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New karyotypes and some considerations about the chromosomal diversification of *Ctenomys minutus* (Rodentia: Ctenomyidae) on the coastal plain of the Brazilian state of Rio Grande do Sul

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Abstract

The dominant mammals occupying the subterranean niche in South America are rodents of the genus *Ctenomys*, which form a large group of 56 species with chromosome numbers ranging from $2n = 10$ to 70. In southern Brazil, *Ctenomys minutus* is the species with the widest geographic distribution, inhabiting sandy fields and dunes extending from Jaguaruna beach in the state of Santa Catarina to the town of São José do Norte in the state of Rio Grande do Sul. Eleven karyotypes ($2n = 42$; $2n = 46$a; $2n = 46$b; $2n = 47$a; $2n = 47$b; $2n = 48$a; $2n = 48$b; $2n = 49$a; $2n = 49$b; $2n = 50$a and $2n = 50$b) were described for this species and zones of hybridization are also known. A sample of 51 *C. minutus* specimens was collected from five sampling sites about 20 km apart along the coastal plain of Rio Grande do Sul between the municipalities of Tavares (31°23′S 51°09′W) and São José do Norte (31°52′S 51°54′W). We were able to extend the known geographic distribution of *C. minutus* by 90 km, from Tavares southwards to São José do Norte. During our study we found five karyotypes ($2n = 46$b, 47b, 48b, 49b and 50b), four of which ($2n = 47$b, 48b, 49b and 50b) have not previously been described for this species.

Introduction

The genus *Ctenomys* is a large group of 56 species (Reig et al., 1990; Lacey, Patton, & Cameron, 2000) of strongly territorial fossorial rodents with low vagility and a patchy distribution (Massarini et al., 1991; Freitas, 1994). This genus is considered as one of the most speciose mammalian genera (Mascheretti et al., 2000), presenting high karyotypic diversity and chromosome numbers ranging from $2n = 10$ to 70 (Freitas, 1997) and are the dominant mammals occupying the subterranean niche in South America.

In southern Brazil, the species with the widest geographic distribution is *Ctenomys minutus*, Nehring, 1887, which inhabits sandy fields and dunes and has a hitherto reported range extending from Jaguaruna beach in the state of Santa Catarina to the town of Tavares in the state of Rio Grande do Sul. In Rio Grande do Sul, *C. minutus* has been used extensively in genetic and population studies and has been shown to exhibit high karyotypic variability, having 11 karyotypes ($2n = 42$; $2n = 46$a; $2n = 46$b; $2n = 47$a; $2n = 47$b; $2n = 48$a; $2n = 48$b; $2n = 49$a; $2n = 49$b; $2n = 50$a and $2n = 50$b) as well as $2n = 46$a–48a and $2n = 42$–48a hybrid forms (Freitas, 1997; Gava & Freitas, 2002; Gava & Freitas, 2003). Such variability makes *C. minutus* a good example of chromosome speciation and work by Freitas (1997) on genetic polymorphisms suggests that this species is undergoing speciation due to geographic isolation.

This species deserves special attention in studies related to conservation and in this paper we describe an expansion of the geographic distribution of *C. minutus* and the detection of four new karyotypes for the species. We also compare the G-band patterns described by Freitas (1997) with the new karyotypes

Table 1. Number of female and male *C. minutus* captured at each sampling site with their autosomic and diploid chromosome numbers

Site details		Sex			Chromosomes			
Site	Map reference	Females	Males	Total	2*n*	AN[a]	Metacentric	Acrocentric
Tavares 1	31°23′S, 51°09′W	10	3	13	46b	76	32	12
Tavares 2	31°25′S, 51°10′W	2	1	3	47b	76	31	14
Tavares 2		2	0	2	46b	76	32	12
Bojuru 1	31°38′S, 51°26′W	5	3	8	48b	76	30	16
Bojuru 1		1	2	3	48b	78	32	14
Bojuru 2	31°44′S, 51°34′W	3	1	4	49b	76	29	18
Bojuru 2		1	0	1	49b	77	30	17
Bojuru 2		3	0	3	50b	76	28	20
São José do Norte	31°52′S, 51°53′W	9	3	12	50b	76	28	20
São José do Norte		1	1	2	50b	77	29	19
Total animals		36	15	51				

[a] Autosomic number.

and record a new distribution of constitutive heterochromatin among the karyotypes. The phylogenetic analysis described in this paper showed that *C. minutus* is evolving on the southern Brazilian Coastal Plain and is producing different karyotypes and hybrid zones.

