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Research Article

Cytogenetics of three Brazilian species of *Eleutherodactylus* (Anura, Leptodactylidae) with 22 chromosomes and re-analysis of multiple translocations in *E. binotatus*

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Abstract

In this paper, we provide a cytogenetic analysis of *Eleutherodactylus guentheri, E. parvus* and *E. binotatus*. All of the species had a diploid chromosomal number of 2n = 22. The karyotypes of *E. guentheri* and *E. parvus* were very similar and differed only slightly in the morphology of pair 2. These two species also had an NOR-bearing secondary constriction on the long arms of pair 6. The karyotype of *E. binotatus* differed from those of *E. guentheri* and *E. parvus* in the morphology and size of the chromosomes, in the number of chromosomal arms, in the NOR location (detected on the short arms of pair 1), and in the pattern of heterochromatin. These results reinforce the differences between *E. guentheri* and *E. binotatus* and support the existence of two species group. Five individuals of *E. binotatus* showed morphs for pairs 2 and 3. These morphs probably arose from the translocation of a segment from one chromosome of pair 3 to a homologue of pair 2. In addition, some mitotic metaphases of *E. binotatus* showed spontaneous chromosomal breaks which suggested that there were sites of fragility. Meiotic diakinesis showed multiple chromosomal rings, indicating the occurrence of multiple translocations, as previously reported by other investigators. These data suggest that, in addition to fission and fusion, other chromosomal rearrangements were probably involved in the differentiation of the karyotypes of these species of *Eleutherodactylus,* especially *E. binotatus*.

Key words: Eleutherodactylus, heterochromatin, karyotype, nucleolus organizing region (NOR), reciprocal translocation.

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Introduction

The genus *Eleutherodactylus* contains nearly 700 species (Frost, 2002), making it the most speciose group of vertebrates. Because of the high diversity among *Eleutherodactylus* species neither the systematic relationships within the genus nor its detailed taxonomy have been satisfactorily resolved (Kaiser *et al.*, 1995). The genus has been divided into five subgenera (*Euhias, Graugastor, Eleutherodactylus, Pelorius* and *Syrrhopus*) (Lynch and Duellman, 1997), with the subgenus *Eleutherodactylus* consisting of more than 20 species groups. According to Bogart and Hedges (1995), *Eleutherodactylus* occurs in a wide variety of habitats throughout the Americas, from Argentina to southern North America, with very extensive speciation in the Antilles. Of more than 30 known Brazilian species, 22 occur in southern and southeastern Brazil, mainly in the Atlantic forest (Castanho and Haddad, 2000). The lack of biological data for South American species of *Eleutherodactylus* hampers our understanding of the origin of the species in this genus and their phylogenetic relationships to species in other regions.

The heterogeneity and diversity of *Eleutherodactylus* has led to the proposal of many species groups based only on morphological characters (Garcia, 1996). The species occurring in southern and southeastern Brazil were included in three species groups by Lynch (1976): *binotatus*, *lacteus* and *parvus.* Heyer (1984) proposed an *E. guentheri* "cluster" with six closely related species, three of which were included in the *E. binotatus* group by Lynch (1976). All members of the cluster occur in the central and southern regions of the Atlantic forest.

In contrast to their relatively conserved morphology, *Eleutherodactylus* species show considerable variation in their chromosomal number (2n = 18 to 36) and chromosomal morphology (Bogart, 1973, 1991; Bogart and Hedges, 1995; Kuramoto, 1990). Such variation is uncommon in anurans which, in general, have conserved karyo-

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types within a genus (Bogart, 1973; Morescalchi, 1973). *Eleutherodactylus* is a rapidly speciating genus and an example of a high rate of chromosomal evolution in frogs (Bogart and Hedges, 1995).

The phylogeny of the South American species of *Eleutherodactylus* is still poorly known, and studies combining morphological, biochemical, molecular and cytogenetic data are necessary to revise the classification of *Eleutherodactylus* in order to understand the phylogenetic relationships of this genus better. Whereas several karyological studies have been reported, especially for Central American species of *Eleutherodactylus* (Bogart, 1970a,b, 1981, 1991; De Weese, 1975; Myamoto, 1983; Schmid *et al.,* 1992; Kaiser, 1995, 1996; Kaiser and Green 1994), little is known about Brazilian species. The karyotypes of *E. lacteus* (De Lucca and Jim, 1974), *E. holti* (De Lucca *et al.*, 1974) (2n = 20), *E. guentheri* (Beçak, 1968) and *E. binotatus* (Beçak and Beçak, 1974) (2n = 22) have been determined by conventional staining.

In this study, we describe the karyotype of *E. parvus* and re-analyze the chromosomes of *E. guentheri* and *E. binotatus*. We also provide a more complete cytogenetic analysis of the three species, which represent three different species groups of *Eleutherodactylus* in southeastern Brazil, and compare the karyotypes of continental populations with those from an ocean island.

