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CARACTERIZAÇÃO DA DIVERSIDADE E ESTRUTURA GENÉTICA DE UMA POPULAÇÃO INVASORA E INVESTIGAÇÃO DE POSSÍVEIS HÍBRIDOS ENTRE AS ESPÉCIES AMEAÇADAS *Leontopithecus rosalia* E *Leontopithecus chrysomelas*

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DISSERTAÇÃO DE MESTRADO

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RESUMO

A introdução de espécies não nativas é um evento que pode pôr em risco a conservação das espécies nativas. A introdução de espécies alóctones geralmente se baseia em poucos indivíduos, e está atrelada aos impactos relacionados ao efeito fundador e à perda da diversidade genética, a qual é dependente do tamanho populacional efetivo inicial, da taxa de crescimento populacional e das características do novo ambiente colonizado, as quais podem propiciar ou não a adaptação ao mesmo. Espécies invasoras podem também favorecer a hibridação com espécies nativas, quando ambas as espécies se encontram e conseguem se acasalar, podendo ser prejudicial para biodiversidade quando uma ou ambas as linhagens ou espécies puras progenitoras é extinta. A criação de zonas de contato entre espécies que naturalmente não co-ocorrem pode, portanto, se constituir em uma ameaça premente à conservação biológica. Esse é o caso de uma região específica no Parque Estadual da Serra da Tiririca, localizada no município de Niterói, no estado do Rio de Janeiro (RJ), na qual foram liberados na década de 90 dois casais da espécie ameaçada *Leontopithecus chrysomelas* (o mico leão-de-cara-dourada, MLCD, endêmico da Mata Atântica da Bahia), e um casal de *Leontopithecus rosalia* (o mico-leão-dourado, MLD, endêmico da Mata Atlântica do Rio de Janeiro). Devido às condições favoráveis presentes nesta área, estes animais conseguiram se adaptar e se reproduzir com sucesso, gerando uma nova população fora da área de ocorrência natural de *L. chrysomelas*. Ao longo de duas décadas, essa população aumentou sua densidade de forma exponencial, sendo estimada a existência de cerca de 200 indivíduos com fenótipo de *L. chrysomelas* naquela área em 2009, nenhum indivíduo com fenótipo de *L. rosalia* foi observado. Em vista deste evento, e do fato de que na época só se tinha a suspeita sobre a possível introdução ilegal de *L. chrysomelas* na região de Niteroi, uma medida alternativa para conter o crescimento e avanço dessa população para áreas adjacentes de ocorrência natural de *L. rosalia* foi translocar indivíduos com fenótipo de *L. chrysomelas* para um fragmento de Mata Atlântica, localizado na RPPN (Reserva Particular do Património Natural) Veracel Celulose, em Belmonte, Bahia. Em 2015, porém, foi confirmada a introdução ilegal de *L. chrysomelas* e de *L. rosalia.* Assim, apesar dos indivíduos da população de Niteroi terem fenótipo de *L. chrysomelas,* sabe-se que híbridos, além de possuírem fenótipos intermediários às espécies inter-cruzantes, podem se assemelhar mais a apenas uma das espécies parentais. Considerando que ambas as espécies são classificadas pela IUCN como "em perigo", investigar a existência de híbridos nesta população e caracterizar sua diversidade e estrutura genética é de fundamental relevância para que medidas adequadas de manejo sejam tomadas nas regiões onde atualmente existem descendentes desta população. Dentro deste contexto, o presente trabalho teve como objetivo principal realizar análises genômicas e genéticas para investigar a ocorrência de hibridização e para caracterizar a diversidade e estrutura genética desta população. Os dados mostraram ausência de sinais de hibridização na população de Niteroi, confirmando que os indivíduos são da espécie *L. chrysomelas.* Adicionalmente, os valores de diversidade genética mostraram-se comparáveis ao que vem sendo observado para outras populações de *Leontopithecus,* evidenciando ausência das consequências negativas de efeito fundador e excelente capacidade da espécie para restabelecer sua diversidade na região de Niteroi. Estes achados são relevantes para que os programas de conservação de ambas as espécies possam continuar manejando adequadamente as populações envolvidas nesse estudo.

