PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

CARACTERIZAÇÃO DO GENOMA MITOCONDRIAL DE ONÇA-PINTADA (*PANTHERA ONCA*) E ELUCIDAÇÃO DA FILOGENIA MITOGENÔMICA DO GÊNERO *PANTHERA*

Laura Moretti Heidtmann

DISSERTAÇÃO DE MESTRADO

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SUMÁRIO

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RESUMO

Genomas mitocondriais (mitogenomas) geralmente são obtidos através do sequenciamento de DNA realizado com uma série de *primers* conservados desenhados de maneira a se sobreporem, completando assim todo o DNA mitocondrial. Esta estratégia é bastante eficaz para alguns organismos. Entretanto, a translocação de segmentos do DNA mitocondrial citoplasmático (*cymt*DNA) para o genoma nuclear (*numt*) é um fenômeno conhecido para muitos táxons, incluindo os felinos pertencentes ao gênero *Panthera*. Algumas estratégias foram desenvolvidas para evitar a amplificação indesejada do *numt*, como por exemplo o isolamento de DNA mitocondrial seguido de PCR ou de PCR longo. Recentemente, as técnicas de sequenciamento de alto desempenho vêm sendo amplamente utilizadas. Dentre estas, o sequenciamento de RNA (RNA-seq) parece ser extremamente útil para gerar mitogenomas e evitar *numts*, uma vez que captura com alta cobertura apenas DNA mitocondrial transcrito, evitando as cópias nucleares pseudogenizadas. Quando este estudo foi iniciado, genomas mitocondriais de todas as espécies do gênero *Panthera* exceto *P. onca* estavam disponíveis em bases de dados como o GenBank. Tendo em vista a importância deste marcador molecular para estudos populacionais de onça-pintada (*Panthera onca*) e para estudos filogenéticos entre as espécies do gênero *Panthera*, os objetivos deste trabalho foram (i) caracterizar o mitogenoma de *Panthera onca* de forma a eliminar a possibilidade de amplificação errônea de *numt* e (ii) realizar a primeira análise mitogenômica do gênero *Panthera*. O genoma mitocondrial da onça-pintada foi caracterizado utilizando dados de RNA-seq. Os transcritos cobriram cerca de 95% do mitogenoma, sendo os demais segmentos cobertos por sequenciamento de DNA baseado em PCR, através da utilização de *primers* específicos desenhados para esta finalidade. Todos os quatro tipos principais de análises filogenéticas do mitogenoma (Maximum Likelihood, Máxima Parcimônia, Neighbor-Joining e Inferência Bayesiana) suportaram uma topologia congruente (((*N. nebulosa* ((*P. tigris* (*P. onca* (*P. uncia*, (*P. leo*, *P. pardus*))))). Esta topologia é inédita para o gênero, porém os resultados indicam ser esta a verdadeira história evolutiva do DNA mitocondrial dentro deste grupo. Neste trabalho, demonstrou-se que através de RNA-seq é possível obter-se praticamente todo o genoma mitocondrial de um indivíduo. Além disso, esta abordagem parece ser bastante promissora especialmente em casos onde grandes e recentes *numts* ocorrem, como é o caso das espécies pertencentes ao gênero *Panthera*.

ABSTRACT

The characterization of the mitochondrial genome of the jaguar (*Panthera onca*) and the elucidation of the mitogenomic phylogeny from *Panthera* genus

Mitochondrial genomes (mitogenomes) are usually obtained through DNA sequencing produced by a set of conserved PCR primers that are designed to generate overlapping segments, thus completing the whole mitochondrial DNA. This may be a good strategy for some organisms. However, the translocation of cytoplasmic mitochondrial DNA (*cymt*DNA) into the nuclear genome (*numt*) is known to be a frequent phenomenon in many taxa, including the felid genus *Panthera*. Some strategies have been developed to avoid the unwanted amplification of *numt*, such as mitochondrial isolation followed by PCR or long-PCR. Recently, next-generation sequencing (NGS) approaches have begun to be extensively used in this field. Among these, RNA sequencing (RNA-seq) seems to be extremely useful to generate mitogenomes and to avoid *numts*, as it allows the efficient capture at high coverage of mtDNA transcripts, avoiding pseudogenized nuclear copies. When we initiated this study, mitochondrial genomes of all species of the *Panthera* genus except the jaguar (*P. onca*) were available in public databases such as GenBank. Given the importance of this molecular marker for jaguar population studies and for phylogenetic analyses within the *Panthera* genus, the goals of this project were to (i) characterize the *Panthera onca* mitogenome, eliminating the possibility of erroneous amplification of *numt*; and (ii) to conduct the first mitogenomic analysis of the *Panthera* genus. We have characterized the mitochondrial genome of the jaguar employing RNA-seq data. The transcripts covered about 95% of the mitogenome, with the remaining gaps being complemented by PCR-based DNA sequencing, using specific primers designed for this purpose. All mitogenomic phylogenetic analyses (Maximum Likelihood, Maximum Parsimony, Neighbor-Joining and Bayesian Inference) supported a congruent topology (((*N. nebulosa* ((*P. tigris* (*P. onca* (*P. uncia*, (*P. leo*, *P. pardus*))))). This topology is unprecedented for the genus, but our results indicate that it correctly reflects the evolutionary history of mitochondrial DNA in this group. This study demonstrated that, with RNA-seq approach, almost the entire mitochondrial genome from an individual can be quickly characterized. Furthermore, this approach holds great promise especially in the case of groups plagued by the presence of large and recent *numts*, as is the case of *Panthera* species.

APRESENTAÇÃO

Esta dissertação, intitulada "Caracterização do genoma mitocondrial de onça-pintada (*Panthera onca*) e elucidação da filogenia mitogenômica do gênero *Panthera*", é um dos requisitos exigidos pelo Programa de Pós-Graduação em Zoologia, da Faculdade de Biociências, da Pontifícia Universidade Católica do Rio Grande do Sul, para a obtenção do título de mestre.

Os resultados aqui apresentados foram gerados em sua maioria no Laboratório de Biologia Genômica e Molecular, vinculado a esta Universidade. Os dados brutos de sequenciamento de RNA foram gerados pelo Laboratório de Biotecnologia Animal, da Escola Superior de Agricultura Luiz de Queiroz, da Universidade de São Paulo.

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Este trabalho teve como objetivos (i) realizar a primeira caracterização completa do genoma mitocondrial de onça-pintada (*Panthera onca*), através do sequenciamento de alto desempenho aplicado à técnica de sequenciamento de RNA; e (ii) elucidar a filogenia mitogenômica do gênero *Panthera*.

A dissertação será apresentada no formato de artigo científico a ser submetido à revista GENE, respeitando as normas de submissão da mesma, disponíveis em: http://www.elsevier.com/journals/gene/0378-1119/guide-for-authors. Antecedendo o artigo há uma introdução geral sobre os temas abordados neste trabalho e, sucedendo o artigo, uma conclusão geral e as referências às fontes citadas nestas seções gerais.

INTRODUÇÃO GERAL

1. Genomas mitocondriais: estrutura, organização e transcrição

O DNA mitocondrial (DNAmt) da maioria dos animais se apresenta como uma molécula de fita dupla e circular. Apenas algumas classes de cnidários (Cubozoa, Scyphozoa e Hydrozoa) possuem o DNAmt arranjado linearmente (Bridge et al., 1992). As duas fitas do DNAmt podem ser distinguidas com base em suas composições nucleotídicas de guanina e timina (G+T), que fazem com que as fitas apresentem pesos moleculares distintos em gradientes alcalinos de cloreto de césio, sendo por este motivo uma das fitas chamada de H ("heavy", que em inglês que significa pesado) e a outra de L ("light", que traduzido do inglês é leve) (Clayton, 1991; Wolstenholme, 1992). A fita pesada é rica em guanina enquanto que a fita leve é pobre em guanina (Clayton, 1991).

Outra característica marcante do DNAmt animal é que este é geralmente muito compacto, possuindo de 15 a 20 Kb, e se apresenta organizado em 37 genes (Anderson et al., 1981). Tipicamente, todo o genoma mitocondrial está distribuído em duas subunidades de rRNA, 22 tRNAs, 13 genes codificadores de proteínas envolvidos na cadeia respiratória, e uma região não codificadora (região controle), envolvida na regulação da replicação e transcrição mitocondrial (Fig. 1) (Anderson et al., 1981; Clayton, 1991; Wolstenholme, 1992; Taanman, 1999; Asin-Cayuela & Gustafsson, 2007). Dessa maneira, existem poucos ou nenhum nucleotídeo entre os genes, não há íntrons e ocorre sobreposição de alguns genes, como *atp8* com *atp6* e *nad4* com *nad4L* (Clayton, 1991; Wolstenholme, 1992). Em muitos casos, os códons de terminação não são codificados, mas gerados posteriormente à transcrição pela poli-adenilação dos mRNAs (Bobrowicz et al., 2008).

Cada um dos 22 tRNAs codificador tem especificidade para carregar um aminoácido distinto, exceto os aminoácidos leucina e serina, cada um dos quais possui duas cópias do respectivo tRNA: trnL(TAG), trnL2(TAA), trnS(GCT), trnS2(TGA) (Wolstenholme, 1992). Os 13 genes codificadores de proteínas pertencem a quatro grandes complexos enzimáticos envolvidos na fosforilação oxidativa: citocromo b; citocromo oxidase c (*cox1-3*); NADH desidrogenase (*nad1-6*, *4L*) e as duas subunidades de ATP sintetase (*atp6*, *atp8*). Os genes *rrnS* e *rrnL* codificam as subunidades pequena e grande do ribossomo mitocondrial, respectivamente.

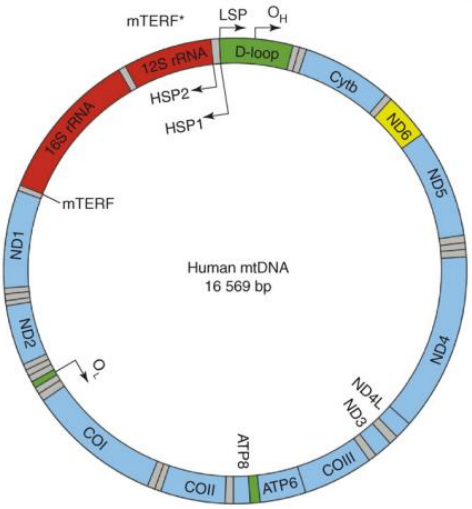

Figura 1 Organização dos 37 genes mitocondriais em humanos. OH e OL – origem de replicação da fita pesada e leve, respectivamente. HSP1 e 2 – referem-se aos dois promotores responsáveis pela transcrição da fita H; LSP- corresponde ao promotor da fita L; mTERF parece ser o responsável pelo bloqueio da transcrição da fita L e da transcrição da fita H iniciada pelo promotor HSP1. mTERF parece ser importante no estímulo da transcrição iniciada pelo promotor HSP1. Em azul, os genes codificadores de proteínas transcritos na fita H; em amarelo, ND6 o único gene codificante transcrito na fita L. Em cinza, os tRNAs. Em vermelho, os genes codificadores das duas subunidades ribossomais mitocondriais. Em verde, regiões não codificadoras e a região controle ou D-loop. Fonte: Asin-Cayuela & Gustafsson, 2007.*

A região controle (CR - *control region*) ou D-loop é a única porção significativa do DNAmt que não é codificante. Em mamíferos, encontra-se localizada entre os genes *trn*P e *trn*F (Fig. 2) e apresenta cerca de 1000pb (Anderson et al., 1981). Esta região está associada a diversos mecanismos celulares como crescimento e ciclo de vida celular além de apresentar papel fundamental na regulação da transcrição e da replicação mitocondrial (King & Low, 1987; Clayton, 1991). Além disso, esta porção é a principal responsável pelas variações de tamanho encontradas dentro da mesma espécie e até no mesmo indivíduo, sendo este último um processo conhecido como heteroplasmia (Hayasaka et al., 1991; Hoelzel et al., 1994).

A CR em mamíferos encontra-se organizada em três grandes domínios: a região conservada central (CCR) e dois domínios ricos em AT que cercam a CCR, os domínios direito e esquerdo (Anderson et al., 1981; Hoelzel et al., 1994; Jae-Heup et al., 2001). Nestes domínios encontram-se segmentos hipervariáveis (HVS), regiões com sequências repetitivas (RS) e blocos de sequencias conservados (CSB). O número de HVS, RS e CSB varia conforme a espécie (ver Hoelzel et al., 1994). A Figura 2 mostra a caracterização da região controle do gênero *Panthera*, onde ocorre o HVS-1 e 2, RS-2 e RS-3 e CBS-1-

3, seguindo as numerações propostas por Hoelzel et al. (1994). No gênero *Panthera*, RS-2 e RS-3 são as principais responsáveis pela variação de tamanho do genoma mitocondrial, tanto inter- quanto intra-especificamente (Jae-Heup et al., 2001).

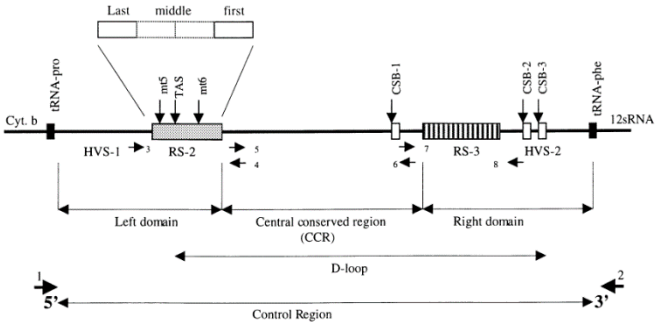

Figura 2 Representação esquemática da região controle do gênero Panthera. *tRNA-pro e tRNA-phe são os limites da região controle. HVS – corresponde aos segmentos hipervariáveis; RS-2 e RS-3 são regiões repetitivas; CSB são os blocos de sequência conservada. Fonte: Jae-Heup et al., 2001.*

O DNAmt possui duas origens de replicação, uma para cada fita (Clayton, 1991). A fita H tem sua origem de replicação na região controle (O_H) e a origem de replicação da fita L (O_L) está localizada dentro de um agrupamento de cinco tRNAs (Fig.1) (revisado por Taanman, 1999).

