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Reassessing the causal connection between satDNA dynamics and chromosomal evolution in *Ctenomys* (Rodentia, Ctenomyidae): Unveiling the overlooked importance of the Y chromosome

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Abstract

Satellite DNAs (satDNA) have long been recognized as a major driving force in karyotypic repatterning, owing to their ability to recombine between non-homologous chromosomes. A quite extensively studied model is the Repetitive PvuII *Ctenomys* Sequence (RPCS), the main component of constitutive heterochromatin in rodents of the genus *Ctenomys* (Rodentia, Ctenomyidae). At the genus level, fluctuations in RPCS copy number have been previously associated with karyotypic instability. However, when a microevolutionary approach was assayed in the most karyotypically variable lineage of the genus, the vast population-level copy number variation precluded any possibility of analyzing it in a phylogenetic framework. The existence of sex-related differences as a source of variability was not considered until later, when chromosomal banding suggested that the Y chromosome may be a significant reservoir of RPCS. This study aimed to investigate the bias associated with the presence of the Y chromosome in RPCS copy number variation in the Corrientes group of *Ctenomys*. The results revealed that the Y chromosome harbors almost twice the amount of RPCS compared to the rest of the chromosome complement, explaining the high levels of intrapopulation variation. The evolution of RPCS copy number in males and females showed independent patterns, attributable to the presence/absence of the Y chromosome. The correlation between RPCS dynamics and diploid number fluctuations was also investigated, concluding that some karyotypic repatterning events could be explained by satDNA amplification/deletion, but not

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all of them. This study highlights the importance of considering differences resulting from the differential accumulation of satDNA in the heterogametic chromosome.

Keywords

Ctenomys – diploid number – phylogeny – Repetitive PvuII Sequence of *Ctenomys* – SatDNA – Y chromosome

Introduction

Satellite DNA (satDNA) represents a major organizational component of eukaryotic genomes, that together with transposable elements (TEs) contribute significantly to genome size variation between taxa, reaching more than 50% of some species' total DNA (López-Flores & Garrido-Ramos, 2012). SatDNAs can be found in different chromosomal locations, usually arranged in constitutive heterochromatin (CH) blocks in pericentromeric, subtelomeric, and interstitial regions (López-Flores & Garrido-Ramos, 2012; Plohl et al., 2012; Louzada et al., 2020); albeit satDNAs have also been found dispersed throughout euchromatic regions in some species (Ugarković & Plohl, 2002; Garrido-Ramos, 2017). SatDNA is characterized by a concerted pattern of evolution implying that there is an observed higher similarity for repeats than expected if they evolved independently (Dover, 1986; Elder & Turner, 1995). The rapid homogenization of satDNA copies is given due to its tandem nature: unequal crossing over, intrastrand homologous recombination, gene conversion, and rolling-circle replication, are the main mechanisms driving satDNA turnover (Ugarković & Plohl, 2002; Palacios-Gimenez et al., 2020). Some of these mechanisms can produce dramatic changes in satDNA copy number, either gains (amplification) or losses (contraction) (Lower et al., 2018). As these processes involve recombination between tandems located in non-homologous loci, satDNA haves been considered agents of chromosomal evolution (Ruiz-Herrera et al., 2006; Farré et al., 2011, 2011; Vieira-da-Silva et al., 2015). Chromosome fusions (i.e., Robertsonian translocations), fissions (Ferguson-Smith & Trifonov, 2007), and inversions (Dobzhansky, 1970), are the rearrangements with the strongest impact on genome architecture during species evolution. The most frequent rearrangements occurring are Robertsonian translocations, which have been shown to involve the reorganization of satDNA sequences at the centromere level (Chaves et al., 2003; Adega et al., 2009).

