Genetics and Molecular Biology, 26, 2, 139-143 (2003)

www.sbg.org.br

Research Article

Restriction fragment analysis of the ribosomal DNA of *Paratelmatobius* and *Scythrophrys* species (Anura, Leptodactylidae)

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Abstract

Physical maps of the ribosomal RNA gene 28S of species belonging to the genera *Paratelmatobius* and *Scythrophrys* were constructed, using five restriction endonucleases. The restriction sites for *Bam* HI, *Bgl* II, *Bst* EII, and *Eco* RI had similar positions in all species, although there were interspecific differences in the size of the restriction fragments obtained. An additional *Pvu* II site was found in *Scythrophrys* specimens from Piraquara (State of Paraná, Brazil) and from São Bento do Sul (State of Santa Catarina, Brazil), but not in the *Scythrophrys* specimens from Rancho Queimado (State of Santa Catarina, Brazil). This finding is in agreement with the hypothesis regarding the existence of two species in the genus *Scythrophrys*. On the other hand, the extra *Bst* EII site considered in the literature to be a synapomorphy for the subfamilies Leptodactylinae and Telmatobiinae was not observed in the genera *Paratelmatobius* and *Scythrophrys*, which brings new questions about some taxonomic classifications that include *Paratelmatobius* in Leptodactylinae and *Scythrophrys* in Telmatobiinae. Interspecific variation was observed in the size of the restriction fragments analyzed and, in the case of group I *Scythrophrys*, there was also a variation between the individuals of the two populations. These data suggest that sequencing of the rDNA segments studied here may be useful in phylogenetic studies of the genera *Paratelmatobius* and *Scythrophrys*.

Key words: ribosomal DNA, 28S rDNA gene, restriction sites, physical map, *Paratelmatobius*, *Scythrophrys*, Leptodactylidae, Anura.

Received: July 15, 2002; accepted: March 10, 2003.

Introduction

The nuclear ribosomal DNA (rDNA) in the NOR (nucleolar organizer regions) of eukaryotes consists of tandemly repeated copies of the transcriptional unit for the precursor of 18S, 5.8S, and 28S ribosomal RNA (rRNA), separated by an intergenic spacer (IGS). Two internal transcribed spacers, known as ITS 1 and ITS 2, are located between the regions coding for 18S and 5.8S rRNA, and between the latter and the 28S coding region. In addition, an external transcribed spacer (ETS) occurs upstream to the 18S gene (Long and Dawid, 1980; Miller, 1981). The copies of rDNA genes in a species evolve in concert through coordinated mechanisms (Arnheim *et al*., 1980; Dover and Coen, 1981; Krystal *et al*., 1981; Coen *et al*., 1982a,b; Arnheim, 1983). As a result, intra- and interindividual variations in rDNA are small and, when present, affect particularly the intergenic spacer (see Hillis and Dixon 1991 for a review).

The low rate of polymorphism in the rDNA transcription unit allows characterization of the rDNA of each species using only a few specimens, and makes this DNA useful for interspecific comparisons. In addition, the different coding regions of the rDNA repeats usually show distinct evolution rates. As a result, this DNA can provide information about almost any systematic level (see Hillis and Dixon, 1991).

In anurans, restriction mapping of the 28S gene in 54 species identified regions of variability that could be useful in phylogenetic studies (Hillis and Davis, 1987). Additionally, an interesting *Bst* EII site was reported by Hillis and Davis (1987) as a possible synapomorphy for the leptodactylid subfamilies Leptodactylinae and Telmatobiinae, as they are considered by Duellman and Trueb (1986).

The leptodactylid genera *Paratelmatobius* and *Scythrophrys* are closely related, as suggested by morphological, ecological (Lynch, 1971; Heyer, 1975; Garcia, 1996) and cytogenetical (Lourenço *et al*., in press) data. However, many authors have considered them as belonging to two different subfamilies, Leptodactylinae and Telmatobiinae, respectively (see Lynch, 1971; Duellman and Trueb, 1986; Frost, 2000). According to the most recent

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taxonomic review of amphibians, both *Paratelmatobius* and *Scythrophrys* belong to the same subfamily, named Cycloramphinae (Frost, 2002). Actually, the taxonomic relationships of *Paratelmatobius* and *Scythrophrys* with other leptodactylids are still not clear, and further data are needed for their correct allocation in subfamilies.

