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

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Although critical for enabling in-depth evolutionary, ecological, or conservation-orientated 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 geographical distribution are still uncertain. This is the case for 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 reviewed and consolidated 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 nonmetric 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 geographical 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.

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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 prerequisite for downstream studies aimed toward better understanding and conserving biodiversity (Wilson, 1992, 2005; Reeder, Helgen & Wilson, 2007). Even amongst 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 of 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.

Amongst 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 geographical 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 harbouring one of the richest hotspots of mammalian diversity worldwide (Olson *et al*., 2001; Schipper *et al*., 2008). Amongst mammalian species, some carnivoran genera are endemic to the Neotropics, including some groups that have so far received very little taxonomic and biogeographical attention. This is the case for 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: *Galictis vittata* (greater grison) (Schreber, 1776) and *Galictis 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 characterize better 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 that 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 of 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 *Mustela 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 *Viverra vittata*, *Viverra cuja*, and *Viverra 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 that he named *Grison*, with *V. vittata* as the type species. This generic name was followed by some authors over 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, *Galictis allamandi*, based on the museum specimen that he had observed. 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 (1847) considered Molina's *M. quiqui* to be 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*. Subsequently, some authors recognized *M. cuja* and *M. quiqui* to be synonyms of *G. vittata* (Gray, 1865, 1869). A few decades later, Thomas (1907) separated *G. vittata* from the Chilean species and considered the latter two (*G. cuja* and *G. quiqui*) to be synonyms of each other. 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 on-going 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 south-eastern 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 geographical distribution of these taxa. For instance, some authors have considered the southern limit of *G. vittata* to be in north-eastern Brazil (e.g. Mares *et al*., 1981; Emmons & Feer, 1997; Larivière & Jennings, 2009), whereas others have indicated that it is in south-eastern 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 geographical reference source for mammals (Wozencraft, 2005). Similar confusion surrounds the distribution of *G. cuja*, with some sources listing its occurrence as far north as north-eastern Brazil (Yensen & Tarifa, 2003b), whereas others indicate its northernmost limit on the Atlantic coast to be in south-eastern 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; Canevari & Vaccaro, 2007; Cuarón, Reid & Helgen, 2008; Reid & Helgen, 2008; Larivière & Jennings, 2009), highlighting the current lack of knowledge regarding their geographical 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 geographical 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 were 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 geographical distribution.

MATERIAL AND METHODS

In order to achieve a comprehensive assessment of genus *Galictis,* we analysed morphological aspects of museum specimens, as well as molecular data collected from fresh tissue samples. On the basis of the ascertained geographical 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 Appendix 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 geographical 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 geographical 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 (MAL), 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 categorical 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). As we observed a strong pattern of sexual size dimorphism in this genus (see Appendix 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. Initially, all measurements were log-transformed so as to reduce their variance and thus perform a more conservative assessment of group differences. We then performed a principal component analysis (PCA) of the correlation matrix of log-transformed variables. We used a plot of principal component 1 (PC1) against PC2 to visually assess the number of distinctive clusters. Then we conducted discriminant function analyses (DFA) using two different approaches: (1) two-group discriminant function, where we analysed male and female data sets separately, in each case considering the two species-level clusters identified by the PCA (see Results); (2) multiple-group discriminant function, in which we performed a joint analysis of the full data set considering four grouping variables, corresponding to males and females of each species. In both cases, the assumptions of the DFA (normality of the variables, homogeneity amongst the covariance matrices, low impact of multicolinearity, and independence of the samples) were assessed and found to be adequately met by our data sets.

Groups identified by the PCA 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 v. 17 (SPSS Statistics for Windows, Rel. 17.0.0, Chicago, USA).

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 Appendix 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 Appendix S3 for GenBank accession numbers). These four taxa are part of the Ictonychinae, one of the mustelid subfamilies defined on the basis of recent analyses of DNA sequences (Koepfli *et al*., 2008; Wolsan & Sato, 2010; Sato *et al*., 2012).

Mitochondrial gene segments

We amplified a segment of the mitochondrial gene *nicotinamide adenine dinucleotide dehydrogenase subunit 5* (*ND5*) via PCR using the primers ND5-DF1 and ND5-DR1 (Trigo *et al*., 2008). PCR reactions were performed in a 20 μL final volume containing 10–100 ng of genomic DNA, 1 × PCR Buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1 U of Taq Platinum DNA polymerase (Invitrogen), and 0.2 μM of each primer. The PCR conditions were the following: ten touchdown cycles of 94 °C for 45 s, 60–51 °C for 45 s (with a decrease in annealing temperature of 1 °C per cycle), and 72 °C for 1.5 min; followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1.5 min, and a final extension of 72 °C 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 analysed 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 JX570686–JX570694) 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, which was also used to assess genetic distances amongst sequences. Phylogenetic analyses were performed using three different criteria: maximum parsimony (MP); maximum likelihood (ML); and Bayesian inference (BI). The MP and ML analyses were conducted using PAUP* 4.0b10 (Swofford, 2002), whereas 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) was implemented in the ML and BI analyses. The ML analysis employed a heuristic search using ten replicates of random taxon addition (keeping one tree per replicate), followed by tree bisection-reconnection (TBR) branch-swapping. The MP phylogeny was based on a heuristic search using 50 replicates of random taxon addition (also keeping one tree per replicate) and TBR branch-swapping. Nodal support for the ML and MP methods was assessed with 500 pseudoreplicates of nonparametric 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 95 °C for 3 min; followed by six cycles of 94 °C for 15 s, 60 to 50 °C for 30 s, with a decrease in annealing temperature of 2 °C per cycle, and 72 °C for 45 s; followed by 30 cycles of 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 45 s; and one cycle of 72 °C for 30 min. Each 15 μL reaction contained 6.98 μL of sterile double-distilled water, 1.5 μL of 10 × PCR Gold Buffer, 1.2 μL 25 mM $MgCl_2$, 1.2 μL of 10 mM dNTP mix, 1.5 μL of both 2 μM forward and reverse primers, 1 U of AmpliTaq Gold *Taq* polymerase (Applied Biosystems, Foster City, CA, USA), and 100 ng genomic DNA; (B) one cycle of 95 °C for 10 min; followed by 16 cycles of 94 °C for 1 min, 63 to 50.2 °C for 1 min, with a decrease in annealing temperature of 0.8 °C per cycle, and 72 °C for 1.5 min; followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min; and one cycle of 72 °C for 5 min. Each 25 μL reaction contained 14.9 μL of sterile double-distilled water, 2.5 μL of 10 × PCR Gold Buffer, 1.5 μL 25 mM $MgCl_2$, 1.0 μL dimethyl sulphoxide, 2.0 μL of 10 mM dNTP mix, 1.0 μL of both 2 μM forward and reverse primers, 1 U AmpliTaq Gold *Taq* polymerase, and 100 ng genomic DNA. A negative control (no DNA) was included with all PCRs. Amplification products were electrophoresed in 1% agarose/Tris-acetic acid-ethylenediaminetetraacetic acid gels and stained with ethidium bromide. A 100 bp 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) 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 v. 5.3 software package (Drummond *et al.*, 2010). Owing to the presence of two mononucleotide repeats within the *Wilms tumour 1* (*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 KC523394–KC523454). 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: MP and ML implemented in PAUP*, along with BI performed with MrBayes. The best-fit model of nucleotide substitution (selected by the AIC as implemented in MODELTEST) was that of Hasegawa, Kishino & Yano (1985) with a proportion of invariant sites. The ML analysis employed a heuristic search starting with a neighbour-joining tree and followed by TBR branch-swapping. Nodal support was estimated by performing 100 bootstrap pseudoreplicates based on the same search strategy. The MP trees were retrieved with a heuristic search using 50 replicates of random taxon addition (keeping one tree per replicate) and TBR branch-swapping. Branch support for the MP tree was assessed with 500 replicates of bootstrapping incorporating the same search profile. 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% discarded as burn-in.

