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Multiple nucleolus organizer regions in *Leptodactylus mystacinus* (Amphibia, Anura) and comments on its systematic position in the *L. fuscus* group based on cytogenetic and molecular analyses

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Abstract

Specimens of *Leptodactylus mystacinus* from Brazil were karyotyped with conventional and differential staining. The $2n = 22$ karyotype is similar to that found for the majority of the *Leptodactylus*, the karyotypic conservatism also confirmed by the similarity of the replication banding patterns with those previously described. *L. mystacinus* has a small amount of C-banded heterochromatin, located mainly at the centromeres, although telomeric or interstitial bands have also been noticed. With DA/CMA_3 some chromosome regions showed slightly bright fluorescence, and with DA/DAPI, no particular AT-rich repetitive region was observed. Silver staining showed an extensive inter- and intraindividual variation in the number and position of Ag-positive regions, in 1p, 4p, 8p, 8q, and 11p**.** Nevertheless, FISH using rDNA probes confirmed only the signals on the short arms of chromosomes 4 and 8 as true NORs. The remaining silver stained regions are probably due to the heterochromatin with some affinity to the Ag-staining. Phylogenetic analysis based on partial cytochrome b sequence revealed that *L. mystacinus* forms a basal branch, so that the presence of multiple NORs in pairs 4 and 8 in this species indicates an autapomorphy.

Introduction

The identification of cytological markers using chromosome banding may be an important tool for taxonomy and systematics, especially in the groups with conservative external morphology and with highly homogeneous karyotypes under standard staining, which is observed, in general, for anuran species (see King, 1990; Kuramoto, 1990). In many cases, however, the karyotypes are poorly differentiated, almost indistinguishable, with banding techniques (Baldissera Jr, Oliveira, and Kasahara, 1993; Kasahara et al., 1996). The genus *Leptodactylus* belongs to this category, the majority of the species sharing closely similar karyotypic pattern, even when the chromosomes are submitted to differential staining (Silva et al., 2004). Some exceptions have been reported by Silva, Haddad and Kasahara (2000a) and Amaro-Ghilardi, Rodrigues and Yonenaga-Yassuda (2004), who found distinct C-band patterns either at inter- or intraspecific levels in representatives of this genus.

Among the banding techniques, those detecting the nucleolar organizer regions with silver staining (Ag-NORs) are particularly interesting in the analysis of the anuran karyotypes. The species exhibit, in general, a single pair of Ag-NOR (Schmid et al., 1990), but some of them, such as the leptodactylids *Physalaemus cuvieri* (Silva, Haddad and Kasahara, 1999), *P. olfersii* (Silva

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et al., 2000b), and *P. petersi* (Lourenço, Recco-Pimentel and Cardoso, 1998), have additional sites of Ag-NORs in the karyotype, in general, besides one main Ag-NOR pair. A unique pair of NOR in the genome has been considered a basal condition, so that the presence of multiple NORs represents, according to King, Contreras and Honeycutt (1990), an apomorphy in the diploid karyotypes of anuran species.

Since the Ag-NOR technique stains NOR-associated proteins, occasionally the fluorochromes mitramycin or chromomycin A_3, and, usually, the method of *in situ* hybridization with rDNA probes have been efficient to establish the number and the position of all ribosomal sites in the genome irrespective of genetic activity. Hence even those not Ag-stained inactive rDNA sequences are detected. On the other hand, recent data obtained in mammal species have shown that some of the clear Ag-positive signals on the chromosomes are not hybridized with rDNA probes, therefore not corresponding to true NORs (Sánchez et al., 1995; Dobigny et al., 2002).

This paper deals with the Ag-NOR interindividual variability in *Leptodactylus mystacinus*, characterized by multiple nucleolar organizer regions, contrary to what is observed in the species of the genus, which usually present a unique chromosome pair bearing this cytological marker. An exceptional variation in the heterochromatic Ag-positive regions, some of them simulating true Ag-NORs, was also observed. Additionally, we discuss the position of *L. mystacinus* within the *L. fuscus* species group on the basis of cytogenetic and molecular data.

Material and methods

Cytogenetic analysis was carried out on 13 specimens (5 males CFBH 4002, CFBH 3995, CFBH 7603, CFBH 7608-09; 5 females CFBH 4726, CFBH 7604-05-06-07; and 3 juveniles CFBH 4538-39, CFBH 4008) collected in Descalvado (21°54′S 47°37′W), and one male specimen (CFBH 6585) collected in Mogi das Cruzes (23°31′S 46°11′W), two localities in the state of São Paulo, Brazil. The voucher specimens are deposited in the amphibian collection (CFBH) of the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, São Paulo, Brazil.

