

**UNIVERSIDADE ESTADUAL PAULISTA**  
**“JÚLIO DE MESQUITA FILHO”**  
**FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS**  
**CÂMPUS DE JABOTICABAL**

**SEGREGAÇÃO GAMÉTICA DE CROMOSSOMOS**  
**ENVOLVIDOS EM TRANSLOCAÇÕES E SEU PAPEL NO**  
**ISOLAMENTO REPRODUTIVO DE ESPÉCIES DO GÊNERO**  
***Mazama* (MAMMALIA; CERVIDAE)**

**David Javier Galindo Huamán**  
**Médico Veterinário**

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**David Javier Galindo Huamán**

**Orientador: Prof. Dr. José Maurício Barbanti Duarte**

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária, área Reprodução Animal.

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Huamán, David Javier Galindo

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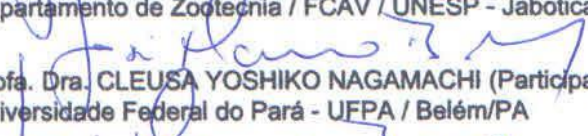
TÍTULO DA TESE: SEGREGAÇÃO GAMÉTICA DE CROMOSSOMOS ENVOLVIDOS EM TRANSLOCAÇÕES E SEU PAPEL NO ISOLAMENTO REPRODUTIVO DE ESPÉCIES DO GÊNERO *Mazama* (MAMMALIA; CERVIDAE)

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Jaboticabal, 05 de abril de 2021

## DADOS CURRICULARES DO AUTOR

**DAVID JAVIER GALINDO HUAMÁN** – Nascido em 18 de fevereiro de 1989, na cidade de Jesús María, Lima, Lima, Peru. Ingressou no curso de graduação em Medicina Veterinária na Universidade Nacional Maior de São Marcos (FMV-UNMSM), em março de 2007; onde participou em diversos grupo de estudos como o “Taller de Biotecnología Reproductiva” (TBR, 2009-2012) e o “Taller de Etología Animal” (TEA, 2010-2012), além de ser membro de órgãos de representação estudantil como o “Centro Federado de Estudiantes” (CEF) em 2009 e o “Consejo de Facultad” (com direito a bolsa) em 2010; concluiu curso superior em Medicina Veterinária em novembro de 2012. Realizou monitoria, *ad honorem*, nas disciplinas de Anatomia Animal e Bases Anatômicas para Cirurgia Veterinária da FMV-UNMSM, entre janeiro e março de 2013, na condição de formado (bacharel vinculado). Em fevereiro de 2014 ingressou na equipe de reprodução animal do Núcleo de Pesquisa e Conservação de Cervídeos (NUPECCE), pertencente ao Departamento de Zootecnia da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP – Câmpus de Jaboticabal; nesta mesma unidade, iniciou o curso de pós-graduação em Medicina Veterinária (área de concentração em Reprodução Animal), em março de 2015, obtendo o título de Mestre em fevereiro de 2017. Membro da “IUCN Species Survival Commission (SSC) - Deer Specialist Group (DSG)” desde fevereiro de 2017. Ingressou em março de 2017 no curso de Pós-graduação em Medicina Veterinária (área de concentração Reprodução Animal), sob orientação do Prof. Dr. José Maurício Barbanti Duarte, sendo bolsista pelo “Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica” (FONDECYT 116-2017) – Peru. Realizou estágio sanduíche no “Veterinary Research Institute”, Brno, República Tcheca durante o período de 29 de agosto de 2019 a 24 de junho de 2020, sob supervisão da Dra. Miluše Vozdová.

*Amar lo que haces es el requisito indispensable para entender lo que haces, y todo aquel que realmente sabe lo que está haciendo es alguien con quien vale la pena conversar más de una vez.*

**Marco Sifuentes Quintana**

**Dedico**

*Ao Andrés Huamán Vera, meu querido avô, que forjou todos os alicerces da pessoa e profissional que espero ter me tornado.*

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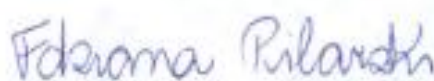
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## CERTIFICADO

Certificamos que o projeto de pesquisa intitulado "**Segregação gamética de cromossomos envolvidos em translocações e seu papel no isolamento reprodutivo de espécies do genero *Mazama* (Mammalia; Cervidae)**", protocolo nº 001930/18, sob a responsabilidade do Prof. Dr. José Mauricio Barbanti Duarte, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 08 de fevereiro de 2018.

Vigência do Projeto	01/03/2018 a 30/11/2020
Espécie / Linhagem	<i>Mazama gouazoubira</i> e <i>Mazama americana</i>
Nº de animais	9 (3 <i>M. gouazoubira</i> , 3 <i>M. americana</i> e 3 híbridos entre diferentes citótipos de <i>M. americana</i> )
Peso / Idade	<i>M. gouazoubira</i> 16.0 a 20.0 Kg / 3 a 6 meses (Quando coletados no campo - vida livre) <i>M. americana</i> 135.0 a 40.0 Kg / 1.5 a 9 anos (animais no plantel)
Sexo	Macho
Origem	Núcleo de Pesquisa e Conservação de Cervídeos - NUPECCE

Jaboticabal, 08 de fevereiro de 2018.

  
Profª Drª Fabiana Pilarski  
Coordenadora - CEUA

**SEGREGAÇÃO GAMÉTICA DE CROMOSSOMOS ENVOLVIDOS EM  
TRANSLOCAÇÕES E SEU PAPEL NO ISOLAMENTO REPRODUTIVO DE  
ESPÉCIES DO GÊNERO *Mazama* (MAMMALIA; CERVIDAE)**

**RESUMO** – A família Cervidae destaca-se por possuir uma das maiores taxas de evolução cariotípica dentre os mamíferos, reflexo de uma fragilidade cromossômica acentuada, que facilita a ocorrência de quebras e rearranjos cromossômicos. Estas, quando vinculadas ao isolamento geográfico em um curto período, poderiam levar as populações a um processo de especiação. Isso é evidenciado na ampla diversificação cariotípica da família, apresentando exemplos extremos de baixo e alto número diploide, como *Muntiacus muntjak* ( $2n = 6/7$ ) e *Capreolus pygargus* ( $2n = 70 + 1 - 14$  Bs), respectivamente. Muitas espécies crípticas estão escondidas pela grande similaridade morfológica, acompanhada de ampla variação cariotípica, intra e interespecífica. Animais portadores de rearranjos cromossômicos podem apresentar diferentes padrões na segregação meiótica durante a espermiogênese, dando passo à formação de gametas desbalanceados. Isso pode estar relacionado com desordens reprodutivas, como evidenciado em animais domésticos, onde é observada a queda da capacidade reprodutiva. A técnica de hibridização *in situ* fluorescente (FISH) permite a marcação, identificação e localização dos cromossomos envolvidos nas translocações a partir de sondas de DNA com marcadores fluorescentes. Dada a falta de sondas específicas para espécies de cervídeos, diversos estudos apontam para o uso de sondas bovinas devido ao conhecido mapa genético bovino e à proximidade filogenética entre as famílias Cervidae e Bovidae. Dessa forma, a aplicação da FISH em células espermáticas (também chamada de sperm-FISH) permite estimar a proporção de gametas normais/balanceados e desbalanceados (portadores de aneuploidias). Assim, o presente estudo teve como objetivo: a) Estimar a proporção dos produtos da segregação meiótica em indivíduos portadores de translocações cromossômicas dentro do gênero *Mazama*, b) Avaliar os potenciais efeitos dos rearranjos cromossômicos na aptidão reprodutiva dos portadores, e c) Avaliar o papel dos rearranjos cromossômicos nos processos de especiação dentro do gênero *Mazama*. Na espécie *M. gouazoubira* foi possível avaliar uma translocação Robertsoniana (TR) em quatro animais, sendo que dois incluíam a presença de uma inversão paracêntrica (IPA). O valor médio dos espermatozoides desbalanceados nos portadores da TR/IPA (6,68%) quase dobrou em relação àquele dos portadores de TR (3,76%), mas não houve diferença significativa. Na espécie *M. americana*, foram avaliadas TRs em diferentes citótipos e fusões em tandem (FT) heterozigotas em híbridos entre citótipos da mesma linhagem cromossômica. Os portadores de TR apresentaram valor médio para a taxa de segregação adjacente de 1,80% e os portadores da FT apresentaram valor médio de 29,07% para os produtos equivalentes a aqueles da segregação adjacente na TR. Nossos resultados indicam um impacto de baixo a moderado do rearranjo cromossômico na aptidão reprodutiva dos machos de *M. gouazoubira* heterozigotos para TR e TR/IPA e um impacto baixo no caso das TRs em *M. americana*. No caso dos híbridos de *M. americana* portadores de FT em heterozigose, os resultados sugerem a formação de uma barreira pós-zigótica eficiente representada pela redução severa da fertilidade dos indivíduos.

**Palavras chaves:** Cervídeos neotropicais, citogenética, polimorfismo cromossômico, segregação meiótica, sperm-FISH

**GAMETIC SEGREGATION OF CHROMOSOMES INVOLVED IN  
TRANSLOCATIONS AND THEIR ROLE IN REPRODUCTIVE ISOLATION OF  
SPECIES OF THE GENUS *Mazama* (MAMMALIA; CERVIDAE)**

**ABSTRACT** – The family Cervidae stands out for showing one of the highest rates of karyotype evolution among mammals, reflecting a marked chromosomal fragility. Thus, the occurrence of chromosomal breaks and rearrangements is facilitated and lead populations to process of speciation when linked to geographic isolation in a short period. Besides, a wide karyotype diversification of the family has been observed in their extreme examples of low and high diploid numbers, such as *Muntiacus muntjak* ( $2n = 6/7$ ) and *Capreolus pygargus* ( $2n = 70 + 1 - 14 Bs$ ), respectively. Many cryptic species are hidden due to the great morphological similarity, accompanied by wide intra and interspecific karyotypic variation. Thus, animals with chromosomal rearrangements may present different patterns of meiotic segregation during spermiogenesis, giving way to the formation of unbalanced gametes. This can be related to reproductive disorders, as observed in domestic animals with reproductive fitness reduction. The fluorescent *in situ* hybridization (FISH) technique allows the marking, identification, and location of the chromosomes involved in translocations using DNA probes with fluorescent markers. Given the lack of specific probes for deer species, several studies point to the use of bovine probes due to the well-known bovine genetic map and the phylogenetic proximity between families Cervidae and Bovidae. Thus, the application of FISH in sperm cells (also called sperm-FISH) allows estimating the proportion of normal/balanced and unbalanced gametes (carriers of aneuploidies). The present study aimed to a) Estimate the mean rate of meiotic segregation products in carriers of chromosomal translocations within the genus *Mazama*, b) Assess the potential effects of chromosomal translocations on the reproductive fitness of carriers, and c) Assess the role of chromosomal polymorphisms in speciation processes within the genus *Mazama*. In the *M. gouazoubira* species, it was possible to evaluate a Robertsonian translocation (RT) in four animals, two of which included the presence of a paracentric inversion (PAI). The mean value of unbalanced gametes in carriers of the RT/PAI (6.68%) almost doubled in relation to that of carriers with the RT (3.76%); however, no significant difference was observed. Regarding *M. americana*, RTs were evaluated in different cytotypes and heterozygous tandem fusion (TF) was evaluated in hybrids from the same chromosomal lineage cytotypes. Carriers of RT showed a mean value for the adjacent segregation rate of 1.80% and carriers of TF showed a mean value of 29.07% for products equivalent to those of the adjacent segregation in RT. Our results indicate low to moderate impacts of chromosomal rearrangement on the reproductive fitness of *M. gouazoubira* heterozygotes for RT and RT/PAI and a low impact in the case of RTs in *M. americana*. In the case of *M. americana* hybrids with heterozygous TF, our results suggest an efficient postzygotic barrier represented by the severe reduction in the individuals' fertility.

**Keywords:** Chromosomal polymorphism, cytogenetics, meiotic segregation, Neotropical deer, sperm-FISH

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## CAPÍTULO 1 – Considerações Gerais

### 1. INTRODUÇÃO E JUSTIFICATIVA

Atualmente a família Cervidae engloba 55 espécies que estão distribuídas amplamente na América, Europa, Ásia, norte da África e Oceania, este último onde diferentes espécies foram introduzidas entre finais do século XVIII e começo do século XIX (JESSER, 2005; IUCN, 2020). Como reflexo de uma acentuada fragilidade cromossômica (DUARTE; GONZÁLEZ; MALDONADO, 2008; VARGAS-MUNAR; SARRIA-PEREA; DUARTE, 2010; TOMAZELLA; ABRIL; DUARTE, 2017), os cervídeos destacam-se por possuir uma das mais elevadas taxas de evolução cariotípica dentre os mamíferos (MUDD et al., 2020; WANG; LAN, 2000). Essa taxa tem sido estimada a partir de observações de algumas espécies do gênero *Muntiacus* (FONTANA; RUBINI, 1990), usando a fórmula de BUSH et al. (1977), onde a taxa de evolução cromossômica no gênero *Muntiacus* era de 1,08 – 2,11 por milhão de anos para o número cromossômico (número diploide,  $2n$ ) e de 0,97 – 1,91 por milhão de anos para o número fundamental (número de braços cromossômicos, NF). Essa estimativa, mesmo que conservadora, foi maior, por exemplo, que a observada no cavalo doméstico (*Equus ferus caballus*; 0,61 por milhão de anos para  $2n$  e 0,79 por milhão de anos para NF), considerado até então a espécie com a taxa de evolução cromossômica mais rápida entre os mamíferos (BUSH et al., 1977; WANG; LAN, 2000). Outro estudo mais recente, estimou ~4,9 milhões de anos de divergência entre o *Muntiacus muntjak* ( $2n = 6/7$ ) e *Muntiacus reevesi* ( $2n = 46$ ), a partir de alinhamentos do genoma nuclear, e com essa informação estimou a taxa de mudança de cariótipo na linhagem de *M. muntjak* em ~5,3 mudanças por milhão de anos (26 fusões após divergência) e na linhagem de *M. reevesi* em ~1,2 mudanças por milhão de anos (6 fusões após divergência) (MUDD et al., 2020).

A fragilidade cromossômica facilita a ocorrência de quebras e rearranjos cromossômicos (GLOVER; STEIN, 1988), como translocações Robertsonianas (fusão centromérica de dois cromossomos acrocêntricos ou telocêntricos), fusões em tandem (fusão telômero-centrômero ou telômero-telômero) e inversões cromossômicas (paracêntrica, não envolvendo o centrômero, ou pericêntrica, envolvendo o

centrômero) (DUARTE; GONZÁLEZ; MALDONADO, 2008; FONTANA; RUBINI, 1990; NEITZEL, 1987; YANG et al., 1997b). Esses rearranjos podem ser fixados e acumulados em uma população como resultado do isolamento geográfico, podendo agir posteriormente como barreira reprodutiva e induzir a formação de novas espécies (ABRIL et al., 2010; YANG et al., 1997a). Além disso, sugere-se que a evolução cariotípica da família Cervidae tenha acontecido por uma redução numérica do cariótipo ancestral, composto por 34 pares de autossomos acrocêntricos, um X acrocêntrico e um pequeno Y metacêntrico ( $2n = 70$ ;  $NF = 70$ ). Isto pode ser corroborado pela presença desse cariótipo em duas espécies distantes filogeneticamente como o *Hidropotes inermis* (Tribu Capreolini) e o *Mazama gouazoubira* (Tribu Rangiferini), inferido a partir de bandas G, C e NOR (FONTANA; RUBINI, 1990; NEITZEL, 1987), e pela demonstração da evolução cariotípica do gênero *Muntiacus* a partir de um ancestral comum com um cariótipo acrocêntrico  $2n = 70$ , em vez de um cariótipo ancestral  $2n = 46$  semelhante ao *M. reevesi*, a espécie com mais alto  $2n$  do gênero (YANG et al., 1997a, 1997b). Além disso, também foi realizada a reconstrução do cariótipo ancestral ( $2n = 70$ ) a partir de um estudo comparativo entre *Capreolus pygargus* ( $2n = 70 + 8 Bs$ ), *Camelus dromedarius* e *Bos taurus*, mediante o uso de sondas dromedárias de pintura cromossômica e comparação com banda G bovina (DEMENTYEVA et al., 2010).

A família Cervidae é marcada por uma ampla diversificação cariotípica, consequência da presença de uma alta taxa de polimorfismos cromossômicos. Dessa forma a família Cervidae apresenta tanto animais com  $2n$  baixo, como o *M. muntjak* ( $2n = 6/7$ ), quanto alto, como o *C. pygargus* ( $2n = 70 + 1 - 14$  cromossomos B – Bs) (BILTUEVA et al., 2020; LIN et al., 1991; NEITZEL, 1987; TRIFONOV et al., 2013). Além disso, tem sido observada a presença constante de cromossomos B, que são cromossomos supranumerários, mitoticamente instáveis, com variação de número entre populações, indivíduos e tecidos de um mesmo indivíduo, e, apesar da presença de genes codificadores duplicados detectados em Bs em algumas espécies, sua função biológica ainda é desconhecida (AQUINO; ABRIL; DUARTE, 2013; CAMACHO; SHARBEL; BEUKEBOOM, 2000; DUARTE et al., 2012; MAKUNIN et al., 2016; TRIFONOV et al., 2013; VALERI; TOMAZELLA; DUARTE, 2018). Isto pode ter relação com o comportamento irregular destes cromossomos durante a meiose e

mitose (PALESTIS et al., 2004; CAMACHO; SCHMID; CABRERO, 2011). Tais características colocam a família Cervidae como alvo de inúmeros impasses taxonômicos (DUARTE; JORGE, 1996) tornando seu estudo muito interessante do ponto de vista citogenético.

Dentre os cervídeos neotropicais, os pertencentes ao gênero *Mazama* caracterizam-se por apresentar uma extensa variação cariotípica, tanto interespecífica quanto intraespecífica (ABRIL et al., 2010; ABRIL; DUARTE, 2008; DUARTE; GONZÁLEZ; MALDONADO, 2008; DUARTE; JORGE, 1996; FIORILLO et al., 2013; VALERI; TOMAZELLA; DUARTE, 2018). Um claro exemplo é a espécie *M. gouazoubira*, única espécie dentro do gênero detentora do cariótipo ancestral da família Cervidae ( $2n = 70$ ,  $NF = 70$ ) (NEITZEL, 1987), na qual tem sido observada a presença de diferentes translocações Robertsonianas em 14 – 26% dos animais em diversos estudos (DUARTE; JORGE, 1996; DUARTE, 1992, 1998; TOMAZELLA, 2016; VALERI; TOMAZELLA; DUARTE, 2018). Translocações Robertsonianas em heterozigose têm sido relatadas em outros mamíferos domésticos e selvagens (BARASC et al., 2018; BONNET-GARNIER et al., 2006, 2008; PARDO-MANUEL DE VILLENA; SAPIENZA, 2001; PINTON et al., 2009; RYBAR et al., 2005; SWITOŃSKI; GUSTAVSSON; PLÖEN, 1987; VOZDOVA et al., 2014), sendo que a presença de mais de uma translocação Robertsoniana em uma população pode ter um impacto negativo na aptidão reprodutiva da população, devido aos erros durante a meiose pela presença de multivalentes complexos alterando a segregação meiótica (BAKER; BICKHAM, 1986; WHITE et al., 1978).

