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Análise Evolutiva da Família *Musashi*: Possíveis Implicações para a Zika

Gabriela Barreto Caldas Garcia

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Orientadora: Profa. Dra. Maria Cátira Bortolini

Coorientadora: Profa. Dra. Vanessa Rodrigues Paixão-Côrtes

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À minha família.

“Evolução não é uma força,

mas um processo: não uma causa, mas uma lei.”

(John Morley, *On Compromise*, 1874)

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Sumário

Abreviaturas

BLAST: *Basic Local Alignment Search Tool*

CDC: *Centers for Disease Control and Prevention* / Centros de Controle e Prevenção de Doenças

CZS: *Congenital Zika Syndrome* / Síndrome Congênita da Zika

DAZAP1: *DAZ Associated Protein 1* / DAZ proteína associada 1

DNA: Ácido Desoxirribonucleico

Fox-1 (RBFOX1): *RNA Binding Fox-1 Homolog 1*

Fox-2 (RBFOX2): *RNA Binding Fox-1 Homolog 2*

GBS: *Guillain-Barré Syndrome* / Síndrome de Guillain-Barré

HNRNPAB: *Heterogeneous Nuclear Ribonucleoprotein A/B* / Ribonucleoproteína nuclear heterogênea A/B

HNRNPD: *Heterogeneous Nuclear Ribonucleoprotein D* / Ribonucleoproteína nuclear heterogênea D

HNRNPDL: *Heterogeneous Nuclear Ribonucleoprotein D Like* / Ribonucleoproteína nuclear heterogênea D-like

HNRNPH1: *Heterogeneous Nuclear Ribonucleoprotein H1* / Ribonucleoproteína nuclear heterogênea H1

HNRNPK: *Heterogeneous Nuclear Ribonucleoprotein K* / Ribonucleoproteína nuclear heterogênea K

HNRNPM: *Heterogeneous Nuclear Ribonucleoprotein M* / Ribonucleoproteína Nuclear Heterogênea M

HNRNPU: *Heterogeneous Nuclear Ribonucleoprotein U* / Ribonucleoproteína nuclear heterogênea U

MEGA: *Molecular Evolutionary Genetics Analysis*

mRNA: RNA mensageiro

MSI1: Musashi 1

MSI2: Musashi 2

MUSCLE: *Multiple Sequence Comparison by Log-Expectation*

NCBI: *National Center for Biotechnology Information*

NWMs: New World Monkeys / Macacos de Novo Mundo

PAHO: *Pan American Health Organization* / Organização Panamericana de Saúde

PAML 4.9: P*hylogenetic analysis by maximum likelihood, version* 4.9 / Análise filogenética por máxima verossimilhança, versão 4.9

PCBP2: *Poly(RC) Binding Protein 2* / Proteína de ligação Poly(RC) 2

pré-mRNA: pré RNA mensageiro

PTBP2: *Polypyrimidine Tract Binding Protein 2* / Protéina de ligação ao trato de polipirimidina 2

RBD1: *RNA-binding domain 1* / Domínio 1 de ligação ao RNA

RBD2: *RNA-binding domain 2* / Domínio 2 de ligação ao RNA

RBPs: *RNA-binding proteins* / Proteínas de ligação ao RNA

RNA: Ácido ribonucleico

SRSF1: *Serine and Arginine Rich Splicing Factor 1* / Fator de *splicing* rico em arginina e serina 1

SRSF10: *Serine and Arginine Rich Splicing Factor 10* / Fator de *splicing* rico em arginina e serina 10

SRSF11: *Serine and Arginine Rich Splicing Factor 11* / Fator de *splicing* rico em arginina e serina 11

SRSF3: *Serine and Arginine Rich Splicing Factor 3* / Fator de *splicing* rico em arginina e serina 3

SRSF6: *Serine and Arginine Rich Splicing Factor 6* / Fator de *splicing* rico em arginina e serina 6

SRSF7: *Serine and Arginine Rich Splicing Factor 7* / Fator de splicing rico em arginina e serina 7

STAT2: *Signal Transducer And Activator Of Transcription 2* / Transdutor de Sinal e Ativador da Transcrição 2

TARDBP: *TAR DNA Binding Protein* / Proteína de ligação TAR-DNA

TRA2A: *Transformer 2 Alpha Homolog*

WHO/OMS: *World Health Organization* / Organização Mundial da Saúde

ZIKV: Zika vírus

Resumo

Apesar dos esforços de pesquisas ao redor do mundo desde 2016, as consequências da infecção pelo vírus Zika (ZIKV) ainda não são completamente compreendidas. Inicialmente, a Zika era considerada uma doença leve, semelhante à gripe, endêmica da África. Repentinamente, a doença espalhou-se para outras áreas tropicais através de picadas do mosquito vetor, relações sexuais e durante a gravidez, da mãe para o feto. Em 2015, o nordeste brasileiro descreveu os primeiros casos de microcefalia em recém-nascidos que foram expostos ao ZIKV dentro do útero. Atualmente, sabe-se que a microcefalia é apenas um dos desfechos possíveis após a infecção pelo ZIKV durante os primeiros estágios de vida. Musashi 1 (MSI1) é uma proteína de ligação ao RNA que participa da regulação pós-transcricional através do reconhecimento de sítios específicos presentes nos transcritos-alvo. Além disso, Musashi 1 que está envolvida em processos de neurodesenvolvimento, é também utilizada pelo ZIKV (RNA de fita simples, senso positivo) para sua replicação. Neste trabalho, realizou-se uma análise evolutiva da sequência codificadora da MSI1 e seus parálogos em vertebrados. 16 espécies de macacos de Novo Mundo (clado Platyrrhini), conhecidas por terem altas taxas evolutivas, foram adicionadas à análise através do sequenciamento de regiões de interesse. A família Musashi inclui MSI2, TARDBP, DAZAP1, HNRNPD, HNRNPDL e HNRNPAB, que aparentemente não interagem com o ZIKV, mas são proteínas de ligação ao RNA importantes que atuam em diversos processos regulatórios ubiquamente. Todos os primatas sequenciados apresentaram o domínio 1 de ligação ao RNA do *MSI1* totalmente conservado. Similarmente, as sequências dos genes da família Musashi encontram-se sob seleção purificadora (valores $\omega < 1$). No entanto, a evolução dos mecanismos regulatórios, principalmente o *splicing* alternativo, parece ser mais dinâmica entre os vertebrados. Existem diferentes isoformas que diferem na região N-terminal e afetam o tamanho da proteína dos animais. Entretanto, como a sequência da principal isoforma que contém dois domínios de ligação ao RNA é preservada, mesmo entre os primatas de Novo Mundo, nós sugerimos que o ZIKV é capaz de interagir com a MSI1 de todos os primatas analisados. Esse fato sinaliza que o vírus pode se replicar nesses potenciais

hospedeiros silvestres, ao menos no que depender da MSI1. Portanto, propõe-se que o ZIKV pode estabelecer um ciclo silvestre fora da África, pois os primatas de Novo Mundo e outros mamíferos provavelmente podem suportar a sua manutenção em áreas onde mosquitos infectados circulam.

Palavras-chave: Zika, Musashi 1, Macacos de Novo Mundo, Síndrome Congênita da Zika, Evolução

Abstract

Despite worldwide research efforts in the last three years, Zika virus infection and its consequences are not fully understood yet. Zika was firstly considered a mild, flu-like disease endemic to Africa. Suddenly, it has shown its high capability to spread to other tropical areas through mosquito bites, sexual activity and from mother to fetus. In 2015, the Brazilian Northeast described the first cases of microcephaly in newborns caused by ZIKV intrauterine exposure. Nowadays, it is known that microcephaly is only one of the possible outcomes of being infected by ZIKV during the early stages of life. Musashi 1 (MSI1) is a RNA-binding protein that participates in post-transcriptional regulation through the recognition of specific binding sites present in transcribed sequences. It is known that MSI1 is involved in neurodevelopmental processes. In addition, MSI1 interacts with the ZIKV genome (a single-stranded positive-sense RNA) and allows its replication. Here we perform an evolutionary analysis of MSI1 code sequence and their paralogs in vertebrates. We added sixteen New World Monkey species (NWMs), known to have higher evolutionary rates, to this analysis by sequencing the region of interest. The Musashi family includes MSI2, TARDBP, DAZAP1, HNRNPD, HNRNPDL, and HNRNPAB, which apparently do not interact with the virus, but are important RNA-binding proteins that act on many regulatory processes ubiquitously. We found that all sixteen primate species have the RNA-binding domain 1 of MSI1 totally conserved. Whilst the general sequences of Musashi family are under purifying selection (ω values < 1), the evolution of regulatory mechanisms, especially alternative splicing, seem to be more dynamic among vertebrates. There are different isoforms that differ at N-terminal region and affect the protein size of animals. However, as the principal isoform that contains two RNA-binding domains is preserved, even amongst NWMs, we suggest that ZIKV can interact with MSI1 of all primates analyzed. This fact signals that ZIKV can replicate in these potential wild hosts, at least in what depends on MSI1. Thus, we propose that ZIKV may establish a sylvatic cycle since NWMs and other mammals probably can support its maintenance in areas where infected mosquitoes surround.

Keywords: Zika, Musashi 1, New World Monkeys, Congenital Zika Syndrome, Evolution

1. Introdução

1.1. Zika Vírus: Infecção e Síndrome Congênita

Inicialmente considerada uma infecção leve, a Zika é caracterizada por sintomas semelhantes aos da dengue, como febre, dor nas articulações, erupção cutânea, dores de cabeça, dores musculares e conjuntivite (CDC, 2017). No entanto, cerca de 80% das pessoas infectadas desenvolvem a doença de maneira assintomática (Klase et al., 2016, Oeser & Ladhani, 2018).

A transmissão do vírus em adultos ocorre principalmente através da picada de mosquitos vetores do gênero *Aedes* (*A. aegypti* e *A. albopictus*) infectados (Nugent et al., 2016; Huerta et al., 2017), embora pesquisadores tenham relatado que mosquitos *Culex quinquefasciatus* portadores do vírus também são potenciais transmissores (Guedes et al., 2017). Alternativamente, a Zika pode ser transmitida por transfusão de sangue, relação sexual e, para fetos, via placenta (Chan, Choi, Yip, Cheng, & Yuen, 2016). O vírus pode ser detectado na urina, saliva, lágrimas e leite materno de indivíduos infectados, mas o potencial de transmissão associado a esses fluidos corporais ainda é desconhecido e pouco estudado (Newman et al., 2017; Zuanazzi et al., 2017). Newman e cols. sugerem que o risco de transmissão pelo contato com secreções orais é baixo (Newman et al., 2017). Em revisão recente, a possibilidade de transmissão via mordidas de animais, amamentação ou durante o parto permanece inconclusiva (Runge-Ranzinger, Morrison, Manrique-Saide, & Horstick, 2019).

O vírus Zika (ZIKV) foi isolado pela primeira vez em 1947, em Uganda, de um macaco rhesus (*Macaca mulatta*) sentinela da floresta Zika. O primeiro caso da infecção descrito em humanos, por sua vez, foi registrado alguns anos mais tarde, na Nigéria em 1954. Durante os anos anteriores a 2007, quando ocorreu o primeiro grande surto da doença na ilha do pacífico Yap, Micronesia, a dispersão do vírus no continente africano foi considerada baixa. Antes de chegar ao Brasil, entre 2013 e 2014, o vírus havia causado surtos em quatro outras ilhas do pacífico – Polinésia Francesa, Ilha de Páscoa, Ilhas Cook e Nova Caledônia (WHO, 2017).

No primeiro semestre de 2015, o Brasil informou à Organização Mundial da Saúde (OMS) aproximadamente 7.000 casos de Zika (Kindhauser, Allen, Frank, Santhana, & Dye, 2016). Ainda em 2015, ocorreu a associação da infecção à Síndrome de Guillain-Barré (GBS; PAHO-WHO, 2015). Essa condição autoimune incomum do sistema nervoso faz com que as células nervosas sejam atacadas, o que resulta em fraqueza muscular e, às vezes, paralisia (CDC, 2017).

Em 2016, de acordo com o Ministério da Saúde, foram contabilizados 130.701 casos de Zika no país (Ministério da Saúde, 2017). Em fevereiro do mesmo ano, foi confirmada a associação de casos de microcefalia e outras desordens neurológicas à infecção pelo vírus ZIKV (Kindhauser, Allen, Frank, Santhana, & Dye, 2016). A partir dessa constatação, a OMS declarou a infecção pelo ZIKV uma emergência de saúde pública de preocupação internacional até 18 de novembro de 2016 (WHO, 2016). Nos anos posteriores houve uma queda no número de infecções registradas no Brasil, 17.593 em 2017 e 8.024 em 2018 (Ministério da Saúde, 2018). Até o último relatório emitido pela OMS, datado em 10 de março de 2017, 84 países tinham anunciado evidência de transmissão da Zika pelo mosquito vetor. Outros 64 países registraram a presença do vetor, mas sem evidência de transmissão constatada (WHO, 2017).

As malformações congênitas que foram associadas ao ZIKV inicialmente no nordeste do Brasil (Eickmann et al., 2016) foram: microcefalia severa, diminuição do tecido cerebral, danos à parte posterior dos olhos, excesso de tônus muscular e juntas com movimento de alcance limitado (Eickmann et al., 2016; CDC, 2017). As ocorrências desses defeitos congênitos transformaram a infecção em um importante tema de pesquisa mundial, pois o ZIKV foi considerado um novo agente teratogênico humano (Schüler-Faccini et al., 2016). Enquanto não há vacinas e tratamentos antivirais disponíveis, a prevenção da infecção, o combate do vetor, uso de métodos contraceptivos e planejamento familiar são essenciais no controle dos casos (Schüler-Faccini et al., 2016).

