FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

REVISÃO DO GÊNERO *GALICTIS* (MAMMALIA, CARNIVORA, MUSTELIDAE) UTILIZANDO MÉTODOS MORFOLÓGICOS E MOLECULARES

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TESE DE DOUTORADO

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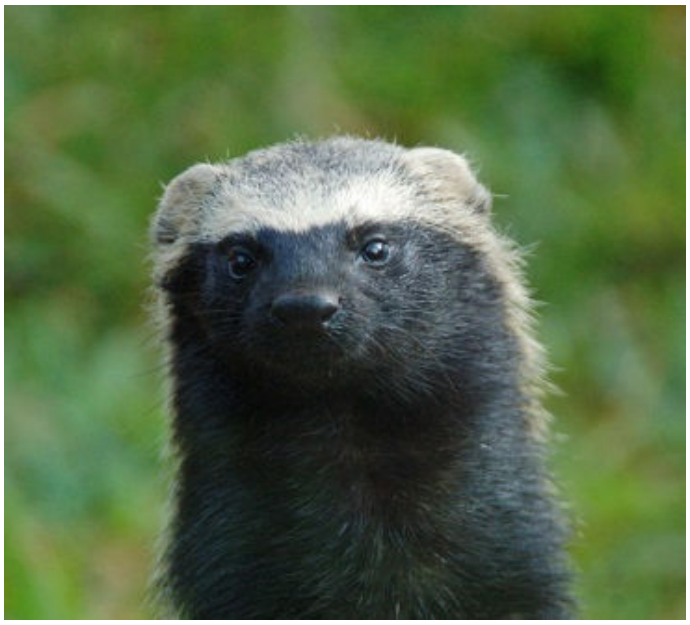

Essa tese é sobre furões e dedicada aos furões. Em quatro anos me tornei uma amiga deles, mesmo sem eles saberem...

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Resumo A taxonomia forma a base para todas as demais ciências da Biologia, uma vez que é através dessa disciplina que as unidades de vida na natureza são reconhecidas e descritas. Sem a correta delimitação das espécies, pesquisas de outras áreas não poderiam reportar seus resultados, pois não teriam a certeza da identificação das unidades de estudo. A estimativa de que existem milhões de espécies ainda para serem descritas reforça a importância da taxonomia, pois é utilizando as ferramentas dessa disciplina que as espécies são descobertas, delineadas e descritas. Mamíferos carnívoros são animais tidos como bem conhecidos; contudo, alguns táxons desse grupo ainda não foram descritos e tantos outros receberam pouca atenção biogeográfica e taxonômica, impedindo estimativas corretas de riqueza, abundância e status de conservação. O gênero *Galictis* (Mustelidae, Carnivora) é um exemplo de táxon ainda pouco estudado na região Neotropical, justamente uma das mais ricas e mais ameaçadas regiões do mundo. Informações básicas sobre esses mustelídeos, tais como número exato de espécies, delimitação entre elas, diagnose e distribuição geográfica das mesmas, não foram ainda investigadas com rigor, o que acarreta incertezas sobre alguns tópicos e compromete a sua taxonomia. Para realizar uma revisão taxonômica ampla de *Galictis*, foram realizadas análises morfológicas e moleculares com base em registros provenientes de toda a distribuição geográfica do gênero. Para a primeira técnica, foram analisados crânios e peles tombados em 22 instituições científicas, e os testes morfológicos assim como a visualização das peles evidenciaram a presença de dois conjuntos de espécimes de *Galictis*, representando as duas espécies usualmente reconhecidas, *G. cuja* e *G. vittata*. Análises filogenéticas com base em segmentos mitocondriais e nucleares corroboraram os resultados morfológicos, com a presença de dois clados bem apoiados que correspondem também a *G. cuja* e *G. vittata*. Nenhum outro agrupamento morfológico ou mesmo indícios de um terceiro clado no gênero foi identificado. Esses resultados confirmam a existência de duas espécies e possibilitam o reconhecimento de caracteres morfológicos diagnósticos para elas. Esses caracteres são de utilidade para a identificação de espécimes de museu e podem também auxiliar a identificação de indivíduos na natureza. Com o correto delineamento das espécies, foi possível definir a distribuição geográfica das mesmas e resolver algumas questões atualmente controversas, como a definição da ocorrência exclusiva de *G. cuja* na região Nordeste do Brasil e o limite austral de *G. vittata* para a Bacia Amazônia. Por fim, a partir da definição confiável e robusta das espécies, obtida na primeira etapa desta tese, foi possível realizar um estudo intra-específico mais detalhado com foco em *G. cuja*, englobando análises filogeográficas de marcadores moleculares, bem como variação morfológica ao longo de sua distribuição. Essa espécie se caracteriza de forma geral por considerável variabilidade genética e morfológica, em que se observam alguns padrões geográficos interessantes. Entre estes, pode-se destacar a diferenciação morfológica de populações do Sul do Chile e Argentina, bem como, uma estruturação genética significativa entre três grandes domínios geográficos, e evidência de uma expansão demográfica relativamente recente no sudeste brasileiro. Todos esses resultados embasam o conhecimento sobre o gênero e propiciam ferramentas para estudos futuros que visem a entender a história evolutiva de *Galictis* na região Neotropical.

Abstract

Revision of genus *Galictis* (Mammalia, Carnivora, Mustelidae) using morphological and molecular methods

Taxonomy forms the basis for all biological sciences, since it is through this discipline that natural units are recognized and described. Without the correct delimitation, researches from other disciplines would be unable to report their results because they would not be sure about the identity of their study units. The current estimate that millions of species are still to be described reinforces the centrality of taxonomy, because is through its use that species are found, delimited and described. Carnivores are usually thought to be well-known mammals, but some of these taxa have not been described yet, while others have received little biogeographic and taxonomic attention, preventing a correct assessment of their richness and conservation status. The genus *Galictis* (Carnivora, Mustelidae) is an example of a little-studied mustelid from the Neotropics, one of the richest and most endangered regions of the world. Basic information, such as number of species, delimitation between them, diagnosis and geographic distribution, have never been thoroughly tested before, leading to uncertainties regarding the taxonomy of this genus. In order to perform a comprehensive revision of *Galictis*, morphological and molecular approaches were applied on the basis of records encompassing all the distribution of the genus. For the former approach, we analyzed skulls and skins from 22 zoological collections and the statistical tests showed the presence of two clusters of *Galictis* specimens, representing *G. cuja* and *G. vittata*. Phylogenetic analyses of mitochondrial and nuclear segments supported morphological results showing two monophyletic groups, corresponding again to *G. cuja* and *G. vittata*. No other morphological grouping or evidence of a third clade was recognized with our data. All these results corroborate the existence of only two species and indicate morphological characters that effectively diagnose them. These are very useful to identify museum specimens and should also help field-based work in some situations. The correct delimitation between these units allowed the investigation of some long-standing issues about the geographic distribution of *Galictis* species. For example, we demonstrate the exclusive presence of *G. cuja* in the northeastern region of Brazil, and established the southernmost limits of *G. vittata* in the Amazon basin. Finally, as species were well identified and characterized, it was possible to conduct phylogeographic inferences as well as analyses of intra-specific morphological variation in *G. cuja*. This species contains moderate to high levels of variability and some interesting geographic patterns. These included the morphological distinction of southern Chile and Argentina, the significant genetic structuring among three broad geographic domains, and the evidence of recent demographic expansion in the Brazilian southeast. The results presented here contribute to substantially enhance our knowledge on the genus *Galictis*, and should help enable further studies focusing on the evolutionary history of these carnivores in the Neotropics.

Capítulo I: Introdução Geral

Apresentação

Esta tese é apresentada em forma de artigos científicos. Portanto, consiste de um capítulo introdutório **(Capítulo I)**, dois capítulos em forma de manuscrito científico (**Capítulo II e III**) e um capítulo de conclusões gerais **(Capítulo IV)**. Os capítulos introdutório e conclusivo foram redigidos de acordo com as normas da revista científica *Zoological Journal of the Linnean Society* (ZJLS). O primeiro manuscrito (Capítulo II) foi submetido para a revista ZJLS no dia 21 de janeiro de 2012 e foi redigido de acordo com as exigências deste periódico. O segundo manuscrito (Capítulo III) foi formulado de acordo com as regras e escopo da revista científica *Genetica*, para qual se tem interesse em submetê-lo. Todas as seções dessa tese contêm suas próprias referências bibliográficas.

Contexto da tese

A taxonomia é a ciência que identifica, descreve, nomeia e classifica a diversidade da vida. Através dessa ciência as unidades da biodiversidade são ordenadas, criando o inventário da vida e formando a base para as demais ciências da Biologia. Por exemplo, projetos de conservação da biodiversidade dependem, em uma ou outra instância, do esforço básico da taxonomia (Wilson, 1992; Wilson, 2005; Reeder, Helgen & Wilson, 2007). Do contrário, como poderíamos identificar *hot-spots* da biodiversidade se as espécies que lá habitam não são delineadas e descritas? Como identificar endemismo sem identificar as espécies? Sem a taxonomia, biólogos de diferentes disciplinas seriam provavelmente incapazes de reportar seus resultados empíricos ou acessar a informação disponível dos seus organismos de estudo, pois não teriam a certeza de suas identidades. A inabilidade ou dificuldade no reconhecimento das espécies pode esconder processos ecológicos e evolutivos e ter consequências negativas como, por exemplo, a subestimação de níveis de riqueza das espécies, entre outros. Assim, fronteiras equívocas entre as espécies conduzem diretamente a estimativas equívocas de biodiversidade. Wilson (2004) argumenta que, ao passo que diferentes vertentes da Biologia têm caráter predominantemente vertical (poucos táxons, estudados em diferentes patamares), a taxonomia se caracteriza por ser essencialmente horizontal, cuja importância central é a delimitação das diversas espécies.

A taxonomia mais tradicional é baseada na morfologia dos táxons. As espécies são identificadas e descritas com base nas suas características fenotípicas, recebendo um nome e uma classificação que refletem suas relações de parentesco. Nas últimas décadas, o advento de

ferramentas moleculares e a velocidade com a qual os resultados são gerados por estas contribuíram para a revitalização da taxonomia clássica, que recebeu uma nova injeção de investimento e popularidade. Por outro lado, surgiram também divergências entre metodologias e resultados gerados pela taxonomia clássica e aqueles gerados pela taxonomia molecular (para revisão sobre taxonomia molecular ver Tautz *et al*., 2003). Adicionalmente, as estimativas estrondosas de que existem aproximadamente dez milhões de espécies para serem ainda descobertas e descritas (Wilson, 2004) fez com que definitivamente a taxonomia geral enfrentasse uma grande crise, muitas vezes identificada como "*taxonomic crisis*" (Gewin, 2002; Dayrat, 2005). Infelizmente, a falta de especialistas em certos grupos taxonômicos bem como a falta de financiamento adequado (Wheeler, Raven & Wilson, 2004) para trabalhos com morfologia clássica também são apontados como contribuintes para o estabelecimento dessa crise (Godfray, 2002; Mallett & Willmott, 2003).

Contudo, é possível avaliar positivamente que a discussão entre morfologia e análise molecular, muitas vezes colocada como antagônica, trouxe à comunidade científica importantes debates sobre as metodologias atualmente utilizadas em estudos que objetivam a delimitação das espécies. Alguns autores especulam que as decisões taxonômicas devem ser baseadas unicamente em estudos com DNA, como, por exemplo, aqueles que defendem a utilização exclusiva de barcodes (ex. Blaxter, 2004, Packer *et al*., 2009). Outro grupo de autores propõe uma teoria integradora entre as abordagens morfológica e molecular (e outras disciplinas, como, por exemplo, a ecologia) conhecida como "*integrative taxonomy*", uma ciência que busca a delimitação e identificação das espécies através de perspectivas múltiplas e complementares (Dayrat, 2005). Neste contexto, há diversos estudos científicos que utilizam os dois tipos de abordagens, usufruindo das vantagens de uma sem negar também o valor da outra, e se detêm na discussão dos resultados, equivalentes ou não, gerados por elas (ex. Malhotra & Thorpe, 2004; Renaud, Chevret & Michaux, 2007; Cardoso, Serrano & Vogler, 2009). Esses debates tendem a qualificar a escolha da metodologia na hora da elaboração de estudos taxonômicos, pois os pontos favoráveis e desfavoráveis, assim como a aplicabilidade de cada ferramenta, são explicitados e discutidos.

Além do recente debate sobre as metodologias mais adequadas para se aplicar à taxonomia, um debate antigo e ainda mais controverso persiste por anos entre os cientistas, aquele que justamente discute *o que é* espécie. Uma das principais problemáticas acerca das definições de espécie é que nenhum conceito, dentre os mais de 20 descritos na literatura, consegue com exclusividade ser compatível e aplicável a todas as unidades da natureza. Como resultado, observam-se anos e anos de debates que levantam opiniões muitas vezes opostas,

mas que objetivam a definição de qual seria *o melhor* conceito de espécie. A unanimidade que permeia as diferentes opiniões é a de que espécie é a unidade viva fundamental de comparação para as inúmeras disciplinas da Biologia. Ao passo que vários autores defendem suas ideias sobre os conceitos de espécie e passam a traçar as diferenças entre o seu conceito e os demais propostos na literatura, Mayden (2002), de Queiroz (2007) e Naomi (2011) se dedicam a buscar uma definição mais norteadora que se constitua das semelhanças entre as definições, desatando *conceito* de *delimitação* de espécies, que na opinião dos autores estão misturados trazendo ruído na busca pela melhor definição (Mayden, 2002; de Queiroz 2007).

Resumidamente, Mayden (2002) propõe uma visão hierárquica entre os conceitos, traçando o evolutivo (ver Wiley 1978) como aquele realmente válido que ocupa uma posição primária no sentido de conceituação, e os demais ocupam uma posição secundária de valor operacional. Assim, pesquisadores podem utilizar outros conceitos (biológico, ecológico, fenético, e assim por diante) para identificar o que é definido pelo conceito evolutivo. Em outras palavras, espécies são metapopulações com histórias evolutivas únicas e bem definidas (conceito), mas a ocupação de diferentes nichos ecológicos ou zonas adaptativas, o isolamento reprodutivo, as diferenças quantitativas num cluster fenético, etc., podem ser utilizados para a identificação das unidades na natureza (critério para delimitação). De Queiroz (2007), semelhantemente à Mayden (2002), busca uma reconciliação entre os diversos conceitos de espécie, cujo principal foco é cunhar uma definição mais ampla e genérica contendo aquilo que todos tem em comum. Portanto, em sua opinião, os conceitos modernos compartilham a ideia de que espécies são linhagens evolutivas em nível de metapopulações (há novamente a aproximação do conceito evolutivo), sendo que as demais propostas por trás dos outros conceitos não precisam necessariamente estar contidas nas linhagens para serem caracterizadas como espécie. Com o objetivo de revisar as duas abordagens descritas acima, bem como uni-las, Naomi (2011) resume pontos positivos e negativos de Mayden (2002) e de Queiroz (2007) e sugere uma terceira abordagem moderna sobre espécie, extraindo ideias de ambos os autores. Assim, Naomi propõe uma versão em que o conceito evolutivo de espécie (revisado por Wiley [1978]) é tratado como aquele válido e adota as propriedades biológicas das espécies (ex. monofilia recíproca, diagnose morfológica, isolamento reprodutivo, etc.) como critérios objetivos para delimitar as espécies (linhagens evolutivas em nível de população).

Interessante perceber que há um esforço por parte dos três autores em não bloquear as ideias contidas nos muitos conceitos de espécie, pois parece haver uma concordância geral de que um conceito específico único não abrange todas as diferentes unidades da natureza. O mais aplicável realmente parece ser a busca por um conceito mais amplo, mas que aceite as

propriedades contidas nas espécies como ferramenta de identificá-las. Por razões práticas, os pesquisadores necessitam delimitar as espécies e por isso, necessitam entender como *delimitá-las* bem como *o que são*. De uma forma geral, todas essas questões estão também conectadas às questões da taxonomia abordadas anteriormente e assim, todas se somam num alicerce único para as demais áreas da Biologia e permitem que essas sejam comparáveis. Uma analogia para esses tópicos poderia ser uma pirâmide, na qual a base é constituída pela taxonomia e os demais níveis, pelas outras ciências. Uma base bem constituída aumenta as chances de que cada subsequente degrau seja também bem formulado. Um desses degraus que depende de um conceito assim como de uma clara delimitação das espécies é o estudo da variação intraespecífica. Uma vez bem definidas as unidades taxonômicas, os estudos das variações internas se tornam uma ferramenta importante para o entendimento dos processos evolutivos que conduzem à diversidade atual.

Muitos táxons são surpreendentemente pouco conhecidos. Nesses casos, as informações sobre a delimitação desses com outros táxons próximos, suas características diagnósticas e distribuição geográfica (base da pirâmide) tem um nível insuficiente de conhecimento para garantir que outros estudos mais específicos se tornem viáveis (demais degraus da pirâmide). O gênero *Galictis* (Mammalia, Carnivora, Mustelidae) compreende os furões neotropicais e é um exemplo de táxon ainda pouco conhecido. A literatura existente sobre o gênero é restrita e inclui questionamentos sobre a delimitação e distribuição das espécies. Uma revisão atenta da literatura atual sobre essas espécies revela algumas lacunas de conhecimento básico ou mesmo controvérsias de informação, principalmente no que se refere à distribuição das espécies. Este cenário faz com que os furões neotropicais sejam um dos grupos de mustelídeos menos estudados nas Américas e a obtenção das informações básicas sobre o gênero persista como uma das grandes prioridades para os estudos de carnívoros neotropicais (ex. IBAMA, 2004; Oliveira, 2009).

Unidade de estudo: gênero *Galictis*

Atualmente são reconhecidas duas espécies do gênero *Galictis*: o furão pequeno (*G. cuja,* Molina, 1782) e o furão grande (*G. vittata,* Schreber, 1776) (Wozencraft, 2005). Esses animais ocorrem em toda a extensão da região Neotropical, ou seja, da metade sul do México até a patagônia chilena e argentina. O tempo de divergência entre as duas espécies do gênero foi estimado em 2,8 milhões de anos atrás, durante a segunda e maior onda de diversificação de mustelídeos no Plioceno (Koepfli *et al*., 2008). Registros fósseis indicam que provavelmente o ancestral comum dos furões neotropicais tenha se originado na América do Norte (Yensen &

Tarifa, 2003a), o que pode sugerir uma posterior invasão na América do Sul através do Istmo do Panamá. Contudo, estudos genéticos visando a entender as dinâmicas de especiação dessas espécies nas Américas do Norte, Central e do Sul não foram ainda desenvolvidos. O parente mais próximo das espécies de *Galictis* é um pequeno mustelídeo patagônico, *Lyncodon patagonicus* (Sato *et al*. in press) e esses dois gêneros (*Galictis* e *Lyncodon*), por sua vez, são grupo irmão das espécies também pouco conhecidas *Ictonyx libyca, Ictonyx striatus, Poecilogale albinucha* (africanos) e *Vormela peregusna* (asiático) (Koepfli *et al*., 2008; Wolsan & Sato, 2010; Sato *et al*. in press). Koepfli *et al*. (2008) sugere que a entrada dos demais mustelídeos sul-americanos (como os representantes do gênero *Mustela* na América Central e do Sul e as lontras, ariranhas e iraras) ocorreu através de eventos independentes de dispersão para a América Central e do Sul.

Embora pouco estudado, algumas informações sobre *Galictis* estão disponíveis na literatura. Um levantamento sobre essas informações propicia um panorama geral desse gênero, como, por exemplo, sua caracterização ecológica. O nome "furão grande" para *G. vittata* se refere ao seu congênere, pois esses mustelídeos são de pequeno porte: *G. cuja* tem cerca de 50 – 60 cm de comprimento total e peso aproximado de 1,2 – 2,5 kg, enquanto *G. vittata* mede 65 – 75 cm e pesa 2,5 a 3,5 kg (Yensen & Tarifa, 2003a,b; Bornholdt *et al*. unpublished data). A dieta desses animais ainda é pouco conhecida. A maioria dos dados sobre ecologia foi publicada apenas para *G. cuja*. Contudo, devido à sua grande semelhança fenotípica com *G. vittata* e às pontuais informações disponíveis sobre este, parece que os furões ocupam nichos ecológicos bastante próximos e usufruem de recursos alimentares muito semelhantes. Assim, *G. cuja* se alimenta basicamente de outros vertebrados e, embora seja um predador eclético e inclua em sua dieta os répteis, algumas pequenas aves e seus ovos (Redford & Eisenberg, 1992; Zapata *et al*., 2005), são os pequenos mamíferos os principais representantes de sua alimentação e, dentre esses, primordialmente os roedores (Delibes *et al*., 2003; Zapata *et al*., 2005). Provavelmente a dieta de *G. vittata* se aproxime muito dessa descrita para *G. cuja*, mas, devido ao porte superior, deve ser constituída também de mamíferos e répteis maiores do que aqueles consumidos por *G. cuja*. Com relação aos hábitos, os furões são predominantemente diurnos, se deslocam em duplas ou pequenos grupos (Yensen & Tarifa 2003a,b) e ocupam uma considerável variedade de ambientes, embora ainda exista incerteza quanto ao contorno da distribuição desses táxons (para diferentes versões, anteriores a este estudo, sobre os limites geográficos dos furões ver Emmons & Feer, 1997; Eisenberg & Redford, 1999; Yensen & Tarifa, 2003a,b; Cuarón, Reid & Helgen, 2008; Reid & Helgen, 2008; Larivière & Jennings, 2009). De forma geral, o que se depreende da literatura é que *G. vittata* parece ocorrer na porção norte da

distribuição do gênero (México até norte da América do Sul) e *G. cuja* na porção sul (centro da América do Sul até a Patagônia), mas uma definição mais clara destes limites ainda não estava disponível.

Justificativa do estudo

O número de espécies atuais existentes no gênero *Galictis* nunca foi testado de forma sistemática ou rigorosa. Esse fato não parece ser uma grande preocupação para outros táxons bem estudados e, portanto, bem conhecidos, mas esse não é o caso dos furões neotropicais. Embora a existência de duas espécies para o gênero seja o mais comumente citado na literatura, uma série de livros de referência para a taxonomia de mamíferos (Eisenberg, 1989; Redford & Eisenberg, 1992; Eisenberg & Redford, 1999) menciona a existência de uma possível terceira espécie, *G. allamandi*. Eisenberg (1989) inclusive fornece um mapa de distribuição para esse terceiro táxon, o que ressalta a necessidade de testar o número de espécies e prover uma revisão taxonômica detalhada do gênero *Galictis*. Além do número, a delimitação das espécies ainda é precária. O reconhecimento de limites bem definidos entre essas espécies viabilizará o reconhecimento de caracteres de diagnose que as identifiquem corretamente. Uma vez bem identificadas, a revisão da distribuição geográfica, assim como das principais variações dentro das espécies, se tornará possível e de grande valia para futuros estudos sobre esses mustelídeos. O conhecimento acerca de todos esses aspectos é muito importante para que se compreenda a história destes organismos na região Neotropical e sua relação ecológica com outros elementos da comunidade de mamíferos nos biomas onde ocorrem. Além disso, a falta de clareza ou mesmo ausência dessas informações impede que a biologia, ecologia e evolução deste gênero sejam investigadas de forma adequada, e que seja avaliado seu estado de conservação e ameaças atuais. Espécies cuja biologia e história evolutiva são amplamente desconhecidas, como neste caso, podem sofrer de uma subestimativa de seu risco de extinção, o que prejudica a elaboração de estratégias adequadas para sua conservação em longo prazo.

Objetivos

Os objetivos dessa tese de doutorado são: **(i)** identificar o número de espécies do gênero *Galictis*, testando a ocorrência de descontinuidades morfológicas e genéticas; **(ii)** revisar a distribuição geográfica de cada espécie confirmada, com base na identificação de espécimes tombados em coleções zoológicas; **(iii)** analisar a ocorrência de variações morfológicas intraespecíficas que possam caracterizar dimorfismo sexual e variação geográfica para cada espécie identificada; **(iv)** realizar análises filogeográficas com base no DNA mitocondrial de ao

menos uma das espécies identificadas, investigando seus padrões de estruturação genética e demografia histórica; **(v)** comparar as inferências baseadas em dados morfológicos e moleculares, buscando uma compreensão da história evolutiva do gênero *Galictis*, a qual será utilizada para embasar uma revisão taxonômica do mesmo.

Ferramentas de estudo

As ferramentas de estudo para essa tese seguirão as ideias desenvolvidas na síntese dos trabalhos de Mayden (2002), de Queiroz (2007) e Naomi (2011), ou seja, as características próprias das espécies serão utilizadas justamente para identificá-las e delimitá-las (critério para delimitação). Para reconhecer essas propriedades das espécies, serão utilizadas análises **morfológicas** e **moleculares**.

Para os estudos morfológicos, as coleções científicas de museus e instituições acadêmicas são valiosas fontes de amostras da biodiversidade. Tratando-se de um caso como o de *Galictis* (além de pouco conhecido, também pouco avistado na natureza), as coleções passam a ser uma das principais documentações oficiais de seus registros. Para explorar esses registros (muitas vezes antigos, com materiais coletados no século 19) será aplicada a morfometria tradicional nos sincrânios dos espécimes de *Galictis* a fim de unir informações que poderão ser testadas para o reconhecimento de unidades morfologicamente distintas, assim como serão analisadas as peles de todos os espécimes identificados. A escolha dessa metodologia se justifica não apenas por ela propiciar uma ferramenta importante para identificação de espécies, mas também por sua aplicabilidade. O trabalho nas coleções científicas poderá trazer respostas de diagnose para a identificação das espécies. Assim, cada vez que um novo registro nas coleções é avaliado, possivelmente se poderá identificar o espécime usando esses caracteres de diagnose (critério para delimitação). Adicionalmente, a compilação dessas informações de tamanho e pele pode ajudar também a identificação de espécimes na natureza, embora esse não seja o foco primordial da tese.

Para reconhecer as propriedades genéticas das unidades de *Galictis*, serão também analisadas variações em sequências de DNA. Dessa forma, será possível testar principalmente se há monofilia recíproca (critério para delimitação) entre unidades que sejam compatíveis com as espécies descritas para o gênero. Adicionalmente, o estudo da filogeografia pode trazer informações interessantes sobre a diversidade intraespecífica e os processos históricos que a influenciaram. Importante salientar que tanto os registros da análise morfológica quanto os registros da análise molecular serão utilizados para atualizar os mapas de distribuição das

espécies reconhecidas, refinando limites geográficos e identificando áreas de possível simpatria entre os táxons.

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Capítulo II

Taxonomic revision of genus *Galictis* (Carnivora: Mustelidae): species delimitation, morphological diagnosis and refined mapping of geographic distribution

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RUNNING TITLE: Taxonomic revision of genus *Galictis*

ABSTRACT:

Although critical for enabling in-depth evolutionary, ecological or conservation-oriented studies, taxonomic knowledge is still scarce for many groups of organisms, including mammals of the order Carnivora. For some of these taxa, even basic aspects such as species limits and geographic distribution are still uncertain. This is the case of the Neotropical mustelid genus *Galictis*, considered one of the least studied carnivoran genera in the Americas. To address this issue, we performed a comprehensive assessment of morphological and molecular characters to test the number of species within *Galictis*, and to characterize their distinctiveness and evolutionary history. In addition, we review and consolidate the available information on the taxonomy of this genus, so as to provide a historical framework upon which we could interpret our data. Our analyses demonstrated that two *Galictis* species can be clearly delimited and diagnosed using metric and non-metric morphological characters as well as DNA sequences from mitochondrial and nuclear gene segments. On the basis of this clarified species-level delimitation, we reassessed the geographic range of each *Galictis* taxon, identifying possible areas of sympatry between them. These results provide a solid taxonomic framework for *Galictis*, enabling the development of additional studies focusing on this poorly known taxon.

KEYWORDS: America – distribution – greater grison – lesser grison – morphometrics – Neotropical region – phylogeny - skin variation – skull variation – taxonomy

INTRODUCTION

Taxonomy forms the basis for all biodiversity sciences, as it provides the overall framework upon which one can describe and characterize spatial or temporal patterns of population or community changes. The science of taxonomy is thus a pre-requisite for downstream studies aimed toward better understanding and conserving biodiversity (Wilson, 1992; Wilson, 2005; Reeder, Helgen & Wilson, 2007). Even among some of the best-known organisms, such as mammals, there is still much to be done in terms of basic taxonomy (Patterson, 2000, 2001), which is illustrated by the rate of discovery of new species observed in the last few decades (Collen, Purvis & Gittleman, 2004; Reeder *et al*., 2007). Interestingly, the spatial pattern of mammalian species description in the last two decades indicates that such findings derive in many cases from a combination of poor historical knowledge on regional faunas (relative to their diversity) and recently increased taxonomic efforts (Schipper *et al*., 2008). This implies that an improved understanding of mammalian biology is still positively related with taxonomic engagement, especially in megadiverse regions whose biotas remain poorly characterized.

Among mammals, carnivores are supposed to be well known, but is that really the case? Some genera and species have received minimal study, which means that basic knowledge such as species limits and geographic distribution are still uncertain (Collen *et al*., 2004). The paucity of such information implies that the biology and conservation status of these species are also unclear, which poses a serious impediment to the design of management strategies for a group that tends to be particularly susceptible to anthropogenic threats (Miller *et al*., 1999; Valenzuela-Galván, Arita & Macdonald, 2007). Curiously, much of the recent improvement in species-level taxonomic knowledge on carnivores derives from revisions based on previously available museum specimens, instead of resulting from actual field-based discoveries of new taxa (Patterson, 2000). This is illustrated by the recent recognition of new carnivoran species using morphological and/or molecular data sets (Helgen, Lim & Helgen, 2008; Helgen *et al*., 2009; del Cerro *et al*., 2010; Goodman & Helgen, 2010) derived at least partially from museum material. In addition to the description of new species, other aspects of basic carnivoran taxonomy may be clarified with thorough revisions of museum specimens, especially in the case of poorly known genera from understudied regions of the world.