Geographical analyses

The recorded localities for all reliably identified *Galictis* specimens (see above for exclusion criteria) were used to build a geographical database of species occurrence. For this purpose, we used the exact locality information available in all specimen tags that included geographical coordinates. However, in most cases we had to assign coordinates based on other

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

Abbreviated gene name	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 tumour 1	GAGAAACCATACCAGTGTGA	GTTTTACCTGTATGAGTCCT	Exon–intron	Venta *et al.*, 1996

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 v. 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 geographical information (e.g. a large country or a very long river) that they could not be reliably used for geographical referencing and were thus excluded from this analysis. The resulting database was then analysed with a geographical 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 geographical range of each species, to assess possible areas of sympatry between them, and to investigate the presence of these taxa across Neotropical biomes.

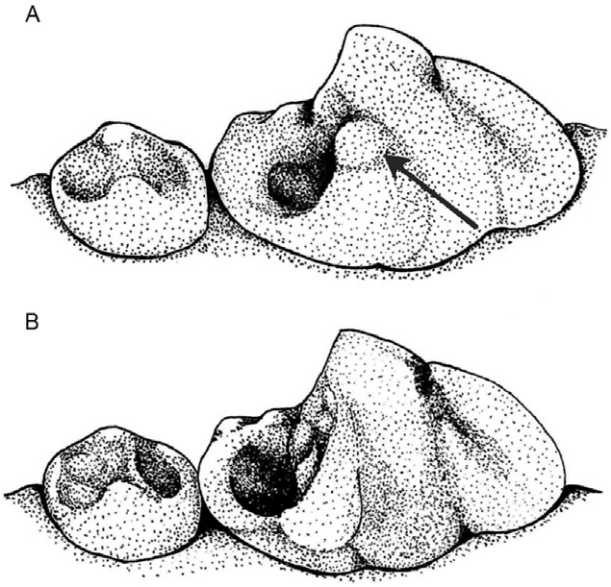

Figure 1. First and second lower molars from (A) *Galictis vittata* and (B) *Galictis cuja* in lingual view, emphasizing the presence of the metaconid (indicated by an arrow) in the former species. The metaconid constitutes a diagnostic character to distinguish the two *Galictis* species (see main text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

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 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, MAL, and c-m2 (males) and GLS, ZB, MAL, and c-m2 (females), with this separation thus reflecting differences in overall skull size.

The DFA corroborated and extended the results of the PCA. The means of 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 classification analysis (using the conservative cross-validation procedure) with the two-group approach (which considered each sex separately) was 100% accurate in correctly classifying the males In the case of females, there was a single *G. cuja* misclassified as *G. vittata* (see Appendix 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 signifi-