The available information about *Paratelmatobius* and *Scythrophrys* includes cytogenetic analyses showing interspecific variation in the NOR location in three karyotypes of *Paratelmatobius* (Lourenço *et al*., 2000; Lourenço *et al*., 2003) and two of *Scythrophrys* (Lourenço *et al*, in press). However, the organization of the rDNA genes in these genera is not known yet.

In this paper, we present a preliminary analysis of the 28S ribosomal RNA gene in *Paratelmatobius* and *Scythrophrys*.

Materials and Methods

Specimens

Three *Paratelmatobius poecilogaster* specimens, four *Paratelmatobius cardosoi*, four *Paratelmatobius* sp (aff. *cardosoi*) (see Lourenço *et al*, 2003), eight *Scythrophrys* from the karyotype group I (five from Piraquara, State of Paraná, and three from São Bento do Sul, State of Santa Catarina, Brazil), and six *Scythrophrys* specimens from the karyotype group II (from Rancho Queimado, State of Santa Catarina, Brazil) (see Lourenço *et al*, in press) were studied. The *P. poecilogaster* and *P. cardosoi* specimens were collected at Paranapiacaba (State of São Paulo, Brazil), and the *Paratelmatobius* sp (aff. *cardosoi*) specimens came from Piraquara (State of Paraná, Brazil). For comparison, specimens of two Cycloramphinae genera (sensu Frost, 2002), *Cycloramphus izecksohni* (previously in the subfamily Leptodactylinae) (one specimen from Corupá, State of Santa Catarina, Brazil) and *Hylodes asper* (previously in the subfamily Hylodinae) (one specimen from Paranapiacaba) were also used. All the specimens studied were deposited at the Célio F.B. Haddad collection (CFBH), Departament of Zoology, Universidade Estadual Paulista, Rio Claro, SP, Brazil, and at the "Prof. Adão José Cardoso" Museum of Natural History (ZUEC), Universidade Estadual de Campinas, Campinas, SP, Brazil.

Southern blot

Genomic DNA was extracted from fresh or frozen liver and muscle tissue, using a standard phenol/chloroform method (Sambrook *et al.*, 1989). About 1-2 µg of genomic DNA from each specimen were digested with the restriction enzymes *Eco* RI, *Bam* HI, *Bgl* II, *Bst* EII, and *Pvu* II, used singly or in combination. The resulting fragments were separated on 1.2% agarose gels at about 1.5 V/cm in 1x TBE, and transferred onto positively charged nylon membranes (Hybond) (Sambrook *et al.*, 1989).

28S rDNA probe

An *Eco* RI-*Bam* HI fragment (~2,5 kb) of the *Xenopus laevis* 28S gene extracted from the plasmid HM 123 (Meunier-Rotival *et al*., 1979) was subcloned into the phagemid pBlueScript (pBS 28) (Figure 1) and used as probe, after labeling with digoxigenin by the random primer method, according to the manufacturer's instructions (Boehringer Mannheim).

Hybridization

The membranes were pre-hybridized for 2-3 h in 5x SSC, 50% formamide, 0.1% lauroylsarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer Mannheim), and then hybridized overnight with the pBS 28 probe, at 42 °C in pre-hybridization solution. After hybridization, the membranes were washed twice in 2x SSC and 0.1% SDS solution at room temperature, and twice in 0.1x SSC and 0.1% SDS solution at 60 °C. Each wash lasted 15 min. The probe was detected by using a chemiluminescence system from Boehringer Mannheim, with CSPD as the chemiluminescent substrate. After incubation with the substrate, the membranes were exposed to X-ray films (Hyperfilm ECL, Amersham). The bands were documented, and the length of the restriction fragments was inferred using a Kodak Digital Science System.

Results

Restriction site maps of the 28S ribosomal gene were constructed for *P. poecilogaster*, *P. cardosoi*, *Paratelmatobius* sp (aff. *cardosoi*), and for three *Scythrophrys* populations (Figure 2). The restriction sites for *Bam* HI, *Bgl* II, *Bst* EII and *Eco* RI had similar positions in all species, although there were interspecific differences in the size of the restriction fragments obtained (Figures 2-4). The digestions using *Pvu* II showed that the individuals from the two group I *Scythrophrys* populations had an extra site for this enzyme, that was absent in the *Scythrophrys* specimens from Rancho Queimado and in the other species analyzed. *Hylodes asper* had three *Pvu* II sites (Figures 5 and 6).

