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The library model for satellite DNA evolution: a case study with the rodents of the genus *Ctenomys* (Octodontidae) from the Iberá marsh, Argentina

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Abstract On the basement of the library model of satellite DNA evolution is the differential amplification of subfamilies through lineages diversification. However, this idea has rarely been explored from an experimental point of view. In the present work, we analyzed copy number and sequence variability of RPCS (repetitive PvuII *Ctenomys* sequence), the major satellite DNA present in the genomes of the rodents of the genus *Ctenomys*, in a closely related group of species and forms inhabiting the Iberá marsh in Argentina. We studied the dependence of these two parameters at the intrapopulation level because in the case of interbreeding genomes, differences in RPCS copy number are due to recent amplification/contraction events. We found an inverse relationship among RPCS copy number and sequence variability: amplifications lead to a decrease in sequence variability, by means of biased homogenization of the overall satellite DNA, prevailing few variants. On the contrary, the contraction events that involve tandems of homogeneous monomers contribute—by default—minor variants to become "evident", which otherwise were undetectable. On the other hand, all the RPCS sequence variants are totally or partially shared by all the studied populations. As a whole, these results are comprehensible if these RPCS variants preexisted in the common ancestor of this *Ctenomys* group.

Keywords Satellite DNA · Library model · Ctenomys

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Introduction

Satellite DNA (formerly "junk DNA") is a conspicuous component of most eukaryotic genomes, being their overall sequence evolution driven by stochastic processes, dependent on the molecular and population levels. Satellite DNAs are involved in chromatin structure (Henikoff and Dalal 2005; Grewal and Elgin 2007), centromeric function (Henikoff et al. 2001; Lam et al. 2006; Plohl et al. 2008), gene expression (Shestakova et al. 2004) and chromosomal evolution (Fronicke and Scherthan 1997; Li et al. 2000; Hartmann and Scherthan 2004; Capanna and Castiglia 2004). Some of the molecular mechanisms underlying chromosomal evolution involve unequal recombination between monomers of satellite DNA sequences from homologous or non-homologous chromosomes (non-allelic, paralogous) (Dobigny et al. 2005; Castiglia et al. 2006). Intragenomic identity among units of satellite DNA sequences is on the base of mechanisms, such as unequal cross-over or rolling-circle replication, that lead to drastic decreases or increases in copy number (Ellingsen et al. 2007). For these reasons, the relation between intragenomic sequence variability and copy number could unveil stasis or dynamic (expansion/contraction) evolution of a satellite DNA. One of the first ideas about satellite DNA evolution proposed that closely related lineages share a "library" of satellite DNA variants present in the hypothetical ancestor of the group and that biases in turnover mechanisms could amplify or delete a particular sequence variant over the remainders, causing lineages-specific satellite DNA profiles (Fry and Salser 1977). During subsequent decades, several authors have taken this idea as a framework model in satellite DNA evolution (Ugarković and Plohl 2002); however, only in few cases, this model was comprehensively contrasted with experimental data

(Meštrović et al. 1998; Meštrović et al. 2006; Ellingsen et al. 2007).

In the present work, we deeper analyzed the model of the ancestral "library" by studying at the population level, the relation between sequence and copy number variability of the major satellite DNA, named RPCS (repetitive PvuII *Ctenomys* sequence) in a group of rodents of the genus *Ctenomys* (popularly known as "tuco-tucos"). Tuco-tucos are the most specious group of extant subterranean rodents, counting more than 60 species (Woods and Kilpatrick 2005), having the widest range of diploid numbers (2n) in mammals. Diploid and fundamental numbers (FN) vary from 10 to 70 and from 16 to 84, respectively (Kiblisky et al. 1977; Anderson et al. 1982; Ortells et al. 1990; Reig et al. 1992). *Ctenomys* has been considered an example of explosive cladogenesis triggered by chromosomal repatterning (Reig et al. 1992).