Em mamíferos, a transcrição do DNAmt inicia-se em três pontos de origem localizados dentro ou adjacentes à região controle (revisado por Asin-Cayuela & Gustafsson, 2007). Existe um único promotor de transcrição da fita L (LSP) e dois promotores para a fita H (HSP1 e HSP2). A maioria das informações mitocondriais são codificadas na fita H, sendo o promotor HSP2 o principal responsável pela transcrição de praticamente toda a fita. Este promotor se localiza dentro do *trn*F e produz um único grande transcrito com 12 genes codificadores de proteínas e as duas subunidades ribossomais de rRNA (Montoya, 1982; Christianson & Clayton, 1988; Taanman, 1999; revisado por Asin-Cayuela & Gustafsson, 2007). O promotor HSP1 se encontra localizado a aproximadamente 100 pb do HSP2, estando mais próximo da região controle (Fig. 1). Este promotor, HSP1, gera um transcrito mais curto, que cobre os dois genes codificadores de rRNA e termina no fim do gene *rrnL*. O promotor LSP é responsável pela transcrição de oito tRNAs e do gene *nad6*, e está localizado dentro da região controle (Montoya, 1982; Clayton, 1991; Taanman, 1999; revisado por Asin-Cayuela & Gustafsson, 2007). Até o momento pouco se sabe a respeito da terminação da transcrição.

Apenas, sabe-se que a transcrição iniciada pelo promotor HSP1 possui um sítio de terminação provável chamado mTERF (Fig.1), este mesmo sítio parece influenciar também o término da transcrição iniciada pelo promotor LSP (ver Asin-Cayuela & Gustafsson, 2007).

O processamento dos transcritos primários é guiado pela localização dos tRNAs, os quais servem de sinal de clivagem para a produção de mRNAs maduros dos genes codificadores de proteínas e para a produção de moléculas de rRNA. Este mecanismo é possível uma vez que estes segmentos encontram-se espaçados por tRNAs - processo este conhecido como modelo de pontuação de tRNA (traduzido do inglês *tRNA punctuation model*) (Ojala et al., 1981). Tipicamente, a maturação dos mRNAs envolve ainda a poli-adenilação, sendo que moléculas de rRNA também passam por este processo (Bobrowicz et al., 2008). Em vertebrados, todos os mRNAs maduros são monocistrônicos, exceto os mensageiros de *nad4*/*nad4L* e *atp8*/*atp6*, que são bicistrônicos (Bernt et al., 2012).

A organização dos genes mitocondriais difere muito pouco dentro dos principais grupos de animais, porém em grupos filogeneticamente distantes os arranjos dos genes podem ser bastante diferentes. Por este motivo, os rearranjos de genes mitocondriais são utilizados como informação filogenética em estudos que buscam resolver os ramos mais basais dos metazoários, por exemplo (Boore, 1999). Porém, como os rearranjos são raros, esta abordagem apresenta a limitação de fornecer poucos caracteres para gerar reconstruções filogenéticas satisfatórias (Curole & Kocker, 1999).

2. Genomas mitocondriais: importância

O DNA mitocondrial é amplamente empregado em análises intra-específicas de estruturação populacional e história demográfica, bem como em estudos evolutivos mais profundos (filogenias supra-específicas) (Avise et al., 1987; Harrison, 1989; Avise, 1994). Este marcador mostrou-se eficaz, sendo frequentemente utilizado para inferir as relações filogenéticas de diversos organismos (Avise, 1994). Atualmente, na era de sequenciamento de alto desempenho, as análises de genomas mitocondriais inteiros têm sido empregadas para inferir relações filogenéticas interespecíficas (por exemplo Nabholz et al., 2010; Fabre et al., 2013; Wang et al., 2013). Para alguns artrópodes e equinodermos, análises de genomas mitocondriais envolvendo aspectos como o seu

tamanho e estrutura, os arranjos dos genes, o código genético e as estruturas secundárias de rRNAs e tRNAs, proveram os únicos caracteres capazes de esclarecer filogenias obscuras (Boore, 1999, 2006).

Os principais motivos que induziram a popularização do DNAmt como marcador molecular em animais são: ausência de recombinação, herança exclusivamente materna, taxas de substituição nucleotídica aceleradas em relação ao DNA nuclear, tamanho compacto e organização conservada em vertebrados (Harrison, 1989; Wolstenholme, 1992; Avise, 1994, Curole & Kocher, 1999). Tais características facilitaram a aplicação e o desenvolvimento de diversos tipos de análise, e são ainda relevantes, mesmo quando se pode também caracterizar locos nucleares (que apresentam padrões evolutivos mais complexos).

3. Genomas mitocondriais: como são obtidos

Os genomas mitocondriais (mitogenomas) podem ser gerados de várias maneiras. O método mais comumente empregado, em organismos-modelo ou em um organismo onde um mitogenoma de uma espécie filogeneticamente próxima já foi caracterizado, é o método de sequenciamento baseado em PCR ou em PCR longo (por exemplo Cheng et al., 1994; Lei et al., 2010; Xu et al., 2012). Para aplicá-lo, uma série de *primers* conservados são desenhados em posições adjacentes ou sobrepostas, com o intuito de amplificar regiões complementares. Para outras espécies, onde não existem muitos trabalhos realizados e se tem pouco conhecimento sobre a estrutura e a composição do DNAmt, a abordagem de *primer-walking* é mais recomendada. Esta técnica consiste em desenhar um primer em uma região conhecida e fazê-lo amplificar um grande número de pares de base; em seguida o fragmento gerado é sequenciado e usado como base para desenhar outro primer que fará a mesma coisa que o primeiro e assim sucessivamente até que se "ande" todo o genoma mitocondrial (por exemplo Imanishi et al., 2013).

Inserções de DNAmt em cromossomos nucleares, conhecidas como *numts*, são comuns em diversos organismos (Bensasson et al., 2001; Hazkani-Covo et al., 2010). Os *numts* são um problema difícil de lidar na hora de reconstruir genomas mitocondriais. Alguns métodos e kits foram desenvolvidos para isolar o DNAmt e assim evitar que as inserções nucleares sejam um problema na hora de caracterizar um mitogenoma (ver Kim

et al., 2006; Wei et al., 2011). Porém, estes métodos de isolamento de DNAmt ainda não foram testados quanto a sua eficácia em remover totalmente o DNA nuclear. Sendo assim, é necessária uma análise mais aprofundada com relação à confiabilidade de genomas mitocondriais gerados através deste método, pelo menos em grupos onde se sabe que existem grandes e recentes *numts*. Além disso, novas abordagens devem ser avaliadas visando a contornar este problema.

O desenvolvimento do sequenciamento de alto desempenho vem revolucionando o campo da genética e da biologia molecular (ver revisões: Mardis, 2008; Metzker, 2009). Dentre estes, o sequenciamento de RNA (RNA-seq) vem revolucionando o ramo de pesquisa de transcriptomas (Wang et al., 2009). Além de ser uma excelente técnica para o esclarecimento da arquitetura da transcrição nuclear em organismos não-modelo, estima-se que 25% dos transcritos gerados por esta técnica são provenientes de DNA organelar (Wang et al., 2009; Torres et al., 2009; Nabholz et al., 2010; Neira-Oviedo et al., 2011; Smith, 2013). Ou seja, esta técnica parece ser bastante promissora para gerar genomas mitocondriais. Além disso, os dados gerados por RNA-seq eliminam a contaminação por *numt* (cópias nucleares de DNA mitocondrial) uma vez que estes segmentos tendem a ser pseudogenes, não sendo transcritos e tampouco gerando mRNAs maduros (Bensasson et al., 2001; Hazkani-Covo et al., 2010).

4. *Numt* e suas complicações

Acredita-se que a mitocôndria originalmente era uma α-proteobacteria que foi incorporada na origem das células eucarióticas através de um processo que conhecemos como endossimbiose (Lang et al., 1999). Parte do material genético desta proteobacteria endossimbionte foi perdida e grande parte foi transferida para o núcleo, motivo pelo qual a grande maioria das proteínas mitocondriais são codificadas atualmente no núcleo (para mais detalhes a respeito da evolução mitocondrial, ver Gray, 2012). Em contrapartida, não há relatos de transferência recente de material genético funcional do DNA mitocondrial para o núcleo em animais (revisado por Bensasson et al., 2001). Possivelmente, isto ocorre porque apenas genes vitais foram mantidos no DNA organelar e também porque os códigos genéticos do DNA nuclear e mitocondrial em animais são distintos. Sendo assim, transferências recentes de sequencias mitocondriais para o núcleo,

conhecidas como *numts*, se assemelham a pseudogenes (Bensasson et al., 2001; Hazkani-Covo et al., 2010).

Na família Felidae existem casos bem documentados de *numts* (Lopez et al. 1994, 1996; Johnson et al., 1996; Cracraft et al., 1998; Kim et al., 2006; Antunes et al., 2007). Um caso bem conhecido e comentado na literatura é a translocação de 7,9 kb do genoma mitocondrial para o cromossomo D2 de *Felis catus* (Lopez et al., 1994, 1996; Antunes et al. 2007). Outro caso em que ocorre tal translocação é no gênero *Panthera*, onde foi detectado em *P. tigris* 12.536 pb do genoma mitocondrial em um único bloco no cromossomo F2 (Cracraft et al., 1998; Kim et al., 2006). A similaridade entre *numt* e *cymt* (DNA mitocondrial citoplasmático) ocasionam inúmeros problemas quando se almeja utilizar marcadores provenientes de DNAmt. Estes problemas variam desde a identificação errônea de espécies (no caso de *DNA barcode*, por exemplo) até problemas relacionados a estudos populacionais ou a reconstruções filogenéticas (Song et al., 2008; Davis et al., 2010; Hazkani-Covo et al., 2010).

5. Relações filogenéticas do gênero *Panthera*

O início da radiação dos felinos modernos é marcado pela divergência da linhagem *Panthera*, formada por *Panthera tigris*, *P. uncia*, *P. onca*, *P. pardus*, *P. leo*, juntamente com as espécies irmãs *Neofelis nebulosa* e *N. diardi* (Johnson et al., 2006). Diversos trabalhos moleculares buscaram resolver a filogenia do gênero *Panthera*, porém, não há sequer dois trabalhos que reconstituam a filogenia do gênero com a mesma topologia (por exemplo, Janczewski et al., 1995; Johnson et al., 1996; Yu & Zhang, 2005; Johnson et al., 2006). Isto está relacionado principalmente à divergência rápida e recente do gênero, o que dificulta a escolha de marcadores moleculares capazes de detectar o sinal filogenético de maneira conclusiva (Johnson et al., 2006).

Atualmente, a filogenia do gênero *Panthera* mais aceita é a proposta por Davis et al. (2010). Neste trabalho, os autores realizaram uma busca exaustiva por diversas sequências disponíveis em base de dados como o GenBank e ainda geraram novas sequências para algumas espécies. O resultado foi a compilação de genes mitocondriais (6.141 pb), autossômicos (19.124 pb), ligados ao cromossomo X (3.223 pb) e ligados ao cromossomo Y (19.140 pb). A filogenia encontrada apresenta concordância com clados

propostos por dados morfológicos e até o momento parece ser a que melhor representa as relações filogenéticas do gênero (Fig. 3). Este trabalho detectou uma série de falhas em estudos filogenéticos anteriores, desde sequencias que estavam mal identificadas - diziam ser de uma espécie mas nas análises filogenéticas eram agrupadas com outra - até amplificação errônea do *numt* ao invés do *cymt*.

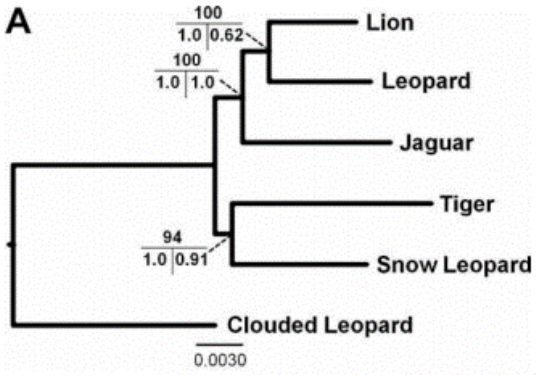

Figura 3 - Filogenia atualmente aceita do gênero Panthera. *Topologia obtida através do método de Maximum likelihood. Os valores de suporte dos ramos estão dispostos da seguinte maneira: em cima valor de bootstrap obtido por Maximum Likelihood, na porção inferior temos na esquerda a probabilidade posterior obtida pela análise de Inferência Bayesiana e na direita a probabilidade posterior estimada pelo método BEST (*Bayesian estimation of species trees*). Fonte: Davis et al., 2010.*

Com o crescimento da era genômica, muitos trabalhos têm buscado resolver as relações filogenéticas do gênero *Panthera* com genomas mitocondriais, como é o caso de Wei et al. (2011) e Bagatharia et al. (2013). Apesar do valor científico destes dois trabalhos, ambos apresentam falhas notáveis. No trabalho de Wei et al. (2011), foram gerados os primeiros genomas mitocondriais de espécies do gênero *Panthera* (*P. tigris*, *P. pardus* e *P. uncia;* n° acessos do GenBank, EF551003, EF551002, EF551004, respectivamente). No entanto, nas análises filogenéticas foram utilizadas apenas partições do DNA mitocondrial, uma vez que as demais espécies não possuíam seus genomas mitocondriais disponíveis, sendo que utilizaram uma sequência disponível no GenBank (n° de acesso AY170043) que já havia sido identificada por Davis et al. (2010) como sendo um erro de identificação de espécie. Esta sequência foi depositada como sendo de *P. leo*, porém as análises filogenéticas posteriores demostraram que esta sequência era na realidade de *P. uncia*. Talvez por este motivo a topologia encontrada por estes autores possua suportes muito baixos e difira tanto das demais filogenias do gênero, principalmente pela ausência do clado *P. leo* e *P. pardus*, o qual que é fortemente

suportado por diversos trabalhos (Janczewski et al., 1995; Johnson et al., 1996; Bininda-Emonds et al., 1999, 2001; Davis et al., 2010).