Mitotic chromosomes were obtained from direct preparations of bone marrow, liver, and spleen of animals previously treated with 0.01% colchicine at a proportion of 0.1 ml/10 g body weight, as described in Baldissera Jr, Oliveira and Kasahara (1993). In order to improve the mitotic index, phytohemagglutinin-P (Difco) was injected at the same proportion as colchicine, 48–72 h before sacrifice. Some specimens were submitted to *in vivo* treatment with a solution of 5-bromodeoxyuridine (BrdU) + 5-fluorodeoxyuridine (FudR) (Silva, Haddad and Kasahara, 2000a). The following techniques were used: standard staining with 2% Giemsa in phosphate-buffered saline, pH 6.8, Fluorochrome plus Giemsa (FPG) staining (Dutrillaux and Couturier, 1981) for differentiation of replication bands, C-banding (Sumner, 1972), Ag-NOR staining (Howell and Black, 1980), fluorescence *in situ* hybridization (FISH) (Viegas-Péquignot, 1992) using the HM 123 and HM 456 rDNA probes (Meunier-Rotival et al., 1979), chromomycin A_3 (CMA_3) and 4′,6-diamidino-2-phenylindole (DAPI) staining, both with the distamycin A (DA) counterstain (Schweizer, 1980).

Results

All the specimens of *L. mystacinus* have $2n = 22$ karyotype (Figure 1), including one large metacentric pair (1), four submetacentric pairs (2, 3, 4, and 7) and two metacentric pairs (5 and 6) of medium size, and four small metacentric or submetacentric pairs (8, 9, 10, and 11). A distal secondary constriction is clearly seen in the short arms of both homologues of pair 4. No distinct heteromorphic sex chromosomes were noticed in the male or female karyotypes.

Replication bands after BrdU incorporation were differentiated in the chromosomes of *L. mystacinus* with FPG staining, allowing a precise homologous pairing of the chromosomes (Figure 2).

C-banding revealed positive stained heterochromatin at centromeric region of the chromosomes (Figure 3). In addition, the chromosomes of pair 1 have a conspicuous C-banded block in the terminal short arms, which is characteristic of *Leptodactylus* species. In some metaphases, slightly stained telomeric or interstitial C-bands could be noticed occasionally in various chromosomes, including those presenting Ag-positive sites.

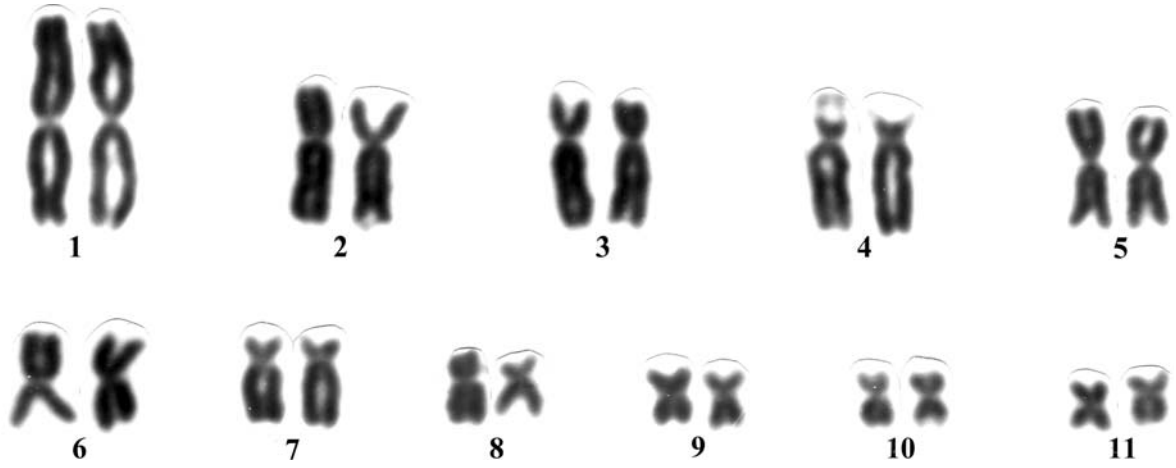

Figure 1. Giemsa stained karyotype of *L. mystacinus* (CFBH 3995), showing an interstitial secondary constriction in the short arms of pair 4.

