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Non invasive methods for genetic analysis applied to ecological and behavioral studies in Latino-America

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ABSTRACT - Documenting the presence and abundance of the neotropical mammals is the first step for understanding their population ecology, behavior and genetic dynamics in designing conservation plans. The combination of field research with molecular genetics techniques are new tools that provide valuable biological information avoiding the disturbance in the ecosystems, trying to minimize the human impact in the process to gather biological information. The objective of this paper is to review the available non invasive sampling techniques that have been used in Neotropical mammal studies to apply to determine the presence and abundance, population structure, sex ratio, taxonomic diagnostic using mitochondrial markers, and assessing genetic variability using nuclear markers. There are a wide range of non invasive sampling techniques used to determine the species identification that inhabit an area such as searching for tracks, feces, and carcasses. Other useful equipment is the camera traps that can generate an image bank that can be valuable to assess species presence and abundance by morphology. With recent advances in molecular biology, it is now possible to use the trace amounts of DNA in feces and amplify it to analyze the species diversity in an area, and the genetic variability at intraspecific level. This is particularly helpful in cases of sympatric and cryptic species in which morphology failed to diagnose the taxonomic status of several species of brocket deer of the genus ***Mazama***.

Key Words: cryptic species, fecal DNA, non invasive sampling

Introduction

In a world that biodiversity is loss in an alarming rate many large mammals species have at present, different levels of risk in the diverse ecosystems. Documenting the presence and abundance of the neotropical mammals is the first step for understanding their population ecology, behavior and genetic dynamics in designing conservation plans. Large mammals in Latin America have been affected by human activities. Many deer and carnivore species have been decline their populations owed to habitat fragmentation and urbanization.

The objective of this paper is to review the available non invasive sampling techniques that have been used in neotropical mammal studies to apply to determine the presence and abundance, population structure, sex ratio, taxonomic diagnostic using mitochondrial markers, and assessing genetic variability using nuclear markers.

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Traditional techniques

Traditional direct individual observation or survey tracks or other signals that suggest the species occurrence is the first step to monitor the species presence. However, opportunities to observe mammals in the field are usually limited, due to their small size and nocturnal and elusive habits. Additionally, most of the species have low densities in contrast to the expansion of domestic species. Large diurnal animals are evasive and cannot be observed directly.

Traces or tracks of mammals can support data on distribution, behavior, age, social status and method of movement and can facilitate animal identification. Photography is one of the most effective ways of obtaining a permanent registry of animal tracks.

Knowledge of identification, interpretation and preservation for tracks and other signs left by mammals can support information on the habits of these species that cannot be obtained by any other method. Tracks can be found in wet or muddy areas

near lakes, puddles and creeks where animals eat and drink and along the paths used to go between different habitats

Remote camera traps

Remote camera traps are ideal for identifying species that inhabit in close forest habitat. The equipment is based on an active infrared sensor should be fitted with a wide angle autofocus lens. A quartz crystal data-back will automatically record time and date information on each photograph. They also provided us information about the abundance and activity patterns. Previously the monitor area need to be examined looking for tracks, faeces to install the equipment.

The advantages of this equipment are they are relatively non invasive, an area can be monitored with minimal human disturbance and the animals do not have to be captured. It is possible to survey large areas with a small crew and with not constant attendance (Wemmer *et al*., 1996).

Molecular ecology

The combination of field research with molecular genetics techniques are new tools that provide valuable biological information avoiding the disturbance in the ecosystems, trying to minimize the human impact in the process to gather biological information. In several occasion species determination using photographs to analyze morphology was not possible as is the case of the neotropical brocket deer, in which there are cryptic species.