Material and Methods

Animals

Eleven specimens (eight males and three females) of *Eleutherodactylus guentheri*, ten specimens (six males and four females) of *E. parvus* and eight specimens (five males and three females) of *E. binotatus* were collected in the Parque Natural Municipal da Serra do Itapety (PNMSI), Mogi das Cruzes (23°31'20" S, 46°11'52" W), São Paulo State, Brazil; one female of *E. parvus* and two males of *E. binotatus* were collected at Ubatuba (23°26'09" S, 45°04'10" W), São Paulo State, and two females of *E. guentheri* and three females of *E. binotatus* were collected in the Parque Estadual de Ilha Bela (PEIB) (23°46'40" S, 45°21'28" W), São Paulo State. The specimens were collected in 2001 and 2002, with authorization from the Instituto Brasileiro do Meio Ambiente e Recursos Renováveis (IBAMA - Proc. 02001.008866/01-20). All of the animals were deposited in the "Prof. Adão José Cardoso" Natural History Museum (ZUEC), at the Universidade Estadual de Campinas (UNICAMP) in Campinas, São Paulo State, under the accession numbers 12159-12189 (*E. guentheri*), (12105-12129) (*E. binotatus*) and 12130-12158 (*E. parvus*).

Chromosome preparation and techniques

Mitotic chromosomes were obtained from intestinal epithelium, testis and bone marrow cell suspensions, as described by Schmid (1978) and Schmid *et al.* (1979), after treatment with colchicine for about 3 h. Conventional staining with 10% Giemsa solution, C-banding (Sumner, 1972), Ag-NOR staining (Howell and Black, 1980) and fluorescence *in situ* hybridization (FISH) (Viegas-Péquignot, 1992) were used to analyze the chromosomes. The rDNA probe used for FISH consisted of a recombinant plasmid HM123 containing a fragment of *Xenopus laevis* rDNA (Meunier-Rotival *et al.*, 1979) that was biotin-labeled by a nick translation reaction according to the manufacturer's protocol. The slides were examined with a BX60 Olympus microscope and some of the photographs were obtained using the software Image-Pro Plus, Version 4. The chromosomes were classified according to Green and Sessions (1991).

Results

Karyotype description

All individuals of the three species studied had a diploid number of 22 chromosomes. The karyotype of *E. guentheri* consisted of seven pairs of metacentric chromosomes (1, 5, 6, 8, 9, 10 and 11), two pairs of submetacentrics (2 and 3) and two pairs of subtelocentrics (4 and 7). In some metaphases, secondary constrictions were observed on the long arms of pairs 2, 3, 6 and 8 (all adjacent to the centromere) and on the short arms of pairs 5 and 9 (Figure 5a). In ten specimens, there was size heteromorphism in the secondary constriction between the homologues of pair 6 (Figures 1a and 5a, Table 1).

The karyotype of *E. parvus* was very similar to that of *E. guentheri*, differing in the morphology of pair 2, which was metacentric in the former species. Secondary constrictions were found on the short arms of pair 2 and on both arms of pair 6 and were heteromorphic between the long arms of the homologues of pair 6 (Figures 1c and 5b, Table 1).

Eleutherodactylus binotatus had a morphologically distinct karyotype in which the chromosomes were 3-4 times bigger than those of the other two species. The karyotype of *E. binotatus* showed 2n = 22 chromosomes which consisted of two pairs of metacentrics (4 and 7), four pairs of submetacentrics (1, 2, 6 and 9), two pairs of subtelocentrics (3 and 5) and three pairs of telocentrics (8, 10 and 11). In five specimens - two from the continent (one male and one female) and three from Ilha Bela island (two males and one female) - pairs 2 and 3 showed size heteromorphism in the relative length between homologues and differed strongly in their relative size and arm ratios. One of the homologues of pair 2 was metacentric because of the considerable increase in the size of its short arm, and one of the homologues of pair 3 was submetacentric because of the reduction in the size of the long arm (Figures 1e,f and 5c, Table 1). A secondary constriction was observed adjacent to the centromere on the short arm of pair 1