Segundo a Organização Panamericana de Saúde, vinculada à OMS, casos da síndrome congênita associada ao ZIKV foram registrados em 26 países e territórios no continente Americano desde outubro de 2015 até 26 de julho de

2017. Brasil, Colômbia, Costa Rica, Equador, Guadalupe, Guatemala, Guiana Francesa, Martinica, México, Panamá, Porto Rico, Ilha de São Martinho e Estados Unidos da América tiveram registros até a data final supracitada (PAHO-WHO, 2017). Além de casos registrados na América, outros países ao redor do mundo também relataram casos de microcefalia – Cabo Verde, Tailândia, Polinésia Francesa, Ilhas Marshall e Vietnã. Espanha e Eslovênia tiveram casos de microcefalia, porém a infecção das mães pelo vírus provavelmente ocorreu na América (WHO, 2017). É importante considerar que nem todos os bebês nascidos após infecção da mãe pelo ZIKV apresentaram a síndrome e, também, que alguns recém-nascidos desenvolveram a microcefalia, e outras malformações relacionadas, somente após o nascimento (CDC, 2017). Em dezembro de 2018, foram confirmados 3.279 casos da chamada “Congenital Zika Syndrome” (CZS) no Brasil (Ministério da Saúde, 2018).

Três anos após o nascimento dos primeiros bebês afetados pela CZS, definiu-se um amplo espectro de fenótipos resultantes da infecção além da microcefalia inicialmente observada. Estima-se que entre 1 a 13% dos fetos em contato intrauterino com o ZIKV apresentem malformações físicas no momento do nascimento (G. V. A. França et al., 2016; Nem de Oliveira Souza et al., 2018). No entanto, outras manifestações clínicas podem aparecer nos meses ou anos posteriores, em bebês expostos ao vírus, sejam eles afetados com a microcefalia ao nascer ou não (van der Linden et al., 2016; Cardoso et al., 2018). Merece nota que bebês infectados durante a gestação e nascidos com o tamanho da cabeça normal podem desenvolver microcefalia pós-natal (Silva et al., 2016; van der Linden et al., 2016).

Além disso, outros problemas podem acometer fetos expostos ao ZIKV: comprometimento motor, artrogripose, convulsões e epilepsia (Pessoa et al., 2018); disfagia, pé torto, síndrome piramidal (espasmos musculares, hipertonia, hiperreflexia e reflexos primitivos aumentados), calcificações cerebrais, malformações corticais, hipoplasia (do cerebelo, tronco cerebral e corpo caloso), ventriculomegalia secundária (Silva et al., 2016; van der Linden et al., 2016; Cardoso et al., 2018), menores taxas de crescimento da circunferência cefálica e de ganho de peso (França et al., 2018); desordens oculares (de Paula Freitas et

al., 2016; Ventura et al., 2016) e auditivas (Leal et al., 2016; Peloggia, Ali, Nanda, & Bahamondes, 2018). Além destas, alterações neurocomportamentais possíveis incluem irritabilidade, déficit cognitivo, problemas de memória e interação social; risco de autismo e esquizofrenia – esses dois últimos sugeridos por Nem de Oliveira Souza *et al*. em estudo de camundongos expostos ao ZIKV e acompanhados até a idade adulta. (Nem de Oliveira Souza et al., 2018; Paul et al., 2018; Saad et al., 2018).

1.2. Animais Modelos

Espécies de animais que são reservatórios para o vírus ainda não estão bem determinadas. No entanto, primatas não humanos como o macaco rhesus (*Macaca mulatta),* macaco vervet *(Cercopithecus aethiops),* macaco de cauda vermelha *(Cercopithecus ascanius schmidti),* macaco mona *(Cercopithecus mona denti),* macaco mangabei *(Cercopithecus albigena johnstoni),* macaco-verde *(Chlorocebus sabaeus),* macaco colobus (*Colobus abyssinicus),* macaco-pata (*Erythrocebus patas)* e orangotango-de-bornéu (*Pongo pygmaeus),* além de outros mamíferos – ovelhas, cabras, zebras, cavalos, vacas, elefantes, roedores, morcegos e búfalos – e o pato doméstico têm sido sugeridos como possíveis hospedeiros com base na detecção de anticorpos e/ou identificação do vírus nesses organismos (Chan et al., 2016; Vorou, 2016).

O ZIKV infecta células embrionárias humanas progenitoras do córtex neural, prejudica a sua proliferação, induz morte celular e evidencia que neurônios humanos são susceptíveis à infecção (Tang et al., 2016, Cugola et al.,2016). Além disso, o vírus pode infectar e danificar o sistema nervoso periférico humano, o que pode ocasionar a GBS em indivíduos adultos (Cao-Lormeau et al., 2016; Parra et al., 2016). Menos comumente, a infecção pode evoluir para um quadro de encefalite (Ugarte et al., 2017). A infecção também causa efeitos em modelos animais. O camundongo (*Mus musculus*) “nocauteado” para Interferon α/β (o camundongo selvagem não desenvolve a doença) apresenta alta vulnerabilidade à infecção com perda de peso, sinais de doenças neurológicas (fraqueza e paralisia dos membros posteriores) e, por fim, morte. Há aumento da carga viral

principalmente no cérebro, corda espinhal e testículos (Lazear et al., 2016). Grant et al. mostraram que o STAT2 humano (fator de transcrição regulado pelo interferon) é degradado pela expressão da proteína NS5 do ZIKV, mas o STAT2 de camundongos não é vulnerável a este mecanismo, o que pode explicar a necessidade da deficiência de interferon no camundongo para que a doença provocada pelo vírus seja evidenciada (Grant et al., 2016).

Nesses camundongos nocauteados, há infecção da placenta e do cérebro do filhote, no início da gestação, com desfecho em uma síndrome congênita que se assemelha à restrição de crescimento intrauterino e aborto espontâneo observado em mulheres grávidas afetadas pelo ZIKV (Miner et al., 2016). Filhotes de camundongos da linhagem SJL infectados pelo vírus também apresentaram malformações congênitas similares às dos recém-nascidos humanos. Entretanto, filhotes da linhagem C57BL/6 de camundongos infectados não apresentaram alterações corporais. Causas que expliquem a incapacidade do vírus de atravessar a placenta dos camundongos C57BL/6 não estão claras, mas este resultado pode ser devido à resposta imune robusta característica desta linhagem, que secreta níveis significantes de interferon dos tipos I e II, possibilitando assim, resistência ao vírus. Esses resultados sugerem que diferenças genéticas poderiam explicar, em parte, o nascimento de alguns bebês sem malformações congênitas detectáveis, mesmo após a infecção de suas mães (Cugola et al., 2016).

O macaco rhesus (*Macaca mulatta*) e o macaco rabo-de-porco (*Macaca nemestrina*) são vulneráveis à doença, com RNA viral detectado na urina, saliva, sangue, sêmen, fluidos vaginais e fluido cerebrospinal (Adams Waldorf et al., 2016; Dudley et al., 2016). O porco (*Sus scrofa domesticus*) e o porquinho-da-índia (*Cavia porcellus,* linhagem *Dunkin-Hartley*) desenvolvem a Zika sintomaticamente com fraqueza nas pernas, ataxia, tremor; e febre, letargia, corcunda, pelo arruinado, diminuição da mobilidade, respectivamente (Darbellay et al., 2017; Kumar, Krause, Azouz, Nakano, & Nerurkar, 2017). Em outro estudo, o porquinho-da-índia (*Cavia porcellus*, linhagem '*strain 13*') não apresentou viremia detectável e poucos indivíduos desenvolveram anticorpos neutralizantes ao ZIKV (Miller et al., 2018). O hamster-sírio (*Mesocricetus auratus*) nocauteado

para o *STAT2* apresenta morbidade e mortalidade elevadas quando são infectados pelo vírus, no entanto, seus filhotes aparentemente não tiveram restrição de crescimento intrauterino ou microcefalia (Siddharthan et al., 2017). O macaco cinomolgo (*Macaca fascicularis*) e o mico (*Callithrix jacchus*) analisados demonstraram susceptibilidade à infecção, porém, de maneira assintomática, com detecção do vírus nos fluidos corporais, linfonodos, entre outros (Koide et al., 2016; Osuna et al., 2016; Chiu et al., 2017).

As espécies que apresentam malformações congênitas, desordens neurológicas e/ou microcefalia, além da humana e do camundongo (nocauteado para os receptores de Interferon α e β), são o macaco-rabo-de-porco (*Macaca nemestrina)*, a galinha (*Gallus gallus*) (Adams Waldorf et al., 2016; Goodfellow et al., 2016), o porco doméstico (*Sus scrofa*) (Schreur, Van Keulen, Anjema, Kant, & Kortekaas, 2018), furão (*Mustela putorius furo*) (Hutchinson et al., 2019), babuíno-anúbis (*Papio anubis)* (Gurung et al., 2019) e sagui-de-tufos-brancos (*Callithrix jacchus*) (Seferovic et al., 2018). Os demais organismos até então permanecem indeterminados quanto à susceptibilidade às malformações congênitas provocadas pela Zika.

Outro possível resultado à exposição ao ZIKV que tem se mostrado frequente nos estudos é a morte fetal, aborto ou ocorrência de natimorto. Fetos/filhotes de sagui-de-tufos-brancos, macaco rhesus, babuíno-anúbis, hamster-sírio, galinha, furão e camundongos não resistiram à infecção, similar ao que também pode acontecer em bebês humanos (Goodfellow et al., 2016; Lazear et al., 2016; Dudley et al., 2018; Seferovic et al., 2018; Szaba et al., 2018; Gurung et al., 2019; Hutchinson et al., 2019).

Há muito a ser esclarecido sobre a transmissão vertical do vírus, pois, em gravidez de gêmeos dizigóticos, foi constatado que o ZIKV afetou com a síndrome somente um dos fetos. Em contrapartida, o irmão gêmeo apresentou exame clínico normal, com ausência do vírus nos testes de sangue e do líquido cérebro-espinhal (Linden et al., 2017). Apesar dos esforços de pesquisas realizados durante os três anos após a epidemia, os mecanismos moleculares que causam a CZS ainda são pouco compreendidos.

1.3. A Família Gênica Musashi e o ZIKV

A família Musashi é um grupo evolutivamente conservado de proteínas que se ligam ao RNA e participam da regulação pós-transcricional a partir do reconhecimento de sítios de ligação específicos presentes nas sequências transcritas (Okano et al., 2005; Okano, Imai, & Okabe, 2002). A classe de proteínas de ligação ao RNA atua na regulação de células saudáveis ou não através de diversos mecanismos, como o capeamento, *splicing,* clivagem e poliadenilação – etapas do processamento de pré-RNA mensageiro; e pode atuar na exportação, estabilidade e tradução do RNA mensageiro maduro (Sutherland, Siddall, Hime, & McLaughlin, 2015). *MSI1* e *MSI2,* genes da família Musashi presentes em mamíferos, caracterizam-se pela presença de motivos de reconhecimento de RNA em *tandem* altamente conservados. Ambos os genes compartilham especificidades de ligação similares, que têm como alvo RNAs que possuem uma sequência mínima UAG presente em uma grande diversidade de motivos de ligação (Kharas & Lengner, 2017). Outros parálogos da família são *TARDBP*, *DAZAP1*, *HNRNPD*, *HNRNPDL* e *HNRNPAB*, de acordo com o *Ensembl*, versão 93, publicada em julho de 2018. Aparentemente, as proteínas de ligação ao RNA codificadas pelos demais parálogos não interagem com o ZIKV.

Diversas proteínas do tipo Musashi foram descritas em vários animais multicelulares e, em geral, a estrutura primária e padrão de expressão são fortemente conservados entre eles (Horisawa, Imai, Okano, & Yanagawa, 2010). A proteína MSI1 é muito expressa nos tecidos neurais de vertebrados, tanto no sistema nervoso central quanto no sistema nervoso periférico (Kaneko et al., 2000; Okano et al., 2005, 2002). Chavali e colaboradores (2017) sugerem que a alta expressão de MSI1 em células precursoras neurais pode ser um fator de importante contribuição para o neurotropismo exibido pelo vírus Zika, visto que há interação dessa proteína com o genoma do vírus e o favorecimento da sua replicação. A MSI1 também é intensamente expressa em outros tecidos considerados vulneráveis à infecção, como a retina e os testículos (Chavali et al., 2017).

Chavali et al. identificaram *in silico*, no genoma viral da cepa brasileira PE243, três sítios de ligação na região 3’UTR para as proteínas MSI1 e MSI2. No

entanto, eles comprovaram experimentalmente a interação direta da MSI1, mas não da MSI2, à região 3'UTR do vírus PE243. Mutações nos três sítios consenso para a MSI1 na mesma região enfraqueceram significativamente esta interação. Depois, os pesquisadores confirmaram a ligação da MSI1 à região 3'UTR da cepa de Uganda MR766 (Chavali et al., 2017).

A infecção pelo ZIKV perturba a ligação da MSI1 aos seus alvos originais e desregula a expressão de fatores responsáveis pelo funcionamento de células tronco neurais (Chavali et al., 2017). A MSI1 está mutada (Ala184Val) em indivíduos com microcefalia primária autossômica recessiva, o que mostra a sua importância durante o neurodesenvolvimento (Chavali et al., 2017). Além disso, o peixe-zebra (*Danio rerio*) com *MSI1* silenciado por morfolino apresenta microcefalia e o camundongo mutante exibe um córtex cerebral estreito, com número reduzido de células neurais maduras, entre outras anormalidades cerebrais (Sakakibara et al., 2002; Shibata et al., 2012).

