UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
CENTRO DE CIÊNCIAS HUMANAS E NATURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Estágios iniciais de divergência em um roedor neotropical com marcante variação genética e cromossômica

Fernanda Couto Zaidan

Vitória - ES

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Orientadora: Valéria Fagundes

Tese submetida ao Programa de Pós-Graduação em Ciências Biológicas (Biologia Animal) da Universidade Federal do Espírito Santo como requisito parcial para a obtenção do grau de Doutora em Biologia Animal.

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Vitória, 12 de dezembro de 2019.

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Desejo que meu filho e as gerações futuras tenham acesso à educação superior pública, gratuita, de qualidade e laica.

"*The important thing is not to stop questioning; curiosity has its own reason for existing. One cannot help but be in awe when contemplating the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of the mystery every day. The important thing is not to stop questioning***."**

Albert Einstein

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RESUMO

Akodon cursor é uma espécie de roedor terrestre endêmica da Mata Atlântica Bbrasileira, com ampla ocorrência, sendo comumente encontrado em áreas abertas e antropizadas desde a Paraíba no norte até o Paraná no sul. Essa espécie vem sendo estudada desde a década de 70 e passou por diversas alterações taxonômicas até que se conhecesse toda a sua diversidade cariotípica e geográfica. Uma importante característica desse roedor é apresentar grande variação cromossômica, com três numeros diplóides, 2n= 14, 15 e 16 e número de braços autossômicos de 18 a 26, o que faz com que a espécie tenha quase 30 cariótipos descritos. Além da ampla variação cromossômica, observa-se estruturação da diversidade genética da espécie em dois grandes agrupamentos principais (norte e sul), que coincidem com o rio Jequitinhonha, no leste do Brasil. Devido a sua ampla distribuição, variação macro e microestruturais do DNA e por ser uma espécie jovem (possivelmente com menos de 2 milhões de anos), fazem de *A. cursor* um excelente modelo para se investigar o papel relativo da geografia e das variantes genômicas estruturais (i.e. cromossomos) nos estágios iniciais de especiação. Nessa tese foram feitas coletas em duas populações ao norte e uma ao sul da distribuição da espécie, o que permitiu a realização de mais de 400 cruzamentos experimentais, que geraram mais de 500 descendentes, que foram analisados citogeneticamente e parte da amostra teve a fertilidade estimada por meio de análises histológicas. Além disso, foram feitas pela primeira vez análises genômicas de mais de 10.000 SNPs de *A. cursor, A. montensis* e híbridos naturais interespecíficos. Foi apresentado um panorama da espécie por meio da integraçao de dados genômicos obtidos pela metodologia de *ddRAD sequencing,* aliados à informações citogenéticas e de distribuiçao geográfica da espécie. Também foram investigadas estimativas de fertilidade com relação aos diferentes números diploides e níveis de isolamento geográfico entre populações. Foi observado que a geografia é preponderante na distribuição genética de *A. cursor*, recuperando os clados norte e sul, com representantes dos três números diploides em ambas as regiões. Além disso, sugiro que mais precisamente a região da nascente do rio Jequitinhonha seja a responsável pela quebra filogeográfica de *A. cursor*, uma vez que houve ali atividade geológica recente que sobrepõe com o possível período em que *A. cursor* divergiu nas duas linhagens principais. Os três números diploides podem ter surgido mais de uma vez durante a evolução da espécieuma vez que representantes desses números diploides não compartilham um ancestral comum mais recente. Isso tem implicações nas teorias de evolução cariotípica da espécie e da possível região de sua origem, que acreditamos ser na região central do Brasil e não ao sul, como se pensava. Os dados mostraram que em uma população do norte na Bahia, onde indivíduos dos três cariótipos estão em simpatria, indivíduos de 2n= 14 formam um clado sem sinais de mistura com o clado 2n= 15 + 2n= 16. O heterocariótipo 2n= 15 é um cariótipo com rearranjos dos pares 1 e 3 em heterozigose e o 2n= 14 apresenta esse rearranjo em homozigose. Portanto, pensava-se que os indivíduos 2n= 15 seriam fruto de mistura entre 2n= 14 e 2n= 16. No entanto, os dados do presente estudo mostraram que, pelo menos nessa população, o 2n= 15 é uma variação de 2n= 16. Esses dados levantaram a possibilidade de que haveria isolamento reprodutivo entre linhagens cariotípicas de *A. cursor*. Refutamos essa ideia, por meio de cruzamentos experimentais entre as formas 2n= 14 e 2n= 16 da população da Bahia, que intercruzou e gerou prole viável (porém subfértil), indicando que potencialmente não há isolamento pós-zigótico entre indivíduos desses cariótipos Dessa forma sugerimos que

possa estar ocorrendo reforço nessa população, com aumento de barrerias pré-zigóticas devido a um possível contato secundário das linhagens 2n= 14 e 2n= 15+16. Além disso, pudemos estimar taxas de sucesso reprodutivo (SR) e tamanho médio de ninhadas (TN) dos cruzamentos entre homocariótipos 2n=14 x 2n= 14 e 2n= 16 x 2n=16, servindo como referências para a espécie. Cruzamentos entre casais de cariótipo 2n= 15 da população da Bahia apresentaram taxas significativamente inferiores de SR em relaçao aos homocariótipos, mas foi recuperado um elevado SR quando 2n= 15 foi retrocruzado com os homocariótipos, sendo esse possivelmente o principal mecanismo em que a forma 2n=15 é mantida na natureza. Os cruzamentos entre indivíduos de uma mesma população e de populações adjacentes (mesmo que pertencentes à clados distintos) indicaram não haver isolamento reprodutivo completo entre as linhagens norte e sul, talvez pela recenticidade do processo geológico que gerou esse padrão genético. No entanto, os cruzamentos entre indivíduos de populações distantes geograficamente e alopátricas (Espírito Santo e Pernambuco, distantes mais de 2 mil km) geraram prole 2n= 15 (15NS) que se mostrou estéril tanto em cruzamentos como em retrocruzamentos. Esses resultados evidenciaram que o isolamento geográfico pode estar levando à incompatibilidades genéticas entre as linhagens dos extremos da distribuição da espécie. A esterilidade observada foi corroborada pelas análises histológicas, que mostraram que os machos de 15NS não apresentam lúmen nos túbulos seminíferos e nem espermatozoides, assim como híbridos interespecíficos entre *A. cursor* e *A. montensis*. Dessa forma, pudemos verificar que *A. cursor* estaria em uma situação análoga a de uma espécie em anel, uma vez que as populações adjacentes têm potencial de intercruzamento, porém representantes das extremidades da distribuição se mostraram incompatíveis. Integrando-se os resultados de genômica e de estimativas de fertilidade entre populações, tem-se um cenário em que existem linhagens norte e sul de *A. cursor,* que não estão associadas a cariótipos específicos, e que formam híbridos estéreis quando são intercruzadas as linhagens do extremo da distribuição. Esse roedor apresenta características de uma espécie biológica, mas apresenta sinais de estar nos estágios iniciais de divergência. Não é possível prever se a evolução caminhará para que essas linhagens se tornem espécies biológicas distintas. Com muito poucos trabalhos utilizando essas abordagens integrativas, o presente estudo se mostra inovador e contribui para o conhecimento evolutivo, mostrando que a geografia é o palco principal para os diferentes cariótipos de *A. cursor* surgirem e interagirem.

Palavras-chave: *Akodon cursor*, especiação, cromossomos, cruzamentos experimentais, genômica, linhagens

ABSTRACT

The terrestrial rodent *Akodon cursor* is endemic to the Brazilian Atlantic Forest, with broad range, easily found in open grasslands and disturbed areas from Paraíba in the north to the state of Paraná in the South of Brazil. This species has been under scientific investigation since the 70's and has passed through various taxonomic alterations until all its range and all its karyotypic diversity was known. One of its most remarkable characteristics is its chromosomal diversity, presenting three diploid numbers, 2n= 14, 15 and 16 and number of autosomal arms varying from 18 to 26, what makes it have nearly 30 described karyotypes. Despite ample chromosomal variation, it is also observed structuring of genetic diversity of the species in two main geographic groups (north and south), which are coincident with the Jequitinhonha river, in the east of Brazil. Due to its broad geographic distribution, macro and micro variation in its DNA and for being a young species (with possibly less than 2 million years old), makes A. cursor an excellent model for investigating the relative roles of geographic constraints and structural genomic variants (i.e. chromosomes) in the early stages of speciation. During this PhD project we collected individuals from two populations in the north and one in the south of the species' range, what made possible carrying out more than 400 experimental crosses, which in turn generated more than 500 animals that were analysed though cytogenetics and a sub sample had its fertility estimated using histological analyses. Besides that, genomic analyses were performed generating more than 10,000 SNPs of *A. cursor, A. montensis* and natural interspecific hybrids. Firstly, we presented an overview of the diversity of the species by integrating chromosome information with geographic distribution and genomic data obtained through ddRAD-sequencing. Later we emphasized investigating fertility estimates regarding the different diploid numbers and levels of geographic isolation between populations. We could observe that geographic structure outstands in the genetic distribution of lineages of *A. cursor*. In addition to that, the region of the source of the Jequitinhonha river is related to the phylogeographic break of this rodent, once it is reported for that region recent geological activity that overlaps with the putative period when *A. cursor* split into two main lineages. The different diploid numbers could have appeared more than once during the evolution of *A. cursor*, since representatives of each karyotype do not share a most recent common ancestor. This assumption has implications on the theories of chromosomal evolution of *A. cursor* and the putative region of origin of the species, suggesting it was actually in its central portion and not in southern Brazil. Our data showed that in a particular population, where individuals of the three diplopid numbers live in simpatry, they do not form distinct genomic clusters, as previously hypothesized, with the invidividuals 2n= 14 as a monophyletic clade, without signs of admixture with the clade of individuals 2n= 15+16. The heterokaryotype 2n= 15 presents rearrangements in pais 1 and 3 in heteorzigosis and the 2n= 14 has its rearrangement in homozygosis, therefore, it was believed that the heterokaryotype individuals of *A. cursor* would be a result of crosses between 2n= 14 and 2n= 16. However, genomic data pointed that, at least in this particular population, 2n= 15 is a variation of 2n= 16. This information raised the possibility that there could be reproductive isolation between karyotypic lineages of *A. cursor.* Through experimental crosses we refuted this suggestion since crosses between 2n= 14 and 2n= 16 from this population produced viable litter (though subfertile), indicating that there is no potential prezygotic isolation between such homokaryotype individuals. Thus, it was suggested that reinforcement could be happening in this population, with an increase of prezygotic

barriers due to a putative secondary contact of the lineages 2n= 14 and 2n= 15+16. Moreover, in the present work it was estimated rates of reproductive success (RS) and average litter size of same-homokaryotype crosses, which can be used as references for the species. Crosses between heterokaryotype individuals from the same population presented significantly inferios rates of RS compared to homokaryotyoes, but it was recovered a high RS when these individuals were backcrossed with honokaryotypes. Though, backcrossings of 2n= 15 with homokaryotypes could be the principal means that this form is maintained in nature. Crosses between individuals from the same population and from adjacent populations (even the ones from distinct clades) pointed that there is no complete reproductive isolation between lineages north and south of *A. cursor*, maybe due to the recency of the geological process that triggered the genetic differentiation of lineages. Nonetheless, crosses between individuals from geographically distant and allopatric popultions (Espírito Santo and Pernambuco, more than 2,000 km apart) produced litter 2n= 15 (15NS) that was sterile in crosses and backcrosses. These results evidenced that geographic isolation might be taking these lineages to accumulate epistatic incompatibilities. The observed sterility was corroborated by histological analyses which demonstrated that the 15NS males do not present lumen in their seminiferous tubules nor spermatozoa, such as interspecific hybrids. Hence, we could verify that *A. cursor* is in a situation analogous to a ring species, once its adjacent populations have the potential to breed, but representatives of the tips of the distribution are reproductively incompatible. By integrating genomic results with estimates of fertility between populations we have a scenario with *A. cursor* split into two main groups which are not correlated to specific karyotypes and that form sterile intraspecific hybrids when are put together. The cursor grass mouse can still be considered a biological species having signs of being in the initial stages of divergence and one can not predict if evolution will follow towards these main lineages to become two species. With very few works that integrated such approaches the present work adds to our knowledge showing that geography is the main stage for the karyotypes of *A. cursor* to emerge and interact.

Keywords: *Akodon cursor***,** speciation, chromosomes, experimental crosses, genomics, lineages

APRESENTAÇÃO

O roedor brasileiro *Akodon cursor* Winge, 1887 é uma espécie terrestre, comumente capturada em ambientes antropizados, como pastos, bordas de fragmentos florestais e florestas em estágios primários de regeneração. Sua distribuição geográfica coincide com a Mata Atlântica, da Paraíba ao norte do Paraná, mas também foi registrada em áreas de Cerrado e Caatinga no estados de Minas Gerais e interior da Bahia. Além de ampla distribuição, a espécie pode ser encontrada desde o nível do mar até 1.170 metros de altitude (Geise 2012).

Essa espécie caracteriza-se pela alta diversidade cariotípica, com 2n= 14, 15 e 16, grande variação do número de braços autossômicos (NFa) variando de 18 a 26 e marcante estruturação genética, representando um bom modelo para estudos evolutivos e biogeográficos. Desde o início da década de 70, quando iniciaram-se os primeiros estudos, foram obtidos muitos avanços sobre a história natural, citogenética, biogeografia e evolução de *A. cursor*, assim como do gênero *Akodon,* de forma geral. Grande parte desses avanços foram possíveis devido ao esforço amostral, com ampliação das áreas de ocorrência, assim como a aplicação de novas tecnologias e análises. Vale destacar que muitas perguntas científicas e inferências sobre a evolução e diversificação do grupo surgiram tendo como ponto de partida as informações cariotípicas de animais obtidos da natureza.

No final da década de 60, espécimes de *A. cursor* eram nominados de *Akodon arviculoides* Wagner 1842, sendo que o primeiro cariótipo da espécie apresentou 2n= 14 em indivíduos coletados no estado de São Paulo (Yonenaga 1972). Desde a primeira publicação já foi possível verificar a grande diversidade cariotípica da espécie, pois em oito indivíduos analisados, foram observados quatro cariótipos diferentes.

Um novo estudo, alguns anos depois, revelou os cariótipos com 2n= 15 em amostras de São Paulo e do Rio de Janeiro (Yonenaga-Yassuda 1979). Análises de bandas cromossômicas demonstraram que se tratava de uma forma variante de 2n= 14, explicada pela fissão de um dos cromossomos homólogos do par 1 em dois cromossomos, seguida

de inversões pericêntricas, que foram chamados de cromossomos 1a e 1b (Figura 1a). Por se tratar de um cariótipo com número ímpar de cromossomos (heterocariótipo), a autora realizou análises meióticas em machos com 2n=15 e observou a formação de figuras de um grande trivalente (Figura 1b), confirmando a proposta de homologia dos três cromossomos, sendo também observado que não houve formação de alças de inversão nas porções invertidas e redução na frequência de quiasmas nos segmentos invertidos.

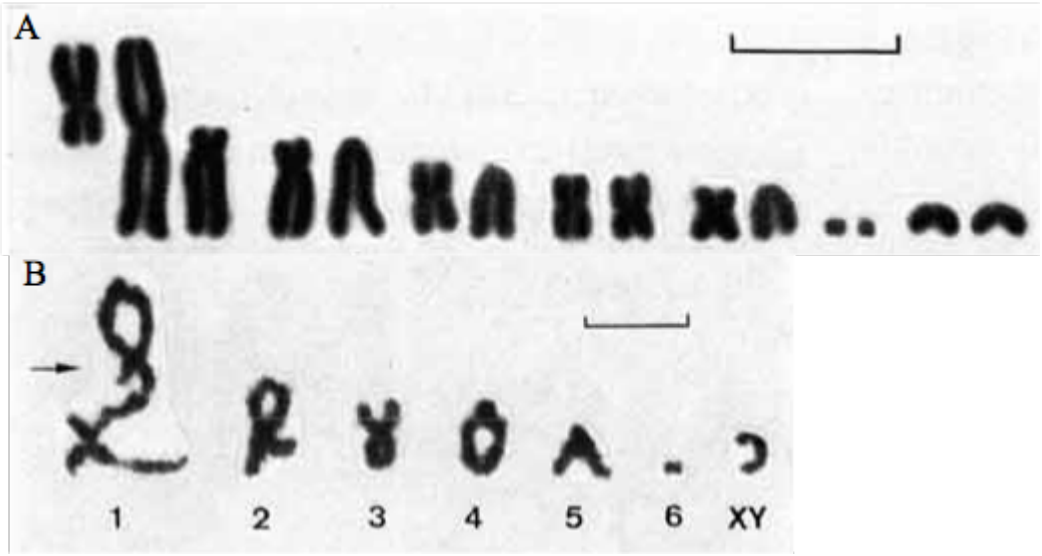

Figura 1. A) Cariótipo de uma fêmea de 2n= 15 de A. cursor, mostrando o par 1 composto por três elementos: 1 grande cromossomo metacêntrico e dois cromossomos submetacêntricos diferentes (1a e 1b). B) Figuras meióticas de cada de um macho de 2n= 15, com a seta indicando um trivalente e sem formação de alças nos cromossomos com inversões pericêntricas (aqui representados como 2, 3 e 5). Extraído de Yonenaga-Yassuda (1979).

Quase 10 anos após a descrição do cariótipo 2n= 14 foram coletados 25 indivíduos de *Akodon arviculoides* em duas localidades no estado de Pernambuco e todos apresentaram o cariótipo com 2n= 16, com os pares 1a e 1b em homozigose (Maia e Langguth 1981) (Figura 2). O padrão de bandeamento confirmou a homologia de todos os cromossomos com aqueles dos cariótipos com 2n=14 e 2n= 15 de São Paulo e Rio de Janeiro. Devido ao rearranjo nos cromossomos 1a e 1b estar em homozigose foi utilizada uma nova nomenclatura referente aos pares cromossômicos, diferente da proposta por Yonenaga-Yassuda (1979).

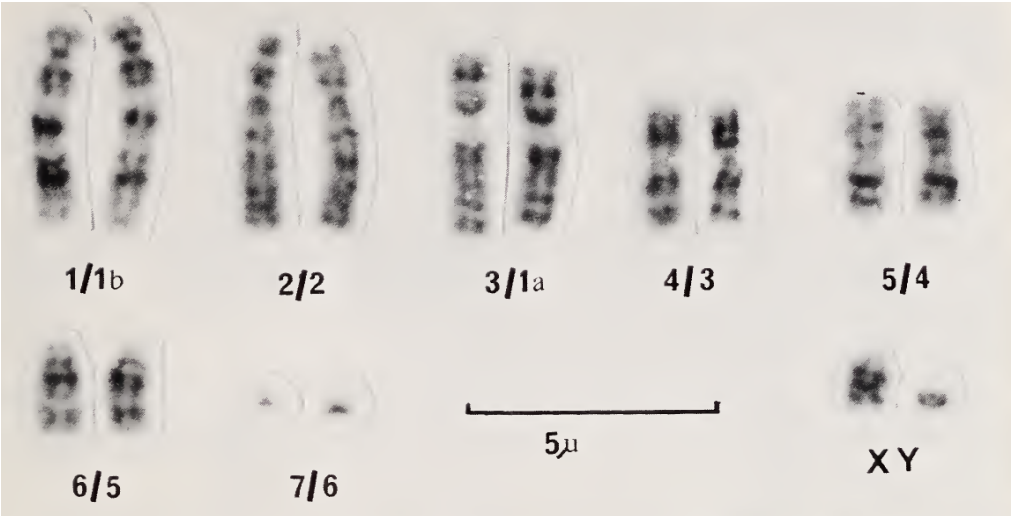

Figura 2. Cariótipo banda G de um macho de A. cursor de 2n= 16 coletado em Pernambuco. O primeiro número abaixo do cromossomo corresponde a nomenclatura dos pares cromossômicos proposta por Maia e Langguth (1981) e o segundo refere-se a nomenclatura proposta por Yonenaga-Yassuda (1979). Extraído de Maia e Langguth (1981).

Liascovich e Reig (1989) realizaram uma revisão taxonômica envolvendo dados de cariótipos de algumas espécies de *Akodon*, e avaliaram que, tanto indivíduos com 2n=24-25 quanto os com 2n=14-16, previamente identificados como *A. arviculoides*, teriam sido equivocadamente identificados, pois *arviculoides* era sinônimo júnior de *Bolomys lasiurus* (Lund, 1841) (atualmente conhecido como *Necromys lasiurus*). Assim, resgataram o epíteto específico *cursor* e atribuíram o nome *A. cursor montensis* aos indivíduos com cariótipo 2n=24-25 provenientes do Uruguai, Paraguai, Argentina e São Paulo; enquanto que os exemplares com 2n=14 de São Paulo e Minas Gerais seriam chamados de *A. cursor cursor.*

As relações evolutivas das espécies do grupo cursor foram estudadas utilizando ferramentas moleculares inicialmente por Rieger e colaboradores (1995). Os autores utilizaram polimorfismo de alozimas em indivíduos com 2n=16 da Paraíba, 2n=14/15 do Espírito Santo e 2n=24 de Santa Catarina e Rio Grande do Sul. Devido à divergência observada entre os três grupos (2,2 a 9,5%), os táxons foram considerados como espécies irmãs e, assim, foram atribuídos os nomes *A.* aff. *cursor* para indivíduos de 2n= 16 do norte da Mata Atlântica; *A. cursor* para os indivíduos de 2n=14/15, conhecidos até então para os estados de São Paulo, Minas Gerais e Espírito Santo, e *A. montensis* com

2n=24/25. Soma-se à proximidade genética, o fato das espécies *A. montensis* e *A. cursor* serem morfologicamente muito semelhantes e ocorrerem em simpatria nos estados de Minas Gerais, São Paulo e Paraná (Geise et al. 2005; Astúa et al. 2015). Híbridos interespecíficos com 2n= 19 já foram coletados no estado de São Paulo (Fagundes *et al.* 1997a,b), sendo que análises histológicas de machos gerados em cativeiro apontaram a infertilidade desses animais (Yonenga et al. 1975).

Em 1996 foram registrados pela primeira vez exemplares com 2n=16 no extremo sul da Mata Atlântica, no estado do Paraná, que foram coletados em simpatria os indivíduos com 2n=14 e 15 (Sbalqueiro e Nascimento 1996). Dessa forma, foi verificado que indivíduos de 2n=16 não tinham ocorrência exclusiva no extremo norte da Mata Atlântica, assim como as análises comparativas de banda G indicaram homologia dos cromossomos do cariótipo de 2n= 16 de Pernambuco com o 2n= 16 encontrado no Paraná. Além disso, nesse estudo foram relatados cruzamentos experimentais nos quais casais com 2n= 15 geraram filhotes recuperando na prole os três números diplóides da espécie (2n=14, 15 e 16).

Em 1998 foi feita uma revisão dos dados cariotípicos da espécie (Fagundes et al. 1998) utilizando dados da literatura e incluindo novas amostras, totalizando 311 indivíduos e um total de 28 cariótipos conhecidos para a espécie, incluindo a descrição de fêmeas X0 para a espécie pela primeira vez. Neste trabalho foi padronizada a numeração dos cromossomos de *A. cursor* (Fig. 3). Ainda, foi possível observar que 100% dos indivíduos do extremo norte (Pernambuco e Paraíba) apresentam 2n= 16, na Bahia correspondem a 44% e em São Paulo, Rio de Janeiro e Paraná somente 2,3% dos indivíduos são 2n= 16. O oposto foi observado para 2n= 14, sendo essa forma ausente no extremo norte, com 5.5% na Bahia e correspondendo a 72% das amostras de São Paulo, Rio de Janeiro e Paraná. A forma 2n= 15 foi encontrado em cerca de 50% dos indivíduos da Bahia e 25% do Sul. Ainda nesse trabalho reforçou-se a hipótese proposta por Fagundes et al. (1997a,b) de que 2n= 16 seria o cariótipo ancestral em *A. cursor*, pois essa forma compartilha com sua espécie irmã *A. montensis* (2n= 24/25) menor número de rearranjos cromossômicos, quando comparado a 2n= 14.

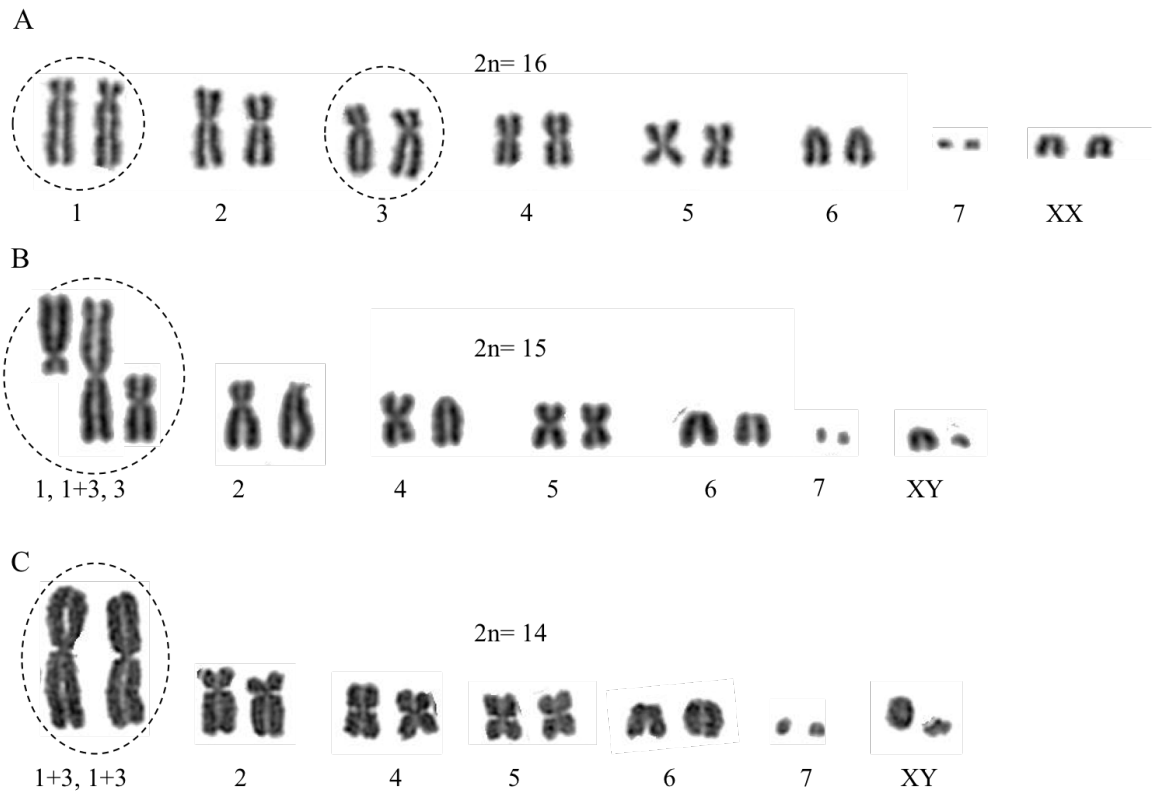

Figura 3. Cariótipos de A. cursor, destacando-se com a linha tracejada os pares 1 e 3 que sofreram rearranjos do tipo fusão, formando um metacêntrico grande (1+3). A) 2n= 16, com par 2 submetacêntrico e par 4 metacêntrico; B) 2n= 15 quando em heterozigose, com um cromossomo 1+3, um de cada um dos pares 1 e 3, com pares 2 e 4 heteromórficos; e C) 2n= 14, com par 1+3 e pares 2 e 4 com dois braços. Ressalta-se que a variação do NFa se deve a morfologia variada nos pares 2, 4 e 6, devido a presença em homozigose ou heterozigose de inversões pericêntricas ou sua ausência. Foto obtida do acervo do Laboratório de Genética Animal.