ABSTRACT

The introduction of non-native species is an event that can jeopardize the conservation of native species. The introduction of alien species is usually based on a few individuals and is linked to impacts related to the founder effect and the loss of genetic diversity, which is dependent on the initial effective population size, the population growth rate, and the characteristics of the new colonized environment, which may or may not provide adaptation to it. Invasive species can also favor hybridization with native species, when both species meet and manage to mate, which can be detrimental to biodiversity when one or both pure progenitor strains or species become extinct. The creation of contact zones between species that do not naturally co-occur can therefore pose a pressing threat to biological conservation. This is the case of a specific region of Serra da Tiririca State Park, located in the municipality of Niterói, Rio de Janeiro (RJ), where two pairs of the threatened species *Leontopithecus chrysomelas* (the golden headed lion tamarin, GHLT, endemic to the Atlantic Forest of Bahia) and a pair of *Leontopithecus rosalia* (the golden lion tamarin, GLT, endemic to the Atlantic Forest of Rio de Janeiro) were released in the 1990s. Due to the favourable conditions present in this area, these animals were able to adapt and reproduce successfully, generating a new population outside the natural range of *L. chrysomelas*. Over two decades, this population increased its density exponentially, and it was estimated that there were about 200 individuals, with phenotype of *L. chrysomelas* in that area in 2009, no individuals with the phenotype of *L. rosalia* were observed. In view of this event, and the fact that at that time there was only the suspicion of a possible illegal introduction of *L. chrysomelas* into the Niteroi region, an alternative measure to contain the growth and advance of this population to adjacent areas of natural occurrence of *L. rosalia*, was to carry out the translocation of individuals with phenotype similar of *L. chrysomelas* to a fragment of Atlantic Forest, located on the RPPN (Private Natural Heritage Reserve) Veracel Celulose in Belmonte, Bahia. In 2015, however, the illegal introduction of *L. chrysomelas* and also *L. rosalia* was confirmed. Although these individuals had the phenotype of *L. chrysomelas*, it is known that hybrids, besides having phenotypes intermediate to the inter-crossing species, can be more similar to only one of the parental species. Considering that both *L. rosalia* and *L. chrysomelas* are species classified by the IUCN as "endangered", investigating the existence of hybrids in this population and characterizing their genetic diversity and structure is relevant for appropriate management measures in regions where currently there are descendants of this population. In this context, the main objective of this study was to carry out genomic and genetic analyses to investigate the occurrence of hybridization and to characterize the genetic diversity and structure of this population. The data showed no signs of hybridization in the Niteroi population, confirming that the individuals are from the species *L. chrysomelas*. Additionally, genetic diversity values were comparable to those observed for other *Leontopithecus* populations, showing the absence of negative founder effect consequences and the excellent capacity of the species to re-establish its diversity in the Niteroi region. These findings are relevant to adequately manage individuals from the invasive population and for the conservation programs of both pure species.

1. INTRODUCTION

1.1. Alien species and hybridization

Non-native species have been introduced into distribution areas of closely species (PARK, 2004; RHYMER; SIMBERLOFF, 1996). The introduction process consists of moving an organism from one area to another, and although it can be used for conservation purposes (IUCN/SSC, 2013), it can negatively impact local biodiversity and is considered as a cause of biodiversity loss (WALKER; STEFFEN, 1997). Introduced species, alien or not, could bring new diseases (MACK; D'ANTONIO, 1998) and intensify competition for resources (BLANCHET et al., 2007; RUIZ-MIRANDA et al., 2006), promoting changes in habitat and ecosystem disturbances, and increasing the risk of extinction of endangered species. In addition, introduced species have to adapt to a new environment, where they were probably not previously adapted, and occasionally several invasive populations outcompete native species (ALLENDORF; FLATHEAD; AITKEN, 2013). The success of these invasive populations is that they are probably better competitors or due to the lack of selective pressure, in which native species would demonstrate better performance, such as for resistance to local specific pathogens (ALLENDORF; FLATHEAD; AITKEN, 2013).