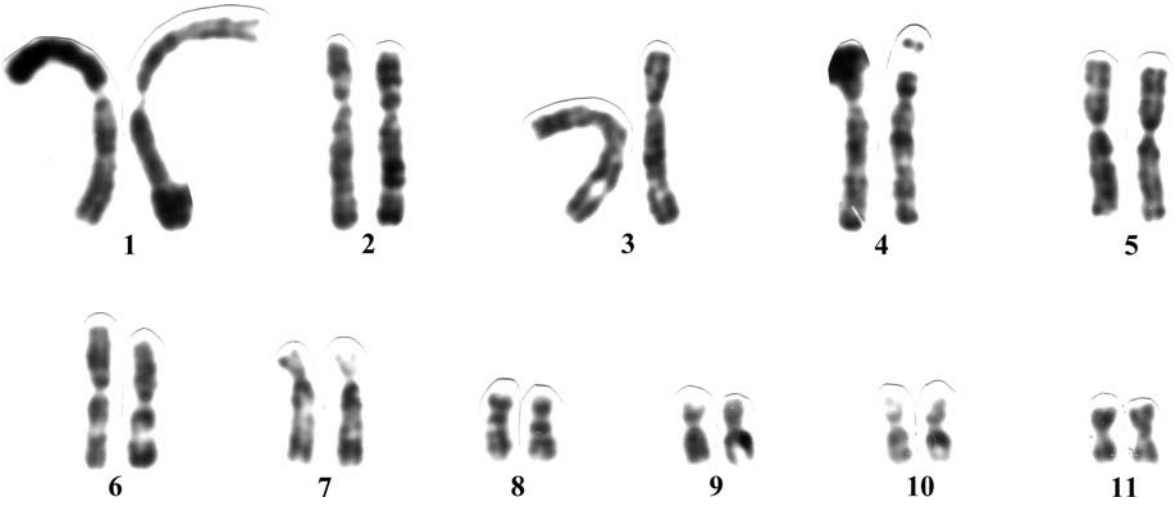

Figure 2. BrdU replication banding patterns in *L. mystacinus* (CFBH 3995) with FPG staining.

Silver staining revealed a wide inter and intra-individual variability in the number as well as in the position of the Ag-stained regions in the 14 specimens of *L. mystacinus* (Figures 4 and 5 and Table 1). Five of them provided few Ag-stained metaphases, but for the majority of the specimens, 20 to 90 metaphases were available for analysis. The chromosomes with Ag-positive signals belong to the pair 1 (interstitial short arms), pair 4 (distal short arms), pair 8 (distal short arms and interstitial long arms), and pair 11 (distal short arms). The number of Ag-positive signals per metaphase was 2 (4p4p, 4p8p), 3 (4p4p8p, 4p4p11p), 4 (4p4p8p8p, 1p1p4p4p), 5 (4p4p8p8p11p), 7 (4p4p8pq8pq11p), and 8 (4p4p8pq8pq11p11p), totalling 9 distinct patterns, as summarized in the Table 1. Some metaphases (Figure 5) also exhibited sporadically some chromosomes with silver stained centromeric region.

FISH with rDNA probes revealed in the metaphases of the specimen CFBH 4726 two fluorescent signals, one in the chromosome 4 and other in the chromosome 8 (Figure 6(a)). Two signals were also observed in the interphase nucleus (Figure 6(b)). In the metaphases of the specimen CFBH 3995, *in situ* hybridization occurred in both homologues of pair 4 and in one of the chromosomes of pair 8 (Figure 6(c)), which is

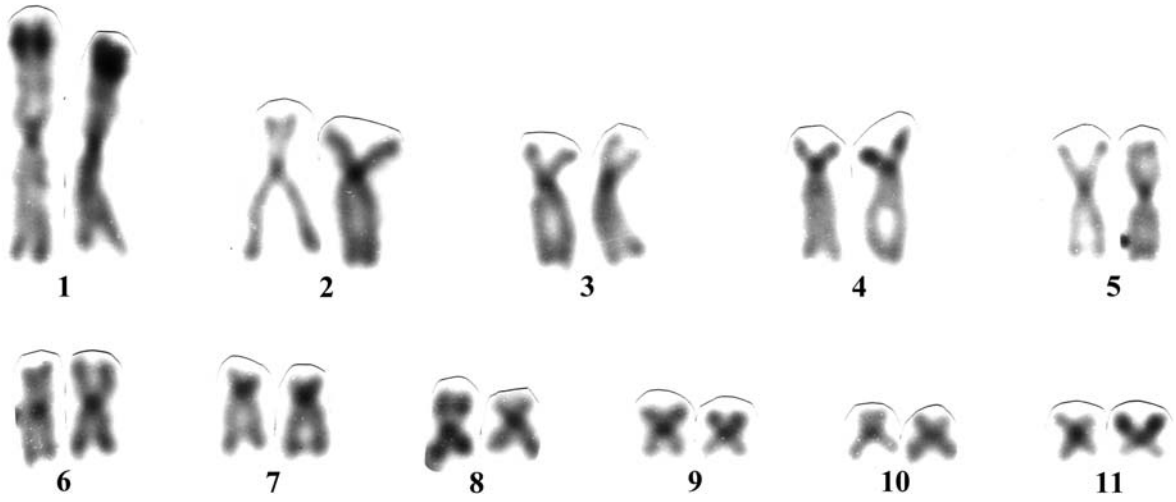

Figure 3. C-banding karyotype of *L. mystacinus* (CFBH 3995). Note additional band in the terminal short arms of the chromosomes of pair 1.

in accordance to the three fluorescent signals seen in the interphase nucleus.

