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Cytogenetics of *Eupemphix nattereri* Steindachner, 1863 (Anura: Leiuperidae) and karyotypic similarity with species of related genera: taxonomic implications*

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Abstract *Eupemphix nattereri* Steindachner, 1863 from three localities in Brazil showed 2n = 22 karyotype, with metacentric or submetacentric chromosomes and one telocentric pair 11. The specimens have a single pair of Ag-NOR, at the distal region of the long arms of chromosomes 11, although Ag-positive labeling was frequently observed in the proximal region of these chromosomes. The true Ag-NOR was confirmed by FISH with an rDNA probe, which never hybridized in any other chromosome site. C banding pattern is predominantly centromeric, with some interstitial or telomeric bands. Chromosomes 8 presented either gain or loss of interstitial heterochromatin, leading to size variation between the homologs in some specimens. Since the heteromorphism occurred in specimens from the three localities and in both male and female collected in one of them, at present, the possibility of geographical karyotypic variation and of cytologically differentiated sex chromosomes was ruled out, due to the small sample size. An increased amount in the centromeric heterochromatin of the chromosome 11 was seen, but only in specimens from one of the localities. CMA_3 staining revealed some GC-rich repetitive regions, whereas with DAPI no particular bright AT-rich site was detected. BrdU replication banding was obtained for the first time, but karyotypic comparisons with leiuperids of related genera were based on standard stained chromosomes. The karyotype of *Eupemphix nattereri* is indistinguishable of those presented by the representatives of *Physalaemus signifer* group analyzed so far. Moreover, the species is karyologically very close to the species of *Pleurodema* Tschudi, 1838, so that our cytogenetic data agree with the proposal based on morphological characters of including *Eupemphix nattereri*, *Pleurodema diplolister* (Peters, 1870) and *P. brachyops* (Cope, 1869) in the same clade [*Acta Zoologica Sinica* 53 (2): 285 – 293, 2007].

Key words *Eupemphix nattereri*, Chromosome, Ag-NOR, C banding, Fluorochrome staining

纳特竖蟾(无尾目:滑背蟾科)的细胞遗传、与相关属物种的核型相似性并兼论其分类地位*

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摘 要 本文报道纳特竖蟾 (*Eupemphix nattereri* Steindachner, 1863) 巴西 3 个产地标本的核型，均为 2n = 22，由 1 对端部和 10 对中或亚中部着丝点染色体组成。仅一对 NORs 位于 No.11 染色体对长臂端部，而其位置有别于常见的具有标志性意义的近着丝点位置。该 NORs 的位置为 rDNA 为探针的荧光原位杂交 (FISH) 所确认。各染色体对中，着丝点 C-带明显，插入或端带偶见。某些标本的 No.8 同源染色体对间 C-带的大小随异染色质化的程度不同而变化。居于较小的实验标本量，这种在 3 个产地的雌或雄性标本之一中观察到的 C-带异型现象可能为种下细胞地理学变异，亦或为细胞学意义的性染体分化。3 产地之一的标本核型的 No.11 着丝点 C-带异染色质化的程度较高。CMA_3 染色检测到部分 GC-rich 区域，DAPI 染色未显示任何 AT-rich 区。成功获得 BrdU 复制带，并将其与滑背蟾类动物 (leiuperid) 中近缘属及物种进行对比分析。比较结果表明，纳特竖蟾的核型与斑符泡蟾种组 (*Physalaemus signifer* group) 难以相互区别，而与肿肋蟾属 (*Pleurodema* Tschudi, 1838) 极为相似。核型数据支持形态学上将纳特竖蟾、二光肿肋蟾 [*Pleurodema diplolister* (Peters, 1870)] 和短头肿肋蟾 [*P. brachyops* (Cope, 1869)] 划为同一分支的观点 [动物学报 53 (2): 285 – 293, 2007]。

关键词 纳特竖蟾 染色体 Ag-NOR C-带 荧光染色 荧光原位杂交

Until recently, Leptodactylidae was the most diversified family among anurans with species distributed in 57 distinct genera (Frost et al., 2006). According to the revision carried out by these authors in the whole class of amphibian, based predominantly on molecular data, this number was reduced to 11, and soon after seven of them, i.e. *Edalorhina* Jiménez de la Espada, 1871, *Engystomops* Jiménez de la Espada, 1872, *Eupemphix* Steindachner, 1863, *Physalaemus* Fitzinger, 1826, *Pleurodema* Tschudi, 1838, *Pseudopaludicola* Miranda Ribeiro, 1926, and *Somuncuria* Lynch, 1978, were transferred to the revalidated family Leiuperidae by Grant et al. (2006).