Por outro lado, a espécie *Mazama americana* exibe uma ampla variação cariotípica com marcada coerência geográfica, onde existem citótipos de duas linhagens. A linhagem de número diploide alto (citótipos Paraná, Carajás, Santarém e Jarí) e uma outra de número diploide baixo (citótipos Juína e Rondônia) (ABRIL et al., 2010). Ambas linhagens com presença de portadores de translocações Robertsonianas em heterozigose, Bs e sistema sexual múltiplo XX/XY1Y2, este último devido a uma fusão tandem X-autossômica, onde o Y2 é o autossomo homólogo ao fundido com o X (ABRIL et al., 2010; AQUINO; ABRIL; DUARTE, 2013). Existem relatos de produção de híbridos entre os citótipos, os quais quando derivados de parentais de diferentes linhagens cromossômicas são inférteis por interrupção da

espermatogênese, mas quando derivados de parentais da mesma linhagem cromossômica não tem gerado efeitos significativos na fertilidade da prole, sendo considerados sub-férteis (SALVIANO et al., 2017). As avaliações dos híbridos entre citótipos de *M. americana* foram realizadas desde o ponto de vista de morfologia e funcionalidade gonadal, mas não foi avaliado o efeito dos diferentes tipos de rearranjos cromossômicos no balanceamento cromossômico dos gametas (SALVIANO et al., 2017).

Uma das metodologias utilizadas para estimar a proporção de gametas desbalanceados é a técnica conhecida como hibridização fluorescente *in situ*, que quando usada nos espermatozoides é denominada de “sperm-FISH” (CASSUTO et al., 2011; MANIEU et al., 2014; MASSIP et al., 2010; PINTON; DUCOS; YERLE, 2004; RUBES; VOZDOVÁ; KUBÍCKOVÁ, 1999). As sondas, quando construídas de cromossomos ou regiões cromossômicas envolvidas em translocações, podem detectar o número de cromossomos marcados em cada espermatozoide, permitindo saber se há um perfeito balanceamento gamético ou não (PINTON; DUCOS; YERLE, 2004). Esta estimativa da proporção de gametas desbalanceados pode ser usada como um preditor do potencial efeito dos rearranjos cromossômicos na aptidão reprodutiva do portador, além de estimar a proporção de aneuploidias nos portadores (PINTON; DUCOS; YERLE, 2004).

## **2. REVISÃO DA LITERATURA**

### **2.1. Rearranjos cromossômicos na especiação dos mamíferos**

Alterações estruturais cromossômicas devido a quebras e rearranjos estruturais, ou como resultado de recombinações genéticas acidentais entre cromossomos homólogos ou não homólogos, podem permitir a formação de inversões, fusões em tandem e translocações Robertsonianas (Figura 1), entre outras (FERGUSON-SMITH; TRIFONOV, 2007; SHAKOORI; AFTAB; AL-GHANIM, 2017). A presença desses rearranjos cromossômicos tem sido descrita na evolução cariotípica dos mamíferos, embora o número de fixações observadas (principalmente as translocações Robertsonianas) sejam surpreendentemente pequeno (FERGUSON-

SMITH; TRIFONOV, 2007). Contudo, entre os mamíferos, muitas espécies apresentam diferenças fixadas no cariótipo, sugerindo que diferentes rearranjos cromossômicos aparecem, se espalham por linhagens e são fixados em táxons terminais, desempenhando um papel significativo no processo de especiação (DOBIGNY; BRITTON-DAVIDIAN; ROBINSON, 2017; FARIA; NAVARRO, 2010).

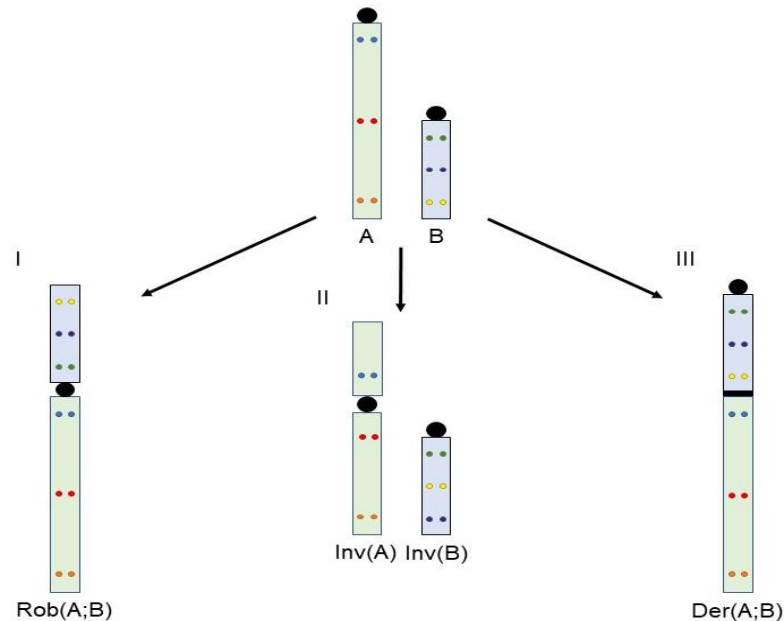


Figura 1. Rearranjos cromossômicos comuns dentro da evolução cariotípica da Família Cervidae, representados em dois cromossomos acrocêntricos não homólogos (A e B). I) Translocação Robertsoniana dos cromossomos A e B; II) Inversão Pericêntrica do cromossomo A e Inversão Paracêntrica do cromossomo B; III) Fusão em tandem entre a região centromérica do Cromossomo A e a região telomérica do cromossomo B. Os pontos aproximam-se das regiões centromérica, medial e terminal dos cromossomos.

A presença de portadores de formas heterozigotas desses polimorfismos cromossômicos poderia levar à formação de barreiras eficientes ao fluxo gênico e subsequente processo de especiação (FARIA; NAVARRO, 2010). Ainda que a presença do rearranjo cromossômico em heterozigose possa não causar alterações morfológicas no portador, ela poderá significar um risco na sua gametogênese, uma vez que os rearranjos cromossômicos heterozigotos podem levar à formação de gametas com aneuploidias e, como consequência, à morte gamética (DOBIGNY;



BRITTON-DAVIDIAN; ROBINSON, 2017; KING, 1993; SHAKOORI; AFTAB; AL-GHANIM, 2017). Além disso, pode levar à formação de gametas desequilibrados que provavelmente levarão a anormalidades no desenvolvimento dos embriões e à subfertilidade ou esterilidade do portador (DOBIGNY; BRITTON-DAVIDIAN; ROBINSON, 2017; MARY et al., 2016). O impacto na aptidão reprodutiva do portador vai depender do tipo de rearranjo cromossômico apresentado, onde tem que ser considerado um fator individual, como observado principalmente em estudos de translocações Robertsonianas em humanos (WILAND et al., 2020).

Esses polimorfismos cromossômicos, quando fixados nas populações, podem significar eficientes mecanismos de barreira de fluxo gênico, dependendo do impacto da forma heterozigota na aptidão reprodutiva dos híbridos. Podem ainda significar uma adaptação a variações ambientais ou de nicho ecológico, facilitando a proteção de combinações alélicas, favoráveis para a população, dos efeitos da recombinação em indivíduos heterozigotos (DOBIGNY; BRITTON-DAVIDIAN; ROBINSON, 2017; FARIA; NAVARRO, 2010). Contudo, a fixação de um rearranjo cromossômico em uma população irá depender de fatores tais como condições demográficas particulares, como a perda de hábitat, que possam levar a um decréscimo populacional e permitam um maior efeito da deriva genética, a taxa de fragilidade cromossômica do táxon, o impulso meiótico, a recombinação e expressão gênica (DOBIGNY; BRITTON-DAVIDIAN; ROBINSON, 2017).

## 2.2. Família Cervidae e o gênero *Mazama*

A família Cervidae, entre os mamíferos, é um dos táxons com maior grau de evolução cariotípica, representado por uma ampla diversidade cromossômica (FONTANA; RUBINI, 1990; NEITZEL, 1987). Diversos estudos de citogenética clássica e molecular tem demonstrado que a grande divergência cariotípica parece ser irrelevante em termos de fenótipo, como observado nos gêneros *Muntiacus* (YANG et al., 1995) ou *Mazama* (ABRIL et al., 2010; DUARTE; GONZÁLEZ; MALDONADO, 2008; GONZÁLEZ; DUARTE, 2020). Por outro lado, acredita-se que a evolução cariotípica da família, tenha ocorrido pela redução do cariótipo ancestral  $2n = 70$ ;  $NF = 70$ , retido por espécies de diferentes gêneros filogeneticamente distantes, como *M.*

*gouazoubira* e *H. inermis* (FONTANA; RUBINI, 1990; NEITZEL, 1987). Isto tem sido corroborado em diversos estudos de comparação cariotípica entre diferentes gêneros da família (FROHLICH et al., 2017; HUANG et al., 2006a; KULEMZINA et al., 2009; YANG et al., 1997a, 1997b), onde são observadas as homologias cromossômicas conservadas dentro de seu processo evolutivo, assim como homologias com outras espécies dentro da ordem Cetartiodactyla (Figura 2) (FROHLICH et al., 2017). Também tem sido descrita a presença de diversas variantes na apresentação do cromossomo sexual X, tendo uma orientação acrocêntrica, submetacêntrica ou metacêntrica, sendo parte de um sistema sexual simples XX / XY ou um sistema sexual múltiplo XX / XY1Y2 (ABRIL et al., 2010; AQUINO; ABRIL; DUARTE, 2013; FIORILLO et al., 2013; FONTANA; RUBINI, 1990; FROHLICH et al., 2017; NEITZEL, 1987; PROSKURYAKOVA et al., 2017), assim como a presença de Bs (ABRIL; DUARTE, 2008; AQUINO; ABRIL; DUARTE, 2013; FIORILLO et al., 2013; MAKUNIN et al., 2016; TOMAZELLA; ABRIL; DUARTE, 2017; TRIFONOV et al., 2013; VALERI; TOMAZELLA; DUARTE, 2018).

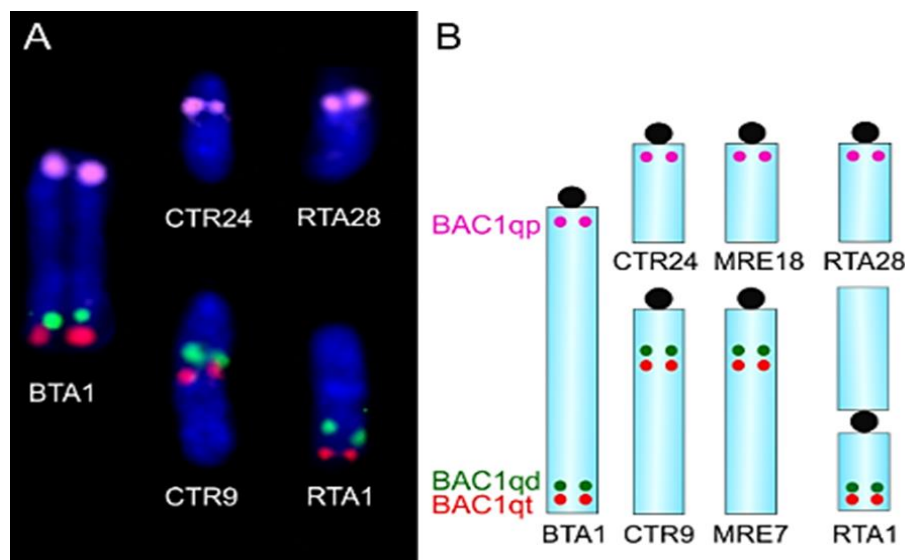


Figura 2. FISH mostrando hibridização de sondas BAC bovinas do cromossomo 1 e sua ilustração esquemática em espécies de cervídeos. (A) Hibridação de sondas bovinas BAC1qp (rosa), BAC1qd (verde) e BAC1qt (vermelho) em cromossomos de *Bos taurus* (BTA), *Cervus timorensis russa* (CTR) e *Rangifer tarandus* (RTA). (B) Ilustrações esquemáticas apresentando os rearranjos dos ortólogos de BTA1 em *Cervus timorensis russa* (CTR), *Muntiacus reevesi* (MRE) e *Rangifer tarandus* (RTA). Os pontos se aproximam das posições das sondas BAC1qp (rosa), BAC1qd (verde) e BAC1qt (vermelho). Fonte: FROHLICH et al. (2017), com modificações.

Um dos gêneros que engloba a maioria dos polimorfismos cromossômicos tratados previamente é o gênero *Mazama*, o qual atualmente está constituído por 10 espécies e é caracterizado não só por uma ampla diversidade cariotípica interespecífica, mas também intraespecífica (ABRIL et al., 2010; ABRIL; DUARTE, 2008; DUARTE; GONZÁLEZ; MALDONADO, 2008; DUARTE, 1992, 1998; FIORILLO et al., 2013; GONZÁLEZ; DUARTE, 2020). Esse alto polimorfismo cromossômico é atribuído a uma maior fragilidade cromossômica no gênero, que tem sido corroborada pela maior taxa de mutação induzida pela doxorubicina (TOMAZELLA; ABRIL; DUARTE, 2017; VARGAS-MUNAR; SARRIA-PEREA; DUARTE, 2010). Essa tendência à quebra poderia estar relacionada com os rearranjos cromossômicos, que quando fixados em uma população podem levar a processos de especiação (DUARTE; JORGE, 1996; TOMAZELLA; ABRIL; DUARTE, 2017; VALERI; TOMAZELLA; DUARTE, 2018). O gênero *Mazama* é composto por espécies de pequeno a médio porte e de ampla distribuição na região Neotropical (DUARTE; GONZÁLEZ, 2010). Atualmente, o gênero *Mazama* é dividido em dois clados, o clado cinza (*M. gouazoubira*, *Mazama nemorivaga*, *Mazama chunyi* e *Mazama pandora*) e o clado vermelho (*M. americana*, *Mazama bororo*, *Mazama nana*, *Mazama temama*, *Mazama rufina* e *Mazama bricenii*), devido a uma classificação morfológica (cor da pelagem), citogenética e molecular (ALLEN, 1915; DUARTE; GONZÁLEZ; MALDONADO, 2008). Além disso, diversos estudos de filogenia molecular postulam que o gênero *Mazama* é um grupo polifilético, dividindo-se em várias linhagens, motivo pelo qual algumas espécies pertencentes ao clado cinza como *M. gouazoubira* e *M. nemorivaga* futuramente deveriam deixar de ser considerados como membros do gênero *Mazama* e passar a formar os próprios gêneros (DUARTE; GONZÁLEZ; MALDONADO, 2008; HECKEBERG, 2020; HECKEBERG et al., 2016).

Por outro lado, a evolução cariotípica dentro do gênero *Mazama* está relacionada com a presença de translocações Robertsonianas, fusões em tandem e inversões cromossômicas como observado nas espécies mais derivadas, sendo que *M. gouazoubira* é o único retentor do cariótipo ancestral da família Cervidae (FONTANA; RUBINI, 1990; NEITZEL, 1987). No Brasil, atualmente, ocorrem 5 espécies, duas do clado cinza *M. gouazoubira* ( $2n = 70 + Bs$ ;  $NF = 70$ ) e *M.*

*nemorivaga* ( $2n = 67 - 70 + Bs$ ;  $NF = 70 - 72$ ) e três do clado vermelho *M. americana* ( $2n = 42 - 53 + Bs$ ;  $NF = 46 - 56$ ), *M. bororo* ( $2n = 32 - 34$ ;  $NF = 46$ ) e *M. nana* ( $2n = 36 - 39 + Bs$ ;  $NF = 58$ ) (DUARTE; GONZÁLEZ, 2010). Assim, a existência de formas heterozigotas de alguns rearranjos cromossômicos na natureza aumenta a diversidade cariotípica intraespecífica, que somado a outros fatores, como a perda de habitat, isolamento geográfico, decréscimo populacional e maiores taxas de endogamia poderia impulsionar processos de especiação. Para exemplificar melhor esses processos, serão apresentados os dados das duas espécies mais estudadas do gênero em termos de polimorfismo cromossômico: *M. gouazoubira* e *M. americana*.

### **2.3. *Mazama gouazoubira***

*Mazama gouazoubira* (Figura 3), conhecido também como veado-catingueiro, é um cervídeo de pequeno a médio porte, de coloração lateral e dorsal do pescoço marrom, com o pelame dorsal e lateral do corpo geralmente marrom acinzentado, às vezes salpicado ou lavado com laranja (VIEIRA ROSSI; VIVO, 2000), com algumas partes do corpo brancas, como o interior das orelhas, as quais são arredondadas e uma mancha superciliar esbranquiçada, que pode ser vista na maioria dos indivíduos (DUARTE; MERINO, 1997). Como na maioria de cervídeos, os machos apresentam chifres não ramificados (padrão do gênero) e estão inclinados póstero-dorsalmente (BLACK-DECIMA et al., 2010; PEREIRA, 2010). A espécie possui ampla distribuição entre Brasil, Bolívia, Paraguai, Argentina e Uruguai (FIGURA 4), apresentando a maior plasticidade ecológica entre as espécies do gênero devido a sua presença em todos os biomas brasileiros, exceto Amazônia, demonstrando sua adaptação a ambientes modificados (BLACK-DECIMA et al., 2010; DUARTE et al., 2012; RODRIGUES; CERVEIRA; DUARTE, 2014). A espécie é classificada como “Pouco Preocupante” pela Lista Vermelha de Espécies Ameaçadas da União Internacional para a Conservação da Natureza (UICN) (BLACK-DECIMA; VOGLIOTTI, 2016).



Figura 3: Macho adulto de *Mazama gouazoubira*. Fonte: Acervo NUPECCE.

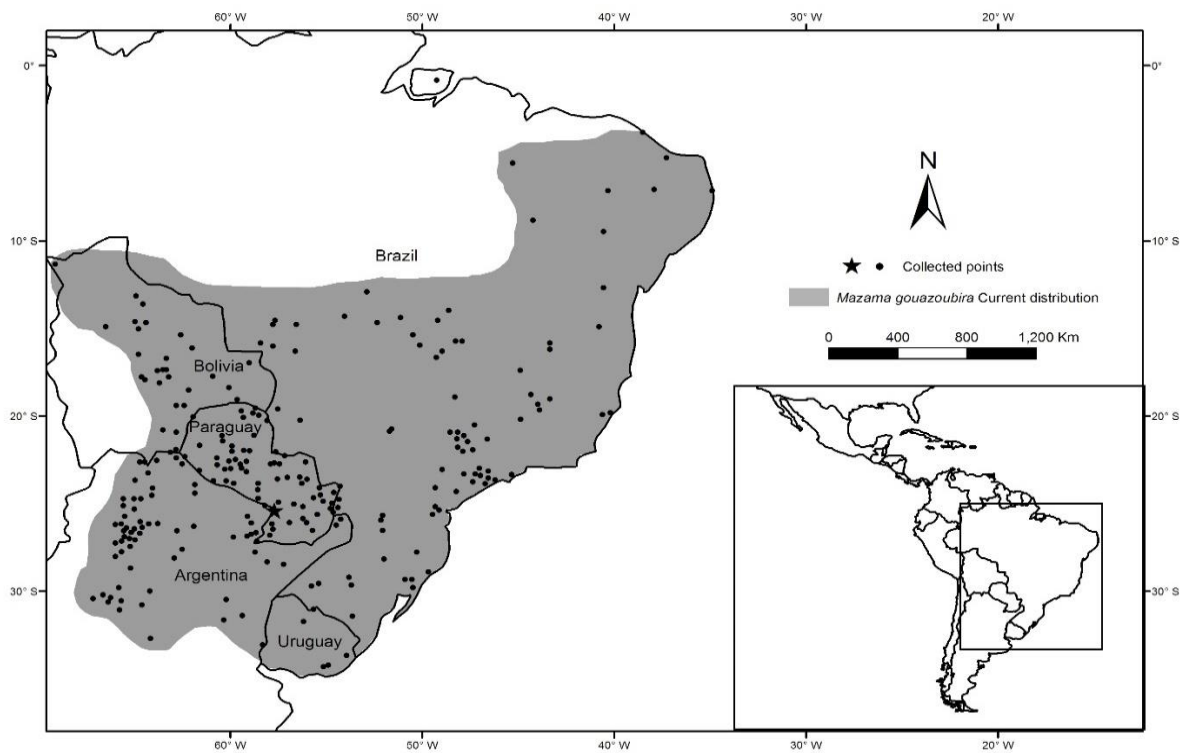


Figura 4. Área de ocorrência de *Mazama gouazoubira* na América do Sul. Fonte: BLACK-DÉCIMA et al. (2010).