Apesar de existir previamente um mitogenoma de *P. leo persica* disponível no GenBank (n° de acesso JQ904290), este não possuía um artigo de referência associado, de modo que Bagatharia et al. (2013) foram os primeiros a publicarem o genoma mitocondrial de *P. leo persica* (n° de acesso: KC834784). Analisando-se este trabalho, nota-se que primeiramente ele menciona *P. onca* como uma espécie pertencente ao gênero *Panthera* (o que é amplamente conhecido), porém nas análises realizadas com os mitogenomas completos os autores excluem esta espécie, não mencionando em momento algum o porquê deste fato. Uma possível explicação é o fato de que até há pouco tempo não existia qualquer genoma mitocondrial de onça-pintada disponível no GenBanK. Entretanto, em novembro de 2013, quando nosso estudo já se encontrava em fase de finalização, uma sequência de mitogenoma de onça-pintada se tornou disponível no GenBank (n° de acesso KF483864), porém sem haver qualquer artigo relacionado à mesma até o momento.

OBJETIVOS

1. Caracterizar o genoma mitocondrial da onça-pintada (*Panthera onca*), utilizando dados de sequenciamento de RNA, certificando-se da especificidade de um mitogenoma gerado sem *numts*.

2. Conduzir a primeira análise filogenética dos mitogenomas completos do gênero *Panthera*.

ARTIGO CIENTÍFICO

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A complete mitochondrial genome of the jaguar (*Panthera onca*) generated on the basis of RNA-sequencing data

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Abstract

Mitochondrial genomes are usually obtained by using a set of conserved PCR primers, which may be a good strategy for several species. However, translocations of cytoplasmic mitochondrial DNA segments into the nuclear genome (*numts*) are known to be a common phenomenon in many taxa, including the felid genus *Panthera*. Available strategies to avoid contamination with *numt* segments include the isolation of mitochondrial DNA followed by PCR, long-PCR, primer walking and, more recently, the use of next-generation sequencing (NGS) approaches. Among these, RNA sequencing (RNA-seq) may be particularly useful, as it allows the efficient capture at high coverage of mtDNA transcripts, avoiding pseudogenized nuclear copies. Using transcriptome data, we have characterized the complete mitochondrial genome of the jaguar (*Panthera onca*), the only species of this genus whose mitogenome had not yet been published. Our results demonstrate that the RNA-seq approach is very efficient for full mtDNA sequencing in mammals, and holds great promise especially in the case of groups plagued by the presence of large and recent *numts*, as is the case of *Panthera* species. We use our jaguar sequence and previously available data on other species to conduct the first complete mitogenomic phylogenetic analysis of the *Panthera* genus.

Keywords: *numt*, felids, mitogenome, transcriptome, next-generation sequencing.

1. Introduction

Mammalian mitochondria contain a very compact, circular, double-stranded genome, spanning 15 – 20 kb and containing 37 genes (Anderson et al., 1981). A typical mammalian mitochondrial genome (mitogenome) encodes 13 protein-coding genes, two mitochondrial ribosomal RNA subunit genes (rRNAs) and 22 transfer RNAs (tRNAs) (Anderson et al., 1981; Wolstenholme, 1992). The mammalian mitochondrial DNA (mtDNA) has no introns and there are few or no nucleotides between genes, with a single non-coding segment called the control region (CR) or displacement loop (D-loop) (Clayton, 1991; Wolstenholme, 1992). The D-loop exhibits the greatest length variation among mammalian mitogenomes (Hoelzel et al., 1994) and has been shown to contain the replication origin of the H strand and the promoters for RNA transcription of both mtDNA strands (Clayton, 1991; Taanman, 1999; Asin-Cayuela and Gustafsson, 2007).

Mitogenomes are usually sequenced with the use of conserved PCR primers that amplify overlapping fragments, which jointly span the entire mtDNA (e.g. Lei et al., 2010). Although this can be a good strategy for many species, in some cases there may be issues posed by the translocation of cytoplasmic mitochondrial DNA (*cymt*DNA) segments into the nuclear genome (i.e. *numt* copies) (Lopez et al., 1994; Bensasson et al., 2001). Homologous segments of the mitochondrial DNA that are inserted into the nuclear genome have been reported in numerous organisms (e.g. Bensasson et al., 2001; Hazkani-Covo et al., 2010), and seem to be quite common in general. In addition to posing challenges to accurate mitogenome reconstruction, the sequence similarity between *numt* and *cymt*DNA copies (especially in the case of recent integrations) can cause other problems. These include the misidentification of species when using mitochondrial markers (*e.g*. DNA barcodes), or errors in phylogenetic reconstruction induced by the comparison of non-orthologous segments (Song et al., 2008; Davis et al., 2010; Hazkani-Covo et al., 2010).

Seeking to avoid such issues, several methods have been developed to prevent the unintentional sequencing of *numt* copies while targeting mtDNA segments or full mitogenomes. These approaches include long-PCR (e.g. Cheng et al., 1994; Machida et al., 2002) or mitochondrial isolation/purification followed by PCR with multiple overlapping primers (e.g Wei et al., 2011). More recently, with the development of next-generation sequencing (NGS) technologies, RNA sequencing (RNA-seq) has emerged as a promising

tool to overcome this problem (e.g. Wang et al., 2009; Nabholz et al., 2010; Fabre et al., 2013; Wang et al., 2013). Of the transcripts resulting from RNA-seq experiments, a substantial fraction (up to 25%) is derived from organelle genomes, which enables a variety of studies addressing mitochondrial gene organization and expression in non-model organisms (Torres et al., 2009; Nabholz et al., 2010; Neira-Oviedo et al., 2011; Smith, 2013; Wang et al., 2013). In addition, transcript data are expected to exclude *numt* contamination, since nuclear translocations tend to become pseudogenized and therefore cease to undergo effective transcription (Bensasson et al., 2001; Hazkani-Covo et al., 2010).

In the family Felidae, there are well-known examples of *numts*, including two different episodes of large-scale transposition (e.g. Lopez et al. 1994, 1996; Johnson et al., 1996; Cracraft et al., 1998; Kim et al., 2006; Antunes et al., 2007). The first large *numt* reported for felids was the translocation of 7.9 kb of the mitochondrial genome into chromosome D2 of the domestic cat (*Felis catus*) and its close relatives (Lopez et al., 1994, 1996). Subsequently, a different insertion was identified in the five cat species belonging to the *Panthera* genus: tiger (*P. tigris*), lion (*P. leo*), leopard (*P. pardus*), jaguar (*P. onca*) and snow leopard (*P. uncia*) (Johnson et al., 1996; Cracraft et al., 1998; Kim et al., 2006). This *Panthera*-specific *numt* turned out to be one of the largest known nuclear integrations in eukaryotes, comprising 74% of the mitochondrial genome copied into chromosome F2 (Kim et al., 2006).

Given the recent age of this *numt* insertion (estimated to have occurred *ca*. 3.5 million years ago, prior to the divergence of extant *Panthera* species), there is still considerable sequence similarity with respect to the paralogous *cymt*DNA segments (Kim et al., 2006). Such similarity, combined with the extent of this translocated segment, has led to difficulties in avoiding spurious amplification of *numt* fragments in PCR-based studies, including efforts to resolve the phylogenetic relationships among *Panthera* species (Davis et al., 2010).

The resolution of this phylogenetic problem has been notoriously difficult, not only because of *numt* contamination of mtDNA data sets, but likely also due to lineage sorting effects induced by the rapid diversification of this group, as well as homoplasy or lack of phylogenetic signal affecting some sets of characters (Johnson et al., 2006; Davis et al., 2010). Although the full resolution of this problem will depend on a multi-locus approach (as independent loci may indeed have different genealogical histories in such a recent radiation), settling the mitochondrial phylogeny of this genus is a step forward. Such a step has not yet been accomplished, as different mtDNA partitions have supported different phylogenies

(Janczewski et al., 1995; Johnson et al., 1996; Wei et al., 2011), and the only available study on full *Panthera* mitogenomes (Bagatharia et al., 2013) did not include the jaguar in its analysis. It is therefore necessary to generate and analyze a full jaguar mtDNA sequence, while striving to completely avoid *numt* contamination.

Although it is known that organelle-derived content is abundant in transcriptome data sets (e.g. Neira-Oviedo et al. 2011; Smith, 2013), so far only a few studies have reported the reconstruction of full mitogenomes with such an approach (e.g. Nabholz et al., 2010; Wang et al., 2013). In particular, the use of this method with mammals is still quite rare, as to our knowledge so far only two published studies have reported it (Hampton et al., 2011; Fabre et al., 2013). In this study, we present the complete mitochondrial genome of the jaguar (*Panthera onca*), whose sequence was based almost entirely on RNA-seq data. We also present the first phylogenetic analyses of the full mitogenomes of all five *Panthera* species (including their sister-species *Neofelis nebulosa* as an outgroup), and show that the resulting topology differs from the most recent, supermatrix-derived phylogeny of this genus. Our study demonstrates that RNA-seq is very efficient to generate mitogenome sequences, especially in cases where large and complex *numts* are present, such as the *Panthera* genus.

2. Materials and methods

2.1. *Sample collection*

Samples of blood, muscle and four areas of the skin were collected from a single jaguar individual as part of the Jaguar Genome Project currently being conducted by a consortium of Brazilian institutions. The target individual was a healthy, wild-caught male housed at the Sorocaba Municipal Zoo in southeastern Brazil. Certified veterinarians and other zoo personnel performed all anesthesia and sampling procedures, which followed protocols approved by the Sorocaba Zoo. Upon collection, blood and tissue samples were immediately stabilized with RNAlater (Invitrogen®), with a 10:1 proportion of this buffer relative to the sample. In addition, we collected *ca*. 5 ml of whole blood separately for use in genomic DNA extractions. This sample was placed in a vacutainer tube containing the anticoagulant K_2EDTA, and stored at 4ºC for less than 48 hours before DNA extractions were carried out.

2.2. *Laboratory procedures and mitogenome assembly*

Total RNA was extracted from blood and tissue samples with a standard Trizol RNA extraction protocol (Chomczynsky & Sacchi, 1987). RNA quantification and quality assessment (integrity and purity) were performed using a NanoDrop spectrophotometer (Thermo Scientific®). We constructed a cDNA library for each sample using the TruSeq RNA Sample Prep Kit v2 (Illumina®), following the manufacturer's instructions. The cDNA libraries were then used to perform multiplexed/barcoded RNA-seq with an Illumina HiScan sequencer.

We performed initial analyses of the Illumina sequence reads using the software package Galaxy (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). Only reads presenting a minimum Phred quality scores $\geq$ 20 per base were included in downstream analyses. *De novo* assembly was conducted with the software package Trinity (Grabherr et al., 2011), applying default parameters. The contigs produced from assembled transcripts were used to perform pairwise BLAST searches against the *Panthera tigris* mitogenome (accession EF551003; Wei et al., 2011), which was used as a reference to identify the mitochondrial segments covered by our transcript data set. We also performed this assessment by using the 'Map to reference' tool implemented in the software package Geneious 6.1.2 (Kearse et al. 2012).

Short segments that were not covered by the transcript data (herein referred to as 'transcript gaps' [TGs]) were bridged with a PCR-based approach. We extracted genomic DNA from the blood sample using the DNeasy Blood & Tissue Kit (Qiagen®) following the manufacturer's instructions. We designed PCR primers (Tab. 1) flanking each TG, applying two criteria to maximize their specificity to the *cymt*DNA copy: (i) high melting temperatures; and (ii) at least 100 bp of sequence on either side of the TG, which could be directly compared to the transcript-based data set. In addition, primers were designed and PCR experiments were conducted based on the same individual whose transcripts had been sequenced, which should further improve the specificity of *cymt*DNA amplification. Furthermore, each pair of primers was tested with the Primer-BLAST software to ensure their high probability of success with *Panthera* mitochondrial DNA (Ye et al., 2012)

PCR reactions were conducted in a total volume of 10µl containing 10-15ng total genomic DNA, 1X Buffer, 1.5mM $MgCl_2$, 100µM dNTPs, 0.25µM of each primer and 0.1U Platinum® Taq DNA Polymerase (Invitrogen®). PCR cycling was performed under the

following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 45s, 64 - 67°C for 45s, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium) and purified with exonuclease I and shrimp alkaline phosphatase before sequencing on a MegaBACE 1000 automated sequencer (GE Healthcare®).

To characterize one of the transcript gaps (TG1), which contained a complex repetitive region (see Results), PCR products were cloned into a pCR®2.1-TOPO® vector using a TOPO TA kit (Invitrogen®), following the manufacturer's protocol. Chemocompetent *E. coli* were transformed with the recombinant plasmids following standard protocols (Sambrook et al., 2001). To obtain sequences spanning the full inserts, plasmids were sequenced using the vector's specific M13 forward (-20) primer (5' GTAAAACGACGGCCAG 3') and M13 reverse primer (5' CAGGAAACAGCTATGAC 3').

2.3. *Jaguar mitogenome annotation*

We employed two different strategies to annotate the jaguar mitogenome: (i) direct comparison to the domestic cat reference mtDNA sequence (accession NC001700; Lopez et al., 1996); and (ii) use of specific online software packages designed for organellar genome annotation: DOGMA (Wyman et al., 2004) and MITOS (Bernt et al., 2012). We assessed the performance of each of these approaches in the accurate prediction of mtDNA content and exact gene boundaries, and selected the strategy whose result best matched well-annotated mammalian mtDNA genomes.

2.4. *Phylogenetic analyses*

We used our jaguar mitogenome, along with mtDNA sequences from the other *Panthera* species obtained from GenBank (Tab. 2), to perform multiple sets of phylogenetic analyses. We initially analyzed a data set containing all *Panthera* mitogenomes, along with mtDNA sequences from the immediate outgroup *Neofelis nebulosa* and the domestic cat (*Felis catus*), as well as the two known large felid *numts* (see Tab. 2). We aligned these sequences using the ClustalW algorithm implemented in the software package MEGA 5.2.2 (Tamura et al., 2011), and refined the alignment by hand. All sites that presented ambiguous alignment (most of which were located in the control region) were visually detected and

removed from the data set. The remaining 15,940 bp were analyzed as single partition in this initial set of phylogenetic analyses.