The genus *Physalaemus* encompassed at first one single species, *P. cuvieri* Fitzinger, 1826, from Brazil; later, the genera *Eupemphix* and *Engystomops* were recognized with the descriptions of *Eupemphix nattereri* Steindachner, 1863 and *Engystomops petersi* Jiménez de la Espada, 1872, respectively (see Nascimento et al., 2005). Since the 1970s, these three genera were unified under *Physalaemus* by Lynch (1970), who recognized four distinct species groups, based on the variation of morphological characters. According to this arrangement, *Physalaemus nattereri* was assigned to the *P. biligonigerus* species group, whereas *Physalaemus petersi* to the *P. pustulosus* species group. Subsequently, species of *Physalaemus* were described or redescribed, but some of them could not be included in any of the species group (Nascimento et al., 2005), showing the taxonomic complexity of this genus. Heyer (1974, 1975) had considered the genus *Physalaemus* as monophyletic, which might form a sister group to *Pleurodema* or to *Pseudopaludicola*, but studies based on diverse characters, including those obtained by mitochondrial gene sequencing, ruled out subsequently the monophyly of the genus (Canatella and Duellman, 1984; Cannatella et al., 1998; Túrano and Ryan, 2002).

All these facts lead Nascimento et al. (2005) to carry out a taxonomic revision in the genus *Physalaemus*, and according to it the species groups were redefined on the basis of morphometrics, external morphology, color patterns, and osteological characters, totaling now seven groups. *Eupemphix* and *Engystomops* were revalidated in this study, the first genus including the single species *Eupemphix nattereri* and the latter, the representatives of the former *Physalaemus pustulosus* group.

To date, only the standard stained karyotype of *Eupemphix nattereri* was described by Beçak (1968). For this reason, further cytogenetic analyses, using Giemsa staining, as well as the techniques of Ag-NOR, C banding, CMA_3 and DAPI fluorochrome stainings, FISH with an rDNA probe, and incorporation of BrdU for obtaining RBG replication banding patterns in the chromosomes were performed. The objective of this study was to evaluate the karyotypic similarity among *Eupemphix nattereri* and some species of related genera, with emphasis in the contribution of the cytogenetic data to the taxonomy of the family Leiuperidae.

1 Material and methods

A sample of 29 specimens of *Eupemphix nattereri* was collected in three localities in the states of São Paulo (SP) and Minas Gerais (MG), southeastern Brazil: one male (CFBH09618), three females (CFBH09616, 09127-28) and eight juveniles (CFBH09617, 09619-23, 09625-26) from Descalvado, SP (21°54′14″S 47°37′10″W); four males (CFBH06473, 06573, 06589-90), one female (CFBH06579) and one juvenile (CFBH09624) from Itirapina, SP (22°15′10″S 47°49′22″W); and eight males (CFBH09631-38) and three females (CFBH09639-41) from Gurinhatã, MG (19°12′48″S 49°47′11″W). The specimens were deposited in the Collection Célio F. B. Haddad, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brazil.

For obtaining mitotic chromosomes, two different

procedures were used, namely direct cytological preparations from bone marrow and liver of animals previously submitted to phytohemagglutinin treatment, 0.01% colchicine injection, and sometimes 5-bromodeoxyurine + 5-fluorodeoxyuridine treatment (Baldissera Jr et al. 1993; Silva et al., 2000), and from intestine epithelial cells of animals injected with 1% colchicine solution (Schmid, 1978). From the majority of male specimens, testis preparations were also made. Standard staining was undertaken with Giemsa solution in phosphate buffer, pH 6.8. The Ag-NOR staining and C banding followed Howell and Black (1980) and Sumner (1972), respectively. Fluorochrome staining was obtained with AT-specific 4′, 6-diamidino-2-phenylindole (DAPI) and GC-specific chromomycin A_3 (CMA_3), according to two distinct techniques: one of them in which both fluorochromes were combined with the counterstain distamycin A (DA), following Schweizer (1980); and the other with no counterstaining, but with previous treatment of the cytological preparations with formamide, according to Christian et al. (1998) with minor modifications. Fluorescence *in situ* hybridization (FISH) was performed with a 18S probe from *Oreochromis niloticus* (Pisces, Cichlidae), according to Martins and Galetti Jr (1999), and RBG replication banding was differentiated by FPG staining, as described by Dutrillaux and Couturier (1981). Chromosome morphology was established by measurements and followed the nomenclature of Green and Sessions (1991).