Materials and methods

The area studied was the coastal plain region of Rio Grande do Sul between the towns of Tavares (31°23′S 51°09′W) and São José do Norte (31°52′S 51°54′W) which are within the Patos and Mirim Lagoon complex multiple barriers system. A total sample of 51 *C. minutus* (Table 1) were collected from five distinct sites on a transect of the study area, the animals being caught alive with the help of an Oneida Victor no. 0 trap.

Chromosome preparations were obtained from bone marrow according to the method of Ford and Hamerton (1956), G-banding patterns being produced by Seabright's (1971) technique, C-banding by Sumner's (1972) method and characterization of the nucleolus organizer regions (NOR) by Howell and Black's (1980) technique. The all human telomeres' probe (ONCOR, 1994) was used for fluorescence *in situ* hybridization (FISH) according to the manufacturer's protocol.

The diploid number was established using 10 metaphases per animal, while for the autosomal number (AN) only the number of autosomal arms was considered. For the karyotypes, the chromosomes were separated by size and the position of their centromere. Phylogenetic analysis was performed by the outgroup method using TREE GARDENER 2.2 software and *Ctenomys lami* as outgroup, since this species is ancient in relation to *C. minutus* (Freitas, 2001).

Results

With this study, we were able to extend the geographic distribution of *C. minutus* from Tavares 90 km southwards to São José do Norte and also to detect five karyotypes ($2n = 46$b, 47b, 48b, 49b and 50b), four of which had not been previously described for the species ($2n = 47$b, 48b, 49b and 50b).

The sex chromosome pair was the same as that found in the other karyotypes of this species, with a sub-metacentric X and an acrocentric Y chromosome in all karyotypes. The $2n = 46$b karyotype, already described by Freitas (1997), presented 16 biarmed chromosomes and six acrocentric pairs (Figure 1(a)). Animals with $2n = 47$b the karyotype is formed by 15 biarmed chromosomes, six acrocentric pairs and a heteromorphic pair (Figure 1(b)). The $2n = 48$b karyotype was present as two forms, the most frequent being composed of 16 biarmed chromosomes and seven acrocentric pairs and the other of 15 biarmed chromosomes and eight acrocentric pairs (Figure 1(c)). Specimens with a $2n = 49$b the karyotype is formed by 14 biarmed chromosomes, eight acrocentric pairs and a heteromorphic pair (Figure 1(d)), while the $2n = 50$b karyotype was formed from 14 biarmed chromosomes and 10 acrocentric pairs (Figure 1(e)).