One particularly interesting model for studying satDNA and chromosomal evolution is the subterranean rodents of the genus *Ctenomys*. Popularly known as tuco-tucos, these rodents are distributed in the austral cone of South America, from Perú and Brazil to Tierra del Fuego, Argentina (D'Elía et al., 2021). The origin of the genus has been estimated to be from 10 to 3 Ma, depending on the study (Parada et al., 2011; Upham & Patterson, 2015; Caraballo & Rossi, 2018; Santi et al., 2021), but there is agreement in that extensive and rapid cladogenesis took place around 3–1 Ma with the development of eight different species groups (Parada et al., 2011; Santi et al., 2021) and more than 60 extant species, constituting one of the most species-rich genera among mammals (D'Elía et al., 2021).

Ctenomys is also characterized by the broadest range of chromosomal numbers known for a mammalian genus, from 2n = 10 to 2n = 70 (Buschiazzo et al., 2022). This extremely high karyotypic variability is the result of several concomitant chromosomal rearrangements, and although Robertsonian translocations have prevailed, other rearrangements have been described, such as pericentric/paracentric inversions, tandem fusions, and/or heterochromatic blocks addition/loss (Reig et al., 1990; de Freitas, 2006; Novello & Villar, 2006; Caraballo et al., 2015). Within *Ctenomys*, the most karyotypically diverse lineage is that of the Corrientes group, which comprises at least 26 related populations, distributed in the area under the influence of the Iberá wetland in the Corrientes Province in Argentina. Some populations are alleged to belong to three species: *C. dorbignyi*, *C. roigi*, and *C. perrensi*, while a fourth lineage, *iberá*, was distinguished based on an integrative approach (Caraballo & Rossi, 2017). In this group, diploid numbers range from 41 to 70 and fundamental numbers (FN) from 76 to 84, mainly due to Robertsonian (Rb) rearrangements, and to the increase/decrease in the number of small acrocentric chromosomes, although other complex rearrangements have also been proposed (Ortells et al., 1990; Giménez et al., 2002; Caraballo et al., 2015).

The major satDNA of *Ctenom*ys, named RPCS (repetitive PvuII *Ctenomys* sequence), is organized in arrangements consisting of monomeric units of 348 bp. RPCS is localized in different chromosomal regions (whole arms, pericentromeric blocks, as well as telomeric and interstitial bands) varying across karyotypes (Rossi et al., 1995). *In situ* hybridization showed that RPCS invariably co-localizes with C-positive heterochromatic blocks, being the major component of constitutive heterochromatin (Rossi et al., 1995). RPCS copy number varies drastically between species, ranging from a few hundred copies (e.g., *C. tucumanus*) to millions of copies (e.g., *C. haigi*) (Rossi et al., 1995; Slamovits et al., 2001). When a phylogenetic approach was employed to analyze such variability, it was proposed that lineages with stable RPCS copy numbers showed karyotype stability, while in karyotypically variable clades, RPCS contracted or amplified independently along branches (Slamovits et al., 2001). The main conclusion of that study was that it is not the high or low copy number, but instead the change in RPCS copy number, that determines chromosomal variability in this group of rodents.

With this background, it was intended to study the variation in RPCS copy number under a phylogenetic approach in the most karyotypically variable lineage of tuco-tucos, the Corrientes group (Caraballo et al., 2010). In that study an unexpectedly wide range of intrapopulation variability in RPCS copy number was observed, leading to the conclusion that amplifications and deletions were ongoing processes, precluding the possibility to analyze such variation in a phylogenetic context. However, further findings would make rethinking those results imperative. Crucial for this reinterpretation was the study of constitutive heterochromatin (CH) blocks via C- and DAPI banding (Buschiazzo et al., 2018), in which it was found that the tuco-tucos from the Corrientes group showed relatively low CH content, with blocks concentrated in a few specific autosomal pairs involved in Rb translocations, and -although variable between populations- a high CH content in the Y chromosome. The intense accumulation of satDNA on the heterogametic sex chromosomes has been reported in a variety of animals (Giovannotti et al., 2018; Escudeiro et al., 2019; Utsunomia et al., 2019; Ferretti et al., 2020), leading to the notion that these chromosomes may function as major reservoirs for satDNA.

