UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

O papel dos telômeros e da metilação global do DNA na evolução cromossômica e na conservação em *Ctenomys* (Rodentia-Ctenomyidae)

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“The only thing that you absolutely have to know, is the location of the library.”

Albert Einstein

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RESUMO

Os telômeros tem sido considerados os novos biomarcadores em trabalhos evolutivos, principalmente através do sinal de sequencias teloméricas intersticiais (ITSs), e de mutagenicidade, através da medição do comprimento telomérico (TL). Neste trabalho utilizamos os experimentos de hibridização *in situ* fluorescente (FISH) em conjunto com a medição do comprimento telomérico para o estudo de evolução cariotípica em uma espécie de *Ctenomys*. O gênero *Ctenomys* tem sido bastante utilizado em estudos de evolução cariotípica devido a uma alta frequência de rearranjos cromossômicos principalmente na espécie *Ctenomys minutus*, considerada modelo para a investigação de mecanismos de instabilidade genômica que se originaram e continuam a originar diferentes cariótipos nessa espécie. Outra abordagem realizada neste trabalho foi a quantificação da metilação global do DNA (5-mdC), utilizada, também, em estudos evolutivos e de mutagênese. A metilação é um dos mecanismos epigenéticos que atuam na regulação da expressão gênica. Neste trabalho podemos perceber como a metilação global do DNA atua em relação ao comprimento telomérico no caso da análise evolutiva dos cariótipos de *C. minutus*, e no caso da análise de mutagênese realizada na espécie *Ctenomys torquatus*.

C. torquatus, tem uma parte da sua distribuição geográfica coincidente com regiões de mineração e exploração do carvão. Assim, foi despertado o interesse em conhecer os efeitos de uma possível exposição ao carvão no DNA nesta espécie. Para isso ambas metodologias mencionadas acima foram aplicadas neste estudo de mutagênese.

Esta tese está dividida em três capítulos principais, apresentados na forma que segue. No primeiro capítulo, é realizado uma pequena revisão de estudos sobre os efeitos de poluentes no DNA em mamíferos subterrâneos, com ênfase no gênero *Ctenomys*. Além disso, apresentaremos possíveis biomarcadores moleculares para futuros trabalhos. Na área de genética da conservação, essas análises realizadas em *Ctenomys* representam uma nova abordagem para avaliar o efeito antropogênico em populações naturais de mamíferos. Esses estudos são importantes devido à ênfase na viabilidade de ensaios genotóxicos e ferramentas epigenéticas em estudos de conservação.

O segundo capítulo discorre sobre as análises de danos ao DNA na espécie *C. torquatus* onde sua distribuição geográfica coincide com regiões de mineração e a Usina Termelétrica de Candiota, no Rio Grande do Sul (RS), que é uma das maiores do Brasil. O carvão é um combustível fóssil que causa impactos ambientais desde sua extração até a combustão devido à liberação de diferentes agentes, tais como hidrocarbonetos aromáticos policíclicos (HPA) e metais pesados. Através do comprimento telomérico, da quantificação da metilação global do DNA e o ensaio genotóxico conseguimos avaliar o efeito da exposição ao carvão no DNA e na estrutura populacional de *C. torquatus*.

E por fim, no terceiro capítulo, a evolução dos cariótipos de *C. minutus* é discutida com o auxílio dos dados obtidos a partir do comprimento telomérico, da quantidade de metilação global do DNA em cada cariótipo e do experimento de FISH realizados nos cariótipos parentais da espécie. Neste trabalho, pela primeira vez, devido à escassez de estudos com técnicas de citogenética molecular para explicar essas diferentes formas cariotípicas que ocorrem nesta espécie, FISH, comprimento telomérico e metilação global do DNA foram analisados em conjunto para melhorar o entendimento da evolução desses cariótipos em toda a sua distribuição geográfica.

ABSTRACT

Telomeres have been considered the new biomarkers in both evolutionary studies, mainly through the signal of interstitial telomeric sequences (ITSs) and mutagenicity, through the measurement of the telomeric length (TL). In this work, we use fluorescent *in situ* hybridization (FISH) experiments with the measurement of telomeric length for a study of karyotype evolution in a species of *Ctenomys*. The genus *Ctenomys* has been widely used in karyotype evolution studies due to a high frequency of chromosomal rearrangements, mainly in the species *Ctenomys minutus*, considered a study model for the investigation of genomic instability mechanisms that originated and continue to originate different karyotypes in this species. Another approach carried out in this work was the quantification of global DNA methylation (5-mdC), also used in evolutionary and mutagenesis studies. Methylation is one of the epigenetic mechanisms that act in the regulation of gene expression. In this work, we can see how the global DNA methylation acts with to the telomeric length in the case of the evolutionary analysis of the karyotypes of *C. minutus*. In the case of the mutagenesis analysis performed in the species *Ctenomys torquatus*.

C. torquatus has part of its geographic distribution coinciding with mining and coal mining regions. Thus, the interest in knowing the effects of possible exposure to coal in DNA in this species was aroused. For that, both methodologies mentioned above were applied in this mutagenesis study.

This thesis is divided into three main chapters where the studies are presented as follows. In the first chapter, a small review of studies on the effects of pollutants on DNA in subterranean mammals is carried out, with an emphasis on the genus *Ctenomys*. This chapter will present some studies on environmental mutagenesis that have been carried out with species of *Ctenomys* in South America. In addition, we will present possible molecular biomarkers for future studies. In the area of conservation genetics, these analyzes carried out in *Ctenomys* represent a new approach to assess the anthropogenic effect in natural mammal populations. These studies are important due to the emphasis on the feasibility of genotoxic assays and epigenetic tools in conservation studies.

The second chapter discusses the analysis of DNA damage in the species *C. torquatus* where its geographic distribution coincides with mining regions and the Candiota Thermoelectric Plant, in Rio Grande do Sul (RS), which is one of the largest in Brazil. Coal is a fossil fuel that causes environmental impacts from its extraction to combustion due to the release of different agents, such as polycyclic aromatic hydrocarbons (PAH) and heavy metals. Through the telomeric length, the quantification of the global DNA methylation and the genotoxic assay we were able to evaluate the effect of the exposure to coal in the DNA and in the population structure of *C. torquatus*.

Finally, in the third chapter, the evolution of *C. minutus* karyotypes is discussed with the help of data obtained from the telomeric length, the amount of global DNA methylation for each karyotype and the FISH experiment carried out on parental karyotypes of the species. In this study, for the first time, due to the lack of studies with molecular cytogenetic techniques to explain these different karyotypic forms that occur in this species, FISH, telomeric length and global DNA methylation were analyzed together to improve the understanding of the evolution of these karyotypes throughout their geographic distribution.

INTRODUÇÃO

Telômeros, o que são?

Telômeros são estruturas nucleoproteícas que protegem as extremidades dos cromossomos da degradação, por ação de nucleases e da fusão com outros cromossomos, mantendo assim, a integridade do genoma (Blackburn 2001). Durante a divisão celular, o DNA também é replicado e, nesse processo, o DNA telomérico termina em uma saliência de fita simples na extremidade 3', por conta da remoção do *primer* de RNA durante esse processo, a fim de não comprometer a integridade do genoma. Sem um mecanismo de replicação especial, as extremidades 3' das fitas recém-replicadas diminuem a cada replicação do DNA. Desse modo, a cada ciclo celular, os telômeros encurtam progressivamente, desencadeando a senescência celular (Zakian 2012; Armanios and Blackburn 2012).

Em mamíferos e outros vertebrados, a sequência de DNA telomérico são repetições em tandem, não codificantes, de um hexâmero (5'-TTAGGG-3')$_n$. São associadas à um complexo proteico chamado *shelterin*, que permite as células distinguirem a extremidade natural dos cromossomos das quebras de DNA (danos), além de ter função na regulação do comprimento e na proteção dos telômeros. O complexo *shelterin* inclui 6 proteínas: TRF1, TRF2, RAP1, TIN2, TPP1 e POT1 (POT1a e POT1b em camundongos) (Schmutz e De Lange 2016). Ele se liga aos telômeros através das TRF1 e TRF2, que interagem com o DNA telomérico de fita dupla, enquanto as proteínas POT1 se associam ao DNA telomérico de fita única. A POT1 está ligada ao TRF1 e TRF2 por meio de uma interação entre a proteína de ligação ao POT1, TPP1 e TIN2, que se liga ao TRF1 e ao TRF2. A sexta subunidade, Rap1, interage exclusivamente com o TRF2 (FIGURA 1). Resumidamente, a subunidade TRF2 reprime a via de sinalização de danos ao DNA bem como o reparo do DNA telomérico, a POT1 protege o DNA telomérico de fita simples da ação de proteínas ativadoras do reparo fortemente ligado pelo TPP1 e TIN2, e o TRF1 tem um papel especializado na promoção da replicação semiconservativa de telômeros (Xin et al. 2008).

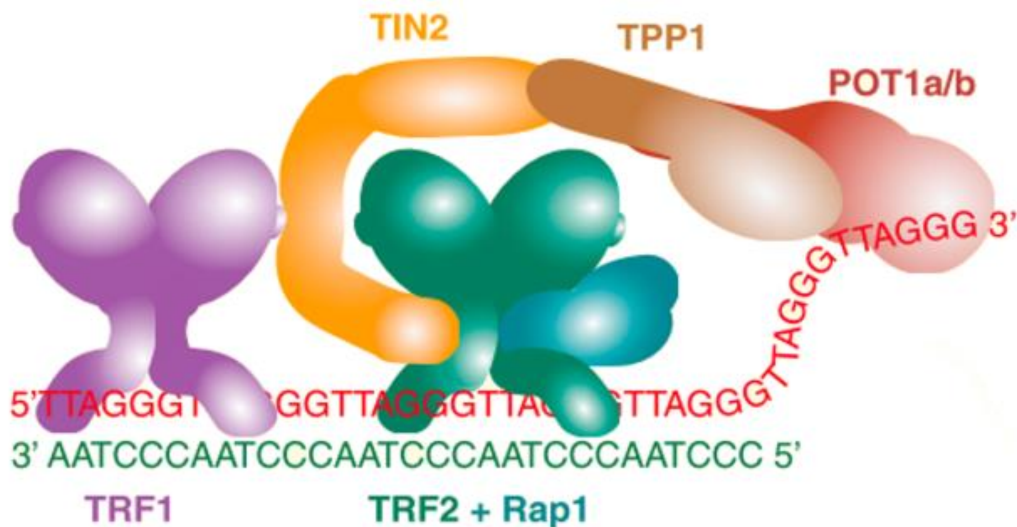

Figura 1. O complexo *shelterin* garante a integridade do genoma, protegendo os telômeros da sinalização de danos e reparo do DNA, promovendo a replicação semiconservativa do DNA telomérico e regulando a manutenção dos telômeros mediada pela telomerase. Modificado de Schmutz e De Lange 2016.

O funcionamento adequado dos telômeros requer um comprimento mínimo de repetições de TTAGGG e a integridade do complexo *shelterin*. O comprimento do telômero (TL) é mantido pela telomerase, uma transcriptase reversa que adiciona repetições teloméricas *de novo* após cada divisão celular. A telomerase é a DNA polimerase especializada que sintetiza novas sequências de telômeros nas extremidades dos cromossomos. Possui dois componentes conservados que desempenham a função de adição repetida de telômeros: a proteína TERT, que contém o domínio da transcriptase reversa da telomerase, e um componente essencial de RNA (TERC), que junta-se com a TERT e fornece o molde para a síntese da sequência telomérica (Armanios e Blackburn 2012). Neutralizando, assim, o problema de replicação nas extremidades dos cromossomos nos tipos de células em que a enzima é expressada, como linhagens celulares imortais, células germinativas, células-tronco, linfócitos ativados e na maioria das células tumorais analisadas até o momento (Blasco 2007).

O alongamento dos telômeros também pode ocorrer na ausência de telomerase através do mecanismo chamado alongamento alternativo dos telômeros (ALT), que envolve recombinação homóloga entre os telômeros e foi

descrita em várias células tumorais e linhagens celulares imortalizadas (Blasco 2007; Shay e Wright 2019).

O encurtamento das sequências teloméricas de DNA às vezes está associado a um aumento nas aberrações numéricas dos cromossomos (FIGURA 2) (Tamayo et al. 2011; Bolzán 2012; Sakellariou et al. 2013). Além disso, o encurtamento de telômeros é acelerado por espécies reativas de oxigênio e radiação ultravioleta, consideradas as principais causas ambientais do encurtamento telomérico (Tchirkov e Lansdorp 2003; Kepinska et al. 2015; Boonekamp et al. 2017).

Em algumas espécies de vertebrados, as repetições de TTAGGG estão presentes não apenas nas regiões terminais dos cromossomos, mas também nos locais intersticiais de alguns cromossomos. Essas sequências são chamadas sequências teloméricas intersticiais (ITSs) e incluem as repetições localizadas próximas aos centrômeros, ou nos centrômeros, e aquelas encontradas entre o centrômero e o telômero (Bolzán 2012).

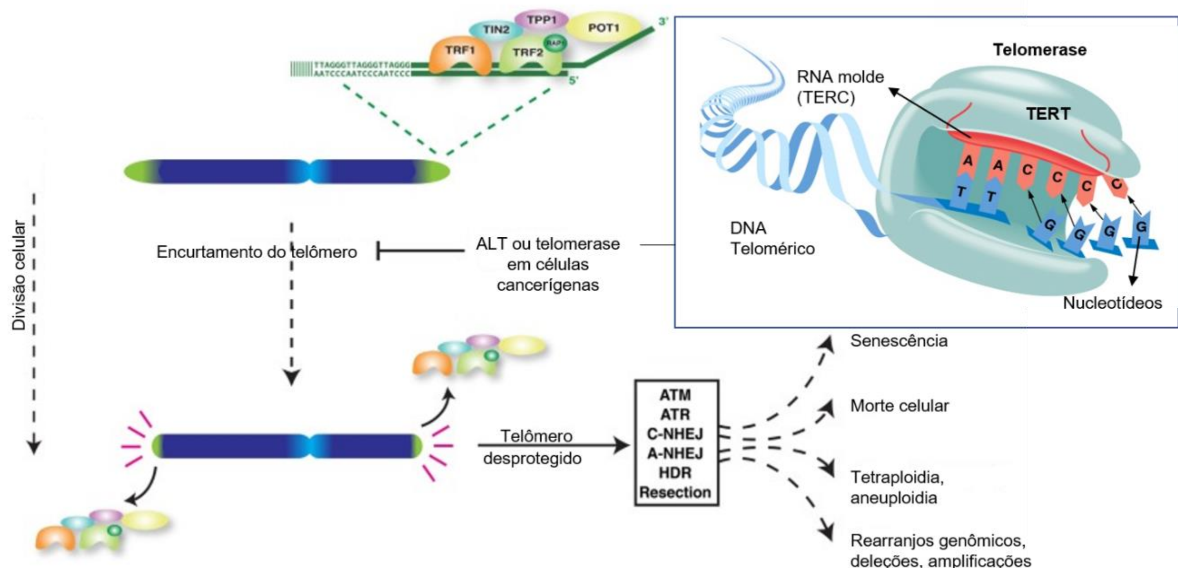

Figura 2. O comprimento do telômero é mantido, a cada divisão celular, pela telomerase, formada pela proteína TERT e um componente essencial de RNA (TERC). Na ausência de telomerase, o alongamento dos telômeros ocorre através do alongamento alternativo dos telômeros (ALT). O encurtamento telomérico progressivo apresenta consequências como senescência ou morte celular. Modificado de Jacobs 2013.

Comprimento telomérico na conservação e em estudos evolutivos

Nas células somáticas, o mecanismo alternativo não baseado na telomerase pode ser ativado para manter a integridade do TL quando ele se torna criticamente curto devido a algum tipo de estresse, ou exposições ambientais e ocupacionais. O DNA telomérico é dinâmico e o TL é considerado o resultado de um equilíbrio entre os processos de redução e manutenção dos telômeros (Shay e Wright 2019).

O TL tem sido medido, como uma forma de biomarcador, para avaliar efeitos no genoma quando o animal passa por eventos de estresse, como por exemplo, a competição por alimento. Os indivíduos dentro de uma população passam por momentos de estresse considerado natural, este estresse ambiental é sentido pelas células através do estresse oxidativo, uma das vias para o encurtamento do TL (Kepinska et al. 2015; Boonekamp et al. 2017). McLennan et al. (2016) em um estudo com salmão (*Salmo salar*), descobriu que os peixes juvenis tinham o TL mais curto quando o crescimento corporal era mais rápido e em condições ambientais mais severas. Um estudo de Stier et al. (2015), também encontrou resultado semelhante em aves chapim-real (*Parus major*). Essas aves são altriciais e a assincronia de eclosão cria uma hierarquia competitiva entre irmãos dentro da ninhada, com filhotes da primeira eclosão desfrutando de vantagens em comparação aos filhotes da última eclosão. Os pesquisadores compararam, então, processos de crescimento e de automanutenção em relação ao TL, esperando haver um estresse pela competição, já que a disponibilidade e a alocação de recursos provavelmente diferem entre filhotes de diferentes eclodições. Descobriram que apesar da desvantagem competitiva inicial, os filhotes nascidos por último exibiram taxa de crescimento semelhantes aos que nasceram primeiro. No entanto, os filhotes recém-nascidos sofreram mais em termos de estresse oxidativo e terminaram o crescimento com telômeros mais curtos do que os nascidos primeiro. Entre os filhotes nascidos por último, aqueles que exibiram um crescimento mais rápido do tamanho do corpo também foram os que exibiram a maior erosão dos telômeros.

Vários estudos também vincularam a exposição a produtos químicos ambientais, com riscos aumentados para várias doenças crônicas e ocupacionais, como câncer e doenças cardiovasculares (Sharifi-Sanjani et al. 2017; de Souza et

al. 2018). Também foi demonstrado que mutações genéticas que alteram a sequência de DNA e fatores epigenéticos, que não envolvem alterações na sequência de DNA, estão envolvidas em doenças relacionadas a produtos químicos ambientais (Pavanello et al. 2010; Dioni et al. 2011). O estresse oxidativo e a inflamação, as duas principais vias para essas doenças, também são fatores de risco para encurtamento de TL (Tchirkov e Lansdorp 2003; Coluzzi et al. 2017). Portanto, o TL pode servir como um indicador de exposição a produtos químicos ambientais e ocupacionais, e o encurtamento do TL pode ser um fator adicional que vincula esses produtos químicos a doenças relacionadas.

Com relação a vida selvagem, poucos estudos relacionam exposição ambiental e contaminantes, como organo-halogenados encontrados em pesticidas, em águias (*Haliaeetus albicilla*) (Sletten et al. 2016) e gaivotas (*Rissa tridactyla*) (Blévin et al. 2017) e metais pesados, em chapim-real (*Parus major*) (Stauffer et al. 2017), e o comprimento telomérico. Mas menos ainda se sabe sobre essa relação em mamíferos subterrâneos.

Além da região terminal dos cromossomos, as repetições teloméricas podem ser observadas em locais intracromossomais, como já mencionado acima, as sequências teloméricas intersticiais (ITSs). De acordo com a organização dessas sequencias e localização, os ITSs podem ser classificados, para fins evolutivos, em dois tipos: ITS heterocromático (het-ITS) e ITS curto (s-ITS). Os het-ITS são blocos de repetições teloméricas dentro ou na margem da heterocromatina, observados principalmente na região pericentromérica ou centromérica do cromossomo. Os s-ITS são pequenas repetições em tandem distribuídas em posições internas nos cromossomos (Ruiz-Herrera et al. 2009).

Os het-ITS são remanescentes de rearranjos cromossômicos como inversões, translocações, fusões e fissões, que contribuem para reorganizar a arquitetura do genoma, fornecendo novas formas cromossômicas em que a seleção natural pode atuar, fazendo, então, parte da evolução do cariótipo. Dentre os rearranjos cromossômicos, a fusão Robertsoniana (Rb) é considerada a mais importante por sua ocorrência em táxons diversos como mamíferos, répteis, insetos ou moluscos. Fusões Rb, juntamente com inversões, contribuem para a especiação cromossômica por sub dominância e / ou pela supressão da recombinação quando

presentes na forma heterozigótica (Nergadze et al. 2007; Ruiz-Herrera et al. 2009; Sánchez-Guillén et al. 2015). Quando, na fusão Rb, não há perda de telômeros originais, a fusão resultante no novo cromossomo apresenta o he-ITS na região pericentromérica (FIGURA 3). Essas regiões apresentam instabilidade por frequentemente ocorrerem fissões e inversões. Assim, o he-ITS pode representar o ponto de fissão onde um novo telômero surgiria a partir das repetições teloméricas pré-existentes, devido a presença de uma micro-homologia para o hexâmero telomérico 3', que é a criação de um novo telômero no local de quebra pela telomerase. Através do mecanismo de deslocamento e conversão da DNA polimerase, é formado também o novo centrômero no cromossomo derivado da fusão Rb. A plasticidade do he-ITS pode resultar em sua redução progressiva e eventual perda por causa da reorganização cromossômica, em menor escala, e mutações pontuais que causariam a degeneração progressiva da sequência (Ruiz-Herrera et al. 2009).

Alguns s-ITS fazem parte das repetições das regiões subteloméricas e são compostos por sequencias em tandem "da cabeça à cauda" (*head-to-tail*) contendo muitas unidades degeneradas, que provavelmente surgiram de eventos de recombinação envolvendo extremidades cromossômicas. As inserções de s-ITS no genoma se dão, principalmente, durante o reparo de quebras duplas do DNA por recombinação não homóloga (NHEJ, em inglês). Essas inserções podem ocorrer de duas maneiras, ou através da telomerase, que utiliza a "ponta solta" 3´da quebra como *primer* para sintetizar a fita rica em G (guanina) de uma repetição telomérica e a DNA polimerase sintetiza a outra fita, restaurando a dupla hélice pela DNA ligase; ou um fragmento pré existente do hexâmero é transposto no lugar da quebra. Esse fragmento pode vir da excisão de uma porção do telômero verdadeiro ou da retrotranscrição de uma repetição telomérica contendo RNA (TERRA) (Ruiz-Herrera et al. 2009).