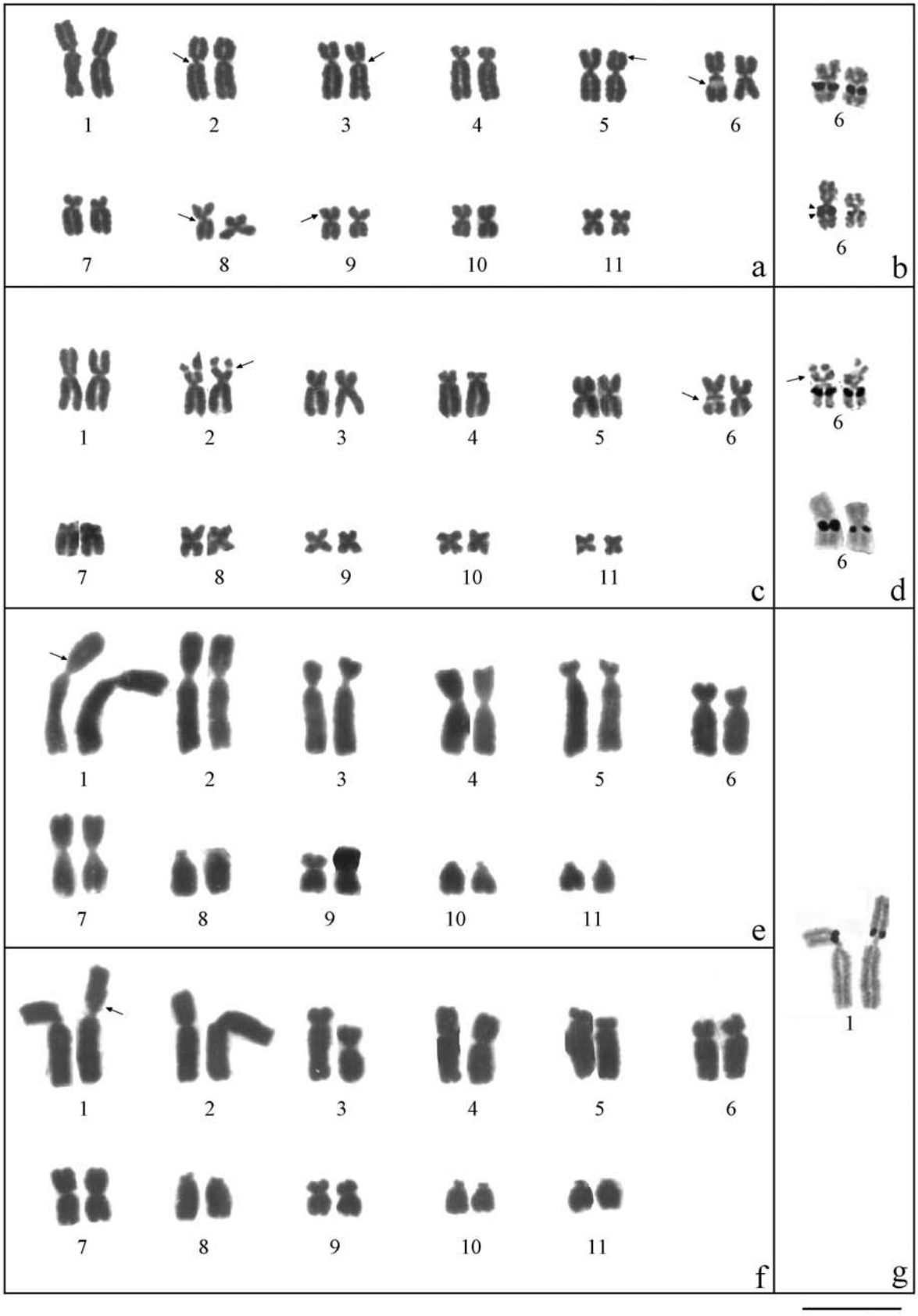

Figure 1 - Karyotypes of *E. guentheri* (**a**), *E. parvus* (**c**) and *E. binotatus* (**e** and **f**) and NOR-bearing chromosomes of each species (**b**, **d**, and **g** respectively). The arrows indicate secondary constrictions and the arrowheads in **b** indicate the double blocks of NOR. Bar = 10 µm.

Table 1 - Morphometric data for mitoic chromosomes of *Eleutherodactylus guentheri, E. parvus* and *E. binotatus.* In *E. binotatus,* the letters **a** and **b** indicate the different morphs of chromosomes 2 and 3 found in five specimens.

Eleutherodactylus guentheri												
	N.	1	2	3	4	5	6	7	8	9	10	11
	RL	14.25	11.92	11.04	10.23	10.10	9.23	7.49	7.25	6.50	6.32	5.68
	CR	1.48	1.72	1.98	3.08	1.44	1.28	3.34	1.52	1.46	1.20	1.18
	CP	M	SM	SM	ST	M	M	ST	M	M	M	M
Eleutherodactylus parvus												
	Nº	1	2	3	4	5	6	7	8	9	10	11
	RL	14.97	11.98	11.86	11.36	9.71	9.25	8.03	6.36	6.02	5.67	4.77
	CR	1.34	1.44	1.85	3.90	1.27	1.09	3.92	1.16	1.21	1.40	1.16
	CP	M	M	SM	ST	M	M	ST	M	M	M	M
Eleutherodactylus binotatus												
N.	1	2a	2b	3a	3b	4	5	6	7	8	9	10	11
RL	15.20	13.68	16.68	11.60	8.26	10.87	9.97	9.28	8.17	6.21	5.14	5.12	4.53
CR	1.73	1.75	1.05	3.50	1.77	1.52	4.62	2.40	1.05	12.22	1.76	10.16	12.82
CP	SM	SM	M	ST	SM	M	ST	SM	M	T	SM	T	T

RL = relative length (%), CR = centromeric ratio, CP = centromeric position, M = metacentric, SM = submetacentric, ST = subtelocentric, T = telocentric. Morphometric data were based on measurements of 20 metaphases from 13 specimens of *E. guentheri*, 18 metaphases from 11 specimens of *E. parvus* and 19 metaphases from 13 especimens of *E. binotatus*.

(Figures 1e,g and 5c). Some mitotic metaphases of *E. binotatus* showed spontaneous chromosome breaks, especially in the first six larger chromosomes of the complement (Figure 4e).

Nucleolus organizer region (NOR)

In *E. guentheri* and *E. parvus*, the NORs were present on the long arms of pair 6, adjacent to the centromeric region and coincident with a secondary constriction observed in Giemsa-stained karyotypes. The NORs were heteromorphic in nine of the 13 specimens (70%) of *E. guentheri* and in eight of the 11 specimens (73%) of *E. parvus* (Figures 1b,d and 5a,b). FISH in five specimens of *E. guentheri* and four of *E. parvus* revealed the same regions detected by the silver staining method (Figure 2a,b).