Em 2001 foi feito o primeiro estudo evolutivo sobre o grupo cursor, incluindo *Akodon cursor*, *A.* aff. *cursor* e *A. montensis*, utilizando-se sequências de um gene mitocondrial (Geise *et al*. 2001). Neste estudo foram incluídos animais de 2n=14 e 15 de São Paulo e Rio de Janeiro e indivíduos de 2n=16 da Paraíba e Norte de Minas Gerais. Não foram incluídas amostras de indivíduos de 2n=16 de São Paulo ou Paraná, no sul da Mata Atlântica. Um dos principais resultados foi a observação de dois clados, um correspondendo aos indivíduos com 2n=16 (chamado de *A.* aff. *cursor)* da Paraíba, Bahia e norte de Minas Gerais, e o outro clado com exemplares com 2n= 14 e 15 da Bahia, Espírito Santo, centro-sul de Minas Gerais, Rio de Janeiro e São Paulo (Fig. 4A). Nesse estudo, os autores reforçam que *A. cursor* seria composta por duas entidades taxonômicas

influenciadas diretamente por sua constituição cariotípica e região geográfica, com *A*. aff. *cursor* (indivíduos de 2n= 16) sendo a espécie do Norte da Mata Atlântica e exemplares de 2n= 14 e 15 correspondentes a espécie *A. cursor*, encontrada ao Sul da Mata Atlântica.

Em 2008 foram utilizadas sequências de fragmentos de um gene mitocondrial para verificar se *A. cursor* seria uma ou duas espécies (Nogueira e Fagundes 2008). Nesse trabalho foram incluídas amostras de indivíduos representantes dos três números diplóides, de localidades correspondentes tanto ao Clado Norte quanto ao Sul da Mata Atlântica, descritos por Geise et al. (2001). As análises filogenéticas recuperaram dois clados, com os três números diplóides 2n= 14, 15 e 16 em ambos os clados (Fig. 4B). Dessa forma, foi refutada a existência de uma forma *A. cursor* do norte associada ao cariótipo 2n= 16 e outra do sul, com os 2n= 14/15, não havendo correlação do número diploide na determinação dos clados principais. Nesse estudo foi sugerido que *A. cursor* fosse considerada uma única espécie, com os três números diplóides encontrados ao longo da distribuição geográfica, sendo a diversidade genética subdividida em dois clados principais (chamados de norte e sul).

Uma das perguntas que surgiu após esses trabalhos foi se a cladogênese que gerou os clados norte e sul em *A. cursor* teria correspondência biogeográfica e se poderia ser coincidente com alguma barreira física. Inicialmente pensava-se que o Rio Doce poderia ser essa barreira, uma vez que ele coincide com padrões genéticos norte-sul em diversas espécies de vertebrados (Costa e Leite 2013). No entanto, análises de genética de populações refutaram essa proposta (Colombi et al. 2010) e posteriormente foi conhecido que a dicotomia norte-sul de *A. cursor* tem correspondência filogeográfica com o Rio Jequitinhonha (Maestri et al. 2016).

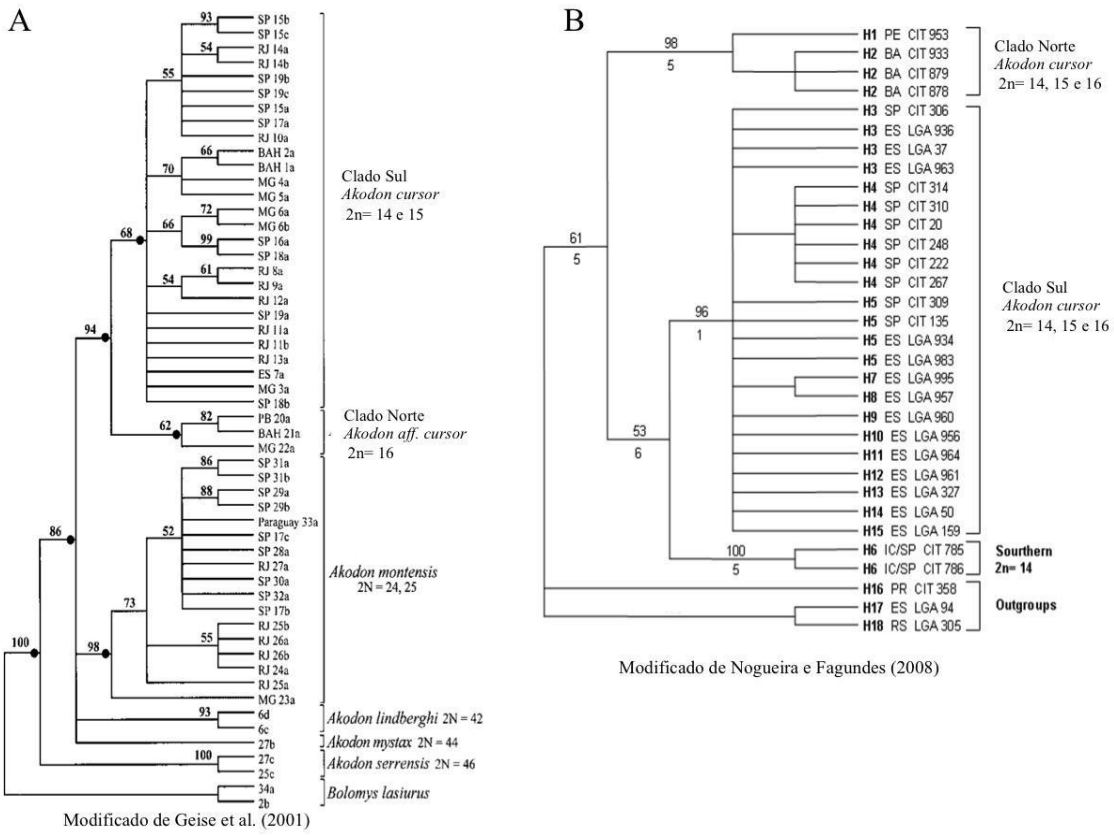

Figura 4. Árvores filogenéticas indicando os clados norte e sul de A. cursor, associados a respectivos números diplóides, segundo Geise et al (2001) e Nogueira e Fagundes (2008). O clado norte está em amarelo claro e o clado sul em cinza. Ambas as árvores foram geradas pela análise de máxima parcimônia e os números sob os ramos são os valores de bootstrap. Em A) foram sequenciados o gene mitocondrial citocromo B e utilizadas amostras de 2n= 14 e 15 do sul e de 2n= 16 do norte e em B) foram usados polimorfismo gerados por enzimas de restrição em amostras de 2n= 14, 15 e 16 de localidades tanto do norte como do sul.

Deste modo, os estudos realizados até o momento, envolvendo análises moleculares de marcadores mitocondriais, sugerindo a distribuição da divergência genética organizada em dois clados; assim como a presença de três números diplóides, amplamente distribuídos geograficamente, exceto por exclusividade de 2n=16 em Pernambuco e 2n=14 no Espírito Santo; assim como sobreposição morfológica entre indivíduos com 2n= 14, 15 e 2n=16 (Geise et al. 2005), mantém algumas questões abertas: a) o padrão de distribuição norte-sul, verificado no genoma mitocondrial também pode ser recuperado no genoma como um todo? b) o número de amostras/caracteres poderia estar influenciando os resultados das análises evolutivas? c) há alguma barreira ao fluxo gênico

entre indivíduos de clados distintos que poderia ser associada a algum fator abiótico? d) a distribuição dos cariótipos nos dois clados é aleatória? e) as formas 2n=14, 15 e 16 são intercruzantes na natureza? f) os cruzamentos 2n=15 x 2n=15 têm potencial de recuperar as formas basais 2n=14 e 2n=16? g) há sinais de isolamento reprodutivo que possam ser atribuídos a um processo incipiente de especiação? h) indivíduos com 2n=15 apresentam características reprodutivas análogas a de um híbrido interespecífico? i) há isolamento reprodutivo entre indivíduos de clados distintos e se diferentes combinações de cariótipos teriam influência na aptidão reprodutiva da espécie? Pretende-se responder algumas dessas questões nos próximos dois capítulos da presente tese.

No Capítulo 1, de forma pioneira, foram obtidos dados genômicos de indivíduos representativos da distribuição geográfica e cariotípica de *A. cursor,* a fim de se inferir as relações tocogenéticas entre populações e verificar se a diversidade genética da espécie está particionada geograficamente em dois clados; assim como verificar o padrão de distribuição dessa diversidade genética entre os cariótipos de uma população. Pretende-se também, verificar se os indivíduos com 2n= 15 apresentam características genéticas intermediárias às formas 2n= 14 e 2n=16.

No Capítulo 2 pretendeu-se verificar se haveria isolamento reprodutivo entre indivíduos de populações adjacentes ou alopátricas, e se diferentes combinações de cariótipos teriam influência na aptidão reprodutiva da espécie, por meio de realização de cruzamentos experimentais entre indivíduos coletados ao longo da distribuição geográfica da espécie. Além disso, devido ao fato de 2n= 15 ser um heterocariótipo, com rearranjos cromossômicos intermediários às formas 2n= 14 e 2n= 16 (homocariótipos), e por ser encontrado em baixas frequências na natureza, foi especulado que essa forma poderia ser um híbrido intraespecífico. Desta forma, nesse capítulo foi avaliada se a condição cariotípica intermediária de 2n= 15 de *A. cursor* apresenta características reprodutivas e genômicas equivalentes a híbridos interespecíficos e foi avaliado se há indícios de um processo incipiente de especiação de linhagens norte e sul em *A. cursor.*

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Capítulo 1

The strength of geographic and karyotype constraints in populations of the cursor grass mouse

A força de restrições geográficas e cariotípicas em populações do rato do mato

The strength of geographic and karyotype constraints in populations of the cursor grass mouse

ABSTRACT

Understanding if chromosomal rearrangements act as barriers to reproductive isolation and drive lineages divergence is challenging, especially when the focal species shows notable influence of geographic constraints. An integrative approach using information on chromosomal rearrangements, geographic distribution and genomics was used to untangle the relative influences of geography and karyotype variants in the evolution of the neotropical rodent *Akodon cursor*. The cursor grass mouse has three diploid numbers (2n= 14, 15 and 16) that occur throughout the Brazilian Atlantic Forest, with a known north-south phylogeographic break, with 2n=15 as an intermediary karyotype. Our genomic results showed that the overall genetic diversity of the species is partitioned in these two geographic groups and the three diploid numbers are embedded into these two main groups with the primary phylogeographic break being concordant with the Jequitinhonha river area, in central-east Brazil. However, we discuss that neither the river itself nor pleistocenic refuges could have imposed constraints that explain the north-south genetic dichotomy, but we found arguments to hypothesize that recent tectonic manifestations in the Quaternary, near the source of this river, tmight have created abiotic conditions that somehow affected the gene flow between populations of the species. However, genomic analysis of a northern population in Bahia, where the three diploid numbers were found in sympatry, signaled a complex scenario, with two distinct groups. One is composed of 2n= 14 individuals only and the other has 2n= 15 and 2n= 16 individuals sharing similar genomic background. However, there are signs of "pure" 2n= 16 individuals and also, these two sympatric groups do not share a most recent common ancestor. Our findings also showed that the 2n= 15 individuals, which so far was considered a hybrid-like form between the other two karyotypes, may not be a byproduct of admixture between 2n= 14 and 2n= 16 and is more closely related to 2n= 16 than to 2n= 14 individuals. In fact, we believe that these two groups (2n= 14 and 2n= 15+16) could present some form of reproductive isolating mechanism. Also, despite not having a replicate population with the three karyotypes in sympatry in the south, in our analysis the southern 2n= 15 individuals grouped with 2n= 14, pointing that the 2n= 15 individuals might have appeared in nature through different means more than once and it has reproductive and phylogenetic consequences. Besides confirming the primary role of geography in shaping the genetic diversity of *A. cursor*, our study demonstrated that despite new technological approaches, diploid number information still as a major source of information since it brought evolutionary insights about the reproductive interaction of individuals of the same species but with different diploid numbers and gave a new perspective on the means by which heterokaryotypes are generated and maintained in nature.

Keywords: *Akodon cursor***,** karyotypes, Atlantic Forest, phylogeographic break, lineages divergence

INTRODUCTION

Differences in chromosome number and form were some of the earliest data available on genetic differences among species (Darlington 1958; White 1973). Because of their influence on how genetic variation is passed and redistributed within and among populations (Potter et al. 2017), attention has focused on the restructuring of the genome in chromosomal rearrangements (CRs) as a cause of reproductive isolation, and hence speciation (Sturtevant 1938; Dobzhansky 1950; Grant 1964; White, 1973), as well as adaptive evolution within species (e.g. Kirkpatrick and Barton 2006, but see Charlesworth and Barton 2018). For example, the role of CRs in reproductive isolation (Coyne and Orr 2004; Nosil et al. 2005; Lowry et al. 2008) is linked directly to the physical pairing of chromosomes during meiosis. Fixation of different CRs across local populations of the same species is a key aspect of the chromosomal speciation hypothesis in promoting divergence without geographical isolation of populations (White 1978).

The suggestion that CRs may play a direct role in speciation comes from the general observation that heterokaryotype individuals may have reduced fertility, either because of various meiotic irregularities or their role in suppressing recombination (Faria and Navarro 2010; Basheva et al. 2014). However, our understanding of the evolutionary consequences of the early stages of karyotypic differentiation in which populations are polymorphic for CRs is still limited (Dobigny 2017). Some examples of non-meiotic impacts (i.e. production of unbalanced gametes) in heterokaryotype individuals are known, such as heterosynapsis followed by suppressed recombination around rearranged

breakpoints in heterokaryotype individuals (Hale 1986; Livingstone and Rieseberg 2004; Faria and Navarro 2010).

Although geographic isolation may not be mandatory in models of speciation, it is an empirical question of whether geography might be important in the differentiation of lineages that present CRs before isolation. If a geographic barrier reduces gene flow between lineages that present CRs, then it is expected that the CRs frequencies would not follow Mendelian rules. Thus, the frequency of karyotypic variation among populations (or nascent species) would be shaped mostly by selection and genetic drift than the constraints imposed by karyotypic incompatibilities *per se* (i.e. karyotypes are not the isolating factor). Addressing the relative role of geography versus karyotype in shaping diversification is quite challenging, since it requires that one investigates populations in the early stages of incompatibilities before they reach the full species status (Charron et al. 2014).

The neotropical rodent *Akodon cursor* (Winge, 1887) offers an outstanding opportunity to study the early stages of lineage divergence. Mitochondrial analyses have demonstrated that the species diversity is distributed in two major clades (Geise et al. 2001; Nogueira and Fagundes 2008), which coincide with the Jequitinhonha river (Maestri et al. 2016). The species presents an emblematic high karyotypic polymorphism with three diploid numbers (2n= 14, 15 and 16) found in populations of both clades, some in sympatry (Fagundes et al. 1998; Nogueira and Fagundes 2008). The different diploid numbers are due to complex CRs in biarmed pairs 1 and 3 (present in the 2n=16 form), involving centric fusion and pericentric inversions resulting in a large metacentric pair, present in

2n= 14 and 2n= 15 individuals when the CRs occur in homozygosis and heterozygosis, respectively. Compiled data showed higher frequencies of 2n= 16 individuals in the north, while 2n= 14 is predominant in the south (Fagundes et al. 1998).

Thus, we aimed to understand the pattern of genetic diversity in *A. cursor*, whether it is shaped mostly by geography or if chromosomal constraints play an important role in the diversification process as well. To verify the relative contribution of karyotypes in population divergence we take advantage of the co-distribution of multiple karyotypes within a given geographic area and test hypotheses about their divergence history using thousands of random genomic SNPs. Specifically, if the karyotypic differences were the drivers of divergence, we would expect most genomic variation to be partitioned by karyotype. However, if not, we predict that geography will explain most of the genetic differentiation observed in the species. Under the latter scenario, karyotype differences might still contribute to genetic differentiation, even if their effects are secondary to those of geography.

MATERIALS AND METHODS

Karyotype and genomic data processing

Genomic data was collected for 66 individuals of *A. cursor* from 19 localities from six populations in eastern Brazil, herein called Pernambuco, Bahia, Minas Gerais (North and South), Espírito Santo and São Paulo (Fig. 1A), for which 76% were karyotyped. Samples of *A. cursor* were collected across its distribution from the three karyotypes from Brazil (Table S1 on Supplementary Material). Chromosome preparations were obtained either from bone marrow or spleen cells by the standard colchicine method (Ford and Hamerton 1956 with modifications) or from fibroblast cultures (Freshney 1986). Karyotypes were analyzed from the best well-spread metaphases for each animal using a Nikkon microscope equipped with a Cytovision® Analyser and diploid number (2n) was determined after conventional Giemsa staining.

Genomic DNA was extracted from muscle or liver using the DNeasy® blood and Tissue kit Qiagen. We used a double-digest RADseq (ddRAD) protocol (Peterson et al. 2012). Before digestion reactions, double-stranded DNA concentrations were quantified using the Qubit dsDNA Assay Kit (Invitrogen) and all samples were adjusted to equal molar concentration. The initial amount of DNA varied from 350 to 500 ng per sample. Briefly, the DNA was double digested with the restriction enzymes EcoR1 and MseI (New England Biolabs), and unique barcodes (10 bp) and Illumina adapter sequences were ligated to the digested fragments. Samples were pooled together and 300-450 bp DNA fragments were size-selected using Pippin Prep (Sage Science) followed by PCR

amplification. The library was sequenced using the Illumina 2500 platform at The Center for Applied Genomics (Hospital for Sick Kids, Toronto, Canada) to generate 150bp single-end reads.

We used the STACKS 1.45 pipeline (Catchen et al. 2011; Catchen et al. 2013) for *de novo* assembly of loci from the fastQ files obtained from the Illumina sequencing. Sequences were demultiplexed using *process_radtags*, individuals with less than 70K reads were excluded from further analysis. Loci and polymorphic nucleotide sites were identified in each individual using the ustacks program with a minimum coverage depth (m = 5), a removal algorithm (-r 0), a deleveraging algorithm (-d) and a maximum distance (in nucleotides) of two allowed between stacks (-M 2). An error rate (ε) was set conservatively (--bound_high 0.1) to avoid underestimating heterozygotes (Catchen et al. 2013). The mean coverage was 12x. A catalog of consensus loci among individuals was constructed with the cstacks program, where loci were merged across individuals if the distance between them (n) was ≤ 2. This catalog was used to determine the allele(s) present in each individual at each homologous locus with the sstacks program. SNP data was exported as Variant Call Format (–vcf) and processed with an R-script in RStudioVersion 1.0.153 (R Studio Team 2016) using the PLYR (Wickham 2011) and PEGAS packages (Paradis 2010) to delete the last 10 bp positions to avoid sites with presumed sequencing errors (i.e., sites from the upper 95th percentile of segregating sites). All STACKS modules were run in parallel with 8 threads on the HPC Linux-based cluster from the University of Michigan.

More than 170 million reads was produced on one lane of Illumina sequencing. After bioinformatics processing and filtering based on read quality, almost 155 million reads were retained in 54 individuals (Table S1 on Supplementary Material). After applying the filters of populations program, a total of 640,215 SNPs in 226,632 loci (maximum of 9 SNPs per locus) were obtained. The software PLINK 1.9 (Purcell et al. 2007) was used to filter the sequences and individuals based on the frequency of missing data. Specifically, two datasets with different levels of missing data were generated given the varying requirements of the respective analyses described below. A dataset with up to 50% of missing data and a second dataset with 10% of missing data per unliked SNP, contained 77,809 and 10,100 loci, respectively.

Genomic data analyses

Population genetic structure were assessed using Bayesian clustering implemented in the software STRUCTURE 2.3.4 (Pritchard et al. 2000) and a Principal Components Analysis (PCA) using the Adegenet package (Jombart et al. 2008) and Plyr (Wickham 2011) in RStudioVersion 1.0.153 (R Studio Team 2016) with the dataset with 10% missing data. For Principal components and STRUCTURE analyses, we first used all the 54 individuals and six populations, and then secondly, we used a subset comprising exclusively the Bahia population with 22 individuals, because it presents individuals of the three diploid numbers in sympatry. PCA were performed on RStudioVersion 1.0.153 (R Studio Team 2016). We used the Adegenet package (Jombart et al. 2008) and Plyr (Wickham 2011). Missing data were replaced by the mean frequency of the corresponding allele, which is recommended for centered PCAs (Jombart et al. 2008).

Major axes for genome-wide SNP data were identified using the R Dudi.pca function (centre = T, scale = F). The Structure analyses were performed under the "Admixture model" and the "Correlated allele frequency model" for a range of *K*-values (i.e., 1 to *n* + 1, where *n* is the number of populations). Ten independent runs were performed for each *K*-value with 500,000 burnin steps and 1,000,000 Markov Chain Montecarlo (MCMC) iterations. A hierarchical STRUCTURE analysis (Massatti and Knowles 2014) was run to evaluate substructure within the identified genetic groups. The populations were firstly defined by a geographic distance criterion (individuals in a range up to 150 km were considered from the same population) and in the Bahia population the individuals were assigned to each karyotype. The optimal *K* for each dataset was chosen using the delta-K method (Evanno et al. 2005) as implemented on STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt 2012). The cluster membership coefficients (posterior probabilities of individual assignments to *K* genetic clusters) were permuted across ten independent runs using CLUMPAK (Kopelman et al. 2015) and plotted using DISTRUCT (Rosenberg 2004).

The phylogenetic relationships among all 54 individuals of *A. cursor* were estimated using the coalescent-based method to accommodate different genealogical histories among loci using the program SVDquartets (Chifman and Kubatko 2014). The SVDquartets algorithm was implemented in PAUP v4.0a157 (Swofford 2002) with exhaustive quartet sampling, using two *A. montensis* samples as outgroup, and 20 threads using the multispecies coalescent with 1000 bootstrap replicates. The dataset with 50% missing data per individual was analyzed (77,809 loci in 54 individuals), because

increasing the amount of loci, despite increasing missing data, demonstrated to provide important phylogenetic content (Huang and Knowles 2014).

For genetic diversity analysis, population genetic statistics, including nucleotide diversity (π), Wright's *F*-statistic (F_{is}), observed heterozygosity (H_{obs}) and pairwise F_{st}-values between populations were calculated using the populations program in the STACKS pipeline (Catchen et al. 2013).

RESULTS

Structuring of genetic variation

Genetic diversity was shaped predominantly by geography, with secondary clustering by diploid numbers in a local scale. Geographic structuring of genetic variation was consistent across PCA, STRUCTURE and phylogenetic analyses. Geography defined the major axes of variation on PCA (Fig. 5B), with populations from the north clustered separately from the southern populations, regardless of the karyotype of the individuals. Despite the geographic proximity, individuals from Minas Gerais presented a north-south subdivision (MG/N and MG/S), not forming a cohesive grouping. Although geography explained most of the genetic variation, the PCA analysis using specimens from Bahia (where the three karyotypes are sympatric in the north, Fig. 5C) revealed most individuals 2n= 14 and 2n= 16 forming distinct clusters, but some of them were genetically mixed with the 2n= 15 group, that was in an intermediate position on PC1.