Os ITSs são visualizados com mais frequência usando a hibridização fluorescente *in situ* (FISH). As técnicas de FISH para visualizar esses ITSs subteloméricos e pericentroméricos requerem células metafásicas, que são difíceis de usar em amostras coletadas de animais selvagens. Experimentos que utilizaram FISH com a sequência $(TTAGGG)_n$ de telômeros, realizados em diversas espécies

de vertebrados, mostraram a presença dessa sequência em posições teloméricas e não-teloméricas (ITS). Por essa sequência ser extremamente conservada entre diferentes grupos, é considerada uma sonda importante para estudos de evolução cromossômica (Meyne et al. 1990).

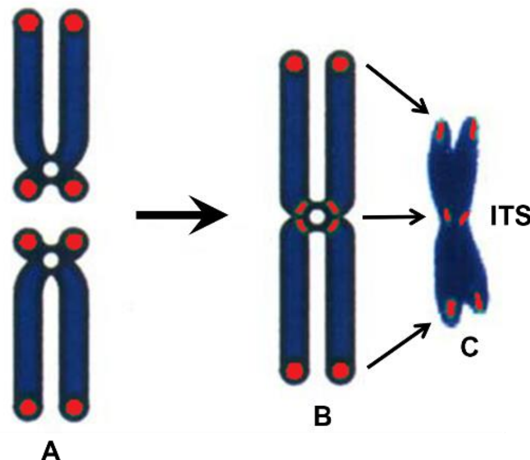

Figura 3. Sequências teloméricas intersticiais (ITS) como evidência de rearranjos Robertsonianos. Dois cromossomos acrocêntricos (A) sofrem fusão originando um cromossomo metacêntrico contendo sinais ITS (B, C), sem perda de sequências teloméricas (em vermelho) O cromossomo (C) é a representação de uma metáfase após hibridização fluorescente *in situ* com uma sonda telomérica. Modificado de Pisano e Ozouf-Costaz 2000.

Metilação do DNA

A metilação do DNA é um tipo de modificação química do DNA que pode ser herdada e subsequentemente removida, sem alterar a sequência original da molécula. É também o mecanismo epigenético mais bem caracterizado (Marsit 2015). Epigenética se refere a mudanças hereditárias na expressão gênica sem a mutação na sequência do DNA. Existem três mecanismos moleculares de regulação da expressão gênica mediada pela epigenética (Cheng et al. 2012). Dois desses mecanismos serão abordados de maneira simples e rapidamente a seguir, e o terceiro mecanismo, sendo parte deste estudo e ter relação com tamanho telomérico, será abordado em maior detalhamento. São os mecanismos: modificações em histonas, expressão do RNA não codificante e metilação do DNA.

Histonas são proteínas nucleares que empacotam o DNA em um nucleossomo, que é a unidade da estrutura da cromatina. A região caudal N-

terminal das histonas estão sujeitas a uma variedade de modificações pós traducionais como acetilação, metilação, fosforilação entre outros (FIGURA 4) (Muñoz-Najar e Sedivy 2011). As modificações nas histonas podem agir sequencialmente ou em combinação para modificar eventos como ativação ou repressão da transcrição. Isto é, o código da histona com essas modificações modula o resultado da transcrição (Bommarito e Fry 2019).

Outro modulador epigenético é o RNA não codificante (ncRNA), que pode ser longo ou curto (de 21 a 25 nucleotídeos) (Muñoz-Najar e Sedivy 2011). O ncRNA curto são o micro RNA (miRNA) e o RNA de interferência curto (siRNA). A regulação gênica mediada pelo ncRNA curto tem dois mecanismos, a repressão da tradução (através do miRNA) e a degradação do mRNA (através do siRNA) (Muñoz-Najar e Sedivy 2011).

O papel da metilação do DNA é crucial para o desenvolvimento normal, proliferação e estabilidade do genoma (Luzhna et al. 2013). A metilação do DNA envolve a adição de um grupo metil (-CH3) no carbono posição 5 da citosina (C), para formar a base 5-metilcitosina (5-mC), em um dinucleotídeo citosina-guanina (CpG). A metilação é catalisada pelas enzimas dimetiltransferses DNMT3a e DNMT3b, e o doador do grupo metil é S-adenosilmetionina (SAM) (Marsit 2015). Uma vez que a metilação está estabelecida, ela deve ser mantida durante a divisão celular. Essa manutenção é realizada pela DNMT1, garantindo, assim, o padrão de metilação do DNA a permanência desse padrão nas gerações celulares seguintes (Cheng et al. 2012).

Uma das maneiras de alterar a expressão genética é quando a metilação do DNA ocorre dentro de promotores do gene, impedindo a maquinaria da transcrição acessar o DNA e iniciar a transcrição. Embora nem todos os fatores de transcrição sejam sensíveis a metilação do DNA ocorrendo dentro dos sítios de ligação (Bommarito e Fry 2019). Outra maneira é através das proteínas de ligação de metil CpG. Essas proteínas se ligam ao DNA metilado e medeiam interações com modificações da histona, que resulta em uma mudança na conformação da cromatina, tornando-a mais condensada e, assim, repressiva. Essa ligação entre a metilação do DNA e modificações epigenéticas garantem a manutenção estável

das mudanças dependentes de metilação através das replicações celulares (Cheng et al. 2012).

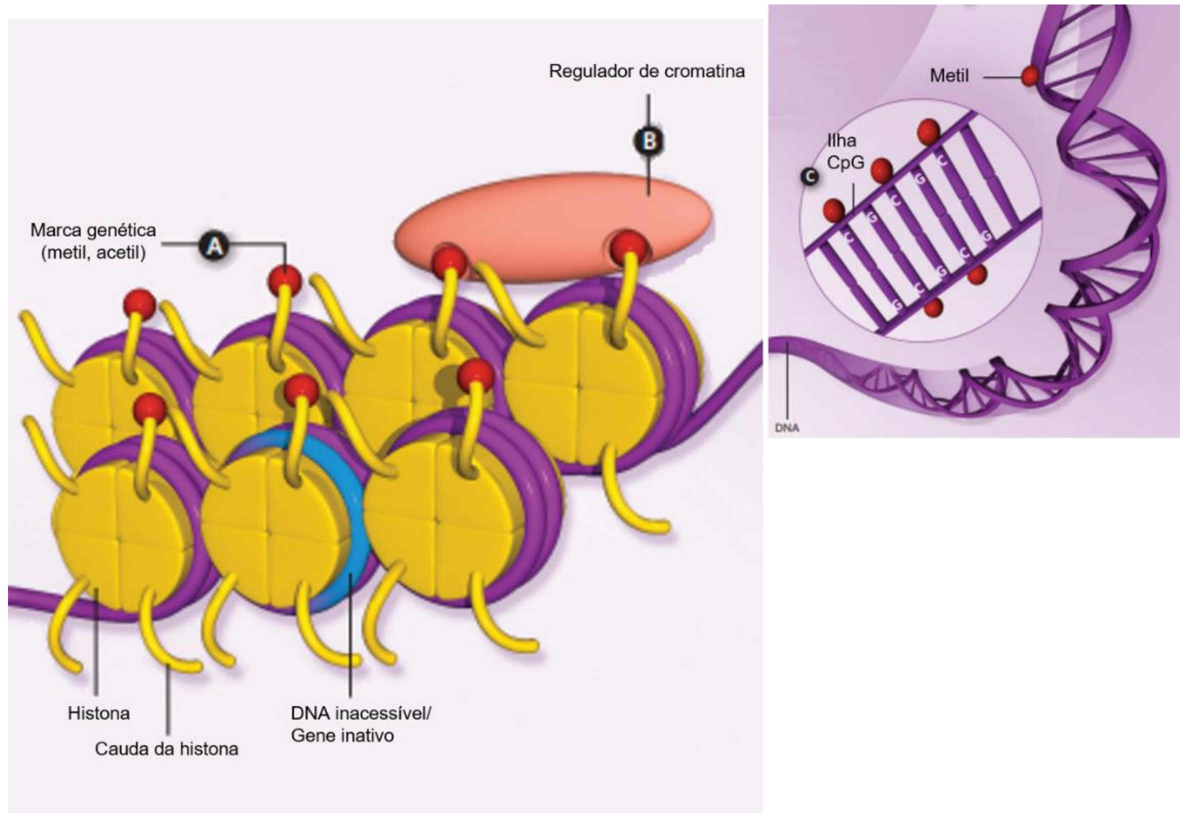

Figura 4. Regulação da expressão gênica mediada pela epigenética: (A) metilação/ acetilação em certas posições nas caudas das histonas. Essas modificações químicas são feitas por várias enzimas modificadoras de histonas e depois reconhecidas por outros reguladores de cromatina (B). (C) metilação do DNA geralmente ocorre em grupos de citosina ou "ilhas" (ilhas CpG), que geralmente ocorrem em promotores de genes. Modificado de https://web.whoi.edu.

Metilação e o ambiente

A metilação do DNA é um mecanismo de memória celular que inclui exposições ambientais presentes e passadas, permitindo ao organismo uma modificação na expressão genética em resposta a essa exposição (Marsit 2015). Uma grande variedade de fatores ambientais já foi ligada a mudanças no metiloma como indução ao estresse (Dowen et al. 2012), exposição a pesticidas (Kahl et al. 2018), metais pesados entre outros (Santoyo et al. 2011; Liou et al. 2017; Shoeb et al. 2017).

Fatores ambientais podem alterar características regulatórias epigenéticas, como a metilação do DNA (Marsit 2015). Existem muitas maneiras pelos quais os efeitos epigenéticos regulam a ativação ou repressão dos genes. Dentro da epigenética ambiental, uma importante consideração é a possibilidade para a herança transgeracional de um padrão de metilação alterado. Enquanto a exposição ambiental pode levar a mudanças no organismo exposto, ela também pode impactar a prole deste organismo através de efeitos diretos da exposição em gametas ou embriões (Cheng et al. 2012; Marsit 2015).

Dowen et al. (2012) estudaram a metilação do DNA como regulador de resposta imune em *Arabidopsis thaliana*. A planta foi exposta, entre outros, ao hormônio ácido salicílico (AS). Em resposta ao AS, as regiões diferencialmente metiladas associadas a elementos transponíveis foram frequentemente acopladas a alterações transcricionais do transposon e/ ou do gene proximal. Assim, mudanças na metilação do DNA em sequências ou transposições repetitivas podem regular os genes vizinhos em resposta ao estresse do AS. Childebayeva et al. (2019) realizaram um interessante estudo para saber se respostas adaptativas andinas em grandes altitudes têm um componente epigenético. Para isso, analisaram a metilação do DNA da região promotora do gene EPAS1 (fator de transcrição responsivo à hipóxia) e do elemento repetitivo LINE-1 entre 572 indivíduos de Quechua, de alta altitude (4.388 m), e do Peru, de baixa altitude (0 m). Os indivíduos de alta altitude tiveram menor metilação do DNA da região promotora do EPAS1 e maior metilação do LINE-1. O LINE-1 é altamente metilado em situação padrão (em ausência de doença). A metilação do LINE-1 pode afetar a expressão de genes próximos, e a sua diminuição no LINE-1 mostrou estar associada à instabilidade genômica, risco de câncer e exposição a substâncias tóxicas (Ogino et al. 2008; Woo e Kim 2012).

Padrões de metilação do DNA são estabelecidos logo no início do desenvolvimento, com duas ondas críticas de metilação e desmetilação que ocorre durante a embriogênese. Esse processo é altamente regulado, e a inibição da maquinaria responsável por estabelecer este padrão resulta em um desenvolvimento anormal ou até mesmo na morte do organismo. Exemplos da função da metilação do DNA na expressão gênica durante o início do

desenvolvimento são a inativação do cromossomo X, o *imprinting* genômico e a repressão dos elementos transponíveis (Bommarito eFry 2019).

O gênero *Ctenomys*

Pertencentes à família Ctenomyidae e a subordem Hystricognathi, o gênero *Ctenomys* Blainville, 1826, compreende roedores subterrâneos endêmicos do sul da região Neotropical. Sua origem é atribuída ao noroeste da Argentina, local onde foi encontrado o fóssil mais antigo do gênero datando de aproximadamente 3.6 Ma (Verzi et al. 2010), idade semelhante a encontrada através de análises de relógio molecular com citocromo b, 3.7 Ma, (Castillo et al. 2005). Durante o Plio-Pleistoceno, na origem do gênero, ocorreu uma explosiva cladogênese atribuída ao gênero *Ctenomys,* que refletiu em estudos filogenéticos utilizando marcadores moleculares mitocondriais e nucleares, os quais costumam resultar em uma politomia basal (Lessa e Cook 1998; D'Elía e Lee 1999; Mascheretti et al. 2000; Slamovits et al. 2001; Castillo et al. 2005; Parada et al. 2011). O estudo filogenético mais recente para o gênero *Ctenomys* é também o mais completo, considerando o maior número de espécies reconhecidas e com ampla cobertura geográfica (Parada et al. 2011). Os resultados permitiram o reconhecimento de 8 clados bem suportados, compondo grupos de espécies de tuco-tucos, apesar de algumas espécies não pertencerem a nenhum grupo e as relações entre elas e os demais grupos não estarem bem resolvidas na base da filogenia.

Atualmente, o gênero é composto por aproximadamente 65 espécies (Teta eD'Elía 2020), apresentam adaptações morfológicas para o hábito de viver em tocas, como corpo fusiforme e redução dos olhos e pavilhão auditivo (Lacey et al. 2000). As tocas proporcionam um ambiente homogêneo – escuro, menor disponibilidade de O_2 e maior concentração de CO_2 - possibilitando que as espécies ocupem diversas formações, sendo limitadas por fatores como densidade do solo e disponibilidade de alimento (Marcy et al. 2016). Com exceção de *C. sociabilis*, as espécies do gênero são solitárias (Lacey et al. 1997), sendo as populações constituídas de pequenos demes populacionais (Freitas 2016). Possuem taxas diferenciais de dispersão, sendo os machos mais propensos a migração (Lopes

2007). Eles são conhecidos popularmente como tuco- tucos devido ao som que emitem dentro das galerias, que se assemelha a "tuc-tuc".

Distribuindo-se desde regiões de altitude, nos Andes peruanos, até as costas do Rio Grande do Sul e sul de Santa Catarina, no Brasil, Uruguai e Argentina; estendendo-se da Patagônia argentina até o Centro-Oeste brasileiro (Reig et al. 1990; Parada et al. 2011; Leipnitz et al. 2020), os tuco-tucos possuem grande diversidade cariotípica, com números diploides variando de 2N= 10, *C. steinbachi*, até 2N=70 em *C. pearsoni* (Reig et al. 1990). Algumas de suas características, tais como baixas taxas de dispersão de adultos (Busch et al. 2000) e sua distribuição em populações relativamente pequenas e fragmentadas, promovem a criação de pequenas unidades genéticas, onde a variação genética é baixa e a divergência interpopulacional é alta (Wlasiuk et al. 2003). Estas características tornam as espécies mais vulneráveis a fatores demográficos estocásticos e a mudanças em seu habitat e também favorecem a fixação de novos rearranjos cromossômicos, os quais foram considerados como tendo um papel importante na diversificação de *Ctenomys* (Reig et al. 1990; Lessa eCook 1998).

No Brasil, são descritas oito espécies, três delas na região Centro-Oeste (*C. bicolor*, *C. nattereri* e *C. rondoni*) – esta última ainda carece da identificação da região de ocorrência. As cinco restantes ocorrem no Estado do Rio Grande do Sul (RS): *Ctenomys minutus*, *C. torquatus*, *C. lami*,*C. flamarioni* e *C. ibicuensis* (Freitas e Lessa 1984; Freitas 1995; Freitas 1997; de Freitas et al. 2012) (FIGURA 5) *Ctenomys lami*, *C. minutus* e *C. flamarioni,* vivem ao longo da Planície Costeira, ocupando habitats distintos. *C. minutus* ocupa a região mais antiga, denominada Coxilha das Lombas, que vai de Porto Alegre até a Lagoa dos Barros. *C. minutus*, distribui-se pelos campos arenosos, paralelamente à Coxilha das Lombas. *C. lami* vive exclusivamente na primeira linha de dunas do Litoral (Cômoros de areia). *C. torquatus*, ocorre na região da Campanha do Estado, sendo delimitada pelos municípios de Candiota, Minas do Leão, Pelotas, Rio Grande, Taim e a região norte do Uruguai, ocupando campos abertos (Freitas 1995) e *C. ibicuiensis* é endêmica da região dos municípios de Manoel Viana e Maçambara, no centro-oeste do Rio Grande do Sul (de Freitas et al. 2012). Nos próximos tópicos as espécies *C. torquatus* e *C. minutus* serão mais bem descritas, pois são objeto deste estudo.

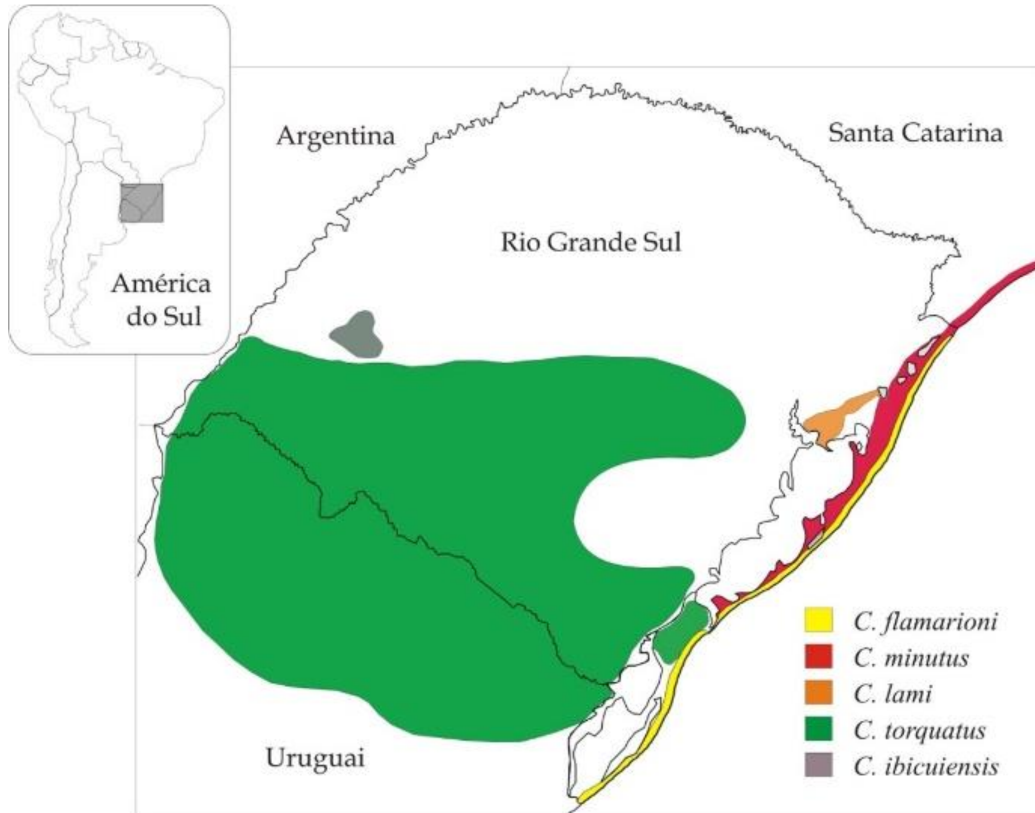

Figura 5. Distribuição geográfica das cinco espécies de tuco- tucos que ocorrem no estado do Rio Grande do Sul. Modificado de Carla Lopes, 2011.

Ctenomys torquatus Lichtenstein, 1830

Ctenomys torquatus (FIGURA 6) possui coloração acastanhada no dorso e flancos, esbranquiçada no ventre e um colar branco (Freitas e Lessa 1984), que dá à espécie o nome popular de tuco-tuco de colar. Entre os grupos de espécies recuperados pela análise filogenética de Parada et al. (2011), o grupo torquatus é composto por espécies que além de compartilharem um ancestral comum, habitam a mesma região no sul do Brasil, Uruguai e nordeste da Argentina.

Kiblisky et al. (1977) descreveram os primeiros cariótipos 2n = 44 de *C. torquatus* para amostras uruguaias de El Aguila, Guabiyú e Salto Nuevo. Posteriormente, Freitas e Lessa (1984) descreveram duas formas cromossômicas para *C. torquatus* no sul do Brasil. Os cariótipos dos espécimes de *C. torquatus* apresentaram dois números diploides diferentes: 2n=44 e número de braços (NB=72), em Laranjal, município de Pelotas, e 2n=46 (NB=72) em Barra Falsa,

Pelotas (localizada a 11 Km de Laranjal), e na Estação Ecológica do Taim (localizada a 82 Km de Barra Falsa). Acredita-se que o evento responsável pela origem da forma 2n=46, seja uma fissão centromérica no par cromossômico número 7 da forma 2n=44, originando os pares 14 e 16 na forma 2n=46 (Freitas e Lessa, 1984).

As populações de *C. torquatus* distribuem-se pelos campos de baixa altitude de toda a metade sul do RS e norte do Uruguai, distante das planícies costeiras afetadas por transgressões marinhas, bem como de outras regiões alagadas, exceto por uma pequena região ao sul de sua ampla distribuição geográfica. A distribuição geográfica de *C. torquatus* no Brasil, descrita por Freitas (1995), estava restrita a pontos isolados na depressão central do Rio Grande do Sul (Minas do Leão, Butiá e Rio Pardo), na região da Campanha (Candiota) e porção sudoeste do Estado (Pelotas, Rio Grande e Taim).

