schmid (1978) and Schmid et al. (1979) for chromosomal preparations. Giemsa staining, C-banding (King 1980) and Ag-NOR staining (Howell and Black 1980) were done. All of the specimens studied were deposited in the Célio F. B. Haddad collection (CFBH, Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, SP, Brazil) or in the Museu de História Natural, Universidade Estadual de Campinas, Campinas, SP, Brazil (ZUEC). Permits to collect the specimens were issued by the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (Licence IBAMA/DIFAS/DIREC-072/2001). The chromosomal characteristics described here were compared with the other karyotypes of *Paratelmatobius* and *Scythrophrys* described elsewhere (Lourenço et al. 2000, 2003a, b).

Table 2 A summary of the results obtained for the phylogenetic inferences

	Inference 1	Inference 2	Inference 3	Inference 4
Number of MPT	16	4	8	8
MPT score	10698	15031	11414	15510
CI	0.24	0.23	0.25	0.23
RI	0.57	0.59	0.59	0.59

In inferences 1 and 2, the indels were treated as missing data, while in inferences 3 and 4 they were treated as a fifth state. In inferences 1 and 3, the transversions and transitions were equally weighted, while in inferences 2 and 4, the transversions were over weighted (weighting function transversion/transition = 2/1). CI: consistency index; RI: retention index; MPT: most parsimonious trees; MPT score: length of the most parsimonious trees

Results

Phylogenetic analysis

The results obtained in each analysis are described in Table 2 and the strict consensus cladogram of all the trees inferred is shown in Fig. 1. In all of the inferences, *Paratelmatobius* and *Scythrophrys* formed a clade supported by a bootstrap value of 100%. The relationships among the *Paratelmatobius* species were fully resolved and two clades were recovered. One of them included *P. poecilogaster* and *P. gaigeae* and the other consisted of *P. cardosoi* and the three undescribed species analyzed here (Fig. 1). A clade composed of *Leptodactylus* species + *Pseudopaludicola falcipes* was inferred as the sister group of *Paratelmatobius* + *Scythrophrys* in all analyses, but this relationship was weakly supported (Fig. 1).

Chromosomal analysis

The karyotype of the specimen of *Paratelmatobius* sp. 2 showed diploid chromosome number 2n = 24 and consisted of 7 pairs of metacentric (m), 4 pairs of submetacentric (sm) (Nos.7, 8, 10 and 11) and one pair of subtelocentric (st) chromosomes (No. 4) (Fig. 2). Secondary constriction was observed in the long arm of both homologues of chromosome pairs Nos. 8 and 4 (Fig. 2A); these constrictions were identified as NORs using the Ag-NOR method (Figs. 2B and 3). C-banding detected small amounts of C-positive constitutive heterochromatin in the centromeric region of all the chromosomes of this complement. Such heterochromatin blocks of constitutive heterochromatin were also observed to be situated interstitially in both arms of chromosome pair No. 1, in the long arm of chromosome pairs Nos. 4, 5, and 9, and in the telomeric region of the long arm of chromosome pair No. 4. In addition, the entire long arm of chromosome pair No. 8, except for the NOR, showed positive C-banding pattern (Fig. 2C). The interstitial C-band in the long arm of chromosome pair No. 5 was associated to a secondary constriction seen in Giemsa-stained metaphases (Fig. 2A) but was not detected as an NOR by the Ag-NOR method (Fig. 2B). A faint C-band was also seen to be situated interstitially in the short arm of chromosome pair No. 5.

The diploid complement of *Paratelmatobius* sp. 3 consisted of 24 m and sm chromosomes (Fig. 4). A secondary constriction was observed in the long arm of chromosome pair No. 8 in Giemsa-stained metaphases (Fig. 4A) and it was identified as an NOR site in silver-stained chromosomes (Figs. 4B and 5). All of the centromeric regions of the chromosomes in the karyotype of *Paratelmatobius* sp. 3 showed a small amount of C-positive constitutive heterochromatin. Non-centromeric heterochromatic bands were easily identified in chromosome pairs Nos. 1, 2, 3, 4 and 5, and in nearly the entire short arm of chromosome pair No. 8 (Fig. 4C). The non-centromeric bands in chromosomes 2, 3, and 4 were small and they were hardly seen in some of the C-banded metaphases. In some of the metaphases, a small C-band was also seen in the short arm of chromosome pair No. 7 and in the short arm of chromosome pair No. 5.

