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Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil

T. C. TRIGO[*], **T. R. O. FREITAS**[*], **G. KUNZLER**[†], **L. CARDOSO**[†], **J. C. R. SILVA**[‡,§,¶], **W. E. JOHNSON**[**], **S. J. O'BRIEN**[**], **S. L. BONATTO**[†], **E. EIZIRIK**[†,**,††]

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

[†] Faculdade de Biociências, Pontificia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, prédio 12, sala 172. Porto Alegre, RS 90619-900, Brazil

[‡] Centro Brasileiro para Conservação dos Felinos Neotropicais, Associação Mata Ciliar, Jundiaí, SP, Brazil

[§] Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, PE, Brazil

[¶] Instituto Brasileiro para Medicina da Conservação – Tríade, São Paulo, SP, Brazil

[**] Laboratory of Genomic Diversity, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[††] Instituto Pró-Carnívoros, Brazil

Abstract

Natural hybrid zones between distinct species have been reported for many taxa, but so far, few examples involve carnivores or Neotropical mammals in general. In this study, we employed mitochondrial DNA (mtDNA) sequences and nine microsatellite loci to identify and characterize a hybrid zone between two Neotropical felids, *Leopardus geoffroyi* and *L. tigrinus*, both of which are well-established species having diverged from each other *c.* 1 million years ago. These two felids are mostly allopatric throughout their ranges in South America, with a narrow contact zone that includes southern Brazil. We present strong evidence for the occurrence of hybridization between these species and identify at least 14 individuals (most of them originating from the geographical contact zone) exhibiting signs of interspecific genomic introgression. The genetic structure of Brazilian *L. tigrinus* populations seems to be affected by this introgression process, showing a gradient of differentiation from *L. geoffroyi* correlated with distance from the contact zone. We also corroborate and extend previous findings of hybridization between *L. tigrinus* and a

Correspondence: Tatiane C. Trigo, Fax: 55-51-3308-7311; tatiane.trigo@gmail.com; and Eduardo Eizirik, Fax: 55-51-3320-3568; eduardo.eizirik@pucrs.br.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

third related felid, *L. colocolo*, leading to an unusual situation for a mammal, in which the former species contains introgressed mtDNA lineages from two distinct taxa in addition to its own.

Keywords

Carnivora; hybridization; introgression; *Leopardus geoffroyi*; *Leopardus tigrinus*; South America

Introduction

The role of hybridization in the evolution of living organisms has been extensively discussed among evolutionists (e.g. Arnold 1992; Harrison 1993; Dowling & Secor 1997; Barton 2001; Fitzpatrick 2004). The classical view of zoologists is that the evolutionary significance of hybridization is small, in most cases consisting of occasional sterile hybrid individuals with no relevant contribution to future generations. In contrast, botanists frequently see hybridization as a common phenomenon, acting as an important source of new variation and potentially new species (Harrison 1993). This apparent dichotomy has been challenged in recent decades, with the development and implementation of diverse molecular techniques allowing for in-depth genetic analyses of natural populations. These approaches have led to the conclusion that interspecies hybridization is quite common in animals and frequently include the production of fertile hybrids which may have considerable importance for future adaptation and even speciation (Barton & Hewitt 1985; Harrison 1993; Allendorf *et al.* 2001). Several hybrid zones have recently been documented in vertebrates, ranging from cases in which few hybrid individuals are detected (Schwartz *et al.* 2004) to extensive introgressive zones leading to the production of hybrid swarms (Nolte *et al.* 2006), or even suggested as possibly responsible for the formation of new species (Roy *et al.* 1994; Reich *et al.* 1999).

Even though it appears that natural hybridization is more common in animals than previously thought, it is still unclear how widespread its occurrence is, and whether some zoological groups or biogeographical regions may be more prone to foster such processes. Moreover, very few cases of animal hybridization have been described in detail, so that the investigation of the underlying causes and evolutionary significance of these processes remains in its infancy. A more thorough understanding of this phenomenon would be important not only to assess its evolutionary relevance (e.g. in terms of illuminating the various aspects of the speciation process), but also as a conceptual basis to design adequate conservation strategies for endangered populations showing signs of admixture with other taxa.

Several cases of hybridization involving mammalian carnivores (Mammalia, Carnivora) have been reported. So far, the examples that have received the most attention are those of North American wild canids (e.g. Lehman *et al.* 1991; Wayne & Jenks 1991; Roy *et al.* 1994; Reich *et al.* 1999; Miller *et al.* 2003), domestic dogs vs. wild canids (e.g. Gottelli *et al.* 1994; Vilà & Wayne 1999; Randi & Lucchini 2002; Adams *et al.* 2003; Vilà *et al.* 2003) and domestic cats vs. European wildcats (e.g. Beaumont *et al.* 2001; Randi *et al.* 2001; Lecis *et al.* 2006). The latter example, as well as most cases involving domestic dogs, describes

situations in which the hybridizing populations are conspecific, i.e. domestic and wild forms of the same species. With the exception of the complex case of North American canids, little attention has been devoted to interspecific hybridization in carnivores, although some interesting examples have recently emerged in the literature (e.g. Schwartz *et al*. 2004; Lancaster *et al*. 2006). Virtually nothing is known about the occurrence of hybridization among Neotropical carnivores, a very diverse assemblage including several sets of closely related species. At least some of these sets are likely the product of rapid radiations following a single invasion after the closure of the Panama Isthmus and, so far, the ecological, evolutionary and biogeographical processes underlying their diversification have not been thoroughly characterized.

Of the 10 species of wild cats occurring in the Neotropics (Sunquist & Sunquist 2002), seven are known to comprise a monophyletic lineage endemic to this region (Johnson *et al*. 2006), herein referred to as the genus *Leopardus* (Wozencraft 2005; Johnson *et al*. 2006; E. Eizirik *et al*., unpublished data). The basal divergence among these seven species has been estimated to have occurred *c.* 2.9 million years ago (Johnson *et al*. 2006), which is consistent with a rapid radiation following a single invasion of South America via the Panama isthmus in the Pliocene. Within this clade, a well-supported subgroup includes the little spotted cat or oncilla (*Leopardus tigrinus*), Geoffroy's cat (*L. geoffroyi*), the kodkod (*L. guigna*), the pampas cat (*L. colocolo*) and possibly also the Andean mountain cat (*L. jacobita*). The species-level delimitation among these felids has been supported by reciprocal monophyly in mitochondrial DNA (mtDNA) analyses including multiple individuals (Johnson *et al*. 1999), corroborating the classical, morphology-based definition of these taxa. Interestingly, our previous analyses using mtDNA and Y-chromosome sequences identified some individuals that appeared to be natural hybrids between *L. tigrinus* and *L. colocolo* (Johnson *et al*. 1999). In spite of the intriguing nature of this finding, further analyses of this issue have so far been hampered by the difficulty in sampling a larger number of individuals from natural populations of these species, especially *L. colocolo*.

Within this group, *L. tigrinus* and *L. geoffroyi* are morphologically similar, exhibiting similar body proportions and general appearance. The main characters used to distinguish them are body size and coat colour, with *L. geoffroyi* being larger and more robust (total length: 690–1250 mm; weight 2.2–7.8 kg) and usually showing a gray background colour with solid black spots (Ximenez 1971, 1973, 1975; Sunquist & Sunquist 2002; Lucherini *et al*. 2006). *L. tigrinus* tends to be smaller (total length: 710–936 mm; weight 1.75–3.5 kg) and more gracile in appearance, with usually yellowish/ochre pelage bearing rows of dark spots and open rosettes (Kitchener 1991; Oliveira 1994; Eisenberg & Redford 1999; Sunquist & Sunquist 2002).

These two species show an essentially allopatric distribution in South America (Fig. 1): *L. geoffroyi* occurs from Bolivia, Paraguay, northern Argentina and southern Brazil to the southern tip of South America (Oliveira 1994; Eisenberg & Redford 1999; Wozencraft 2005), overlapping with the distribution of *L. tigrinus* at the northern end of its range. The latter species ranges from Costa Rica to southern Brazil and northeastern Argentina (Oliveira 1994; Nowell & Jackson 1996; Eisenberg & Redford 1999), but its current distribution is not completely defined and may be discontinuous, mainly due to the lack of

detailed evidence of its occurrence throughout the Amazon basin (Nowell & Jackson 1996; Oliveira 2004).

Field-based surveys of the precise geographical distribution of *L. tigrinus* and *L. geoffroyi* in Rio Grande do Sul (RS) state, southernmost Brazil, conducted between 1993 and 2004, revealed a narrow contact zone (≤100 km in width) between these species (Eizirik *et al.* 2006). In this region, we and others have observed individuals bearing atypical coat colour patterns, seemingly 'intermediate' between the two species (Mazim *et al.* 2004; Eizirik *et al.* 2006). Many of these animals were recorded in the Central Depression region of RS state (which consists of a mosaic of grassland, riparian forests and marshland fragments amidst a matrix of agricultural landscapes), exactly where contact between the two species occurs (Eizirik *et al.* 2006). This atypical colour pattern has led us to hypothesize the existence of a hybrid zone between these species in this area.

In this context, the goals of the present study were (i) to test the field-based hypothesis that *L. tigrinus* and *L. geoffroyi* hybridize in the wild, and to investigate whether the genetic patterns are consistent with a hybrid zone; (ii) if this hypothesis was supported, to characterize this hybrid zone, assessing the magnitude of admixture and the occurrence of genomic introgression in one or both directions; (iii) to further investigate the evidence of hybridization between *L. tigrinus* and *L. colocolo*; and (iv) to test the possibility of hybridization between *L. geoffroyi* and *L. colocolo.* We employed mtDNA sequences and nuclear microsatellite markers to address these issues, and interpreted these molecular data in a comparative fashion, as well as in the light of the morphologically-based identification of each sampled individual. Our results corroborate and expand the previous inference of hybridization between *L. tigrinus* and *L. colocolo* and strongly support the hypothesis of a hybrid zone between the former and *L. geoffroyi* in southern Brazil, leading to interesting inferences regarding the evolutionary history of these species in South America.

