Genetica
https://doi.org/10.1007/s10709-018-0032-0

ORIGINAL PAPER

Integrative analysis of chromosome banding, telomere localization and molecular genetics in the highly variable *Ctenomys* of the Corrientes group (Rodentia; Ctenomyidae)

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Received: 1 March 2018 / Accepted: 27 July 2018

Abstract

The genus *Ctenomys* comprises about 70 species with great chromosome diversity. The Corrientes group is one of the most chromosomally variable lineages in the genus, where the diploid number (2n) varies from 41 to 70. In this group, three nominal species and numerous polymorphic and polytypic populations have been described. In order to get insight into the chromosomal evolution of this species complex, we applied different banding and molecular cytogenetic techniques. The results were interpreted in an evolutionary context, based on mitochondrial cytochrome *b* analyses. Studied samples are representative of the broad chromosomal variability in the group, including specimens with 2n = 42 to 2n = 70. Heterochromatin was scarce but concentrated in a few chromosomes. Centromeric DAPI-negative heterochromatin was observed in some autosomal pairs, which differed among populations. Location and amount of DAPI-neutral heterochromatin within the Y chromosome varied among populations. The variable distribution of heterochromatin indicates its dynamic behavior. NORs were detected in one pair of autosomes, which also differed among some populations. Telomeric FISH signals were observed in all complements only at the chromosome ends. The Corrientes group belongs to a clade that also includes *C. pearsoni, C. lami, C. minutus, C. ibicuiensis* and *C. torquatus*. Almost all of these species are variable at the chromosomal level, suggesting that this is the ancestral condition of the clade. Within the Corrientes group, the observed low genetic divergence, in contrast with its high chromosomal variability, is indicative of decoupling between the rates of chromosomal and mitochondrial evolution.

Keywords Chromosome rearrangements · Banding techniques · Telomeres · Cytochrome *b* · Rodents · South America

Introduction

Subterranean rodents of the genus *Ctenomys* are endemic to South America and comprise about 70 described species, and numerous populations of uncertain taxonomic status (Bidau 2015; de Freitas 2016). At the chromosomal level, *Ctenomys* species are highly variable. Diploid numbers range from 2n = 10 in *C. steinbachi* to 2n = 70 in two related species: *C. dorbignyi* and *C. pearsoni* (Kiblisky et al. 1977; Anderson et al. 1987; Ortells et al. 1990; Argüelles et al. 2001). In addition to the interspecific variability, several polymorphic and/or polytypic species with different chromosomal rearrangements have been described (Ortells et al. 1990; Novello and Lessa 1986; Massarini et al. 1998, 2002; Lanzone et al. 2007; de Freitas et al. 2012; Caraballo et al. 2015).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10709-018-0032-0) contains supplementary material, which is available to authorized users.

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Published online: 03 August 2018

One of the most chromosomally variable groups in this genus is a species complex named "Corrientes group" or "*Ctenomys perrensi* superspecies" (Giménez et al. 2002; Lanzone et al. 2007; Caraballo et al. 2016), which comprises at least 26 related populations, distributed in the vast area under the influence of the Iberá wetland in the Corrientes Province in Argentina. In this group diploid numbers vary from 41 to 70 and autosomal fundamental numbers (FNa) from 72 to 84, mainly due to Robertsonian (Rb) rearrangements, and to the increase/decrease in the number of small acrocentric chromosomes (Ortells et al. 1990; Giménez et al. 2002; Caraballo et al. 2015). Three nominal species were formerly described in the Corrientes group: *C. dorbignyi* with 2n = 70/FNa = 80 and 84, *C. roigi* with 2n = 48/FNa = 76, and *C. perrensi* with 2n = 50/FNa = 80. Additionally, numerous populations of uncertain specific status were reported (Ortells et al. 1990; Giménez et al. 2002; Caraballo et al. 2015). However, although most populations had been characterized by conventional staining methods (Caraballo et al. 2015 and references therein), only a small proportion was studied using banding techniques (Ortells 1995; Garcia et al. 2000a; Argüelles et al. 2001).

Recently, under the application of an integrative approach combining mitochondrial and microsatellite (SSR) variability, together with chromosomal morphology, the existence of seven independent lineages within the Corrientes group was proposed (Caraballo and Rossi 2018a). In the most inclusive phylogenetic analyses using mitochondrial markers, the Corrientes group was found to be sister to *C. pearsoni*, a species complex that inhabits the eastern part of the distribution area of the genus (Parada et al. 2011; Caraballo et al. 2016). Both, the Corrientes group and *C. pearsoni* were included in the torquatus group: a higher clade that includes species distributed in the southeast of Brazil and Uruguay: *C. torquatus, C. ibicuiensis, C. minutus* and *C. lami* (Parada et al. 2011; de Freitas et al. 2012; Caraballo and Rossi 2018a, b).

At the karyotypic level, several studies suggested that specific genomic regions such as heterochromatic blocks, NORs, and telomeres are frequently involved in chromosomal rearrangements (Garagna et al. 1993; Bolzán 2012, 2017 and references therein). In this paper, we performed DAPI (4′,6-diamidino-2-phenylindole), C and Ag-NORs (nucleolus organizing regions) chromosome banding, and fluorescent *in situ* hybridization (FISH) for telomeric repeats, in order to better understand karyotypic diversity and evolution in the *Ctenomys* Corrientes group. Additionally, we revised and contrasted chromosomal and cytochrome *b* (cyt-*b*) variability in this group, and from closely related species, in an evolutionary context.

Materials and methods

Chromosomal studies were conducted in samples which have been previously described through conventional Giemsa staining and analyzed at the molecular level (Caraballo et al. 2015, 2016, respectively). Karyotypes were prepared from bone marrow suspensions according to Ford and Hamerton (1956). For each specimen a minimum of 20 metaphases was analyzed. The fundamental number of autosomal arms (FNa) was established following de Freitas (2007). The distribution of constitutive heterochromatin (CH) was studied according to Sumner (1972). DAPI fluorochrome staining (Schweizer et al. 1978) and Ag-NORs technique (Howell and Black 1980) were performed. A total of 22 individuals (10 females and 12 males) belonging to 10 localities in the Corrientes province of Argentina were studied performing the aforementioned banding techniques (Fig. 1 and Supplementary Table 1).