Em algumas destas localidades, onde ocorriam áreas de extração de carvão (Candiota e Butiá), foi realizado um estudo de biomonitoramento que demonstrou que *C. torquatus* é um excelente organismo bioindicador por ser sensível a danos biológicos causados pela extração de carvão e seus derivados (da Silva et al. 2000; Da Silva et al. 2000). Nestes estudos foram utilizados marcadores mutagênicos como Teste de micronúcleos (MN) e Ensaio cometa (EC) em indivíduos amostrados nas áreas de exposição, Butiá e Candiota, e na área de controle, Pelotas. Além de terem sido utilizadas amostras biológicas dos espécimes, foram analisados também o solo das tuqueiras quanto a presença de metais. Todos os trabalhos mostraram um aumento no dano ao DNA nas amostras expostas em relação ao controle. Esta informação possibilitaria classificar esta espécie de tuco-tuco como potencial biomonitor ambiental e possivelmente ameaçada pela atividade extrativista e todos os danos advindos desta (Freitas, 1995).

Figura 6. *Ctenomys torquatus*. Foto de Cláudio Dias Timm.

***Ctenomys minutus* Nehring, 1887**

Ctenomys minutus (FIGURA 7) é uma espécie endêmica da planície costeira do Sul do Brasil, e pertence filogeneticamente ao grupo Torquatus no gênero *Ctenomys* (Freitas 1995; Parada et al. 2011). Os indivíduos desta espécie apresentam coloração castanha claro com tons da cor areia na parte inferior e uma coloração castanho-médio/ escura. Apresentam dimorfismo sexual, com os machos sendo significantemente maiores que as fêmeas (Rui, A. M. 1997; Marinho e Freitas 2000). A razão sexual observada em *C. minutus* apresentou uma tendência a um maior número de fêmeas, o que estaria provavelmente relacionado a uma maior mortalidade e maior deslocamento dos machos para fixação de território e procura de fêmeas no período de acasalamento (Marinho 1997).

Esta espécie habita os campos arenosos e as dunas da Planície Costeira do Sul do Brasil, ocupando o maior gradiente latitudinal dentre as espécies de tuco-tucos no sul do Brasil, sendo sua distribuição desde a praia de Jaguaruna no estado de Santa Catarina (SC), até o município de São José do Norte no estado do Rio Grande do Sul (RS) (Freygang et al. 2004). Essa espécie ocupa do norte de sua distribuição a primeira linha de dunas da beira da praia, até as proximidades de

Capão da Canoa (RS), e a partir de Capão da Canoa em direção ao sul a espécie passa a ocupar o segundo e terceiro sistemas de barreiras-lagunas, correspondente aos campos arenosos da segunda linha de dunas, interiorizando-se na costa até as proximidades das margens da Lagoa dos Patos (Freitas 1995).

Figura 7. *Ctenomys minutus*. Foto de Tatiane Noviski.

As populações de *C. minutus* possuem notável variação cariotípica (Gava e Freitas 2004). Estudos realizados por Freitas (1997), Gava e Freitas (2003), Freygang et al. (2004) demonstraram a existência de sete cariótipos parentais distribuídos parapatricamente (2n = 50a, 48a, 46a, 42, 46b, 48b e 50b), entre os quais se tem a formação de quatro zonas híbridas intraespecíficas que dão origem a cariótipos intermediários entre os parentais: I) 2n = 46a x 2n = 48a → 2n = 47a; II) 2n = 42 x 2n = 48a → 2n = 43, 44, 45, 46, 47 (foram encontrados 5 números diploides, porém 25 combinações cariotípicas diferentes); III) 2n = 46b x 2n = 48b → 2n = 47b; IV) 2n = 50b x 2n = 48b → 2n = 49b (Freitas 1997; Gava e de Freitas 2002; Freygang et al. 2004; De Freitas 2006). Cada cariótipo pode

ser designado por uma área geográfica particular, com distribuição contígua ou separada por barreiras geográficas, sendo que nos dois extremos da distribuição tem-se 2n = 50, enquanto que em direção ao centro os cariótipos variam seguindo uma clina, e o número diploide é progressivamente reduzido até 2n = 42, através de rearranjos Robertsonianos, fusões/fissões *in tandem*, inversões paracêntricas e pericêntricas (FIGURA 8). No caso de um mesmo número diploide, com cariótipos descritos como “a” ou “b”, o que os diferencia são rearranjos em cromossomos distintos (Freitas 1997; Freygang et al. 2004).

Os rearranjos cromossômicos envolvidos em cada cariótipo foram identificados através da técnica de banda G, e compreende, a partir de 2n = 50a, os cromossomos 2, 16, 17, 19, 20, 22, 23 e 24 (Freygang et al. 2004; Freitas 2006). Por exemplo, em todos os espécimes de cariótipo “a” (2n = 46a, 48a e 50a) e em 2n = 42, o par cromossômico 2 encontra-se integro, enquanto que nos cariótipos “b” (2n = 46b, 48b e 50b), o par 2 encontra-se fissionado, originando dois cromossomos, correspondentes aos braços 2p e 2q, sendo que o braço 2p sofreu uma inversão pericêntrica, originando um cromossomo metacêntrico (Freygang et al. 2004; Freitas 2006).

Freygang et al. (2004) compararam o cladograma obtido pela distribuição dos cariótipos com a distribuição geográfica de *C. minutus*, e inferiram que, inicialmente, toda a planície costeira era colonizada pelo cariótipo 2n = 50a. O rio Araranguá pode ter dividido a distribuição de 2n = 50a, originando 2n = 50b do lado sul do rio. Também foi inferido que, através do cladograma, os cariótipos 2n = 48b, 46b, e 48a surgiram do cariótipo 2n = 50b. Sugeriram, então, duas explicações, uma que os cariótipos surgiram independentemente, e a outra, deduzida por parcimônia, que o 2n = 48b apareceu dentro da área de distribuição de 2n = 50b, e através de uma inversão, produziu o 2n = 48a, e independentemente, por uma fusão, surgiu o 2n = 46b.

Assim, a distribuição das diferentes formas cariotípicas ao longo da distribuição de *C. minutus*, parece estar relacionada com eventos de formação da planície costeira (Freitas 1997; Freygang et al. 2004).

Assim como o tuco-tuco, outras espécies como o *Muntiacum muntjak* 2n = 6 e 7, e *Muntiacus reveesi* 2n = 46 apresentam cariótipos muito diferentes apesar de

possuírem o conteúdo genético idêntico. A fusão entre as extremidades cromossômicas tem sido sugerida como a origem dessa grande variação de cariótipo dentro de uma espécie (Tsipouri et al. 2008). A fusão na região terminal dos telômeros poderia ser a causa de muitas fusões em mamíferos (Azzalin e Nergadze 2001; Nergadze et al. 2004). Este fator é gerado por uma redução da região telomérica, que normalmente é relacionada ao envelhecimento celular (Zakian 2012), mas que diferentes mecanismos podem estar envolvidos, incluindo fatores ambientais e de fisiologia (Daniali et al. 2013).

Freitas (1997) e Freygang et al. (2004) observaram que a variabilidade cromossômica de *C. minutus* pode ser consequência de rearranjo Robertsoniano e em fusões *in tandem*. A partir desses estudos, tem-se a hipótese de que os eventos de fusão *in tandem* cromossômicos ocorreram durante a evolução da linhagem dessa espécie de tuco-tuco. Isto poderá ser demonstrado pela presença de reduções teloméricas nas posições intersticiais de cromossomos de *C. minutus* por hibridação *in situ* fluorescente. Estudos de mapeamento com cromossomos de *C. minutus* irão ajudar na orientação de outros locais de fusão cromossômica ancestral no tuco-tuco.

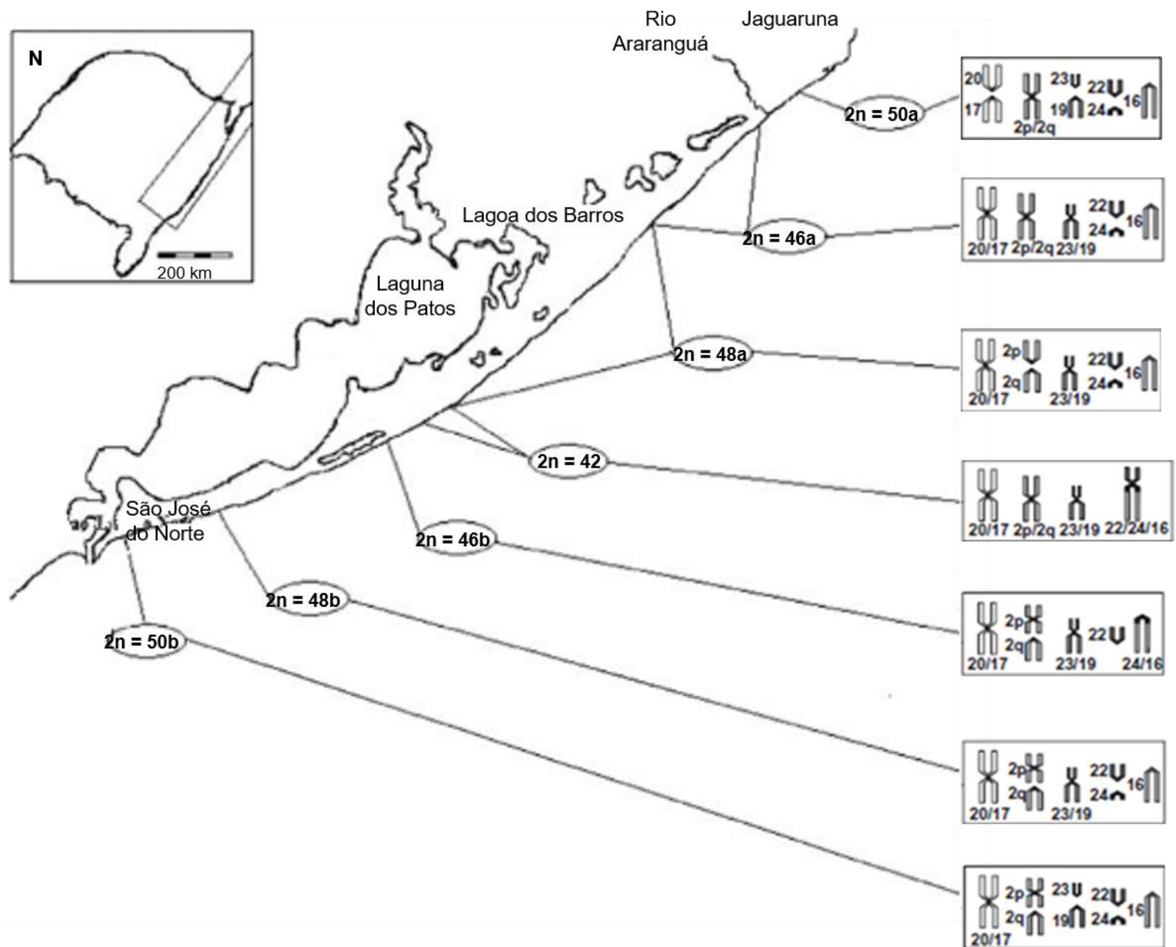

Figura 8. Distribuição geográfica e cariotípica de *C. minutus*. Dentro dos quadros, os rearranjos que os diferenciam. Modificado de De Freitas 2006 e Lopes et al. 2013.

OBJETIVO

Avaliar o comprimento dos telômeros e a metilação global do DNA em *C. torquatus* a fim de relacionar o papel na conservação, bem como a influência de fatores ambientais sobre os mesmos parâmetros. E utilizar a mesma abordagem em *C. minutus* a fim de conhecer os eventos evolutivos que levam à diversidade cariotípica nesta espécie.

Objetivos específicos:

- Revisar sobre os efeitos da poluição ambiental na conservação de *Ctenomys*;
- Determinar o comprimento telomérico em *C. torquatus* a fim de comparar esses valores entre populações expostas e não expostas ao carvão;
- Determinar a porcentagem de metilação global do DNA nas populações de *C. torquatus* através da técnica de HPLC a fim de relacionar com o comprimento telomérico e danos ao DNA.
- Determinar o tamanho do telômero nos diferentes cariótipos de *C. minutus* por reação em cadeia de polimerase em tempo real quantitativo (*q*PCR) e correlacionar o comprimento de telômero com as fusões e instabilidade cromossômica
- Determinar as marcações teloméricas, incluindo sequências teloméricas intersticiais (ITS) através da identificação de região telomérica por hibridização fluorescente *in situ* (FISH);
- Determinar a porcentagem de metilação global do DNA nos genomas de *C. minutus* através da técnica de HPLC e relacionar esse resultado ao comprimento telomérico.

CAPÍTULO 1

Effects of Environmental Pollution on the Conservation of *Ctenomys*

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EFFECTS OF ENVIRONMENTAL POLLUTION ON THE CONSERVATION OF *CTENOMYS*

Cristina A. Matzenbacher and Juliana da Silva

ABSTRACT

Environmental pollutants are related to genetic and epigenetic changes with different effects on natural populations, including the size of the population reduction due to loss of variability and loss of reproductive capacity. In the past few decades, interest in using biomarkers or bioindicators as monitoring tools to assess environmental pollution has increased considerably. This is because biomarkers are suitable not only to provide information on the health status of the exposed organisms, but also on the quality and / or quantity of the exposure situation. The genus *Ctenomys*, popularly known as tuco-tucos, is endemic to South America and is being evaluated as a possible bioindicator of its habitat. Animals such as tuco-tucos, which have reduced the size of the population and still suffer from the impact on their environments, such as habitat fragmentation, are more susceptible to suffering DNA damage from exposure to pollutants and have a more catastrophic effect on the structure of the its population. This chapter will present some studies on environmental mutagenesis that have been carried out with species of *Ctenomys* in South America. In addition, we will present possible molecular biomarkers for future studies. In conservation genetics, these analyzes performed with *Ctenomys* represent a new approach to assess the anthropogenic effect on natural mammal populations. These studies are important due to the emphasis on the feasibility of genotoxic assays and epigenetic tools in conservation studies.

INTRODUCTION

All species on the planet have developed adaptations, in different ways, that promote enough aptitude to sustain different populations for long periods of time and environmental changes. This is the result of the interaction between the genome and the environment. The ability of species or population to adaptations from a polluted environment is called toxicology evolutionary (Bickham et al. 2000). Besides mutations and natural selection or genetic drift (dependent of population size), recently has been discussed epigenetic factors. Epigenetics involves meiotically and mitotically stable alterations in gene expression that are not based on DNA sequence changes but involve processes that impact the packaging of DNA (chromatin structure) (Kalisz and Purugganan 2004). Although epigenetic variation can occur in the absence of genetic variation, genetic variation can influence epigenetic variation and the epimutation rate in several ways. Epigenetic variation can be a significant source of natural phenotypic variation; therefore, it has the potential to play a major role in adaptation to environmental change. Modern evolutionary theory is primarily based on the inheritance of random genetic variation, so there has been widely discussed whether evolutionary theory requires revision considering epigenetics (Jablonka and Raz 2009; Richards et al. 2010; Jablonka 2017).

Environmental pollutants are related with genetic and epigenetic alterations with different effects on populations, between them population reduction size through loss of variability and loss of reproductive capacity, for example. Pollution is any change in the environment that can be a natural or agrarian ecosystem, an urban system or even a micro scale. It can cause changes in the proportions or characteristics of one of the elements that make up the environment itself, such as increased carbon dioxide concentration. It may be the result of the introduction of natural substances, although foreign to certain ecosystems, or artificial substances such as the deposition of pesticides in the soil (Maximillian et al. 2019).

To assess complex chemical-biological interactions and predict the potential for chemical substances that cause harmful effects and impact ecological communities and populations, chemical and ecotoxicological methods are used to assess responses at both the organism and cellular levels (Schaeffer 1991). This kind of research integrates data from the field and laboratory biomarkers to understand and predict adverse effects; this way of study looks at the whole system. Adverse outcome pathways (AOPs) are conceptual frameworks that bring together what is known about chemical agents - with limited safety data - and their adverse effects on human health and ecosystems (Groh et al. 2015).

Contaminants transferred through the food chain play an important role in their action and persistence in the ecosystem and can slowly accumulate in the tissues of individuals over time. Many toxic substances such as metals and organic compounds can be transferred from individuals' tissues to their predators and reach a higher concentration at higher trophic levels. Pollutants can also act on DNA leading to teratogenic effects, mutations in germ cells, premature aging or induce neoplasia in somatic cells. In addition, DNA damage (mutations) can affect population structure, in which genetic imbalance (change in genetic variability and allelic frequency) has a direct effect on biodiversity decline, increasing vulnerability to environmental stress and, consequently, may lead species to reduced survival and extinction (Hemminki et al. 1979; Groh et al. 2015).

Toxicology is the study of chemical substances of environmental pollutants effects over living organisms, since on the individual organisms, tissues or cells used in the test and extrapolate the results to populations and other species. Environmental toxicology represents a complex triangular interaction between anthropogenic chemicals, the environment and biota. A biomarker approach also involves the identification of molecules capable of responding to anthropogenic (xenobiotic) chemicals by positive or negative regulation, in order to use them to assess all environmental quality. In recent decades, interest in using biomarkers or

bioindicators as monitoring tools to assess environmental pollution has increased considerably. This is because biomarkers are suitable not only for providing information on the health status of exposed organisms, but also on the quality and/or quantity of the exposure situation. Thus, the biomarkers can be used as toxicity measurements or as fingerprints for exposure to chemicals. Depending on the character of the selected biomarkers, the evaluator will receive more data to the adverse effects on organisms or about the exposure situation (Sturla et al. 2014).

BIOMARKERS AND BIOINDICATORS

Different approaches are used to assess effects and risks of exposure to chemicals, physical, and biological agents. Biomarker is a term for analyzing the interaction between a biological system and an environmental agent. In order to study the effects of exposure to environmental genotoxins in wild life species, many biomarkers have been applied which have been derived from human cancer risk assessment studies, including markers of chemical DNA modifications such as DNA adducts, markers of cytogenetic effects such as chromosomal aberrations and micronucleate cells. Studies of markers of genotoxic effects in somatic or germ cells with wild animal populations can help in the analysis of exposure to environmental mutagens. It evaluates whether agent is affecting biodiversity and the chance of survival, inducing changes or selecting critical environments for the survival of current environmental levels of pollution contributing to the assessment of ecological risks (Kleinjans and Van Schooten 2002).

Many studies suggest that exposure to pollutants is associated with genotoxicity biomarkers such as chromosomal aberrations, sister chromatic exchange (SCE), micronuclei (MN; See Figure 12.1) and DNA damage observed by the comet assay (CA; See Figure 12.2) (León-Mejía et al. 2011; Rohr et al. 2013; da Silva 2016; Espitia-Pérez et al. 2018). MN

formation is widely used in molecular epidemiology as a biomarker of chromosomal damage, genome instability and eventually cancer (Fenech 2002). The occurrence of MN represents an integrated response to phenotypes of chromosomal instability and altered cell viability caused by genetic defects and/or exogenous exposures to genotoxic agents. The MN test detects aneugens (numerical chromosomes aberrations) and clastogens (inducing breakages of chromosomes) in the cytoplasm of interphase cells that have undergone cell division during or after exposure to different agents (Fenech 2002, 2007; da Silva 2016).

The CA is a procedure for evaluating DNA lesions (i.e., strand breaks, DNA adducts, excision repair sites, cross-links, and alkali-labile sites), involves application of an electrical current to cells, which results in the transport of DNA fragments out of the nucleus. The image of DNA migration obtained resembles a comet with a head and a tail, hence the term comet assay (Singh and Stephens 1998; Silva et al. 2000). Since the DNA damage induced by toxic agents is often tissue and cell-specific, CA is especially useful because it can detect DNA lesions in individual cells obtained under a variety of experimental conditions. The technique can also be used to evaluate DNA repair (Tice et al. 2000). The CA has already been used to detect DNA damage in several native animals, especially small mammal species, living near or in polluted areas (Petras, M., Vrzoc, M., Pandrangi, R., Ralph, S. and Perry 1995; Salagovic et al. 1996).

Another biomarker that is being used recently is the telomere length (TL). Telomeres are found at both ends of each chromosome, protect genome from nucleolytic degradation, unnecessary recombination, repair, and interchromosomal fusion. Telomeres are nucleoprotein structures that protect the ends of eukaryotic chromosomes with a non-coding repeatable DNA sequence (TTAGGG). It has a single strand 3'G adjacent nucleotide overhang linked by the shelterin hexameric protein complex (Figure 12.3), which makes them sensitive to oxidative stress and single stranded DNA breaks. Repeating units and shelter complexes

harbor differences between species, but their homology is established in almost every respect. In normal somatic cells, telomeres decrease with each round of cell division due to limitations of the replication mechanism to replicate the ends of linear DNA molecules (De Lange 2005; Zakian 2012). When telomere length reaches a critical limit, the cell undergoes senescence and/or apoptosis. Telomere length may therefore serve as a biological clock to determine the lifespan of a cell and an organism, since telomere length decreases with age. Once telomeric DNA is less capable of repair, some agents associated with specific habits may expedite telomere shortening by inducing damage to DNA (Kahl and da Silva 2016).

