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Chromosome evolution in three Brazilian *Leptodactylus* species (Anura, Leptodactylidae), with phylogenetic considerations

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Karyotypic analyses on three species of the *Leptodactylus* from Brazil showed 2n = 24 in *L.* cf. *marmoratus*, 2n = 23 in *Leptodactylus* sp. (aff. *bokermanni*), and 2n = 26 in *L. hylaedactylus*, with distinct numbers of bi and uni-armed chromosomes. *Leptodactylus* cf. *marmoratus* presented a variation as regard to the morphology of pair 12. All specimens of *L.* cf. *marmoratus* had Ag-NOR in pair 6, confirmed by FISH, but the sample from one of the localities presented additional Ag-NOR, in one of the chromosomes 8. In *Leptodactylus* sp. (aff. *bokermanni*) and *L. hylaedactylus* the chromosome pairs bearing Ag-NOR are 11 and 7, respectively. The C banding patterns are predominantly centromeric, but only in *L. marmoratus* this heterochromatin appeared very brilliant with DAPI. On the other hand, bright labelling was noticed with CMA_3 in the three species, on the Ag-NOR site. The data obtained here are in accordance with the proposed phylogeny to the genus, and the chromosomal analyses in these *Leptodactylus* showed that the karyotype evolution was based mainly in centric fusion and pericentric inversion.

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The species previously identified as belonging to the genus *Adenomera* were allocated in *Leptodactylus* after the revision of Frost et al. (2006), but the relationships of both genera have long been a target of many discussions. Firstly, the species *A. marmorata* was renamed *L. marmoratus* by Heyer (1973), due to its geographic distribution and several morphological characteristics shared with some *Leptodactylus*, as size, shape, texture and colour pattern. Soon after, Heyer (1974) carried out an extensive revision on the relationships within the subfamily Leptodactylinae, using primitive and derived states for 50 morphological characters. The study showed that the relationships in the *marmoratus* group were better explained by placing the species in a distinct genus, so the name *Adenomera* was revalidated. Several other papers have dealt with this question using distinct parameters (Heyer 1975, 1977, 1998; Heyer and Maxson 1982; Angulo et al. 2003; Kokubum and Giaretta 2005), and in all of them the genera *Adenomera* and *Leptodactylus* were accepted, although their separation was not very clear. Heyer (1998) and Kokubum and Giaretta (2005) presented evidence that *Adenomera* renders *Leptodactylus* paraphyletic, and that the genus *Lithodytes* was the sister-taxon of *Adenomera*.

The revision of Frost et al. (2006), based predominantly on molecular data, introduced great changes in all the Amphibia class, and according to it, the number of genera in the family Leptodactylidae, was reduced from 57 to 11, and soon after, to four, by Grant et al. (2006). *Leptodactylus*, the most speciose of them with 85 species, includes currently the former *Leptodactylus* and the representatives of *Adenomera*, *Lithodytes* and *Vanzolinius* (Frost 2008). Some of the remainder genera were distributed into other families, some of them already recognised and others revalidated or created. Nevertheless, many questions about the new taxonomy still exist.

Leptodactylus marmoratus is a species-complex and the correct identification of the specimens probably is hindered by the very similar morphology (Angulo et al. 2003). In fact, Kwet (2006) suggested that populations of *L. marmoratus* from southern Brazil belong to, at least, four distinct species, so that these specimens must be treated as *L.* cf. *marmoratus*.

Relatively few species of *Leptodactylus* have hitherto been karyotyped and almost all of them are characterised by 2n = 22 chromosomes (revisions in King 1990; Kuramoto 1990; Amaro-Ghilardi 2005). The exceptions are *L. andreae* and *L. hylaedactylus* with 2n = 26, *L. marmoratus* and *L. silvanimbus* with

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2n = 24, and *L. lineatus* with 2n = 18 (Bogart 1970, 1973, 1974; Amaro-ghilardi et al. 2004). Only *L. silvanimbus* was also analysed with differential staining (Amaro-ghilardi et al. 2004).