Weak bands were detected after double digestion with *Bgl* II and *Pvu* II, and were considered to be the product of partial digestions (Figure 5). This conclusion agreed with the pattern obtained in other experiments and by some

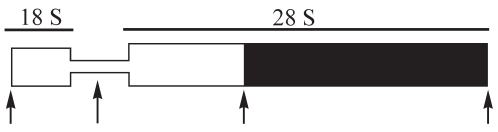

Figure 1 - Representation of the *Xenopus laevis* rDNA fragment in the plasmid HM 123 (Meunier-Rotival *et al*., 1979). The arrows and arrowheads indicate the restriction sites of *Eco* RI and *Bam* HI, respectively. The solid block represents the fragment of about 2.5 kb subcloned in pBueScript and used as probe.

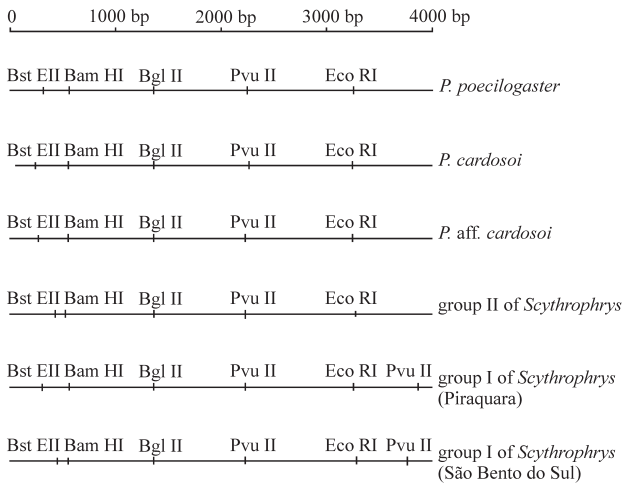

Figure 2 - Restriction maps of *Paratelmatobius* and *Scythrophrys* 28S rDNA for the enzymes *Bst* EII, *Bam* HI, *Bgl* II, *Pvu* II and *Eco* RI. (The presumed *Pvu* II site of *Scythrophrys* from group II experiments is not shown).

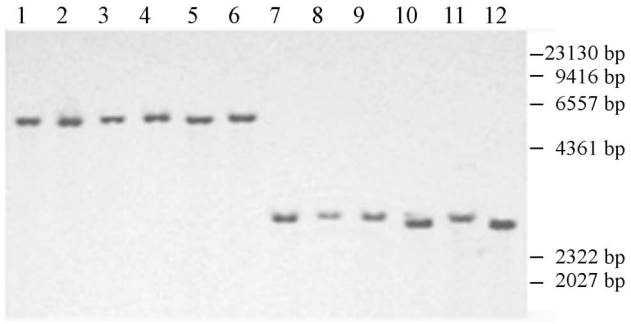

Figure 3 - Fragments of genomic DNA obtained after digestion with *Eco* RI (**1-6**) and *Eco* RI-*Bst* EII (**7-12**) and hybridization with the pBS 28 probe. **1-2**, **7**. *Paratelmatobius poecilogaster*. Lanes 1 and 2 contain DNA from two individuals, both of which showed the same pattern. **3, 8**. *P. cardosoi*. **4**, **9**. *Paratelmatobius* sp (aff. *cardosoi*). **5**, **11**. *Scythrophrys* of group I from Piraquara. **6**, **12**. *Scythrophrys* of group I from São Bento do Sul. **10**. *Scythrophrys* of group II from Rancho Queimado.

single digestions not shown here. Comparison of these data with the restriction sites in the rDNA sequence of *Xenopus laevis* (GenBank/X02995) indicated that the ~1 kb fragment in lanes 1 and 5 of Figure 5 was a *Bgl* II-*Pvu* II segment derived from partial digestion of *Pvu* II, since this hypothetical site is present in *X. laevis*. The total digestion of this fragment probably gave rise to two fragments, one of ~900 bp and another of about 100 bp, not detected in our hybridization experiments. Since partial digestions were not done for all of the species studied, the hypothetical *Pvu* II site mentioned above was not considered in interspecific comparisons.