Discussion

Phylogenetic relationships

Since the present analyses sampled several representatives of the Leptodactylidae, which currently includes *Paratelmatobius* and *Scythrophrys*, as well as representatives of other families related to it, we interpret the consistent clade *Paratelmatobius* + *Scythrophrys* inferred here as additional evidence of the close phylogenetic relationship of these genera, in agreement with the findings initially reported by Frost et al. (2006). Although our analyses did not include *P. lutzii* and *P. matiqueira*, species that are not being found in the field and that may be extinct (see Pombal Jr. and Haddad 1999), two clades were recognized in *Paratelmatobius* (*P. poecilogaster* + *P. gaigeae* and *P. cardosoi* + *Paratelmatobius* sp. 1 + *Paratelmatobius* sp. 2 + *Paratelmatobius* sp. 3) and corroborated the *P. cardosoi* and *P. lutzii* groups proposed by Pombal Jr. and Haddad (1999).

Although many genera were sampled, it was not possible to properly identify the sister group of *Paratelmatobius* + *Scythrophrys*. Even though all of the analyses indicated that the clade formed by *Leptodactylus* species + *Pseudopaludicola falcipes* was closest to the *Paratelmatobius* + *Scythrophrys* group, this relationship was not strongly supported. This inference differs from that reported by Frost et al. (2006) in which *Pseudopaludicola falcipes* was the sister group of a clade containing *Paratelmatobius* + *Scythrophrys* and the *Leptodactylus*