Materials and methods

Sample collection and laboratory procedures

Biological material (blood and tissue samples) of Neotropical felids was obtained from captive animals of known origin, road-killed individuals or wild animals captured by farmers. Samples of 57 *Leopardus tigrinus* were obtained from three major Brazilian regions, comprising eight Brazilian states: the southern region, including RS (n = 16), Paraná (PR) ($n = 9$) and Santa Catarina (SC) ($n = 1$) states; the southeastern region, including Sao Paulo (SP) ($n = 23$), Rio de Janeiro (RJ) ($n = 1$) and Espirito Santo (ES) ($n = 1$) states; and the center-west region, including Mato Grosso do Sul (MS) ($n = 2$) and Goiás (GO) ($n = 4$) states (see Fig. 1 for sample collection locales and Supplementary material for details). In addition, two samples from Paraguay, one from Costa Rica and one Brazilian sample with unknown state origin were included. Samples of 41 *L. geoffroyi* individuals were obtained from Argentina ($n = 5$), Bolivia ($n = 7$), Uruguay ($n = 5$) and RS state in Brazil ($n = 22$), along with two samples of unknown origin. Seven samples of *L. colocolo* were also included in the study (two from Argentina, one from Bolivia, one from Chile, two from Brazil and one with unknown origin). Finally, two samples of *L. guigna* and one each of ocelot (*L. pardalis*) and margay (*L. wiedii*) were used for comparison in some of the

analyses. DNA extraction from all samples was performed using standard phenol/chloroform protocols (Sambrook *et al.* 1989; Palumbi *et al.* 1991; Hillis *et al.* 1996).

Three mtDNA segments were amplified by polymerase chain reaction (PCR) from these samples, using primers developed or adapted for improved performance in carnivores: a segment of the *ND5* gene including *c.* 750 bp [using primers ND5-DF1 (TTGGTGCAACTCCAAATAAAAGT) and ND5-DR1 (AGGAGTTGGGCCTTCTATGG)]; a ~400-bp segment including the *ATP8* gene and part of the *ATP6* gene [using primers ATP8-DF1 (AGAAGCTAAATAAG-CATTAACCTTTTA) and ATP6-DR1 (CCAGTATTTGTTTT-GATGTTAGTTG)], and the 5′ portion of the control region (CR) [using primers MTLPRO2 and CCR-DR1 (Tchaicka *et al.* 2007)]. For all three segments, PCR reactions were performed in a 20–25 μL final volume containing 1.5–2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Taq DNA polymerase (Invitrogen) or Taq GOLD (ABI), and 0.2 μM each of the forward and reverse primers. Thermocycling used a touchdown profile as described in Tchaicka *et al.* (2007), with the annealing temperature decreasing from 60 °C to 51 °C in 10 cycles, followed by 30–35 cycles in which it was kept constant at 50 °C.

PCR products were analyzed on an ethidium-bromide-stained 1% agarose gel and then purified using either Polyethyleneglycol-8000 or the enzymes exonuclease I and Shrimp alkaline phosphatase. Purified PCR products were sequenced using either the DYEnamic ET kit (Amersham) or Big Dye chemistry (ABI) and subsequently analyzed in a MegaBACE 1000 or an ABI-PRISM 3700 automated sequencer, respectively. Sequence electropherograms were verified and corrected by eye using SEQUENCHER (Gene Codes) or CHROMAS (http://www.technelysium.com.au/chromas.html) and then aligned using the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar *et al.* 2004); the alignment of each mtDNA segment was checked and edited by hand separately.

In addition to the mtDNA sequences, nine microsatellite markers [six tetranucleotide (FCA391, FCA424, FCA441, FCA453, F42, F124), two trinucleotide repeat loci (F98 and F146) and one dinucleotide (FCA723)], developed originally for the domestic cat (Menotti-Raymond *et al.* 1999, 2005) were selected for use in this study. Each microsatellite locus was amplified individually by PCR (Saiki *et al.* 1985); reactions were performed in a 15 μL volume containing 1.5–3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Taq DNA polymerase, and 0.1 μM each of the forward and reverse primers. The thermal profile was: 94 °C for 3′, and 30 cycles of 45″ at 94 °C, 45″ at 48–60 °C (annealing temperature varied among loci), and 1′ at 72 °C, followed by 10′ of final extension.

PCR products for microsatellite loci were analyzed by vertical electrophoresis in 6% nondenaturing polyacrylamide gels, and the microsatellite alleles were detected by silver nitrate staining (Tegelstrom 1992). Genotypes were scored manually using a 25-bp size ladder (Gibco BRL), as well as an allelic ladder constructed with all alleles found for each locus in this study. Aiming to thoroughly verify and confirm the observed genotypes, 25% to 30% of the samples were reanalyzed two to three times per locus, resulting in 100% concordance among replicates. This effort also included five cases in which the same captive animal was collected twice by different people at different times, and each of the samples was genotyped separately, again resulting in complete concordance of results. Finally, part

of our data set (15 *L. tigrinus* and 29 *L. geoffroyi* individuals) was generated using fluorescently labelled primers and an ABI 373 A automated sequencer, employing the computer programs GENESCAN 2.1 (ABI) and GENOTYPER 2.1 (ABI) to precisely calibrate allele sizes. Of these individuals, seven of each species were also genotyped in silver-stained polyacrylamide gels to verify and calibrate the allelic correspondence between the two detection methods, resulting in full agreement of all replicated genotypes. All genotypes were thus integrated into a single data set, with the exact size of each allele based on the more precise estimation using the automated sequencer.

Data analysis

mtDNA data.—Exploratory phylogenetic analyses were initially performed for each mtDNA segment separately using the distance-based neighbour-joining (NJ) algorithm (Saitou & Nei 1987) implemented in MEGA, to assess any occurrence of incongruence among these data sets. Support for inferred nodes was assessed using 100 replicates of nonparametric bootstrap. As no incongruence was identified at any well-supported node, the three segments were concatenated into a single data set, which was used for all subsequent analyses.

Phylogenetic analyses of the final data set were performed using four optimality criteria: maximum likelihood (ML), maximum parsimony (MP), distance-based (with the NJ algorithm) and Bayesian Inference (BI). The ML, MP and NJ approaches were performed using PAUP*4.b10 (Swofford 1998), while the BI method employed MRBAYES 3.1 (Huelsenbeck & Ronquist 2001). The best-fit model of nucleotide evolution for the concatenated data set was estimated using the Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada & Crandall 1998). This model (or an approximation of it) was implemented in the ML and BI analyses, as well as the NJ search (which used ML distances). The ML analysis employed a heuristic search started from a NJ tree and followed by NNI branch-swapping. The final MP phylogeny was based on a heuristic search using simple taxon addition and TBR branch-swapping, limiting the procedure to store a maximum of 10 000 trees. Nodal support for the ML, MP and NJ methods was assessed with 100 replicates of bootstrapping (in the case of MP limiting the search to store a maximum of 1000 trees per replicate). The Bayesian analysis used two independent replicates of the Metropolis-Coupled Markov chain Monte Carlo procedure, each containing four chains (one cold, three heated) run for 3 000 000 generations, with trees and parameters sampled every 100 steps, and the first 25% of the samples discarded as burn-in. Trees were rooted using *L. pardalis* and *L. wiedii* as outgroups (see Johnson *et al.* 2006).

In addition to phylogenies, haplotype networks were built using the median-joining approach (Bandelt *et al.* 1999) implemented in NETWORK 4.2.0 (www.fluxus-engineering.com), allowing for ambiguous connections as well as direct ancestor-descendent relationships among haplotypes. Measures of mtDNA diversity were calculated with DnaSP 4.10.0.8 (Rozas *et al.* 2003), which was also employed to make inferences regarding historical changes in population size using a mismatch distribution analysis and several neutrality tests (Tajima's D, Fu & Li' D* and F*, Fu's Fs). In addition, ARLEQUIN 3.11

(Excoffier *et al.* 2005) was used to assess the magnitude of mtDNA-based species-level differentiation, employing an Analysis of Molecular Variance (AMOVA) approach (Excoffier *et al.* 1992), the results of which were tested for statistical significance with 10 000 permutations.

The age of each of the observed mtDNA clades was estimated using the Bayesian approach implemented in the program BEAST 1.4.4 (Drummond *et al.* 2002; Drummond & Rambaut 2006), employing a molecular calibration point for the divergence between *L. tigrinus* and (*L. geoffroyi* + *L. guigna).* The age of this node was estimated in a previous study (using a Bayesian relaxed clock method applied to a 18.7-kb nuclear supermatrix and incorporating multiple fossil constraints; Johnson *et al.* 2006) to be 930 000 years ago, with a credibility interval of 560 000–1 480 000 years ago. To apply this molecular calibration in a conservative fashion, we used the minimum and maximum ages in this credibility interval as boundaries in a uniform prior distribution for this node's age. The MCMC procedure was run for 50 million generations, with samples taken every 1000 steps; results were analyzed with the program TRACER (Rambaut & Drummond 2004) removing the initial 5 million steps as burn-in.

Microsatellite data.