There was no intraindividual or intrapopulational variation either in the number or in the size of the fragments generated in any of the experiments (Figures 3-6 and data not shown). In contrast, an interpopulational variation was

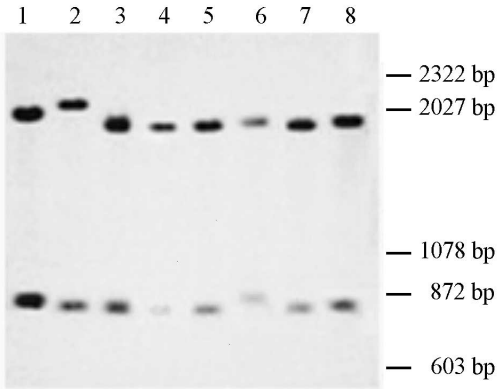

Figure 4 - Fragments of genomic DNA from *Hylodes asper* (**1**), *Cycloramphus izechsoni* (**2**), *Paratelmatobius poecilogaster* (**3**), *P. cardosoi* (**4**), *Paratelmatobius* sp (aff. *cardosoi*) (**5**), *Scythrophrys* from Rancho Queimado (**6**), *Scythrophrys* of group I from Piraquara (**7**), and of group I from São Bento do Sul (**8**) hybridized with the pBS 28 probe, after digestion with *Eco* RI-*Bam* HI-*Bgl* II.

observed in the size of the digested fragments of *Scythrophrys* group I (Figure 3: lanes 10-12; Figure 4: lanes 6-8; Figure 5: lanes 5-7). Interspecific variation in the size of the digested fragments was also found. The three *Paratelmatobius* species, for example, could easily be distinguished from each other by variations in the size of the *Bgl* II-*Pvu* II fragment (Figure 5: lanes 2-4) and of the Bst EII-Bam HI fragment (Figure 2), whose size was inferred from the analysis of the experiments shown in Figures 3 (lanes 7-9) and 4 (lanes 3-5).

Discussion

The restriction sites of *Bam* HI, *Bgl* II, and *Eco* RI found in all the specimens studied here were also detected in the anurans analyzed by Hillis and Davis (1987). The same applies to the *Pvu* II site located between the *Bgl* II and the *Eco* RI sites. On the other hand, the *Bst* EII site observed in all *Paratelmatobius* and *Scythrophrys* species was absent in some of the anurans investigated by Hillis and Davis, although it was present in the three leptodactylids analyzed by them (*Ceratophrys ornata*, *Leptodactylus wagneri* and *Telmatobius niger*).

Moreover, Hillis and Davis (1987) detected an interesting extra *Bst* EII site between the *Bgl* II and the *Pvu* II sites mentioned earlier. It was found only in the leptodactyline *Leptodactylus wagneri* and in the telmatobine *Telmatobius niger* and was therefore considered to be a possible synapomorphy for these Leptodactylidae subfamilies. None of the *Paratelmatobius* and *Scythrophrys* species studied here had this extra *Bst* EII site. So, if we consider the classification adopted by Duellman and Trueb (1986) and followed by Hillis and Davis (1987), our results refute the hypothesis of Hillis and Davis about the interpretation of the extra Bst EII site, since, by that classification, *Scythrophrys* belonged to the