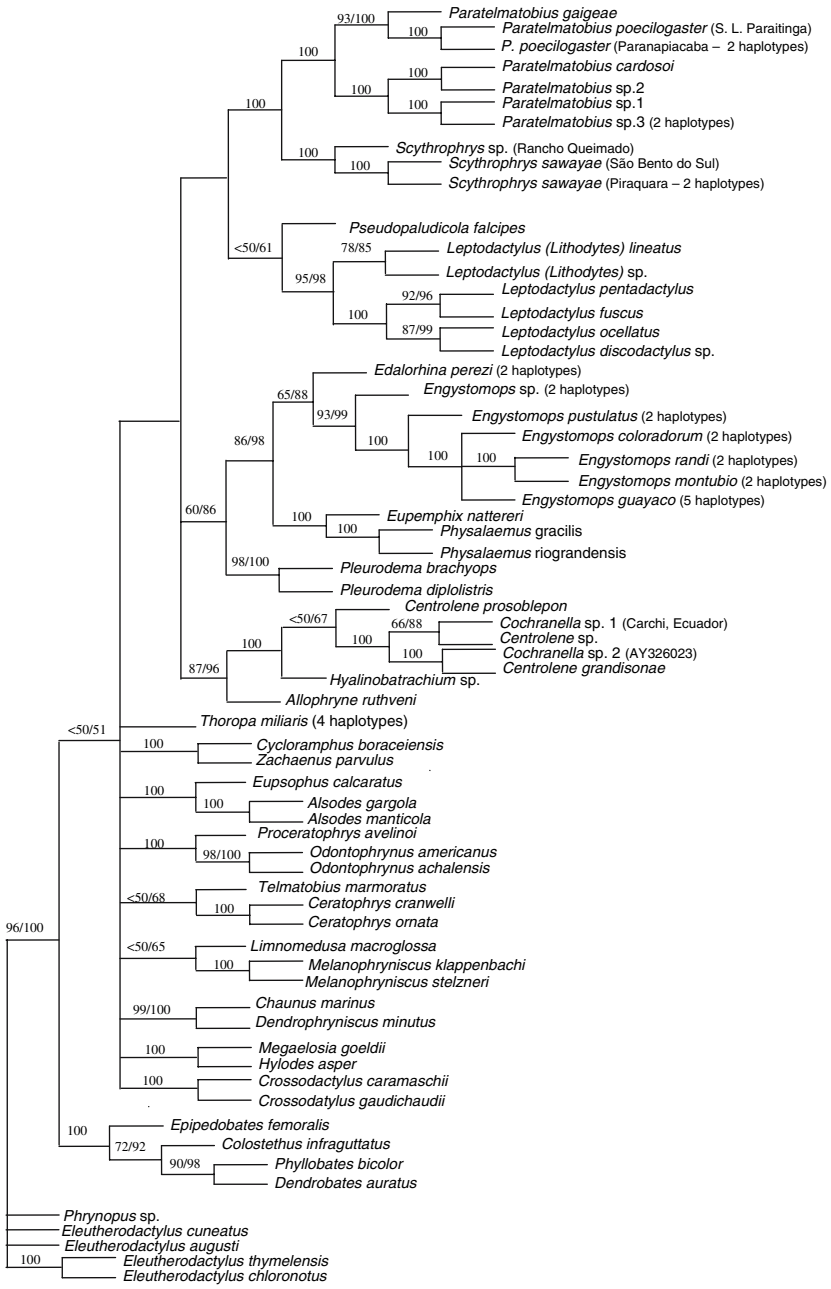

Fig. 1 A strict consensus cladogram of the most parsimonious trees. The numbers indicate the range of bootstrap values obtained in the different inferences. Branches without numbers had bootstrap values <50. Different haplotypes of the same species/locality are represented in the same branch, although each haplotype was considered as one OTU

species. Our results also differ markedly from Grant et al. (2006) who proposed a distant relationship between *Paratelmatobius* + *Scythrophrys* and *Pseudopaludicola*. However, these inferences were still only weakly supported, and the relationships of *Paratelmatobius* and *Scythrophrys* with other genera remain to be clarified.

Karyotype differentiation within the Paratelmatobius + Scythrophrys group

The karyotype of *Paratelmatobius* sp. 2 showed many similarities with that of *P. cardosoi* (Lourenço et al. 2000) (Fig. 6). Despite the poor quality of C-banded metaphases of *Paratelmatobius* sp. 2, it was possible to see that all of the C-positive heterochromatic bands found in the chromosomes of the karyotype of *P. cardosoi* were also detected in the karyotype of *Paratelmatobius* sp. 2, except for the small C-band near the telomere of the long arm of chromosome pair No. 6 seen in some metaphases of *P. cardosoi*. The failure to consistently detect this band can be related to its small size, since even in *P. cardosoi* it could not be easily detected. Some of the C-bands shared by these karyotypes differed in size, such as the interstitial C-band on the long arm of pair 1. Another noticeable difference was seen in the heterochromatic block adjacent to the NOR; this block was

