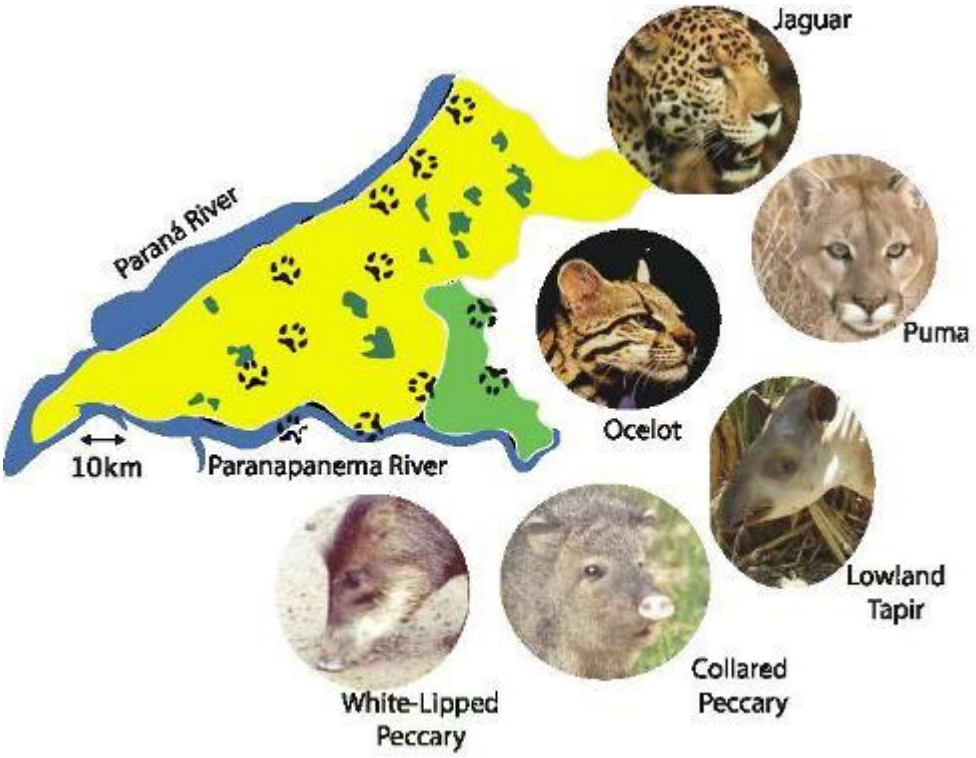
Final Report to the Rufford Foundation:

Conservation Genetics of Large Mammals in the Atlantic Forest, BRAZIL



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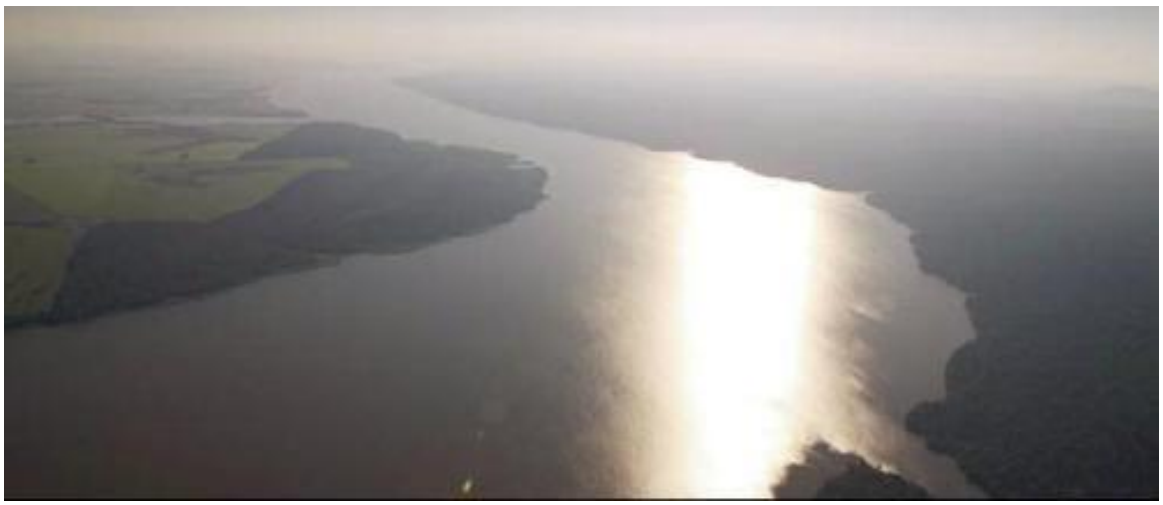


