Scat detection dogs, DNA and species distribution modelling reveal a diminutive geographical range for the Vulnerable small red brocket deer *Mazama bororo*

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Abstract The small red brocket deer *Mazama bororo* is endemic to the Brazilian Atlantic Forest, a biome that has been greatly fragmented and altered by human activities. This elusive species is morphologically similar to the red brocket deer *Mazama americana* and the Brazilian dwarf brocket deer *Mazama nana*, and genetic typing is necessary for reliable identification. To determine the geographical range of *M. bororo* more accurately, we conducted non-invasive genetic sampling using scat detection dogs trained to locate deer faeces. We surveyed 46 protected areas located within the species' potential distribution and collected a total of 555 scat samples in 30 of the protected areas. Using a polymerase chain reaction–restriction fragment length polymorphism approach, we genotyped 497 scat samples (89%) and detected *M. bororo* in seven localities in three Brazilian states. The results support a range extension of the small red brocket deer to latitudes 23 and 28°S and longitudes 47 and 49°W. We show that the species' distribution is associated with 37,517 km² of the Ombrophilous Dense Forest in the Brazilian Atlantic Forest, and this conclusion is supported by species distribution modelling. The small red brocket deer is the largest endemic species in Brazil and may have the smallest geographical distribution of any Neotropical deer species. This species occupies fragmented landscapes and is threatened by human encroachment, poaching, and predation by dogs, and based on our findings we recommend policy intervention for conservation planning of the Ombrophilous Dense Forest.

Introduction

The small red brocket deer *Mazama bororo* is one of the most threatened, elusive and poorly known Neotropical deer. It is categorized as Vulnerable on the IUCN Red List (Duarte, 2008). The species inhabits remnants of the Atlantic Forest (Duarte & Jorge, 2003; Weber & González, 2003; Duarte, 2008; Duarte et al., 2008), the second largest rainforest of the American continent, which once stretched almost continuously along the Brazilian coast, extending inland in the south and into eastern Paraguay and north-eastern Argentina (Tabarelli et al., 2005). This biome may hold up to 8% of all species of flora and fauna (Silva & Casteleti, 2003), and has a history of intensive habitat loss and fragmentation caused by human activity. More than 88% of the original forest cover has already been cleared, and the land converted to non-forest use (Ribeiro et al., 2009).

The small red brocket deer is difficult to observe because it inhabits dense forests. In addition, morphological similarities with sympatric brocket deer species preclude the use of traditional methodologies for detection and species identification (Duarte & Jorge, 2003; Vogliotti & Duarte, 2009, 2010). Skins and skulls from Brazilian scientific collections revealed size, coloration and pelage differences between specimens taken from within the extent of occurrence of *M. bororo* and specimens of red brocket deer *M. americana* from other regions of Brazil (Rossi, 2000). Although some morphological characteristics (weight, height, length of body, circumference of thorax, and length of metacarpus and metatarsus) may be potential discriminators of the species in living individuals or when entire carcasses are available (Duarte & Jorge, 2003), these characteristics are difficult to evaluate in the field using camera traps or even visual encounter data.

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Because of these morphological similarities, it was only through karyotype characterization and subsequent molecular confirmation (Duarte et al., 2008) that the small red brocket deer was identified and described as a distinct species from the red brocket deer (2n = 32 and 2n = 52, respectively; Duarte & Jorge, 2003). Karyotyping has been proposed as the most reliable tool to distinguish *M. bororo* from other sympatric species of brocket deer (Duarte, 1996; Duarte & Merino, 1997; Duarte & Jorge, 2003; Vogliotti & Duarte, 2010). However, to obtain karyotypes using cytogenetic protocols it is necessary to capture live individuals and collect fresh samples (blood and skin), which is difficult for this elusive species (Duarte et al., 1999). Efforts to locate and capture the deer are expensive, time consuming and infeasible. We conducted intensive field work surveys for 4 years and were able to locate and capture only five free-living *M. bororo* (Vogliotti & Duarte, 2009). This difficulty prompted us to develop faster, more efficient methods to detect this rare and elusive species and determine its geographical range, using non-invasive sampling of faecal material in combination with molecular markers (polymerase chain reaction–restriction fragment length polymorphism, PCR–RFLP; González et al., 2009).