Fluorescent *in situ* hybridization (FISH) was performed with a Cy3-conjugated PNA pantelomeric probe [Cy3-(CCCTAA)3] obtained from PNABio Inc. (California, USA), according to the protocol provided by the supplier, as previously described (Lanzone et al. 2015). Slides were mounted in an antifade reagent containing DAPI as the counterstain. Fluorescence microscopy was performed on a Nikon Eclipse 50i epifluorescence microscope equipped with an HBO 100 mercury lamp, a Nikon high-resolution digital color camera (DS-Ri-U3), and filters for DAPI and Cy3 (Chroma Technology Corp., Rockingham, VT, USA). A total of 10 specimens from the following localities were studied by FISH: Loreto (2n = 42/FNa = 72; N = 1), San Miguel (2n = 44/FNa = 72; N = 3), Paraje Caimán (2n = 46/FNa = 74; N = 1), Estancia San Luis (2n = 48/FNa = 76; N = 1), Saladas Centro (2n = 54/FNa = 80; N = 1), Chavarría (2n = 56/FNa = 80; N = 1) and Santa Rosa (2n = 66/NF = 82; N = 1). In FISH experiments we also included a sample from Paraje Sarandicito (2n = 70/FNa = 80) to encompass the complete chromosomal diversity observed in the group.

We also revised all 2n and FNa described in the Corrientes group and in its closest phylogenetically related species: *C. pearsoni, C. lami, C. minutus, C. torquatus* and the recently described *C. ibicuiensis* (Supplementary Table 1). We performed molecular analyses (p distances, a phylogenetic tree, and a network) to contrast them with chromosomal data, including 2n, FNa, banding patterns, telomeric signals and chromosomal rearrangements found in each group. A Bayesian phylogenetic analysis was conducted including 95 cytochrome *b* (cyt-*b*) sequences representing all populations of the torquatus group available in GenBank (Supplementary Table 1). In order to evaluate the degree of molecular divergence within the torquatus

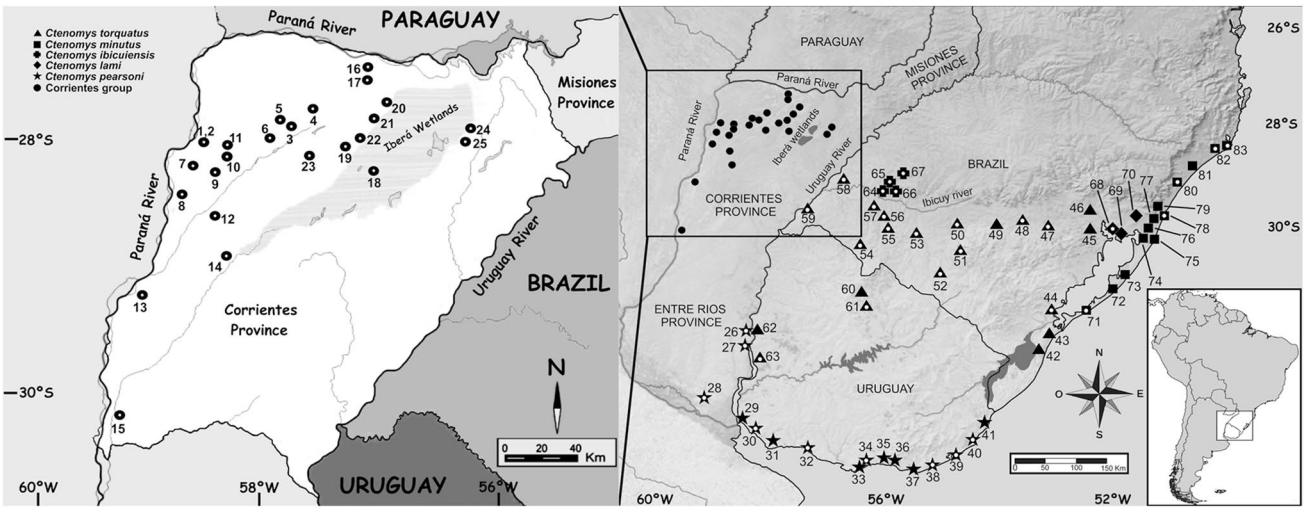


Fig. 1 Map showing the populations of *Ctenomys* from the Corrientes group and its related species. Cytogenetically analyzed localities are: Estancia San Luis (2), Saladas Centro (10), Chavarría (14), Paraje Caimán (19), Loreto (20), Curuzú Laurel (21), San Miguel (22), Santa Rosa (23) Contreras Cué (25) and Paraje Sarandicito (15). Localities included in the phylogenetic analysis are marked with a white central dot. Additional information concerning samples and localities are included in Supplementary Table 1

group, pairwise genetic distances (p) were calculated with MEGA 6.0 (Tamura et al. 2013). Sequences from the Corrientes group and from the sister species *C. pearsoni* (N = 58, Supplementary Table 1) were transformed into haplotypes using DNASP 5.1 (Librado and Rozas 2009). Haplotypes were employed to construct a median network containing all possible maximum parsimony trees using Network 4.6 (Bandelt et al. 1999). We used the Median Joining algorithm and a value of zero for the epsilon parameter. All characters were treated with equal weight.

In the phylogenetic analysis, in addition to the 95 cyt-*b* sequences mentioned above, three sequences from the sister taxon *C. rionegrensis* (Parada et al. 2011), as well as *C. conoveri, C. sociabilis, C. leucodon* and *Octodon degus* were included as candidate outgroups. We forced *O. degus* as the outgroup, inasmuch it is a member of the superfamily Octodontoidea, but external to the radiation of the family composed by the only living genus *Ctenomys*. The analysis was performed in MrBayes 3.2.6 (Ronquist et al. 2012), partitioning the cyt-*b* sequence in 1st, 2nd and 3rd codon positions separately. For each codon position, instead of using *a priori* model testing, we sampled across the GTR model space in the Bayesian MCMC analysis, integrating out the uncertainty about the correct substitution model. Across-sites rate heterogeneity was modeled with gamma-distributed rates and a proportion of invariable sites. Two independent runs for 1×10^7 MCMC generations sampling every 1.000 generations were carried out. Convergence was assessed analyzing the potential scale reduction factor (PSRF), and the average standard deviation of split frequencies (ASDSF). The burnin phase was set up in the generation which fulfilled PSRF values of 1.00–1.02 for all estimated parameters and standard deviations lower than 0.01.

Results

Specimens from Curuzú Laurel (locality 21), Contreras Cué (locality 25) and Loreto (locality 20) showed similar chromosome complements with 2n = 42/FNa = 72, composed by 16 pairs of biarmed and four pairs of acrocentric autosomes, plus the sex pair (Fig. 2a). The X chromosome was large and metacentric, while the Y chromosome was smaller and submetacentric (Figs. 2a, 3a, d; Supplementary Fig. 1). A secondary constriction Ag-NOR-positive was identified in pair 7 (Figs. 2a, 3o). DAPI bands allowed the homologation of chromosome complements in all specimens from these localities. Chromosome pairs 1 and 4 displayed large pericentromeric DAPI-negative/C-positive blocks. A conspicuous terminal DAPI-neutral/C-positive band was observed in the long arm of the Y chromosome (Fig. 3a, d).