In *E. binotatus*, the NORs were located adjacent to the centromere on the short arms of pair 1, and were detected by silver staining and fluorescence *in situ* hybridization (Figures 1g and 5c). One of the specimens with a karyotype containing morphs of chromosomes 2 and 3 had an additional fluorescent label in the telomere of the long arm of subtelocentric pair 3, that was not seen with silver staining (Figure 2c).

C-Banding

Blocks of strongly stained heterochromatin were located in the centromeric region of all chromosomes of *E. guentheri* and *E. parvus* and in a few interstitial regions, whereas *E. binotatus* showed a smaller amount of centromeric heterochromatin. In *E. guentheri,* small blocks of heterochromatin were also observed adjacent to the centromere on the long arms of pairs 2, 3 and 8 coincident with secondary constrictions. In *E. parvus,* interstitial bands were detected on the short arms of pairs 2 and 6, coincident with secondary constrictions (Figures 3a,b and 5a,b). *Eleutherodactylus binotatus* showed bands adjacent to the centromere on the short arms of pair 7, on the long arms of pairs 7, 8, 10 and 11, and interstitially on the long arms of pairs 7, 10 and 11 (Figures 3c and 5c).

Meiosis

Multiple rings were observed in diakinesis of all male specimens of *E. binotatus* (Figure 4a-c). Most rings showed three or four pairs of large chromosomes involved in these multiple associations (Figure 4a-c). The same individual of *E. binotatus* always had the same number of chromosomes in all multivalent rings. In individuals with a karyotype containing morphs of chromosomes 2 and 3, the proportion of cells with only bivalents versus cells with multiple rings was 1:15, the reverse of normal karyotypes. In some cases, there were differences in the size of homologous bivalents in pairs 2 and 3 (Figure 4d). In *E. guentheri* and *E. parvus*, only bivalents were observed.

Discussion

The diploid number of 22 chromosomes observed in *E. guentheri, E. parvus* and *E. binotatus* is common among species of the Leptodactylidae (Kuramoto, 1990), and has been described in about 20 species of *Eleutherodactylus.* The species analyzed here did not show the large variation in chromosomal numbers reported for some species of *Eleutherodactylus* (De Lucca *et al.*, 1974; De Lucca and