Considering that cytogenetic data in *Leptodactylus* with diploid numbers different of 2n = 22 are scarce, chromosome analyses were carried out in *L.* cf. *marmoratus*, *Leptodactylus* sp. (aff. *bokermanni*), and *L. hylaedactylus* using, for the first time, Ag-NOR and C-banding techniques, FISH using an rDNA probe, and base pair-specific fluorochrome staining. Besides a better evaluation on the karyotype diversity within this group, these data were useful to explain its relationships with the species of *Leptodactylus* with 2n = 22.

MATERIAL AND METHODS

Cytogenetic analyses were performed in 34 specimens of *Leptodactylus* cf. *marmoratus* and in one *Leptodactylus* sp. (aff. *bokermanni*) from southeastern Brazil, and in 15 *L. hylaedactylus* from northern Brazil (Table 1). The localities where the species were collected are showed in Fig. 1. One of the five collection sites of *L.* cf. *marmoratus* is Ilha dos Alcatrazes, municipality of São Sebastião, about 35 km from the coast of state of São Paulo. All the voucher specimens were deposited in the Coleção de Anfíbios (CFBH) of the Depto de Zoologia, Inst. de Biociências, UNESP, Rio Claro, São Paulo, Brazil.

Chromosome spreads were obtained from bone marrow, liver, spleen and testes, according to the procedures described in Baldissera Jr et al. (1993), or from intestinal epithelium, following Schmid (1978), with minor modifications. Conventional staining was performed with Giemsa diluted in phosphate-buffered saline, pH 6.8. Ag-NOR impregnation and C-banding were obtained by the techniques of Howell and Black (1980) and Sumner (1972), respectively. Fluorochrome staining was performed with AT-specific 4′,6-diamidino-2-phenylindole (DAPI) and GC-specific chromomycin A_3 (CMA_3), both combined with distamycin A (DA) by the method of Schweizer (1980), or without DA counterstain according to Christian et al. (1998). Fluorescence in situ hybridisation (FISH) with rDNA probe, the HM123 (Meunier-Rotival et al. 1979) was carried out following the method of Viegas-Péquignot (1992). The bi-armed chromosomes were classified as metacentric or submetacentric, and the uni-armed, as telocentric or subtelocentric, by visual inspection, following the nomenclature of Green and Sessions (1991).

RESULTS

Karyotype description

All males and females of *Leptodactylus* cf. *marmoratus* showed 2n = 24 chromosomes, distributed into four large, two medium and six small-sized pairs (Fig. 2a–b). The karyotype of specimens from Santa Branca, and Ilha dos Alcatrazes, is formed by two metacentric pairs (1 and 5), three submetacentric pairs (2–4), and seven telocentric pairs (6–12). The remainder specimens from

Table 1. *Species, number and sex, voucher number, and collection locality of specimens of Leptodactylus.*

Species	Number and sex	Voucher number	Collection locality
L. cf. *marmoratus*	1J	CFBH13650	Salesopólis, SP 23°33′S 45°50′W
	19F, 5M	CFBH11511-12 CFBH11514-25 CFBH11532-37	Santa Branca, SP 23°23′S 45°53′W
	1M, 1J	A499, A370	São Luís do Paraitinga, SP 23°13′S 45°17′W
	4F, 1M, 1J	CFBH17137-43	Ilha de Alcatrazes, SP 24°05′S, 45°41′W
	1M	CFBH13651	Ubatuba, SP 23°26′S 45°04′W
Leptodactylus sp. (aff. *bokermanni*)	1M	CFBH11531	Santa Branca, SP 23°23′S 45°53′W
L. hylaedactylus	2F, 2M	CFBH17155-58	Amapá, AP 02°57′N 50°47′W
	5F, 3M, 1J	CFBH17146-54	Macapá, AP 00°02′N 51°03′W
	2F	CFBH17159-60	Porto Velho, RO 08°45′S 63°54′W

F: female; M: male; J: juvenile.