In general, introduced species has small population sizes in the beginning, due to the reduced number of founder individuals. Thus, non-native populations can suffer from the extreme founder effect, rapid loss of genetic diversity and increased inbreeding (ALLENDORF; LUNDQUIST, 2003; PÉREZ et al., 2006). The founder effect is the result of random sampling of alleles from a few individuals belonging to a certain population in order to establish a new one (NEI; MARUYAMA; CHAKRABORTY, 1975). This leads to genetic diversity that is not representative of the original (ALLENDORF, 1986). Such founder effect may rapidly change allele frequency from one generation to another, causing an increase in the frequency of certain deleterious alleles (ALLENDORF; FLATHEAD; AITKEN, 2013). Further, genetic drift, that is stronger than selection in small populations, may facilitate the fixation of these alleles (KRATZER et al., 2020). In the case of small invasive founder populations with no subsequent new introductions, the individuals tend to lose fitness (KRATZER et al.,

2020), since the populations usually have a small effective size, which increases the genetic drift and the loss of genetic diversity (BRAMBILLA et al., 2014).

The establishment of new isolated populations may also lead to a genetic differentiation caused by the lack of gene flow among populations and random effects of the genetic drift. Indeed, the founder effect is considered a case of genetic drift that leads to a strong and rapid differentiation between populations. On the other hand, invasive species can colonize other areas designated for the conservation of native species (RUIZ-MIRANDA et al., 2006), enabling mixing between lineages or historically isolated species. This can establish possible hybridization zones (RHYMER; SIMBERLOFF, 1996), which could result in hybrids, compromising the integrity of the inbred pure species or lineages (IUCN/SSC, 2013). Hybrids can present heterosis or *hybrid vigor*, which can lead to a superiority over pure lineages or species. Conversely, hybridization can result in offspring with lower fitness, facilitating the extinction of the hybrid population (ALLENDORF et al., 2001; ALLENDORF; FLATHEAD; AITKEN, 2013). Therefore, programs and actions involving translocation, introduction and/or formation of artificial hybrid zones must be efficiently managed and monitored to ensure that conservation goals are achieved without compromising the integrity of native and introduced species.

Hybridization can be caused naturally or by human activity. This last one is known as anthropogenic hybridization and may be caused not only by the introduction of non-native species to the area, but also by habitat modifications caused by human activities (ALLENDORF; FLATHEAD; AITKEN, 2013). Hybridization have been reported for several taxa, including amphibians, birds, fishes, felid, canids, deer (BOHLING; WAITS, 2011; EVA; YAMAZAKI, 2018; FITZPATRICK et al., 2010; GALAVERNI et al., 2017; LE ROUX et al., 2015; SCHUMER et al., 2018; TOEWS et al., 2016; TRIGO et al., 2008; VALLENDER et al., 2007); and primates (BUNLUNGSUP et al., 2017; CORTÉS-ORTIZ et al., 2007; JADEJAROEN et al., 2016; SCHWITZER et al., 2013; SUPRIATNA; MOOTNICK; ANDAYANI, 2010). In Neotropical primates (Parvorder Platyrrhini), hybridization events have been detected in Brazil for different taxa, such as *Alouatta* (CORTÉS-ORTIZ et al., 2007; MOURTHE et al., 2019) and *Callithrix* (MALUKIEWICZ et al., 2014). Moreover, the presence of non-native primates has been reported for different biomes, including the Atlantic Forest, where eight non-native species were found only in the state of Rio de Janeiro, including *Saimiri sciureus,*

Callithrix jacchus, Callithrix penicillata, Ateles paniscusm, Alouatta caraya and *Leontopithecus chrysomelas* (OLIVEIRA; GRELLE, 2012).

1.2. The local study species

Leontopithecus species are primates popularly known as lion tamarins, which are endemic to the Brazilian Atlantic Forest. Currently there are four recognized species found in allopatric in different regions of Brazil. The black-faced lion tamarin (BFLT), *L. caissara,* is found in the state of Paraná (PR), in the southeast of São Paulo; and the black lion tamarin (BLT), *L. chrysopygus,* is endemic to the state of São Paulo (SP). The golden lion tamarin (GLT), *L. rosalia*; and the golden-headed lion tamarin (GHLT), *L. chrysomelas,* are endemic to the state of Rio de Janeiro (RJ) and of Bahia (BA), respectively (KIERULFF et al., 2008a, 2008b, 2008c; RUIZ-MIRANDA et al., 2019a; RYLANDS et al., 1996).