Essa ampla distribuição, somada à bem relatada presença de polimorfismos cromossômicos e cromossomos B nas diferentes populações de *M. gouazoubira*

levanta a questão sobre a possível existência de processos de especiação em andamento. Diversos estudos tem relatado formas heterozigotas e homozigotas de translocações Robertsonianas com uma incidência entre 14 e 26% dos indivíduos estudados (DUARTE; JORGE, 1996; DUARTE, 1992, 1998; TOMAZELLA, 2016; VALERI; TOMAZELLA; DUARTE, 2018).

A classificação das diversas translocações Robertsonianas tem sido prejudicada pela dificuldade na obtenção de bandeamento G de alta qualidade na espécie, além da difícil classificação dos cromossomos que são todos acrocêntricos e de tamanhos semelhantes (34 pares), sendo o Y metacêntrico (NEITZEL, 1987; TOMAZELLA, 2016). Contudo, em uma população do Pantanal da Nhecolândia (no município de Corumbá, Mato Grosso do Sul, Brasil (19 ° 00 ' 33 ' S, 57 ° 39 ' 12 ' W), foi descrita a presença de pelo menos 3 diferentes translocações Robertsonianas em heterozigose (Figura 5) (VALERI; TOMAZELLA; DUARTE, 2018), embora seja necessária uma confirmação mediante técnicas de citogenética molecular para a identificação exata dos cromossomos envolvidos.

A presença de uma translocação Robertsoniana é compatível com a vida do portador, uma vez que não existe perda ou excesso de material genético na apresentação desses rearranjos cromossômicos (BARASC et al., 2018). O problema é apresentado quando o portador entra na etapa reprodutiva, quando pode apresentar alterações no pareamento cromossômico durante a gametogênese devido à formação de trivalentes entre os cromossomos envolvidos na translocação e seus pares homólogos durante a prófase da Meiose I (ROUX et al., 2005; SWITONSKI; GUSTAVSSON; PLÖEN, 1987). Os portadores heterozigotos podem produzir gametas geneticamente desbalanceados, apresentando nulissomia ou dissomia para os cromossomos envolvidos na translocação como produto das segregações adjacentes e de 3:0 no final da meiose I (Figura 6), os quais são responsáveis pela mortalidade embrionária inicial quando fecundarem o oócito (ROUX et al., 2005). Contudo, o impacto na aptidão reprodutiva do portador vai depender de cada tipo de translocação, sendo que em humano tem sido relatada desde leve até severa alteração da aptidão reprodutiva dos portadores com variações individuais entre portadores de uma mesma translocação Robertsoniana (WILAND et al., 2020). Já em

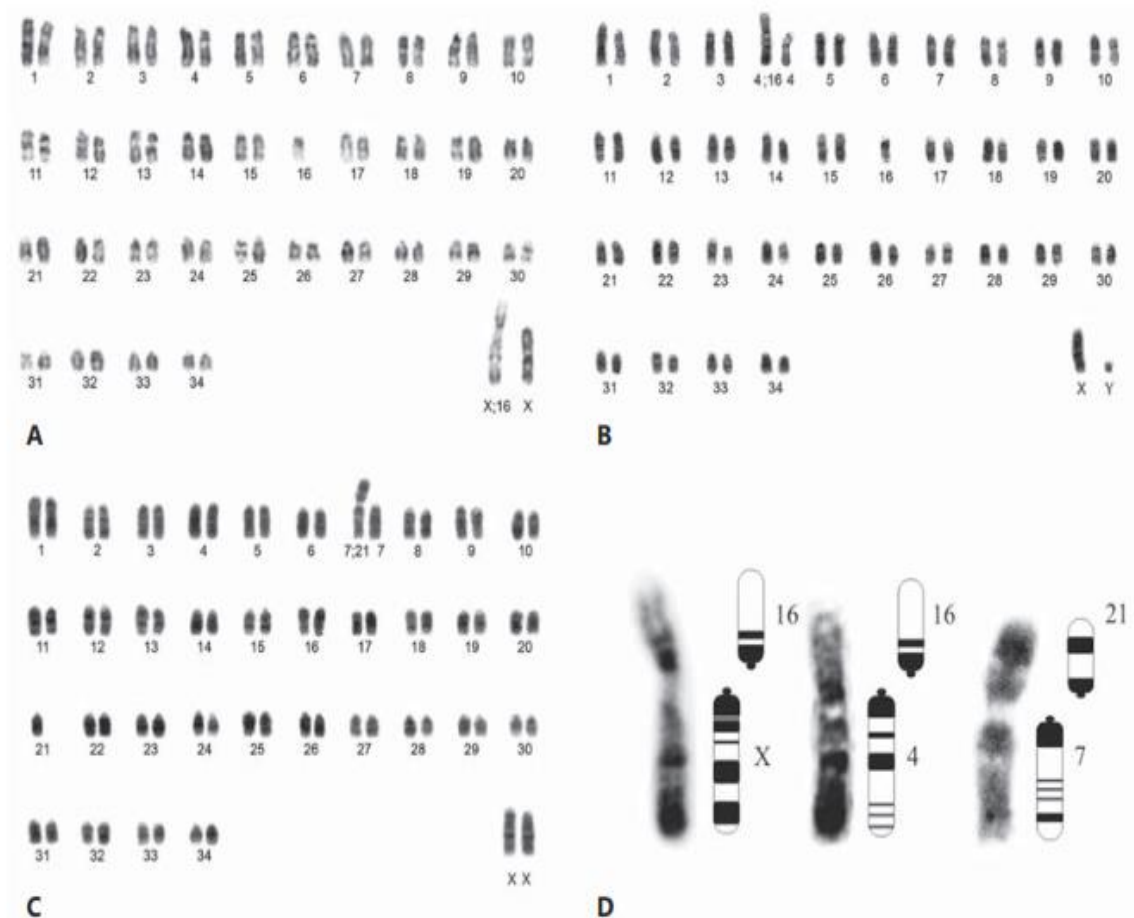


Figura 5. Cariótipos em banda G de *Mazama gouazoubira*. A) Cariótipo com presença de translocação Robertsoniana rob(X;16). B) Cariótipo com presença de translocação Robertsoniana rob(4;16). C) Cariótipo com presença de translocação Robertsoniana rob(7;21). D) Cromossomos com banda G e ilustração esquemática das respectivas translocações. Fonte: VALERI et al. (2018).

animais domésticos, têm sido observados impactos leves a moderados na aptidão reprodutiva dos portadores (BARASC et al., 2018; BONNET-GARNIER et al., 2006; DUCOS et al., 2007; PINTON et al., 2009). Um maior problema, no caso de *M. gouazoubira*, será entender as consequências de uma provável fixação de diferentes rearranjos cromossômicos nas populações. Os híbridos gerados entre estas populações terão uma maior incidência em erros durante o pareamento meiótico, levando à sub-esterilidade ou infertilidade do híbrido heterozigoto para as configurações cromossômicas diferentes entre as populações, favorecendo assim um provável processo de especiação (RIBAGORDA et al., 2019).

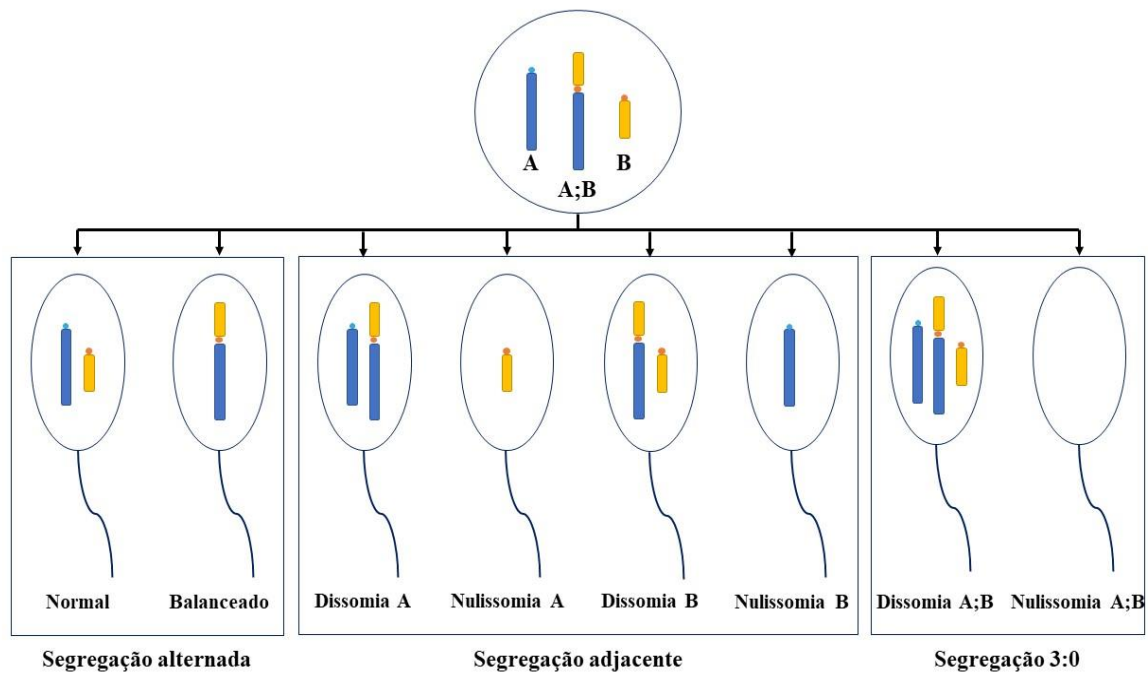


Figura 6. Cromossomos envolvidos na translocação Robertsoniana. Resultados dos gametas produzidos nos diferentes modos de segregação meiótica nesse rearranjo cromossômico.

#### 2.4. *Mazama americana*

*Mazama americana* (Figura 7), também conhecido como veado-mateiro, é a maior espécie do gênero, chegando a pesar entre 30 – 40 kg (DUARTE; JORGE, 1996). Apresenta predominante cor marrom avermelhada na maioria das partes do corpo, enquanto o pescoço e o rosto são geralmente cinza. A parte interna dos membros posteriores e cauda, região submandibular, ponta da maxila superior e borda interna das orelhas são brancas (DUARTE; JORGE, 1996; VARELA et al., 2010). Os chifres dos machos têm pontas curtas e retas, voltadas para trás, sem ramificações (padrão do gênero). A espécie tem uma área de distribuição ampla na América do Sul, cobrindo território de quase todos os países do continente, com exceção do Chile e o Uruguai (Figura 8). Contudo, atualmente é classificada como “Dados Deficientes” pela Lista Vermelha de Espécies Ameaçadas da UICN (DUARTE; VOGLIOTTI, 2016), devido a problemática de classificação taxonômica decorrente da ampla diversidade cariotípica intraespecífica com coerência geográfica ao longo da sua distribuição (ABRIL et al., 2010; DUARTE; GONZÁLEZ; MALDONADO, 2008).





Figura 7. Macho adulto de *Mazama americana*. Fonte: Acervo NUPECCE.

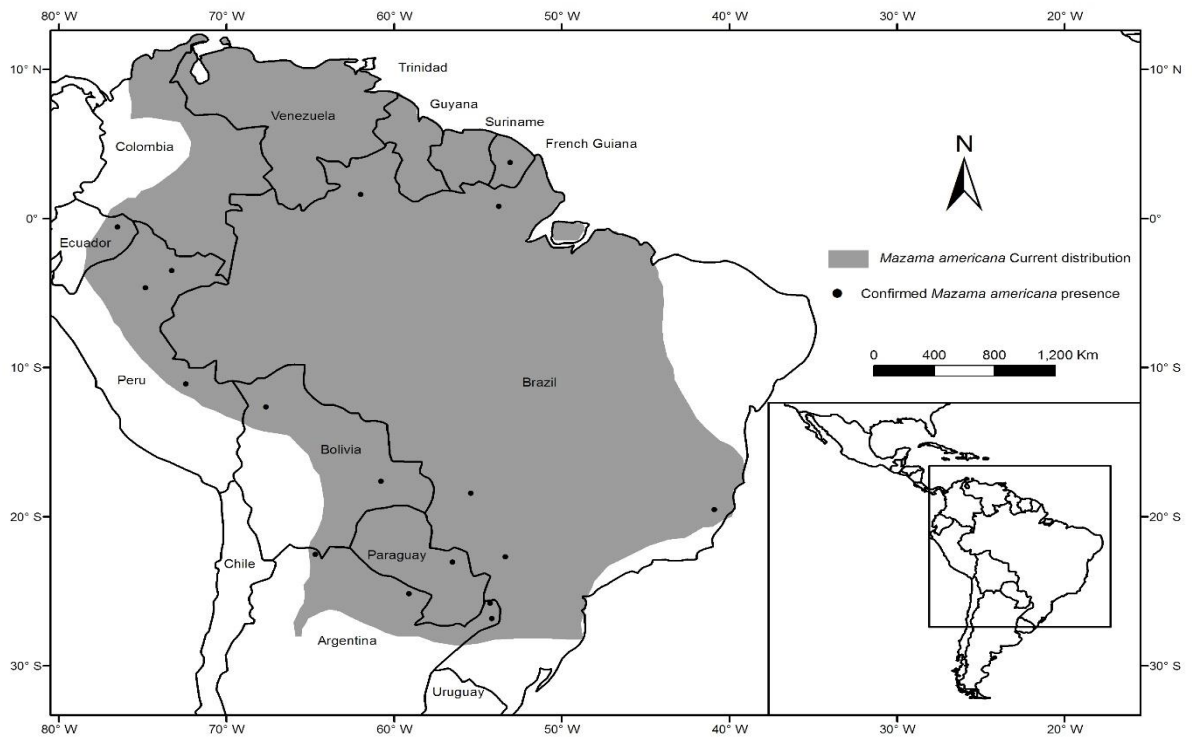


Figura 8. Área de ocorrência de *Mazama americana* na América do Sul. Fonte: VARELA et al. (2010).

Nesse sentido, atualmente dentro do “complexo” *M. americana* são reconhecidos 6 citótipos divididos em duas linhagens cromossômicas evolutivas, a linhagem de número diploide baixo (Citótipo Rondônia:  $2n = 42/43 + Bs$ , NF = 46; Citótipo Juína:  $2n = 44/45 + Bs$ , NF = 48) e a linhagem de número diploide alto (Citótipo Carajás:  $2n = 48/49 + Bs$ , NF = 56; Citótipo Santarém:  $2n = 50/51 + Bs$ , NF = 56; Citótipo Carajás:  $2n = 50/51 + Bs$ , NF = 54; Citótipo Paraná:  $2n = 52/53 + Bs$ , NF = 56) (ABRIL et al., 2010) (Figura 9). Estudos que avaliaram a produção de híbridos entre citótipos de *M. americana* têm comprovado que grandes diferenças cromossômicas podem gerar animais inférteis por interrupção da espermatogênese (SALVIANO et al., 2017) ou oogênese (CURSINO et al., 2014). Essa redução da aptidão reprodutiva foi atribuída aos diferentes tipos de pareamento cromossômico durante a meiose I, devido principalmente ao acúmulo de rearranjos cromossômicos entre as linhagens, tais como translocações Robertsonianas, fusões em tandem e inversões (ABRIL et al., 2010; CURSINO et al., 2014; SALVIANO et al., 2017). Entretanto, diferenças menores no cariótipo dos parentais não geram efeitos significativos na fertilidade da prole, desde o ponto de vista de morfologia e funcionalidade gonadal, sendo considerados sub-férteis. Contudo, nesse último caso, os mecanismos de segregação meiótica podem levar a um desbalanço da ploidia espermiática de alguns cromossomos envolvidos nos rearranjos que levaram a diferenciação dos parentais. Assim, não seriam identificados problemas na meiose e espermatogênese, mas após fertilização estes gametas certamente inviabilizariam o zigoto formado, portando trissomias ou monossomias.

Na literatura, já tem sido descritas diferenças entre os citótipos do complexo *M. americana*, assim como a presença de Bs e sistema múltiplo sexual XY1Y2 (ABRIL et al., 2010; AQUINO; ABRIL; DUARTE, 2013; CURSINO et al., 2014; SALVIANO et al., 2017). Dentre os citótipos da linhagem de número diploide baixo (citótipos Rondônia e Juína) existe a diferença de uma fusão em tandem. Já no caso dos citótipos da linhagem de número diploide alto existe a diferença de uma fusão em tandem do citótipo Paraná para o citótipo Carajás e a diferença de uma translocação Robertsoniana do citótipo Paraná para o citótipo Santarém, e desse último para o citótipo Jarí (ABRIL et al., 2010) (Figura 10).

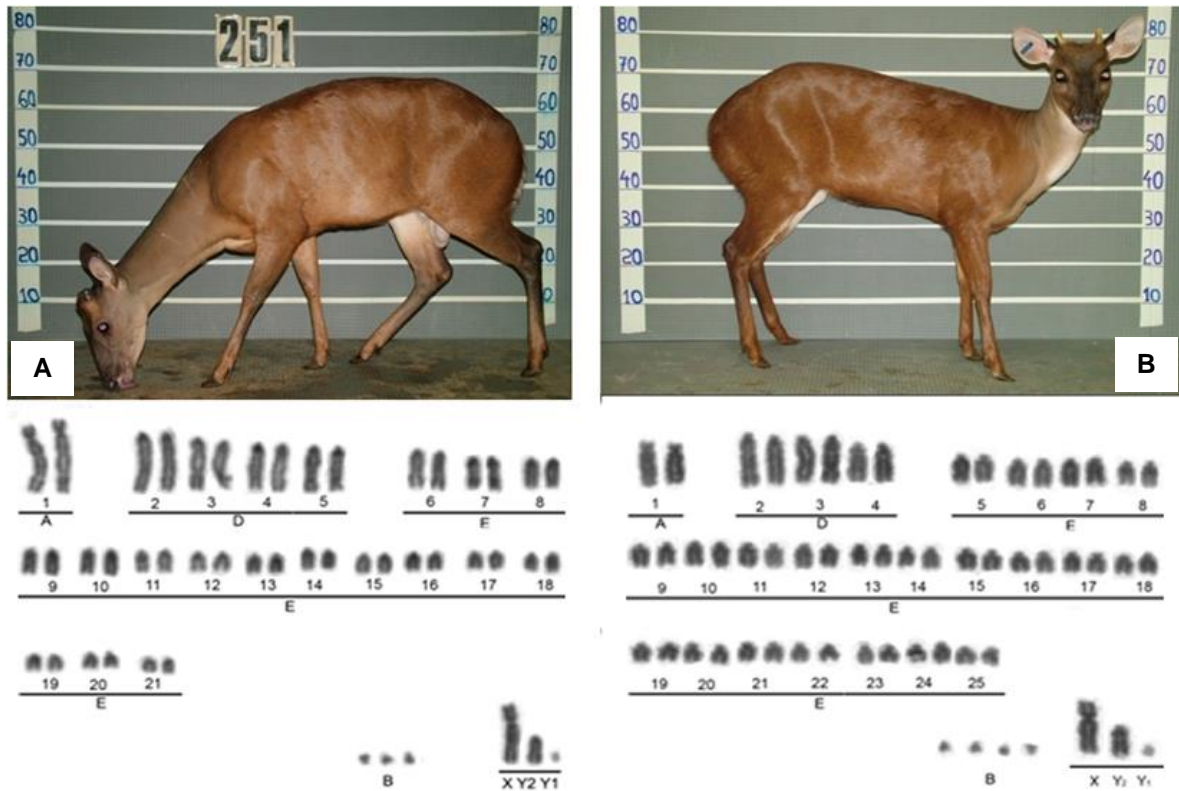


Figura 9. Machos adultos de *Mazama americana*. A) Citótipo Juína,  $2n = 45 + 3Bs$ ,  $NF = 48$ ; B) Citótipo Paraná,  $2n = 53 + 4Bs$ ,  $NF = 56$ . Fonte: Acervo NUPECCE.