It is known that DNA methylation has a function in telomere length variability (Blasco 2007; Yehezkel et al. 2008). DNA methylation patterns are formed by a family of DNA methyltransferases enzymes that transfer a methyl group from s-adenosyl-1-methionine (SAM) to the 5-position of cytosine forming 5-methyl-2-deoxycytidine (5-mdC), primarily found in CpG dinucleotides (Liou et al. 2017). Epigenetic mechanisms, such as DNA, RNA and histones modifications, and microRNAs, have been shown to be potential links between the genetic and environmental exposure, which can be determinant to health life or reduction in life span. Environmental toxicants can alter epigenetic regulatory processes, and mediate specific mechanisms of toxicity and responses. Particularly, epigenetic modifications can alter genome expression and function under exogenous influence (Kahl et al. 2019). Growing evidence suggests that at least fifteen environmental chemicals may lead directly to diseases via epigenetic mechanism-regulated gene expression changes, such as hydrocarbons and inorganic elements (Santoyo et al. 2011; Liou et al. 2017). Other studies have brought evidence of the connection of global DNA methylation and mechanisms such as oxidative stress (Pavanello et al. 2010).

As important as knowing some biomarkers used to know and evaluate the effects of contaminants in free-living animals, it is knowing which are the best bioindicators for each

assessment. The bioindicator is an organism that provides information about the environmental conditions of its habitat by its presence or absence or by its behavior (Van Gestel and Van Brummelen 1996), and are defined as species or group of species that readily reflect the abiotic or biotic state of an environment, representing the impact of environmental changes on a habitat, community or ecosystem or are indicative of the diversity of a subset of taxa or of all diversity within an area (Gerhardt 2000). According to Altenburger et al. (2003), bioindicators detect a biochemical aspect of toxic action (for example, damage to the membrane, inhibition of enzymes and damage to DNA), providing rapid and direct indications of toxic impact on the environment. Bioindicators can reveal a lot about the mechanism of toxic action, which allows the extrapolation of related contaminants. However, bioindicators tend to be specific for toxic substances, as not all compounds inhibit the same biological processes. Thus, it is important to choose bioindicators relevant to the mechanisms of action to be known or evaluated.

The main anthropogenic activities studied using rodents refer to exhaustion of motor vehicles (Degrassi et al. 1999; Heuser et al. 2007), industrial emissions (Ieradi et al. 1998; Hazratian et al. 2017), polluted mining dump area (Andráš et al. 2006) and coal mining area(León et al. 2007). In general, contaminating compounds are complex mixtures and contain heavy metals and hydrocarbons. However, studies using subterranean rodents as bioindicators are rare. Subterranean rodents are interesting as bioindicators of soil pollution, mainly because they maintain the same territory for long periods and consume large amounts of vegetation. They play an important role in the dynamics of the ecosystem, due to their ability to modify the availability and dynamics of soil nutrients and resources for other species, because of their excavation system (Nevo 1979; Busch et al. 2000; Reichman and Seabloom 2002; Kerley et al. 2004; Dacar et al. 2010). Thus, studies with subterranean rodents could provide better information on contamination of the environment, the food chain

and, principally, on bioaccumulation. As well as data on potential exposures to wildlife and humans and on the effects of exposure to organic and metallic pollutants, mainly due to their ecology.

The Role of Environmental Pollution in *Ctenomys* Adaptation

The genus *Ctenomys*, popularly known as tuco-tucos, is composed of about 65 species of subterranean rodents (Teta and D'Elia 2012, Chapter 2 this volume) that live in burrows, but come to the surface to vocalize, clear the burrows, seek food and have morphological characteristics adapted to this lifestyle, such as back paw bristles, reduced pinna, incisor teeth outside the mouth, as they use these teeth to aid excavation, not letting sand in your mouth (Parada et al. 2011). They share some common characteristics, including their solitary and territorial habits, small patchily distributed populations, and small effective population sizes, which associated with low rates of adult dispersal lead to a pattern of low genetic variation within populations and high genetic divergence among populations. Furthermore, the species commonly show high levels of karyotypic variation (Reig et al. 1990; Nowak 1999; Lacey et al. 2000).

The genus has a large geographic distribution extending from the extreme south of the Neotropical region to the south of Peru, spreading all over the Patagonic region with a great latitudinal variation, and recorded from the sea level up to 4000 m high in the Andean region (Reig, O.A.; Busch, C.; Ortellis, M.O.; Contreras 1990). In Brazil, eight species of tuco-tucos are described. Three of them, *Ctenomys rondoni* Miranda Ribeiro, 1914, *C. bicolor* Miranda Ribeiro, 1914, and *C. nattereri* Wagner, 1848, are still poorly investigated. All the other five species of tuco-tucos, *Ctenomys torquatus* Lichtenstein, 1830, *C. minutus* Nehring, 1887, *C. flamarioni* Travi, 1981, *C. lami* Freitas, 2001, and *C. ibicuensis*, de Freitas et al., 2012, occur in southern Brazil, in the States of Rio Grande do Sul (RS) and Santa Catarina (SC).

C. torquatus is endemic to southern South America (Freitas and Lessa 1984). Despite the wide geographic range, there is a more common chromosomal form (2n = 44), and karyotype polymorphisms in the southern regions, restricted to the limits of the Atlantic Ocean (2n = 46), and Western (2n = 40 and 42) of its distribution (Freitas and Lessa 1984; Fernandes et al. 2009; Gonçalves et al. 2009). Its geographic distribution in RS almost coincides with the distribution of coal reserves (Freitas 1995). This coincidence took our research group to these regions to analyze possible genetic damage in the tuco-tucos that lived there. Because damage to DNA is not immediately recognized in organisms and has broad-ranging effects, this rodent could be an important system to monitor changes in environmental genotoxicity.

Since late 1990s, our research group has been studying *C. torquatus* (Da Silva et al. 2000b; a; Silva et al. 2000; Matzenbacher et al. 2019) and *C. minutus* (Heuser et al. 2002) as an environmental quality bioindicators in Rio Grande do Sul.

Silva et al (2000; a; b) conducted a two-year study to detect the effects of coal, comparing the results with MN assay and CA to *C. torquatus* (2n= 44). At the end of 2 years, 240 rodents had been analyzed (capture-mark-recapture method). The locations studied covered three locations in RS: Candiota, a region about 2 km from the Presidente Médici coal power plant, Butiá, a region approximately 5 km from a strip coal mine, and Pelotas, a region without a coal mine and power plant. Biological hazards associated with Candiota coal field were investigated in a pilot study. The results showed that coal and derivatives induced DNA and chromosomal lesions in *C. torquatus* cells that were demonstrated by CA and MN test. The CA was more sensitive and showed a direct relationship between age and damage, and an inverse relationship between temperature and damage index. In addition to the authors demonstrated higher concentrations of heavy metals for soil samples from coal regions (Zn, Ni, Pb, Cd, V and Cu), as well as hydrocarbons, and a relation of these concentrations and

DNA damage. Other studies using free living rodents, yellow-necked mouse (*Apodemus flavicollis*) and bank vole (*Clethrionomys glareolus*), which were exposed to a coal mining area (Czech Republic) also demonstrated higher levels of DNA damage (Degrassi et al., 1999). Besides, León et al. (2007), using CA assay in wild rodents *Rattus rattus* and *Mus musculus*, exposed in a coal mining area (Cordoba, Colombia), show that mice and rats originating from the coal mining area exhibited a significantly higher extent of DNA damage as assessed by length of DNA migration, damage index and percentage of damaged cells compared to animals from a control area.

Another study was conducted at the same sites of Silva et al (2000 a; b), with the same species of tuco-tucos, to evaluate the effect of exposure to coal and its derivatives and to examine the relationship of coal exposure with variations in TL, global DNA methylation and genotoxicity (Matzenbacher et al. 2019). The study showed a significant reduction in the TL of the exposed tuco-tucos compared to the unexposed ones. Moreover, was demonstrated no association to factors such as sex and age with the coal exposition. In this study, no relationship was found between global DNA methylation and exposure to coal, as well as no correlation between TL and DNA methylation, probably due to our small sample size. But a relation between more damaged cells in adults may be related with the reduction in the adults' number from Candiota. The reduction in TL is normal and expected, but this reduction is greater in exposed animals, which means that something is accelerating this loss in this region of the chromosome, leading to a premature senescence. Our results demonstrated that *C. torquatus* suffer DNA damage/instability, as observed in the CA (DNA damage), and telomere shortening, likely as a consequence of the oxidative damage that results from their exposure to a complex mixture, including inorganic and organic elements. To prove the possibility of this mechanism, metal analyzes were carried out and Zn, Ni, Pb, Cd, V and Cu were detected in soil. Martiniaková et al. (2010) studies determine the concentrations of heavy metal in the

liver, kidney and bone of yellow-necked mice (*A. flavicollis*) and bank voles (*C. glareolus*) trapped in a region with chemical plant, coal power station and coal mines in Nováky, Slovakia. Highest concentrations of Cd and Zn were found in the bone of both yellow-necked mice and bank voles. Cu and Fe accumulated mainly in kidney and liver. There are many studies that report the relation between some coal exposure and the high concentration of chemical elemental and oxidative stress indexes such as the highest contents of S, Cl, Fe, Zn, and Br in frog *Hypsiboas faber* (Zocche et al. 2014); the reduced survival to metamorphosis in exposed larvae in grass shrimp (*Palaemonetes pugio*) (Kuzmick et al. 2007); a reduction in DNA repair capacity in *Mytilus edulis* with increasing duration of exposure to genotoxic agents (Steinert et al. 1998).

The other specie studied by our group was *C. minutus* which has a wide distribution, occurring from the south of “Farol de Santa Marta” (SC) to “São José do Norte” (RS). Its distribution is related to the formation of the coastal plain in southern Brazil and presents patterns of karyotype diversity that correspond to regions where there were paleochannels limiting the dispersion between parapatric locations (Lopes et al. 2013). They have seven parental karyotypes (2n = 50a, 46a, 48a, 42, 46b, 48b and 50b) and current hybridization zones with intermediate karyotypes (Freygang et al. 2004; Marinho and De Freitas 2006; Lopes et al. 2013). This species has been used in genetic and population studies because exhibit high karyotypic variability and deserves special attention in studies related to conservation.

Heuser et al. (2002) evaluated a possible genotoxic effects of vehicles emissions in *C. minutus* (2n = 46) in both sides of a highway on the coastal region (Amaral and Weber), and Maribo, a control area. Peripherical blood of *C. minutus* was used to perform MN test and CA, and the soil from their burrows to analyze the hydrocarbon concentration and the presence of some metals related to vehicle emission. In addition, concentration of NO_2 in the air also was

measure. The study showed that the DNA damage rate was higher near the highway, as well as the average NO_2 concentration. Adult females showed greater DNA damage. The metals found in the soil of the highway with higher concentrations were Cr, Ni, Cu and Zn, and the hydrocarbons were also shown higher in the two studied points in the highway in relation to the control region. This study provided chemical and biological data from areas exposed to automobile exhaust, indicating the association among environmental agents with levels of damaged cells observed in the wild rodent *C. minutus*. Similarly, Degrassi et al (1999) found in the three parameters of measurement of the MN test (total number, average and frequency) increase in the exposed area of Muro Torto, Rome (Italy) compared to the control area of the zoo under study with house mice (*Mus musculus domesticus*), however, this difference was not statistically significant. Vehicular emission also demonstrated affect the vegetation growth as shown in the study of Wagh et al. (2006) that evaluate pollution impact on the vegetation along the road in Jalgaon City, Maharashtra (India) and observed that vegetation at roadside with heavy traffic had less leaf area, total chlorophyll and total proteins in leaves. Hazratian et al. (2017) to assess the potential use of the Norway rat, *Rattus norvegicus,* as a bioindicator for lead and cadmium accumulation in 10 urban zones in Tehran, Iran. The anthropogenic activities and vehicular emissions contribute to the entry of toxic metals to humans and other animal's food chains. The accumulation of heavy metals in some free-living rodents has been extensively studied (Šumbera et al. 2003; Guerrero-Castilla et al. 2014). Other studies also showed relation between inorganic elements present in vehicles exhaustion and DNA damage in different species (Meireles et al., 2009; Brito et al., 2013).

Schleich et al. (2010) evaluated the concentrations of four heavy metals (Pb, Zn, Fe and Cu) in muscle and liver from the subterranean rodent *Ctenomys talarum* to from natural dunes, cultivated area and military area of Buenos Aires Province, Argentina. The study revealed a higher concentration of metals in the livers than in the *C. talarum* muscles in the

military (Fe and Cu) and cultivation areas (Pb and Cu). In soil samples, the highest concentrations of metals found were Fe in both military and cultivated area. In the vegetation samples, low levels of metals were found, with Cu being most abundant in the military area, Pb in the cultivation area and Zn in both cultivation and dunes areas.

In addition to the biomarkers that assess the effects of contamination on free-living animals, other markers of equal importance for the conservation and adaptation of these animals are interesting to be considered for a more comprehensive assessment of these effects. Knowledge of the genetic structures of these populations is an example. Lopes and de Freitas (2012) studied the effects of habitat reduction and fragmentation in four *C. lami* populations, due to human occupation, progressive urbanization and expansion of agriculture and livestock. *Ctenomys* have a limited geographical distribution, as they are small populations distributed irregularly and low levels of adult dispersal (Reig, O.A.; Busch, C.; Ortellis, M.O.; Contreras 1990; Nowak 1999; Lacey 2001). In this study, was demonstrated that the populations had no genetic structure associated with the distinct karyotype groups. However, molecular mitochondrial and nuclear markers demonstrated the existence of two populations instead of the four populations at the beginning of the study. These two populations are not completely isolated but are probably reinforced by a geographical barrier. The vulnerability of *C. lami* was greater than the authors previously assumed, then the author suggested the designation evolutionarily significant units (ESU) to one population, based on their genetic differentiation for both molecular markers (nuclear and mitochondrial), and a Management Unit (MU) to another one, which considers statistically significant divergence in allele frequencies (nuclear or mitochondrial), no matter the phylogenetic differentiation of the alleles. In addition they suggested the inclusion of the conservation status of this species as vulnerable (Lopes and de Freitas 2012).

Another interesting study involving conservation and habitat loss was carried out with Magellan tuco-tuco. Magellan tuco-tuco (*Ctenomys magellanicus*) is the southernmost Patagonian-Fueguian rodent, with a small distribution, which was categorized as vulnerable due to a strong population decline caused by overexploitation and loss and degradation of habitats produced by grazing sheep. In a study of Lazo-Cancino et al. (2020), the authors estimated the appropriate habitat distribution for *C. magellanicus* and predicted the appropriate habitat distribution and potential range changes under future climate change. According to the study, seven climatic variables, associated with the water regime and temperature variation between seasons in Patagonia, were the most important for the distribution of species. Under most future climate change scenarios, suitable habitat for *C. magellanicus* would decline mainly in its current continental distribution, with drastic loss and fragmentation of suitable habitats. This information is important for predicting a bottleneck, which results in a decrease in genetic diversity, which is extremely important for the viability of a population.

Animals such as tuco-tucos, which have reduced population size and still suffer from the impact on their environments, such as habitat fragmentation, are more susceptible to suffering DNA damage from exposure to pollutants and have a more catastrophic effect on your population structure. The figure 4 shows us a summary of the way that some chemical agents, in the form of a complex mixture, and fragmented habitats could be affecting small populations of mammals such as the tuco-tucos. These habitats are also a source of reduced genetic diversity, due to the degradation of the environment, and the emergence of new mutations that are leading them to an evolutionary adaptation to these effects (Matson et al. 2006; Pedrosa et al. 2017). Species have developed adaptations that promote enough aptitude to sustain different populations for long periods of time and environmental changes. The

adaptations reflect responses to the selection imposed by toxins that characterized the environment (Brady et al. 2017).

FUTURE

Little is really known about the physiology of subterranean mammals, and much less about the effects to which they are subjected in relation to the contamination that their habitats are exposed to every day. Although the underground environments are considered more stable and simpler than those above ground, its characteristics are important ecologically and evolutionarily at the level of development of the species. The characteristics of underground niches have led to similar evolutionary pressures that have resulted in morphological, physiological, and behavioral adaptations converging on underground life, wherever they occur in the world. Subterranean rodents (e.g., *Geomys*, *Thomomys*, *Ctenomys*) play an important role in the dynamics of the ecosystem. They are considered ecosystem engineers because of their ability to modify the availability of resources directly or indirectly for other species. Its large excavation systems affect texture, water holding capacity, soil nutrient dynamics and vegetation composition and abundance (Nevo 1979; Jones et al. 1994; Busch et al. 2000; Reichman and Seabloom 2002; Kerley et al. 2004; Dacar et al. 2010).

Studies with conservation genetics or landscape genetics, conducted with populations of tuco-tucos, concluded that contemporary habitat fragmentation increases population differentiation. Genetic analysis of the landscape suggests that habitat quality and longitude were the most strongly associated environmental factors (Lopes and de Freitas 2012; Mora et al. 2017). It may be time by now, given that our knowledge of genetic toxicology has improved and that we also technically are better able to investigate DNA damage making use of modern molecular biological techniques, to start thinking on a new test strategy. Some examples are *in silico* methods, transcriptome approach as well as next generation screening

and sequencing tests (Perkins et al. 2003; Barzon et al. 2011). Transcriptomic profiles obtained from samples of wild animals considered to be environmental bioindicators may highlight candidate genes associated with pollution tolerance. Searching selection signatures using single nucleotide polymorphisms (SNP) genotyping offers a complementary route to explore adaptive evolution and has the gain of being able to provide information on selective pressures that affect all kinds of tissues (Hamilton et al. 2016). *In silico* toxicology can complement the predominant *in vitro* and *in vivo* toxicity tests, predicting toxicity and prioritizing chemicals or drugs in order to minimize harmful effects (Parthasarathi and Dhawan 2018). New DNA sequencing techniques, known as "next generation sequencing" (NGS), offer high speed and throughput that can produce a huge volume of DNA sequences with many applications in research and diagnosis. The benefit is the determination of sequence data from single DNA fragments from a library that are secreted into chips, preventing the need for cloning into vectors before the acquisition of the sequence. At This Time, NGS technologies are applied for complete genomic sequencing, research of genomic, metagenomic, epigenetic diversity, discovery of non-coding RNAs and protein-binding sites and gene expression profile by RNA sequencing (Barzon et al. 2011).

CONCLUSIONS

In conservation genetics, the analyses performed with *Ctenomys* described in this chapter represent a new approach to assess the anthropogenic effect on natural mammal populations. These studies are important due to the emphasis on the viability of genotoxic assays and epigenetic tools in conservation studies. We are currently watching a growing trend of cooperation between ecotoxicologists and conservationists, as evidenced by the rising number of studies with this approach, which can improve research in the areas of conservation and evolution. Genotoxicity and alteration in the methylation patterns in *Ctenomys* can result in

environmentally induced phenotypic plasticity, which may be transgenerationally inherited. More studies, including modern molecular biological techniques, will always be needed in the field of ecogenotoxicity and epigenetics to continue to shape our understanding of the mechanisms of controlling and creating phenotypic variation and its implications for evolution.

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Figure legends

Figure 1. Images of the *C. torquatus* bone marrow cells representing micronuclei (MN). Images from the author's archive.

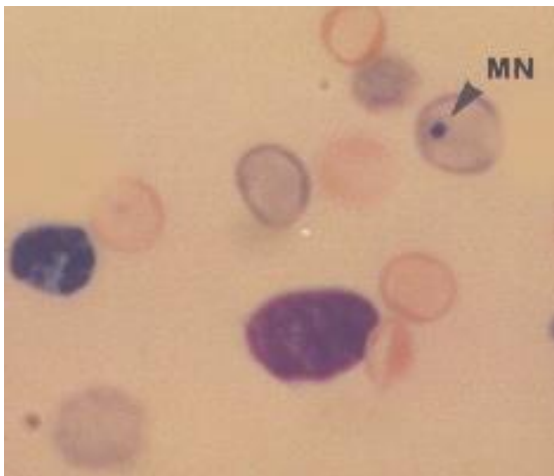


Figure 2. Images of comets assay of *C. torquatus* in blood cells representing damage classes. A, Class 0, undamaged; B, class 1; C, class 2; D, class 3; E, class 4, maximum damage. Images from the author's archive.

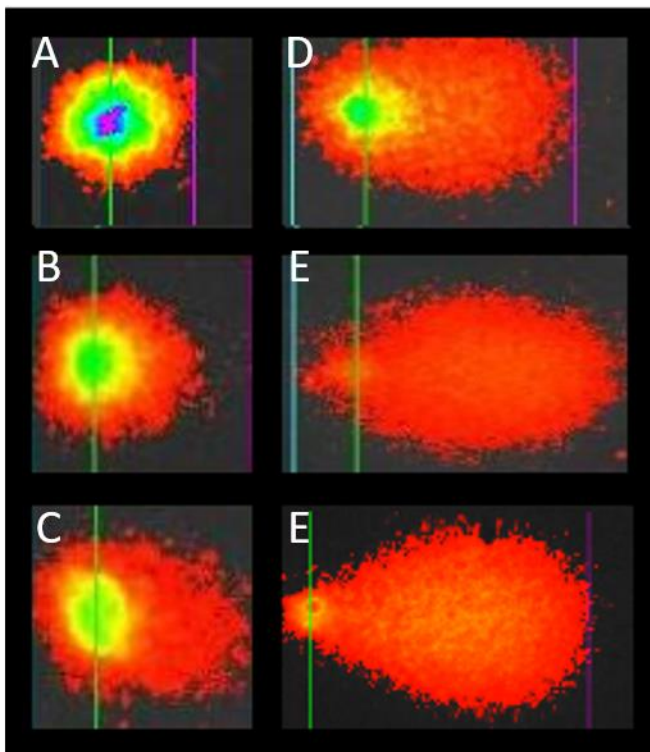


Figure 3. Telomere with complex shelterin and telomerase. When TL reaches a critical limit, the cell undergoes senescence or apoptosis. Modify from Jacobs (2013) and Alenalee (2008) .