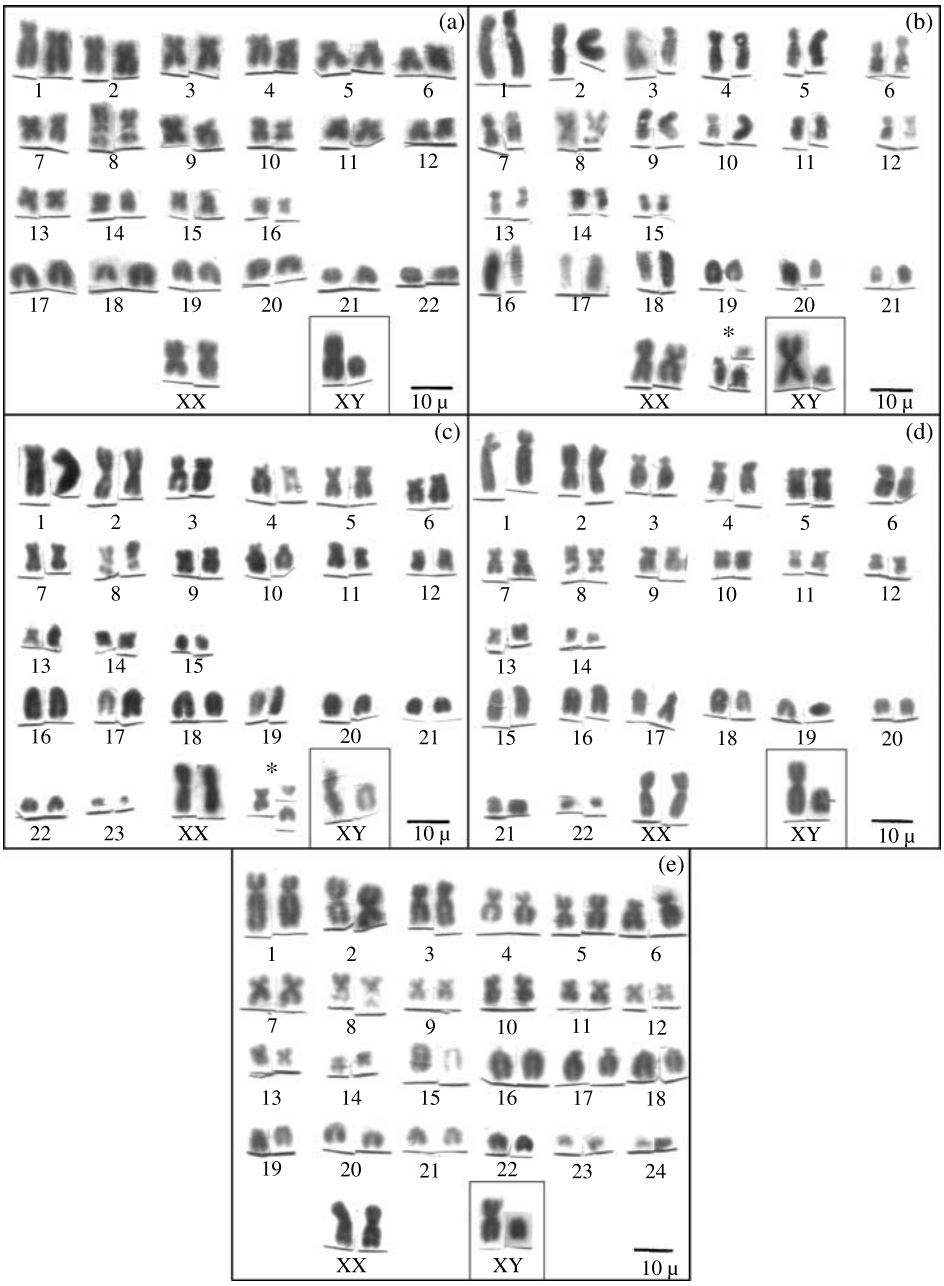

Figure 1. Karyotypes found in this study. (a) $2n = 46$b, (b) $2n = 47$b, (c) $2n = 48$b, (d) $2n = 49$b, and (e) $2n = 50$b. The asterisks in (b) and (c) indicate the heterozygote pair.

Figure 2 compares the G-band patterns with those described by Freitas (1997). In karyotypes 50a and 50b, pairs 17 and 20 of the Jaguaruna karyotype are fused in the São José do Norte karyotype, while pair 2 identified in the $2n = 50$ karyotype from Jaguaruna is fissioned in the São José do Norte karyotype. The $2n = 48$b karyotype differs from the $2n = 48$a karyotype because of the presence of an inversion in chromosome 2p, which transforms it into a metacentric chromosome.

Regarding the hybrid forms, the difference between the $2n = 47$b and $2n = 47$a karyotypes stems from the heterozygote pair formed by a 24/16 rearrangement in the $2n = 47$b karyotype and a 2p/2q

