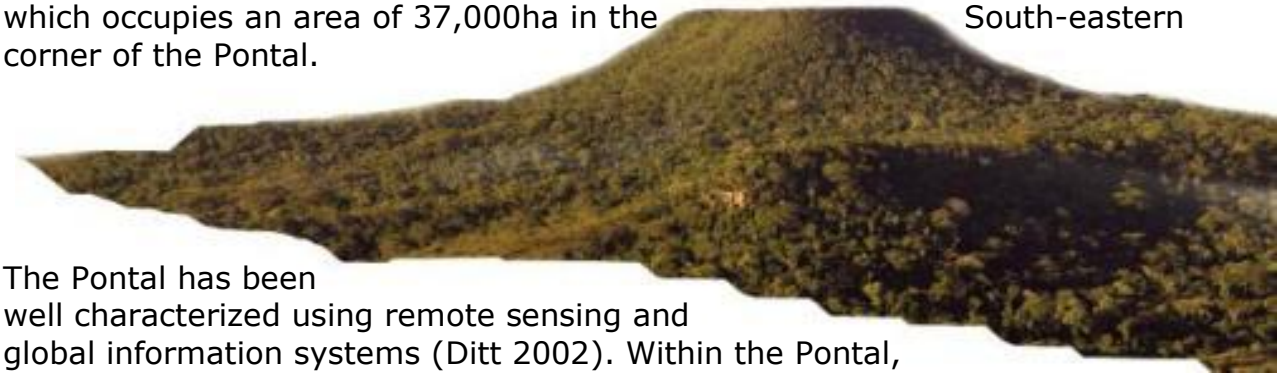
The Landscape

The proposed study will be carried out in the Pontal do Paranapanema Region of Brazil, hereafter called the Pontal. More specifically, the study will be contained within the area of roughly 270,000ha that was once the Grande Reserva do Pontal do Paranpanema (The Great Reserve of the Pontal do Paranapanema; Ditt 2002, and references therein). This region is located at the western tip of the State of São Paulo (Brazil), and is delimited by the Paranpanema River in the South, the Paraná River in the West, the Anhumas Brook in the North, and the Dividing Ridge of the Paranapanema-Paraná River Basins in the East (Ditt 2002). The area is part of the Atlantic Forest Complex (Morellato 2000), and its original vegetation cover is specifically classified as Atlantic Forest of the Interior (Ditt 2002).

The original forest cover has been reduced to roughly 5% of its extent in the last 50 years (Valladares-Padua et al. 1997), subdividing the forest into hundreds of relatively small fragments (~345 fragments with areas of 5-2000 ha each) interspersed among agriculture and pasture land (Ditt 2002). The unique exception is the Parque Estadual Morro do Diabo (Devil's Hill State Park - DH), which occupies an area of 37,000ha in the South-eastern corner of the Pontal.



The Pontal has been well characterized using remote sensing and global information systems (Ditt 2002). Within the Pontal, 13 fragments of 300 to 2000ha each, representing 21% of forested area have been the focus of several ecological studies (Ditt 2002, Jacob 2002, and Bassi 2004) and one population genetic study (Perez-Sweeney 2001), and are considered the main refugia for the native fauna and flora of the area (Ditt 2002).

The Organisms

The Pontal is home to many mammalian species. In a survey of the 10 forest fragments included in this study and DH, Bassi (2004) found an average of 14.76 (range: 11-21) species of non-volant mammals per fragment. Of these, six species (listed above) were chosen for this study, comprising three orders (Carnivora, Artiodactyla and Perissodactyla) and two trophic levels (carnivores and herbivores). All six species have been the focus of intense ecological studies in the area, some of which were undertaken from a landscape perspective (jaguars and pumas, Cullen Jr, unpublished data; ocelots, Jacob 2002; tapirs, Medici unpublished data; peccaries, Nava unpublished data).

Among the species chosen, the Felids are the best studied, with phylogeographic and population genetic studies undertaken for all three species (Eizirik et al. 1998, Ernest et al. 2000, Eizirik et al. 2001). These studies suggest high gene flow among populations of all three species, with a few important barriers. However, not much is known about movement patterns in these three species. Studies

suggest that jaguars and pumas move widely, and have the capacity to disperse long distances (>50km, Quigley and Crawshaw 2002; and, observed by Cullen Jr. in the Pontal), and that males disperse farther from their natal sites than females (Beier 1995, Quigley and Crawshaw 2002). Ocelots, on the other hand, seem to have more restricted movement patterns, preferring to move under the dense cover provided by riparian forests and dense bushes (Jacob 2002).