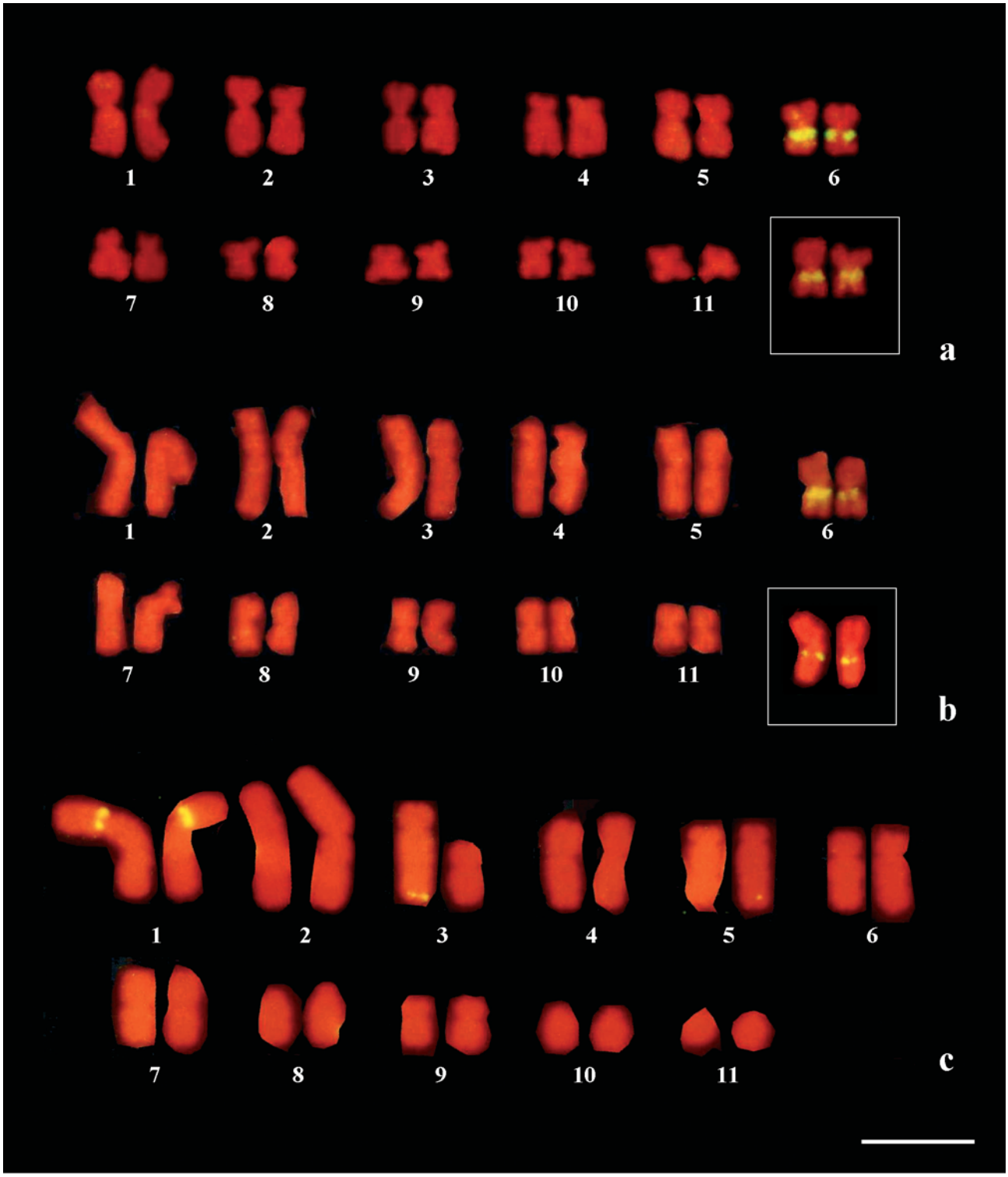

Figure 2 - Metaphases of *E. guentheri* (**a**), *E. parvus* (**b**) and *E. binotatus* (**c**) after fluorescence *in situ* hybridization with an rDNA probe. Note the additional labeling in one of the homologues of pair 3 in *E. binotatus*. Bar = 10 µm.

Jim, 1974; Bogart and Hedges, 1995; Kaiser, 1995). As stated by Bogart (1991), although centric fusions and fissions are the most likely mechanism for changes in chromosomal number in *Eleutherodactylus*, they are not the only mechanism. There are instances when identical chromosomal numbers have been derived independently (Bogart, 1991). Other mutational events, such as pericentric inversions, translocations, insertions, and dele-

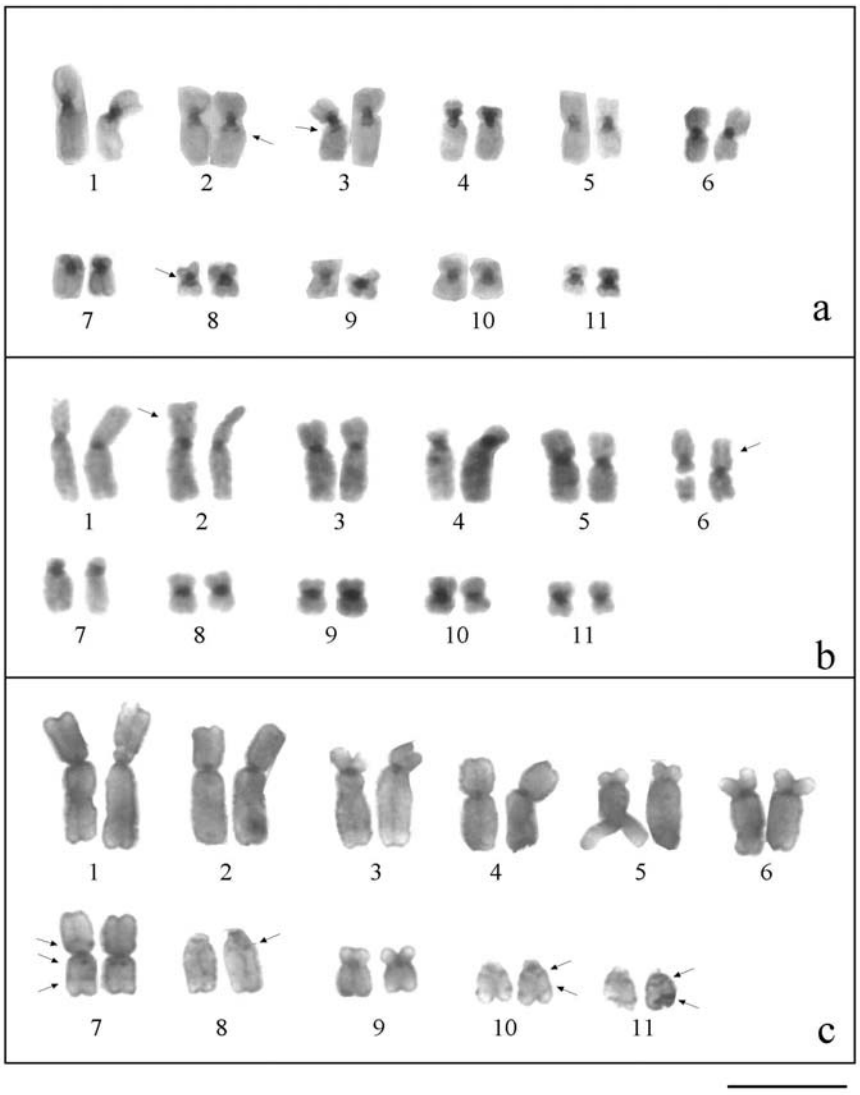

Figure 3 - Karyotypes of *E. guentheri* (**a**), *E. parvus* (**b**) and *E. binotatus* (**c**) after C-banding. The arrows indicate the interstitial heterochromatin. Bar = 10 μm.

tions could contribute to the chromosomal variation in *Eleutherodactylus.* Additionally, if centric fusions and fissions are equally likely, then the same number of chromosomes could easily be derived by the convergence of separate lineages (Bogart and Hedges, 1995).