Specimens from San Miguel (locality 22) had identical 2n = 44/FNa = 72 karyotypes (Fig. 2b), while in Paraje Caimán (locality 19) a variable 2n = 45–46/FNa = 74 complement was found (Fig. 2c). The 2n = 44 and 46 cytotypes shared 15 biarmed autosomal pairs and the heteromorphic sex pair. The most conspicuous difference between them was the presence of six small telocentric pairs in samples from San Miguel, compared to the seven found in Paraje Caimán (Supplementary Fig. 1). In this last locality, one individual was heterozygous for a Rb rearrangement involving the acrocentric pairs 16 and 17 and thus depicted a 2n = 45 (data not shown). An Ag-NOR-positive secondary constriction

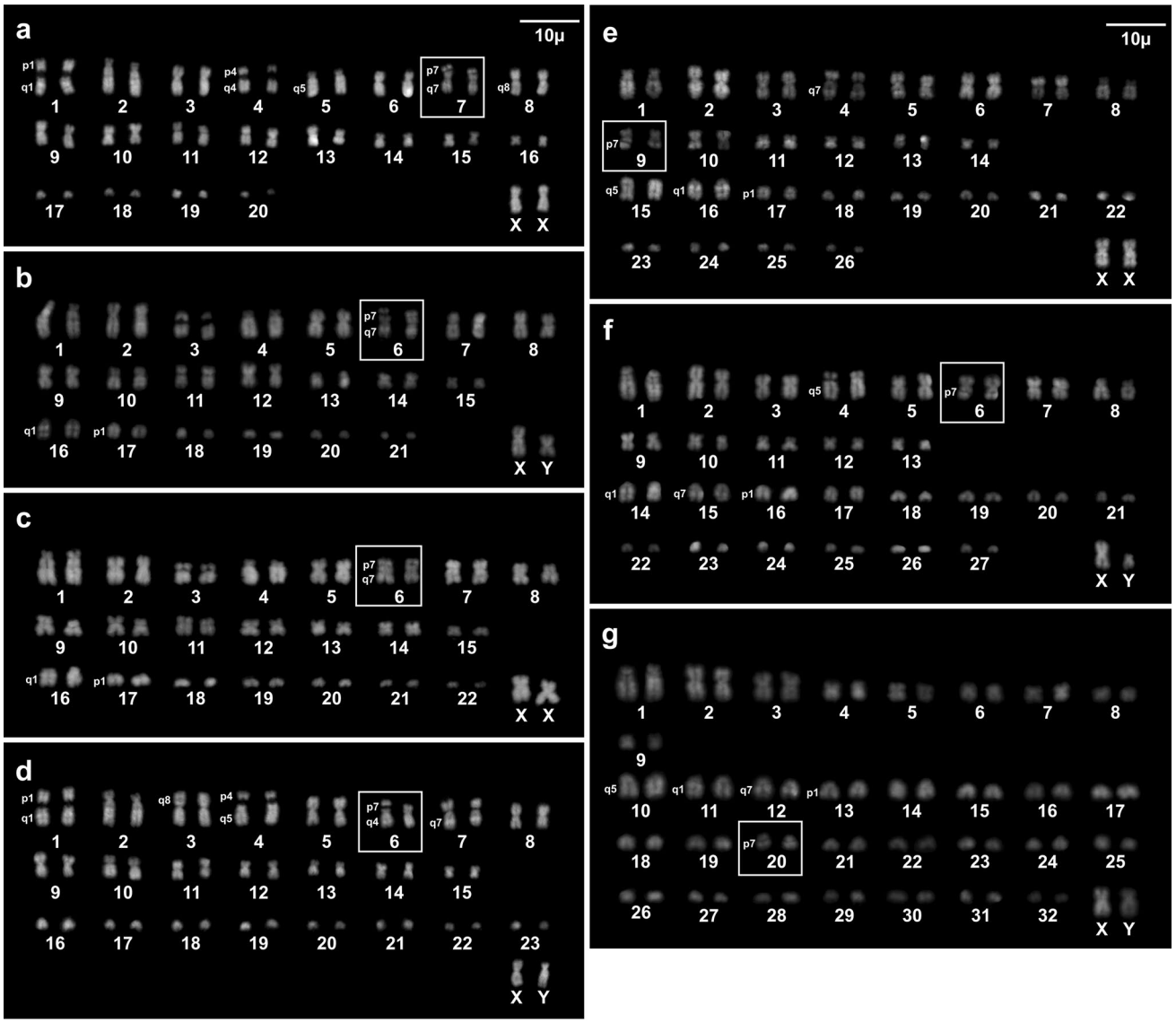


Fig. 2 DAPI stained karyotypes of specimens from the Corrientes group with different diploid and fundamental numbers: **a** female with 2n = 42, FNa = 72; **b** male with 2n = 44, FNa = 72; **c** female with 2n = 46, FNa = 74; **d** male with 2n = 48, FNa = 76; **e** female with 2n = 54, FNa = 80; **f** male with 2n = 56 FNa = 80; **g** male with 2n = 66, FNa = 82. NOR-bearing chromosomal pairs are depicted with white frames. Short (p) and long (q) chromosomal arms involved in identified rearrangements between karyotypes are indicated. Bar = 10 µm

was observed in pair 6 in all specimens from both localities (Figs. 2b, c, 3o). Large DAPI-negative/C-positive pericentromeric blocks were observed in pair 3 (San Miguel: Fig. 3b, e; Paraje Caimán: Fig. 3c, f). Additionally, acrocentric pairs 16 and 17 were larger (medium sized) than the remaining acrocentric chromosomes. According to DAPI banding patterns, acrocentric pairs 16 and 17 of specimens from San Miguel and Paraje Caimán are homologous to the long (q) and short (p) arms, respectively, of chromosome pair 1 observed in the complements with 2n = 42/FNa = 72 (Fig. 2a–c; Supplementary Fig. 1). The X chromosome in specimens from San Miguel and Paraje Caimán was indistinguishable from that of specimens with 2n = 42. The male sex chromosome from Paraje Caimán showed a DAPI-neutral/C-positive terminal block in the long arm (Fig. 3c, f). In contrast, males from San Miguel depicted a Y chromosome with no visible CH marks (Fig. 3e).

Specimens from Estancia San Luis (locality 2) had 2n = 48/FNa = 76, composed of 15 biarmed pairs, eight acrocentric pairs, and the sex XX/XY pair (Fig. 2d; Supplementary Fig. 1). The Y chromosome was larger than those previously described, while the X chromosome remained conserved. An Ag-NOR-positive secondary constriction was observed in pair 6 (Figs. 2d, 3o). Chromosome pairs 1 and 4 showed distinctive DAPI-negative/C-positive pericentromeric blocks (Fig. 3g, j). Pair 7 also presented these blocks

but were smaller in size. The long arm of the Y chromosome showed two DAPI-neutral/C-positive blocks: one was large and terminal, while the other was smaller and interstitial (Fig. 3g, j).