The Neotropical region spans a large portion of the Americas, extending from central Mexico to southern Argentina and Chile, and harboring one of the richest hotspots of mammalian diversity worldwide (Olson *et al*., 2001; Schipper *et al*., 2008). Among mammalian species, some

carnivoran genera are endemic to the Neotropics, including some groups that have so far received very little taxonomic and biogeographic attention. This is the case of the grisons (genus *Galictis*, Carnivora, Mustelidae) for which very little information has so far been published, rendering it one of the least studied carnivoran genera in the Americas. Not surprisingly, the acquisition of basic information about *Galictis* has already been cited as a priority for carnivore studies in the Neotropics (Oliveira, 2009).

Currently, there are two species recognized in this genus: *G. vittata* (greater grison) (Schreber, 1776) and *G. cuja* (lesser grison) (Molina, 1782) (Wozencraft, 2005), but the taxonomic history of these forms is relatively complex. The name *Galictis* first appeared in 1826 in a short publication by the English naturalist Thomas Bell (Bell, 1826). This publication contained a brief description of the habits of a living animal caught in Guyana that the author himself observed for several months. Bell, however, used the name *Galictis* without providing any detailed description of the precise locality of collection or its distinctive characteristics. Finally, in the last lines of the publication, Bell mentioned his intention to better characterize the animal and designate it as the type for a new genus. Eleven years after this report, Bell published a more detailed description of this carnivoran taxon, in which he examined a specimen from the collection of the Zoological Society and recognized essential similarities between this individual and the one he had observed previously, thus officially validating the generic name *Galictis* (Bell, 1837). However, prior to the establishment of Bell's genus, these Neotropical carnivores were already known to science, as previous authors had described several taxa under different generic names.

The first reference to grisons in the natural history literature was that of the Swiss naturalist Jan Nicolaas Allamand, who presented in 1771 a drawing with a new mammal from Suriname, for which he coined the name 'grison' (Buffon, 1776). The official description of that species came some years later with the work of Johann Christian von Schreber, who described it based on Allamand's drawing, and named it *Viverra vittata* (Schreber, 1776). In parallel, Chilean naturalist Juan Ignacio Molina described two species of carnivores from Chile, *Mustela cuja* and *M. quiqui* (Molina, 1782). The author, however, presented these species without any specification of their type locality. Nevertheless, it was not until the beginning of the 19th century that the species described by Schreber (1776) and by Molina (1782) were recognized as belonging in the same genus. Shaw (1800) combined the species described by both authors in the genus *Viverra* using the names *V. vittata*, *V. cuja* and *V. quiqui*. This author considered Molina's *M. cuja* and *M. quiqui* to be synonyms of *V. cuja* and *V. quiqui*, respectively. In addition, he considered the name 'grison' to remain attached only to *V. vittata*.

Some years later, Oken (1816) described a new genus he named *Grison*, with *V. vittata* as the type species. This generic name was followed by some authors during many years (Thomas, 1907; Ihering, 1911; Goldman, 1920; Osgood, 1943; Goodwin, 1946), but Oken's (1816) names have subsequently been ruled invalid by the International Commission on Zoological Nomenclature (ICZN, 1956). Various other generic appellations were also applied to the grisons over the years, such as *Gulo* (Desmarest, 1820), *Ursus* (Thunberg, 1820), and *Lutra* (Traill, 1821), names formerly employed in much broader taxonomic contexts, as well as the names *Grisonia* (Gray, 1865) and *Grisonella* (Thomas, 1912), which currently represent synonyms of *Galictis* (see Yensen & Tarifa [2003a] for a recent review).

Although the validity of *Galictis* as a taxonomic entity has been solidly established, much more uncertainty has surrounded its species-level composition. When Bell (1837) officially described *Galictis*, he recognized Schreber's (1776) *V. vittata* as a member of the new genus, and also described a second species, *G. allamandi*, based on a single museum specimen. Bell did not include in the new genus the two Chilean species described by Molina (1782). In a comprehensive zoological list from Chile, Claudio Gay considered Molinas' *M. quiqui* as a synonym of *G. vittata*, but did not mention *M. cuja*. As far as we know, this is the first synonymy list that included any of Molina's Chilean species in the genus *Galictis* (Gay, 1847). Subsequently, some authors recognized *M. cuja* and *M. quiqui* to be synonyms of *G. vittata* (Gray, 1865, 1869). A few decades later, Thomas separated *G. vittata* from the Chilean species, while considering the latter two (*G. cuja* and *G. quiqui*) synonyms of each other (Thomas, 1907). However, in 1912 Thomas again raised the issue of *G. cuja* and *G. quiqui* potentially being separate species, but regarded the issue as unresolved (Thomas, 1912). In addition to this ongoing discussion with respect to the status of the two Chilean species of *Galictis*, several other species were described for this genus during the 20th century, leading to considerable variation in the taxonomic literature addressing this group (see Yensen & Tarifa, 2003a,b for exhaustive synonym lists). Overall, although some authors have continued to recognize *G. allamandi* as a potentially valid species (e.g. Eisenberg, 1989), most current sources only recognize *G. vittata* and *G. cuja* (e.g. Yensen & Tarifa, 2003a,b), although their exact limits remain poorly defined (see below).

Modern systematic assessments of grison morphology and species delimitation may be seen as beginning only with Thomas's contributions in the early 20th century (e.g. Thomas 1907, 1912), as this author was the first to recognize diagnostic characters that are still perceived as valid today (e.g. Yensen & Tarifa, 2003a,b). For example, he noted that specimens from southeastern Brazil (Minas Gerais state) lacked a "supplementary internal cusp" in their first lower molar that seemed to be present in grisons from northern South America (Thomas, 1907: 162).

Five years later, he proposed that there were two forms of grisons: a larger one presenting this internal cusp in the first lower molar (and occurring mostly in the northern Neotropics), and a smaller one lacking this internal cusp and inhabiting more southerly areas of the region (Thomas, 1912). Although these ideas have formed the basis for present-day recognition of *G. vittata vs. G. cuja* (e.g. Yensen & Tarifa, 2003a), to our knowledge they have never been thoroughly reassessed since Thomas's original studies, so that their effectiveness and general applicability remain unknown.

The uncertainty surrounding the exact morphological distinction between the two *Galictis* species is a challenge to the identification of specimens collected or observed throughout the Neotropics. As a consequence, there is considerable controversy in the literature regarding the geographic distribution of these taxa. For instance, some authors have considered the southern limit of *G. vittata* to be in northeastern Brazil (e.g. Mares *et al*., 1981; Emmons & Feer, 1997; Larivière & Jennings, 2009), while others have indicated that it is in southeastern Brazil (e.g. Nowak, 1991; Eisenberg & Redford, 1999) or even southern Brazil (Yensen & Tarifa, 2003a). In stark contrast, Brazil is not even listed as a country of occurrence for this species in a recent taxonomic and geographic reference source for mammals (Wozencraft, 2005). Similar confusion surrounds the distribution of *G. cuja*, with some sources listing its occurrence as far north as northeastern Brazil (Yensen & Tarifa, 2003b), whereas others indicate its northernmost limit on the Atlantic coast to be in southeastern Brazil (e.g. Eisenberg & Redford, 1999). Such discrepancies are apparent when one compares the distribution maps for these species published in recent years (e.g. Emmons & Feer, 1997; Eisenberg & Redford, 1999; Yensen & Tarifa, 2003a,b; Cuarón, Reid & Helgen, 2008; Reid & Helgen, 2008; Larivière & Jennings, 2009), highlighting the current lack of knowledge regarding their geographic range and actual areas of known or potential sympatry.

Given the long and convoluted taxonomic history of *Galictis*, as well as the controversial views about the geographic distributions of *G. vittata* and *G. cuja*, it is critical to address and characterize in detail the species limits within this genus, which should also clarify the actual range of the emerging taxa. Our goals here are thus to test whether two or more species of *Galictis* can be recognized based on morphological and molecular characters, as well as to provide reliable diagnostic features for them and a thorough reassessment of their geographic distribution.

MATERIALS AND METHODS

In order to achieve a comprehensive assessment of genus *Galictis,* we analyzed morphological aspects of museum specimens, as well as molecular data collected from fresh tissue samples. On the basis of the ascertained geographic records derived from the morphological and molecular data sets, we produced an updated map of the distribution of each grison species.

MORPHOLOGICAL ANALYSES

We examined skins and skulls of *Galictis* specimens deposited in 22 mammalian collections: Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil (MCT-PUCRS); Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Brazil (FZB/RS); Museu de Ciências Naturais da Universidade Luterana do Brasil, Canoas, Brazil (ULBRA); Laboratório de Mamíferos Aquáticos da Universidade Federal de Santa Catarina, Florianópolis, Brazil (LAMAq-UFSC); Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP); Museu Nacional de História Natural, Rio de Janeiro, Brazil (MNHN); Coleção de Mamíferos da Universidade Federal de Pernambuco, Recife, Brazil (UFPE); Museu Paraense Emilio Goeldi, Belém, Brazil (MPEG); Museo Nacional de Historia Natural y Antropologia, Montevideo, Uruguay (MNHNA); Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina (MACN); Museo de La Plata, La Plata, Argentina (MLP); National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA (USNM); American Museum of Natural History, New York, USA (AMNH); The Academy of Natural Sciences of Philadelphia, Philadelphia, USA (ANSP); Museum of Comparative Zoology, Harvard University, Cambridge, USA (MCZ); The Field Museum of Natural History, Chicago, USA (FMNH); Yale Peabody Museum, Yale University, New Haven, USA (YPM); Natural History Museum from the University of Kansas, Lawrence, USA (NHMKU); Museum of Vertebrate Zoology, Berkeley, USA (MVZ); The Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMNS); Natural History Museum, London, England (BMNH); and Staatliches Museum für Tierkunde, Dresden, Germany (SMT). The complete list of specimens is provided in the Supporting Information S1.

For each of these institutions, all *Galictis* specimens were examined, especially those represented by skulls and/or skins. Skulls were categorized as adult *vs.* non-adult, and also assessed in terms of their integrity (i.e. whether they were intact or broken, and if measurements could be accurately taken). Only adult skulls with known sex and sufficient integrity to undertake

measurements were included in statistical analyses. These skulls, along with all others that could be reliably identified (i.e. including those that presented diagnostic features [see Results] but belonged to non-adults and/or were broken, as well as those with unknown sex) were used for geographic analyses aimed at reassessing the distribution of the *Galictis* species. Skin variation was also surveyed and characterized, especially in cases where the associated skull could provide reliable species identification (see Results). Specimens represented only by a skin were not included in the geographic analysis (see below).

For the morphological analyses, we defined 15 craniodental measurements: skull - greatest length of skull (GLS), nasal length (NL), zygomatic breadth (ZB), mastoid breadth (MB), braincase breadth (BB), interorbital constriction (IC), postorbital constriction (PC), palatal width (PW), braincase height (BH), mandible length (ML), and mandible height (MH); teeth - length of maxillary toothrow (C-M2), external alveolar distance between upper canines (C-C), external alveolar distance between upper molars (M2-M2), and length of mandible toothrow (c-m2). Adult specimens were recognized as those presenting a fully erupted permanent dentition along with a total fusion of the skull sutures. All measurements were taken by the first author, except for the specimens from BMNH and SMT, which were measured by the second author. One discrete variable was recorded for all specimens: the presence or absence of a metaconid in the first lower molar.

In addition to the skull measurements, we also recorded the total body length (TL) from several individuals that contained this information in their skin tags. To increase the sample size for this external variable, we combined our data with TL measurements reported in previous studies focusing on *Galictis* (Thomas, 1903, 1907, 1912, 1921; Goodwin, 1946; Greer, 1966; Husson, 1978).

Considering that sexual size dimorphism is prominent in many mustelids, with males being larger than females (Dayan *et al*., 1989; Dayan & Simberloff, 1994; Thom, Harrington & Macdonald, 2004; Rozhnov & Abramov, 2006; Monakhov, 2009), we performed *t*-test comparisons (with a Bonferroni correction) to assess whether any measurement differed significantly between the sexes within each putative *Galictis* species (defined *a priori* based on a meristic character – see Results). Since we observed a strong pattern of sexual size dimorphism in this genus (see Supporting Information S2), we performed all subsequent statistical analyses separately for males and females, as described below.

To investigate whether skull measurements contained information that supported segregation between different *Galictis* clusters, we conducted multivariate analyses using all craniodental measurements. We initially performed a Principal Component Analysis (PCA), and

then conducted Discriminant Function Analyses (DFA). The latter were performed using two different approaches: (i) two-group discriminant function, where we analyzed male and female data sets separately, in each considering the two species-level clusters identified by the PCA (see Results); (ii) multiple-group discriminant function, where we performed a joint analysis of the full data set considering four grouping variables, corresponding to males and females of each species. Prior to these multivariate analyses, all measurements were log-transformed so as to reduce their variance and thus perform a more conservative assessment of group differences.

Groups defined by these analyses were then used for subsequent comparisons. We initially calculated standard descriptive statistics (mean, standard deviation and range) for each measurement in each of the identified groups, and assessed whether they overlapped between them. We then compared the mean of each skull measurement and the TL between the groups using *t*-tests with a Bonferroni correction for multiple comparisons (with an adjusted significance level of $P = 0.05/16$). These analyses were performed separately for males and females. All statistical procedures based on morphological data were performed with SPSS version 17 (SPSS Statistics for Windows, Rel. 17.0.0, Chicago).

MOLECULAR ANALYSES

Tissue samples were obtained from ten individuals of *G. cuja* (five obtained from different Brazilian states [Rio Grande do Sul, Paraná, São Paulo, Minas Gerais, and Bahia] and five from Argentina) and three individuals of *G. vittata* from Peru (see Supporting Information S1 for sample details). Genomic DNA was extracted from all samples using a standard phenol-chloroform protocol (Sambrook, Fritsch & Maniatis, 1989) or the DNeasy Blood and Tissue Kit (Qiagen), followed by quality checking on an agarose gel. Available sequences from two related species were used as outgroups: *Ictonyx striatus* and *Poecilogale albinucha* (see Supporting Information S3 for GenBank accession numbers). These four taxa are part of the Galictinae, one of the mustelid subfamilies defined on the basis of recent analyses of DNA sequences (Koepfli *et al*., 2008; Wolsan & Sato, 2010).

Mitochondrial gene segments

We amplified a segment of the mitochondrial gene *NADH dehydrogenase subunit 5* (*ND5*) via the Polymerase Chain Reaction (PCR) using the primers ND5-DF1 and ND5-DR1 (Trigo *et al*., 2008). PCR reactions were performed in a 20µL final volume containing 10-100 ng of genomic DNA, 1x PCR Buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq Platinum DNA polymerase (Invitrogen), and 0.2 µM of each primer. The PCR conditions were the

following: 10 touchdown cycles of 94ºC for 45s, 60-51ºC for 45s (with a decrease in annealing temperature of 1ºC per cycle), and 72ºC for 1.5 min; followed by 30 cycles of 94ºC for 45s, 50ºC for 45s, and 72ºC for 1.5 min, and a final extension of 72ºC for 3 min. PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium) and purified by precipitation using ammonium acetate. Purified PCR products were sequenced in both directions using the *DYEnamic ET Dye Terminator Sequencing Kit* (GE Healthcare®) and subsequently analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare®).

The forward and reverse chromatograms were assembled, visualized and checked using the *Phred/Phrap/Consed* package (Ewing *et al*., 1998; Gordon, Abajian & Green, 1998). Consensus sequences were deposited in GenBank (accession numbers **XXXXX-XXXXX**) and aligned using the ClustalW algorithm implemented in MEGA 4.0 (Tamura *et al*., 2007). The alignment was checked and edited by hand using MEGA. Phylogenetic analyses were performed using four different criteria: maximum parsimony (MP); maximum likelihood (ML); distance-based with the neighbor-joining (NJ) algorithm; and Bayesian Inference (BI). The MP, ML and NJ analyses were conducted using PAUP* 4.0b10 (Swofford, 2002), while MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) was used to reconstruct the BI tree. The best-fit model of nucleotide substitution for the data was estimated using the Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada & Crandall, 1998). The selected model (General-Time-Reversible with a proportion of invariable sites [GTR+I]) was implemented in the ML and BI analyses, as well as in the NJ search (which used ML distances). The ML analysis employed a heuristic search using random taxa addition followed by TBR branch-swapping. The MP phylogeny was also based on a heuristic search using random taxon addition and TBR branch-swapping. Nodal support for the ML, MP and NJ methods was assessed with 500 replicates of bootstrapping. The Bayesian analysis used two independent replicates of the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) procedure, each containing four chains (one cold, three heated) run for 10^6 generations, with trees and parameters sampled every 100 steps, and the initial 25% discarded as burn-in.

Nuclear gene segments

We amplified 12 nuclear gene segments (Table 1) with either one of two (A and B) PCR touchdown protocols: A) one cycle of 95ºC for 3 min; followed by 6 cycles of 94ºC for 15s, 60ºC to 50ºC for 30s, with a decrease in annealing temperature of 2ºC per cycle, and 72ºC for 45s; followed by 30 cycles of 94ºC for 15s, 50ºC for 30s, and 72ºC for 45s; and one cycle of 72ºC for 30 min. Each 15µl reaction contained 6.98µl of sterile double-distilled water, 1.5µl of 10X PCR

Gold Buffer, 1.2µl 25mM $MgCl_2$, 1.2µl of 10mM dNTP mix, 1.5µl of both 2µM forward and reverse primers, 1 U of AmpliTaq Gold *Taq* polymerase (Applied Biosystems, Foster City, CA, USA), and 100 ng of genomic DNA. B) one cycle of 95ºC for 10 min; followed by 16 cycles of 94ºC for 1 min, 63ºC to 50.2ºC for 1 min, with a decrease in annealing temperature of 0.8ºC per cycle, and 72ºC for 1.5 min; followed by 30 cycles of 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 1.5 min; and one cycle of 72ºC for 5 min. Each 25µl reaction contained 14.9µl of sterile double-distilled water, 2.5µl of 10X PCR Gold Buffer, 1.5µl 25mM $MgCl_2$, 1.0µl DMSO, 2.0µl of 10mM dNTP mix, 1.0µl of both 2µM forward and reverse primers, 1 U of AmpliTaq Gold *Taq* polymerase, and 100 ng of genomic DNA. A negative control (no DNA) was included with all PCRs. Amplification products were electrophoresed in 1% agarose/Tris-acetic acid-EDTA gels and stained with ethidium bromide. A 100bp molecular weight marker (Promega, Madison, WI, USA) was run with all PCR products to check that the correct product size was amplified. PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (Affymetrix, Cleveland, OH, USA). Purified products were then cycle sequenced in both directions using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the original amplification primers. Cycle sequencing reactions were purified using Agencourt Cleanseq (Beckman Coulter Inc., Brea, CA, USA) and then run on an Applied Biosystems 3730xl DNA Analyzer.

Forward and reverse sequence chromatograms were assembled, checked and edited using the Geneious v5.3 software package (Drummond *et al*., 2010). Due to the presence of two mononucleotide repeats within the *WT1* gene segment, forward and reverse sequences could not be assembled and were therefore checked individually on either side of the repeats. Sequences for some locus-species combinations were taken from Koepfli *et al*. (2008) and added to their respective alignments (a summary of the sequence sources for nDNA analyses is provided in Table 1). Newly generated sequences were deposited in Genbank (accession numbers **XXXXXX – XXXXXX**). Haplotype networks were built by hand for each nuclear gene segment, and phylogenies were reconstructed from a concatenated supermatrix combining all fragments. These analyses employed the same criteria used for the mitochondrial data: maximum parsimony (MP), maximum likelihood (ML), distance-based (with NJ algorithm), all implemented in PAUP*, and Bayesian Inference (BI) performed in MrBayes. Model parameters were selected by AIC in Modeltest for use in ML, NJ and BI analyses. In this case the most appropriate model of evolution was that of Hasegawa, Kishino & Yano (1985) with a proportion of invariant site (HKY+I). The ML analysis employed a heuristic search started with a NJ tree and followed by NNI branch-swapping. Nodal support was estimated by performing 100 bootstrap replicates. The MP trees

were retrieved with a heuristic search using simple taxon addition and TBR branch-swapping. Branch support was assessed with 100 replicates of bootstrapping. The Bayesian analysis used two independent replicates of the MCMCMC procedure, each containing four chains (one cold, three heated) run for 10^6 generations, with trees and parameters sampled every 100 steps, and the initial 25% were discarded as burn-in.

GEOGRAPHIC ANALYSES

The record localities for all reliably identified *Galictis* specimens (see above for exclusion criteria) were used to build a geographic data base of species occurrence. For that purpose, we used the exact locality information available in all specimen tags that included geographic coordinates. However, in most cases we had to assign coordinates based on other types of locality data available on the tag (which often consisted of information restricted to municipality, state, or even country, as well as the name of a lake, river or mountain for some specimens). This assignment was performed using Global Gazetteer Version 2.2 (available at http://www.fallingrain.com) for all data points containing municipality data. In a few cases where this was not available, Google Earth 6.1.0 (available at http://www.googleearth.com) was used to identify other landscape features within a delimited country or region. In cases where the locality of origin was not precise, but could still provide a reasonable region of placement within an approximate radius of 500 km (e.g. unspecified state-level records in Brazil, or tags referring to countries such as Uruguay or Panama) we used the central coordinates of the identified region. Still, some specimens contained such vague geographic information (e.g. a large country or a very long river) that they could not be reliably used for geographic referencing and were thus excluded from this analysis. The resulting data base was then analyzed with a geographic information system (GIS) using the biome map reported by Olson *et al*. (2001) and the software ArcGIS 9.3 (ESRI, 2009). We then used the distributional maps to characterize in detail the geographic range of each species, to assess possible areas of sympatry between them, and to investigate the presence of these taxa across Neotropical biomes.

RESULTS

MORPHOLOGICAL DATA

Our first assessment of cranial material consisted of a survey for the presence of the metaconid in the first lower molar that has been proposed to be a diagnostic character distinguishing these species (see Introduction). These initial surveys revealed that indeed there

was a pool of specimens bearing such a feature (almost all of which had been sampled in the northern portion of the genus's range), and another lacking the metaconid (most of which had been collected in the southern portion of the *Galictis* range) (Fig. 1). Based on this character, along with prior identification of museum specimens, we provisionally assigned each individual of the former group to *G. vittata*, and the latter to *G. cuja*, so as to provide a taxonomic hypothesis that could be tested with statistical morphometrics.

We then performed a principal component analysis (PCA) based on the 15 craniodental measurements, aiming to test whether two or more distinct clusters could be distinguished, and if such groups would be congruent with the *a priori* taxonomic partition outlined above. The PCA showed the presence of two distinct clusters in both male (Fig. 2A) and female (Fig. 2B) data sets, which corresponded very well to the *G. cuja* and *G. vittata* partitions previously hypothesized on the basis of the metaconid character. For both sexes, the distinction between the two groups was essentially along the first principal component, which explained 85.15% of the total variance for males, and 88.64% for females (Table 2). Taken together, the first two components explained 89.19% (males) and 92.38% (females) of the total variation. The variables with the greatest contribution to the first component were GLS, ZB, MB, ML, and c-m2 (males) and GLS, ZB, ML, and c-m2 (females), with this separation thus reflecting differences in overall skull size.

The Discriminant Function Analysis (DFA) corroborated and extended the results of the PCA. The means from all variables (craniodental measurements) were statistically different between the groups (species), indicating that all of them were highly significant with respect to their ability to segregate the species (Table 3). The two-group approach (which considered each sex separately) correctly classified all individuals, which means that if the sex of a specimen is known, the resulting classification analysis is 100% accurate (see Supporting Information S4 for details of the classification statistics).

The multiple-group DFA yielded three discriminant functions, but the Chi-square test indicated that only the first two canonical variates (or roots) were significant (Table 2). The first canonical variate (CV1) accounted for 90.8% of the discriminatory power between clusters, while the second (CV2) accounted for 7.9%. Based on the standardized coefficients for the 15 variables, the most relevant variables for group segregation along CV1 were: c-m2, BB, and MB. The means of population groups represented by the group centroid indicated two clear clusters, again corresponding to *G. cuja* and *G. vittata* (Fig. 3). The segregation between males and females within each species was mainly seen along the CV2 axis, although this segregation was not perfect and there was considerable overlap between males and females within each species.

The classification analysis performed in the context of the multiple-group DFA yielded an overall correct classification of 88.1%. The percentage of inefficient classification occurred only between sexes of the same species, and never between *G. cuja* and *G. vittata*. Within *G. cuja*, four (9.1%) males and five (14.3%) females were not classified correctly, while within *G. vittata* two (12.5%) males and three (13.0%) females were misclassified. The complete classification function extracted from the classification coefficients is shown in Supporting Information S4.

Given the identification of distinct clusters by the multivariate analyses, we then surveyed the range of all linear measurements observed in each group, while keeping male and female data sets separate. We observed that some variables do not overlap between the two groups (Table 4), highlighting the size distinction between these clusters. Although the magnitude of the distinction was in some cases modest, the following variables enabled the direct segregation of *Galictis* specimens into two species-level clusters (*G. cuja* and *G. vittata*): BB, c-m2 and TL for males; and GLS, ZB, BB, IC, ML, and c-m2 for females (see Table 4). In addition to the observation of non-overlapping ranges in some measurements, we noted for both males and females that the mean of all variables was clearly different between *G. cuja* and *G. vittata*, with the latter being considerably larger (skull size comparison is represented in Fig. 4). The *t*-test results showed significant differences in all linear measurements (craniodental and TL), even after applying the conservative Bonferroni correction (Table 5).

The analysis of pelage features revealed a trend which seems to fit the species-level distinction demonstrated by the linear measurements, although we did not perform statistical tests here due to the high variability in skin age and preparation that could bias this assessment. We observed that all specimens identified as *G. cuja* presented a denser and/or longer pelage, leading to a 'furrier' appearance. This feature was never observed in any individual identified as *G. vittata*, whose fur was always shorter (Fig. 5). In addition, *G. vittata* individuals were always grayish (varying from pale gray to medium-dark gray), never exhibiting yellowish hues (see Fig. 5). In contrast, *G. cuja* specimens tended to be yellowish, although some individuals could be as grayish as *G. vittata*, thus overlapping in coloration with the latter species. Therefore, a combination of fur length/density with coloration may be a reliable external character to diagnose these species, although caution should be taken given the observed overlap in the latter.

MOLECULAR DATA

Mitochondrial and nuclear gene segments provided clear evidence of differentiation between two distinct *Galictis* clades (Fig. 6). All phylogenetic analyses performed with the mtDNA data set resulted in high support for these two clades, whose geographic occurrence indicated

that they corresponded to *G. cuja* and *G. vittata* (Fig. 6A). Likewise, all 12 nuclear segments provided concordant evidence for this separation, even when analyzed individually (Fig. 7 and Supporting Information S5). There was no nuclear haplotype sharing between the two geographic groups corresponding to *G. cuja* and *G. vittata*, which were found to be reciprocally monophyletic in every case. Depending on the locus analyzed, these two groups were differentiated by one (*JAK1* and *MACF1*), two (*TRHDE*, *AAMP2, ADORA3, PFKFB1* and *PTPN4*), three (*GNAT1*, *APOB* and *RHO1*) or even six mutational steps (*RAG1* and *WT1*). In addition to the individual-gene assessments, we also concatenated the 12 segments for each individual (ignoring heterozygous sites), and observed very high support for the two *Galictis* clades with all phylogenetic methods employed (Fig. 6B). Overall, considering both the mtDNA and nuclear data sets, nodal support for these clades was >90% with all analytical methods.

GEOGRAPHIC DISTRIBUTION

Since the morphological and molecular approaches corroborated the recognition of two clusters in *Galictis*, corresponding to *G. vittata* and *G. cuja*, we employed our data sets to reassess and refine the geographic distribution of each of these species. The resulting maps indicated that *G. vittata* is distributed in the northern range of the genus, from the extreme north of the Neotropics, in Mexico, to the central region of South America. In contrast, *G. cuja* is distributed in the southern range of the genus, from the extreme south of Peru, southern Bolivia, and northeastern Brazil to southern Chile and Argentina (Fig. 8 and Supporting Information S6).

The refined distributional maps allowed an assessment of potential areas of sympatry between the two species. The range of *G. vittata* extends southwards to Peru (throughout Loreto, Amazonas, Ucayali, Pasco, Junín, and Cuzco departments), Bolivia (Santa Cruz department), Paraguay (Guaira department), and northwestern Brazil (Amazonas, Amapá, Pará, and Rondônia states). The occurrence in these countries suggests some overlap with the northwestern limit of the *G. cuja* range, documented in Peru (Puno department), Bolivia (Cochabamba, Santa Cruz, and Tarija departments), and throughout central-western to northeastern Brazil (including Alagoas, Bahia, Ceará, Goiás, Paraíba, and Pernambuco states and the Distrito Federal) (see Fig. 8 and Supporting Information S6).

A finer-scale analysis reveals less evidence of geographic overlap, although some instances may remain. When the presence of each species was assessed with respect to different biomes or ecoregions, we observed that in Peru *G. vittata* seems to occur in the broad region of the Peruvian Amazon, but not in the mountainous region of the Andes or the drier region of the coast. In contrast, the *G. cuja* records from that country are from the south, in the Montane

Grassland and Scrublands (Puna Grasslands) region, up in the Andes. In Bolivia, the only record of *G. vittata* originates from the Tropical and Subtropical Dry Broadleaf Forest (Chiquitano), whereas those for *G. cuja* include this same biome as well as the Amazonian region (Tropical and Subtropical Moist Broadleaf Forest). In Paraguay, both species seem to co-exist in the Tropical and Subtropical Moist Broadleaf Forests (Atlantic Forest) in the southern region of the country, although *G. cuja* also occurs in the Savanna (Chaco). Within Brazil, there seems to exist a clear biogeographic division between the species, with *G. vittata* occurring exclusively in the Amazonian region and *G. cuja* occurring in the other biomes, including the drier Cerrado (Savanna) and Caatinga (Deserts and Xeric Shrublands) of the northeast, as well as the Atlantic Forest throughout the eastern seaboard, and the pampas grasslands towards the south (see Fig. 8).