The karyotypes of *E. guentheri* and *E. binotatus* differed from those described by Beçak (1968) for specimens of a population from Campos do Jordão, São Paulo State, Brazil, particularly in the classification of some chromosomal pairs and in the number and location of secondary constrictions. Such divergence in chromosome classification may be a technical artifact that reflects the different methods of classification adopted in these karyotypic descriptions. If the classification of Green and Sessions (1991) is applied to the karyotype of *E. guentheri* described by Beçak (1968), then pairs 5 and 7 are subtelocentrics and pair 9 is metacentric. The same argument applies to pairs 3 and 5 of *E. binotatus*. Since pairs 4 and 5 of *E. guentheri* have almost the same size, if their position in the karyogram is reversed then the karyotypes become identical. The differences in the number and locations of the secondary constrictions in *E. guentheri* and *E. parvus* may reflect interpopulational chromosomal variation or could be related to the high degree of chromosomal condensation, which would hamper visualization of the constrictions.

We also compared continental specimens of *E. guentheri* and *E. binotatus* with those from an island (Ilha Bela) located about 1.76 km off the coast of São Paulo State. Ilha Bela is a continental island that originated approximately 11,000 years ago (Vanzolini, 1973). A sea water barrier is particularly interesting since the permeability of amphibian skin makes these animals sensitive to salty water, thereby limiting their ability to travel between islands (Kaiser, 1995) and to the continent. A karyotypic

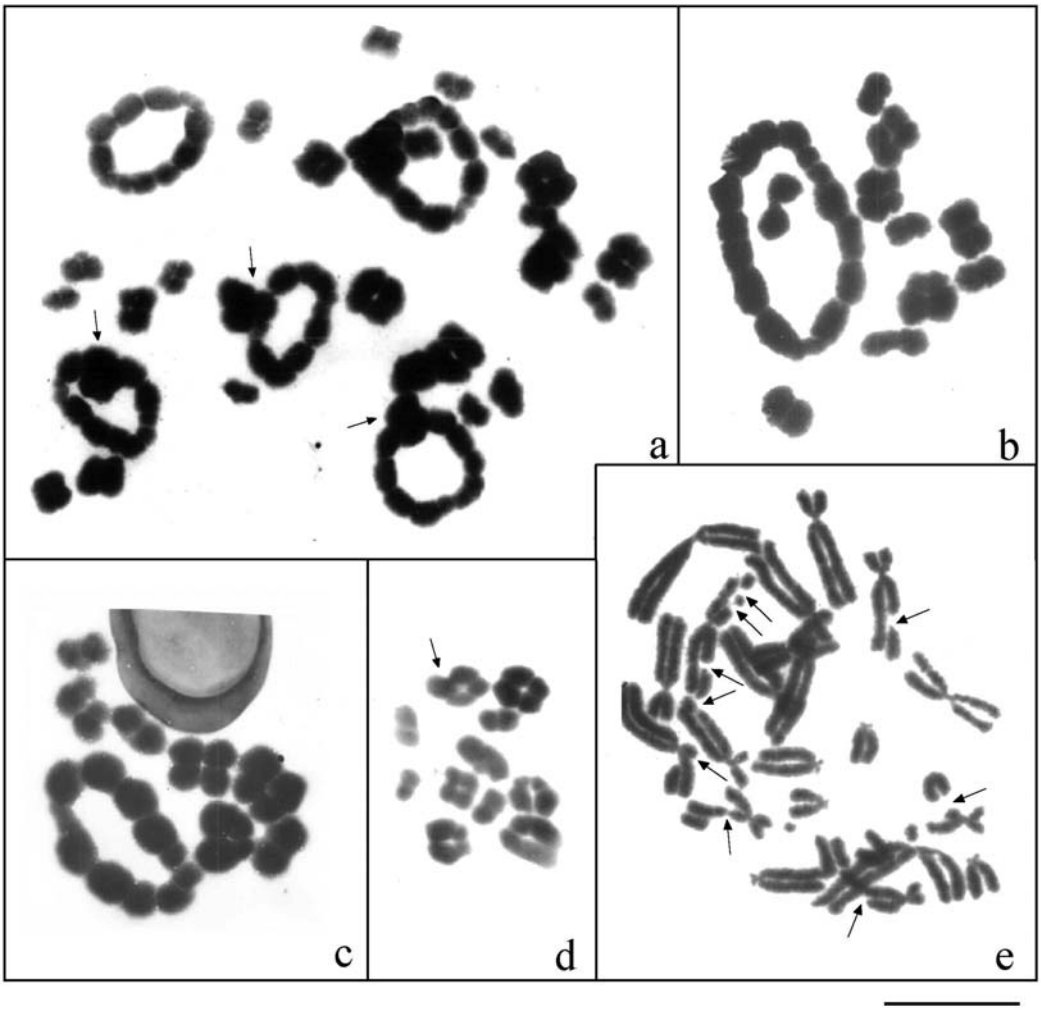

Figure 4 - Diakinesis (**a** - **d**) showing multivalent rings in male specimens of *E. binotatus.* In **a** and **c**, the rings have three associated chromosomal pairs, whereas in **b**, the ring has four associated pairs. The arrows indicate a bivalent that is laterally associated with the multivalent rings. In **d**, 11 bivalents and their association are shown. The arrow in **d** indicates the difference in size between the homologues of pair 3. In **e**, the mitotic metaphase of *E. binotatus* shows spontaneous chromosomal breaks (arrows). Bar = 10 μm.

analysis of populations separated by a geographic barrier can contribute to studies of speciation and chromosomal evolution. Hass and Hedges (1991) and Gascon *et al.* (1996, 1998) used morphology and allozymes to study groups of amphibians separated by a river and found no differences between the populations. A river barrier can diminish, but may not completely hinder, gene flow. Our hypothesis that the sea barrier would effectively lead to cytogenetic divergences was not confirmed since no karyotypic variation was observed between mainland and island populations of *E. guentheri* and *E. binotatus.* However, further populational analyses are necessary since one specimen of *E. binotatus* from Queimada Grande island did not show multiple translocations (Beçak and Beçak, 1974).