Little information is available on the three herbivores apart from their diets (e.g. Salas and Fuller 1996) and their roles as seed dispersers (e.g. Galetti et al. 2001). The lowland tapir, the largest of the three (Macdonald 1995), displays movement patterns closely associated with water and wooded areas (Padilla and Dowler 1994). In the Pontal region it is known to leave forest fragments to feed in sugar cane plantations, and pastures in search for salt licks (Médici pers. comm.).

The collared and the white-lipped peccaries have different habitat requirements (Fragoso 1999). Collared peccaries require forest cover, while white-lipped peccaries need a variety of habitats varying from forests to marshlands to survive (Fragoso 1999). Not much is known about their movement patterns, except that in natural habitat they can range over wide areas (Judas and Henry 1999, Carrillo et al. 2002), but usually avoid roads and areas of dense human populations (Bellantoni and Krausman 1993).

Therefore, there is a general lack of information about dispersal distances and movement patterns in these species. Because of this, we chose to evaluate relative dispersal capacity among species using the algorithms proposed by Sutherland et al. (2000) (Figure 1), and published average weights for each species (Nowak 1999). According to average weight jaguars, pumas and ocelots are large, medium and small carnivores, respectively; and tapirs, white-lipped peccaries and collared peccaries are large, medium and small herbivores, respectively.

Field Collection and Laboratory Analysis

Due to the nature of the species being studied (i.e. large and elusive), most of the sampling for DNA analysis will be based on faecal collection. Non-invasive sampling based on faeces has been used in many studies of population genetics of mammals (for review, Taberlet and Waits 1998), both in carnivores (e.g. Ernest et al. 2000, Sacks et al. 2004) and in herbivores (e.g. Garnier et al. 2001, Fernando et al. 2003a). Therefore, DNA from faeces is a reliable source of information for population genetics. Blood and tissue samples will also be obtained from local researchers that regularly capture these animals to conduct their ecological studies.

Sampling will be carried out in or immediately around 10 of the 13 fragments of Atlantic Forest of the Interior in the Pontal and the DH. Focusing sampling on

these fragments has two advantages: (1) it affords this study a wealth of previous and invaluable knowledge of the landscape and the animals being studied; and (2) it allows the study to provide supplementary information for conservation of a severely fragmented landscape. Samples will be handled as described by Fernando et al. (2003b) and preserved in RNAlater (Ambion, Inc.). Both blood and tissue samples will be preserved in Easy Blood buffer (Tris HCl, EDTA and SDS) at room temperature until reaching the lab, where the samples will be kept at -20 oC until DNA extraction.

DNA extraction will follow the CTAB based method described in Ferreira and Grattapaglia (1998), with modifications. First, 100mg of wet sample will be separated and washed with 500µl of PBS preceding the extraction, then after re-suspension of DNA pellets in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) the extract will be further cleaned using the QIAquick Gel Extraction Kit (Qiagen). All extractions will be performed with at least two negative extraction controls, one at the beginning of the series and one at the end. To avoid issues of contamination and genotyping errors extraction and PCR reaction safety protocols will be followed as recommended by Fernando et al. (2003b) and Taberlet et al. (1999). Isolation of DNA from blood and tissue samples shall be carried out using the DNeasy Tissue Kit (Qiagen) following manufacturer's protocol. Species identification will be done following the protocol delineated by Farrell et al. (2000), using reference sequences amplified from blood samples.

To quantify neutral genetic variability within our six study species at this fine a scale, we will employ nuclear DNA microsatellite markers. Mitochondrial sequences were not chosen because it is highly unlikely that they will be informative at this scale for such large mammals (e.g. Eizirik et al. 2001). Microsatellites, on the other hand, are hyper variable tandem repeats spread across the genome, with very high mutation rates (Tautz 1989). Alleles at each locus are defined by the number of repeats and scored by their size differences. Microsatellite markers have been widely used in population genetics, and in particular in fine-scale studies (Manel 2003). A battery of species-specific and cross-specific primers will be used. For tapirs, specific loci have been developed (Norton and Ashely 2004). Primers have also been successfully transferred from domestic cats (Menotti-Raymond et al. 1999) to jaguars (Eizirik et al. 2001); pumas (Ernest et al. 2003) and ocelots (see Preliminary Results). Finally, primers have been successfully transferred from the domestic pig (Archibald et al. 1995) to collared and white-lipped peccaries (Gongora et al. 2002, Lowden et al. 2002). PCR optimization will be conducted for all study species until a total of 7 polymorphic loci are identified for each species. Fecal samples that have the same genotype for all loci will be conservatively considered to have come from the same individual.