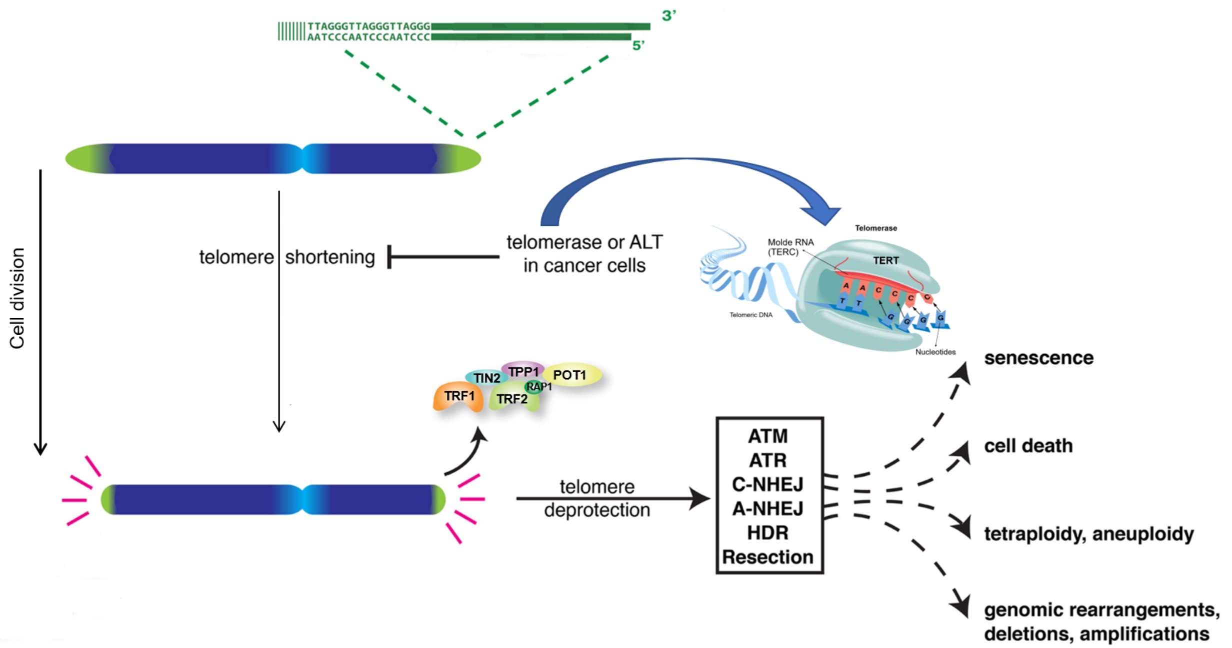
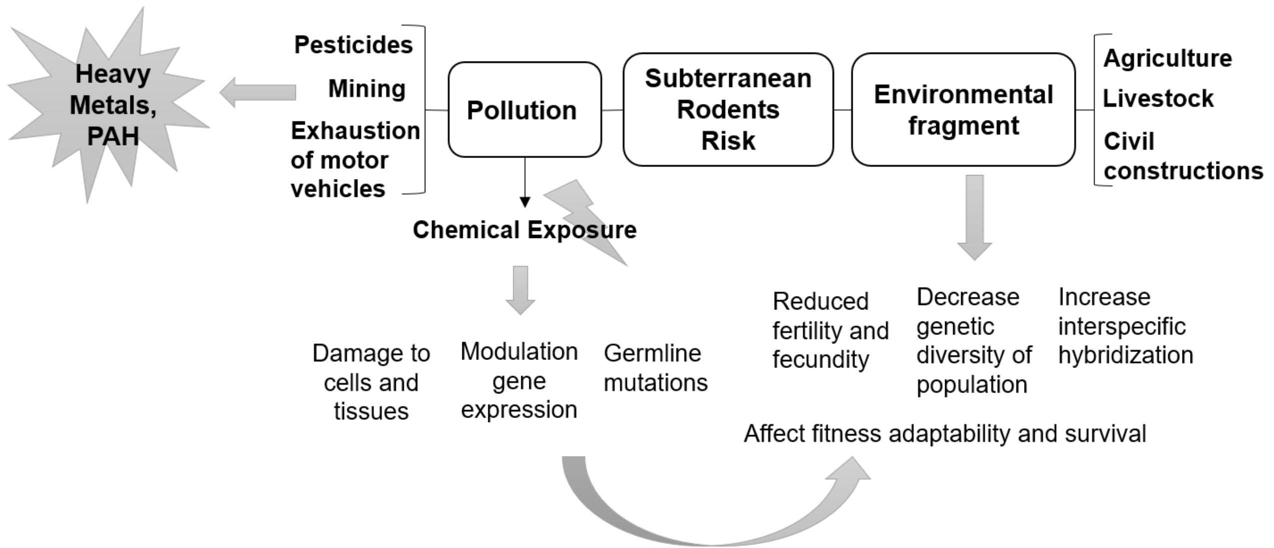


Figure 4. Pressures that subterranean mammals suffer from anthropogenic actions.



CAPÍTULO 2

Anthropogenic Effects on Natural Mammalian Populations: Correlation Between Telomere Length and Coal Exposure

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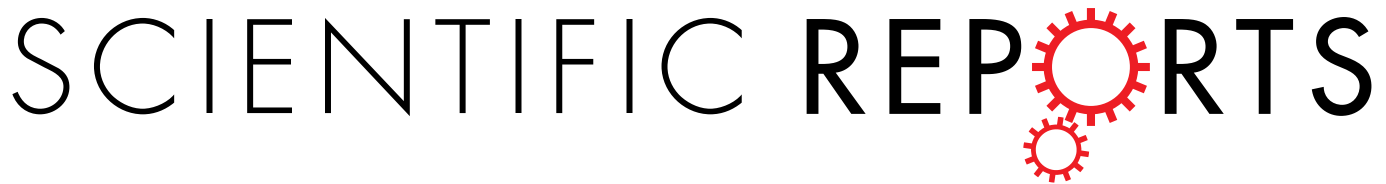

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Anthropogenic Effects on Natural Mammalian Populations: Correlation Between Telomere Length and Coal Exposure

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The Candiota coal mine in Rio Grande do Sul (RS) is one of the largest in Brazil. Coal is a fossil fuel that causes environmental impacts from its extraction to combustion due to the release of different agents, such as polycyclic aromatic hydrocarbons (PAH) and heavy metals. C*tenomys torquatus* are herbivorous and subterranean rodents that dig tunnels with their paws and teeth and can be exposed to coal through contaminated food. Exposure to pollutants can cause DNA damage and affect different tissues, inducing alterations in the population structure and genetic diversity. Our study aimed to evaluate the effect of exposure to coal and its derivatives on the *C. torquatus* population and to examine the relationship of coal exposure with variations in absolute telomere length (aTL), global DNA methylation and genotoxicity. Our study showed an inverse correlation between telomere length and coal exposure in addition to an increase in DNA damage. The results indicate that coal and its byproducts can contribute to the alteration of the *C. torquatus* population structure, as evidenced by a reduction in the number of adults.

The purpose of ecotoxicological studies is to detect a cause-effect relationship between different ecosystems, from the molecular level to the entire ecosystem, and complex chemical mixtures of pollutants[1]. Many of the contaminants in environment affect organisms directly, which present acute effect and fast detection, and can cause physiological disorders, such as endocrine disruption, abnormal development and/or decreasing the lifetime, reducing reproductive parameters and altering the sex ratio[2,3]. In large populations, mutations can be eliminated by selection; however, in small populations, selection is less effective, and genetic drift is one of the factors that can eliminate a mutation[4] that is more common in small populations than selection and gene flow[5]. The continuous progression and decline of "fitness" in small populations due to the fixation of characteristics are not changes that occur without consequences[6]. Since the mutations induced by pollutants generally do not occur in a single individual in the exposed population, the detection of changes in the genome plays a fundamental role in species conservation. These exposure-induced mutation can lead to incorrect gene expression and/or damage to somatic cells, which can reduce an individual's health and reproductive capacity, disrupt development, alter the sex ratio by reducing the effective population size, decrease the genetic diversity, and lead to the end of the viability of the population[7].

Coal is a fossil fuel that causes impacts through environmental pollution from its extraction to its combustion. A wide range of coal dust and coal byproducts contribute to the pollution of the atmosphere[8], water and soil[9,10]. Inorganic elements (metals) and polycyclic aromatic hydrocarbons (PAH), which are found in coal, can induce DNA damage by introducing a cellular oxidative imbalance by the generation of several reactive oxygen species (ROS)[11–13]. Telomere, a noncoding DNA sequence at the end of eukaryotic chromosomes, has a sequence of adjacent triple-guanine nucleotides (TTAGGG) that makes them highly sensitive to oxidative stress and

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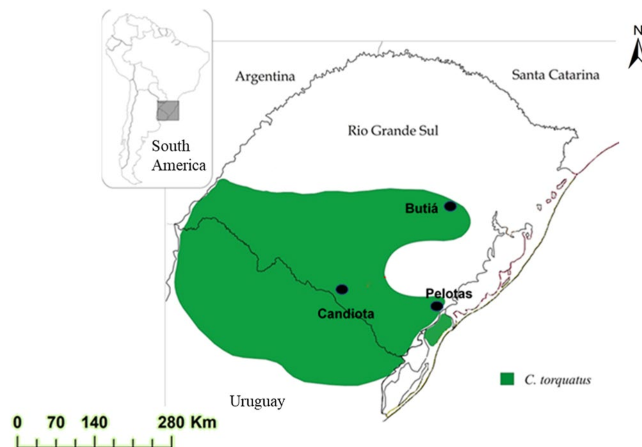

Figure 1. Geographic distribution of *C. torquatus*, which coincides with the geographic distribution of coal reserves and power plant Candiota (modified from Fernandes *et al.*[62]).

single-strand breaks[14]. It is known that DNA methylation has a function in telomere length variability[15,16]. DNA methylation patterns are formed by a family of DNA methyltransferases enzymes that transfer a methyl group from s-adenosyl-1-methionine (SAM) to the 5-position of cytosine forming 5-methyl-2′-deoxycytidine (5-mdC), primarily found in CpG dinucleotides[17]. Previous studies[18,19] showed an inverse correlation between PAH and telomere length in different types of human cells and tissues. Stauffer *et al.*[14] studied the association among exposure to heavy metal pollution, oxidative stress and telomere damage in two passerines, the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*). Recently, studies concerning the effects of environmental pollution on DNA methylation in human and animal, found an association between levels of DNA methylation and exposure to hydrocarbons and inorganic elements[17,20]. An *in vitro* study suggested a relationship between ROS and global DNA methylation[18].

The Presidente Médici thermoelectric plant is a complex composed of three plants: Candiota I, inaugurated in 1961; Candiota II, which has been operating since 1974; and Candiota III, which entered into commercial operation in 2011. All the coal used in this plant comes from the Candiota coalfield, one of the largest coal reserves in Brazil, which is located in the municipality of Candiota, RS, 400 kilometers from Porto Alegre (State Capital)[21]. Several studies have been conducted characterizing coal and its byproducts on power plants in Candiota (RS, Brazil) in addition to evaluating their effects on different organisms. Including a study performed by Siva *et al.*[22–24], who observed an increase in DNA damage in *C. torquatus*.

The collared tuco-tuco, *C. torquatus* Lichtenstein 1830, is a subterranean rodent with a diploid number (2n) of 40, 42, 44 and 46 chromosomes and a fundamental number (FN) of 72 (Refs.[25,26]). This species is present in the fields of the Pampas ecoregion, from the central area of the RS, in southern Brazil, to the central-north region of Uruguay[27]. The geographical distribution of *C. torquatus* in the RS almost coincides with the geographic distribution of the state's coal reserves[28] (Fig. 1). The presence of PAH was found in its coal, bottom and fly ash samples[29], metals were detected in soil samples[30] and Zn, Ni, Cu, Cd, V and Pb were detected in the tissues of the subterranean rodent *C. torquatus*[23]. In addition, studies with organisms have shown DNA damage in plants (*Baccharis trimera*)[9,31], snail (*Helix aspersa*)[10] and coal workers' blood[30,32,33]. To our knowledge, no previous studies have examined the effects of coal and its byproducts on telomere length or DNA global methylation in tuco-tuco.

In this study, we aimed to evaluate the effect of coal exposure and its derivatives on the *C. torquatus* population using absolute telomere length and global DNA methylation measurements, as well as correlate these results with DNA damage obtained previously by Silva *et al.*[19–21]. We hypothesized that the animals living near coal reserves and coal exploration regions that previously reflected in high levels of DNA damage[22] would show high levels of genomic instability now measured by molecular techniques.

Results

Population Size Monitoring. To monitor the population size, we used 198 rodents trapped over two years by Silva *et al.*[22] in three regions: (i) Pelotas, with 77 animals captured and 18% recaptured; (ii) Butiá, with 61 captures and 20% recaptured; (iii) Candiota with 60 animals captured but only 10% recaptured. The female: male ratio was 2:1 in the Pelotas field, 3:1 in the Butiá field and 4:1 in the Candiota field. When comparing the regions with coal derivative exposure and without coal exposure, we verified that among the three age classes, the results were different (Table 1).

Comet Assay. Regarding the Comet assay (CA) results (data obtained by Silva *et al.*[20]), 30 rodents were analyzed as the control group (Pelotas), and 36 rodents were analyzed as the exposure group (14 Butiá and 22 Candiota), considered here are only the individuals utilized for this study. Figure 2 shows a significant increase in DI (damage index, details see Material and Methods) in the Candiota rodents compared with the Pelotas rodents. Males and females from Candiota demonstrated a DI significantly higher than males and females from the control location, Pelotas, but no difference between the sexes of each group was observed (Fig. 3). Regarding age,

Age	Pelotas (%)	Butiá (%)	Candiota (%)
Juveniles	10	11	5
Subadults	34	35	49
Adults	53	54	46
Ne (±SD)[a]	22 (7)	17 (11)	37 (16)*

Table 1. Proportion of individuals by age from region with and without coal and derivatives exposition and population size estimative (Ne). [a]By Lincoln-Petersen method; *Significant in relation to Pelotas and Butiá at P < 0.05, ANOVA.

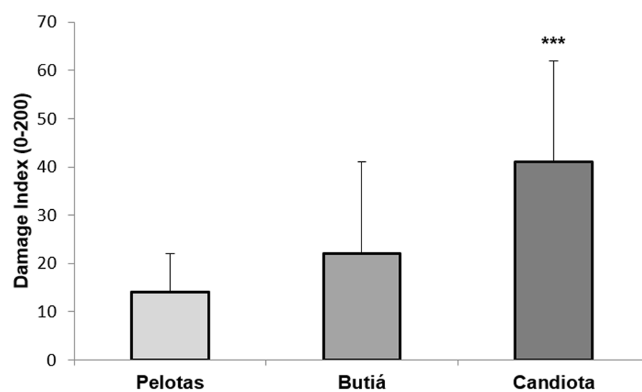

Figure 2. Detection of DNA damage (mean ± SD) in the blood leukocytes of *C. torquatus* from Pelotas (N = 30), Butiá (N = 14), and Candiota (N = 22). ***Significant compared to Pelotas at P < 0.001, ANOVA (Dunnett).

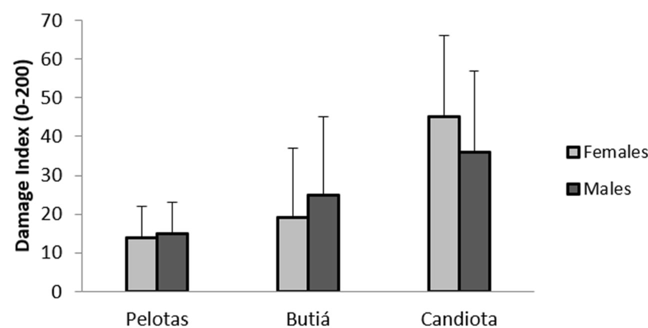

Figure 3. Mean values (mean ± SD) of the damage index in the leukocyte cells of *C. torquatus* by site and sex. Pelotas (female = 22; male = 8), Butiá (female = 11; male = 3) and Candiota (female = 19; male = 3). No significant differences were found.

Fig. 4 shows that juveniles have a lower DI than subadults and adults at each site. Adults from Butiá and Candiota displayed a DI significantly different from the juveniles of the same site (P < 0.05).

Telomere Length. To evaluate absolute telomere length (aTL), 30 rodents were used as the control group (22 females and 8 males), and 36 rodents were used as the exposure group (30 females and 6 males). There is an inverse relationship between the absolute telomere length and the exposure to coal (P = 0.01): the control group had a higher aTL than the exposure group (Fig. 5). Moreover, we dismissed sex (r = 0.042; P = 0.731) and age (r = 0.049; P = 0.706) as confounding factors in the correlation between aTL and coal effects. Additionally, we did not find significant differences in the telomere length of the different tissue samples evaluated.

Global DNA Methylation. We measured global DNA methylation levels in tissues from tuco-tuco in Pelotas, including 30 (22 females and 8 males) individuals as the control group, and in Butiá or Candiota, including 35 (30 females and 5 males) as the coal-exposed individuals, by comparing the relative levels of 5-mdC. We did not find a significant difference in the global DNA methylation between the cities. The average (±SD) methylation levels were 2.3% (±0.19) in Pelotas, 2.27% (±0.18) in Butiá and 2.41% (±0.2) in Candiota (Fig. 6). We also dismissed sex (r = 0.095; P = 0.456) and age (r = 0.006; P = 0.965) as confounding factors in the correlation between the levels of 5-mdC and coal effects, and we did not find significant differences in the levels of 5-mdC of the different tissue samples evaluated.

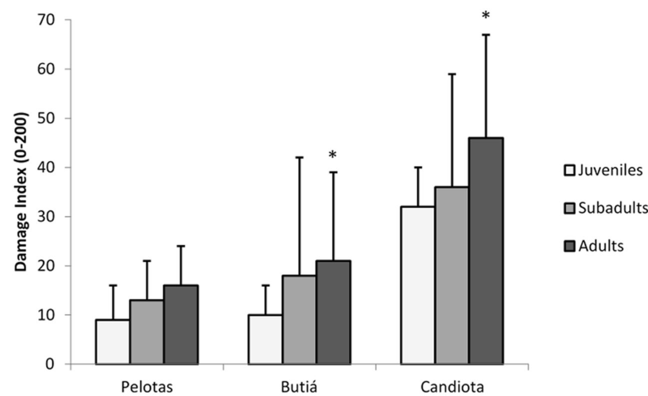

Figure 4. Comparisons between age, site, and damage index - DI (mean ± SD) of blood samples from *C. torquatus*. *Significant compared to juveniles from the same site at $P < 0.05$; ANOVA (Dunnet). Pelotas (5 juveniles, 8 subadults and 17 adults), Butiá (1 juvenile, 4 subadults and 9 adults) and Candiota (1 juvenile, 18 subadults and 3 adults).

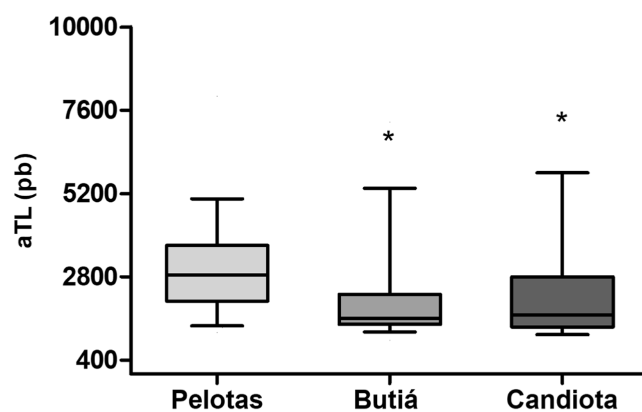

Figure 5. Telomere length of nonexposed (Pelotas) and exposed (Butiá and Candiota) *C. torquatus* individuals. *Significantly different from the nonexposed group at $P = 0.01$; ANOVA (Kruskal Wallis).

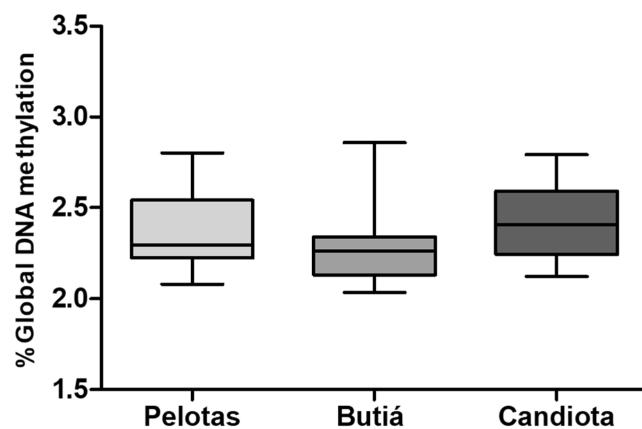

Figure 6. Proportion Global DNA methylation of nonexposed (Pelotas) and exposed (Butiá and Candiota) *C. torquatus* individuals.

Discussion

Previous[22,23] and current studies indicate that coal and its byproducts induce DNA damage in tuco-tucos with no a difference between males and females. Silva *et al.*[23] demonstrated an association between DNA damage and hydrocarbons level in soil. This finding coincides with Zitko *et al.*[34], who measured PHA in harp seal beaters (*Phoca groenlandica*) and did not find a difference between males and females. The main cellular mechanism of DNA damage discussed by the authors was the oxidative lesions caused by PHA[9,18,35].