Lion tamarins primarily inhabit primary forest (KLEIMAN, 1988), however, due to the decline and destruction of their natural habitats, these species are now found in regenerating secondary forests as well (Kierulff et al., 2002). According to the International Union for Conservation of Nature (IUCN, 2020), all four *Leontopithecus* species are under some degree of threat of extinction. The GLT and GHLT are categorized as Endangered (EN), and have their population sizes estimated, respectively, around 3,700 individuals, with 1,400 of mature age (RUIZ-MIRANDA et al., 2019b); and 6,000-15,000 individuals, with a maximum of 2,500 of mature age (ICMBIO/MMA, 2018; PINTO; RYLANDS, 1997).The conservation of the lion tamarins has currently been considered a great challenge, mainly due to human demographic growth and the irresponsible use of natural resources (COIMBRA-FILHO, 2004), which includes deforestation and the illegal commercialization of these species. Although human actions are negatively impacting the biodiversity of our planet, paradoxically, human intervention can also promote its conservation (CABALLERO, 2014).

1.3. Conservation Genetics

Conservation Biology is a field of knowledge dedicated to the study of strategies aimed at the conservation of biodiversity, through the establishment of management and biological conservation programs based on knowledge of the species, including behavioral, demographic, ecological and genetic aspects (BERTORELLE et al., 2009). Conservation Genetics is a discipline within Conservation Biology that aims to apply the theoretical knowledge of Quantitative and Population Genetics to the conservation of species and their evolutionary potential (FRANKHAM; BALLOU; BRISCOE, 2008).

Studying and quantifying the genetic diversity of species and their populations can provide relevant information for species adaptability in the face of environmental changes and anthropogenic impacts (ALLENDORF; HOHENLOHE; LUIKART, 2010). Maintaining adequate levels of genetic diversity minimizes detrimental effects related to increased inbreeding and eventual inbreeding depression, especially, in small and/or threatened populations, in which fitness and persistence capacity are more drastically affected by evolutionary events (FRANKHAM et al., 2017).

Different DNA and molecular-based approaches have been used by Conservation Genetics, aiming to produce data for clarifying aspects relevant to biological conservation (FRANKHAM; BALLOU; BRISCOE, 2008). The study of phenomena involving loss of genetic diversity and increased inbreeding, as well as the characterization of the genetic structure of species' populations, including the detection of hybridization, for example, can aid to identify groups that are under threat, and to understand processes that may lead species and/or populations to extinction (FRANKHAM; BALLOU; BRISCOE, 2008).

Among the main methodological approaches employed to estimate genetic parameters relevant to the understanding of issues related to the maintenance or extinction of populations and species are the use of molecular markers, especially microsatellites (SERGE; ANDREW, 2016). These markers, also called SSRs (Single Sequence Repeats), are short tandem repetitive sequences of one to six nucleotides (ALLENDORF; FLATHEAD; AITKEN, 2013). The number of the repetitive motif of a microsatellite locus varies among individuals of the same species due to its high mutation rate, which generates variations in the length of microsatellite and confers the high level of polymorphism of this marker (FERREIRA; GRATTAPAGLIA, 1998). For this reason, SSRs are very useful to study genetic, ecological, historical, and demographic events,

such as the presence of inbreeding, population structure, dispersal, and hybridization (ALLENDORF; FLATHEAD; AITKEN, 2013; BEAUMONT; NICHOLS; B, 1996; FRANKHAM; BALLOU; BRISCOE, 2008; TORRES et al., 2017). For the genus *Leontopithecus*, the use of microsatellites has been effective to assess genetic diversity and population differentiation of different species (AYALA-BURBANO et al., 2017; GALBUSERA; GILLEMOT, 2008; GRATIVOL; BALLOU; FLEISCHER, 2001; MARTINS; GALETTI JUNIOR, 2010; MORAES et al., 2017; PEREZ-SWEENEY et al., 2005). Microsatellite markers have also been efficient for hybrid identification in primates in the genus *Alouatta* (CORTÉS-ORTIZ et al., 2007; MOURTHE et al., 2019) and *Callithrix* (MALUKIEWICZ et al., 2014).

More recently, the development of modern technologies for large-scale molecular analysis has enabled the employment of robust genomic approaches to study population diversity and genetic structure through the use of next-generation sequencing (NGS) and identification of thousands of Single Nucleotide Polymorphisms (SNPs) in the genome of species (SERGE; ANDREW, 2016). SNPs are single base pair variations of DNA, widely distributed in the genome, being present in both coding and non-coding regions (BROOKES, 1999; TURCHETTO-ZOLET et al., 2017). The identification of SNPs has allowing robust estimates of genetic diversity and population structure (KELLER et al., 2013), in addition to hybrid detection (HOHENLOHE et al., 2011; KELLER et al., 2013), and differential selection analyses, since these loci can be located in genes related to fitness (TURCHETTO-ZOLET et al., 2017).