Fig. 1. Collection localities of the species: 1-Amapá, AP; 2-Macapá, AP; 3-Porto Velho, RO; 4-Santa Branca, SP; 5-São Luíz do Paraitinga, SP; 6-Salesópolis, SP; 7-Ubatuba, SP; 8-Ilha dos Alcatrazes, SP. *Leptodactylus* cf. *marmoratus* was collected in 4, 5, 6, 7, and 8, *Leptodactylus* sp. (aff. *bokermanni*) in 4, and *L. hylaedactylus* in 1, 2 and 3.

the other three localities have a very similar karyotype, but the homologues of pair 12 are metacentrics. Frequently, the chromosomes 6 showed a prominent proximal secondary constriction (Fig. 2a). *Leptodactylus* sp. (aff. *bokermanni*) presented an odd diploid number of 2n = 23 (Fig. 2c), with two metacentric pairs (1 and 5), three submetacentric pairs (2–4), one subtelocentric pair (8), four telocentric pairs (6, 10–12), and three unpaired chromosomes, one of them being metacentric, equivalent in size to the chromosome pair 5, and two telocentrics, probably, chromosomes 7 and 9. Males and females of *L. hylaedactylus* from the three localities showed 2n = 26 (Fig. 2d), with three large submetacentric pairs (1–3), one medium metacentric pair (4), and nine telocentric pairs (5–13), one of them medium-sized (6) and the remainder of small size. Secondary constriction can be visualised in a variable number of small telocentric chromosomes, always at the proximal region.

Male specimens of *L.* cf. *marmoratus* and *L. hylaedactylus* exhibited, respectively, 12 and 13 bivalents in diplotene/metaphase I, and 12 and 13 chromosomes in metaphase II cells. The male of *Leptodactylus* sp. (aff. *bokermanni*) showed 10 bivalents and one trivalent in diplotene/metaphase I (Fig. 3a–b). No cells in metaphase II were observed in the meiotic cytological preparation.

Differential staining

In *Leptodactylus* cf. *marmoratus*, Ag-NOR is located at the proximal region of both homologues of the telocentric pair 6, in the same site of the secondary constriction (Fig. 4a). Nevertheless, differently from what was observed in the majority of the samples, the specimens from Ilha dos Alcatrazes showed a very subtle silver impregnation. Within the same specimen only one Ag-NOR was visualised in some metaphases; and an additional Ag-positive site at the proximal region of a small telocentric chromosome, probably the 8, could be noticed in rare metaphases (Fig. 4b). The NOR site was confirmed by FISH in the specimen from Salesópolis (Fig. 5). In *Leptodactylus* sp. (aff. *bokermanni*), the Ag-NOR is at the terminal long arms of the telocentrics 11 (Fig. 4c), and in *L. hylaedactylus* at the proximal region of the telocentrics 7 (Fig. 4d). C-banding showed heterochromatin at the centromeric