Telomeres are known to be highly susceptible to oxidative damage and telomere attrition can be accelerated by different environmental factors, such as coal[32]. The ability of ROS to induce 8-oxodG within the GGG triplet found in the telomeric sequence (TTAGGG) is why telomeres are particularly susceptible to oxidative stress-induced damage and chromosome instability, as explained in Coluzzi *et al*.[36]. Our results revealed a significant reduction in aTL in the rodents exposed to coal (Candiota and Butiá) compared with the aTL of the nonexposed rodents (Pelotas). Many variables can influence the loss of telomere length, such as age[37], sex[38], and tissue[39]. Thus, we performed a correlation analysis in the evaluated population to test whether some of those factors could be influencing the reduction in telomere length; however, our results did not support this correlation. We observed that exposure to coal was significantly associated with a decrease in aTL even after adjusting for these variables. Daniali *et al*.[39], in a study examining telomere shortening in human somatic tissues, found that in the tissues analyzed (leukocytes, muscle, skin and fat), there were similar rates of age-dependent aTL attrition in adulthood, and the aTL among tissues did not show significant changes with age. This variation in aTL across somatic tissues is also observed in other mammals[40]. Kirby *et al*.[41] studied the relationship between environmental variables, such as habitat productivity, human development, elevation, and latitude, and individual characteristics, like age, sex, body size and genetic kinship, in *Ursus americanus* and did not find a relationship between telomere length and individual characteristics. Another study found a relationship between shortened telomeres in the blood and exposure to persistent organic pollutants (POPs) using *Rissa tridactyla*, an arctic seabird species, and the most extensive shortening was observed in females[38]. We did not find a relationship between aTL and sex, despite the trend of more telomere shortening (not significant) in male tuco-tucos than in female tuco-tucos in a region exposed to coal. These results indicate that coal and coal byproducts may contribute to telomere shortening in the *C. torquatus* population, and these results can also affect other mammals in the region.

DNA global methylation is an epigenetic alteration that plays a role in regulating cellular processes, including genomic instability and gene expression[42]. Many human and animal studies have investigated the effects of environmental pollution and have reported an association between DNA methylation levels and environmental exposures, including lead, arsenic, benzene, Cr(VI), POPs and air pollutants[17,20,43]. Additionally, the methylation of the sub-telomeric region is correlated with aTL and can be an important region for the epigenetic regulation of telomere length[16,44]. In this study, we did not find a relationship between global DNA methylation and coal exposure, and no correlation was found between aTL and DNA methylation. De Souza *et al*.[32] observed DNA instability and an inverse correlation between telomere length and coal exposure in the mine workers but did not detect correlations between aTL and DNA methylation. We also performed correlations examining the relationship of the percentage of global DNA methylation with sex, age and tissues; however, no correlation was found, probably due to our small sample size, despite detecting more global DNA methylation, with no significant difference, in exposed males than in exposed females. The effect of sex, age and tissue variations in the levels of DNA methylation has been widely discussed, as different studies report different findings, especially when considering the possibilities of inter-individual differences[45–48]. These variations can occur due to the differences in the methods used to quantify DNA methylation, the heterogeneity of tissue types, the sample size and the type of analysis used to evaluate the global methylation content or the locus specific methylation.

Coal extraction and burning have caused environmental and human health problems in South Brazil, in both mining regions and nearby regions (e.g., acid rain in Uruguay)[49,50]. However, analytical techniques that characterize the levels of known contaminants do not provide insight into the biological risks associated with pollution[51] nor the effect on natural population structure. Although *C. torquatus* are subterranean rodents, they normally come up to the surface to collected food and clean their burrows. They are herbivorous animals, digging their den with their paws and teeth. Some animals that are bred in contaminated environments are directly exposed to these contaminants, but herbivores, such as the subterranean rodents analyzed in this study, can be exposed through contaminated food, as observed by Menezes *et al*.[31] in plants from Candiota, which had hight level of inorganic elements. Thus, these animals expose themselves to the contaminants in the soil and plants, in their microenvironment and in the atmosphere in regions with or without pollutants.

Our results demonstrated a possible relationship between age and DNA damage. Adults and juveniles of tuco-tucos from Candiota had higher DNA damage than rodents of the same age group from Butiá (Fig. 4). Increase genetic alterations, such as chromosomal structural aberrations and aneuploidies, are associated with aging[52]. Increase as these, in the levels of cell damage associated with age, were found in whales exposed to PHAs, with the adults showing significantly more cytochrome P450 expression than juveniles[53]. The data reveal that the estimated size of the population of Candiota (the region where the rodents had higher DNA damage) is larger than the population sizes at Pelotas and Butiá but there was lower frequency of recaptures in Candiota (Table 1). However, it can also be noted that the genotoxic damage was the greatest in the adult organisms, of which there were fewer in Candiota, leading to the assumption that - among others - there is an increase in mortality rate as a result of the probable cumulative damage. Additionally, Roratto *et al*.[54] examined the phylogeographic pattern of *C. torquatus* and found approximately 40 haplotypes. In the regions where the populations were analyzed that were under the influence of coal, four haplotypes were found in Minas do Leão, a region that only extracts coal and is also at the center of the distribution range. In Pelotas, the location that served as control over the other two populations, there were two haplotypes found in a peripheral geographical region. Candiota, which is the center of our toxicology analysis, showed only one haplotype and is also located centrally in the geographical distribution of the species. These data indicate low genetic variability in Candiota. Regarding genetic variability, the same relationship was observed as with DNA damage; however, in the ascending order was reversed, i.e., Candiota $<$ Pelotas $<$ Butiá. In a review by Hamilton *et al*.[7], the disappearance of 16 fish species in Belews Lake in the USA was reported, which was assigned to selenium contamination from a coal ash disposal basin.

The results of our study describing the population size in the three evaluated cities suggest that coal exposure does not interfere with *C. torquatus* population size or sex ratio, but instead impacts the structure of the population, as reflected by adults representing the largest number of recaptured individuals. Furthermore, *C. torquatus*

suffer DNA damage/instability, as observed in the comet assay (DNA damage), and telomere shortening, likely as a consequence of the oxidative damage that results from their exposure to a composite mixture, including inorganic elements such as those found in the soil[23,33] and lung of *C. torquatus* and organic elements such as hydrocarbons[23]. The Candiota population is impacted by coal, and certainly other mammal populations could also be affected. Almeida and Freitas[55] wrote about the effects of coal on the cranial morphology of *C. torquatus*. This study reports a relationship of environmental influences and pressures with ontogenetic trajectory. These results indicate that the vertebrate fauna should be constantly monitored, and here we suggest that species with a low "home-range" such as *Ctenomys* can be affected.

In conservation genetics, these analyses that were conducted with *C. torquatus* represent a new way to evaluate the anthropogenic effect on natural mammalian populations. We analyze three new points of view (genotoxicity, telomere length and methylation) as well as addressing conservation genetics, which is a field that is always concerned about the genetic diversity of small populations. Thus, these results, which showed a correlation of coal exposure with damage to the genome and decreased telomere length, suggest that such effects underlie the effects that have always been analyzed in conservation genetics, including are population parameters and genetic variability. Our study is important due to the emphasis on the viability of genotoxic assays and epigenetic tools in conservation studies. Currently, we are observing an increasing trend of cooperation among ecotoxicologists and conservationists, as evidenced by the growing number of studies with this approach, which can improve both conservation and evolution studies. More studies are needed to estimate the population size and increase the sample sizes to know the current status of these populations.

Materials and Methods

Approval and Accordance. *Approval.* This study was performed with the permission of the official Brazilian Environmental Protection Agency - IBAMA (14690-1).

Accordance. The sampling methodology used in this study was in accordance with all regulations and guidelines from the Ethics Committee on the Use of Animals of the Universidade Federal do Rio Grande do Sul (CEUA - 31925).

Sampling. The samples used in this study were collected by Silva *et al.*[22–24]. *Ctenomys torquatus* (Ctenomyidae-Rodentia) individuals were captured at two locations in different environments in RS: the individuals in the control group were captured in Pelotas (31°S, 52°W), a region without a coal mine and power plant; and the individuals in the exposed group were captured at two locations, Butiá (30°S, 51°W), a region approximately 5 km from a strip coal mine, and Candiota (31°S, 54°W), a region about 2 km from the Presidente Médici coal power plant. All populations presented the same diploid number, 2n = 44 (Ref.[25]) (Fig. 1).

The natural populations of the rodents were monitored by Silva *et al.*[22–24]. The *C. torquatus* age groups were determined according to the[56] method, which is based on body weight. Three age classes for *C. torquatus* were determined by weight: juveniles, females up to 125 g and males up to 135 g; subadults, females from 125 to 185 g and males from 135 to 225 g; and adults, above the limits of the subadults[22,23].

The tissue samples used in this study to determined absolute telomere length (aTL) and global DNA methylation were obtained from the collection of the Laboratório de Citogenética e Evolução of the Universidade Federal do Rio Grande do Sul, and we used the samples that were available.

DNA Extraction. DNA extraction was performed on the tissues from 30 controls (24 females and 6 males) and 36 exposed individuals (30 females and 6 males). Total DNA was extracted from liver, kidney, muscle and skin tissue samples using a standard phenol: chloroform protocol[57].

Genotoxicity Measurement by Comet Assay. The alkaline comet assay (CA) in blood cells was performed as described by Singh and Stephens[58] as modified in Silva *et al.*[24] for field work. Images of 25 randomly selected cells (in duplicate) were analyzed from each animal. Cells were visually classified according to tail size into five classes: undamaged (0) to maximally damaged (4), resulting in a single DNA damage score for each animal and consequently to each studied group. Therefore, the group's damage index (DI) could range from 0 (completely undamaged, 50 cells X 0) to 200 (maximum damage, 50 cells X 4).

Telomere Length Analysis by qPCR. Telomere length was measured from the total genomic *C. torquatus* DNA of 30 controls (22 females and 8 males) and 36 exposed individuals (30 females and 6 males) by using a real-time quantitative Polymerase Chain Reaction (qPCR) method following the protocol described by Callicott and Womack[59], with slight modifications. The forward and reverse primer sequences for the telomeric region gene were 5′ CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG TTT GGG TT 3′ and 5′ GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3′, respectively. Primers for the mouse 36B4 gene corresponded to the acidic ribosomal phosphoprotein PO (36B4) gene, which is well conserved. The forward and reverse primers used in the 36B4 portion of the assay were 5′ ACT GGT CTA GGA CCC GAG AAG 3′ and 5′ TCA ATG GTG CCT CTG GAG ATT 3′, respectively[59]. Each reaction examining the telomere and 36B4 fragments included 12.5 μl SYBR Green PCR Master Mix (Quatro G), 300 nM telomere primers (forward and reverse), 300 nM 36B4 forward primer and 500 nM 36B4 reverse primer, 20 ng genomic DNA, and enough water was added to yield a 25-μl reaction. Three 20-ng samples of each DNA mixture were placed in adjacent wells of a 96-well plate for the telomere and 36B4 assays and analyzed using the Step One Plus TM Real Time PCR System (Applied Biosystems, Foster City, CA, USA). For the telomere amplicons, qPCR was performed using the following reaction conditions: set at 95 °C for 10 min; followed by 30 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 56 °C for 1 min. For the 36B4 amplicons, the reaction conditions were an initial step at 95 °C for 10 min followed by 35 cycles of data collection at 95 °C for 15 s, with 52 °C annealing for 20 s, followed by extension at 72 °C for 30 s.

Serially diluted DNA standards ranging from 0.103 to 25 ng/μL (3-fold dilution; six data points) were used to generate the standard curves for the telomere and 36B4 fragments on each 96-well plate. The Tel STD curve was used to measure the telomeric content per sample in kilobases (kb), while the 36B4 STD curve was used to measure the number of diploid genome copies per sample. The qPCR method used to evaluate aTL was adapted in our laboratory, and our results showed reproducible and consistent standard curves for both the telomere and 36B4 (single copy gene) standards (see in Supplementary Information Figures S1 and S2). The cycle threshold (Ct) of the telomere qPCRs ranged from 6 to 15, and all target samples were within the standard linear range. All samples were analyzed in triplicate, with negative and reference controls as well as standard curves. The Ct point of each sample was used to calculate the total aTL in kb per *C. torquatus* diploid genome. Individual samples with a standard deviation of $Ct < 1$ for the triplicate samples were included in the full analysis.

Global DNA Methylation Analysis by HPLC. Global DNA methylation (5-mdC) levels were quantified in the isolated DNA of 30 controls (22 females and 8 males) and 35 exposed individuals (30 females and 5 males) based on the proportional quantification of 5-mdC using high performance liquid chromatography (HPLC) as detailed elsewhere[60,61]. Briefly, DNA was hydrolyzed with nuclease P1 and alkaline phosphatase to yield 2′-deoxymononucleosides, which were isolated by HPLC and detected by ultraviolet (UV) light. A mixture of deoxyadenosine, deoxythymidine, deoxyguanosine, deoxycytidine, 5-methyl-2′-deoxycytidine and deoxyuridine (Sigma-Aldrich) was used as a standard. The percentages of global genomic DNA methylation were calculated by the integration of the 5-mdC peak area (obtained from the HPLC analysis) relating to global cytidine (methylated or not). The average for each sample was calculated and duplicated samples, showing a difference in 5-mdC greater than 3% or with low HPLC resolution, were removed.

Statistical Analysis. The Bartlett-Box test was used to evaluate DNA damage (CA) and the variation in the homogeneity of each site group. The damage index (DI) was calculated based on the number of cells with tails versus those without tails. The statistical evaluation was performed using ANOVA (Dunnet). The Kruskal Wallis test was used to compare significant differences in aTL among the Pelotas, Butiá and Candiota groups. The correlations between telomere length and the other characteristics (sex, age and tissue) were evaluated by Spearman's test. To statistically analyze the methylation data, we applied nonparametric tests, such as the Mann–Whitney-Wilcoxon test to identify differences between the exposed and control individuals in binary variables or the Kruskal-Wallis test for variables with more than two states. Spearman's test was also used to examine the correlation of methylation data with tissue, age and sex. Additionally, we evaluated whether there was any correlation between aTL and the methylation data. $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed with GraphPad PRISM software, version 5.01 (GraphPad Inc., San Diego, CA).

Data Availability

The datasets generated during and current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). Readers are welcome to comment on the online version of the paper.

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Author Contributions

T.R.O.F. and J.S. conceived of the original idea. C.A.M., J.S., A.L.H.G. and M.C. conceived and planned the experiments. C.A.M., A.L.H.G. and J.S. conducted the experiments. C.A.M. wrote the manuscript with support from J.S. and T.R.O.F. All authors provided critical feedback and helped shape the research, discussed the results, analysis and manuscript.

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CAPÍTULO 3

Using telomeric length measurements and methylation to study the karyotype evolution of small fossorial mammals.

Artigo em preparação para ser submetido.

Using telomeric length measurements and methylation to study the karyotype evolution of small fossorial mammals

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ABSTRACT

The genus *Ctenomys* has been widely used in karyotype evolution studies due to the variation in their diploid numbers (2n), which range from 2n = 10 to 2n = 70. Among them, *Ctenomys minutus* is characterized by intraspecific variation in 2n, which makes it an interesting model to investigate the genomic instability mechanisms that have led to different cytotypes in this species. Despite the role of telomeres in genomic rearrangements, studies concerning telomere length and global DNA methylation techniques have never been applied to *Ctenomys* to explain their karyotype evolution. Hence, we aimed to contribute to the knowledge about telomeres' role in chromosomal instability and global DNA methylation in the genome evolution of *C. minutus*. Interstitial telomere sequence (ITS) are short array blocks of telomeric- type DNA in intrachromosomal sites, which can be produced by chromosomal rearrangements, among other mechanisms. The most common epigenetic mark, DNA methylation, is correlated with telomere length regulation in subtelomeric regions. In this study, we found that telomere length differs between cytotypes sex-dependent in relation to females and that global DNA methylation differs between age groups. In general, we observe that the cytotype with more rearrangement has the largest telomere length and is more unstable because of its poor methylation. Instead, the cytotype with less rearrangement has the shortest telomere length and would be the most stable cytotype. These findings may help us to elucidate the evolution of cytotypes in *C. minutus*.

KEYWORDS: Telomere length; Epigenetic; Chromosome evolution; FISH; Interstitial telomere sequence; *Ctenomys*.

INTRODUCTION

The telomere is a nucleoprotein complex that protects the ends of the chromosomes against the action of nucleases, preventing DNA degradation, recombination, or fusion with other chromosomes (Blackburn et al. 2006). In vertebrates, the telomere is composed of a tandem repetition of hexamer (TTAGGG)n. It is located adjacent to

subtelomeric regions poor in genes, which are also enriched in repetitive DNA (De Lange 2005). Telomerase reverse transcriptase (TERT), associated with telomerase RNA subunit (TERC) and other proteins, forms an enzymatic ribonucleoprotein complex that synthesizes telomeres at the ends of the chromosome, controlling the length of the telomeric repetitions (Blackburn and Collins 2011; Zakian 2012). The shortening of telomeric length may be related to the increase in numerical chromosomal aberrations (Tamayo et al. 2011).

Several methods can be used to analyze telomere length, one of which is the Quantitative PCR (qPCR) methods, which denature DNA prior to labeling or amplification and, consequently, detect true telomeres and interstitial telomeric sequence (ITS) (Foote et al. 2013). Several mammalian species present repetitions of short blocks of telomeric-like DNA in intrachromosomal sites (ITSs) (Nergadze et al. 2004), including those repeats located close to the centromeres and those found between the centromeres and the telomeres (Lin and Yan 2008). These sequences can be generated by mechanisms of mutation and repair mistake, inadequate crossing over, transposition, or they may be indicative of a chromosomal rearrangement such as inversion, centric or tandem fusion, which can arise during karyotype evolution (Meyne et al. 1990; Pagnozzi et al. 2000; Blasco et al. 2004). Chromosomal sites composed of ITSs have been detected in widely different vertebrate species (Meyne et al. 1990), fishes (Ocalewicz 2013), anuran (Nanda et al. 2009; Schmid and Steinlein 2016), squamate reptiles (Rovatsos et al. 2015), birds (Nanda et al. 2002), marsupials (Metcalfe et al. 1998, 2002, 2004), rodents (Ventura et al. 2006; Rovatsos et al. 2011), and plants (Tek and Jiang 2004; He et al. 2013). The function of the telomerase enzyme, together with a variety of telomere-binding proteins, is required to maintain adequately long telomeres, certifying stability to the linear eukaryotic chromosomes. Nergadze et al. (2007) proposed that telomerase was used, in some cases, to repair double-stranded DNA breaks that arose in the rodent and primate genomes during chromosomal evolution, that the ITSs were inserted into the repair and that the ITSs could arise from the capture of telomeric fragments or telomerase action and that they were fixed during genome evolution.

Gonzalo et al. (2006) showed that mouse telomeric and subtelomeric chromatin contains histone modifications commonly found in heterochromatin and that subtelomeric DNA can be methylated. DNA methylation, the most common epigenetic mark, is the addition of a methyl group (CH_3) to cytosine at the CpG dinucleotide, forming 5-methyl-2'-deoxycytidine, or 5-mdC by a DNA (cytosine-5)-methyltransferase 1 (DNMT1) enzyme (Armstrong et al. 2011). Histone modifications in telomeric chromatin or DNA methylation in subtelomeric regions are correlated with telomere length deregulation (Blasco 2004). Increasing evidence indicates the existence of functional links between these epigenetic marks and homeostasis of telomere length (Blasco 2007).

Rodents have strongly contrasting genomic organizations and are examples to study the role of chromosomal rearrangements in speciation. Studies by classical cytogenetics show that rodents display large karyotypic diversity, with diploid numbers ranging from 2n = 10 in *Ctenomys steinbachi* (Ctenomyidae) (Anderson et al. 1987) to 2n = 102 in *Tympanoctomys barrerae* (Octodontidae) (Gallardo et al. 1999; Svartman et al. 2005). The genus *Ctenomys* has about 65 species described (Teta and D'Elía 2020) with high rates of chromosomal variation that vary from 2n = 10 to 2n = 70 (Reig et al. 1990). Additionally, there are species with significant chromosomal variation such as *C. pearsoni* (2n = 56, 64 and 70), *C. boliviensis* (2n = 42 - 46), *C. rionegrensis* (2n = 48, 50, 56 and 58), *C. minutus* (2n = 42-50), *C. lami* (2n = 54-58), *C. talarum* (2n = 44 - 48) and *C. perrensis* (2n = 50, 54, 56 and 58) (Novello and Lessa 1986; Cook et al. 1990; Reig et al. 1992; Freitas 1997; Massarini et al. 2002; De Freitas 2007). Populations of *C. minutus* have autosomal chromosomes characterized by the numerical variation between biarmed (from 14 to 17 pairs) and acrocentric (from 3 to 10 pairs) chromosomes, thus forming different karyotypes (De Freitas 2006). This species has chromosomal variation, comprising seven parapatrically dispersed parental karyotypes (2n = 50a, 46a, 48a, 42, 46b, 48b and 50b) and six intraspecific hybrid zones with transitional karyotypes: (I) 50a x 48a = 49a; (II) 46a x 48a = 47a; (III) 48a x 42 = 43,

44, 45, 46; (IV) 42 x 46b = 43, 44, 45; (V) 46b x 48b = 47b; and (VI) 48b x 50b = 49b (Freitas 1997; Gava and de Freitas 2002, 2004; Gava and Freitas 2003; Freygang et al. 2004; Castilho et al. 2012; Lopes et al. 2013). The karyotypes show an instigating geographic distribution, where 2n = 50 are found at the extremes of the distribution, and this number is reduced to approximately the central region of the distribution, presenting 2n = 42. Studies show Robertsonian rearrangements, fissions and fusions or tandem fissions, and fusions, and a pericentromeric inversion, which occurred in the chromosomes, originating even the 'a' and 'b' systems (Freitas 1997; Gava and Freitas 2003; Freygang et al. 2004).