Microsatellite diversity was evaluated separately for *L. tigrinus*, *L. geoffroyi* and *L. colocolo* based on the number of polymorphic loci, alleles per locus and private alleles. We used ARLEQUIN to compute values of observed (H_O) and expected (H_E) heterozygosity and to test for deviations from Hardy–Weinberg Equilibrium (HWE) for each locus, using the exact test of Guo & Thompson (1992). The microsatellite data set was tested for genotyping errors due to stuttering, short allele dominance and null alleles using a Monte Carlo simulation of expected allele-size differences using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Allele size-difference frequencies were determined to deviate from expectations if they fell outside the Bonferroni-corrected 95% confidence interval generated by the simulation. The distributions of allele frequencies, presence of private alleles and linkage equilibrium (LE) at all loci for each of the species were evaluated using GENEPOP 3.1d (Raymond & Rousset 1995). Significance levels of HWE and LE were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice 1989). To test for the occurrence of genetic introgression, the same methodology was conducted with the *L. tigrinus* sample subdivided into regional groups (see Results). The level of genetic differentiation between species and among regional groups of *L. tigrinus* was assessed with an AMOVA, as implemented in ARLEQUIN, using an F_{ST} analogue (Weir & Cockerham 1984). Statistical significance of the observed values was tested using 10 000 permutations.

We applied the Bayesian clustering method implemented in the program STRUCTURE 2.2 (Pritchard *et al.* 2000), incorporating the 'correlated allele frequencies' model (Falush *et al.* 2003), to assign individuals to populations and to identify hybrids between *L. tigrinus*, *L. geoffroyi* and *L. colocolo*. Three different sets of analyses were performed, in every case using 500 000 MCMC iterations following a burn-in period of 200 000 steps. We initially evaluated the approximate probability of each of a varying number of K populations for the pooled data, by empirically setting prior values of $K = 1–10$, and evaluating the Ln

likelihood of the data (for each value of *K*, five independent runs were performed to confirm the stability of the likelihood estimates). For this analysis, we did not use the phenotypic information for species assignment, so that the most likely number of populations was thus determined from the genetic data alone. This approach was employed for two separate data sets; one of them including all three species and a second one focusing exclusively on the comparison between *L. geoffroyi* and *L. tigrinus.* A third set of STRUCTURE analyses, conducted only for the *L. geoffroyi* + *L. tigrinus* data set, employed the phenotype-based species identification; in this case the program estimates the probability of each individual belonging to one of the assumed clusters, or to have partial ancestry in one of them in previous generations (see Results for more details on the different sets of STRUCTURE runs).

Results

mtDNA

Genetic variation and phylogenetic relationships.—The three investigated species exhibited moderate to high levels of variability in the concatenated mtDNA data set (Table 1). Haplotype (gene) diversity was highest in *Leopardus tigrinus* and lowest in *L. colocolo*, correlating with the included sample size and number of variable sites for each species. In contrast, nucleotide diversity was considerably higher in *L. colocolo* than in the other two species, in spite of the small sample size available for the former, which only partially represented its geographical range.

All phylogenetic analyses performed with various methods led to congruent results with respect to major topological features of the mtDNA tree (Fig. 2). Clades representing *L. geoffroyi* and *L. colocolo* were strongly supported, as was the placement of the kodkod (*L. guigna*) as the sister-group of *L. geoffroyi*. All Brazilian samples of *L. tigrinus* also formed a highly supported clade, corresponding to a morphologically well-defined species occurring in this region. The deep divergence between these lineages and the Central American sample of *L. tigrinus* (Lti13), which had been observed in a previous study (Johnson *et al.* 1999), was corroborated by this larger mtDNA data set. Moreover, in several of the analyses performed here (e.g. Fig. 2), the Lti13 lineage was found to be more closely related to the (*L. geoffroyi* + *L. guigna*) clade than to the remaining *L. tigrinus*, which would challenge the monophyly of this species. Additional sampling of *L. tigrinus* individuals in Central America and northern South America will be required to further investigate the possibility of a species-level distinction involving these populations.

Identification of hybridization and introgression events.

The phylogenetic trees revealed the presence of several ‘misplaced’ individuals, i.e. animals whose morphological identification did not match their respective mtDNA clade (Fig. 2). These included eight *L. geoffroyi* individuals (bLge01, 02, 06, 07, 08, 11, 13, 60) placed in the *L. tigrinus* clade; one *L. colocolo* individual (Lco02) also placed in the *L. tigrinus* clade; six *L. tigrinus* individuals (bLti01, 09, 49, 65, 77, 79) placed in the *L. geoffroyi* clade; and five *L. tigrinus* samples (bLti24, 28, 81, 85, 88) placed in the *L. colocolo* clade. Several of these misplaced individuals were available for re-inspection (e.g. frozen carcass from road-

killed animals); in all such cases the original morphological identification was affirmed by congruent re-assessments by the authors as well as independent scrutiny by other experts. To verify if contamination could explain the results, DNA from these individuals was re-extracted, and the relevant mtDNA segments were independently amplified and sequenced, in every case confirming the original result. Interestingly, four of the *L. geoffroyi* found to contain 'misplaced' mtDNA haplotypes (bLge02, 07, 08, 11) presented coat colour patterns that seemed to be ambiguous or intermediate with respect to *L. tigrinus*.

Given the observation of clear species-level distinction on the basis of tree topology, combined with individual 'swaps' suggestive of secondary contact, we performed a pairwise AMOVA to assess the magnitude of overall mtDNA differentiation among the samples defined by morphology as belonging to each species. This approach aimed (i) at assessing the net mtDNA differentiation among species; and (ii) to serve as a baseline for comparison with the microsatellite data set (see below). Various sequence-based distance measures were explored, as well as a traditional F_{ST} using only haplotype frequencies. The latter approach led to a severe underestimate of species-level differentiation (overall $F_{ST} = 0.06$) likely due to the high intraspecific haplotype diversity (see Table 1) masking the evident occurrence of distinct clades (see Fig. 2). In contrast, all AMOVA comparisons incorporating haplotype differences produced high and significant ($P < 0.001$) F_{ST} values, in spite of the inclusion of 'swapped' individuals in their morphology-based cluster. For example, an AMOVA using p-distances led to F_{ST} estimates of 0.61 for *L. geoffroyi* vs. *L. colocolo;* 0.53 for *L. tigrinus* vs. *L. geoffroyi* and 0.47 for *L. tigrinus* vs. *L. colocolo*.

Demographic history and implications for hybridization.

The phylogenetic trees indicated the existence of very little internal structure within the *L. tigrinus* and *L. geoffroyi* clades, with shallow branches, no robust support for any grouping and no clear geographical clustering of haplotypes (Fig. 2). This very shallow and unstructured pattern, which approximates a star-like appearance in an unrooted tree, is suggestive of a recent population expansion. In contrast, the *L. colocolo* clade did present some internal structure, in agreement with our previous results (Johnson *et al.* 1999). Interestingly, there was a well-supported inner group joining the single Brazilian sample of *L. colocolo* included in the mtDNA data set (Lco13) and all *L. tigrinus* samples that clustered in this clade.

The next set of mtDNA-based analyses focused exclusively on the *L. tigrinus* and *L. geoffroyi* clades and aimed to investigate two issues: (i) the occurrence and age of historical population expansions in these groups; and (ii) the relationship between these inferred past events and the haplotype-swaps indicative of interspecies hybridization. To test the hypothesis of recent population expansions in these groups, we performed mismatch distribution analyses and neutrality tests for each of these mtDNA clades. The mismatch distribution was smoothly unimodal for the *L. tigrinus* clade (see Supplementary material), supporting the inference of a recent demographic expansion in this species. Although the pattern was also roughly unimodal for the *L. geoffroyi* clade, in this case, the curve was not completely smooth, suggesting a more complex demographic history for this felid. All neutrality tests produced negative values for both clades, but most were nonsignificant ($P >$

0.05). The only exception was Fu's Fs test for the *L. tigrinus* clade (−12.179), which was significantly negative ($P < 0.05$). Overall, these results are supportive of the hypothesis of a recent population expansion in both species, especially in *L. tigrinus*.

We then performed a molecular dating analysis to assess the age of the haplotype coalescence (Time to the Most Recent Common Ancestor, TMRCA) in each of the two focal clades. This coalescence age would be an upper bound to the time since the inferred expansions occurred. Moreover, given the shallow, unstructured pattern observed in the phylogeny, it can be assumed that the coalescence age approximates the expansion age, especially in the case of *L. tigrinus* (for which the signal of recent expansion is clearer). The Bayesian molecular dating analyses performed with BEAST yielded a smooth posterior probability distribution (indicating a reliable parameter estimate) for the TMRCA of both the *L. tigrinus* and *L. geoffroyi* mtDNA clades. Interestingly, the ages were remarkably concordant for the two clades, with median TMRCA estimates of 75 700 and 70 000 years ago, respectively. The credibility interval around these estimates, indicated by the 95% highest probability density interval, was also very similar in the two species: 28 700–157 000 years ago for the *L. tigrinus* clade, and 26 800–144 000 years ago for the *L. geoffroyi* clade.

Finally, to gain a more detailed understanding of the genealogical relationships among mtDNA haplotypes in these two focal clades, we constructed a median-joining network, using a concatenation of the *ND5* and control region segments (the *ATP8* segment was excluded due to its having more missing data) and removing from the analysis all sites with missing or ambiguous information (Fig. 3). The two species-level groups were again apparent, separated by 48 substitutions and one synapomorphic indel. A star-like pattern could be observed in a portion of the *L. tigrinus* cluster, with one common haplotype in a central position connected by short branches (one mutational step each in all but one case) to multiple rarer sequences. No such pattern could be discerned in the *L. geoffroyi* phylogroup, which is congruent with the other results, indicating that the signal for a recent sudden expansion is less clear in this species than in *L. tigrinus*. Five haplotypes were shared between the two species, and four others seemed to be 'swapped' between them (i.e. they were only sampled in the species which did not correspond to their containing cluster). Interestingly, these haplotypes were always nested within each of the well-defined groups, i.e. they occupied internal rather than basal positions within each cluster, supporting the interpretation that the 'swaps' are due to secondary contact. In the case of the *L. tigrinus* clade, all haplotypes sampled in *L. geoffroyi* individuals were associated with the star-like portion of the cluster (see Fig. 3).