Interagir com a MSI1 é uma vantagem evolutiva para a replicação do ZIKV, pois mutações próximas à região de reconhecimento do genoma do vírus foram selecionadas, o que resultou no aumento no número de sítios de ligação de dois (cepa MR766 ZIKV) para três (cepas asiáticas e PE243) (Klase et al, 2016; Chavali et al, 2017). Similarmente, em relação ao hospedeiro, poderiam variações interespecíficas no *MSI1* proteger algumas espécies da infecção pelo ZIKV ou CZS?

Uma hipótese é que variantes em genes importantes relacionados ao desenvolvimento embrionário poderiam influenciar no resultado clínico da infecção ou amplo espectro da CZS. Portanto, aqui é investigado se alterações na sequência do *MSI1* (principalmente no *RNA-binding domain 1*, domínio 1 de ligação ao RNA), selecionado aqui como um gene candidato, poderiam conferir diferentes desfechos nos animais expostos ao vírus. Adicionalmente, os outros 6 parálogos da família Musashi foram também analisados para estudos comparativos.

2. Objetivos Gerais

Descrever a evolução molecular da família dos genes Musashi através de um estudo de genômica comparativa. A partir dos resultados obtidos, verificar se há diferenças interespecíficas no gene *MSI1* que possam contribuir no entendimento dos diferentes resultados à exposição ao ZIKV e síndrome congênita associada.

2.1 Objetivos Específicos

- Obter e comparar as taxas evolutivas dos genes Musashi (7 genes) nas ≅ 75 espécies de vertebrados disponíveis nos bancos de genomas;
- Sequenciar o domínio principal de ligação ao RNA (RBD1) do *MSI1* em 16 espécies de Platyrrhini (*Cacajao melanocephalus, Chiropotes albinasus, Chiropotes utahickae, Callicebus coimbrai, Saguinus niger, Saguinus martinsi, Saguinus bicolor, Callimico goeldii, Callithrix geoffroyi, Callithrix humeralifera, Callithrix melanura, Callithrix saterei, Callithrix humilis, Callithrix pygmaea, Sapajus xanthosternos e Sapajus nigritus*);
- Investigar as divergências encontradas na família Musashi, com ênfase no *MSI1*, e suas implicações para a Zika.

3. Resultados

Manuscrito em Preparação

Evolutionary analysis of the Musashi family: what can it tell us about Zika?

Gabriela Barreto Caldas Garcia[1], Lavínia Schüler-Faccini [1,3], Vanessa Rodrigues Paixão-Côrtes[2], Maria Cátira Bortolini[1]

[1]Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Instituto de Biologia, Universidade Federal da Bahia, Salvador, BA, Brazil

[3]Instituto Nacional de Genética Médica Populacional, Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

Abstract

Despite worldwide research efforts in the last three years, Zika virus infection and its consequences are not fully understood yet. Zika was firstly considered a mild, flu-like disease endemic to Africa. Suddenly, it has shown its high capability to spread to other tropical areas through mosquito bites, sexual activity and from mother to fetus. In 2015, the Brazilian Northeast described the first cases of microcephaly in newborns caused by ZIKV intrauterine exposure. Nowadays, it is known that microcephaly is only one of the possible outcomes of being infected by ZIKV during the early stages of life. Musashi 1 (MSI1) is a RNA-binding protein that participates in post-transcriptional regulation through the recognition of specific binding sites present in transcribed sequences. It is known that MSI1 is involved in neurodevelopmental processes. In addition, MSI1 interacts with the ZIKV genome (a single-stranded positive-sense RNA) and allows its replication. Here we perform an evolutionary analysis of MSI1 coding sequence and their orthologs in vertebrates. We added data from sixteen New World Monkey species (NWMs), known to have higher evolutionary rates, to this analysis by sequencing selected regions of interest (RNA-binding domains-RBDs). The Musashi family includes MSI2, TARDBP, DAZAP1, HNRNPD, HNRNPDL, and HNRNPAB, which apparently do not interact with the virus, but are important RNA-binding proteins that act on many regulatory processes ubiquitously. We found that all sixteen primate species have the RNA-binding domain 1 of MSI1 totally conserved. Whilst

the general sequences of Musashi family are under purifying selection (ω values < 1), the evolution of regulatory mechanisms, especially alternative splicing, seem to be more dynamic among vertebrates. There are different isoforms that differ at N-terminal region and affect the protein size. However, as the principal isoform that contains two RNA-binding domains is preserved, even amongst NWMs, we suggest that ZIKV is able to interact with MSI1 of all primates analyzed. This fact signals that ZIKV can replicate in these potential wild hosts, at least in what depends on MSI1. Thus, we propose that ZIKV may establish a sylvatic cycle since NWMs and other mammals probably can support its maintenance in areas where infected mosquitoes surround.

Keywords: Zika, Musashi 1, New World Monkeys, Congenital Zika Syndrome, Evolution

Introduction

Zika Virus Infection

In the beginning of 2015, the Brazilian Northeast suffered an outbreak of Zika virus (ZIKV), a single-stranded positive-sense RNA flavivirus transmitted mainly by *Aedes* mosquitoes (Schüler-Faccini et al., 2016). ZIKV also can be transmitted through other routes, such as blood transfusion, sex, and from mother to child (Gregory et al., 2017). According to Brazilian Ministry of Health, in 2016, Brazil confirmed 130.701 Zika cases (*Ministério da Saúde*, 2017). In 2017 and 2018, the number of people infected registered in this country decreased for 17.593 and 8.024, respectively (*Ministério da Saúde*, 2018). Until March 10, 2017, 84 countries worldwide have stated evidence of vector-borne ZIKV transmission, while other 64 countries have a competent vector established, but there was no sign of transmission (WHO, 2017).

ZIKV infection is a mild disease characterized by Dengue-like symptoms as fever, rash, joint and muscle pain, headache, and red eyes (CDC, 2019). However, about 80% of infected individuals are asymptomatic (Klase et al., 2016, Oeser & Ladhani, 2018).

Although Zika first cases in humans happen 65 years ago, in Uganda and Tanzania, it was only in 2016 that Brazilian Northeast begun to describe the worst side of this disease for pregnant women and their babies (Kindhauser, Allen, Frank, Santhana, & Dye, 2016). The rise of microcephaly cases associated with ZIKV after the last outbreak, in early 2015, changed the virus concept from just a mild fever etiologic agent to a new human teratogen (Schüler-Faccini et al., 2016). Neuropediatricians initially recognized the Congenital Zika Syndrome (CZS) by severe microcephaly, and other brain abnormalities, such as calcifications, lissencephaly, and ventriculomegaly (Eickmann et al., 2016; Schüler-Faccini et al., 2016; Victora et al., 2016). Further, ZIKV has been associated with Guillain-Barré syndrome (GBS), as a new trigger of this adult autoimmune condition, and other serious neurologic complications, such as encephalitis, and transverse myelitis (PAHO-WHO, 2015; Cao-Lormeau et al., 2016; Parra et al., 2016, Rocha Ferreira da Silva et al., 2017; Ugarte et al., 2017).

An important differential characteristic of ZIKV is its broad tropism for different tissues, including the brain, eyes, testis, uterus and vagina, placenta, and body fluids such as saliva or semen (Miner and Diamond, 2017). The preference of ZIKV to infect and produce apoptosis in neural progenitor cells (NPC) was evidenced by several studies and explains the effects of infection on neurodevelopment (Li et al., 2016; Qin et al., 2016).

Research efforts worldwide continually are elucidating that CZS has a wide spectrum beyond microcephaly, including hearing loss, ocular abnormalities, severe motor impairment, hypertonia, dysphagia, behavioral disorders, memory issues, epilepsy, and more. Therefore, CZS has different degrees of gravity varying from mild developmental delay to severe microcephaly (Leal et al., 2016; van der Linden et al., 2016; del Campo et al.,2017; Moore et al., 2017; Oeser & Ladhani, 2018). Moreover, the infection also can lead to miscarriage, fetal death, and stillbirth (van der Eijk et al., 2016; Hoen et al., 2018; Holtzman, Golden, & Sheffield, 2018). In addition, the long-term consequences of intrauterine exposition to ZIKV are yet to be investigated, since affected children are very young, being at most four years old in 2019. On December 2018, the Secretary of Surveillance confirmed 3.279 CZS cases in Brazil (*Ministério da Saúde,* 2018). Because there

is no vaccine available, it is essential to prevent transmission and keep doing research to support the patients.

Animal Models

Working with animal models to understand ZIKV infection is very helpful since such studies cannot be performed in humans. Despite the strong experimental data published, it is interesting to evaluate the genetic differences among model species to identify possible factors that may help to explain why Zika outcomes are so diverse even among individuals of the same species. CZS presents phenotypes that can include the onset of microcephaly after birth, as well as neurodevelopmental abnormalities (Moura da Silva et al., 2016; Shao et al., 2016). Recent reviews show the complexity of studying ZIKV phenotypes on animal models (Bradley & Nagamine, 2017; Caine, Jagger, & Diamond, 2018; Pena et al., 2018; Nazerai et al., 2019). In summary, we highlight that except for the wild type mouse, which is efficient on its immune response preventing ZIKV replication, all the studied species is somehow susceptible to Zika infection (Figure 1).

ZIKV has a nonstructural protein (NS5) that target human STAT2, a transcription factor involved in type I interferon signaling, for proteasomal degradation. Thereby, this virus evades the host antiviral responses disturbing an essential signaling pathway. However, because mouse STAT2 is not vulnerable to this interaction, the murine immune reaction to ZIKV is efficient (Grant et al., 2016). Furthermore, immunocompromised mouse models were developed and, under these circumstances, they are affected by several fetal disorders such as intrauterine growth restriction, neurological conditions, and fetal demise (Cugola et al., 2016; Lazear et al., 2016; Miner et al., 2016). Recently, Gorman and colleagues developed an immunocompetent model by generating a mouse-adapted ZIKV strain, in combination with the knock-in of the human *STAT2* gene, resulting in infection similar to that of ZIKV in humans, including placental and fetal brain invasion (Gorman et al., 2018). This example illustrates how taxon-specific

genetic variants (characterizing a particular species, family, genus, etc.) may result in the differential response to infectious diseases in the animal kingdom.

Nonhuman primates (NHP) are essential animal models to study ZIKV infection because there is an idea about the establishment of a sylvatic cycle in the wild involving monkeys and mosquitoes as occurs with Yellow Fever, Dengue, and Chikungunya viruses (Haddow et al., 2012; Vasilakis et al., 2012; Hanley et al., 2013; Althouse et al., 2018). *Macaca mulatta*, *M. fascicularis*, *M. nemestrina*, and *Papio anubis* are Old World Monkeys that support ZIKV infection (Adams Waldorf et al., 2016; Osuna et al., 2016; Hirsch et al., 2017; Gurung et al., 2019). In addition, *Callithrix jacchus, C. penicillata, Sapajus libidinosus*, *Aotus spp*., and *Saimiri spp*. are some New World Monkeys (NWMs) that have been tested for ZIKV virulence and/or its teratogenicity so far (Favoretto et al.,2016; Nehete el at., 2017; Seferovic et al., 2018; Terzian et al., 2018).

Other studied animal models are *Sus scrofa domesticus, Mesocricetus auratus*, strain 13 *Cavia porcellus*, Dunkin-Hartley *Cavia porcellus*, *Mustela putorius furo*, and *Gallus gallus* (Goodfellow et al., 2016; Kumar et al., 2017; Bavari et al., 2018; Schreur et al., 2018; Hutchinson et al., 2019). We portray an overview of these species when infected with ZIKV in figure 1.

The specific mechanisms of the ZIKV entry into a cell in humans and other vertebrates, the evasion of the host immune system, its tropisms, and its ability to cross the placentas is not fully known. One of the biggest questions regarding ZIKV is its teratogenic effects. In other words, which are the mechanisms of neural damage in the embryo? Chavali and colleagues (2017) showed that Musashi 1 (MSI1), a causal gene of autosomal recessive primary microcephaly, interacts with ZIKV single-stranded RNA, and enables virus replication. The interaction between MSI1 and ZIKV may directly affect the neurodevelopmental process since MSI1 is highly required in neural progenitor cells. Thus, the Musashi family genes may be involved in the teratogenic effect of the ZIKV.