2 Results

2.1 Karyotype description

All the 29 specimens of *Eupemphix nattereri* from the three localities exhibited 2n = 22 and karyotype formed by seven pairs of chromosomes of large or medium size, and four of small size (Fig.1). The chromosomes 1, 2, 5, 6, 8 and 10 are metacentric, whereas the chromosomes 3, 4, 7 and 9 are submetacentric and chromosomes 11 are telocentric. In the majority of the 22 specimens, pair 8 was homomorphic (Table 1, Fig.1a). The remaining specimens, one female and one juvenile from Descalvado,

Table 1 Morphometric data of mitotic chromosomes of *E. nattereri*

Chromosome number	Total lenght	Short arm	Long arm	Centromeric ratio	Classification
01	2.21	1.0	1.21	1.21	M
02	1.82	0.74	1.08	1.45	M
03	1.66	0.53	1.13	2.13	SM
04	1.59	0.45	1.14	2.53	SM
05	1.59	0.71	0.88	1.23	M
06	1.47	0.66	0.81	1.22	M
07	1.29	0.45	0.84	1.86	SM
08	0.99	0.42	0.57	1.35	M
09	0.9	0.32	0.58	1.81	SM
10	0.87	0.36	0.51	1.41	M
11	0.71		0.71	0	T

Classification by Green and Session (1991).

M = metacentric; SM = submetacentric; T = telocentric.

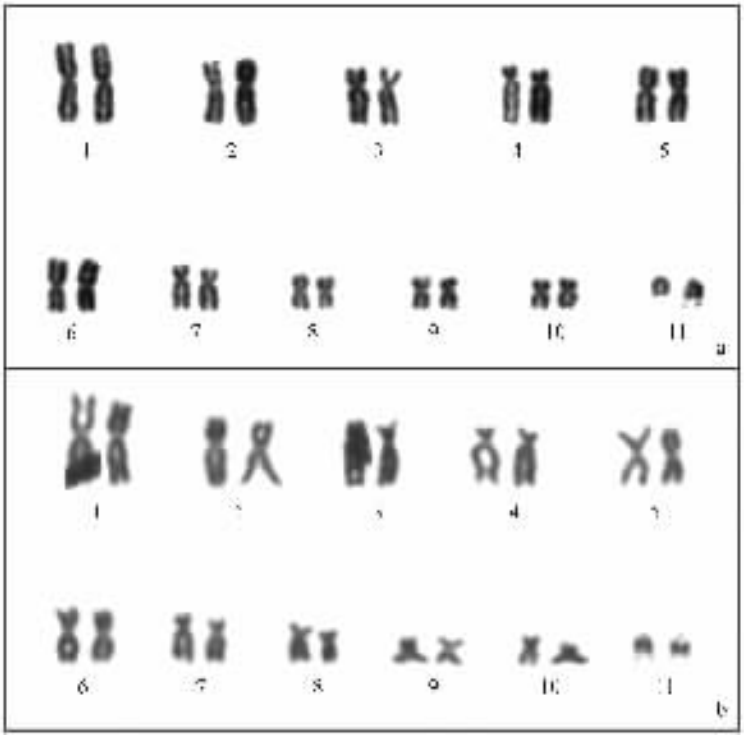

Fig.1 Giemsa stained karyotypes of *Eupemphix nattereri*

a. Male CFBH06589 from Itirapina, SP, with homomorphic pair 8. b. Female CFBH09616 from Descalvado, SP, with heteromorphic pair 8. Note distal secondary constriction in the chromosomes of pair 11.

SP, the juvenile from Itirapina, SP, and three males and one female from Gurinhatã, MG, exhibited an heteromorphic pair 8, in which the homologous chromosomes are of different sizes (Fig.1b). The chromosomes of pair 11 disclosed frequently a distal secondary constriction (Fig.1, Fig.8a), appearing associated in some metaphases.

Despite the heteromorphism of chromosome pair 8 observed in some specimens, no cytologically differentiated sex chromosome pair was could be ascribed to *Eupemphix nattereri*. Meiotic analysis in males, which exhibited eleven bivalents in diplotene and metaphase I cells, also provided no cytological evidence of XY bivalent.

2.2 Analyses of chromosomes with differential staining techniques

Chromosome preparations of all specimens were submitted to silver staining technique (Fig.2, Fig.8b), revealing Ag-NOR at the distal region of the telocentrics 11, coincident to the site of the secondary constriction. The proximal region of these same chromosomes appeared also silver stained, frequently with an intensity comparable to that presented by true NORs, but in some metaphases, sometimes within the same individual, this labeling at the proximal centromeric region was very subtle, or even completely absent in both homologs.