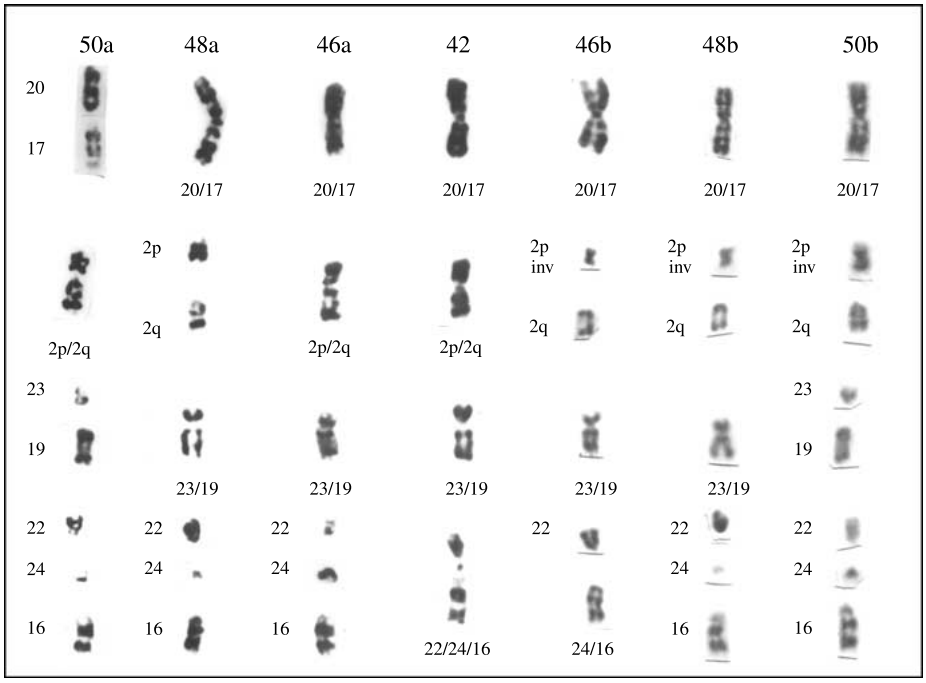

Figure 2. Comparison of *C. minutus* karyotypes $2n = 50$b, 48b and 46b, found between Tavares and São José do Norte and the *C. minutus* karyotypes $2n = 50$a, 48a, 46a and 42 reported by Freitas (1997).

rearrangement in the $2n = 47$a karyotype. The $2n = 49$a and $2n = 49$b karyotypes differ not because of the heterozygote pair but because of the 2p/2q rearrangement, which is fused in the $2n = 49$a karyotype and fissioned in the $2n = 49$b karyotype and by the 20/17 rearrangement which is fissioned in the $2n = 49$a karyotype and fused in the $2n = 49$b karyotype.

The constitutive heterochromatin was located in three chromosomes, occupying the entire length of the short arm and in the Y chromosome in $2n = 50$b and 48b males (Figure 3(a)). In the $2n = 46$b karyotype just one autosomal chromosome and the Y-chromosome presented C-banding, while the X showed no positive C-banding.

The NOR was found to be located in the secondary constriction of the long arm of pair 08 (Figure 3(b)).

The FISH technique was used to try to identify chromosome rearrangements in the karyotypes. Probe hybridization was found in the telomeric regions of both short and long arms of all chromosomes of the $2n = 50$b karyotype but there was no ectopic signal around the centromeres of the chromosomes studied (Figure 2). In both the 48b and 46b karyotypes the degree of denaturation of the chromosomes prevented analysis.

Only 15 characters (Table 2) were used for phylogenetic analysis and to build the coded matrix shown in Table 3. Four trees were obtained, the consensus tree being shown in Figure 4(a).

The consensus tree separated the karyotypes into four groups: the most basal group being $2n = 50$a, the next $2n = 50$b, the third $2n = 46$b, 48b and the last $2n = 42$ and 46a, showing more apomorphic characteristics. The consistency index was 0.61 and the retention index 0.60. Both these indices are generally considered low but due to the low number of characters used the tree can be considered relatively consistent. Characters 1 (chromosome 20/17), 2 (chromosome 20) and 3 (chromosome 17) were the main ones responsible for separating the 50a karyotype from the other karyotypes.