Por outro lado, também tem sido relatada a presença de variantes heterozigotas para translocações Robertsonianas nos diferentes citótipos de *M. americana* (ABRIL, 2009). Contudo, só existe um relato sobre a avaliação da aptidão reprodutiva de um portador de translocação Robertsoniana do citótipo Rondônia (AQUINO; ABRIL; DUARTE, 2013). Nesse estudo foi realizada a avaliação do pareamento meiótico mediante a análise do complexo sinaptonémico e foi sugerida a estabilidade da segregação gamética na presença desse rearranjo. Assim, cada um dos citótipos locais poderiam ser considerados como espécies distintas se houvesse um mecanismo de isolamento reprodutivo do tipo infertilidade do híbrido. A partir daí, surge uma questão importante, que seja, qual nível de diferença cromossômica entre os parentais gera infertilidade ou sub-fertilidade nos seus descendentes? Qual o efeito dos diferentes tipos de rearranjos cromossômicos no balanceamento cromossômico dos gametas e conseqüentemente na sua fertilidade? De posse deste conhecimento, será possível definir os limites taxonômicos que são gerados pelas diferenças cromossômicas entre populações e conseqüentemente avançar na reorganização

taxonômica do gênero *Mazama* e de outros grupos que tem alta variação cromossômica.

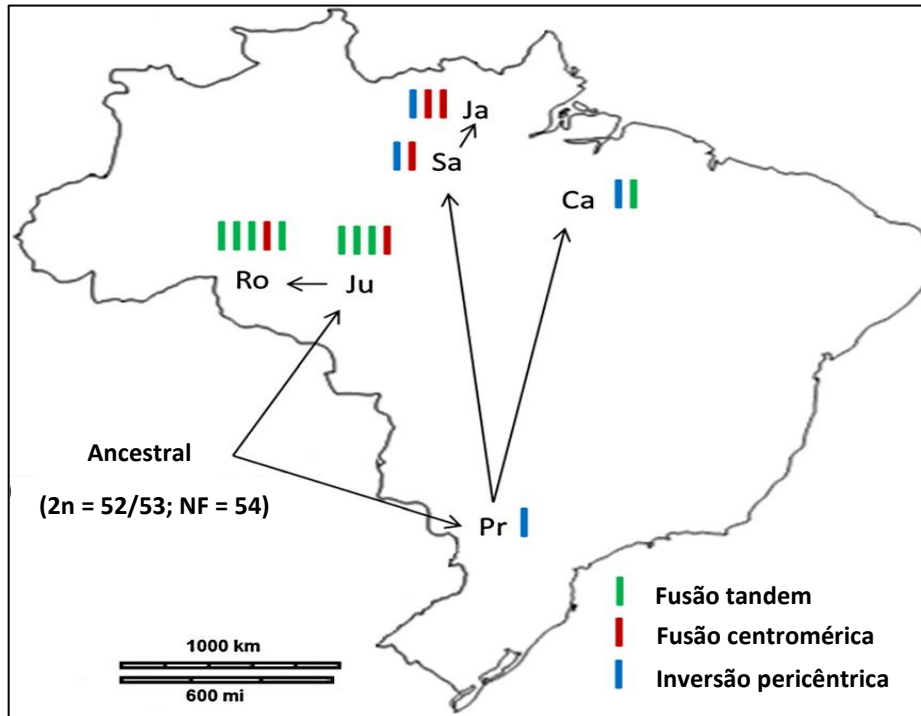


Figura 10. Relações dos 6 citótipos de *Mazama americana* analisados e sua distribuição geográfica, modificados de Abril et al. (2010). O cariótipo ancestral originou a linhagem de número diploide baixo (noroeste do Brasil; Rondônia – RO e Juína – JU) e a linhagem de número diploide alto (sul e norte do Brasil; Paraná – PA, Carajás – CA, Santarém – SA e Jarí – JA). A diferenciação cromossômica de cada citótipo do ancestral comum hipotético ( $2n = 52-53$ ;  $NF = 54$ ) foi alcançada pela fixação de diferentes rearranjos. As barras de cores indicam os rearranjos cromossômicos acumulados pelo citótipo. Fonte: CURSINO et al. (2014), com modificações.

## 2.5. Citogenética molecular e Sperm-FISH

As metodologias clássicas para a detecção e descrição de rearranjos cromossômicos têm sido os bandamentos cromossômicos (Giemsa, C, Ag-NOR), que possibilitaram a construção do cariótipo ancestral dos cervídeos e diversas análises da evolução cariotípica da família Cervidae (FONTANA; RUBINI, 1990; JORGE et al., 1977; NEITZEL, 1987; TAYLOR; HUNGERFORD; SNYDER, 1969). Contudo, nas últimas décadas, a citogenética clássica tem sido complementada com técnicas

moleculares mediante a confecção de sondas de pintura cromossômica por citometria de fluxo ou microdissecção e sondas de cromossomos artificiais bacterianos – BACs (FRÖHLICH et al., 2017; KUBICKOVA et al., 2002). Estas metodologias (hibridização fluorescente *in situ* – FISH) permitiram a caracterização de rearranjos cromossômicos complexos intra e interespecíficos na família Cervidae (CHI et al., 2005a, 2005b; DEMENTYEVA et al., 2010; FROHLICH et al., 2017; HUANG et al., 2006b; PROSKURYAKOVA et al., 2017; VOZDOVA et al., 2021; YANG et al., 1995, 1997b). Assim, a FISH é uma técnica onde o DNA-alvo é fixado em uma lâmina e o DNA da sonda é marcado com um fluorocromo, ambos os DNAs são desnaturados em DNAs de fita simples e unificados em uma mistura de hibridização para a reação (LIEHR; WEISE, 2017). Dessa forma, pela produção de sondas a partir de material da espécie de interesse ou de material de espécies domésticas filogeneticamente próximas, tem sido possível a realização de estudos de evolução cariotípica entre os diferentes gêneros de cervídeos (DEMENTYEVA et al., 2010; FROHLICH et al., 2017; PROSKURYAKOVA et al., 2017; YANG et al., 1995, 1997b).

A técnica de FISH, quando aplicada na análise de processos meióticos, permite a rápida identificação dos cromossomos durante a meiose, permitindo a caracterização de anomalias meióticas (OLIVER-BONNET, 2017). A FISH quando usada no núcleo espermático é denominada de sperm-FISH e tem sido usada para avaliar a aneuploidia dos gametas e para determinar o padrão de segregação de rearranjos cromossômicos no humano e espécies domésticas (BARASC et al., 2018; BONNET-GARNIER et al., 2006; DI DIO et al., 2020; MANIEU et al., 2014; MARTIN et al., 2000; PINTON; DUCOS; YERLE, 2003; RUBES; VOZDOVÁ; KUBÍCKOVÁ, 1999; WILAND et al., 2020). Assim, as sondas, quando construídas de cromossomos ou regiões cromossômicas envolvidas em translocações, podem detectar o número de cromossomos marcados em cada espermatozoide, permitindo saber se há um adequado balanceamento gamético ou não (Figura 11) (BONNET-GARNIER et al., 2006; PINTON; DUCOS; YERLE, 2004; ROUX et al., 2005). Esta estimativa da proporção de gametas desbalanceados pode ser usada como um preditor do potencial efeito dos rearranjos cromossômicos na aptidão reprodutiva do portador (PINTON; DUCOS; YERLE, 2004).

No caso do gênero *Mazama*, há uma falta de sondas de pintura cromossômica específicas para as diversas espécies do gênero. Diversas tentativas têm sido realizadas no Núcleo de Pesquisa e Conservação de Cervídeos (NUPECCE) para a produção de sondas cromossômicas para FISH a partir de material de *M. gouazoubira*, considerado o único retentor do cariótipo ancestral dentro do gênero, com o intuito de poder utilizá-las para estudos de evolução cariotípica dentro do gênero. Porém, problemas têm ocorrido independente da técnica escolhida, citometria de fluxo ou microdissecção (ABRIL, 2009; TOMAZELLA, 2016). Nesse sentido, tem sido avaliada a possibilidade de uso de sondas de pintura cromossômica e BAC de bovino (*B. taurus*), as quais tem se mostrado adequadas e eficientes na marcação de cromossomos de diversas espécies de cervídeos, fazendo uso das sequencias ortólogas conservadas entre a espécie bovina e as espécies dentro da família Cervidae (FROHLICH et al., 2017; PROSKURYAKOVA et al., 2017).

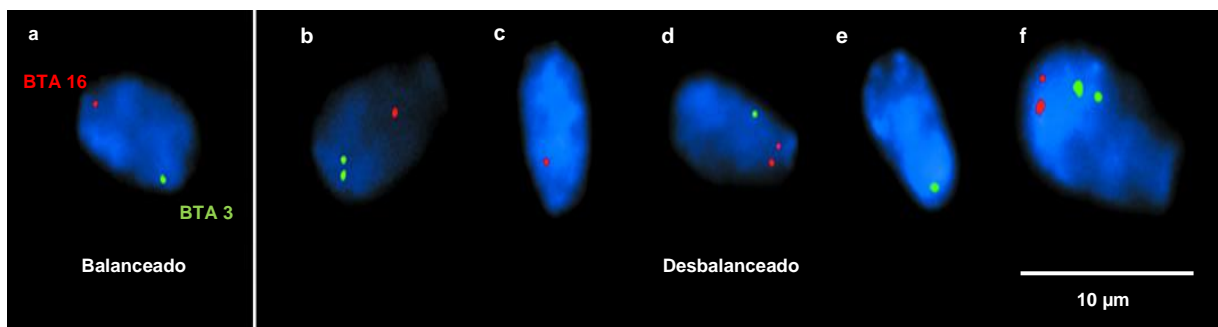


Figura 11. Hibridização das sondas BAC do cromossomo 3 (verde) e 16 (vermelho) em núcleos de espermatozoides descondensados de touro (*Bos taurus* – BTA) portador heterozigoto rob(3;16). a) Núcleo espermático normal: 3/16 (segregação alternada). b) Núcleo espermático dissômico para cromossomo 3. c) Núcleo espermático nulissômico para o cromossomo 3. d) Núcleo espermático dissômico para o cromossomo 16. e) Núcleo espermático nulissômico para o cromossomo 16. f) Núcleo espermático dissômico para ambos os cromossomos 3 e 16. Fonte: BARASC et al. (2018), com modificações.

### 3. OBJETIVOS

#### 3.1. Objetivo Geral

- Realizar uma análise citogenética molecular em indivíduos heterozigotos para rearranjos cromossômicos no gênero *Mazama* e definir o efeito deles na

produção de espermatozoides aneuplóides e no isolamento reprodutivo entre as populações e espécies.

### 3.2. Objetivos Específicos

- Estimar a proporção de espermatozoides normais/balanceados e desbalanceados em indivíduos do gênero *Mazama* portadores de rearranjos cromossômicos e híbridos entre citótipos de *M. americana*.
- Avaliar os potenciais efeitos de alguns rearranjos cromossômicos sobre a aptidão reprodutiva em machos do gênero *Mazama*.
- Contribuir na resolução das incertezas taxonômicas do gênero *Mazama* e demais cervídeos.

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## CAPÍTULO 2 – “Sperm chromosome segregation of rob(4;16) and rob(4;16)inv(4) in the brown brocket deer (*Mazama gouazoubira*)”<sup>1</sup>

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**ABSTRACT** – The genus *Mazama* stands out among the Neotropical deer due to their wide intra and interspecific karyotypic diversification, which is associated with an accentuated chromosomal fragility. There are reports of heterozygous Robertsonian translocation (RT) carriers in a free-range population of *Mazama gouazoubira* (brown brocket deer), as well as in captive animals of this and other species of the genus. To analyze possible negative impacts of heterozygous chromosome rearrangements on reproductive fitness of the carriers, we performed an analysis of sperm meiotic segregation in four brown brocket bucks, carriers of a rob(4;16), and compared the results with those of a normal buck. We established a reliable FISH and sperm-FISH protocol for the brown brocket deer using bovine (*Bos taurus*; diploid number, 2n = 60) whole chromosome painting (WCP) and BAC probes. Using BAC probes, we revealed the presence of a paracentric inversion (PAI) of the fused chromosome 4 in two of the four analyzed RT carriers. The mean frequency of normal/balanced sperm in the translocation carriers was significantly lower than in the normal buck (94.78% vs 98.40%). The mean value of total unbalanced spermatozoa was almost doubled in the RT/PAI carriers (6.68%) when compared to RT carriers (3.76%), but the difference was not statistically significant. This study demonstrated the efficiency of FISH with bovine

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WCP and BAC probes in the characterization of chromosome rearrangements and gametic segregation patterns in brown brocket deer. Our results indicate a low to moderate increase in the rates of unbalanced meiotic segregation products in brown brocket bucks heterozygous for RT and RT/PAIs.

**Keywords:** Meiotic segregation; neotropical deer; paracentric inversion; Robertsonian translocation; sperm-FISH; animal cytogenetics.

## 1. INTRODUCTION

Currently, the family Cervidae includes about 55 deer species [1] and it stands out for showing one of the highest rates of karyotypic evolution among mammals, potentially generated by chromosomal fragility [2,3]. This feature allows the occurrence of chromosomal rearrangements, which if combined with geographic isolation, a significant alteration or loss of habitat between populations, could induce the formation of new species [4–7]. Thus, the family Cervidae is marked by a wide variety of karyotypes, presenting species with low diploid number, such as *Muntiacus muntjak* ( $2n = 6/7$ ), and high diploid number, such as *Capreolus pygargus* ( $2n = 70 + 1$  to 14 B chromosomes – Bs) [8,9].

Among the 17 genera currently recognized in the family Cervidae [1], the genus *Mazama* deserves special attention. Not only because its fossil records are scarce and incomplete making evolutionary studies difficult [10–12], but also because it represents one of the most surprising examples of morphological convergence among mammals [2,10,13]. This feature does not correlate with the wide intra and interspecific karyotype diversification observed in the species conforming the genus [2,7,14–16].

Karyotype variations within the genus *Mazama* were likely originated through complex rearrangements, such as inversions, tandem chromosomal fusions and Robertsonian translocations (RT), which occurred in lineages originated from a common ancestor of Cervidae as observed in other genera [5,8,17–19]. Chromosomal rearrangements such as those found in the genus *Mazama* are quite common genetic abnormalities in domestic and wild species, as well as in human [6,20,21]. However, the high rate of inter- and intraspecific chromosome variation within the genus *Mazama*

is probably related to a high chromosome fragility index, which has already been suggested by doxorubicin-induced chromosomal aberrations, and also to the presence of Bs [3,5,16,22].

In the genus *Mazama*, the occurrence of heterozygous chromosomal rearrangements has already been evidenced in *Mazama americana* [23], *Mazama nana* [7], and *M. gouazoubira* (brown brocket deer). In fact, Valeri et al. (2018), described the presence of more than one RT in the same natural population of brown brocket deer, which is the sole holder of the putative ancestral karyotype ( $2n = 70$ ; fundamental number,  $FN = 70$ ) within the genus *Mazama* [8,16,24]. This brought up the possibility of a speciation event within a free-living population of *M. gouazoubira*.

However, fusions between non-homologous chromosomes appearing in heterozygosity can be directly related to reproductive disorders that result in decreased fertility due to meiotic segregation problems during prophase I and a subsequent impaired spermatogenesis [20,25,26]. In meiosis, the fused chromosomes pair with their non-translocated homologues, and meiotic segregation of such trivalents leads to the formation of both normal/balanced (alternating segregation mode) and unbalanced gametes (adjacent and 3:0 segregations) [20,27,28]. Unbalanced gametes presenting aneuploidies, despite having normal fertilizing capacity, usually lead to an early embryonic death, as they generate embryos with trisomies and monosomies of the chromosomes involved, directly affecting the animal's reproductive success [20,29–31].

To estimate the proportion of balanced or unbalanced gametes, the method of fluorescent *in situ* hybridization (FISH), has been frequently used, which is called "sperm-FISH" when used in spermatozoa [29,32]. Using FISH probes specific to chromosomes involved in translocations, we can assess the frequency of normal/balanced and unbalanced gametes, which can be used as a predictor of the potential effect of chromosomal rearrangements on the reproductive fitness of the carrier [26,29]. Currently, there is a lack of specific FISH probes for species of the genus *Mazama*. However, recent studies point to the use of cattle (*Bos taurus*, BTA) whole-chromosome painting (WCP) probes and bacterial artificial chromosome (BAC) probes in deer species to study chromosome homologies, and karyotype evolution



within Cetartiodactyla, since both families Bovidae and Cervidae are evolutionally closely related [18,19].

In this study, we used bovine WCP and BAC probes to establish a reliable FISH and sperm-FISH protocol for the brown brocket deer. The specific goal of the present study was to estimate the proportion of normal/balanced and unbalanced gametes in brown brocket deer Robertsonian translocations carriers through the use of bovine BACs.

## **2. MATERIAL AND METHODS**

### **2.1. Location and Ethics statement**

This study was carried out in two institutions: (1) The Deer Research and Conservation Center (NUPECCE), Jaboticabal, São Paulo, Brazil. All samples belong to the NUPECCE's germplasm bank. All animal procedures were approved by the Ethics Committee on Animal Use (CEUA) of the School of Agricultural and Veterinarian Sciences from São Paulo State University (Unesp), Jaboticabal, São Paulo, Brazil (protocol number 1930/18). This is in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA); (2) Veterinary Research Institute (VRI), Brno, Czech Republic. Cytogenetic procedures were performed at the VRI.

### **2.2. Animals**

Five adult brown brocket bucks (*M. gouazoubira*), one control (T376) and four heterozygous RT carriers (T300, T302, T307, and T381) were analyzed in this study. Animals T300, T302, and T307 were from Nhecolândia Pantanal in the municipality of Corumbá, Mato Grosso do Sul, Brazil. Animals T376 and T381 came from the Metropolitan Region of Ribeirão Preto, São Paulo, Brazil.

### **2.3. Tissue culture**

Skin biopsies from brown brocket deer were collected and used for fibroblast culture to obtain chromosomal preparations at NUPECCE [33]. Metaphase chromosome slides were prepared, and C-banding was performed using the standard techniques [34,35]. Tissue cultures from cattle (BTA,  $2n = 60$ ) were used for flow sorting or laser microdissection of bovine chromosomes to obtain WCP, as previously described [36].

#### **2.4. Semen samples and sperm nuclei preparation**

Cryopreserved semen samples were obtained from NUPECCE's germplasm bank. Ejaculates were collected and cryopreserved with Tris-egg yolk-glycerol extender [37]. Sperm nuclei decondensation was accomplished by a modification of the method described by Rubes et al. (1999) [38]. Briefly, semen samples were thawed at 37 °C for 20 s, then washed with 500 µl of phosphate-buffered saline (PBS, pH 7.2), centrifuged for 5 min ( $g = 380$ ), and the supernatant was discarded (repeated 3x). Washed spermatozoa were resuspended in 500 µl of PBS containing 5 mM dithiothreitol (DTT) and incubated for 60 min (room temperature). Samples were washed again with 300 µl of PBS (3x), and then washed in Carnoy solution (3:1 methanol:acetic acid) (3x). Finally, samples were fixed in Carnoy solution for 30 min at -20 °C. For dropping onto clean microscope slides, samples were diluted to a desired concentration.