Given the precedent findings, it is plausible to consider that the observed differences in satDNA abundance at the population level in the Corrientes group could have been erroneously attributed to interindividual variability of autosomal clusters when the greatest contribution to that variability is given by the differences between sex chromosomes. Two predictions can be made from this background: 1) Within a population, RPCS copy number should be higher in males than in females since the Y chromosome should harbor extra RPCS copies; 2) When analyzing females (removing noise from Y-related fluctuations) in a phylogenetic context, there should be a correlation between copy number fluctuation and karyotypic repatterning. This study aims to test both predictions by analyzing RPCS copy number of males and females separately, quantifying the relative contribution of the Y chromosome to the global RPCS profile, and analyzing variation in copy number together with karyotypic repatterning along the phylogeny of the *Ctenomys* Corrientes group.

Materials and methods

Data acquisition

RPCS copy number of the Corrientes group was classified according to the specimens' sex, using previously quantified samples (Caraballo et al., 2010). Diploid numbers were obtained from the literature (Ortells et al., 1990; Caraballo et al., 2015). Sequences for the phylogenetic inference were downloaded from Genbank (Accession numbers JX275502-JX275655 and KT818638-KT818684). For transparency and reproducibility of the results, the scripts used in this study are available at: https://github.com/d-caraballo/RPCS-scripts. Supplementary data is publicly available at: 10.6084/m9.figshare.22243669 under a CC BY 4.0 license.

To estimate the proportion of RPCS that the Y chromosome contributes to the total amount of an individual in a given population, the mean copy number in females was subtracted from the mean copy numbers observed in males, and then relativized to the total copy number in females. The rationale for this calculation is that the main difference between males and females should be the amount of RPCS found in the Y chromosome.

Mitochondrial phylogeny

To analyze the evolution of RPCS copy number and diploid number a Bayesian phylogenetic tree was obtained based on a 2,178 bp alignment of partial mitochondrial (mtDNA) loci: cytochrome b (cytb), cytochrome oxidase I (CO1) and the control region (CR) (supplementary file S1) (Caraballo et al., 2012, 2016). One specimen's sequence set was retained for localities that met reciprocal monophyly (20 localities). The haplotypes from Loma Alta and Curuzú Laurel were removed from the analysis because both localities fail to form monophyletic groups due to incomplete lineage sorting (Caraballo et al., 2012, 2016; Caraballo & Rossi, 2017), besides that there is no karyotypic data from Loma Alta. Curuzú Laurel has been postulated as part of the *iberá* lineage, given that it shares the same karyotype (2n = 42) as the majority of populations of this subgroup. It is noteworthy that in both Loma Alta and Curuzú Laurel males have markedly higher amounts of RPCS than females, and that in the latter it is verified that this copy number reaches overlapping values with the remaining populations of the *iberá* lineage. The locality of Manantiales was also removed from the analysis because it has neither available RPCS copy number nor karyotypic information. Finally, both haplotypes from San Miguel were retained in the analysis, because these are closely related sequences that fall within the *iberá* lineage.

A total of 1.5×10^7 Markov Chain Monte Carlo (MCMC) generations were run in MrBayes 3.2.7 (Ronquist et al., 2012) through the Cipres Gateway (Miller et al., 2010) under the general time reversible, plus a proportion of invariant sites and gamma-distributed rate heterogeneity among sites (GTR+I+G) model, sampling every 5×10^3 generations. A sequence from the sister group *Ctenomys pearsoni* (from Médanos, Entre Ríos, Argentina) was included as outgroup. The potential scale reduction factor (PSRF) and the average standard deviation of split frequencies (ASDSF) were used for convergence diagnostics. The *burn-in* phase was set as the MCMC generation that reached standard deviations of <0.01 and PSRF values of 1.00 to 1.02 for all estimated parameters, corresponding to the 6.36% of the run. Trees were visualized in FigTree v1.4.4 (Rambaut, 2014).

RPCS reconstruction

Ancestral RPCS copy numbers of males and females separately were inferred with the phytools package (Revell, 2012) using the R (R Development Core Team, 2021) environment and RStudio (RStudio Team, 2020). RPCS copy number was treated as a continuous character and mapped along the obtained mtDNA phylogeny. In those localities where there was more than one specimen of the same sex quantified, the mean value was used for this reconstruction. The function anc.ML was used to fit RPCS copy number in the phylogeny, while the projection of the reconstruction onto the edges of the tree was made with the function contMap.