We reconstructed the phylogenetic relationships of this data set using four different optimality criteria: (i) minimum evolution using the Neighbor-joining (NJ) algorithm; (ii) Maximum Parsimony (MP); (iii) Maximum Likelihood (ML); and Bayesian Inference (BI). We performed NJ and MP analyses using MEGA, considering only transversions and employing Maximum Composite Likelihood distances (Tamura et al., 2004) in the former, while conducting 10 replicates of random taxon addition combined with tree-bisection-reconnection (TBR) branch-swapping in the latter. We also used MEGA to reconstruct the ML tree of this data set, initially by estimating its best-fitting model of nucleotide substitution based on the Bayesian Information Criterion (BIC). The Tamura-Nei model with gamma correction for rate heterogeneity among sites (TN93+G) was selected and used the subsequent analysis, which employed a NJ starting tree and nearest-neighbor-interchange (NNI) branch swapping. For these NJ, MP and ML analyses, nodal support was assessed with 500 nonparametric bootstrap replicates. The BI phylogeny was reconstructed with BEAST 1.8.0 (Drummond et al., 2012), using a random starting tree and assuming the TN93+G model of substitution, a strict molecular clock and a Yule process for the tree prior. We ran the MCMC algorithm for 10,000,000 generations and sampled trees and parameters every 1,000 steps. Resulting trees were visualized using FigTree 1.4.

We then conducted a final set of phylogenetic analyses including only the *Panthera* mitogenomes and the *N. nebulosa* outgroup, in which we assessed three different data sets: (i) full mitogenomes; (ii) full mitogenomes excluding the control region; and (iii) only protein-coding genes. We performed the NJ analyses using the same approach described above, except that both transitions and tranversions were included in the data sets. The MP analyses were performed with PAUP* 4.0b10 (Swofford, 2003), using heuristic searches incorporating 500 replicates of random taxon addition and TBR branch-swapping. MP nodal support was assessed with 500 bootstrap replicates, each consisting of a heuristic search with 50 iterations of random taxon addition followed by TBR.

The ML trees of these final data sets were inferred with the RAxML-VI-HPC (Stamatakis, 2006) platform, using the graphic user interface raxmlGUI (Silvestro & Michalak, 2012). We used the software jModelTest2 (Darriba, 2012) to estimate the best-fitting model of nucleotide substitution for each data set, based on the Akaike Information

Criterion (AIC). The general-time-reversible model with gamma correction and a proportion of invariant sites (GTR+G+I) was selected for the first two data sets (complete mitogenomes with and without the control region). For the data set including only protein-coding genes, GTR+G was found to be the best model. The ML trees were assessed with the rapid bootstrap option, based on 1000 bootstrap replicates.

The BI analyses of these data sets were also performed with BEAST, but employing a data partitioning approach to improve model fitting. We divided the mitogenome into 16 partitions, comprising the CR, the tRNAs joined together, the two rRNAs combined, and each of the 13 protein-coding genes, respectively. For each partition, the best-fitting model of nucleotide substitution was estimated with MEGA (see Supplementary Tab. 1) and incorporated into the analysis. We unlinked substitution models and clock rates among partitions, and assumed a strict molecular clock and a Yule process tree prior. The MCMC algorithm was started from a random tree and ran for 20,000,000 generations, with parameters and trees sampled every 1,000 steps. The first 10,000 trees were discarded as burn-in, and we used the latter 10,000 samples to infer the consensus topology and the posterior probability of its included clades.

3. Results and Discussion

3.1. Transcript coverage and PCR-based complementary data

A total of 17,703,707 single-end reads (spanning 50 bp each) were generated from six samples (blood, muscle and four different skin biopsies) of the same individual. The *de novo* assembly resulted in a total of 19,728 contigs (average length 201 – 6,107 bp). Of these, 79 contigs (ranging in length from 260 to 3,903 bp each, and resulting from the overall clustering of 576,024 raw reads) yielded positive results with a pairwise BLAST against the *Panthera tigris* mitogenome, and were therefore used to reconstruct the mitochondrial genome of *P. onca*. Fifteen contigs were derived from blood transcripts, 14 from muscle and 50 from the skin (skin samples 1, 2, 3 and 4; see Supplementary Tab. 2). The set of contigs obtained from each sample presented similar start and end positions across the mitogenome (Supplementary Tab. 2), so that we obtained an almost complete mtDNA assembly independently for each of six replicates. The coverage of raw reads for each base pair included in the transcript data sets was as follows: blood: 1-821; muscle: 1-1,496; skin1: 1-5,807; skin2: 1-1,707; skin3: 1-1,925; and skin4: 1-2,221. All samples presented very good coverage from the *cox1* to the *cox3*

coding genes (Supplementary Fig. 1). The remaining portions presented variable coverage, which may be due to differences in transcript stability or other post-transcriptional processes (Torres et al., 2009).

When the data from all six samples were pooled, the number of raw reads per base of the jaguar mitogenome ranged from 1 to 10,950 (Fig. 1). Moreover, this combined transcript data set covered *ca*. 96% of the complete *P. onca* mitochondrial genome. Read abundance in protein-coding genes was greater than in other portions of the mitochondrial genome, but we also mapped some reads to tRNA genes, both rRNA subunit genes and even stretches of the control region (see Fig. 1).

Typically, tRNAs are lost from main transcripts during post-transcriptional events, as their structures act as signals for the cleavage of the polycistronic transcript in a process known as tRNA punctuation model (Ojala et al., 1981; Torres et al., 2009). This excision of tRNAs is part of the process leading to mature mRNA and rRNA molecules, after which the mRNAs are typically polyadenylated (Bobrowicz et al., 2008).

Since our approach to cDNA library construction used a purification step with poly-T oligo beads (that bind to poly-A tails of mature mRNAs), this may be the cause of the observed lower abundance of transcript reads mapping to tRNAs genes, and even the lack of coverage of some tRNA segments. It may also account for the observed difference in read abundance between the two rRNA subunits (see Fig. 1), which might be unexpected since they are co-transcribed. However, there is evidence indicating that, after cleavage of the rRNA molecules, the 12S rRNA receives a shorter poly-A tail relative to the 16S rRNA (Torres et al., 2009; Neira- Oviedo et al., 2011), which would account for the much lower capture of the former segment using the poly-T beads employed in this study.

Six short segments of the mitogenome were not covered by the RNA-seq data, and were thus identified as ‘transcript gaps’ (TGs). Two of the TGs corresponded to the felid repetitive segments known as RS-2 and RS-3, located in the control region (Hoelzel et al., 1994; Jae-Heup et al., 2001), while the others comprised portions of tRNAs.

The repetitive sequence RS-3 is known to present considerable length variation within and among individuals owing to constant duplication, deletion or substitution of the short (6 – 10 bp) tandem repeat units (Jae-Heup et al., 2001). To characterize this region, we cloned and sequenced 18 PCR products spanning this segment, whose length ranged from 201 bp to 503

bp (Supplementary Fig. 2). Seven clones exhibited flanking sequences (*i.e*. immediately outside the repetitive array) that were identical to the mitogenome generated with RNA-sequencing data, and were thus considered to reliably represent the *cymt*DNA copy of this segment. These seven clones showed internal variation within the repetitive array, and we selected the longest one (*i.e*. the one containing the most repeat units) to include in the full mitogenome sequence reported here.

In addition to these seven clones, three others also contained flanking sequences that were very similar to the transcript-based data set, with the only observed differences being two nucleotide substitutions e one nucleotide deletion (located within a poly-T stretch). As these minor differences may be due to heteroplasmy or to Taq error in the PCR step prior to cloning, we find it likely that these clones were also derived from the *cymt*DNA copy. With their inclusion as likely *cymt*DNA-derived sequences, we had a total of 10 clones representing this segment of the mitogenome, whose length varied from 293 to 503 bp due to differences in RS-3 repeat composition.

The eight remaining clones comprised two very distinct sequences, represented by two and six copies each. The sequence represented by two clones was quite short (201-bp long) and very distinct from all others, presenting no identifiable similarity with any mtDNA-related entry in GenBank. Since they presented some similarity with apparently random domestic cat genomic segments, we infer that they derive from either spurious amplification of nuclear DNA stretches, or from old *numt* insertions that can still be captured with the mtDNA-targeting primers designed here. On the other hand, the sequence represented by six clones was quite similar to the *cymt*DNA copy described above, but shorter (238-bp long) and bearing 17 nucleotide substitutions relative to the latter on the 5' flank of the repetitive array, as well as a deletion in a conserved segment on the 3' flank of the repeats. We infer that these six clones represent the jaguar homolog of the large, recent *Panthera numt*, which is still expected to retain considerable sequence similarity with the *cymt*DNA copy (Kim et al., 2006). Interestingly, the tiger version of this *numt* translocation (the only homolog that had been characterized so far) also displays a rather large (340-bp long) deletion affecting this repetitive segment and its flanking regions (Kim et al., 2006). Assuming that we have indeed captured the same *numt*, and given that these deletions have different start and end positions in this region, we infer that they occurred independently in each lineage, after the original integration of the mtDNA segment.

3.2 Panthera onca *mitogenome organization*

The complete mitogenome sequence of *P. onca* was deposited in GenBank under accession number XXXXXX. Due to differences in the control region length, mostly caused by the RS-3 repetitive sequences, the jaguar mitogenome ranged in size from 16,856 bp to 17,066 bp. As expected, the jaguar mitogenome comprises 13 protein-coding genes, two mitochondrial ribosomal RNA subunits genes (rRNAs), 22 transfer RNAs (tRNAs) genes and the control region (Tab. 3).

To annotate gene boundaries, we tested two frequently used online organellar genome software packages, DOGMA and MITOS. The latter program performed better in the prediction of gene boundaries, but neither was 100% accurate. At the same time, orientation of gene transcription (L-strand or H-strand) was accurate with both programs. MITOS predicted 20 tRNA gene boundaries precisely, but the remaining edges were not perfectly identified (Supplementary Tab.3). We therefore decided to perform the final annotation of the jaguar mitogenome by hand, using the *Felis catus* mitogenome (accession NC001700; Lopez et al., 1996) as a reference, validated by additional comparisons to other mammals.

The jaguar mitochondrial genome presented the same content and the same transcriptional orientation for homologous genes as other mammals (Tab. 3). The *nad1*, *nad2*, *nad3*, *nad4*, *nad6* and *cox3* genes did not contain complete termination codons, which should be generated post-transcriptionally by the polyadenilation of their respective mRNAs (Ojala et al., 1981; Bobrowicz et al., 2008; Torres et al., 2009). Overall, we detected 48 transcripts with 3'-end poly-A stretches (Supplementary Tab. 2), which likely represent polyadenilated mature mRNAs. Transcripts from all six tissue samples presented poly-A stretches after the *nad4*, *nad2* and trnS2 genes. In five of the six samples, we also observed a poly-A tail after *nad3*, *cyt*. B, and *cox2*. Finally, we also observed poly-A stretches after the trnE and *atp6* genes for four different tissue samples. Such observations should be useful to document patterns of polyadenilation in different mammalian groups, and should become more common if the use of RNA-seq for mtDNA characterization is expanded.

3.3 Phylogenetic analyses

Before we performed phylogenetic analyses, we assessed the composition and information content of several subsets of the mitogenome alignments to check for potential biases that could affect the results. Among the 13 protein-coding genes, we verified the nucleotide composition of each locus and compared it to the overall average. We observed minor differences in base composition in the *atp8* and *nad6* genes (Supplementary Fig. 3), but did not find evidence that they could bias the phylogenetic results. Although other studies (e.g. Paton et al., 2002; Nabholz et al., 2010) have removed the *nad6* gene because it often presents an outlier nucleotide composition and is encoded in the L-strand (see Fig. 1), in this case we saw no reason to exclude it from the analysis, and therefore kept it in all data sets described below.

The first set of phylogenetic analyses, including all *Panthera* mitogenomes plus those of *Neofelis nebulosa* and *F. catus*, as well as the *F. catus* and *P. tigris numts* (see Tab. 2), and excluding ambiguously aligned sites, resulted in a topology that was congruently supported by almost all methods (Fig.2A). The only exception was MP, which showed a sister-group relationship between *P. onca* and *P. uncia*, albeit with low (52%) bootstrap support. All other methods tended to provide moderate to strong support to all nodes in the consensus tree (Tab. 4). As expected, the *F. catus* mitogenome and *numt* grouped together, and the *P. tigris numt* grouped with the *Panthera* mitogenomes, which is consistent with previous analyses assessing the respective origins of these nuclear insertions (Lopez et al., 1996; Kim et al., 2006). However, our topology did not reconstruct the *P. tigris numt* as a sister-lineage to a monophyletic group of *Panthera* mitogenomes, which would be expected given the inference that this nuclear translocation occurred only once, before the divergence of extant *Panthera* species (Kim et al., 2006). Rather, our trees provided high support for the tiger mitogenome being the most basal lineage in the clade formed by node 5 in Fig. 2A (see Tab. 4). The most likely explanation for this result is that the tiger mitogenome presents an accelerated rate of nucleotide substitution relative to the other *Panthera* species, leading it to undergo long-branch-attraction with the outgroup *N. nebulosa* , and thus drawing it to the base of this clade. The same conclusion was reached by Davis et al. (2010) when discussing the discrepancy observed between the topologies reconstructed with their nuclear *vs*. mtDNA data sets.

The final set of analyses, including only the mitogenomes of *Panthera* spp. and the *N. nebulosa* outgroup, yielded strong and congruent support to a single phylogenetic topology (Fig. 2B). This tree was consistent with the results obtained in the initial set of analyses (Fig. 2A), but support values for the *Panthera* nodes tended improve with the exclusion of the

additional sequences (*F*. *catus* and *numts*). There was maximum support for the sister-group relationship between lion and leopard with all methods and all data sets (Tab. 5). The same was observed with respect to the basal position of the tiger within the *Panthera* genus. The node uniting the snow leopard (*P*. *uncia*) with the lion/leopard clade (node 2 in Fig. 2B) continued to present less support than the other two, but this set of analyses revealed an increased robustness of this relationship. Of the four optimality criteria, MP was the only one that consistently failed to recover this relationship with high support, but at the same time it did not support any alternative topology. Furthermore, the MP support increased (as did the NJ and ML support) when only protein-coding genes were analyzed (see Tab. 5). This observation indicates that homoplasy in the control region and in tRNA and rRNA segments may be a source of noise that negatively affects the resolution if this node. The protein-coding data set should be less affected by homoplasy, and thus provides a more conservative assessment of the phylogeny. Although it included less information (*i.e*. fewer nucleotide sites), this data set consistently yielded higher support values for the inferred topology, suggesting that its signal-to-noise ratio was better than those of the other two data sets.