Among the currently most used methodologies for SNP characterization is Genotyping by Sequencing (GBS). The GBS technique is based on the next-generation sequencing of small fragments of the genome generated by restriction enzymes (ELSHIRE et al., 2011). The use of restriction enzymes produces a reduced representation of the species' genome for sequencing. In addition, the digested DNA samples are bound to adapters and barcodes, making possible the sequencing of several individuals at the same time, and then their subsequent identification (TURCHETTO-ZOLET et al., 2017). GBS-based studies in primates are recent but have already been successfully employed for phylogenetic analyses (VALENCIA et al., 2018), detection of hybrids in *Macaca* (BUNLUNGSUP et al., 2017; JADEJAROEN et al., 2016), and assessment of genetic diversity in *Callithrix* (MALUKIEWICZ et al., 2017), proving to be extremely efficient when compared to the use of microsatellites in small scale due to the greater number of

loci analyzed simultaneously and the possibility of identifying non-neutral polymorphisms (FISCHER et al., 2017).

1.4. Background to the problem

Based on historical records, personal communications, and according to the Pri-Matas Institute, entity responsible for the management of the species *L. chrysomelas*, in 1994 there was an illegal release of two males and two females of *L. chrysomelas*, along with one male and one female of *L. rosalia*, in the Serra da Tiririca State Park, in Niteroi, RJ (23˚70'40.55"E; 74˚65'35.3"S). However, this information was not available in 2009, when a census estimated the existence of around 200 lion tamarins, all of them with phenotype of *L. chrysomelas,* occurring in that area (KIERULFF C, personal communication). For that reason, the Pri-Matas Institute initiated the capture of these individuals to prevent the advance of the invasive population to the region where *L. rosalia* naturally occurs. In the subsequent years, there was a growth of the invasive population of Niteroi, and, in 2012, 293 animals were transferred to a fragment forest (24˚45'76.67"E; 82˚30'65.0"S) in the RPPN (Private Natural Heritage Reserve) Veracel Celulose, located in Belmonte (BA), a region where *L. chrysomelas* is endemic. Most of the remaining individuals from Niteroi were transferred to the Primatology Center of Rio de Janeiro (CPRJ). The animals transferred to Bahia adapted very well to the local conditions in Belmonte, however, no genetic analyses were performed to confirm if the animals of Niteroi were indeed pure *L. chrysomelas* (KIERULFF C., personal communication). Hence, in 2015, when information on the introduction of both species in the Niteroi region was obtained, it was raised the concern that the population present in Niteroi and transferred to Bahia could be constituted by hybrid individuals.

Despite the fact of the animals from Niteroi have their phenotype like the pure species *L. chrysomelas*, complementary analysis to the morphological traits must be implemented to verify the existence of hybridization in the managed areas, since, although hybrids may present intermediate phenotypes to those of the parental species (CORTÉS-ORTIZ, 2017; SMITH, 1992), a greater representation of genes from just one of the parents can generate hybrids morphologically very similar to a single species (LEARY; GOULD; SAGE, 2011), that could depend on backcrosses into one parental specie (MCFARLANE; PEMBERTON, 2019). Further, is important to consider that in

backcross's individual, the proportion of expected invasive genome is reduced in 50% each generation (BOECKLEN; HOWARD, 1997).

2. JUSTIFICATIONS, QUESTIONS AND OBJECTIVES

Considering the context presented here, two issues relevant to biodiversity conservation must be considered.