The group formed by $2n = 42$, 46a, 48a, 46b and 48b was supported by three synapomorphies of character 7 (chromosome 23/19), 8 (chromosome 23) and 9 (chromosome 19) and the group formed by $2n = 46$a and 42 was supported by the synapomorphies of character 4, whilst character 10 (chromosomes 22/24/26)

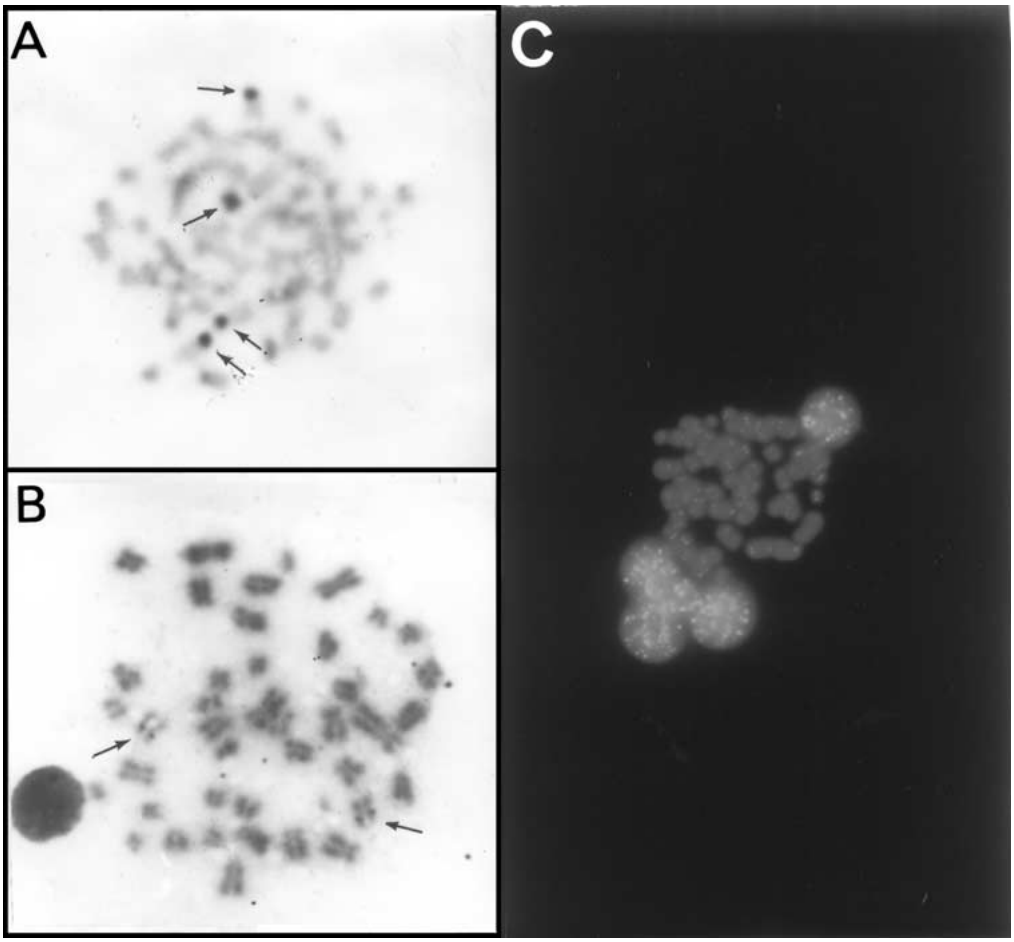

Figure 3. Chromosome banding patterns for *C. minutus*. (a) C-banding obtained from a $2n = 50$b male. The arrows indicate the demarcation points, the one in the center indicating the Y chromosome. (b) NOR-banding obtained with karyotype $2n = 50$b. The same pattern was observed in other karyotypes. (c) Telomeric signal obtained using FISH in karyotype $2n = 50$b.