Introduction

The interaction between an animal and the landscape it inhabits has a significant impact on the spatial distribution of the species' genetic variation. The amount and distribution of suitable habitat relative to the species' ability to move through the habitat affects the rates of dispersal among populations. In turn, this ecological process affects evolutionary rates, shaping the spatial distribution of genetic diversity. Here, we propose to study the effect of two fundamental characteristics explaining variation in dispersal distance in mammals, size and trophic level, on the spatial genetic structure of six mammalian species (jaguar – *Panthera onca*; puma – *Puma concolor*; ocelot – *Leopardus pardalis*; lowland tapir – *Tapirus terrestris*; collared peccary – *Tayassu tajacu*; white-lipped peccary – *Tayassu pecari*).

In mammals, two characteristics are essential in determining dispersal distances: trophic level and body size; these characteristics have been shown to explain together 88% and 74% of variation in median dispersal distance in mammalian carnivores and herbivores, respectively (Sutherland et al. 2000). Trophic level correlates with spacing behaviour and home range size (Harestad and Bunnell 1979). Thus, carnivores disperse, on average, relatively farther than do herbivores of similar size (Sutherland et al. 2000). Body mass correlates with longevity and resource search time (Brown et al. 2000), leading to bigger animals generally dispersing over larger distances than smaller animals (Sutherland et al. 2000). However, dispersal distances alone do not explain the spatial distribution of genetic variation. Rather there is a fundamental interaction between dispersal and the surrounding landscape, known as functional landscape connectivity (Merriam 1984). Landscape connectivity implies an interaction between landscape and organism (Merriam 1984). As such, the degree of landscape connectivity for any given species is dependent on the scale of spatial heterogeneity (Li and Reynolds 1995), relative to the animal's ability to disperse over the landscape (Lima and Zollner 1996, Sutherland et al. 2000).

Landscape connectivity has a significant impact over dispersal, influencing how individuals are distributed within a landscape (e.g. With and Crist 1995), how they disperse among suitable habitat patches (e.g. With and King 1999), and the structure of dispersal (i.e. sex-biased dispersal, Stow et al. 2001). If we assume

dispersal and gene flow are intimately associated, landscape connectivity would affect gene flow, and ultimately the spatial genetic structure (e.g. Rousset 2000, Coulon et al. 2004). This assumption, although relatively controversial (Bohonak 1999), is believed to be valid (Epperson 2003), in particular for natal dispersal (i.e. dispersal by juveniles away from their natal areas, Wiklund 1996), and numerous examples exist where genetically inferred dispersal rates (i.e. through estimates of gene flow) conformed well to observed rates (Bohonak 1999). Therefore, it is conceivable that a landscape, through the interaction with a species' biological characteristics, can influence gene flow, and thus the distribution of a species' genetic variation in geographical space.

To study this effect, we have to first show that gene flow, and thus dispersal, is an important force shaping the distribution of genetic variation. This will be done by evaluating the degree of association of the inferred genetic structure with physical distances that take into account most plausible routes of dispersal (as in Keyghobadi et al. 1999). Next, we have to describe the spatial structure of genetic variation in each species. In most instances, this structure is inferred from a model of population structure based on our best knowledge of the species and its surrounding landscape (Hartl and Clark 1997). However, since we are interested in how species react to landscapes, it is important that structure be inferred from the data, rather than by imposition of an a priori structure. Using a landscape genetic approach (Manel 2003), we are able to describe the spatial structure of genetic variation with few a priori assumptions about the effect of the landscape or about the number of populations (Guillot et al. 2005a). After the underlying distribution of genetic variation is inferred for each species we will test for differences among the species using bootstrap methods to construct confidence intervals (Manly 2001). Finally, using a series of regression analyses we intend to assess the relationship between the degree of spatial dependence of genetic variation and these two characteristics. This final step is only possible because we are examining genetic variation across several species, inhabiting the same landscape.