Feces collection and preservation

Preservation methods are simple and require minimal field equipment (Wasser *et al*., 1997). The equipment consists on: centrifuge 50 ml plastic tubes, gloves, ethanol 70 or 95%, plastic bags, and permanent marker to label the sample. Each feces sample needs to be collected using gloves to avoid contamination. Centrifuge tubes are one of the best containers for storage feces with ethanol. Also using plastic bags in the field and preserving refrigerated is an effective method. Other alternatives for sample transport and storage in dry environments, for instance, is to change the ethanol by silica gel. Last but not least it is important to label the sample with all the information related to the collection site. If you have a GPS device, you will have the precise and detailed information about geographical coordinates, date, hour, and climate conditions. Other important observations are concerning the sample state: if it is fresh, or dried; if you saw dung beetles or parasites, etc. All these record will be useful to replicate the sampling and interpreter the results in the laboratory.

Other tested alternative in some neotropical deer species was the used of trained dogs, that can be very useful to search feces in tropical forest areas (Duarte, 2005). Previously, dogs need to be trained to identify the deer feces. This requires a professional trainee to learn the dog to identify the feces smell and to be educated to look for the feces in field conditions without destroy or contaminate the samples (Smith *et al*., 2001; 2003).

DNA extraction

Common DNA extraction protocols would be useful but, in most cases, the PCR inhibitors present in feces are not totally removed. Feces contain cells shed from the gut and a complex mixture of other compounds (microorganisms, undigested food, digestive enzymes, mucus, bile salts and bilirubin). In addition herbivore feces contain plant polysaccharides and phenolic compounds. Further more addition of bovine serum albumin (BSA), cellulose or other substance to DNA extraction and PCR circumvented inhibition (Kohn & Wayne, 1997).

Genetic variation

The mtDNA control region is commonly variable on the intraspecific level and is suitable for studies of genetic variability, phylogeography, assignment to management units, and forensics (Kohn & Wayne, 1997). Studies conducted in some Neotropical deer using this region as genetic marker, as in the pampas deer and the gray brocket deer, showed that this is very polymorphic and informative (González *et al*., 1998; Bidegaray *et al.*, 2003). But in other species as the huemul and the marsh deer it showed discrete variation, being informative to conduct phylogeographic studies (Jara *et al*., 2005; Márquez *et al*, 2006).

Taxonomic determination

The taxonomy, distribution, ecology, and status of Brocket deer (*Mazama)* are unclear showing a

wide distribution in the Neotropical region from southern Mexico to Argentina (Weber & González, 2003). They are secretive and difficult to detect and observe, due especially the close habitat they live.

In Brazil there are five species of brocket deer *M. americana*, *M gouazoubira*, *M nemorivaga*, *M nana* and the recently described *M bororo*. (Duarte & Jorge, 2003).

The small red brocket deer *Mazama bororo* is one of the most endangered and unknown neotropical deer that inhabit in small fragments of the remaining Atlantic forest, extending from the southeastern part of the state of Sao Paulo to the north eastern part of the state of Paraná-Brazil (Weber & González, 2003; Duarte & Jorge, 2003). The morphological similarities with other simpatric brocket species, and the difficult to detect and observe, due especially the habitat they live, prevent the use of traditional methodologies for the species identification (Duarte & Jorge, 2003).

In order to improve the detection of endangered small red brocket deer among the other brocket deer species that are in sympatry, and therefore enhance conservation and management strategies, we developed a new specific sets of primers and a system of restriction enzyme digestion of PCR – amplified mitochondrial DNA that can be easily and reliable applied.

Sequences of the complete cytochrome b were obtained from individuals belonging to: *Mazama americana* (24), *M. bororo* (5), *M. gouazoubira* (14), *M. nana* (6). The sample contains information about morphology, cytogenetics, and biochemical genetics. This information was taking into account for the sample selection of 30 individuals from the sympatric area, to design a new set of cytochrome b primers adequate to amplify a small fragment from DNA isolated from feces.

In the field feces were collected using the aid of a trained dog in Conservation Units (CU) from south and southeast Brazil's regions. Positive identification of the species occurred only from four localities of São Paulo and Paraná States.

The designed set of primers were useful to amplified a sequence of 224 bp, in which we found unique enzyme restriction sites using Sequencher software, that can easily differentiated among the four sympatric species. Previously the primers designed and the RFLP reactions were tested in the entire control sample.