The GC-specific fluorochrome CMA_3 counterstained with DA showed in the metaphases of the specimen CFBH 4002 slightly bright fluorescence in the short arms of the two chromosomes of pair 4, in one of the chromosomes of pair 8, in both the short and long arms, and in the short arms of the homologues of pair 11 (Figure 7(a)). The centromeric region of some chromosomes is also slightly bright fluorescent; besides, a clear-cut CMA_3-negative region was noticed distally in the short arms of the homologues of pair 1. The AT-specific fluorochrome DAPI counterstained with DA produced a uniform staining, with no particular bright region in any of the chromosomes of the *L. mystacinus* (Figure 7(b)). Nevertheless, there is an unequivocally negative band in the short arms of the chromosomes of pairs 1 and 4.

In Figure 8, we present the phylogenetic tree that was obtained by us (Silva et al., 2004), with the partial sequencing of the mitochondrial cytochrome b gene of *L. mystacinus, L. fuscus* from two distinct localities, *L. plaumanni, L. gracilis delattini*, and *L. gracilis gracilis*, as well as of *P. cuvieri* (outgroup).

Discussion

The standard stained karyotype of *L. mystacinus* is similar to those reported for the great majority of the species belonging to the genus *Leptodactylus* (Beçak, 1968; Beçak, Denaro and Beçak, 1970; Denaro, 1972; Bogart, 1974; Lisanti et al., 1990; Silva, Haddad and Kasahara, 2000a; Amaro-Ghilardi, Rodrigues and Yonenaga-Yassuda, 2004; Silva et al., 2004). The exception is *L. podicipinus* with a divergent karyotypic pattern, including four pairs of telocentrics among the 22 chromosomes (Bogart, 1974; Silva, Haddad and Kasahara, 2000a).

The karyotypic similarity of *L. mystacinus* with other species of *Leptodactylus* is also supported by the same replication banding patterns presented by their chromosomes, mainly those of large and of medium size, obtained in the present study and previously in Silva, Haddad and Kasahara (2000a) and Silva et al. (2004). In fact, the homeology of RBG- banding patterns among the six largest pairs of three species of *Leptodactylus* was recently established (Amaro-Ghilardi, Rodrigues and Yonenaga-Yassuda, 2004), which support the idea of conservatism of the *Leptodactylus* karyotypes.

L. mystacinus exhibited a C-banding pattern with faintly stained centromeric heterochromatin, similarly to those observed in some species of the genus (Silva, Haddad and Kasahara, 2000a; Amaro-Ghilardi, Rodrigues and Yonenaga-Yassuda 2004). By contrast, there are species with very marked C-bands, either at the centromeric and telomeric regions or interstitially. In the particular case of *L. ocellatus* (Silva, Haddad and Kasahara 2000a), the differences in the distribu-