#### **2.5. Painting probes**

Bovine whole chromosomes for the construction of WCP probes were isolated by flow sorting using MoFlo XDP Cell Sorter (Beckman Coulter, USA) [36] or microdissected by PALM Microlaser system (Carl Zeiss MicroImaging GmbH, Munich, Germany) [39]. DOP-PCR (degenerate oligonucleotide primed polymerase chain reaction) was used to amplify the chromosomal DNA [40]. Probe labelling was performed during the secondary PCR with Green-dUTP or Orange-dUTP (Abbott Park, IL, USA) [39].

## 2.6. BAC clones

Bovine BAC clones were selected from the CHORI-240 cattle library based on NCBI ARS-UCD1.2 Assembly data and obtained from the BACPAC Genomics, Emeryville, CA, USA. BAC DNA was labelled with biotin16-dUTP or digoxigenin-11-dUTP (Roche, Mannheim, Germany) using BioPrime Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA, USA). Two BAC clones were selected from the telomeric region of BTA19 and combined into a single probe to improve hybridization signals. Detailed list of BACs used in the present study appears in Table 1.

Table 1. List of bovine BAC clones used in the present study for detection of BTA homologies with brown brocket deer chromosomes involved in translocations and for sperm-FISH.

Chromosome region		Cattle location (Mb)	BAC clone (probe name)
Cattle	<i>Mazama</i>		
BTA 7	MGO 4	1.360 – 1.566	CH240-57O13 (BAC 7C)
		38.880 – 39.135	CH240-117P3 (BAC 7P)
BTA 19	MGO 16	55.619 – 55.852	CH240-106P5 (BAC 19T)
		55.911 – 56.097	CH240-188A20 (BAC 19T)

**BTA = *Bos taurus*; MGO = *Mazama gouazoubira*.**

## 2.7. Fluorescent *in situ* hybridization

FISH analysis of metaphase chromosomes from *M. gouazoubira*'s fibroblast cultures was carried out with bovine WCP probes to identify chromosomes orthologous to the chromosomes involved in the chromosomal translocations in the brown brocket bucks. Sperm-FISH analysis was carried out with bovine BAC probes to assess the rates of normal/balanced and unbalanced sperm. FISH and sperm-FISH procedures were carried out as described in Vozdova et al. 2019 [41], with slight modification for sperm denaturation. Briefly, spermatozoa were denatured in 1 M NaOH for 4 – 5 min.

The BAC probes labelled with biotin-16-dUTP were detected with Streptavidine-Cy5 (Invitrogen/Molecular Probes Camarillo, CA, USA) for FISH and Avidin-FITC (Vector Laboratories, Inc., Burlingame, CA, US) for Sperm-FISH. The BAC probes labelled with digoxigenin-11-dUTP were detected with antidigoxigenin rhodamine (Roche Diagnostics, Indianapolis, IN, USA). A Zeiss Axio Imager Z2 fluorescence microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) equipped with appropriate fluorescent filters and the Metafer Slide Scanning System (MetaSystems, Altlusheim, Germany) was used for image capture of well-spread metaphase chromosomes and scoring of normal/balanced and unbalanced gametes. Images were analyzed using ISIS3 software (MetaSystems, Altlusheim, Germany).

Only intact, non-overlapping gametes were scored using strict scoring criteria. The sperm was considered disomic if it showed two signals of the same color, size, and intensity, separated by a distance of at least one signal domain size. Diploid spermatozoa were differentiated from the double disomic ones by their larger size, as it is generally accepted in human and animal sperm-FISH studies [42-44].

## **2.8. Statistical analysis**

Non-parametric Mann-Whitney exact test was used to compare the frequencies of the different segregation products between individual sharing the same translocation. Non-parametric Wilcoxon signed-rank test was used to compare FISH phenotypes per each chromosome. Alternate segregation (normal/balanced products), adjacent segregation, and total abnormal products were analyzed using the Kruskal-Wallis test and the difference between groups was obtained using the Dunn's multiple comparison test, adjusted by Bonferroni method. All analyzes were performed using Software R (R Foundation for Statistical Computing, Vienna, Austria) and  $P < 0.05$  was considered significant.

## **3. RESULTS**

The fused chromosomes present in the studied heterozygous RT carriers could be divided into two different types according to their morphology and C-banding (Fig.

1 A – D). Using FISH with bovine WCP and BAC probes, we confirmed the rob(4;16)

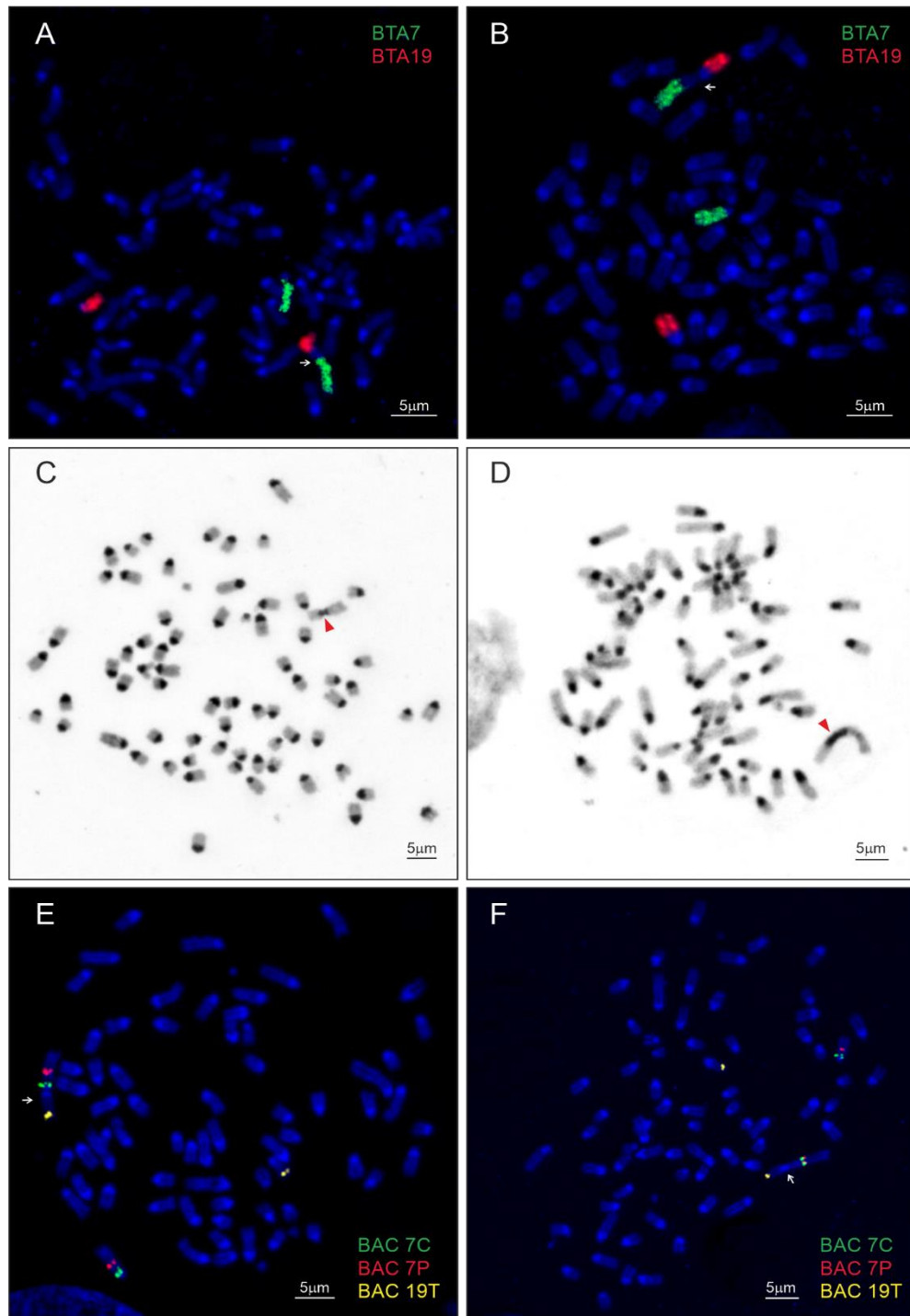


Fig.1. Metaphase chromosomes of the rob(4;16) (A, C, and E) and rob(4;16)inv(4) carriers (B, D, and F), arrows point to the fused chromosomes. (A – B) FISH with bovine WCP probes orthologous to MGO chromosome 4 (green – BTA 7) and MGO chromosome 16 (red – BTA 19). (C – D) C-banding. (E – F) FISH with bovine BAC probes orthologous to MGO chromosome 4 (BAC 7C – green, BAC 7P – red) and MGO chromosome 16 (BAC 19T – yellow).

in all of the carriers. These chromosomes are orthologous to bovine chromosomes BTA7 and BTA19. An apparent polymorphism represented mainly by large centromeric heterochromatin blocs was observed in two of the carriers (T300 and T307) (Fig. 1 D). Using bovine BAC probes, we revealed the presence of a RT with paracentric inversion (PAI) of the proximal part of the fused chromosome 4 in these animals [ $rob(4;16)inv(4)$ ] (Fig. 1 F). A schematic presentation of the rearrangement and an assumed process of its formation is displayed in Fig. 2.

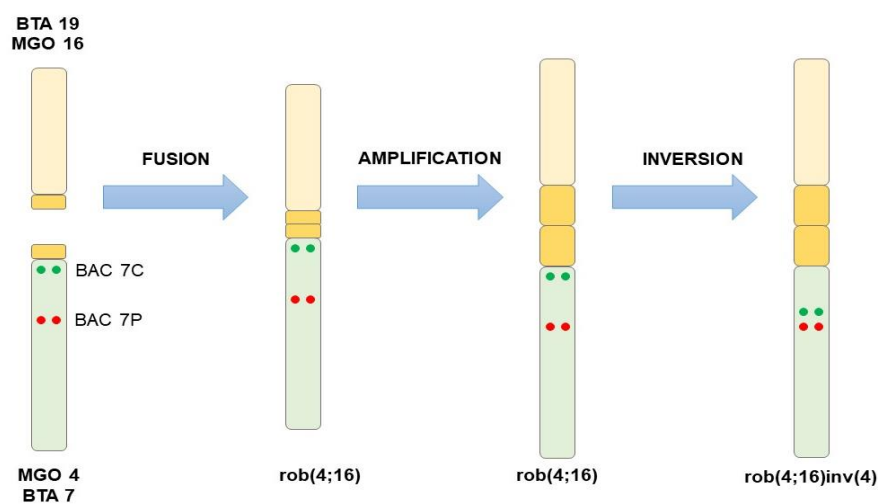


Fig. 2. Schematic illustration demonstrating the formation of the RT/PAI in the  $rob(4;16)inv(4)$ . The dots approximate the positions of BAC 7C (green) and BAC 7P (red) probes.

Regarding sperm-FISH technique, bovine BAC probes showed excellent specificity and sensitivity in the brown brocket deer sperm nuclei with hybridization rates higher than 99% in all cases [38]. A total of 5000 sperm nuclei were scored for each animal. Examples of fluorescent phenotypes observed in the carriers of the  $rob(4;16)$  are shown in Fig. 3. The results are presented in Table 2 and summarized below.

In the translocation carriers, the mean frequency of meiotic products resulting from the alternate (normal or balanced – Fig. 3a and f) and adjacent (Fig. 3b – e) segregation modes was 94.78% and 3.90%, respectively. A significant difference in the meiotic segregation patterns (normal/balanced, total adjacent, and total abnormal)

was detected between the translocation carriers and the control ( $P = 0.02$ ), but no significant difference was observed when comparing between individuals sharing the same translocation ( $P > 0.05$ ). We observed a difference between the RT and the RT/PAI carriers in the mean frequency of normal/balanced spermatozoa which was lower in the case of the RT/PAI carriers (93.32% vs. 96.24%), and, correspondingly, the adjacent segregation modes were more frequent in the RT/PAI carriers (4.98% vs. 2.81%). Also, a slightly higher frequency of disomic and diploid, as well as other signal combinations not listed, was observed in the carriers of the RT/PAI. However, none of these differences was statistically significant. There was no significant difference between nullisomies and disomies for any one of the chromosomes ( $P > 0.05$ ). The frequencies of diploid gametes (2n) and 3:0 segregation products were also not significantly different ( $P > 0.05$ ).

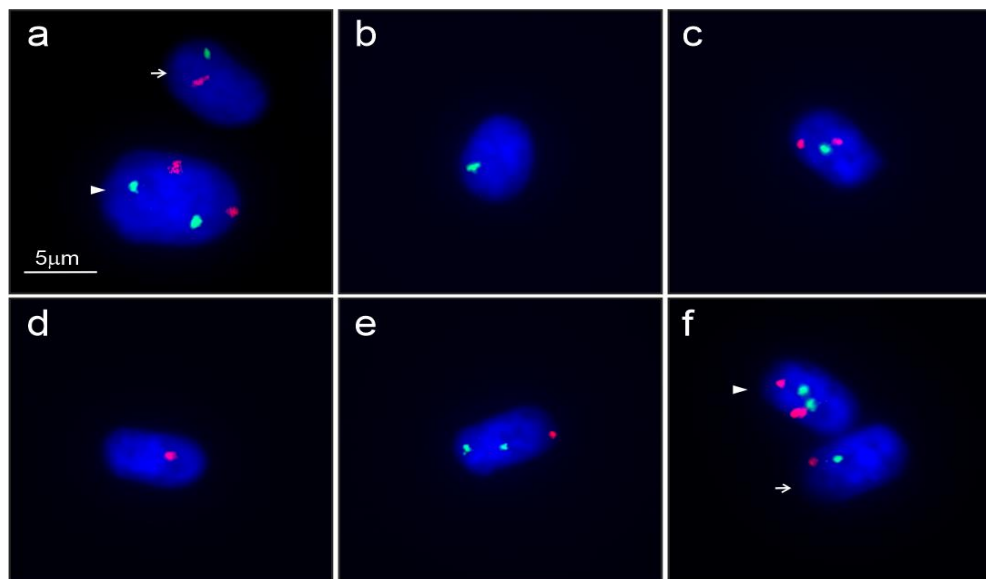


Fig. 3. Examples of sperm nuclei of heterozygous rearrangement carriers after hybridization with bovine BAC probes. Chromosome 4 (red) and 16 (green). (a) Normal or balanced sperm (arrow) and diploid (2n) sperm (arrowhead). (b) Nullisomic sperm for chromosome 4. (c) Disomic sperm for chromosome 4. (d) Nullisomic sperm for chromosome 16. (e) Disomic sperm for chromosome 16. (f) Disomic sperm for both chromosomes 4 and 16 (arrowhead) and normal sperm (arrow).

Table 2. Sperm meiotic segregation in the rearrangement carriers and a control animal with normal karyotype.

Segregation mode	Chromosomal constitution	FISH phenotype	Gametes investigated (%)										
			T302 <sup>a</sup>		T381 <sup>a</sup>		T300 <sup>b</sup>		T307 <sup>b</sup>		<i>P</i> value <sup>e</sup>	T376 <sup>c</sup>	
<b>Alternate</b>	4/16 or rob(4;16)	Normal / Balanced	<b>4874</b>	<b>(97.48)</b>	<b>4750</b>	<b>(95.00)</b>	<b>4707</b>	<b>(94.14)</b>	<b>4625</b>	<b>(92.50)</b>	<b>0.02</b>	<b>4920</b>	<b>(98.40)</b>
<b>Adjacent</b>	16	Nullisomy 4	30	(0.60)	52	(1.04)	52	(1.04)	67	(1.34)		22	(0.44)
	4/rob(4;16)	Disomy 4	18	(0.36)	17	(0.34)	50	(1.00)	91	(1.82)		3	(0.06)
	4	Nullisomy 16	18	(0.36)	54	(1.08)	39	(0.78)	19	(0.38)		16	(0.32)
	16/rob(4;16)	Disomy 16	20	(0.40)	72	(1.44)	88	(1.76)	92	(1.84)		19	(0.38)
<b>Total of adjacent products</b>			<b>86</b>	<b>(1.72)</b>	<b>195</b>	<b>(3.90)</b>	<b>229</b>	<b>(4.58)</b>	<b>269</b>	<b>(5.38)</b>	<b>0.02</b>	<b>60</b>	<b>(1.20)</b>
	4/16/rob(4;16) or 4/4/16/16	Disomy 4+16	3	(0.06)	6	(0.12)	6	(0.12)	15	(0.30)		4	(0.08)
	Nor 4 or 16	Nullisomy 4+16	11	(0.22)	17	(0.34)	11	(0.22)	11	(0.22)		7	(0.14)
	4/16/rob(4;16) or 4/4/16/16	Diploidy	21	(0.42)	20	(0.40)	29	(0.58)	50	(1.00)		7	(0.14)
	Others <sup>d</sup>		5	(0.10)	12	(0.24)	18	(0.36)	30	(0.60)		2	(0.04)
<b>Total of abnormal products</b>			<b>126</b>	<b>(2.52)</b>	<b>250</b>	<b>(5.00)</b>	<b>293</b>	<b>(5.86)</b>	<b>375</b>	<b>(7.50)</b>	<b>0.02</b>	<b>80</b>	<b>(1.60)</b>
<b>TOTAL</b>			<b>5000</b>	<b>(100)</b>	<b>5000</b>	<b>(100)</b>	<b>5000</b>	<b>(100)</b>	<b>5000</b>	<b>(100)</b>		<b>5000</b>	<b>(100)</b>

<sup>a</sup> rob(4;16); <sup>b</sup> rob(4;16)inv(4); <sup>c</sup> Normal karyotype; <sup>d</sup> Other, less frequent signal combinations. <sup>e</sup> Significant differences between the group of carriers and the control animal.



#### 4. DISCUSSION

Previous studies have reported the occurrence of RTs in 14 – 26% of the animals studied in *M. gouazoubira* [14,16,24,45,46], revealing a chromosomal fragility with a high rate of polymorphism [3,22]. Thus, it is necessary to estimate how these chromosomal polymorphisms can affect the rates of chromosomally unbalanced gametes, the reproductive fitness of the carriers, and population stability in natural areas. Chromosomal translocations are common in the evolutionary history of the genus *Mazama*, as also observed in other members of the family Cervidae [8,47–49]. Besides, the presence of heterozygous chromosomal translocations carriers has also been reported for other species within the genus *Mazama* [5,7,23]. However, in *Mazama* species, there are only two studies in *M. americana* about RT effect on meiotic chromosome segregation [50] and the impact on the fertility of a carrier in terms of synaptonemal complex analysis [23].

To the best of our knowledge, this is the first report on the use of sperm-FISH technique to estimate the proportion of normal/balanced and unbalanced spermatozoa in bucks from *M. gouazoubira* with heterozygous RT. In this study, the use of molecular cytogenetic methods allowed an accurate evaluation of chromosomal rearrangements, as well as the analysis of meiotic segregation products in the carriers. In fact, it was revealed that animals T300 and T307, previously described as rob(X;16) carriers by G-banding [16], were actually carriers of a rob(4;16) with a PAI of the chromosome 4 involved in the translocation (Fig. 1 and Fig. 2). Unfortunately, cytogenetic studies based only on banding techniques may present inaccuracies, even more so in the case of *M. gouazoubira*, presenting 34 pairs of acrocentric autosomes of similar size in its karyotype, and by the difficulty in obtaining a high-quality G-banding which makes proper identification of the chromosomes difficult [8,16].