Microsatellites

Patterns of allelic diversity.—All nine microsatellite loci were polymorphic for *L. tigrinus*, *L. geoffroyi* and *L. colocolo*. All individuals presented unique multilocus composite genotypes. Levels of genetic diversity and allele frequency distributions were similar for the three species (Table 2; see Supplementary material for details). There were 36 private alleles, 14 of which in *L. tigrinus*, 12 in *L. geoffroyi* and 10 in *L. colocolo*. A significant

departure from HWE was observed at two loci each for *L. tigrinus* and *L. geoffroyi* after a Bonferroni correction ($\alpha = 0.05$) (Table 2).

These deviations indicated the possible presence of null alleles or other locus-specific genotyping errors. Analysis of the microsatellite data set with MICRO-CHECKER showed no evidence for genotyping errors due to stuttering or largeallele dropout, suggesting the presence of nonamplifying alleles as a probable source of genotyping errors. Estimated null frequencies varied by population and locus, with *L. geoffroyi* presenting two significant results of general excess of homozygotes for most allele size classes (F124, FCA723), *L. tigrinus* three (F98, F146, FCA723) and *L. colocolo* one (FCA391). The only locus with evidence of null alleles detected at more than one population was FCA723, suggesting that this locus is prone to genotyping errors that may lead to deviations from HWE. All pairwise locus combinations were in LE for *L. geoffroyi* and *L. colocolo* (no information was available for the analysis of the combinations including loci F42, F124 and F146 for *L. colocolo;* $\alpha = 0.05$, after Bonferroni correction for 36 comparisons). However, two combinations of loci were in linkage disequilibrium for *L. tigrinus* (FCA424 × F42 and FCA441 × F98). Additional analyses were performed after exclusion of inferred hybrids (see below), leading to a better understanding of the mechanisms underlying the observed departures from equilibrium.

The microsatellite-based AMOVA indicated that genetic diversity was significantly partitioned between the three species, although the magnitude of interspecies differentiation was modest. There was a higher degree of differentiation observed for *L. colocolo* relative to the other two ($F_{ST} = 0.162$ with *L. geoffroyi* and $F_{ST} = 0.140$ with *L. tigrinus*; $P < 0.001$). The genetic differentiation between *L. tigrinus* and *L. geoffroyi* was remarkably low for an interspecies comparison ($F_{ST} = 0.064$; $P < 0.001$), especially given that these two species clearly form separate evolutionary units on the basis of the mtDNA data (see Fig. 2 and text above for mtDNA-based AMOVA).

Admixture analyses.

To investigate whether hybridization among these species could be an underlying cause for the low levels of observed interspecific microsatellite divergence, we performed a more detailed assessment using the Bayesian approach implemented in the program STRUCTURE. In the initial sets of analyses we utilized all genotyped samples of *L. tigrinus* and *L. geoffroyi*, as well as six *L. colocolo* individuals (see Supplementary material). We began by evaluating the most likely subdivision scenario without using the phenotype-based information, and the probability of the observed data was minimal with $K = 10$ and maximal with $K = 3$ (see Supplementary material for mean ± SD values of –Ln Likelihoods). Evaluating the results with $K = 3$, we observed that each species was assigned predominantly to one of the three clusters. However, while the *L. colocolo* population was assigned to Cluster 3 with a mean probability higher than 0.9, the *L. geoffroyi* and *L. tigrinus* populations were assigned to Clusters 1 and 2 with mean probabilities of 0.558 and 0.642, respectively. These results suggested that it was more difficult to assign the *L. geoffroyi* and *L. tigrinus* individuals to exclusively one cluster based on the genetic information alone. One *L. tigrinus* individual (bLti81) was assigned to the *L. colocolo*

cluster with a probability of 0.95, in agreement with the mtDNA results (Fig. 2). Intriguingly, four other individuals (bLti24, 28, 85, 88) whose mtDNA indicated a hybrid ancestry with *L. colocolo*, were instead simultaneously (and partially) assigned to the *L. tigrinus* and *L. geoffroyi* clusters.

The exclusion of these five *L. tigrinus* individuals identified by the mtDNA data as hybrids with *L. colocolo* eliminated the linkage disequilibrium for the former species at all nine analyzed loci, even though the hybrids with *L. geoffroyi* still remained in the data set. In contrast, HW disequilibrium persisted in the *L. tigrinus* and *L. geoffroyi* population, even after the exclusion of all individuals putatively identified as hybrids by the present analyses (see below).

The next sets of analyses were focused on investigating the hybridization between *L. tigrinus* and *L. geoffroyi*, and therefore we excluded all *L. colocolo* individuals as well as the five samples (mentioned above) inferred to be hybrids with this species. To dissect the genetic composition of all individuals in this data set ($n = 96$), we performed a detailed analysis using STRUCTURE. In the main set of runs, we did not use phenotype-based prior information on species assignment, so as to let the clusters be assessed solely on the basis of the genetic data. We initially investigated the most likely number of distinct populations included in this data set. For this set of analyses we only tested $K = 1$–4, since we had observed a substantial decrease in the likelihood of the data with $K > 4$ in the previous runs (which had also included *L. colocolo).* The probability of the data was maximal for $K = 3$ (–Ln likelihood = 2738.44; see Supplementary material for mean ± SD of all likelihood values), followed closely by $K = 2$ (2743.84). Since the correlated frequencies model has been reported to overestimate K in some cases, reflecting deviations from random assortment that are not caused by genuine population subdivision (Falush *et al.* 2003), we followed Pritchard *et al.*'s (2000) recommendation that, when different values of K have similar probability estimates, we should be sceptical about the reliability of the ones implying a higher degree of subdivision. This would especially be the case when the assignments were roughly symmetrical to multiple populations, with almost no individuals strongly assigned to one of the three clusters, and with no clear biological interpretation for these assignments. As this pattern was clearly recognizable in our case when assuming $K = 3$, we chose to employ $K = 2$ for this set of analyses, as this seemed to capture most of the genetic structure in the sampled individuals and was biologically more reasonable.

With $K = 2$, the *L. geoffroyi* samples were assigned predominantly to Cluster 1 (with $q1 = 0.85$), and those of *L. tigrinus* to Cluster 2 (with $q2 = 0.82$) (Table 3). Of the 41 *L. geoffroyi* individuals, 29 were assigned to Cluster 1 ('geoffroyi' cluster) with $q1 \geq 0.9$, while 11 had intermediate $q1$ values between 0.818 and 0.396 (Fig. 4), i.e. they had a considerable portion of their genome inferred to be of *L. tigrinus* ancestry (see Supplementary material for a full list of q-values for all individuals). One additional individual (bLge67, from Bolivia) had a probability of 0.92 of belonging to the *L. tigrinus* population instead of its own, indicating that it also possessed hybrid ancestry. The 12 samples with $q1 < 0.90$ were all from RS state, mostly from its central region, with only one exception from Argentina (bLge50) (see Fig. 1). Of the 55 *L tigrinus* samples, 34 were assigned to Cluster 2 ('tigrinus' cluster) with $q2 \geq 0.9$, while 19 had intermediate $q2$ values (between 0.897 and 0.113) and two were highly

associated with the *L. geoffroyi* cluster (bLti09: $q2 = 0.099/q1 = 0.901$ and bLti49: $q2 = 0.072/q1 = 0.928$) (Fig. 4). Of the 21 individuals assigned to Cluster 2 with $q2 < 0.90$, 12 (57%) were from RS state, while the remaining ones originated in different Brazilian states: Paraná (6), São Paulo (2) and Goiás (1).

All samples from both species with $q \leq 0.90$ showed very broad credibility intervals for their *q*-values, often encompassing both ends of the 0–1 range (see Fig. 4 and Supplementary material). This type of pattern has also been reported for other highly hybridizing species (e.g. Beaumont *et al.* 2001; Nielsen *et al.* 2003). Assuming a *q*-value of 0.9 as the threshold for distinguishing pure from hybrid individuals, as reported in similar hybridization studies (e.g. Lancaster *et al.* 2006; Vähä & Primmer 2006; Oliveira *et al.* 2008), and considering the broad intervals for *q* found in the intermediate individuals in our sample, our microsatellite data set identified 12 putative hybrids among the morphologically defined *L. geoffroyi* individuals, and 21 among the *L. tigrinus* (see Supplementary material).

To further assess the genetic ancestry of the inferred hybrids so as to determine the occurrence and extent of genomic introgression in our sample, we performed an additional set of STRUCTURE analyses using the phenotype-based information. In this case, the analysis allows the inference, for each individual, of the probability that its ancestry lies in a different population in the first, second or third past generations (Pritchard *et al.* 2000). In this case, these three possibilities are equivalent to the sample originating from a misidentified individual (i.e. totally belonging in the other genetic population), or an F_1 hybrid, or a second-generation hybrid, respectively. As expected, the use of phenotype-based priors greatly enhanced the assignment of individuals to their assumed population (Table 3): the *L. geoffroyi* sample was now assigned with $q1 = 0.97$ to Cluster 1 and the *L. tigrinus* sample with $q2 = 0.95$ to Cluster 2. All individuals inferred to be hybrids in the previous set of analyses were carefully inspected, revealing no case suggestive of misidentification. The majority of the hybrid individuals presented high probabilities of belonging to their assumed phenotype-based population while having admixed ancestry from the other species, predominantly in the second and third past generations. Five of the twelve *L. geoffroyi* presenting *q*-values ≤0.90 of belonging to its own cluster in the previous set of analyses (without phenotype-based information), and ten of the 21 *L tigrinus*, still presented evidence of admixed ancestry when maintaining a threshold of $q = 0.9$ for these runs. In addition, none of the inferred hybrid individuals presented a very high probability of being an F_1, indicating the occurrence of advanced introgression and complex patterns of admixed ancestry (see Table 3 and Supplementary material).