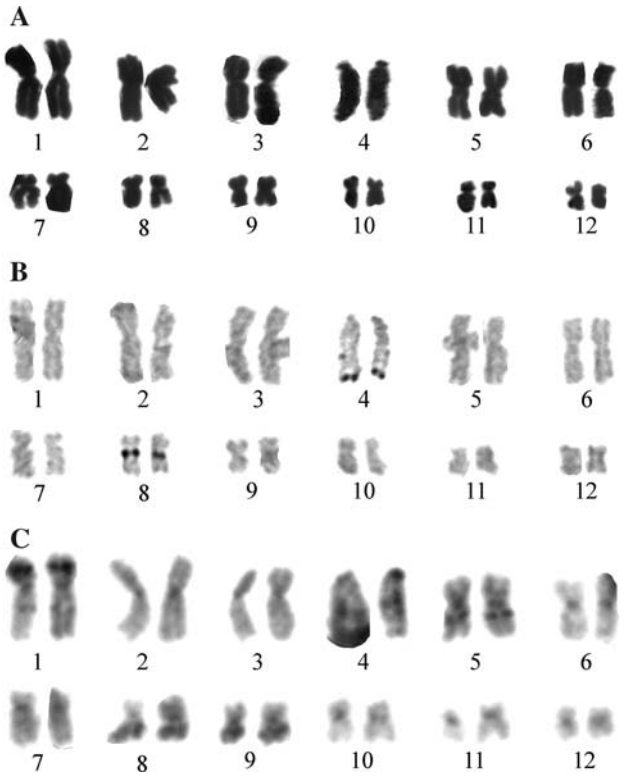

Fig. 2 Karyotype of *Paratelmatobius* sp. 2 arranged from Giemsa-stained (**A**), Ag-NOR stained (**B**) and C-banded (**C**) chromosomes

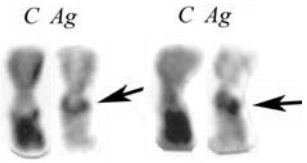

Fig. 3 Chromosome pair No. 8 of *Paratelmatobius* sp. 2 stained sequentially with C-banding (*C*) and Ag-NOR (*Ag*). Note the NOR (arrow) adjacent to a large C-band

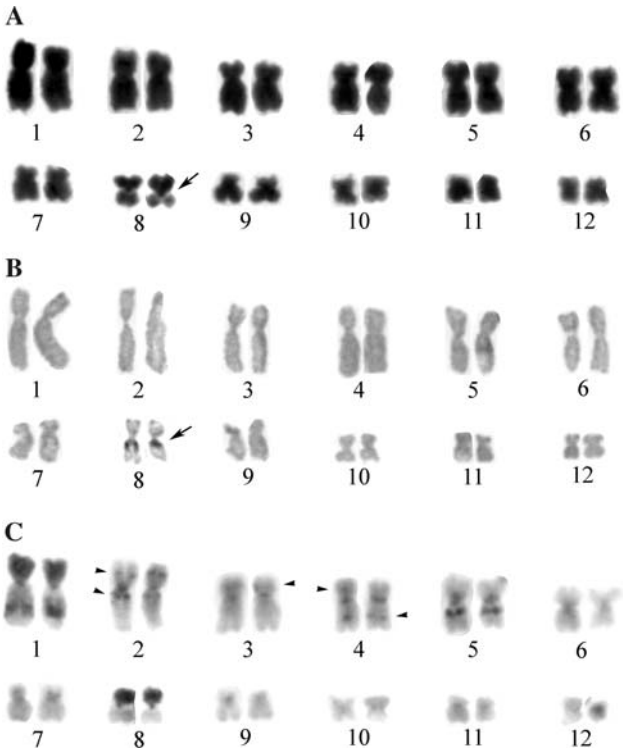

Fig. 4 Karyotype of *Paratelmatobius* sp. 3 arranged from Giemsa-stained (**A**), Ag-NOR stained (**B**) and C-banded chromosomes (**C**). Arrows indicate the NOR and arrowheads indicate small C-bands. See the text for additional information

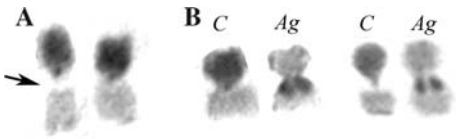

Fig. 5 (**A**) C-banded chromosomal pair No. 8 of *Paratelmatobius* sp. 3 in which the NOR was seen as a secondary constriction (arrow). (**B**) Chromosome 8 of *Paratelmatobius* sp. 3 was processed sequentially for C-banding (*C*) and Ag-NOR (*Ag*). Note the NOR in the long arm while the short arm is fully heterochromatic

much smaller in the karyotype of *Paratelmatobius* sp. 2 than in the karyotype of *P. cardosoi*, which explained the larger size of the corresponding chromosome in the karyotype of *P. cardosoi* and its classification as the seventh pair of this karyotype whereas in the karyotype of *Paratelmatobius* sp. 2 it formed pair 8.