Chromosome complements with 2n = 48 and 2n = 42 are remarkably divergent (Fig. 2a, d; Supplementary Fig. 1). However, monobrachial homologies could be established among several chromosomes. Homology in DAPI bands was observed between the long arm of pair 5 from the 2n = 42 cytotype and the long arm of pair 4 from the 2n = 48 cytotype; between the long arm of pair 4 from the 2n = 42 and the long arm of pair 6 from the 2n = 48; between the long arm of pair 8 (2n = 42) and the short arm of pair 3 (2n = 48). Additionally, the long arm of pair 7 was the same in both cytotypes, while the short arm was the NOR´s carrier in the 2n = 42, but not in the 2n = 48 cytotype (Fig. 2a, d).

Specimens from Saladas Centro (locality 10) had 2n = 54/FNa = 80, composed of 14 biarmed and 12 acrocentric pairs of autosomes, and the sex pair (Fig. 2e; Supplementary Fig. 1). A secondary constriction was identified in pair 9. Acrocentric autosome pairs 15 and 16 were medium sized, while the remaining acrocentrics were small. Biarmed pair 4 showed a DAPI-negative/C-positive pericentromeric block. In addition, pair 9 also exhibited a pericentromeric C-positive block and the secondary constriction, which was Ag-NOR-positive (Fig. 3h, k, o).

Chromosome complements of samples from Chavarría (locality 14) had 2n = 56/FNa = 80, composed of 13 biarmed and 14 acrocentric autosomal pairs, plus the sex pair (Fig. 2f; Supplementary Fig. 1). The X chromosome was indistinguishable from those observed in previously described cytotypes. The Y chromosome was medium sized. An Ag-NOR-positive secondary constriction was located in pair 6 (Figs. 2f, 3o). Chromosome pairs 1 and 4 showed DAPI-negative/C-positive pericentromeric blocks. In addition, chromosome pair 6 presented a DAPI-neutral/C-positive pericentromeric block (Fig. 3i, l). As observed in Saladas, specimens from Chavarría had two distinctively large acrocentric pairs (14 and 15), which also showed characteristic DAPI banding patterns. The Y chromosome had three C-positive blocks (terminal, interstitial and pericentromeric), which were DAPI-neutral (Fig. 3i, l).

Chromosome banding comparison between the populations of Saladas Centro and Chavarría (2n = 54/FNa = 80 and 2n = 56/FNa = 80, respectively) indicated that despite their close 2n and FNa, these karyotypes were quite different (Fig. 2e, f; Supplementary Fig. 1). Chromosomal homologies could be established based on DAPI banding patterns between the long (q) arm of pair 4 (2n = 56/FNa = 80 cytotype from Chavarría) and the acro-telocentric pair 15 (2n = 54/NF = 80 cytotype from Saladas), and between the acro-telocentric pair 15, from Chavarría, and the long q arm of pair 4 from Saladas. Chromosome comparison among samples of Saladas, Chavarría and the remaining populations showed higher levels of divergence, hindering chromosomal homologation (Supplementary Fig. 1).

Specimens from Santa Rosa (locality 23) had 2n = 66/FNa = 82, composed of nine pairs of biarmed and 23 pairs of acrocentric autosomes, plus the sex pair (Fig. 2g; Supplementary Fig. 1). The X chromosome remained conserved, while the Y chromosome was subtelocentric and remarkably large (comparable to the X chromosome). An Ag-NOR-positive secondary constriction was observed in acrocentric chromosome pair 20 (Figs. 2g, 3o). As occurred in Chavarría, a DAPI-negative/C-positive pericentromeric block was observed in pair 1. Additionally, a medium sized acrocentric pair showed a DAPI-neutral/C-positive block. The Y chromosome exhibited a large DAPI-neutral/C-positive heterochromatic block along its q arm (Fig. 3m, n).

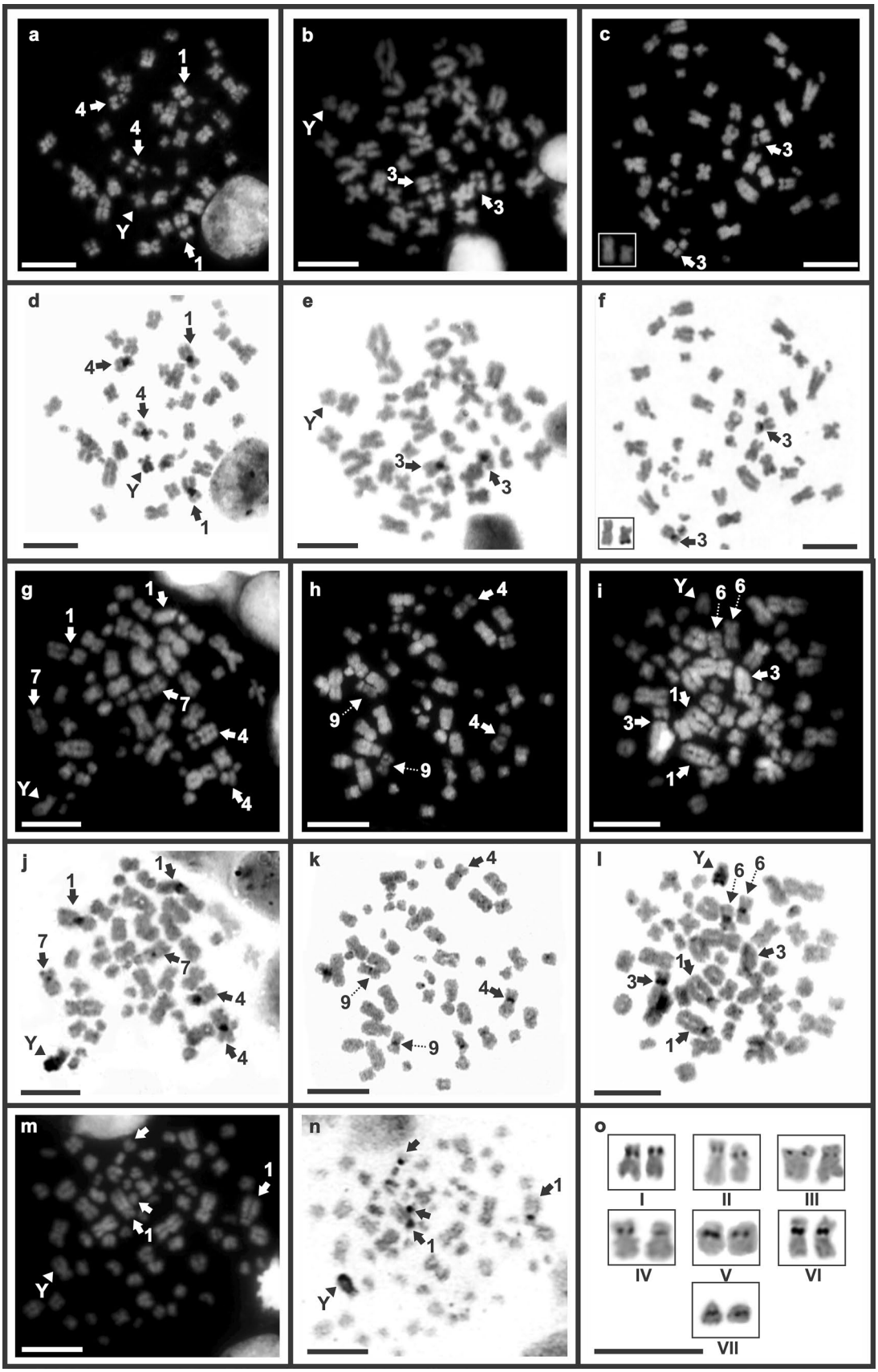
Silver impregnation confirmed that NORs were present in single chromosome pairs in all studied karyotypes; although the NOR-bearing pair varied among cytotypes (Fig. 3o).