Considering the high frequency of chromosomal rearrangements among *C. minutus* populations, this species is an excellent model for studying the mechanism of genomic instability that originated and continues to originate different karyotypes in this species. In addition, there is a lack of studies with molecular cytogenetic techniques to explain these different cytotypes that occur in this species. Thus, this study aims to contribute to the knowledge about the role of telomeres in chromosomal instability and global DNA methylation in the karyotype diversity of *C. minutus* throughout its geographic distribution.

MATERIAL AND METHODS

Specimens studied

In this study, we used fresh biological material to obtain chromosome preparations and biological material from the animal captured using Oneida-Victor number 0 Snap traps under the guidelines of the American Society of Mammologists' Animal Care Committee (Sikes 2016). The animals were collected in the municipalities of Bacupari, Bojuru, Jaguaruna, Mostardas, Praia do Barco, São José do Norte and Tavares.

The experiments were performed following all regulations and guidelines from the Ethics Committee on the Use of Animals of the Universidade Federal do Rio Grande do Sul (CEUA -31925). The sampling methodology was performed following the permission of the official Brazilian Environmental Protection Agency - IBAMA (14690-1).

C. minutus age groups were established according to the Wilks (1963) method, which is centered on body weight. Three age groups for *C. minutus* were defined by weight: juveniles, females up to 125 g and males up to 135 g; subadults, females from 125 to 185 g and males from 135 to 225 g; and adults, above the limits of the subadults (da Silva et al. 2000).

Cell culture and chromosome preparations

Lung biopsies were collected from one adult *C. minutus* per cytotype in the geographic regions related to the different cytotypes, except in Tavares (two adults) (see Fig1). To execute cell culture, according to Verma and Babu (1996), the fibroblast cultures were grown at 37 °C in medium-high glucose (Dulbecco's Modified Eagle's ,Gibco) enriched with 15% fetal bovine serum (GIBCO), penicillin (100 units/ml) and streptomycin (100 mg/ml). Chromosome preparations were made following standard methods, which consist of 1 hour in colchicine, 15 minutes in hypotonic solution (0.075 M KCl), and fixation. The diploid number and chromosomal morphology were established from at least 50 metaphase chromosomes per individual, stained with Giemsa 10% followed by air drying.

To determine telomere length and global DNA methylation, we used tissue samples available in the animal collection of the Laboratório de Citogenética e Evolução of the Universidade Federal do Rio Grande do Sul for DNA extraction.

DNA Extraction

DNA extraction was performed on the tissues from 150 individuals (90 females and 60 males). Total DNA was extracted from muscle tissue samples using the standard phenol: chloroform protocol (Green and Sambrook 2018).

Fluorescence in situ hybridization (FISH)

Biotin-labeled (TTAGGG)n probes were obtained by PCR using the DNA of *C. minutus* as DNA template and detected with Cy3-streptavidin (Ijdo et al. 1991) to detect the location of telomeric sequences on the chromosomes of seven different karyotypic forms of *C. minutus* (2n=50a and b; 48a and b; 46a and b, and 42). At least 10 metaphase spreads per individual were analyzed to confirm the FISH results. The slides were analyzed using a Zeiss Imager 2 microscope, 63x objective, and Axiovision 4.8 software (Zeiss, Germany).

Telomere Length Analysis by qPCR

Telomere length (TL) was quantified from the total *C. minutus* genomic DNA by using a real-time quantitative Polymerase Chain Reaction (qPCR) method following the protocol according to Callicott and Womack (2006), with minor modifications. The forward and reverse primer sequences for the telomeric region gene were 5' CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG TTT GGG TT 3' and 5' GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3', respectively. The mouse 36B4 gene primers related to the acidic ribosomal phosphoprotein PO (36B4) gene, which is well conserved. The forward and reverse primers used in the 36B4 fraction of the assay were 5' ACT GGT CTA GGA CCC GAG AAG 3' and 5' TCA ATG GTG CCT CTG GAG ATT 3', respectively. Each reaction analyzing the telomere and 36B4 fragments comprised 12.5 µL SYBR Green PCR Master Mix (Quatro G), 300 nM telomere primers (forward and reverse), 300 nM 36B4 forward primer and 500 nM 36B4 reverse primer, 20 ng genomic DNA, and an adequate amount of water was added to yield a 25 µl reaction. Three 20 ng samples of each DNA mixture were put in adjacent wells of a 96-well plate for the telomere and 36B4 assays and analyzed using the Step One Plus TM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For the telomere amplicons, qPCR was done using the following reaction conditions: set at 95 °C for 10 min; followed by 30 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 56 °C for 1 min. For the 36B4 amplicons, the reaction conditions were an initial step at 95 °C for 10 min followed by 35 cycles of data collection at 95 °C for 15 s, with 52 °C annealing for 20 s, followed by extension at 72 °C for 30 s.

Serially diluted DNA standards ranging from 0.384 to 37.5 ng/µL (3-fold dilution; six data points) were used to produce the standard curves for the telomere and 36B4 fragments on each 96-well plate. The Tel STD curve was used to quantify the telomeric content per sample in kilobases (kb), while the 36B4 STD curve was used to quantify the number of diploid genome copies per sample. The qPCR method used to assess aTL was adapted in our laboratory, and our results presented reproducible and consistent standard curves for both the telomere and 36B4 (single-copy gene) standards (see in Online Resource 1). The cycle threshold (Ct) of the telomere qPCRs ranged from 7 to 13, and all target samples were within the standard linear range. All samples were evaluated in triplicate, with negative and reference controls as standard curves. The Ct point of each sample was used to calculate the total aTL in kb per *C. minutus* diploid genome. Individual samples with a standard deviation of Ct < 1 for the triplicate samples were included in the complete evaluation.

Global DNA Methylation Analysis by HPLC

Global DNA methylation (5-mdC) levels were quantified in the isolated DNA based on the proportional quantification of 5-mdC using high-performance liquid chromatography (HPLC) as described in Berdasco M, Fraga MF (2009) and Cappetta et al. (2015). Quickly, DNA was hydrolyzed with nuclease P1 and alkaline phosphatase to yield 2-

deoxymononucleosides, which were isolated by HPLC and detected by ultraviolet (UV) light. A mixture of deoxyadenosine, deoxythymidine, deoxyguanosine, deoxycytidine, 5-methyl-2-deoxycytidine, and deoxyuridine (Sigma-Aldrich) was used as a standard. The percentages of global genomic DNA methylation were calculated by integrating the 5-mdC peak area (obtained from the HPLC analysis) relating to global cytidine (methylated or not). The median for each sample was calculated, and duplicated samples, indicating a difference in 5-mdC greater than 3% or with low HPLC resolution, were eliminated.

Chromosome Rearrangements

Freygang et al. (2004) observed specific chromosomal rearrangements that differentiate the *C. minutus* karyotypes, and we analyzed them for TL and 5-mdC. The analyzed rearrangements are characterized according to the study by Freygang et al. (2004): Character 01 (pairs 20 and 17 separated in the karyotype 2n = 50a and fused in the other karyotypes); character 04 (metacentric chromosome 2 in karyotypes 2n = 50a, 46a and 42; acrocentric showing inversion in arm 2p in karyotypes 2n = 46b, 48b and 50b; chromosome 2 acrocentric in karyotype 2n = 48a); character 07 (pairs 23 and 19 separated in karyotypes 2n = 50a and 50b and fused in the other karyotypes); character 10 (pairs 22, 24 and 16 fused in karyotype 2n = 42; pairs 24 and 16 fused in karyotype 2n = 46b; separate pairs in karyotypes 2n = 50a, 48a, 46a, 48b and 50b); character 15 (positive signal of constitutive heterochromatin (CH) of chromosome pair 08 in karyotypes 2n = 50a, 48a, 46a and 42).

Data Analysis

The telomere length and 5-mdC data presented asymmetric distribution and/or heterogeneity of variances. Then we used a one-way ANOVA with Brown-Forsythe (B-F) robust test for equality of means and Tamhane test for multiple comparisons to evaluate differences among karyotypes, sex, and age groups. We checked the results using Kruskal-Wallis (KW) nonparametric ANOVA, followed by pairwise Wilcoxon-Mann-Whitney (WMW) tests. Next, we employed Generalized Linear Models (GZLM) to evaluate the effects of the three factors karyotype, sex, and age group in the same model, to investigate possible interaction effects. Based on the deviance results, we chose a gamma distribution with log link for global DNA methylation, and telomere length values were transformed into natural Log (Ln). All interactions between the factors were initially considered in the models but dropped in sequence if statistically non-significant. Using the GZLM approach, adjustments for multiple comparisons between groups were performed using the sequential Bonferroni procedure. We evaluated whether there was any correlation between TL and the 5-mdC data using the nonparametric Spearman rank correlation coefficient. To analyze the rearrangements, we used Kruskal-Wallis, ANOVA (Tukey), Mann- Whitney, and t-test. P values ≤ 0.05 were considered statistically significant. The analyses were performed with SPSS version 18 and GraphPad PRISM software, version 5.01 (GraphPad Inc., San Diego, CA)

RESULTS

Karyotype description and telomere sequence mapping

A tuco-tuco specimen 2n = 45b was collected in Tavares, Rio Grande do Sul, where 2n = 48b is commonly found; however, there is a contact zone between 2n = 48b and 42 karyotypic forms in this local (Freitas 1997; Gava and Freitas 2003).

The signals of the (TTAGGG)n sequences were observed on both telomeric ends of all chromosomes of seven different karyotypic forms of *Ctenomys minutus* (2n = 50a, 50b; 48a,48b; 46a, 45b and 42) tested here (Fig2. a-g).

Interstitial signals (ITS) were only found in the pericentromeric region of chromosome 1 of cytotype 2n = 50b (Fig2 c). There were no ITSs in the sex chromosomes.

Telomere Length (TL)

To analyze telomere length in different cytotypes, a total of 134 individuals of *C. minutus* DNA were used. As the distribution of the data was asymmetric, we used log transformation in the first comparisons among cytotypes, sexes, and age groups. No difference between the cytotypes was statistically significant (ANOVA P = 0.473) (Fig3 a). Age groups and sexes did not either differ (ANOVA p values 0.410 and 0.076, respectively) (Fig3 b and c). Using GZLM analysis, however, we observed statistically significant interactions between cytotype and sex (P = 0.015), indicating sex-dependent differences among cytotypes in TL length. In the multiple pairwise comparisons, no differences between cytotypes were observed in males (P > 0.20 in all comparisons). However, in females, the telomere length of 2n = 50a was shorter than that of 2n = 48a (P = 0.008), 2n = 46a (P = 0.048) and 2n = 42 (P = 0.001) (Fig4).

Concerning rearrangement characters, no statistically significant difference was found between them and the telomere length: character 01 (P = 0.09), character 04 (P = 0.937), character 07 (P = 0.289), character 10 (P = 0.85) and character 15 (P = 0.757) (Fig5 a-e).

Global DNA Methylation

We measured global DNA methylation in a total of 124 individuals of *C. minutus* DNA by quantifying 5-mdC in different cytotypes. In the first analysis, we did not observe significant differences among cytotypes (ANOVA KW P = 0.088) (Fig6 a) or between sexes (ANOVA KW P = 0.097) (Fig6 c). The difference among age groups was statistically significant (ANOVA Kruskal-Wallis P = 0.048) (Fig6 b). Pairwise WMW indicated a significant difference between juveniles and subadults (P = 0.011) and between juveniles and adults (P = 0.048). When analyzing all factors together in the GZLM model, we found no suggestion of interaction effects. The P values of the main effects were karyotype (P = 0.187), sex (P = 0.199), and age group (P = 0.087).

Regarding the rearrangements, there were no statistically significant differences between the characters and global DNA methylation: character 01 (P = 0.05), character 04 (P = 0.269), character 07 (P = 0.847), character 10 (P = 0.367) and character 15 (P = 0.283) .MW and ANOVA (Kruskal-Wallis) (Fig7. a-e).

We did not observe a correlation between TL and 5-mdC values (Spearman´s r = 0.065; P = 0.501).

DISCUSSION

In this study, for the first time, FISH, telomeric length and global DNA methylation were analyzed together in the different cytotypes of *C. minutus*, to better understand the evolution of these cytotypes.

We observed that all the cytotypes of *C. minutus* included in our study showed telomeric FISH signals at the end of all chromosomes. In addition, contrary to the finding by Freygang et al. (2004), an ITS signal was found in the pericentromeric region on chromosome 1 of cytotype 2n= 50b (Fig2 g). This ITS region may indicate a fusion between chromosomes 7 and 5 of *C. flamarioni*, found recently in the cytotype 2n = 46a of *C. minutus* by Kubiak et al. (2020). De Freitas (2006) also observed a fusion between chromosomes 23 and 13 of *C. lami* in chromosome 1 from a hybrid individual of *C. minutus* and *C. lami*. They may be a remnant of the chromosomal rearrangement produced during karyotype evolution, as ITS regions are considered fragile sites and susceptible to spontaneous and induced chromosomal breaks (Slijepcevic et al. 1996). The absence of ITS in the other cytotypes suggests that the telomeric repetitions were lost due to a progressive reduction or degeneration through small chromosomal organizations and point mutations (Ruiz-Herrera et al. 2009). This absence of ITS after chromosomal rearrangements has also been reported in

Ctenomys magellanicus (Lizarralde et al. 2003) and in species of *Oligoryzomys* (De Jesus Silva and Yonenaga-Yassuda 1997), *Nectomys* (De Jesus Silva and Yonenaga-Yassuda 1998), and *Rhipiidomys* (Silva 1999).

Telomeres protect the ends of chromosomes to stabilize the nuclear genome with high fidelity during early adulthood but usually decrease that fidelity in normal cell aging and disease progression (Shay 2018). Studies have been observed in several species, with greater attrition in the telomeres in males than in females (Barrett and Richardson 2011), including this study (Fig3 c). We found that the differences between the cytotypes are sex- dependent (Fig4). Although chromosomal rearrangements that differentiate cytotypes also occur in males, differences in TL were observed only in some cytotypes in females. There are some hypotheses to this, the inactivation on the X chromosome (Surrallés et al. 1999) and sex size dimorphism, where males are larger and suffer more duplication of cells (Stindl 2004). The relationship between TL shortening and aging was not observed in this study, although juvenile organisms presented larger telomeres than subadults and adults (Fig3 b). The relationship between telomere length and aging has been demonstrated in avian (Heidinger et al. 2012; Tricola et al. 2018; Whittemore et al. 2019), cats (McKevitt et al. 2003) and other rodents (Coviello-McLaughlin and Prowse 1997).

Although not having a significant difference in TL among the different karyotypes, Fig3 a show that the cytotype 2n = 42 has the highest TL mean and is the cytotype with more fusions. In Fig5, we can observe that the cytotypes with the highest number of fusions have the highest TL mean. According to Blasco (2004), the promoter region of TERT, which regulates telomerase, is a CpG- rich island and consequently regulates TL. Lin and Yan (2008) suggested that DNA methylation could protect ITS-rich chromosome regions from breakage and play important roles in gene expression regulation. They proposed that hypermethylation favors ITS stability, whereas demethylated or hypomethylated ITS tends to be unstable. As we can conclude, due to the characteristics of the lowest global DNA methylation average, the highest TL average, and the greatest number of recombinations, the cytotype 2n = 42 is the most unstable among the different cytotypes studied.

Concerning the cytotype 2n = 50a, although not significant, it has the highest global DNA methylation average. According to Freitas (1997), this cytotype has 54% autosomal chromosomes with constitutive heterochromatin. Heterochromatin is highly methylated and negatively regulates TL. Moreover, the decrease in methylation in the subtelomeric region leads to an increase in telomeric recombination, such as sisters' chromatid exchange (Gonzalo et al. 2006). Thus, we suggest that cytotype 2n = 50a is the most stable among those studied.

In this study, we found a significant decrease in global DNA methylation in subadults and adults compared to juveniles (Fig6 b). This decrease is explained because, during aging, mammalian cells undergo a DNA methylation deviation, which alters the 5-methyl-cytosine distribution, resulting in this decrease. This decline occurs mainly in domains with repetitive sequences and constitutive heterochromatin, facilitating heterochromatin decondensation. It has been proposed that this occurs because of the loss of effectiveness of DNMT1 (Muñoz-Najar and Sedivy 2011).

In this study, signals of ITSs were observed in chromosome one of cytotype 2n = 50b. A chromosomal fusion in chromosome one has recently been identified in cytotype 2n = 46a (Kubiak et al. 2020). It is likely that ITSs in 2n = 50b indicate remnants of a chromosomal fusion. However, future studies are needed to address the chromosomal mechanism that maintained ITSs in the cytotype 2n = 50b and not in the 2n = 46a. In *C. minutus* species, we found significant differences between the karyotypes and telomere length sex-dependent - and an interesting difference in the general DNA methylation between ages. There are reasons to think that there are some advantages to *C. minutus* that present high rates of chromosomal variation. One obvious advantage seems to be the possibility of rapidly accruing a phenotypic effect from combinatorial acquisition of smaller-effect genetic changes. Telomeres may play a role in modulating the genome's evolution across species, and here we demonstrated some relation with the chromosome number, sex, and age of individuals.

The results presented in this study add knowledge about the roles of repetitive telomeric sequences and DNA methylation in the chromosomal evolution in the *Ctenomys minutus* species.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ana Leticia H. Garcia, Cristina A. Matzenbacher, Rafael Kretschmer and Mónica Cappetta. The first draft of the manuscript was written by Cristina A. Matzenbacher and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figure Captions

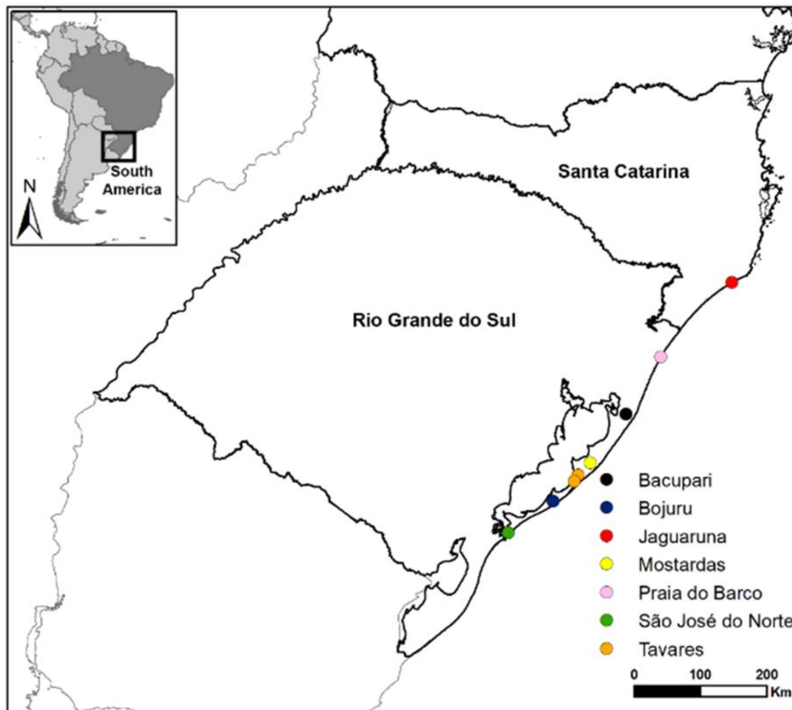

Fig 1 Locations where collections were made for the FISH experiment. Bacupari (2n = 48a), Bojuru (2n = 48b), Jaguaruna (2n = 50a), Mostardas (2n = 42), Praia do Barco (2n = 46a), São José do Norte (2n = 50b) and Tavares (2n = 46b)

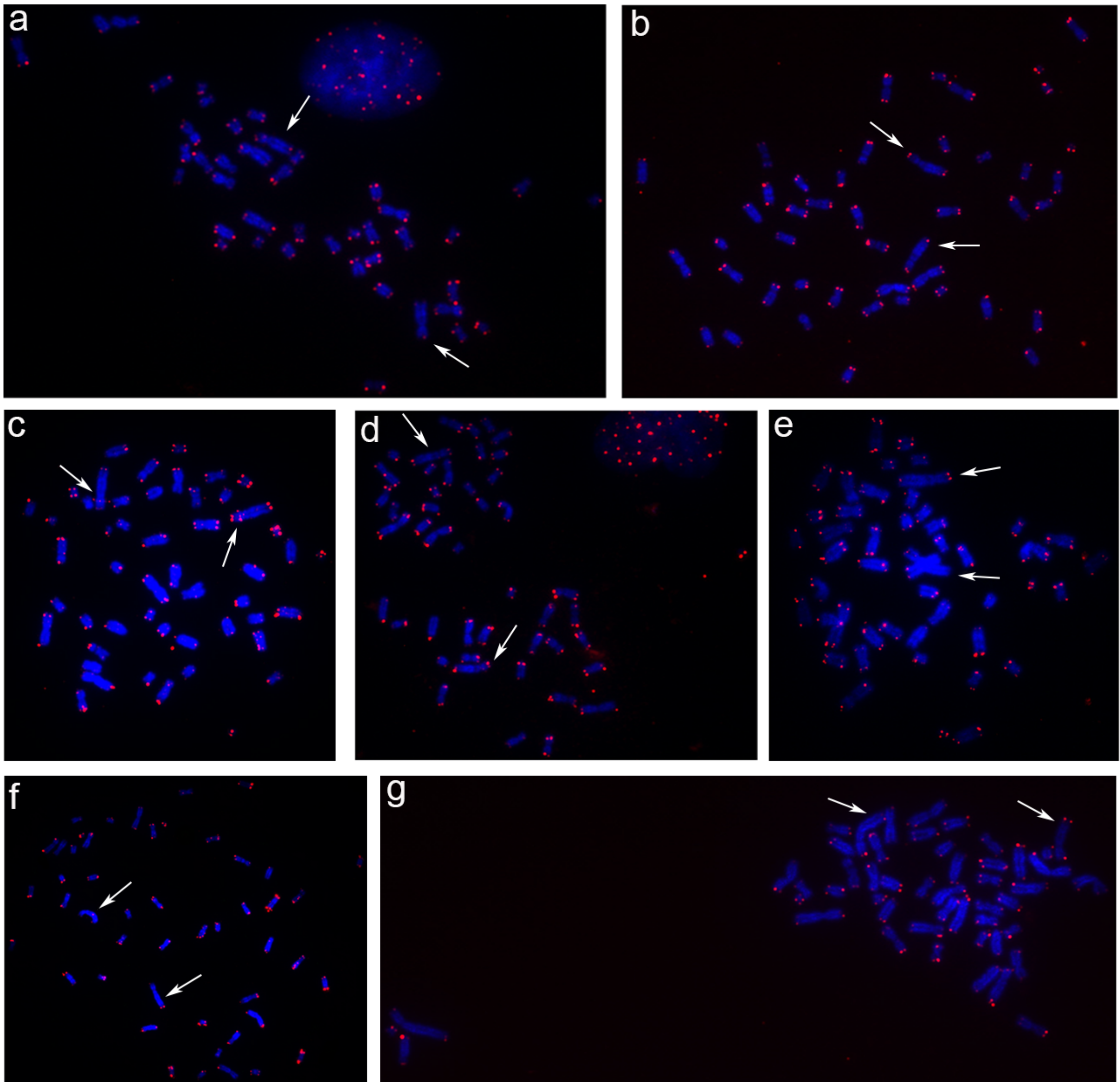

Fig 2 Fluorescence *in situ* localization of the telomeric sequence (TTAGGG)n in *C. minutus* with different diploid numbers: a) 2n = 42; b) 2n = 46a; c) 2n = 50b; d) 2n = 45b; e) 2n = 48b; f) 2n =48a; g) 2n = 50a. Arrows indicate chromosome 1 in all karyotypes.