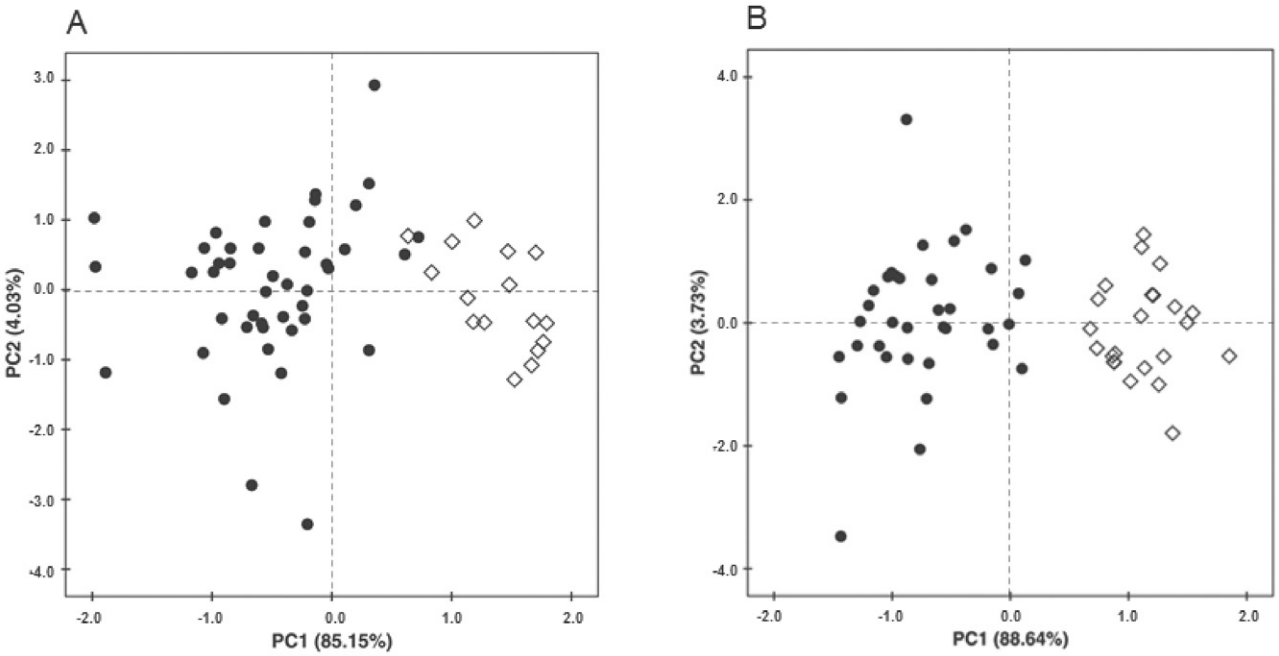

Figure 2. Principal component analysis projection based on scores from the first (PC1) and second (PC2) principal components for 15 craniodental measurements (see Table 2 for details) from specimens of genus *Galictis*: *Galictis cuja* (black circles) and *Galictis vittata* (white diamonds). A, male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

cant (Table 2). The first canonical variate (CV1) accounted for 90.8% of the discriminatory power between clusters, whereas 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 (varying along CV1), 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 with the multiple-group DFA yielded an overall correct classification of 76.3% (using the cross-validation procedure). Almost all cases of misclassification occurred between sexes of the same species, with a single event of exchange between *G. cuja* and *G. vittata*. In this case, one *G. cuja* female was classified as a *G. vittata* female. The complete function extracted from the classification coefficients is shown in Appendix S4.

Given the identification of distinct clusters by the PCA, we surveyed the range of all linear measurements observed in each group, 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, MAL, 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 that seems to fit the species-level distinction demonstrated by the linear measurements, although we did not perform statistical tests here because of 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 greyish (varying from pale grey to medium-dark grey), never

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 Fig. 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 Fig. 3)

	PCA					
	Males		Females		Multiple-group DFA	
Variable	PC1	PC2	PC1	PC2	CV1	CV2
GLS	**0.977**	–0.059	**0.982**	–0.064	–0.328	–0.111
NL	0.808	0.212	0.817	–0.357	0.242	0.127
ZB	**0.969**	–0.117	**0.983**	–0.035	0.024	0.489
MB	**0.969**	–0.132	0.979	–0.065	**0.673**	–0.516
BB	0.918	0.177	0.962	0.139	**0.807**	–0.936
IC	0.928	–0.136	0.967	0.014	0.453	–0.243
PC	0.746	0.622	0.771	0.580	–0.361	0.537
PW	0.880	0.094	0.844	0.159	–0.415	–0.470
BH	0.932	0.095	0.949	0.106	–0.076	0.045
MAL	**0.972**	–0.085	**0.983**	–0.091	–0.437	0.515
MH	0.882	–0.096	0.955	–0.037	–0.609	0.985
C-M2	0.939	–0.016	0.960	–0.067	0.019	–0.220
C-C	0.959	–0.190	0.973	–0.096	–0.512	0.319
M2-M2	0.961	–0.076	0.978	–0.019	0.269	0.154
c-m2	**0.969**	–0.111	**0.984**	–0.077	**1.045**	–0.083
Wilks' lambda	–	–	–	–	0.052	0.504
Chi-square statistic	–	–	–	–	18.684	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	8.778	0.760
Cumulative variance (%)	85.15	89.19	88.64	92.38	90.8	98.7

Values highlighted in bold represent those with the greatest contribution to the first principal component and first canonical variate. Asterisks indicate results that are statistically significant with the Chi-square statistic of the DFA. GLS, greatest length of skull; NL, nasal length; ZB, zygomatic breadth; MB, mastoid breadth; BB, braincase breadth; IC, interorbital constriction; PC, postorbital constriction; PW, palatal width; BH, braincase height; MAL, mandible length; MH, mandible height; C-M2, length of maxillary toothrow; C-C, external alveolar distance between upper canines; M2-M2, external alveolar distance between upper molars; c-m2, length of mandible toothrow.

exhibiting yellowish hues (see Fig. 5). In contrast, *G. cuja* specimens tended to be yellowish, although some individuals could be as greyish 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 geographical occurrence indicated that they corresponded to *G. cuja* and *G. vittata* (Fig. 6A). The genetic distance between these groups was substantial, e.g. 12.7% mean uncorrected divergence (p-distance) with the *ND5* data set. All 12 nuclear segments provided concordant evidence for this separation, even when analysed individually (Fig. 7, Appendix S5). There was no nuclear haplotype sharing between the two geographical groups corresponding to *G. cuja* and *G. vittata*, which were found to be reciprocally monophyletic in every case. Depending on the locus analysed, these two groups were differentiated by one (*JAK1* and *MACF1*), two (*TRHDE, AAMP2*, *ADORA3*, *PFKFB1*, and *PTPN4*), three (*GNAT1*, *APOB*, and

Table 3. Equality test of group mean results for two-group (males and females analysed separately) and multiple-group discriminant function analysis (DFA): independent contribution (differentiation power) of each variable to discriminate the groups

	Two-group DFA								
	Males			Females			Multiple-group DFA		
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
MAL	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

F = *F*-value; *P*-value = significance.
Asterisks indicate morphometric variables that are statistically significant between the groups.
GLS, greatest length of skull; NL, nasal length; ZB, zygomatic breadth; MB, mastoid breadth; BB, braincase breadth; IC, interorbital constriction; PC, postorbital constriction; PW, palatal width; BH, braincase height; MAL, mandible length; MH, mandible height; C-M2, length of maxillary toothrow; C-C, external alveolar distance between upper canines; M2-M2, external alveolar distance between upper molars; c-m2, length of mandible toothrow.

RHO1), or even six mutational steps (*RAG1* and *WT1*) (see Table 1 for full names of genes). 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.