Visually locating deer faeces in dense forest is challenging (Rivero et al., 2004). To maximize the number of faecal samples detected during surveys we used scat detection dogs, which have been proven to significantly improve the detection of faeces from rare and elusive species (Smith et al., 2001; Oliveira et al., 2012), and have been used effectively to locate faecal samples from a diversity of carnivores (Smith et al., 2003, 2005; Harrison, 2006; Long et al., 2007; Reed et al., 2011; Vynne et al., 2011), Xenarthrans (Vynne et al., 2011), and North Atlantic right whales *Eubalaena glacialis* (Rolland et al., 2006).

It is challenging to predict the ranges of rare and elusive species precisely but this information is critical for conservation planning. One approach that has become increasingly important is species distribution modelling (Guisan et al., 2013), which is used to derive spatially explicit predictions of environmental and ecological suitability for a particular species. This is achieved by the identification of statistical relationships between observations of species occurrence and environmental or ecological descriptors that characterize the species' ecological niche. Species distribution modelling has the potential to play a critical role in supporting spatial conservation decision making (Addison et al., 2013). It has proven to be particularly useful when occurrence data are limited, and the results can be used to inform planning for new surveys (Pearson, 2007).

To determine the geographical range of *M. bororo* more accurately we conducted intensive non-invasive surveys using scat detection dogs in all potential areas of occurrence, and used the occurrence data in species distribution modelling. Our results confirmed that the small red brocket deer

has the smallest geographical distribution of any deer species described to date, and its distribution appears to have decreased significantly over the past 2 centuries. We therefore suggest there is an urgent need to develop conservation and restoration actions for all remnants of the Atlantic Forest, and recommend that conservation efforts should target the reduction of the edge effects of forest fragments, palm heart exploitation and poaching.

Study area

The sampling sites were selected based on the proposed distribution of *M. bororo* in the Serra do Mar coastal forests ecoregion (Duarte, 1996). We focused on sampling in the Ombrophilous Dense Forest, and we also included other associated formations, such as the Mixed Ombrophilous Forest (Araucaria moist forest), Semideciduous Seasonal Forest (Paraná–Paraíba interior forests) and some ecotones. The chosen areas were mainly government protected areas and private reserves > 1,000 ha. Additionally, we surveyed buffer areas surrounding protected areas where the scat detection dogs were forbidden from entering. These buffer areas were adjacent to the protected areas and had similar ecological characteristics. In total, we surveyed 46 sites in the states of São Paulo, Paraná, Santa Catarina, Bahia, Espírito Santo and Minas Gerais (Fig. 1; Table 1).

Methods

Sample collection and storage

Searches for faecal samples were conducted during June 2003–October 2012 by one or more observers and a handler with two scat detection dogs. The dogs were trained by the military police of São Paulo State narcotics detection programme and worked freely off-lead. The searches were conducted along natural trails and the borders of rivers and forests. Although the Atlantic Forest is continually affected by deforestation and exploitation (Galindo-Leal & Câmara, 2005), we had to assume that no changes in habitat or deer distribution occurred over the time period of the sampling. As we aimed only to record presence data, the sampling effort was not recorded in detail (e.g. time in hours, or meters surveyed) and the duration of surveys varied from 1 day in some areas to up to 1 week in others. This sampling scheme precluded gathering true absence data.