We take as case study the group of species of *Ctenomys* from Corrientes province, Argentina. This group is conformed by populations mainly distributed within the area under the influence of the Iberá marsh, a temporary and spatially fragmented habitat. Some of these populations are alleged to belong to the species *Ctenomys roigi*, *Ctenomys dorbignyi* and *Ctenomys perrensi*, but most of them have an undefined taxonomic status. The molecular phylogeny of the Corrientes group could not yet been clarified (Giménez et al. 2002). The high karyotypic variability of tuco-tucos from Corrientes—even at the intrapopulation level—with diploid numbers ranging from 40 to 70 (Ortells et al. 1990; García et al. 2000; Argüelles et al. 2001; Giménez et al. 2002; Lanzone et al. 2002), together with their patchy distribution of isolated or semi-isolated demes, encourages the hypothesis that chromosomal evolution is an ongoing active and recurrent process in this group.

RPCS is arranged in long tandems of 348-bp monomers, as many satellite DNAs (Rossi et al. 1990), but with a remarkable sequence and structural similarity with a retroviral long-terminal repeat (Rossi et al. 1993a). In situ hybridization showed that RPCS localizes in different chromosomal regions (whole arms, pericentromeric blocks, as well as telomeric and interstitial bands) in different karyotypes (Rossi et al. 1995). RPCS also varies drastically in copy number among species, ranging from 2,000 to 6.66×10^6 copies per haploid genome (Rossi et al. 1993b; Slamovits et al. 2001). When a phylogenetic approach was employed, it could be demonstrated that stable karyotypes (shared among related species) exhibit stasis for RPCS copy number, while in karyotypically variable clades, RPCS contracted or amplified independently along branches (Slamovits et al. 2001). RPCS sequence variability measured as *genomic consensus sequences* showed a mutational profile shared by distantly related *Ctenomys* lineages, whose range of variation depends on copy number and karyotypic stability (Ellingsen et al. 2007).

The observed evolutionary patterns of a satellite DNA can be explained by the concourse of factors as de novo mutation, vertical and horizontal spreading of new mutants within a group of reproductively related organisms (molecular drive, gene conversion) (Dover and Tautz 1986), differential amplification or deletion of ancestral sequence variants throughout species, (ancestral library) (Fry and Salser 1977; Ugarković and Plohl 2002) as well as selective constraints (Ellingsen et al. 2007; Plohl et al. 2008). In the present paper, we analyze RPCS evolution within populations of a group of closely related and probably recently diverging *Ctenomys* species. Particularly, we would experimentally test the "library" hypothesis of satellite DNA evolution in our model, by evaluating the relationship between copy number and intragenomic nucleotide variability. If satellite DNA profiles observed in a group of closely related populations or species (as is the case of the Corrientes group) were the result of amplifications and deletions of ancestral variants of a preexisting "library", copy number should be inversely related to sequence variability.

Materials and methods

Ctenomys tissue samples

Liver and phalanx tissues were preserved in ethanol 100%. Samples were collected between October 2007 and October 2009, from a total of 18 populations: Estancia la Tacuarita (27°58′42.7″S; 56°33′40.6″W), 3 specimens; Pago Alegre (28°8′50.7″S; 58°21′44.3″W), 4 specimens; Saladas (28°14′23.3″S; 58°37′50.6″W), 2 specimens; Saladas Km Sur (28°17′37.5″S; 58°41′19.2″W), 4 specimens; San Alonso (28°17′6.3″S; 57°24′43.4″W), 4 specimens; Rincón de Ambrosio (28°15′05.2″S; 58°53′40.9″W), 2 specimens; Estancia San Luis (28°6′43.9″S; 58°51′48″W), 4 specimens; Colonia 3 de Abril (28°23′26.9″S; 58°53′37.5″W), 2 specimens; Goya (29°11′15.2″S; 59°12′33.1″W), 4 specimens; San Roque (28°41′S; 58°42′W), 3 specimens; Santa Rosa (28°10′45.6″S; 58°08′07″W), 3 specimens; Chavarría (28°58′S; 58°35′W), 5 specimens; Curuzú Laurel (27°55′24.4″S; 57°29′23.5″W), 2 specimens; Loma Alta (28°5′02.5″S; 58°19′29.9″W), 2 specimens; Loreto (27°44′43.7″S; 57°14′35.2″W), 2 specimens; Contreras Cué (28°01′35.4″S; 56°36′02.3″W), 1 specimen; Mbarigüí (27°33′S; 57°31′W), 1 specimen; and Paraje Angostura (27°33′S; 57°31′W), 1 specimen. Specimens' numbers were assigned at field.