Telomeric FISH signals at both chromosome ends were observed in all studied cytotypes (Fig. 4). Robertsonian biarmed chromosomes, bearing DAPI-negative centromeres, also showed telomeric signals strictly at their ends (Fig. 4). Some variation in the intensity of fluorescent signals was detected at both, intra- and inter-chromosomal levels (see Fig. 4). Sexual chromosomes exhibited an equivalent amount of telomeric repeats than autosomes. Despite the wide 2n and FNa ranges analyzed, none of the studied specimens showed interstitial telomeric sequences (ITS).

Ctenomys of the Corrientes group has the widest range of 2n observed when compared with other lineages from the torquatus species group, being 2n = 41–70, but it should be considered that the group includes three nominal species: *C. dorbignyi* with 2n = 70, *C. roigi* with 2n = 48 and *C. perrensi* with 2n = 50. All possible even diploid numbers within the observed range were registered. *Ctenomys dorbignyi* together with *C. pearsoni* share the higher 2n recorded for the entire genus. In this last species, different chromosomal complements, which vary from 2n = 56 to 70 and FNa from 76 to 80, were also described (references in Supplementary Table 1).

Molecular analysis

Cytochrome *b* genetic distances were very low among all samples of *Ctenomys* from the Corrientes group, ranging from 0 to 2.28% (p distance; Supplementary Table 2). The highest genetic distance values were observed between samples of *C. roigi* from Costa Mansión (locality 1) and several other populations. Surprisingly, distances were also low between samples from the Corrientes group and those from the remaining species of the torquatus group (Supplementary Table 2). The network showed a complex haplotype (H)


a
1
4
4
Y
1
b
Y
3
3
c
3
3
d
1
4
4
Y
1
e
Y
3
3
f
3
3
g
1
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7
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3
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1
m
1
1
Y
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1
1
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o
I
II
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V
VI
VII

◂**Fig. 3** Sequential chromosome staining with DAPI (**a–c, g–i, m**) and C-bands (**d–f, j–l, n**) in metaphases from the *Ctenomys* of the Corrientes group with different diploid and fundamental numbers. **a, d** Male with 2n = 42, FNa = 72; **b, e** male with 2n = 44, FNa = 72; **c**, **f** female with 2n = 46, FNa = 74 (male sex chromosomes are shown in frames); **g, j** male with 2n = 48, FNa = 76; **h, k** female with 2n = 54, FNa = 80; **i, l** male with 2n = 56 FNa = 80; **m, n** male with 2n = 66, FNa = 82. **o** Autosomal pairs with positive Ag-NOR bands in cytotypes with 2n = 42 (I), 2n = 44 (II), 2n = 46 (III), 2n = 48 (IV), 2n = 54 (V), 2n = 56 (VI) and 2n = 66 (VII). Full arrows indicate autosomal pairs with DAPI-negative/C-positive centromeres. Dashed-line arrows indicate additional autosomal pairs with C-positive centromeres. Arrowheads indicate Y chromosomes. Bar = 10 μm

distribution pattern in the Corrientes group, without a coherent taxonomic cut between species pairs (Fig. 5). Samples of *C. roigi* from Costa Mansión (locality 1; H23) and Estancia San Luis (locality 2; H16), both with 2n = 48/FNa = 76, were related to samples from Manantiales (locality 4; H14), Loma Alta (locality 5; H14), and Pago Alegre (locality 6; H15; this is the only population cytogenetically studied and had 2n = 56/FN = 80) by a hypothetical haplotype. Samples from the type locality of *C. dorbignyi* (Mbarigüí, locality 16) and from the close locality Paraje Angostura (locality 17), both with 2n = 70/FNa = 80, shared a haplotype (H5). This shared haplotype (H5) was connected to a hypothetical haplotype, which in turn was related to samples from Contreras Cué (locality 25) and Estancia La Tacuarita (locality 24) with 2n = 41–42/FNa = 72 (H6). Samples from Paraje Sarandicito (locality 15), also with 2n = 70/FNa = 80, shared a divergent haplotype (H22), which was connected through a hypothetical haplotype to samples from Paraje Caimán [locality 19; 2n = 45–46/FNa = 74 (H17)] and to haplotype 4 from San Miguel (locality 22). Haplotypes from the Corrientes group were separated from those of *C. pearsoni* (localities from 26 to 41) by at least 11 substitutions. This last species showed larger genetic discontinuities among haplotypes (Fig. 5).

The resulting Bayesian phylogenetic tree is shown in Supplementary Fig. 2. Posterior probabilities for the averaged substitution models for each partition are shown in Supplementary Table 3. The overall topology is congruent with previous studies (Caraballo et al. 2016; Caraballo and Rossi 2018a). Namely, the torquatus group is monophyletic and its internal relationships are: {*C. ibicuiensis* [(*C. torquatus* (*C. lami, C. minutus*)], (*C. pearsoni* complex, Corrientes group))}.

Discussion

Cytogenetic characterization of the Corrientes group

Rodents are the most species-rich mammalian order and their high speciation rates have been accompanied by elevated levels of chromosome diversity (Patton and Sherwood 1983); the *Ctenomys* of the Corrientes group is an example of extreme karyotypic heterogeneity with diploid numbers ranging from 2n = 41 to 70. In this group (and in all species of the torquatus group where data are available), there is only one chromosome pair carrying the NORs (de Freitas and Lessa 1984; Argüelles et al. 2001; de Freitas 2006, 2007), but this pair varies among populations as confirmed by Ag-NORs, C, G and DAPI bands (Argüelles et al. 2001; this study). This finding suggests that independent rearrangements would have occurred in these chromosome complements. Available data indicate that several Rb rearrangements (and/or whole-arm reciprocal translocations) occurred in the Corrientes group (and in the rest of the lineages within the torquatus group) involving different chromosome arms (Villar et al. 2014 and Supplementary Table 1). This is consistent with the observation that several cytotypes share the same FNa while showing a great dispersion in their 2n.