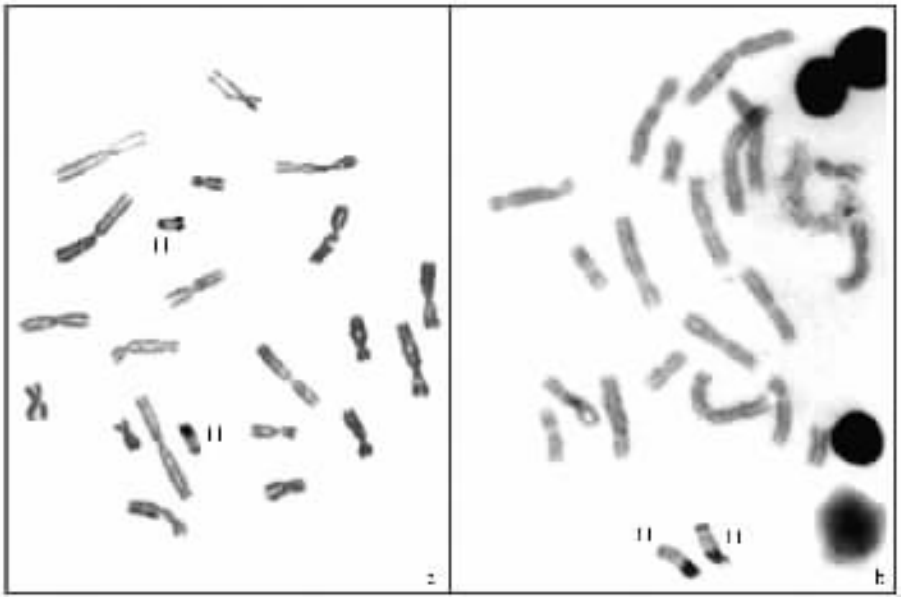

Fig.2 Silver stained metaphases of *Eupemphix nattereri*, showing Ag-NOR at the distal region of the chromosomes of pair 11. Note silver staining in the centromeric region of the same chromosomes

C banding was obtained in seven specimens of *Eupemphix nattereri* from the three localities (Fig.3), revealing positively stained centromeric heterochromatin; some interstitial bands, like in the short arms of the chromosome pairs 1, 2, 3, and 4, or telomeric bands in the case of the specimens from Gurinhatã, MG, were also observed. In the sample, three distinct types of C banded chromosomes 8 and 11, respectively, could be recognized (Fig.4, Fig.8c), so that interindividual variations concerning to these pairs were observed. One male from Itirapina, SP, and one male and one female from Descalvado, SP, had both homologs of pair 8 with identical C banding pattern, with a larger C banded block in the short arms than in the long arms (Fig.3a, Fig.4a and 4d). Chromosome 8 with this same C banding pattern is exhibited by one of the juveniles from Descalvado, SP, bearing heteromorphic pair 8 (Fig.3b, Fig.4b and 4e), but its larger homolog had an increased C band in the long arms. On the other hand, this large chromosome 8 occurred in one female from Descalvado, SP (Fig.4c and 4f), and one male and one female from Gurinhatã, MG, also bearing heteromorphic pair, but the smaller homolog had C band only in the short arms, beeing absent in the long arms. The three other specimens of the sample, in which heteromorphic pair 8 was noticed with standard staining, provided no C banding data. As regard to the chromosome 11, an additional amount of centromeric heterochromatin was observed in one of the homologs in two specimens from Gurinhatã, MG (Fig.3a). Sporadically, a slight positive staining in the site of the secondary constriction was seen (Fig.3b). In the C banded karyotypes of the remaining specimens, chromosomes 11 had centromeric C positive block of normal size.

Cytological preparations of three specimens, from Itirapina, SP, and Descalvado, SP, were submitted to CMA_3 and DAPI fluorochrome stainings. With CMA_3, both with or without DA counterstain, bright fluorescence was always observed in the heterochromatin of centromeric region of the chromosomes 1 and 11 (Fig.5a and 5c, Fig.8d). However, only with the staining procedure without DA (Fig.5c), bright fluorescence was also seen