The collected faecal samples were stored in absolute ethanol in 50 ml sterile polypropylene conical tubes. The samples were identified by location name, collection number, geographical coordinates and date. When multiple faecal pellets were found accumulated in the same location they were considered to belong to a latrine. In these cases, to avoid resampling the same individuals, we collected only
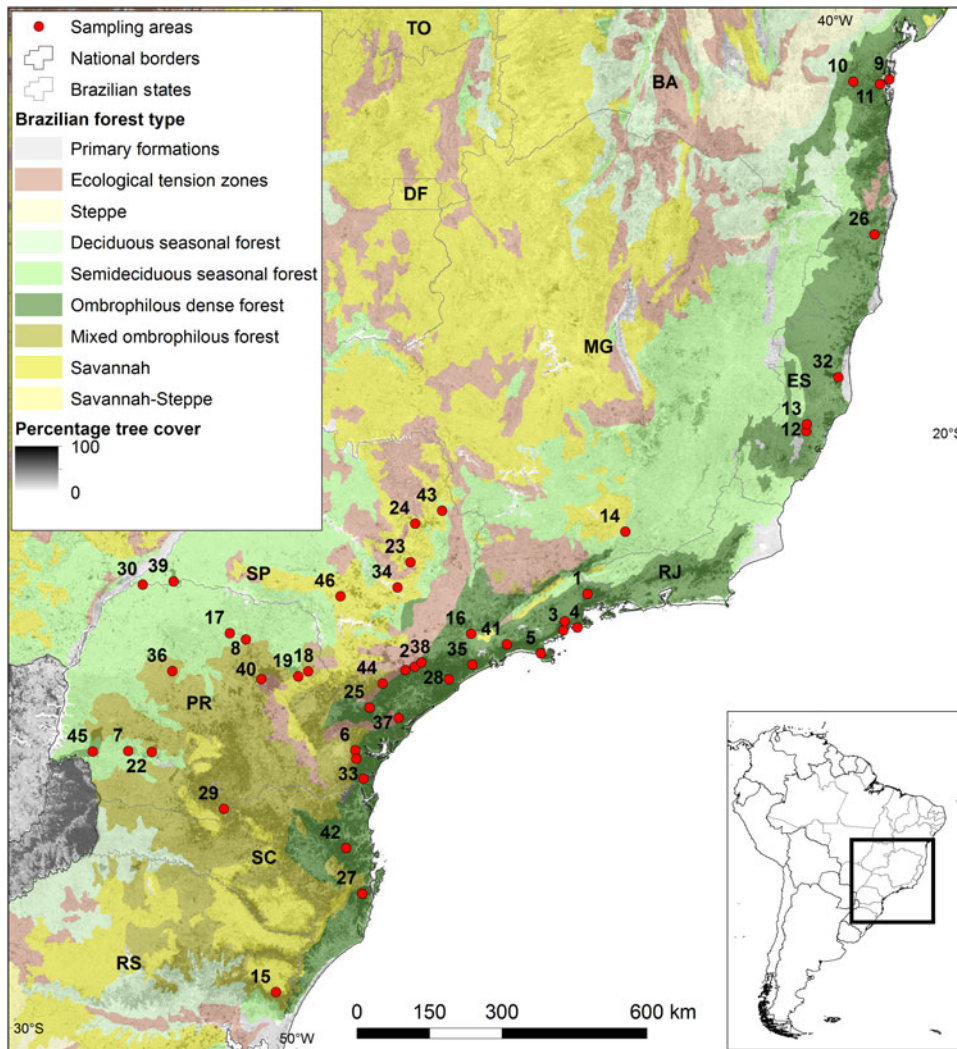
FIG. 1 Sampling sites (Table 1) for deer scat across the Brazilian Atlantic Forest. Forest type is categorized according to Veloso et al. (1991), and percentage tree cover is based on Hansen et al. (2003).

representative samples from clusters of pellets found at separate locations.

DNA extraction and PCR–RFLP

Faecal DNA extractions were performed using a Qiamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Extractions were carried out in a dedicated room free of PCR products to prevent contamination.