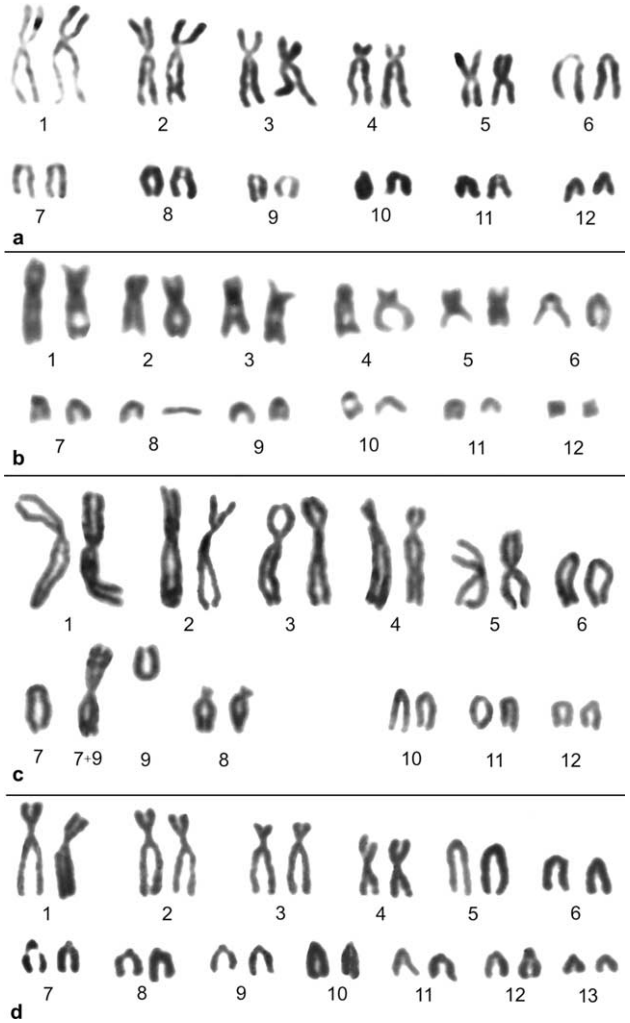

Fig. 2a-d. Giemsa stained karyotype. *Leptodactylus* cf. *marmoratus*, karyotype A (**a**) and karyotype B (**b**); *Leptodactylus* sp. (aff. *bokermanni*) (**c**); *L. hylaedactylus* (**d**). Note secondary constriction in the chromosomes 6 in (**a**) and in some small sized telocentrics, e.g. 7, 8, 9 and 12 in (**d**).

region of the chromosomes in the three species (Fig. 6). In addition, a C-positive band was observed at the same site of Ag-NOR in *L.* cf. *marmoratus* (Fig. 6a), and in *Leptodactylus* sp. (aff. *bokermanni*) the telocentric 7 exhibited a pericentromeric C band (Fig. 6b).

With DAPI, counterstained or not with DA, a very brilliant fluorescence was observed in the centromeric region of the chromosomes in *L.* cf. *marmoratus* from Santa Branca, whereas with CMA_3 only the Ag-NOR site appeared with slight fluorescence with both procedures (Fig. 7a–b). In *Leptodactylus* sp. (aff. *bokermanni*) no fluorescent band with DAPI/DA was visualised, but with CMA_3/DA the site of the Ag-NOR was extremely bright (Fig. 7c). In *L. hylaedactylus* no particular fluorescent band was observed with DAPI/DA, but with CMA_3/DA the NOR bearing chromosomes, as well as some of the small telocentrics showed a slight fluorescence in the site of the secondary constriction (Fig. 7d).

DISCUSSION

The species of *Leptodactylus* of the present paper showed distinct diploid numbers of 2n = 23, 24, and 26, but the FN = 34 is observed in all three species, with exception of some individuals with 2n = 24, FN = 36. Differently to that observed in the majority of *Leptodactylus* analysed so far presenting 2n = 22, FN = 44, with no telocentric or subtelocentric chromosomes, the species exhibited a high and variable number of uni-armed chromosomes in their karyotypes.

Specimens of *L.* cf. *marmoratus,* from two localities, including those from island, showed karyotype A with 2n = 24, FN = 34, characterised by seven telocentric pairs, the same that had been found by Bogart (1974) in specimens from a not mentioned locality, but also in the state of São Paulo. The karyotype B with 2n = 24, described in specimens from the remainder localities is equivalent to karyotype A, regarding the morphology of almost the totality of the chromosome pairs. The remarkable difference is the smallest chromosome pair that is of the metacentric type, explaining the FN = 36.

The two morphological types of chromosomes 12 probably resulted from a pericentric inversion. Considering that the metacentric chromosome pair 12 has been found in three distinct localities, the possibility of intra-specific geographical karyotypic differences might be suggested, despite the few analysed specimens in each sample. Nevertheless, taking into account that *L. marmoratus* has long been considered a species-complex, the hypothesis that the different karyotypes here observed could be ascribed to distinct species, is not ruled out. In this case, it would be

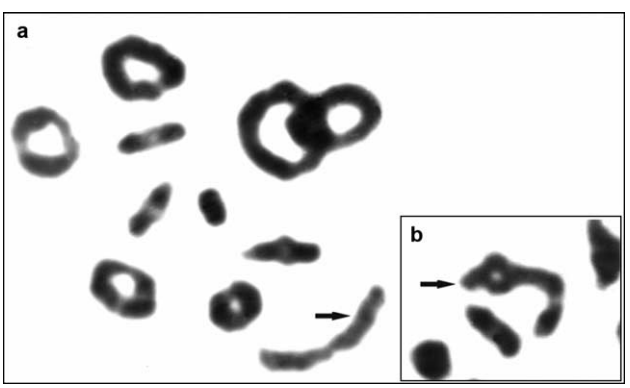

Fig. 3a-b. Diplotene/Metaphase I of *Leptodactylus* sp. (aff. *bokermanni*), showing one trivalent and ten bivalents (**a**) and partial Diplotene/Metaphase I of the same animal (**b**). Arrow indicates the trivalent.