Diploid number reconstruction

Ancestral diploid numbers were estimated using RevBayes (Höhna et al., 2016), under the ChromEvol model, a continuous-time Markov chain (CTMC) model of chromosome number evolution. As this model assumes that cladogenesis events are always strictly bifurcating (i.e. there are no hard polytomies), the two polytomies in the mtDNA tree were resolved under all possible combinations to check if this affected the inferred ancestral states. The maximum chromosome number was set to 90, no polyploidization rate was considered (rho = 0), and the run was set to 200 MCMC generations, discarding the first 25% as burnin.

The RevGadgets (Tribble et al., 2022), ggplot2 (Wickham, 2016), and ggtree (Yu, 2020) R packages were used to plot ancestral chromosome number estimates and their associated posterior probability values.

Results

RPCS copy number in males and females

Within any given locality males show a substantially higher RPCS copy number than females (figs 1 and 2, supplementary table S1). There is only one exception to this pattern: a female from Santa Rosa with 1.3×10^6 copies, but it is likely to be the product of an artifact since another female from the same locality has 6.8×10^5 copies. However, it must be noted that this lower value is comparable to the copy number found in a male in this locality. Whereas in most localities females' RPCS range between ~1.2 and 5.5×10^5 copies, in males this range is between 5.0×10^5 and 1.3×10^6 copies. The specimens from Goya depict the highest RPCS copy number in all the Corrientes group. Even when females have >1.5 10^6 copies (more than any male from any other locality), males have more than 2.0 10^6 copies, confirming again that the heteromorphic sex chromosome contributes a significant amount of SatDNA in low- and high-copy number profiles. Notably, the net difference between males and females ranges from 1.6×10^5 to 1.2×10^6 copies, suggesting

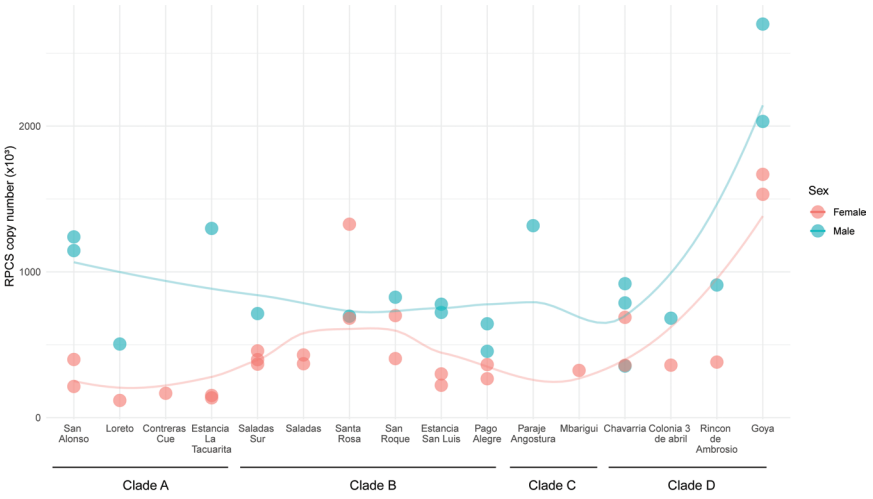

FIGURE 1 Differences in RPCS copy number in males and females. Scatter plot showing differences in RPCS copy numbers between males and females of the *Ctenomys* Corrientes group, expressed as thousands of copies. Clades A-D correspond to the four different clades of the phylogeny (figs 3 and 4). A smoothing function was applied with the package ggplot2.

high levels of variation in the copy number of the Y chromosome. The only exception to this rule is that of Santa Rosa, but this is expectable due to the presence of an outlier as mentioned above. The relation between the RPCS content of the Y chromosome and the rest of the chromosomal complement ranges between 0.3 and 8.0, with a mean value of 1.9 (S.D. = 2.25).