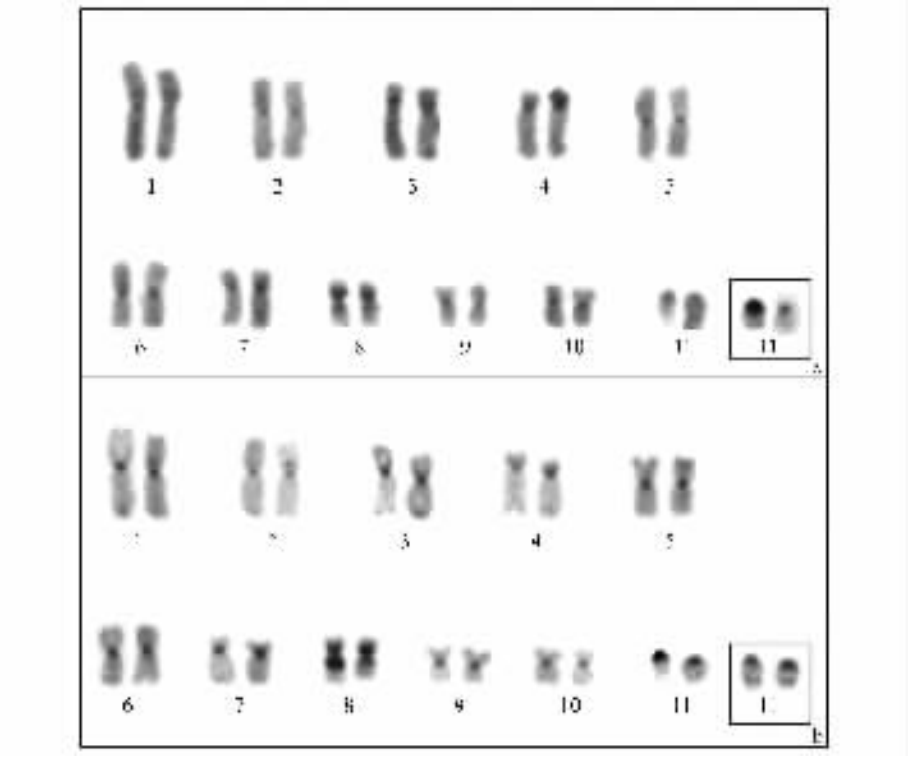

Fig.3　C banded karyotypes of *Eupemphix nattereri*

a. Male CFBH06590 from Itirapina, SP, with homomorphic pair 8. Inset: pair 11 from male CFBH09625 from Gurinhatã, MG, with an increased centromeric C band in one of the homologs. b. Juvenile CFBH09625 from Descalvado, SP, with heteromorphic pair 8. Inset: pair 11 from another metaphase, showing slight C banding in the distal region of the chromosomes.

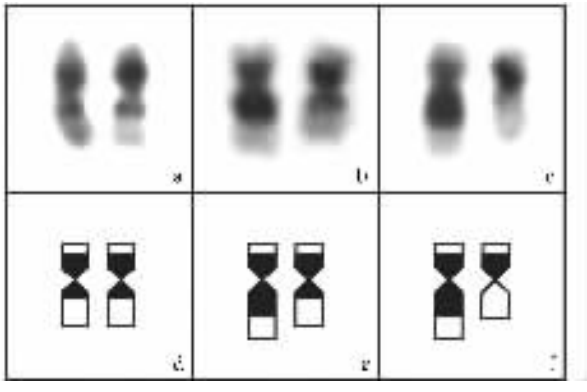

Fig.4　C banded chromosomes of pair 8 (a, b and c) and corresponding schematic representation (d, e and f)

a and d. Homomorphic pair 8 of male CFBH09618 from Descalvado, SP. b and e. Heteromorphic pair 8 of juvenile CFBH09625 from Descalvado, SP. c and f. Heteromorphic pair 8 of female CFBH09616 from Descalvado, SP.

in the distal region of one of the chromosomes 11, corresponding to the NOR, as well as in both short and long arms of chromosomes 8, and, less markedly, in the proximal region of the short arms of chromosomes 4. With DAPI, both with or without DA counterstain, no particular brilliant region was observed in any of the karyotypes (Fig.5b, Fig.8e).

In situ hybridization with an 18S probe confirmed the occurrence of ribosomal sequences just at the distal region of the telocentric pair 11, coincident with the true Ag-NORs (Fig.6, Fig.8f). RBG replication banding using BrdU incorporation was obtained in some specimens from Descalvado, SP, allowing the precise pairing of some chromosomes (Fig.7, Fig.8g).

3　Discussion

The karyotype of *Eupemphix nattereri* agrees thoroughly with that described by Beçak (1968), for three specimens from São José do Rio Preto, SP (23°11′15″S 45°56′15″W), but the novelty in the present study is the size variation in the chromosomes 8. With standard staining, the heteromorphism was not promptly recognized, because morphologically similar small-sized chromosomes were tentatively paired in the karyograms, according to their decreasing size. Nevertheless, it was unequivocally evidenced after C banding, which revealed either gain or loss of heterochromatin in the long arms of the chromosomes 8. If this technical procedure had been carried out in the cytological preparations of all individuals of the sample, most probably the heteromorphic pair 8 would have been detected in a greater number of specimens.