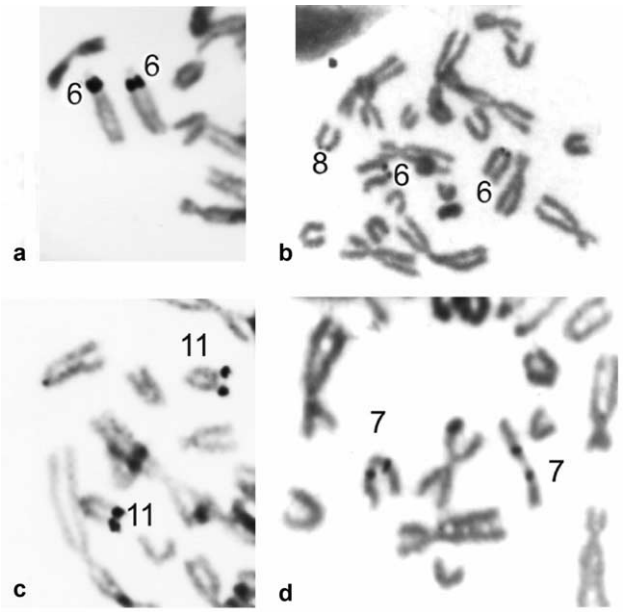

Fig. 4a-d. Partial metaphases after Ag-NOR technique. *Leptodactylus* cf. *marmoratus* from Santa Branca (**a**), *L.* cf. *marmoratus* from Ilha dos Alcatrazes (**b**), *Leptodactylus* sp. (aff. *bokermanni*) from Santa Branca (**c**), and *L. hylaedactylus* from Macapá (**d**).

important to analyse other characters, such as vocalisation, external and internal morphologies, geographic distribution, and mainly sequencing of nuclear and mitochondrial genes, in addition to cytogenetic studies.

The Ag-NOR pattern in *L.* cf. *marmoratus* is the same among the individuals from the all five localities showing the chromosomes 6 labelled at the proximal region, confirmed by FISH with an rDNA probe. It would be interesting to use this technique in order to verify whether the subtle labelling in the chromosomes 6 in the specimens from Ilha dos Alcatrazes is due to a differential gene activity or may represent, in fact, a less amount of repetitive sequences. This technique will also be useful to confirm the third Ag-positive site in one of the chromosomes 8, as true NOR or not, since associated proteins in heterochromatic regions with silver affinity have already been described in some amphibian species (Kasahara et al. 1996; Silva et al. 2006; Ananias et al. 2007). Undoubtedly, both techniques of Ag-NOR and FISH should be extended to a larger number of specimens, from Ilha dos Alcatrazes and from Santa Branca, in order to evaluate if the NOR is a good cytological marker to differentiate karyotype A from these two geographical regions.

The specimen of *Leptodactylus* sp. (aff. *bokermanni*) showed a peculiar karyotype constitution, highly indicative of a centric fusion. This rearrangement involves two telocentric chromosome pairs, among those of larger size, probably, the 7 and the 9, because the odd metacentric is a large sized chromosome. Less probable, the centric fission occurred in a $2n = 22$ karyotype, because the chromosomal evolution in this group seems to be towards the reduction in the chromosome numbers (Heyer and Diment 1974). This rearrangement in heteromorphic condition was fully confirmed by the presence of a meiotic trivalent.