Geographical distribution

As 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 geographical distribution of both 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 north-eastern Brazil to southern Chile and Argentina (Fig. 8, Appendix 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 north-western Brazil (Amazonas, Amapá, Pará, and Rondônia states). The occurrence in these countries suggests some overlap with the north-western limit of the *G. cuja* range, documented in Peru (Puno department), Bolivia (Cochabamba, Santa Cruz, and Tarija departments), and throughout central-western to north-eastern Brazil (including Alagoas, Bahia, Ceará, Goiás, Paraíba, and Pernambuco states and the Distrito Federal) (see Fig. 8 and Appendix S6).

A finer-scale analysis reveals less evidence of geographical overlap, although some instances may

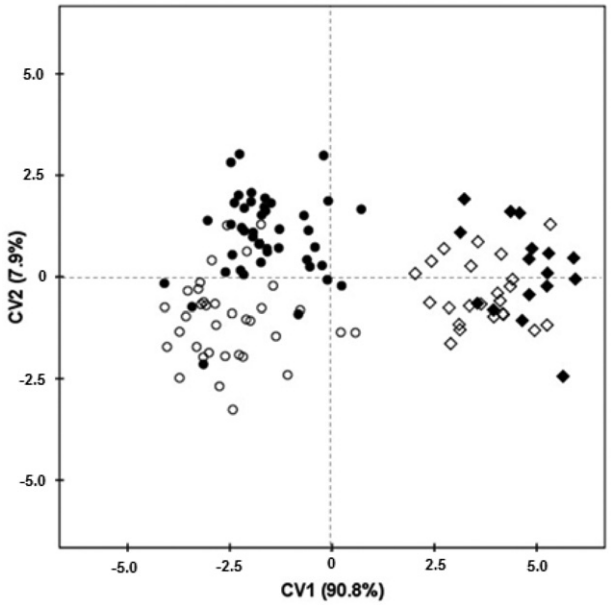

Figure 3. Multiple-group discriminant function analysis plot based on scores from the first and second canonical variates (CV1 and CV2) for 15 craniodental measurements (see Table 2 for details) from specimens of genus *Galictis*: *Galictis cuja* males (black circles); *G. cuja* females (white circles);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

remain. When the presence of each species was assessed with respect to different biomes or ecoregions (Olson *et al.*, 2001), 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 biogeographical 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 north-east, 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 nonmetric 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 geographical 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 extant 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. As 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 *V. vittata* based on the drawing by Allamand (Buffon, 1776) of an animal originating from Suriname, he seems not to have analysed 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 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

Table 4. Descriptive statistics (mean, standard deviation, range, and sample size) for 15 craniodental measurements and one external variable comparing *Galictis cuja* and *Galictis vittata*, with males and females treated separately (measurements in mm)

Variable	*G. cuja* ♂	*G. vittata* ♂	*G. cuja* ♀	*G. vittata* ♀
GLS	76.28 ± 4.03	88.80 ± 3.93	69.50 ± 3.49	85.44 ± 3.12
	64.93–83.52	81.85–94.76	**63.40–77.14**	**79.10–92.90**
	N = 60	*N* = 19	*N* = 44	*N* = 26
NL	21.45 ± 1.76	24.74 ± 1.22	19.83 ± 1.36	23.96 ± 1.48
	17.20–26.47	22.81–27.41	17.40–23.63	21.13–27.76
	N = 67	*N* = 22	*N* = 43	*N* = 31
ZB	43.02 ± 3.12	52.87 ± 2.86	39.21 ± 2.18	50.13 ± 2.40
	35.0–50.09	47.18–56.81	**34.16–44.04**	**44.72–54.50**
	N = 60	*N* = 20	*N* = 44	*N* = 31
MB	39.76 ± 2.75	50.04 ± 2.47	35.62 ± 2.33	46.89 ± 2.61
	32.52–46.83	44.80–54.36	31.28–41.74	41.61–52.29
	N = 63	*N* = 20	*N* = 43	*N* = 31
BB	34.68 ± 1.83	40.68 ± 1.20	33.10 ± 1.54	39.70 ± 1.39
	30.34–37.98	**38.34–43.05**	**29.70–36.51**	**37.34–42.37**
	N = 63	*N* = 21	*N* = 45	*N* = 31
IC	16.55 ± 1.30	21.88 ± 1.62	15.26 ± 1.21	20.29 ± 0.97
	13.20–19.50	18.50–26.98	**13.0–18.17**	**18.41–21.90**
	N = 66	*N* = 22	*N* = 44	*N* = 31
PC	17.72 ± 1.33	19.92 ± 0.76	16.83 ± 1.40	19.28 ± 0.98
	14.39–21.66	18.81–21.36	12.96–19.76	17.81–20.88
	N = 64	*N* = 21	*N* = 45	*N* = 31
PW	11.68 ± 0.96	13.58 ± 0.90	11.07 ± 0.87	13.05 ± 0.77
	9.51–13.50	12.05–15.60	9.62–12.73	10.77–14.71
	N = 65	*N* = 22	*N* = 46	*N* = 31
BH	25.43 ± 1.69	30.73 ± 1.15	23.17 ± 2.08	29.06 ± 1.30
	21.91–28.94	28.81–33.14	18.67–29.35	26.90–32.23
	N = 63	*N* = 20	*N* = 44	*N* = 30
MAL	44.79 ± 2.98	54.11 ± 2.50	40.32 ± 2.22	50.83 ± 2.63
	36.73–51.70	48.74–57.80	**36.02–45.35**	**46.76–58.38**
	N = 59	*N* = 20	*N* = 39	*N* = 28
MH	21.80 ± 1.84	25.10 ± 1.95	19.05 ± 1.58	23.62 ± 1.20
	17.16–25.99	20.47–27.94	16.02–22.77	21.27–26.65
	N = 72	*N* = 21	*N* = 47	*N* = 31
C-M2	20.98 ± 1.50	25.94 ± 1.14	19.08 ± 1.66	24.44 ± 1.11
	17.22–27.96	23.82–27.56	16.20–25.90	22.01–26.86
	N = 69	*N* = 22	*N* = 47	*N* = 31
C-C	16.75 ± 1.41	21.23 ± 1.42	14.91 ± 1.29	19.41 ± 1.24
	13.30–21.49	17.40–23.47	12.30–17.91	17.03–23.03
	N = 67	*N* = 22	*N* = 47	*N* = 30
M2-M2	23.97 ± 1.64	29.76 ± 1.29	21.90 ± 1.55	28.11 ± 1.16
	20.02–27.91	27.81–31.51	19.64–27.55	26.20–30.51
	N = 67	*N* = 22	*N* = 45	*N* = 31
c-m2	26.14 ± 1.52	33.24 ± 1.49	23.74 ± 1.56	30.86 ± 1.50
	21.71–29.18	**30.30–35.72**	**20.0–27.40**	**28.51–35.34**
	N = 69	*N* = 21	*N* = 46	*N* = 30
TL	601.66 ± 40.62	722.16 ± 19.18	531.36 ± 55.67	658.12 ± 38.62
	525.0–657.0	**700.0–755.0**	443.0–645.0	600.0–706.0
	N = 12	*N* = 6	*N* = 11	*N* = 8