The karyotype of *Paratelmatobius* sp. 3 was very similar to that of *Paratelmatobius* sp. 1 described previously (Lourenço et al. 2003b). One remarkable difference between these karyotypes involved the NOR site, since in the karyotype of *Paratelmatobius* sp. 1 the NOR was adjacent to a large heterochromatic block of the short arm of the chromosome classified as No. 9. Other difference between these karyotypes regards the interstitial C-band on the long arm of chromosome 3 of the karyotype of *Paratelmatobius* sp. 1, which was absent in that of *Paratelmatobius* sp. 3. On the other hand, the heterochromatic non-centromeric bands of chromosomes 1 and 5 were detected in both karyotypes. The non-centromeric C-bands seen on the chromosome 2 of the karyotype of *Paratelmatobius* sp. 3 seem also to be present in the karyotype of *Paratelmatobius* sp. 1, although too smaller and hardly seen. Since these bands could not be unambiguously detected previously, they were not mentioned in the description of the karyotype of *Paratelmatobius* sp. 1 (Lourenço et al. 2003b). The small telomeric band seen on the short arm of chromosome 8 in some metaphases of the karyotype of *Paratelmatobius* sp. 1 was not found in chromosome 9 of the karyotype of *Paratelmatobius* sp. 3, considered to be homeologous of that chromosome 8.