The heteromorphism of pair 8 seems not to be an intraspecific geographical chromosome variation, because it was shown by specimens from the three localities. Moreover, the hypothesis of cytologically differentiated sex chromosomes was, at present, also ruled out, since both male and female specimens collected in one of the

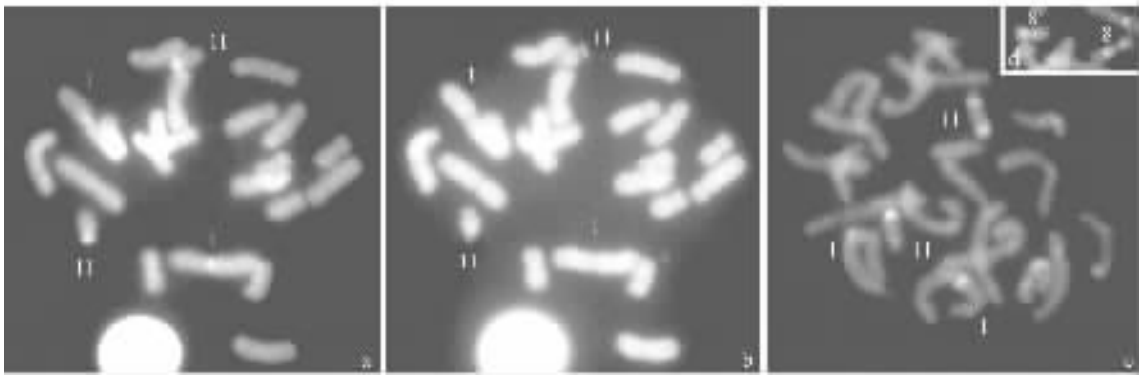

Fig.5 a and b. CMA_3/DA and DAPI/DA stained metaphases, respectively, of female CFBH09628 from Descalvado, SP. c. CMA_3 stained metaphase of juvenile CFBH09625 from Descalvado, SP. d. CMA_3 stained partial metaphase of the same specimen of c, showing chromosomes of heteromorphic pair 8.

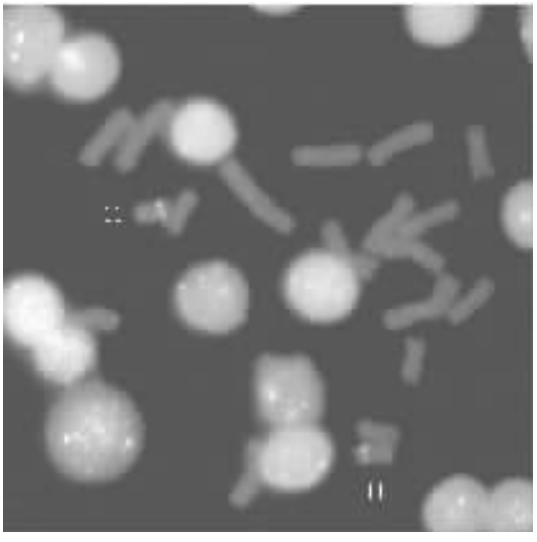

Fig.6 Fluorescence *in situ* hybridization (FISH) with an rDNA probe in male CFBH06590 from Itirapina, SP, showing hybridization signal in the distal region of the chromosomes 11

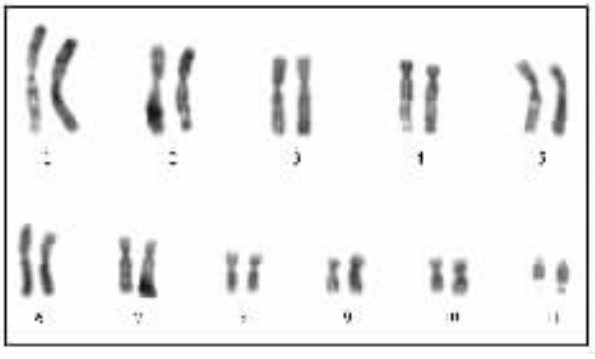

Fig.7 RBG replication banding patterns after BrdU incorporation in the chromosomes of *Eupemphix nattereri*

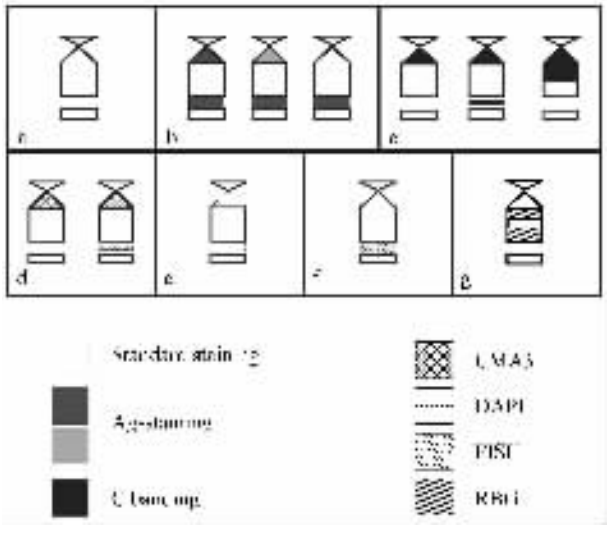

Fig.8 Schematic representation of distinct patterns in chromosomes 11, according to Giemsa staining (a), silver staining (b), C banding (c), CMA_3 staining with and without DA counterstain (d), DAPI staining with and without DA counterstain (e), FISH with an rDNA probe (f), and RBG replication banding after BrdU incorporation (g)