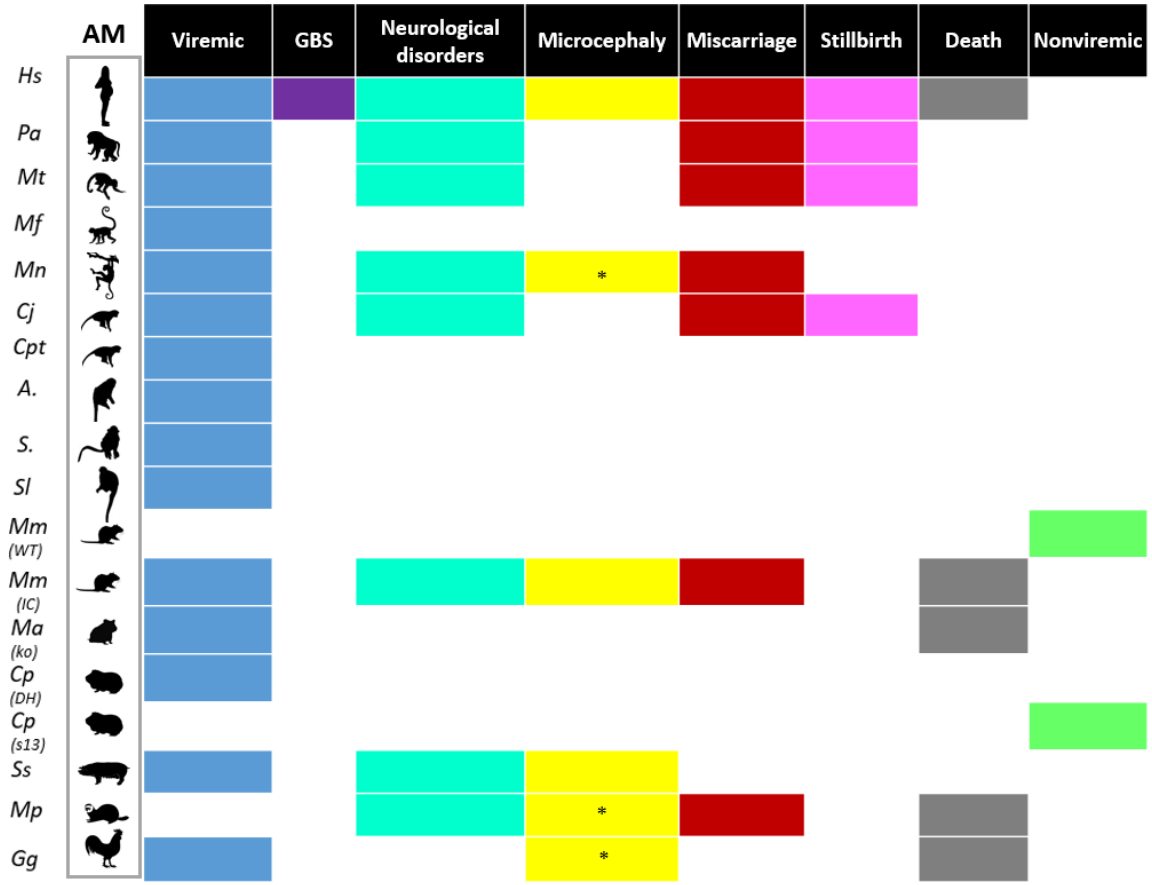

Figure 1. Human and animal models (AM) tested for ZIKV infection: a simplified overview of clinical outcomes. *Microcephaly-like phenotype or neurological abnormalities leading to microcephaly. 'Death' refers to postnatal death or in adulthood. Abbreviations: Hs: *Homo sapiens*; Pa: *Papio anubis*; Mt: *Macaca mulatta*; Mf: *M. fascicularis*; Mn: *M. nemestrina*; Cj: *Callithrix jacchus*; Cpt: *C. penicillata*; A.: *Aotus sp*; S.: *Saimiri sp*; Sl: *Sapajus libidinosus*; Mm(WT): wild type *Mus musculus*; Mm(IC): immunocompromised *M. musculus*; Ma(ko): *STAT2* knock-out *Mesocricetus auratus*; Cp(DH): Dunkin-Hartley *Cavia porcellus*; Cp(s13): strain 13 *C. porcellus*; Ss: *Sus scrofa domesticus*; Mp: *Mustela putorius furo*; Gg: *Gallus gallus*. GBS: Guillain-Barré syndrome.

Musashi Family

Musashi 1 (MSI1) is an RNA-binding protein (RBP; essential regulators of mRNA stability and translation) highly enriched in the developing central nervous system (CNS). MSI1 plays an essential role in the development of the CNS since it is highly expressed in fetal and adult neural stem cells (Shibata et al., 2012). At this environment, undifferentiated dividing cells give rise to several cell types such as neurons, astrocytes, and oligodendrocytes (Kaneko et al., 2000). Indeed, MSI1 and its paralog MSI2 post-transcriptionally regulate the expression of genes involved in cell fate resolution, specifically meiotic cycle advancement, stem cell self-renewal, and cancer growth (Iwaoka et al., 2017).

The Musashi family includes five paralogs (genes belong to the same species and result from a duplication event, whereas orthologs are homologous genes in distinct species derived from a speciation event). Besides *MSI1* and *MSI2*, according to Ensembl v.93 (July 2018), are found the genes *TARDBP, DAZAP1, HNRNPD, HNRNPDL*, and HNRNPAB. They also code RNA-binding proteins, ubiquitously expressed, that plays redundant (post-transcriptional regulation) as well as independent roles when compared with MSI1 and MSI2 (Table 1). Musashi proteins can recognize target RNAs through two RNA-binding domains (RBDs). The RBD1 binds to RNA more strongly than RBD2 and, for mouse MSI1, the RBD1 does the recognition of targets, while the RDB2 adds affinity to the link (Zearfoss et al., 2014). For that reason, RBD1 is an excellent candidate region for studies which seek to identify the characteristics involved in this virus-host interaction.

Chavali et al. (2017) identified that the Brazilian ZIKV RNA genome (PE243 strain) has three consensus binding sites in the 3’ UTR region for MSI1 and MSI2 proteins. Besides, they showed that only MSI1 interacts with ZIKV single-stranded RNA to enable its replication (Chavali et al., 2017). The hijack of MSI1 by the virus, consequently the alterations of binding to its original targets may directly affect the neurodevelopmental process since it is highly required in neural progenitor cells. Besides, individuals affected by autosomal recessive primary microcephaly have their MSI1 mutated (p. Ala184Val; Chavali et al., 2017). More

interesting, retina and testis, places where MSI1 is overexpressed, have been described as tissues infected by ZIKV (Chavali et al., 2017; Salinas et al., 2017; Matusali et al., 2018).

Then, interacting with MSI1 is probably an essential evolutionary advantage for ZIKV replication. Indeed, mutations close to ZIKV genome region of recognition have been selected resulting in the increase of binding sites from two (MR766 ZIKV strain) to three (Asian-lineage strains, PE243) (Klase et al., 2016; Chavali et al., 2017).

We hypothesize that taxon-specific variants in essential genes related to embryonic development can influence the outcome of ZIKV infection and its teratogenicity. Comparative genomics allows us to study gene sequences available in genome browsers in several species and afterward correlate the findings with experimental data already published. Thus, this study aims to investigate if there are taxon-specific variants among the RBD1 sequences of the MSI1 vertebrate orthologs, including Zika-tested species, whose genome is available. Additionally, we sequenced the same candidate region of 16 NWM species, which allowed the introduction of original RBD1 primate data in the analyzes. With this in mind, it is possible to ask: at least, on behalf of the host, would taxon-specific variants in the RBD1 region of gene MSI1 protect any species against ZIKV infection or CZS?

The answer to this question might help to explain the full spectrum of CZS, and/or different replication capabilities that could affect clinical phenotypes.

Table 1. Genes of Musashi family

GENE	General Function	Expression	*MSI1* % ID	References
MSI1 (Musashi 1) [O43347]	RNA-binding protein that acts on regulation of target mRNAs post-transcriptionally. It is involved in proliferation and maintenance of stem cells in the CNS [1].	Ubiquitous expression. The tissues enhanced are cerebral cortex, testis, and retina [2].	100%	[1] UniprotKB [2] The Human Protein Atlas
MSI2 (Musashi 2) [Q96DH6]	RNA-binding protein that acts on regulation of target mRNAs post-transcriptionally. It is involved in proliferation and maintenance of stem cells in the CNS [1].	Ubiquitous cytoplasmic expression [2].	75.91%	[1] UniprotKB [2] The Human Protein Atlas
TARDBP (TAR DNA Binding Protein) [Q13148]	DNA and RNA-binding protein that participates in transcription regulation and splicing [1].	Ubiquitous nuclear expression [2].	19.57%	[1] UniprotKB [2] The Human Protein Atlas
DAZAP1 (DAZ Associated Protein 1) [Q96EP5]	RNA-binding protein related to spermatogenesis [1].	Ubiquitous nuclear expression [2]. Mainly expressed in testis [1].	29.98%	[1] UniprotKB [2] The Human Protein Atlas

HNRNPD (Heterogeneous Nuclear Ribonucleoprotein D) [Q14103]	DNA and RNA-binding protein that works as a transcription factor and may be involved in regulation of translation [1]. It acts on pre-mRNA processing, mRNA metabolism, transport, and stability [3].	General nuclear expression [2]. Ubiquitously expressed [3].	30.42%	[1] UniprotKB [2] The Human Protein Atlas [3] RefSeq NCBI
HNRNPDL (Heterogeneous Nuclear Ribonucleoprotein D-Like) [O14979]	DNA and RNA-binding protein that works as a transcriptional regulator. It promotes transcription repression [1]. This protein acts on pre-mRNA processing, mRNA metabolism, and transport [3].	Nuclear expression in most cells at variable levels. Ubiquitously expressed [3].	27.14%	[1] UniprotKB [2] The Human Protein Atlas [3] RefSeq NCBI
HNRNPAB (Heterogeneous nuclear ribonucleoprotein A/B) [Q99729]	RNA-binding protein that binds to single-stranded RNA [1]. This protein acts on pre-mRNA processing, mRNA metabolism, and transport [3].	Nuclear and cytoplasmic expression [2]. Ubiquitously expressed [3].	31.33%	[1] UniprotKB [2] The Human Protein Atlas [3] RefSeq NCBI

[1] UniprotKB (https://www.uniprot.org/)

[2] The Human Protein Atlas (https://www.proteinatlas.org/)

[3] RefSeq NCBI (https://www.ncbi.nlm.nih.gov/refseq/)

Materials and methods

DNA samples, PCR, and sequencing

The “Centro de Primatologia do Rio de Janeiro”, RJ, Brazil (CPRJ) provided blood samples from 16 New World Monkey species. Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the protocol recommended by the producer. We designed primer sets for three DNA fragments that constitute the RNA-binding domain 1 (RBD1) of MSI1, including six exons. Primer sets were obtained from Primer3 v.4.1.0 (http://bioinfo.ut.ee/primer3/) (Untergasser et al., 2012), and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012).

The following primer sets have amplified our region of interest: 5'-CAGCCTCCCTCTCCCAAAG-3' (Forward), and 5'-CCCGATGGAGACTGACGC -3' (Reverse) to amplify the very end of exon 1 and further exons 2, 3, and 4; 5'-CCCTTTCTCCCAGCACAC-3' (F), and 5'-GAGGCTTTTGGGGTTTCTGG-3' (R) for exon 5; 5’-GAGGCTGCAAGGTGGATT-3' (F), and 5'-CAGGACTCAGATGCCCACTT-3' (R) for exon 6. PCR standard conditions were applied [95 ℃, 5 min; 35 cycles of (95 ℃, 30 sec; 55 ℃, 30 sec; 72 ℃, 1min 30 sec); 72 ℃, 7 min; and storage at 4 ℃]. For sequencing reactions, the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the sequencer ABI PRISM 3730XL Analyzer (96 capillary types) were used by an external service provider.

In silico analysis: alignment and evolutionary rates

DNA sequences from all seven genes that constitute the family were recovered from vertebrates available in Ensembl v.93 (https://www.ensembl.org/index.html) and/or through BLAST on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), according to each sequence's quality and confidence. The number of useful orthologs ranges from 70 to 86, prioritizing the best quality of the alignment. We chose the human canonical sequence of each

Musashi paralog as the reference for BLAST searches and alignments. The transcript or isoform of each ortholog was obtained considering the length (closer to the human canonical sequence size) and by the similarity of the amino acids that compose the peptide sequences.

Next, we used MEGA 7 (Kumar et al. 2016), MUSCLE algorithm (Edgar, 2004), to align the sequences. Besides, we performed manual adjustments when it was necessary; for instance, we removed incomplete sequences and those that were unaligned due to sequencing and/or annotation errors. Indeed, it is critical to ensure that Zika-tested species present in genome databases are involved in this analysis.

To estimate evolutionary rates, we applied the NSsites test, accessible in the CODEML program of PAML 4.9 package (Yang, 2007). The phylogenetic analysis by maximum likelihood allows testing positive selection and provides the ratio between nonsynonymous (dN) and synonymous (dS) nucleotide substitutions (dN/dS = ω). This evolutionary rate describes possible types of selection depending on ω values. Thus, $\omega < 1$ indicates negative or purifying selection, $\omega = 1$ indicates neutrality, and $\omega > 1$ indicates positive selection. As the CODEML program requires a phylogenetic tree as one of its input files, we used TimeTree (http://www.timetree.org/) (Kumar et al., 2017) to build them.

After, we run neutral models (M1a and M8a) to compare with selection models (M2a and M8), respectively. With this in mind, we performed likelihood ratio tests (LRTs) available in HyPhy v2.2.4 package (Pond et al., 2005). In detail, we tested the log-likelihood values of the evolutionary models (M1a versus M2a, 2 degrees of freedom; M8a *vs*. M8, 1 degree of freedom) to evaluate which model is the best to describe each gene of the Musashi family.

Results and Discussion

Seven genes that integrate the Musashi family were analyzed among vertebrates: 86 species for *MSI1*, 70 for *MSI2*, 79 for *TARDBP*, 82 for *DAZAP1*, 75 for *HNRNPD*, 74 for *HNRNPDL*, and 70 for *HNRNPAB*. The inclusion criteria of sequences prioritized the identity and harmony of the alignment among the

orthologs. The phylogenetic trees we used in these analyses, and a table with the databases used to retrieve the sequences is available in the supplementary material.