Objectives

The objective of this study is to understand the impact of a well-studied landscape on the spatial distribution of genetic variation in six mammalian species varying in both trophic level and body mass. As proposed, the study constitutes a natural experiment, in which we hold the landscape constant and evaluate its impact on mammalian species with differing degrees of landscape connectivity. This will allow us to test the degree of association between landscape connectivity, ecological adaptation, and evolutionary genetic processes. In pursuit of this objective we will address three specific questions:

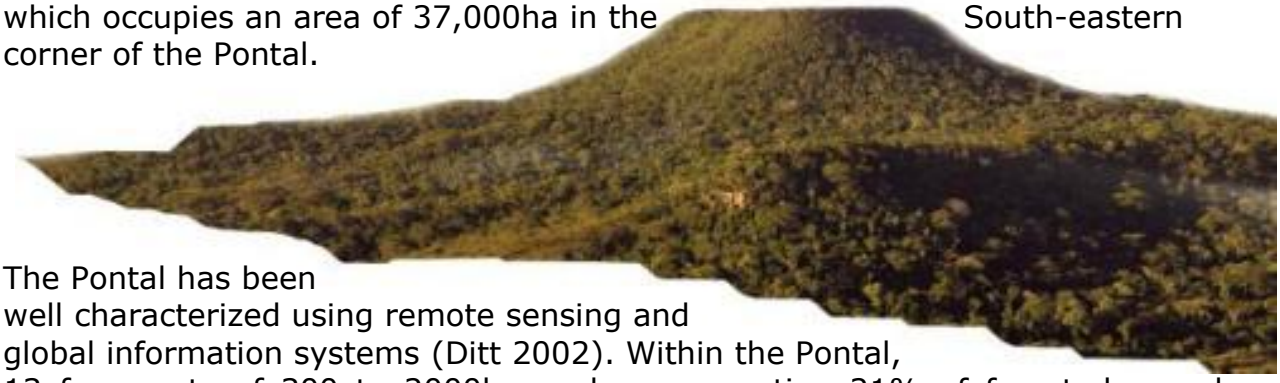
(1) How does the studied landscape affect gene flow in each one of the six species?
(2) Are there differences in the spatial genetic structure among the six species?
(3) What is the effect of trophic level and body mass on the distribution of genetic variation?

Initially all samples will be used to answer these questions. However, if sampling size permits, subsets exclusively of males and females will be analyzed separately to evaluate the effect of gender.

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).

Achieved objectives to date

So far, I have been able to:

- Genotyped all collared and white-lipped peccary samples.
- Genotyped all blood samples from tapirs, jaguars, pumas and ocelots.
- Compiled a database of geographic coordinates for most samples – missing coordinates have been requested from the original collector.
- Run preliminary analysis on collected genotypes, which suggests mild, but statistically significant, inbreeding levels for tapirs, collared peccaries and ocelots.
- Presented preliminary analysis to IPÊ at its staff meeting on June 6th, 2006. This was the first feedback on the project to the general staff of IPÊ. The staff seemed excited about the prospects.
- Methods developed in this project are being used in conservation genetics projects of mountain tapirs in Peru and Colombia, spectacled bears in Peru, Baird's tapir in Costa Rica and Mexico (2), on the lowland tapir in Peru, and in a preliminary Malay tapir study is being undertaken with captive animals by the Atwerp Zoo using dung DNA. Additionally, there is a preliminary inquiry about using the same techniques in Honduras with Baird's tapir; and the method will be soon replicated in the Brazilian Pantanal with the Lowland Tapir.

Jannet Cisneros, DVM (Peru) with a captive baby mountain tapir. Dr. Cisneros and her colleague Jorge Rodriguez, DVM are collecting mountain tapir faeces in Peru to measure current levels of genetic diversity in Peruvian populations of the species.