DNA extraction, PCR amplification, sequencing

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). RPCS internal primers were designed considering conserved regions from

preliminary sequences obtained with those described in Ellingsen et al. (2007) for other *Ctenomys* species. Since the whole monomer cannot be obtained using only one set of internal primers, we designed two pairs of primers. Our main goal in PCR amplifications was to recover most RPCS variants, even minor ones. Hence, to reduce potential bias, we employed degenerated positions at the 3′ end of two of the primers and low annealing temperatures, as well as 2.5 mM $MgCl_2$. PCR were performed using the following parameters: one denaturation cycle at 94°C (5 min); 35 cycles at 94°C denaturation (1 min), 48°C annealing (1 min), 72°C extension (45 s); one final extension cycle at 72°C (5 min). In cases where PCR products were scarce, we performed 55 looping cycles, instead of 35. Primers were named U-175 (5′ CAACTTYGCMTATACTTTCTCTT3′), L-157 (5′CAAAAGAGATACAGGCACAWN3′), U-48 (5′ GASCACAAGMAATBMCMTCTTG3′) and L-346 (5′ GC TCCAGCTTCWTTGGGAAN3′). The two amplified fragments were named A (using primers U-175 and L-325) and C (using primers U-48 and L-346). The combination of primers U-175 and L-346 yielded a third amplicon, named B, but since its sequence did not recover extra variability, we decided to exclude it from the analysis.

Amplicons A (329 bp) and C (298 bp) are overlapped in 279 bp and cover the RPCS-348-bp entire monomer. PCR products showed discrete multimer ladders in agarose gels, corresponding to the amplicon length plus multiple integers of the 348-bp repeat unit. PCR products were purified with Purelink PCR Purification Kit (Invitrogen) and directly sequenced with the same primers used for PCR amplification. For each amplicon, both strands were sequenced, and the electropherogram on one strand was corroborated in the complementary one using Contig Express (Vector NTI 10.3.0. Invitrogen), obtaining the *amplicon sequence*. We call *intragenomic polymorphisms* (*ips*) to the occurrence of two (rarely three) peaks in the same position, as defined in Ellingsen et al. (2007). *Intragenomic polymorphisms* reveal the existence of monomers with different nucleotides for a given position, where the relative heights of the peaks that compose each *ip* are indicative of the relative amount of those variants in an individual genome. Minor peaks were considered only if their presence was corroborated in both strands. *Intragenomic polymorphisms* were pointed out using IUPAC nomenclature code. One *genomic consensus sequence* per individual was constructed aligning both *amplicon sequences* using Bioedit. The *gcs* represents the variants of RPCS monomers in one individual.

Dot blot analyses

Sample DNA concentration was measured using an UV spectrophotometer. For dot blot experiments, 20, 200 and 2,000 ng of genomic DNA were fixed to a nylon membrane using The Convertible Filtration Manifold System (GIBCO) and hybridized to ^{32}P-labeled RPCS probe (obtained from a mix of amplicons A and C of all Correntinean specimens and labeled by random priming). Afterward, the hybridized membranes were washed under high- (6X SSC, 60°C) and low-stringency (0.1X SSC, 60°C) conditions. Since we did not find differences between both conditions, we discarded bias in the RPCS probe. Autoradiographs were scanned, and hybridization signals were estimated using the Dot Blot Analyzer for ImageJ (Carpentier 2008). RPCS copy number was estimated loading two calibration curves with 6 points each, which ranged between a total of 9.18×10^7 and 3.06×10^{10} RPCS copies: one of the calibration curves contains unlabeled RPCS probe and the other one was made from genomic DNA of a specimen whose RPCS copy number assessed in a previous work (Slamovits et al. 2001). The resulting paired points of both calibration curves did not differ significantly in the copy number. Absolute copy number was estimated assuming the C-value for *Ctenomys* (Rossi et al. 1993b) and rodents in general is 7 pg (Ruedas et al. 1993). In order to gain more accuracy, the data were obtained from two independent dot blot experiments.