Evolutionary analysis – alignment, sequencing and rates

In agreement with previous studies, the RNA-binding proteins of Musashi family are much conserved among vertebrates (Kaneko et al., 2000; Okano et al., 2002; Okano et al., 2005). Because MSI1 is proven related to ZIKV replication, our results will be more detailed in sequence variations of this protein, especially in RBDs. Interestingly, even among NWMs, which have been described as species with high evolutionary rates (Moorjani et al., 2016), including in highly conserved genes (see oxytocin case in Vargas-Pinilla et al., 2015) the RBD1 of MSI1 and the beginning of RBD2 are conserved when compared with other primates, including human.

20 NWM species were aligned (4 species from Ensembl, and 16 from our sequenced samples). We do not find nonsynonymous changes in the NWM samples. The exons 1 to 4, which code RBD1, are identical to the homologous human sequence, except for the synonymous c.C162T found in *Aotus nancymaae* [ENSANAT00000036520.1]. Moreover, we found a few synonymous substitutions in exon 5 as follow c.T276C, c.G279A, and c.C306T in all NWMs. Part of *MSI1* that encodes RBD2 we also sequenced. It is preserved likewise, though exon 6 has some synonymous substitutions as well: there are c.G354C in *Chiropotes albinasus* and *C. utahickae*; c.G363A in 17 NWM species (except *Chiropotes albinasus*, *C. utahickae*, and *Callicebus coimbrai* that at this position are equal to human sequence); c.C372T in *Saguinus niger*, *S. martinsi*, *S. bicolor*, *Callimico goeldii*, *Callithrix jacchus*, *C. geoffroyi*, *C. pygmaea*, *C. saterei*, *C. melanura*, *C. humeralifera*, and *C. humilis*. Ultimately, there is c.G393A in all NWM species.

The Musashi's evolutionary rates indicate negative (purifying) selection (Table 2), which results in their relatively high conservation in Vertebrata. The models (M1a and M8a) have better described our data since all LRTs performed that allow positive selection (M2a and M8) were non-significant ($p > 0.05$). These findings agree with precedent publications that show developmental genes are

well conserved and under functional restriction in metazoans (Bates et al., 2005; Paixão-Côrtes et al., 2013). More specifically, the class of RNA-binding proteins are historically known to be highly conserved, principally their RBDs (Kuroyanagi, 2009; Shibata et al., 2012; Gerstberger et al., 2014).

Table 2. Evolutionary rates and Likelihood Ratio Test (LRT) for Musashi's family

GENE	Models	M1a		M8a		
	ω	Proportion (p)	*LRT (M1a vs. M2a)*	ω	Proportion (p)	*LRT (M8a vs. M8)*
MSI1	< 0.03964	0.98	*p* > 0.99	< 0.19049	0.99	*p > 0.99*
MSI2	< 0.03803	0.93	*p > 0.89*	< 0.24996	0.95	*p > 0.07*
TARDBP	< 0.02447	0.99	*p > 0.99*	< 0.12509	0.99	*p > 0.99*
DAZAP1	< 0.04599	0.95	*p > 0.99*	< 0.23753	0.98	*p > 0.99*
HNRNPD	< 0.03777	0.84	*p > 0.99*	< 0.38757	0.94	*p > 0.10*
HNRNPDL	< 0.03660	0.88	*p > 0.99*	< 0.22039	0.91	*p > 0.54*
HNRNPAB	< 0.04923	0.88	*p > 0.99*	< 0.37927	0.96	*p > 0.99*

Note: Degrees of freedom: M1a *vs*. M2a = 2; M8a *vs*. M8 = 1. The omega values and proportions refer to neutral models (M1a and M8a).

Animal Models Comparison

Regarding Zika-tested species, we observed that in general *MSI1* follows the pattern of conservation seen in other animals. There are just a few amino acid substitutions in RBD1, Cis20Ala in *Cavia porcellus* [H0VL96]; Gli41Ser in *Gallus gallus* [F1NSZ7], and in RBD2, Glu127His in *Mus musculus* [Q61474]. Variants found proximal to N-terminal protein region, as Cis20Ala in *C. porcellus*, need to be further studied because apparently, that region is suffering alternative splicing. In some species seem to have lost the N-terminal region of MSI1 and other

paralogs' proteins, resulting in shortening of them. As a result, the different composition of amino acids seen in the N-terminal region of the *C. porcellus* MSI1, for instance, may be due to differential splicing and/or may represent a signal peptide removed later (more details in the next session).

Thus, despite of the conservation of RBD1 and RBD2, we found 2 changes present only in the wild type *Mus Musculus* and absent in the humans and all other animals that showed signals of ZIKV teratogenicity (Glu127His in the RBD2, and further Tre251Ser out of this domain). Additionally, *Sus Scrofa* has a singleton (Ala323Tre) and *Gallus gallus* has two more amino acid changes out of the domains (Tre225Ala and Leu235Iso) and it looks like it has an alternative exon, which encodes the amino acid chain at position 245 to 298.

Evolution of post-transcriptional regulation – alternative splicing and new isoforms

In this analysis, we searched for the most homologous isoform of each gene in the species included. Nevertheless, it was possible to realize that while the two functional domains of the Musashi family remained highly conserved, the evolution of regulatory mechanisms, especially alternative splicing, seem to be more dynamic among vertebrates. We identified different isoforms. They differ in the N-terminal region and affect the size of the protein, although keeping conserved the two functional RBDs. Therefore, all species here analyzed have at least one isoform that contains both functional domains but can diverge in its size after pre-mRNA processing, at the transcript level.

Through BLAST searches on NCBI, other transcripts containing different sizes were also found concomitantly with the wanted longer isoform of reference. These findings suggest that while the Musashi's DNA sequences are preserved, regulatory modifications can alter the resulting proteins. In other words, it seems that the evolvability (*i.e.* potential to evolve) of this kind of system is more related to regulation than to structural alterations in the coding region of functional domains, at least in Vertebrate.

The vast majority of RBPs in human and mouse appears to be owing to further duplications of ancestral RBP-coding genes in Vertebrate lineage because of whole-genome duplications occurred throughout early vertebrate evolution (Panopoulou & Poustka, 2005). Thus, the RBP types present in modern metazoans were already present in the last common ancestor of metazoans; consequently, the collection of RBPs has been firmly maintained during metazoan evolution, which suggests these proteins are critical for the origin and maintenance of multicellular condition since the beginning of animal kingdom (Kerneret al., 2011).

The members of Musashi family and other RBPs share the same binding sites, sometimes overlapping functionally (Gerstberger et al., 2014). The last authors suggest that the conserved function of RBP paralogs may be an alternative to increase protein production and/or favor regulation amongst cell types (Gerstberger et al., 2014). Notably, Iwaoka et al. (2017) propose that because MSI1 and MSI2 have amino acids which can recognize the same minimum RNA sequences, [r(GUAG) and r(UAG)], conserved into their RBDs, they are expected to bind to similar targets (Iwaoka et al., 2017).

On the other hand, we observed that some species presented a gap or highly divergent amino acids composing the N-terminal region of MSI1, MSI2, DAZAP1, HNRNPD, HNRNPDL, and HNRNPAB when compared to human canonical sequence and/or other orthologs. TARDBP was the only protein that kept conserved its N-terminal portion interspecifically. Different amino acids that compose some of the N-terminal regions in the Musashi family proteins (or their absence) can illustrate a process of evolution of post-transcriptional regulation acting (Figure 2). N-terminal is considered as an intracellular postal code, which provides adequate signaling/transport/movement of the protein for organelles and compartments of the cell.

Even considering that there are possible errors or lack of annotation of some genomes used here, it is unreasonable to think that this type of problem always occurred in the N-terminal region of the six paralogs, in several Vertebrate species. Therefore, we suggest that different Musashi isoforms are occurring

interspecifically, or at least this diversification is occurring at different tissues, even though some animals or close related phylogenetic groups can share some of them. Noteworthy, that modifications affecting the N-terminal region usually take place co-translationally when the protein is still very short and yet bound to the ribosome, is considered the earliest changes which a protein undergoes (Giglione et al., 2015). Thus, N-terminal regions can be responsible for an essential part of the Musashi genes' differential functionality at tissue level or even of their evolvability.

Supporting this assumption, FOX1 family composed of three coding RBP paralogs is evolutionary conserved and plays a role in alternative splicing in metazoans. This family shares a unique preserved RBD that is responsible for identifying target RNAs (Nakahata & Kawamoto, 2005). In mammals, FOX1 and FOX2 present many isoforms, due to alternative splicing. Also, the N-terminal and C-terminal regions are less conserved than the RBD; for instance, some FOX1 splice variants have a frame-shift in the C-terminal portion and are not localized preferentially to the nucleus. However, most of the FOX1 and FOX2 isoforms that have a conserved nuclear localization signal at the C-terminal end are restricted to nucleus (Kuroyanagi, 2009). In summary, the emergence of different isoforms of mammalian FOX1 paralogs relates to tissue-specific splicing activities (Nakahata & Kawamoto, 2005). Interestingly, many of FOX2 target RNAs encode splicing regulator RBPs essential for human embryonic stem cell survival. This finding suggests that FOX2 may be an upstream regulator of a splicing network necessary for stem cells (Yeo et al., 2009).

Similarly, Musashi transcripts might be under the influence of different splicing events, tissue and/or taxon-specific, which justify the occurrence of potentially divergent isoforms regarding their sizes or composition (for instance, with or without the N-terminal portion). Further, Shibata et al. (2018) detected a series of MSI1 splice variants in zebrafish (Shibata et al., 2012). Likewise, recently, Ma et al. (2018) have identified a novel splice variant of the human MSI1. MSI1 variant 2, as they named, does not have both of RBDs (Ma et al., 2018).

Chen and cols. (1016) compared alternative splicing patterns between human and mouse orthologs and found that alternative splicing is divergent

interspecifically since human orthologs could encode more isoforms than the mouse orthologs (Chen et al., 2016). In a recent review concerning RBPs in general, Hentze et al. (2018) discuss the fact that proteome-wide approaches have shown the existence of hundreds of RBPs lacking traditional RBDs and how they could function to acknowledge their RNA targets (Hentze et al., 2018), suggesting that RBPs have more plasticity than previously thought. Summing up, those findings indicate that the regulatory processes surrounding RBPs are yet to be fully understood since they are much more dynamic than RBPs' DNA coding sequences.

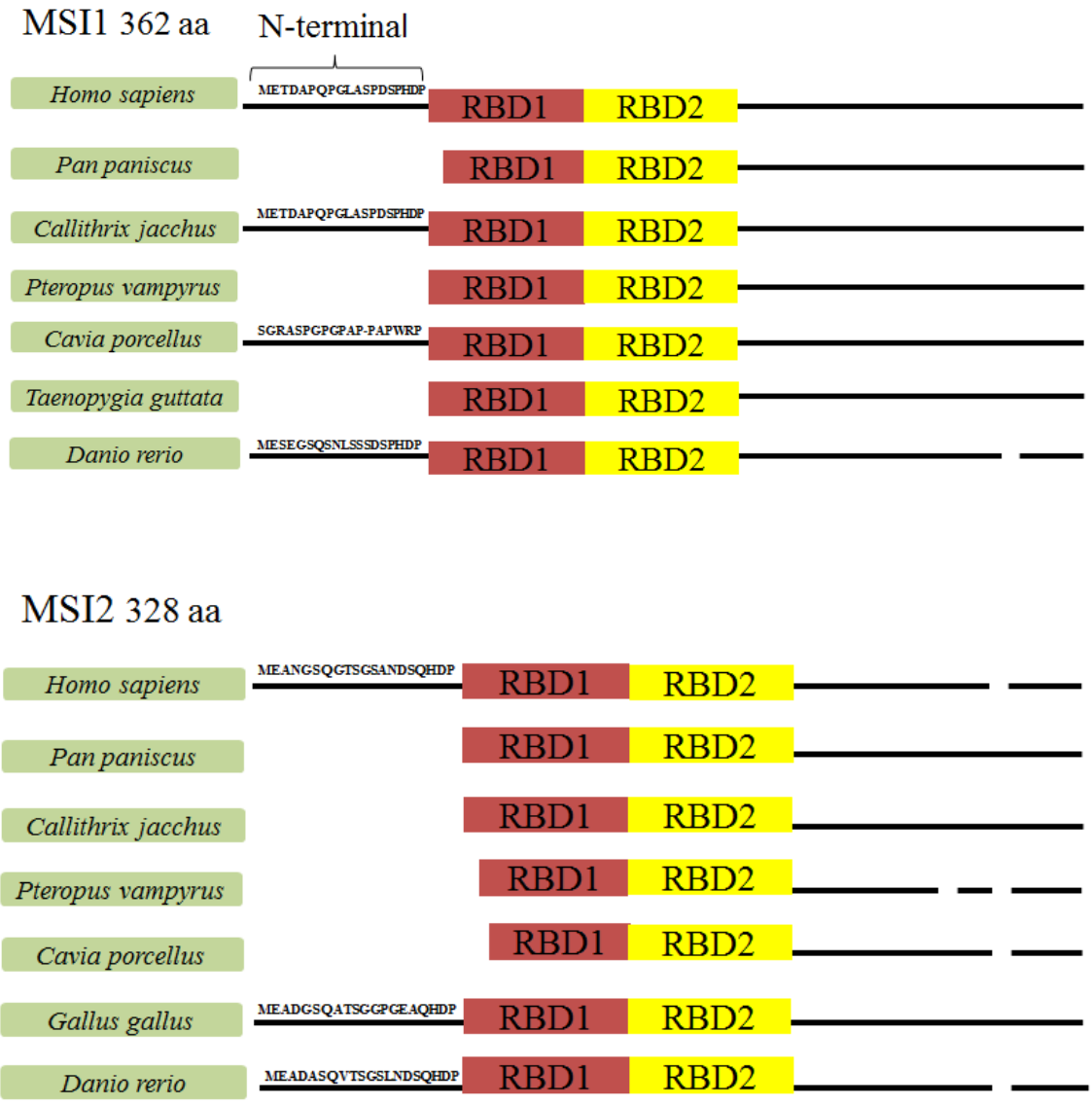

Figure 2. A simplified representation of the MSI1 and MSI2 alignments. The N-terminal region is highly variable among vertebrates. Some of them do not have this part of a protein. This pattern is present in 6 of the 7 Musashi genes investigated here. Other modifications occur in the MSI2 C-terminal region, where a small region of approximately 18aa is absent in humans, but present in other animals. Here we show that the significant protein modifications in the Musashi genes seem to be related to different splicing rather than to occurrence of nonsynonymous changes.