When comparing the female RPCS copy number there is some degree of geographic/lineage-specific correlation (fig. 2). *Ctenomys roigi*, *C. dorbignyi* and *iberá*, depict relatively low levels of RPCS copy number. In contrast, the *C. perrensi* complex depicts moderate to high copy number of this satDNA, including Goya, with the highest copy numbers of the Corrientes group as mentioned above. The pattern is similar among males, except that *C. dorbignyi* and *iberá* show higher levels of RPCS copy number, suggesting there is a differential contribution of the Y chromosome in these lineages. It is important to note that the *C. perrensi* complex comprises two mtDNA clades that have shown to be interconnected by karyological and microsatellite data (Caraballo & Rossi, 2017).

RPCS dynamics

The ancestral reconstruction of RPCS copy number showed different patterns between males and females (fig. 3). In males, the basal copy number was intermediate-low (1.0×10^6 copies), and remained relatively stable in clades A and C, while there was a reduction in clade B. On clade D there was a predominant reduction, but in the branch leading to Goya, there is an impressive amplification leading to the highest RPCS copy numbers observed in the Corrientes group.

The ancestral RPCS copy number of females was also intermediate-low (4.1×10^5 copies), however, it showed some differences with respect to the evolution of this trait in males (fig. 3). In clade A, it suffered a gradual reduction that continued occurring along

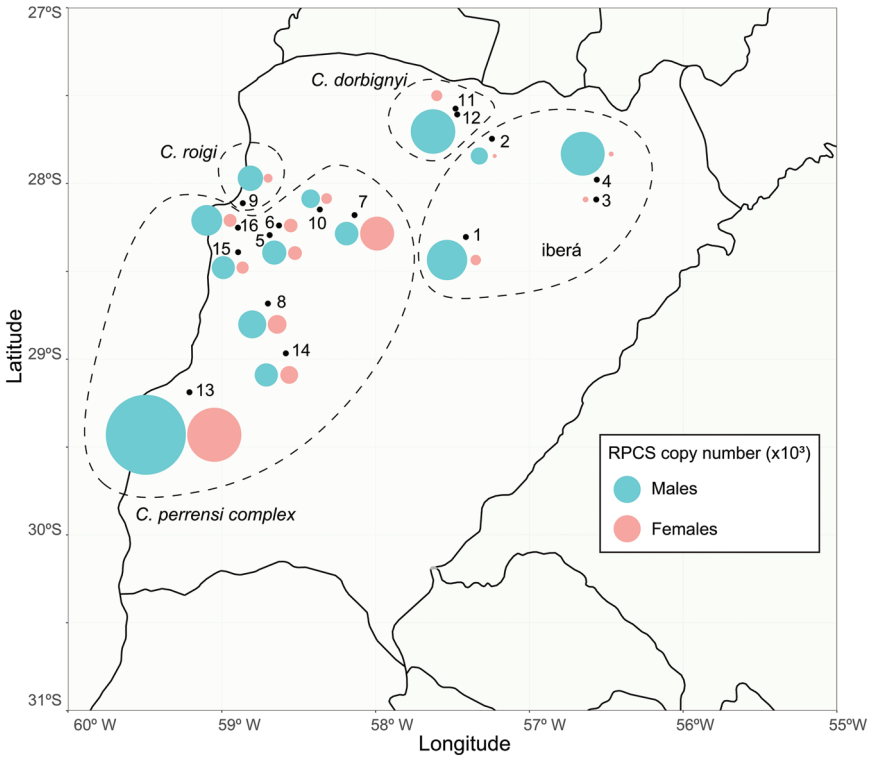

FIGURE 2 Geographic distribution of mean RPCS copy number. The Corrientes group lineages are surrounded by dashed lines. Locality numbers are: 1 – San Alonso, 2 – Loreto, 3 – Contreras_Cué, 4 – Estancia La Tacuarita, 5 – Saladas Sur, 6 – Saladas, 7 – Santa Rosa, 8 – San Roque, 9 – Estancia San Luis, 10 – Pago Alegre, 11 – Mbarigüí, 12 – Paraje Angostura, 13 – Goya, 14 – Chavarría, 15 – Colonia 3 de abril, 16 – Rincón de Ambrosio.