Genomic consensus sequences data treatment and analysis

Since there is no software available to compute genetic distances with DNA sequence characters other than A, C, G and T, we had considered major and minor variants as K, W, M, etc., we ought to consider only one of them. In most cases, an *ip* is composed of two variants. Almost all the *gcs* (except, specimen s199 at position 9, see Fig. 1) share the major variant of the *ip*s; thus, if we would had kept the major variants for all *ip*s, it would have resulted in an underestimation of overall variability of *gcs*. For this reason, for AMOVA and genetic distance calculations, we have decided to consider the second variant in abundance. Gene conversion was tested by AMOVA performed using Arlequin software (Excoffier et al. 2005), assuming only one genetic structure level (each population is independent and encloses only individuals, but not subgroups), running 20,000 permutations. We used Jukes–Cantor 1 parameter model for genetic distances calculations, since it does not assume different transformation costs. The input data—*genomic consensus sequences* where *ip*s were replaced by the second variants in abundance—emerge from the consensus of thousands of monomer sequences, where changes in mutational profiles do not occur by single nucleotide substitutions, but instead by replacement of whole monomers due to losses and gains of RPCS tandems. After recoding *gcs* by decomposing each position into four binary characters (A, C, G, T; presence/absence), we performed

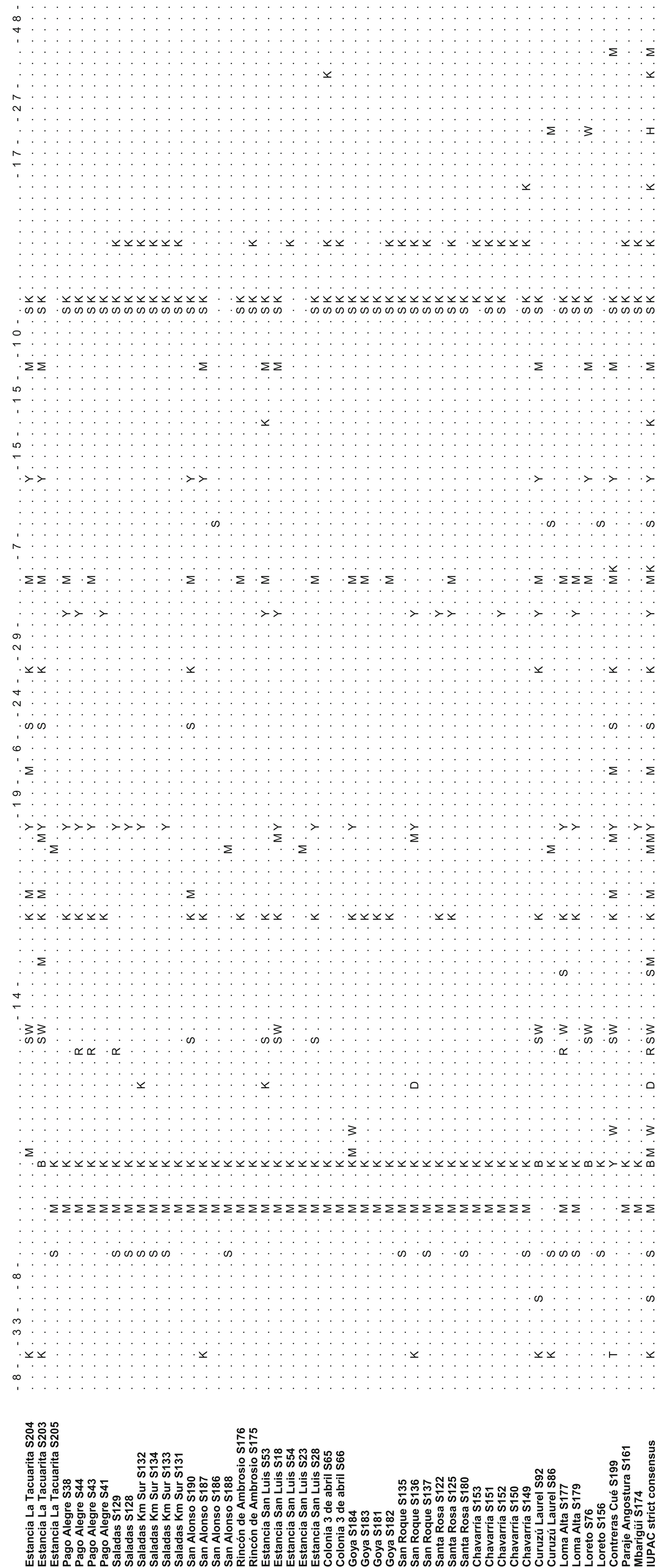

Fig. 1 RPCS genomic consensus sequences of 50 *Ctenomys* specimens from Corrientes. In the *first line*, the *numbers* indicate the length of invariable tracks, whereas *dots* indicate at least one variable position for any of the 50 *gcs*. In the *lines below,* one *gcs* per specimen is shown. For each *gcs*, *letters* represent *ip*s (expressed using IUPAC convention) and *dots* represent invariant sites

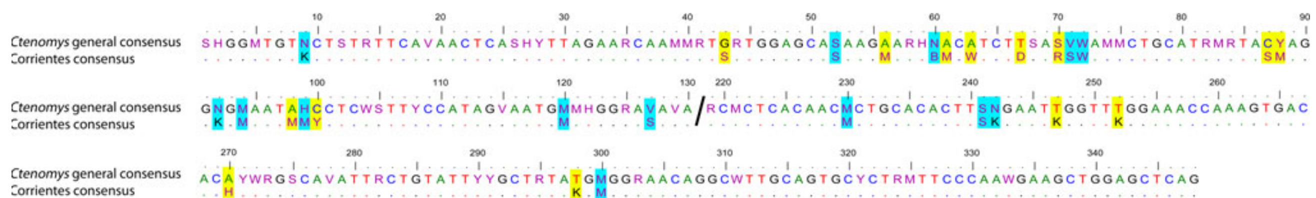