Minimum and maximum values in bold indicate variables whose range does not overlap between the species.
GLS, greatest length of skull; NL, nasal length; ZB, zygomatic breadth; MB, mastoid breadth; BB, braincase breadth; IC, interorbital constriction; PC, postorbital constriction; PW, palatal width; BH, braincase height; MAL, mandible length; MH, mandible height; C-M2, length of maxillary toothrow; C-C, external alveolar distance between upper canines; M2-M2, external alveolar distance between upper molars; c-m2, length of mandible toothrow; TL, total body length.

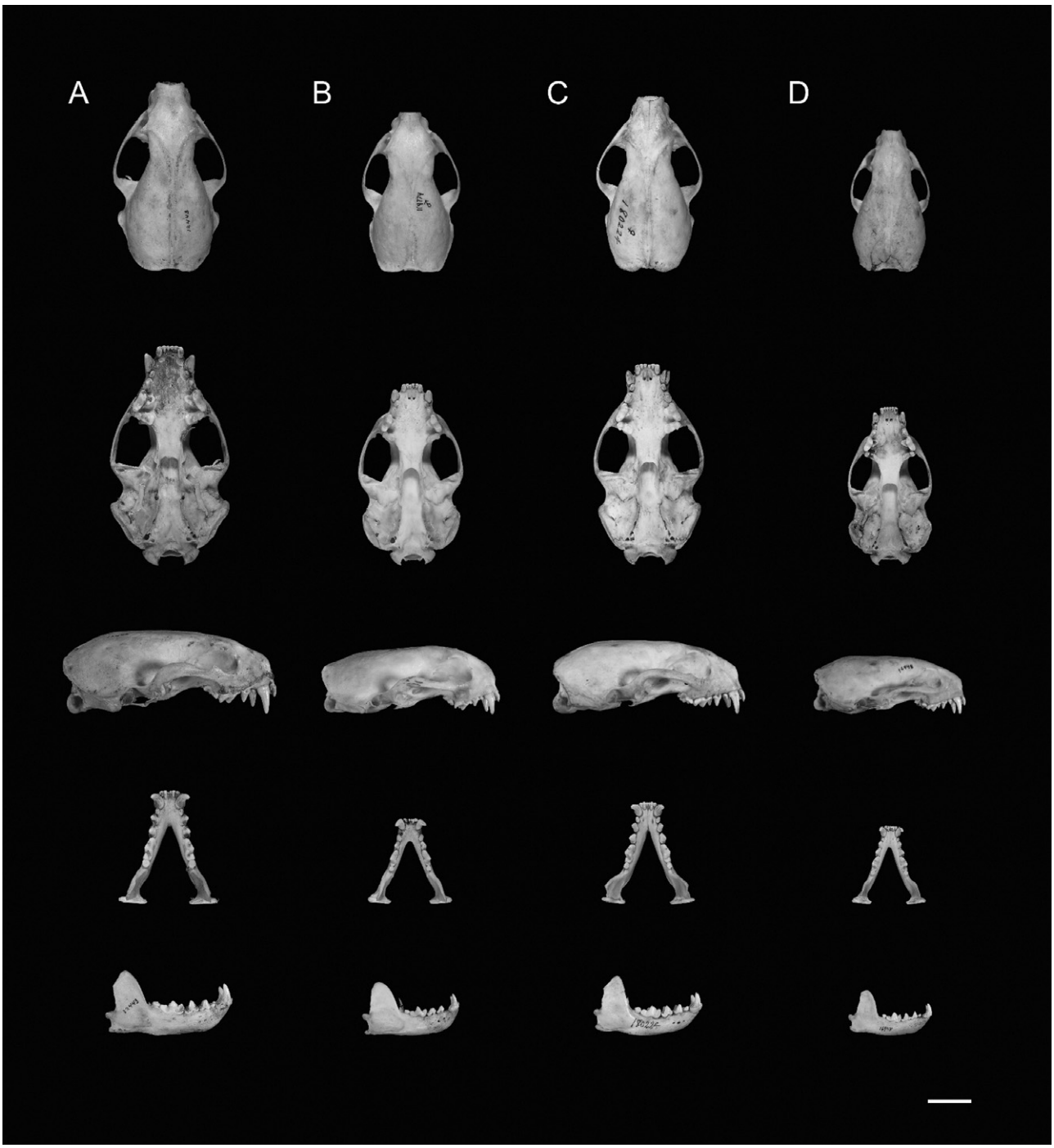

Figure 4. Representative skull views (dorsal, ventral, and lateral skull views and dorsal and lateral mandible views) from *Galictis* specimens, emphasizing size differences amongst the groups: A, *Galictis vittata* male, LSUMNS 2443; B, *Galictis cuja* male, MVZ 114774; C, *G. vittata* female, USNM 180224; and D, *G. cuja* female, LSUMNS 16948. Scale bar = 10 mm.