Overall, these results indicate that the topology recovered here is quite robust, and reflects the mitochondrial genealogy of genus *Panthera*. Interestingly, this topology differs from the recent reconstruction of *Panthera* relationships based on a large supermatrix including some mtDNA segments along with substantial information from autosomal, X-linked and Y-linked genes (Davis et al., 2010). Both studies strongly support a sister-group relationship between lion and leopard, but here the snow leopard (*P*. *uncia*) is the sister-group to the lion/leopard clade. In contrast, Davis et al. (2010) found strong support for the jaguar (*P*. *onca*) as a sister lineage to lion/leopard, which is consistent with morphological data supporting lion/leopard/jaguar as a monophyletic subgenus *Panthera* (Hemmer 1978). Our interpretation of this difference is that the lion/leopard/jaguar clade most likely reflects the correct genomic/organismal phylogeny, while the distinct mitochondrial topology represents a case of true genealogical discordance induced by incomplete lineage sorting during the rapid diversification of *Panthera* lineages. Another factor that may have affected the mtDNA topology is homoplasy at fast-evolving sites. As mentioned above, our results (as well as the observation by Davis et al. [2010]) suggest that the tiger mitogenome presents an accelerated rate of substitution that induces long-branch-attraction towards the outgroup *N*. *nebulosa*. Such a process would tend to place the tiger mitogenome at the base of *Panthera*, and contribute to disrupt a sister-group relationship between this lineage and the snow leopard,

which was detected with the supermatrix data (Davis et al., 2010) with high support. In combination, these two processes can account for the differences observed between the supermatrix results (vastly dominated by the nuclear signal) and the mitogenome genealogy resolved here.

As we were finalizing this article for submission, an independently generated sequence of the jaguar mitogenome became available in GenBank (accession number KF483864). We compared it to our sequence and found it to be 98% identical, and to bear essentially the same average nucleotide composition. Phylogenetic analyses showed that the two jaguar sequences clustered together with maximum support in every case, and that their joint position within *Panthera* was the same as the one we observed with our data alone. At the same time, we observed that an additional lion mitogenome has recently also become available in GenBank (accession KF776494), and performed the same exercise. Again, we observed that the two lions grouped together with maximum support, and their position was the same as we report here.

Conclusions

The complete sequencing of the jaguar mitochondrial genome enabled for the first time phylogenetic analyses of the *Panthera* genus based on full mtDNA data and including all five extant species of this group. These analyses provided robust and congruent support for a topology that should settle the long-standing effort to resolve the *Panthera* mitochondrial genealogy. Although this topology differs from the most supported genomic/organismal phylogeny inferred from multi-locus data, it should still be useful as an evolutionary framework for further studies employing mtDNA data in this group. In addition to aiding in the resolution of the *Panthera* mitochondrial phylogeny, the jaguar mitogenome should also be useful as a reference to studies on the species itself. For example, it will now be possible to design high-stringency and species-specific PCR primers targeting any segment of the jaguar mtDNA, which should decrease the probability of *numt* co-amplification and its downstream analytical problems in phylogeographic and population genetic studies. Finally, our results demonstrate that RNA-seq is a powerful tool for characterizing complete mammalian mitogenomes, and may be particularly helpful for studies targeting lineages with large and recent *numts* that pose challenges to traditional approaches of full mtDNA sequencing.

References

Anderson, S., Bankier, A. T., Barrell, B. G., De Bruijn, M. H. L., Coulson, A. R., Drouin, J., ... & Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature*, 290, 457 – 465.

Antunes, A., Pontius, J., Ramos, M. J., O'Brien, S. J., & Johnson, W. E. (2007). Mitochondrial introgressions into the nuclear genome of the domestic cat. *Journal of Heredity*, *98*(5), 414-420.

Asin-Cayuela, J., & Gustafsson, C. M. (2007). Mitochondrial transcription and its regulation in mammalian cells. *Trends in biochemical sciences*, *32*(3), 111-117.

Bagatharia, S. B., Joshi, M. N., Pandya, R. V., Pandit, A. S., Patel, R. P., Desai, S. M., ... & Saxena, A. K. (2013). Complete mitogenome of asiatic lion resolves phylogenetic status within *Panthera*. *BMC genomics*, *14*(1), 572.

Bensasson, D., Zhang, D. X., Hartl, D. L., & Hewitt, G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution*, *16*(6), 314-321.

Bernt, M., Donath, A., Jühling, F., Externbrink, F., Florentz, C., Fritzsch, G., ... & Stadler, P. F. (2013). MITOS: Improved *de novo* Metazoan Mitochondrial Genome Annotation. *Molecular Phylogenetics and Evolution*, 69(2), 313-319.

Blankenberg, D., Kuster, G. V., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., ... & Taylor, J. (2010). Galaxy: A Web-Based Genome Analysis Tool for Experimentalists. *Current protocols in molecular biology*, 19-10.

Bobrowicz, A., Lightowlers, R., & Chrzanowska-Lightowlers, Z. (2008). Polyadenylation and degradation of mRNA in mammalian mitochondria: a missing link?. *Biochemical Society Transactions*, *36*(Pt 3), 517.

Cheng, S., Higuchi, R., & Stoneking, M. (1994). Complete mitochondrial genome amplification. *Nature genetics*, *7*(3), 350-351.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, *162*(1), 156-159.

Clayton, D. A. (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annual review of cell biology*, *7*(1), 453-478.

Cracraft, J., Feinstein, J., Vaughn, J., & Helm-Bychowski, K. (1998). Sorting out tigers (*Panthera tigris*): mitochondrial sequences, nuclear inserts, systematics, and conservation genetics. *Animal Conservation*, *1*(2), 139-150.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, *9*(8), 772-772.

Davis, B. W., Li, G., & Murphy, W. J. (2010). Supermatrix and species tree methods resolve phylogenetic relationships within the big cats, *Panthera* (Carnivora: Felidae). *Molecular Phylogenetics and Evolution*, *56*(1), 64-76.

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular biology and evolution*, *29*(8), 1969-1973.

Fabre, P. H., Jønsson, K. A., & Douzery, E. J. (2013). Jumping and gliding rodents: Mitogenomic affinities of Pedetidae and Anomaluridae deduced from an RNA-Seq approach. *Gene*, *531*(2), 388-397.

Giardine, B., Riemer, C., Hardison, R. C., Burhans, R., Elnitski, L., Shah, P., ... & Nekrutenko, A. (2005). Galaxy: a platform for interactive large-scale genome analysis. *Genome research*, *15*(10), 1451-1455.

Goecks, J., Nekrutenko, A., Taylor, J., & Team, T. G. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*, *11*(8), R86.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., ... & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology*, *29*(7), 644-652.

Hampton, M., Melvin, R. G., Kendall, A. H., Kirkpatrick, B. R., Peterson, N., & Andrews, M. T. (2011). Deep sequencing the transcriptome reveals seasonal adaptive mechanisms in a hibernating mammal. *PloS one*, *6*(10), e27021.

Hazkani-Covo, E., Zeller, R. M., & Martin, W. (2010). Molecular poltergeists: mitochondrial DNA copies (*numts*) in sequenced nuclear genomes. *PLoS Genetics*, *6*(2), e1000834.

Hemmer, H. (1978). The evolutionary systematics of living Felidae: present status and current problems. *Carnivore*, 1, 71‑79.

Hoelzel, A. R., Lopez, J. V., Dover, G. A., & O'Brien, S. J. (1994). Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. *Journal of molecular evolution*, *39*(2), 191-199.

Jae-Heup, K., Eizirik, E., O'Brien, S. J., & Johnson, W. E. (2001). Structure and patterns of sequence variation in the mitochondrial DNA control region of the great cats. *Mitochondrion*, *1*(3), 279-292.

Johnson, W. E., Dratch, P. A., Martenson, J. S., & O'Brien, S. J. (1996). Resolution of recent radiations within three evolutionary lineages of Felidae using mitochondrial restriction fragment length polymorphism variation. *Journal of Mammalian Evolution*, *3*(2), 97-120.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software

platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647-1649.

Kim, J. H., Antunes, A., Luo, S. J., Menninger, J., Nash, W. G., O'Brien, S. J., & Johnson, W. E. (2006). Evolutionary analysis of a large mtDNA translocation (*numt*) into the nuclear genome of the *Panthera* genus species. *Gene*, *366*(2), 292-302.

Lei, R., Shore, G. D., Brenneman, R. A., Engberg, S. E., Sitzmann, B. D., Bailey, C. A., ... & Louis Jr, E. E. (2010). Complete sequence and gene organization of the mitochondrial genome for Hubbard's sportive lemur (*Lepilemur hubbardorum*). *Gene*, *464*(1), 44-49.

Lopez, J. V., Cevario, S., & O'Brien, S. J. (1996). Complete Nucleotide Sequences of the Domestic Cat (*Felis catus*) Mitochondrial Genome and a Transposed mtDNA Tandem Repeat (*Numt*) in the Nuclear Genome. *Genomics*, *33*(2), 229-246.

Lopez, J. V., Yuhki, N., Masuda, R., Modi, W., & O'Brien, S. J. (1994). Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. *Journal of Molecular Evolution*, *39*(2), 174-190.

Machida, R. J., Miya, M. U., Nishida, M., & Nishida, S. (2002). Complete mitochondrial DNA sequence of Tigriopus japonicus (Crustacea: Copepoda). *Marine Biotechnology*, *4*(4), 406-417.

Nabholz, B., Jarvis, E. D., & Ellegren, H. (2010). Obtaining mtDNA genomes from next-generation transcriptome sequencing: a case study on the basal Passerida (Aves: Passeriformes) phylogeny. *Molecular phylogenetics and evolution*, *57*(1), 466-470.

Neira-Oviedo, M., Tsyganov-Bodounov, A., Lycett, G. J., Kokoza, V., Raikhel, A. S., & Krzywinski, J. (2011). The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect molecular biology*, *20*(2), 141-152.

Ojala, D., Montoya, J., & Attardi, G. (1981). tRNA punctuation model of RNA processing in human mitochondria. *Nature* **290**, 470 – 474.

Paton, T., Haddrath, O., & Baker, A. J. (2002). Complete mitochondrial DNA genome sequences show that modern birds are not descended from transitional shorebirds. *Proceedings of the Royal Society of London*. *Series B: Biological Sciences*, *269*(1493), 839-846.

Sambrook, J., Russell, D. W., & Russell, D. W. (2001). Molecular cloning: a laboratory manual 1, 3.ed., New York: Cold Spring Harbour Laboratory Press.

Silvestro, D., & Michalak, I. (2012). raxmlGUI: a graphical front-end for RAxML.*Organisms Diversity & Evolution*, *12*(4), 335-337.

Smith, D. R. (2013). RNA-Seq data: a goldmine for organelle research. *Briefings in functional genomics*.

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, *105*(36), 13486-13491.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*(21), 2688-2690.

Swofford, D. L. (2003). PAUP*: phylogenetic analysis using parsimony, version 4.0 b10.

Taanman, J. W. (1999). The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1410*(2), 103-123.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(30), 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, *28*(10), 2731-2739.

Torres, T. T., Dolezal, M., Schlötterer, C., & Ottenwälder, B. (2009). Expression profiling of Drosophila mitochondrial genes via deep mRNA sequencing. *Nucleic acids research*, *37*(22), 7509-7518.

Wang, H. L., Yang, J., Boykin, L. M., Zhao, Q. Y., Li, Q., Wang, X. W., & Liu, S. S. (2013). The characteristics and expression profiles of the mitochondrial genome for the Mediterranean species of the Bemisia tabaci complex. *BMC genomics*, *14*(1), 401.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57-63.

Wei, L., Wu, X., Zhu, L., & Jiang, Z. (2011). Mitogenomic analysis of the genus *Panthera*. *Science China Life Sciences*, *54*(10), 917-930.

Wolstenholme, D. R. (1992). Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol* 141: 173-216.

Wu, X., Zheng, T., Jiang, Z., & Wei, L. (2007). The mitochondrial genome structure of the clouded leopard (*Neofelis nebulosa*). *Genome*, *50*(2), 252-257.

Wyman, S. K., Jansen, R. K., & Boore, J. L. (2004). Automatic annotation of organellar genomes with DOGMA. *Bioinformatics*, *20*(17), 3252-3255.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*, *13*(1), 134.

Tables:

Table 1 – PCR primers developed in this study to amplify and sequence transcript gaps (TG) in the jaguar mitogenome (i.e. regions not covered by the RNA-seq data). The table indicates the size and location of each TG in the jaguar mitogenome, as well as the respective PCR product size and the melting temperature (Tm) of each primer pair.

TG	Location	TG size (bp)	PCR size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)
1	309–532 bp	~250	~ 500	TATTCTCTATGCGGGGGTTCCACAC	GGGGGTAAGGGGGGTTTGTTTAAGC	66°C
2	888–957 bp	70	541	CCTTACCCCCCGTTAACCCTATTTG	CGCTTTACGCCGTAGGTCCATTGG	66°C
3	1887–1949 bp	63	351	GCCATCTTCAGCAAACCCTAAAAAG	TTTCTTTCATCTTTCCCTTGCGGTA	64°C
4	5844– 5862 bp	19	312	ATACCTACACTGCTGGCTATAACCG	CCACCAATCTAGTAAGGGCTTAGC	64°C
5	12457–12607 bp	151	463	TTATCACAACCCAACGAGGTAAGTACC	CGGAATTATGCTGATGGTAAAGGCATA	64°C
6	16686 – 16794 bp	109	524	GTGCTTGCCCAGTATGTCCTCA	CGGAGCGAGAAGAGGTACACG	67°C

Table 2- Mitogenomes and *numt* sequences used for phylogenetic reconstruction.