The first issue concerns the existence of hybrids and its potential negative impact on the allopatric native pure species in both Rio de Janeiro and Bahia regions, where animals from the invasive population are currently occurring. In Neotropical primates, the natural hybridization process generating individuals with intermediate and unique morphology has been well reported in the literature for species of the genus *Alouatta*, naturally occurring in areas where the geographic distribution of two species overlap (CORTÉS-ORTIZ et al., 2007). In marmosets, the translocation and introduction of the species *Callitrhix jacchus* and *Callithrix penicillata*, naturally occurring in northeaster and central Brazil, into the southeaster region of the country has promoted rapid population expansion and hybrid formation, negatively impacting the genetic integrity of the pure species (MALUKIEWICZ et al., 2014). In addition, the presence of *Callithrix* in Rio de Janeiro may impact the native *L. rosalia* due to increased competition for resources (RUIZ-MIRANDA et al., 2006). In the genus *Leontopithecus*, hybrids between *L. rosalia* and *L. chrysomelas* have already been described in captivity (COIMBRA-FILHO; MITTERMEIER, 1976). However, in the wild, there are no published reports in the scientific literature describing hybrids for the genus to date, either by the formation of natural or artificial hybridization zones.

The second issue is related to the inexistence of hybridization and consequently the origin of the whole population from only four founders (two females and two males of *L. chrysomelas*). This scenario may affect the genetic diversity of the invasive population, since this usually has a small effective size, enhancing the genetic drift and leading to the loss of genetic diversity (BRAMBILLA et al., 2014). Moreover, such populations when isolated may result in high rates of inbreeding (BRAMBILLA et al., 2014). Also, the introduction of a population into an isolated environment or that is not

its natural habitat can lead to a strong genetic structuring due to the lack of gene flow in addition to the strong genetic drift effect (FULLER et al., 2020).

Considering both hypothesized scenarios and their implications, the main objectives of this project were:

1.1. To investigate the existence of hybridization in an invasive population of *Leontopithecus* from the State Park of Serra da Tiririca, located in the region of Niteroi municipality (RJ);

1.2. To characterize the genetic diversity and structure of the invasive population of *Leontopithecus* in the State Park of Serra da Tiririca, located in the region of Niteroi municipality (RJ);

1.3. To infer on relevant aspects related to the rapid adaptation and expansion of this invasive population, with views to conservation proposes of the pure species *L. rosalia* and *L. chrysomelas*.

3. MATERIALS AND METHODS

3.1. Collection of biological material and DNA extraction

Biological samples from the invasive population of lion tamarins from the Serra da Tiririca, Niteroi, RJ (N=30) were collected in different sites as shown in Figure 1. We also collected biological samples from both pure species *L. chrysomelas* (N=34) and *L. rosalia* (N=19) from the Primatology Center of Rio de Janeiro (CPRJ), the Sao Paulo Zoological Park Foundation (FPZSP) and the wild, and captive hybrids (N=4) kept at Primatology Center of Rio de Janeiro (CPRJ). Information about the samples used in this study are available in details in Supplementary Table 1. Tissue and blood samples were stored in 1.5 ml tubes containing 70% alcohol and EDTA (3.6 mg), respectively, and kept at -20°C. Hair samples were conditioned in paper bags containing silica at room temperature. DNA extractions were performed following the phenol/chloroform protocol proposed by SAMBROOK; FRITSCH and MANIATIS (1983). Subsequently, the integrity and quantify of DNA was evaluated using a Nanodrop spectrophotometer

(NanoVue Plus, GE Healthcare, Chicago, United State), and a Qubit fluorometer (Life Technologies, Carlsbad, California, United State).