Fig. 2 Strict consensus of 50 Corrientinean *gcs* aligned with the strict consensus of 10 *gcs* from other *Ctenomys* lineages (Ellingsen et al. 2007). Corrientinean *gcs* consensus invariant sites are shown as *dots*, while intragenomic polymorphisms are showed following IUPAC codes. A 260-bp sequence is shown. The sequences at both sides of the oblique *bar* (/) are not contiguous, because *Ctenomys* general consensus does not include a central segment of 88 bp

phylogenetic analyses. Neighbor-joining and maximum parsimony trees were constructed with PAUP 4 (Swofford 2002). A total of 1,000 bootstrap replicas were performed and visualized with MEGA 3.1.

Results

RPCS sequence variability within and among *Ctenomys* populations from Corrientes

We obtained the whole 348-bp RPCS genomic consensus sequences for 50 specimens belonging to 18 *Ctenomys* populations from Corrientes. The strict consensus of the 50 *gcs* showed 35/348 (10.06%) variable positions, most of them (23/35 positions, 77.14%) shared by at least two individuals (Fig. 1). All the observed *ip*s overlap totally or partially at a given position. The 98.9% of *ips* are composed by 2 variants, while the remaining 1.1% corresponds to *ips* with 3 variants. As happened with other *Ctenomys* lineages, RPCS variability is not uniformly distributed along the monomer; some segments are identical in all specimens (Fig. 1), suggesting a potential constraint in the evolution of these regions (Ellingsen et al. 2007). Content in A + T (56%) of RPCS from Corrientinean tuco-tucos also coincides with that of other species of the genus (Ellingsen et al. 2007).

The strict consensus of 50 *gcs* from Corrientinean tuco-tucos was compared with the strict consensus of 10 *gcs* from other *Ctenomys* lineages (Ellingsen et al. 2007) (Fig. 2). All the *ips* from the Corrientes group partially or totally share variants, with the strict consensus of the species external to Corrientes, and none of Corrientes RPCS variable sites exhibit complete fixation, i.e. when two *gcs* posses different fixated variants, other *gcs* have *ips* with both variants (Fig. 1).