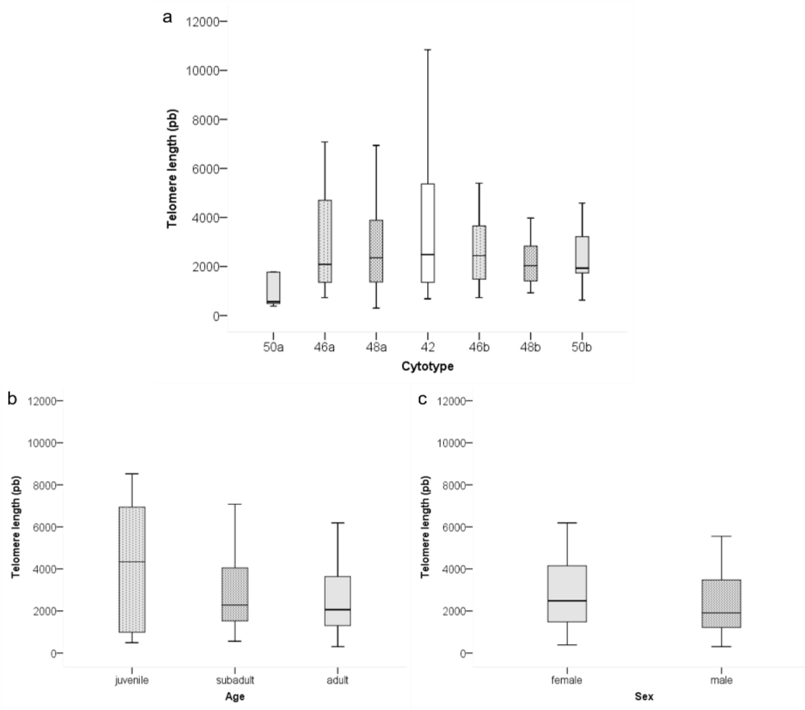

Fig 3 Mean values, standard deviation, minimum and maximum telomere length among the *C. minutus* cytotypes: a): 2n = 50a (04), 2n = 46a (22), 2n = 48a (34), 2n = 42 (11), 2n = 46b (16), 2n = 48b (17) and 2n = 50b (18) N of each karyotype; b) age classes: juvenile (09), subadult (51) and adult (62) N of juvenile, subadult and adult, respectively; and c) sex: female (71) and male (51) N of female and male, respectively

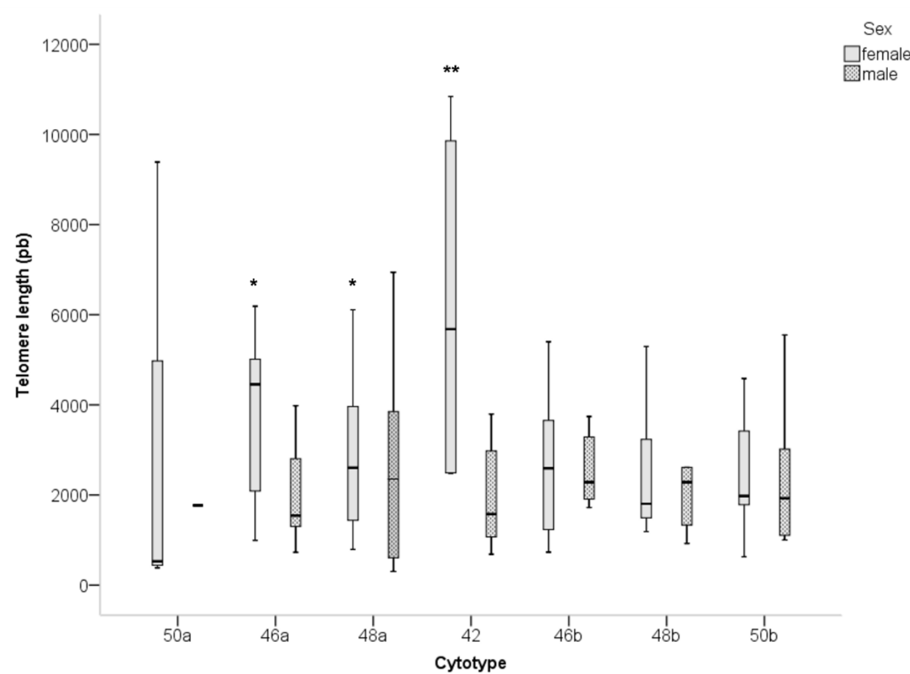

Fig 4 Telomere length among the *C. minutus* cytotype. Mean values, standard deviation, minimum and maximum by sex: 2n = 50a (04, 01), 2n = 46a (10, 14), 2n = 48a (22, 17), 2n = 42 (04, 07), 46b (13, 05), 2n = 48b (11, 06) and 2n = 50b (14, 05). N of females and males, respectively. * P < 0.05 and ** P < 0.001 in relation to 2n = 50a

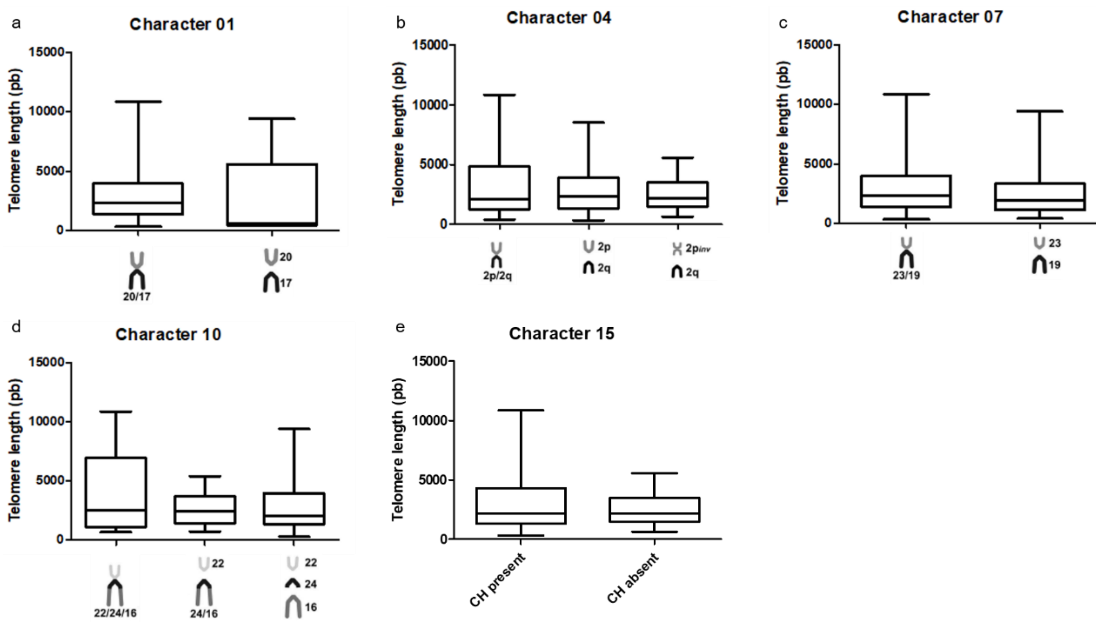

Fig 5 Comparison between telomere length and different rearrangements found in *C. minutus* cytotypes. a) chromosomes 20 and 17 fused (N = 129), separated (N =5); b) chromosome 2 metacentric (N = 41), acrocentric (N = 39) and 2p with inversion (N = 54); c) chromosomes 23 and 19 fused (N = 110), separated (N = 24); d) chromosomes 22, 24 and 16 fused (N = 11), 24 and 16 fused (N = 18), separated (N = 105); e) heterochromatin present (N = 80), absent (N =54). Adapted from Lopes et al., 2013

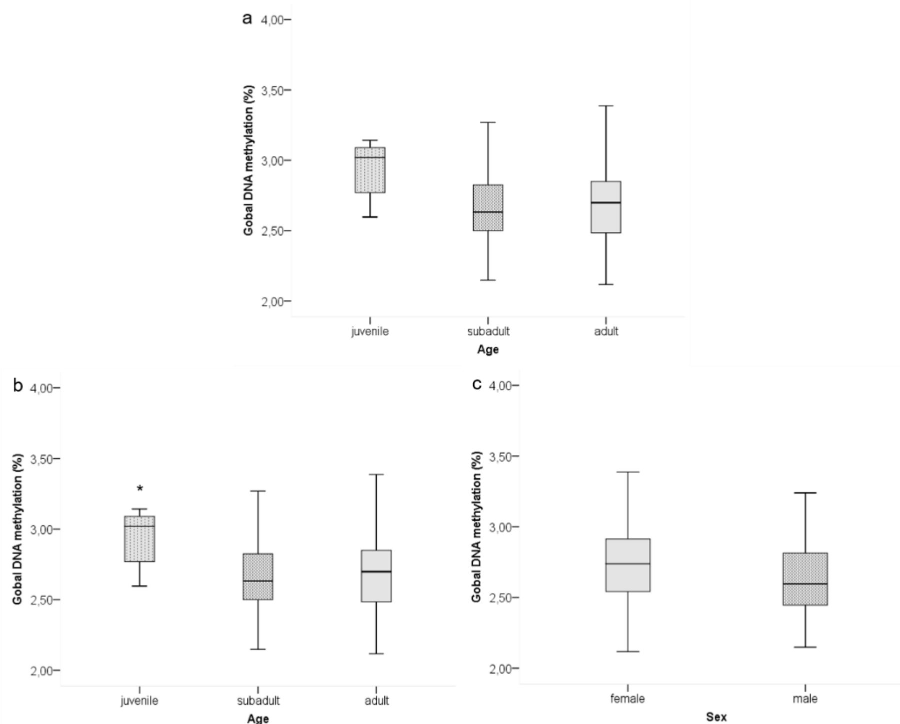

Fig 6 Mean values, standard deviation, minimum and maximum of 5-mdC. a) among the *C. minutus* cytotypes: 2n = 50a (06), 2n = 46a (19), 2n = 48a (35), 2n = 42 (13), 2n = 46b (14), 2n = 48b (14) and 2n = 50b (16) N of each karyotype; b) among age groups: juvenile (08), subadult (48) and adult (61); c) between sexes: female (73) and male (44).* $P < 0.005$ in relation to subadults (P WMW = 0.011) and adults (P WMW = 0.048)

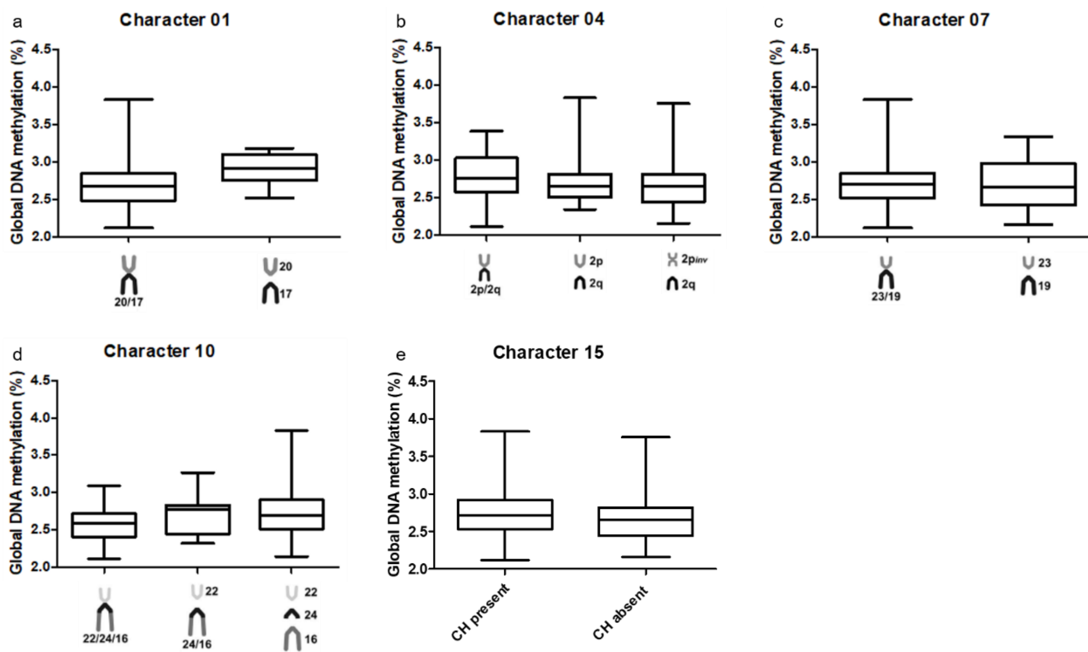

Fig 7 Comparison between global DNA methylation and different rearrangements found in *C. minutus* cytotypes. a) chromosomes 20 and 17 fused (N = 117), separated (N =7); b) chromosome 2 metacentric (N = 40), acrocentric (N = 38) and 2p with inversion (N = 46); c) chromosomes 23 and 19 fused (N = 100), separated (N = 24); d) chromosomes 22, 24 and 16 fused (N = 13), 24 and 16 fused (N = 15), separated (N = 96); e) heterochromatin present (N = 78), absent (N =46). Adapted from Lopes et al., 2013

Using telomeric length measurements and methylation to study the karyotype evolution of small fossorial mammals

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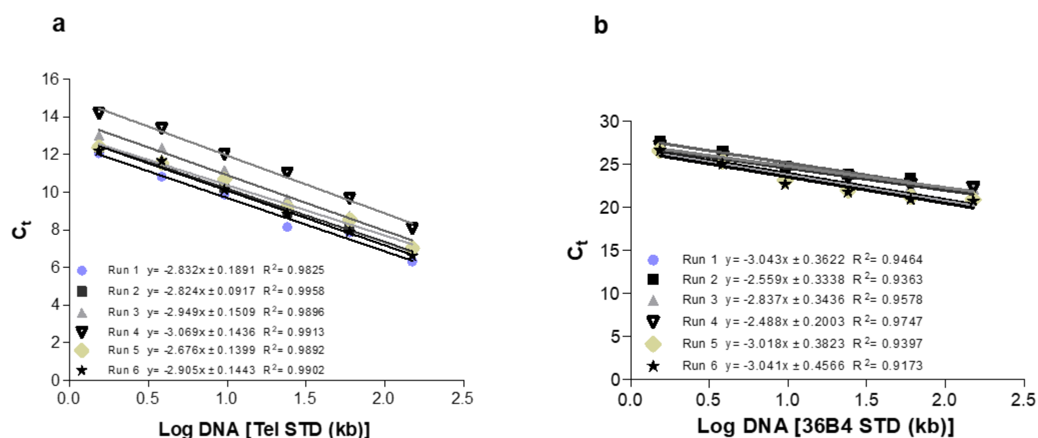
The telomere standard curve (Tel STD) was used to measure the telomeric content per sample in kilobases (kb). The cycle threshold (Ct) of the telomere qPCRs ranged from 7 to 13, and all target samples were within the standard linear range. The 36B4 STD curve was used to measure the number of diploid genome copies per sample.



Standard curves used to calculate TL. a) The log of kb of telomere standard oligomer DNA; b) The log of kb of 36B4 standard oligomer DNA

CONSIDERAÇÕES FINAIS

Este trabalho foi realizado a partir das abordagens de medição do comprimento telomérico e da metilação global do DNA, para tentar responder dois problemas distintos: os efeitos dos danos ao DNA induzidos por exposição ao carvão em *C. torquatus*, e uma melhor compreensão da evolução das diferentes formas cariotípicas na espécie *C. minutus*.

Os tuco-tucos são animais herbívoros, que cavam suas tocas usando as patas e os dentes. Logo, se esses animais vivem em ambientes poluídos, eles serão diretamente expostos a contaminantes, tanto através do solo como pelos alimentos. Na revisão dos estudos que tratam de efeitos da poluição em *Ctenomys*, foi possível observar que pouco se conhece sobre os efeitos dos diferentes poluentes neste nicho. Nos estudos compilados, a maioria mostrou o acúmulo de metais, principalmente no solo e em diferentes tecidos de mamíferos, sendo a maioria roedores. Das 65 espécies de *Ctenomys*, apenas em duas, *C. torquatus* e *C. minutus*, foram realizados estudos de avaliação dos efeitos da exposição a agentes genotóxicos em nível celular e molecular. Nessas espécies foram realizados ensaios crônicos como Teste de micronúcleos, Ensaio Cometa, e uma nova avaliação de instabilidade cromossômica, a medição do comprimento telomérico. Em *C. talarum* foi realizada a quantificação de metais nos solos e tecidos dos tuco-tucos, sem a avaliação do efeito desses metais na biologia dos indivíduos. Esses roedores subterrâneos mostraram ser bons sentinelas para biomonitoramento do solo, e estudos como este podem auxiliar estudos de genética de populações na avaliação do efeito antrópico nas populações naturais de mamíferos.

O estudo realizado em *C. torquatus*, no segundo capítulo, que tratou da exposição aos poluentes derivados de carvão, foi o primeiro realizado em um roedor subterrâneo considerando metais, danos celulares e moleculares. Nossos resultados mostraram que os animais expostos tiveram consequências relativas à diminuição na média do comprimento telomérico e aumento de danos ao DNA, mostrados através dos dados do Ensaio cometa. O resultado deste estudo indica que os componentes de carvão, encontrados no solo das regiões de mineração e

da Termoelétrica (Butiá e Candiota, respectivamente) onde são encontrados os tuco-tucos, estão relacionados com a diminuição do comprimento telomérico nesses indivíduos. Esses dados são um passo muito importante na pesquisa de mutagenicidade em um nicho ecológico pouco explorado. Com cada vez mais seu habitat natural sendo fragmentado por avanços na agropecuária e especulação imobiliária, e degradado por poluentes de agroquímicos, além da mineração, a população de tuco-tucos, que é naturalmente pequena, se torna cada vez mais vulnerável, como pode ser observado no nosso estudo. Por isso que avaliações de ecogenotoxicidade e de genética de populações são complementares na conservação de espécies.

O estudo realizado em *C. minutus*, no terceiro capítulo, também foi o primeiro considerando as técnicas de FISH, comprimento telomérico e metilação global do DNA para buscar compreender melhor a história evolutiva das diferentes formas cariotípicas desta espécie. Através do experimento de FISH com sonda telomérica, conseguimos observar um sinal positivo de ITS no cromossomo 1 na forma cariotípica 2n = 50b. Com relação ao cromossomo 1, já se tinha conhecimento da ocorrência de fusão entre dois cromossomos homólogos formando este cromossomo. Porém, mais estudos são necessários para compreendermos por que só conseguimos observar ITS em 2n = 50b e não nas outras formas cariotípicas, como seria o esperado. Dados interessante foram encontrados nas diferentes formas cariotípicas de *C. minutus* em relação a uma média maior do comprimento telomérico nas fêmeas pertencentes aos cariótipos com maior número de fusões (2n = 42> 46a > 48a). Neste estudo, sugerimos que o cariótipo mais estável seria o 2n = 50a, devido a maior média na porcentagem de metilação do DNA (diferenças não significativas). E sugerimos também, que o cariótipo mais instável seria o 2n = 42, por ter a menor na porcentagem de metilação do DNA. Para confirmarmos essas hipóteses, seria interessante medir, futuramente, a expressão da TERT nos indivíduos.

Dentre as metodologias utilizadas nestes trabalhos, a medida do comprimento telomérico apresentou mais relações significativas com diferentes parâmetros que a metilação global do DNA. Contudo ambas avaliações são importantes tanto no estudo de ecotoxicologia quanto em estudos de evolução. O

comprimento telomérico nos informa muito sobre a instabilidade genômica em uma população sob efeito de algum agente genotóxico, ou em relação a rearranjos cromossômicos, juntamente com a técnica de FISH. A metilação global do DNA nos mostra o quanto temos de metilação na sequência de DNA em CpG. A metilação nos dá dados importantes referente a regulação genica.

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