At first sight, the specimen *Leptodactylus* sp. (aff. *bokermanni*) shares several morphological characters with *L. bokermanni*, also occurring in São Paulo state. Nevertheless, a more detailed analysis showed that is a representative of an unknown species, not described so far. Considering that only one specimen was karyotyped, it is not possible to know if the rearrangement is a sporadic variant or if is present in the other individuals. This question might be investigated increasing the sample size from the same locality, which is also relevant to ascertain the basic chromosome number of *Leptodactylus* sp. (aff. *bokermanni*). If bearing $2n = 24$, with seven telocentric pairs, the karyotype would be very similar to the karyotype A of *L.* cf. *marmoratus*, occurring in the same locality of Santa Branca. Nevertheless some clear differences can be pointed out, such as the presence of the subtelocentric pair 8, and Ag-NOR site located on the terminal region of the telocentric 11 in *Leptodactylus* sp. (aff. *bokermanni*).

The karyotype of *L. hylaedactylus*, the most discrepant among the analysed species, has also been described by Bogart (1974) for specimens from Peru. In *L. hylaedactylus* there are four bi-armed chromosome pairs, but the large metacentric pair 1, char-

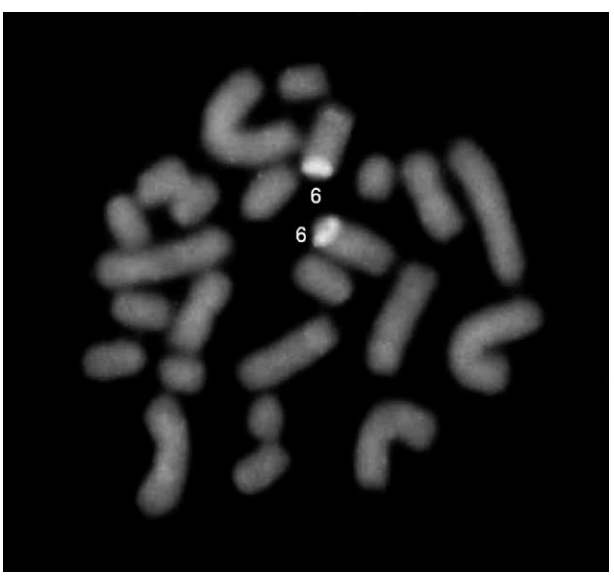

Fig. 5. Metaphase of *Leptodactylus* cf. *marmoratus* with FISH using the rDNA probe HM123.

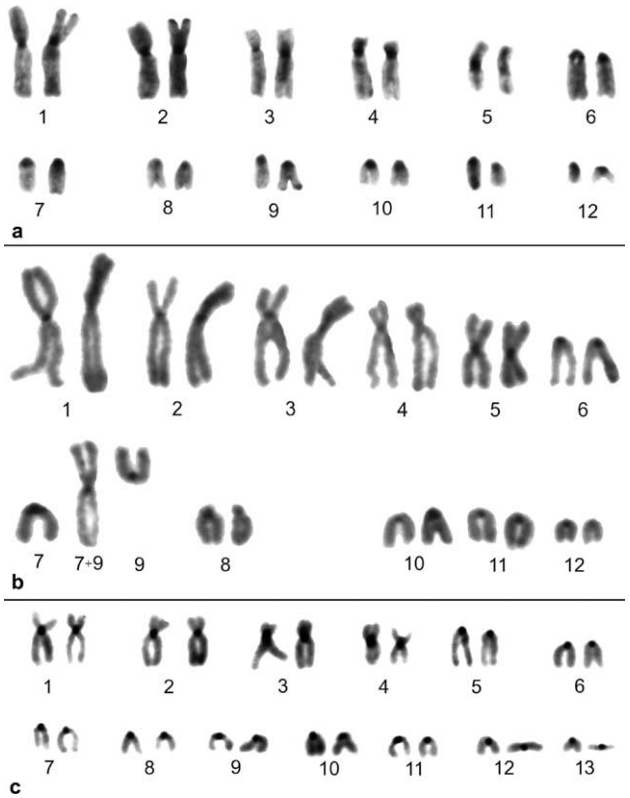

Fig. 6a-c. C banded karyotype of *Leptodactylus* cf. *marmoratus* (**a**), *Leptodactylus* sp. (aff. *bokermanni*) (**b**), and *L. hylaedactylus* (**c**). Observe the proximal heterochromatin in the odd telocentric 7 of *Leptodactylus* sp. (aff. *bokermanni*).