Regarding the centromeric fusion, the proportion of meiotic products from adjacent segregation modes in *M. gouazoubira* is consistent with that reported in the literature for RT in domestic species such as bulls – 2.58% to 5.42% [26,51], boars – 3.16% [25] and mice – 8.00% to 11.50% [52]. In humans, where several investigations have been carried out on various types of translocations, common and rare RTs show a bigger range of adjacent products, varying from 0.20% to 49.01%, depending on the

chromosomes involved in the RT [20]. In the case of *M. gouazoubira*, a slightly higher frequency of adjacent products was observed in animals carrying the *rob(4;16)inv(4)* compared to those with the simple *rob(4;16)* (4.98% vs. 2.81%). Also, a higher contribution of diploids (2n) and 3:0 segregation products was observed in males carrying *rob(4;16)inv(4)*. However, it is important to note that those errors may be a consequence of mis-segregation in both the first and second meiotic divisions [20]. The present study did not evaluate interchromosomal effects (ICE) of the rearrangements. The ICE is defined as a disturbance in meiosis, where rearranged chromosomes disrupt disjunction and distribution of other chromosome pairs not involved in the rearrangement, including also both sexual chromosomes, X and Y [20,53,54]. This was previously observed in human RT carriers with a high frequency of aneuploid sperm, and, recently, also in cattle, in heterozygous *rob(3;16)* carrier [20,51,53,55]. Although there is still controversy about whether this phenomenon would represent a factual reproductive genetic risk for carriers [20,51,53–56].

Although the mean frequency of unbalanced products was not significantly different between the RT/PAI and RT carriers, the observation of a slightly higher percentage of chromosomally abnormal products might suggest a higher occurrence of meiotic errors in both the first and second meiotic division in association with the presence of the RT/PAI [57,58]. Literature in humans about PAI points out that the synapsis of homologous chromosomes depends on the length of the inverted segment and the configuration adopted by the bivalent [58,59], or trivalent in our specific case. Thus, long inverted regions tend to form a hair-pin loop configuration to align the homologous regions by twisting and folding the inverted segment, while a short inversion can remain as an asynaptic balloon [58,59]. However, synaptonemal complex and meiotic segregation analyses in pigs showed no correlation between the sizes of the inverted fragments and the percentages of unbalanced gametes produced [60,61]. This suggests that meiotic behavior might vary from one species to another. On the other hand, taking into account that meiotic synapsis is initiated at the telomeres and proceeds towards the centromere [62], the regions proximal to the centromere might not undergo complete synapsis or might finally pair through heterosynapsis or synaptic adjustments, as it was observed in meiotic studies in RT carriers [23,61,63–65]. If meiotic recombination occurs within the inversion loop, it results in a formation

of acentric and dicentric chromosomes. High frequency of such events might further affect the reproductive fitness of the PAI carriers [59].

Regarding the RT in *M. gouazoubira*, a slightly higher frequency of adjacent and total abnormal meiotic products was observed when compared with the well-known rob(1;29) in cattle (2.76% of unbalanced products) [26,66,67]. This suggests that the fertility in RT carriers of *M. gouazoubira* might be affected, as it was previously observed in other studies in animal and human heterozygous RT carriers [20,68,69]. Studies in cattle have reported exceptionally low rates of success for *in vitro* oocyte fertilization, early and advanced embryo development using semen from a heterozygous rob(16;20) bull [30,31]. On the other hand, a rob(1;29) carrier bull showed *in vitro* oocyte fertilization and early embryo development rates similar to non-translocated animals, and only reduced advanced embryo development rates [30]. Besides, studies in cattle have shown the rob(1;29) in 50% of embryos [31]. This confirmed a 50% rate of embryo carrying RTs, as theoretically predicted for this and other RTs in cattle [70,71]. However, the female carriers could produce greater rates of unbalanced meiotic products, as already reported in cattle [67] and pigs [25]. This might have significant consequences for the reproductive fitness of RT carriers in general.

In this study, we observed up to 7.50% chromosomally abnormal gametes in the *M. gouazoubira* rearrangements carriers. This is more than in a recent study in *M. americana*, with heterozygous rob(5;11) (Carajás cytotype) and heterozygous rob(7;20) (Rondônia cytotype) showing 2.34% and 2.20% chromosomally abnormal gametes, respectively, with no significant difference with the control [50]. A previously published study focused on the synaptonemal complex analysis in the above mentioned rob(7;20) red brocket (*M. americana*) buck suggested that the formation of unbalanced gametes was highly unlikely for this RT carrier [23]. This was supported by a fact that the buck fathered four fawns, not exhibiting any obvious reproductive impairment. Studies carrying out the synaptonemal complex analysis have had similar results in evaluating the effect of centromeric fusions on meiosis and reproduction of cattle [64], goitered gazelle [72], and impala [63]. From the above, we can infer that the trivalent behavior in meiosis depends on the species studied and the chromosomes

involved in the rearrangement [63]. Thus, every single RT need to be evaluated in order to understand its potential effect on reproduction of the carriers.

In addition to the rearrangements evaluated in this study, the presence of at least one more translocation was reported within the *M. gouazoubira* population [16]. These findings suggest an ongoing process of speciation in *M. gouazoubira*, as a result of possible reproductive isolation [16]. However, a more robust population sampling in Pantanal, as well as in other natural populations, would be necessary to draw adequate conclusions. The hypothetical coexistence of two or more different RTs in the same population would increase the risk of producing individuals heterozygous for 2 different fusions, causing serious meiotic disturbances in the carrier [73]. Therefore, the necessity of gametic segregation assessment in heterozygous carriers of chromosomal translocations becomes evident for a better understanding of their impact within and between natural populations of the genus *Mazama*, as well as for setting up a systematic karyotype control of captive populations in order to avoid the propagation of chromosomal abnormalities to the progeny and to natural populations when reintroduction programs are performed.

## 5. CONCLUSION

The present study demonstrated the efficacy of bovine WCP and BAC probes for the establishment of a FISH and a sperm-FISH protocols for the species *Mazama gouazoubira*. The sperm-FISH has been shown to be an adequate technique for the study of meiotic segregation in carriers of Robertsonian translocations in the genus *Mazama*. *Mazama gouazoubira* Robertsonian translocation carriers showed similar rates of normal/balanced and unbalanced gametes to those already reported for cattle and pigs. Besides, meiotic and sperm studies in carriers of different chromosomal rearrangements are still necessary in the genus *Mazama*, in order to analyze their impact on reproduction. Cytogenetic monitoring of *ex-situ* and *in-situ* conservation programs is highly recommended for the management of threatened species in captivity or free-ranging.

## **CRedit authorship contribution statement**

David Javier Galindo: Conceptualization, Forma analysis, Funding acquisition, Investigation, Project administration, Visualization, Writing – original draft. Miluse Vozdova: Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing. Svatava Kubickova: Investigation, Methodology, Writing – review & editing. Halina Cernohorska: Investigation, Methodology. Agda Maria Bernegossi: Investigation. Dita Kadlcikova: Investigation Jiri Rubes: Supervision. José Maurício Barbanti Duarte: Conceptualization, Funding acquisition, Resources, Writing – review & editing.

## **Declaration of competing interest**

The authors declare no conflict of interest to disclose.

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### CAPÍTULO 3 – “Chromosomal Polymorphism and Speciation: the Case of the Genus *Mazama* (Cetartiodactyla; Cervidae)”<sup>1</sup>

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**Abstract:** Chromosomal polymorphism plays a major role in speciation processes in mammals with high rates of karyotypic evolution, as observed in the family Cervidae. One remarkable example is the genus *Mazama* that comprises wide inter- and intra-specific chromosomal variability. To evaluate the impact of chromosomal polymorphisms as reproductive barriers within the genus *Mazama*, inter-specific hybrids between *Mazama gouazoubira* and *Mazama nemorivaga* (MGO x MNE) and intra-specific hybrids between cytotypes of *Mazama americana* (MAM) differing by a tandem (TF) or centric fusion (Robertsonian translocations — RT) were evaluated. MGO x MNE hybrid fertility was evaluated by the seminal quality and testicular histology. MAM hybrids estimation of the meiotic segregation products was performed by sperm-FISH analysis. MGO x MNE hybrids analyses showed different degrees of fertility reduction, from severe subfertility to complete sterility. Regarding MAM, RT,

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and TF carriers showed a mean value for alternate segregation rate of 97.74%, and 67.23%, and adjacent segregation rate of 1.80%, and 29.07%, respectively. Our results suggested an efficient post-zygotic barrier represented by severe fertility reduction for MGO x MNE and MAM with heterozygous TF. Nevertheless, RT did not show a severe effect on the reproductive fitness in MAM. Our data support the validity of MGO and MNE as different species and reveals cryptic species within MAM.

**Keywords:** cytogenetics; hybrids; post-zygotic barrier; sperm-FISH; Neotropical deer

## 1. INTRODUCTION

Chromosomal polymorphisms have played a meaningful role in speciation [1], by leading to the formation of efficient barriers to gene flow and subsequent differentiation process [2,3]. Among mammals, the family Cervidae stands out as one of the families with the highest presence of chromosomal polymorphisms, which is demonstrated in genera such as *Muntiacus*, whose diploid number ranges from  $2n = 6/7$  (*Muntiacus muntjak*) to  $2n = 46$  (*Muntiacus reevesi*) [4,5], and *Mazama*, whose diploid number ranges from  $2n = 32 - 34 + Bs$  (*Mazama bororo* — MBO) to  $2n = 70 + Bs$  (MGO) [6–8]. Intra-specific polymorphism is also present in several *Mazama* species (*Mazama nana* — MNA,  $2n = 36 - 39 + Bs$ ; MAM,  $2n = 42 - 53 + Bs$ ; MNE,  $2n = 67 - 69 + Bs$ ) [9–11] which, in part, justifies the great complexity in the species taxonomic definition and classification.

Regarding the genus *Mazama*, the occurrence of chromosomal rearrangements, mainly heterozygous RT, has been observed in MGO, the only holder of the ancestral karyotype within the genus [7,12,13]. Presence of these RTs denotes a high index of chromosomal fragility in this species, which has been previously tested and corroborated by doxorubicin-induced chromosomal aberrations [14,15]. Thus, we can hypothesize an ongoing speciation process in MGO [12]. Studies on deer mitochondrial DNA have suggest that MNE and MGO would not belong to the genus *Mazama*. Both of them standing in independent clades and distant from other *Mazama*, sharing the gray clade with other genera such as *Blastocerus*, *Ozotoceros*, and *Hippocamelus* [16,17]. In the meantime, this group is characterized by low levels of



inter-specific chromosome difference and karyotypes with a high diploid number [11,16,17]. Due to their parapatric distribution [18] and morphological similarity, the differentiation between these two species has been the subject of extensive debate over the years, being demonstrated only recently through morphological [19,20], cytogenetic [11,20,21], and phylogenetic [20,22] analyzes.

Indeed, the comparison between MGO and MNE karyotypes have demonstrated that despite notable cytogenetic similarities, two chromosomal differences separate these species: (a) The presence of a MNE population with a rob(4;32), regarding to the base karyotype for the species, with a sex chromosome system XX/XY and a submetacentric X, different from the acrocentric X of MGO, and (b) The presence of an X-autosome TF in other MNE population, which resulted in a multiple sex chromosome system XX/XY1Y2 [7,8,11,20]. The occurrence of these rearrangements, by themselves, is already a strong indication of their possible role in the separation of these two species, although more evidence is needed to corroborate this statement [11,23].

Chromosomal polymorphisms are potent promoters of reproductive isolation since they can trigger a series of errors during meiosis in hybrids of different species or lineages, such as incorrect pairing of parental chromosomes, errors in chromosome segregation, and during crossing-over. These so-called meiosis defects have a deleterious effect on the individual's reproductive fitness, leading to subfertility or sterility [1,24]. Although a description of morphophysiological evidence for reproductive isolation needs further investigation within the gray clade, this does not seem to be the case of the second clade of the genus *Mazama*, the red clade.

Regarding the red clade, what was traditionally reported as MAM today is considered a complex of cryptic species with two chromosomal lineages, one with high diploid number (Cytotypes Paraná — PR,  $2n = 52/53$ , FN = 56; Santarém — SA,  $2n = 50/51$ , FN = 56; Jarí — JA,  $2n = 48/49$ , FN = 56; and Carajás — CA,  $2n = 50/51$ , FN = 54) and one with a low diploid number (Cytotypes Juína — JU,  $2n = 44/45$ , FN = 48; and Rondônia — RO,  $2n = 42/43$ , FN = 46), all of them with wide geographical coherence [10]. Comparisons between cytotypes of the same lineage by G-banding showed minimal differences, such as TF or RT, from one cytotype to another [10].

A reproductive study on MAM showed that hybrids produced by crossbreeding of the two different chromosomal lineages are sterile [25]. This indicated the occurrence of post-zygotic reproductive isolation between the MAM lineages, which was associated with errors in meiotic recombination and gametic segregation due to several chromosomal differences, such as TF, RT, and inversions [25,26]. Hybrids between cytotypes of the same chromosomal lineage, with a chromosome number difference being equal to or less than 3 between the parents, were considered subfertile. Nonetheless, spermatogenesis was only evaluated in morphological and histological terms, without assessing the presence of chromosomally balanced or unbalanced gametes [25]. On the other hand, the presence of heterozygous RT in MAM probably only has a low effect on the reproductive fitness of the carrier [27].

This study aimed to assess the role of chromosomal polymorphism as a reproductive barrier and speciation mechanism within the genus *Mazama*. Thus, inter-specific hybrids between *M. gouazoubira* and *M. nemorivaga* (MGO x MNE) and intra-specific hybrids between *M. americana* (MAM) cytotypes differing by TF or RT were evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Species and Samples

Fibroblast tissue cultures prepared from skin biopsies according to standard protocols, testicular tissue, and sperm of *M. gouazoubira* (MGO), *M. nemorivaga* (MNE), *M. americana* (MAM) cytotypes and hybrids, available at NUPECCE (Jaboticabal, São Paulo, Brazil), were used in the present study. For the inter-specific hybridization experiment, two hybrids between *M. gouazoubira* and *M. nemorivaga* and five pure bucks ( $n = 3$ , *M. gouazoubira* and  $n = 2$ , *M. nemorivaga*) were used and are described in Table 1.

For the intra-specific hybridization experiment, two heterozygous Robertsonian translocation hybrids, three heterozygous TF hybrids, and two Carajás cytotype bucks from *M. americana* (MAM) were used. A detailed data of the animals is described in Table 2.

Table 1. Summary of chromosomal data from *M. gouazoubira*, *M. nemorivaga*, and inter-specific hybrids

Animal	Species	2n	FN	Translocations		B
				RT	Multiple Sexual System	
PG1	<i>M. gouazoubira</i>	70	70	–	No	0 – 2
PG2	<i>M. gouazoubira</i>	70	70	–	No	0 – 2
PG3	<i>M. gouazoubira</i>	70	70	–	No	0 – 2
PN1	<i>M. nemorivaga</i>	68	72	rob(4;32)(4;32) <sup>a</sup>	No	1 – 9
PN2	<i>M. nemorivaga</i>	67	70	rob(4;32)(4;32) <sup>a</sup>	Yes <sup>a</sup>	2 – 5
H1	MGO♂ × MNE♀	69	72	rob(4;32) <sup>a</sup>	No	0 – 3
H2	MNE♂ × MGO♀	70	70	–	No	0 – 2

2n = chromosome number; FN = fundamental number; RT = Robertsonian translocation; TF = tandem fusion; B = supernumerary chromosomes. <sup>a</sup> Chromosome classification according to standard karyotype for *M. nemorivaga* [11].

## 2.2. Whole-Chromosome Painting and Bacterial Artificial Chromosomes (BAC) Probes

Bovine whole-chromosome painting (WCP) probes were used for identification of chromosomes involved in the Robertsonian and Tandem fusions in animals analyzed in this study. Bovine whole chromosomes were isolated by flow sorting using MoFlo XDP Cell Sorter (Beckman Coulter, Indianapolis, IN, USA) [29] or microdissected by PALM Microlaser system (Carl Zeiss MicroImaging GmbH, Munich, Germany) [30]. Once isolated, bovine chromosomes were used to produce WCP probes by DOP-PCR [31]. Probe labeling was performed during the secondary PCR with Green-dUTP or Orange-dUTP (Abbott, Abbott Park, IL, USA) [30].

**Table 2.** Summary of chromosomal data from *M. americana* carriers of chromosomal translocations, hybrids of different MAM cytotypes, and non-translocated animals.

Animal	Cytotypes	2n	FN	Translocations		B
				RT	TF	
<b>T297</b> <sup>a</sup>	Carajás	51	54	–	–	2 – 3
<b>T274</b>	Carajás	50	54	rob(5;11) <sup>b</sup>	–	3
<b>T326</b>	Carajás	49	54	rob(5;11)(5;11) <sup>b</sup>	–	3 – 4
<b>T269</b>	Rondônia	42	46	rob(7;20) <sup>c</sup>	–	3 – 5
<b>T343</b>	Juína♂ × Rondônia♀	43	47	rob(7;20) <sup>c</sup>	der(7;10) <sup>d</sup>	2 – 3
<b>T347</b>	Rondônia♂ × Juína♀	44	47	–	der(7;10) <sup>d</sup>	2 – 4
<b>T421</b>	Paraná♂ × Carajás♀	52	55	–	der(5;10) <sup>e</sup>	–

2n = chromosome number; FN = fundamental number; RT = Robertsonian translocation; TF = tandem fusion; B = supernumerary chromosomes. <sup>a</sup> Control bucks, normal karyotype. <sup>b</sup> Chromosome classification according to Carajás cytotype [10]. <sup>c</sup> Chromosome classification according to Rondônia cytotype [10,27]. <sup>d</sup> Chromosome classification according to Juína cytotype [28]. Rondônia chromosome 4 = tandem fusion of Juína chromosomes 7 + 10. <sup>e</sup> Chromosome classification according to Paraná cytotype [28]. Carajás chromosome 3 = tandem fusion of Paraná chromosomes 5 + 10.

For sperm-FISH, bovine BAC clones localized to the chromosomes involved in translocations were selected from the CHORI-240 cattle library (BACPAC Genomics, Emeryville, CA, USA). BAC DNA labeling with digoxigenin-11-dUTP or biotin-16-dUTP (Roche, Mannheim, Germany) was performed using BioPrime Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA, USA). Detailed list of BACs used in the present study appears in Table S1.

### 2.3. FISH

FISH and sperm-FISH procedures were carried out as described in Vozdova et al. 2019 [32]. BAC probes labeled with digoxigenin-11-dUTP were detected with antidigoxigenin rhodamine (Roche Diagnostics, Indianapolis, IN, USA). BAC probes labeled with biotin-16-dUTP were detected with Avidin-FITC (Vector Laboratories, Inc., Burlingame, CA, USA). Hybridization signals were examined using Zeiss Axio Imager.Z2 fluorescence microscope (Carl Zeiss Microimaging GmbH, Jena Germany) equipped with appropriate fluorescent filters and the Metafer Slide Scanning System (MetaSystems, Altlussheim, Germany). Images of well-spread metaphase cells were captured and analyzed using ISIS3 software (MetaSystems, Altlussheim, Germany).

### 2.4. *Inter-Specific Hybrids (MGO x MNE) Reproductive Assessment*

#### 2.4.1. Spermogram

All animals went through at least one semen collection procedure once they achieved adulthood (<12 months of age). Electroejaculation procedure followed Favoretto et al. (2012) [33]. In short, all animals were anaesthetized intramuscularly with a combination of xylazine (1 mg/kg) and ketamine hydrochloride (7 mg/kg). Following sedation, a probe was inserted into the rectum and placed against the anterior wall close to the seminal vesicles. Each animal was submitted to sequential electroshocks increasing from 250 mA to 750 mA, with a mean duration of 3 s (and 3 s of rest). Three stimulation sequences of 10 shocks each were performed at intervals of 1 – 2 min [34]. Collected samples were maintained in microtubes (2 mL) at 37 °C in

water bath until the beginning of analysis. Ejaculate color was determined by a single researcher to avoid any individual biases. Volume, total motility, sperm vigor, and sperm count were evaluated as described by Alvarez et al. (2020) [35]. Morphological analysis of the ejaculate was performed through the examination of wet preparations of fixed spermatozoa under phase contrast microscope. Morphological defects were classified according to their origin, to detect defects arising from an anomalous spermatogenesis (primary defects, resulting from testicular and secondary defects, resulting from inadequate maturation) [36].