ZIKV, MSI1 and New World Monkeys relationship

The wild type MSI1 isoform contains two RBDs and binds to ZIKV genome, promoting its replication (Chavali et al., 2017). As all twenty NWM species here analyzed has the RBD1 sequence of the MSI1 orthologs conserved, we suggest that they can serve as wild hosts of ZIKV because they have a genetic potential to enable ZIKV replication similar than occur with humans. Thus, we predicted that these primate species once infected might be carriers of the virus, contributing to the maintenance of Zika infection in tropical areas they inhabit. Nevertheless this suggestion, and based on our results indicating a potentially large variability of the N-terminal portion in the Vertebrate Musashi orthologs, this region naturally becomes a candidate for further studies with the same Platyrrhini species investigated here for the RBDs´ coding sequence.

Other factors such as intra or interspecies immunological specificities doubtless influence the clinical outcomes in response to Zika disease, as it occurs with the naturally protected wild-type mice.

By contrast, due to the high level of MSI1 evolutionary restriction in vertebrates, indeed the broad spectrum of CZS and ZIKV dissimilar susceptibility do not occur because of possible MSI1 code sequence variants. As a result, to our concern, ZIKV binding to universal MSI1 must be very advantageous for its prevalence and fitness, since there can be several hosts, at least among mammals, that can be stung by infected mosquitoes.

Conclusion

In conclusion, the evolutionary restriction characterizing the Musashi family seems to be counterbalanced by a higher dynamism in their post-transcriptional regulatory processes, such as alternative splicing, resulting in larger or lesser molecules (*e.g*. with or without the N-terminal variation). An increased number of transcripts that express different protein isoforms can overcome the apparent uniformity of MSIs across vertebrate species. Relating to Zika, MSI1 binds to ZIKV

and promotes its replication. Besides, it is highly conserved and present in all animals included in this analysis.

In addition, New World monkeys, known for their general high evolutionary rates, have the RBD1 of MSI1 conserved. This fact signals that ZIKV can replicate in these potential wild hosts, at least in what depends on MSI1. Thus, we suggest that ZIKV may establish a sylvatic cycle since NWMs and other mammals probably can support its maintenance in tropical areas. Other factors are worth to be investigated, such as immunological particularities intra or interspecies, that might be determinant for ZIKV infection susceptibility, and CZS wide-spectrum clinical outcomes.

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Supplementary Material

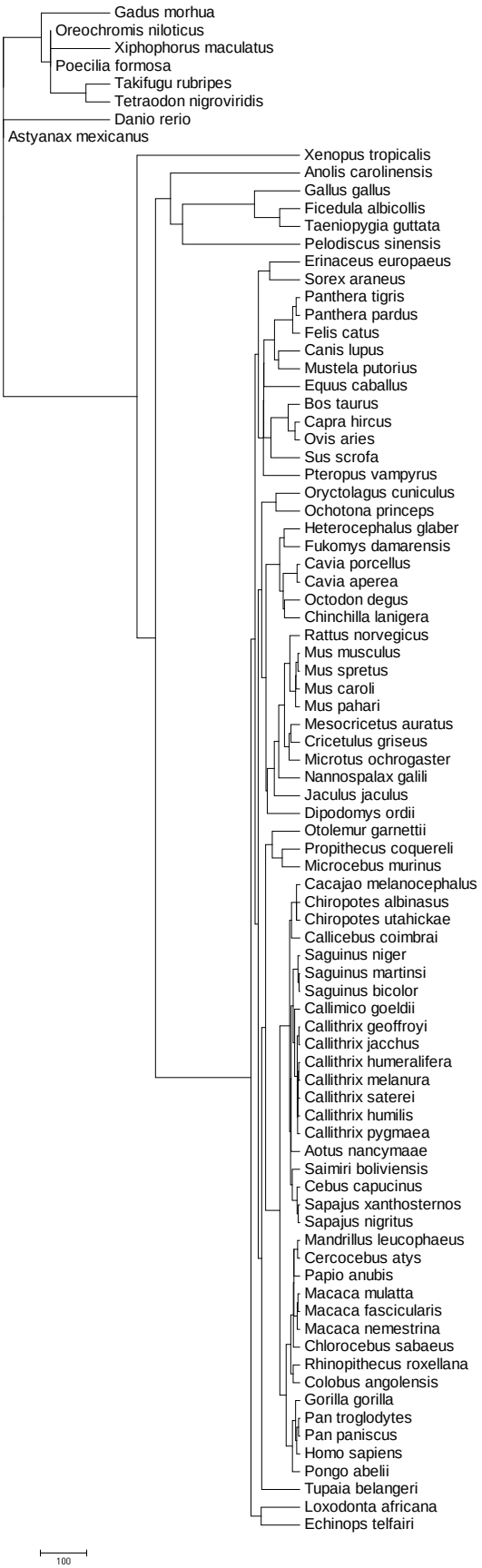

Figure 1. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *MSI1*.

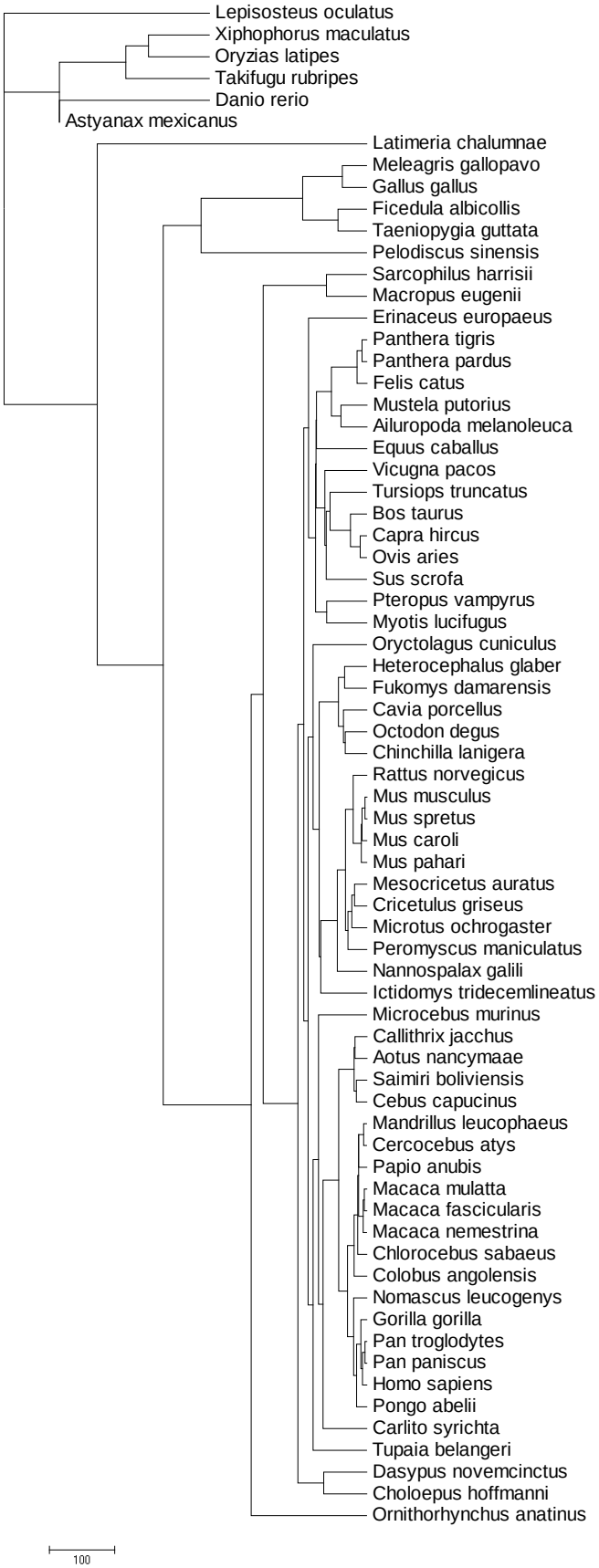

Figure 2. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *MSI2*.

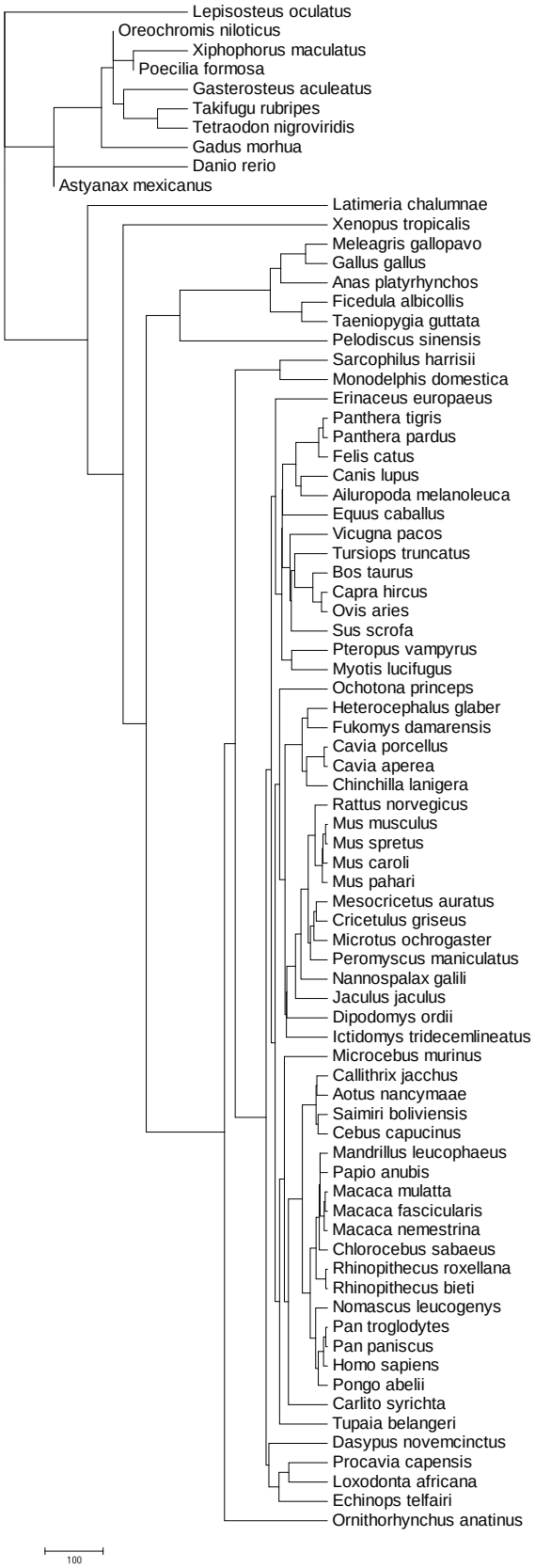

Figure 3. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *TARDBP*.

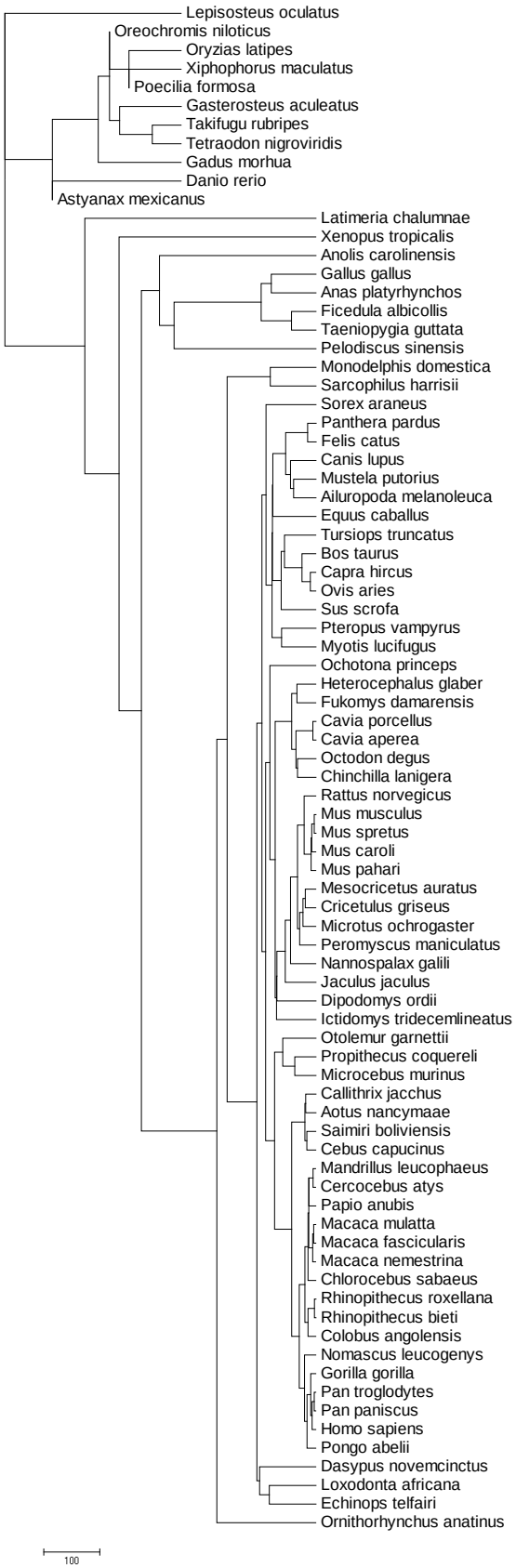

Figure 4. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *DAZAP1*.