PCR amplifications of a 224 bp fragment of Cytochrome b were performed in a final volume of 25 μl and contained 1 × Invitrogen buffer, 1.5 mM MgCl$_2$, 0.1 mM of each dNTP (deoxynucleotide triphosphate), 1 pmol μl^{-1} of each primer (IDMAZ224L and IDMAZ224H; González et al., 2009), 1.0 U Taq DNA Polymerase (Invitrogen), and 60–100 ng μl^{-1} of faecal DNA. For the thermal profile we followed González et al. (2009). We included a negative control in each set of PCRs performed, to detect any possible contamination.

The amplified 224 bp fragment of Cytochrome b contains three informative restriction sites that can discriminate between *M. bororo* and its sympatric brocket deer species *M. americana*, *M. nana* and *M. gouazoubira* (González et al., 2009). The presence of the restriction site for *ECORII* or *BSTNI* in position 160 was used to confirm species identification because it has been demonstrated to be unique to *M. bororo* (González et al., 2009). PCR products that failed to digest were subjected to cycle sequencing to verify species identification. PCR products were purified using a Zymoclean™ DNA Recovery Kit (Zymo Research, Irvine, USA), and sequenced using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA) with the IDMAZ224L and IDMAZ224H (forward and reverse) primers described in González et al. (2009). Sequences were analysed using *MEGA 5.1* (Tamura et al., 2011).

Species distribution modelling procedures

We developed species distribution models using *MaxEnt v. 3.3.3k* (Phillips et al., 2006), the most adequate and frequently used software for species distribution modelling (Baldwin,

TABLE 1 Sampling localities (see numbered locations in Fig. 1), total number of faecal samples collected, and number of samples identified, by *Mazama* species.

Locality (State*)	No. of samples identified					
	Total	*M. bororo*	*M. americana*	*M. nana*	*M. gouazoubira*	Unknown
1. Serra da Bocaina National Park (SP)	0	0	0	0	0	0
2. Parque do Zizo Private Reserve (SP)	0	0	0	0	0	0
3. Serra do Mar State Park, Santa Virgínia (SP)	0	0	0	0	0	0
4. Serra do Mar State Park, Picinguaba (SP)	0	0	0	0	0	0
5. Serra do Mar State Park, São Sebastião (SP)	0	0	0	0	0	0
6. Pico do Marumbi State Park (PR)	0	0	0	0	0	0
7. Rio Guarani State Park (PR)	0	0	0	0	0	0
8. Mata do Barão Private Reserve (PR)	0	0	0	0	0	0
9. Pratigi Environmental Protection Area (BA)	0	0	0	0	0	0
10. Fazenda Fugidos (BA)	0	0	0	0	0	0
11. Fazenda Michelin (BA)	0	0	0	0	0	0
12. Santa Lucia Biological Station (ES)	0	0	0	0	0	0
13. Fazenda Lombardia (ES)	0	0	0	0	0	0
14. Serra do Ibitipoca (MG)	0	0	0	0	0	0
15. FLONA São Francisco de Paula (RS)	0	0	0	0	0	0
16. Jaraguá State Park (SP)	0	0	0	0	0	0
17. Mata dos Godoy State Park (PR)	1	0	0	0	0	1
18. Fazenda Barra Mansa (PR)	3	0	0	0	0	3
19. Fazenda São Nicolau (PR)	4	0	0	0	0	4
20. Serra do Mar State Park, Cunha-Indaiá (SP)	1	0	1	0	0	0
21. Pau-Oco State Park (PR)	1	0	0	1	0	0
22. ARAUPEL (PR)	1	0	1	0	0	0
23. Itirapina Ecological Station (SP)	1	0	0	0	1	0
24. Jataí Ecological Station (SP)	21	0	0	0	1	20
25. Lauraceas State Park (PR)	2	0	0	2	0	0
26. Pau-Brasil National Park (BA)	2	0	0	0	2	0
27. Serra do Tabuleiro State Park (SC)	3	2	0	0	0	1
28. Serra do Mar State Park, Pedro de Toledo (SP)	3	0	0	0	3	0
29. Araucárias State Park (PR)	4	0	0	3	0	1
30. Caiuá Ecological Station (PR)	5	0	5	0	0	0
31. Carlos Botelho State Park (SP)	10	5	0	0	0	5
32. Fazenda Boa Sorte (ES)	6	0	0	0	6	0
33. Guaratuba Environmental Protection Area (PR)	6	2	0	4	0	0
34. Fazenda Barreiro Rico (SP)	8	0	8	0	0	0
35. Serra do Mar State Park, Curucutu (SP)	8	0	3	0	5	0
36. Lago Azul State Park (PR)	9	0	5	0	4	0
37. Jacupiranga State Park (SP)	9	6	0	0	3	0
38. Fazenda João XXIII (SP)	11	10	1	0	0	0
39. Morro do Diabo State Park (SP)	13	0	8	0	5	0
40. Monte Alegre Private Reserve (PR)	13	0	1	0	12	0
41. Biritiba-Mirim (SP)	19	0	0	0	19	0
42. Serra do Itajaí National Park (SC)	28	4	0	21	0	3
43. Cajuru Legal Reserve (SP)	52	0	45	0	0	7
44. Intervales State Park (SP)	87	87	0	0	0	0
45. Iguaçu National Park (PR)	112	0	80	20	0	12
46. Santa Bárbara Ecological Station (SP)	112	0	0	0	111	1
Total samples	555	116	158	51	172	58