There were several cases of congruence in the detection of hybrids using the mtDNA and microsatellite data sets (e.g. bLge07, bLge11, bLge13, bLti01, bLti09, bLti49 and bLti79; see Table 3). However, some individuals bearing ‘misplaced’ mtDNA haplotypes were only identified as admixed in the microsatellite-based STRUCTURE analysis without phenotypic information, while others were not recognized as being admixed by any microsatellite-based assessment. Finally, some individuals presented intermediate *q*-values in the microsatellite-based analyses, suggestive of admixture, but there was no evidence of hybridization with mtDNA. Given this complex set of patterns, which is expected under a scenario of multigenerational admixture, we conservatively defined as inferred hybrids only the 14

individuals bearing conclusive mtDNA-based evidence (Table 3), while the actual number may be much higher; up to 33 considering the microsatellite data alone (see above and Supplementary material for details).

To investigate whether we could detect a geographical pattern in this observed genetic introgression, we focused on the *L. tigrinus* sample, for which a broader spatial coverage was available. We hypothesized that admixture with *L. geoffroyi* would be more prevalent in the vicinity of their geographical contact zone than farther north in Brazil, as indicated by the individual-based analyses described above. Our goal was to test whether this trend could be detected at the population level with our microsatellite data set. For this analysis, we only used *L. tigrinus* samples with a known geographical origin and excluded regions for which our sample size was very small (Brazilian center–west and Paraguay). This resulted in a sample of 50 *L. tigrinus*, which were subdivided into three geographical subpopulations, arranged in a south-to-north sequence: (i) RS state; (ii) SC + PR states; and (iii) SP + RJ + ES states (see Fig. 1 and Supplementary material). We assessed the genetic differentiation among these regions, as well as each of them vs. the entire sample of *L. geoffroyi* ($n = 41$), using an AMOVA-based estimate of F_{ST}. All comparisons yielded significant F_{ST} values, except between the two northernmost *L. tigrinus* populations (Table 4). The RS partition was thus the most divergent among the three *L. tigrinus* subgroups and was found to be more similar genetically to the *L. geoffroyi* sample than to the SP + RJ + ES population of its own species. There was a clear trend of increased differentiation from *L. geoffroyi* as the sampling got more distant from the contact zone between the two species, supporting the inference of a geographical gradient of introgression affecting the genetic composition of *L. tigrinus* in the surveyed areas.

Discussion

A hybrid zone between Leopardus tigrinus and L. geoffroyi in southern Brazil

The results presented here provide strong evidence for the occurrence of hybridization between *Leopardus tigrinus* and *L. geoffroyi*, which seems to be concentrated in their region of geographical contact in southern Brazil (see Fig. 1). The joint inference from the mtDNA and microsatellite data sets, in combination with the morphology-based assessments, suggests that a hybrid zone between these species occurs in this area. Our analyses also identified a geographical gradient of introgression of *L. geoffroyi* genomic components into *L. tigrinus* populations sampled at varying distances from the inferred hybrid zone.

The magnitude of *L. tigrinus* and *L. geoffroyi* differentiation based on our microsatellite data set was quite low, relative to what is usually observed in other studies focusing on carnivores (e.g. Johnson *et al.* 1999; Randi *et al.* 2001; Randi & Lucchini 2002). This pattern might be explained by incomplete evolutionary separation between these two cat species, due to recent common ancestry. However, this hypothesis is rejected by the mtDNA results presented here (see Fig. 2), as well as previous analyses indicating that these species do present clear evolutionary distinctiveness (Johnson *et al.* 1999, 2006). The extensive interspecific allele sharing observed here could instead be influenced by the occurrence of rampant homoplasy at the examined loci, leading to the co-occurrence of alleles that are identical in state, though not by descent (Jarne & Lagoda 1996). This phenomenon tends to

homogenize allele frequencies between distantly related species or populations and has been reported even at the intraspecific level (Nauta & Weissing 1996; Culver *et al.* 2001). It is probable that homoplasy does play a role in the low microsatellite-based interspecies divergence observed here (as may be inferred from the low F_{st} differentiating *L. colocolo* from *L. geoffroyi*, in spite of the lack of any evidence of hybridization between them). However, it seems unlikely that the magnitude of interspecies allele overlap observed here could be achieved by homoplasy alone, especially in light of the geographical pattern seen in Table 4, as well as the mtDNA-based analyses supporting hybridization. More likely, the observed pattern of extensive allele sharing and weak genetic differentiation between the two species reflects a combination of some level of homoplasy and considerable introgressive hybridization between these species, which has eroded their allelic differences at these markers.

The evidence of hybridization and introgression between these two cat species is supported by the observation of 'misplaced' mtDNA haplotypes and by a typical cline in microsatellite-based genetic differentiation between them (see Fig. 4). However, the precise identification of all hybrid individuals is challenging, due to the complex pattern of admixture inferred by the combination of the two types of molecular markers. Fourteen individuals were identified as hybrids due to their 'swapped' positions in the mtDNA phylogeny (see Fig. 2), including animals from both species (based on morphological criteria). Although most individuals could be unambiguously assigned to one species using morphological criteria, some of the animals identified as putative hybrids (e.g. bLge08 and bLge11) bore unusual coloration patterns, seemingly intermediate between the two taxa. With the microsatellite data, several individuals of both species presented intermediate *q*-values assigning them to either cluster, with very broad credibility intervals that hampered a conclusive inference of their allocation (see Table 3 and Supplementary material). There was no clear correlation between the extent of microsatellite-based admixture and the presence of an introgressed mtDNA lineage (e.g. when comparing both types of data for bLge01, bLge02, bLti65 and bLti77 in Table 3 and Fig. 2). Nevertheless, there were some cases of concordance between mtDNA introgression and a high level of nuclear admixed ancestry, which possibly indicate individuals descended from recent (and/or multiple) episodes of hybridization.

Considering the combined evidence from the mtDNA and microsatellites, up to 33 individuals may be identified as hybrids between *L. tigrinus* and *L. geoffroyi* (assuming $q = 0.9$ as a threshold; see Supplementary material), which represents 34% of the final sample ($n = 96$). This would be one of the most extensive levels of hybridization reported for carnivores up to now, similar to what has been observed in the intensely hybridizing populations of wild and domestic cats in Hungary (Lecis *et al.* 2006). However, in the case of the European wild and domestic cats (*Felis silvestris*), the hybridizing populations are very closely related and currently regarded as conspecific (Driscoll *et al.* 2007), whereas the Neotropical pair described here consists of distinct species separated *c.* 1 million years ago (Johnson *et al.* 2006). Our mtDNA data support the evolutionary distinction of the two lineages (see Fig. 2), in stark contrast with the extremely low level of differentiation observed with the microsatellite markers.

Most of the individuals identified here as probable hybrids based on both molecular markers have a geographical origin compatible with the presence of a hybrid zone. Of the 14 cats identified as hybrids by the mtDNA data, 12 originate from RS state, particularly from its central region near parallel 30°S, the only area in Brazil where sympatry of the two species has been documented (Eizirik *et al.* 2006). Despite the difficulty in precisely defining hybrids with our microsatellite data, we could observe a similar pattern of concentration of intermediate individuals in this geographical contact zone, with few implicated animals sampled far from it. As a whole, these observations indicate that the admixture between these species is quite concentrated in the areas surrounding their contact zone, suggesting either that the hybridization process is extremely recent or that there is some selective restriction on the geographical spread of admixed descendants. In-depth studies involving ecological as well as genetic analyses will be required to further understand the underlying causes of this pattern.

Although we could only document the clear occurrence of genomic introgression from *L. geoffroyi* to *L. tigrinus*, it remains plausible that it also occurs in the opposite direction, given the observation that morphologically defined *L. geoffroyi* bear genomic segments and/or mtDNA haplotypes originating from *L. tigrinus.* If affirmed by further scrutiny, it would remain to be determined whether the magnitude of introgression is symmetrical between these species. This would produce an interesting comparison to the patterns found in several studies of hybridization between other pairs of carnivore taxa, which often identify asymmetric introgression (e.g. Roy *et al.* 1994; Vilà & Wayne 1999; Randi & Lucchini 2002; Lancaster *et al.* 2006; Lecis *et al.* 2006). A common and plausible explanation for the asymmetry observed in other systems is the difference in local density between the two hybridizing populations, leading to the increased pressure of genomic introgression in one direction vs. the other. The uneven presence of males and females from different hybridizing populations may also affect the directionality of the process (e.g. Lancaster *et al.* 2006), especially if associated with mating systems that favour one of the possible hybrid pairs. Although very little is known about the mating system of *L. geoffroyi* and *L. tigrinus* in the wild, or their relative densities in this hybrid zone, preliminary field observations suggest that both are relatively common in RS state (the former in the south and the latter in the north, Eizirik *et al.* 2006), suggesting that the genomic influx may be similar in both directions.