DISCUSSION

Our analyses demonstrated that two *Galictis* species can be clearly delimited and diagnosed using metric and non-metric morphological characters (Table 6), as well as DNA sequences from mitochondrial and nuclear gene segments. On the basis of this clarified species-level delimitation, we reassessed the geographic range of each *Galictis* taxon in the Neotropics, identifying possible areas of sympatry between them. Each of these topics will be discussed in detail below.

NUMBER OF SPECIES IN GENUS *GALICTIS*

Although the presence of two grison species has been commonly mentioned in literature sources reviewing mammalian taxonomy (e.g. Yensen & Tarifa, 2003a,b; Wozencraft, 2005; Larivière & Jennings, 2009) this hypothesis has never been formally tested. In addition, the possible existence of a third species (*G. allamandi*) in northern South America and/or Central America is mentioned by some sources, including an influential set of reference books on Neotropical mammals (Eisenberg, 1989; Redford & Eisenberg, 1992; Eisenberg & Redford, 1999). In these publications, the authors mentioned that *G. allamandi* might be a synonym of *G. vittata*, but tentatively recognized it as a distinct taxon. Moreover, in Eisenberg (1989), a range map for *G. allamandi* is provided, implying that such a taxon might indeed warrant recognition. Since our morphometric analyses support the recognition of only two *Galictis* species, with no evidence for a third cluster in Central America or northern South America, we review below the taxonomic history of *G. allamandi*, so as to clarify its status.

When Schreber (1776) described *Viverra vittata* based on the drawing made by Allamand (Buffon, 1776) of an animal originated from Suriname, he seems not to have analyzed any actual specimen (museum material or living animal), implying that the grison features discussed were originally tied to the drawing alone. When Bell (1837) described the new species *G. allamandi* as distinct from *G. vittata* (albeit in a new genus), he also tied the description of the former to the drawing made by Allamand, along with a single museum specimen that he had examined personally, saying that both "may perhaps be identical" (Bell, 1837: 45). For the latter species, however, he took the name 'vittata' (originally tied to the Allamand drawing) and associated it with the living animal from Guyana that he had observed (see Introduction), suggesting that it exhibited a "distinct specific difference" (Bell, 1837: 45) from *G. allamandi*. Therefore, we can conclude that Bell (1837) untied the Allamand drawing from the Schreber description in order to recognize *G. allamandi* and *G. vittata*, whose distinction was ultimately based on a single museum specimen compared to a single living animal. Given this context, it is apparent that there was no strong evidence that distinguished these species upon their proposition by Bell (1837). Indeed, our results, which are based on a large sample of individuals collected throughout the distribution of *Galictis*, did not reveal any evidence for a third cluster in that region. Thus, the uncertainty involving *G. allamandi* seems to have been due to taxonomic confusion based on intraspecific variation, supporting the view that this species should be in fact considered a junior synonym of *G. vittata*.

SPECIES DELIMITATION – *GALICTIS CUJA VS. G. VITTATA*

The magnitude of morphological and genetic differentiation between the two *Galictis* species was similar to results reported by recent studies targeting the taxonomic status of other mustelid genera (e.g. Helgen *et al*., 2008; Harding & Smith, 2009; Jacques *et al*., 2009; del Cerro *et al*., 2010). Our observed discrimination between the two clusters in both types of multivariate analysis (PCA and DFA) was almost perfect, with the exception of two male records of *G. cuja* (one from Rio Grande do Sul state, southern Brazil [MZUSP 1044], and another from the coast of Uruguay [MNHNA 2696]) that were located within the *G. vittata* cluster in the PCA (see Fig. 2A). Observing these two individuals in detail, we noticed that both were exceptionally large, exhibiting all linear measurements longer (in some cases much longer) than the mean for all *G. cuja* males. Given that these specimens were indeed very large, it is not surprising that they would deviate towards the *G. vittata* cluster. This observation is rather interesting, and illustrates that the smallest *G. vittata* males could overlap in size with the largest *G. cuja* males, possibly leading to misidentification if only some linear measurements are employed. It is noteworthy that several

other male specimens from Uruguay and southern Brazil were also analyzed, all of which lay within the *G. cuja* cluster, thus indicating that the large size of those two specimens may not be due to any particular geographic trend, but rather be attributed to within-population inter-individual variation. In spite of these two exceptions, the multivariate classification analyses exhibited extremely high (100%) accuracy when distinguishing the two species, even when the individual's gender was unknown (see Results and Supporting Information S4). Another line of evidence that supported clear species-level delimitation derived from the univariate analyses. For all 16 linear measurements, the differences between species were highly significant, corroborating the interpretation that their distinctiveness is strongly related to divergence in size. Such a pattern was also observed in the external measurement compared here (TL), indicating that this size-based discrimination may also be feasible for identifying live animals.

In addition to the morphological data that supported clear-cut species-level separation, the molecular data sets also indicated that the two *Galictis* taxa are substantially differentiated. All trees portrayed two reciprocally monophyletic lineages, one of which included only samples from Peru (almost certainly corresponding to *G. vittata*) and the other comprising lineages sampled in Brazil and Argentina (including areas where only *G. cuja* was found to occur). The magnitude of evolutionary differentiation between these two groups (e.g. 12.7% mean uncorrected divergence in our mtDNA *ND5* data set) was sufficient to induce reciprocal monophyly at all 12 surveyed nuclear loci, a pattern which is often not observed in other closely related taxa (e.g. Syring *et al*., 2007; Degnan & Rosenberg, 2009). Such nuclear differentiation is compatible with the estimate of divergence time between *G. cuja* and *G. vittata*, which was inferred to be *ca.* 2.8 million years ago, during the second (and largest) burst of mustelid diversification (Koepfli *et al*., 2008). The fossil record indicates that grisons likely originated in North America, where they may have shared a common ancestor with the Pliocene (Blancan land mammal age) genus *Trigonictis* (Yensen & Tarifa, 2003a). The depth of evolutionary divergence estimated by Koepfli *et al*. (2008), which is consistent with the results from this study, suggests that a single *Galictis* ancestor may have invaded South America via the Panamanian isthmus during the Great American Biotic Interchange (Hunt, 1996; Eizirik, in press), soon afterwards giving rise to the two extant species. Further genetic analyses addressing the tempo and mode of this speciation process should yield interesting insights into the history of this lineage and associated components of the Neotropical biota.

MORPHOLOGICAL DIAGNOSIS

Skull

The results from this study corroborated the usefulness of a non-metric character as a diagnostic feature between these species (Fig. 1): the metaconid in m1 was present in 100% of the *G. vittata* skulls, and absent in virtually all *G. cuja* skulls (the single potential exception was one individual from Chile [AMNH 33281] that bore a very small metaconid on the left m1). In addition, we observed that the two grisons were also very different with respect to size. The PCA revealed that the split between *G. cuja* and *G. vittata* occurred along the first principal component (PC1), highlighting size as the main factor of overall cranial differences. All subsequent analyses supported the importance of this factor in the segregation between species, and in all cases *G. vittata* was larger than *G. cuja*. When all analyses were assessed together, we noticed some linear measurements that contributed the most to such size segregation: greatest length of skull (GLS), zygomatic breadth (ZB), mastoid breadth (MB), braincase breadth (BB), mandible length (ML), and length of mandible toothrow (c-m2). Of these variables, GLS and ML describe overall skull size, yielding very clear differences between species. The measurements ZB, MB and BB are the most important to describe general skull width, indicating that *G. vittata* not only has a longer but also a broader skull, leading to a more robust cranial design than its congener (see Fig. 4).

Skin

Pelage variation may also be used to aid in species diagnosis within the genus *Galictis*, although our results indicate that it is not as clear-cut as the skull characters. The marked difference in fur length/density between *G. cuja* and *G. vittata* seems to be a potentially reliable character to distinguish these species, since all *G. cuja* had long and dense fur and all *G. vittata* had a short coat. The same precision was not observed with respect to fur color, given the intra-specific variation observed in *G. cuja*. Still, the tendency of the latter species to bear yellowish fur and of *G. vittata* to have grayish fur can contribute to species diagnosis, but only when used in combination with fur density/length. This may be especially relevant in the context of visual (or photographic) identification of specimens in the field. Our results indicate that it may be possible to perform reliable identification of *Galictis* species in cases where overall body size (e.g. TL) can be assessed in combination with coat density/length, leaving pelage coloration as a third and less clear-cut criterion. Further studies focusing on live individuals whose reliable identification is available (e.g. based on the molecular characters we report here) should help ascertain the error rate associated with such a field-oriented diagnosis strategy.

GEOGRAPHIC DISTRIBUTION

The records obtained in this study cover the complete range of genus *Galictis*, from the northern limit of the Neotropical region, in Mexico, to Patagonia in Chile and Argentina (except for the southernmost recorded points [for a review focusing on Patagonia, see Prevosti & Travaini, 2005]). The northernmost limit of *G. vittata* corroborated the data provided in the literature, i.e. the Mexican provinces of San Luis Potosi and Veracruz (Wozencraft, 2005). This region is the boundary between humid and semi-humid forests in southern Mexico to drier and more open regions in northern Mexico (where no *G. vittata* was recorded). This is an interesting distributional pattern because it is very similar to that observed in the southernmost limits of the species. The range of *G. vittata* extends from mid-southern Mexico through Central America into northern South America, where it occupies tropical and subtropical forests, including the entire Amazon basin. This pattern excludes adjacent biomes (such as savannas, deserts and montane grasslands), except for a single record in southern Paraguay (AMNH 77695, skull only, with locality given as "east of Villarica", Guaira). Interestingly, Yensen & Tarifa (2003a) did not include Paraguay in the range of *G. vittata*, likely because this single record was not analyzed in the literature sources reviewed by those authors. This specimen did not have gender information, thus it was excluded from the morphometric analyses. However, its large size (e.g. BB= 40.4mm and c-m2= 33.96mm) and the presence of the metaconid in the lower carnassial identified it as *G. vittata*. The fact that this is the only specimen departing from the distributional pattern otherwise observed for *G. vittata* suggests that it might be a mislabeled individual with actual origin different from that stated on its tag. Otherwise, it would indicate that *G. vittata* does indeed extend its range into southern Paraguay, implying considerable range overlap with *G. cuja* in that region. Further scrutiny of the extent of overlap between these species in this area is warranted, so as to clarify the actual range overlap and degree of habitat segregation between *Galictis* species.

In addition to Paraguay, other potential areas of distributional overlap may include southern Peru, central Brazil and portions of Bolivia. The observed range of these taxa in Peru was in accordance with literature sources (e.g. Pacheco *et al*., 1995; Eisenberg & Redford, 1999; Yensen & Tarifa, 2003b; Larivière & Jennings, 2009), i.e. *G. cuja* occurring exclusively in the extreme south, associated with the Andes, and *G. vittata* occurring throughout the tropical forests northward. In Brazil, very few data points were available from the Cerrado biome, hampering any in-depth assessment of the exact boundary between these species in this region. However, some records from eastern Amazonia (Maranhão state) indicate that *G. cuja* and *G. vittata* do coexist in this region (Oliveira, 2009), although the geographic extent of this overlap is presently unknown. In Bolivia, Cuéllar & Noss (2003) reported both species in the south, but our results extend

northward the range of *G. cuja*, leading to a distributional pattern similar to that presented by Yensen & Tarifa (2003b) and Eisenberg & Redford (1999) (see Fig. 8).

In spite of these remaining uncertainties, our results clarified the southern limits of the *G. vittata* distribution and allowed us to provide a more precise geographic perspective of its range. Our data indicate that *G. vittata* is adapted to tropical forests in Central and South America rather than dry and open landscapes or high-elevation vegetation associated with colder temperatures. We examined a total of 67 specimens of *G. vittata*, whose identification was confirmed based on morphological characters. We found no evidence corroborating the occurrence of *G. vittata* in northeastern, southeastern or southern Brazil, in contrast to species lists and range maps reported in previous studies (e.g. da Fonseca *et al*., 1996; Emmons & Feer, 1997; Eisenberg & Redford, 1999; Guedes *et al*., 2000; Briani *et al*., 2001; Yensen & Tarifa, 2003a; Cherem *et al*., 2004; Larivière & Jennings, 2009). Such a clarification is important, as it indicates that *G. cuja* is the only grison species occurring throughout eastern Brazil, where it occupies a wide variety of biomes, including the Atlantic Forest, the Cerrado and the Caatinga.

Overall, our analysis of the *G. cuja* distribution produced a map that was very similar to that reported by Yensen & Tarifa (2003b), but different from those presented by other authors (e.g. Eisenberg & Redford, 1999), mainly with respect to the occurrence of this species in northeastern Brazil. The presence of *G. cuja* in this region has been controversial and/or poorly documented, with some reference maps ignoring its occurrence in the area (e.g. Larivière & Jennings, 2009), while other sources (e.g. de Freitas & Silva, 2005; Oliveira, 2009) reported that it does exist in at least some of the included biomes, such as the Caatinga. Our data corroborate this view, and provide conclusive evidence that *G. cuja* indeed occurs throughout northeastern Brazil.

EVOLUTIONARY CONSIDERATIONS

The two species of *Galictis* are segregated mainly by size, with *G. vittata* being consistently larger and *G. cuja* smaller. It is thus interesting to hypothesize about the evolutionary pressures that have shaped such a size-based distinction between them. Considering that the two grison species do not show extensive range overlap (see Fig. 8 and Supporting Information S6), which could induce and maintain pervasive character displacement between them, we postulate that other evolutionary processes underlie this observed pattern of size segregation. One possibility is that their geographic ranges overlapped much more in the past than they do today, implying that their size distinction was indeed generated by character displacement between them in sympatry, followed by more recent range shifts in one or both species (Davies *et*

al., 2007). Another hypothesis is that their size difference is mostly influenced by trophic competition with other mustelids, and not with each other. It may be noted that *G. vittata* is one of the largest mustelids in the Neotropical region, and particularly so if we consider sympatric species that are exclusively terrestrial (*Mustela africana, M. felipei, and M. frenata*). A notable exception is the tayra (*Eira barbara*), which likely occupies a particularly distinct niche due to its arboreal habits and more omnivorous diet (Presley, 2000). Under this scenario, after speciation *G. cuja* might have maintained the ancestral size for the genus, while *G. vittata* would have evolved larger size due to competition with these smaller mustelids. A third hypothesis would postulate that the differences in size and geographic range between *G. cuja* and *G. vittata* are induced by ecological sorting, with each species being adapted to a distinct set of environments. *G. vittata* seems to be rather restricted to humid rain forests, while *G. cuja* occurs in a much broader array of habitats (see below). Adaptation to these different environments and their constituent prey (along with competition with other carnivore species within these different habitats, as postulated in the previous hypothesis) could have acted in concert to shape the observed body-size patterns. In addition, both species might compete for resources at the boundary of their ranges, which would inhibit pervasive geographic overlap (Davies *et al*., 2007). Testing these hypotheses with diverse approaches should provide an interesting avenue of research in the future.

The size segregation between the two species corresponds very well to the occurrence of the metaconid in the lower carnassial, which is exclusive to *G. vittata*. There is no evidence that any other mustelid from the Galictinea lineage (Koepfli *et al*., 2008; Wolsan & Sato, 2010) possesses this peculiar feature (Larivière, 2001, 2002; Gorsuch & Larivière, 2005), indicating that its presence is derived. In general, due to the consumption of meat, carnivorans have developed skull modifications capable of processing the muscle and skin of their prey. These modifications include, for example, the development of blade-like teeth and the reduction or even loss of the metaconid in the first lower molar (Biknevicius & Van Valkenburgh, 1996). Among the carnivorans, some species consume not only the meat but also the bones of their prey (e.g. mustelids) and the blade-like teeth have often been replaced by a stout tooth design that helps breaking and crushing more rigid materials (Biknevicius & Van Valkenburgh, 1996). Interestingly, the greater grison carries a metaconid on m1, suggesting that this tooth is not only robust enough to process bones but is pointed and conical as well, possibly associated with consumption of meat and bones of larger preys. Given the robust appearance of its skull and the presence of the metaconid, we infer that *G. vittata* has evolved these features in response to some dietary pressure.

The geographic distribution of *G. vittata* harbors a very rich terrestrial fauna, including many potential prey items for a small carnivore. Given the range overlap with smaller mustelids of genus *Mustela* (which tend to specialize in hunting small rodents [King, 1989]), as well as some areas of likely sympatry with *G. cuja* (which mainly preys on mammals, but also includes lizards, birds and their eggs in its diet [Delibes *et al*., 2003; Zapata *et al*., 2005]), we hypothesize that *G. vittata* has evolved these features as adaptations to prey on larger items, which minimizes niche overlap with those other species. This hypothesis could be addressed with detailed studies focusing on the trophic ecology of these species, especially *G. vittata*, for which no such analysis has been found. Likewise, additional ecological studies targeting *G. cuja* are required, especially in its areas of sympatry with *G. vittata* in the north and the Patagonian weasel (*Lyncodon patagonicus*) in the south. Given its smaller size than the former, and larger size than the latter, it could be hypothesized that it may exhibit an intermediate range of prey sizes, with opposite pressures for character displacement acting in areas of sympatry with each of these other mustelids.

Another aspect that could be explored in future evolutionary studies targeting *Galictis* is coat color variation. *G. cuja,* which usually exhibits yellowish and dense fur, occurs mainly in open and drier landscapes, such as the barren Caatinga and the Cerrado in northeastern Brazil, the savannas and the grasslands throughout Argentina and the dry coast of Uruguay. It could be hypothesized that the yellowish fur provides an amber appearance that could be favored as camouflage in such landscapes. However, *G. cuja* also occupies forests, and adaptation to different habitats might have led to its observed variation in coat color. Additionally, this species often reaches high altitudes (Osgood, 1943; Greer, 1966; Lucherini M., Tellaeche C., Reppucci J., Luengos Vidal E., unpubl. data) and latitudes (Quintana, Yañez & Valdebenito, 2000; Parera, 2002), which may have led historically to selective pressures for denser pelage. In contrast, *G. vittata* exhibits more constant skin color and density. The mixture between black and white fur, producing a pale gray pelage, might be favored in dense vegetation and darker landscapes, such as tropical forests, the main type of biome occupied by *G. vittata*. Additionally, the shorter and sparser fur in this species is likely an adaptation to the warm temperatures that are prevalent throughout its geographic range (see Fig. 8). As in the case of the size-based differences, in-depth ecological work is required to test these hypotheses, so as to shed light onto the evolutionary processes that have shaped these phenotypes. As the first step towards this goal would be to robustly delimit and diagnose these species, as well as to better define their geographic range, the present study should contribute to establish such baseline aspects, and to

identify patterns that have the potential to spur additional research on behalf of these little-known carnivorans.

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Figure Legends:

Figure 1. First and second lower molars from (**A**) *Galictis vittata* and (**B**) *G. cuja* at lingual view, emphasizing the presence of the metaconid (indicated by an arrow) in the former species. The metaconid constituted a diagnostic character to distinguish the two *Galictis* species (see main text for details).

Figure 2. Principal Component Analysis (PCA) projection based on scores from the first (**PC1**) and second (**PC2**) principal components for 15 craniodental measurements (see Table 2 for details) from specimens of genus *Galictis*: *G. cuja* (black circles) and *G. vittata* (white diamonds). **A:** male data set**; B:** female data set.

Figure 3. Multiple-group Discriminant Function Analysis (DFA) plot based on scores from the first (**CV1**) and second (**CV2**) canonical variates for 15 craniodental measurements (see Table 2 for details) from specimens of genus *Galictis*: *G. cuja*, males (black circles); *G. cuja*, females (white circles); *G. vittata*, males (black diamonds), and *G. vittata*, females (white diamonds).

Figure 4. Representative skull views (dorsal, ventral, and lateral skull views and dorsal and lateral mandible views) from *Galictis* specimens, emphasizing size differences among the groups:

(**A**) *G. vittata* male, LSUMNS 2443; (**B**) *G. cuja* male, MVZ 114774; (**C**) *G. vittata* female, USNM 180224; and (**D**) *G. cuja* female, LSUMNS 16948. Scale bar: 10 mm.

Figure 5. Dorsal view from representative skins for the two *Galictis* species, emphasizing size and general appearance differences between the groups: (**A**) *G. vittata* and (**B**) *G. cuja*. **A:** AMNH 76630, adult female from Peru. **B:** AMNH 38983, adult female from Bolivia. Scale bar: 10 cm.

Figure 6. Maximum likelihood (ML) phylogenies depicting the evolutionary relationships among *Galictis cuja* (bGcu or Gcu sample IDs) and *G. vittata* (Gvi sample IDs) individuals (see Supporting Information S1 for more details on sample IDs). **A:** mitochondrial DNA (*ND5* gene segment) data set; **B:** concatenated nuclear data set containing 12 different segments (see Table 1 for more information on nuclear segments).Values at internodes indicate support based on ML/MP/NJ/BI methods, respectively (Bayesian posterior probabilities are indicated as percentages). Support is represented only for major nodes. See text for additional details.

Figure 7. Representative haplotype network for one nuclear gene segment (*RAG1*). Each rectangle represents a distinct haplotype. Haplotypes sampled only in *Galictis cuja* are depicted in grey, while that sampled only in *G. vittata* is shown in white. The number of copies sampled for each haplotype is indicated inside the respective rectangle. Hatches across branches indicate mutational steps. The arrow indicates the position of the root, based on comparison with two outgroup species (*Poecilogale albinucha* and *Ictonyx striatus*). Haplotype networks for the remaining 11 nuclear segments, as well as the underlying sequence information, are given in Supporting Information S5.

Figure 8. Map showing the distribution of *Galictis vittata* (red squares) and *G. cuja* (black circles) based on the geographic origin of individuals with ascertained species-level identification. All *G. vittata* records are based on skulls and skins. *G. cuja* records based on skulls and skins are indicated by black circles with a white border, while those based on DNA samples are depicted as fully black circles. The distributional points are overlaid on a map of Neotropical biomes (defined according to Olson *et al*., 2001) to allow a visual assessment of the species' ranges. Records indicate distinct locations where specimens were found (i.e. repeated coordinates were collapsed into a single point). See text for additional details.

Table 1: Gene symbol and name, primer sequences (forward and reverse), description of gene segments and literature source for each nuclear segment used in the study.

Gene	Gene name	Forward primer	Reverse primer	Region	Reference
AAMP2	Angio-associated, migratory cell protein	AGCTGCTCTTTGAGTGTGC	CAGCACAAGTAACAGAGTC	exon-intron	Väli *et al*., 2008
ADORA3	A3 adenosine receptor	ACCCCCATGTTTGGCTGGAA	GATAGGGTTCATCATGGAGTT	exon	Murphy *et al*., 2001
APOB	Apolipoprotein B	GTGCCAGGTTCAATCAGTATAAGT	CCAGCAAAATTTTCTTTTACTTCAA	exon	Amrine-Madsen *et al*., 2003
GNAT1	Rod transducin alpha-subunit	AGCACCATCGTCAAGCAGA	CTGGATACCCGAGTCCTTC	exon-intron	Brouillette, Andrew & Venta, 2000
JAK1	Janus kinase 1	GATCTCTTCATGCACCGGAA	CATTTCCATGGACCAGGTCTT	exon-intron	Housley *et al*., 2006
MACF1	Microtubule-actin crosslinking factor 1	CCATCTGCTGAGTATAAAGTGGTGAA	GCCTCCTTCTGCTTGAAGCA	exon-intron	Housley *et al*., 2006
PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	CAGAGAACGACGGTCACTGAT	GGTCATTACAAATGGACTCAATGA	exon-intron	Housley *et al*., 2006
PTPN4	Protein tyrosine phosphatase, non-receptor type 4	CCAGTATTTTTTGCAAATTAAACAAGA	AGGAATGAAAGAATAATCTGAGAGGT	exon-intron	Housley *et al*., 2006
RAG1	Recombination activating protein 1	GCTTTGATGGACATGGAAGAAGACAT	GAGCCATCCCTCTCAATAATTTCAGG	exon	Teeling *et al*., 2000
RHO1	Rhodopsin	TACATGTTCGTGGTCCACTT	TGGTGGGTGAAGATGTAGAA	exon-intron	Venta *et al*., 1996
TRHDE	Thyrotropin-releasing hormone degrading enzyme	CTGGATGAGGATGTCTGGGA	TGAAAAACTTCCAGGCAAGGTC	exon-intron	Housley *et al*., 2006
WT1	Wilms tumor 1	GAGAAACCATACCAGTGTGA	GTTTTACCTGTATGAGTCCT	exon-intron	Venta *et al*., 1996

Table 2. Summary of results from the Principal Component Analysis (PCA) and multiple-group Discriminant Function Analysis (DFA): male and female factor loading for each craniodental measurement, eigenvalue and cumulative variance of the first two principal components in the PCA (represented in Figure 2), and discriminant loadings for each craniodental measurements, Chi-square statistics, canonical correlation, eigenvalue and cumulative variance of the first two canonical variates in the DFA (represented in Figure 3). Values highlighted in bold represent those with greater contribution to the first principal component and first canonical variate. See main text for variable abbreviations.

PCA					**Multiple-group DFA**		
	Males		Females				
Variable	**PC1**	**PC2**	**PC1**	**PC2**	**Variable**	**CV1**	**CV2**
GLS	**0.977**	-0.059	**0.982**	-0.064	GLS	-0.328	-0.111
NL	0.808	0.212	0.817	-0.357	NL	0.242	0.127
ZB	**0.969**	-0.117	**0.983**	-0.035	ZB	0.024	0.489
MB	**0.969**	-0.132	0.979	-0.065	MB	**0.673**	-0.516
BB	0.918	0.177	0.962	0.139	BB	**0.807**	-0.936
IC	0.928	-0.136	0.967	0.014	IC	0.453	-0.243
PC	0.746	0.622	0.771	0.580	PC	-0.361	0.537
PW	0.880	0.094	0.844	0.159	PW	-0.415	-0.470
BH	0.932	0.095	0.949	0.106	BH	-0.076	0.045
ML	**0.972**	-0.085	**0.983**	-0.091	ML	-0.437	0.515
MH	0.882	-0.096	0.955	-0.037	MH	-0.609	0.985
C-M2	0.939	-0.016	0.960	-0.067	C-M2	0.019	-0.220
C-C	0.959	-0.190	0.973	-0.096	C-C	-0.512	0.319
M2-M2	0.961	-0.076	0.978	-0.019	M2-M2	0.269	0.154
c-m2	**0.969**	-0.111	**0.984**	-0.077	c-m2	**1.045**	-0.083
					Wilks' lambda	0.052	0.504
					Chi-square statistic	318.684	73.575
					d.f.	45	28
					P-value	0.000*	0.000*
					Canonical correlation	0.947	0.657
Eigenvalue	12.77	0.60	13.29	0.56	Eigenvalue	8.778	0.760
Cumulative variance (%)	85.15	89.19	88.64	92.38	Cumulative variance (%)	90.8	98.7

Table 3. Equality test of group mean results for two-group (males and females analyzed separately) and multiple-group Discriminant Function Analysis (DFA): independent contribution (differentiation power) of each variable to discriminate the groups (see main text for variable abbreviations). Wilk's lambda; F= F value; *P*-value= significance. Asterisks indicate morphometric variables that are statistically significant between the groups.

	Two-group DFA						Multiple-group DFA		
	Males			Females					
Variable	**Wilks' lambda**	**F**	***P*-value**	**Wilks' lambda**	**F**	***P*-value**	**Wilks' lambda**	**F**	***P*-value**
GLS	0.338	113.413	0.000*	0.171	270.764	0.000*	0.226	130.359	0.000*
NL	0.616	36.111	0.000*	0.416	78.666	0.000*	0.464	43.929	0.000*
ZB	0.356	104.882	0.000*	0.161	292.247	0.000*	0.233	125.126	0.000*
MB	0.289	142.597	0.000*	0.166	281.393	0.000*	0.209	143.734	0.000*
BB	0.300	135.408	0.000*	0.173	267.036	0.000*	0.229	127.746	0.000*
IC	0.256	168.879	0.000*	0.187	243.061	0.000*	0.216	137.752	0.000*
PC	0.665	29.197	0.000*	0.575	41.318	0.000*	0.604	24.902	0.000*
PW	0.589	40.441	0.000*	0.461	65.578	0.000*	0.518	35.323	0.000*
BH	0.339	113.105	0.000*	0.287	139.093	0.000*	0.296	90.212	0.000*
ML	0.385	92.564	0.000*	0.167	280.263	0.000*	0.238	121.628	0.000*
MH	0.649	31.319	0.000*	0.257	161.867	0.000*	0.363	66.654	0.000*
C-M2	0.331	117.029	0.000*	0.229	189.052	0.000*	0.261	107.477	0.000*
C-C	0.308	130.511	0.000*	0.226	191.826	0.000*	0.243	118.482	0.000*
M2-M2	0.319	123.720	0.000*	0.154	306.761	0.000*	0.218	136.472	0.000*
c-m2	0.219	207.258	0.000*	0.149	319.806	0.000*	0.172	183.571	0.000*

Table 4. Descriptive statistics (mean, standard deviation, range, and sample size) for 15 craniodental measurements and one external variable comparing *Galictis cuja* and *G. vittata*, with males and females treated separately (measurements in mm). See main text for variable abbreviations. Minimum and maximum values in bold indicate variables whose range does not overlap between the species.