	Accession number	Reference
Panthera tigris	EF551003	Wei et al., 2011
P. pardus	EF551002	Wei et al., 2011
P. uncia	EF551004	Wei et al., 2012
P. leo	KC834784	Bagatharia et al., 2013
P. onca	XXXXXXX	this study
P. tigris numt	DQ151551	Jae-Heup et al., 2001
Neofelis nebulosa	DQ257669	Wu et al., 2007
Felis catus	NC001700	Lopez et al., 1996
F. catus numt	U20754	Lopez et al., 1996

Table 3 – Annotation of the jaguar mitogenome, including the location and length of mitochondrial genes, as well as their coding strand (heavy [H] or light [L]). The start and stop codons used in each gene are also indicated; in the stop codons, '*a*' indicates that it is completed by the post-transcriptional addition of one or two 3'-A nucleotides. 'Poly-A detection' indicates genes for which we observed contigs with poly-A stretches after the coding end.

	Position				Codon			
Name	Start	Stop	Length	Strand	Start	Stop	5′ intervening spacer	Poly-A detection
trnF(GAA)	886	955	70	H				
rrnS	956	1915	960	H				
trnV(TAC)	1916	1983	68	H				
rrnL	1984	3558	1575	H				
trnL2(TAA)	3559	3633	75	H				
nad1	3636	4591	956	H	ATG	TA*a*	AC	
trnI(GAT)	4592	4660	69	H				
trnQ(TTG)	4658	4731	74	L				
trnM(CAT)	4733	4801	69	H			A	
nad2	4802	5843	1042	H	ATC	T*aa*		Detected
trnW(TCA)	5844	5912	69	H				
trnA(TGC)	5929	5997	69	L			CATACCAACTAACCCT	
trnN(GTT)	5999	6071	73	L			A	
OLR	6072	6103	32					
trnC(GCA)	6104	6169	66	L				
trnY(GTA)	6170	6235	66	L				
cox1	6237	7781	1545	H	ATG	TAA	T	
trnS2(TGA)	7780	7849	70	L				Detected
trnD(GTC)	7854	7922	69	H			TCAA	
cox2	7923	8606	684	H	ATG	TAA		Detected
trnK(TTT)	8610	8677	68	H			ATT	
atp8	8679	8882	204	H	ATG	TAA	C	
atp6	8840	9520	681	H	ATG	TAA		Detected
cox3	9520	10303	784	H	ATG	T*aa*		Detected
trnG(TCC)	10304	10372	69	H				
nad3	10373	10719	347	H	ATA	TA*a*		Detected
trnR(TCG)	10720	10788	69	H				
nad4L	10789	11085	297	H	ATG	TAA		
nad4	11079	12456	1378	H	ATG	T*aa*		Detected
trnH(GTG)	12457	12525	69	H				
trnS1(GCT)	12526	12584	59	H				
trnL1(TAG)	12585	12654	70	H				
nad5	12655	14475	1821	H	ATA	TAA		
nad6	14459	14986	528	L	TTA	T*aa*		
trnE(TTC)	14987	15055	69	L				Detected
cytB	15059	16201	1143	H	ATG	AGA	TTA	Detected
trnT(TGT)	16202	16271	70	H				
trnP(TGG)	16272	16338	67	L				
CR	16339	885	1,403 – 1,613					

Table 4 – Nodal support for the tree reconstructed in the first set of phylogenetic analyses. Node numbers refer to Fig. 2A. Values represent the percent bootstrap support in the Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. Values on the BI column represent the posterior probability of the respective clade, derived from the Bayesian Inference of phylogeny.

Nodes	Nodal support			
	NJ	MP	ML	BI
1	90	84	86	1
2	76	<50	84	1
3	59	67	79	1
4	95	98	96	1
5	100	100	99	1
6	-	-	-	1
7	100	100	100	1

Table 5 –

Nodal support for the trees reconstructed in the final set of phylogenetic analyses. Node numbers refer to Fig. 2B. Values represent the percent bootstrap support in the Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. Values on the BI column represent the posterior probability of the respective clade, derived from the Bayesian Inference of phylogeny. Three data sets were analyzed: (i) all data, comprising full mitogenomes; (ii) full mitogenomes excluding the control region (no CR); and (iii) concatenation containing only the 13 protein-coding genes (coding only).

	All data				No CR				Coding only			
Nodes	NJ	MP	ML	BI	NJ	MP	ML	BI	NJ	MP	ML	BI
1	100	100	100	1	100	100	100	1	100	100	100	1
2	78	50	75	1	84	46	93	1	87	68	98	1
3	100	100	100	1	100	100	100	1	100	100	100	1

Figures:

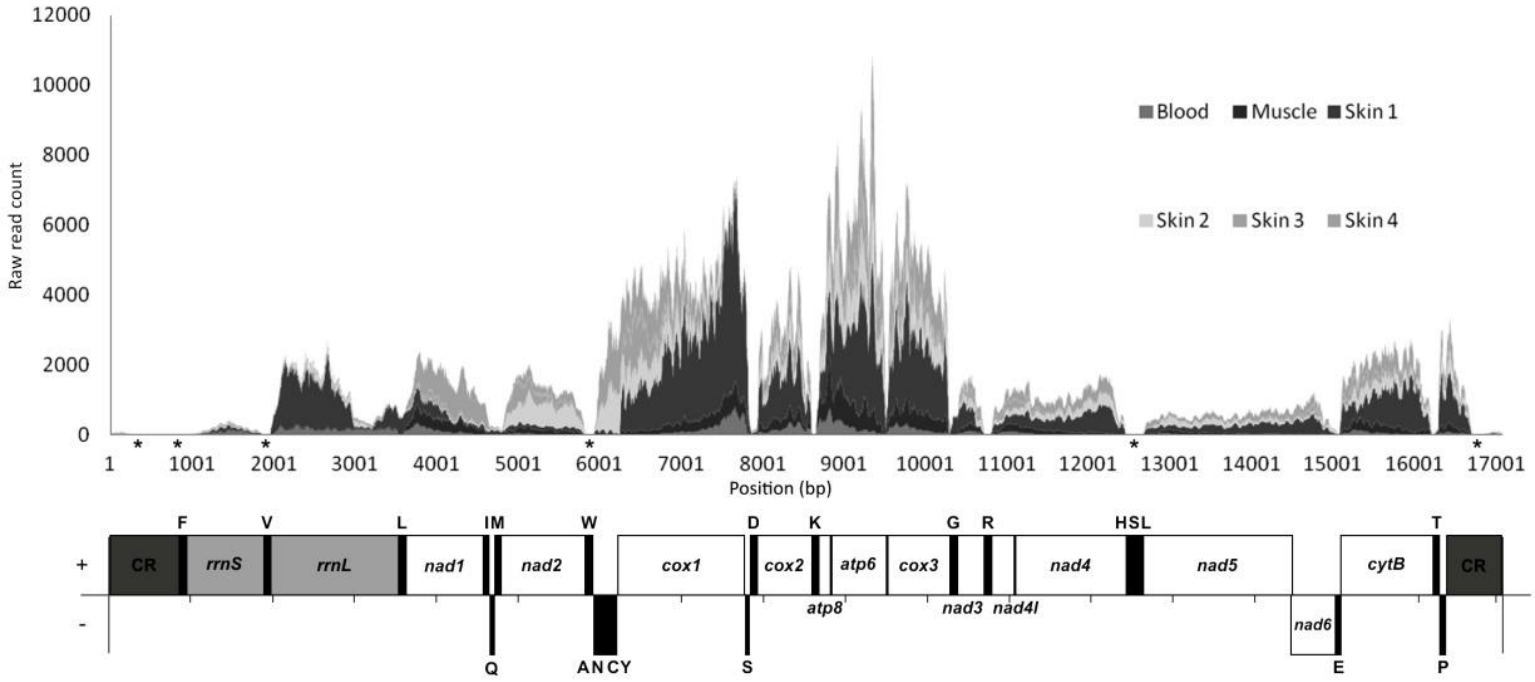


Figure 1 – Cumulative number of raw RNA-seq reads that covered each base pair of the jaguar mitogenome. The pooled data from all tissue samples (represented by different shades of gray) are shown here (see Supplementary Figure 1 for a separate representation of each tissue sample). The bar at the bottom indicates the jaguar mtDNA annotation. White boxes represent the 13 protein-coding genes; light gray boxes are the two rRNA subunit genes; dark gray boxes are the two sections of the control region; and black boxes are the tRNA genes. Boxes shown in the top (+) portion are genes included in the H-strand polycistronic transcript, while those in the bottom (-) are transcribed from the L-strand. Asterisks below the X-axis indicate the location of the six transcripts gaps (TGs) that were covered with PCR-based experiments (see text).

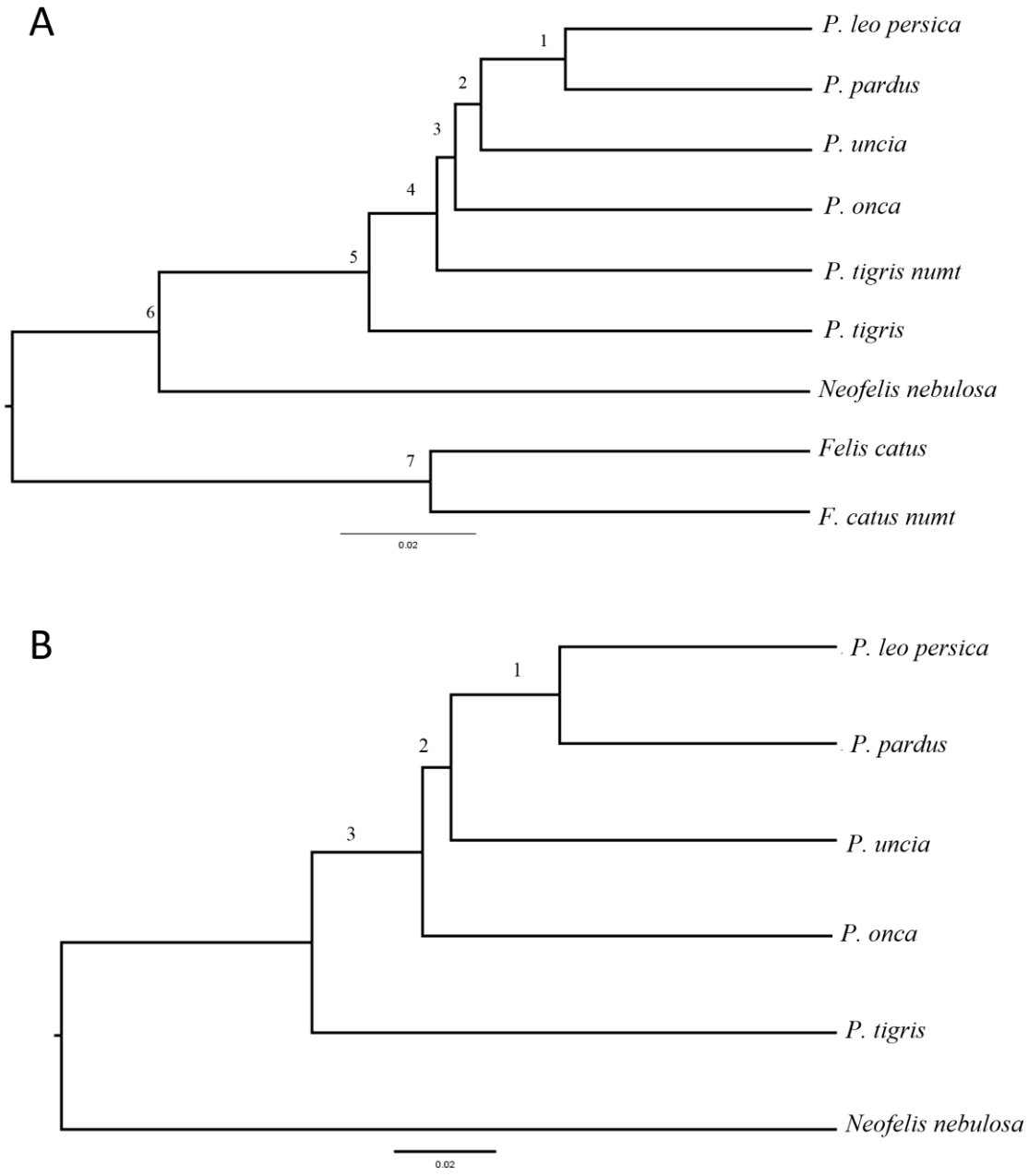


Figure 2 – Phylogenetic relationships of *Panthera* mitochondrial genomes. A) Bayesian phylogeny inferred from the initial data set (broader taxon sampling [Tab. 2] and exclusion of ambiguously aligned sites). ML and NJ topologies were identical to one shown here, while the MP tree did not confidently resolve node 2. Node numbers are cross-referenced to Tab. 4, which shows the support values observed for each clade with each optimality criterion. B) Bayesian phylogeny inferred from the final data sets, including only *Panthera* mitogenomes and the *N. nebulosa* outgroup. All three data sets (see text for details) produced the same topology with BI, ML and NJ, while MP only retrieved it consistently with the third data set (coding genes only). Numbers on braches are refer to support values for the adjacent node, which are shown in Table 5.

Supplementary material:

Supplementary Table 1 – Best-fitting nucleotide substitution models estimated for each mtDNA partition and employed in the final BEAST analyses. All models incorporating a gamma correction for rate heterogeneity among sites were run assuming four gamma categories.

Partition	Substitution model
apt6	HKY + G
atp8	HKY + G
cox1	HKY + G
cox2	HKY + I
cox3	HKY + G
cytB	HKY + I
nad1	HKY + G
nad2	HKY + G
nad3	HKY + G
nad4	HKY + G
nad4L	HKY + I
nad5	HKY + G
nad6	HKY + G
tRNAs	HKY + G
rRNAs	TN93 + G
CR	HKY + G

Supplementary Table 2 – List of RNA-seq contigs (grouped by sample) spanning the jaguar mitogenome. The table indicates the start and stop position of each contig (relative to the full mitogenome reported here), as well as its length. An 'a' after the stop position indicates that the contig contained a poly-A stretch.