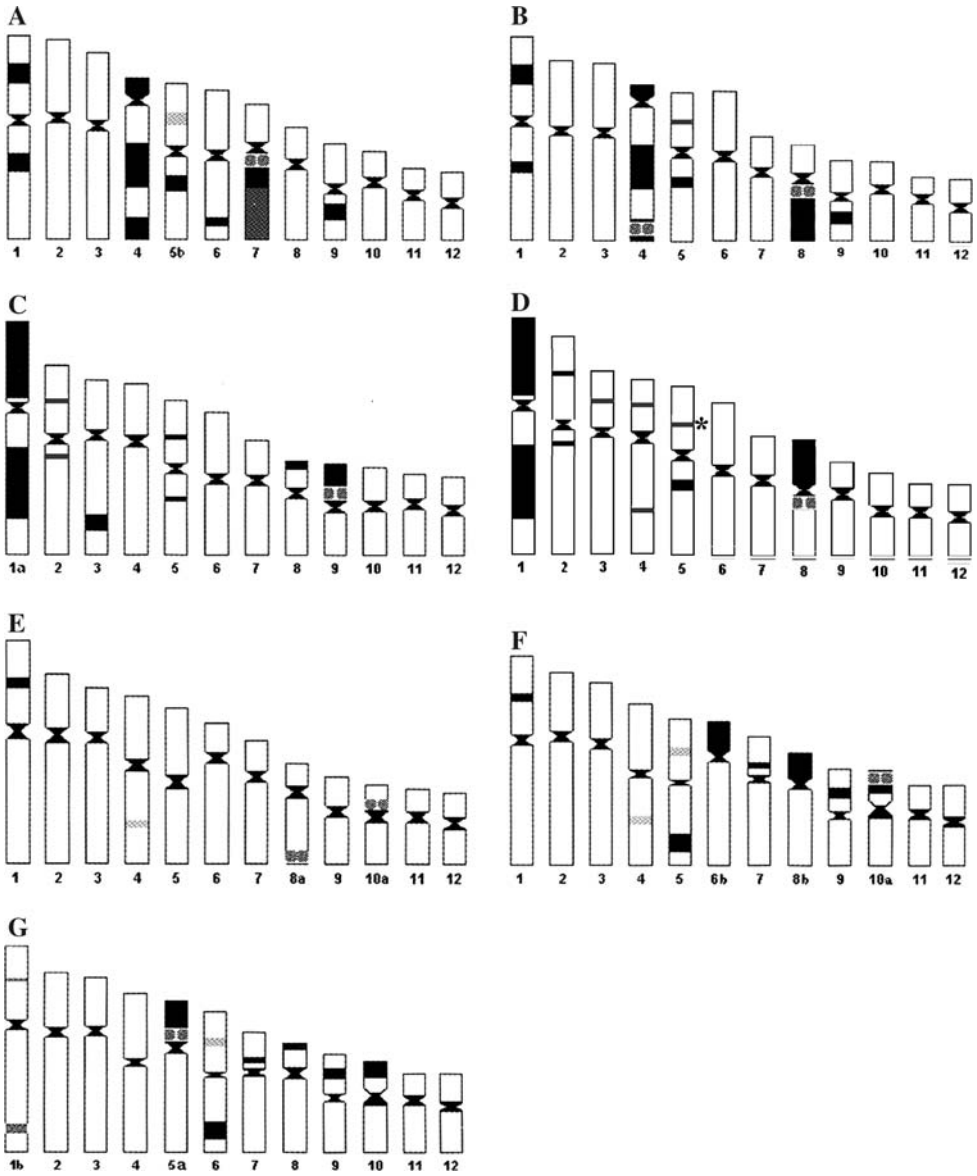

Fig. 6 Idiograms of the karyotypes of *Paratelmatobius* and *Scythrophrys*. (**A**) *Paratelmatobius cardosoi* (based on Lourenço et al. 2000). (**B**) *Paratelmatobius* sp. 2. (**C**) *Paratelmatobius* sp. 1 (based on Lourenço et al. 2003b). (**D**) *Paratelmatobius* sp. 3. (**E**) *Paratelmatobius poecilogaster* (based on Lourenço et al. 2000). (**F**) *Scythrophrys* sp. (based on Lourenço et al. 2003a). (**G**) *Scythrophrys sawayae* (based on Lourenço et al. 2003a). Solid blocks: dark C-bands. Gray blocks: faint C-bands. Checkered circles: NORs. *C-band that could be hardly seen only in some metaphases

However, also in this case we do not discard that the very small size of this band in pair 8 could have prevented its detection in the karyotype of *Paratelmatobius* sp. 3. Consequently, this discrepancy could be a technical artifact (related to the limit of detection) rather than a real difference between these karyotypes.

These new karyological data and the phylogenetic relationships of the *Paratelmatobius* and *Scythrophrys* species corroborated previous suggestions regarding cytogenetic homeologies and synapomorphies among these species (Lourenço et al. 2003a,b) and enabled other inferences about the karyotype differentiation in this group. With regard to the changes of the NOR-bearing chromosomes, we considered chromosome 10 of the karyotypes of *P. poecilogaster* and *Scythrophrys* sp., chromosome 9 of the karyotype of *Paratelmatobius* sp. 1, chromosome 8 of the karyotypes of *Paratelmatobius* sp. 2 and sp. 3, and chromosome 7 of the karyotype of *P. cardosoi* to be homeologous (see Figs. 6 and 7). In the karyotype of the common ancestor of *Paratelmatobius* and *Scythrophrys* species, the NOR was probably near the centromere and hypothetical rearrangements in this chromosomal arm rearranged the NOR to a telomeric position during the evolution of *Scythrophrys* species. It could have originated that NOR-bearing chromosome No. 10 found in the karyotype of *Scythrophrys* sp., as previously proposed by Lourenço et al. (2003a).

With respect to the karyotypic differentiation in the genus *Paratelmatobius* we hypothesized that it involved gains in heterochromatin adjacent to the NOR after the divergence of *P. poecilogaster*, with a resulting increase in chromosome arm length and enlargement of the NOR-