Variable	*G. cuja* ♂	*G. vittata* ♂	*G. cuja* ♀	*G. vittata* ♀
GLS	76.28 ± 4.03 64.93 – 83.52 N= 60	88.80 ± 3.93 81.85 – 94.76 N= 19	69.50 ± 3.49 **63.40 – 77.14** N= 44	85.44 ± 3.12 **79.10 – 92.90** N= 26
NL	21.45 ± 1.76 17.20 – 26.47 N= 67	24.74 ± 1.22 22.81 – 27.41 N= 22	19.83 ± 1.36 17.40 – 23.63 N= 43	23.96 ± 1.48 21.13 – 27.76 N= 31
ZB	43.02 ± 3.12 35.0 – 50.09 N= 60	52.87 ± 2.86 47.18 – 56.81 N= 20	39.21 ± 2.18 **34.16 – 44.04** N= 44	50.13 ± 2.40 **44.72 – 54.50** N= 31
MB	39.76 ± 2.75 32.52 – 46.83 N= 63	50.04 ± 2.47 44.80 – 54.36 N= 20	35.62 ± 2.33 31.28 – 41.74 N= 43	46.89 ± 2.61 41.61 – 52.29 N= 31
BB	34.68 ± 1.83 **30.34 – 37.98** N= 63	40.68 ± 1.20 **38.34 – 43.05** N= 21	33.10 ± 1.54 **29.70 – 36.51** N= 45	39.70 ± 1.39 **37.34 – 42.37** N= 31
IC	16.55 ± 1.30 13.20 – 19.50 N= 66	21.88 ± 1.62 18.50 – 26.98 N= 22	15.26 ± 1.21 **13.0 – 18.17** N= 44	20.29 ± 0.97 **18.41 – 21.90** N= 31
PC	17.72 ± 1.33 14.39 – 21.66 N= 64	19.92 ± 0.76 18.81 – 21.36 N= 21	16.83 ± 1.40 12.96 – 19.76 N= 45	19.28 ± 0.98 17.81 – 20.88 N= 31
PW	11.68 ± 0.96 9.51 – 13.50 N= 65	13.58 ± 0.90 12.05 – 15.60 N= 22	11.07 ± 0.87 9.62 – 12.73 N= 46	13.05 ± 0.77 10.77 – 14.71 N= 31
BH	25.43 ± 1.69 21.91 – 28.94 N= 63	30.73 ± 1.15 28.81 – 33.14 N= 20	23.17 ± 2.08 18.67 – 29.35 N= 44	29.06 ± 1.30 26.90 – 32.23 N= 30
ML	44.79 ± 2.98 36.73 – 51.70 N= 59	54.11 ± 2.50 48.74 – 57.80 N= 20	40.32 ± 2.22 **36.02 – 45.35** N= 39	50.83 ± 2.63 **46.76 – 58.38** N= 28
MH	21.80 ± 1.84 17.16 – 25.99 N= 72	25.10 ± 1.95 20.47 – 27.94 N= 21	19.05 ± 1.58 16.02 – 22.77 N= 47	23.62 ± 1.20 21.27 – 26.65 N= 31

	20.98 ± 1.50	25.94 ± 1.14	19.08 ± 1.66	24.44 ± 1.11
C-M2	17.22 – 27.96	23.82 – 27.56	16.20 – 25.90	22.01 – 26.86
	N= 69	N= 22	N= 47	N= 31
	16.75 ± 1.41	21.23 ± 1.42	14.91 ± 1.29	19.41 ± 1.24
C-C	13.30 – 21.49	17.40 – 23.47	12.30 – 17.91	17.03 – 23.03
	N= 67	N= 22	N= 47	N= 30
	23.97 ± 1.64	29.76 ± 1.29	21.90 ± 1.55	28.11 ± 1.16
M2-M2	20.02 – 27.91	27.81 – 31.51	19.64 – 27.55	26.20 – 30.51
	N= 67	N= 22	N= 45	N= 31
	26.14 ± 1.52	33.24 ± 1.49	23.74 ± 1.56	30.86 ± 1.50
c-m2	**21.71 – 29.18**	**30.30 – 35.72**	**20.0 – 27.40**	**28.51 – 35.34**
	N= 69	N= 21	N= 46	N= 30
	601.66 ± 40.62	722.16 ± 19.18	531.36 ± 55.67	658.12 ± 38.62
TL	**525.0 – 657.0**	**700.0 – 755.0**	443.0 – 645.0	600.0 – 706.0
	N= 12	N= 6	N= 11	N= 8

Table 5. Male and female results of the *t*-test comparison between *Galictis cuja* and *G. vittata* based on the mean of 15 craniodental measurements and the external variable (see main text for variable abbreviations). t= t value; d.f.= degrees of freedom; *P*-value= significance. Results emphasized by asterisk represent those that are statistically significant after sequential Bonferroni correction.

	Males			**Females**		
Variable	**t**	**d.f.**	***P*-value**	**t**	**d.f.**	***P*-value**
GLS	-11.862	77	0.000*	-19.153	68	0.000*
NL	-8.099	87	0.000*	-12.375	72	0.000*
ZB	-12.464	78	0.000*	-20.458	73	0.000*
MB	-14.868	81	0.000*	-19.488	72	0.000*
BB	-13.991	82	0.000*	-19.024	74	0.000*
IC	-15.595	86	0.000*	-19.097	73	0.000*
PC	-7.141	83	0.000*	-8.373	74	0.000*
PW	-8.119	85	0.000*	-10.137	75	0.000*
BH	-13.035	81	0.000*	-13.704	72	0.000*
ML	-12.549	77	0.000*	-17.638	65	0.000*
MH	-7.146	91	0.000*	-13.637	76	0.000*
C-M2	-14.144	89	0.000*	-15.711	76	0.000*
C-C	-12.882	87	0.000*	-15.078	75	0.000*
M2-M2	-15.062	87	0.000*	-18.876	74	0.000*
c-m2	-18.751	88	0.000*	-19.746	74	0.000*
TL	-6.817	16	0.000*	-5.526	17	0.000*

Table 6. Summary of non-metric (skull and external aspects) and metric (craniodental and external measurements) morphological features that distinguish *Galictis cuja* from *G. vittata*. Six linear measurements were of major importance to diagnose these species (see main text for additional details), but in this table we only include those that show no overlap between the two species.

Character	***Galictis cuja***	***Galictis vittata***
Metaconid	Absent	Present
Dominant pelage color	Usually yellowish	Grayish
General pelage appearance	Dense/long fur	Short fur
Greater length of skull	♀ 63.4 – 77.1 mm	♀ 79.1 – 92.9 mm
Zygomatic breath	♀ 34.1 – 44.0 mm	♀ 44.7 – 54.5 mm
Braincase breath	♂ 30.3 – 37.9 mm ♀ 29.7 – 36.5 mm	♂ 38.3 – 43.0 mm ♀ 37.3 – 42.3 mm
Mandible length	♀ 36.0 – 45.3 mm	♀ 46.7 – 58.3 mm
Length of mandible toothrow	♂ 21.7 – 29.1 mm ♀ 20 – 27.4 mm	♂ 30.3 – 35.7 mm ♀ 28.5 – 35.3 mm
Total length of the body	♂ 525 – 657 mm	♂ 700 – 755 mm

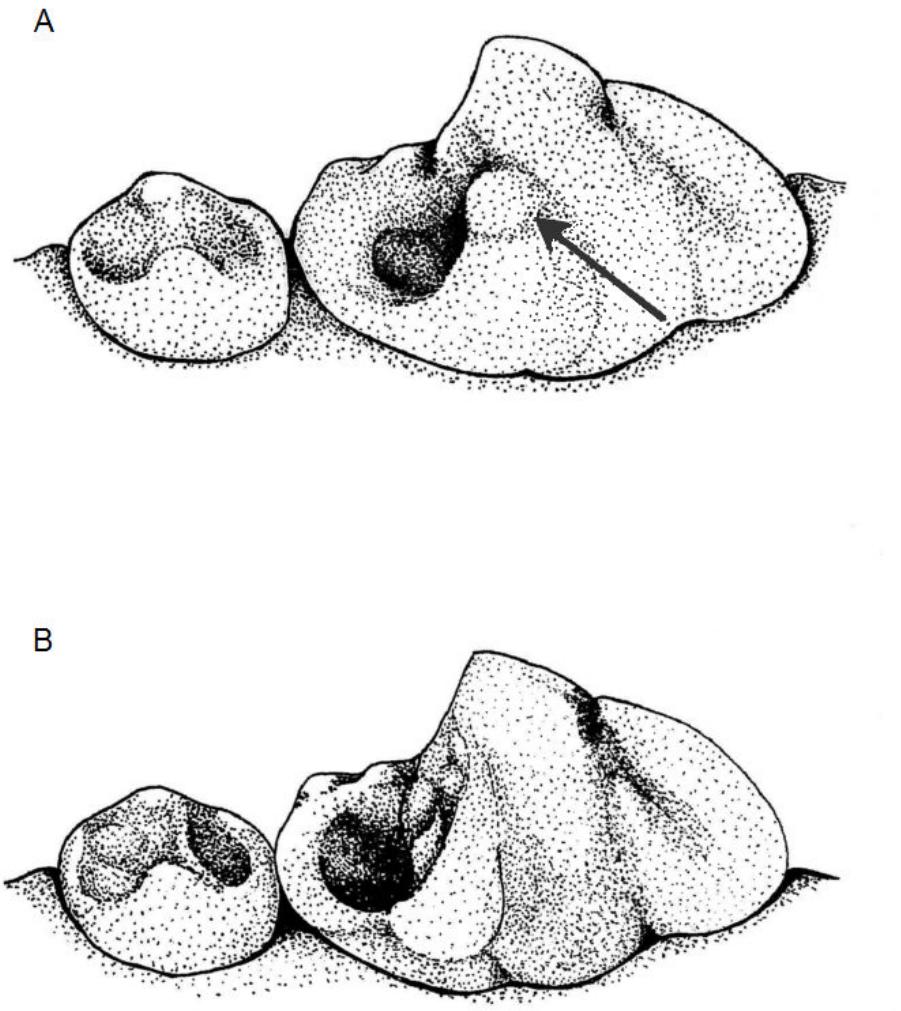

Figure 1

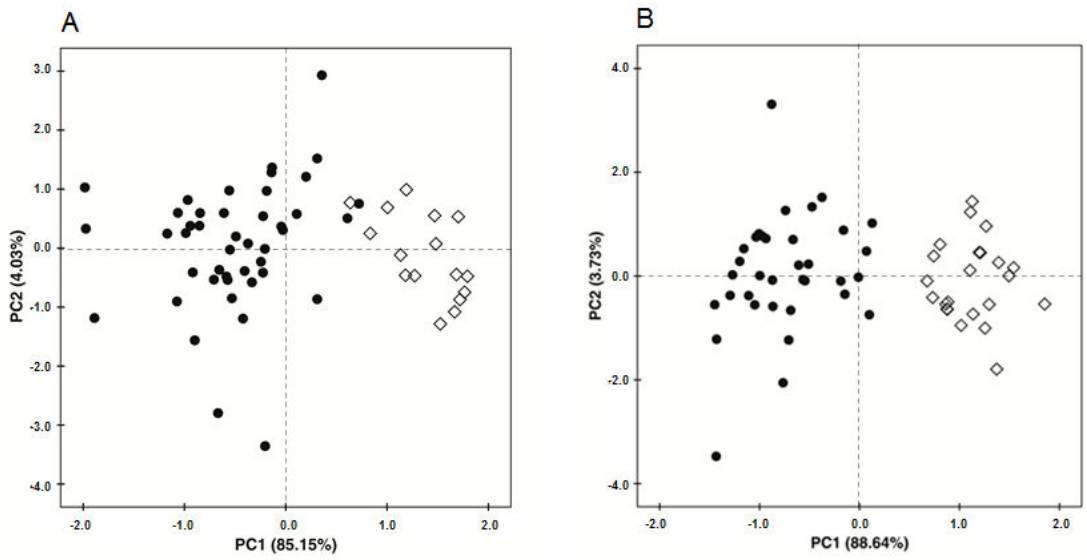

Figure 2

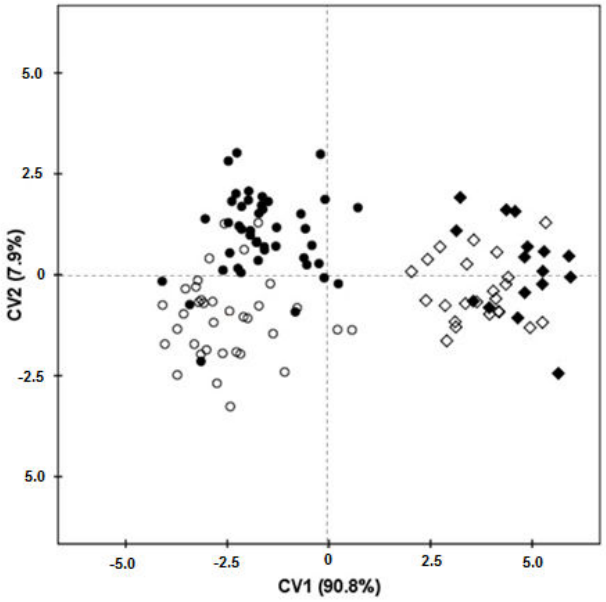

Figure 3

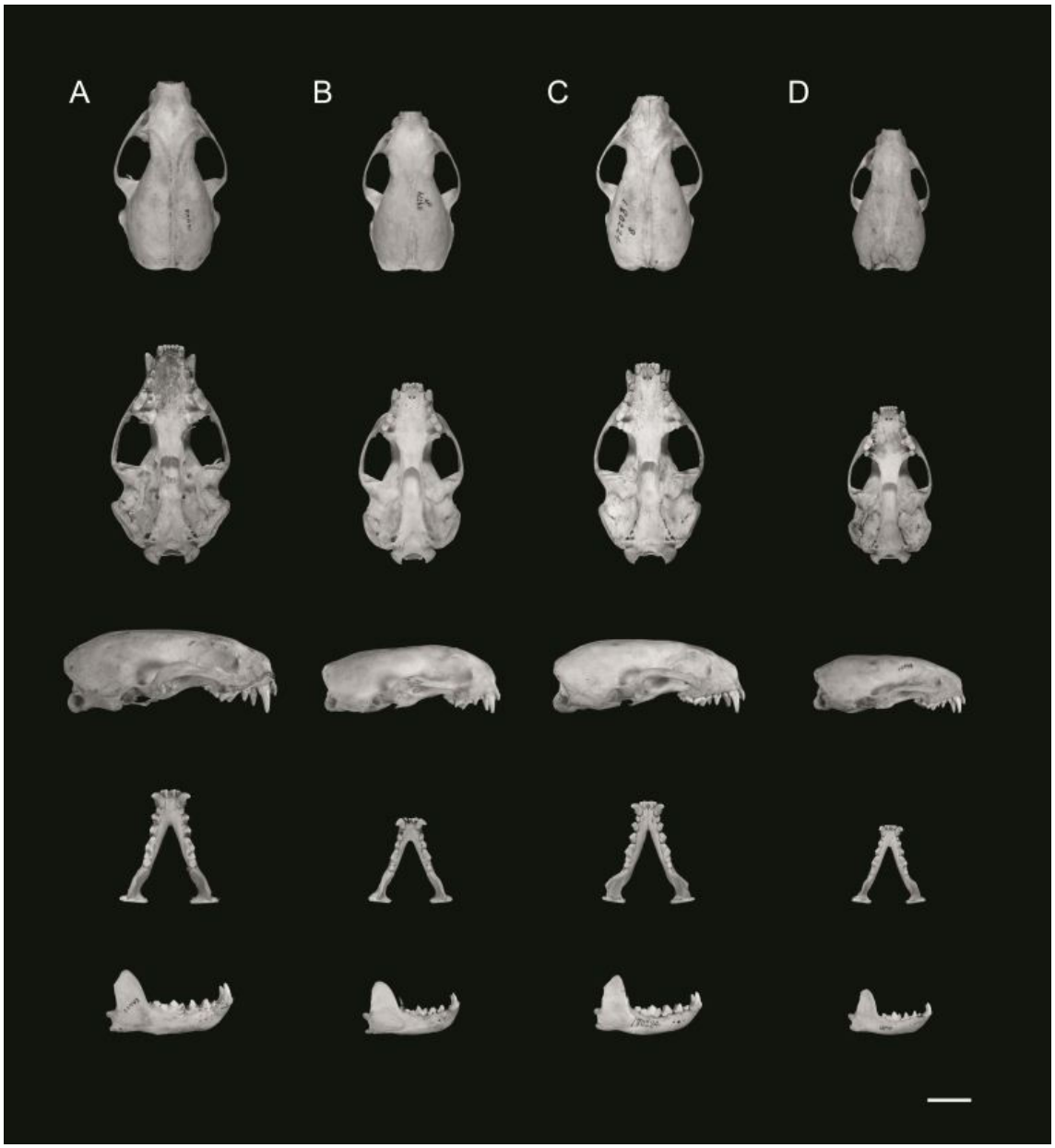

Figure 4

Figure 5

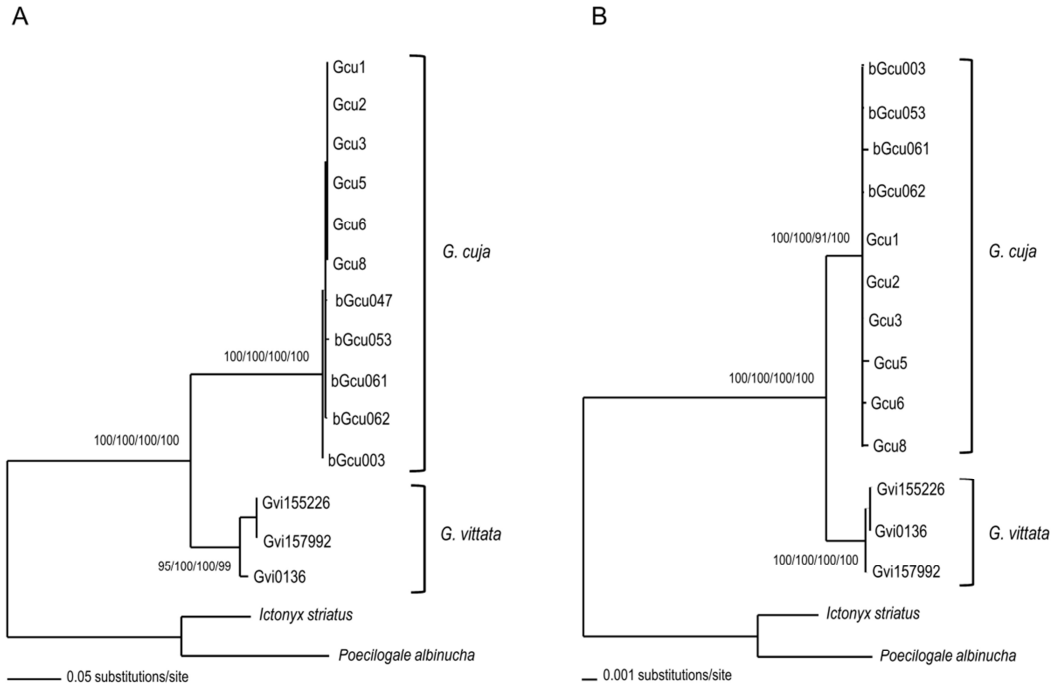

Figure 6

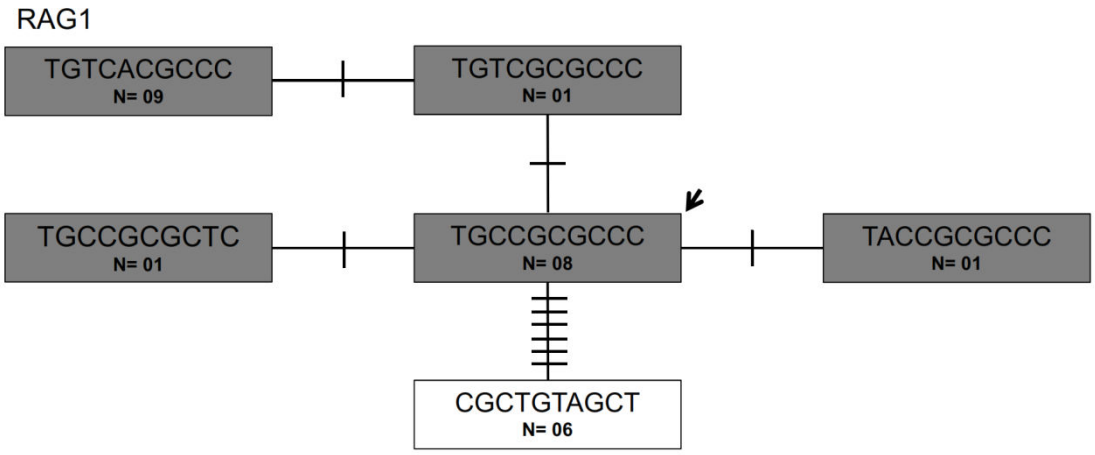

Figure 7

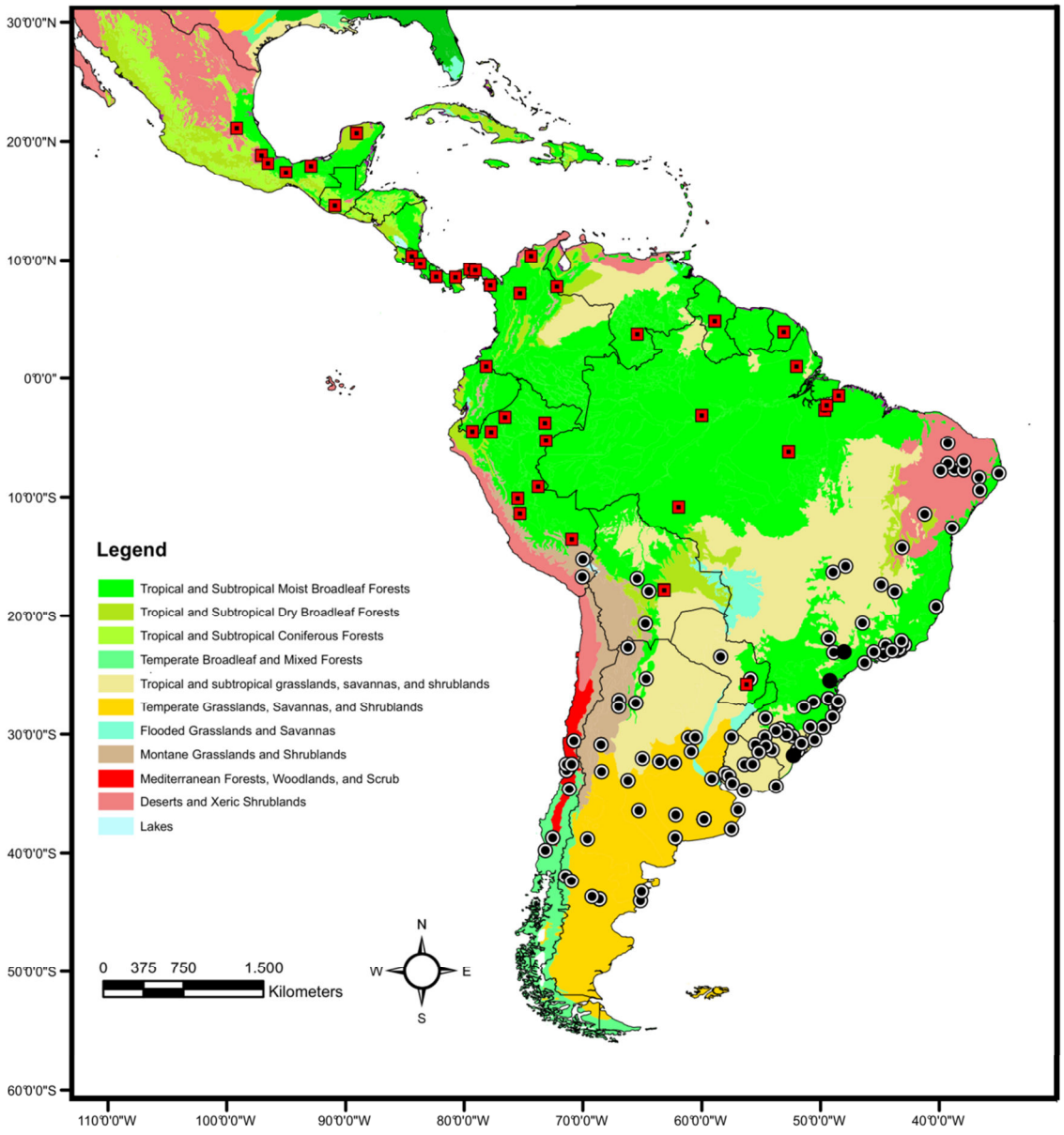
30°0'0"N
20°0'0"N
10°0'0"N
0°0'0"
10°0'0"S
20°0'0"S
30°0'0"S
40°0'0"S
50°0'0"S
60°0'0"S
110°0'0"W
100°0'0"W
90°0'0"W
80°0'0"W
70°0'0"W
60°0'0"W
50°0'0"W
40°0'0"W
Legend
Tropical and Subtropical Moist Broadleaf Forests
Tropical and Subtropical Dry Broadleaf Forests
Tropical and Subtropical Coniferous Forests
Temperate Broadleaf and Mixed Forests
Tropical and subtropical grasslands, savannas, and shrublands
Temperate Grasslands, Savannas, and Shrublands
Flooded Grasslands and Savannas
Montane Grasslands and Shrublands
Mediterranean Forests, Woodlands, and Scrub
Deserts and Xeric Shrublands
Lakes
0 375 750 1.500
Kilometers
N
W
E
S

Figure 8

SUPPORTING INFORMATION S1. *Galictis* specimens used in the morphological, genetic and geographic analyses listed per species per institution.

Species assignment is based on the diagnostic morphological criteria described in the main text. Abbreviations: m = male; f = female; nd = no data on gender.

Note: Individuals marked with an asterisk indicate specimens bearing no detailed geographic origin, which were thus included in the morphometric analysis but not in the geographic assessment (see text).

Galictis cuja (n= 197: m= 78; f= 62; nd= 57):

MCT-PUCRS: nd – 0177 (30°27'S, 50°31'W).

FZB/RS: m – 2660 (31°19'S, 54°06'W), 3034 (30°56'S, 54°45'W), 3035 (30°53'S, 54°51'W), 3065 (29°25'S, 49°48'W); **f –** 2765 (31°19'S, 54°06'W), 3066*; **nd –** 2512 (29°19'S, 49°43'W).

ULBRA: m – 73 (30°10'S, 52°22'W), 483 (30°01'S, 52°53'W), 627 (28°37'S, 54°40'W), 748 (30°13'S, 54°43'W), 899 (30°52'S, 55°31'W), 928 (30°59'S, 54°37'W); **f –** 31 (29°32'S, 53°23'W), 71 (30°13'S, 57°32'W), 746 (29°40'S, 53°47'), 749 (30°45'S, 51°38'W), 757 (31°19'S, 54°06'W).

LAMAq-UFSC: m – 399 (27°14'S, 48°37'W), 3182 (27°09'S, 48°32'W); **f –** 785 (28°30'S, 49°01'W); **nd –** 746 (27°40'S, 48°46'W), 870 (26°55', 49°21'W).

MZUSP: m – 1044 (31°22'S, 51°58'W), 1247 (22°06'S, 43°12'W), 3066 (17°21'S, 44°55'W), 6463 (21°54'S, 49°21'W), 8454 (7°01'S, 37°58'W), 9633 (23°56'S, 46°19'W), 10331 (23°04'S, 48°55'W), 13468 (12°35'S, 38°58'W); **f –** 227 (31°22'S, 51°58'W), 978 (21°54'S, 49°21'W); **nd –** 230 (31°22'S, 51°58'W), 10193 (21°54'S, 49°21'W).

MNHN: **m –** 1882 (22°54'S, 43°33'W), 3114 (37°12'S, 59°50'W), 4845 (16°19'S, 48°58'W), 25684 (7°12S, 39°19'W), 29981 (8°22'S, 36°42'W), 29985 (22°45'S, 43°26'W), 29998 (7°46'S, 39°55'W), 29999 (7°43'S, 38°0'W), 30001 (20°37'S, 46°30'W); **f –** 1498 (7°40'S, 38°45'W), 3127 (22°30'S, 44°34'W), 5809 (22°47'S, 43°18'W), 8236 (23°13'S, 44°43'W), 8238 (23°13'S, 44°43'W), 29983 (11°24'S, 41°16'W), 29984 (23°01'S, 45°32'W), 29988 (9°25'S, 36°37'W); **nd –** 3129 (22°25'S, 42°58'W), 3131 (29°32'S, 53°23'W), 7258 (22°25'S, 42°58'W), 29986 (5°29'S, 39°19'W).

UFPE: f – 977 (8°00'S, 35°02'W).

MPEG: m – 22188 (27°40'S, 51°25'W); **f –** 538*, 22229 (30°01'S, 52°53'W), 22230 (29°23'S, 50°53'W); **nd –** 22027 (14°16'S, 43°10'W).

MNHNA: m – 2333 (34°44'S, 56°27'W), 2690 (34°08'S, 57°27'W), 2696 (34°44'S, 56°27'W); **f –** 296 (33°30'S, 57°44'W), 1158 (32°32'S, 56°26'W), 2548 (32°31'S, 55°45'W); **nd –** 6412 (34°23'S, 53°46'W), 6433 (31°28'S, 55°13'W).

MACN: m – 3095 (27°19'S, 65°34'W), 3096 (27°19'S, 65°34'W), 16520 (42°22'S, 71°01'W); **f –** 13498 (38°49'S, 69°40'W), 13939 (43°41'S, 69°16'W); **nd –** 284 (37°12'S, 59°50'W), 2680 (33°52'S, 66°14'W), 2875 (43°17'S, 65°05'W), 3784 (22°39'S, 66°14'W), 13075 (37°12'S, 59°50'W), 13963 (41°58'S, 71°31'W), 15584 (36°28'S, 65°19'W), 26166 (37°12'S, 59°50'W), 29191 (32°01'S, 65°02'W), 29795 (30°52'S, 68°31'W), 29929 (33°52'S, 66°14'W), 31171 (22°39'S, 66°14'W), 31200 (22°39'S, 66°14'W), 47373 (23°26'S, 58°26'W).