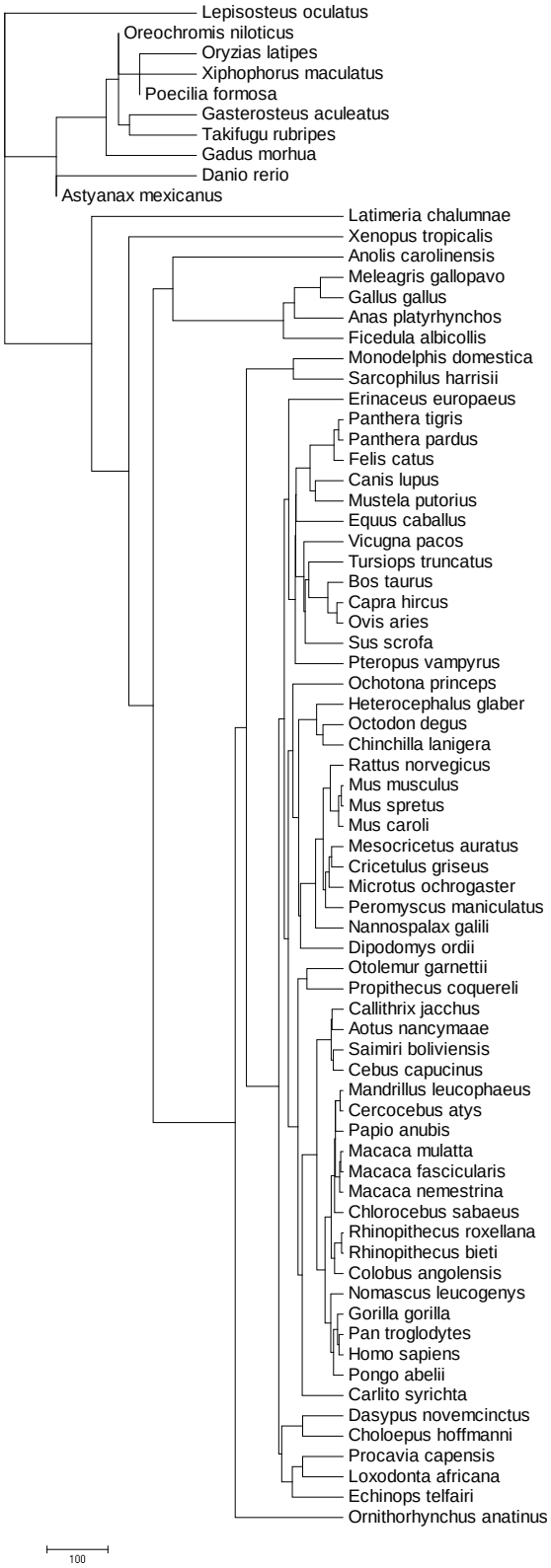

Figure 5. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *HNRNPD*.

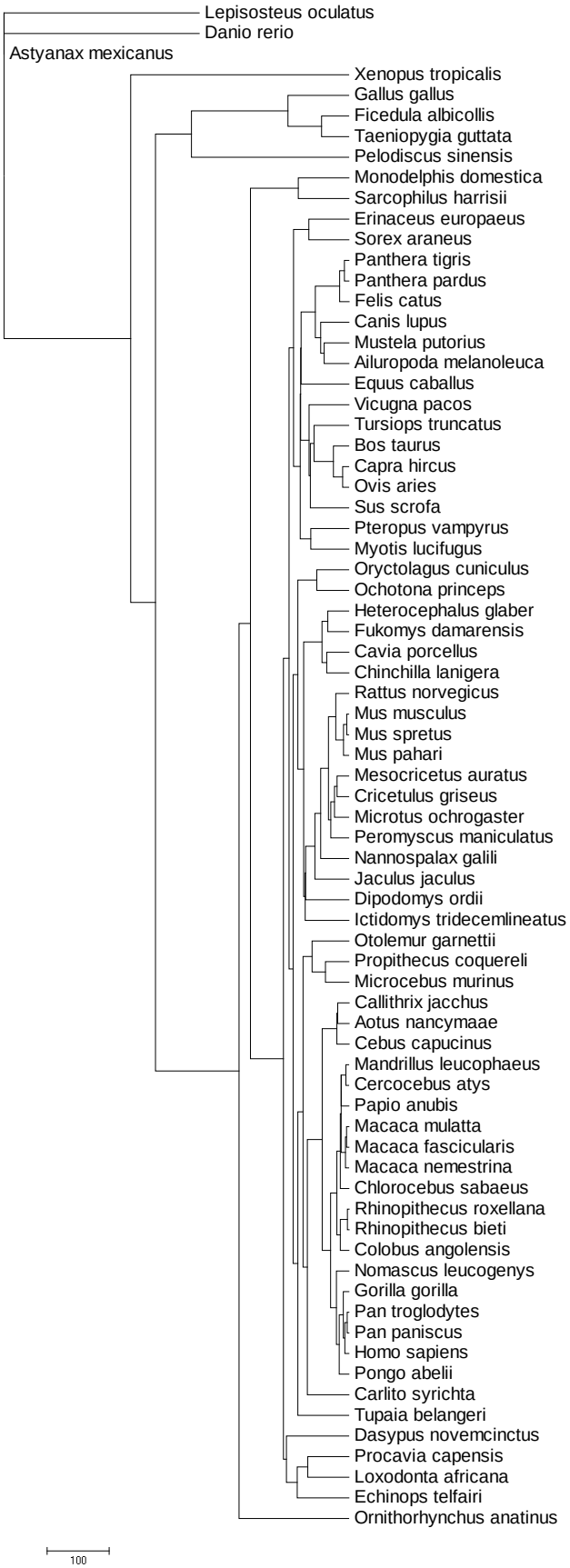

Figure 6. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *HNRNPDL*.

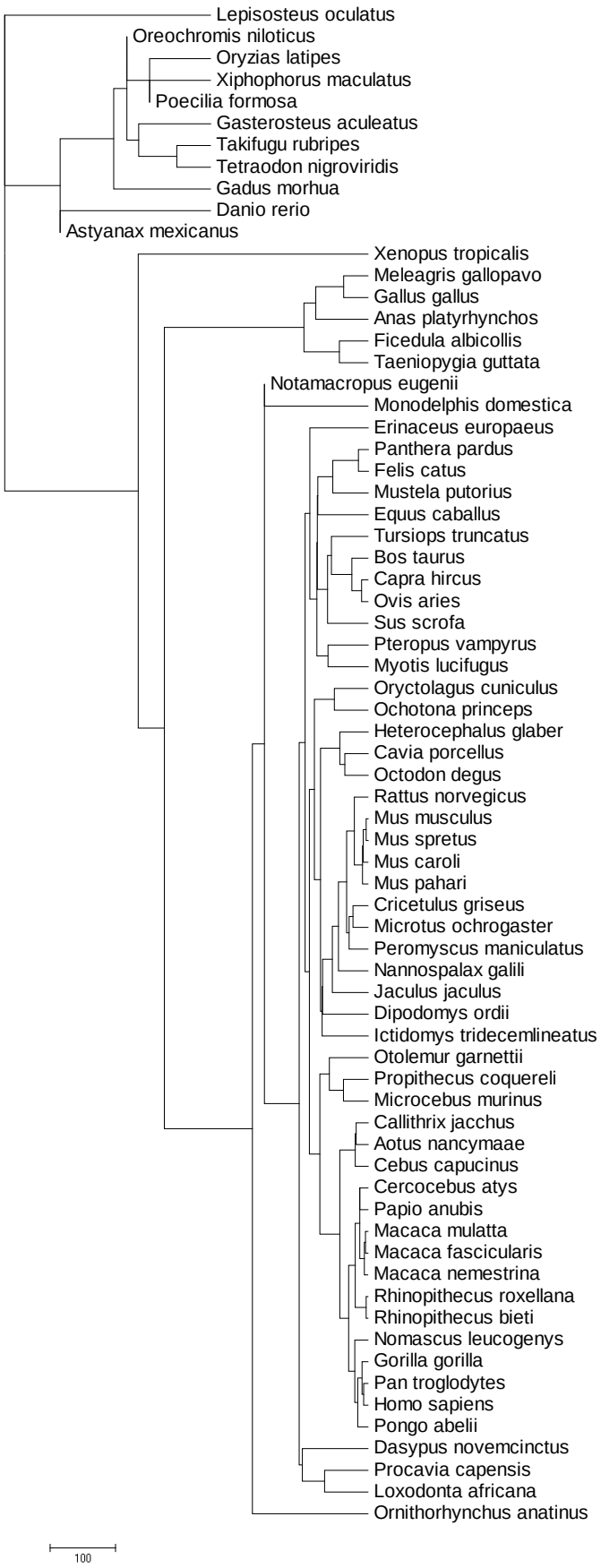

Figure 7. Phylogenetic tree of vertebrate species used in the evolutionary analysis of *HNRNPAB*.

Table 1. Databases used to retrieve the sequences of Musashi family

Genes	*MSI1*	*MSI2*	*TARDBP*	*DAZAP1*	*HNRNPD*	*HNRNPDL*	*HNRNPAB*
Vertebrate species							
Lepisosteus oculatus		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Astyanax mexicanus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Danio rerio	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Gadus morhua	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Tetraodon nigroviridis	ENSEMBL		ENSEMBL	ENSEMBL			ENSEMBL
Takifugu rubripes	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Gasterosteus aculeatus			ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Oreochromis niloticus	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Oryzias latipes		ENSEMBL		ENSEMBL	NCBI		ENSEMBL
Xiphophorus maculatus	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Poecilia formosa	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Latimeria chalumnae		NCBI	ENSEMBL	ENSEMBL	ENSEMBL		
Xenopus tropicalis	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Pelodiscus sinensis	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	
Gallus gallus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Meleagris gallopavo		ENSEMBL	ENSEMBL		ENSEMBL		ENSEMBL
Anas platyrhynchos			ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Ficedula albicollis	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Taeniopygia guttata	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL
Anolis carolinensis	ENSEMBL			NCBI	ENSEMBL		
Ornithorhynchus anatinus		ENSEMBL	ENSEMBL	NCBI	ENSEMBL	NCBI	ENSEMBL
Sarcophilus harrisii		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	
Notamacropus eugenii		ENSEMBL					ENSEMBL
Monodelphis domestica			NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL

Loxodonta africana	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Procavia capensis			ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL
Echinops telfairi	ENSEMBL		NCBI	ENSEMBL	ENSEMBL	ENSEMBL	
Dasypus novemcinctus		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Choloepus hoffmanni		ENSEMBL			ENSEMBL		
Oryctolagus cuniculus	ENSEMBL	ENSEMBL				ENSEMBL	NCBI
Ochotona princeps	ENSEMBL		ENSEMBL	NCBI	ENSEMBL	NCBI	ENSEMBL
Cavia aperea	ENSEMBL		ENSEMBL	ENSEMBL			
Cavia porcellus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL
Chinchilla lanigera	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	
Octodon degus	ENSEMBL	ENSEMBL	NCBI	ENSEMBL	ENSEMBL		ENSEMBL
Heterocephalus glaber	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	ENSEMBL
Fukomys damarensis	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	
Ictidomys tridecemlineatus		ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL
Dipodomys ordii	ENSEMBL		ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL
Jaculus jaculus	ENSEMBL		ENSEMBL	ENSEMBL		ENSEMBL	NCBI
Nannospalax galili	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Cricetulus griseus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Mesocricetus auratus	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	
Peromyscus maniculatus bairdii		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	ENSEMBL
Microtus ochrogaster		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Rattus norvegicus	NCBI	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Mus pahari		ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL
Mus caroli	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL
Mus spretus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Mus musculus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Tupaia belangeri	ENSEMBL	ENSEMBL				ENSEMBL	
Propithecus coquereli	NCBI			ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Microcebus murinus	ENSEMBL	NCBI	ENSEMBL	ENSEMBL		NCBI	ENSEMBL

Otolemur garnettii	ENSEMBL			ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Carlito syrichta	NCBI	ENSEMBL	ENSEMBL		ENSEMBL	NCBI	
Saimiri boliviensis	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		
Cebus capucinus	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Callithrix jacchus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	ENSEMBL
Aotus nancymaae	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	ENSEMBL
Pan paniscus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	
Pan troglodytes	NCBI	ENSEMBL	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL
Homo sapiens	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Gorilla gorilla	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Pongo abelii	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI
Nomascus leucogenys	ENSEMBL	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Rhinopithecus roxellana			ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Rhinopithecus bieti	NCBI		NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Colobus angolensis palliatus		ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	
Chlorocebus sabaeus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	
Mandrillus leucophaeus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	
Cercocebus atys	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Papio anubis	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Macaca mulatta	NCBI	NCBI	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Macaca fascicularis	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Macaca nemestrina	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Erinaceus europaeus	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL	NCBI
Sorex araneus	NCBI			NCBI		NCBI	
Tursiops truncatus	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Capra hircus		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Ovis aries	ENSEMBL	ENSEMBL	ENSEMBL	NCBI	ENSEMBL	NCBI	ENSEMBL
Bos taurus	NCBI	NCBI	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL
Vicugna pacos	ENSEMBL	NCBI	ENSEMBL		ENSEMBL	ENSEMBL	

Sus scrofa		ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Equus caballus	NCBI	NCBI	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	NCBI
Panthera pardus	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Panthera tigris altaica	ENSEMBL	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL	
Felis catus	ENSEMBL	NCBI	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Canis familiaris	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	
Mustela putorius furo	ENSEMBL	ENSEMBL		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL
Ailuropoda melanoleuca	NCBI	NCBI	ENSEMBL	ENSEMBL		NCBI	
Pteropus vampyrus		ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	ENSEMBL	NCBI
Myotis lucifugus	NCBI	NCBI	ENSEMBL	NCBI		ENSEMBL	ENSEMBL
Total	70	70	79	82	75	74	70

4. Discussão

Elementos “ultraconservados” no genoma humano são considerados aqueles maiores de 200 pares de bases, com 100% de identidade em regiões ortólogas de humanos, ratos e camundongos; entre 95 a 99% de identidade nos genomas de galinhas e cães; muitos significativamente conservados também no genoma de peixes (Bejerano et al., 2004). Esses elementos incluem regiões codificadoras de proteínas de ligação ao RNA como por exemplo, HNRNPK, HNRNPH1, HNRNPU, **HNRNPDL**, HNRNPM, SRSF1, SRSF3, SRSF6, SRSF7, SRSF10, SRSF11, TRA2A, PCBP2 e PTBP2 (Bejerano et al., 2004). De acordo os últimos autores, todos os genes desse grupo, e anotados pela base de dados GO (*Gene Ontology*), exibem clara evidência de marcador de sequência expressa ou mRNAs de *splicing* alternativo que se sobrepõem ao elemento ultra conservado (Bejerano et al., 2004).