Table 2. Character list for chromosome banding in *C. minutus*

Character	State 0	State 1	State 2
01 – chromosome 20/17	Absent	Present	/
02 – chromosome 20	Absent	Present	/
03 – chromosome 17	Absent	Present	/
04 – chromosome 2p/2q	Absent	Present	/
05 – chromosome 2p	Absent	Present	Present with inversion
06 – chromosome 2q	Absent	Present	/
07 – chromosome 23/19	Absent	Present	/
08 – chromosome 23	Absent	Present	/
09 – chromosome 19	Absent	Present	/
10 – chromosome 22/24/16	Absent	Present	/
11 – chromosome 24/16	Absent	Present	/
12 – chromosome 22	Absent	Present	/
13 – chromosome	Absent	Present	/
14 – chromosome 16	Absent	Present	/
15 – Heterochromatin of chromosome pair 08	Absent	Present	/

Table 3. Character matrix for chromosome banding in *C. lami and C. minutus*

Character	*C. lami*	*C. minutus*					
	L58	M50a	M48a	M46a	M42	M46b	M48b	M50b
01 – chromosome 20/17	0	0	1	1	1	1	1	1
02 – chromosome 20	1	1	0	0	0	0	0	0
03 – chromosome 17	1	1	0	0	0	0	0	0
04 – chromosome 2p/2q	0	1	0	1	1	0	0	0
05 – chromosome 2p	1	0	1	0	0	2	2	2
06 – chromosome 2q	1	0	1	0	0	1	1	1
07 – chromosome 23/19	0	0	1	1	1	1	1	0
08 – chromosome 23	1	1	0	0	0	0	0	1
09 – chromosome 19	1	1	0	0	0	0	0	1
10 – chromosome 22/24/16	0	0	0	0	1	0	0	0
11 – chromosome 24/16	0	0	0	0	0	1	0	0
12 – chromosome 22	1	1	1	1	0	1	1	1
13 – chromosome	1	1	1	1	0	0	1	1
14 – chromosome 16	1	1	1	1	0	0	1	1
15 – Heterochromatin of chromosome pair 08	1	1	1	1	1	0	0	0

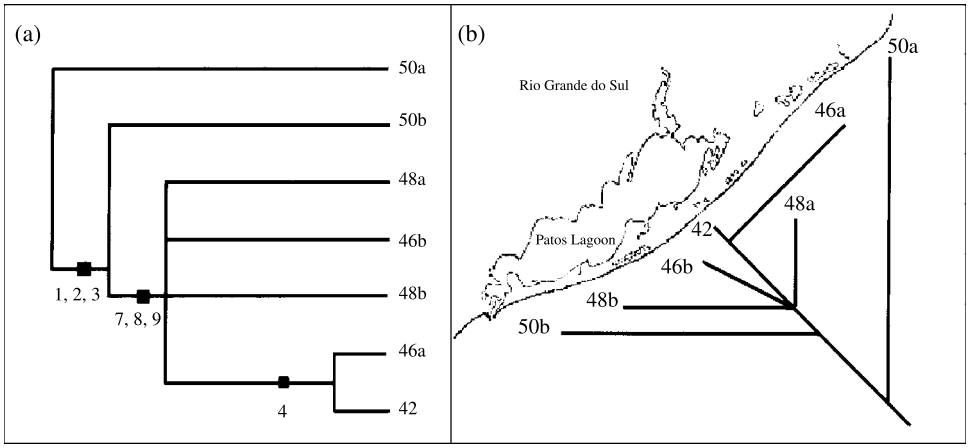

Figure 4. (a) Consensus tree obtained from the chromosome banding data for *C. minutus*, using *C. lami* as external group (not shown). The black squares represent the characters responsible for the separation of groups. (b) Geographic distribution of the karyotypes and their respective phylogenetic relationships.

represented an autopomorphy for the respective karyotype.

Discussion

The new karyotypes found between Tavares and São José do Norte present the basic characteristics of the karyotypes described by Freitas (1997) and Gava and Freitas (2002). It is interesting to observe that the rearrangements detected for karyotypes $2n = 46$b, 47b, 48b, 49b and 50b also occur in the chromosomes described by Freitas (1997) and chromosome variability is a consequence of Robertsonian rearrangements and *in tandem* fusions.

In total, *C. minutus* has 11 different karyotypic forms ($2n = 50$a and b; 49a and b; 48a and b; 47a and b; 46a and b and 42) throughout its geographic distribution from Santa Catarina to the south of Rio Grande do Sul. The geographic distribution of these karyotypes is interesting, because the $2n = 50$ karyotype is found at both ends of the range of *C. minutus*

but the karyotype is reduced through chromosomal rearrangements to $2n = 42$ more or less in the center of the range, suggesting that this distribution only occurs because the coastal plain of southern Brazil is narrow.