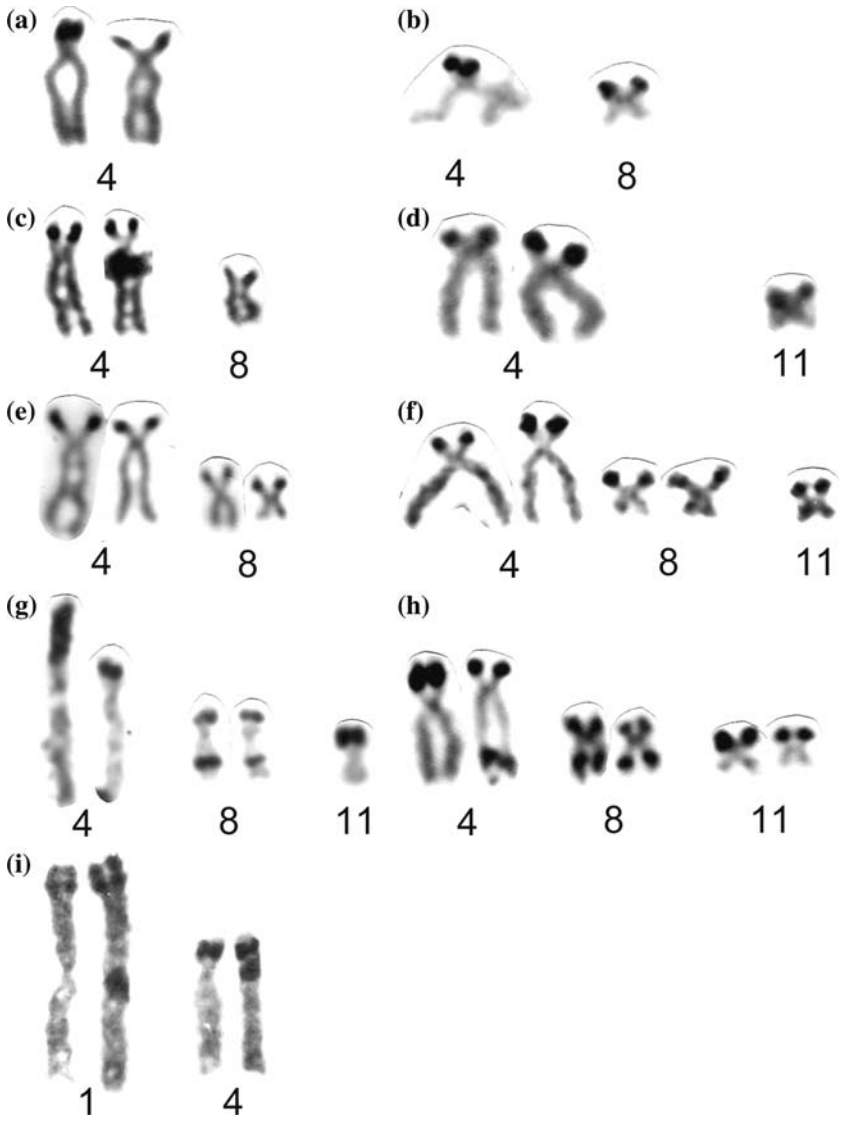

Figure 4. (a–i) Chromosomes of *L. mystacinus* with Ag-positive stained regions: (a) 4p4p, (b) 4p8p, (c) 4p4p8p, (d) 4p4p11p, (e) 4p4p8p8p, (f) 4p4p8p8p11p, (g) 4p4p8pq8pq11p, (h) 4p4p8pq8pq11p11p, (i) 1p1p4p4p.

tion of some landmark C-positive bands are suggestive of geographical variation of the karyotypes or even might support the idea of a complex of species, since representatives of *L. ocellatus* can also be distinguished by external morphology, bioacoustics, and habitat (Haddad, unpublished data).

Based on the chromosome analysis of one specimen of *L. mystacinus* with CMA_3/DA/DAPI staining, no special fluorescent AT-rich repetitive region with DA/DAPI was noticed, but with GC-specific CMA_3 the centromeric heterochromatin of some chromosomes appears with a slightly bright fluorescence, as well as the sites in 4p, 8p, 8q, and 11q. Taking into account the relatively weak intensity of the fluorescent signals in these three chromosomes, we are prone to consider them not as nucleolar organizer regions, but as due to the CMA_3 staining of the heterochromatin, associated or not to the NORs. Indeed, we had observed, in some anuran species, that NORs identified with DA/CMA_3 appeared usually with a very brilliant staining, whereas the adjacent heterochromatin itself has a less marked fluorescence (Kasahara

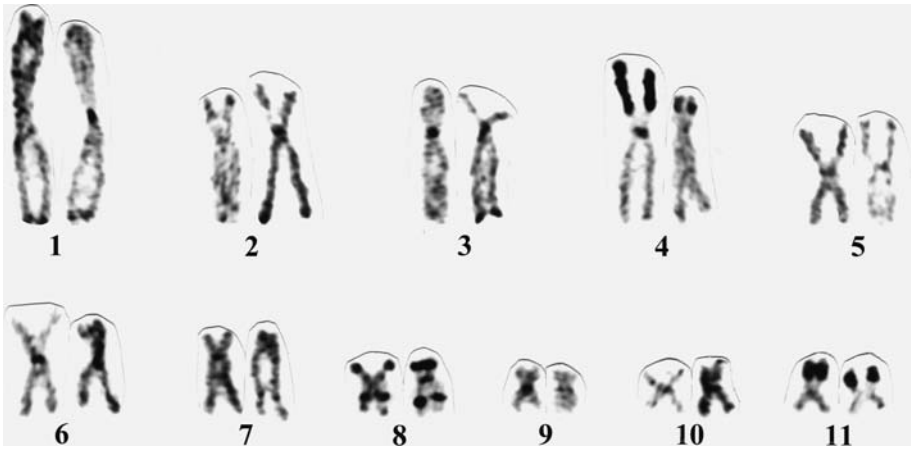

Figure 5. Silver stained karyotype of *L. mystacinus* (CFBH 3995). Note some chromosomes with Ag-positive centromeric heterochromatin.

et al., 2003). The DAPI-negative band in the short arms of the chromosomes of pair 1 seems to mark the boundary of the euchromatic region and the heterochromatic C-positive block. On the other hand, the CMA_3-negative band observed in these same chromosome arms seems to be more distally located. This means that the C-banded terminal heterochromatin on the chromosomes 1 is apparently not homogeneous, but the CMA_3/DA/DAPI data give no confirmation about its molecular content. Nevertheless, some clue on this subject is observed in the metaphases analyzed after FISH technique, in which the terminal heterochromatin of chromosome 1 appeared slight positively stained. Considering that DAPI was the fluorochrome used for counterstaining in that technique, most probably such heterochromatic region is AT-rich in a great extension.