Almost all species of the torquatus group (with the exception of *C. ibicuiensis* which has 2n = 50/FNa = 68) are also chromosomally variable: *C. lami* 2n = 54–58/FNa = 74–84, *C. minutus* 2n = 42–52/FNa = 74–80, *C. torquatus* 2n = 40–46/FNa = 72. *Ctenomys lami* presents the widest FNa range found in the genus (references in Supplementary Table 1). The presence of different chromosome complements in almost all species of the torquatus group suggests that its ancestor could have been also variable at the chromosomal level.

Extensive levels of variation in the amount and distribution of CH among *Ctenomys* species, populations, and even individuals have been reported (Reig et al. 1992; Massarini et al. 1998; Lizarralde et al. 2003). Our results show that cytotypes of the Corrientes group have low CH content, with heterochromatic blocks concentrated in a few specific pairs, as revealed by C-banding patterns. This is a shared condition for the Corrientes group and its related species: *C. pearsoni, C. minutus, C. lami* and *C. torquatus* (de Freitas and Lessa 1984; Reig et al. 1992; Garcia et al. 2000a, b; Argüelles et al. 2001; Freygang et al. 2004; de Freitas 2007).

Sequential DAPI staining and C-banding revealed at least two types of heterochromatin in the Corrientes group, which differ in location and composition. In the Y chromosome DAPI-neutral (with no predominance of AT or CG) heterochromatic blocks were found in variable amounts and locations across populations. These blocks could either be restricted to interstitial, proximal or terminal localizations, to combinations of them or absent at all, as described for other *Ctenomys* species (Reig et al. 1992). The observed heterogeneous patterns of heterochromatin distribution in the male sex chromosome may be due to differential amplifications and deletions of these regions, as previously described in other mammals (Marshall Graves 2006). Additionally,

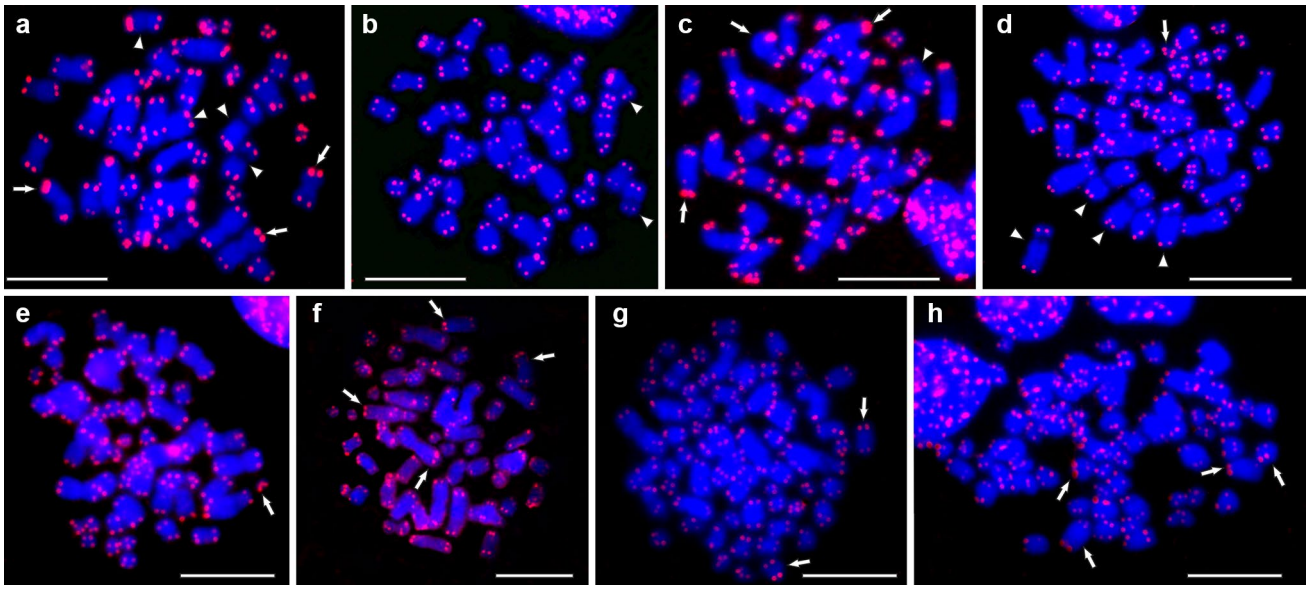

Fig. 4 Fluorescence in situ localization of the telomeric sequence (TTAGGG)n in *Ctenomys* with different chromosome complements: **a** 2n = 42, FNa = 72; **b** 2n = 44, FNa = 72; **c** 2n = 46, FNa = 74; **d** 2n = 48, FNa = 76; **e** 2n = 54, FNa = 80; **f** 2n = 56 FNa = 80; **g** 2n = 66, FNa = 82; **h** 2n = 70. Arrowheads indicate the presence of telomeric signals at the ends of Rb biarmed chromosomes. Arrows indicate chromosomes with heterogeneous signals at chromosome ends. Bar = 10 μm

large DAPI-negative (with low AT content) pericentromeric CH blocks were observed in specific biarmed autosomes. These pairs were involved in whole-arm chromosomal rearrangements, but interestingly, CH blocks were absent in the respective homologous acrocentric arms (Garcia et al. 2000a; this study). A central role of CH (and of repetitive sequences located in these regions), in the occurrence of chromosomal rearrangements was claimed for several taxa, especially in chromosomally variable rodents such as *Mus musculus domesticus* (Garagna et al. 1993 and references therein; Kalitsis et al. 2006). In *Ctenomys*, the major satellite DNA named RPCS has shown high levels of variation in copy number, which correlates with karyotypic variability (Slamovits et al. 2001; Ellingsen et al. 2007; Caraballo et al. 2010), indicating a complex dynamics of amplification and loss of RPCS sequences throughout the evolution of the genus.

Besides true telomeres, vertebrate chromosomes are usually enriched in interstitial telomeric sequences that may promote chromosomal rearrangements (Bolzán 2017). In this study, we found exclusively strict terminal telomeric signals in all studied karyotypes of the Corrientes group. This same pattern was described in the closely related species *C. minutus* (Freygang et al. 2004). Garcia et al. (2000a) suggested that the variation of 2n = 50 to 57 in specimens of the *Ctenomys* Corrientes group was due to chromosome fissions. In this type of rearrangement, centromeric ITS in biarmed chromosomes are not expected to be produced. However, ITS were undetectable in taxa among which several fusions have occurred, because of loss or degeneration of these sequences (Nanda et al. 1995; Rogatcheva et al. 2000; Lanzone et al. 2015), showing that the absence of ITS is not necessarily indicative of chromosome fissions. Some authors suggested that the chromosome complement with 2n = 70 could be the ancestral karyomorph of the group (Ortells et al. 1990; Caraballo et al. 2016). This hypothesis is based on the presence of two variants of this karyotype in more than one species (*C. dorbignyi* and *C. pearsoni*), and its disjunct distribution at the extremes of the geographic range of the Corrientes group. If the ancestral karyotype had 2n = 70, then fusions should have been frequent. However, in the *Ctenomys* Corrientes group both fusions and fissions are likely to have occurred. Phylogenetic analyses are central to test these hypotheses, but in this case the cyt-b marker does not provide enough resolution to determine the polarity of the rearrangements that took place in the evolution of the group (Caraballo et al. 2016; present study).