Freitas (1997) analyzed the C-bands of *C. minutus* and found differences in the amount of constitutive heterochromatin in animals from the north of the distribution in relation to those from the south, northern animals having large amounts of heterochromatin and southern animals small amounts. This pattern is also present in the karyotypes described here. Slamovits et al. (2001) suggest that deletions and amplifications of satellite DNA (located in the chromosome as positive C-bands) can cause such variation, but this does not seem to be related to the variations in euchromatin found in our study. According to Gallardo (1991) and Garcia et al. (2000), increases or decreases in chromosome number and changes in euchromatin content are independent of the changes in the amount of heterochromatin. The location of the NOR found in the present study is the same as that found for the karyotypes described by Freitas (1997). The $2n = 50$b karyotype did not present pericentromeric signs of a telomeric sequence, however Garagna et al. (1995) and Metcalfe et al. (1998) suggest that the telomeric sequence is not always retained during fusion events.

There is a possibility that two hybrid zones occur in the range of this species: between Tavares 1 ($2n = 46$b) and Bojuru 1 ($2n = 48$b), where there is a population with $2n = 46$b and 47b and the second between Bojuru 1 ($2n = 48$b) and São José do Norte ($2n = 50$b), due to the occurrence of $2n = 49$b and 50b in Bojuru 2. However, $2n = 47$b and $2n = 49$b might be product of chromosomic polymorphism. Patton (1993) observed that many hybrid zones are characterized by a patch of different karyotypes, as is the case with the hybrid zone described for *C. minutus* by Gava (1996) and Gava and Freitas (2002). A hybrid zone with $2n = 47$b would be the result of a secondary contact after regression of the sea level about 2300 years ago and the closing of a connection between Patos Lagoon and the Atlantic Ocean at a location near the Barra Falsa. Regarding the $2n = 49$b karyotype, evidence suggests that the region near the Estreito locality (between São José do Norte and Bojuru) may have acted also as a barrier.

In our phylogenetic analyses there were two polytomy events in the consensus tree. Polytomy was first observed by Lara, Patton, and Silva (1996) in Echimyidae rodents. This was confirmed soon after for *Ctenomys* by Lessa and Cook (1998), Mascheretti et al. (2000) and Slamovits et al. (2001), who observed that several basic relationships between species of the genera are poorly understood and suggested that polytomy due to simultaneous radiation had occurred, with the majority of the lineages being formed simultaneously. When we compare the cladogram obtained from the distribution of karyotypes in the southern Brazilian coastal plain (Figure 4(b)) with the geographic distribution of *C. minutus*, we can infer that, initially, the whole coastal plain was colonized by the $2n = 50$a karyotype. The Araranguá river formed about 13–15 million years ago (Dillenburg, personal communication) may have divided the distribution of the $2n = 50$a karyotype to form the $2n = 50$b karyotype on the southern side.

It can be also inferred from the cladogram (Figure 4(b)) that the $2n = 48$b, $2n = 46$b and $2n = 48$a karyotypes arise from the 50b karyotype. Two explanations are possible, one being that all the karyotypes appeared independently and the second, deduced by parsimony, being that the $2n = 48$b karyotype initially appeared inside the area of distribution of the $2n = 50$b karyotype and that an inversion produced the $2n = 48$a karyotype and an independent fusion gave origin to the $2n = 46$b karyotype.

It is probable that gene flow between the $2n = 48$a and $2n = 46$b karyotypes could not have occurred because inversion would have hindered balanced segregation, the absence of hybrid zones between these two karyotypes reinforcing this supposition. For the same reason, we believe that the two derived karyotypes, $2n = 42$ and $2n = 46$a have appeared independently of the $2n = 48$a karyotype but not of the $2n = 46$b karyotype. It is generally thought that karyotypic differences between *Ctenomys* species are due to geographic barriers, however our study found that there are two different karyotypes with no geographic barriers between them.

This evolutionary scenario seems to agree with White's cascade model (King, 1993), because new races appear inside the distribution of the parental race by accumulation of different chromosomal rearrangements, with some races being genetically closer than other races.

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