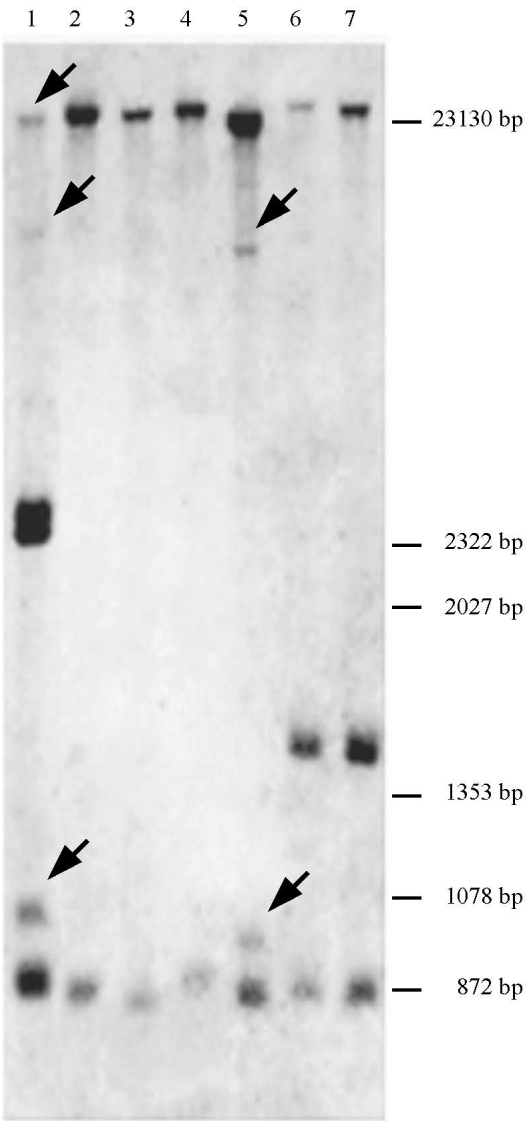

Figure 5 - Southern blotting of the genomic DNA of *Hylodes asper* (**1**), *Paratelmatobius poecilogaster* (**2**), *P. cardosoi* (**3**), *Paratelmatobius* sp (aff. *cardosoi*) (**4**), *Scythrophrys* from Rancho Queimado (**5**), *Scythrophrys* of group I from Piraquara (**6**), and *Scythrophrys* of group I from São Bento do Sul (**7**) with the pBS 28 probe, after double digestion with *Bgl* II and *Pvu* II. The arrows indicate partially digested fragments.

Telmatobiinae and *Paratelmatobius* to the Leptodactylinae subfamily.

The monophyly of Leptodactylinae and Telmatobiinae (sensu Duellman and Trueb, 1986) was already refuted in a study by Ruvinsky and Maxson (1996), in which *Eleutherodactylus cuneatus* (currently allocated to the Eleutherodactylinae subfamily) was the representative of Telmatobiinae, and *Lithodytes lineatus* the representative of Leptodactylinae. In that paper, the results also suggested Leptodactylidae polyphyly.

On the other hand, if we consider the current classification presented by Frost (2002), in which *Paratelmatobius* and *Scythrophrys* are allocated together and apart from the

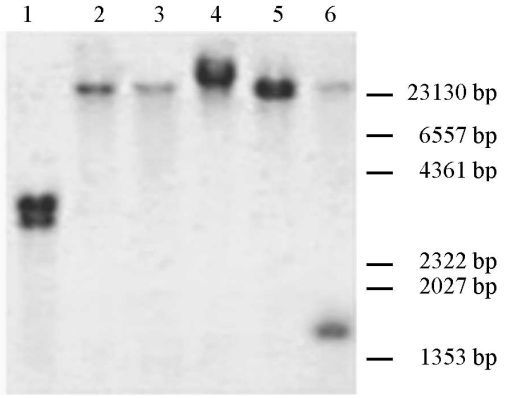

Figure 6 - Southern blotting of the genomic DNA of *Hylodes asper* (**1**), *Paratelmatobius poecilogaster* (**2**), *P. cardosoi* (**3**), *Paratelmatobius* sp (aff. *cardosoi*) (**4**), *Scythrophrys* from Rancho Queimado (**5**), and *Scythrophrys* of group I from Piraquara (**6**), after digestion with *Pvu* II and hybridization with the pBS 28 probe.

Leptodactylinae and Telmatobiinae species, our results do not confront with the hypothesis brought by Hillis and Davis (1987). So, although a study including various Leptodactylidae genera and several characters is necessary for a conclusive analysis of their taxonomy and intergeneric relationships, our results suggest that an investigation of the *Bst* EII site in the 28S gene may be useful.

The other informative region was the additional *Pvu* II site found in the two group I *Scythrophrys* populations, which was absent in the *Scythrophrys* specimens from Rancho Queimado, in the *Paratelmatobius* species, and in other anurans of this study and others (Hillis and Davis, 1987). Since intraspecific variation in the 28S gene is not common (see Hillis and Dixon, 1991), and the existence of two species in the genus *Scythrophrys* has already been proposed based on studies of the same populations sampled here (Lourenço *et al*., in press), it is possible that the variation in the Pvu II restriction site described above represents an interspecific divergence.

It is also interesting to note that there was no intraindividual variability in *P. poecilogaster*, despite the two NOR-bearing chromosome pairs in this karyotype (chromosome pairs 8 and 10; Lourenço *et al*., 2000). Moreover, all the species studied, as well as the individuals from the two group I *Scythrophrys* populations, could be distinguished from each other by the size of the digested fragments. This finding and the restriction site variation discussed above suggest that sequencing of the rDNA segment focused in this study could provide useful data for phylogenetic studies of *Paratelmatobius* and *Scythrophrys*.

Acknowledgments

The authors thank Dr. Anete Pereira de Souza for helping with the subcloning experiments, Dr. Wanderley Dias da Silveira for important suggestions, and FAPESP (Foundation for the Support of Research of the State of São

Paulo) for financial support (Proc. 97/00459-9 and 98/06087-9).

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Editor: Yatiyo Yonenaga-Yassuda