The results presented here allow us to propose a hypothesis for the genesis of this hybrid zone, which postulates that the two species evolved in allopatry from a common ancestor that lived *c.* 1 million years ago, and they only recently entered in geographical contact due to a population expansion in one or both of them. Our analyses are compatible with the inference of recent population expansions in both species, though the signal is stronger and clearer for *L. tigrinus.* The shapes of the mtDNA phylogeny and haplotype network, along with results from the mismatch distribution analysis and Fu's Fs test, all indicate that this species bears the signature of a recent demographic expansion, which was inferred to have occurred near the coalescence of its haplotypes *c.* 76 000 years ago. An intriguing finding was the very concordant coalescence date of the *L. geoffroyi* clade, raising the possibility that the demography of both species was similarly affected by the same historical events. Most interestingly, the network positions of all *L. tigrinus* mtDNA haplotypes introgressed

into the sampled *L. geoffroyi* were associated with this inferred expansion (see Fig. 3), supporting the speculation that the hybrid zone may be a consequence of this historical process of population growth.

There are several examples of hybrid zones that seem to be the result of secondary contact between previously allopatric populations that meet due to demographic expansions caused by responses to climatic and habitat changes (Barton & Hewitt 1985; Harrison 1993). Although this scenario is plausible for the case of *L. tigrinus* and *L. geoffroyi*, with a demographic expansion playing an important role in the geographical encounter between the two species, the exact age of the hybridization process still cannot be determined. Although the observed pattern is consistent with a natural hybrid zone formed *c.* 70 000 years ago, it is still possible that the actual admixture between the species is much more recent and could have been influenced by human activities. Anthropogenic habitat alteration has been rampant in some areas of RS state for over two centuries, and it is conceivable that these populations were not in direct contact prior to human disturbance. Depending on the intensity of interspecies breeding per generation, it is not impossible that two centuries of hybridization could lead to the observed pattern of admixture (e.g. see Mank *et al*. 2004). The distinction between these two historical scenarios is one of the major challenges ahead in the effort to characterize this hybrid zone.

The broader picture: hybridization among L. tigrinus, L. geoffroyi and L. colocolo

Hybridization between *L. tigrinus* and *L. colocolo* had been previously reported (Johnson *et al*. 1999), and three of the implicated individuals identified in that paper (bLti24, 28 and 85) are included in this study, corroborating our initial findings with an expanded data set. In addition, here we identified two more *L. tigrinus* individuals that share *L. colocolo* ancestry, and one animal (Lco02) that seems to be a hybrid in the opposite direction (*L. tigrinus* mtDNA introgressed into a *L. colocolo*). The latter individual was a captive animal, whose sample was collected in 1981, so it is difficult to ascertain whether the implied hybridization event (involving a female *L. tigrinus* and a male *L. colocolo*) happened in the wild. The remaining animals involved in this hybrid combination can be ascertained to have a wild origin, implying that the underlying events did occur *in situ*. Interestingly, the mtDNA data supported a phylogenetic connection between these individuals and the *L. colocolo* sample collected in central Brazil, whose regional origin (Goias state) is the same as that of three of these hybrid animals (see Fig. 2 and Supplementary material). We can thus infer that a hybrid zone between *L. tigrinus* and *L. colocolo* occurs in central Brazil, even though more sampling is still required to characterize it in more detail.

These results therefore reveal a remarkable pattern of complex interspecies admixture, which can be graphically observed in our mtDNA-based phylogenetic tree (see Fig. 2): individuals identified morphologically as *L. tigrinus* may bear one of three very distinct mitochondrial lineages, i.e. (i) that of their own species; (ii) that of *L. geoffroyi;* or (iii) that of *L. colocolo*. This situation of a double hybrid zone is quite unusual, especially involving medium-to-large mammals. Among the few reported cases of hybridization involving wild populations of three different mammal species, two include carnivores. The case of North American canids includes the observation that coyotes (*Canis latrans*) have expanded their

range in the last century and in that process have hybridized at different levels with congeneric species, namely the grey wolves (*C. lupus*) and the eastern wolf-like populations often recognized as separate taxa (*C. rufus* and/or *C. lycaon*) (e.g. Lehman *et al.* 1991; Roy *et al.* 1994; Kyle *et al.* 2006). The second case is that of three species of fur seals of the genus *Arctocephalus* re-colonizing a subantartic island after historical extinction in the 19th century (Lancaster *et al.* 2006). The influence of human disturbance is clear in the latter case (as the historical extinction occurred due to over-hunting), and the three-species hybridization that can currently be observed on that single island is likely to decrease over time (Lancaster *et al.* 2006). In the case of canids, human impact has also played a major role, in the form of habitat alteration fostering coyote expansion, and as persecution of wolves leading to their decline in eastern North America (Kyle *et al.* 2006). The main source of complexity in this case lies in the persisting debate over the historical distinctiveness of eastern/red wolves, and whether they did represent a unique taxon prior to hybridization with coyotes (Roy *et al.* 1994; Murray & Waits 2007). The case reported here thus seems to present some relevant differences with respect to one or both of these other examples: (i) the lineages involved seem to be distinct enough to represent well-accepted species; (ii) the historical population expansion that may have been involved in this process is much older than the human presence in the region; (iii) there seem to be two geographically defined hybrid zones instead of a single site of admixture (as is the case in fur seals); (iv) bidirectional introgression may be occurring (although not yet demonstrated conclusively), at least in the case of *L. tigrinus* vs. *L. geoffroyi*; and (v) the causative role of recent human impact on the genesis of the hybrid zone is not as clear (or likely) as in the other two cases, although it remains a possibility.

Finally, our analyses showed no evidence of hybridization between *L. geoffroyi* and *L. colocolo*, in sharp contrast with the observed admixture of *L. tigrinus* with both of those species. If this observation is affirmed by further sampling of these felids, it is likely to reflect an important difference with respect to the reproductive isolation mechanisms acting in the various possible pairs formed by these species. Since *L. colocolo* and *L. geoffroyi* are sympatric over most of their geographical ranges (see Fig. 1), it can be hypothesized that they have evolved fully effective mechanisms for avoidance of hybridization with each other. The fact that this is not the case of *L. tigrinus* with respect to either of these two other species supports the inference that it has evolved in allopatry and only more recently entered in contact with these congeners, inducing the formation of a double hybrid zone.

Implications for conservation

The elucidation of hybridization processes between wild species in nature is critically important for the conservation of the involved taxa (Allendorf *et al.* 2001). Both *L. tigrinus* and *L. geoffroyi* are listed in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), while *L. colocolo* is in Appendix II (UNEP-WCMC 2004); all three species are considered to be nearthreatened by the World Conservation Union (IUCN; IUCN/SSC Cat Specialist Group 2002). In Brazil, *L. tigrinus* and *L. colocolo* are considered vulnerable and *L. geoffroyi* nearthreatened (IBAMA 2003), while in RS state, *L. colocolo* is listed as endangered and the others two as vulnerable (Marques *et al.* 2002). For the three species, lack of information on their biology, ecology,

genetic structure and evolutionary history pose challenges to the design and implementation of adequate management and conservation strategies (Nowell & Jackson 1996).

In this context, our results allow several recommendations regarding the conservation of these felids. Captive breeding of animals originating from areas where hybridization has been detected should be managed carefully, so as not to artificially increase the representation of introgressed genomic segments in the *ex situ* gene pool. In particular, we showed here that RS state constitutes a genetically distinct *L. tigrinus* population, which is more similar to *L. geoffroyi* than to conspecific populations located farther from the contact zone. Since it is still conceivable that human-induced habitat alteration has exacerbated (or even caused) this hybridization process, we recommend that this population be managed separately (e.g. in captive breeding programs and possible translocation operations), so as to not to compromise the genetic integrity of *L. tigrinus* in areas located farther from the hybrid zone. Furthermore, it is critical to perform in-depth ecological and genetic studies attempting to dissect the causes and current consequences of these hybridization processes, including the possible influence of human-induced habitat change and the role of natural selection in restricting the spread of introgression beyond the hybrid zone detected in southern Brazil.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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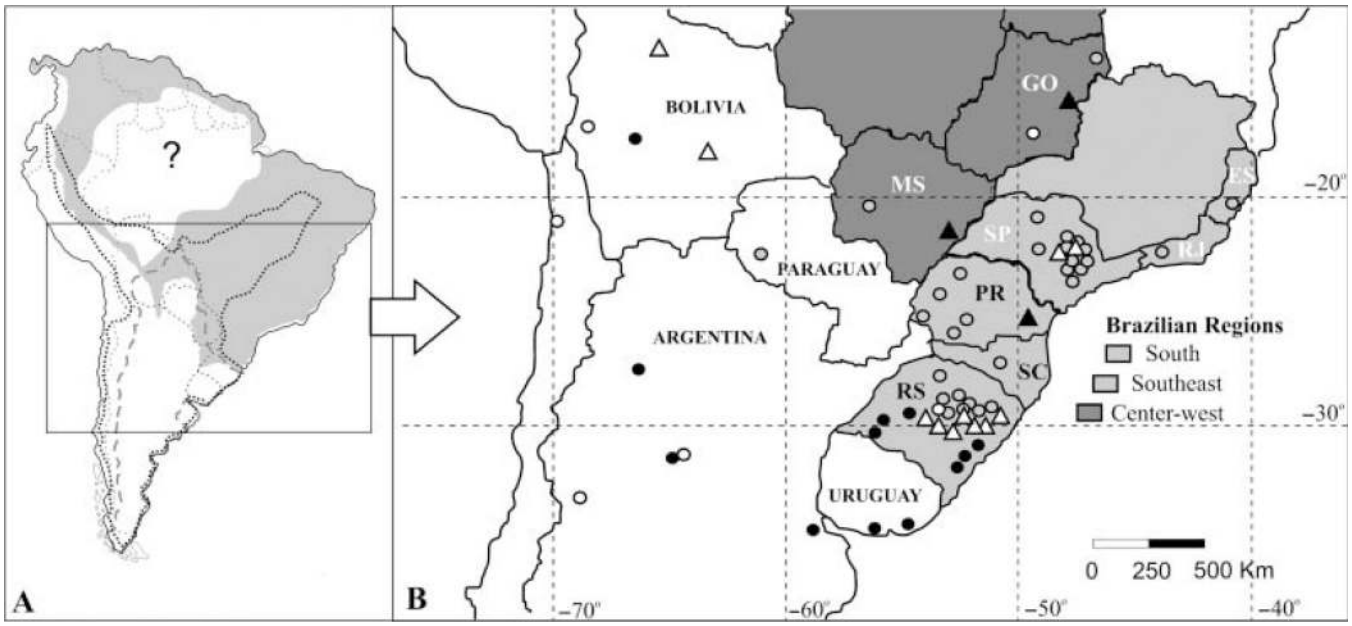

Fig. 1.