Proposed objectives for the coming year

- Finish genotyping faecal samples (see below in Lessons Learnt).
- Undertake a paternity analysis of the jaguar populations in the Pontal, as Laury Cullen Jr. suggested that one of two-sampled jaguar males could be the father of one of the sampled cubs.
- Undertake spatial genetic analysis to identify the spatial distribution of genetic variation in the landscape and potential dispersal routes used by the animals. Unfortunately, these analyses have not been carried out yet because of lab complications (see Lessons Learnt).
- Compare across species, and attempt to identify trends in the spatial distribution of genetic variation that might be related to body size and trophic level (see Lessons Learnt).
- Defend thesis by May/07, and subsequently publish 3 articles relative to the thesis in leading scientific journals.
- Submit final report to IPÊ containing findings of this project, and assist IPÊ in implementing recommendations outlined in final report.

Lessons learnt

- Dung DNA can be a valuable source of genetic information, however genotyping individuals from dung samples can be problematic and time consuming. Its advantages are great, for instance, it is possible (with enough work) for one person to collect 1 to 2 good samples a day with dung, meanwhile it takes many more people and time to collect a blood sample from large endangered mammals. However, the technique does not come without its limitations. It is possible, for instance, for one person to successfully genotype 60+ blood samples in one week. Yet, the same amount of dung samples can take 4 to 5 months, sometimes even longer. This is mainly due to the poor quality and low quantity of the sampled DNA. Nevertheless, better techniques to handle dung samples in the lab are in development, which promise to reduce man-hours in the lab and produce higher quality genotypes.
- Undertaking the lab work in Brazil, while feasible from a technical standpoint, and desirable in terms of capacity building and the fact that samples are kept within the country, logistically it can be complicated. Twice the DNA sequencer necessary for the genotyping of samples broke down, which meant 3-4 weeks down time in processing samples because of the distance from qualified technical support and the need to import machine parts from overseas, which faced increased delays because of strikes among the custom officials. Additionally, acquiring necessary chemicals can be problematic because of, again, the necessity to import them. However, this scenario is expected to change, as Brazil is starting to build its own biotechnology industry. Therefore, most of these chemicals will begin to be produced in country, or imported without the levying of import duties, making them as cheap as in US and EU markets.

Preliminary results

From a genetics perspective there are two important measures of diversity. First, there is the average number of alleles per locus. This is simply the count of alleles over all analyzed loci divided by the total number of loci. From a conservation standpoint, this measure is important because it is an estimate of the number of different variants in a population, which is an assessment of the populations ability to respond to selective pressures; therefore, the more alleles the better. Additionally, the distribution of alleles in each locus can be used to identify and measure the extent of past bottlenecks in the population.

Second, there is heterozygocity, which is a measure of the proportion individuals that are heterozygotes for a given locus. This allows us to gauge how variation is distributed within and among populations. Understanding how variation is distributed within populations is important, for instance, when inbreeding occurs. In inbreeding, observed heterozygocity (Ho) decreases relative to the expected heterozygocity (He) if the population was breeding exclusively with non-relatives. While, heterozygote deficiency can be an indication of inbreeding, heterozygote excess can be an indication of a small effective population size (Balloux 2004). As such, heterozygocity can be an important tool to evaluate the degree of inbreeding in a natural population, which generally lack pedigrees; and serve as a proxy to estimate effective population sizes.