localities exhibited heteromorphic pair 8. Before additional cytogenetic data on a greater number of individuals of both sexes from the same or another localities are available, we are prone to consider the present finding as an autosomal heteromorphism, occurring in all three populations. Nevertheless, it is important to emphasized that although descriptions of morphologically recognized sex chromosomes are relatively rare among anurans, reports on geographical variability of sex chromosomes, as well as unusual types of mechanisms of sex determination, have been more and more frequent in the literature (Schmid et al., 1991).

The variability in the chromosome pair 11, due to an increased amount of the centromeric heterochromatin, was observed only in specimens from one of the localities, but we may not discard the possibility of specimens from the other regions exhibit this variation, because C banded karyotypes are still scanty. It is worth to remark that the heterochromatin in the chromosomes 8 and 11, as well as that of the chromosomes 1 and 4 of *Eupemphix nattereri* is particularly rich in GC base pairs, as indicated by bright

fluorescence with CMA_3 staining.

Quantitative variation in the amount of heterochromatin, like observed in *Eupemphix nattereri*, is a relatively common occurrence in the eukaryote genomes and it may be produced, among other mechanisms, as consequence of non-homologous pairing of the chromosomes, followed by unequal crossing-over (John, 1988). It is remarkable that not a single individual was observed bearing both homomorphic pair 8, in which the two homologs had either increased or deleted C bands, as well as bearing heteromorphic pair 8, with one chromosome with deleted C band and the other with the usual pattern. Similarly, chromosomes 11 with an increased centromeric C band never appeared in homomorphism. These findings may rather be due to the relatively small number of karyotyped specimens with C banding than to possible deleterious combinations of chromosomes 8. Variations in the amount of heterochromatin have been considered well tolerated, but the idea of its genetic inactivity can no longer be assumed, as evidences on its several functions have been accumulated (Pardue and Hennig, 1990; Redi et al., 2001).

The silver stained metaphases of *Eupemphix nattereri* displayed two Ag-labeled sites in the chromosomes of pair 11, leading us to the presumption of multiple Ag-NORs, as described before in *Engystomops petersi* (Lourenço et al., 1998), *Physalaemus cuvieri* (Silva et al., 1999), and *P. olfersii* Lichtenstein and Martens, 1856 (Silva et al., 2000). The possibility of multiple nucleolar organizer regions was discarded, because the Ag-staining in the proximal region was, generally, slight or even absent in some metaphases of the same individual and mostly because the rDNA probe labeled only the distal region of chromosomes 11. The lack of fluorescent hybridization signal is rather due to the absence of ribosomal genes than to the limitation of the FISH technique to detect a low copy number of rDNA repeats. *In situ* hybridization procedures using fluorescent probes have a high sensitivity and specificity (Viegas-Péquignot, 1992), so that the silver stained proximal region of the chromosome 11 is not considered true NORs. Most probably, this labeling must be due to the presence of C-positive heterochromatin with affinity to the silver impregnation, similarly to what was described in *Chaunus schneideri* (Werner, 1894) (as *Bufo paracnemis* in Kasahara et al., 1996). In this species, the centromeric heterochromatin of all chromosomes was silver stained, like the single pair of NORs. More recently, another description of silver stained sites not corresponding to true NORs, but to heterochromatic regions, was confirmed by FISH experiments in the anuran *Leptodactylus mystacinus* (Burmeister, 1861) (Silva et al., 2006). Similar findings have been reported in mammals (Sánchez et al., 1995; Dobigny et al., 2002) and also in fish species (Gromicho et al., 2005), so that Ag-staining technique is not so reliable for detecting previously active NOR sites in the eukaryotic genomes.

Although preliminarly, the replication banding obtained after BrdU incorporation in *Eupemphix nattereri* is very promising, as comparisons with other species can be more effective. Nevertheless, the evaluation of the karyotypic proximity with other leiuperids was performed on the basis of standard stained chromosomes.