internal branches (except for San Alonso, where a slight amplification would have taken place). Along clade B it showed both amplifications and deletions, although in a narrow range of values (except for Santa Rosa, which is likely to be inflated by an artificial cause, but even when removing the candidate outlier, the model postulates an amplification in the branch leading to this locality – data not shown). Copy number remained stable in clade C, but it was amplified in the branch leading to clade D. Within this clade, a slight reduction took place in one branch, while in the other there was a conspicuous amplification, owing to the highest observed values in Goya (1.6×10^6 copies), as occurred in males.

Chromosomal dynamics

The ancestral reconstruction of chromosomal numbers yielded an ancestral 2n = 70, in agreement with previously published literature (Caraballo et al., 2012; Buschiazzo et al., 2022), also congruent with the fact that this karyotype is shared with *C. pearsoni*, the sister species of the Corrientes group (fig. 4). This ancestral 2n remained stable until the divergence of the four main clades of the Corrientes group and, except for the branch leading to *C. dorbignyi* (clade C) and Sarandicito, all branches experienced significant diploid number reductions (>6 chromosomes). The most abrupt reduction is that leading to the *iberá* lineage (clade A), where the net

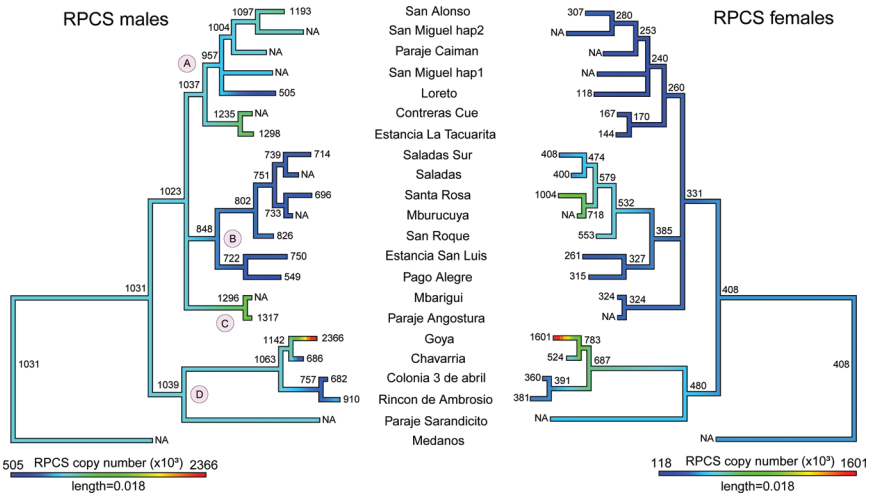

FIGURE 3 Ancestral RPCS copy number reconstruction. RPCS copy number was mapped along the mtDNA phylogeny using the phytools function anc.ML, for males and females of the *Ctenomys* Corrientes group separately. The projection of the reconstruction onto the edges of the tree was made with the function contMap. RPCS copy number is expressed as thousands of copies. The scale of the tree is expressed in substitutions per site. Letters A–D correspond to the four main clades of the group.

difference is 16 chromosomes. There are two secondary reductions, in terminal branches leading to *C. roigi* (Estancia San Luis, clade B) and Goya, the type locality of *C. perrensi* (clade D). Interestingly, the branch leading to Santa Rosa is the only branch where an increase in the diploid number took place. As mentioned in Materials and Methods, the possible effect of the different resolutions of polytomies was tested, but no difference in the ancestral 2n of those polytomies was found.

Discussion

The first prediction that derives from the background of this study implies that since males have a greater amount of heterochromatin for having a CH-rich-heterogametic chromosome, and that the main component of heterochromatin in *Ctenomys* is RPCS, then males are expected to have a greater number of copies of this satDNA. This prediction is fulfilled in all the localities that comprise this case study (except for one outlier female, see Results) (figs 1 and 2). On average, males have three times the amount of RPCS than females, which suggests a striking contribution of the Y chromosome to a specimen's satDNA profile. In other words, two parts of the RPCS satellitome in males are harbored in the Y chromosome.