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

Table 5. Male and female results of the *t*-test comparison between *Galictis cuja* and *Galictis vittata* based on the mean of 15 craniodental measurements and one external variable

	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
MAL	–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

t = *t*-value; d.f. = degrees of freedom; *P*-value = significance.
GLS, greatest length of skull; NL, nasal length; ZB, zygomatic breadth; MB, mastoid breadth; BB, braincase breadth; IC, interorbital constriction; PC, postorbital constriction; PW, palatal width; BH, braincase height; MAL, mandible length; MH, mandible height; C-M2, length of maxillary toothrow; C-C, external alveolar distance between upper canines; M2-M2, external alveolar distance between upper molars; c-m2, length of mandible toothrow; TL, total body length.

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 a result of 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 – *G. 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 their size, 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 analysed, all of which lay within the *G. cuja* cluster, thus indicating that the large size of those two specimens may not be because of any particular geographical trend, but rather be attributed to within-population inter-individual variation. In spite of these two exceptions, the multivariate classification analyses exhibited extremely high accuracy when distinguishing the two species, and remained effective even with individuals whose gender was unknown (see Results and Appendix S4). Another line of evidence that supported clear species-level delimitation derived from the univariate analyses. For all 15 craniodental 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

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

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% p-distance with *ND5*) was sufficient to induce reciprocal monophyly at all 12 surveyed nuclear loci, a pattern that is often not observed in 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 *c.* 2 to 3 million years ago (Mya) (Koepfli *et al*., 2008; Sato *et al*., 2012). The fossil record indicates that grisons probably originated in North America, where they may have shared a common ancestor with the Pliocene genus *Trigonictis* (Reig, 1957; Yensen & Tarifa, 2003a). Representatives of genus *Galictis* then colonized South America during the Great American Biotic Interchange (GABI), with their first record in this sub-continent appearing in Argentina in the Vorohuean subage of the late Pliocene (Reig, 1957; Hunt, 1996; Woodburne, 2010), dated at 3.0–2.5 Mya (Cione & Tonni, 1995; Prevosti & Soibelzon, 2012). Integrating our results with the available fossil and molecular information, we can hypothesize that a single *Galictis* ancestor invaded South America via the Panamanian isthmus early during the GABI (Hunt, 1996; Woodburne, 2010; Eizirik, 2012), soon afterwards giving rise to the two extant species, as well as additional extinct members of the genus (Reig, 1957). Further 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 of *G. cuja* and G. vittata

Skull

Historically, various literature sources (e.g. Thomas, 1912; Yensen & Tarifa, 2003a, b) have mentioned that the two extant *Galictis* species can be distinguished on the basis of size differences along with the presence/absence of the metaconid in m1. However, this distinction had so far not been formally tested with a broad sample from across the genus's range. The results from this study corroborated the usefulness of the metaconid as a diagnostic feature between these species (Fig. 1), as it was present in 100% of the *G. vittata* skulls, and absent in virtually all *G. cuja* skulls. The single potential exception was a very small *G. cuja* male (AMNH 33281) from Temuco, Chile, that bore a subtle cusp on the left m1, which was similar but smaller than the metaconid seen in *G. vittata*. In addition to the presence/absence of the

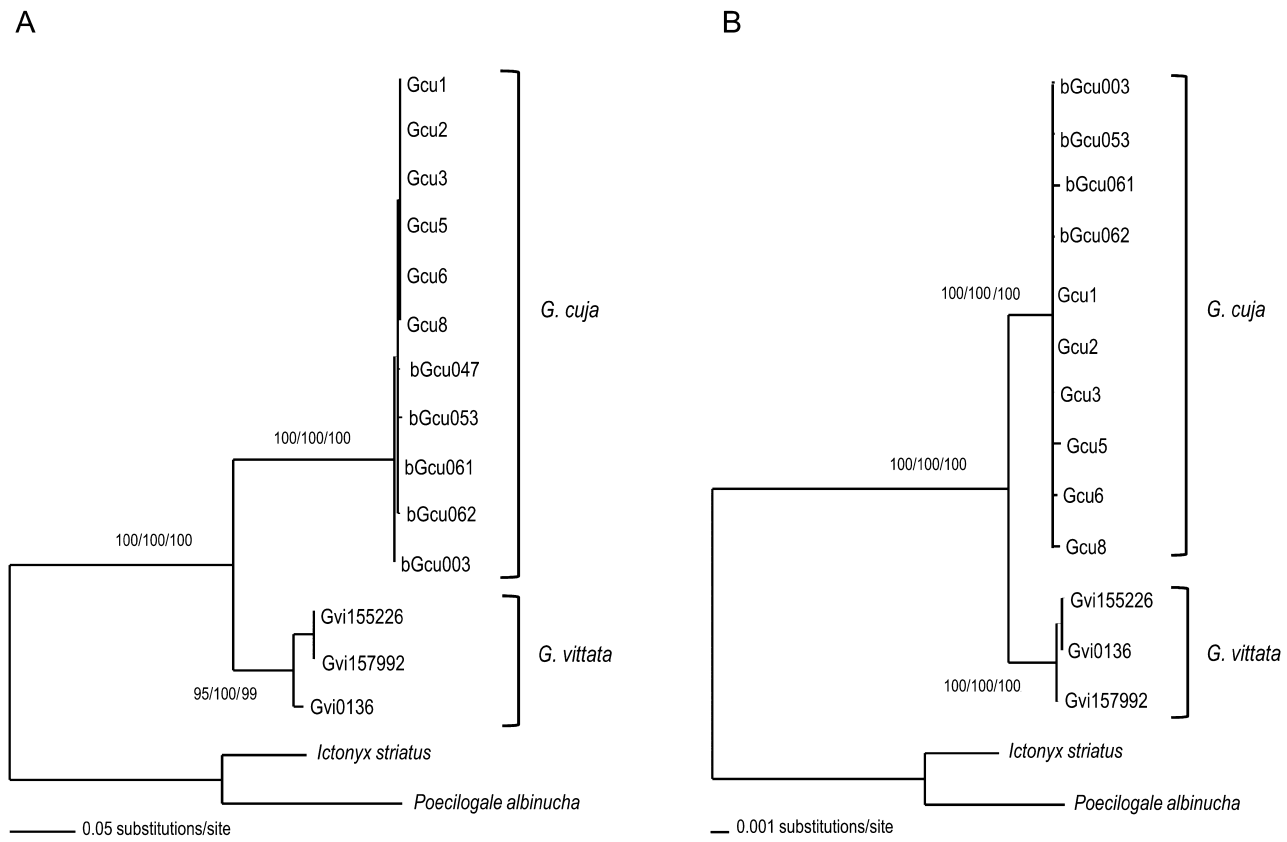

Figure 6. Maximum likelihood (ML) phylogenies depicting the evolutionary relationships amongst *Galictis cuja* (bGcu or Gcu sample IDs) and *Galictis vittata* (Gvi sample IDs) individuals (see Appendix S1 for more details on sample IDs). A, mitochondrial DNA (*nicotinamide adenine dinucleotide dehydrogenase subunit 5* 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/maximum parsimony/Bayesian inference methods, respectively (Bayesian posterior probabilities are indicated as percentages). Support is represented only for major nodes. See text for additional details.