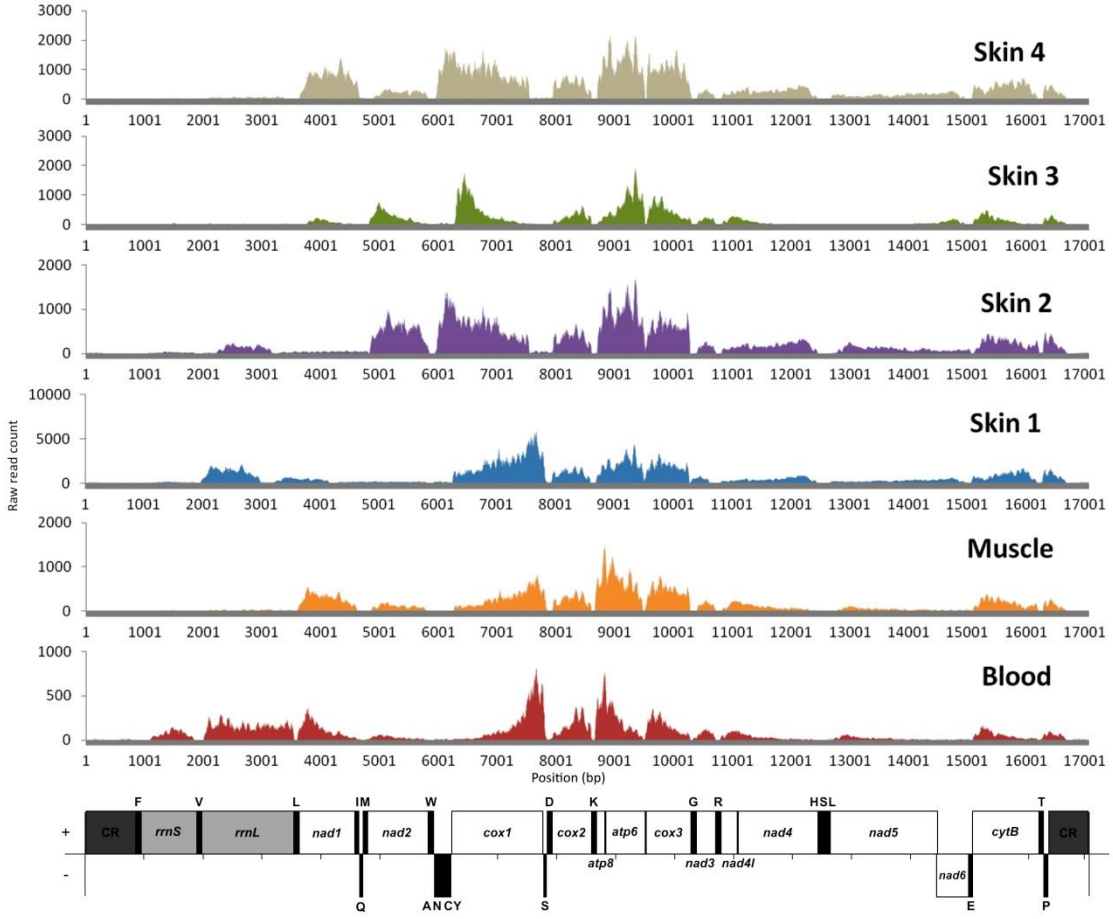
Contig	Start	Stop	Length (bp)
#comp1630 blood	529	887	359
#comp795 blood	1079	1850	772
#comp31 blood	1994	3534	1541
#comp137 blood	3576	5844a	2278
#comp657 blood	5927	6186	260
#comp130 blood	8636	9520	893
#comp133 blood	6165	7849a	1697
#comp140 blood	7830	8606a	793
#comp134 blood	9497	10303a	818
#comp158 blood	10297	10719a	431
#comp171 blood	10770	12456a	1697
#comp228 blood	12608	15055a	2458
#comp149 blood	15071	16201a	1135
#comp168 blood	16207	16686	480
#comp1479 blood	16795	292	564
#comp574 Muscle	1023	1757	735
#comp354 Muscle	1994	2409	416
#comp27 Muscle	2388	3534	1147
#comp94 Muscle	3576	5843a	2277
#comp91 Muscle	6245	7849a	1617
#comp95 Muscle	7830	8606a	788
#comp87 Muscle	8636	9520a	892
#comp90 Muscle	9497	10303a	816
#comp106 Muscle	10297	10719a	429
#comp111 Muscle	10793	12456a	1673
#comp166 Muscle	12669	15055a	2397
#comp99 Muscle	15071	16201a	1135
#comp107 Muscle	16207	16686	480
#comp1065 Muscle	16811	40	296
#comp1637 Skin1	544	845	302
#comp344 Skin1	968	1880	913
#comp160 Skin1	1950	5843a	3903
#comp153 Skin1	5863	7849a	1998
#comp154 Skin1	7830	9520a	1698
#comp156 Skin1	9497	10303a	817
#comp173 Skin1	10282	10719a	445
#comp178 Skin1	10696	12456a	1770
#comp161 Skin1	12635	16201a	3572
#comp164 Skin1	16214	16686	473
#comp752 Skin1	16795	292	564
#comp678 Skin2	968	1844	877
#comp207 Skin2	1978	4819	2842
#comp160 Skin2	4786	5843a	1067
#comp157 Skin2	5927	7849a	1934
#comp166 Skin2	7830	8606a	788
#comp154 Skin2	8630	9520a	898
#comp159 Skin2	9497	10303a	816
#comp182 Skin2	10294	10719a	432
#comp183 Skin2	10784	12456a	1681
#comp66 Skin2	12669	15055a	2397
#comp172 Skin2	15071	16222	1152
#comp169 Skin2	16197	16686	490

Supplementary Table 2. Continued.

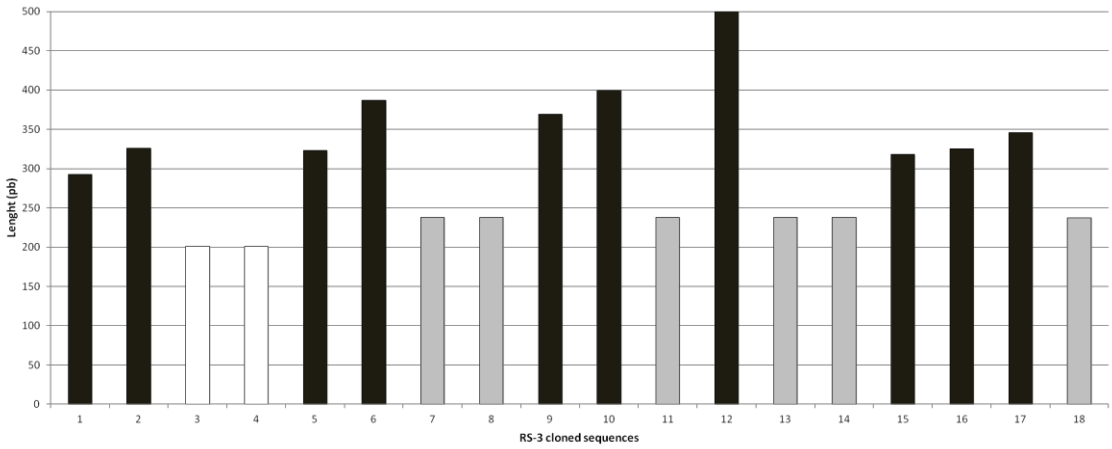
Contig	Start	Stop	Length (bp)
#comp1631 Skin2	16696	311	583
#comp335 Skin3	968	1886	919
#comp435 Skin3	1997	3538	1542
#comp142Skin3	3622	4710	1089
#comp106 Skin3	4792	5843a	1061
#comp288 Skin3	5911	6229	319
#comp99 Skin3	6248	7849a	1614
#comp107 Skin3	7830	8606a	789
#comp98 Skin3	8687	9520a	841
#comp103 Skin3	9524	10303a	789
#comp129 Skin3	10369	10719a	357
#comp128 Skin3	10800	12456a	1666
#comp1852 Skin3	12667	12941	275
#comp154 Skin3	13079	15055a	1987
#comp110 Skin3	15071	16201a	1137
#comp118 Skin3	16215	16686	472
#comp1352 Skin3	16877	160	350
#comp750 Skin4	952	1834	883
#comp426 Skin4	1979	3395	1417
#comp194 Skin4	3608	5843a	2245
#comp192 Skin4	5927	7849a	1935
#comp197 Skin4	7830	8606a	789
#comp189 Skin4	8630	10303a	1680
#comp217 Skin4	10288	12456a	2175
#comp198 Skin4	12666	16201a	3541
#comp208 Skin4	16197	16686	490
#comp2269 Skin4	16837	257	487

Supplementary Table 3- Gene boundaries annotated in the jaguar mitogenome. The left columns indicate the start and stop positions based on the *Felis catus* (NC001700) mitogenome annotation; the right columns indicate the equivalent results derived from the gene boundary prediction performed with the software MITOS.

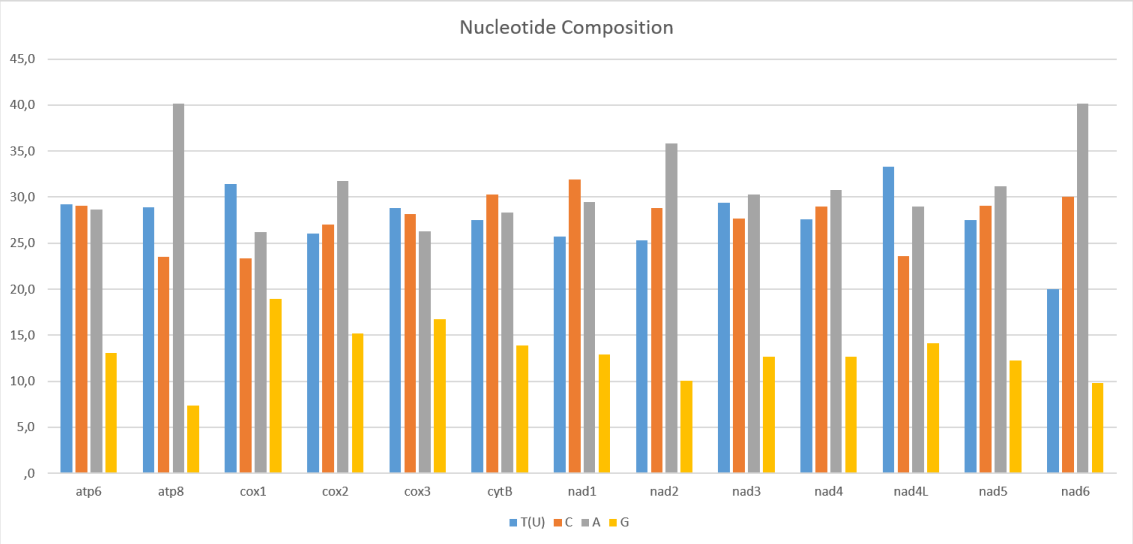
Name	*F. catus*		MITOS	
	Start	**Stop**	**Start**	**Stop**
trnF(GAA)	886	955	886	955
rrnS	956	1915	956	1917
trnV(TAC)	1916	1983	1916	1983
rrnL	1984	3558	1982	3557
trnL2(TAA)	3559	3633	3559	3633
nad1	3636	4591	3636	4586
trnI(GAT)	4592	4660	4592	4660
trnQ(TTG)	4658	4731	4658	4731
trnM(CAT)	4733	4801	4733	4801
nad2	4802	5843	4796	5830
trnW(TCA)	5844	5912	5844	5912
trnA(TGC)	5929	5997	5929	5997
trnN(GTT)	5999	6071	5999	6071
OLR	6072	6103		
trnC(GCA)	6104	6169	6105	6169
trnY(GTA)	6170	6235	6170	6235
cox1	6237	7781	6228	7769
trnS2(TGA)	7780	7849	7779	7847
trnD(GTC)	7854	7922	7854	7922
cox2	7923	8606	7923	8603
trnK(TTT)	8610	8677	8610	8677
atp8	8679	8882	8679	8876
apt6	8840	9520	8840	9514
cox3	9520	10303	9520	10302
trnG(TCC)	10304	10372	10304	10372
nad3	10373	10719	10370	10717
trnR(TCG)	10720	10788	10720	10788
nad4L	10789	11085	10789	11082
nad4	11079	12456	11079	12446
trnH(GTG)	12457	12525	12457	12525
trnS1(GCT)	12526	12584	12526	12584
trnL1(TAG)	12585	12654	12585	12654
nad5	12655	14475	12646	14460
nad6	14459	14986	14465	14989
trnE(TTC)	14987	15055	14987	15055
cyt B	15059	16201	15059	16192
trnT(TGT)	16202	16271	16202	16271
trnP(TGG)	16272	16338	16272	16338
CR	16339	885		



Supplementary Figure 1 - Number of raw reads that covered each base pair per sample. The bar at the bottom indicates the jaguar mtDNA annotation. White boxes represent the 13 protein-coding genes; light gray boxes are the two rRNA subunit genes; dark gray boxes are the two sections of the control region; and black boxes are the tRNA genes. Boxes shown in the top (+) portion are genes included in the H-strand polycistronic transcript, while those in the bottom (-) are transcribed from the L-strand.

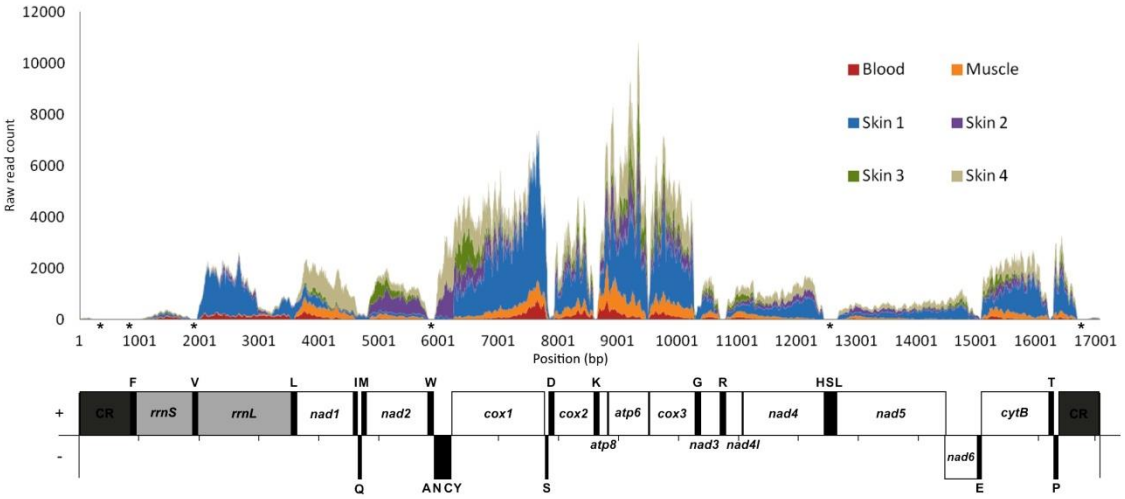


Supplementary Figure 2 – Length variation observed in cloned PCR products spanning the RS-3 repetitive segment of the jaguar control region. Black columns represent clones that contained sequences flanking the repeats that were identical (or almost identical) to the transcript data (see main text), and were thus considered to represent the true cytoplasmatic mitochondrial DNA (*cymt*DNA). Grey columns represent clones that could still be easily aligned to the *cymt*DNA, but presented several nucleotide differences on the repeat flanks, leading us to conclude that they were derived from the *Panthera numt*. White columns represent very divergent sequences that may be derived from older *numt* insertions (see text for details).