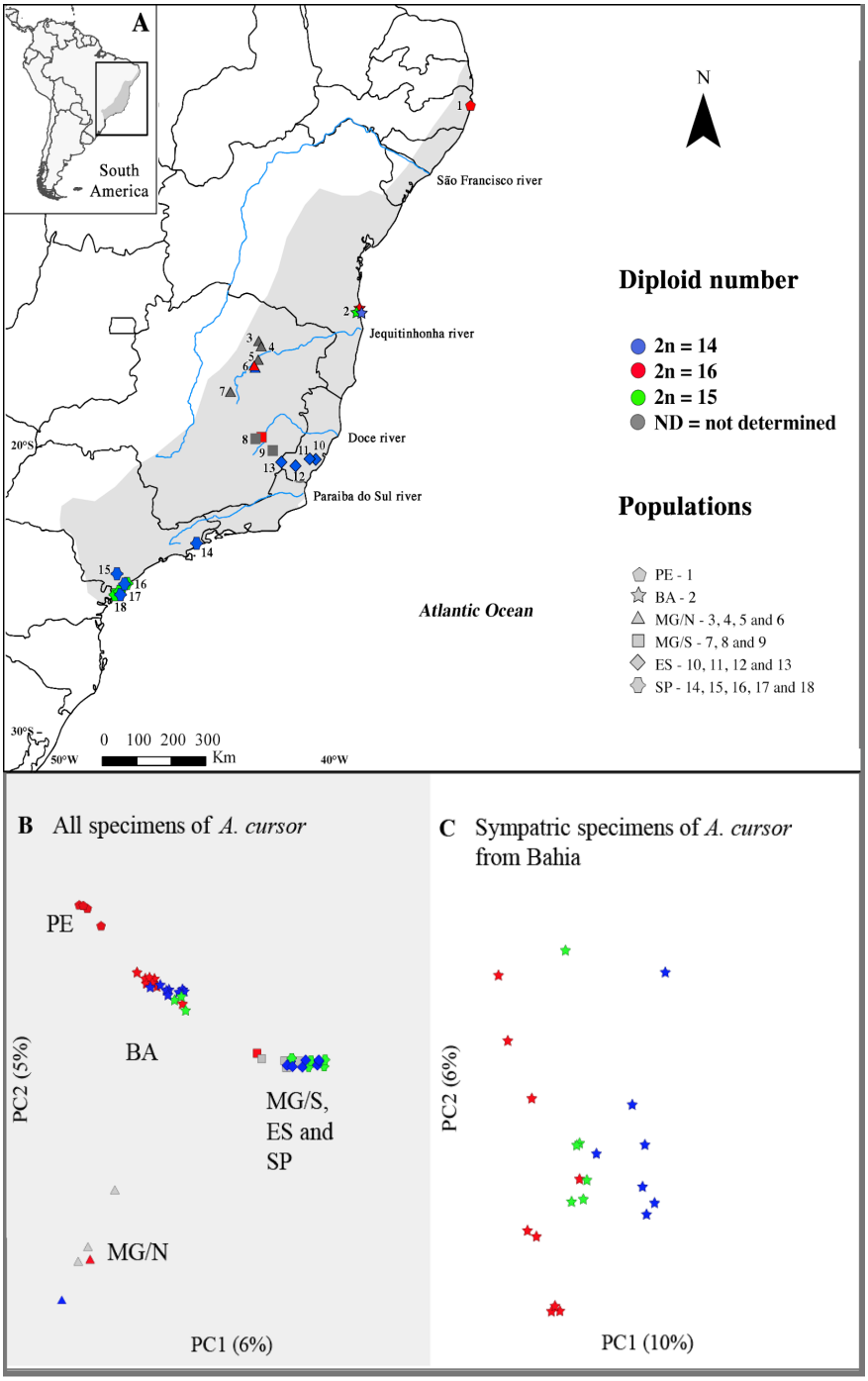

Figure 5. A) Map of Akodon cursor distribution in grey according to the IUCN showing sampled populations with unique symbols and respective karyotypes given in different colors (ND marks individuals without karyotypic data). Numbered localities are described in the Supplementary Material. The main coastal rivers rivers are represented in light blue lines and the populations, often named after the respective Brazilian states where they were sampled are shown in the legend: specifically, Pernambuco (PE), Bahia (BA), Minas Gerais (MG), Espirito Santo (ES) and Sao Paulo (SP). B) Principal component analysis of 54 specimens of A. cursor. C) PCA of individuals from Bahia, where representatives of the three karyotypes are found in sympatry.

Both STRUCTURE analyses showed a K= 2 (the most probable number of genetic clusters) (Table S2 in Supplementary Material for details about the most probable K). The all-individuals analysis confirmed a sharp North-South divergence, with individuals from Minas Gerais being split into MG/N or MG/S (Fig. 6A). It is noteworthy to point that one individual from a northern locality in Minas Gerais (Fig 5A), in the region of the source of the Jequitinhonha river, was grouped with those from southern Minas Gerais, with the closest locality situated more than 350 km apart, suggesting the main break between clades is in the area of the source of the Jequitinhonha river.

Likewise, sequential STRUCTURE analyses recovered distinct genetic clusters of sympatric individuals from Bahia that differed in their karyotypes (Fig 6B), such as K=2 separating 2n= 14 (blue) and 2n=16 (orange) while individuals with 2n=15 and some 2n=16 showed an intermediary conditions (orange/blue). This result are in accordance with the PCA depicted on Fig. 5C, because not only 2n= 15 individuals presented an intermediary genetic pattern. There were some individuals 2n= 16 that presented similar genetic background to that observed in 2n= 15 samples, suggesting backcrossing might happen among them. Also, our STRUCTURE results suggest that 2n= 15 individuals can endure beyond F1 generations, since there is was one individual presenting more proportions of orange than blue (and not 50% of each as expected in F1 individuals). On the other hand, the 2n= 14 group does not have signs of genetic admixture with the 2n= 16+15 group. Thus, the karyotypes of the individuals seem to have an influence on reproduction, however, the primary division separating individuals was geographical (Fig. 6A).

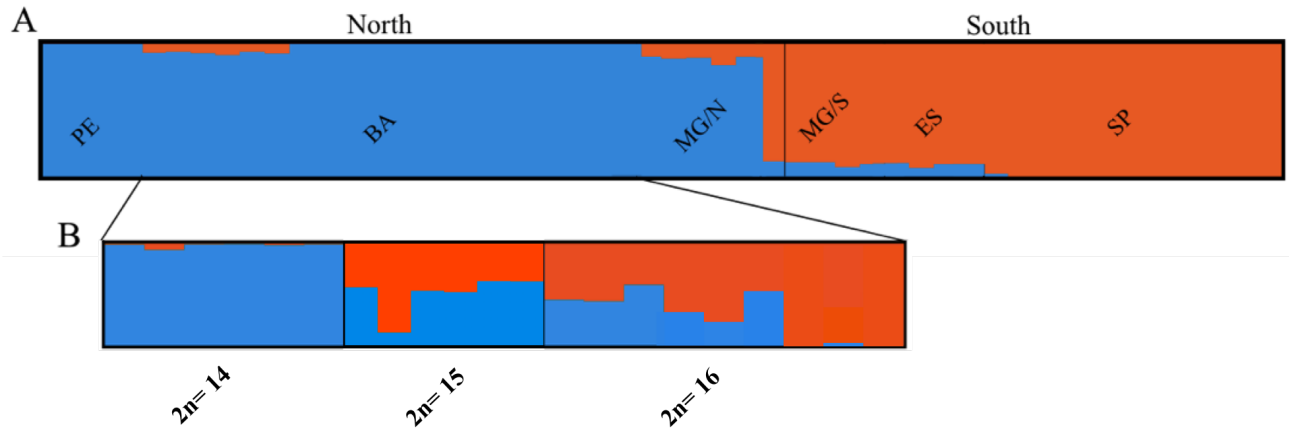

Figure 6. Population STRUCTURE analyses with posterior probability plots of individual assignments to the inferred genetic clusters shown for the most probable K-value of K=2 for A. all Akodon cursor populations (which contains all three different karyotypes; see Fig. 1 for distribution of populations), and B. for analysis of individuals from the Bahia population, which has representatives of the three karyotypes in sympatry, and for which K = 2 was most probable (see Table S2 in the Supplementary Material for details regarding the posterior probability of different numbers of K genetic clusters).

The tree from SVDquartets recovered the three karyotypes in both north and south groups (Fig. 7). The south group is monophyletic and the north is paraphyletic. In the north the individuals from the same locality in Bahia do not form a monophyletic group. There is a clade composed of only 2n= 14/Bahia which shares the most recent common ancestor with populations from Pernambuco and MG/N and not with the other clade containing Bahia individuals (2n=15 and 16). The south clade also follows a clinal distribution, having individuals from ES, MG/S and north of SP forming a subclade and samples from the southernmost SP as another group. Despite the fact that we did not have 2n= 16 individuals from SP, differently from Bahia, the southern individuals of 2n= 15 grouped with 2n= 14 individuals. Lastly, the phylogenetic tree showed similar results compared to STRUCTURE and PCA about the overall north-south split of the species and the peculiar groupings of the sympatric individuals from Bahia, showing that

the 2n= 15 individuals are not byproducts of admixture between 2n= 14 and 2n= 16 individuals, and are closely related with the 2n= 16 individuals.

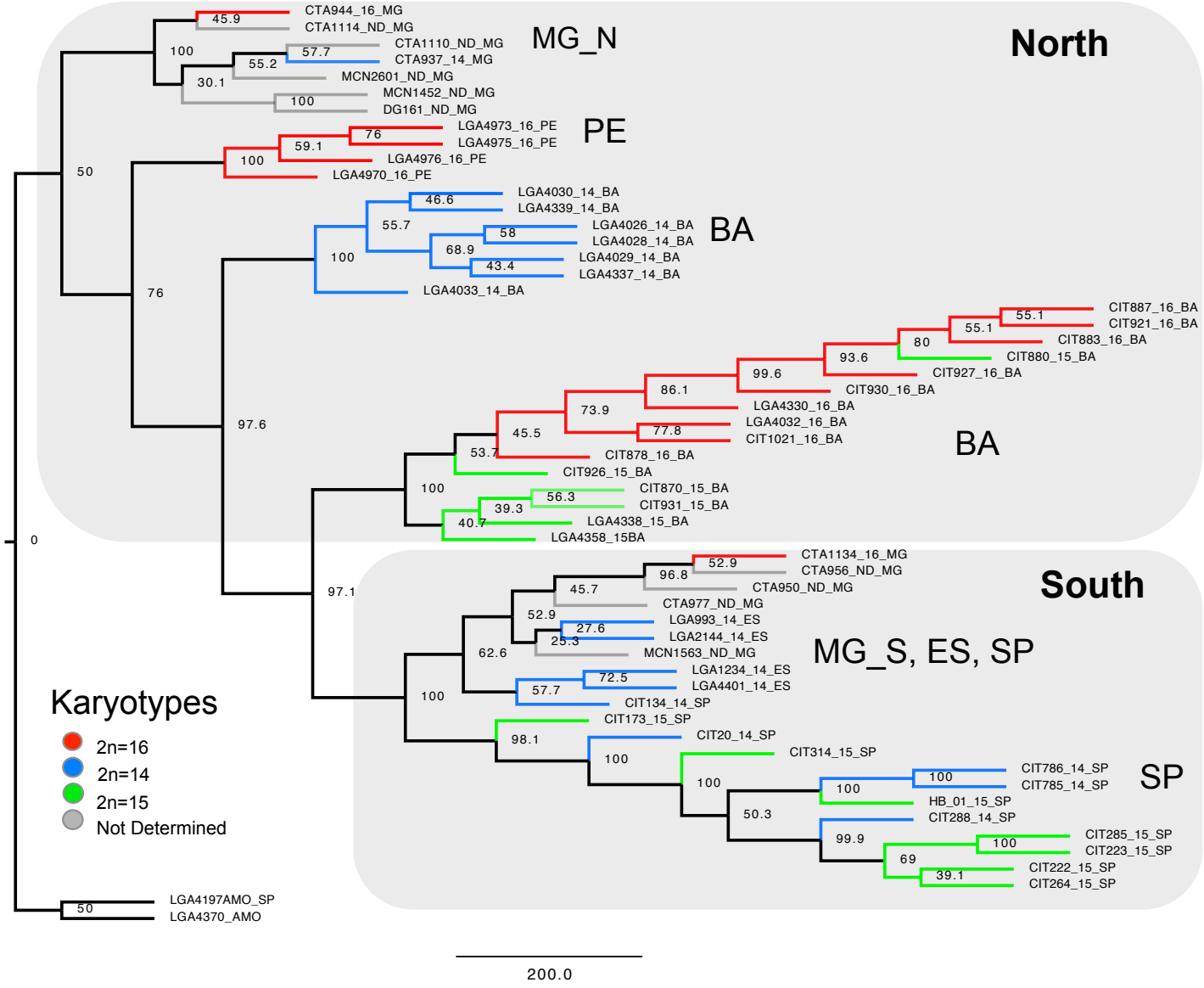

Figure 7. Phylogenetic tree of A. cursor, with 54 individuals and 77,809 SNPs inferred under the coalescente model using SVDquartet, using A. montensis as outgroup. The tree shows the divergence between populations that are located in the north and in the south of the Jequitinhonha river. The branches are color coded by the sample's karyotype. Bootstrap values are shown. Populations are named after the states in which the individuals were collected: Pernambuco (PE), Bahia (BA), Minas Gerais (MG/N and MG/S for the individuals with a more northern versus southern phylogenetic affinity, respectively), Espirito Santo (ES), and Sao Paulo (SP).

In general, geographic structuring was also apparent in F_{ST} analyses (Table S11 on Supplementary Material), with values ranging from 0.07 to 0.27 between populations of the same geographic region to 0.12 to 0.28 between populations of different regions. Genetic diversity (Table S12 on Supplementary Material) shows broadly overlapping values among populations and karyotypes.

DISCUSSION

In our study we integrated evidence from karyotype polymorphisms, genomics and geographic distribution aiming to investigate the process of lineage diversification, reproductive isolation and putative speciation in a vertebrate species. The use of complementary approaches allowed us to infer about the relative influence of geographic constraints and karyotype variants in shaping the process of genetic diversification in a widely distributed neotropical rodent species. Our results corroborated previous mtDNA results, with geographic isolation as the main factor separating the populations of *A. cursor* into north and south lineages (Geise et al. 2001; Nogueira and Fagundes 2008; Maestri et al. 2016). Each clade presents the same three karyotypes defining secondary levels of genetic structure – that is, individuals with similar karyotypes are not similar by descent from a common ancestor. Instead, we can say that divergence associated with different karyotypes is more recent than geographic structure across the species range. Also, the karyotypic variants arose repeatedly within different regions. We found no clear evidence of clustering based on karyotypes in the north and south groups, although some populations present individuals with one exclusive diploid number, such as Pernambuco with 2n= 16 (north) and Espírito Santo with 2n= 14 individuals (south).

However, the predominance of geographic structuring of genetic variation does not necessarily mean that karyotypic differences do not also contribute to reduce gene flow in *A. cursor*. When we analyzed the genetic background of a northern population (i.e. Bahia) where individuals with the three distinct karyotypes live in simpatry, we observed that independent lineages are defined by karyotypes and somehow the karyotype

constitution would have an influence on mate choice or some other reproductive characteristic of individuals in this particular population.

The north-south division of biodiversity in the Atlantic Forest

The genomic data in this study recovered the primary divergence in *A. cursor* as a division of a paraphyletic north group and south clade, having representatives of the three karyotypes in both main groups. This split in *A. cursor* was already suggested in previous studies using analyses of mtDNA (Geise et al. 2001; Nogueira and Fagundes 2008). Firstly, a riverine barrier in the north Espírito Santo state (i.e. the Doce river, represented on Fig. 5A) was refuted by Colombi et al. (2010), and later a more comprehensive study suggested the phylogeographic break of *A. cursor* along the Jequitinhonha river (Maestri et al. 2016).

The north-south pattern of genetic diversity is also reported for other vertebrates from the Brazilian Atlantic Forest and most of them are coincident with coastal rivers (Costa and Leite 2013; Paz et al. 2018). Although some of these studies tried to explain the observed north-south distribution of the fauna from the Atlantic Forest considering rivers as barriers, as postulated by Wallace (1852), most of them have proposed the combination of rivers with climatic and vegetation changes using the theory of pleistocenic refuges (Carnaval and Moritz 2008). Pleistocenic refuges of the Atlantic Forest are considered stable remnants of forest patches that may have existed during the Pleistocene (2.58 – 0.01 MYA [million years ago]), where populations of many groups of organisms were confined and, because of the period of isolation these patches exhibit high levels of

endemism and species richness (Carnaval and Moritz 2008; Carnaval et al. 2009; Carnaval et al. 2014). However, a recent new hypothesis has been proposed arguing that during the last glacial maximum (21 kybp) favorable climatic conditions were found in the emerged area of the continental shelf, promoting forests expansion and benefiting forest-dependent species from the Atlantic Forest (Leite et al. 2016).

Nonetheless, divergence time estimates obtained by fossils and mutation rates of mtDNA pointed the oldest *Akodon* fossil (*Akodon lorenzinii*), known from east-central Argentina, to be about 2.1 million years old (Reig 1987) and the divergence of *A. cursor* and *A. montensis* was estimated to have occurred during the Pleistocene at about 1.0-2.6 MYA (Coyner et al. 2013). Although the divergence time of the north and south lineages of *A. cursor* was not estimated here, we considered that it could have happened right after *A. cursor* separated from *A. montensis* or more recently. Nonetheless, this putative divergence period of the species suggests there is no synchronicity of the north-south splitting window of time with the Jequitinhonha river age, once its geological groups date the Proterozoic (Saadi 1995).

Although tempting, the use of rivers as barriers and pleistocenic refuges as proxy biogeographical explanations for the divergence patterns observed in numerous species, we found support for the idea that the river and the confinement of populations in refuges were not necessarily the causative agents of genetic divergence in *A. cursor*. It is likely that the river itself did not have a role promoting lineages of *A. cursor* to diverge because the river is too old (its geology dates the Proterozoic, see Saadi 1995). In addition to that, long-term ecological studies reporting habitat preferences of *A. cursor* pointed that the

species is more abundant in forest edges and seldom caught in the interior of mature forests, preferring open grass fields and disturbed areas (Geise 2012). Thereby, neither the forest remnants that formed the pleistocenic refuges nor the forested exposed continental shelf would be suitable areas for this species to thrive. Otherwise, the matrix between the forest refuges would present better conditions for expanding the populations of the cursor grass mouse, connecting once allopatric populations, blurring the north-south genetic biogeographical sign of the species. Similar pattern, where the grasslands offered optimal conditions for populations expansion during the climatic oscillations of the Pleistocene, was observed for *Oxymycterus nasutus* (rodent endemic to open areas in the Pampas and Atlantic Forest biomes) (Peçanha et al. 2017).

Searching for processes that might have influenced the primary genetic divergence in *A. cursor*, we found that Ribeiro et al. (2006) was the first to explicitly use arguments of recent tectonic activity (during the Quaternary) to explain the biogeographical distribution of fishes in southern Atlantic Forest. Such geological events have also been mentioned as probable causes for the north-south split in species distribution of bees (Batalha-Filho et al. 2010), frogs (Thomé et al. 2010; Brunes et al. 2014; Paz et al. 2018) and marsupials (Sartorato Zanchetta et al. 2019). These recent geological manifestations on the Brazilian Platform were mapped by Saadi (1993) and Saadi et al. (2002) and many of them are coincident with important coastal rivers and delimit known areas of phylogeographic endemism of the Atlantic Forest biodiversity (Silva et al. 2012; Carnaval et al. 2014; DaSilva et al. 2015). Moreover, these geological maps also show faults and lineaments in the country side of Brazil that are not always coincident with rivers, that could be tested as hypothetical barriers in further phylogeographic studies.

Thus, we argue that recent tectonic manifestations during the Quaternary (less than 1.6 MYA according to Saadi et al. 2002) in the area of the Jequitinhonha river might have influenced gene flow between populations of *A. cursor*, producing the north-south genetic pattern. One locality in the north of Minas Gerais (e.g. Diamantina number 7 in Fig. 8), helped localizing with more accuracy the area of the primary split observed in *A. cursor*. Despite being geographically closer to the other samples from the north of Minas Gerais (numbers 3, 4, 5 and 6), our genomic analysis grouped the specimen from Diamantina with individuals from the southern populations of Minas Gerais (numbers 8 and 9 in Fig. 5). This city is inserted in the southern border of the Espinhaço mountain range, near the source of the Jequitinhonha river, a region characterized by the encounter of two geological groups, the Macaubas group, where the sample from Diamantina is located, and the Supergrupo Espinhaço, where all other samples from the north of Minas Gerais are inserted (Fig. 5). This geological enclave reflects in a complex landscape, with varied types of soils and consequently diverse vegetation types, including highland field formations (*campos rupestres*), flooded areas (*veredas*) and cerrado *stricto sensu* (Benites et al. 2003; Schaefer et al. 2016). Also the mountain range of Espinhaço separates two important Brazilian biomes in its central and southern portions: the Atlantic Forest on the eastern slope, and the Cerrado on the western slope (Melo-Junior et al. 2001). The Jequitinhonha river geology dates the precambrian, nonetheless the Espinhaço range has been through periods of tectonic stability in the Miocene (5-20 MYA), but also a recent strong compressional tectonic event in the Pleistocene (2-5 MYA) resulted in an eastward tilting of the plateau and in the capture of several valley heads of the São Francisco drainage basin by the Jequitinhonha hydrographic system (Saadi 1995). Even more recent

neotectonic reactivation was reported in the right margin of the Jequitinhonha river (less than 1.6 MYA), generating the Araçuai river fault (Fig. 5) which defined the eastern border of the "Serra do Espinhaço Setentrional" (Saadi 2002). This long fault (639 km) has its geomorphology expressed differently along its southern and northern parts. In the south (near the source of the river) it forms a strongly entrenched, linear (NE-SW) fault line-valley (Upper Araçuai River). On the north, the fault controls the confluence of the Jequitinhonha and Araçuai rivers.

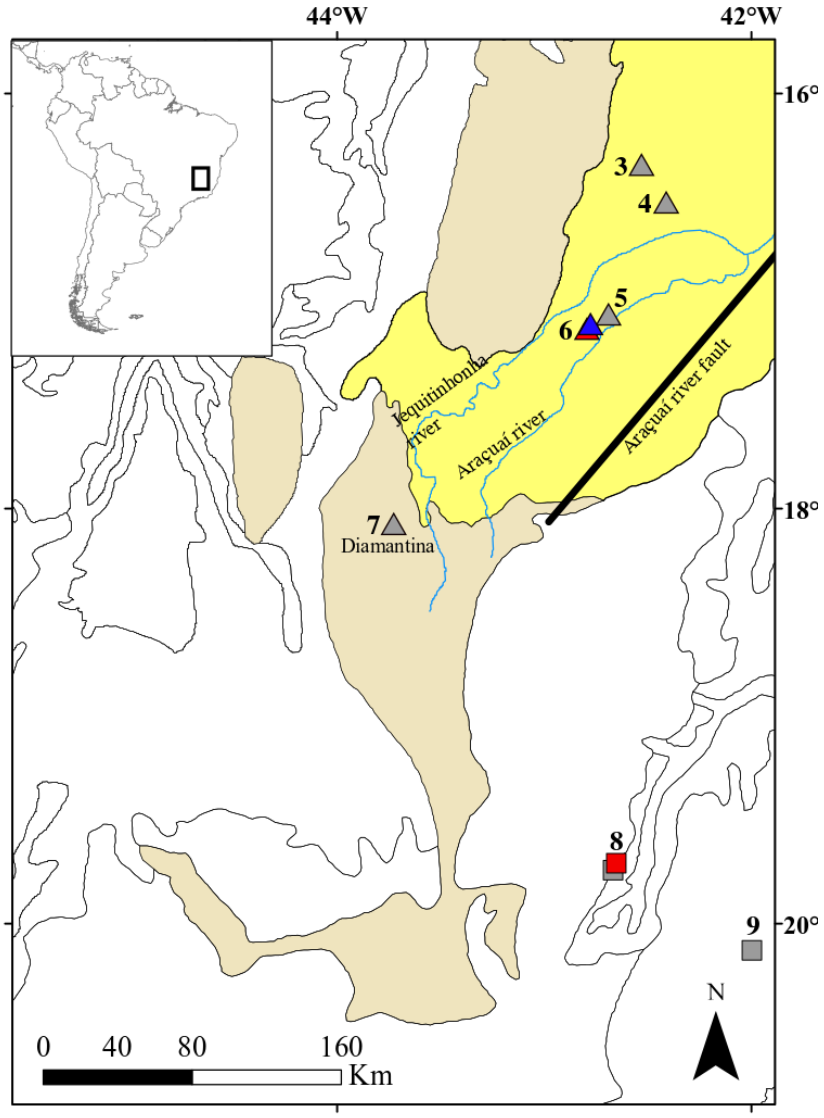

Figure 8. Representation of the main geological groups in the area of the source of the Jequitinhonha river, with the bright yellow area as the Macaubas Group and the pale yellow as the Espinhaço Supergroup. Localities are all from Minas Gerais state (localities names are referred in Fig. 5), but numbers 3, 4, 5 and 6 are from the North clade and localities 7, 8 and 9 are belong to the South clade. The thick black line represents the south portion of the Araçuaí River fault. Modified of Saadi (1995) and Saadi et al. (2002).

However, to corroborate our hypothesis it would be necessary that the age of these recent geological events overlapped with the time of divergence of the lineages. If we consider the standard error of the estimated divergence of *A. cursor* and *A. montensis* between 1.0-2.5 MYA (Coyner et al. 2013), the putative period of the *A. cursor* lineages splitting is synchronic with neotectonic activity reported as less than 1.6 MYA in the Jequitinhonha valley (Saadi 1995; Saadi et al. 2002). Although the range of time of this north-south split coincides with the Pliocene-Pleistocene period, the forest refuges would not favor the biology of *A. cursor* and ultimately we could suggest climate would not operate as a trigger for vicariance in this open field/grass species. Therefore, neotectonic activity is a plausible vicariant event that may explain the main diversification pattern observed in *A. cursor.*

Karyotypes and genetic divergence

Our data showed that the overall genetic diversity in *A. cursor* is primarily nested within the northern and southern regional groups. Thus, we believe that chromosomal rearrangements arose more than once in the evolution of this species, and consequently, should not be associated with vicariant events that accumulated, with reduced gene flow across the Jequitinhonha river. Each major clade presents shared karyotypes defining secondary levels of genetic structure – that is, in some cases, individuals with similar diploid number are not similar by descent from a most recent common ancestor. Instead, divergence associated with different karyotypes is more recent than geographic structure across the species range and arose repeatedly within different regions.

This places the general role of such chromosomal changes in *A. cursor* secondary to geographic/historic factors, which show the strongest degree of genetic structure, as highlighted by the most probable number of clusters divided by abiotic factor in the region of the source of the Jequitinhonha river. The concordance across taxa in their phylogeographic breaks is not unheard of, and in fact, is evidence of abiotic factors structuring species divergence (Papadopoulou and Knowles 2015). However, such geographic structuring of genetic variation when considered jointly with the evolutionary history of karyotypic differences reveals whether or not such abiotic factors might work in tandem with biotic factors (i.e. karyotypic change is coincident with the geographic division). Our results could be related to the recentness and repetitive chromosomal differentiation compared with the genetic differentiation acquired in allopatry during the last inter glaciation periods, and suggests that in the studied taxon karyotypic differences played a minor role in structuring the populations relative to historical and/or geographical factors.