Of all the secondary constrictions, only that on the long arms of pair 6 was located at the same position and showed the same heteromorphism in *E. guentheri* and *E. parvus*. This constriction correspond to the NOR-bearing region of this chromosome pair in both species. In addition to the marked similarity between the chromosomal morphologies of the *E. guentheri* and *E. parvus* karyotypes, the same NOR location may also denote a very close relationship between the species, as proposed by Schmid (1982) and Schmid *et al.* (1990). The NOR heteromorphism seen in some individuals of both species apparently involved a tandem duplication of the NOR since two distinct blocks were observed with FISH and silver staining. As stated by Schmid (1982) and Schmid *et al.* (1990), unequal meiotic crossing-over and sister chromatid exchanges could give rise to heteromorphic NOR, including duplications and deletions.

In one individual of *E. guentheri* and two of *E. parvus,* only one chromosome of pair 6 was silver-stained. Similar cases have been described for *Xenopus laevis* (Elsdale *et al.,* 1958) and *Bufo fowleri* (Schmid, 1982), and may have resulted from a deletion, although in these cases

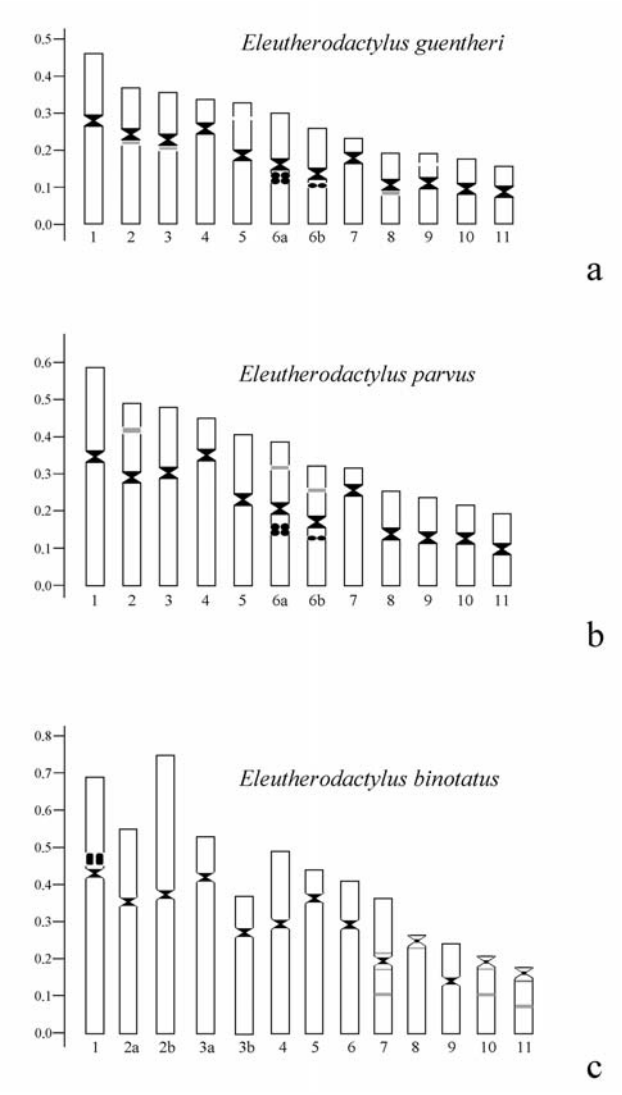

Figure 5 - Ideograms of the karyotypes of *E. guentheri* (**a**), *E. parvus* (**b**) and *E. binotatus* (**c**). Solid areas: dark C-bands. Gray areas: faint C-bands. Dark circles: NORs. Open regions: secondary constrictions. In **c**, the letters **a** and **b** indicate the different morphs of chromosomes 2 and 3.

the absence of rDNA was not confirmed by molecular techniques. In *E. parvus* and *E. guentheri*, both homologues were labeled by FISH, although the labeling was very small in one homologue. This observation suggests that the NOR was not completely deleted and that silver staining was not effective in detecting such a small amount of rDNA, in contrast to FISH.

Blocks of strongly stained heterochromatin were located in the centromeric region of all chromosomes of *E. guentheri* and *E. parvus* and in a few interstitial regions, whereas *E. binotatus* showed a smaller amount of centromeric heterochromatin. Bogart (1973) suggested that species with very similar karyotypes had a common ancestry. This may be the case for *E. guentheri* and *E. parvus,* which differed only slightly in the morphology of pair 2 and in the pattern of the interstitial heterochromatin. This chromosome was submetacentric in *E. guentheri* and metacentric in *E. parvus*, with the short arm being bigger in *E. parvus*, probably because of the presence of a secondary constriction associated with a heterochromatic block. Bogart (1981) and King (1991) suggested that reciprocal translocations, inversions and additions of heterochromatic blocks may have occurred during the recent phylogenesis of this genus.