Supplementary Figure 3 – nucleotide composition of each protein-coding gene contained in the *Panthera onca* mitogenome.

Online version Figure 1 -



CONCLUSÕES GERAIS

- O genoma mitocondrial de onça-pintada (*Panthera onca*), obtido através de uma técnica que, pelo conhecimento atual, parece eliminar o problema das inserções de DNA mitocondrial no genoma nuclear (*numt*), é um fato inédito para o gênero, e ainda raro para mamíferos em geral;

- Esta caracterização irá auxiliar no desenvolvimento de marcadores que possam ser utilizados em estudos genéticos desta espécie;

- O DNA mitocondrial citoplasmático (*cymt*) possui informações valiosas, podendo ser empregado tanto em estudos populacionais de uma espécie, que podem auxiliar no delineamento de medidas de manejo adequadas, quanto para investigar as relações filogenéticas entre espécies do mesmo grupo;

- Até o momento, o uso deste marcador para estudos populacionais de onça-pintada estava limitado pela falta de esclarecimento a respeito do genoma mitocondrial da espécie e pela ausência de confiabilidade do DNAmt representar, sem equívocos, o *cymt* e não o *numt*;

- Os resultados encontrados apontam para a alta eficácia em gerar praticamente todo o genoma mitocondrial de um indivíduo através do sequenciamento de RNA (RNA-seq). Esta técnica é mais rápida e mais barata em relação ao sequenciamento convencional através de múltiplos *primers*. Além disso, empregando esta técnica, os pseudogenes de origem mitocondrial encontrados no núcleo (*numts*) são evitados, uma vez que RNA-seq recupera apenas genes que são transcritos;

- Esclarecemos a filogenia mitogenômica do gênero *Panthera*, com valores de *bootstrap* e de probabilidade posterior confiáveis, os quais revelaram uma filogenia inédita para o gênero. Esta realmente parece ser a história evolutiva do genoma mitocondrial do gênero, a qual difere da topologia recuperada por um estudo recente baseado em uma grande super-matriz de dados, dominada por marcadores nucleares. As diferenças entre estas topologias parecem ser devidas ao efeito de segregação incompleta de polimorfismos durante a rápida diversificação do gênero, podendo também terem sido influenciadas pela ocorrência de homoplasia no DNA mitocondrial;

- Ressaltamos que o uso de segmentos de DNAmt deve ser realizado com cautela, tanto em estudos populacionais dentro das espécies do gênero *Panthera* quanto para estudos que buscam inferir as relações filogenéticas entre as espécies do gênero;

- Em ambos os casos deve-se tomar todas as precauções necessárias para evitar a amplificação errônea do *numt* ao invés do *cymt* e deve-se buscar regiões informativas para cada um dos objetivos. Isto porque até mesmo genes mitocondriais diferentes apresentam diferentes reconstruções da história evolutiva do grupo, tendo sido necessário o sequenciamento completo do mitogenoma para se se atingir uma topologia estável e robustamente apoiada para este sistema genético.

REFERÊNCIAS

Anderson, S., Bankier, A. T., Barrell, B. G., De Bruijn, M. H. L., Coulson, A. R., Drouin, J., ... & Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature*, 290, 457 – 465.

Antunes, A., Pontius, J., Ramos, M. J., O'Brien, S. J., & Johnson, W. E. (2007). Mitochondrial introgressions into the nuclear genome of the domestic cat. *Journal of Heredity*, *98*(5), 414-420.

Asin-Cayuela, J., & Gustafsson, C. M. (2007). Mitochondrial transcription and its regulation in mammalian cells. *Trends in biochemical sciences*, *32*(3), 111-117.

Avise, J. C. (1994). *Molecular markers: natural history and evolution*. Springer.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., ... & Saunders, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual review of ecology and systematics*, *18*, 489-522.

Bagatharia, S. B., Joshi, M. N., Pandya, R. V., Pandit, A. S., Patel, R. P., Desai, S. M., ... & Saxena, A. K. (2013). Complete mitogenome of asiatic lion resolves phylogenetic status within *Panthera*. *BMC genomics*, *14*(1), 572.

Bensasson, D., Zhang, D. X., Hartl, D. L., & Hewitt, G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution*, *16*(6), 314-321.

Bernt, M., Braband, A., Schierwater, B., & Stadler, P. F. (2012). Genetic aspects of mitochondrial genome evolution. *Molecular phylogenetics and evolution*.

Bininda-Emonds, O. R., Decker-Flum, D. M., & Gittleman, J. L. (2001). The utility of chemical signals as phylogenetic characters: an example from the Felidae. *Biological Journal of the Linnean Society*, *72*(1), 1-15.

Bininda-Emonds, O. R., Gittleman, J. L., & Purvis, A. (1999). Building large trees by combining phylogenetic information: a complete phylogeny of the extant Carnivora (Mammalia). *Biological Reviews*, *74*(2), 143-175.

Bobrowicz, A., Lightowlers, R., & Chrzanowska-Lightowlers, Z. (2008). Polyadenylation and degradation of mRNA in mammalian mitochondria: a missing link?. *Biochemical Society Transactions*, *36*(Pt 3), 517.

Boore, J. L. (1999). Animal mitochondrial genomes. *Nucleic Acids Research*,*27*(8), 1767-1780.

Boore, J. L. (2006). The complete sequence of the mitochondrial genome of Nautilus macromphalus (Mollusca: Cephalopoda). *BMC genomics*, *7*(1), 182.

Bridge, D., Cunningham, C. W., Schierwater, B., DeSalle, R., & Buss, L. W. (1992). Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure. *Proceedings of the National academy of Sciences*, *89*(18), 8750-8753.

Cheng, S., Higuchi, R., & Stoneking, M. (1994). Complete mitochondrial genome amplification. *Nature genetics*, *7*(3), 350-351.

Christianson, T. W., & Clayton, D. A. (1988). A tridecamer DNA sequence supports human mitochondrial RNA 3'-end formation in vitro. *Molecular and cellular biology*, *8*(10), 4502-4509.

Clayton, D. A. (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annual review of cell biology*, *7*(1), 453-478.

Cracraft, J., Feinstein, J., Vaughn, J., & Helm-Bychowski, K. (1998). Sorting out tigers (Panthera tigris): mitochondrial sequences, nuclear inserts, systematics, and conservation genetics. *Animal Conservation*, *1*(2), 139-150.

Curole, J. P., & Kocher, T. D. (1999). Mitogenomics: digging deeper with complete mitochondrial genomes. *Trends in Ecology & Evolution*, *14*(10), 394-398.

Davis, B. W., Li, G., & Murphy, W. J. (2010). Supermatrix and species tree methods resolve phylogenetic relationships within the big cats, *Panthera* (Carnivora: Felidae). *Molecular Phylogenetics and Evolution*, *56*(1), 64-76.

Fabre, P. H., Jønsson, K. A., & Douzery, E. J. (2013). Jumping and gliding rodents: Mitogenomic affinities of Pedetidae and Anomaluridae deduced from an RNA-Seq approach. *Gene*, *531*(2), 388-397.

Gray, M. W. (2012). Mitochondrial evolution. *Cold Spring Harbor Perspectives in Biology*, *4*(9).

Harrison, R. G. (1989). Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends in Ecology & Evolution*, *4*(1), 6-11.

Hayasaka, K., Ishida, T., & Horai, S. (1991). Heteroplasmy and polymorphism in the major noncoding region of mitochondrial DNA in Japanese monkeys: association with tandemly repeated sequences.*Molecular biology and evolution*, *8*(4), 399-415.

Hazkani-Covo, E., Zeller, R. M., & Martin, W. (2010). Molecular poltergeists: mitochondrial DNA copies (*numts*) in sequenced nuclear genomes. *PLoS genetics*, *6*(2), e1000834.

Hoelzel, A. R., Lopez, J. V., Dover, G. A., & O'Brien, S. J. (1994). Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. *Journal of molecular evolution*, *39*(2), 191-199.

Imanishi, Y., Tanaka, M., & Fujiwara, M. (2013). Complete mitochondrial genome sequence of Japanese cockle Fulvia mutica (Cardiidae). *Fisheries science*, *79*(6), 949-957.

Jae-Heup, K., Eizirik, E., O'Brien, S. J., & Johnson, W. E. (2001). Structure and patterns of sequence variation in the mitochondrial DNA control region of the great cats. *Mitochondrion*, *1*(3), 279-292.

Janczewski, D. N., Modi, W. S., Stephens, J. C., & O'Brien, S. J. (1995). Molecular evolution of mitochondrial 12S RNA and cytochrome b sequences in the pantherine lineage of Felidae. *Molecular Biology and Evolution*, *12*(4), 690-707.

Johnson, W. E., Dratch, P. A., Martenson, J. S., & O'Brien, S. J. (1996). Resolution of recent radiations within three evolutionary lineages of Felidae using mitochondrial restriction fragment length polymorphism variation. *Journal of Mammalian Evolution*, *3*(2), 97-120.

Johnson, W. E., Eizirik, E., Pecon-Slattery, J., Murphy, W. J., Antunes, A., Teeling, E., & O'Brien, S. J. (2006). The late Miocene radiation of modern Felidae: a genetic assessment. *Science*, *311*(5757), 73-77.

Kim, J. H., Antunes, A., Luo, S. J., Menninger, J., Nash, W. G., O'Brien, S. J., & Johnson, W. E. (2006). Evolutionary analysis of a large mtDNA translocation (*numt*) into the nuclear genome of the *Panthera* genus species.*Gene*, *366*(2), 292-302.

King, T. C., & Low, R. L. (1987). Mitochondrial DNA displacement loop structure depends on growth state in bovine cells. *Journal of Biological Chemistry*, *262*(13), 6214-6220.

Lang, B. F., Gray, M. W., & Burger, G. (1999). Mitochondrial genome evolution and the origin of eukaryotes. *Annual review of genetics*, *33*(1), 351-397.

Lei, R., Shore, G. D., Brenneman, R. A., Engberg, S. E., Sitzmann, B. D., Bailey, C. A., ... & Louis Jr, E. E. (2010). Complete sequence and gene organization of the mitochondrial genome for Hubbard's sportive lemur (< i> Lepilemur hubbardorum</i>). *Gene*, *464*(1), 44-49.

Lopez, J. V., Cevario, S., & O'Brien, S. J. (1996). Complete Nucleotide Sequences of the Domestic Cat (*Felis catus*) Mitochondrial Genome and a Transposed mtDNA Tandem Repeat (*Numt*) in the Nuclear Genome. *Genomics*, *33*(2), 229-246.

Lopez, J. V., Yuhki, N., Masuda, R., Modi, W., & O'Brien, S. J. (1994). Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. *Journal of Molecular Evolution*, *39*(2), 174-190.

Mardis, E. R. (2008). The impact of next-generation sequencing technology on genetics. *Trends in genetics*, *24*(3), 133-141.

Metzker, M. L. (2009). Sequencing technologies—the next generation. *Nature Reviews Genetics*, *11*(1), 31-46.

Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., & Attardi, G. (1982). Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *Proceedings of the National Academy of Sciences*, *79*(23), 7195-7199.

Nabholz, B., Jarvis, E. D., & Ellegren, H. (2010). Obtaining mtDNA genomes from next-generation transcriptome sequencing: a case study on the basal Passerida (Aves: Passeriformes) phylogeny. *Molecular phylogenetics and evolution*, *57*(1), 466-470.

Neira-Oviedo, M., Tsyganov-Bodounov, A., Lycett, G. J., Kokoza, V., Raikhel, A. S., & Krzywinski, J. (2011). The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect molecular biology*, *20*(2), 141-152.

Ojala, D., Montoya, J., & Attardi, G. (1981). tRNA punctuation model of RNA processing in human mitochondria. *Nature* **290**, 470 – 474.

Smith, D. R. (2013). RNA-Seq data: a goldmine for organelle research. *Briefings in functional genomics*.

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences*, *105*(36), 13486-13491.

Taanman, J. W. (1999). The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1410*(2), 103-123.

Torres, T. T., Dolezal, M., Schlötterer, C., & Ottenwälder, B. (2009). Expression profiling of Drosophila mitochondrial genes via deep mRNA sequencing. *Nucleic acids research*, *37*(22), 7509-7518.

Wang, H. L., Yang, J., Boykin, L. M., Zhao, Q. Y., Li, Q., Wang, X. W., & Liu, S. S. (2013). The characteristics and expression profiles of the mitochondrial genome for the Mediterranean species of the Bemisia tabaci complex. *BMC genomics*, *14*(1), 401.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57-63.

Wei, L., Wu, X., Zhu, L., & Jiang, Z. (2011). Mitogenomic analysis of the genus *Panthera*. *Science China Life Sciences*, *54*(10), 917-930.

Wolstenholme, D. R. "Animal mitochondrial DNA: structure and evolution."*Int Rev Cytol* 141 (1992): 173-216.

Xu, X., Wu, X., & Yu, Z. (2012). Comparative studies of the complete mitochondrial genomes of four< i> Paphia</i> clams and reconsideration of subgenus< i> Neotapes</i>(Bivalvia: Veneridae). *Gene*, *494*(1), 17-23.

Yu, L., & Zhang, Y. P. (2005). Phylogenetic studies of pantherine cats (Felidae) based on multiple genes, with novel application of nuclear β-fibrinogen intron 7 to carnivores. *Molecular phylogenetics and evolution*, *35*(2), 483-495.