MLP: f – 15v97 (37°12'S, 59°50'W); **nd –** 388 (37°12'S, 59°50'W), 671 (30°14'S, 60°34'W), 674 (30°14'S, 60°34'W), 704 (30°14'S, 60°34'W), 1014*, 1588*, 1706 (33°43'W, 59°10'W), 3v994 (43°53'S, 68°40'W), 8v596 (33°08'S, 68°28'W).

USNM: m – 282248*, 35259*; **f –** 271309 (32°30'S, 71°0'W), 293164 (25°17'S, 55°56'W); **nd –** 172795 (38°0'S, 57°32'W), 172796 (37°12'S, 59°50'W), 271573*.

AMNH: m – 235992 (29°40'S, 52°47'W), 33281 (38°43'S, 72°35'W), 70339*, 80037*; **f –** 38983 (20°40'S, 64°46'W), 205832 (32°32'S, 56°26'W); **nd –** 212544 (44°02'S, 65°11'W).

ANSP: f – 841*

MCZ: m – 19219 (36°23'S, 56°58'W).

FMNH: m – 23919 (32°31'S, 71°27'W), 94316 (21°54'S, 49°21'W); **f –** 23440 (30°32'S, 70°48'W), 23441 (32°31'S, 71°27'W), 23445 (37°12'S, 59°50'W), 51882 (16°51'S, 65°28'W), 94317 (21°54'S, 49°21'W); **nd –** 23918 (32°31'S, 71°27'W), 52418 (15°14'S, 70°03'W).

YPM: nd – 7027*

MVZ: m – 114774 (16°43'S, 69°66'W), 85164 (22°56'S, 44°01'W), **nd –** 162287 (33°98'S, 70°72'W).

LSUMNS: m – 16949 (43°53'S, 68°40'W); **f –** 16947 (41°58'W, 71°31'W), 16948 (41°58'S, 71°31'W).

BMNH: m – 1223 (32°30'S, 71°0'W), 1681 (32°52'S, 71°16'W), 1682 (33°02'S, 71°26'W), 5244 (38°43'S, 72°35'W), 11152 (32°30'S, 71°0'W), 17531 (30°16'S, 61°08'W), 17532 (30°16'S, 61°08'W), 28591 (27°19'S, 65°34'W), 28592 (27°19'S, 65°34'W), 29664 (26°55'S, 49°21'W), 122176 (38°0'S, 57°32'W), 127121 (33°02'S, 71°26'W), 138164 (33°02'S, 71°26'W), 141274 (26°55'S, 49°21'W), 161034 (36°49'S, 62°13'W), 171254 (32°17'S, 63°34'W), 171255 (32°17'S, 63°34'W), 203171 (27°04'S, 66°59'W), 216191 (30°52'S, 68°31'W), 261291 (27°19'S, 65°34'W), 261292 (27°19'S, 65°34'W), 294291 (17°55'S, 43°47'W), 349254 (17°53'S, 64°28'W), 992229 (43°53'S, 68°40'W), 1110165 (38°43'S, 72°35'W), 1810116 (26°55'S, 49°21'W), 2810115 (26°55'S, 49°21'W), 3411415 (25°15'S, 64°42'W), 44376210f (17°55'S, 43°47'W); **f –** 12831*, 16626 (21°54'S, 49°21'W), 127122*, 141275 (26°55'S, 49°21'W), 161035 (37°12'S, 59°50'W), 171256 (32°22'S, 62°19W), 171257 (32°22'S, 62°19W), 1610395 (37°12'S, 59°50'W), 17533

(30°16'S, 61°08'W), 17534 (30°16'S, 61°08'W), 191216 (32°17'S, 63°34'W), 249184 (33°15'S, 58°01'W), 261061 (27°19'S, 65°34'W), 261062 (27°19'S, 65°34'W), 261251 (27°19'S, 65°34'W), 261293 (27°19'S, 65°34'W), 269132 (27°19'S, 65°34'W), 885161 (37°12'S, 59°50'W), 2512133 (27°19'S, 65°34'W), 2810117 (27°13'S, 50°37'W), 34111416 (27°38'S, 67°01'W), 88113019 (31°22'S, 51°58'W), **nd –** 12411 (31°26'S, 60°55'W), 12413 (31°26'S, 60°55'W), 12414 (31°26'S, 60°55'W), 103113 (38°43'S, 72°35'W), 111184 (32°30'S, 71°0'W), 122177 (38°0'S, 57°32'W), 961075 (38°43'S, 62°16'W), 2811141 (27°13'S, 50°37'W), 3610181 (15°49'S, 47°55'W), 88113018 (31°22'S, 51°58'W).

SMT: m – B3962 (39°47'S, 73°13'W); **nd –** B5348 (19°11'S, 40°18'W), B5424 (21°54'S, 49°21'W), B7092 (27°13'S, 50°37'W).

Galictis vittata (n= 67: m= 23; f= 36; nd= 8):

MZUSP: f – 19826 (1°27'S, 48°30'W).

MNHN: f – 29993 (0°58'N, 52°02'W); **nd –** 3093 (10°49'S, 61°58'W), 29989 (3°06'S, 60°01'W).

MPEG: m – 1139 (6°12'S, 52°42'W), 2344 (1°27'S, 48°30'W), 4222*, 6524*, 22603*, 22604*; **f –** 4221*, 5651 (6°12'S, 52°42'W), 6520*, 7230 (3°06S', 60°01'W).

MACN: f - 5091 (17°48'S, 63°10'W).

USNM: m – 159562 (20°42'N, 89°05'W), 314590 (9°10'N, 79°05'W), 395079 (451'N, 58°55'W); **f –** 180224 (7°52'N, 77°50'W), 281481 (10°24'N, 74°24'W), 307043 (7°52'N, 77°50'W), 362246 (4°51'N, 58°55'W), 395077 (4°51'N, 58°55'W), 443720 (7°46'N, 72°13'W); **nd –** 1221 (9°44'N, 83°44'W), 339878 (8°32'N, 80°46'W), 395076 (4°51'N, 58°55'W), 541323 (8°35'N, 82°23'W).

AMNH: m – 91188 (20°42'N, 89°05'W), 98571 (3°44'S, 73°14'W), 173910 (8°58'N, 79°17'W), 267055 (3°56'N, 53°07'W); **f –** 15157 (5°15'S, 73°09'W), 76630*, 95292*, 96285 (2°40'S, 49°40'W), 768556 (3°46'N, 65°26'W); **nd –** 77695 (25°43'S, 56°15'W).

MCZ: m – 20233 (8°32'N, 80°46'W); **f –** 6420 (18°51'N, 97°05'W), 27487 (9°13'N, 79°34'W), 30733 (2°15'S, 49°30'W), 43487 (7°11'N, 75°20'W).

FMNH: m – 98231 (3°15'S, 76°35'W) 123657*, 127293*; **f –** 21395 (17°45'S, 63°12'W), 64460 (14°36'N, 90°55'W).

NHMKU: m – 29994 (19°15'N, 96°34'W); **nd –** 32222 (17°25'N, 95°01'W).

MVZ: f – 155226 (4°27'S, 78°10'W), 155227 (4°27'S, 78°80'W), 157992 (4°30'S, 77°46'W).

LSUMNS: m – 6238 (18°52'N, 96°49'W), 12443*; **f –** 2769 (21°19'N, 98°58'W), 6239 (18°52'N, 96°49'W), 7632 (17°58'N, 92°55'W).

BMNH: m – 37141 (10°04'S, 75°31'W), 41248 (13°35'S, 70°58'W) sk87a*; **f –** 71128 (8°32'N, 80°46'W), 71129 (8°32'N, 80°46'W), 76154 (11°20'S, 75°20'W), 94202 (4°51'N, 58°55'W), 116723 (4°51'N, 58°55'W), 551224246*.

Galictis specimens (n= 14: *G. vittata* n= 3; *G. cuja* n= 11) used in the genetics and geographic.

Note: Individuals marked with an asterisk indicate specimens bearing no detailed geographic origin, which were thus included in the genetics analysis but not in the geographic assessment (see text).

Laboratory of Genomic Diversity (LGD): Gcu1*, Gcu2*, Gcu3*, Gcu5*, Gcu6*, Gcu8* (Argentina); Gvi155226*, Gvi157992*, Gvi136* (Peru).

PUCRS: bGcu003 (31°46'S, 52°19'W), bGcu0047 (25°25'S, 49°15'W), bGcu0053 (23°01'S, 48°04'W), bGcu061* (Minas Gerais state, Brazil), bGcu062* (Bahia state, Brazil).

SUPPORTING INFORMATION S2. Results of the *t*-test comparison for sexual size dimorphism within *Galictis cuja* and within *G. vittata*, based on 15 craniodental measurements and one external measurement (see main text for variable abbreviations). t= t value; d.f. = degrees of freedom; *P*-value = significance. Asterisks represent comparisons that are statistically significant after sequential Bonferroni correction.

	Galictis cuja			***Galictis vittata***		
Variable	**t**	**d.f.**	***P*-value**	**t**	**d.f.**	***P*-value**
GLS	-8.950	102	0.000*	-3.193	43	0.003*
NL	-5.125	108	0.000*	-2.022	51	0.048
ZB	-6.936	102	0.000*	-3.691	49	0.001*
MB	-8.066	104	0.000*	-4.296	49	0.000*
BB	-4.688	106	0.000*	-2.645	50	0.011
IC	-5.225	108	0.000*	-4.465	51	0.000*
PC	-3.365	107	0.001*	-2.491	50	0.016
PW	-3.370	109	0.001*	-2.305	51	0.025
BH	-6.147	105	0.000*	-4.658	48	0.000*
ML	-7.993	96	0.000*	-4.335	46	0.000*
MH	-8.397	117	0.000*	-3.405	50	0.001*
C-M2	-6.382	114	0.000*	-4.742	51	0.000*
C-C	-7.062	112	0.000*	-4.911	50	0.000*
M2-M2	-6.697	110	0.000*	-4.850	51	0.000*
c-m2	-8.208	113	0.000*	-5.566	49	0.000*
TL	-3.481	21	0.002*	-3.706	12	0.003*

SUPPORTING INFORMATION S3. GenBank accession numbers for previously published mitochondrial and nuclear sequences of two mustelids used as outgroups in the current study (*Ictonyx striatus* and *Poecilogale albinucha*).

	Ictonyx striatus	***Poecilogale albinucha***
ND5	EF472385.1	EF472382.1
ADOARA3	EF987512	EF987489
APOB	EF472297	EF472295
GNAT1	EF472370	EF472367
RAG1	EF472413	EF472411
RHO1	AF498225	EF472429
WT1	EF472459	EF472456

SUPPORTING INFORMATION S4. Classification coefficients from the classification analyses for each *Galictis* species (*G. cuja* and *G. vittata*) extracted from the two-group and multiple-group discriminant function analysis (DFA).

	Coefficients: Two-group DFA				**Coefficients: Multiple-group DFA**			
	Males		Females		Males		Females	
Variable	***Galictis cuja***	***Galictis vittata***	***Galictis cuja***	***Galictis vittata***	***Galictis cuja***	***Galictis cuja***	***Galictis vittata***	***Galictis vittata***
GLS	11544.948	11373.248	13781.381	13666.509	11437.827	11463.365	11352.827	11359.133
NL	-24.984	35.820	1439.165	1479.254	511.802	496.344	541.428	549.129
ZB	-895.849	-1018.679	-2479.309	-2448.312	-778.835	-817.856	-795.487	-794.023
MB	133.101	357.538	2393.709	2516.638	1050.633	1067.879	1217.737	1199.515
BB	2299.781	2591.005	6868.670	7201.516	3907.010	3962.694	4179.744	4212.676
IC	102.286	201.0	-216.492	-114.859	122.298	128.679	232.883	201.734
PC	-848.861	-970.995	-466.118	-532.198	-773.224	-795.117	-846.307	-870.242
PW	-287.748	-330.613	185.223	91.269	-102.389	-62.790	-163.805	-150.198
BH	550.657	598.806	-1803.808	-1896.980	-628.769	-630.880	-651.413	-641.047
ML	-737.205	-912.367	-1687.771	-1611.183	-1607.459	-1638.298	-1750.505	-1712.564
MH	-3410.086	-3582.308	-2878.950	-2951.915	-3007.010	-3053.044	-3152.737	-3141.092
C-M2	-1863.927	-1804.429	-1812.673	-1834.458	-1377.193	-1377.459	-1382.809	-1377.193
C-C	-1078.630	-1142.557	-3305.335	-3445.203	-1738.886	-1739.711	-1823.268	-1852.254
M2-M2	1419.897	1476.976	570.393	684.819	642.446	617.218	680.140	700.693
c-m2	-853.371	-512.045	-763.633	-610.647	-721.677	-745.914	-429.760	-508.185
Constant	-7331.975	-7752.211	-10753.199	-11376.072	-7999.708	-7934.320	-8447.762	-8416.592

In order to classify a *Galictis* specimen at species level it is necessary to calculate the classification function and group the specimen according to the highest classification scores (as in the example described below). When the gender of a specimen is known (the case explored by the two-group discriminant function analysis), it is necessary to calculate the classification function using the first four columns of the table above (according to the gender). The highest score will indicate with 100% accuracy the group (species) that the specimen belongs to. On the other hand, if the gender of the *Galictis* specimen is unknown (the case explored by the multiple-group discriminant function analysis) one may use the last four columns of the same table. In this case, the highest score will have an 88.1% probability of correctly indicating the group (species and gender) in which the specimen fits.

General classification function=

Classification function coefficient from Variable 1(Variable1) + Classification function coefficient from Variable 2(Variable2) + Classification function coefficient from Variable 3(Variable3) + Constant.

Example: classification analysis of a male *Galictis* specimen.

Score for *Galictis cuja* ♂=

11544.948(GLS) - 24.984(NL) - 895.849(ZB) + 133.101(MB) + 2299.781(BB) + 102.286(IC) - 848.861(PC) - 287.748(PW) + 550.657(BH) - 737.205(ML) - 3410.086(MH) - 1863.927(C-M2) - 1078.630(C-C) + 1419.897(M2-M2) - 853.371(c-m2) - 7331.975.

Score for Galictis vittata ♂=

11373.248(GLS) + 35.820(NL) - 1018.679(ZB) + 357.538(MB) + 2591.005 (BB) + 201.0 (IC) - 970.995(PC) - 330.613(PW) + 598.806 (BH) - 912.367(ML) - 3582.308(MH) - 1804.429(C-M2) - 1142.557(C-C) + 1476.976(M2-M2) - 512.045(c-m2) - 7752.211.

The highest of these two scores will indicate the species in which this male specimen belongs.

SUPPORTING INFORMATION S5.

A) Table depicting the variable sites found within and between *Galictis cuja* and *G. vittata* in 12 nuclear gene segments. Nucleotide positions (vertical notation) correspond to numbering in the concatenated alignment (7066 nucleotides total length). Columns shaded gray are sites that show fixed differences between *G. cuja* and *G. vittata*. Nucleotide positions shown for *Ictonyx striatus* and *Poecilogale albinucha* are only those that are variable in *Galictis*; many additional sites are distinct between these species and *Galictis*, which are not shown. "."= nucleotide identical with the top (reference) sequence; R= A/G; Y= C/T; W= A/T; "-"= deletion; "?" = missing sequence.

	AAMP2			***ADORA 3***		***APOB***					***GNAT1***								
	0	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**2**
	0	**3**	**5**	**5**	**7**	**7**	**1**	**1**	**1**	**3**	**4**	**7**	**7**	**9**	**9**	**9**	**0**	**0**	**1**
	4	**4**	**1**	**6**	**2**	**8**	**3**	**8**	**9**	**3**	**6**	**3**	**4**	**0**	**7**	**9**	**3**	**9**	**1**
	8	**0**	**4**	**8**	**0**	**6**	**2**	**2**	**3**	**1**	**4**	**5**	**5**	**8**	**7**	**4**	**2**	**7**	**5**
Galictis cuja 003	T	A	C	A	Y	G	C	T	A	C	G	T	C	G	A	G	C	T	G
Galictis cuja 053	.	.	.	.	C	.	.	.	.	.	R	.	.	.	.	.	A	.	.
Galictis cuja 061	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	A	A	.	C
Galictis cuja 062	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Galictis cuja 1	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Galictis cuja 2	.	.	.	.	C	.	.	.	.	.	.	-	.	.	.	.	A	.	.
Galictis cuja 3	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	A	.	.
Galictis cuja 5	?	?	?	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Galictis cuja 6	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Galictis cuja 8	.	.	.	.	T	.	.	.	.	.	.	-	.	.	.	.	A	.	.
Galictis vittata 155226 Peru	C	G	.	G	C	A	A	.	G	T	.	.	.	C	G	.	A	C	.
Galictis vittata 157992 Peru	C	G	.	G	C	A	A	.	G	T	.	.	Y	C	G	.	A	C	.
Galictis vittata 0136 CPT	C	G	Y	G	C	A	A	.	G	T	.	.	.	C	G	.	A	C	.
Ictonyx striatus 880085	C	G	.	.	C	.	A	.	.	T	.	.	.	C	G	.	A	C	.
Poecilogale alibinucha 6991	C	G	.	.	C	.	A	.	.	T	.	.	.	C	G	.	A	C	.

Continued.

JAK1			*MACF1*				*PFKFB1*								*PTPN4*		*RAG1*								
2	**2**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**4**	**4**	**4**	**4**	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
1	**2**	**3**	**8**	**8**	**3**	**4**	**8**	**8**	**8**	**9**	**9**	**0**	**3**	**7**	**8**	**9**	**0**	**0**	**1**	**1**	**4**	**5**	**7**
4	**2**	**5**	**1**	**3**	**6**	**2**	**3**	**4**	**4**	**2**	**3**	**8**	**3**	**3**	**8**	**0**	**4**	**4**	**0**	**9**	**3**	**3**	**0**
8	**6**	**0**	**2**	**4**	**7**	**2**	**2**	**5**	**6**	**4**	**6**	**9**	**5**	**4**	**7**	**2**	**0**	**1**	**9**	**9**	**3**	**5**	**3**
C	C	T	C	A	Y	C	G	G	T	A	T	G	A	T	G	C	C	G	C	G	C	C	C
.	Y	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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?	.	C	.	.	C	T	.	.	.	C	C	A	G	C	.	.	T	.	T	A	G	.	T
T	.	C	T	.	C	T	.	.	.	C	C	A	G	C	.	.	T	.	T	A	G	.	T
.	.	C	T	.	C	T	.	.	.	C	C	A	G	C	.	.	T	.	T	A	G	.	T
C	.	.	.	.	T	.	-	.	.	C	C	.	.	C	.	.	.	.	.	.	.	.	.
T	.	.	.	.	T	G	.	.	.	C	C	.	.	C	C	.	.	.	.	.	.	.	.

Continued.

RHO1				*TRHDE*				*WT1*						
5	**5**	**5**	**5**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
7	**7**	**7**	**8**	**0**	**0**	**1**	**1**	**6**	**6**	**6**	**6**	**6**	**7**	**9**
1	**8**	**9**	**4**	**2**	**5**	**1**	**3**	**0**	**5**	**6**	**7**	**8**	**6**	**6**
0	**6**	**1**	**6**	**1**	**3**	**5**	**5**	**4**	**4**	**1**	**8**	**5**	**3**	**8**
C	C	G	C	T	A	C	T	T	-	C	G	G	G	G
.	.	.	.	.	.	.	.	?	?	?	?	?	?	?
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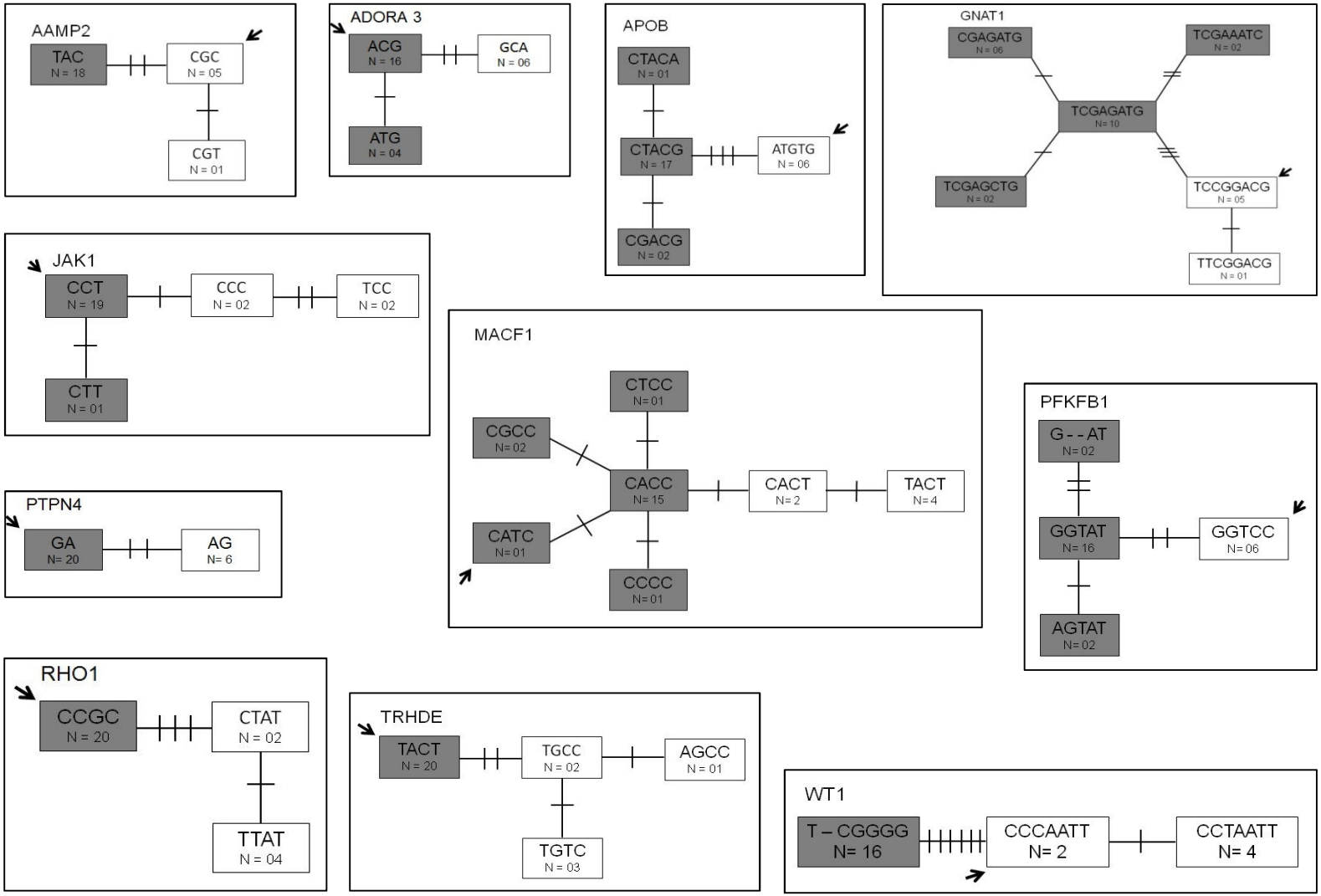
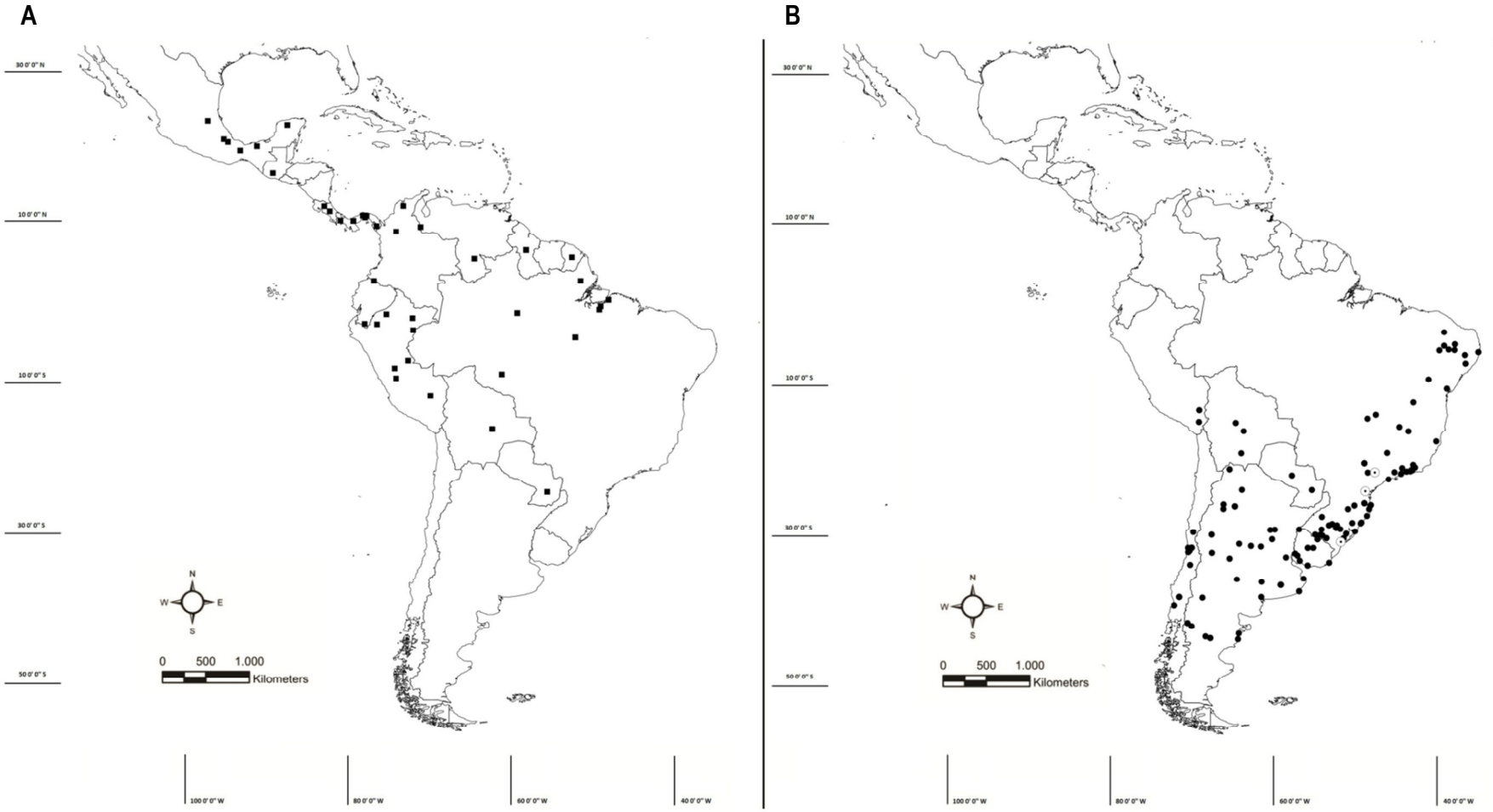
T	T	A	T	.	G	Y	C	C	C	T	A	A	T	T
.	T	A	T	W	G	Y	C	C	C	.	A	A	T	T
T	T	A	T	.	G	T	C	C	C	T	A	A	T	T
.	.	.	.	.	.	T	.	C	C	.	A	A	T	T
.	.	.	.	.	.	T	.	C	C	.	A	A	T	T

B) Haplotype networks derived from the nucleotide information described in (A) for 11 of the 12 nuclear segments (the network for *RAG1* is shown in Figure 7). Details are as in Figure 7



SUPPORTING INFORMATION S6. Geographic distribution map of *Galictis vittata* (**A**) and *G. cuja* (**B**) throughout the Neotropical region based on skulls and skins (solid shapes) and DNA samples (open shapes). The records (squares for *G. vittata* and circles for *G. cuja*) represent all specimens suitable for inclusion in the geographic analysis (see main text and Supporting Information S1 for additional details).



Capítulo III

Phylogeography, demographic history, and morphological variation of the little-known South American mustelid *Galictis cuja* (Mammalia, Carnivora)

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Abstract

Studies of intraspecific variation can shed light on various aspects of the evolutionary history of present-day populations, including past demographic changes, geographic structuring and adaptive features. Although this is true for all taxa, it is of most important concern for those that are not well documented. *Galictis cuja* is a little-known small mustelid from South America with a broad range covering a diverse array of landscapes. To investigate the spatial structure of this species and infer about historical processes that may have shaped within-species diversity, we conducted genetic and morphological analyses in *G. cuja* throughout most of its range. For the former approach, we analyzed two segments of the mitochondrial DNA resulting in a total alignment of 1,108 bp and evaluated phylogeographic patterns as well as demographic history. For the latter approach, we analyzed 117 skulls of *G. cuja* to estimate skull variation related to sex and geographic distribution. Molecular results indicated the presence of two main monophyletic groups that are not clearly associated with present-day geography. We infer that they may reflect past population isolation followed by subsequent reconnection, or long-term retention of ancestral polymorphism associated with a rather deep coalescence and stable population size in southern Brazil. Neutrality testes suggested some recent demographic expansion in the Brazilian southeast. Skull morphology indicated strong pattern of sexual size dimorphism and indicated interesting and localized association between environmental variation and skull variation. The results may help guide further research targeting the evolutionary history of this mustelid.