All the *Leptodactylus* species karyotyped thus far after silver staining exhibited one single pair of Ag-NORs, located invariably in the chromosomes of pair 8 (Lisanti et al., 1990; Silva, Haddad and Kasahara, 2000a; Amaro-Ghilardi, Rodrigues and Yonenaga-Yassuda, 2004). The novelty in *L. mystacinus* is the presence of multiple Ag-NORs, occurring in the chromosomes 4 and 8, as shown by the FISH data with rDNA probes, as well as pair 4 with the status of main Ag-NOR pair. In fact, all the 14 individuals had Ag-NOR in, at least, one of the homologues of pair 4; besides, three of them had solely the homologues of pair 4 as the Ag-NOR bearing chromosomes. Nevertheless, while CFBH 4538 and CFBH 4539 had a very reduced amount of analyzed metaphases (3 and 4, respectively), CFBH 6585 showed a considerable sample (35 metaphases), thus reinforcing pair 4 as the main Ag-NOR chromosome pair in *L. mystacinus*. This last specimen is from Mogi das Cruzes very far away from the another collecting site in Descalvado. So, the possibility that some populations of *L. mystacinus* have just a single pair of Ag-NOR cannot be ruled out, before more individuals are karyotyped.

According to our data, it is evident that the elements of pair 8 keep, in a great extension, the condition of NOR-bearing chromosomes, because the majority of the specimens of *L. mystacinus* exhibited, sometimes in a considerable number of cells, at least one of the homologues 8 with Ag-NOR. Moreover, the specimen CFBH 7603 showed Ag-NOR in both pairs 4 and 8 in almost the totality of the metaphases (59 out 60). In the species of *Leptodactylus* previously karyotyped, the chromosomes 8 showed Ag-NORs in the short arms, at the interstitial region, at the telomere or at the distal region, as well as interstitially in the long arms, but, for the first time, two sites, in the short and the long arms, appeared simultaneously silver stained in pair 8 of four specimens of *L. mystacinus*.

Interindividual variation in single or multiple Ag-NOR patterns has been found in some anurans, as *Hyla chrysoscelis* and *H. versicolor* (Wiley et al., 1989), *Bufo terrestris* (Foote et al., 1991), *Rana japonica* (Miura, 1994), *Agalychnis callidryas* (Schmid et al., 1995), *Hyla ebraccata* (Kaiser et al., 1996), and *P. cuvieri* (Silva, Haddad and

Table 1. Patterns of Ag-positive patterns in the metaphases of the 14 specimens of *L. mystacinus*

Ag-positive patterns	CFBH 4538	CFBH 4539	CFBH 4008	CFBH 4726	CFBH 4002	CFBH 3995	CFBH 7603	CFBH 7604	CFBH 7605	CFBH 7606	CFBH 7607	CFBH 7608	CFBH 7609	CFBH 6585
4p4p	3	4	3		7		1	32	85	4	17	5	45	35
4p8p				12						3	2			
4p4p8p			1		1	3		2	4	24	17		1	
4p4p11p					5	3								
4p4p8p8p					7	15	53	1		9			5	
4p4p8p8p11p					3	6	3	1		2		2		
4p4p8pq8pq11p					4	7								
4p4p8pq8pq11p11p						10	3						3	
1p1p4p4p									3					
Total of metaphases	3	4	4	12	27	44	60	36	92	42	36	7	54	35

p = short arms; q = long arms.

Kasahara, 1999). With exception of this last species showing additionally variation within the same individual, one particular Ag-NOR pattern is, in general, constant within each specimen. *L. mystacinus* might, then, illustrate another rare case with Ag-NOR intraindividual variation, in which ten specimens have more than one Ag-NOR pattern and only four have one invariable pattern among their metaphases. Nevertheless, some considerations can be made on this assumption on the basis of our FISH data.

The presence of one fluorescent signal in two distinct chromosomes, in all the analyzed metaphases of the specimen CFBH 4726, is according to its invariable 4p8p Ag-NOR pattern, and indicates the lack of ribosomal sequence in one of the homologues of both pairs 4 and 8. On the other hand, in the case of CFBH 3995, with six distinct patterns of silver stained regions in the sample of 44 metaphases, and supposedly more than one Ag-NOR pattern, only three FISH signals were noticed. This result may then indicate that in this specimen only the 4p4p8p is the true and invariable Ag-NOR pattern, the others due to heterochromatin staining, even the patterns including more than one Ag-stained 8p region. This hypothesis is more probable, otherwise we have to admit that in our FISH screening the other 8p NOR site, in the homologous chromosome, was not present just in all the analyzed cells, but it should be genetically active in the majority of the Ag-stained metaphases.