The *Ctenomys* Corrientes group is a lineage with several unique chromosomal complements (Giménez et al. 2002; Parada et al. 2011; Caraballo et al. 2015). Its most recent common ancestor has been dated to 630,000 years before present (400,000–900,000 ybp) according to a multi-calibrated relaxed clock analysis (Caraballo and Rossi 2018b). This indicates that the ample number of chromosomal changes that distinguish the group should have taken place in a short evolutionary period of time. Hence, the absence of ITS is not likely to be a consequence of a gradual loss, but instead of a rapid one simultaneous to/after the occurrence

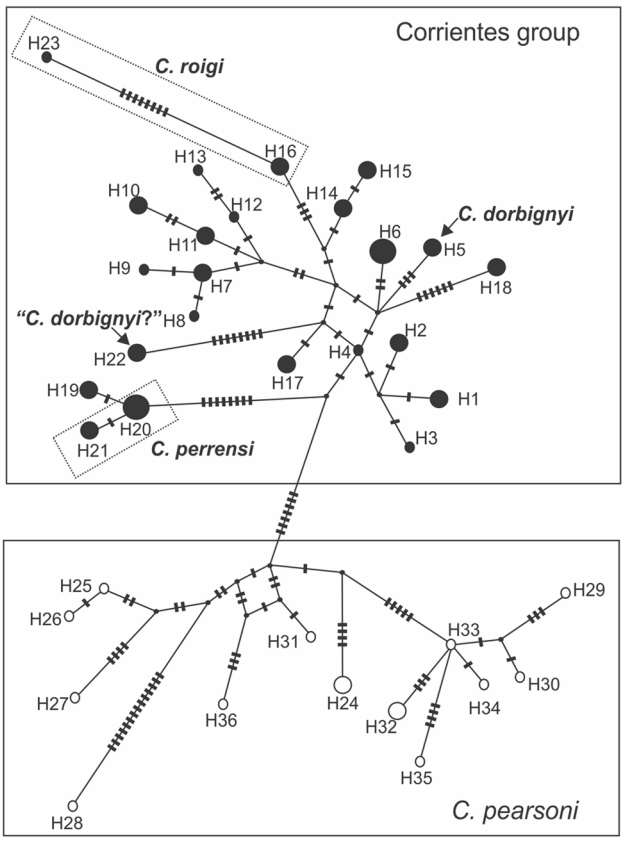

Fig. 5 Median network of *Ctenomys* haplotypes from the Corrientes group and *C. pearsoni*. Circle sizes are proportional to haplotypic frequencies. Small perpendicular lines represent mutational steps between haplotypes. The correspondence between haplotypes, Genbank Accession numbers and specimens are shown in Supplementary Table 1. Species groups are delimited with frames

of chromosomal rearrangements. A rapid elimination of ITS may favor the stabilization of rearranged chromosomes and thus serve to the maintenance of chromosomal diversity (Kilburn et al. 2001; Bolzán 2017).

Population differentiation

The populations with 2n = 42/FNa = 72, namely Loreto, Curuzú Laurel and Contreras Cué, included in this study share a highly conserved karyotype as revealed by NORs, C, and DAPI banding. This cytotype was also described in specimens from the close locality Estancia La Tacuarita (Caraballo et al. 2015). To a large extent, DAPI and C-banding patterns (this study) coincide with G- and C-bands published by Ortells (1995) and Reig et al. (1992) in samples from Curuzú Laurel, respectively. The most conspicuous difference resides in the description of a completely heterochromatic Y chromosome (Reig et al. 1992), in contraposition with small CH blocks found in the present study.

At the molecular level, microsatellite (SSR) studies (Mirol et al. 2010), as well as cyt-*b* phylogenetic analysis (Caraballo et al. 2016) agree in that the populations of Contreras Cué and Estancia La Tacuarita form a well-supported clade. These localities are situated in the eastern margins of the Iberá basin, a big depression that passes through the Corrientes province in Northeast-Southwest direction. The remaining populations with 2n = 42/NF = 72 (Curuzú Laurel and Loreto), located in the western margin of the Iberá, were grouped in different clades (Caraballo et al. 2016, this study). The high level of homology among these karyotypes supports the hypothesis of the existence of a common ancestral population with 2n = 42, which went through a subsequent process of molecular differentiation originated by the physical separation of the eastern Iberá's populations, *via* a founder event or a population bottleneck (Mirol et al. 2010; Gómez Fernández et al. 2012).

It should be noted that a 2n = 42/FNa = 72 cytotype is also found in Brazilian populations of *C. torquatus* (Fernandes et al. 2009). However, conventional chromosome morphology, as well as G and DAPI banding patterns, revealed strong differences between both cytotypes, indicating that both 2n and FNa are convergent. Molecular evidence also supports this hypothesis (Fernandes et al. 2009; this study), inasmuch as *C. torquatus* and the Corrientes group are reciprocally monophyletic and their genetic distances are among the highest in interspecific pairwise comparisons (Supplementary Table 1). Geographically, *C. torquatus* and the populations in Corrientes are closer but separated by the Uruguay River, which has probably acted as an effective barrier to gene flow (Fernandes et al. 2009).

On the other hand, the localities of San Miguel (2n = 44/FNa = 72) and Paraje Caimán (2n = 45–46/FNa = 74) are geographically close and exhibit closely related karyotypes. The most conspicuous difference between them corresponds to the presence/absence of a small acrocentric pair, but no specific rearrangements explaining this discrepancy could be determined using banding techniques.

At the mitochondrial level, samples from San Miguel are polyphyletic but fall into a major clade that, with the exception of Curuzú Laurel (see above), groups all Iberanan populations (Supplementary Fig. 2; Caraballo et al. 2012). Samples from Paraje Caimán (2n = 45–46/FNa = 74) are monophyletic and fall together within the same clade as the former. The polyphyletic condition concerning haplotypes from San Miguel (and those from other populations), could be the result of gene flow between populations as well as to incomplete lineage sorting (Giménez et al. 2002; Caraballo et al. 2016).