metaconid, we observed that the two extant grisons were also very different with respect to size. The PCA revealed that the split between *G. cuja* and *G. vittata* occurred along 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: GLS, ZB, MB, BB, MAL, and c-m2. Of these variables, GLS and MAL 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, as 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 colour, given the intraspecific variation observed in *G. cuja*. Still, the tendency of the latter species to bear yellowish fur and of *G. vittata* to have greyish 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

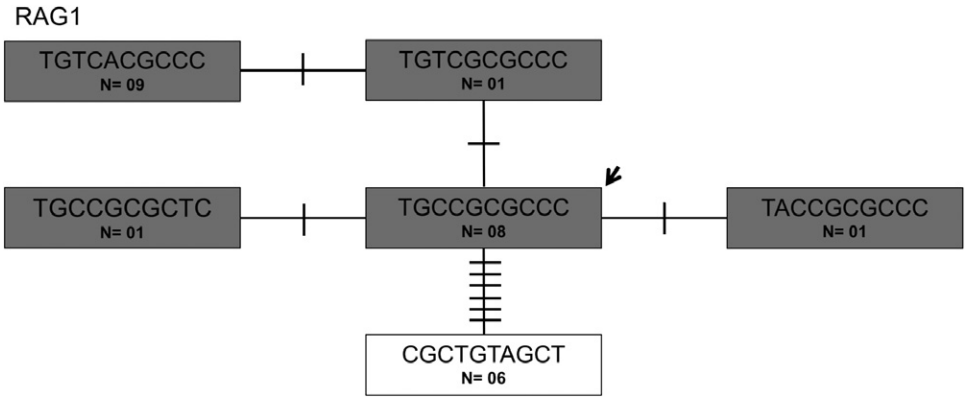

Figure 7. Representative haplotype network for one nuclear gene segment (*recombination activating protein 1*, *RAG1*). Each rectangle represents a distinct haplotype. Haplotypes sampled only in *Galictis cuja* are depicted in grey, whereas that sampled only in *Galictis vittata* is shown in white. The 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 Appendix S5.

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. Another potential feature that may distinguish these species is tail length, as suggested by some previous authors (e.g. Emmons & Feer, 1997; Yensen & Tarifa, 2003a, b). We did observe that *G. cuja* tended to have a proportionally longer tail (e.g. see Fig. 5), as previously suggested, but did not test this feature statistically given the small sample size of complete skeletons and potential biases induced by skin preparations. Further studies focusing on live individuals whose reliable identification is available (e.g. based on the molecular characters that we report here) should help ascertain the error rate associated with such field-orientated diagnosis strategies.

Geographical distribution of *G. cuja* and G. vittata

The records obtained in this study cover the complete range of the 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 view 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 semihumid forests in southern Mexico to drier and more open regions in northern Mexico (where no *G. vittata* were 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 savannahs, deserts, and montane grasslands, as well as most of the Atlantic Forest in eastern South America (see Fig. 8). In our data set, the latter biome is marginally represented by a single record in southern Paraguay (AMNH 77695, skull only, with specific locality given as 'east of Villarica', Guaira). Interestingly, Yensen & Tarifa (2003a) did not include Paraguay in the range of *G. vittata*, probably because this single record was not analysed in the literature sources reviewed by those authors. This specimen did not contain gender information; thus it was excluded from the morphometric analyses. However, its large size (e.g. BB = 40.4 mm and c-m2 = 33.96 mm) and the presence of the metaconid in the lower carnassial identified it as *G. vittata*. In addition to this individual, there has been a report of a *G. vittata* specimen from Misiones province, northeastern Argentina (Chebez & Massoia, 1996; Díaz & Lucherini, 2006; F. Prevosti, pers. comm.). Although this individual was not analysed in the present study, its presence in the south-western edge of the Atlantic Forest (see Fig. 8) lends support to the hypothesis that *G. vittata* may indeed extend its range into this area, implying potentially considerable geographical overlap with *G. cuja* in the region. Further scrutiny of the geographical limits of these species in this area is warranted, so as to clarify their actual range overlap and degree of habitat segregation when in sympatry.