In the field 246 scats were collected using the aid of a training dog, and 95 % of them were successfully amplified. The PCR-RFLP reaction showed that 43% were determined as *M bororo*. The RFLP reaction failed in 23 cases (9%). The remaining sample was distributed among grey brown brocket deer (30%), red brocket deer (10%) and the Brazilian dwarf brocket deer (8%).

The designed molecular tool showed to be efficient for taxonomic distinction of the three brocket deer species that are in simpatry with the small red brocket deer. The sampling strategy used showed to be effective and we map the occurrence area of the small red brocket deer. These results suggest a distribution restricted for *Floresta Ombrófila Densa*, between 24º16'06"S to 48º24'41"W.

Population estimation

Estimates population sizes based on fecal DNA recently has been developed based on that each individual is characterized by a unique multilocus genotype to determine the number of animals sampled, and through the use of statistical models to estimate population size (Belleiman, *et al.*, 2005). However these methods have several limitations, mainly due to the low quantity and quality of the DNA isolated. Microsatellites have two main scoring errors: allelic dropout and false alleles that can lead to incorrect genotyping and biased estimates (Taberlet *et al*., 1996; 1999).

The use of microsatellites to estimate population size can be based on: Capture Mark Recapture method (Seber, 1982) and rarefaction analysis (Kohn *et al*., 1999). Few studies have compared genetic estimation with field data estimations to test reliability and accuracy only in bear (Belleiman *et al*., 2005) not yet in neotropical mammals.

The small red brocket deer population size from Intervales National Park-Brazil was estimated based on fecal DNA amplified with 3 microsatellites primer set (Duarte, 2005). In the study first they obtained the density value in a Km^2, and afterward this value was extrapolated to the entire area Park. Subsequently was applied the models of capture, mark and recapture (Petersen and Schnabel/Schumacher models) to estimate the population size in the area using the software Ecological Methodology. Finally the species estimation in all the range area was performed based on the density value obtained in the Intervales Park.

Based on this data, the authors concluded that the population of *M. bororo* at the Intervales Park should be up to 615 animals. Crossing this information with the knowledge of the conservation status of other Atlantic Forest reminiscent, they conclude that this Park can be the last and the most important conservation unit for this species (Duarte, 2005).

Sexing sampl es

Sex determination based on DNA extracted from the feces of elusive wildlife animals has recently become feasible. Previous studies amplified a partial sequence of the sex determining region Y (SRY) gene, which is present only on the Y chromosome. Since feces have been shown to contain inhibitors substances of the polymerase chain reaction (PCR), an internal positive control was necessary in order to make sure that PCR took place normally (González *et al.*, 2004). The new molecular strategies developed are based on amplification of gene located in the X and Y chromosome. The amelogenin (AMEL) gene, which exists on both X and Y chromosomes, has been used to determine gender in cattle and in humans was tested in wild sika deer (*Cervus nippon*) (Yamauchi *et al*., 2000).

Conservation implications

Now it is possible to survey wild mammals using several tools as the traditional direct observation and newly molecular genetic techniques. These last techniques are develop a new ecology the *molecular ecology* that use the trace amounts of DNA from feces to amplify with mitochondrial markers and analyze the species diversity and richness in an area. Furthermore monitor the genetic variability at intraspecific level and population structure applying molecular sexing. These set of techniques are particularly helpful in cases of sympatric and cryptic species in which morphology failed to diagnose the taxonomic status of several species of brocket deer of the genus *Mazama.*

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Literature cited

BELLEIMAN, S.; SWENSON, J.E.; TALLMON, D. *et al.* Estimating population size of elusive animals with DNA from hunter-collected feces: Four methods for brown bears. **Conservation Biology**, v.19, n.1, p.150-161, 2005.

BIDEGARAY, L.; MALDONADO, J.; DUARTE, J.M.B. *et al.* **Variabilidad genética en *Mazama gouazoubira* (MAMMALIA:CERVIDAE)**. IV Taller de Cérvidos del Uruguay-VII Jornadas de Zoología del Uruguay. Sociedad Zoológica del Uruguay**.** Pp 173. 2003.