(A) Geographical distribution of *Leopardus tigrinus* (grey-shaded area), *L. geoffroyi* (area defined by the grey broken Line) and *L. colocolo* (area defined by the black dotted line) in South America (modified from Oliveira 1994; Nowell & Jackson 1996; Eisenberg & Redford 1999). (B) Map of the study area showing approximate sample collection sites for *L. tigrinus* (grey circles), *L. geoffroyi* (black circles) and *L. colocolo* (white circles). Each symbol represents one sampling locale and may include one or more individuals (only individuals with known collection locales are included). The Central American sample of *L. tigrinus* (Lti13) and the samples from other felid species (*L. guigna*, *L. pardalis*, *L. wiedii*) are not shown in the figure. White triangles indicate the sampling sites of hybrids between *L. tigrinus* and *L. geoffroyi*, while black triangles indicate collection locales for hybrids between *L. tigrinus* and *L. colocolo.* Abbreviations of Brazilian states: RS (Rio Grande do Sul), SC (Santa Catarina), Paraná (PR), MS (Mato Grosso do Sul), SP (Sao Paulo), RJ (Rio de Janeiro), ES (Espírito Santo), GO (Goiás).

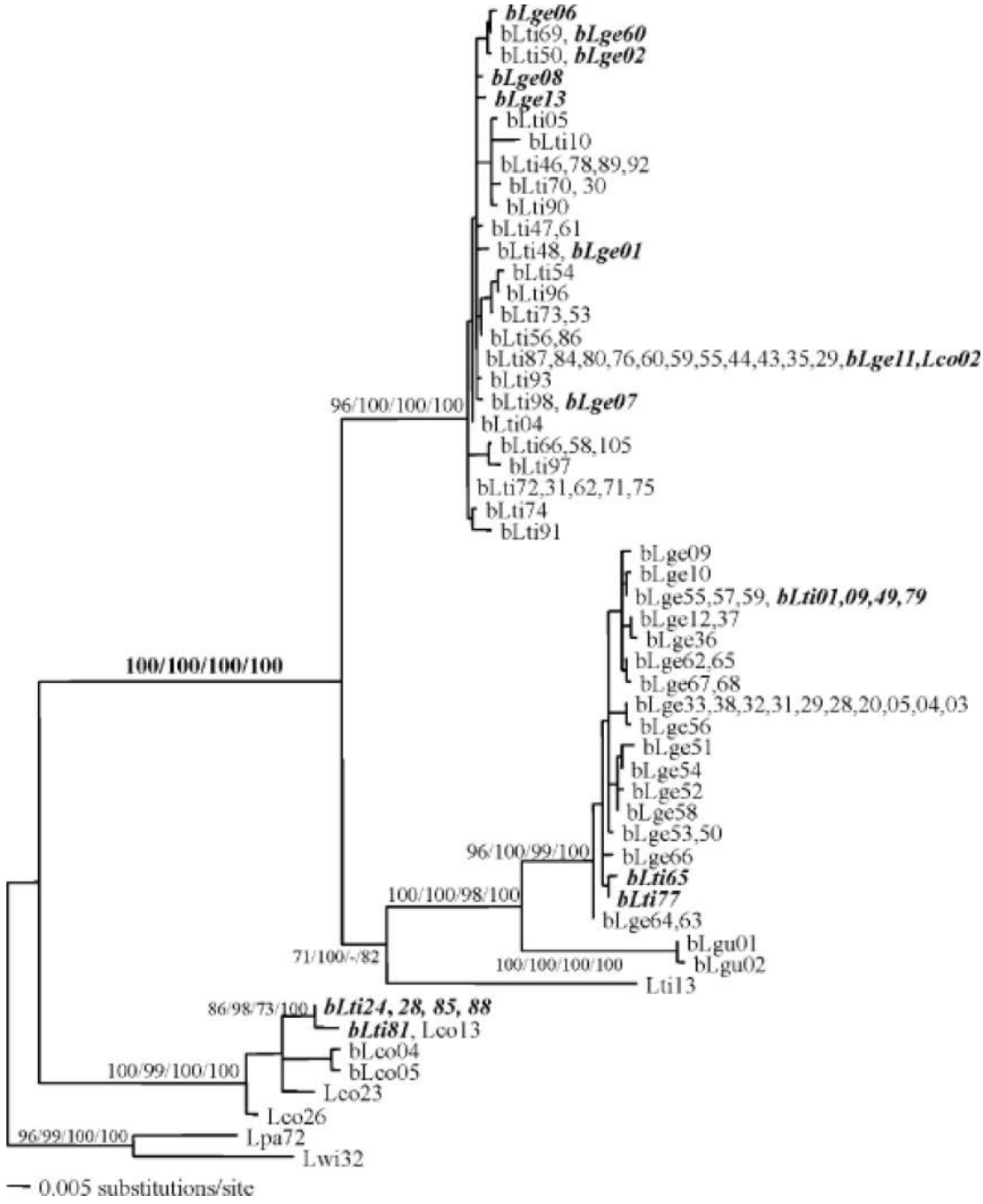

Fig. 2.

Maximum likelihood (ML) phylogeny of mitochondrial DNA haplotypes (concatenated control region, *ND5* and *ATP8* segments, totaling 1024 bp) sampled in multiple individuals of *Leopardus tigrinus* (bLti), *L. geoffroyi* (bLge), *L. colocolo* (Lco), *L. guigna* (bLgu), *L. wiedii* (Lwi) and *L. pardalis* (Lpa) (see Supplementary material for details on sample ID and collection data). Individuals shown in bold italic fonts are inferred to be interspecific hybrids carrying an introgressed mtDNA haplotype from a different species. Values above or below branches indicate support for the subsequent node based on ML/MP/NJ/BI (Bayesian

posterior probabilities are indicated as percentages); support is depicted only for nodes defining major clades relevant for our analyses.

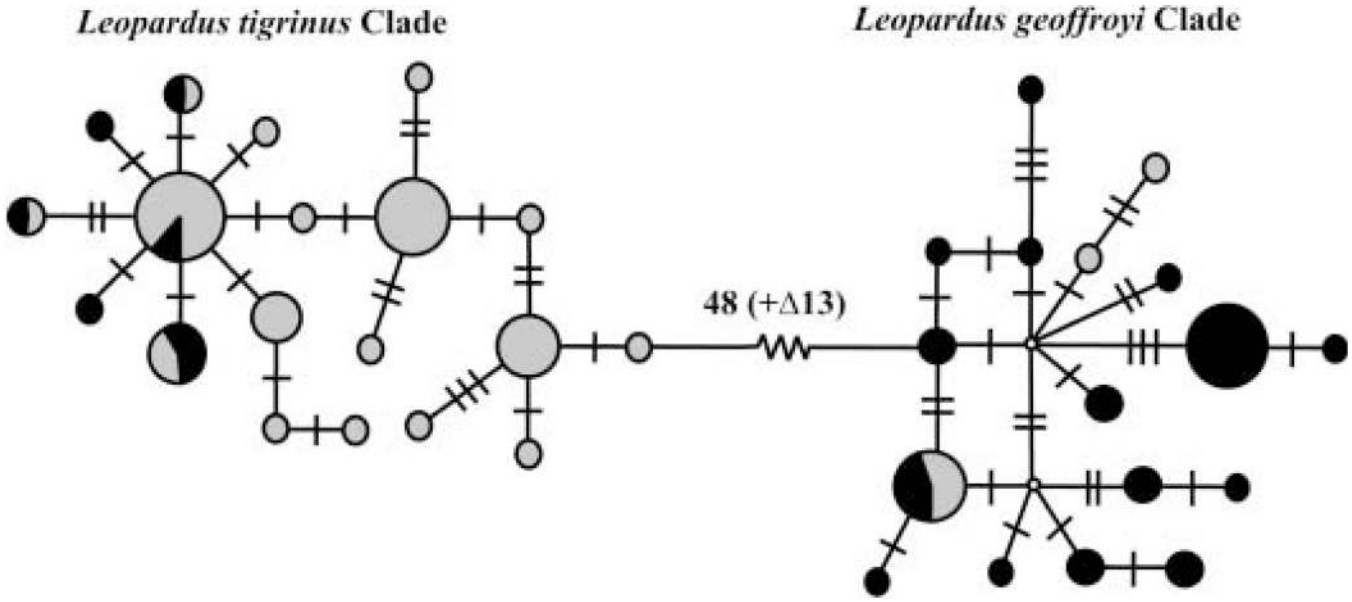

Fig. 3.

Median-joining network of mtDNA haplotypes sampled in *Leopardus tigrinus* (grey) and *L. geoffroyi* (black) individuals. Only control region (CR) and *ND5* sequences were used in this analysis (totaling 795 bp), and all sites containing missing information or gaps were excluded. The area of each circle is roughly proportional to the haplotype frequency. Haplotypes shared between the two species are represented by circles with mixed colours, in which the relative frequency is indicated by the proportion of black and grey. Bars placed on connecting lines indicate the exact number of nucleotide differences between haplotypes. The branch connecting the two main clades contains 48 nucleotide differences and a synapomorphic deletion of 13 nucleotides in the CR defining the *L. geoffroyi* clade.