Finally, all DNA extractions and amplification products will be checked on standard Agarose gels with Ethidium Bromide staining, and quantified by comparison to High DNA Mass Ladder (Invitrogen Corporation). Genotyping of individuals shall be carried out using standard fluorescent techniques on an ABI PRISM® 3730 Automated Sequencing machine (Applied Biosystems). All sequencing shall be carried out with ABI PRISM® BigDye™ Terminators v 3.1 Cycle Sequencing Kit 3.1 (Applied Biosystems), and an ABI PRISM® 3730 Automated Sequencing machine (Applied Biosystems). Direct cycle sequencing reactions of PCR products shall be preceded by cleanup step with the QIAquick PCR Purification Kit (Qiagen).

Preliminary results

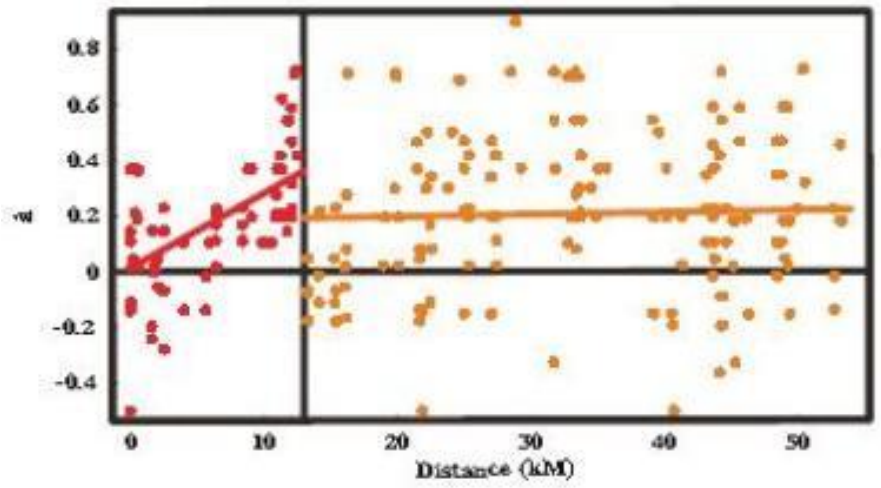
A total of 360 samples have been collected in 10 forest fragments in the Pontal do Paranapanema region (for distribution of tapir samples see Figure 3). These include 60 feline, 48 collared peccary, 45 white-lipped peccary, and 206 lowland tapir blood, tissue and dung samples.

Distribution of tapir samples in the Pontal do Paranapanema (n=260)

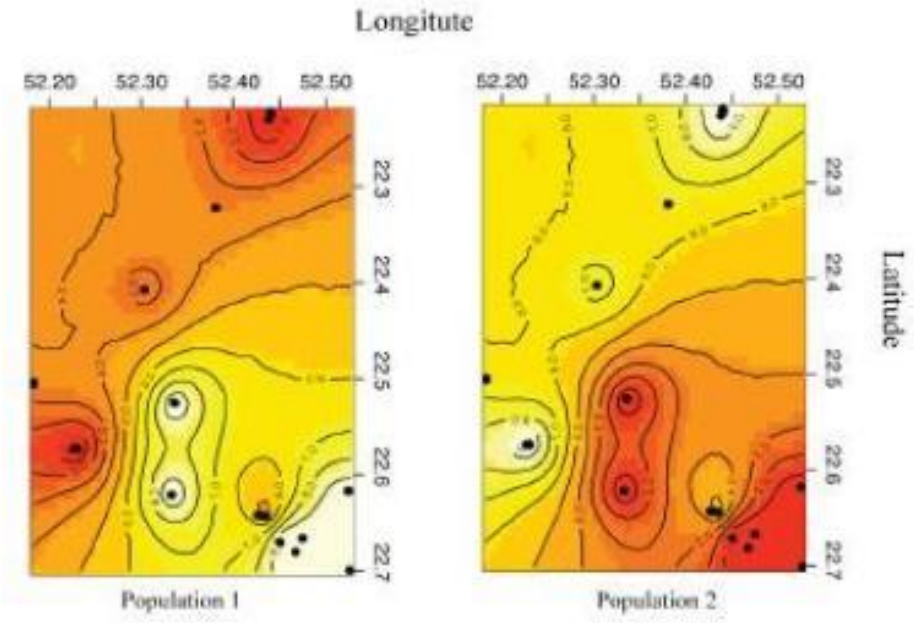
A total of 40 domestic pig primer pairs were tested on peccaries, 15 domestic cat primers were tested on the felines, and the 7 most polymorphic were kept for each species for further analyses. All 7 tapir specific primers have proven to be polymorphic. In samples of 10 individuals for jaguars, ocelots, and white-lipped and collared peccaries, multilocus tests for Hardy-Weinberg Equilibrium were performed (Raymond and Rousset 1995). Heterozygote deficiency was found only in collared peccaries (p=0.001). Pumas have not been analyzed yet, because of the small number of samples processed. And, tapirs were found to have significant heterozygote deficiency in a sample of 29 individuals analyzed (p=0.0). Number of observed alleles among all loci analyzed varied from 4 to 10, with an average of 7.2 alleles per locus.