DUARTE, J.M.B. **Ecologia e distribuição de *Mazama bororo* (MAMMALIA: CERVIDAE)**. Relatório Final de Pesquisa. FUNEP, Jaboticabal. 104 pp. 2005.

DUARTE, J.M.B.; JORGE, W. Morphologic and cytogenetic description of the small red brocket (*Mazama bororo* Duarte,1996) in Brazil. **Mammalia**, v.67, n.3, p.403-410, 2003.

GONZÁLEZ, S.; MALDONADO, J.E.; LEONARD, J.A. *et al.* Conservation genetics of the endangered Pampas deer (***Ozotoceros bezoarticus***). Molecular Ecology, v.7, p.47-56, 1998.

GONZÁLEZ, S.; MALDONADO, J.E.; BIDEGARAY, L. *et al.* **Determinación de *Mazama bororo* utilizando métodos no-invasivos.** VI International Conference on Wildlife Management in Amazonia and Latinamerica, 5-11 Septiembre, Iquitos-Perú. 2004.

JARA, A.; VICTORIANO S.P.; ORTIZ, J.C. *et al.* **Estudios de variabilidad genética en poblaciones de Hippocamelus bisulcus (Molina, 1782): Alcances en la conservación de la especie**. Informe proyecto Universidad de Concepción. 55 pp. 2005.

KOHN, M.H.; WAYNE, R.K. Facts from feces revisited. **Tree**, v.12, n.6, p.223-227, 1997.

KOHN, M.H.; YORK, E.C.; KAMRADT, D.A. *et al.* Estimating population size by genotyping feces. **Proc. R. Soc. Lond B. Biol. Sci.**, v.7, n.266(1420), p.657-63, 1999.

MÁRQUEZ, A.; MALDONADO, J.E.; GONZÁLEZ, S.; *et al.* Phylogeography and Pleistocene demographic history of the endangered marsh deer (*Blastocerus dichotomus*) from the Río de la Plata Basin. **Conservation Genetic**, v.7, p.563-575, 2006.

SEBER, G.A.F. **The estimation of animal abundance and related parameters**. 2nd edition Macmillan, New York. 1982.

SMITH, D.A.; RALLS, B.K.; DAVENPORT, B. *et al.* Canine assistants for conservationists. **Science**, v.291, p.435, 2001.

SMITH, D.A.; RALLS, A.K.; HURT, B. *et al.* Detection and accuracy rates of dogs trained to find scats of San Joaquin kit foxes (Vulpes macrotis mutica). **Animal Conservation**, v.6, p.339-346, 2003.

TABERLET, P.; GRIFFIN, S.; GOOSSENS, B. *et al.* Reliable genotyping of samples with very low DNA quantities using PCR. **Nucleic Acids Research**, v.24, p.3189-3194, 1996.

TABERLET, P.; WALTS, L.P.; LUIKART, G. Noninvasive genetic sampling: look before you leap. **Trends in Ecology and Evolution**, v.14, n.8, p.323-327, 1999.

WASSER, S.K.; HOUSTON, C.S.; KOEHLER, G.M. *et al.* Techniques for application of faecal DNA methods to field studies of Ursids. **Molecular Ecology**, v.6, p.1091-1097, 1997.

WEBER, M.; GONZALEZ, S. Latin American deer diversity and conservation: A review on status and distributions. **Ecoscience**, v.10, n.4, p.443-454, 2003.

WEMMER, C.; KUNZ, T.H.; LUNDIE-JENKINS, G. *et al.* Mammalian Sign. In: Measuring and Monitoring Biological Diversity. Standar Methods for Mammals. (ed. Wilson, D. E. *et al.*). p.157-176, Smithsonian Inst. Press. Washington and London. 1996.

YAMAUCHI, K.; HAMASAKI, S.; MIYAZAKI, K.*et al.* Sex determination based on fecal DNA analysis of the amelogenin gene in sikadeer (*Cervus nippon*). **J. Vet. Med. Sci.**, v.62, n.6, p.669-71, 2000.