Nonetheless, the predominance of geographic structuring of genetic variation does not necessarily mean that karyotypic differences do not also contribute to reduce gene flow. Genomic data, at least in Bahia population, showed that intermediate forms (heterokaryotypes) are not necessarily the direct product of admixture of two main gene pools (homokaryotypes), but is primarily associated with 2n= 16. In Bahia, where the three karyotypes are found in sympatry, the genetic data showed that the sampled 2n= 15 individuals are more closely related with 2n= 16 than to 2n= 14 individuals, which appears as a distinct group. It indicates that some reproductive isolating mechanism may be operating among these sympatric 2n=14 and 2n=15/16 lineages in the wild. We

believe that, at least in the Bahia population, it might exist some reproductive isolating mechanism between 2n=14 individuals and the 2n=16+15 group (i.e. sexual interactions such as divergent mating preferences or courtship behaviors reducing interbreeding). It is likely that the homokaryotypes from Bahia belong to distinct lineages that came into secondary contact and reproductive isolation was enhanced when the allopatric lineages became sympatric.

Our findings are concordant with the *Drosophila* studies conducted by Coyne and Orr (1989; 1998), where they observed that mating discrimination and postzygotic isolation evolve at similar rates in allopatric species, but among sympatric species strong mating discrimination appears well before severe sterility or inviability. This process, known as reinforcement, suggests that prezygotic reproductive isolation may be enhanced during sympatric speciation or following secondary contact between populations derived in allopatry (Dobzhansky 1940; Servedio and Noor 2003; Rosser et al. 2019).

Furthermore, despite the fact that our southern samples did not contain a replicate population with individuals of the three karyotypes in sympatry, we could observe in the phylogenetic tree that the southern 2n= 15 individuals grouped with the 2n= 14 individuals, which is the opposite situation observed with the northern 2n= 15. This suggests that in the past, the heterokaryotype individuals may have had some reproductive compatibility with 2n=16 (in the north) and also with 2n= 14 (in the south), as seen by the shared genetic background. These findings signal that heterokaryotypes may have different reproductive interactions with distinct homokaryotypes and that they could be generated and maintained in nature by different means.

Herein we integrated different perspectives on the DNA of *A. cursor*, by studying the role of its genomic structural variants allied to the phylogeographic background they comprise. Because of the astonishing karyotypic diversity of the species (Fagundes et al. 1998), many theories have been proposed to understand how all these variants emerged and if they have any role in promoting the species' diversification. Based on G-banding and FISH data, Fagundes et al. (1997a,b) have proposed that 2n = 16 would be the primitive karyotype of *A. cursor* and that the 2n = 14 karyotype is its most derived form. Rieger et al. (1995) have proposed that *A. cursor* (2n= 14 and 2n= 15) would have originated from *A. montensis* (which occurs in the South and Southeast Brazil) and that 2n= 16 (*A.* aff. *cursor*) have originated in the extreme northern end of the *A. cursor*'s distribution range. We argue that *Akodon* sp., with 2n= 10, from Central Brazil and not *A. montensis*, is more closely related to *A. cursor* (Silva et al. 2006) and the interpretation of chromosomal evolution should consider its closest relatives to get a hint to the direction of the evolution (Schubert and Lysak 2011). Moreover, all these assumptions imply that the different karyotypes of *A. cursor* had one single origin and that each karyotype shares the same common ancestor. With genomic data we could see that the same karyotypes do not come from a most recent common ancestor and that the different karyotypes probably appeared more than once in different populations, demonstrating the ephemerality of such structural variants. And the region where *Akodon* sp. (2n= 10) occurs is close to the region where *A. cursor* have probably split initially into two main fronts, leading us to suggest that the species probably originated in Central Brazil (and not in the South as previously hypothesized) with the different karyotypes emerging, being diferentially fixated in populations and disappearing more than once.

Conclusions and future directions

The evolutionary effects of chromosomal rearrangements are indisputable. However, as just one mechanism with the potential to contribute to species divergence, the evolutionary history of such changes is integral to understanding their contribution in the divergence process, and specifically, the extent to which such changes are the drivers or consequence of other factors that promote species divergence. Geographic sampling across the range of *A. cursor* in our work suggests that chromosomal changes are common, appearing more than once in divergent lineages. As such, we argue that other factors, and primary geographic isolation, that is responsible for the distribution of chromosomal variants within taxa and their potential role in species divergence.

Recent geological manifestations in the area near the source of the Jequitinhonha river can be associated to the vicariant event that caused the species to split into north and south lineages. However, some questions still need deeper investigation. A more precise dating of neotectonic events in Brazil and estimating the divergence times of the clades would provide a fruitful scenario to be explored by evolutionary biologists that work with recent lowland vertebrate species, that are not forest-environment-dependent. Also, some questions still need to be answered, such as: Is there a role of the karyotypes in the species fertility? Does the north-south genetic division of the species reflect into reproductive isolation of individuals? Do they all have the potential do mate and produce viable offspring? Do they all form viable gametes? Are the heterokaryotypes (intermediary karyotypic condition) equivalent to interspecific hybrids, regarding

reproductive parameters? Understanding the mechanisms of how heterokaryotype individuals of *A. cursor* are generated and maintained in nature would highlight how the early stages of incompatibilities are triggered and help hypothesizing how that could ultimately lead to speciation. Our study emphasizes that despite new technological approaches, cytogenetic characterization was important for bringing up evolutionary insights such as if the chromosomal rearrangements could ultimately have a role in speciation. Because of that, it is important that cytogenetics continue to be done in the field and to train a new generation of cytogeneticists (Di-Nizo et al 2017; Deakin et al. 2019).

Our findings corroborate that an integrative taxonomy, coupling research areas of ecology, morphology, genomics, cytogenetics, cell biology and bioinformatics could benefit evolutionary understanding as a whole (Padial et al. 2010; Deakin et al. 2019). Complementary criteria, such as biogeographic patterns, molecular divergence and phenotypes (including morphology and karyotype) make the species scenario more complex, such as it probably is in nature.

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SUPPLEMENTARY MATERIAL

Table S1. Information about the specimens of Akodon (A. cursor and A. montensis) used in the genomic library, including diploid number (2n), quality control processing and geographic coordinates.

Voucher	2n	State	Locality	Lat	Long	Total Reads	No RadTag	Low quality	Retained	Mean coverage	St dev	Max	Missing data	Missing data after Plink
Akodon cursor														
LGA 4339	14	Bahia	Una	-15.18	-39.04	901667	12374	484	847133	7.51117	7.34217	639	0.9006	0.525
LGA 4030	14	Bahia	Una	-15.18	-39.04	1744902	15879	886	1696291	10.7083	5.04161	496	0.6144	0.01723
LGA 4026	14	Bahia	Una	-15.18	-39.04	1999592	13906	1017	1935081	12.0575	6.15398	172	0.5975	0.01248
LGA 4033	14	Bahia	Una	-15.18	-39.04	2400245	11273	1215	2318198	12.065	5.76923	147	0.5391	0.0104
LGA 4028	14	Bahia	Una	-15.18	-39.04	3106401	14348	1602	3014949	15.1418	7.95441	811	0.4786	0.003861
LGA 4029	14	Bahia	Una	-15.18	-39.04	3575180	42495	1758	3370617	13.954	7.27161	170	0.4802	0.005941
LGA 4337	14	Bahia	Una	-15.18	-39.04	4858796	17786	2527	4690901	20.2613	11.645	454	0.426	0.003168
LGA 4338	15	Bahia	Una	-15.18	-39.04	1051916	4512	535	1020098	8.55363	6.64817	922	0.799	0.2019
CIT 926	15	Bahia	Una	-15.18	-39.04	1094283	9676	555	1058378	8.88052	4.27413	435	0.7934	0.1559
LGA 4358	15	Bahia	Una	-15.18	-39.04	1238179	6794	633	1186484	8.73811	4.41333	154	0.7782	0.2117
CIT 880	15	Bahia	Una	-15.18	-39.04	1592052	12361	816	1484099	7.78218	19.2867	2427	0.8915	0.5064
CIT 931	15	Bahia	Una	-15.18	-39.04	2886148	13041	1492	2821061	14.1277	7.57847	448	0.4975	0.005347
CIT 878	16	Bahia	Una	-15.18	-39.04	1636958	10944	837	1575763	9.5975	5.31714	752	0.651	0.07653
CIT 887	16	Bahia	Una	-15.18	-39.04	3383014	21782	1788	1815411	10.454	4.13884	244	0.5818	0.02267
CIT 883	16	Bahia	Una	-15.18	-39.04	1903642	17503	991	1834068	10.5206	4.96879	189	0.5953	0.02564
CIT 927	16	Bahia	Una	-15.18	-39.04	2151178	13341	1142	2077719	11.461	5.30258	203	0.5551	0.01505
LGA 4330	16	Bahia	Una	-15.18	-39.04	2615560	10256	1373	2537342	13.0616	6.52577	719	0.519	0.005743
CIT 930	16	Bahia	Una	-15.18	-39.04	3043652	11712	1566	2977736	15.8593	9.02241	396	0.5101	0.003663
CIT 921	16	Bahia	Una	-15.18	-39.04	3218858	18411	1682	3115207	14.6767	7.05472	246	0.4785	0.003762
LGA 4032	16	Bahia	Una	-15.18	-39.04	4599662	21234	2388	4495308	22.5211	14.7195	822	0.4824	0.01842

CIT 1021	16	Bahia	Una	-15.18	-39.04	5069030	17315	2567	4954371	22.7604	12.6916	388	0.4289	0.002376
LGA 993	14	Espírito Santo	Castelo	-20.6	-41.18	1830089	5583	993	1788649	11	4.83187	177	0.5805	0.02564
LGA 4401	14	Espírito Santo	D. Martins	-20.37	-40.67	2300317	11865	1197	2233511	12.312	6.73355	773	0.5447	0.01059
LGA 1234	14	Espírito Santo	Ibitirama	-20.48	-41.72	2108889	15299	1056	2052424	11.7072	5.83861	208	0.5783	0.01337
LGA 2144	14	Espírito Santo	Viana	-20.38	-40.45	3157486	15795	1623	3080145	14.6815	7.4895	225	0.4718	0.006337
MCN-M 1563	ND	Minas Gerais	Diamantina	-18.09	-43.75	2474412	14859	1271	2377933	12.0377	5.66904	131	0.5414	0.01406
MCN-M 2601	ND	Minas Gerais	Grão Mogol	-16.62	-42.81	790697	5647	428	763929	8.18445	5.341	628	0.8683	0.3236
MCN-DG 161	ND	Minas Gerais	J. Gonçalves de Minas	-16.62	-42,81	518525	3862	266	504761	7.4223	7.53234	707	0.9376	0.5939
MCN-M 1452	ND	Minas Gerais	Leme do Prado	-17.08	-42.69	3114163	14314	1551	3036511	15.8131	9.8847	430	0.5181	0.006733
UFES-CTA 1134	16	Minas Gerais	Marliéria	-19,71	-42,65	1423986	8989	719	1390050	10.6002	5.35513	180	0.7021	0.06356
UFES-CTA 956	ND	Minas Gerais	Marliéria	-19,71	-42,65	1913661	9337	996	1864929	16.0104	8.52498	257	0.4702	0.00505
UFES-CTA 950	ND	Minas Gerais	Marliéria	-19,71	-42,65	2282325	9252	1199	2206161	13.1238	6.06926	284	0.5524	0.03277
UFES-CTA 977	ND	Minas Gerais	Simonésia	-20.13	-42	3706304	30259	1964	3580155	16.1993	8.33728	147	0.4518	0.004554
UFES-CTA 937	14	Minas Gerais	Turmalina	-17,13	-42,77	3667347	18115	1865	3588583	12.0602	7.35452	223	0.5459	0.01406
UFES-CTA 944	16	Minas Gerais	Turmalina	-17,13	-42,77	1913661	9337	996	1864929	10.8143	5.08581	332	0.6074	0.02188
UFES-CTA 1114	ND	Minas Gerais	Turmalina	-17,13	-42,77	1399597	8796	674	1350906	9.24035	4.01742	166	0.6976	0.07604
UFES-CTA 1110	ND	Minas Gerais	Turmalina	-17,13	-42,77	1828450	9711	946	1781040	10.8635	4.60099	110	0.607	0.02356
LGA 4973	16	Pernambuco	Camaragibe	-7.97	-34.98	1728282	9077	880	1666570	10.398	5.03137	549	0.6112	0.04386
LGA 4975	16	Pernambuco	Camaragibe	-7.97	-34.98	2047866	8375	992	1981187	11.3902	5.49069	542	0.567	0.01733
LGA 4970	16	Pernambuco	Camaragibe	-7.97	-34.98	3045455	15819	1596	2952275	14.7183	7.31116	199	0.5079	0.01149
LGA 4976	16	Pernambuco	Camaragibe	-7.97	-34.98	4781656	22157	2509	4654470	21.9253	12.401	518	0.4493	0.005842
CIT 314	15	São Paulo	Ariri	-25.2	-48.03	1070126	8223	523	1040260	8.91071	3.70269	74	0.7728	0.1314
CIT 288	14	São Paulo	Iguape	-24.72	-47.55	4072967	17900	2065	3953959	18.8717	10.0908	247	0.4544	0.003861
CIT 222	15	São Paulo	Iguape	-24.72	-47.55	1647245	10306	878	1606166	10.2974	4.73778	607	0.6059	0.03188

CIT 173	15	São Paulo	Iguape	-24.72	-47.55	1925084	6004	925	1883300	10.9339	5.25624	750	0.5704	0.02574
CIT 264	15	São Paulo	Iguape	-24.72	-47.55	2802532	12874	1447	2736821	13.954	8.67166	294	0.5535	0.006436
CIT 285	15	São Paulo	Iguape	-24.72	-47.55	3001567	16014	1451	2904153	15.3797	7.77589	220	0.4944	0.004356
CIT 223	15	São Paulo	Iguape	-24.72	-47.55	4754912	17153	2439	4629931	21.8329	11.5889	211	0.4326	0.003069
CIT 786	14	São Paulo	Ilha do Cardoso	-25.17	-47.93	2591074	256897	1257	2291404	13.2788	7.80091	696	0.5519	0.004059
CIT 785	14	São Paulo	Ilha do Cardoso	-25.17	-47.93	4059396	19652	2000	3940462	19.1854	10.2431	818	0.4477	0.001089
UERJ-HB 01	15	São Paulo	Ilha do Cardoso	-25.17	-47.93	3238237	12586	1678	3151816	15.3755	7.44789	202	0.4817	0.003366
CIT 20	14	São Paulo	Sete Barras	-24.38	-47.85	3764603	21198	1916	3646404	16.5465	8.4464	204	0.4486	0.005347
CIT 134	16	São Paulo	Ubatuba	-23.37	-44.83	1886907	10483	951	1850991	11.7692	7.31683	801	0.6058	0.01802
CIT 870	15		Una	-15.18	-39.04	2270668	11248	1167	2189931	11.4757	5.36883	315	0.5543	0.02455
Akodon montensis														
LGA 4197	24	São Paulo	Cananéia	-25.01	-47.93	3885464	14722	2008	3756272	18.1686	9.07282	222	0.5517	0.0603
LGA 4370	24	São Paulo	Cananéia	-25.01	-47.93	4101858	15665	2135	4007959	21.1304	10.9423	365	0.563	0.06257

Table S2. Results of STRUCTURE analyses on the dataset containing all populations of A. cursor and only the Bahia population, that has representatives of the three karyotypes of the species

Level	Group	Inds	1st K	Delta K	2nd K	Delta K	MCMC	Burn in
1	All *A. cursor*	54	2	1555.108038	3	149.986577	1.000,000	500,000
1.1	Bahia	22	2	32.438048	3	3.104967	1.000,000	500,000

Table S3. Pairwise F_{st} estimates between sampled populations and their karyotypes. Dark grey headings are for northern populations and light grey for southern populations.

Population and karyotype	Pernambuco (2n= 16)	Bahia (2n=16)	Bahia (2n=14)	Bahia (2n=15)	MG/N (ND)	MG/S (ND)	Espirito Santo (2n= 14)	Sao Paulo (2n=14)	Sao Paulo (2n=15)
Pernambuco (2n= 16)		0.187187	0.218255	0.259666	0.277294	0.238479	0.24602	0.280423	0.267006
Bahia (2n= 16)			0.101512	0.0779078	0.161087	0.148983	0.151492	0.177565	0.177379
Bahia (2n= 14)				0.0987067	0.175122	0.127008	0.131485	0.159882	0.162666
Bahia (2n= 15)					0.208326	0.163329	0.16801	0.201234	0.196056
MG/N (ND)						0.180965	0.185529	0.21842	0.210731
MG/S (ND)							0.0975467	0.131095	0.134792
Espirito Santo (2n= 14)								0.134534	0.137107
Sao Paulo (2n= 14)									0.0843043
Sao Paulo (2n= 15)									

Table S4. Summaries of genetic diversity, percentage of polymorphic sites (% pol), (average observed heterozygosity (H_{obs}), average nucleotide diversity (π), and Wright's inbreeding coefficient (F_{IS}) per sampled population and for each karyotype when information was available, as well as sample sizes and percentage of sites that were polymorphic within each population.

Population	n	% pol	π	H_{obs}	F_{is}
Pernambuco (2n= 16)	4	0.3168	0.0475	0.0408	0.0126
Bahia (total)	16	0.9508	0.0886	0.066	0.0621
2n= 16	8	0.6931	0.0789	0.0637	0.037
2n= 14	5	0.6654	0.0845	0.0698	0.0318
2n= 15	3	0.4939	0.0807	0.0712	0.0176
MG/N (ND)	4	0.6206	0.0881	0.0691	0.0353
MG/S (ND)	4	0.6766	0.0923	0.0755	0.0327
Espirito Santo (2n= 14)	4	0.6469	0.0905	0.0736	0.0324
Sao Paulo (total)	11	0.7462	0.0734	0.0542	0.0538
2n= 14	5	0.5298	0.0712	0.0535	0.0387
2n= 15	6	0.5577	0.0694	0.0555	0.0331

Capítulo 2

Closing the ring: the early stages of a speciation process in a neotropical rodent

Fechando o anel: os estágios iniciais de um processo de especiação em um roedor neotropical

Closing the ring: the early stages of a speciation process in a neotropical rodent

ABSTRACT

The rodent *A. cursor* is a neotropical species, that occurs mostly in grasslands and disturbed areas and has a broad range, from latitudes 9 to 26 ºS. One remarkable characteristic is its polymorphic diploid numbers (2n= 14, 15 and 16), being found in two major clades that coincide with the Jequitinhonha river, an important coastal river in Brazil. Herein we investigated the fertility of the species regarding combinations of diploid numbers and geographic distance of parentals (same population, adjacent or allopatric); genomics of the species; specially the heterokaryotype 2n= 15, contrasting data on natural hybrids between *A. cursor* and *A. montensis*. Experimental crosses showed higher reproductive success (RS) in crosses between same-homokaryotype individuals. We also observed high RS between different-homokaryotype individuals (northern 2n= 14 x 2n= 16), pointing there is no pre or postzygotic isolation within these groups under lab conditions. However, these karyotypes form distinct groups in a particular population in nature, therefore we proposed that reinforcement might be happening and these lineages could be facing secondary contact. Northern heterokaryotype couples had significantly inferior RS, nonetheless they recovered moderate fitness when backcrossed with homokaryotype parentals and could be the principal means they are maintained in nature. We testes reproductive isolation across adjacent populations (Bahia x Espirito Santo and Bahia x Pernambuco) as well as geographically distant and allopatric populations (Pernambuco 16N x Espirito Santo 14S), generating heterokaryotype individuals, herein called 15NS. The resulting heterokaryotype individuals 15NS were sterile and indicated the accumulation of genetic incompatibilities with the increase of geographic distance and isolation. The results from experimental crosses were corroborated by histological analyses of gonads, with normal gametogenesis in all groups of males and females of *A. cursor*, except in the 15NS males, which present similar anomalies on testis similar to the interspecific hybrids between *A. cursor* and *A. montensis*. Therefore, we believe that the incompatibilities in *A. cursor* are a consequence of the combination of divergent genetic backgrounds associated to the reorganization of the macrogenomic structure (that originates the diploid number variants). We also assumed that the different genomic structural variants (i.e. diploid numbers) originated more than once in the evolution of the species, and have a secondary role in reproductive involving diferente lineages. *A. cursor* could be facing the early stages of a speciation process. The combination of genomic data with experimental crosses results allowed us to assume that *A. cursor* could represent a species in the early stages of speciation and for now it should still be considered as one specie.

Keywords: *Akodon cursor*; speciation; karyotypes; experimental crosses; genomics

INTRODUCTION

Species are a moving target, thus is a major challenge in evolutionary biology understanding the processes that drive differentiation and genetic incompatibilities that lead to the formation of new species (Coyne and Orr 2004). The idea that structural chromosomal rearrangements (CRs) have a role in accelerating genetic differentiation, and therefore promoting speciation, has been central to the debate on chromosomal speciation (King 1993; Rieseberg 2001; Ortiz-Barrientos et al. 2016). Species with high karyotypic diversity that present broad geographic distribution are good examples for speciation investigation and raise questions about the early stages in generating a new species. There are those who advocate for a causative role of chromosomal rearrangements, claiming that alterations in chromosome structure (i.e. chromosome number, chromosomal rearrangements) cause meiotic problems in heterozygosis, producing unbalanced gametes and consequently reducing fertility and reproductive fitness of heterozygous individuals (Sturtevant and Beadle 1936; White 1978; King 1993). A more recent model predicts that suppression of recombination around rearrangement breakpoints in heterokaryotypes would diminish gene flow, allowing the accumulation of genomic incompatibilities within rearranged regions, and thus would be the main factor determining the role of CRs in speciation (Rieseberg 2001; Navarro and Barton 2003; Feder and Nosil 2009; Faria and Navarro 2010).

However, it is very difficult to empirically test the exact sequence of processes that lead to reproductive isolation, because either a CR arises and chromosomal incompatibilities

facilitate speciation, or reproductive isolation occurs for some reason (i.e. vicariance or geographic isolation) and facilitate speciation, regardless of the karyotype variants (Basset et al. 2019). Especially complicated is discriminating between incompatibilities generated by genes only, or by a combination of genes and CRs. It is relevant to highlight that karyotypic differences could contribute to speciation without being the exclusive driving force in this complex evolutionary process (Basset et al. 2019).

The formation of new optimal variants (or species), without sterile or inviable intermediates, found theoretical explanation by epistatic interaction of genes of different lineages. In this case, a hybrid would be unfit if two or more loci interact negatively. Therefore, reproductive isolation, and perhaps speciation, would not be a difficult process, and could evolve without populations having to pass through an adaptive valley of low fitness. This idea was proposed by Bateson (1909), Dobzhansky (1936, 1937), and Muller (1942) and is referred to as the "Bateson-Dobzhansky-Muller incompatibilities theory" (BDMI). In this model, populations that evolve in allopatry would accumulate new mutations during time and, if reunited in sympatry at some point, the incompatibility of ancestral and derived genes would lead to reproductive isolation and if isolation is strong enough, the lineages will now be different species (Fishman and Willis 2001).

Intraspecific reproductive variations have the potential to be a powerful tool in investigating the early stages of genetic incompatibilities within lineages (Barnard-Kubow et al. 2016). To capture that perspective one should characterize patterns of reproductive isolation across the full geographic and genetic range of a species. However,

while there are studies reporting the existence of variation in reproductive isolation within a species (Charron et al. 2014; Cutter 2012; Snoek et al. 2014), are generally lacking detailed investigations characterizing multiple components of intraspecific reproductive isolation considering the full geographic and genetic range of a species (Peterson et al. 2013; Barnard-Kubow and Galloway 2017; Martin et al. 2017). Particularly, one fruitful approach from the empirical perspective, would be to apply genome-scale analyses to systems that exemplify chromosome change among closely related taxa (Potter et al. 2017).

The neotropical rodent species *Akodon cursor* (Winge, 1887) can be used as a model for studying the early stages of genetic incompatibilities in a chromosomal and geographic context. The cursor grass mouse is a young species (< 2 million years; Coyner et al 2013; Steppan et al. 2017), having three diploid numbers that can be evenly found in two major clades, occupying a wide geographic distribution in the Brazilian Atlantic Forest (Fagundes et al. 1998; Nogueira and Fagundes 2008). One of this species most remarkable characteristic is its karyotypic diversity, presenting 2n= 14, 15 and 16 as a consequence of complex CRs in pairs 1 and 3 involving pericentric inversions and fusions (forming 2n= 14 when in homozygosis and 2n= 15 in heterozygosis) and also much variation in autosomal arms (FN) due to pericentric inversions in pairs 2, 4 and 6, having more than 30 karyotypes being described (Fagundes et al. 1998; Colombi et al. 2010). This terrestrial rodent can be found mostly throughout the Brazilian Atlantic Forest and also in Central Brazil, in part of Cerrado (savannah) and Caatinga (semi arid), having preferences for disturbed environments, such as grasslands and forest borders

(Geise et al. 2012). Genetic studies have demonstrated its genetic diversity is distributed in two major clades (hereafter, north and south) (Geise et al. 2001; Nogueira and Fagundes 2008) and that the main split is coincident with the area of the source of the Jequitinhonha river, located in the north of the state of Minas Gerais, in central Brazil (Maestri et al. 2016; Chapter 1). The three diploid numbers are present in both clades and there are populations where the three karyotypes are in sympatry (i.e. municipality of Una, in Bahia - north clade), whereas other localities present individuals with one exclusive diploid number (i.e. Espírito Santo state – south clade – with only 2n= 14 individuals).

The rodent *A. cursor* is morphologically cryptic with its sister species, *A. montensis* Thomas, 1913, and they occur in sympatry in southeast Brazil in Rio de Janeiro, Minas Gerais, São Paulo and north of Paraná. However, they have strikingly different diploid numbers (*A. montensis* has 2n= 24 as its most frequent karyotype) and genetic divergence (Rieger et al. 1995; Yonenaga- Yassuda et al. 1975; Geise et al. 2001; Fagundes and Nogueira 2007; Soares et al. 2018). Also, the presence or absence of the gallbladder or cranial morphometric analyses can be used to distinguish them (Geise et al. 2005; Astúa et al. 2015). Interspecific hybrids have been generated in lab crosses and histological analysis pointed infertility of males, that did not present any spermatozoa and their meiosis was abnormal (Yonenaga et al. 1975). Later, interspecific hybrids were collected in São Paulo and were first identified because of their intermediary karyotype (2n= 19) (Fagundes et al. 1997a, b).