According to classical theory, sex chromosomes are believed to originate from a pair of homologous autosomes that accumulate genetic differences during the early stages of this process due to recombination suppression (Charlesworth, 1991). As a result, deleterious mutations that accumulate on the heterogametic chromosome cannot be eliminated by recombination with a beneficial copy of the chromosome, which leads to a gradual loss of genetic diversity on the heterogametic chromosome (Charlesworth

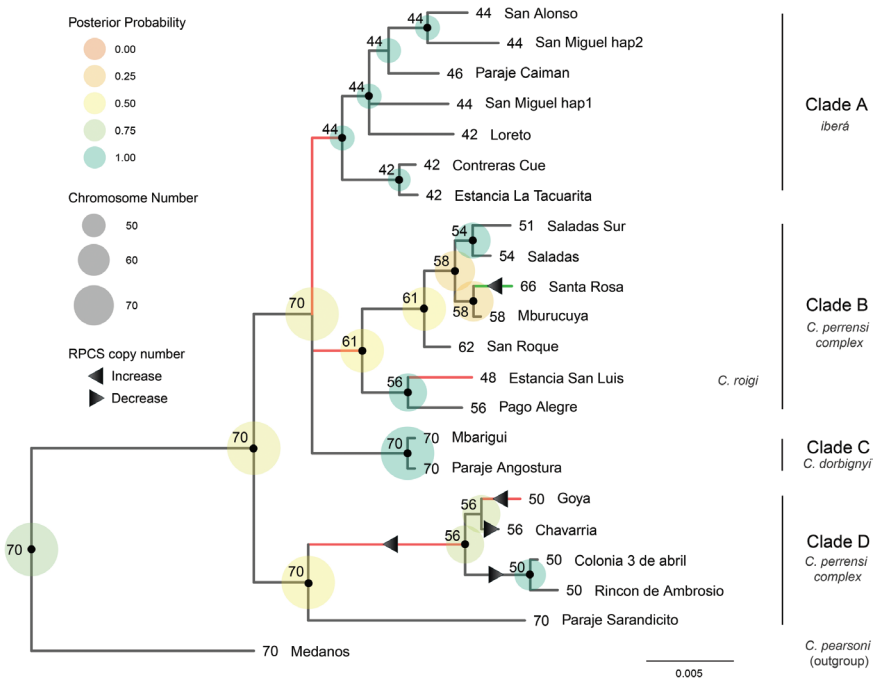

FIGURE 4 Ancestral reconstruction of diploid numbers (2n) and main RPCS reductions and amplifications in the *Ctenomys* Corrientes group. Ancestral diploid numbers were inferred with the ChromEvol model implemented in RevBayes, over the mtDNA Bayesian phylogeny of the Corrientes group. Numbers in internal nodes/terminals represent inferred/observed 2n. Colored circles depict 2n (size) and posterior probability of the inferred value (color). Red and green branches depict significant reductions and amplifications in diploid numbers, respectively. Smaller equal-sized black circles show well-supported nodes (posterior probability > 0.75). Black arrowheads denote a marked increase/decrease in RPCS copy numbers (inferred from females). The scale bar is expressed in substitutions per site.

& Charlesworth, 2000). However, despite the loss of genes, the heterogametic chromosome has preserved its role in sex determination and is essential for male development (Furman et al., 2020). In mammals, the evolution of sex chromosomes has been studied extensively, and the process is believed to have involved several rounds of recombination suppression and gene loss on the Y chromosome (Bellott et al., 2010). The accumulation of repetitive DNA elements, such as transposable elements and satDNA, has also been implicated in the differentiation of sex chromosomes in mammals (Charlesworth, 1991; Charlesworth & Charlesworth, 2000; Cabral-de-Mello et al., 2021). The observation emanating from the present study – the differential accumulation of RPCS in males (in the Y chromosome) – is coherent with the empirical and theoretical background. Five of the 13 localities where male RPCS copy numbers could be measured have been screened for the presence of HC blocks in the Y chromosome (Buschiazzo et al., 2018). In congruence with the present findings, in all studied localities the Y chromosome depicted one to three conspicuous CH blocks, which would provide evidence of the existence and the physical location of large amounts of RPCS in the male sex chromosome.