Analysis of isolation-by-distance with 23 individual tapirs spread out across the landscape suggests that there is significant positive relationship between physical distance and genetic distance (as measured by â, eq 5 in Rousset 2000) with individuals located up to 13kM from each other (a=0.0268, p=0; Figure 4). However, at larger distances no significant relationship was found (a=0.001, p=0.67; Figure 4). This suggests that gene flow is an important force at small distances of up to 13kM, after which genetic drift becomes more important in shaping the distribution of genetic variation. The observed pattern resembles what Hutchison and Templeton (1999) hypothesize would happen in a non-equilibrium situation (as assumed by the island model; Wright 1931) where gene flow is important at small spatial scales, but drift takes over at larger spatial scales (case IV, fig 1; Hutchison and Templeton 1999). This could be caused by flow is possible across the landscape (Hutchison and Templeton 1999).

A preliminary analysis of spatial structure in lowland tapirs (n=29) using Geneland (Guillot et al. 2005b) suggests that there at least two populations in the Pontal region, one contained within the Morro do Diabo State Park, and another composed by the outside fragments (Figure 5). It also shows what could possibly be migrants from the fragments into the Park. However, the probabilities of population membership for each individual are quite similar across populations. Therefore, a larger number of samples will have to be processed before a better estimate of the structure of genetic variation for the lowland tapir can be obtained. Nevertheless, it is encouraging to find that with these two forms of analysis we were able to detect some level of genetic structuring in the lowland tapir.



Correlation of physical and genetic distance between individuals (vertical line indicates 13kM cut-off).



Plot of population membership for 23 individual tapirs in the Pontal do Paranapanema. Lighter colouring indicates increasing probability of membership. Population 1, in the lower right of the map, is the Morro do Diabo State Park population. Population 2, on the left and upper part of the map, is the population located outside of the Park. Arrow indicates possible migrants into Population 1.

Dissemination of results

Preliminary results were orally presented at the III International Tapir Symposium, held in Buenos Aires from January 26th to 31st, 2006. The presentation drew the attention of group members from Colombia and Ecuador who are eager to reproduce the study in their own study sites.

Acknowledgments

This project is highly indebted to Patrícia Medici, Laury Cullen Jr., Alessandra Nava, George Velastin, Christina Tófoli, José Maria do Aragão, Arnaud Desbiez, Robin Elliott and Reem Hajjar for help during sampling and sharing of samples. Furthermore, AGS would like to thank Claudio Valladares-Pádua for opening the doors to the Instituto de Pesquisas Ecólogicas (IPÊ), and making its infrastructure available to this project. Additionally, AGS would also like to thank the great assistance given to him by Juliana José, Gonçalo Pereira, Vera Solferini, Eva Mamani, Juliano Pádua, Marília Pappas, Nathália Bueno, and Dario Gratappaglia during the lab phase of this project. Finally, this project would not be possible without the financial support of the Rufford Foundation, the Disney Wildlife Conservation Fund, the Explorers Club, the American Society of Mammalogists, the Graduate School of Arts and Sciences (Columbia University) and the Department of Ecology, Evolution and Environmental Biology at Columbia University.

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