Keywords

Galictis cuja, geographic variation, mtDNA, phylogeography, sexual size dimorphism, within-species variation

Introduction

The analysis of current levels of intraspecific variation can help understand historical processes shaping spatial and temporal patterns of population structure (e.g. Moussalli et al. 2009; Vianna et al. 2011). Within-species changes comprise the first step for evolutionary divergence, and may influence several other levels of the biological hierarchy. The diversity present in an organism can be understood in the context of genetic, morphological, physiological or behavioral variation, and their clarification is crucial to characterize species and their containing communities. Indeed, the initial factor allowing evolution to occur is mutation, and genetic diversity within species can be assessed with molecular approaches. In comparison, the phenotype reflects the underlying genetic diversity but also adaptations to external factors, so that the study of morphological variation may help understand the responses of the species to diverse environments.

Up to now, however, few studies have addressed the complexity of intraspecific variation of South American taxa from a perspective that combines molecular phylogeography and morphological assessments. Interestingly, South America is one of the most diverse and one of the most threatened regions on Earth (Schipper et al. 2008). It may thus be stated that evolutionary factors influencing the current genetic and morphological diversity of species remain insufficiently explored in one of the most important biodiversity hotspots of the world. The scarcity of studies jointly addressing these topics is even more extreme when one considers analyses that cover the vast majority of the geographic range of the analyzed taxon, allowing one to perform broad biogeographic inferences.

The lesser grison, *Galictis cuja* (Molina, 1782), is a little-known mustelid with a relatively broad distribution, occurring from northeastern Brazil to the Patagonian region in Argentina and Chile (Yensen and Tarifa 2003; Bornholdt et al. unpublished data). It is a small mustelid (total length *c*. 56 cm and weight *c*. 1.2-2.5 kg) exhibiting an almost exclusively carnivorous diet, feeding mainly on small mammals, but also on lizards and birds (Delibes et al. 2003; Zapata et al. 2005). Throughout its range, *G. cuja* occupies a considerable variety of landscapes, such as subtropical and temperate forests, open savannas and grasslands, high montane grasslands, and arid xeric shrublands (Bornholdt et al. unpublished data), as well as human-altered areas (Dotta and Verdade 2007). Field-based approaches have indicated that the lesser grison has a tendency to be rare, with few records (such as camera trapping) obtained for this species when compared to the records obtained for other mammals (Santos et al. 2004; Vaz 2005; Trolle et al. 2007). This observation may not be an indicator of threat (the IUCN categorized it as “Least Concern” [Reid

and Helgen 2008]), but may be the result of the combination of a broad distribution and low density, as already suggested for its single congener, *G. vittata* (greater grison) (Arita et al. 1990). The main threat for the lesser grison seems to be road-killing (Vieira 1996), although the exact demographic consequences of it remain unclear. Overall, few studies have ever been published about this mustelid, and the gathering of even basic information about it persists as one of the most important priorities for the carnivore research in South America (e.g. IBAMA 2004; Oliveira 2009).

The two grison species (*G. cuja* and *G. vittata*) along with the South American mustelid *Lyncodon patagonicus*, the African *Ictonyx libyca*, *I. striatus*, and *Poecilogale albinucha* and the Eurasian *Vormela peregusna* are part of the subfamily Ictonychinae (Sato et al. in press) whose common ancestor likely occurred in Eurasia, an inferred center of origin for many lineages in the Mustelidae (Koepfli et al. 2008). As for grisons specifically, it is likely that a common ancestor for genus *Galictis* arrived in South America, coming from North America, and then gave rise to the two extant species during the Pliocene (Eizirik, in press). Studying the patterns of the current diversity within these taxa, including both genetics and morphological variation, should help to shed light on the evolutionary history across different biogeographic areas, and consequently add information to compound the history of the grisons in the Neotropics. Therefore, here we focus on the amount of genetic and phenotypic variation within *G. cuja* and speculate about spatial and temporal causes leading to the intraspecific patterns.

In the genetic context, studies aiming to better understand within-species variation, mitochondrial (mtDNA) is often employed, mainly because it is a highly polymorphic, expressing prominent intraspecific variation and commonly absence of recombination (Avise 2000). Specifically among the mtDNA, the control region (CR) is well recognized to exhibit high genetic diversity in many vertebrate species, as it contains the main regulatory elements for the replication and expression of the mitochondrial genome (Avise 1994). Not surprisingly, this segment has been extensively employed in phylogeographic studies in various vertebrates. However, contrasting this well-known scenario, previous studies with mustelids have demonstrated low levels (and in some cases very low) of genetic variability in the mtDNA CR in Europe (Effenberger and Suchentrunk, 1999; Mucci et al. 1999; Cassens et al. 2000; Ferrando et al. 2004; Pérez-Haro et al. 2005; Pertoldi et al. 2008) and more recently, in Argentina (Centrón et al. 2008), compounding an interesting issue to be tested in other mustelid species.

In the present study, we addressed genetic and morphological variability of *G. cuja* based on the analysis of DNA sequences from three mitochondrial segments, as well as on the assessment of skull linear measurements from individuals sampled throughout most of the

species' range. On the basis of these data sets, we investigated the spatial structure of the observed variation, and inferred historical processes that may have shaped the observed patterns.

Materials and methods

Genetic analyses

Population sampling and laboratory procedures

Biological material was collected from 71 *G. cuja* individuals sampled across Brazil (Table 1, Fig. 1). The material consisted of tissue samples obtained from road-killed individuals preserved in 96% ethanol. Additionally, mtDNA sequences from seven individuals sampled in Argentina were contributed by collaborators (K.P. Koepfli, M. Lucherini, unpublished data) and also added in the analyses. Finally, six samples from *G. vittata* (three from Peru and three from French Guiana) were included in the study to be used as outgroups in phylogenetic and network-based analyses.

Genomic DNA was extracted from tissue samples using a phenol-chloroform-isoamyl alcohol protocol (Sambrook et al. 1989). Two fragments of the mtDNA were amplified via the polymerase chain reaction (PCR): (i) 438 bp of the 5' portion of the control region (CR), containing the first hypervariable segment, using primers MTLPRO2 and CCR-DR1 (Tchaicka et al. 2007); and (ii) 645 bp of the gene *NADH dehydrogenase subunit 5* (*ND5*) using primers ND5-DF1 and ND5-DR1 (Trigo et al. 2008). PCR was performed in 20 µL final volume containing 10-100 ng of genomic DNA, 1x PCR Buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq Platinum DNA polymerase (Invitrogen), and 0.2 µM of each primer. Thermocycling conditions for the CR and *ND5* amplification began with 10 cycles (touchdown) each including a 45s denaturing step at 94ºC, 45s annealing at 60-51ºC (with a decrease in temperature of 1ºC per cycle), and 1.5 min extension at 72ºC, followed by 30 cycles of 45 s at 94ºC, 45s at 50ºC, 1.5 min at 72°C, and a final extension of 3 min at 72ºC. Products were visualized on a 1% agarose gel stained with GelRed (Biotium) and purified either by precipitation with ammonium acetate or by employing the enzymes Exonuclease I and Shrimp Alkaline Phosphatase. Purified PCR products were sequenced in both directions using the DYEnamic ET Dye Terminator Sequencing Kit (GE Healthcare®), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare®). Sequences were deposited in GenBank under accession numbers **XXXX-XXXX**.

Initial data analysis

Forward and reverse sequence electropherograms were inspected, edited and aligned (with the Muscle algorithm) using the Geneious version 5.5 software package (Drummond et al. 2010). Initially, sequence analyses were performed considering three mtDNA data sets: (i) control region (CR); (ii) *ND5*; and (iii) concatenated alignment of CR + ND5. For each of these data sets, we estimated basic measures of diversity using DnaSP version 5 (Librado and Rozas 2009) and ARLEQUIN version 3.01 (Excoffier et al. 2005) and also generated haplotype networks using the median-joining approach (Bandelt et al. 1999) implemented in Network version 4.6.1.0 (available on http://fluxus-engineering.com). These assessments provided initial information on the variability and phylogeographic information contained in each data set. Given the lower amplification success and lower variability observed for the CR relative to *ND5* (see Results), we focused on the latter segment as well as on the concatenated data set for all subsequent analyses.

In-depth DNA sequence analyses

To determine the appropriate model of nucleotide evolution for both the *ND5* and concatenated data sets, we used the Akaike Information Criterion (Akaike 1978) as implemented in jModelTest (Guindon and Gascuel 2003; Posada 2008). The TrN + G nucleotide substitution model was found to provide the best fit to the *ND5* (-lnL = 1423.9755) and the concatenated (-lnL = 2270.5378) data sets, and was applied in all subsequent model-based analyses. Phylogenetic relationships among haplotypes were estimated using maximum likelihood (ML) and Bayesian inference (BI) approaches. The ML analysis was conducted using the software PhyML 3.0 (Guindon et al., 2010) with a BioNJ (Gascuel 1997) starting tree and support assessed by 1,000 bootstrap replicates. The BI analysis was performed using BEAST version 1.6.2 (Drummond and Rambaut 2007) with two independent runs for 5 million iterations and samples taken every 500 steps. Initial 10% chains were discarded as burn-in. Both trees were rooted using *G. vittata* as the outgroup.

To further investigate population structure, we hypothesized three geographic groups in *G. cuja* on the basis of the observed phylogeographic patterns (see Results): (i) southern Brazil; (ii) southeastern Brazil; (iii) Argentina. First, we estimated fixation indices (F_{ST}) (Wright 1965) as measures of differentiation among these groups using an Analysis of Molecular Variance (AMOVA) approach (Excoffier et al. 1992) implemented in ARLEQUIN. Then, evidences for demographic changes were investigated through the neutrality tests Fu's Fs (Fu 1997) and Tajima's *D* (Tajima 1989), performed in DnaSP and ARLEQUIN.

Morphological analyses

Craniodental variation within *G. cuja* was assessed using a total sample of 117 (71 males and 46 females) adult skulls and/or mandibles with known sex (see Table 2 for the complete list of the scientific collections visited and specimens ID). This sample covers almost the entire *G. cuja* range (Fig. 2). Adult specimens were defined by fully erupted permanent dentition and complete fusion of the skull fissures. We took 15 linear measurements for all specimens: skull - greatest length of skull (GLS), nasal length (NL), zygomatic breadth (ZB), mastoid breadth (MB), braincase breadth (BB), interorbital constriction (IC), postorbital constriction (PC), palatal width (PW), braincase height (BH), mandible length (ML), and mandible height (MH); teeth - length of maxillary toothrow (C-M2), external alveolar distance between upper canines (C-C), external alveolar distance between upper molars (M2-M2), and length of mandible toothrow (c-m2). The measurements were taken by the first author, with exception of the specimens from BMNH and SMT, performed by the second author.

In order to evaluate intraspecific variation and thus infer about possible morphological patterns of dissimilarity within *G. cuja*, we first tested the presence of any *a priori* grouping in the species. To achieve this assessment, we performed a Principal Component Analysis (PCA) and plotted the first (PC1) and the second (PC2) components, so as to visualize whether the specimens used in the morphometric analysis tended to form any cluster. Applying this analysis we reduced the dimensionality of our multivariate data and create a new set of uncorrelated variables (the principal components) that more clearly characterize the existing variation of the data set. The results of this ordination approach were used to provide exploratory insights on the general distribution of each individual in the component scores, as well as any segregation in our morphometric variables. To place *G. cuja* specimens into specified groups, we also applied a classification approach described below.

We then analyzed the morphometric data concerning two main topics: variation related to sex (sexual size dimorphism) and variation related to spatial distribution (geographic variation). To assess the first analysis, we compared the means of each linear measurement between males and females using *t*-test comparisons with the Bonferroni correction. As the species demonstrated to be sexual-sized dimorphic (see Results), all subsequent statistical analyses were performed with males and females separated, so as to avoid the noise introduced by sexual differences in the geographic analysis, which could bias the interpretation of the results.

To investigate the geographic distribution of morphological diversity, we first assigned in advance each *G. cuja* specimen to a regional category, following to two approaches: (i)

geographic latitudes, where specimens were considered as belonging to the same group when they were from the same five-degree interval of latitude; and (ii) biomes, where the specimens from the same biome (based on those described by Olson et al., 2001) comprised the same geographic group. Here the classification approach Discriminant Function Analysis (DFA) was conducted to evaluate whether the specimens from these latitudes and biomes could be distinguished. We then plotted the first (CV1) and the second (CV2) canonical variates to assess the discriminant analysis and check the consistency of these geographic groups. Applying this classification approach we grouped *G. cuja* specimens into well-defined classes (geographic groups) and assessed how distinct these classes were. Measurements were log-transformed before performing the PCA and the DFA. All morphological statistics were conducted using SPSS v17 (SPSS Statistics for Windows, Rel. 17.0.0, Chicago).

Results

Molecular phylogeography and demographic history

We obtained a total of 444 bp of the mtDNA control region (CR) for 61 *G. cuja* individuals, and 645 bp of the *ND5* segment for 77 animals. Due to ambiguous alignment in one hypervariable portion of the CR, six sites were excluded from the analyses, resulting in a final CR alignment of 438 bp. *G. vittata* was sequenced for the same sequence length of each fragment. Both segments were concatenated for use in phylogeographic analyses, leading to a total data set of 1,083 bp (Table 3).

The CR data set contained eight distinct haplotypes, while the *ND5* and concatenated data sets contained 18 haplotypes each (see Table 3). The lowest values for gene (haplotype) diversity and nucleotide diversity, considering the entire alignment, were the ones for the CR, indicating that this mtDNA segment contain less variability in this species than the *ND5* gene. Haplotype networks for the *ND5* (Table 4) and concatenated (Table 5) data sets were rather similar (Fig. 3), but distinct from that of the CR (data not shown). The former two networks depicted the presence of two main groups separated by ten (*ND5*) and 15 (concatenated) mutational steps. The same general geographic pattern was found for both sets: one group represented by individuals exclusively from southern Brazilian localities (Rio Grande do Sul, Santa Catarina, and Paraná states) and the other group encompassing these same areas but also including individuals from central and southeastern Brazilian localities (São Paulo, Minas Gerais, and Bahia states and the Federal District) as well as Argentinean samples (see Fig. 3),

which bore a unique haplotype (GC18 for concatenated data set and GN18 for *ND5* data set). Interestingly, one municipality of Rio Grande do Sul state (Dom Pedrito) harbored haplotypes from both phylogroups. Considering only the concatenated network, no haplotype was shared between the three main geographic groups (southern Brazil, central/southeastern Brazil and Argentina), which suggests the existence of genetic structuring within *G. cuja*. When the *ND5* data set was analyzed by itself, we observed one shared haplotype (GN07 [see Fig. 3A]) between states from southern Brazil and central/southeastern Brazil. Also, the *ND5* network revealed an interesting pattern of haplotype relationships, with the *G. cuja* root placed in southern Brazil, and an indication of population expansion in the Brazilian southeastern samples (several localized lineages connected by single mutational steps to a common haplotype shared with the south).

The phylogenetic reconstructions generated with BI and ML were not identical, although major patterns were consistent between the two methods. The BI method depicted two monophyletic groups of *G. cuja* mtDNA lineages, both bearing Bayesian posterior probability of 1.0 and 100% ML bootstrap support (Fig. 4). The first group (Clade 1) was composed by individuals from Rio Grande do Sul, Santa Catarina and Paraná states (southern Brazil), from Minas Gerais and São Paulo states as well as the Federal District (central/southeastern Brazil) and Argentina. The second group (Clade 2) included individuals only from southern Brazilian states. Further analysis within Clade 1 demonstrated some phylogenetic structuring, with the presence of two sub-clades, whose internal phylogenies exhibited short branches and no explicit evidence of geographic substructure. One of these subdivisions from Clade 1 (Sub-clade 1) was weakly supported and comprised haplotypes from São Paulo, Minas Gerais and the Federal District in Brazil, along with the one from Argentina. The other group (Sub-clade 2) comprised haplotypes from Rio Grande do Sul, Santa Catarina, and Paraná states (southern Brazil), as well as São Paulo and Minas Gerais states (southeastern Brazil), and exhibited considerably high support. Finally, there was also a smaller, well-supported clade with individuals that are exclusive from Rio Grande do Sul state, southernmost Brazil (GC01, GC03, GC07, and GC08) (see Fig. 4).

On the basis of the phylogenetic reconstructions and haplotype networks, we defined three main geographic domains in the *G. cuja* distribution that formed three hypothesized population groups: (i) southern Brazilian states (Rio Grande do Sul, Santa Catarina, and Paraná), named “southern Brazil”; (ii) Federal District and southeastern Brazilian states (São Paulo and Minas Gerais), named “southeastern Brazil”; and (iii) Argentina. We then evaluated the genetic differentiation among these groups, as well as the genetic diversity within them. The AMOVA results demonstrated that 23% of the observed genetic variability in *G. cuja* mtDNA was explained by differences among the three geographic groups, leaving 77% of the total variability

for differences within each group. In general, the F_{ST} indices between groups were rather high, and suggested a consistent geographic pattern. The highest F_{ST} value corresponded to the more distant areas, southeastern Brazil *vs.* Argentina (0.45, P = 0.000), followed by southern Brazil *vs.* Argentina (0.26, P = 0.01) and finally southern Brazil *vs.* southeastern Brazil (0.17, P = 0.01). Nucleotide and haplotype diversity within these populations were different. Southern Brazil had high haplotype diversity (0.8752 ± 0.0265) and moderate nucleotide diversity (0.00989 ± 0.0049), while southeastern Brazil had higher haplotype diversity (0.9166 ± 0.092), but lower nucleotide diversity (0.00284 ± 0.00185) (see Table 3). The Argentina group was formed by seven sampled individuals, all of which shared the same haplotype. There was no clear evidence of signatures of a recent expansion for any geographic groups (see Table 3), except for Fu's F_S test for southeastern Brazil (F_S = -2.546, P = 0.04).

Morphological variation

The exploratory PCA did not indicate any separate cluster that could be specifically investigated in more detail (Supplementary Material 1). Without assigning in advance specific groups for *G. cuja*, the specimens analyzed here formed a single cluster with few specimens slightly separated from the main group (AMNH 205832, female from Uruguay; MNHN 29988, female from northeastern Brazil, and USNM 271573, male from Chile). The results of the *t*-test showed significant sexual size dimorphism for all craniodental measurements, even after using the Bonferroni-corrected significance level ($\alpha \leq 0.003$). For all of them, males were larger than the females (Table 6) and the remaining analyses were performed for each sex separately.

The assessment of morphological variation related to spatial distribution in the lesser grison demonstrated quite different patterns for males and females, considering both biomes and latitudes as guiding geographic subdivisions. The biome DFA generated eight (males) and six (females) discriminant functions, but only the first function was significant to discriminate groups for both data sets (Table 7). Considering only the males, on the basis of standardized coefficients it was found that the first canonical variate (CV1) accounted for 50.5% of the total variation. The skull variables that contributed the most to the CV1 were the zygomatic breath (ZB), the mandible height (MH), and the length of mandible toothrow (c-m2). For the females, CV1 was responsible for 69.1% of the total variation, and the external alveolar distance between upper canines (C-C), the postorbital constriction (PC), and the palatal width (PW) were the measurements with the greatest contribution to CV1 (see Table 7).

These results suggested that the main segregation among different biomes in general occurs along the CV1, although this segregation was not perfect in some biomes for both data sets (Fig. 5A-B). The illustration of the male DFA (see Fig. 5A) depicted the two Chilean biomes (Valdivian Temperate Forest and Chilean Matorral) close to each other and representing the smallest among all clusters. Still along the CV1, the Chilean biomes are followed by the Argentinean and the Brazilian savannas (Cerrado), and so the other biomes are pooled together with little segregation among them (see Fig. 5A). The same pattern of Chilean-Savannas-remaining biomes was not observed when focusing only the females. In these data, the dry Brazilian Caatinga was isolated as the smallest group (represented by a single record), followed by a larger cluster containing the Uruguayan and southern Brazilian Pampas, the Brazilian Atlantic Forest, the Chilean Matorral and the Montane Grassland and Shrublands (see Fig. 5B). Following this large grouping (but well segregated with almost no overlap between them) were the Argentinean Savannas and the Valdivian Temperate Forest (see Fig. 5B). Moreover, it is possible to recognize some tendency of grouping along the second canonical variate (CV2), although the Chi-square statistics indicated that this discriminant function was not significant to group specimens (see Table 7).

To evaluate if morphological differences were associated with a latitudinal gradient, specimens were classified according to seven geographic groups (Fig. 5C-D). For males and females, the DFA generated six discriminant functions, and again, only the first of each was significantly important to differentiate latitudes (Table 8). Concerning the males, the CV1 was responsible for 45% of the variance in latitude and there was no clear discrimination among the groups along this axis, except for the 41° to 46° cluster (light green) that comprised a very distinct cluster (see Fig. 5C). The variables that contributed most to the CV1 were the braincase breadth (BB), the interorbital constriction (IC), and the mandible length (ML). In the female data set, 66% of the total variation was explained by the CV1, and there was a more clear-cut grouping, mainly when considering the extreme latitudes (06° to 11° [green] and 41° to 46° [light green]). The postorbital constriction (PC), mandible length (ML), and the external alveolar distance between upper canines (C-C) were the most important measurements for this discrimination pattern (see Fig. 5D).

Discussion

Genetic diversity in *G. cuja*

Our data indicated that the control region (CR) harbors less diversity than the *ND5* gene in *G. cuja* (Table 3), although this CR diversity was not as low as that reported for other mustelids, such as the European *Lutra lutra* (Effenberger and Suchentrunk, 1999; Ferrando et al. 2004) and *Martes martes* (Pertoldi et al. 2008) and the South American *Lontra provocax* (Centrón et al. 2008). Regarding the latter species (*L. provocax*), Centrón et al. (2008), based on 13 CR sequences from two areas of southern Chile, reported levels of haplotype diversity of 0.44 and 0.71 and nucleotide diversity (π) of 0.0013 and 0.0016. Ferrando et al (2004) discussed some factors that may influence such low variability maintained in the CR segment of otters. For example, he mentioned the potential influence of the otter life history (preferential dispersal by males) and the shorter hypervariable flanking region comparing to other carnivores. Still, given our opportunity to compare the CR diversity with that of a mitochondrial coding gene (*ND5*), it is noteworthy that the former does seem to harbor considerably less diversity, a feature that is unusual in most animal groups. Also, this comparison argues against any demographic or ecological explanation, since they would affect both mtDNA regions similarly. Therefore, our data suggest that some specific molecular feature of the *Galictis* mtDNA CR induces lower accumulation of diversity than usual. Such features would include lower mutation rates or negative selection, both of which would be interesting topics to further investigate.

Considering the concatenated data set, the estimated mtDNA diversity cannot be directly compared to those of other mustelids because data on the equivalent segments are unavailable in the literature. However, it is possible to have some insights on the general level of polymorphism in *G. cuja* (e.g. π = 0.00848), which was moderate when compared to other South Americans mustelids. For instance, using the *cytochrome b* and CR segments, Pickles et al. (2011) found levels of nucleotide diversity of 0.015 for the giant otter (*Pteronura brasiliensis*) and Trinca et al. (in press), applying the same CR and *ND5* segments used here but adding the *ATP8/ATP6* segment, reported equivalent nucleotide diversity (π = 0.00840), although Vianna et al. (2011), applying a concatenated set of CR + ND5 + *cytochrome b* reported π = 0.0016 for the Patagonian otter (*L. provocax*). In addition to the moderate levels of polymorphism, the gene diversity (h) within *G. cuja* was high with respect to the same South American mustelids. Pickles et al. (2011) documented h = 0.93 (giant otter), Trinca et al. (in press) h = 0.969 (river otter), and Vianna et al. (2011) h = 0.877 (Patagonian otter), while our study estimated a gene diversity of 0.923. In spite of the moderate to high mtDNA diversity documented here in relation to other carnivores, our analyses demonstrated that most of it came from the *ND5* segment, suggesting that the CR may not be the best mitochondrial marker for phylogeographic studies in *G. cuja*, while the former seems to be very informative in this species.

Phylogeographic structure

The most remarkable outcome observed with the mtDNA data was the presence of two major groups within *G. cuja* (Clades 1 and 2 in Figs 3 and 4). Interestingly, these groups did not clearly reflect two different geographic regions. There was a set of haplotypes whose origin was the southernmost region of Brazil (Rio Grande do Sul, Santa Catarina and Paraná states), which formed a separate group from the remaining individuals. The other group encompassed haplotypes from these same areas (the same southern Brazilian states and in one case the same municipality) as well as haplotypes from central and southeastern Brazil and Argentina. In spite of the absence of a defined phylogeographic break, we observed significant F_{ST} values among the three assumed geographic partitions (southern Brazil, southeastern Brazil and Argentina), indicating that the mtDNA diversity is indeed structured in this species. These three groups were thus considered while visualizing the historical relationships of these areas as reflected in the mtDNA of *G. cuja* (see Fig 3).

The split between the two *G. cuja* major groups (considering the concatenated data) implies at least 15 mutational steps, which is considerably deep mainly if we consider that it is not geographic localized. Lebarbenchon et al. (2010) found a similar within-species break between two well-supported clades in *Mustela nivalis* and Reis et al. (2011) found even more mutations in the amphibian *Salamandra salamandra*, but these reported divergences were explained by geographic structuring between the groups. In contrast, our results may indicate that haplotypes from Southern Brazil represent relatively stable populations that have persisted throughout the years and the presence of these samples in divergent monophyletic clades may represent retention of ancient polymorphism, as suggested for other vertebrates (e.g. Moran and Kornfield 1993; Cantanhere et al. 2005; Dubach et al 2005). Another possibility is that the two major clades do indeed result from historical isolation in separate regions, leading to phylogeographic structuring of mtDNA lineages, but have subsequently been re-connected by more recent gene flow. In such a scenario, dispersal and gene flow would gradually override the original geographic signal, while the phylogenetic break persists until one of the implicated lineages goes extinct by drift. Both hypotheses fit the evidence for an older age of haplotype lineages sampled in southern Brazil, and highlight the interest in pursuing further phylogeographic investigations focusing on this species.

When we assessed molecular variation within each *G. cuja* group, we observed that southeastern Brazil has the highest gene diversity, since we sampled seven haplotypes in only nine individuals. However, these nine samples presented only nine polymorphic sites, leading to lower nucleotide diversity when compared it to southern Brazil (see Table 3). It is interesting to

note that although southern Brazil presented lower gene diversity (h = 0.8752), the haplotypes from this region were split between the two major phylogroups, supporting the inference of more ancient variability retained in this area. The Argentina group (GC18) presented seven identical sequences, possibly indicating a recent origin for the lineages present in this region. Additional sampling will be required to further test this latter hypothesis.

Demographic inferences

The patterns observed with the mtDNA haplotype networks (especially that of *ND5* segment [Fig 3A]) suggest recent population expansion in southeastern Brazil (orange circles), with all haplotypes from this region differing from each other by a single mutational step and smoothly resembling a star-shaped structure. The same suggestion emerges from an inspection of the neutrality tests results (see Table 3), in which, among the three groups, only southeastern Brazil showed support for demographic expansion (significant only for Fu's Fs).

Morphological diversity

Morphometric data can provide robust intraspecific inferences, since the accumulation of morphological divergence allows the recognition of phenotypic patterns within species, often associated to adaptation to different environments. Here we report some morphological variation within *G. cuja* that, combined with molecular inferences, can provide support to subsequent studies aiming to understand the evolutionary history of such taxon.

The absence of a defined group in *G. cuja* demonstrated by the PCA revealed that there was not a strong grouping in the species that could be recognized with this ordination technique. The three specimens that deviated from the main cluster do not suggest any geographic signal that could explain the morphological divergence, since the three areas (Uruguay, Chile and the northeastern Brazil) were also represented in the main group. The specimen MNHN 29988 (northeastern Brazil) was the one that deviated the most with respect to PC1, suggesting a difference in size. However, it is important to note that all three outliers were not placed outside the interval of PC1 (with the slight exception of MNHN 29988), but rather they shift from the central cluster along the PC2 (which is less influenced by size). The female specimen AMNH 205832 had a small dental anomaly, the absence of the right superior canine. Thus, we checked whether this feature could have been responsible for its deviation by looking at the descriptive statistics of the length of maxillary toothrow (C-M2), the linear measurement that could have been directly influenced by the absence of the canine, from all females. The value of C-M2 for this specimen (18.42 mm) was very close to the mean (19.01 mm, ranging from 16.2 – 25.9 mm) and

thus it is not the bias influencing such discrepancy. We therefore conclude that these individuals deviate from the main group due to within-population variation in *G. cuja*.

This mustelid, as well as many others, is sexually dimorphic, with males larger than females. Our morphometric data documented substantial dimorphism in *G. cuja*, with highly significant differences for all 15 measurements. Overall, there are two primary categories of explanation for this intraspecific variation within the family. First, resource partitioning that calls for sexual dimorphism to reduce competition for food (Dayan *et al*., 1989; Gittleman and Van Valkenburgh 1997), and second, sexual selection that predicts different selective pressure on each sex (Ralls 1977; Erlinge 1979). Some authors have been studying sexual size dimorphism associated to the feeding apparatus in mustelids in order to test for niche separation between sexes, and there seems to be a general accordance that this hypothesis may be the cause for sexual dimorphism in mustelids or at least it plays a role in maintaining the observed differences (Johnson and Macdonald 2001; Thom et al. 2004; Rozhnov and Abramov 2006). In contrast, some authors have designated sexual selection as the main cause for this intraspecific variation in the family (e.g. Ralls 1977; Moors 1980). As no single measurement in our data set was particularly relevant in inducing this pattern (which could be important to speculate about possible causes for sexual dimorphism in *G. cuja*), we are unable to further assess the specific processes underlying this result. Still, our observation of strong sexual dimorphism reflected in all measurements may serve as a starting point for further studies focusing, for example, on a particular morphological apparatus or on field-based work targeting the ecology and behavior of this species.