*SP, São Paulo; PR, Paraná; BA, Bahia; ES, Espírito Santo; MG, Minas Gerais; RS, Rio Grande do Sul; SC, Santa Catarina

2009). In comparison to other approaches *MaxEnt* performs well with small sample sizes (Hernandez et al., 2006).

Occurrence data were based on only 19 sample locations, following the exclusion of multiple records in the same pixel (c. 1 km²). We used 70% of the points to train the model and 30% to test the model. They were sampled by bootstrapping 10 random partitions with replacement (Pearson, 2007).
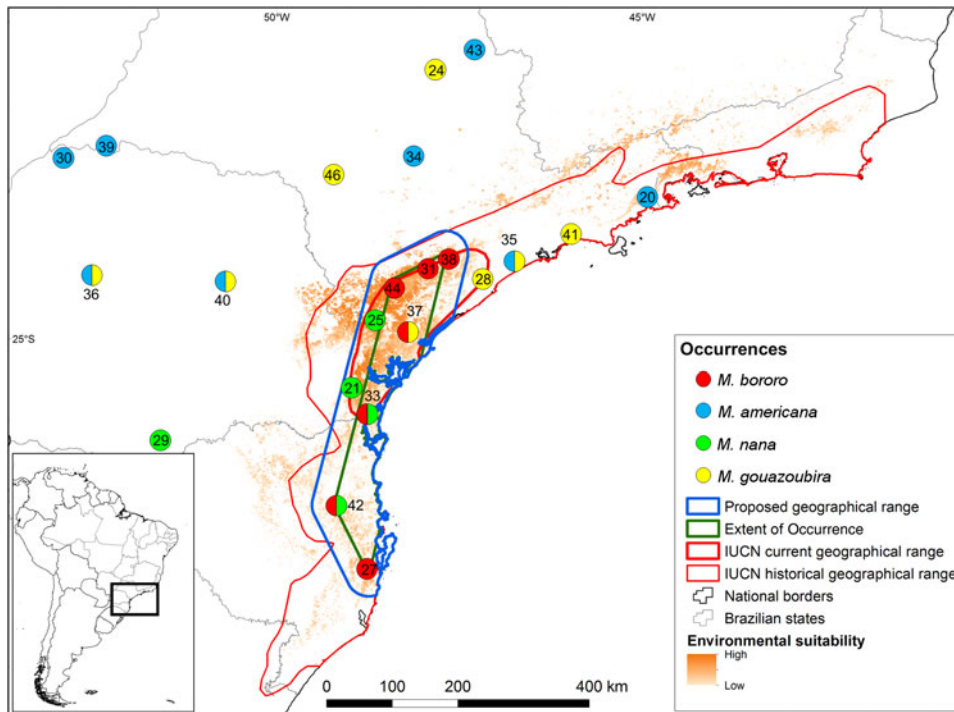
Fig. 2 Species distribution model for *Mazama bororo*, with the extent of occurrence, IUCN geographical ranges, the proposed geographical range, and locations of occurrence (numbered as in Table 1) of *Mazama* species. A circle with two colours indicates that two species were found at the same sampling site.