The C-banding pattern of *L. mystacinus*, along with the FISH data of the specimen CFBH 3995 and the fluorochrome staining of the specimen CFBH 4002, enlighten in a certain extension the variation observed in the positively silver stained regions among the specimens of our sample. Undoubtedly, it is very intriguing that for the majority of these sites in CFBH 3995 no rDNA sequences have been detected with FISH technique, in spite of their silver positive staining, similar to that found in the Ag-NORs. Similar incongruous FISH data with Ag-staining findings were reported in some mammal species by Sánchez et al. (1995) in the hedgehog *Erinaceus algirus*, as well as in the rodents *Akodon cursor* by Fagundes (1997), and *Taterillus* sp. and *T. pygargus* by Dobigny et al. (2002). As also pointed out by these authors, some Ag-positive sites in the genome of *L. mystacinus* are not true Ag-NORs but might be

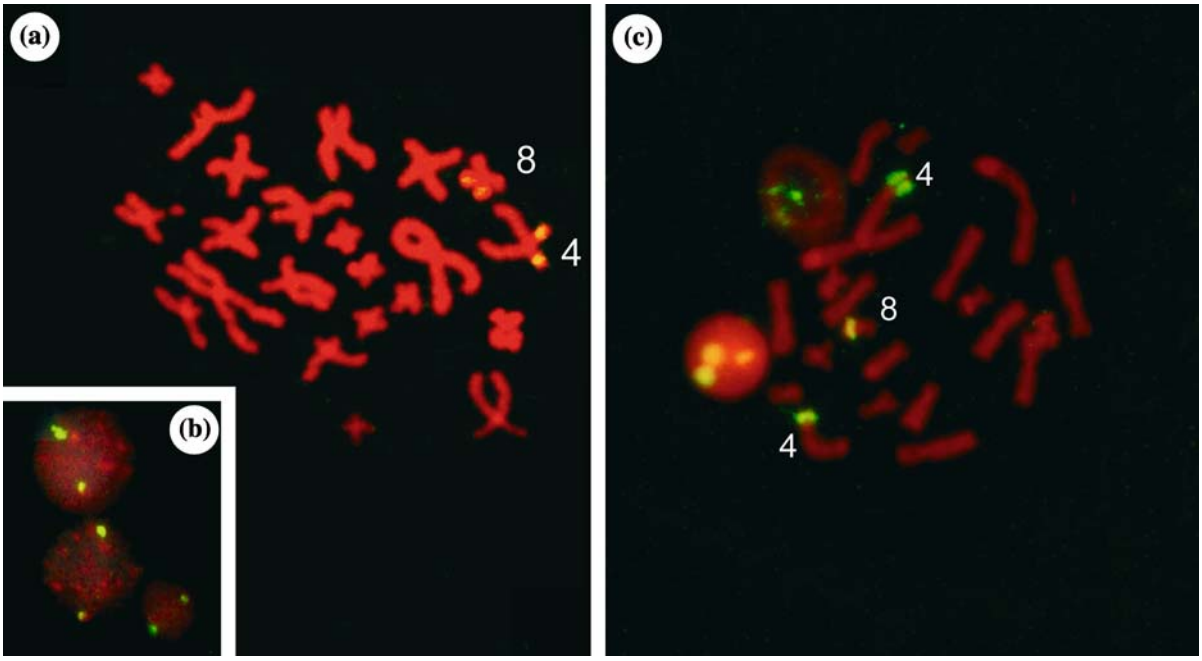

Figure 6. FISH with rDNA probe in *L. mystacinus*. (a, b) metaphase and interphase nucleus of CFBH 4726 with two fluorescent signals. (c) metaphase and interphase nucleus of CFBH 3995 with three fluorescent signals.