Morphological divergence linked to geographic variation did not show a strict pattern of spatial divergence in *G. cuja*. Interestingly, the outcomes from the female data were clearer than those of the males (see Fig 5). Comparisons based on biomes and latitudes supplied similar answers, since both may be related to temperature variation and associated features (e.g. major vegetational types). However, when we considered only the biomes, we could assess morphological variation connected basically to major vegetation domains, while studying the latitudes it was possible to understand patterns more likely associated to broad differences in temperature (although admittedly latitude is not the only variable that influences temperature). Our results indicate that *G. cuja* specimens cannot be well distinguished by differences in biomes and latitudes, suggesting that the most important intraspecific variation are not due to these factors, although some interesting patterns could be observed. For example, in males the groups of biomes and latitudes are concentrated in the middle of the DFA plot (without clear segregation), and some specimens deviated essentially from this main cluster indicating that they

were considerably distinct from the remaining individuals. Chilean specimens (from both Chilean Matorral and Valdivian Temperate Forest) and Argentinean specimens from the savanna (middle-south Argentina) were distinct, as were specimens from the region spanning 36° to 46° of latitude (which mostly coincided with Chilean and middle-southern Argentinean biomes), ultimately suggesting that males of *G. cuja* are morphologically similar, but populations from cooler temperature (and vegetation domains related to it in South America) may have important craniodental features that separate them well.

Overall, in the females it was also recorded a tendency in which there was no clear-cut clustering, with some different groups deviating from the main assemblage. However, some differences between the sexes can be reported. The Caatinga (the dry biome located in northeastern Brazil) and the 06° to 11° of latitude group were placed quite distantly from the main cluster, as were the specimens from the Valdivian Temperate Forest, Argentinean savannas and specimens from 41° to 46°. Although the segregation between the groups was not perfect, females of *G. cuja* demonstrated a clearer segregation than males in both approaches (biomes and latitudes). In conclusion, few groups were indicative of some geographic distinction (such as the populations from the extremes of the *G. cuja* geographic distribution and the population from the Argentinean savannas), because specimens from these DFA assignments were well distinguished. Interesting to note that, for both sexes, the two closer biomes are the Pampas and the Atlantic Forest, demonstrating a slight morphological continuing from Uruguay to middle Brazil.

Geographic variation is important to illustrate adaptive divergence within species and there is often a strong correlation between morphology and measurements of the environment (Gould and Johnston 1972). Based on that several ecogeographic rules have been described to understand the relation between morphology and environmental variation (one of the best known rule is Bergmann's rule, proposing that larger specimens would live in cooler localities). The absence of a defining geographic partitioning with our data is a meaningful outcome, once it shows the relatively weak relationship between craniodental measurements and environmental traits. Other morphological aspects may play a more important role in geographic variation, such as body mass or skin variation (as suggested by Bornholdt et al. unpublished data).

Overall, our molecular and morphological data showed a moderate variability in *G. cuja*, suggested few but interesting and localized association between environmental variation and skull variation (basically middle-south Chile and Argentina and northeastern Brazil), described a significant pattern of sexual size dimorphism, reported a strong mtDNA partitioning into two phylogenetic groups (not geographically delimited), and evidenced a recent expansion in the

Brazilian southeast. This is the first description of within-species variation in *G. cuja* using molecular and morphological approaches, which raised hypotheses that may help guide further research avenues targeting the evolutionary history of this mustelid.

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Figure legends

Figure 1 Map showing the current geographic distribution of *Galictis cuja* (shaded area) with the genetic sample collection sites from the three data sets analyzed in the study: mtDNA control region (star-shaped symbols), *ND5* gene (dark triangles), and concatenated (open circles). Ellipses represent the geographic groups defined in the population structure analysis (southeastern Brazil, southern Brazil, and Argentina). The collection sites from the Argentinean samples are not specified (see Table 1 for sample ID and the detailed geographic origin). The two sites from the outgroup (*G. vittata*) are represented by stars. Map adapted from Yensen and Tarifa 2003 and from Bornholdt et al. (unpublished data).

Figure 2 Map showing the current geographic distribution of *Galictis cuja* (shaded area) with the morphological samples collection sites and the biomes domains or ecorregions (see legend). Records indicate distinct locations (i.e. repeated coordinates were collapsed into a single point). For all zoological collections assessed in the study with the samples ID and detailed information of each sample see Table 2. Map adapted from Yensen and Tarifa 2003 and from Bornholdt et al. unpublished data.

Figure 3 Median-joining networks of *Galictis cuja* mtDNA haplotypes based on the *ND5* gene (**A**) and the concatenated data set (**B**). The dimension of each circle is indicative of the haplotype absolute frequency and bars placed on connecting lines indicate the exact number of nucleotide differences between haplotypes. Green circles correspond to southern Brazilian haplotypes, orange circles to southeastern Brazilian haplotypes, and the blue circle to Argentinean samples. Grey circles correspond to different haplotypes of the outgroup (*G. vittata*). The concatenated network (**B**) indicates two groups separated by 15 mutational steps that agree with the two well-defined clades revealed by the Bayesian inference tree (see text for more details). For more information on haplotypes see Table 4 (*ND5* set) and Table 5 (concatenated set).

Figure 4 Bayesian phylogenetic tree of *Galictis cuja* constructed using the mtDNA concatenated data set haplotypes. Labels are haplotype identification numbers (see Table 5). Nodal support values are presented as Bayesian posterior probabilities. Black bars represent the two main clades (Clade 1 and Clade 2) as well as the selected outgroup (*G. vittata*). Internal grey bars

indicate sub-divisions from the Clade 1 (Sub-clade 1 and Sub-clade 2). The dotted line indicates a well-supported group formed exclusively by Rio Grande do Sul state samples (southern Brazil). Asterisks represent nodal support < 60%.

Figure 5 Discriminant Function Analysis (DFA) plot based on scores from the first (**CV1**) and the second (**CV2**) canonical variates over 15 craniodental measurements (see Table 7 for details) from specimens of *Galictis cuja*. Letters **A** (males) and **B** (females) picture DFA plots from the biome-based analysis; letters **C** (males) and **D** (females) show DFA plots from the latitude-based analysis (see legend for both analyses).

Table 1. Genetic samples of *Galictis cuja* and *G. vittata* analyzed in the study with the corresponding geographic origin. Asterisks represent absence of geographic origin information.

	Sample ID	Geographic origin
G. cuja	bGcu001	Cachoeira do Sul, Rio Grande do Sul state, Brazil
	bGcu002	São Vicente, Rio Grande do Sul state, Brazil
	bGcu003	Pelotas, Rio Grande do Sul state, Brazil
	bGcu004	Rio Grande do Sul state, Brazil
	bGcu005	Rio Grande do Sul state, Brazil
	bGcu006	Rio Grande do Sul state, Brazil
	bGcu008	Rio Grande do Sul state, Brazil
	bGcu009	Rio Grande do Sul state, Brazil
	bGcu010	Rio Grande do Sul state, Brazil
	bGcu011	Rio Grande do Sul state, Brazil
	bGcu012	Viamão, Rio Grande do Sul state, Brazil
	bGcu013	Rio Grande do Sul state, Brazil
	bGcu014	Bom Jardim da Serra, Santa Catarina state, Brazil
	bGcu015	Capão do Leão, Rio Grande do Sul state, Brazil
	bGcu016	Camaquã, Rio Grande do Sul state, Brazil
	bGcu018	Rio Grande do Sul state, Brazil
	bGcu019	Sarandi, Rio Grande do Sul state, Brazil
	bGcu021	Lajeado, Rio Grande do Sul state, Brazil
	bGcu022	Lajeado, Rio Grande do Sul state, Brazil
	bGcu023	Cristal, Rio Grande do Sul state, Brazil
	bGcu024	Arroio do Meio, Rio Grande do Sul state, Brazil
	bGcu025	São Vicente do Sul, Rio Grande do Sul state, Brazil
	bGcu026	Cristal, Rio Grande do Sul state, Brazil
	bGcu028	Cruzeiro do Sul, Rio Grande do Sul state, Brazil
	bGcu029	Pelotas – Jaguarão Road, Rio Grande do Sul state, Brazil
	bGcu030	Pelotas – Jaguarão Road, Rio Grande do Sul state, Brazil
	bGcu031	Campo Belo do Sul, Santa Catarina state, Brazil
	bGcu032	Rosário do Sul, Rio Grande do Sul state, Brazil
	bGcu033	São Pedro, Rio Grande do Sul state, Brazil
	bGcu034	Rosário do Sul, Rio Grande do Sul state, Brazil
	bGcu035	Cachoeira do Sul, Rio Grande do Sul state, Brazil
	bGcu036	Cachoeira do Sul, Rio Grande do Sul state, Brazil
	bGcu037	Cachoeira do Sul, Rio Grande do Sul state, Brazil
	bGcu038	Santa Maria, Rio Grande do Sul state, Brazil

Continued.

	bGcu039	Rio Grande do Sul state, Brazil
	bGcu040	Dom Pedrito, Rio Grande do Sul state, Brazil
	bGcu041	Rio Grande do Sul state, Brazil
	bGcu042	Cachoeira do Sul, Rio Grande do Sul state, Brazil
	bGcu043	Santana do Livramento, Rio Grande do Sul state, Brazil
	bGcu047	Curitiba, Paraná state, Brazil
	bGcu048	Campo Magro, Paraná state, Brazil
	bGcu049	Pinhão, Paraná state, Brazil
	bGcu050	Pinhão, Paraná state, Brazil
	bGcu052	Rio Grande do Sul state, Brazil
	bGcu053	São Paulo state, Brazil
	bGcu054	São Paulo state, Brazil
	bGcu055	Brasília, Distrito Federal, Brazil
	bGcu056	São Paulo state, Brazil
	bGcu058	Araguari Road, Minas Gerais state, Brazil
	bGcu060	Nonoai, Rio Grande do Sul state, Brazil
	bGcu061	Minas Gerais state, Brazil
	bGcu062	Bahia state, Brazil
	bGcu063	Santa Catarina state, Brazil
	bGcu064	Minas Gerais state, Brazil
	bGcu066	Pedro Osório, Rio Grande do Sul state, Brazil
	bGcu067	Arroio Grande, Rio Grande do Sul state, Brazil
	bGcu068	Tapes, Rio Grande do Sul state, Brazil
	bGcu069	Brazil
	bGcu071	*
	bGcu072	Oliveira, Minas Gerais state, Brazil
	bGcu073	Lagoa Santa, Minas Gerais state, Brazil
	bGcu074	Itabira, Minas Gerais state, Brazil
	bGcu075	Itabira, Minas Gerais state, Brazil
	bGcu076	Nanuque, Minas Gerais state, Brazil
	bGcu077	Pantano Grande, Rio Grande do Sul state, Brazil
	bGcu078	Bom Retiro, Santa Catarina state, Brazil
	bGcu079	Fraiburgo, Santa Catarina state, Brazil
	bGcu080	Rio Grande do Sul state, Brazil
	bGcu081	Rio Grande do Sul state, Brazil
	bGcu086	Dom Pedrito, Rio Grande do Sul state, Brazil

Continued.

	bGcu087	Estrada do Sol Road, Rio Grande do Sul state, Brazil
	Gcu001	Argentina
	Gcu002	Argentina
	Gcu003	Argentina
	Gcu004	Argentina
	Gcu005	Argentina
	Gcu006	Argentina
	Gcu008	Argentina
Galictis vittata	bGvi010	Cayenne region, French Guiana
	bGvi011	Cayenne region, French Guiana
	bGvi015	Cayenne region, French Guiana
	bGvi155226	Peru
	bGvi157992	Peru
	bGvi0136	Peru

Table 2. Zoological collection assessed in the study, containing all morphological specimens ID of *Galictis cuja* from each collection, corresponding sex, geographic origin, and the two geographic groups from the morphometric analyses (Biomes and Latitudes). Asterisks represent vague or absence of geographic origin information.

Zoological collection	Specimen ID	Sex	Geographic origin	Biomes	Latitudes
AMNH - American Museum of Natural History, New York, USA	205832	Female	Uruguay	Pampas	*
	235992	Male	Candelária, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	33281	Male	Temuco, Chile	Valdivian Temperate Forest	36° - 41°
	38983	Female	Bolivia	*	*
	70339	Male	*	*	*
	80037	Male	*	*	*
ANSP - The Academy of Natural Sciences of Philadelphia, Philadelphia, USA	00841	Female	Brazil	*	*
BMNH - Natural History Museum, London, England	011152	Male	Valparaiso, Chile	Chilean Matorral	31° - 36°
	1110165	Male	Temuco, Chile	Valdivian Temperate Forest	36° - 41°
	122176	Male	Mar del Plata, Argentina	Argentinean Savannas	36° - 41°
	122177	Female	Mar del Plata, Argentina	Argentinean Savannas	36° - 41°
	1223	Male	Valparaiso, Chile	Chilean Matorral	31° - 36°
	127121	Male	Quilpue, Chile	Chilean Matorral	31° - 36°
	127122	Female	*	*	*
	12831	Female	*	*	*
	1681	Male	Quillota, Chile	Chilean Matorral	31° - 36°
	1682	Male	Quillota, Chile	Chilean Matorral	31° - 36°
	171254	Male	Cordoba, Argentina	Argentinean Savannas	31° - 36°
	171255	Male	Cordoba, Argentina	Argentinean Savannas	31° - 36°
	171257	Female	Noetinger, Argentina	Argentinean Savannas	31° - 36°

Continued.

	17534	Female	San Cristóbal, Argentina	Argentinean savannas	26° - 31°
	216191	Male	Pedernal, Argentina	Montane Grasslands and Shrublands	31° - 36°
	249184	Female	Mercedes, Uruguay	Pampas	31° - 36°
	261291	Male	Concepción, Argentina	Argentinean and Bolivian Forests	26° - 31°
	2810115	Male	Santa Catarina state, Brazil	Atlantic Forest	26° - 31°
	2811141	Female	Santa Catarina state, Brazil	Atlantic Forest	26° - 31°
	28591	Male	Concepción, Argentina	Argentinean and Bolivian Forests	26° - 31°
	28592	Male	Concepción, Argentina	Argentinean and Bolivian Forests	26° - 31°
	294291	Male	Minas Gerais state, Brazil	*	*
	29664	Male	Santa Catarina state, Brazil	Atlantic Forest	26° - 31°
	3411415	Male	Salta, Argentina	Argentinean and Bolivian Forests	21° - 26°
	44376210f	Male	Minas Gerais state, Brazil	*	*
	992229	Male	Chubut, Argentina	Argentinean Savannas	41° - 46°
	103113	Female	Chile	*	*
FMNH - The Field Museum of Natural History, Chicago, USA	23441	Female	Papudo, Chile	Chilean Matorral	31° - 36°
	23445	Female	Buenos Aires, Argentina	Argentinean Savannas	31° - 36°
	23919	Male	Papudo, Chile	Chilean Matorral	31° - 36°
	51882	Female	Chapare, Bolivia	Montane Grasslands and Shrublands	16° - 21°
	94316	Male	São Paulo state, Brazil	*	*
	94317	Female	São Paulo state, Brazil	*	*
FZB/RS - Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Brazil	02660	Male	Bagé, Rio Grande do Sul state, Brazil	Pampas	31° - 36°
	02765	Female	Bagé, Rio Grande do Sul state, Brazil	Pampas	31° - 36°
	03034	Male	BR293 Road, Rio Grande do Sul state, Brazil	Pampas	26° - 31°

Continued.

	03035	Male	BR293 Road, Rio Grande do Sul state, Brazil	Pampas	26° - 31°
	03065	Male	RS389 Road, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	03066	Female	Brazil	*	*
LAMAq-UFSC - Laboratório de Mamíferos Aquáticos da Universidade Federal de Santa Catarina, Florianópolis, Brazil	00399	Male	Tijucas, Santa Catarina state, Brazil	Atlantic Forest	26° - 31°
	00785	Female	Tubarão, Santa Catarina state, Brazil	Atlantic forest	26° - 31°
	03182	Male	Porto Belo, Santa Catarina state, Brazil	Atlantic Forest	26° - 31°
LSUMNS - The Louisiana State University Museum of Natural Science, Baton Rouge, USA	16947	Female	El Bolson, Argentina	Valdivian Temperate Forest	41° - 46°
	16948	Female	El Bolson, Argentina	Valdivian Temperate Forest	41° - 46°
	16949	Female	Chubut, Argentina	Argentinean Savannas	41° - 46°
MACN - Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina	03095	Male	Concepción, Argentina	Argentinean and Bolivian Forests	26° - 31°
	03096	Male	Concepción, Argentina	Argentinean and Bolivian Forests	26° - 31°
	13498	Female	Neuquén, Argentina	Argentinean Savannas	36° - 41°
	13939	Female	Chubut, Argentina	Argentinean Savannas	41° - 46°
	16520	Male	Cushamen, Chubut, Argentina	Argentinean Savannas	41° - 46°
MCZ - Museum of Comparative Zoology, Harvard University, Cambridge, USA	19219	Male	Buenos Aires, Argentina	Argentinean Savannas	31° - 36°
MLP - Museo de La Plata, La Plata, Argentina	01014	Male	Argentina	*	*
	01588	Male	Argentina	*	*
	15v97	Female	Buenos Aires, Argentina	Argentinean Savannas	31° - 36°

Continued.

MNHN - Museu Nacional de História Natural, Rio de Janeiro, Brazil	01498	Female	Monte Alegre, Pernambuco state, Brazil	Caatinga	06° - 11°
	01882	Male	Campo Grande, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	03114	Male	Buenos Aires, Argentina	Argentinean Savannas	31° - 36°
	03127	Female	Itatiaia, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	04845	Male	Anápolis, Goiás state, Brazil	Cerrado	16° - 21°
	05809	Female	Caxias, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	08236	Female	Parati, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	08238	Female	Parati, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	25684	Male	Juazeiro do Norte, Ceará state, Brazil	Caatinga	06° - 11°
	29983	Female	Bahia state, Brazil	*	*
	29984	Female	Taubaté, São Paulo state, Brazil	Atlantic Forest	21° - 26°
	29985	Male	Nova Iguaçu, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	29988	Female	Palmeira dos Indios, Alagoas state, Brazil	Caatinga	06° - 11°
	29998	Male	Bodocó, Pernambuco state, Brazil	Caatinga	06° - 11°
	29999	Male	Princeza Izabel, Paraíba state, Brazil	Caatinga	06° - 11°
	30001	Male	São João da Glória, Minas Gerais state, Brazil	Cerrado	16° - 21°
MNHNA - Museo Nacional de Historia Natural y Antropologia, Montevideo, Uruguay	00296	Female	Soriano, Uruguay	Pampas	31° - 36°
	01158	Female	Tacuarembó, Uruguay	Pampas	31° - 36°
	02333	Male	Playa Pascual, Uruguay	Pampas	31° - 36°
	02548	Female	Uruguay	Pampas	*
	02690	Male	Colônia, Uruguay	Pampas	31° - 36°
	02696	Male	Playa Pascual, Uruguay	Pampas	31° - 36°
MPEG - Museu Paraense Emilio Goeldi, Belém, Brazil	00538	Female	*	*	*
	22188	Male	Barracão, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°

Continued.

	22229	Female	Cachoeira do Sul, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	22230	Female	Gramado, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
MVZ - Museum of Vertebrate Zoology, Berkeley, USA	114774	Male	Puno, Peru	Montane Grasslands and Shrublands	16° - 21°
	85164	Male	Mangaratiba, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
MZUSP - Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil	00227	Female	São Lourenço do Sul, Rio Grande do Sul state, Brazil	Pampas	31° - 36°
	00978	Female	São Paulo, São Paulo state, Brazil	Atlantic Forest	21° - 26°
	01044	Male	São Lourenço do Sul, Rio Grande do Sul state, Brazil	Pampas	31° - 36°
	01247	Male	Entre Rios, Rio de Janeiro state, Brazil	Atlantic Forest	21° - 26°
	03066	Male	Pirapora, Minas Gerais state, Brazil	Cerrado	16° - 21°
	06463	Male	São Paulo, São Paulo state, Brazil	Atlantic Forest	21° - 26°
	08454	Male	Coremas, Paraíba state, Brazil	Caatinga	06° - 11°
	09633	Male	Santos, São Paulo state, Brazil	Atlantic Forest	21° - 26°
	10331	Male	Avaré, São Paulo state, Brazil	Cerrado	21° - 26°
	13468	Male	Cachoeira, Bahia state, Brazil	Atlantic Forest	11° - 16°
SMT - Staatliches Museum für Tierkunde, Dresden, Germany	B3962	Male	Valdivia, Chile	Valdivian Temperate Forest	36° - 41°
UFPE - Coleção de Mamíferos da Universidade Federal de Pernambuco, Recife, Brazil	00977	Female	São Lourenço da Mata, Pernambuco state, Brazil	Atlantic Forest	06° - 11°
ULBRA - Museu de Ciências Naturais da Universidade Luterana do Brasil, Canoas, Brazil	00031	Male	Rio Grande do Sul state, Brazil	*	*
	00071	Female	Barra do Quaraí, Rio Grande do Sul state, Brazil	Pampas	26° - 31°
	00073	Male	Pantano Grande, Rio Grande do Sul state, Brazil	Pampas	26° - 31°

Continued.

	00483	Male	Cachoeira do Sul, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	00627	Male	São Pedro, Rio Grande do Sul state, Brazil	Pampas	26° - 31°
	00746	Female	Santa Maria, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	00748	Male	BR290 Road, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	00749	Female	BR116 Road, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
	00757	Male	Bagé, Rio Grande do Sul state, Brazil	Pampas	31° - 36°
	00899	Male	Santana do Livramento, Rio Grande do Sul state, Brazil	Pampas	26° - 31°
	00928	Male	BR293 Road, Rio Grande do Sul state, Brazil	Atlantic Forest	26° - 31°
USNM - National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA	271309	Female	Valparaiso, Chile	Chilean Matorral	31° - 36°
	271573	Male	Chile	*	*
	282248	Male	Argentina	*	*
	293164	Female	Caaguazú, Paraguay	Atlantic Forest	21° - 26°
	a35259	Male	*	*	*

Table 3. Genetic diversity and neutrality tests assessed for *Galictis cuja* samples using the mtDNA control region (CR), the *ND5* gene (*ND5*), and the concatenated data set comprising these two segments. Indices of concatenated data are shown for the total sampling and for each of the three geographic groups (see main text). Significance for neutrality tests was ***P* < 0.05. Single asterisks represent absence of diversity and neutrality indices.

		N	Length (bp)	No. of variable sites	No. of haplotype	Nucleotide diversity (± SE)	Haplotype diversity (± SE)	Fu's Fs	Tajima's *D*
CR	Total	61	438	8	8	0.00571 (± 0.00077)	0.715 (± 0.050)	0.221	0.830
ND5	Total	77	645	28	18	0.00982 (± 0.00093)	0.898 (± 0.016)	-0.810	0.18
Concatenated	Total	50	1,083	32	18	0.00848 (± 0.00101)	0.923 (± 0.016)	-0.367	0.647
	Southern Brazil	34	1,083	28	10	0.00989 (± 0.00499)	0.8752 (± 0.0265)	4.823	1.899
	Southeastern Brazil	09	1,083	9	7	0.00284 (0.00185)	0.9167 (± 0.0920)	-2.546**	-0.432
	Argentina	07	1,083	0	1	*	*	*	*

Table 4. Haplotype names, corresponding individuals, absolute frequency of each haplotype and their geographic origin from the mtDNA N*D5* data set of *Galictis cuja*.

Haplotype	Individuals	F	Geographic occurrence	Geographic group
GN01	bGcu001, bGcu021, bGcu022, bGcu026, bGcu030, bGcu037, bGCu042, bGcu047, bGcu048, bGcu052, bGcu063, bGcu081, bGcu087	13	Brazil: Rio Grande do Sul / Santa Catarina / Paraná states	Southern Brazil
GN02	bGcu003	1	Brazil: Rio Grande do Sul state	Southern Brazil
GN03	bGcu004, bGCu011, bGcu012, bGcu014, bGcu024, bGcu025, bGCu028, bGcu031, bGcu049, bGcu077, bGcu086	11	Brazil: Rio Grande do Sul / Santa Catarina / Paraná states	Southern Brazil
GN04	bGCu005, bGCu006, bGCu010, bGcu015, bGcu018, bGcu023, bGcu029, bGCu035, bGCu038, bGCu067, bGcu068, bGCu069	12	Brazil: Rio Grande do Sul state	Southern Brazil
GN05	bGCu009, bGcu040, bGCu041, bGcu066, bGCu071	5	Brazil: Rio Grande do Sul state	Southern Brazil
GN06	bGCu013, bGCu039, bGcu080	3	Brazil: Rio Grande do Sul state	Southern Brazil
GN07	bGCu019, bGcu054, bGCu060, bGCu061, bGCu074, bGCu075	6	Brazil: Rio Grande do Sul / Minas Gerais / São Paulo states	Southern Brazil / Southeastern Brazil
GN08	bGcu032, bGcu033, bGCu034, bGCu043	4	Brazil: Rio Grande do Sul state	Southern Brazil
GN09	bGcu036	1	Brazil: Rio Grande do Sul state	Southern Brazil
GN10	bGcu053	1	Brazil: São Paulo state	Southeastern Brazil
GN11	bGCu055	1	Brazil: Federal District	Southeastern Brazil
GN12	bGcu056	1	Brazil: São Paulo state	Southeastern Brazil
GN13	bGcu058	1	Brazil: Minas Gerais state	Southeastern Brazil
GN14	bGCu062	1	Brazil: Bahia state	Southeastern Brazil
GN15	bGcu064	1	Brazil: Minas Gerais state	Southeastern Brazil
GN16	bGcu072	1	Brazil: Minas Gerais state	Southeastern Brazil
GN17	bGCu073	1	Brazil: Minas Gerais state	Southeastern Brazil
GN18	Gcu001, Gcu002, Gcu003, Gcu004, Gcu005, Gcu006, Gcu008	7	Argentina	Argentina

Table 5. Haplotype names, corresponding individuals, absolute frequency of each haplotype and their geographic origin from the concatenated mtDNA data set of *Galictis cuja*.

Haplotype	Individuals	F	Geographic occurrence	Geographic group
GC01	bGcu009, bGcu040, bGcu041, bGcu066, bGcu071	5	Brazil: Rio Grande do Sul state	Southern Brazil
GC02	bGcu011, bGcu025, bGcu028, bGcu031, bGcu086	5	Brazil: Rio Grande do Sul / Santa Catarina states	Southern Brazil
GC03	bGcu015, bGcu018, bGcu023, bGcu029, bGcu038, bGcu069	6	Brazil: Rio Grande do Sul state	Southern Brazil
GC04	bGcu019	1	Brazil: Rio Grande do Sul state	Southern Brazil
GC05	bGcu022, bGcu026, bGcu030, bGcu047, bGcu048, bGcu052, bGcu063, bGcu081	8	Brazil: Rio Grande do Sul / Santa Catarina / Paraná states	Southern Brazil
GC06	bGcu032, bGcu033, bGcu034, bGcu043	4	Brazil: Rio Grande do Sul state	Southern Brazil
GC07	bGcu035	1	Brazil: Rio Grande do Sul state	Southern Brazil
GC08	bGcu036	1	Brazil: Rio Grande do Sul state	Southern Brazil
GC09	bGcu039, bGcu080	2	Brazil: Rio Grande do Sul state	Southern Brazil
GC10	bGcu049	1	Brazil: Paraná state	Southern Brazil
GC11	bGcu053	1	Brazil: São Paulo state	Southeastern Brazil
GC12	bGcu054, bGcu074, bGcu075	3	Brazil: Minas Gerais / São Paulo states	Southeastern Brazil
GC13	bGcu055	1	Brazil: Federal District	Southeastern Brazil
GC14	bGcu056	1	Brazil: São Paulo state	Southeastern Brazil
GC15	bGcu058	1	Brazil: Minas Gerais state	Southeastern Brazil
GC16	bGcu072	1	Brazil: Minas Gerais state	Southeastern Brazil
GC17	bGcu073	1	Brazil: Minas Gerais state	Southeasternl Brazil
GC18	Gcu001, Gcu002, Gcu003, Gcu004, Gcu005, Gcu006, Gcu008	7	Argentina	Argentina

Table 6. Results of the *t*-test comparison for sexual size dimorphism within *Galictis cuja* based on the mean of 15 craniodental measurements. t= t value; d.f.= degrees of freedom; *P*-value= significance. Results emphasized by asterisk represent those that are statistically significant after sequential Bonferroni correction ($\alpha \leq 0.003$). See main text for variable abbreviations.

Variable	t	d.f.	*P*-value
GLS	-9.521	101	0.000*
NL	-5.143	108	0.000*
ZB	-7.418	101	0.000*
MB	-8.613	103	0.000*
BB	-5.154	105	0.000*
IC	-5.416	107	0.000*
PC	-3.372	106	0.001*
PW	-3.384	108	0.001*
BH	-6.705	104	0.000*
ML	-8.660	95	0.000*
MH	-8.703	116	0.000*
C-M2	-6.547	113	0.000*
C-C	-7.503	111	0.000*
M2-M2	-7.065	109	0.000*
c-m2	-8.674	112	0.000*

Table 7. Summary of the biome-oriented Discriminant Function Analysis (DFA) for males and females of *Galictis cuja*: discriminant loadings for each craniodental measurement, eigenvalue, cumulative variance, canonical correlation, and Chi-square statistics of the first two canonical variates (represented in Figure 6A-B). Discriminant loadings highlighted in bold represent those with greater contribution to the first canonical variate. See main text for variable abbreviations.