The environmental layers were composed of bioclimatic variables, including mean annual temperature (bio1), temperature seasonality (the standard deviation, multiplied by 100, of the monthly temperature; bio4), annual precipitation (bio12), precipitation seasonality (bio15), and precipitation of the driest month (bio14), from the WorldClim database (Hijmans et al., 2005), percentage tree cover in 2000–2001 (Hansen et al., 2003), altitude (Valeriano, 2008), and slope obtained from the AMBDATA database. We also produced raster maps with Euclidean distance from maps of the protected areas (Ministério do Meio Ambiente, 2015), forest remnants (Ribeiro et al., 2009) and the Ombrophilous Dense Forest (Veloso et al., 1991). The environmental layers were selected based on variables that had a high likelihood of being associated with *M. bororo* habitat in the humid coastal Atlantic Forest distributed throughout mountain chains.

All layers were trimmed to match the limits of the Atlantic Forest (modelling scope) and rescaled to a resolution of 1 km². All spatial analyses were conducted using *ArcMap v. 10.3.1* (ESRI, Redlands, USA). We evaluated the degree of correlation between environmental variables using *PAST 3.06* (Hammer et al., 2001). Two bioclimatic variables (mean annual temperature and precipitation of the driest month) were removed because of their high correlation ($r > |0.75|$) with altitude and precipitation seasonality, respectively. We performed a principal components analysis to select the most explicative variables. The variables identified and subsequently used in the *MaxEnt* analysis were bio12, bio15, bio4, distance from Ombrophilous Dense Forest, altitude, and percentage tree cover. The resulting model was evaluated using the area under the receiver

operating curve, omission error and a two-proportion binomial test (Fielding & Bell, 1997; Elith et al., 2006; Pearson, 2007; Phillips & Dudík, 2008). To predict the species' distribution we assumed only the probabilistic values over the minimum training presence logistic threshold.

Results

Species identification from scat samples

We collected 555 deer scat samples from 30 of the 46 surveyed areas. PCR reactions amplified 497 (89%) of the samples successfully. The RFLP reaction failed for PCR products from 18 samples (3.2%). For these samples we then proceeded to sequence the PCR products directly, but only 10 of the PCR products yielded sequences of sufficient quality to determine species. The PCR–RFLP reaction and sequencing indicated that 116 (23%) samples were from *M. bororo*. The remaining samples were from *M. gouazoubira* (172; 34%), *M. americana* (158; 31%) and *M. nana* (51; 10%). The small red brocket deer was positively identified in only seven localities within the states of São Paulo, Paraná and Santa Catarina (Fig. 2).

Geographical range

The size of the area obtained by linking external points of positive occurrence (extent of occurrence) was 20,000 km², encompassing the Serra do Mar and Serra de Paranapiacaba from the states of São Paulo, Paraná and

Santa Catarina. This region encompasses the Ombrophilous Dense Forest, an Atlantic Forest formation that is close to the Brazilian coastline, at 8–29°S. We did not detect *M. bororo* samples in the states of Espírito Santo or Bahia. To define the proposed geographical range we added a buffer zone of 30 km to the extent of occurrence, except for the areas that extended to the ocean (Fig. 2). This yielded a geographical range of 41,714 km², which is the best estimate of the actual distribution of the species to date.