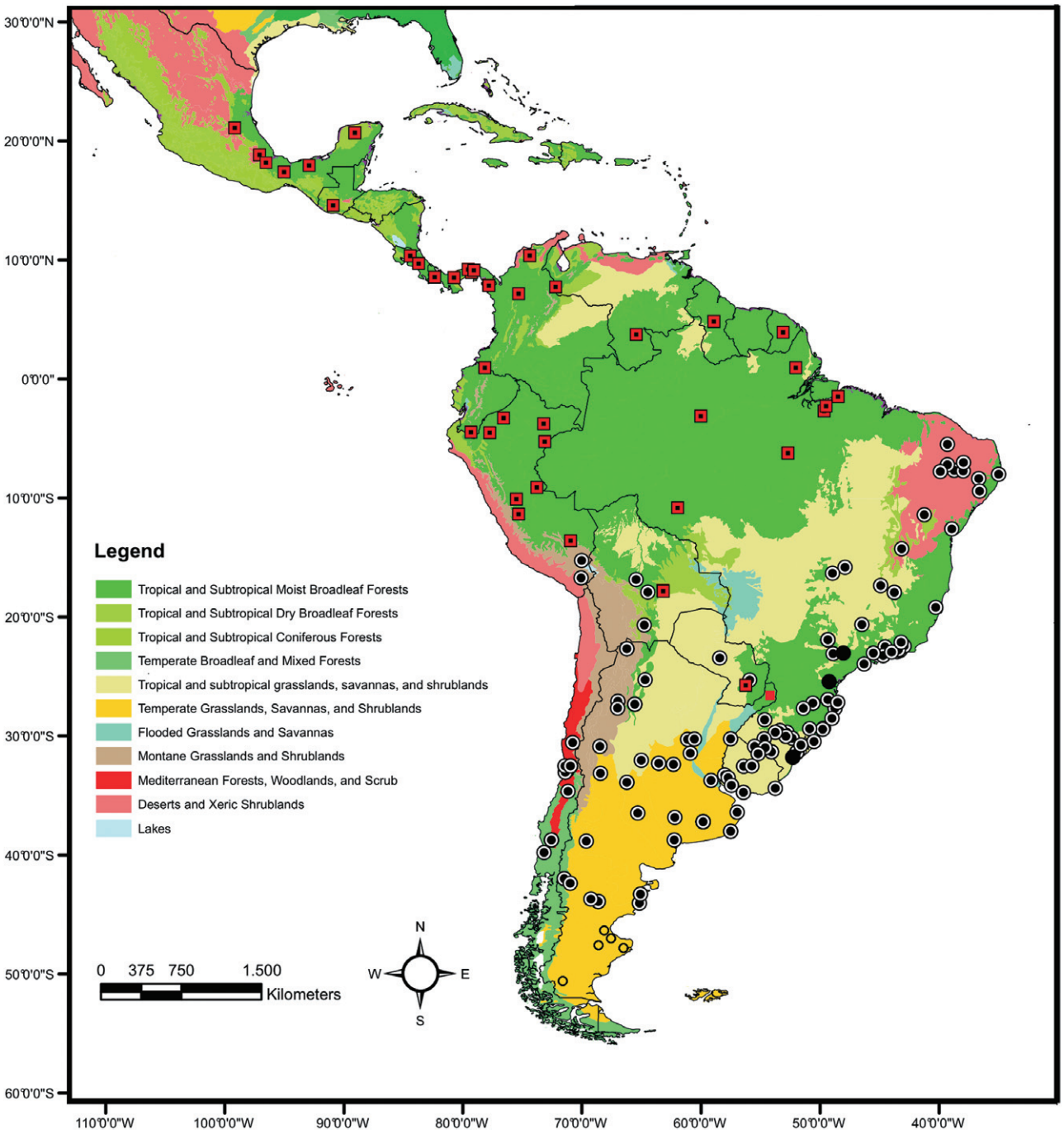

Figure 8. Map showing the distribution of *Galictis vittata* (squares) and *Galictis cuja* (circles) based on the geographical origin of individuals with ascertained species-level identification. *Galictis vittata* records represented by red squares with an inner black dot are based on skulls and skins analysed in this study. The record represented by a solid red square is an individual collected in Misiones, Argentina (Chebez & Massoia, 1996), that was not analysed directly (see text for details). *Galictis cuja* records represented by black circles with a white border are based on skulls and skins analysed here, whereas those based on DNA samples are depicted as solid black circles. In addition, we also show open circles representing *G. cuja* records reported by Prevosti & Travaini (2005) from the southernmost limit of the species' range. 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).

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

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

In addition to Paraguay and north-eastern Argentina, 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 consistent 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 geographical 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 and eastern limits of the *G. vittata* distribution and allowed us to provide a more precise geographical 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 north-eastern, south-eastern, 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 north-eastern 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), whereas 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 north-eastern 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 Appendix 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 geographical 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 living or extinct mustelids, and not with each other. Amongst the extinct taxa that might have driven such size evolution are other species of the genus *Galictis*, whose presence is documented in the South American fossil record (e.g. Reig, 1957; Prevosti & Soibelzon, 2012). Given the age of the separation between *G. cuja* and *G. vittata* (see above), and the concomi-

tant existence of these congeneric species throughout the late Pliocene and Pleistocene, it is plausible to hypothesize that they may have played a competitive role in shaping the size of the two living lineages. A third hypothesis would postulate that the differences in size and geographical range between *G. cuja* and *G. vittata* are induced by ecological sorting, with each species being adapted to a distinct set of environments. *Galictis vittata* seems to be rather restricted to humid rain forests, whereas *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 living or extinct carnivore species within these different habitats) 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 geographical overlap (Davies *et al*., 2007). Testing these hypotheses with diverse approaches should provide an interesting avenue of research in the future.

Another aspect that could be explored in future evolutionary studies targeting *Galictis* is coat colour variation. *Galictis cuja*, which usually exhibits yellowish and dense fur, occurs mainly in open and drier landscapes, such as the barren Caatinga and the Cerrado in north-eastern Brazil, the savannahs 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 favoured 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 colour. Additionally, this species often reaches high altitudes (Osgood, 1943; Greer, 1966; M. Lucherini, C. Tellaeche, J. Reppucci & E. Luengos Vidal, unpubl. data) and latitudes (Quintana, Yañez & Valdebenito, 2000; Parera, 2002; Prevosti & Travaini, 2005), which may have led historically to selective pressures for denser pelage. In contrast, *G. vittata* exhibits more constant skin colour and density. The mixture between black and white fur, producing a pale grey pelage, might be favoured 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 to be an adaptation to the warm temperatures that are prevalent throughout its geographical 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 define better their geographical range, the present study should contribute to establishing such baseline aspects, and to identifying patterns that have the potential to spur additional research into these little-known carnivorans.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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

Appendix S2. Results of the t-test comparison for sexual size dimorphism within *Galictis cuja* and within *G. vittata*.

Appendix 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*).

Appendix 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).

Appendix S5. A. Table depicting the variable sites found within and between *Galictis cuja* and *G. vittata* in 12 nuclear gene segments.

B. Haplotype networks derived from the nucleotide information described in A for 11 of the 12 nuclear segments.

Appendix S6. Geographic distribution map of *Galictis vittata* and *G. cuja* throughout the Neotropical region based on skulls, skins and DNA samples.

Appendix S7. Results from the multiple-group Discriminant Function Analysis (DFA) applying the stepwise method.