The 2n= 15 of *A. cursor* presents the CRs in pairs 1 and 3 in heterozygosis, thus they are considered an intermediate karyotype between the forms 2n= 14 and 2n= 16 (potentially an intraspecific hybrid). Nonetheless, previous genomic results using samples from the north of the species distribution showed that, in nature and in a specific locality, they are not a byproduct of admixture of the different homokaryotypes, sharing a most recent common ancestor with northern 2n= 16 individuals only (Chapter 1).

A. cursor presents suitable characteristics for investigating patterns of the early stages of divergence, such as: it is a very young species with high karyotypic polymorphism; is distributed in a broad range; and has representatives of its different karyotypes distributed in two major clades, that coincide with an important coastal river in Brazil. Thus, in the present work we aimed to investigate the roles of the different karyotypes of *A. cursor* in the fertility of the species and also how geographic distance is influencing the genetic diversity of the species.

MATERIALS AND METHODS

We used three main approaches in this study, all of them considering cytogenetic information: histology of gonads, experimental crosses and genomics. All specimens investigated by histology and experimental crosses were karyotyped and 76% of the genotyped individuals had karyotype information. Our sample is composed of a combination of wild and captive individuals of *A. cursor, A. montensis* and their interspecific hybrids. Wild specimens are represented in Fig. 1. and details can be found in Tables S1 and 3 in Supplementary Material. Chromosome preparations were obtained either from bone marrow or spleen cells by the standard colchicine method (Ford and Hamerton 1956) or from fibroblast cultures (Freshney 1986). Diploid numberss were analyzed from the best well-spread metaphases for each animal using a Nikkon microscope equipped with a Cytovision® Analyser and diploid number (2n) was determined after conventional Giemsa staining.

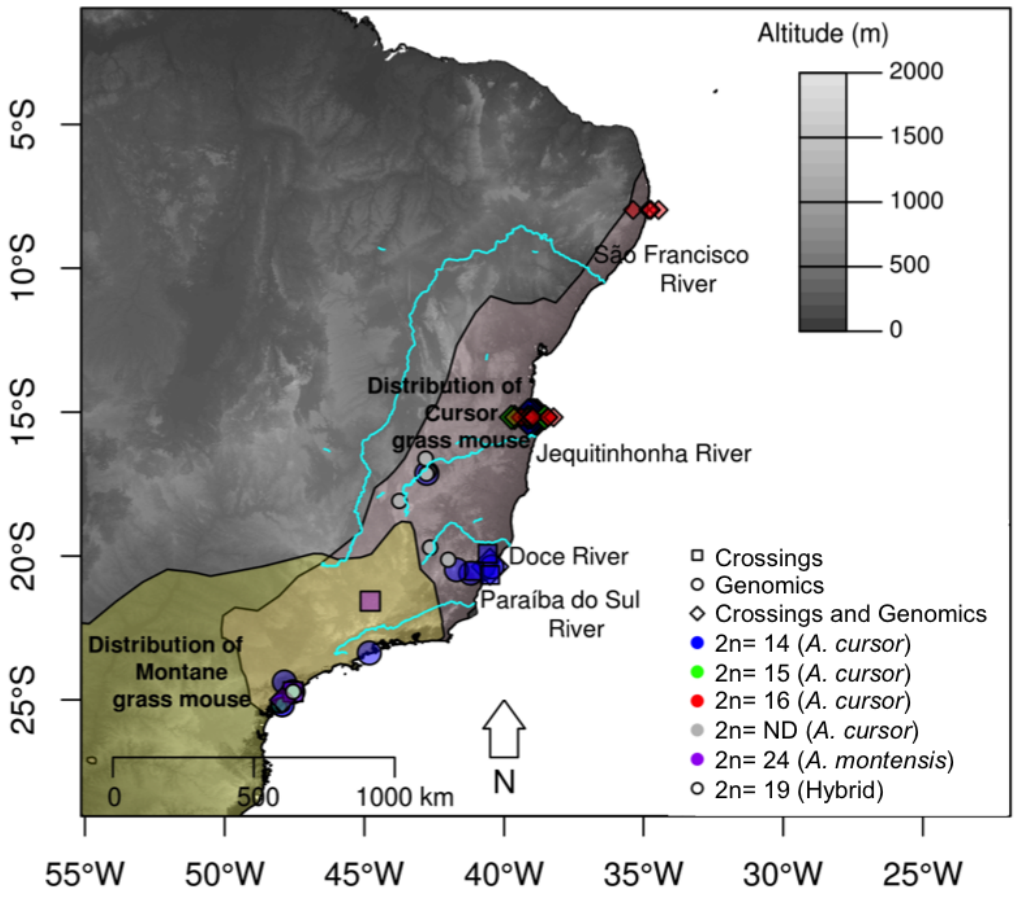

Figure 1. Map of samples of A. cursor, A. montensis and natural interspecific hybrids that were used in the experiments. Symbols represent the locality of samples that were used in the experimental crosses (square), genomic analyses (circle) or both (diamond). Karyotypes are color coded and geographic distributions are according to IUCN (A. cursor - Cursor grass mouse is in pale red and A. montensis – Montane grass mouse is in pale yellow). The main coastal rivers from Brazil are represented and the states are abbreviations for Pernambuco (PE), Bahia (BA), Minas Gerais (MG/N and MG/S), Espírito Santo (ES) and Sao Paulo (SP).

Histology of gonads

The structure of gonads were analyzed from ovaries and testicles of ACU and testicles of AMO and hybrids (ACU x AMO). The ACU sample was divided into five groups of 10 individuals (5 males and 5 females), as follows: 2n= 14 from the north (14N); 2n= 14

from the south (14S); 2n= 14 that resulted from crosses between 2n= 14 individuals from the North and the South (14NS); 2n= 16 from the north (16N); 2n= 15 from the north (15N); and 2n= 15 that resulted from crosses between 2n= 16N and 2n= 14S (15NS). We analyzed 5 males of AMO and 5 hybrid males (HYB) (Table S2 in Supplementary Material).

The structure of gonads was investigated using histological and stereological methods after the application of different fixatives and stains for light microscopy. Briefly, ovaries and testicles were removed from euthanized individuals (ages between 90-120 days old) and fixed in Karnowsky solution (4% paraformaldehyde, 4% glutaraldehyde, 1:1, in phosphate buffer 0.1 mol/L-1, pH 7.2), for 24 hours at 10ºC. Testicles fragments were then washed in phosphate buffer, dehydrated in ethanol, cleared in Xylol®, and embedded in paraplast (Leica Paraplast®, Leica Microsystems Nussloch, Germany). Ovaries fragments were dehydrated in ethanol and embedded in glycol methacrylate resin (Leica Historesin®, Leica Microsystems Nussloch, Germany). The histological sections were made in a rotary microtome (Thermo Scientific; 5 μm thick) and stained with haematoxylin and eosin. Images of the organs were captured in a digital camera attached to the microscope and stereological analyses were performed using Image Proplus® software. For the females, we estimated volumetric proportions (%) of the tissue components of the ovary (tunica albuginea, germinal epithelium, primordial follicle, primary follicle, secondary follicle, tertiary follicle, Graaf follicle, corpora lutea, interstitial cells (IC), connective tissue stroma, blood vessels, atretic follicles and defense cells) using a squared grid of 100 intersections (points) on the captured images (20X

magnification). Points were computed on 10 images of each ovary per animal. Medium values of the stereological parameters were statistically compared between groups using ANOVA or Kruskal-Wallis tests, accordingly to the normality of data ($p<0.05$). Males had the volumetric proportions (%) of the components of the testicular parenchyma estimated by counting 2000 points per animal amplified 400X. Tubular diameter and height of the seminiferous epithelium of each animal were measured by randomly choosing 30 transversal sections of seminiferous tubules that presented round contours. Spermatogenesis was qualitatively evaluated in seminiferous tubules which presented round contours, identifying the presence or absence of the cell types that form the germ epithelium: Sertoli cells, spermatogonial cells, spermatocytes, round spermatids, elongated spermatids and the presence of spermatozoids in the lumen.

Slides were mounted during the removal of the testicle from the individual to analyze the morphology of spermatozoa. One centimeter of the deferent duct was sectioned (near the epididymis) and twisted with forceps, then placed into a sodium phosphate buffer solution (0.1 M, pH 7.2) for 15 minutes. After the spermatozoa were liberated in the solution, we mounted histological slides for morphological analysis. For each animal, we analyzed 200 spermatozoa in an optical microscope (400X magnification) and the cells were characterized as normal or abnormal (Oliveira et al 2009; 2010). The measurements were statistically evaluated using ANOVA test, followed by Tukey test ($p<0.05$).

Experimental crosses

We conducted experimental crosses to estimate fertility parameters such as rates of reproductive success (RS), i.e. ratio between attempts and crossings that produced at least one pup per litter; and the average litter size (LS), i.e. the ratio between the total number of born rats per total number of crossings that produced offspring. The target species is *A. cursor* (ACU), but *A. montensis* (AMO) and ACU x AMO hybrids were also tested for comparative reproductive compatibility parameters. The experiments took place in the Animal Experimental Room at UFES (Universidade Federal do Espírito Santo), Vitória, Brazil using parents collected during field works from 2013 to 2015 in 9 localities in the Brazilian Atlantic Rainforest (Table S1, Supplementary Material), under the authorization of the Animal Care and Use Comitee from UFES (CEUA permits 007/2012 and 037/2015).

Crosses were performed between July 2013 to February 2017. Animals were housed separately in individual propylene containers specific for rodents (30x20x13cm) and in pairs for mating in larger containers (49x34x16cm). Couples were housed together for 5 to 7 days. Pregnant females would start giving birth after 15-16 days, but we kept females under observation until 21 days, following Pereira et al. (1993). During the birth window period, nests were checked twice a day and the litter size and day of birth recorded. We kept the wild caught individuals alive for the maximum time of 2 years and the lab individuals were sacrificed when they were 90-120 days old (which is the desirable age for analyzing gonads). Animals were euthanized to confirm their diploid

number with an overdose of ketamine and xylazine following veterinarian instructions. Chromosome preparations were obtained either from bone marrow or spleen cells by the standard colchicine method (Fagundes et al. 1998). Karyotypes were analyzed using a microscope equipped with the Case Data Manager software (Applied Spectral Imaging®) and diploid number (2n) was determined using conventional Giemsa staining.

Statistical differences of RS and LS between groups were performed on SPSS® v.20 (IBM 2011). Significance of differences of RS between groups of crosses was estimated using the $\chi 2$-test, except when the number of crosses was too small or differed in size, it was evaluated using Fisher's exact test. For the average litter size we used the one-way ANOVA test followed by Tukey test, whenever necessary (p-value < 0.05).

Through experimental crosses we aimed to i) establish standard fertility parameters of *A. cursor* using homokaryotype individuals from within a geographic region, as well as of *A. montensis*; ii) investigate if there is a correlation of reproductive fitness rates and geographic origin of parentals; and iii) verify the reproductive parameters of crosses of heterokaryotypes and iv) estimate reproductive parameters of interspecific hybrids and compare them with the results obtained from heterokaryotypes.

The ACU sample was composed of 2n= 14, 15 and 16 individuals from the north (Bahia and Pernambuco) and 2n=14 from the south, in Espírito Santo, totaling 56 wild caught animals. Southern 2n= 15 and 2n= 16 individuals were unavailable for experiments. In the ACU crosses we used combinations regarding the karyotypes of the individuals , their

geographic region of origin (north or south) and also classified them as belonging to the same population, adjacent or allopatric populations. As adjacent populations we had BAxES (Bahia and Espírito Santo) and BAxPE (Bahia and Pernambuco), with the former being populations from different geographic regions (BA is from the north and ES from the south) and the latter being both populations from the north. As allopatric populations we had the combination ESxPE (Espírito Santo and Pernambuco), which are populations from different regions. We also collected 27 wild caught AMO individuals (all had 2n= 24) from three localities in southeast Brazil (São Paulo and south of Minas Gerais).

To establish species' reproductive standards we crossed pairs of homokaryotype individuals of ACU from within a geographic region, as follows: 16Nx16N using specimens from Pernambuco (2n= 16 females from Bahia were unavailable); 14Nx14N using specimens from Bahia; 14Sx14S mating specimens from Espírito Santo; and pairs of AMO (24x24) from São Paulo and Minas Gerais.

Reproductive compatibility x geographic distance

We expected that crosses within individuals from the same populations would show higher reproductive compatibility, when compared to those from adjacentes and allopatric populations. Thus, reproductive fitness would decrease proportionally with the increase of geographic distance of parentals. We also evaluated if karyotype constitution of parentals would have an influence on reproductive parameters and if resulting individuals from crosses of different populations would have reproductive fitness beyond

F1. For that reason crosses and backcrosses were performed between individuals with the same and different diploid numbers. The combinations of the experimental crosses are summarized in Table 1.

Table 1. Summary of the combinations of A. cursor individuals used in the experimental crosses, considering their geographic region (N- North or S- South), the proximity of the localities ("same populations" are Bahia x Bahia (BA), Espírito Santo x Espírito Santo (ES) and Pernambuco x Pernambuco (PE); "adjacent populations" are Bahia x Pernambuco and Bahia x Espírito Santo; "allopatric populations" are Espírito Santo x Pernambuco) and karyotypes are 2n= 14, 15 or 16.

Origin of parentals	Same-karyotype parentals	Different-karyotype parentals
same population	14N (BA) x 14N (BA)	14N (BA) x 16N (BA)
	14S (ES) x 14S (ES)	15N (BA) x 14N (BA)
	16N (PE) x 16N (PE)	
	15N (BA) x 15N (BA)	
adjacent populations	14N (BA) x 14S (ES)	14N (BA) x 16N (PE)
	14NS (BA/ES) x 14N (BA)	15N (BA) x 16N (PE)
	14NS (BA/ES) x 14S (ES)	15NS (ES/PE) x 14S (ES)
	15N (BA/PE) x 15N (BA/PE)	15 NS (ES/PE) x 16N (PE)
allopatric population	15NS (ES/PE) x 15NS (ES/PE)	14S (ES) x 16N (PE)

Fertility parameters of ACU 2n= 15

In this geographic-distance context we specifically evaluated fertility parameters of ACU heterokaryotype individuals 2n= 15. Thus, we crossed pairs of ACU 2n= 15 individuals both from Bahia. We also generated ACU 2n= 15 in lab by crossing 2n= 14 from Bahia

with 2n= 16 from Pernambuco individuals (hereafter, 15BA/PE), which are adjacent populations. Also, we generated 2n= 15 individuals by crossing allopatric individuals from Espírito Santo (2n= 14) with 2n= 16 from Pernambuco (hereafter 15NS). Pairs of 2n=15 NS were crossed with ng 2n= 15 individuals from the allopatric parentals northern 2n=16 from Pernambuco and southern 2n=14 from Espírito Santo (hereafter 15NS) were also crossed. We backcrossed northern ACU 15N from Bahia with parentals (homokaryotypes 14N from Bahia, 16N from Bahia or 16N from Pernambuco). The ACU 15NS individuals were backcrossed with parentals 14S from Espírito Santo or 16N from Pernambuco.

Interspecific hybrids

Hybrids between ACU and AMO were generated by crossing: ACU 14S from Espírito Santo x AMO; ACU 14N from Bahia x AMO; ACU 15N from Bahia x AMO and ACU 16N from Pernambuco x AMO. Pairs of hybrids were then crossed (HYB x HYB) and also backcrossed with the parental species (HYB x AMO and HYB x ACU).

Genomic data processing

The sample was composed of 54 individuals of *A. cursor* from 19 localities from six populations, 2 individuals of *A. montensis* from one locality and 2 natural hybrids from one locality from the Brazilian Atlantic Forest (Table 3 in Supplementary Material). We had karyotype information from 76% of the *A. cursor* individuals, and from both individuals of *A. montensis* (2n= 24) and the natural hybrids (2n= 19).

Genomic DNA was extracted from muscle or liver using the DNeasy® blood and Tissue kit Qiagen. We followed the double-digest RADseq (ddRAD) protocol (Peterson et al. 2012). Before digestion reactions, double-stranded DNA concentrations were quantified using the Qubit dsDNA Assay Kit (Invitrogen) and all samples had the initial amount of DNA varying from 350 to 500 ng per sample. The DNA was double digested with the restriction enzymes EcoR1 and MseI (New England Biolabs), and unique barcodes (10 bp) and Illumina adapter sequences were ligated to the digested fragments. Samples were pooled together and 300-450 bp DNA fragments were size-selected using Pippin Prep (Sage Science) followed by PCR amplification. The library was sequenced using the Illumina 2500 platform at The Center for Applied Genomics (Hospital for Sick Kids, Toronto, Canada) to generate 150bp single-end reads. We used the STACKS 1.45 pipeline (Catchen et al. 2011; Catchen et al. 2013) for de novo assembly of loci from the fastQ files obtained from the Illumina sequencing. Sequences were demultiplexed using process_radtags and individuals with less than 70K reads were excluded from further analysis. Loci and polymorphic nucleotide sites were identified in each individual using the ustacks program with a minimum coverage depth (m = 5), a removal algorithm (-r 0), a deleveraging algorithm (-d) and a maximum distance (in nucleotides) of two allowed between stacks (-M 2). An error rate (ε) was set conservatively (--bound_high 0.1) to avoid underestimating heterozygotes (Catchen et al. 2013). The mean coverage was 12x. A catalog of consensus loci among individuals was constructed with the cstacks program, where loci were merged across individuals if the distance between them (n) was ≤ 2; this catalog was used to determine the allele(s) present in each individual at each homologous

locus with the sstacks program. SNP data was exported as Variant Call Format (–vcf) and processed with an R-script in RStudioVersion 1.0.153 (R Studio Team 2016) using the PLYR (Wickham 2011) and PEGAS packages (Paradis 2010) to delete the last 10 bp positions to avoid sites with presumed sequencing errors (i.e., sites from the upper 95th percentile of segregating sites). All STACKS modules were run in parallel with 8 threads on the HPC Linux-based cluster from the University of Michigan.

More than 170 million reads were produced on one lane of Illumina sequencing. After bioinformatics processing and filtering based on read quality, almost 155 million reads were retained in 54 individuals of *A. cursor*, 2 individuals of *A. montensis* and 2 natural hybrids (Table S1 in Supplementary Material). After applying the filters of populations program, a total of 640,215 SNPs in 226,632 loci (maximum of 9 SNPs per locus) were obtained. The software PLINK 1.9 (Purcell et al. 2007) was used to filter the sequences and individuals based on the frequency of missing data. In the end of the data filtering we had a dataset with up to 10% of missing data per unliked SNP with 10,100 loci and a genotyping rate of 0.93781.

Structuring of populations

Population genetic structure were assessed using Bayesian clustering implemented in the software STRUCTURE 2.3.4 (Pritchard et al. 2000) and a Principal Components Analysis (PCA) using the Adegenet package (Jombart et al. 2008) and Plyr (Wickham 2011) in RStudioVersion 1.0.153 (R Studio Team 2016). For Principal Component

Analyses and STRUCTURE analyses, we used all the *A. cursor* individuals, the *A. montensis* and the natural hybrids. We also analyzed a subset comprising exclusively the Bahia population with 22 individuals, since it has representatives of the three diploid numbers in sympatry. The STRUCTURE analyses were performed under the "Admixture model" and the "Correlated allele frequency model" for a range of K-values (i.e., 1 to n + 1, where n is the number of populations). Ten independent runs were performed for each K-value with 500,000 burnin steps and 1,000,000 Markov Chain Montecarlo (MCMC) iterations. We first grouped all the *A. cursor* individuals, the *A. montensis* and the natural hybrids and in the other subset we analyzed only the karyotyped individuals from the Bahia population. The optimal K for each dataset was chosen using the delta-K method (Evanno et al. 2005) as implemented on STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt 2012). The cluster membership coefficients (posterior probabilities of individual assignments to K genetic clusters) were permuted across ten independent runs using CLUMPAK (Kopelman et al. 2015) and plotted using DISTRUCT (Rosenberg 2004).

Principal Components Analyses were performed on RStudioVersion 1.0.153 (R Studio Team 2016). We used the Adegenet package (Jombart et al. 2008) and Plyr (Wickham 2011). Missing data were replaced by the mean frequency of the corresponding allele, which is recommended for centered PCAs (Jombart et al. 2008). Major axes for genome-wide SNP data were identified using the R Dudi.pca function (centre = T, scale = F).

Genetic structure was also assessed through population genetic indexes that were

calculated on the *populations* function of STACKS 1.45 pipeline (Catchen et al. 2013): percentages of polymorphic sites (% pol), average observed heterozygosity (H_{obs}), average nucleotide diversity (π) and Wright's inbreeding coefficient (F_{IS}) and F_{st} values (SNP-based F statistics).

Association between genomic variation and geographic distance

We used Mantel tests to verify the correlation between geography (Euclidean geographic distances) and genetic variation (F_{st}) and if an isolation by distance (IBD) would be associated with our data (Mantel 1967). First, we ran a general test with all populations and then successive analyses which excluded one population at a time, in a sequential population drop out procedure, to check if any particular population would be specifically contributing to an IBD pattern. These Mantel tests were performed on RStudio (version 1.0.153), using the VEGAN package (Oksanen et al. 2017), and 1.000,000 permutations.

RESULTS

Histology of gonads

The ovaries of all representatives ACU groups were delimited by the germinal epithelium tissue, adjacent to the tunica albuginea and presented follicles in various stages of maturation, having all expected structures, such as oocytes, granulosa cells, internal and external theca, constituted by androgenic cells and fibroblasts, respectively (Fig. 2). We observed a well-developed hypertrophic mass of interstitial cells (IC) occupying the majority of the organ. These cells are originated in the internal theca of atretic follicles and their function is to secrete steroids. Also, in the cortex we observed the endocrine gland corpora luteum (CL), irrigated by blood vessels. The existence of CL is evidence of ovulation and possibly a sign of female fertility, because once ovulation takes place the cells from the internal theca and the granulosa are luteinized by the luteinizing hormone (LH) inducing the onset of corpora lutea, which maintains the initial phase of pregnancy. In summary, representatives of all groups exhibited the same ovarian morphology, and through qualitative analysis, it was possible to observe follicle development in all individuals. To corroborate these findings, the stereological parameters (Table S5 on Supplementary Material) did not present any statistical differences between groups (p-values > 0.05), confirming that gametogenesis occurs in females of all karyotypes of *A. cursor* and that they are potentially fertile.

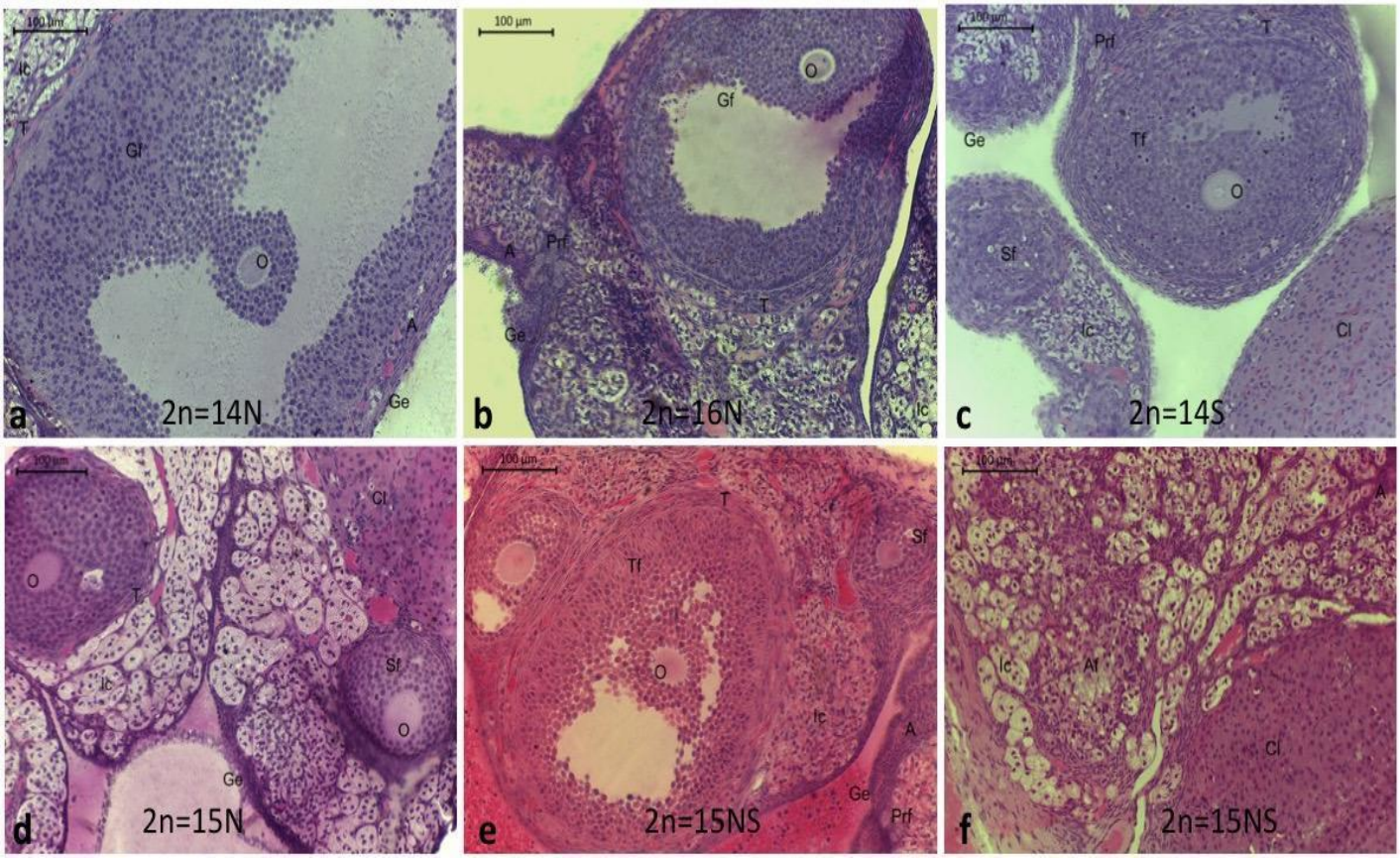

Figure 2. Ovary sections of Akodon cursor, magnified 20x. a) 2n= 14 from the North; b) 2n= 16 from the North; c) 2n= 14 from the South; d) 2n= 15 from the North; e) 2n= 15 North/South and f) 2n= 15 North/South The following structures can be observed: Gf – Graafian follicle; Tf – Terciary follicle; Sf – Secondary follicle; Pf – Primary follicle; Prf - Primordial follicle; Af – Atretic follicle; Cl – Corpus luteum; It – Interstitial cell; T – Theca; Ge – Germinal epithelium; A – tunica albuginea.