As it was discussed by Ellingsen et al. (2007), gene conversion might influence preferentially in the local establishment of new variants (arisen by de novo mutation), while differential expansions/contractions leaded by rolling-circle amplification or unequal cross-over may have a leading role in shaping overall RPCS profiles. The absence of fixated RPCS variants in the Corrientes populations (Fig. 1) suggests a relatively minor role for gene conversion. However, we further evaluated gene conversion by comparing RPCS variability within and among populations (see Materials and methods). When the distribution of the sources of RPCS variability was analyzed, 64.88% corresponded to within population variation and 35.12% to among populations variation, reflecting the weakness of the intrapopulation homogenization events and/or the breeding exchange among these populations.

RPCS copy number and *intragenomic polymorphisms*

As a way to get more insight into RPCS evolution and further testing the library model in these tuco-tucos populations, we analyzed the relationship between *ip*s and copy number. We reasoned that if an ancestral "library" of RPCS variants fluctuate in copy number via amplifications and deletions, it would result in decrease or increase in the number of *ip*s. The background of this reasoning resides in that the main mechanisms postulated for amplifications and/or deletions, rolling-circle and unequal crossing-over, involve contiguous and homogeneous tandems of monomers. If a particular RPCS variant is amplified over the others, resulting over-represented in the whole of the satellite arrays, the proportion of the remainders—particularly minor ones—would be expected to decrease. On the contrary, deletion of homogeneous tandem arrays would be followed by an increase in proportion of the minor variants. Therefore, RPCS copy number increases would be mirrored by lower *ips* values than RPCS copy number decreases.

Measurement of RPCS copy number in the tuco-tucos from Corrientes showed a range from 118 – 2,700 $\times 10^3$ copies per haploid genome (Table 1), which represents intermediate values for *Ctenomys* (Slamovits et al. 2001). Taking into account that RPCS probably constitutes the bulk of the C-heterochromatin present in Corrientes tuco-tucos karyotypes, as happened with other *Ctenomys* groups (Rossi et al. 1995), these values are in agreement with intermediate to low C-heterochromatin amounts found in

Table 1 RPCS copy number × 10^3 and number of *ips* for 46 *Ctenomys* specimens from 15 Corrientinean populations

Locality	Dot Blot	RPCS copy # (*10^3)	IPs
Estancia La Tacuarita S204		136	15
Estancia La Tacuarita S203		152	16
Estancia La Tacuarita S205		1298	4
Pago Alegre S38		267	8
Pago Alegre S44		364	8
Pago Alegre S43		455	8
Pago Alegre S41		644	6
Saladas S129		370	8
Saladas S128		430	7
Saladas Km Sur S132		367	8
Saladas Km Sur S134		398	6
Saladas Km Sur S133		458	7
Saladas Km Sur S131		714	5
San Alonso S190		214	11
San Alonso S187		399	8
San Alonso S186		1146	3
San Alonso S188		1240	4
Rincón de Ambrosio S176		381	6
Rincón de Ambrosio S175		910	5
Estancia San Luis S53		222	11
Estancia San Luis S18		300	11
Estancia San Luis S54		722	3
Estancia San Luis S23		778	3
Colonia 3 de abril S65		360	6
Colonia 3 de abril S66		682	5
Goya S184		1532	9
Goya S183		1669	6
Goya S181		2032	5
Goya S182		2700	7
San Roque S135		405	6
San Roque S136		700	10
San Roque S137		826	6
Santa Rosa S122		682	6
Santa Rosa S125		696	8
Santa Rosa S180		1327	5
Curuzú Laurel S92		118	13
Curuzú Laurel S86		1745	6
Loma Alta S177		196	11
Loma Alta S179		441	9
Loreto S76		118	9
Loreto S156		505	3
Chavarría S153		354	4
Chavarría S151		359	5
Chavarría S152		688	6
Chavarría S150		787	3
Chavarría S149		919	7
Mbarigüí S174		0	6
Paraje Angostura S161		0	5
Estancia San Luis S28		0	8
Contreras Cué S199		0	18