Tapirs

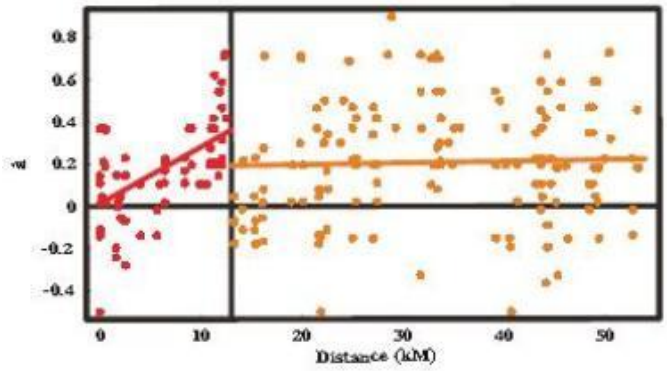
In a sample of 29 tapir samples analyzed, the total number of observed alleles among all loci analyzed varied from 5 to 10, with an average of 7.9 (±1.97) alleles per locus. Preliminary analyses for this report including all genotyped individuals indicate significant heterozygote deficiency, with an observed heterozygocity of 0.72 and an expected of 0.75.

As reported in the preliminary report, I carried out preliminary spatial analysis with the tapir populations. Here, the same results are presented for illustration purposes. However, no advance has yet been made since the preliminary report (see Lessons Learnt). 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

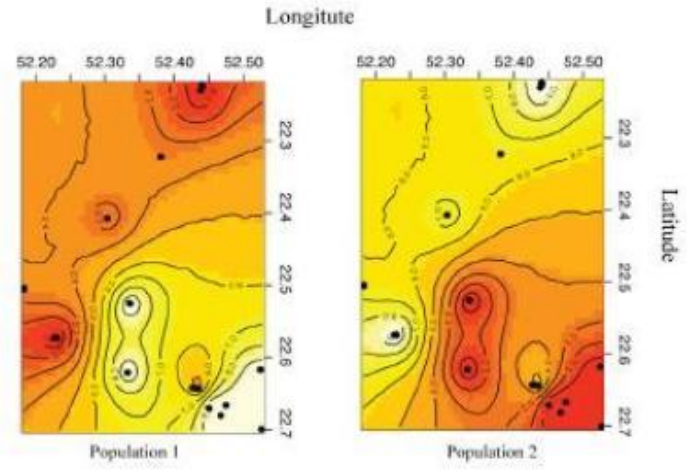
Distribution of tapir samples in the Pontal do Paranapanema (n=260)

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 recent fragmentation of the habitat, and equilibrium can be eventually reached if gene 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).



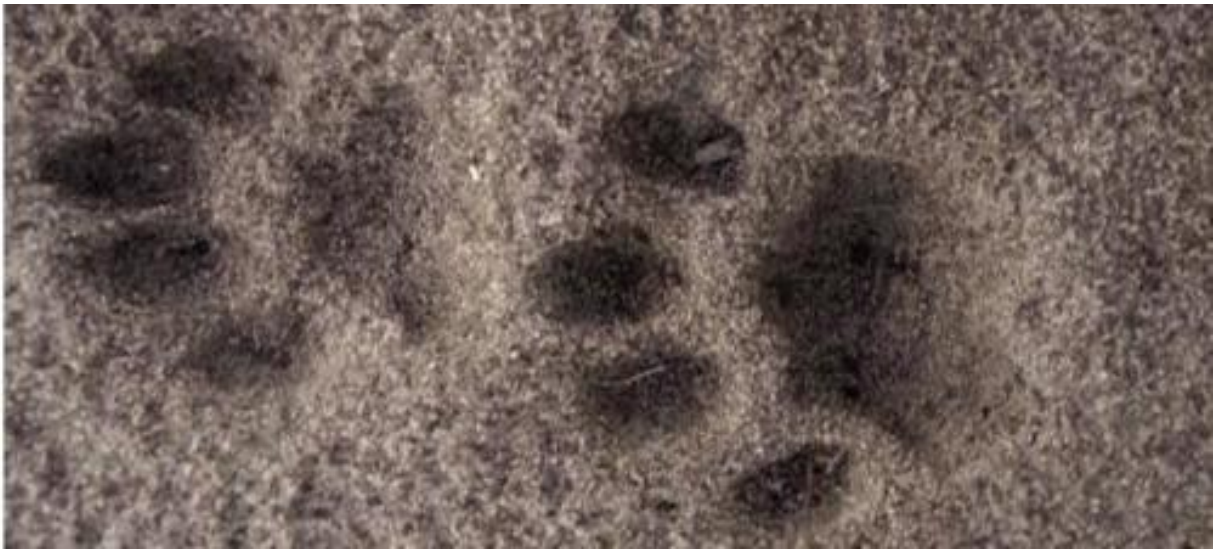
Plot of population membership for 23 individual tapirs in the Pontal do Paranapanema. Lighter colouring indicates increasing probability of membership. Population 1, in the lower right of the map, is the Morro do Diabo State Park population. Population 2, on the left and upper part of the map, is the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