The second prediction that can be drawn from the background is that there should be a correlation between RPCS copy number fluctuations and 2n dynamics. The initial observation in this regard is that RPCS copy number evolution does not coincide between males and females (fig. 3). This is totally plausible since the evolution of the majority of RPCS copies in males is present in the Y chromosome and is independent of the fate of female genomes. Interestingly, the striking rise in RPCS copy number experienced in the ancestor of the specimens from Goya was mirrored by males and females. In this case, male copy number is more strongly influenced by the enormous copy number found in the rest of the chromosomal complement, representing the second lowest (male-female)/female ratio, which means that in this locality the relative contribution of the Y chromosome is diluted. However, there is a still relevant contribution of the Y chromosome to the total RPCS profile, since males have ~8×10^5 extra copies.

Given that the Y chromosome is somehow disconnected from the rest of the genome (it has not been postulated to participate in chromosomal rearrangements in this group), and that it contributes significantly to the total RPCS profile in a genome, the relationship between 2n and RPCS copy fluctuations should be carried out analyzing females solely. This relationship is verified in some cases, but it is not ubiquitous (fig. 4). An especially dynamic lineage is clade D. In this clade there are both reductions and amplifications of RPCS copies, and these fluctuations can be correlated to significant reductions in the diploid number, all of them hypothesized to be the result of centric fusions (Caraballo et al., 2012). Similarly, in the branch leading to Santa Rosa, where a considerable karyotypic repatterning took place, a notable amplification of RPCS occurred. However, high fluctuations in copy number are not observed in other three lineages, two of which underwent the most extensive karyotypic restructuration of the Corrientes group. One of these is the branch leading to the iberanan populations (clade A), which have the lowest 2n and FN of all the Corrientes group, while the other is the branch leading to *C. roigi* (clade B), which together with the former has the highest differentiated karyotypes (Buschiazzo et al., 2018). These results seem counterintuitive given that in the same group of rodents, a general correlation between 2n and RPCS dynamics has been postulated (Slamovits et al., 2001). However, there are two aspects that should be taken into account from that study. In the first place, except for one case, the authors quantified RPCS of one specimen per species, preventing the assessment of intraspecific variation. Second, and most important, there is no distinction between males and females in the study. Given that the majority of RPCS can be found in the Y chromosome, the observed fluctuations can be artifactually produced by including specimens of different sex in the analysis, and therefore the conclusions of Slamovits et al. (2001) should be taken cautiously. As a result, there is currently no conclusive evidence that large copy number fluctuations should be expected to precede large karyotypic repatterning events in *Ctenomys*.

In conclusion, the results of the present study confirm that *Ctenomys* males have several times the number of copies of RPCS than females. As the only chromosomal difference between males and females is the presence of the Y chromosome, it is concluded that this chromosome functions as a significant reservoir of this satDNA, which in turn has the potential to interact with other RPCS arrays located in autosomes generating neo-sex chromosomes (Ferretti et al., 2020). As expected, this produces a different pattern in the evolution of RPCS in males compared

to females, due to the *disconnected* stock that males have. These differences attributable to the heterogametic chromosome are of such relevance that if not taken into account, the interindividual variability could be mistakenly interpreted as evidence of high copy number fluctuations. Finally, although it is not a rule, the dynamics of the major satellite DNA of *Ctenomys* explain some but not the most extensive karyotypic repatterning events in the most chromosomally unstable group of the genus. Apart from these novel results, this study is hoped to provide working hypotheses which can further be tested by different experimental approaches, such as FISH and/or Next Generation Sequencing approaches (Palacios-Gimenez et al., 2020).

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Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.23791842

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