From left to right the three dots correspond to dot blot experiments where 2,000, 200 and 20 ng genomic DNA per specimen were hybridized with the RPCS probe

this group (Reig et al. 1992; García et al. 2000). The result that first attracted our attention was the wide range of RPCS copy number variation found among individuals from the same population (Table 1). Intrapopulation RPCS copy number differences are not so conspicuous as we found among species (3-orders of magnitude) (Slamovits et al. 2001), but reach one order of magnitude (Table 1). The individuals from Curuzú Laurel differenced in 14-folds in RPCS copy number, while Estancia La Tacuarita population differentiated 9.5-folds. Loreto and San Alonso populations ranged fourfolds, while Estancia San Luis and Chavarría populations ranged threefolds. The remaining specimens of the Corrientes populations were differentiated about twofolds. Taking into account the close relatedness of the individuals within a population, RPCS copy number differences found among individuals suggest that amplifications and deletions are ongoing processes in these genomes.

When we analyzed RPCS *ip*s values within populations, we stated that individuals with lower RPCS copy number have higher *ip*s values than those with higher RPCS copy number (Table 1). This relation occurred in all populations with the exception of San Roque and Chavarría, where this relation seemed to be erratic.

Discussion

Since proposed by Fry and Salser in 1977 (Fry and Salser 1977), the library hypothesis has been scarcely tested from an experimental point of view. In nematodes (Meštrović et al. 2006), as well as in beetles (Meštrović et al. 1998; Bruvo-Madaric et al. 2007), the same variants of a satellite DNA were amplified in different proportions in closely related species. These findings suggest that these variants were already present in the common hypothetical ancestor of the group. In an analysis that comprised 10 phylogenetically differentiated *Ctenomys* species, the ancestral library hypothesis had been supported for RPCS satellite DNA by the absence of complete fixation of any nucleotide variant, in any variable position of the *genomic consensus sequence* (Ellingsen et al. 2007).

Although the evolution of RPCS has been explored from several points of view (Rossi et al. 1990, 1995; Slamovits et al. 2001; Ellingsen et al. 2007), the *Ctenomys* Corrientes group has not been included in these studies. In the present work, we focused the study of RPCS evolution at inter and intrapopulation levels, taking as a case of study 18 populations belonging to a particularly interesting group of *Ctenomys* species. As mentioned earlier, the tuco-tucos from Corrientes province constitute an extreme case of karyotypic evolution (mainly fusions and fissions and variability in heterochromatin amount and location) (Ortells et al. 1990; Reig et al. 1992; Ortells and Barrantes 1994; García et al. 2000; Argüelles et al. 2001; Lanzone et al. 2002), probably facilitated by the temporary and spatial isolation/reconnection of populations.

Taking into account that the lineages belonging to the *Ctenomys* Corrientes group probably have diverged in not very different times, this group constitutes an interesting case of study in order to test the "library" model, since there could be found "fresh" fingerprints of the RPCS expansion and contraction processes. RPCS contractions and expansions have been involved in chromosomal evolution in tuco-tucos (Slamovits et al. 2001). In the current work, we measured RPCS copy number as well as estimated sequence variability in this group. Cloning of random genomic monomers has been a usual approach for studying satellite DNA sequences (Pons and Gillespie 2004; Luchetti et al. 2006; Matsubara et al. 2008). As argued elsewhere, this strategy has a weak point that resides in the uncertainty of assessing the number of clones needed to be sequenced to acquire a reliable estimation of total satellite DNA variability (Ellingsen et al. 2007). Regarding that satellite DNAs are composed by hundreds or millons of tandemly arranged copies per genome, the analysis of the nucleotide variability could require an adequate strategy. Direct sequencing of PCR amplicons of a satellite DNA privileges the recovery of the overall nucleotide variability over the distribution of this variability among individual monomers. We think that this approach is suitable for the evaluation of the "library" model. Over the past few years, some research groups assayed PCR amplifications, in order to estimate the overall variability of a satellite DNA (Picariello et al. 2002; Ellingsen et al. 2007). The PCR-based approaches require avoiding biases in the estimation of variability due to primer specificity. Aiming the minimization of primer bias, we employed two sets of primers with degenerated 3′ positions and relaxed annealing conditions in PCR (see Materials and Methods). In spite of these precautions, we cannot rule out that quite minor variants might remain hidden in the overall genomic consensus sequences. However, this seems not to be the case, since no differences were found between high- and low-stringency conditions in dot blot hybridizations.