Spermatozoa was found in seminiferous tubules of AMO and ACU (14S, 14N, 16N and 15N). On the other hand, in interspecific hybrids (HYB) and ACU 15NS, no spermatozoa was observed. Additionally, in hybrids we could observe fagocitary cells and only Sertoli cells and primary spermatocytes in the interstitial compartments of testicles and seminiferous tubules (Fig. 3F).

Seminiferous tubules presented complete spermatogenesis in almost all groups of *A.*

cursor, except in ACU 15NS and interspecific hybrids (Fig. 3). In *A. cursor* we found type A spermatogonium and Sertoli cells in the basal portion of seminiferous tubules; in the intermediary section, we observed primary and secondary spermatocytes, and in the adluminal portion we found spermatids (round and elongated), and spermatozoa were evidenced in the lumen. In the tubules of all groups we visualized Sertoli cells. The seminiferous tubules in the ACU 15NS group presented incomplete spermatogenesis, with only spermatogonium, spermatocytes in pre-leptotene/leptotene and pachytene phase and Sertoli cells (Fig. 3E). There were not any round or elongated spermatids and spermatozoa found in their lumen. Similar results were observed in hybrids, where spermatogonies, Sertoli cells and primary spermatocytes were observed (Fig. 3F).

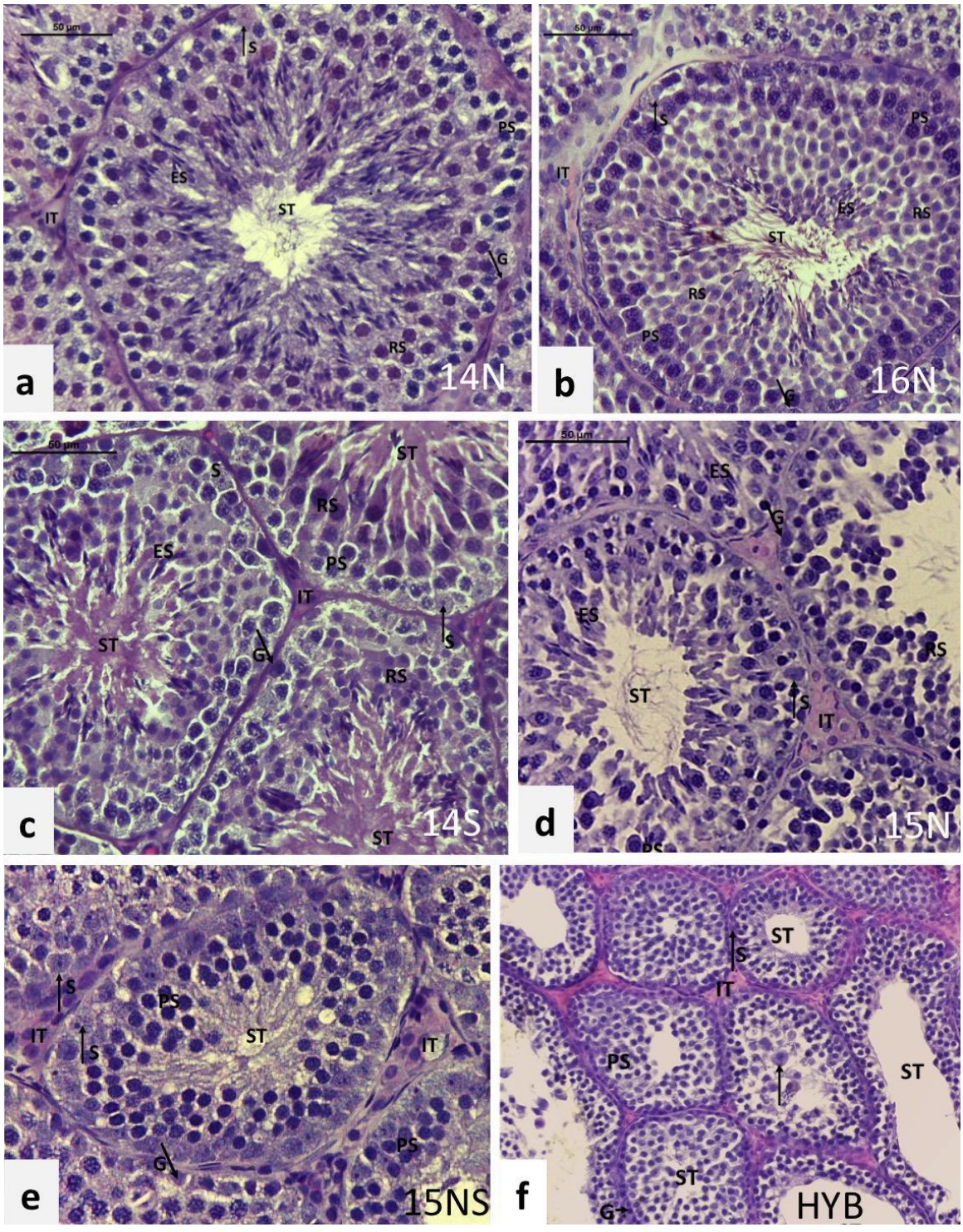

Figure 3. Histological sections of seminiferous tubules of testicles of Akodon cursor, A. montensis and interspecific hybrids. A) A. cursor 2n= 14 from the North; B) A. cursor 2n= 16 from the North; C) A. cursor 2n= 14 from the South; D) A. cursor 2n= 15 from the North; E) A. montensis 2n= 24 and F) interspecific hybrids 2n= 19 (arrow here is to show fagocitary cells). Legend: TS) seminiferous tubules; L) lumen of the tubule; It)

interstitial layer; S) Sertoli cells; ES) elongated spermatids; AS) round spermatids; VS) blood vessel; A) type A spermatogony.

The average count of normal spermatozoa was around 60% in all groups of *A. cursor* (Table S8 on Supplementary Material). No spermatozoa was found in slides of the ACU 2n= 15 NS group, which is concordant with the analysis of its seminiferous tubules (no spermatozoa were observed in the lumen). Besides that, this group differed from all other groups (see Table S5) in the following parameters: epithelium height, proportion of the seminiferous tubules and interstitium. Moreover, the normal count of spermatozoa in the northern 2n=14 group was statistically different from the other groups of the same region (2n= 15 and 2n= 16, p-values 0.0496 and 0.000234, respectively). In summary, all natural *A. cursor* groups (northern 2n=14, 15, 16 and southern 2n= 14) presented complete spermatogenesis and viable spermatozoa, thus, they can be considered potentially fertile animals. The ACU 15NS male group presented results equivalent to interspecific hybrids, that are sterile. We could not collect data on spermatozoa of *A. montensis* and there was no lumen nor spermatozoa in the hybrid's seminiferous tubules. On Table 1 we have compiled information on male gonads of ACU, AMO and HYB.

Table 1. Summary of histological parameters of male gonads of A. cursor (ACU), A. montensis (AMO) and hybrids (HYB). Numbers refers to diploid number of ACU samples and N=North, S=South.

Parameter	14N	16N	14S	14NS	15N	15NS	AMO	HYB
presence of spermatozoa	+	+	+	+	+	-	+	-
complete spermatogenesis	+	+	+	+	+	-	+	-

ND=not determined, + = presence, - = absence; ++ =moderately higher; +++ =the highest values.

Experimental estimates of reproductive success

In general, from the total of 419 experimental crosses (Summary on Table S8 on Supplementary Material) the higher RS was verified in crosses involving ACU homokaryotype individuals from the same geographic region. No reproductive isolation was verified between individuals from different geographic regions, and 2n= 15 showed reduced fertility when compared to homokaryotypes, suggesting that karyotype constitution may not be the main aspect driving reproductive incompatibilities in lineages of *A. cursor*. The average number of pups did not present significant diferences between groups in general.

Species reproductive standards

The highest RS rates were obtained from pairs of ACU with the same diploid number and from the same population (i.e., 14Nx14N from Bahia, 14Sx14S from Espírito Santo and 16Nx16N from Pernambuco) followed by AMO couples, ranging from 55% to 76% of RS (p-value 0.270). These values were used herein as a reference for each species (Fig.

4A).

Reproductive compatibility x geographic distance

Crosses involving individuals of different ACU homokaryotypes (i.e. 14x16), presented statistically different results considering individuals from the same, adjacent or from allopatric populations (p-value 0.016), with higher rates of RS in crosses of individuals from the same population (BA x BA; 63%), followed by crosses between allopatric populations (ES x PE; 50%). On the other hand, the combination of the same diploid number from adjacent populations and from different regions(14Nx14S) had 37.5% of RS (Fig. 4B). The average number of pups in this group (Fig. 5B) was 4.1 pups/litter and they were not statistically different from each other (p-value 0.270).

The resulting 14NS individuals that were generated in crosses between the adjacent populations of ES and BA were crossed (14NS x 14NS) and backcrossed (14NS x 14N and 14NS x 14S), presenting one of the highest rates of RS (66.6-78.9%, p-value 0.126). The average number of pups in these groups was 4.6 and the Tukey HDS test showed significant differences in the number of pups between groups 14NS x 14NS and 14NS x 14S (p-value 0.002).

Our results suggest that individuals from populations that occupy an intermediary geographic position in the range of the species (i.e. Bahia) have the potential to successfully breed with northern or southern populations, therefore they could be a bridge

connecting the extremes of the range of ACU.

Fertility parameters of ACU 2n= 15 - northern and north/south

Northern couples of ACU 2n= 15 (BA x BA and BA/PE x BA/PE) had similar rates of RS ranging from 13% to 16% (p-value 0.822) which were significantly inferior to the values observed in the species' reproductive standards (p-value 0.000) (Fig. 4C). It is relevant to mention that the three successful matings between pairs of northern ACU 15x15 (Table 2) involved F2 and F3 individuals as parentals (generating litter of up to 7 pups), indicating that 2n= 15N fertility may endure beyond F1. Although 2n= 15N individuals have the potential to produce gametes n= 7 and n= 8 and recover descendants with the three karyotypes of ACU (2n=14, 15 and 16), in the 12 born pups of such crosses, 7 had 2n= 15 and 4 had 2n= 16 (one pup died without karyotype information). Even though the number of pups is low, no pup with 2n= 14 was observed, suggesting a bias that would restrict the production of gametes n= 7 in males and females with 2n= 15. Regarding the average number of pups of these groups (Fig. 5C) there was no statistically significant difference between them (p-value= 0.330).

Another test involving crosses of pairs of ACU 15NS showed 0% of RS. It is noteworthy to mention that the ACU 15NS individuals were generated by crossing individuals from allopatric and geographically distant populations (Espírito Santo is almost 2,000 km apart from Pernambuco).

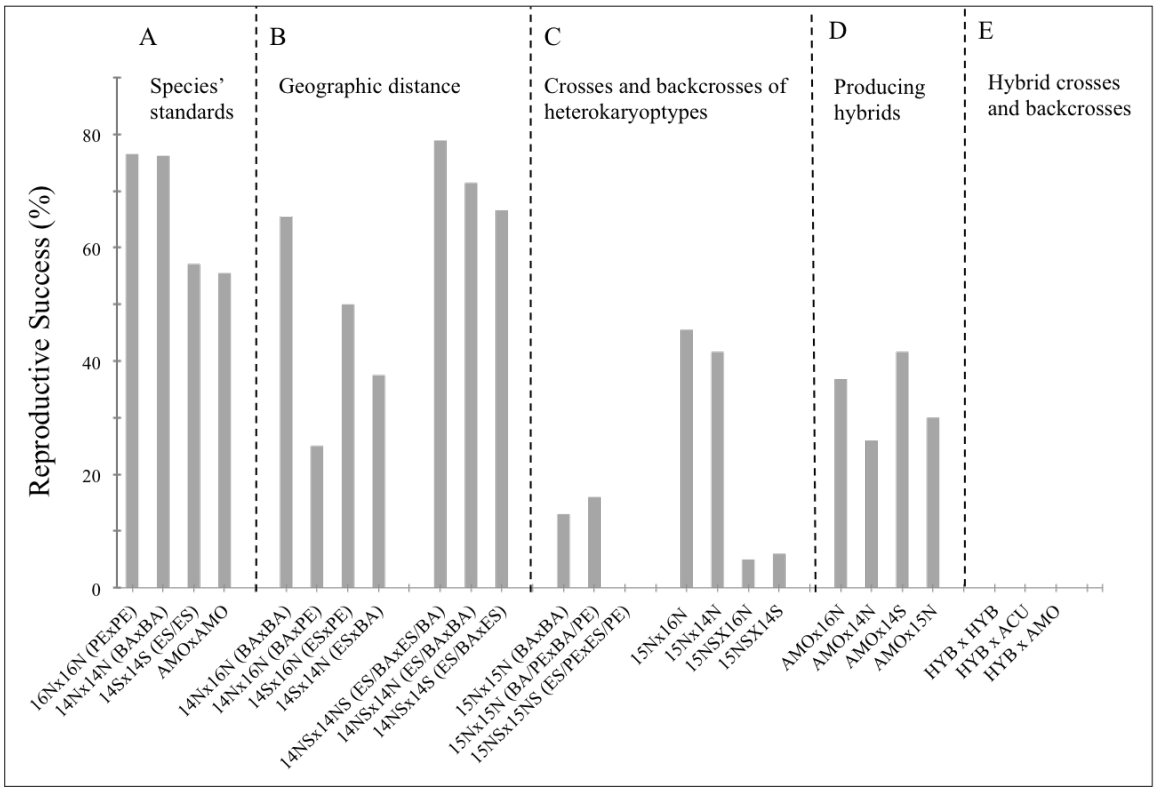

Figure 4. Rates of reproductive success of crosses involving A. cursor (ACU), A. montensis (AMO) and interspecific hybrids. Numbers above the bars are the total number of crosses of each category. A) Standards of pairs of homokaryotype ACU x ACU and AMO x AMO; B) Crosses and backcrosses involving ACU individuals of different karyotypes or from different populations (same population, adjacent or allopatric) ; C) Crosses of pairs of northern ACU 2n= 15 (either from Bahia or generated from Bahia x Pernambuco individuals) and pairs of 2n= 15 North-South individuals (generated by crosses between Espírito Santo and Pernambuco); D) Backcrosses of the different types of ACU 2n= 15, including northern individuals and north-south individuals; E) Crosses between hybrids and backcrosses with parental species (ACU or AMO).

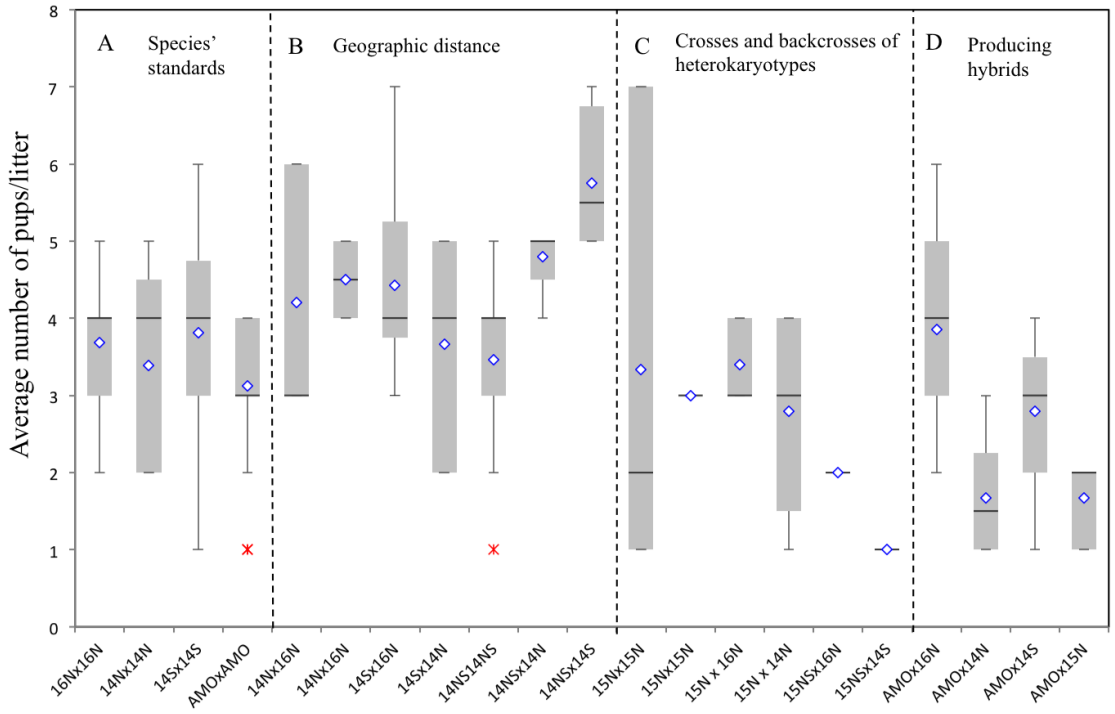

Figure 5. Average number of pups per group of cross involving A. cursor and A. montensis. The blue diamond is the average and the red star indicates a lower outlier value. Groups are the same as in Fig. 4. A) Pups of pairs of homokaryotype ACU x ACU and AMO x AMO; B) pups of crosses and backcrosses involving ACU individuals of different karyotypes or from different populations; C) pups of crosses of pairs of northern ACU 2n= 15 (either from Bahia or generated from Bahia x Pernambuco individuals) and pairs of 2n= 15 North-South individuals (generated by crosses between Espírito Santo and Pernambuco); D) pups of backcrosses of the different types of ACU 2n= 15, including northern and north-south individuals and E) pups of interspecific crosses between ACU variants and AMO. Crosses between 15NS couples and between interspecific hybrids did not produce any litter, thus they were not represented.

Backcrosses involving ACU heterokaryotype individuals

Backcrosses of 15N with homokaryotype individuals (i.e. 14N or 16N) resulted in an average of 44% RS (p-value 0.204), with an average of 3.1 pups/litter (Figs. 4D and 5D). We can say that both sexes of northern 2n= 15 individuals are fertile because they

produced pups when males and females 2n= 15 were involved, reinforcing the histological results in which both sexes presented complete gametogenesis.

The 15NS individuals, on the other hand, presented an average of 6% of RS when backcrossed with parentals, which was significantly inferior compared to the backcrosses involving the 15N individuals (p-value 0.000). The two successful backcrosses of 15NS generated three pups in total (Figs. 4D 5D). The 15NS females were involved in the successful backcrosses, in concordance with histological data. On the other hand, all attempts of backcrosses involving 15NS males failed, which is concordant with no spermatozoa after histological data.

Interspecific hybrids

The crosses involving ACU and AMO produced hybrids in a RS rates from 26.0% to 41.6% (Fig. 4E, p-value 0.783). However, couples of 16NxAMO produced on average more pups than 14NxAMO and the 15NxAMO group (Tukey HSD, p-values 0.0094 and 0.0403, respectively). On the other hand, all crosses involving hybrids as parents (crosses and backcrosses) had 0% of RS, even backcrosses and male or female hybrids, indicating that both sexes of interspecific hybrids are sterile (Fig. 4F).

Genetic structure

Structure analysis using *A. cursor* (n= 54), *A. montensis* (n= 2) and natural hybrids (n= 2)

recovered K= 2 as the most probable number of clusters, with each species as a different group and the hybrids as a mixture between these groups (Fig. 6A, values on Table S9 on Supplementary Material). Genomic structure of hybrids showed that they present 50% of genomic background of each species, what could be indicative that they are F1 generation (Fig. 6A). The Structure analysis of a subset of the total sample, with only population from Bahia individuals with 2n=14, 2n=15 and 2n=16 (Fig. 6B) recovered K= 2, showing one group composed of 2n= 14 individuals and other formed by three out of nine 2n= 16 individuals (i.e. "pure 2n=16"). An intermediate group with mixed genomic composition was formed by six 2n=16 and six 2n=15 individuals.

PCAs with *A. cursor*, *A. montensis* and natural hybrids showed results similar to Structure, with the AMO and ACU species in the extremes and the hybrids in an intermediary position in the PC space (Fig. 7A). The results of the subset involving the Bahia population showed that the 2n= 15 is not a well defined group (Fig. 7B), similarly to the Structure results.

Genetic differentiation between populations was assessed through F_{st} values, which had low to intermediate values, ranging from 0.09 to 0.24 (Table 4) and shallow association between geographic distance and genetic could be observed (i.e. for pairs of populations from the same geographic region we would expect lower values). Summaries of genetic diversity pointed low genetic diversity within populations (nucleotide diversity below 0.090 and H_{obs} below 0.074) and low levels of inbreeding (F_{is} values under 0.06).

The Mantel test with all populations did not show patterns of Isolation by Distance (IBD) (p = 0.126). However, the Mantel test that excluded one population at a time showed that if the Bahia population was removed from the analysis, an IBD pattern would be observed (p-value 0.033, Table S10 in Supplementary Material). This population occupies an intermediary geographic position in the *A. cursor* range.

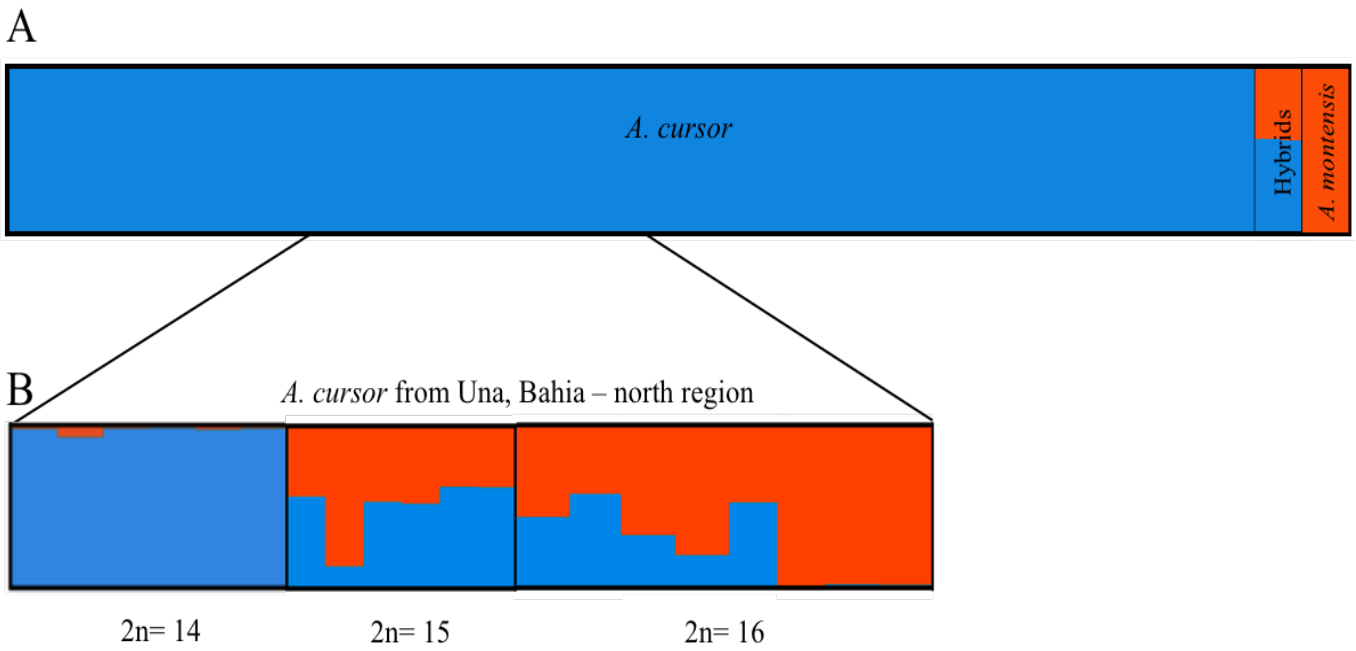

Figure. 6. Population STRUCTURE analyses with posterior probability plots of individual assignments to the inferred genetic clusters shown for the most probable K-value of K=2 for (A) all 54 Akodon cursor individuals (which contains all three different karyotypes from populations from the north and south of the species' distribution), A. montensis individuals (n= 2) and natural interspecific hybrids (n=2); B) 22 individuals from the Bahia population, which has representatives of the three karyotypes in sympatry, and for which K = 2 was the most probable K-value (see Table 2 in the Supplementary Material for details regarding the posterior probability of different numbers of K genetic clusters).