The great similarity between the karyotypes of *E. guentheri* and *E. parvus* may be indicative of their close phylogenetic relationship. Likewise, Kaiser *et al.* (1995) reported that the karyotypes of *E. charlottevilensis* and *E. terraebolivianus* also showed great similarity at the morphological levels and concluded that these species were closely related.

Despite having the same number of chromosomes (2n = 22), the karyotype of *E. binotatus* differed from those of *E. guentheri* and *E. parvus* in the number of chromosomal arms (38 in *E. binotatus* and 44 in *E. guentheri* and *E. parvus*), in the larger size of its chromosomes [Beçak and Beçak (1974) reported that the genome of *E. binotatus* was four times greater than in *E. guentheri*], in the presence of telocentric chromosomes, in the NOR-bearing chromosomes, and in the amount and distribution of heterochromatin. Together, these differences indicate that distinct chromosomal rearrangements occurred during the evolution of these taxa.

The presence of multivalent rings during meiosis in *E. binotatus* indicated multiple translocations, as previously suggested by Beçak and Beçak (1974). The variable relative size and the arm ratios of the different morphs of chromosomes 2 and 3 in *E. binotatus* may have been generated by heterozygous translocation. This hypothesis is supported by the presence of multiple translocations and of unequal pairing between the homologues of pairs 2 and 3 during diakinesis in males. No individuals had both homologues of the translocated morphs of pairs 2 and 3, perhaps because this was a lethal combination that may have led to developmental disturbances in early embryogenesis. The chromosomal breaks seen in some metaphases also indicated that there were fragile sites in the karyotype of *E. binotatus* that allowed chromosomal rearrangements.

Apparently, only pairs 1 to 6 could be involved in the formation of the big multivalent ring, with pair 8 or 9 being the bivalent associated laterally with the multivalent ring. The lack of association of some chromosomes (pairs 7, 10 and 11, which remain as bivalents) with the multivalent rings could be related to the presence of interstitial heterochromatic regions in these chromosomes that could prevent pairing between homologous chromosomal arms. This hypothesis was proposed for *Physalaemus petersi* by Lourenço *et al.* (2000), who described the second case of a multivalent meiotic configuration in Anura.

The rDNA site detected only by FISH in the telomere of one homologue of pair 3 of *E. binotatus* could also be ex-

plained by the translocation of homologous rDNA sequences. The association of the NOR-bearing chromosomal arm of pair 1 with the long arm or pair 3 that carried the additional rDNA site reinforced the hypothesis of translocations as the main mechanism involved in this chromosomal rearrangement. Nevertheless, we cannot exclude the possibility that other mechanisms of dispersion, such as those discussed by Foote *et al.* (1991), Schmid *et al.* (1995) and Lourenço *et al.* (1998, 2000), are also involved, although they are less probable. These mechanisms include the transposition of mobile genetic elements, ribosomal cistron amplification and rDNA reinsertion errors during extra chromosomal amplification of ribosomal cistrons. Cases of additional labels detected in one of the homologues by FISH but not seen by silver-staining have also been described in *Hyla chrysoscelis* and *H. versicolor* (Wiley *et al.,* 1989), *H. nana* (Medeiros *et al*., 2003), and *Colostethus* sp. aff. *marchesianus* (Veiga-Menoncello *et al.*, 2003).

The great similarity in the karyotypic morphology and NOR location of *E. guentheri* and *E. parvus* compared to the larger DNA content and divergent karyotype (size of the chromosomes and number of chromosomal arms and telocentrics) and NOR location in *E. binotatus,* indicates a divergence from the latter species. Beçak and Beçak (1974) suggested that polyploidy combined with interstitial duplications was the most probable explanation for the drastic increase in the DNA content of *E. binotatus*, and that the multivalent meiotic ring observed in this species was caused by translocations that occurred after polyploidy.

Our cytogenetic data do not agree with the placement of *E. guentheri* in the *binotatus* group as proposed by Lynch (1976) based on morphological data. The *E. guentheri* cluster suggested by Heyer (1984) is more consistent with our results. The chromosomal information for the three karyotypes examined here reinforces the differences between *E. guentheri* and *E. binotatus* and supports the existence of two species group, although a detailed cytogenetic analysis of other species of the three groups (*binotatus*, *guentheri* and *parvus*) and of the *E. lacteus* group is necessary to support sister-group relationships or wider affinities of these taxa.

The Giemsa-stained karyotypes of *E. holti* (2n = 20) (De Lucca *et al.*, 1974) and *E. lacteus* (2n = 20) (De Lucca and Jim, 1974) are more similar to *E. guentheri* and *E. parvus* than to *E. binotatus*. The divergence in the karyotypes of *E. guentheri*, *E. parvus*, *E. lacteus* and *E. holti* appears have involved rearrangements in the smaller group of chromosomes since the six largest pairs are morphologically very similar.

Based on the six species analyzed and the proposed groups, southern and southeastern Brazil may have only one group of *Eleutherodactylus* with a low chromosomal number that is apparently fixed at around 20 to 22. However, further cytogenetic studies on other Brazilian species are necessary to confirm this hypothesis. Additional studies are also needed to determine whether the large genome size of *E. binotatus* is also present in other species, especially those of the *binotatus* species group, and to assess whether the karyotypes of other species of the *E. guentheri* and *E. parvus* groups are closely related.

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