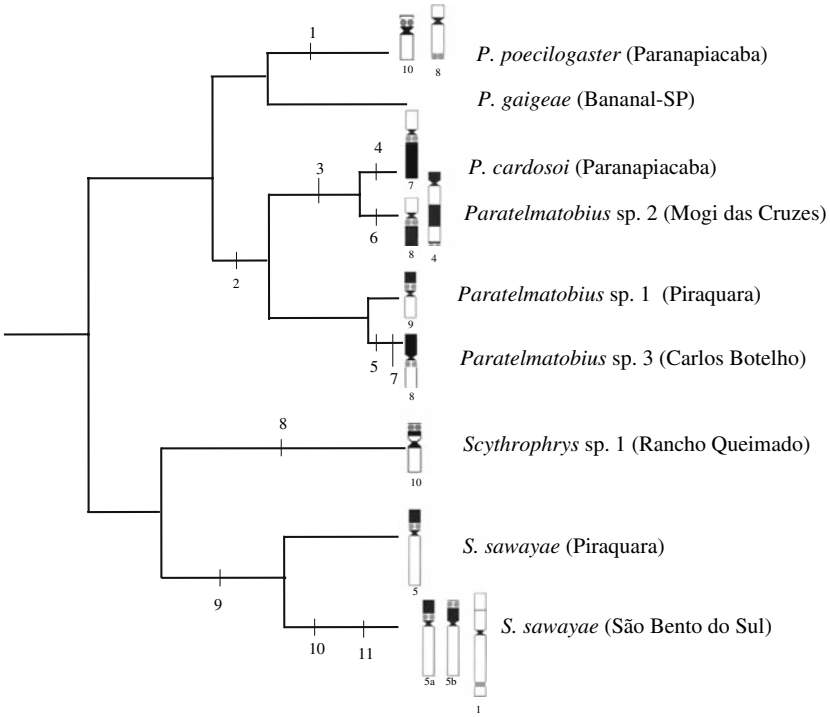

Fig. 7 A partial view of the strict consensus cladogram of Figure 1 showing some of the hypothetical transformations in NOR-bearing chromosomes (numbers in the branches). **1.** An additional NOR in pair 8. **2–5.** Gain in heterochromatin*. **6**. An additional NOR in pair 4. **7.** A pericentric inversion. **8.** Rearrangements in the short arm. **9.** A deletion in the NOR of pair 10 and appearance of an NOR in pair 5. **10.** A paracentric inversion in pair 5. **11.** An additional NOR in pair 1. *Here we present just one hypothesis to explain the gain in heterochromatin, but other alternative equally parsimonious exist and was not represented

bearing chromosome. These phenomena could explain the different classifications of the NOR-bearing chromosomes in the karyotypes of *Paratelmatobius cardosoi*, *Paratelmatobius* sp. 1, *Paratelmatobius* sp. 2, and *Paratelmatobius* sp. 3. In addition to a gain in heterochromatin, other events may also have played a role during the divergence of *Paratelmatobius* sp. 3 and *Paratelmatobius* sp. 1 that could account for the dissociation between the large heterochromatic block and the NOR in the karyotype of the former. One of these hypothetical events could be a pericentric inversion (see Figs. 6 and 7).

The detection of constitutive heterochromatin on the long arm of chromosome 1 in the karyotypes described here (*Paratelmatobius* sp. 2 and sp. 3) confirmed that this is a synapomorphy in species of the *Paratelmatobius cardosoi* group (see Lourenço et al. 2003b). Another interesting feature confirmed here was that the karyotypes of all the species of *Paratelmatobius* and *Scythrophrys* had a heterochromatic band on the short arm of chromosome 1, although there were interspecific differences in the size of these heterochromatic regions.

Other informative chromosomal characters were regarded to the C-band in the long arm of chromosome 5 and the morphology of the chromosome 4. A chromosome 5 with an interstitial C-band in the long arm and near the centromere was exclusively seen in the karyotypes of all species of the *Paratelmatobius cardosoi* group (although with different sizes), and a subtelocentric pair 4 was exclusively found in the karyotypes of *P. cardosoi* and *Paratelmatobius* sp. 2 (see Figs. 6 and 7).

Finally we emphasize that our inferences on chromosomal differentiation were based on the cladogram arisen from mitocondrial data and, therefore, must be taken with some caution. Despite 12S and 16S mtDNA sequences have been largely used in phylogenetic studies of anurans (e.g., Darst and Cannatella 2004; Ron et al. 2006), we must be aware that incongruences between the phylogenetic relationships inferred from mitochondrial and nuclear markers may occur (e.g., Gonçalves et al. 2007; Koblmüller et al. 2007).

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