Peccaries

A total of 35 collared peccary and 54 white-lipped peccary samples have been genotyped. An average of 5.1 (±2.56) alleles per locus and 4.6 (±1.52) alleles per locus were found in the collared and white-lipped peccaries, respectively. In so far as heterozygocity, collared peccaries had a significantly lower proportion of heterozygotes then expected (Ho = 0.58; He = 0.62); on the other hand, white-lipped peccaries had an excess of heterozygotes compared to what would be expected (Ho = 0.73; He = 0.68), however the difference was not statistically significant.

Felines

Twenty-five feline samples have been successfully processed to date, which include 12 jaguar, 3 puma, and 10 ocelot samples. An average of 2.75 (±1.03) alleles per locus were found in the jaguar; 2.62 (±1.06) for the pumas; and 4.25 (±2.05) for the ocelots. Out of the three species, the ocelot and the puma populations were found to have a lower proportion of heterozygotes then what would be expected (Ho = 0.39, He = 0.46; and Ho = 0.59; He = 0.64, respectively); however, the observed difference was only significant for the ocelot population. The jaguar was found to have an excess of heterozygotes (Ho = 0.57; He = 0.55), but it was not found to be statistically significant.



General recommendations

The results imply inbreeding in populations of tapirs, collared peccaries and ocelots. Additionally, pumas are potentially inbreeding, but this remains to be confirmed with larger sample sizes. White-lipped peccaries and jaguars both have significant heterozygote excess, suggesting a very small effective population size (i.e. small number of breeders).

Assuming these results persist, it would be recommended that corridors be built to link the Pontal region to other regions of Atlantic Forest, Cerrado and Pantanal. This would allow animals from other regions to move to the Pontal, and allow for dispersing animals from the Pontal to safely move away. Additionally, while corridors are not fully implemented, plans should be considered to translocate animals from other populations into the Pontal. This has already been successfully undertaken by IPÊ with black faced lion tamarins.

At the moment, the Pontal is the focus of an intense reforestation program, which is connecting all major forest fragments in the region (an example of a corridor in the region can be seen above). While, this is good in that it is increasing the carrying capacity of the region (i.e. allowing for more animals to live in the region), it is still not enough, because the populations of these species in the region seem too small for long-term (e.g. next 100 years) persistence, with some genetic deterioration already happening in some of them. There are already preliminary studies looking at the links between jaguars in northern Argentina and the Pontal through the Paraná River, and the possibilities of building a forest corridor connecting these areas are being considered. The next step would look at building corridors east, towards the remaining Atlantic Forest patches in the southeast portion of the State of São Paulo; and, building a corridor west to northwest to link the area to the Pantanal and Cerrado regions. This would hopefully return the Pontal to its former status as a link between the Atlantic Forest on the coast, and the Pantanal and Cerrado in the interior.

Nevertheless, structuring in the population that has yet not been observed could be causing the heterozygote deficiency. If so, the pattern of structuring relative to landscape features can lead to insights on habitat features deterring movement among different populations. Spatial analysis, which will be conducted in this next year, should help in revealing such barriers. As seen in the preliminary tapir results, this is a possibility. If this is indeed the case, recommendation will be

made to either remove such barriers (if possible) or to encounter ways for the animals to circumvent them.

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.

Additionally, preliminary results were presented to the staff at IPÊ, and are expected to be presented at the Rufford Foundation in London in the coming months. Moreover, at least three articles in leading scientific journals are expected to be submitted in the coming 12 months; and a full report with recommendations will be delivered to IPÊ. Finally, experience gained with this project is being used by other researchers on other species in other regions.

Acknowledgments

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