De modo geral, a localização desses componentes no genoma humano frequentemente se sobrepõe a éxons ou íntrons de genes envolvidos no processamento de RNA ou nas proximidades de genes relacionados à regulação da transcrição e ao desenvolvimento (Bejerano et al., 2004). Esses elementos genéticos são mais altamente preservados do que seria esperado segundo a teoria neutra da evolução, sendo assim, devem estar sujeitos a poderosa ação da seleção purificadora (Bejerano et al., 2004). Isso sugere que os elementos genéticos ultraconservados são fundamentais para a ontogenia de mamíferos e outros vertebrados (Bejerano et al., 2004).

Em concordância com esses dados, foi descrito nesse trabalho que os sete genes codificadores de proteínas pertencentes à classe de ligação ao RNA, constituintes da família Musashi, apresentaram valores $\omega < 1$, o que indica constrição evolutiva. Especificamente, o *HNRNPDL*, um dos parálogos da família relatado no trabalho de Bejerano e *cols*., coincide com a localização de um elemento genético ultraconservado, o que ratifica a restrição para mudanças evolutivas, pelo menos considerando suas regiões codificadoras.

No entanto, apesar da pouca variabilidade em nível de substituições não sinônimas na sequência gênica, ocorre uma diversificação a nível pós-

transcricional. Em busca no algoritmo BLAST dos transcritos homólogos de cada componente da família, percebe-se que existem variantes de mRNA nas diferentes espécies de vertebrados e esse padrão de diversificação se repete em seis dos sete genes. Além disso, mesmo o transcrito da isoforma mais próxima à canônica humana de referência (que foi incluído nas análises) é capaz de conter diferenças em tamanho e/ou composição, principalmente na região N-terminal, com manutenção dos dois domínios de reconhecimento de RNA característicos da família.

Hentze *et al.*, em revisão publicada em 2018, discutem o aumento expressivo na descrição de isoformas de RBPs em geral, inclusive a existência de peptídeos que não contêm os dois principais RBDs, até então considerados essenciais para manter a função dessa classe de proteínas regulatórias (Hentze et al., 2018). Essas descobertas fortalecem a hipótese de que estes elementos de regulação pós-transcricional, embora muitíssimo conservados considerando suas porções codificadoras, são mais plásticos e/ou dinâmicos, considerando o processamento (*splicing*) de seus próprios pré-mRNas. Assim, a coexistência dessas novas proteínas pode capacitar o desempenho de funções diversificadas, mesmo na ausência dos domínios historicamente bem caracterizados, o que pode ter resultado na ocorrência de novidades evolutivas, mesmo na ausência de mutações positivamente selecionadas em suas sequências codificadoras. A região N-terminal das RBPs, como já saleintado, essencial para a sinalização/transporte/mobilidade da proteína dentro da célula, foi encontrada, no presente estudo, como sendo muito variável nas espécies aqui consideradas.

Outro dado interessante diz respeito aos achados do sequenciamento da porção codificadora do domínio RBD1 e parte do RBD2 do *MSI1* em espécies de Platyrrhini. Vale destacar que as taxas evolutivas nesse clado são destacadamente maiores, quando comparadas a de outros primatas (Moorjani et a., 2016), incluindo em genes com extraordinária conservação como é o caso do gene que codifica o neurohormônio ocitocina (*OXT Oxytocin;* Vargas-Pinilla et al., 2015). Conforme demonstrado em estudos prévios do nosso grupo de pesquisa, esses primatas possuem sítios com sinais de seleção positiva em genes relacionados ao comportamento, como por exemplo o já citado *OXT* (*Oxytocin*),

além dos receptores *OXTR* (*Oxytocin Receptor)*, *AVPR1b* (*Arginine Vasopressin Receptor 1B)* e *AVPR2* (*Arginine Vasopressin Receptor 2)* (Vargas-Pinilla et al., 2015; Fam et al., 2018). Todavia, para a região sequenciada do *MSI1,* os macacos de Novo Mundo seguem o padrão de conservação encontrado nos demais vertebrados.

Consequentemente, em relação à Zika, há a possibilidade de que essas espécies de macacos sirvam como hospedeiros e reservatórios do vírus, assim como os primatas de Velho Mundo relatados como susceptíveis à infecção e as suas consequencias. O potencial de interação do ZIKV com a proteína MSI1 poderia justificar a capacidade do estabelecimento de um ciclo silvestre da Zika no Brasil. Primatas não humanos possuem fisiologia, estrutura placentária e desenvolvimento fetal similares aos humanos (Nazerai et al., 2019). Aliada à grande probabilidade de replicação do ZIKV nesses animais, existe a suspeita de que seus filhotes sejam susceptíveis à CZS, em seu amplo espectro.

Nenhuma espécie já testada mostrou-se completamente insensível aos efeitos teratogênicos do ZIKV. Camundongos imunocompetentes, quando expostos por rotas não fisiológicas a altas doses do ZIKV, também sofrem dano fetal e patologia placentária, mesmo na ausência de infecção do feto (Szaba et al., 2018). Esses resultados sugerem que alterações placentárias, mais do que a presença do vírus no filhote, contribuem fortemente para a ocorrência de efeitos prejudiciais à gestação em camundongos. Portanto, a ausência da transmissão vertical pode não garantir a prevenção de um desfecho fetal adverso (Szaba et al., 2018). A resposta imune do camundongo imunocompetente pode, como um efeito colateral, danificar a placenta no decorrer da depuração viral e resultar em imunopatologia e insuficiência placentária sem carga viral detectável (Szaba et al., 2018).

Há muito a ser esclarecido sobre as consequências protetivas ou prejudiciais da resposta imune inata ou adaptativa materna e fetal ao ZIKV durante a gravidez (Szaba et al., 2018). Igualmente, pensa-se que a microcefalia é uma manifestação extrema da CZS e, que outras deficiências neurológicas não diagnosticadas no nascimento podem emergir à medida em que as crianças

nascidas de mães infectadas crescem (Oliveira Melo et al., 2016; Aragao et al., 2017). Em adição, Paul e colaboradores mostraram que mesmo uma infecção leve pelo ZIKV em camundongos imunocompetentes pode levar a déficits pós-natais, como por exemplo, retardo no crescimento e alterações neurocomportamentais. Portanto, evidencia-se a necessidade de monitoramento do crescimento e desenvolvimento de crianças supostamente saudáveis nascidas de mães expostas ao ZIKV (Paul et al., 2018).

Em relação aos primatas discutidos anteriormente, por serem mais próximos filogeneticamente aos humanos do que os camundongos e, por isso, os modelos animais preferidos para deduzir possíveis resultados à infecção, é provável que seus filhotes também sejam afetados, em algum grau, pela CZS. Por isso, são necessários mais estudos para determinar os níveis de susceptibilidade desses animais ao ZIKV, além da capacidade de replicação mediada pela MSI1.

5. Conclusão

É indubitável que existe uma vulnerabilidade diferenciada aos efeitos da Zika e CZS intra e interespecificamente. No entanto, a família Musashi e o principal alvo de replicação do ZIKV, MSI1, aparentemente não são os responsáveis pela diversidade de desfechos à infecção, pelo menos considerando os domínios RBD1 e parte do RBD2. Os Musashis estão sob forte ação de seleção negativa (o que deve ter favorecido evolutivamente a co-optação deste para a replicação do vírus), porém são plásticos em nível pós-transcricional, nos diferentes transcritos que podem ser produzidos após o processamento dos pré-mRNAs. Especificamente, o alto nível de conservação do RBD1 do *MSI1,* nas espécies de macacos de Novo Mundo sequenciadas, permite inferir que esses animais são potenciais hospedeiros do ZIKV e poderiam suportar o estabelecimento de um ciclo silvestre fora da África, ao menos no que depender dessa proteína. Não obstante, como o estudo com os ortólogos indicou grande variabilidade da porção N-terminal, com potencial funcional (atua na sinalização e transporte da proteína para os compartimento celulares; Giglione et al., 2015), essa região torna-se naturalmente candidata para estudos adicionais com as mesmas espécies de

Platyrrhini investigadas aqui para a sequência codificadora de RBD1 e parte de RBD2.

Por fim, sugere-se também que fatores relacionados à resposta imune inata ou adaptativa e alterações da placenta contribuam para os fenótipos de amplo espectro da CZS.

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Anexo I

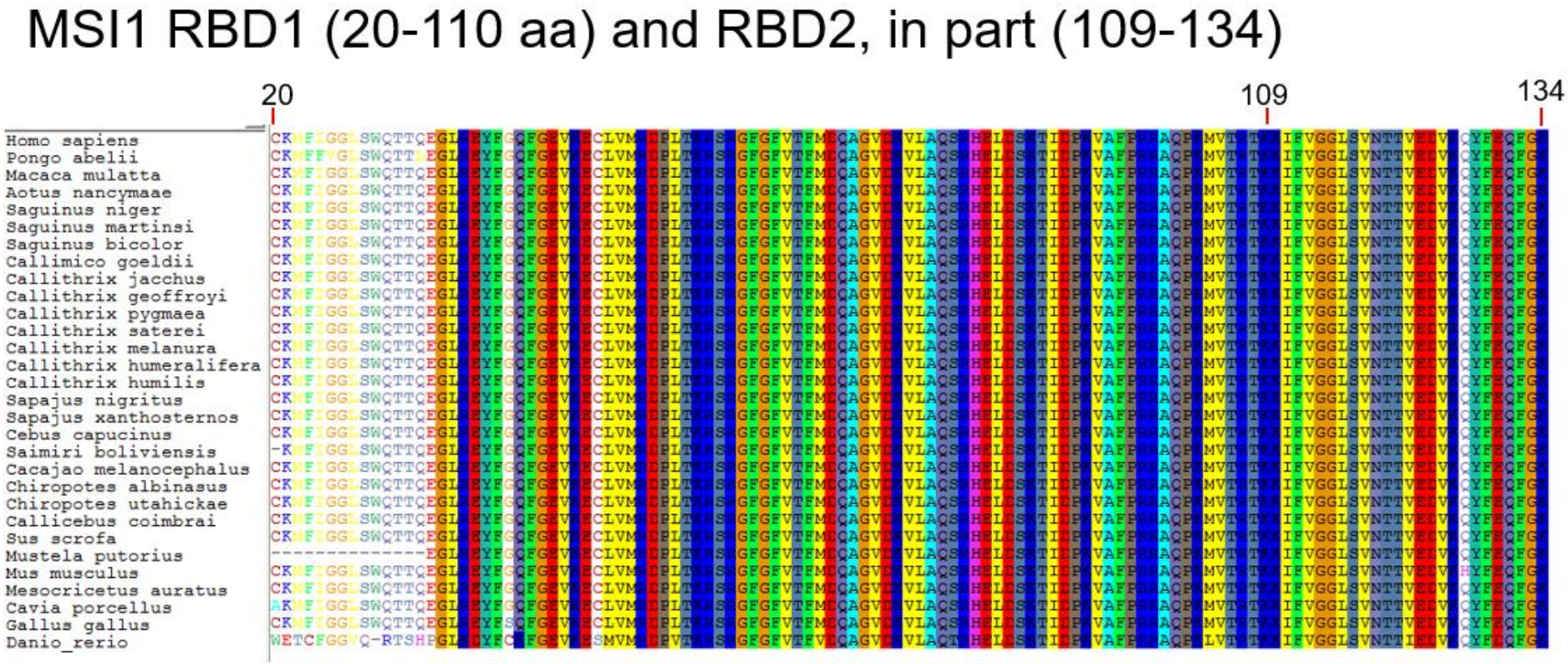

Figura 1. Alinhamento do RBD1 e parte do RBD2 do *MSI1* em 20 espécies de Macacos de Novo Mundo e algumas espécies testadas para a Zika.