One of the first findings that deserved our attention in dot blot experiments was the difference in RPCS copy number (up to 1 order in magnitude) among individuals from the same population (Table 1). The differences in copy number of a satellite DNA among interbreeding individuals of the same population or among individuals from different populations of a species have not been explored before. However, the differences in amount and distribution of C-heterochromatic bands found among individuals from three populations of *Ctenomys talarum*

talarum can be considered as a precedent (Reig et al. 1992). Our results indicate that at least moderated differences in RPCS copy number among individuals with the same karyotype do not constitute a reproductive barrier. On the other hand, although RPCS is active within these genomes, this activity does not imply high values of sequence divergence, which rises up to 6.4% in *gcs* pairwise comparison. Apparently, the amplification/deletion processes are not linked to the extent of global homogenization. Intragenomic polymorphisms are frequently shared among individuals from different populations (but not among all individuals from the same population). This could be due to a recent population diversification and/or weak sequence homogenization.

For most positions, the *ip*s are composed of two variants, the major one being the invariant nucleotide across other *gcs*. Minor variants are frequently shared by members of different populations, suggesting that they belong to a common ancestral stock. In the lack of a resolute phylogeny of this group, we cannot assess the direction of changes. If the change was from an *ip* toward an invariant state, it seems more probable that major variants had been amplified at expense of minor ones. If, on the contrary, an *ip* emerges from an invariant state, the frequent presence of shared variants (even among populations) suggests that this variability had been present in the background of the group. In addition, no positions show totally fixated variants and all the *ip*s partially or totally superpose within the *Ctenomys* Corrientes group. Indeed, the strict *gcs* consensus of the *Ctenomys* Corrientes group also overlaps with the strict consensus of the 10 *Ctenomys* species, previously studied (Ellingsen et al. 2007). These results indicate that the observed RPCS nucleotide variation had already been present in the common ancestor of the Corrientes group, and part of it in the common ancestor of the genus. These results are in complete agreement with the "library" hypothesis. Indeed, although satellite DNA, composed by hundreds or millions of copies per genome, is not a priori a trustable phylogenetic marker because it violates the principle of homology, we have tried some phylogenetic analyses in order to study how *gcs* from different populations were grouped. The obtained phylogenies were poorly supported (not shown), as is expectable regarding the existence of ancestral variants and the observed high intrapopulation variability.

Although in the frame of the "library" model, variation in copy number and nucleotide sequence of a satellite DNA have been claimed to be interdependent parameters, they rarely have been analyzed as a whole. In our case of study, we found an inverse relation between RPCS copy number and nucleotide variability in almost all the studied populations. San Roque and Chavarría populations are the only two exceptions. We cannot certainly assert the reasons that account for RPCS profiles in these cases, but probably they reside in the population level. Chavarría and San Roque are apparently isolated populations, but are located within a poorly studied area (see Materials and Methods for geographic location) and perhaps are reproductively connected with up-to-now undiscovered populations. The observed correlation between RPCS copy number and sequence variability is in agreement with the "library" hypothesis: the stock of RPCS variants of the population, by means of recombinational processes, are amplified, contracted or even deleted. When amplification occurs, an array of monomer variants—which could be under local gene conversion—expands at the expense of other variants, causing a reduction in overall variability. In an analogous manner, when a significant copy number reduction occurs—as it might involve a large tandem of homogeneous repeats—the relative amount of minor variants increases in the overall profile, increasing the *ip*s. It is worth noting that the *ip*s, which turn out "visible" in the *gcs* as a result of RPCS copy number reduction, are the same in different populations, suggesting, again, that this variability is shared by common ancestry.

The "library" hypothesis is a comprehensive framework to study and interpret satellite DNA evolution, as it incorporates the expected results of the main postulated turnover mechanisms for these sequences. However, this hypothesis lacks of experimental detailed validation. The current contribution brings an original methodological approach at the previously unexplored population level that results in a clear validation of the "library" hypothesis.

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