DFA - Males			**DFA - Females**		
Variable	CV1	CV2	Variable	CV1	CV2
GLS	-1.857	0.283	GLS	1.892	-2.573
NL	0.662	-0.13	NL	-0.408	1.107
ZB	5.875	-2.401	ZB	-1.225	0.945
MB	-2.337	-0.61	MB	0.483	0.807
BB	0.097	1.155	BB	-0.834	1.723
IC	-0.668	0.595	IC	-0.688	0.743
PC	0.692	0.041	PC	1.967	-1.322
PW	-0.418	0.609	PW	1.959	-0.664
BH	-1.576	-0.507	BH	1.804	-0.274
ML	-2.674	2.186	ML	-2.776	1.388
MH	2.424	-1.003	MH	-0.908	-0.794
C-M2	0.574	-0.917	C-M2	-1.464	-0.888
C-C	-2.287	0.705	C-C	3.399	-2.151
M2-M2	0.502	0.693	M2-M2	-0.962	0.727
c-m2	1.7	-0.056	c-m2	-0.512	1.852
Eigenvalue	7.207	3.214	Eigenvalue	21.075	5.22
Cumulative variance (%)	50.5	73.1	Cumulative variance (%)	69.1	86.2
Canonical correlation	0.937	0.873	Canonical correlation	0.977	0.916
Wilks' lambda	0.002	0.016	Wilks' lambda	0.001	0.011
Chi-square statistic	156.334	103.708	Chi-square statistic	128.919	76.313
d.f.	120	98	d.f.	90	70
P-value	0.014*	0.327	*P*-value	0.004*	0.283

Table 8. Summary of latitude-orientated Discriminant Function Analysis (DFA) for males and females of *Galictis cuja*: discriminant loadings for each craniodental measurement, eigenvalue, cumulative variance, canonical correlation, and Chi-square statistics of the first two canonical variates (represented in Figure 6C-D). Discriminant loadings highlighted in bold represent those with greater contribution to the first canonical variate. See main text for variables abbreviations.

DFA - Males			**DFA - Females**		
Variable	CV1	CV2	Variable	CV1	CV2
GLS	-.228	-1.607	GLS	-1.541	1.73
NL	0.346	0.069	NL	-0.204	-0.487
ZB	-3.573	1.787	ZB	-1.512	-1.599
MB	-1.404	-1.152	MB	-1.143	0.88
BB	1.404	-0.007	BB	-.855	1.12
IC	1.232	-0.56	IC	0.074	1.364
PC	.059	0.793	PC	1.834	-0.435
PW	0.138	-0.136	PW	0.496	0.647
BH	0.783	0.114	BH	1.72	-0.661
ML	1.328	0.239	ML	1.725	-0.919
MH	-1.185	0.689	MH	0.415	0.579
C-M2	-1.214	-0.283	C-M2	-1.158	-0.525
C-C	1.204	-0.554	C-C	2.495	-1.809
M2-M2	1.148	-0.015	M2-M2	-2.467	2.22
c-m2	0.923	1.31	c-m2	1.312	-1.208
Eigenvalue	3.716	1.724	Eigenvalue	15.887	4.708
Cumulative variance (%)	45	65.9	Cumulative variance (%)	66	85.5
Canonical correlation	0.888	0.796	Canonical correlation	0.97	0.908
Wilks' lambda	0.01	0.046	Wilks' lambda	0.001	0.2
Chi-square statistic	115.549	76.776	Chi-square statistic	97.45	56.46
d.f.	90	70	d.f.	75	56
P-value	0.036	0.271	*P*-value	0.042	0.457

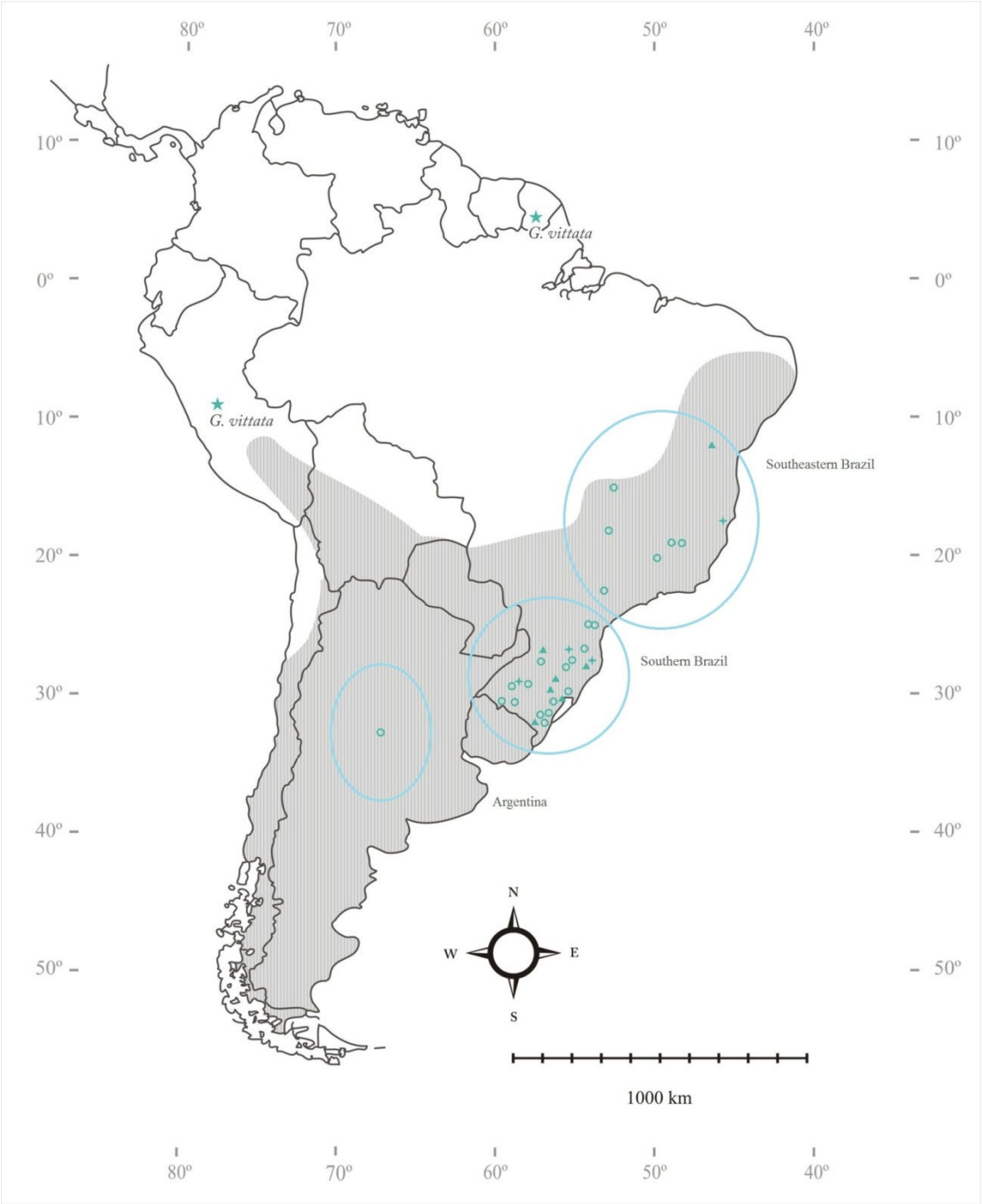

80º
70º
60º
50º
40º
10º
0º
10º
20º
30º
40º
50º
G. vittata
G. vittata
Southeastern Brazil
Southern Brazil
Argentina
N
W
E
S
1000 km

Figure 1

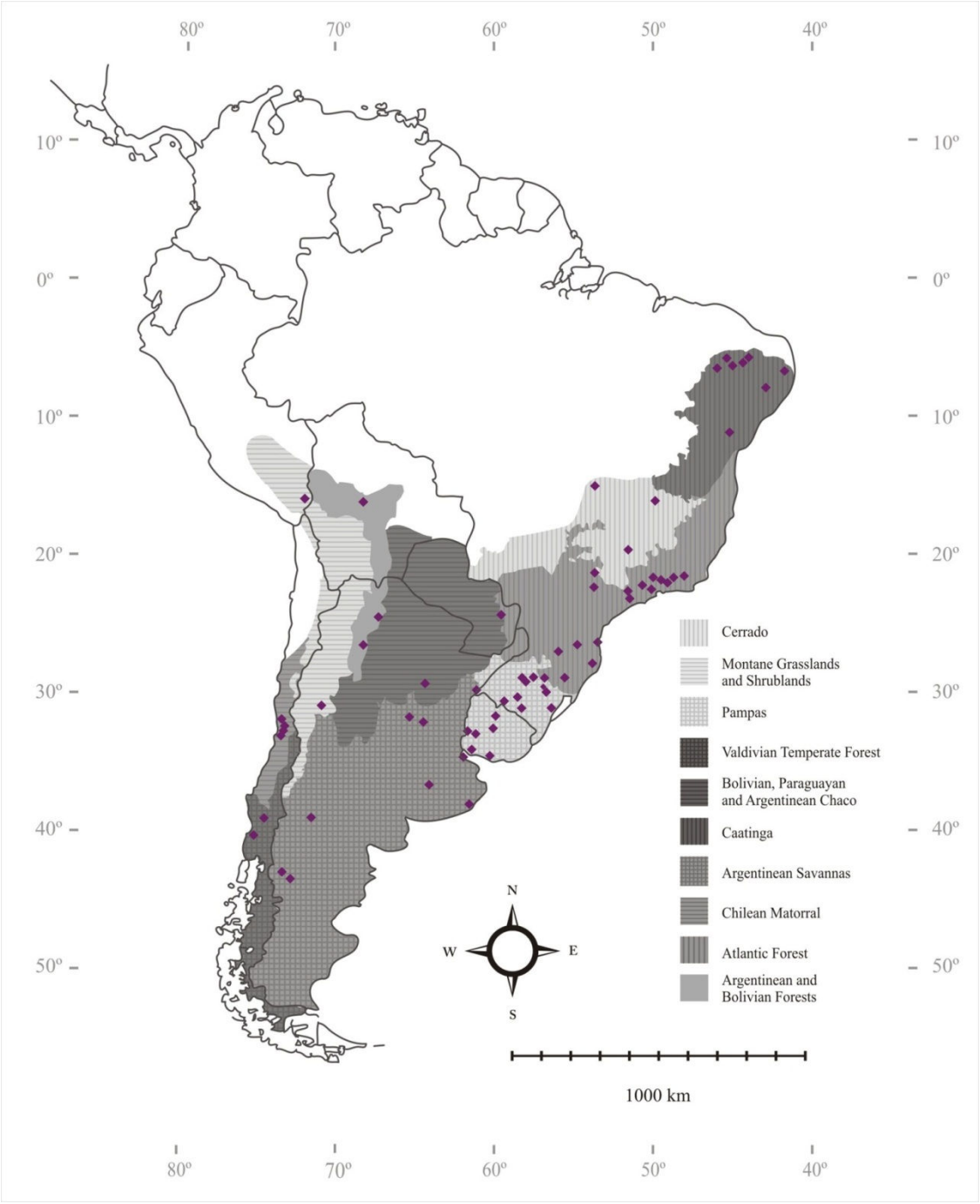
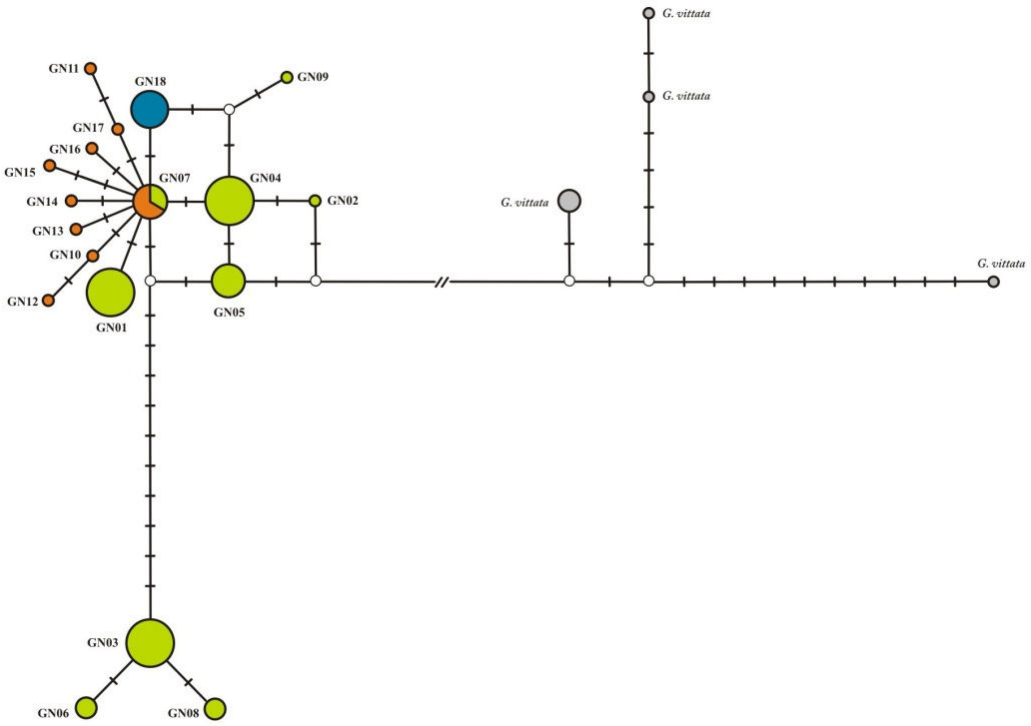

80º
70º
60º
50º
40º
10º
0º
10º
20º
30º
40º
50º
Cerrado
Montane Grasslands and Shrublands
Pampas
Valdivian Temperate Forest
Bolivian, Paraguayan and Argentinean Chaco
Caatinga
Argentinean Savannas
Chilean Matorral
Atlantic Forest
Argentinean and Bolivian Forests
N
W
E
S
1000 km

Figure 2

A


G. vittata
G. vittata
G. vittata
G. vittata
GN11
GN18
GN09
GN17
GN16
GN15
GN07
GN04
GN14
GN02
GN13
GN10
GN12
GN01
GN05
GN03
GN06
GN08

B

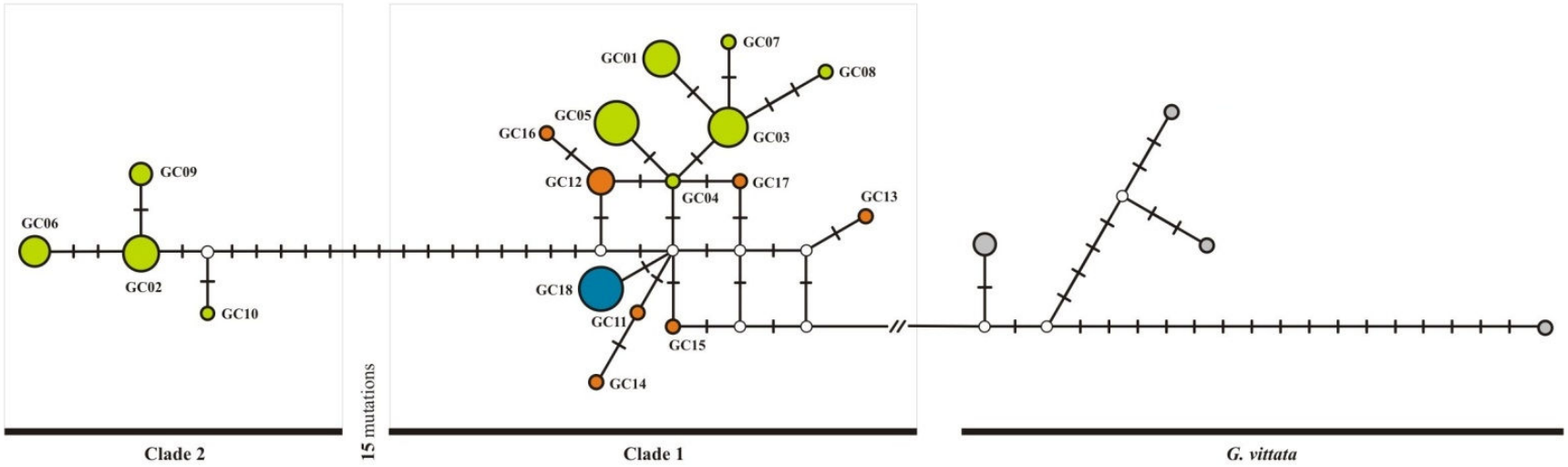

GC01
GC07
GC08
GC05
GC16
GC03
GC12
GC17
GC04
GC13
GC09
GC06
GC02
GC10
GC18
GC11
GC15
GC14
15 mutations
Clade 2
Clade 1
G. vittata

Figure 3

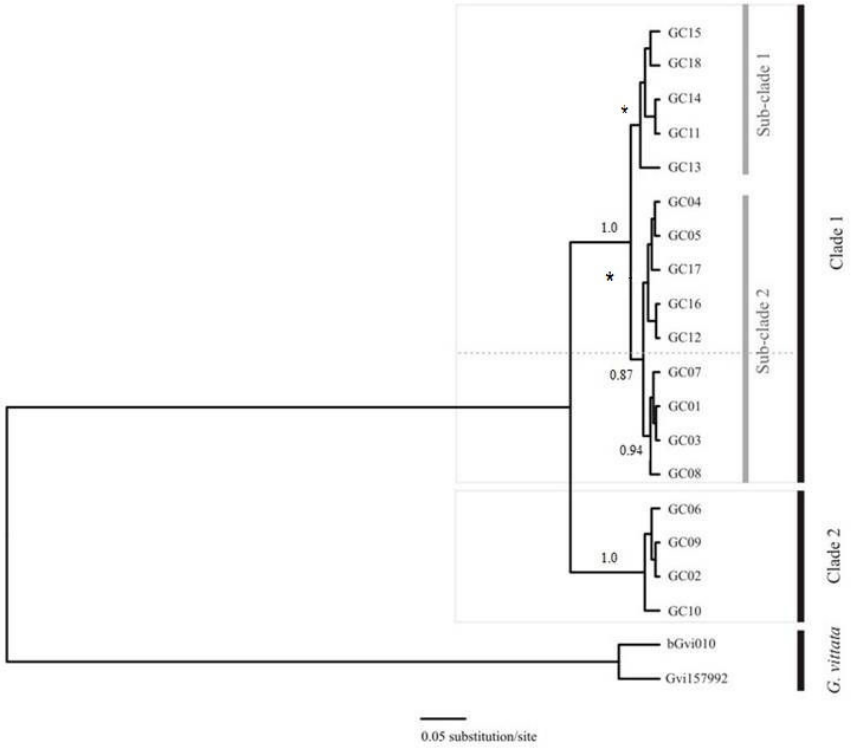

GC15
GC18
GC14
GC11
GC13
GC04
GC05
GC17
GC16
GC12
GC07
GC01
GC03
GC08
GC06
GC09
GC02
GC10
bGvi010
Gvi157992
1.0
*
*
0.87
0.94
1.0
Sub-clade 1
Sub-clade 2
Clade 1
Clade 2
G. vittata
0.05 substitution/site

Figure 4

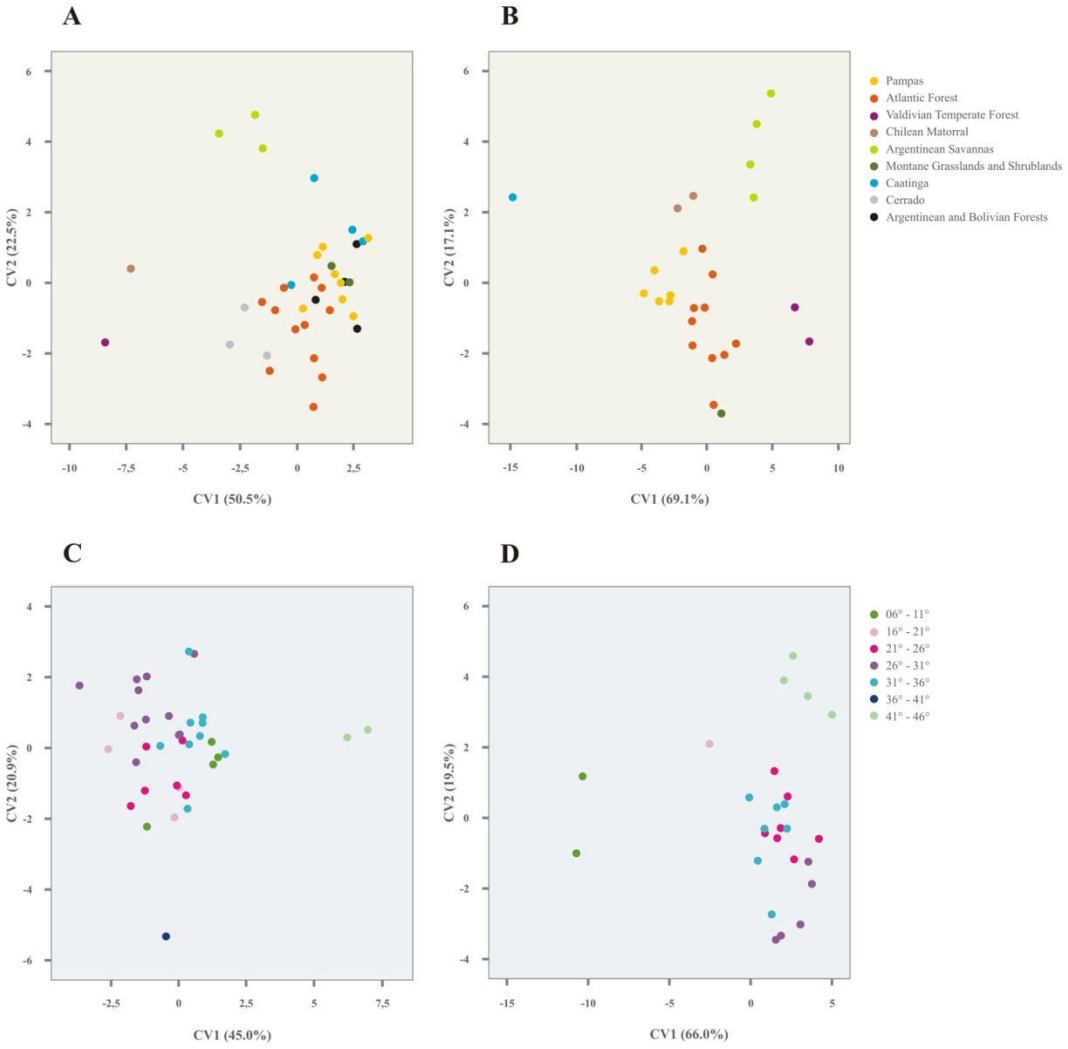
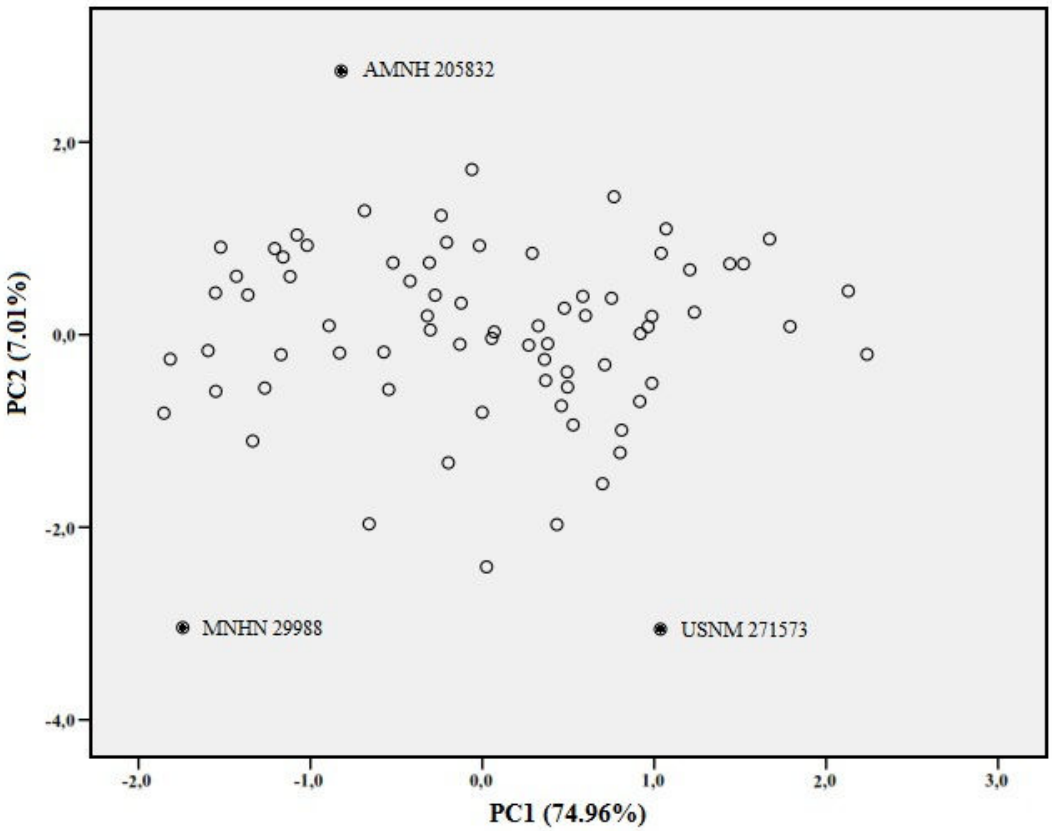

A
B
C
D
CV2 (22.5%)
CV1 (50.5%)
CV2 (17.1%)
CV1 (69.1%)
CV2 (20.9%)
CV1 (45.0%)
CV2 (19.5%)
CV1 (66.0%)
Pampas
Atlantic Forest
Valdivian Temperate Forest
Chilean Matorral
Argentinean Savannas
Montane Grasslands and Shrublands
Caatinga
Cerrado
Argentinean and Bolivian Forests
06° - 11°
16° - 21°
21° - 26°
26° - 31°
31° - 36°
36° - 41°
41° - 46°

Figure 5



Supplementary Material 1. Principal Component Analysis (PCA) projection based on scores from the first (**PC1**) and second (**PC2**) principal components over 15 craniodental measurements from specimens of *Galictis cuja*. Three specimens are emphasized by black circles (with the corresponding ID), representing those with some deviation from the central cluster (see main text for details and Table 2 for a complete sample ID list).

Capítulo IV: Conclusões Gerais

A presença de duas espécies no gênero *Galictis* (*G. cuja* e *G. vittata*) foi confirmada através de métodos morfológicos e moleculares. A existência de dois grupos morfológicos, assim como a existência de dois clados bem definidos e apoiados por segmentos mitocondriais e nucleares corroboram as duas unidades do gênero e descartam a existência de um terceiro táxon nas áreas amostradas. De forma geral, essas duas espécies de mustelídeos neotropicais se assemelham fenotipicamente. Contudo, análises morfológicas mais profundas descrevem principalmente dois caracteres de diagnose substanciais: a presença/ausência do metaconídeo no primeiro molar inferior (m1) e o maior/menor tamanho. Por um lado, *G. vittata* possui essa cúspide adicional no m1 e é significativamente maior, por outro, *G. cuja* é a espécie que não carrega o metaconídeo e é menor em todas as 15 medidas lineares, assim como no comprimento total do corpo. As seguintes medidas lineares se destacam para a diagnose das espécies: comprimento total do crânio, largura zigomática, largura da caixa craniana, comprimento da mandíbula e comprimento da série de dentes inferiores. A análise da pelagem se mostrou um caráter menos eficiente de diagnose, mas padrões gerais podem auxiliar na identificação de espécimes de museus e também daqueles avistados na natureza. *G. cuja* possui coloração densa e com tons amarelados e *G. vittata* possui pêlos curtos e sua coloração é consistentemente acinzentada. Com as espécies definidas, a revisão detalhada da distribuição geográfica de *G. cuja* e *G. vittata* esclarece as controvérsias sobre os limites das espécies e resolve a presença de *G. cuja* no nordeste do Brasil (uma das principais dúvidas sobre a distribuição desses animais). Os pontos de provável contato entre as duas espécies localizam-se na porção sul do Peru, Bolívia, Paraguai e centro do Brasil, contudo a exata área de simpatria ainda não é totalmente clara. A ausência de registros científicos em coleções zoológicas de espécimes de *Galictis* e a pouca amostragem genética para os estados brasileiros de Maranhão, Piauí, Mato Grosso e Mato Grosso do Sul permanecem como um dos grandes complicadores para o entendimento das áreas de contato entre as espécies. Apesar disso, a distribuição de *G. cuja* limita-se ao norte no sul do Peru, metade sul da Bolívia, Paraguai e, no Brasil, essa espécie avança para os estados nordestinos. No sul, os pontos mais austrais foram para a Província de Chubut, na Argentina, embora a literatura mostre registros para áreas mais extremas na Patagônia (ex. Prevosti & Travaini, 2005). Por outro lado, *G. vittata* ocorre desde o extremo norte da Região Neotropical (metade sul do México) até o leste peruano, Bolívia e Paraguai. No Brasil, esta espécie parece estar restrita à Bacia Amazônica. A ocupação das duas espécies entre os biomas Amazônia e Cerrado, no entanto, ainda não está bem determinada. Esses resultados

contribuem para estabelecer aspectos básicos desses mustelídeos pouco conhecidos e contribuir para o entendimento da biologia dos carnívoros neotropicais.

A análise molecular intraespecífica de *G. cuja* identificou considerável variabilidade genética na espécie, e demonstrou um padrão filogeográfico interessante, com a presença de dois grupos bem divergentes e sem padrão geográfico exato. A variação morfológica dessa espécie indicou estrutura geográfica localizada e importante dimorfismo sexual no tamanho, onde os machos são significativamente maiores do que as fêmeas. Esses resultados são os primeiros passos para o entendimento dessa espécie e levanta possibilidades de estudo para a compreensão da história evolutiva de *G. cuja* na América do Sul. O mesmo estudo intraespecífico para *G. vittata* não foi possível devido ao pequeno número de amostras genéticas disponível para essa espécie. Para realizar essa documentação, será necessário um aumento de esforço amostral, o que se apresenta como uma perspectiva importante de trabalho futuro nesta área.

Referência

Prevosti FJ, Travaini A. 2005. New records of *Galictis cuja* (Molina, 1782) (Carnivora, Mustelidae) in Southern Patagonia. *Mammalian Biology* **70:** 317-320.