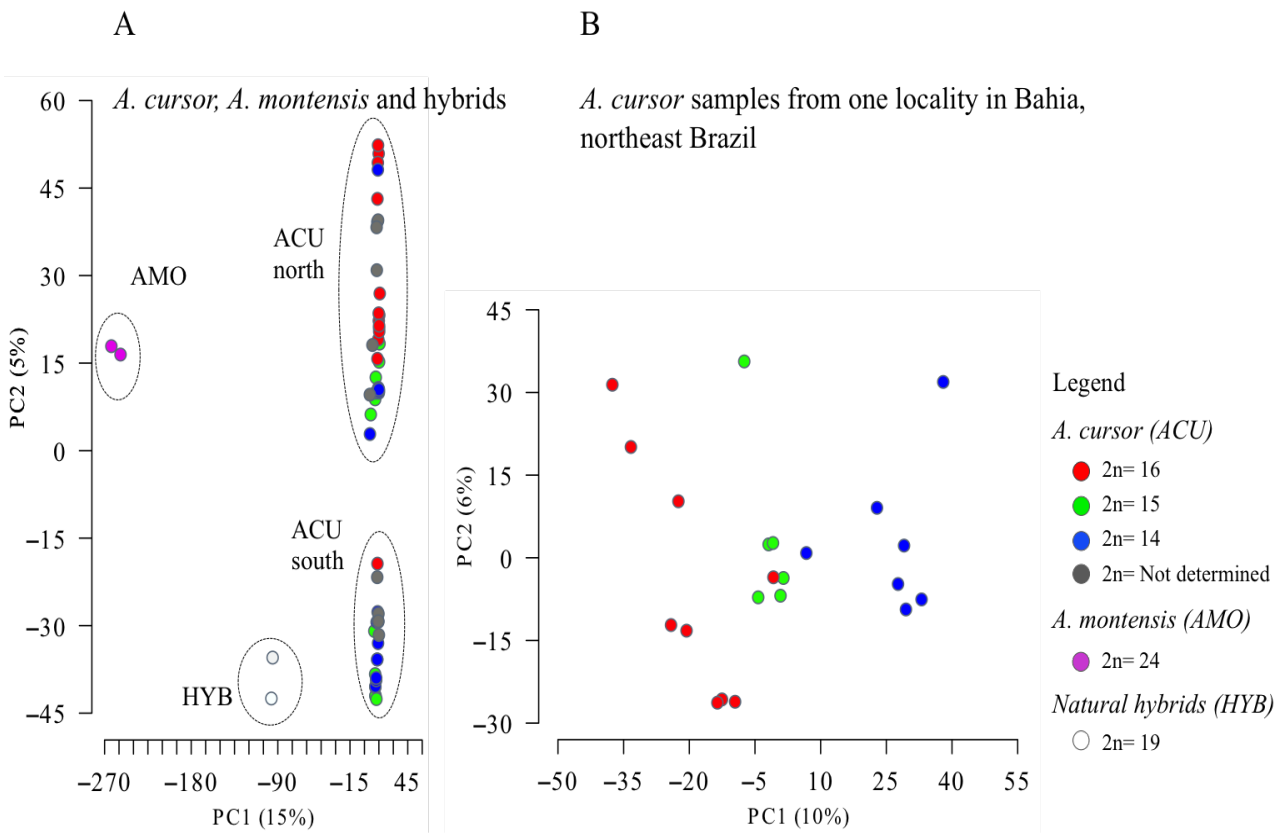

Figure 7. Principal component analyses of SNPs of A) Akodon cursor, A. montensis and natural interspecific hybrids and B) of A. cursor individuals from Bahia, where representatives of the three karyotypes are found in sympatry. Diploid numbers of the samples are color coded.

DISCUSSION

Even after 40 years of research on the cursor grass mouse, a common rodent from the Brazilian Atlantic Forest, the number of questions about its evolution is still increasing. Of special interest is the investigation of the karyotypes as determinant factors in the distribution of the species' genetic diversity. We herein integrated knowledge on experimental crosses, which allowed us to test levels of reproductive compatibility based on different combinations of karyotypes and levels of geographic isolation between individuals, and contrast this information with genomic data, which provided indicatives of what is actually happening in nature. Thus, our results clarified old paradigms about *A. cursor* and set the stage to new evolutionary questions.

In an unprecedented effort we collected and successfully crossed wild individuals of *A. cursor* from localities that were almost 3,000 km apart, comprising the species' geographic range and most of its karyotypic and genetic diversity. Such vast sampling also enabled us to test levels of intraspecific reproductive compatibility regarding the different karyotypes of the species and also the influence of geographic isolation of parentals. It is also the first time that genomic data on natural hybrids and *A. montensis* are presented.

Are there optimal karyotypes in *A. cursor*?

Karyotype combinations are relevant in the reproduction of *A. cursor,* with homokaryotypes being the forms with higher fitness in the species. Our lab crosses data

showed that, in general, pairs of individuals from the same population present higher fitness when they have the same diploid number (i.e. like tends to pair with like), except in the case of pairs of heterokaryotypes. Under confinement we could also observe that different-homokaryotype couples can successfully breed with high fitness, what led us to assume that assortative mating could be taking place in nature, where individuals 2n= 14 or 2n= 16 would preferably mate with another individual of the same karyotype as itself, but occasionally they could cross with an individual of different karyotype. The outstanding reproductive fitness of homokaryotypes is reflected in their higher frequency in nature compared to heterokaryotypes, having the 2n= 16 as the prevalent form in the north, whereas the 2n= 14 is more abundant in the south (Fagundes et al. 1998). According to the canalization model proposed by Bickham and Baker (1979) species evolve optimal karyotypes for a particular adaptive zone. In this model it is predicted that the karyotype contributes significantly to the fitness of the individual and the younger the group the less "canalized" to an optimal karyotype they will be, presenting high levels of polymorphism, such as those observed in Rodentia (King 1985). Though, according to this model a species would present an optimal karyotype for a determined niche during a specific period of time. Allied to small population sizes and stochastic events some karyotypes might be favored and become fixed in a population. During these pulses of chromosomal evolution (including changes in chromosome structure), and extensive genome reorganization new variants (biological novelties) appear initially as polymorphisms that are then subjected to selective and stochastic processes with the potential to contribute to population differentiation and ultimately to speciation (Dobigny 2017).

Therefore, our data on reproductive fitness combined with the known frequencies of karyotypes in nature we believe that the homokaryotypes 2n= 14 and 2n= 16 in represent the most stable or optimal karyotypic variants in *A. cursor*. Nonetheless we are not saying that one of these karyotypes is better than the other, since they can occur in simpatry in some areas and they seem to have appeared more than once in the evolution of the species (see Chapter 1).

Fertility of heterokaryotype individuals

Although lab crosses involving northern 2n= 15 individuals pointed they are subfertile (RS around 16%), histological analyses evidenced that both males and females have normal gametogenesis (producing ovules and spermatozoa), with males producing an average number of normal spermatozoa, equivalent to homokaryotype individuals. Thus, we are prone to assume that some other mechanism might provoking low efficiency in generating viable embryos, either by deficiency in gamete fusion or the zygotes do not follow normal development.

Heterokaryotype individuals present a complex chromosomal rearrangement in heterozygosis, with a big chromosome as a result from fusion of chromosomes 1+3 and its homologues are not fused, with separated chromosomes 1 and 3 (Yonenaga-Yassuda 1979). Meiosis of heterokaryotype males have been studied before and pachytene cells

had no loop formation on the inverted segments of the trivalent, nor a reduction in the frequency of the chiasmas (Yonenaga-Yassuda 1979), suggesting balanced segregation of the trivalent rearrangement involving chromosomes 1, 3 and 1 + 3 (Fagundes et al. 1998).

During meiosis, pairing and segregation is possible through the formation of a trivalent during prophase I. In anaphase I the trivalent segregates into one of the three patterns: alternate segregation, adjacent segregation and 3:0 segregation (Guttenbach et al. 1997). Theoretically, eight types of gametes can be produced with respect to chromosomal constitution, including two balanced gametes generated from alternate segregation (Lamotte et al. 2018; Zhang et al. 2019). In an heterokaryotype individual of *A. cursor* it means that in eight possible gametes only two would be normal (i.e. 25%), having n= 7 or n= 8 (i.e. present the fused 1+3 chromosome or chromosomes 1 and 3 without the rearrangement). The other types of segregation would result in an inviable and unbalanced haploid constitution of n= 6, n= 9, n= 15 or n= 0. Thus, we believe that the low frequency of producing good gametes would be the major cause of the observed lower fertility of heterokaryotype individuals of *A. cursor*.

Beyond the putative low efficiency of segregation of gametes we also observed a peculiar situation in the pups produced by crosses between heterokaryotype individuals. If we consider the alternate segregation aforementioned, heterokaryotype individuals would produce gametes n= 7 and n= 8. Thus, if we follow a Punnet square of phenotype probabilities, then crosses between 2n= 15 individuals would produce litter with proportion of 2:1:1 of karyotypes in the pups. However, this proportion was not observed

in the 11 karyotyped pups derived from such crosses, with individuals being either 2n= 15 or 2n= 16, in a proportion of 1:1. The closer phylogenetic relationship observed between northern 2n= 15 and 2n= 16 may have an influence on the formation of gametes, without balanced production of n= 7 gametes by both sexes. This situation would be explained by the evolution of meiotic drive distortions (reviewed by Lindhom et al. 2016), with a possible situation where one of the sexes of 2n= 15 individuals selfishly produces only n= 8 gametes, whereas the other sex produces both n= 7 and n= 8, causing the pups to present only 2n= 15 or 2n= 16.

Reinforcement in the Bahia population

Reinforcement is the process by which natural selection will directly favor an increase in assortative mating (prezygotic isolation) if two divergent populations produce maladapted hybrids (behaviourally, ecologically or reproductively) where they come into contact, in order to prevent the formation of such individuals (Butlin 1995). This process can lead to reproductive isolation -and possibly speciation- between sympatric populations versus those in allopatry (Dobzhansky 1937; Servedio and Noor 2003; Pfennig 2016).

While the importance of reinforcement in evolution has been historically debated (Paterson 1978; Tempeton 1981; Spencer et al. 1986), many well documented examples now exist in various groups of organisms (Hoskin et al. 2005; Hopkins et al. 2014; Rosser et al. 2019).

The high RS observed in lab crosses between 2n= 14 and 2n= 16 individuals were contrasting with the genomic data, which evidenced absence of admixture between such group of individuals from the Bahia population, with 2n= 14 individuals forming a distinct group from 2n= 15+16 (revealed in the STRUCTURE analysis). Considering that the lab crosses experiments evaluated if matings would produce viable offspring and thatthe genomic data are reflex of the actual matings that took place in nature, our STRUCTURE data suggests that no assortative mating is happening between individuals of 2n=14 and 2n=16 in this particular population.

Our data shows that despite being able to breed, the two lineages in Bahia (2n= 14 and 2n= 15+2n= 16) are facing some kind of reproductive barrier, since they belong to distinct clades (Chapter 1). Yet, considering that the individuals from Bahia with 2n=14, 15 and 16 have normal structure of gonads, producing oocytes and spermatozoids and offspring in the lab, we are inclined to assume that the kind of reproductive barrier is prezygotic, which includes seasonal and habitat preferences, courtship differences, and differences in genitalic structures (Mayr 1963; Dobzhansky 1970). The kind of postzygotic barriers include embryo inviability, hybrid inviability, hybrid sterility and hybrid breakdown in the lab crosses (Mayr 1963; Dobzhansky 1970). We observed that the 2n=15, the "hybrid-like" condition is viable, fertile (at least when representatives of the Bahia population were crossed in lab), thus we believe that there is no postzygotic barrier.

When geographically or ecologically isolated populations or diverging lineages co-occur in the same niche or area, their interaction may lead to different evolutionary outcomes depending on the strength of the reproductive barriers that they have evolved during isolation (Poikela et al. 2018; Rosser et al. 2019). The outcomes of the contact between these lineages depend on the strength of reproductive barriers. If they are not strong, then the lineages can exchange genes through backcrossing and hybridization and end up fused (Servedio and Noor 2003; Abbott et al. 2013). But if there are strong enough isolating mechanisms between them, then the two previously isolated populations may evolve in sympatry (Dobzhansky 1940; Coyne and Orr 1998; Servedio and Noor 2003; Poikela et al. 2018). Reinforcement of sexual or postmating prezygotic barriers promotes reproductive character displacement (i.e. increased differences between lineages in areas of sympatry than in areas of allopatry) in traits like mate discrimination, courtship and gamete recognition. As a consequence, sympatric speciation between populations of the same species may be promoted (Coyne and Orr 1997; Howard 1993; Ortiz-Barrientos et al. 2009).

We can not affirm that reproductive constraints that lead to sympatric speciation are operating in these two lineages of Bahia, but some forces are keeping them apart and stochastic events may be favoring the progressive differentiation of these lineages. Our data on experimental crosses, showing high RS between 14x16 individuals from Bahia contrasting with the phylogenetic relationships between these lineages (in Chapter 1), shed light on the putative absence of postzygotic barriers between sympatric karyotypic

lineages. This result brought up the possibility that reinforcement could be taking place in this particular population in Bahia.

Secondary contact in the Bahia population

Genetic structuring was shallow in populations in general (the higher Fst-value was 0.22) and the inbreeding values within populations, which could signal depressed levels of diversity, were low (< 0.06), indicating good populational sizes (Hedrick and Kalinowsky 2000). These values were similar to those observed in genomic analyses of species of South American marsh rats (Prado et al. 2019). Although not very informative about what evolutionary constraints *A. cursor* might be facing, these values are expected for a young species. This is the case of *A. cursor*, a species with age estimated to be under 2 million years old (Coyner et al 2013; Steppan et al. 2017). Its emergence was in the Pleistocene, a period known for its climatic oscillations, which may have had a significant impact on the dynamics of the species' distribution and consequently in the emergence, fixation and interaction of different chromosomal lineages.

A. cursor prefers habitats of grasslands and forest edges (Geise 2012) and the theory of the "Atlantis Forest" presented a scenario where forests expanded onto the the Brazilian continental shelf (Leite et al. 2016), which may have altered suitable habitats of this species putting once isolated populations of *A. cursor* in contact. Hybrid zones or contact zones between related populations are true “natural laboratories” for the study of speciation as they allow the observation of whether the groups are blending and what

factors maintain discrete lineages in the face of ongoing gene flow (Hewitt 1988; Irwin 2019). The effects of climate changes in vertebrate species which are not forest-dependent are scarce, specially terrestrial mammals of the Atlantic Forest (but see Peçanha et al. 2017) and we believe that the region of Una was as a contact zone between karyotypic lineages of *A. cursor*.

Akodon cursor: a ring species-like example

According to the evolutionary biologist Ernst Mayr (1942), the best example of speciation would come from "*the situation in which a chain of intergrading subspecies forms a loop or an overlapping circle, of which the terminal forms no longer interbreed, even though they coexist in the same localities*". He concisely described one of the most fascinating phenomena in Biology now known as "ring species" (Cain 1954; Moritz et al. 1992; Wake 2001; Irwin et al. 2001). Theoretically, ring species are developed when a single ancestral population expands in two directions around a geographical barrier, connected by gene flow between adjacent populations, which accumulate gradual differences and, finally, when terminal populations are back in contact they can no longer interbreed or they generate inviable or sterile intraspecific hybrids (Irwin and Wake 2016). This biogeographical scenario results in a pattern of isolation by distance around the barrier, as a result of restriction to gene flow with populations from the extremes of the species' distribution (i.e. tips of the ring), that form sterile intraspecific hybrids (Stebbins 1949).

The species *A. cursor* can be used as an analogous example of ring species since it has a broad geographic distribution in Brazil, from Paraíba in the north to Paraná in the south, with its primary genetic diversity split being coincident with the region of the source of the Jequitinhonha river, in northern Minas Gerais (Maestri et al. 2016; Chapter 1). Also, our lab crosses pointed that there is no complete reproductive isolation between individuals from the north and the south of the geographic distribution as well as between individuals from adjacent areas. We could observe that homokaryotype individuals from adjacent (ES x BA and BA x PE) or allopatric populations (ES x PE) present high RS in F1, regardless if they are from the same region (North or South) or if they have the same diploid number or not.

However, specifically the two different groups of 2n= 15 individuals from lab crosses presented contrasting results, depending on the geographic origin (or genetic background) of their parentals. Heterokaryotype individuals produced by both northern and adjacent (BA x PE) or same population (BA or PE) parentals are subfertile compared to the species' reproductive standards. On the other hand, they recover high RS when backcrossing with parentals. We also verified through histological analysis that males and females of heterokaryotypes presented normal gametogenesis and males presented normal rates of spermatozoa, comparable to data of homokaryotype individuals. Pairs of southern heterokaryotype individuals have been crossed in the lab before and it was verified they are fertile (Sbalqueiro and Nascimento 1996).

The other heterokaryotype group, which was originated by crossing allopatric parentals (2n=14 from ES x 2n=16 from Pernambuco), presented an extreme condition of sterility. Males 15NS had abnormal seminiferous tubules, without lumen or spermatozoa, such has interspecific hybrids. The females 15NS apparently have normal gametogenesis. Thus, by contrasting fertility information between individuals of the same diploid number (2n=15), but that were generated through different means (same-population or allopatric-population parentals), we can say that the combination of genomic backgrounds are more important than the macro structure of the chromosomes *per se*.

Although probably recent, the period of isolation that the populations from Pernambuco and Espírito Santo have been facing was enough to build up important genetic incompatibilities, making such North/South heterokaryotype individuals (or intraspecific hybrids) a biological dead-end. It is thus likely that intrinsic barriers, notably due to genomic incompatibilities in second generation hybrids (i.e. Bateson-Dobzhansky-Muller incompatibilities, BDMIs) may be at play (Bateson 1909; Dobzhansky 1936, 1937; Muller 1942). This results also are in concordance with Dobzhansky (1937) prediction about hybrid sterility not being caused by chromosome rearrangements *per se* (since the northern ACU 15 is fertile), but that this effect is probably due to genetic incompatibilities, not to large CRs (Orr 1996). We could also consider that an interesting test would be to cross representatives from the extremes of the species' range of *A. cursor*: 2n= 16 individuals from Paraná or São Paulo, the southernmost of the distribution, with 2n= 16 individuals from Pernambuco, the northernmost population, and then contrast the reproductive fitness of 16NS with 15NS (which we verified to be

basically sterile). These results would provide extra evidence about the evolution of epistatic genetic incompatibilities in allopatric populations of *A. cursor* and would help separating the effect of sterility due to geographic distance over to the CRs in heterozygosis. Also, a further investigation would be to identify and localize the genes involved in such putative genomic incompatibilities in the chromosomes between allopatric lineages of *A. cursor*.

Akodon cursor in the grey zone of speciation

> *"...these forms may still be only ... varieties; but we have only to suppose the steps of modification to be more numerous or greater in amount, to convert these forms into species ... thus species are multiplied." (Darwin 1859, p. 120)*

Species has proven to be one of the most challenging components of biodiversity to be conceptualized. Mayden (1997) listed 24 species definitions each of them using its own criteria (i.e. morphological, ecological, phylogenetic, biological, evolutionary, or genotypic) and variations of them still appear (Wang et al. 2019). The definitions of species and how they should be operationally delimited are central to biodiversity science since it directly affects many research fields, including taxonomy, ecology, conservation, biogeography and macroevolution (Agapow et al. 2004; Fišer et al. 2018).

Because speciation is not an instantaneous event, which involves distinct phases of initiation, structuring and completion a proper identification (or classification) of a species depends on the stage of the speciation spectrum its lineages are at (De Queiroz 2007; Nosil and Feder 2012; Roux et al. 2016). Different criteria evolve at different

paces, and many times do not converge simultaneously, making it difficult to categorize a taxon when it is in the grey zone of the speciation continuum (i.e. between-lineage divergence) (Roux et al. 2016). Therefore, distinct species concepts may not convey to the same conclusions regarding species delineation. One of the main issues nowadays is that many recent taxonomic studies are relying solely on genomic data, which can be powerful at diagnosing structure, which could be interpreted as a result of population isolation or could indicate species boundaries. Nonetheless, not all populations become species and it is recommended that researchers be cautious and use multiple lines of evidence to validate observed genetic structure (Sukumaran and Knowles 2017).

The Biological Species Concept (BSC) puts emphasis on the discontinuity of organic forms based on their capability of interbreeding and leaving fertile descendants (Dobzhansky 1935; Mayr 1963). This situation is true for adjacent populations of *A. cursor*, but not between allopatric populations, which forms sterile intraspecific hybrids. It signaled that although being a young species (Coyner et al. 2013; Steppan et al. 2017), allopatric populations of *A. cursor* are accumulating genetic differences in response to geographic isolation. Considering the genomic data, we could see the major north and south groups, not structured primarily by karyotypes, and with shallow differentiation between populations (the highest F_{st} value was 0.22 between ES and PE). Such homogeneously low F_{st} values are indicatives of the early stages of divergence in the speciation spectrum (Roux et al. 2016).

We consider *A. cursor* as ring species-like species and we believe that given time and

geographic isolation, *A. cursor* could become two species. However, evolution is not a straight forward road, so the outcomes are not always the most parsimoinous or the most reasonable. According to the characteristics we observed (i.e. fertility and genomics) *A. cursor* should still be considered a single species. However, since geography seems to play an important role in these features we believe that the lineages from the north of the Jequitinhonha river are enough distinct from the populations from the south of the river, regardless of their karyotypes (2n= 14, 15 or 16).

Conclusions and future directions

With very few genetic studies comprising reproductive fitness, karyotypic diversity and geographic distribution data on non-model vertebrates the present work added to our knowledge, but there is much that remains unknown. With this study we brought some new insights regarding *A. cursor*, such as: the karyotypes rised more than once in the species; the intermediate karyotype is not equivalent to a hybrid of the other two; mate choice can be dependent on karyotype in nature and geographic isolation may be the main cause of the accumulation of genetic incompatibilities among lineages of this species.

An interesting next step would be to investigate, for example, if hotspots for chromosomal rearrangements exists in the genome of *A. cursor* or the effects of climatic oscillations during the Pleistocene in the dynamics of terrestrial organisms, which are not forest-dependent, such as *A. cursor*. Also, following the recommendations from Deakin et al. (2019) we emphasize the importance of keeping generating cytogenetic data, since

it helped bringing so many evolutionary insights that were only possible because people were trained in this area and cared about this information.

By contrasting information on experimental crosses and genomics we had the opportunity to see how nature is complex and does not follow parsimonious ways (or not the way we expect). And when we think we are approaching a better understanding about the species, more questions come up and more analyses and advanced technologies will help answering some of them. Herein we highlighted the importance of thorough sampling, comprising not only genetic diversity, but also karyotypic diversity, which can be useful in understanding the early stages of intraspecific incompatibilities and can be relevant in the dynamics of reproduction and also influence mate choice.

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SUPPLEMENTARY MATERIAL

Table S1. Individuals of A. cursor (ACU) 2n= 14, 2n=15 and 2n= 16, A. montensis (2n= 24 that were used in the experimental crosses.

Species	Voucher	2n	Locality	Latitude	Longitude	Sex
A. cursor	LGA 4024	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4029	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4030	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4031	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4351	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4352	14	Una, BA	-15.18	-39.04	♀
A. cursor	LGA 4021	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4022	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4023	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4025	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4026	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4028	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4033	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4034	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4034	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4337	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4339	14	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4032	16	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4338	15	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4358	15	Una, BA	-15.18	-39.04	♂
A. cursor	LGA 4972	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4974	16	Camaragibe, PE	-7.97	-34.98	♀

A. cursor	LGA 4984	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4985	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4986	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4989	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4990	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4991	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4993	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4994	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4972	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4974	16	Camaragibe, PE	-7.97	-34.98	♀
A. cursor	LGA 4969	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4976	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4978	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4979	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4980	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4983	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4987	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4988	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4992	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4995	16	Camaragibe, PE	-7.97	-34.98	♂
A. cursor	LGA 4245	14	Castelo, ES	-20.52	-41.08	♀
A. cursor	LGA 4451	14	Domingos Martins, ES	-20.18	-40.83	♀
A. cursor	LGA 4452	14	Domingos Martins, ES	-20.18	-40.83	♀
A. cursor	LGA 4453	14	Domingos Martins, ES	-20.18	-40.83	♀
A. cursor	LGA 4480	14	Santa Teresa, ES	-19.92	-40.60	♀
A. cursor	LGA 4481	14	Santa Teresa, ES	-19.92	-40.60	♀
A. cursor	LGA 5013	14	Domingos Martins, ES	-20.20	-40.83	♀

A. cursor	LGA 5014	14	Domingos Martins, ES	-20.20	-40.83	♀
A. cursor	LGA 4260	14	Castelo, ES	-20.52	-41.08	♂
A. cursor	LGA 4261	14	Castelo, ES	-20.52	-41.08	♂
A. cursor	LGA 4262	14	Castelo, ES	-20.52	-41.08	♂
A. cursor	LGA 4401	14	Domingos Martins, ES	-20.36	-40.66	♂
A. cursor	LGA 4471	14	Domingos Martins, ES	-20.18	-40.83	♂
A. cursor	LGA 4472	14	Domingos Martins, ES	-20.18	-40.83	♂
A. cursor	LGA 4509	14	Guarapari, ES	-20.66	-40.51	♂
A. cursor	LGA 5015	14	Domingos Martins, ES	-20.20	-40.83	♂
A. montensis	LGA 4552	24	Luminárias, MG	-21.57	-44.79	♀
A. montensis	LGA 4547	24	Luminárias, MG	-21.57	-44.79	♀
A. montensis	LGA 4539	24	Luminárias, MG	-21.57	-44.79	♀
A. montensis	LGA 4410	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4411	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4412	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4395	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4396	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4398	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4360	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4207	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4197	24	Cananéia, SP	-25.02	-47.90	♀
A. montensis	LGA 4190	24	Iguape, SP	-24.72	-47.55	♀
A. montensis	LGA 4150	24	Iguape, SP	-24.72	-47.55	♂
A. montensis	LGA 4201	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4203	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4362	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4366	24	Cananéia, SP	-25.02	-47.90	♂

A. montensis	LGA 4369	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4370	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4371	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4386	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4387	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4399	24	Cananéia, SP	-25.02	-47.90	♂
A. montensis	LGA 4538	24	Luminárias, MG	-21.57	-44.79	♂
A. montensis	LGA 4561	24	Luminárias, MG	-21.57	-44.79	♂
A. montensis	LGA 4567	24	Luminárias, MG	-21.57	-44.79	♂

Table S2. Vouchers of captive A. cursor (ACU) 2n= 14, 2n=15 and 2n= 16, A. montensis (2n= 24) and interspecific hybrids (HYB) that were used in histological analyses.

Species	Vouchers	2n	Origin	Sex
ACU	LGA 5027, 5077, 5190, 5192, 5193	16	North	male
ACU	LGA 4799, 5010, 5011, 5194	16	North	female
ACU	LGA 4021, 4419, 4282, 4409, 4511	14	North	male
ACU	LGA 4406, 4407, 4473, 4279	14	North	female
ACU	LGA 4444, 4503, 4508, 4612, 4651	14	South	male
ACU	LGA 5248, 5251, 5256, 5257	14	South	female
ACU	LGA 4648, 4903, 4271, 4967, 5051	15	North	male
ACU	LGA 4901, 4964, 4962, 4961	15	North	female
ACU	LGA 5164, 5165, 5166, 5179, 5180	15	North/South	male
ACU	LGA 5195, 5183, 5178, 5162	15	North/South	female
AMO	LGA 4414, 4447, 4594, 4462, 4477	24		male
HYB	LGA 4458, 4466, 4469, 4737, 4470	19/20		male

Table S3. Quality control of samples of A. cursor, A. montensis and natural hybrids during genome data processing. Samples without karyotype information are coded as ND (not determined).