#### 2.4.2. Testicular Histology

PG1, PG2, PN1, and H1 underwent unilateral orchiectomy after electroejaculation procedure. PG3, PN2, and H2 had their testicles collected immediately post-mortem. Testicular tissue was grossed into 1 cm thick sections, fixed in Bouin's fixative for 24 h, processed for paraffin embedding, microtome-sectioned at 5- $\mu$ m thickness, stained with hematoxylin and eosin, and imaged with a microscope. Then, 60 round or nearly round tubular profiles from each animal were randomly chosen and had diameter and epithelium height measured (Axio Vision v. 4.8.2, Carl Zeiss AG, Feldbach, Switzerland, size measurement tools were used). Ten sections of seminiferous tubules were analyzed to quantify the population of sperm cells. The results were presented with mean  $\pm$  SD.

### 2.5. *Intra-Specific Hybrids (MAM) Reproductive Assessment*

#### 2.5.1. Semen Samples and Sperm Nuclei Preparation

Cryopreserved semen samples were obtained from NUPECCE's germplasm bank. Ejaculates were collected and cryopreserved with Tris-egg yolk-glycerol extender [33]. To perform decondensation of the sperm nuclei, the method described by Rubes et al. (1999) was used, with slight modifications [37]. Briefly, semen samples were thawed at 37 °C for 20 s. Samples were transferred to a 2 mL Eppendorf tube, then washed with 500  $\mu$ L of phosphate-buffered saline (PBS, pH 7.2), centrifuged at

380 g (5 min), and the supernatant discarded (repeated 3x). Pellet was resuspended in 500  $\mu$ L of PBS containing 5 mM dithiothreitol (DTT), and incubated for 40 – 60 min, with slight homogenization every 10 min, then centrifuged. Pellet was washed in 300  $\mu$ L of PBS (3x), and then fixed in Carnoy solution (3:1 methanol:acetic acid) (3x). Finally, samples were stored at -20 °C (30 min) in Carnoy solution. For dropping onto clean microscope slides, samples were diluted to a desired concentration.

### 2.5.2. Sperm-FISH

The FISH protocol described in **Section 2.3** was used with a slight modification for sperm denaturation. Briefly, spermatozoa were denatured in 1M NaOH for 6 – 10 min. BAC probes labeled with digoxigenin-11-dUTP and biotin-16-dUTP were detected with antidigoxigenin rhodamine and Avidin-FITC, respectively. Scoring of normal/balanced and unbalanced gametes was performed using Zeiss Axio Imager.Z2 fluorescence microscope. Only intact, non-overlapping gametes were scored using strict scoring criteria. The sperm was considered disomic if it showed two signals of the same color, size, and intensity, separated by a distance of at least one signal domain size. Diploid spermatozoa were differentiated from the double disomic ones by their larger size.

### 2.6. *Statistical Analysis*

Results for the histological measurements and the percentage of intratubular cells were presented by mean  $\pm$  SD. All the results were submitted to Shapiro–Wilk normality test. Tubular diameter and germinal epithelium height did not present normal distribution therefore individual means were compared using non-parametric Kruskal-Wallis teste followed by pairwise Mann-Whitney U test with Bonferroni correction. Seminal parameters from hybrids between MGO and MNE and “pure” bucks were descriptively compared. Non-parametric Mann-Whitney exact test and Wilcoxon signed-rank test were used to compare frequencies of different segregation products between individuals and to compare FISH phenotypes per each chromosome, respectively. Meiotic segregation patterns were analyzed using the Kruskal-Wallis test

and the difference between groups was obtained using the Dunn's multiple comparison test, adjusted by Bonferroni. All analyzes were performed using Software R (R Foundation, 2020) [38] and  $p < 0.05$  was considered significant.

### 3. RESULTS

Using FISH with bovine WCP probes, we identified homologies between bovine chromosomes and the translocated chromosomes in the analyzed brocket deer hybrids. Chromosome differences between MGO and MNE identified by FISH with bovine WCP probes are shown in Figure 1. The FISH analysis of the hybrids showed that buck H1 obtained the rob(4;32) (Figure 2 A) and the submetacentric X of MNE. The buck H2 did not inherit the X-autosomal fusion of MNE, but the acrocentric X of MGO.

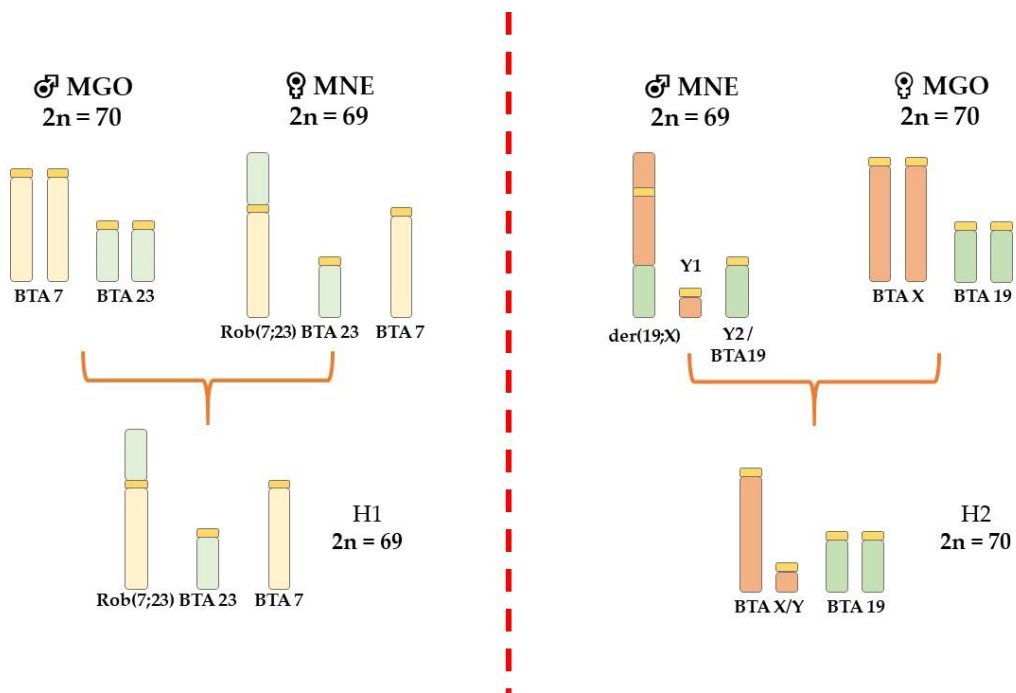


Figure 1. Schematic illustration showing the chromosomal polymorphism involved in the formation of MGO x MNE hybrids with orthologous bovine chromosomes.



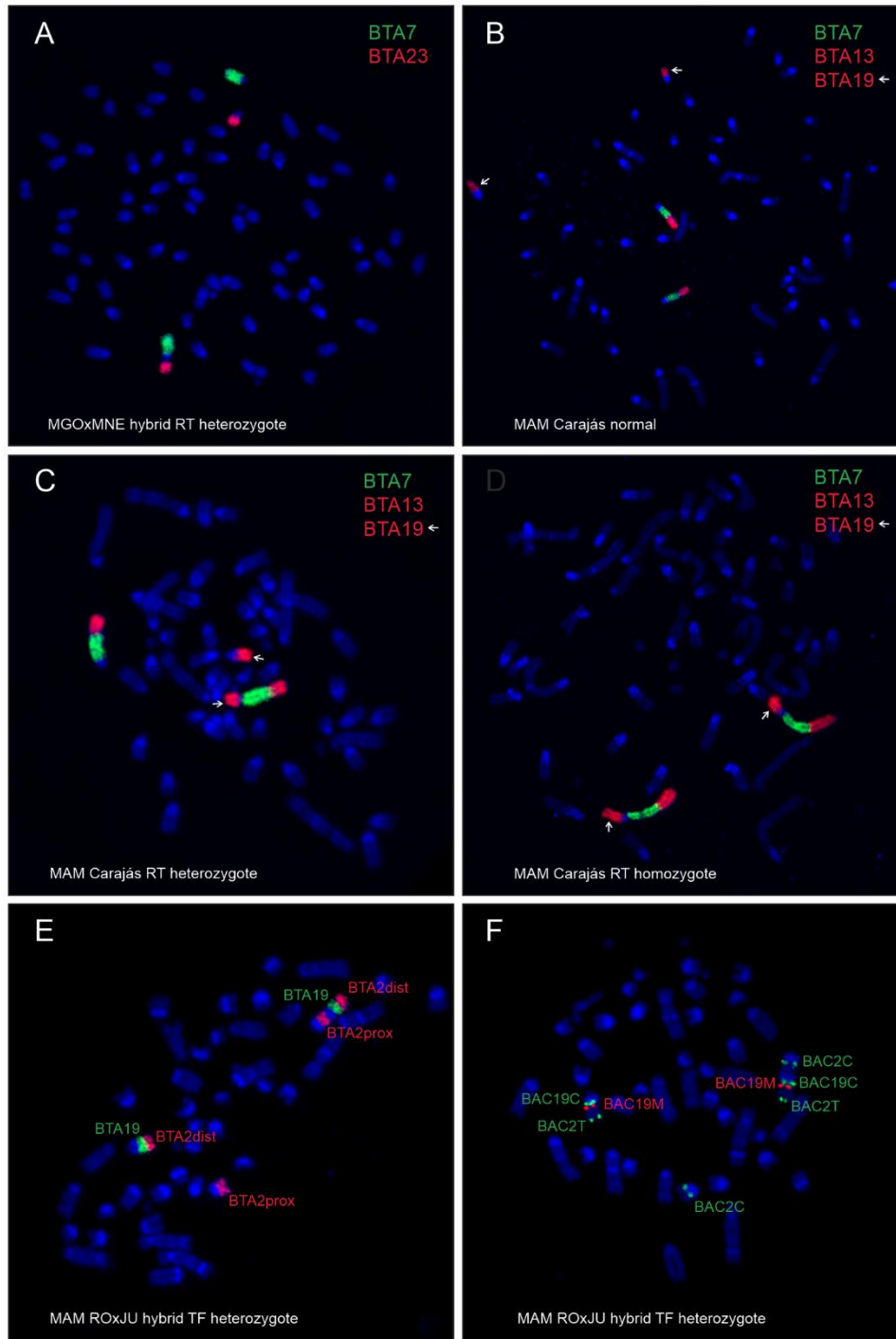


Figure 2. Metaphase chromosomes of the analyzed animals after FISH with bovine WCP (A – E) and BAC (F) probes. (A) Hybrid H1 (*M. gouazoubira* x *M. nemorivaga*) heterozygous for rob(4;32). (B) *M. americana* T297, Carajás cytotype, with normal karyotype. (C) *M. americana* T274, Carajás cytotype, heterozygous for rob(5;11). (D) *M. americana* T326, Carajás cytotype, homozygous for rob(5;11). (E) *M. americana* T343, Rondônia x Juína cytotype hybrid heterozygous for tandem fusion der(7;10) with WCP probes. (F) *M. americana*, Rondônia x Juína cytotype hybrid heterozygous for tandem fusion der(7;10) with BAC probes.

Regarding MAM, differences between a non-translocated, heterozygous, and homozygous rob(5;11) in the Carajás cytotype are shown in Figure 2 B – D. Difference between Rondônia (2n = 42/43) and Juína (2n = 44/45) cytotypes, as well as Carajás (2n = 50/51) and Paraná (2n = 52/53) cytotypes, was confirmed by FISH with bovine WCP and BAC probes, revealing a TF (centromere—telomere) (Figure 2 E,F). Heterozygous TF in the hybrids was classified according to Abril (2009) [28], where a der(7;10) in Juína and a der(5;10) in Paraná are equivalent to the acrocentric chromosomes 4 in Rondônia and 3 in Carajás, respectively.

### 3.1. *Inter-Specific Hybrids*

The fertility of pure and hybrids bucks (MGO x MNE) was assessed by testicular and sperm analysis. Photomicrographs of the testicular tissue revealed three distinct testis histology phenotypes (exemplified in Figure 3 A – D) among pure animals and both hybrids. MGO and MNE testis (A, B) were considered totally functional, with multiple round tubules containing a plush spermatogenic epithelium. Morphometric measurements (Figure 3 E, F) revealed larger tubules and thicker seminiferous epithelium, with PG3 presenting the highest mean values for tubular diameter and epithelium height among all individuals. Spermatogenesis was active and uniform in all sections analyzed, which was later confirmed by the quantification of sperm cells (Table 3) and the higher spermatid-to-spermatocyte ratio (SSR) values (1.50 – 4.19).

Regarding hybrids, histology phenotypes presented different levels of testicular hypoplasia as well as epithelial vacuolization suggestive of apoptosis. In H1, all the seminiferous tubules analyzed were hypoplastic (Figure 3 C) with evidence of spermatogenesis interruption during the first meiosis (SSR = 0, Table 3). H1 showed significant lower mean diameter and epithelium height ( $p < 0.05$ ) among all the animals analyzed. H2, in turn, seemed to be affected to a lesser extent, with the majority (90%) of seminiferous tubules being considered active (Figure 3 D), even though spermatogenesis was complete in only part of them (demonstrated by reduction in later cell types, SSR = 0.84). Morphometric means for H2 did not significantly differ from most pure bucks (PG1, PG2, and PN2).

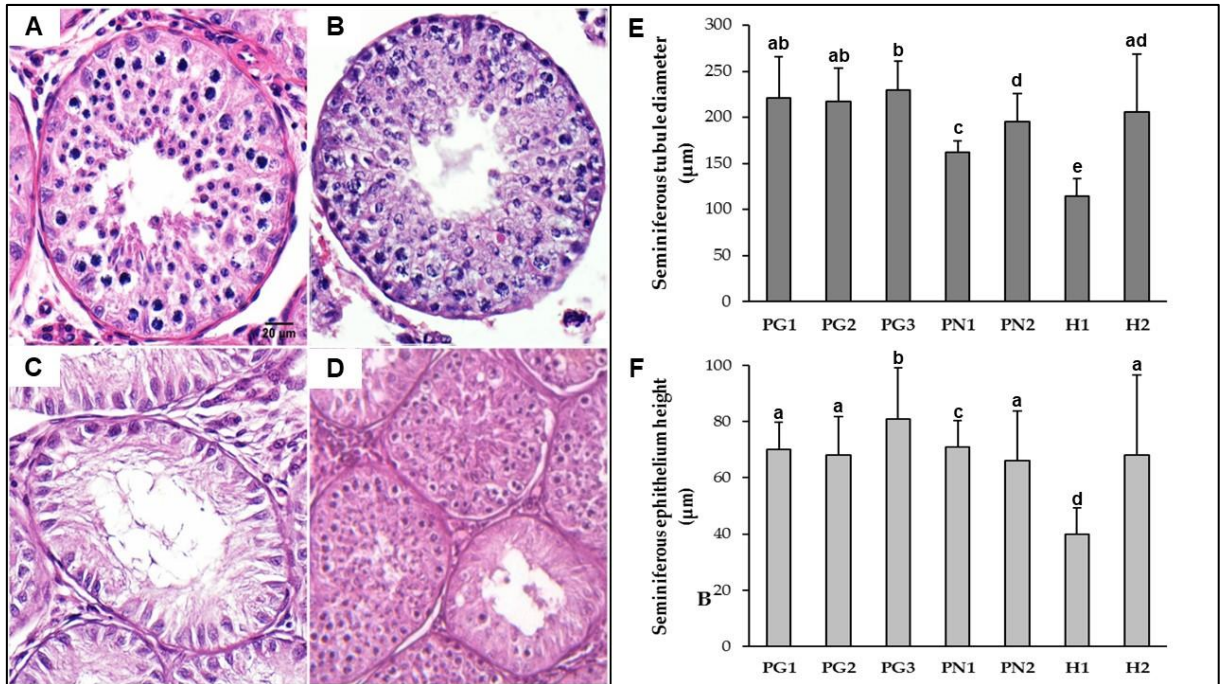


Figure 3. Morphometric differences between *M. gouazoubira* (PG), *M. nemorivaga* (PN) and inter-specific hybrids (H) testes (A – D); Histological sections of testes (scale bar = 20 mm); (A) Fertile PG buck; (B) fertile PN buck; (C) sterile hybrid buck (H1), note hypoplastic aspect of tubules and absence of spermatozoa in tubule lumen; (D) subfertile hybrid buck (H2), note juxtaposition of defective and functional seminiferous tubule cross-sections; (E) mean diameters of seminiferous tubules; and (F) mean seminiferous epithelium height. Columns followed by the same letter do not differ according to the Mann-Whitney U test ( $p < 0.05$ ).

The seminal parameters are presented in Table 4. Overall, despite the species, pure individuals performed better than hybrids in most of the parameters evaluated. Seminal parameters of pure MGO and pure MNE remained rather consistent between both species and within the values of reference for them [39]. Regarding sperm morphology, in general, primary defects were more frequent in most animals. On the other hand, hybrid H1 was azoospermic, while the seminal analysis of hybrid H2 showed a remarkably low concentration, sparse motile sperm cells, and a high percentage of sperm defects (90%). Most of the defects were in the sperm flagellum and head.

Table 3. Mean  $\pm$  standard deviation of the percentages of cell types of the seminiferous epithelium of adult males *M. gouazoubira* (PG), *M. nemorivaga* (PN) and inter-specific hybrids (H).

<b>Animal</b>	<b>Spermatogonia A (%)</b>	<b>Spermatogonia B (%)</b>	<b>Leptotenes/Zygotenes (%)</b>	<b>Pachytene (%)</b>	<b>Round Spermatids (%)</b>	<b>Sertoli Cells (%)</b>	<b>SSR <sup>a</sup></b>
<b>PG1</b>	17.36 $\pm$ 12.70	22.63 $\pm$ 10.49	9.61 $\pm$ 4.30	10.53 $\pm$ 2.75	21.45 $\pm$ 12.34	18.42 $\pm$ 2.91	2.03
<b>PG2</b>	6.34 $\pm$ 2.20	12.67 $\pm$ 7.08	21.42 $\pm$ 22.00	16.16 $\pm$ 5.81	33.40 $\pm$ 15.23	10.01 $\pm$ 2.46	2.06
<b>PG3</b>	11.03 $\pm$ 7.98	12.76 $\pm$ 7.44	16.77 $\pm$ 31.65	14.54 $\pm$ 14.46	33.36 $\pm$ 23.08	11.53 $\pm$ 4.00	2.29
<b>PN1</b>	15.61 $\pm$ 4.69	13.33 $\pm$ 6.81	22.28 $\pm$ 10.90	15.70 $\pm$ 4.43	23.86 $\pm$ 14.72	9.22 $\pm$ 1.43	1.50
<b>PN2</b>	9.42 $\pm$ 7.04	8.22 $\pm$ 2.45	16.38 $\pm$ 13.49	10.47 $\pm$ 5.72	43.95 $\pm$ 20.33	11.56 $\pm$ 4.12	4.19
<b>H1</b>	41.64 $\pm$ 4.55	29.79 $\pm$ 2.39	9.42 $\pm$ 0.00	0.00	0.00	19.15 $\pm$ 3.65	0.00
<b>H2</b>	15.52 $\pm$ 3.39	15.64 $\pm$ 6.82	22.79 $\pm$ 8.36	18.18 $\pm$ 5.23	15.27 $\pm$ 6.17	12.60 $\pm$ 2.01	0,84

<sup>a</sup> Spermatid-to-spermatocyte ratio.

Table 4. Seminal parameters of adult bucks of *M. gouazoubira*, *M. nemorivaga*, and inter-specific hybrids.