Species distribution modelling

The model developed in *MaxEnt* (Fig. 2) was evaluated satisfactorily by the mean area under the curve (0.9782 ± SD 0.0078) external test with 10 random partitions and was significantly different from random (P < 0.01) in a two proportion binomial test. The omission error was 0.14. The model predicted the occurrence of *M. bororo* with the highest values of environmental suitability at Serra de Paranapiacaba, Guaraqueçaba Environmental Protection Area and Serra do Tabuleiro State Park (Fig. 2). The variables that most contributed to the species distribution model were percentage tree cover (62.4%), distance from Ombrophilous Dense Forest (18.6%) and temperature seasonality (10.9%).

Discussion

Species of the genus *Mazama* display a high degree of morphological similarity (Duarte et al., 2008), making it difficult to distinguish them by direct field observations or camera traps (Vogliotti & Duarte, 2010). Our findings confirmed the sympatry of *M. bororo*, *M. americana*, *M. nana* and *M. gouazoubira* by the identification of more than one of the species in nine of 46 localities sampled. Furthermore, we found one or more sympatric *Mazama* species in three of seven localities (42.8%) where *M. bororo* was also detected. We detected *M. nana* in sympatry with *M. bororo* in two separate localities, and with *M. gouazoubira* in another locality (Fig. 2). These two species (*M. nana* and *M. gouazoubira*) are more common than *M. bororo* in human-altered and less pristine environments (Vogliotti, 2008). This suggests that the more suitable habitats used by *M. bororo* are being altered even in the protected areas where our sampling took place. This change in *M. bororo* habitat may be the result of edge effects (Ribeiro et al., 2009), palm heart exploitation, poaching (Vogliotti & Duarte, 2012) or global climatic changes (Allen et al., 2010). We hypothesize that *M. nana* may be invading areas that were formerly occupied only by *M. bororo* and that this invasion may be facilitated by the degradation of the Ombrophilous Mixed Forest. In addition, *M. gouazoubira,* which is known to be one of the brocket species with greater ecological plasticity (Pinder & Leeuwenberg, 1997; Black-Décima et al., 2010) and resistance to anthropogenic habitat alteration (Rodrigues et al., 2014), has been extending its range to include deforested areas that were previously covered with Atlantic Forest. The role of competition in the distribution and survival of sympatric brocket species in this area needs careful evaluation to define and mitigate better the effect of habitat changes in conservation plans for the small red brocket.

Morphometric and morphological studies of skulls and skins from various Brazilian collections (Rossi, 2000) failed to clearly differentiate *M. bororo* from *M. americana*, although the data supported the presence of a partially differentiated population of *M. americana* distributed throughout the south coast of Brazil. These results were considered to be the most reasonable evidence of the historical distribution of *M. bororo* in the Atlantic Forest. It is important to note that the ranges of distribution established here and by Rossi (2000) were based on different methods, and there are many uncertainties involved in the reconstruction of the historical distribution. However, we suspect that the extent of the decline in distribution has been dramatic, as the species would have occupied the mountain ranges of the Serra de Paranapiacaba and the Serra do Mar, from the eastern part of the state of Santa Catarina to the state of São Paulo in the south and south-east regions of Brazil. We estimate that the historical geographical range was 114,120 km² (Fig. 2).

The original proposed extent of the geographical distribution of *M. bororo* was based on the localities of provenance of the captive individuals used in the karyotypic characterization that resulted in the species description. These individuals originated in the cities of Capão Bonito, Barra do Turvo, São José dos Pinhais and Paranaguá and these localities were used to set the distribution limits to the south of São Paulo and east of Paraná states (Duarte, 1992, 1996; Duarte & Jorge, 2003; Vogliotti & Duarte, 2009).