Voucher	2n	State	Locality	Lat	Long	Total Reads	No RadTag	Low quality	Retained	Mean coverage	St dev	Max	Missing data
Akodon cursor													
LGA 4339	14	Bahia	Una	-15.18	-39.04	901667	12374	484	847133	7.51117	7.34217	639	0.9006
LGA 4030	14	Bahia	Una	-15.18	-39.04	1744902	15879	886	1696291	10.7083	5.04161	496	0.6144
LGA 4026	14	Bahia	Una	-15.18	-39.04	1999592	13906	1017	1935081	12.0575	6.15398	172	0.5975
LGA 4033	14	Bahia	Una	-15.18	-39.04	2400245	11273	1215	2318198	12.065	5.76923	147	0.5391
LGA 4028	14	Bahia	Una	-15.18	-39.04	3106401	14348	1602	3014949	15.1418	7.95441	811	0.4786
LGA 4029	14	Bahia	Una	-15.18	-39.04	3575180	42495	1758	3370617	13.954	7.27161	170	0.4802
LGA 4337	14	Bahia	Una	-15.18	-39.04	4858796	17786	2527	4690901	20.2613	11.645		0.426
LGA 4338	15	Bahia	Una	-15.18	-39.04	1051916	4512	535	1020098	8.55363	6.64817	922	0.799
CIT 926	15	Bahia	Una	-15.18	-39.04	1094283	9676	555	1058378	8.88052	4.27413	435	0.7934
LGA4358	15	Bahia	Una	-15.18	-39.04	1238179	6794	633	1186484	8.73811	4.41333	154	0.7782
CIT 880	15	Bahia	Una	-15.18	-39.04	1592052	12361	816	1484099	7.78218	19.2867	2427	0.8915
CIT 931	15	Bahia	Una	-15.18	-39.04	2886148	13041	1492	2821061	14.1277	7.57847	448	0.4975
CIT 878	16	Bahia	Una	-15.18	-39.04	1636958	10944	837	1575763	9.5975	5.31714	752	0.651
CIT 887	16	Bahia	Una	-15.18	-39.04	3383014	21782	1788	1815411	10.454	4.13884	244	0.5818
CIT 883	16	Bahia	Una	-15.18	-39.04	1903642	17503	991	1834068	10.5206	4.96879	189	0.5953

CIT 927	16	Bahia	Una	-15.18	-39.04	2151178	13341	1142	2077719	11.461	5.30258	203	0.5551
LGA4330	16	Bahia	Una	-15.18	-39.04	2615560	10256	1373	2537342	13.0616	6.52577	719	0.519
CIT 930	16	Bahia	Una	-15.18	-39.04	3043652	11712	1566	2977736	15.8593	9.02241	396	0.5101
CIT 921	16	Bahia	Una	-15.18	-39.04	3218858	18411	1682	3115207	14.6767	7.05472	246	0.4785
LGA 4032	16	Bahia	Una	-15.18	-39.04	4599662	21234	2388	4495308	22.5211	14.7195	822	0.4824
CIT 1021	16	Bahia	Una	-15.18	-39.04	5069030	17315	2567	4954371	22.7604	12.6916	388	0.4289
LGA 993	14	Espírito Santo	Castelo	-20.6	-41.18	1830089	5583	993	1788649	11	4.83187	177	0.5805
LGA 4401	14	Espírito Santo	D. Martins	-20.37	-40.67	2300317	11865	1197	2233511	12.312	6.73355	773	0.5447
LGA 1234	14	Espírito Santo	Ibitirama	-20.48	-41.72	2108889	15299	1056	2052424	11.7072	5.83861	208	0.5783
LGA 2144	14	Espírito Santo	Viana	-20.38	-40.45	3157486	15795	1623	3080145	14.6815	7.4895	225	0.4718
MCN-M 1563	ND	Minas Gerais	Diamantina	-18.09	-43.75	2474412	14859	1271	2377933	12.0377	5.66904	131	0.5414
MCN-M 2601	ND	Minas Gerais	Grão Mogol	-16.62	-42.81	790697	5647	428	763929	8.18445	5.341	628	0.8683
MCN-DG 161	ND	Minas Gerais	J. Gonçalves de Minas	-16.62	-42,81	518525	3862	266	504761	7.4223	7.53234	707	0.9376
MCN-M 1452	ND	Minas Gerais	Leme do Prado	-17.08	-42.69	3114163	14314	1551	3036511	15.8131	9.8847	430	0.5181
UFES-CTA 1134	16	Minas Gerais	Marliéria	-19,71	-42,65	1423986	8989	719	1390050	10.6002	5.35513	180	0.7021
UFES-CTA 956	ND	Minas Gerais	Marliéria	-19,71	-42,65	1913661	9337	996	1864929	16.0104	8.52498	257	0.4702
UFES-CTA 950	ND	Minas Gerais	Marliéria	-19,71	-42,65	2282325	9252	1199	2206161	13.1238	6.06926	284	0.5524
UFES-CTA 977	ND	Minas Gerais	Simonésia	-20.13	-42	3706304	30259	1964	3580155	16.1993	8.33728	147	0.4518
UFES-CTA 937	14	Minas Gerais	Turmalina	-17,13	-42,77	3667347	18115	1865	3588583	12.0602	7.35452	223	0.5459
UFES-CTA 944	16	Minas Gerais	Turmalina	-17,13	-42,77	1913661	9337	996	1864929	10.8143	5.08581	332	0.6074

UFES-CTA 1114	ND	Minas Gerais	Turmalina	-17,13	-42,77	1399597	8796	674	1350906	9.24035	4.01742	166	0.6976
UFES-CTA 1110	ND	Minas Gerais	Turmalina	-17,13	-42,77	1828450	9711	946	1781040	10.8635	4.60099	110	0.607
LGA 4973	16	Pernambuco	Camaragibe	-7.97	-34.98	1728282	9077	880	1666570	10.398	5.03137	549	0.6112
LGA 4975	16	Pernambuco	Camaragibe	-7.97	-34.98	2047866	8375	992	1981187	11.3902	5.49069	542	0.567
LGA 4970	16	Pernambuco	Camaragibe	-7.97	-34.98	3045455	15819	1596	2952275	14.7183	7.31116	199	0.5079
LGA 4976	16	Pernambuco	Camaragibe	-7.97	-34.98	4781656	22157	2509	4654470	21.9253	12.401	518	0.4493
CIT 314	15	São Paulo	Ariri	-25.2	-48.03	1070126	8223	523	1040260	8.91071	3.70269	74	0.7728
CIT 288	14	São Paulo	Iguape	-24.72	-47.55	4072967	17900	2065	3953959	18.8717	10.0908	247	0.4544
CIT 222	15	São Paulo	Iguape	-24.72	-47.55	1647245	10306	878	1606166	10.2974	4.73778	607	0.6059
CIT 173	15	São Paulo	Iguape	-24.72	-47.55	1925084	6004	925	1883300	10.9339	5.25624	750	0.5704
CIT 264	15	São Paulo	Iguape	-24.72	-47.55	2802532	12874	1447	2736821	13.954	8.67166	294	0.5535
CIT 285	15	São Paulo	Iguape	-24.72	-47.55	3001567	16014	1451	2904153	15.3797	7.77589	220	0.4944
CIT 223	15	São Paulo	Iguape	-24.72	-47.55	4754912	17153	2439	4629931	21.8329	11.5889	211	0.4326
CIT 786	14	São Paulo	Ilha do Cardoso	-25.17	-47.93	2591074	256897	1257	2291404	13.2788	7.80091	696	0.5519
CIT 785	14	São Paulo	Ilha do Cardoso	-25.17	-47.93	4059396	19652	2000	3940462	19.1854	10.2431	818	0.4477
UERJ-HB 01	15	São Paulo	Ilha do Cardoso	-25.17	-47.93	3238237	12586	1678	3151816	15.3755	7.44789	202	0.4817
CIT 20	14	São Paulo	Sete Barras	-24.38	-47.85	3764603	21198	1916	3646404	16.5465	8.4464	204	0.4486
CIT 134	16	São Paulo	Ubatuba	-23.37	-44.83	1886907	10483	951	1850991	11.7692	7.31683	801	0.6058
CIT 870	15	Bahia	Una	-15.18	-39.04	2270668	11248	1167	2189931	11.4757	5.36883	315	0.5543

Akodon montensis													
LGA 4197	24	São Paulo	Cananéia	-25.01	-47.93	3885464	14722	2008	3756272	18.1686	9.07282	222	0.5517
LGA 4370	24	São Paulo	Cananéia	-25.01	-47.93	4101858	15665	2135	4007959	21.1304	10.9423	365	0.563
Natural hybrids													
CIT 159	19	São Paulo	Iguape	-24.72	-47.55	1965717	7756	942	1922615	10.0812	4.81872	203	0.5722
CIT 265	19	São Paulo	Iguape	-24.72	-47.55	2139006	16921	1105	2050572	10.2799	4.49781	119	0.5817

Table S4. Stereological parameters (volumetric proportions) of the ovaries of A. cursor from groups 2n=14 from the North (14N) and South (14S), 2n= 15 from the North (15N) and 2n= 16 from the North (16N) and 2n= 15 North/South. Values are medium and standard deviation (±). No parameters were significantly different from each other (p-values > 0.05).

Parameter	**14S**	**14N**	**15N**	**15NS**	**16N**
Albuginea	5,30±2,00	3,33±1,82	3,13±0,38	3,33±1,09	3,98±1,85
Germ epithelium	3,88±0,69	3,68±0,61	2,95±0,90	2,08±1,04	2,83±1,27
Primordial follicle	0,18±0,17	0,03±0,05	0,00±0,00	0,55±0,42	1,05±1,32
Primary follicle	0,08±0,15	0,20±0,08	0,50±0,47	0,13±0,19	0,33±0,36
Secondary follicle	3,45±2,21	1,08±0,78	2,95±2,80	3,10±3,51	3,48±1,71
Tertiary follicle	3,20±2,53	5,68±3,76	4,08±3,64	4,28±4,50	4,15±1,69
Graaf follicle	3,85±5,66	6,93±4,64	0,00±0,00	3,73±4,85	7,00±9,27
Corpo Luteo	25,05±28,83	14,73±20,84	24,50±18,13	30,33±28,92	9,98±4,22
Interstitial cells stroma	31,08±19,78	38,08±9,58	42,75±11,91	28,38±14,91	34,25±11,65
Conjunctive stroma	15,88±7,93	15,83±4,91	11,60±5,43	14,50±6,40	21,25±7,54
Blood vessels	1,13±0,86	2,10±0,57	1,63±1,51	3,43±1,35	1,25±0,37
Atresic follicule	6,78±3,50	7,33±1,75	5,55±7,42	5,55±4,04	9,35±3,06
Defense cells	0,18±0,13	1,05±1,08	0,38±0,39	0,23±0,26	0,50±0,00

Table S5. Volumetric proportion (%) of seminiferous tubules and interstitial tissue between groups of Akodon cursor. The groups are: 2n=14 from the North (14N), from the South (14S)and north-south (14NS), 2n= 15 from the North (15N) and 2n= 16 from the North (16N) and 2n= 15 North/South.

Group (2n and region)	**Seminiferous tubules (%)**	**Interstitial tissue (%)**
14 North	**92,88±2,62***	**7,12±2,62***
14 South	**93,88±0,59+**	**6,12±0,59+**
14 North/South	**94,70±1,55#**	**5,30±1,55#**
15 North	**88,87±4,15*,**,+**	**11,13±4,15*,**,+**
15 North/South	**82,51±3,91*, **,+**	**17,49±3,91*,**,+**
16 North	**88,61±1,82****	**11,39±1,82****

Values shown are medium and standard deviation (±). Symbols are for $p<0.05$ between groups. * Group 2n= 14 North x group 2n= 15 North (p=0. 000138) and group 2n= 15 North/South (p=0.00); ** group 2n= 15 North x group 2n=15 North/South (p=0.0.014) and both 2n= 15 groups differ from 2n= 16 North (p= p=0.000140); + group 2n= 14 South differs from groups 2n= 15 North (p=0.01246) and 2n=15 North/South (p= 0.01177); # 2n=14 north/South differs from 2n= 16 north (p= 0,020).

Table S6. Tubular diameter and epithelium height between groups of Akodon cursor. The groups were: 2n=14 from the North (14N), and from the South (14S), 2n= 15 from the North (15N) and 2n= 16 from the North (16N) and 2n= 15 North/South.

Group (2n and region)	**Tubular diameter (µm)**	**Epithelium height (µm)**
14 North	210,54±53,4	**44,77±9,67***
14 South	231,03±22,19	**55,42±5,52**$^{+}$
14 North/South	223,33±26,30	**52,59±7,31**$^{\#}$
15 North	238,07±34,09	**76,30±14,54**$^{*,+,**}$
15 North/South	222,98±10,60	**76,44±5,06**$^{*,+,**}$
16 North	201,11±27,84	**50,87±7,80****

Values shown are medium and standard deviation (±). Symbols are for p<0.05 between groups * Group 2n= 14 North x group 2n= 15 North (p= 0. 000246) and group 2n= 15 North/South (p=0. 000234); ** group 2n= 15 North x 2n= 16 North (p= 0. 001858) and group 2n=15 North/South x 2n= 16 North (p=0. 001757); $^{+}$ group 2n= 14 South x groups 2n= 15 North (p= 0. 01246) and 2n=15 North/South (p=0. 01177); 2n= 14 north/south x 2n= 15 north (p=0.003815) and 2n= 14 north/south x 2n= 15 north/south (p=0.003601)

Table S7. Proportion of normal and abnormal spermatozoids (%) in the deferent ducts of groups of Akodon cursor. The groups were: 2n=14 from the North (14N), from the South (14S), and 2n= 14 North/South (14NS); 2n= 15 from the North (15N) and 2n= 16 from the North (16N). Group 2n= 15NS did not present spermatozoa.

Spermatozoids	**14 north**	**14 south**	**14 nortth/south**	**15 north**	**16 north**
Normal	66.7±7.54%	64.5±21.36%	78,8±29,26%	64.1±37.17%	72,4±21.69%
Abnormals					
Head separated from the flagellum	2.9±2.30%	1.4±1.78%	0,6±1,78% 0,01±0,13%	25.2±40.32%	0.6±1.34%
Head with a tip	--------	--------		----------	---------
Intermediary piece with anomalous insertion	7.2±7.29%	12.3±10.80%	5,10±4,81%	3.7±4.89%	1.6±6.57%
Folded intermediary piece	1.1±0.96%	0.9±1,47%	4,5±2,94%	5.3±7.27%	4.8±26.74%
Intermediary piece with a camber	0.2±0.45%	0.1±0.22%	4,0±4,59% 0,6±0,78%	--------	2.1±3.91%
Short flagellum	0.9±1,24%	0.6±0.82%	**1,6±1,64%** 0,5±2,13%	0.4±0,65%	-----
Curled flagellum	**3.4±1.85%***	**3.3±2.61%**	**4,2±1,64%**	**1.1±0.67%***	**0.5±0.50%**
Fragmented flagellum	3.3±3.05%	3.2±5.79%		0.1±0.22%	0.6±1.34%
Folded flagellum	**14.3±7.82%**$^{\#}$	**13.7±11.69%****		**0.1±0.45%**$^{\#,**}$	**17.4±1.64%**$^{\#}$

Values shown are medium and standard deviation (±).Symbols are for $p<0.05$ between groups. *Group 2n= 14 North x group 2n=15 North (p=0.0496) and 2n=16 North (p=0.000234); # group 2n=14 North x group 2n= 15 North (p= 0. 021069) and group 2n= 16 North (p= 0. 041097); ** group 2n=14 South x group 2n= 15 North (p=0. 02876).

Table S8. Summary of data on experimental crosses of A. cursor (ACU), A. montensis (AMO) and interspecific hybrids (HYB) considering the species' karyotype (diploid number = 2n) and geographic origin of the parentals (North=N; South=S, BA = Bahia, ES = Espirito Santo ; PE = Pernambuco).

Species	2n and origin of parentals	Total of crossings	With progeny	Without progeny	Reproductive success (%)	Total of pups	Average number pups/cross	Min-Max
Species' standards								
ACU x ACU	16N x 16N (PE x PE)	21	16	5	76.2	58	3.6	2-5
ACU x ACU	14N x 14N (BA x BA)	17	13	4	76.5	36	3.3	2-5
ACU x ACU	14S x 14S (ES x ES)	28	16	12	57.1	61	3.8	3-6
AMO x AMO	24 x 24	27	15	12	55.5	50	3.3	1-4
Geographic distances x karyotypes								
ACU x ACU same population	14N x 16N (BA x BA)	8	5	3	62.5	21	4.2	3-6
ACU x ACU adjacent populations	14N x 16N (BA x PE)	8	2	6	25.0	9	4.5	4-5
ACU x ACU adjacent populations	14S x 14N (ES x BA)	16	6	10	37.5	22	3.6	2-5
ACU x ACU allopatric populations	14S x 16N (ES x PE)	28	14	14	50.0	62	4.4	3-7
ACU x ACU adjacent populations	14NS x 14NS (ES/BA x ES/BA)	19	15	4	78.9	52	3.4	1-5
ACU x ACU adjacent populations	14NS x 14S (ES/BA x ES)	6	4	2	66.6	23	5.7	5-7
ACU x ACU Adjacent populations	14NS x 14N (ES/BA x BA)	7	5	2	71.4	24	4.8	4-5
Northern ACU heterokaryotype								
ACU x ACU	15N x 15N (BA x BA)	23	3	20	13.0	10	3.3	1-7
ACU x ACU	15N x 15N (BA/PE x BA/PE)	6	1	5	16.6	3	3.0	3
ACU x ACU	15N x 14N (BA x BA)	12	5	7	41.6	14	2.8	1-4
ACU x ACU	15N x 16N (BA x BA/PE)	11	5	6	45.5	17	3.4	3-4

North/South ACU heterokaryotype								
ACU x ACU	15NS x15NS (ES/PE x ES/PE)	35	0	35	0	0	0	0
ACU x ACU	15NS x 16N (ES/PE x PE)	20	1	19	5.0	2	2	2
ACU x ACU	15NS x 14S (ES/PE x ES)	16	1	15	6.0	1	1	1
Producing hybrids								
AMO x ACU	24 x 14S (ES)	12	5	7	41.6	14	2.8	1-4
AMO x ACU	24 x 14N (BA)	23	6	17	26.0	10	1.6	1-3
AMO x ACU	24 x 16N (BA/PE)	19	7	12	36.8	27	3.8	2-6
AMO x ACU	24 x 15N (BA)	10	3	7	30.0	5	1.6	1-2
Crossing hybrids								
HYB x HYB	19/20 x 19/20	17	0	17	0.0	0	0	0
HYB x parental ACU	19/20 x14/15/16	14	0	14	0.0	0	0	0
HYB x parental AMO	19/20 x 24	16	0	16	0.0	0	0	0
TOTAL		419						

Table S9. Results of STRUCTURE analyses on the dataset containing all populations of A. cursor , A. montensis and natural interspecific hybrids and only the Bahia population, which has representatives of the three karyotypes of the species

Level	Group	Inds	1st K	Delta K	2nd K	Delta K	MCMC	Burn in
1	*All populations*	58	2	1427.072875	3	8.816129	1.000,000	500,000
1.1	Bahia	22	2	32.438048	3	3.104967	1.000,000	500,000

Table S10. Mantel test excluding one population of A. cursor at a time. Significant values are in bold. Abbreviations are for: SP (Sao Paulo), PE (Pernambuco), MG_N (Minas Gerais/North), ES (Espírito Santo), MG_S (Minas Gerais/South) and BA (Bahia).

Removed pop	r statistic	Significance
SP	0.6611	0.11667
PE	0.08489	0.4
MG_N	0.4828	0.21667
ES	0.4979	0.23333
MG_S	0.5755	0.15833
BA	0.7394	**0.033333**

Table S11. F_{st}-values between populations of A. cursor. Abbreviations are for: SP (Sao Paulo), PE (Pernambuco), MG_N (Minas Gerais/North), ES (Espírito Santo), MG_S (Minas Gerais/South) and BA (Bahia). States in bold are from the South.

	SP	PE	BA	**MG_S**	MG_N	**ES**
SP		0.227629	0.118644	0.110953	0.164273	0.117846
PE			0.123758	0.222022	0.220692	0.246012
BA				0.0915113	0.102304	0.0944547
MG_S					0.147186	0.0905855
MG_N						0.157858
ES						

Table S12. Summaries of genetic diversity: average observed heterozygosity (H_{obs}), average nucleotide diversity (π), and Wright's inbreeding coefficient (F_{IS}) per sampled population of A. cursor as well as sample sizes (n) and percentage of sites that were polymorphic within each population (% pol).

Population	n	% pol	π	H_{obs}	F_{is}
Pernambuco	4	0.537	0.0475	0.0408	0.0126
Bahia	16	7.2628	0.0886	0.0668	0.0621
MG/N	4	1.7271	0.0881	0.0708	0.0353
MG/S	4	0.7377	0.0918	0.0742	0.0364
Espirito Santo	4	0.5472	0.0905	0.0736	0.0324
Sao Paulo	10	2.7629	0.0698	0.0537	0.0426

CONSIDERAÇÕES FINAIS

A espécie *Akodon cursor* surpreendeu cientistas desde o primeiro trabalho com análises cromossômicas publicado em 1972, no qual foram observadas 4 composições cariotípicas distintas em 8 indivíduos com 2n= 14 de uma mesma população. Passados 47 anos, mesmo com muitos avanços metodológicos e teóricos, ainda estamos longe de compreender os mecanismos que estão moldando a evolução dessa espécie.

Com o presente estudo pudemos observar que a geografia, por meio de processos vicariantes, teve um papel preponderante na formação de linhagens de *A. cursor*. Também temos subsídios para sugerir que os diferentes cariótipos da espécie não tiveram um surgimento único. Acreditamos que a dispersão diferencial (modo e tempo) de linhagens cariotípicas, aliada à eventos estocásticos que levaram à fixação de algumas variantes em determinadas populações podem ter sido fatores determinantes para definir os padrões observados. Há indícios que eventos vicariantes desempenharam um papel mais determinante na estruturação das populações do norte do que das populações do Sul. Nas linhagens ao sul do rio Jequitinhonha observa-se que populações de diversas áreas geográficas agruparam-se de forma mais coesa.

No entanto, nos questionamos por quais motivos em alguns locais apenas um tipo de cariótipo é encontrado e em outros vemos indivíduos de diversos cariótipos em simpatria. Existiriam ondas de dispersão de cariótipos fundadores? Será que existiriam cariótipos ótimos para determinados habitats? Como seria possível mensurar a otimização de cariótipos em função de um ambiente/habitat?

No entanto, a situação mais surpreendente foi observada em uma população da Bahia, na qual coexistem duas linhagens em uma mesma localidade (2n= 14 e 2n= 15+16), mas que não

compartilham um ancestral comum mais recente. Além disso, nossos dados indicaram que não há mistura genética entre essas duas linhagens, embora em cativeiro esses grupos intercruzaram com alto sucesso reprodutivo e produziram prole fértil. Esse padrão nos leva a pensar que na natureza os indivíduos dessas diferentes linhagens seriam capazes de se reconhecerem como entidades distintas e nao intercruzarem. Se esse mecanismo de fato existe, abre-se uma vasta frente de investigação em busca de explicações ecológicas, comportamentais ou biológicas.

Quando iniciamos esse projeto a hipótese inicial a ser testada era se os indivíduos 2n= 15 seriam híbridos intraespecíficos e que indicariam um processo de especiação em *A. cursor*. Os resultados da Bahia, no entanto, são um exemplo de que na natureza nem tudo é previsível, sendo o 2n= 15 dessa população uma variação de 2n= 16, e não uma mistura dos diferentes homocariótipos. Dessa forma a evolução nos mostra que tem caminhos criativos e que nem sempre são os mais parsimoniosos.

A situação que evidenciou que *A. cursor* pode ser considerada uma espécie em anel mostra que podemos estar diante de um processo inicial de divergência dessa espécie, em que o isolamento geográfico poderá contribuir para que exista um completo isolamento reprodutivo entre linhagens dessa espécie no futuro.

O roedor *A. cursor* é um exemplo de espécie abundante e fácil de ser capturada na natureza. Todos os seus atributos a tornam um organismo que pode vir a ser utilizado como modelo em estudos sobre os estágios iniciais de especiação, com possível localização de áreas genômicas com baixa recombinação; investigação de processos de desvio meiótico e de segregação de trivalentes em heterocariótipos e investigação de fatores ecológicos envolvidos no processo de reforço de barreiras pré-zigóticas em linhagens cariotípicas.

O fato de cariótipos estruturalmente idênticos terem surgido mais de uma vez na história da espécie, torna-se um tema intrigante, levantando questionamentos sobre aspectos acerca da evolução cariotípica, assim como da presença de *hotspots* para rearranjos cromossômicos.

Deve-se abordar mais detalhadamente os períodos de divergência entre as linhagens para tentar explicar com mais robustez os possíveis processos biogeográficos envolvidos na distribuição das linhagens de *A. cursor*. Essas análises poderão contribuir para um melhor entendimento de como as oscilações climáticas afetaram animais terrestres não dependents de florestas e também permitirão correlações mais precisas com processos geológicos que afetaram recentemente a crosta do Brasil.

Por fim, a história filogenética das linhagens de *A. cursor* se mostrou um cenário ainda mais complexo do que já se conhecia e foram levantadas mais questionamentos a serem respondidos. Novas ferramentas e abordagens têm contribuído para o avanço rápido da Ciência, e espera-se que não não sejam precisos mais 50 anos para que consigamos compreender com mais clareza os vários processos que estão por trás da evolução e diversificação dessa espécie.

Esse trabalho mostrou não apenas peculiaridades evolutivas envolvendo os cromossomos de *A. cursor,* mas indicou que estudos biogeográficos, filogenéticos e evolutivos em geral só têm a ganhar com a incorporação de informações cromossômicas. O grande esforço para se coletar animais e mantê-los em cativeiro também não foi em vão e permitiram uma visão que, ao ser integrada com os dados genômicos ampliaram as perspectivas acerca de possíveis processos evolutivos nessa espécie. Ressalta-se com esse trabalho a importância de se utilizar várias abordagens para que seja possível se ter uma noção mais aproximada dos processos que

ocorrem o tempo todo na natureza e que impactam diretamente a biodiversidade, tais como vicariância, dispersão, competição, especiação e extinção.