Eupemphix nattereri, formerly included in the *Physalaemus* by Lynch (1970), is karyologically very similar to the species of this genus. Based on some available karyograms, Silva et al. (2000) had suggested two 2n = 22 karyotypic formulae for the species of *Physalaemus*, differing basically in the morphology of the smallest chromosome pair, which is uniarmed in some species and biarmed in others. *Eupemphix nattereri* has almost identical karyotype to those presented by *P. crombiei* Heyer and Wolf, 1989, *P. signifer* (Girard, 1853), *P. spiniger* (Miranda Ribeiro, 1926), and some non-identified species (Lucca et al., 1974; Silva et al., 2000), all of them exhibiting the first karyotypic formulae, as well as two species recently analyzed by us, *P. atlanticus* Haddad and Sazima, 2004 and *P. moreirae* (Miranda Ribeiro, 1937). The remaining species karyotyped so far, *P. aguirrei* Bokermann, 1966, *P. albifrons* (Spix, 1924), *P. biligonigerus* (Cope, 1861), *P. centralis* Bokermann, 1962, *P. cicada* Bokermann, 1966, *P. cuvieri*, *P. fuscomaculatus* (Steindachner, 1864), *P. gracilis* (Boulenger, 1883), *P. kroyeri* (Reinhardt and Lütken, 1862), *P. olfersii*, and *P. soaresi* Izecksohn, 1965 (Beçak, 1968; Beçak et al., 1970; Denaro, 1972; Lucca et al., 1974; Silva et al., 1999; Amaral et al., 2000; Silva et al., 2000), exhibited karyotypic formulae bearing biarmed chromosomes 11.

It is worth to remark that *Physalaemus biligonigerus* and *P. fuscomaculatus*, which were included with *Eupemphix nattereri* in the morphological group of *P. biligonigerus* of Lynch (1970), have discrepant karyotypes (Amaral et al., 2000; Silva et al., 2000), with regard to this latter species, due to the presence of biarmed chromosome pair 11. On the other hand, the karyotype of *E. nattereri* is indistinguishable of those exhibited by *P. crombiei*, *P. signifer*, *P. spiniger*, *P. atlanticus*, and *P. moreirae*, all of them currently assigned to the *P. signifer* species group, according to the recent taxonomic review of Nascimento et al. (2005). This fact highly strengthens the proposal of this grouping of *Physalaemus* species made by these authors and also indicates a closer proximity with *E. nattereri*. By this reason, the generic position of *E. nattereri* in relation to *Physalaemus* should be reevaluated on the basis of molecular data. The arrow head mark present on the dorsum of all species of *Physalaemus* from the group

signifer is observed too in *E. nattereri* and probably is a synapomorphy of this group (personal observations). If *E. nattereri* belongs to the *P. signifer* group, this species would be the only one from open formation in the group.

With regard to the five other genera of Leiuperidae, *Edalorhina*, *Engystomops*, *Pleurodema*, *Pseudopaludicola*, and *Somuncuria*, the analyzed species have also shown 2n = 22, with exception of the two species of *Pseudopaludicola* (revision in King, 1990; Kuramoto, 1990; Beçak, 1968; Léon, 1970; Veloso and Iturra, 1987; Schmid et al., 1993; Lourenço et al., 1998, 1999; Lourenço et al., 2000). By examining the available karyograms, it was noticed that the karyotype of *Eupemphix nattereri* is somewhat distinct from those presented by *Pseudopaludicola ameghini* Cope, 1887 with 2n = 20 and *P. falcipes* Hensel, 1867 with 2n = 18 (Beçak, 1968), *Engystomops petersi* (Lourenço et al., 1998, 1999) and *Edalorhina perezi* Jiménez de la Espada, 1871 (Lourenço et al., 2000); nevertheless, it matches almost thoroughly with those of *Pleurodema thaul* Lesson, 1827 and *Pleurodema brachyops* Cope, 1869 (Veloso and Iturra, 1987; Schmid et al., 1993), despite of the distinct ordering of the chromosomes in the karyograms presented by the authors. These cytogenetic data indicate a closer proximity of *Eupemphix* with *Pleurodema* than with *Pseudopaludicola*, giving more consistent support to the UPGMA analyses of Nascimento et al. (2005), in which *Eupemphix nattereri*, *Pleurodema diplolister* (Peters, 1870) and *P. brachyops* (Cope, 1869) were allocated in the same clade that is, on the other hand, basal to all *Physalaemus* species. Less probable, seems to be the inclusion of *Eupemphix nattereri* in the same clade of *Engystomops petersi* and *E. pustulosus*.

In conclusion, further cytogenetic studies, with more refined techniques, and mainly analysis using molecular approaches are highly recommended, in order to better establish the phylogenetic relationships of *Eupemphix nattereri* with the leiuperid species.

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