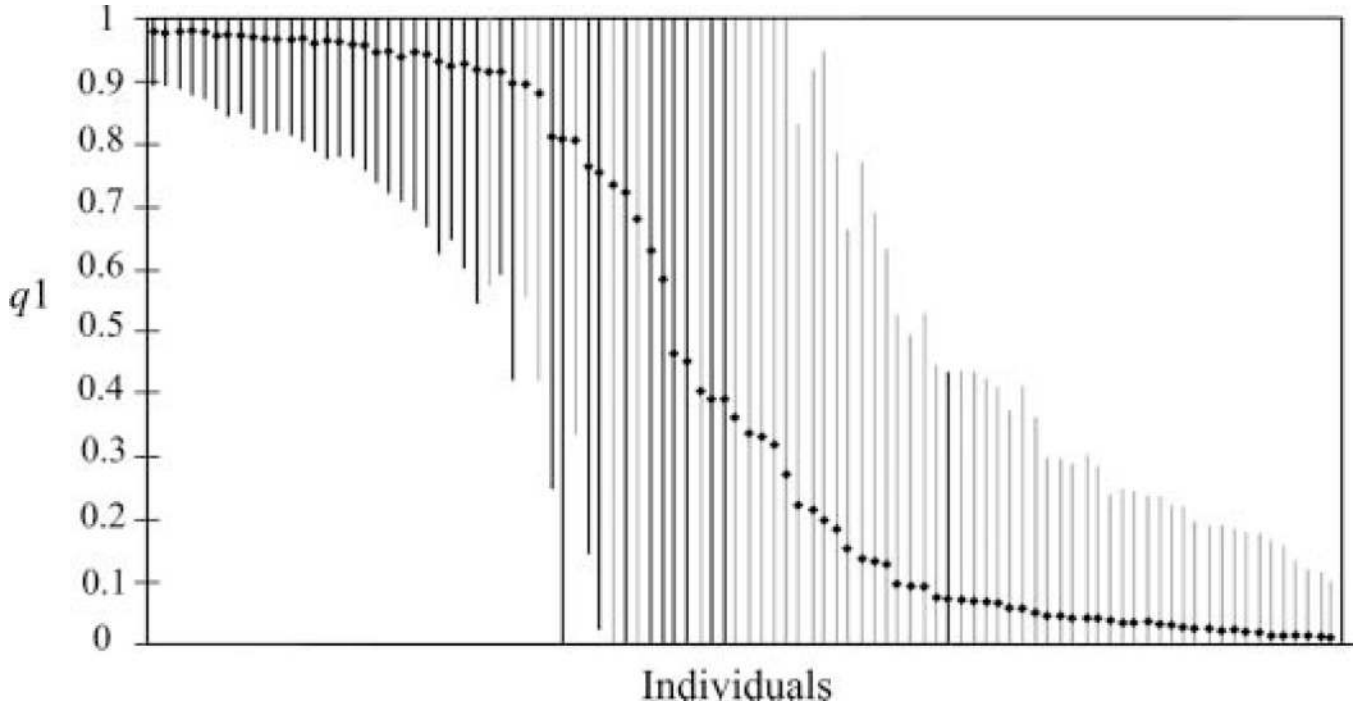

Fig. 4.

Graph depicting the results of the Bayesian admixture analysis focusing on the hybridization between *Leopardus geoffroyi* and *L. tigrinus* (performed with STRUCTURE, with $K = 2$, employing the correlated frequencies model and no use of prior population information, i.e. only the genetic data were used to infer population assignment). All *L. colocolo* individuals and the five identified hybrids with that species were excluded from this data set. Diamonds represent the mean $q1$ value for each individual (averaged over five independent runs). Vertical lines represent a conservative estimate of the credibility interval (CI) for each individual, i.e. the range between the lower and upper bounds of the CIs observed across the five runs. Thicker black lines correspond to individuals morphologically identified as *L. geoffroyi*, while thinner gray lines indicate individuals identified as *L. tigrinus*. Individuals are sorted according to their mean $q1$ value (see Supplementary material for a complete list of q-values and their CIs for all individuals).

Table 1

Genetic diversity assessed for *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* samples using a concatenated mtDNA data set containing segments of the control region and the *ND5* and *ATP8* genes

Clade	N	No. of haplotypes	No. of variable sites	Nucleotide diversity (± SE)	Haplotype diversity (± SE)
L. tigrinus	54	25	27	0.003866 (± 0.002269)	0.9266 (± 0.0247)
L. geoffroyi	38	18	28	0.006247 (± 0.003462)	0.8990 (± 0.0341)
L. colocolo	10	6	18	0.009462 (± 0.005638)	0.8444 (± 0.1029)

Table 2

Characteristics of nine microsatellite loci analyzed in 60 *Leopardus tigrinus*, 41 *L. geoffroyi* and six *L. colocolo* individuals. The size range, number of alleles and expected heterozygosity (H_E) are given for each locus. The mean values across loci are shown in bold at the bottom

		L. tigrinus		*L. geoffroyi*		*L. colocolo*	
Locus	**Size range**	**No. alleles**	H_E	**No. alleles**	H_E	**No. alleles**	H_E
FCA391	207–247	7	0.795	8	0.773	5	0.712
FCA424	166–198	6	0.669	4	0.353	5	0.909
FCA441	131–151	5	0.674	5	0.693	5	0.848
FCA453	186–210	7	0.695	5	0.717	3	0.818
FCA723	243–343	20	0.907*	24	0.932*	4	0.803
F42	219–259	9	0.858	11	0.884	7	0.894
F98	163–187	6	0.402*	3	0.572	5	0.803
F124	160–216	13	0.800	9	0.843*	7	0.924
F146	151–169	7	0.645	5	0.658	5	0.939
Mean		**8.89**	**0.716**	**8.22**	**0.714**	**5.11**	**0.85**

*Significant departure from HWE ($P < 0.05$).

Table 3

Microsatellite-based population assignment and ancestry allocation of inferred hybrids between *Leopardus tigrinus* and *L. geoffroyi*. Only individuals conservatively identified as hybrids based on the mtDNA data set are shown. See text and Supplementary material for discussion on additional individuals whose microsatellite genotypes also suggest a hybrid origin. Abbreviations for individual ID and geographical origin are the same as in Figs 1 and 2

		Without phenotypic information*		With phenotypic information†	
Sample	**Origin**	**Cluster 1 (bLge)**	**Cluster 2 (bLti)**	**Cluster 1 (bLge)**	**Cluster 2 (bLti)**
L. geoffroyi		0.85	0.15	0.97	0.03
bLge01	RS	0.98 (0.89–1.00)	0.02 (0.00–0.11)	0.99	0.00–0.00–0.01
bLge02	RS	0.91 (0.42–1.00)	0.09 (0.00–0.58)	0.98	0.00–0.00–0.02
bLge06	RS	0.73 (0.00–1.00)	0.27 (0.00–1.00)	0.94	0.00–0.02–0.04
bLge07	RS	0.45 (0.00–1.00)	0.55 (0.00–1.00)	0.81	0.01–0.07–0.11
bLge08	RS	0.63 (0.00–1.00)	0.37 (0.00–1.00)	0.91	0.00–0.02–0.07
bLge11	RS	0.40 (0.00–1.00)	0.60 (0.00–1.00)	0.73	0.09–0.08–0.10
bLge13	RS	0.40 (0.00–1.00)	0.60 (0.00–1.00)	0.79	0.05–0.04–0.12
bLge60	RS	0.47 (0.00–1.00)	0.53 (0.00–1.00)	0.93	0.01–0.02–0.04
L. tigrinus		0.18	0.82	0.05	0.95
bLti01	RS	0.37 (0.00–1.00)	0.63 (0.00–1.00)	0.00–0.07–0.10	0.83
bLti09	RS	0.90 (0.54–1.00)	0.10 (0.00–0.46)	0.15–0.61–0.14	0.10
bLti49	RS	0.93 (0.59–1.00)	0.07 (0.00–0.41)	0.59–0.16–0.09	0.16
bLti65	SP	0.04 (0.00–0.24)	0.96 (0.76–1.00)	0.00–0.00–0.02	0.98
bLti77	SP	0.03 (0.00–0.18)	0.97 (0.82–1.00)	0.00–0.00–0.01	0.99
bLti79	RS	0.81 (0.34–1.00)	0.19 (0.00–0.66)	0.24–0.49–0.17	0.10

*These columns contain the '*q*' value (mean across five runs) for each individual in each population cluster, i.e. the probability that its genomic ancestry lies in that group, disregarding any prior assumption based on morphology (see text for details). A conservative estimate of the credibility interval (lowest observed lower bound to highest upper bound among five independent runs) is given in parentheses. The top line for each species indicates the overall assignment of its samples (identified morphologically) to each genetically defined cluster.

†These columns contain the *q* value for each individual in either its own assumed cluster (single number) or as a result of admixture with the other group (three numbers). In the latter case, the numbers are the probabilities that the individual's ancestry lies in the other genetic cluster in the first, second or third past generation, respectively (see text for details).

Table 4

Genetic differentiation among three geographical subpopulations of *Leopardus tigrinus* and the *L. geoffroyi* sample, estimated using an F_{ST} analogue calculated via an AMOVA approach. The overall F_{ST} value for this subdivision scenario was 0.075 ($P < 0.05$). Abbreviations of species designation and Brazilian states are as in Figs 1 and 2

	bLge	bLti-RS	bLti-SC/PR
bLge	—	—	—
bLti-RS	0.049*	—	—
bLti-SC/PR	0.069*	0.027*	—
bLti-SP/RJ/ES	0.117*	0.057*	0.009

*Statistically significant F_{ST} value ($P < 0.05$).