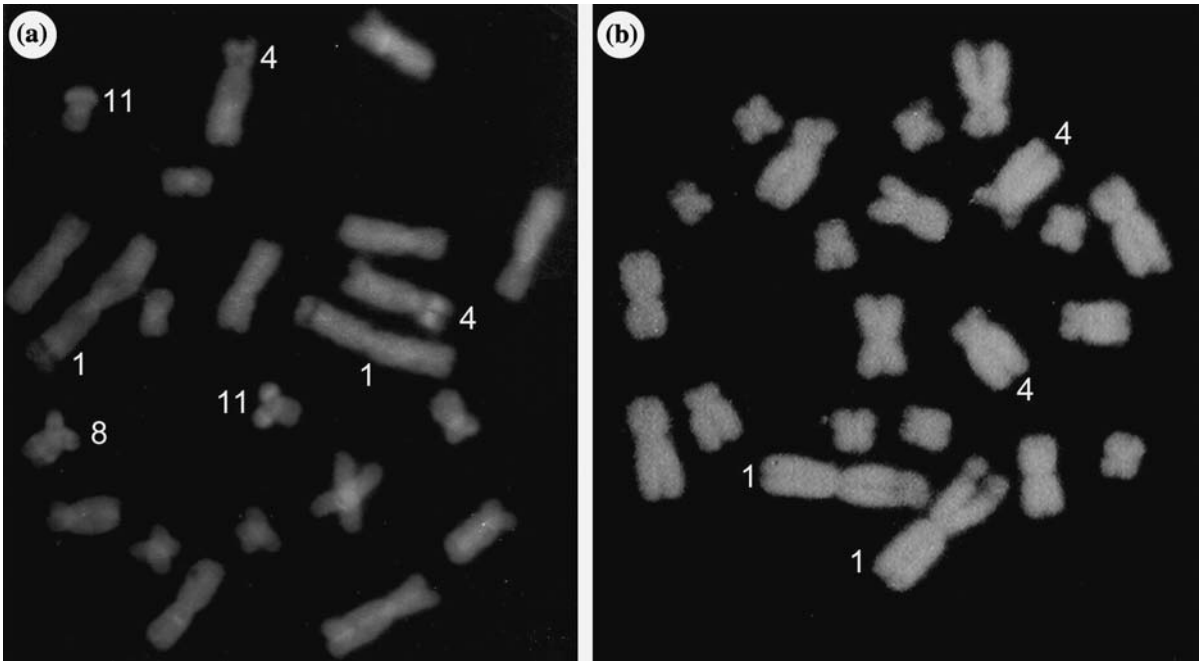

Figure 7. Metaphases of *L. mystacinus* (CFBH 4002) with fluorochrome staining. (a) DA/CMA3. (b) DA/DAPI. Note negatively stained regions in the chromosome pair 1 in (a) and (b).

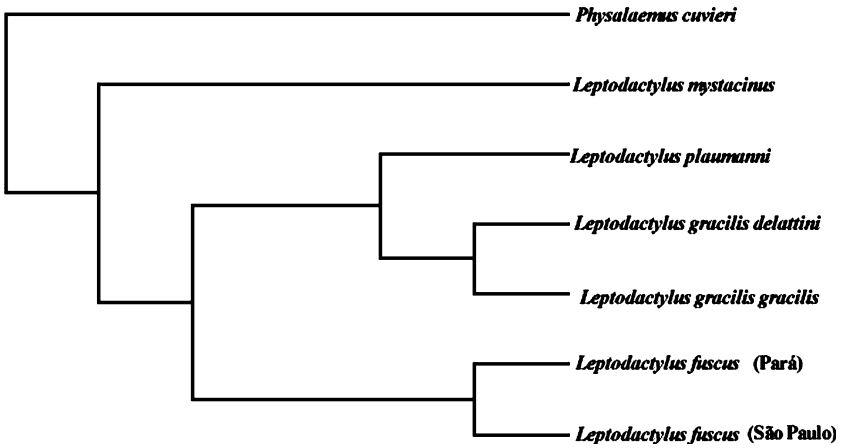

Figure 8. Parsimony phylogenetic tree derived from partial cytochrome b sequence. Reproduced from Silva et al. (2004) with modifications.

related to some peculiarities of the heterochromatin located at these sites, although they are not always identified with C-banding. Formerly, we had observed an unexpected result in *Bufo schneideri* (called *Bufo paracnemis*) in which the centromeric heterochromatin of all chromosomes appeared silver stained, in a similar way to that presented by its single pair of Ag-NORs (Kasahara, Silva and Haddad, 1996). In such case, the authors suggested the presence of proteins in the heterochromatin with affinity to silver, because the staining pattern matched thoroughly the C-banding of the species. We presume that the Ag-staining of the C-banded centromeric heterochromatin eventually observed in some chromosomes of *L. mystacinus* is due to the same reason. It is important to remark that apparently the attribute of silver staining is characteristic of both GC and AT-rich heterochromatin, because while the majority of the silver stained chromosome sites corresponded to CMA_3 positive regions, the Ag-stained signal on the short arms of the chromosome pair 1 is clearly CMA_3-negative.

In conclusion, more FISH data, in a greater number of specimens, are still necessary, in order to make clear whether the intraindividual variability in the silver stained regions in *L. mystacinus* is owing to Ag-NORs variation and in which extension it is due to the heterochromatin staining.

The phylogenetic tree constructed by us (Silva et al., 2004) on the basis of the partial sequences of the mitochondrial cytochrome b gene in some species of *L. fuscus* group, demonstrated three clear distinct groupings. One of them is formed by two *L. fuscus* populations from the states of Pará and São Paulo. The other comprises the cryptic species *L. gracilis gracilis*, *L. gracilis delattinni*, and *L. plaumanni*, from the state of Santa Catarina. The third grouping is formed by *L. mystacinus*, which represents a separate branch, indicating that this species diverged before the other studied *Leptodactylus* species. This result agrees with the cytogenetic data since the Ag-NOR pattern of *L. mystacinus* markedly differs from that of the other species of the *L. fuscus* group. The first report of multiple NORs in the genus *Leptodactylus* corresponds to a derived condition, and might thus be considered, in fact, an autapomorphy in *L. mystacinus*.

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