acteristic of *L.* cf. *marmoratus* and *Leptodactylus* sp. (aff. *bokermanni*), is missing. On the other hand, in *L. hylaedactylus* a higher number of telocentric pairs is observed with two additional pairs, probably, the large telocentric pairs 5 and 6, since their sizes correspond, respectively, to the long and short arms of the metacentric 1 of *L.* cf. *marmoratus* and *Leptodactylus* sp. (aff. *bokermanni*). The seven smallest telocentric pairs in the karyotype A of *L.* cf. *marmoratus* and in *L. hylaedactylus* seem to be homeologous, including the chromosome pairs bearing Ag-NOR, although they were in a different position in the karyograms. All these facts support the hypothesis that the karyotypic differentiation among the species of the present study is based, predominantly, on centric fusion. High-resolution procedures, like replication banding after BrdU treatment or chromosome painting using microdissection, might be used to confirm or not the structural rearrangements occurred in differentiation of these karyotypes.

The three species of the present study exhibited practically the same C banding pattern. The large pericentromeric C band in the odd chromosome 7 of *Leptodactylus* sp. (aff. *bokermanni*) might represent a karyological species-specific character. This marker C band, however, is not visualised in the metacentric 7+9, suggesting its loss during the fusion process. The base-pair contents of some repetitive regions were provided by the fluorochrome staining. This was the case of the centromeric heterochromatin of *L.* cf. *marmoratus* which is AT-rich. The brilliant sites with CMA_3 observed in the negative heteropicnotic regions in some of the conventionally stained small telocentrics of *L. hylaedactylus* are an indication that GC-rich heterochromatin occurs in these chromosomes, although not C-banded. As usually observed the Ag-NOR sites in all three species also showed CMA_3 brilliant labelling.

In 1974, Heyer and Diment established a phylogeny based on the diploid number and chromosome morphology of 23 species of *Leptodactylus*, four of them are currently *L. andreae*, *L. hylaedactylus*, *L. marmoratus* and *L. discodactylus*. According to this phylogeny, $2n = 26$ and the presence of uni-armed chromosomes in the karyotype, as exhibited by *L. andreae* and *L. hylaedactylus*, is the most primitive condition, whereas the karyotype with $2n = 24$, also bearing uni-armed chromosome pairs, as shown by *L. marmoratus*, is derived. The $2n = 22$ was considered as a secondarily derived state, the karyotype with at least one uni-armed chromosome pair being more primitive than the karyotype with exclusively bi-armed chromosomes.

According to Heyer and Diment (1974), the predominant mechanism responsible for the karyotype differentiation is the centric fusion, but pericentric

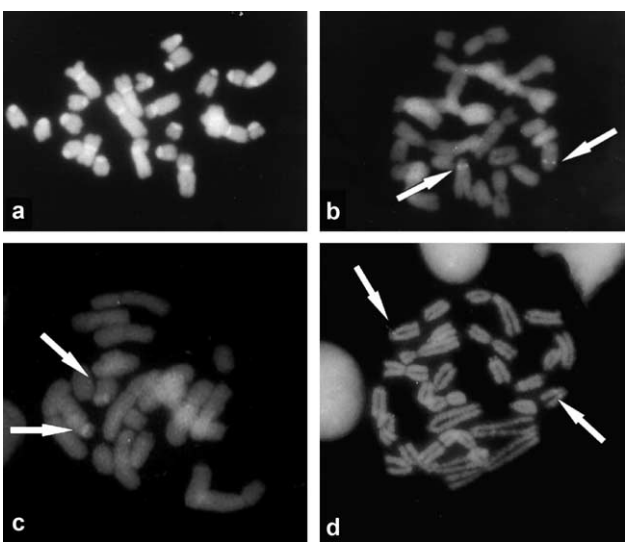

Fig. 7a-d. Metaphases with fluorochrome staining. DAPI (**a**) and CMA_3 (**b**) in *Leptodactylus* cf. *marmoratus*, CMA_3 (**c**) in *Leptodactylus* sp. (aff. *bokermanni*), and CMA_3 (**d**) in *L. hylaedactylus*. Arrow indicates chromosomes bearing Ag-NOR.

inversions must also have occurred. Our chromosome data is in accordance with this hypothesis, since the large metacentric 1 of *L.* cf. *marmoratus* and *Leptodactylus* sp. (aff. *bokermanni*), as well as the odd metacentric of this latter species are produced by centric fusion. On the other hand, pericentric inversions have occurred in chromosomes 12 in karyotype B of *L.* cf. *marmoratus* and 8 of *Leptodactylus* sp. (aff. *bokermanni*), altering their morphology.