Our survey results support an extension of the geographical range of the small red brocket deer to the latitudes 23–28°S and to the longitudes 47–49°W (Fig. 2), encompassing 37,517 km². This range is considered to be the extent of occurrence, similar to the current area of occupancy because it represents an ecological continuum constituted by protected areas. A comparison of the historical distribution of 114,120 km² (Rossi, 2000) with our results suggests that there has been an 83% decline in the distribution over the last 2 centuries. Given its recent description and hypothesized geographical range, there is little historical evidence to facilitate evaluation of the effects of anthropogenic development on the original distribution of the small red brocket deer. Currently, the habitat in the species' area of occupancy is well-preserved, with few barriers to gene flow. If protected, habitat connectivity will help to maintain adequate levels of genetic diversity in the future.

The small red brocket is the largest endemic animal of Brazil, with perhaps the smallest geographical distribution of any Neotropical deer species. It is categorized as Vulnerable on the IUCN Red List because of its small population size and ongoing decline inferred from poaching and habitat loss, and because all of the remaining individuals are from a single subpopulation (Duarte, 2008). It is threatened by human encroachment (70% of the Brazilian population lives within the original limits of the Atlantic Forest), poaching, and predation by dogs (Duarte, 2008).

Species distribution modelling indicates that the current geographical distribution reported by IUCN should be revised to encompass northern areas with high environmental suitability, as well as the southern part of the Serra do Tabuleiro State Park and Serra do Itajaí National Park, where we collected occurrence data. In addition, areas of high suitability where no sampling was carried out or no deer species were detected during sampling should be revisited to confirm presence or absence. We also suggest that future species distribution models incorporate variables based on patch size and connectivity. Species distribution modelling provides more detailed information than traditional distribution estimates as it gives a suitability index for each 1 km² pixel instead of a continuous distribution of values. It increases our understanding of species' needs and gives support to conservation policies such as those for the design and establishment of protected and conservation priority areas.

We have demonstrated the importance of incorporating traditional field techniques with non-invasive sampling and sophisticated molecular tools to elucidate geographical range, extent of occurrence, and areas with the highest suitability for occupancy by the elusive small red brocket deer. Such information is essential in designing strategies for species conservation, particularly in the Neotropics, where new species with limited distributions are being discovered. Conservation efforts should aim more specifically to maintain the last remnants of the Atlantic Forest and to target the reduction of the edge effects of forest fragments, poaching and palm heart exploitation. *M. bororo* should be considered a flagship species and used as an indicator of the quality and restoration of the habitat.

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Author contributions

JMBD was the project coordinator and contributed to writing the article. ACT and JEG conducted laboratory analysis. AV contributed to non-invasive sampling and data management. MLO contributed to non-invasive sampling, laboratory analysis, distribution modelling, and writing the article. JEM provided scientific support and contributed to writing the article. SG provided scientific support, conducted laboratory analysis and contributed to writing the article.

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Biographical sketches

José Maurício Barbanti Duarte has worked on various aspects of the genetics, evolution, ecology and conservation of Neotropical deer species, particularly of the genus *Mazama*. Ângela Cristina Talarico is a biologist, and her MSc research focused on the distribution of the small red brocket deer. Alexandre Vogliotti has worked on small red brocket deer research and conservation, and is currently focused on deer ecology and conservation planning in the Atlantic Forest. José Eduardo Garcia is interested in the conservation genetics of Brazilian wildlife. Márcio Leite Oliveira's research is focused on distribution modelling and molecular ecology of brocket deer. Jesús E. Maldonado applies non-invasive genetic techniques to study conservation genetics, behavioural ecology and evolutionary genetics of a diversity of rare mammals. Susana González has conducted research in conservation biology for over 20 years and is co-chair of the IUCN SSC Deer Specialist Group.