Microsatellite data suggest significant levels of gene flow among Loreto, Curuzú Laurel, San Miguel and Paraje Caimán populations, which share low 2n and FNa and are geographically proximate (Mirol et al. 2010). Comparison

among these three karyotypes indicated that one of the differences between them is the presence of a Rb rearrangement, a type of chromosomal mutation that has been frequently recorded within the Corrientes group (Ortells et al. 1990; Lanzone et al. 2007; Caraballo et al. 2015), and is one of the most common rearrangements observed in rodents (Patton and Sherwood 1983). This type of rearrangement is not expected to produce reproductive isolation (Lanzone et al. 2002; Basheva et al. 2014); its high frequency may be explained by its nearly neutral effects in heterozygosis. But in addition, an extra rearrangement would be necessary to explain the variation in FNa among these karyotypes. Although the involved chromosome is particularly small, the most probable rearrangement involving this element would be a tandem fusion, which is expected to act as a severe reproductive barrier; in fact no heterozygotes for these rearrangements were found in the Corrientes group, in contrast with the high frequency of Rb heterozygotes reported (Ortells 1995; Garcia et al. 2000a; Lanzone et al. 2007; Caraballo et al. 2015). More extensive sampling would be needed to search for possible contact zones and hybridization in this particularly complex lineage (Caraballo et al. 2016; Caraballo and Rossi 2018a).

Chromosome banding supports the cohesion of the Iberá lineage (Giménez et al. 2002; Caraballo et al. 2012, this study). Within this group, Caraballo and Rossi (2018a) postulated the existence of three subgroups over the basis of different genetic markers, but the current analysis based exclusively on cytochrome *b* sequences failed to recover the same groupings. Inter-population genetic distances were extremely low, ranging from 0% (between Tacuarita and Contreras Cué) to 0.85% (between Curuzú Laurel and other populations).

An especially divergent lineage in the Corrientes group is that of *C. roigi*, being Costa Mansión its type locality. Its karyotype was 2n = 48/FNa = 76 (Ortells et al. 1990), which was also found in neighboring populations Colonia Brougnes and Estancia San Luis (Giménez et al. 2002; Caraballo et al. 2015). Previous banding patterns based on specimens from Costa Mansión (Reig et al. 1992; Ortells 1995) are equivalent to our results based on specimens from Estancia San Luis. This species exhibits a marked karyotypic differentiation with respect to members of the Iberá group and to the rest of the Corrientes group. Based on G-banding patterns, Ortells (1995) identified several distinctive rearrangements (four Rb rearrangements, two with monobrachial homologies, a pericentric inversion and one arm without any homology) between the 2n = 42/FNa = 72 and 2n = 48/FNa = 76 cytotypes. DAPI banding patterns (this study) are not resolutive enough to confirm Ortells's results. Nevertheless, we corroborated the existence of monobrachial homologies, possibly reflecting full-arm translocations between non-homologous chromosomes. This type of rearrangement constitutes an important source of chromosomal variability in other rodents, such as *Mus musculus domesticus* (Piálek et al. 2005 and references therein).

Supporting their high level of differentiation, samples with 2n = 48/FNa = 76 form a unique cluster based on microsatellite markers, and were monophyletic in mtDNA phylogenetic analyses (Mirol et al. 2010; Caraballo et al. 2012; Gómez Fernández et al. 2012; Caraballo and Rossi 2018a; this study). Genetic distances between these samples and other members of the Corrientes group varied from 0.38 to 2.3%. The exclusivity in molecular and chromosomal characters supports the species status of the populations assigned to *C. roigi*.

Different chromosome complements were described in Chavarría and Saladas. In Chavarría a monomorphic 2n = 56, FNa = 80 was found. In Saladas, and neighboring populations, a Rb polymorphism producing 2n = 56, 55, 54 and FNa = 80 was described (Ortells et al. 1990; Giménez et al. 2002; Lanzone et al. 2007). In addition, cytotypes with 2n = 52, 51 and 50 in other related populations were registered (rev. in Caraballo and Rossi 2018a). Reig et al. (1992) described pericentromeric heavily stained blocks of CH restricted to a small number of chromosome pairs. We confirmed these observations and determined low levels of AT content (DAPI-negative) in these blocks. In some of these populations, B chromosomes and mosaicisms had been postulated (Garcia et al. 2000a); but none of these findings were recovered in our study. DAPI banding revealed that despite the close 2n and FNa found in these populations, there is a significant chromosomal differentiation between samples from Chavarría and Saladas. Our results agree with molecular evidence supporting the membership of these two populations to distinct evolutionary lineages (Mirol et al. 2010; Gómez Fernández et al. 2012; Caraballo et al. 2012; Caraballo and Rossi 2018a).

Samples from Santa Rosa had a divergent chromosome complement with high 2n and FNa values (66/82 respectively). Although this population was considered a member of the lineage assigned to the *C. perrensi* complex, its relationship with other lineages varied depending on the markers and the methods of analysis used (rev. in Caraballo and Rossi 2018a).

The highest 2n described in the group is 70 and corresponds to *C. dorbignyi*. Two northern populations ascribed to this species were studied, but this 2n was also registered in the southernmost locality of the distribution area of the Corrientes group. However, on the basis of chromosomal data, mtDNA, and SSR markers, it has been suggested that these northern and southern populations correspond to two different lineages (Argüelles et al. 2001; Caraballo and Rossi 2018a). A very similar 2n = 70 cytotype was registered in *C. pearsoni* (Novello and Lessa 1986). Although there are discrepancies about the exact FNa of population ascribed

to *C. dorbignyi* (Garcia et al. 2000b; Argüelles et al. 2001), there is an overall agreement in the high level of homology between chromosome complements of specimens with 2n = 70, even with those described in *C. pearsoni*. Molecular data are in accordance with these observations, showing low cytochrome *b* distances between samples from the Corrientes group, and also between them and *C. pearsoni* (Supplementary Table 2). Most values fall into the expected range of intraspecific variability (Baker and Bradley 2006). The cytochrome *b* haplotype network also showed a pattern which is not entirely compatible with current taxonomy. This indicates that the group deserves a holistic taxonomic revision, including samples from all nominal species and covering intermediate localities.

Finally, it is important to consider that in some cases mitochondrial and nuclear genomes can suffer different evolutionary histories, through differential introgression, in taxa that hybridize at least sporadically (Armella Sierra et al. 2017 and references therein). These processes could account for the high chromosomal divergence and the low mitochondrial variability observed in this complex group of *Ctenomys*.

Acknowledgements This research has been partially funded by PIP 0198, CONICET, RI: CL. PIP 2012–2014 No 0182, CONICET, RI: ADB and funds from the CICPBA and UNLP of Argentina, as well as by The Linnean Society of London (Systematics Research Fund); RI: MSR from the United Kingdom. DAC was supported by a postdoctoral fellowship awarded by CONICET. MSR, ADB, and CL are career investigators of CONICET; LMB, CAL. and JMF are very grateful to CONICET for the granted postgraduate scholarship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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