The chromosome constitution of *L. hylaedactylus* and *L. andreae* is very close, both sharing the same 2n = 26 diploid number (Bogart 1974), without the large metacentric pair 1, characteristic of the remainder species of the present study, but differing in the FN, which is 34 and 40, respectively. In fact, in *L. andreae* the number of telocentric pairs is six, instead of nine like in *L. hylaedactylus*, and they correspond to the small-sized chromosomes 7–10, and 12–13; the bi-armed chromosome pairs 5, 6, and 11 might be, therefore, resulted from a pericentric inversion in chromosomes originally of the telocentric type.

Although Bogart (1970) had already described the karyotype of *Leptodactylus lineatus* (as *Lithodytes lineatus*) with 2n = 18, FN = 36, all of them of the bi-armed type, this information was not considered in the phylogenetic analysis of Heyer and Diment (1974). According to their hypothesis this karyotype would correspond to the most derived in the group. Despite the very different diploid numbers, the karyotypes of *L. lineatus* with 2n = 18 and *L. hylaedactylus* with 2n = 26 are relatively close, presuming four centric fusions in an ancestral 2n = 26 karyotype, among the small-sized chromosomes, and one pericentric inversion in the chromosome 5.

Taking into account the cytogenetic information available in the literature and our own data, a karyotype evolution within the former genera *Adenomera* and *Lithodytes* could be visualised. From an ancestral karyotype with 2n = 26 and nine telocentric pairs, as exhibited by *L. hylaedactylus*, two evolutionary lineages could be suggested. One of them corresponds to that of the species *L. andreae* (2n = 26) and *L. lineatus* (2n = 18), without the large metacentric 1, and the other, of the species *L.* cf. *marmoratus* (2n = 24) and *L.* sp. (aff. *bokermanni*) (2n = 23), both bearing this marker.

The species of *Leptodactylus* with 2n = 22 chromosomes, certainly belong to the second lineage, also retaining the marker metacentric 1, as well as *L. silvanimbus* with 2n = 24 (Amaro-Ghilardi et al. 2004). In order to explain the presence of all chromosomes of the bi-armed type, characteristic of the majority of the 2n = 22 *Leptodactylus* (Heyer and Diment 1974; Silva et al. 2000; Amaro-Ghilardi et al. 2004), it would be necessary to assume one more centric fusion and pericentric inversions in a variable number of telocentrics. In fact, in *Leptodactylus* with 2n = 22, the karyotypes have in general no telocentrics, but some of them, like in *L. latinasus*, *L.* "*natalensis*", *L. wagneri*, and *L. podicipinus* have one to four telocentric pairs (Bogart 1970; Silva et al. 2000). The metacentric morphology of the chromosomes of pair 12 in the Karyotype B of *L.* cf. *marmoratus* reinforces our suggestion.

The present analysis on chromosome evolution showed that the species belonging to the former *Lithodytes* and *Adenomera* are very close. This is supported by previous studies carried out by Heyer (1998) and Kokubum and Giaretta (2005), using morphological and reproductive characters and by Frost et al. (2006), based mainly on molecular data. In the phylogeny proposed by the latter authors, *L. lineatus* and *L. hylaedactylus* form a sister-taxon that is basal, regarding the group of *Leptodactylus* with 2n = 22. Certainly, cytogenetic analysis using high-resolution procedures and nuclear or mitochondrial gene sequencing should be extended to other species of *Leptodactylus*, previously recognised as belonging to the genus *Adenomera*, for a better understanding of phylogenetical relationships within the genus *Leptodactylus*.

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