Animal	Volume ( $\mu\text{L}$ )	Concentration ( $10^9/\text{mL}$ )	Color	Motility (%)	Vigor (0–5)	Defects (%)		Normal Sperm (%)
						Primary	Secondary	
PG1	375	0.57	White	40	2	37.0	5.0	58.0
PG2	240	3.34	White	65	4	29.5	13.5	57.0
PG3	270	2.32	White	60	3	13.5	13.0	73.0
PN1	375	2.25	Reddish <sup>a</sup>	70	3	35.0	13.5	51.5
PN2	60	2.71	Reddish <sup>a</sup>	90	4	4.0	39.0	57.0
H1 <sup>b</sup>	160	–	Clear	–	–	–	–	–
H2	50	0.02	Watery	<1	0	66.0	24.0	10.0

<sup>a</sup> Considered physiologically normal for the species (CURSINO; DUARTE, 2016). <sup>b</sup> Azoospermic.

### 3.2. *Intra-Specific Hybrids*

FISH with bovine BAC probes specific to the chromosomes involved in translocations was used to assess the fertility of heterozygous and homozygous translocation carriers in MAM. A total of 5000 and 2000 sperm nuclei were scored for RT and TF carriers, respectively. The sperm-FISH technique showed high specificity and sensitivity in red brocket deer sperm nuclei with bovine BAC probes hybridization rates higher than 99% in all cases. Results obtained for the RT and TF are presented in Tables 5 and 6 and summarized below.

Regarding the RT carriers, the meiotic segregation patterns were not significantly different among the homozygous and heterozygous carriers and the control (Table 5). No significant differences were observed between the frequencies of nullisomies and disomies for any one of the analyzed chromosomes).

Regarding the heterozygous TF carriers, their segregation profiles (Table6) were noticeably different when compared to RT cases described above. The MAM hybrids with heterozygous TFs showed lower rates of normal/balanced spermatozoa with a mean frequency of 67.23%, as well as higher rates of adjacent products with a mean frequency of 29.07%. Frequencies of nullisomies and disomies were not different for any one of the chromosomes. Hybrid T343 also shared the same RT presented for the Rondônia cytotype *rob(7;20)*, inherited from its mother.

## 4. DISCUSSION

Several studies on chromosomal polymorphism point out its key role in the formation of gene flow barriers between populations or species and, consequently, in the processes of adaptation and speciation [1–3]. In this context, the presence of chromosome heterozygosity is considered the main factor responsible for the formation of these barriers. Thus, a reduction in the reproductive fitness of the carriers might be caused by a hypothetical probability of errors in meiotic segregation and the formation of unbalanced gametes [1,2]. However, the real impact of these chromosomal rearrangements on the reproductive fitness of carriers and their subsequent impact within a population is not always fully understood. This knowledge

Table 5. Sperm meiotic segregation in Robertsonian translocation carriers of *Mazama americana*, including a non-translocated buck as control.

FISH Phenotype	Robertsonian Translocation (A;B) (%)							
	T269 rob(7;20) <sup>a,d</sup>		T274 rob(5;11) <sup>a,e</sup>		T326 rob(5;11) <sup>b,e</sup>		T297 <sup>c,e</sup>	
<b>Normal/Balanced</b>	4890	(97.80)	4884	(97.68)	4949	(98.98)	4926	(98.52)
Nullisomy A	36	(0.72)	21	(0.42)	18	(0.36)	19	(0.38)
Disomy A	7	(0.14)	10	(0.20)	6	(0.12)	3	(0.06)
Nullisomy B	25	(0.50)	33	(0.66)	8	(0.16)	17	(0.34)
Disomy B	29	(0.58)	19	(0.38)	7	(0.14)	7	(0.14)
<b>Total adjacent</b>	97	(1.94)	83	(1.66)	39	(0.78)	46	(0.92)
Disomy A + B	1	(0.02)	9	(0.18)	0	(0.00)	7	(0.14)
Nullisomy A + B	1	(0.02)	9	(0.18)	6	(0.12)	2	(0.04)
Diploidy	7	(0.14)	10	(0.20)	5	(0.10)	19	(0.38)
Others <sup>f</sup>	4	(0.08)	5	(0.10)	1	(0.02)	0	(0.00)
<b>Total unbalanced</b>	110	(2.20)	116	(2.32)	51	(1.02)	74	(1.48)
<b>TOTAL</b>	5000	(100)	5000	(100)	5000	(100)	5000	(100)

<sup>a</sup>Heterozygous carrier. <sup>b</sup>Homozygous carrier. <sup>c</sup>Non-translocated buck (normal karyotype). <sup>d</sup>Buck analyzed with bovine BAC probes 17C and BAC 25M. <sup>e</sup>Buck analyzed with bovine BAC probes 13T and 19T. <sup>f</sup>Other, less frequent signal combinations.

Table 6. Sperm meiotic segregation in heterozygous tandem fusion carrier hybrids of *Mazama americana* cytotypes, including a non-translocated buck as control.

FISH Phenotype	Tandem Fusion (A;B) (%)							
	T343 der(7;10) <sup>a</sup>		T347 der(7;10) <sup>a</sup>		T421 der(5;10) <sup>b</sup>		T297 <sup>b,c</sup>	
<b>Normal/Balanced</b>	1139	(56.95)	1390	(69.50)	1505	(75.25)	1970	(98.50)
Nullisomy A	328	(16.40)	203	(10.15)	166	(8.30)	6	(0.30)
Disomy A	249	(12.45)	170	(8.50)	144	(7.20)	5	(0.25)
Nullisomy B	124	(6.20)	109	(5.45)	56	(2.80)	8	(0.40)
Disomy B	82	(4.10)	67	(3.35)	46	(2.30)	4	(0.20)
<b>Total adjacent</b>	783	(39.15)	549	(27.45)	412	(20.60)	23	(1.15)
Disomy A + B	18	(0.90)	13	(0.65)	26	(1.30)	4	(0.20)
Nullisomy A + B	11	(0.55)	12	(0.60)	11	(0.55)	3	(0.15)
Diploidy	15	(0.75)	11	(0.55)	12	(0.60)	0	(0.00)
Others <sup>d</sup>	34	(1.70)	25	(1.25)	34	(1.70)	0	(0.00)
<b>Total unbalanced</b>	861	(43.05)	610	(30.50)	495	(24.75)	30	(1.50)
<b>TOTAL</b>	2000	(100)	2000	(100)	2000	(100)	2000	(100)

<sup>a</sup> Buck analyzed with bovine BAC probes 2P and BAC 19T. <sup>b</sup> Buck analyzed with bovine BAC probes 3T and 28M. <sup>c</sup> Non-translocated buck (normal karyotype). <sup>d</sup> Other, less frequent signal combinations.



gap worsens in wild species, where studies on the topic are scarce when compared to reports in domestic species [27,40–45].

Regarding the family Cervidae, the occurrence of chromosomal polymorphisms has been reported throughout the karyotype evolution of several species [7,8]. It is assumed that the ancestral karyotype of this family had 34 pairs of acrocentric autosomes, an acrocentric X, and a small metacentric Y ( $2n = 70$ ;  $FN = 70$ ), given its presence in two species with long phylogenetic distance, such as *Hidropotes inermis* (Old world deer) and *M. gouazoubira* (New world deer) [5,7,8]. Thus, the karyotype evolution in the different genera of the family has been developed mainly by the reduction of the diploid number and the accumulation of chromosomal rearrangements such as inversions, RT or TF, as observed in the evolutionary history of the genus *Mazama* [7–11,46,47].

Hybridization evaluation between species or nearby lineages is one of the best approaches for those seeking to understand the diversification process [48]. In this study, we investigated the effect of chromosomal rearrangements on the fertility of hybrids between cytotypes of the same lineage (MAM) and between different species (MGO x MNE), to determine how these chromosomal polymorphisms could act as an effective barrier to genetic flow during parapatric or sympatric speciation in the genus *Mazama*.

#### 4.1. *Inter-Specific Hybrids*

The sterility observed in hybrid animals is a way to irreversibly accelerate genetic divergences, preventing free gene flow between genetically different populations [49]. Traditionally, hybrid sterility is attributed to genetic incompatibilities between parental species, whether of chromosomal or genetic origin [50]. Although in most animals, incompatibilities mediated by deleterious interactions between genes are considered the primary cause of hybrid inaptitude (Dobzhansky-Müller model). The results of cytogenetic analyzes of the MGO x MNE hybrids most likely indicate that the occurrence of post-zygotic reproductive isolation between MGO and MNE is probably linked to numerical and structural chromosomal differences. These differences lead to

the accumulation of heterozygous chromosomal rearrangements in the hybrids and may trigger anomalous pairing during meiosis, resulting in gametogenesis failures and unbalanced gamete production [51].

Even though inter-individual variation among animals was evident, in general, all seminal, morphological, and most histological reproductive parameters observed in pure animals (PG1, PG2, PG3, PN1, and PN2) were superior to those obtained for hybrids, being within expected for their respective species [39,52]. In contrast, evidence of fertility reduction varied between the hybrids, showing different effects of chromosomal differences found between the parent's karyotypes.

The effect of the chromosomal rearrangements accumulation on hybrid reproductive fitness was especially evident in H1, with a rob(4;32) and a submetacentric X inherited from the mother (MNE). In its spermiogram, this animal demonstrated complete interruption of spermatogenesis, which was reflected in azoospermia. In this case, presumed sterility could be attributed to multiple chromosomal pairing failures during meiosis, getting worse when differences between parent karyotypes are greater [53]. Thus, the H1 karyotype ( $2n = 69 + 0 - 3 Bs$ ) was the most discrepant concerning the pattern of parental species among the analyzed MGO x MNE hybrids.

In most cases of hybrid sterility, associations between cell death and meiosis occur between pachytene and spermiogenesis, which results in high attrition rates in the pachytene of meiosis I [54]. Similar patterns in the histological analysis of H1 cell types suggested spermatogenic interruption. Moreover, the total hypoplasia of seminiferous tubules observed in H1, frequently described in infertile hybrids [55–57], is a direct consequence of the spermatogenesis interruption during meiosis I. The absence of differentiated germ cells results in a decrease in tubular diameter and height of seminiferous epithelium, aspects that in H1, obtained the lowest averages among all animals analyzed. Similar conformations have been reported in other hybrid forms such as donkeys [58], rats [57], and within the MAM cytotype complex itself [25].

Despite having obtained better performance than H1 in all reproductive analyzes, mostly functional tubular structure, and no chromosomal translocation, the fertility of the H2 hybrid was also severely affected by chromosomal differences. The severe subfertility showed by H2 reinforces the importance of the role of chromosomes

in the process of reproductive isolation, even when the rearrangements are not so apparent. Seminal analysis of this animal revealed an ejaculate with extremely low volume and concentration, irrelevant motility, and a high prevalence of sperm defects. This low seminal quality is the result of a series of structural, pathological, and functional changes at the testicular level: H2 showed hypoplasia in part of its seminiferous tubules and the presence of cells with a pycnotic nucleus and epithelial vacuolization, suggestive of the occurrence of apoptosis in both functional and hypoplastic tubules. Moreover, H2 also showed a low conversion rate between spermatids and spermatozoa (SRR = 0.84) when compared with pure animals. All of this evidence points to the loss of germinal epithelium and cell degeneration, typically found in hybrid forms [57,59].

Finally, it is worth remembering that although the presence of sperm in a hybrid ejaculate has been described in several inter-specific crossbreeding [55,59–62], it does not guarantee its fertility. Chromosomal non-disjunction during anaphase I is the second leading cause of reduced fertility in these animals since heterozygous configurations of hybrids undergo an anomalous separation process leading to the formation of unbalanced gametes (aneuploidy) and non-viable embryos [59]. Thus, it is likely that, similar to what was observed in intra-specific MAM hybrids in this study, future FISH analysis of H2 also reveals a high rate of unbalanced gametes.

Since the pre-zygotic reproductive barrier between MNE and MGO is fragile [63], the post-zygotic barrier for sterility of the hybrid seems to keep these two species isolated and evolving independently. Even with wide geographical contact between the Amazon (MNE habitat) and the Cerrado (MGO habitat) for more than 2000 km.

#### 4.2. *Intra-Specific Hybrids*

A previous study carried out in MAM, demonstrated that hybrids with the presence of heterozygous TF presented seminal parameters similar to those presented by pure animals of the different lineages (volume: 270  $\mu$ L vs. 135  $\mu$ L; motility: 75% vs. 77.5%; concentration: 2.22 sptz x 10<sup>9</sup>/mL vs. 3.81 sptz x 10<sup>9</sup>/mL; and pathologies: 47.25% vs. 30%, for heterozygous TF hybrids and pure animals, respectively) [25]. Thus, the fertility of the hybrids could not be defined or ruled out,

which is why they were considered subfertile. Because of this, we decided to perform the technique of sperm-FISH to estimate the proportion of normal/balanced and unbalanced spermatozoa in bucks with heterozygous rearrangements and animals from crossbreeding between cytotypes of the same lineage in MAM. The proportion of meiotic products from adjacent segregation modes in RT carriers analyzed in this study is consistent with reports for domestic species such as bulls, boars and mice (2.58 – 5.42%, 3.16%, and 8 – 11.5%, respectively) [64–67]. These findings may suggest a low negative effect on the reproductive fitness of heterozygous carriers of RT reported here for MAM, unlike that reported for several RT in humans where there is a wide variation in reproductive impact (0.2 – 49.1% of adjacent segregation products) [68].

Our results are in agreement with a previous report focused on the synaptonemal complex analysis of the same heterozygous *rob(7;20)* carrier (T269) [27], where results suggested a highly unlikely formation of unbalanced gametes for this RT. Similar results for synaptonemal complex analysis focused on the effect of centromeric fusion on meiosis and reproduction of cattle, goitered gazelle, and impala have been reported [40,69,70]. In fact, the NUPECCE's breeding records indicate that this *rob(7;20)* carrier was used for breeding purposes and produced 4 fawns, not exhibiting any obvious re-productive impairment. Regarding the *rob(5;11)*, the heterozygous carrier produced a non-translocated female fawn, also suggesting no reproductive impairment. On the other hand, no reproductive records were available for the homozygous carrier. However, our findings suggest that the homozygous translocation could offer greater stability during the meiotic segregation, not affecting its reproductive fitness, and showing a meiotic segregation pattern similar to the control values. The presence of homozygous translocation suggests a possible fixation of this chromosomal polymorphism in free-living populations, opening the possibility of future speciation processes. However, our results on the meiotic segregation patterns of carriers, both homozygous and heterozygous *rob(5;11)*, would suggest an apparent gene flow between these populations. Thus, every single RT must be assessed to understand its potential effect on the reproductive fitness of the carriers. Errors in meiosis are the result of the behavior of those chromosomes involved in the translocation and their trivalent during the first meiotic segregation [40,68].

In this study, we also analyzed three heterozygous TF carriers produced in captivity between *Mazama americana* cytotypes of the same chromosomal lineage (n = 2, Rondônia x Juína cytotypes; n = 1, Carajás x Paraná cytotypes) [10]. Although TF are chromosomal rearrangements present in the evolutionary history of cervids, they have been previously related with reduction in fertility in animal [71–73]. A previous study reported subfertile male hybrids from MAM cytotypes of the same chromosomal lineage and azoospermic hybrids from different chromosomal lineages [25]. Azoospermia was attributed to the great karyotypic differences, a meiotic arrest in spermatocyte stage, and errors in meiotic segregation for hybrids between different lineages, providing an adequate post-zygotic reproductive barrier and suggesting the presence of different species [25]. In this study, MAM hybrids heterozygous for TF showed the highest rate of unbalanced spermatozoa of all analyzed *Mazama* males. This can explain the previously reported subfertility of Rondônia x Juína hybrids, carrying a heterozygous TF, which did not show any significant compromise in seminal quality or testicular histology [25].

Also, it is important to mention that hybrid T343 also carried a heterozygous rob(7;20), which might have increased the errors in meiotic chromosome pairing, leading to a greater error in meiotic segregation in this buck. Thus, the red brocket male T269 only heterozygous for the rob(7;20), or hybrid T347 only heterozygous for the der(7;10), showed unbalanced spermatozoa rates of 2.20 and 30.50%, respectively. Regarding hybrids T347 and T421, our data presented about 70% balanced gametes suggesting a subfertility status, similar to Salviano et al. (2017) and contrasting the estimates of 50% aneuploid gametes made by White et al. (1967) [74] for heterozygous TF. However, if we consider a hypothetical 1:1 ratio between gametes carrying or not the TF, we would have a frequency of 35% for each phenotype. This will be, only a 35% chance of successful reproduction in a backcross of the T347 hybrid with a female of cytotype Rondônia or Juína, and the T421 hybrid with a female of cytotype Carajás or Paraná, suggesting virtual sterility of the hybrids similar to the *Otomys irroratus* case [73], and dismissing the previous description of subfertility for hybrids carrying heterozygous TF in MAM, made by Salviano et al. (2017) [25].

We report the first production of hybrids between MGO and MNE, which were viable until maturity, but presumably infertile. There are no reports of hybrids in the wild, although a weak pre-mating isolation barrier between species has been observed in captivity [63]. Regarding MAM, reports of captive crossbreeding between cytotypes already exist [25,75], which are explained by the verified lack of a clear pre-mating barrier [26]. However, it is difficult to say that this can happen in nature, despite the geographical proximity between MGO and MNE, as well as between the MAM cytotypes. Therefore, there is a clear need for a better understanding of chromosomal polymorphisms between species and intra-specific populations to elucidate their role in forming barriers to gene flow within the genus *Mazama*, the isolation from former populations, and subsequent adaptation/speciation. Moreover, meiotic segregation assessment in hybrids and carriers of heterozygous chromosomal translocations is presented as a mandatory tool for estimating the impact of chromosomal polymorphisms in both the reproductive fitness of carriers and in *Mazama* speciation processes. Thus, leaving the morphological evaluation of the gametes as a complementary assessment.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2073-4425/12/2/165/s1>, Table S1: List of bovine BAC clones used in the present study for detection of bovine (*Bos taurus* – BTA) homologies with brocket deer chromosomes involved in translocations and for sperm-FISH.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee on Animal Use of the School of Agricultural and Veterinarian Sciences, São Paulo State University (approval No. 000180/11 and 001930/18). The biological material for tissue culture, semen analysis and testicular histology was obtained by a veterinarian during medical examination of the animals.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the article and supplementary material.

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## SUPPLEMENTARY MATERIAL

**Table S1.** List of bovine BAC clones used in the present study for detection of bovine (*Bos taurus* – BTA) homologies with brocket deer chromosomes involved in translocations and for sperm-FISH.

Chromosome region		Cattle location (Mb)	BAC clone (probe name)
Cattle	<i>Mazama</i>		
		9.510 – 9.718	CH240-42D15 (BAC 2C)
BTA 2	MAM-JU 10	91.323 – 91.543	CH240-186F21 (BAC 2P)
		135.511 – 135.718	CH240-437C7 (BAC 2T)
BTA 3	MAM-PA 5	120.614 – 120.777	CH240-250C11 (BAC 3T)
		120,807 – 120.9.2	CH240-106D7 (BAC 3T)
BTA 13	MAM-CA 5	74.679 – 74.851	CH240-332D20 (BAC 13T)
		75.027 – 75.262	CH240-278J11 (BAC 13T)
BTA 17	MAM-RO 7	3.909 – 4.129	CH240-63H8 (BAC 7C)
		4.140 – 4.334	CH240-175F4 (BAC 7C)
		9.123 – 9.343	CH240-130E10 (BAC 19C)
BTA 19	MAM-JU 7	34.353 – 34.575	CH240-50L8 (BAC 19M)
	MAM-CA 11	55.619 – 55.852	CH240-106P5 (BAC 19T)
		55.911 – 56.097	CH240-188A20 (BAC 19T)
BTA 25	MAM-RO 20	21.271 – 21.511	CH240-89A17 (BAC 25M)
BTA 28	MAM-PA 10	24.741 – 24.943	CH240-108O21 (BAC 28M)

BTA = *Bos taurus*; MAM = *Mazama americana*; JU = Juína cytotype; PA = Paraná cytotype; CA = Carajás cytotype; RO = Rondônia cytotype.