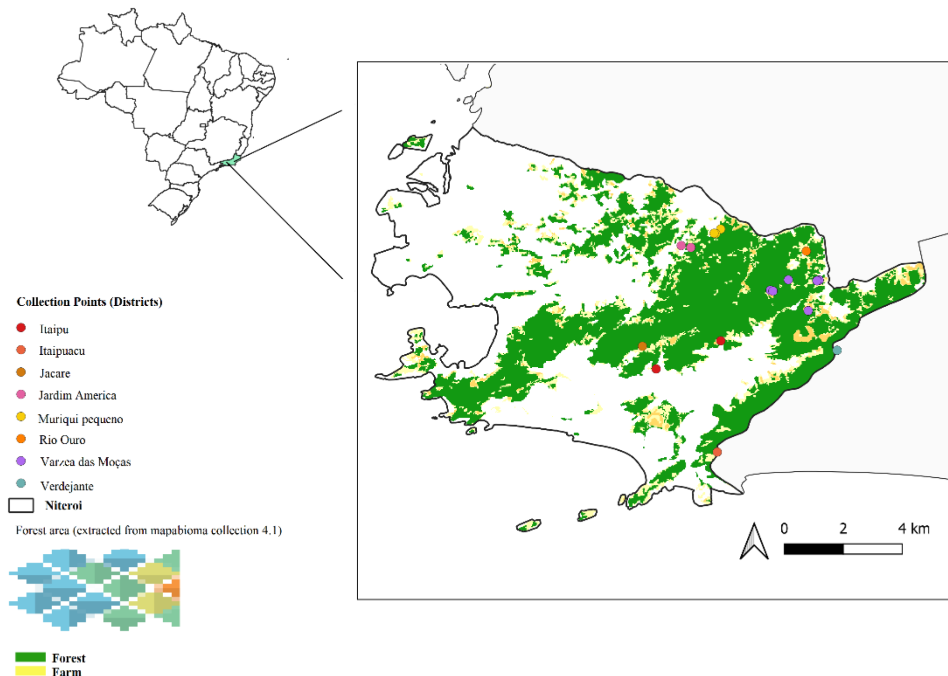

Figure 1. Geographic location of the Municipality of Niteroi in the state of Rio de Janeiro, and sites where the invasive population of *Leontopithecus* were found and biological samples were collected.

3.2. Ethical requirements

Biological collections followed the ethical and legal standards established by the Ethics Committee in Animal Use and Experimentation of the Federal University of São Carlos (CEUA-UFSCar, authorization number 7058110316), the Biodiversity Authorization and Information System (SISBIO-ICMBio, MMA, Federal Government, Brazil, authorization number 53201-1), and the "Code of best practices for field Primatology" (RILEY et al., 2014) from the International Primatology Society and the American Society of Primatology Steering Committee. The access to genetic patrimony was registered at National Genetic Heritage Management System (SisGen, number A411359).

3.3. Genetic analyses

3.3.1. Mitochondrial and Y-chromosome analyses

For determining the maternal contribution, Polymerase Chain Reactions (PCRs) were performed to amplify the mitochondrial gene of subunit I of the enzyme Cytochrome Oxidasse I (COI), using the primers described by (FOLMER et al., 1994). PCRs contained 2.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.2 mM dNTPs and 1.0 U of Taq and 1 µl of DNA at 50 ng, in a final volume of 10 µl. Amplification reactions were carried out on an Eppendorf Mastercycler Gradient® thermocycler (Eppendorf AG, Hamburg, Germany) under the following conditions: an initial denaturation cycle at 95 °C for 6', followed by 35 cycles of 94 °C at 1', 60 °C at 45" and 72 °C at 45" (FOLMER et al., 1994).

For paternal lineage determination, we amplified the SRY (sex-determining region Y) gene using the primer set described by (DI FIORE, 2005). PCRs were performed using 1.5 U of Taq polymerase, 1 uL of dNTP mix at 10mM, 1.5 mM MgCl2 at 25mM and 1 µl of 50 ng DNA in a final volume of 12 µl. The amplification reactions were carried out on an Eppendorf Mastercycler Gradient® thermal cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: an initial denaturation cycle 94°C for 2', followed by 40 cycles of 30" at 94°C, 30" at 58°C, and 30" at 72°C, with a final extension at 72°C for 10'.

PCR products for both markers were checked on a 1% and 3% agarose gel, stained with GelRed (Uniscience, ETX-35.M, Vilmer Lourmat, Collégien France), for the COI and SRY genes, respectively, and visualized with a UV light transilluminator (ETX-35.M, Vilmer Lourmat, Collégien France). PCR products were purified using the protocol of (LIS, 1980) and sequenced on an automatic Sequencer 3730XL (Applied Biosystems, Waltham, Massachusetts, USA). Sequence alignments were performed using the Clustal W tool available in the Geneious software (KEARSE et al., 2012).

3.3.2. Microsatellite analyses

Microsatellite amplifications were performed for four loci described for the species *L. chrysopygus* (PEREZ-SWEENEY et al., 2005), and seven described for *L. chrysomelas* (GALBUSERA; GILLEMOT, 2008) (Table 1). Amplification reactions were performed following the recommendations proposed by (AYALA-BURBANO et al., 2017) and (SCHUELKE, 2000). PCRs were carried out using 1x GoTaq® Promega (Madison, Wisconsin, USA), 1x Buffer, 0.46 pmol of the reverse and M13 primers, 0.12 pmol of the forward primer, 0.30 mg/mL of BSA, 0.75 mM of $MgCl_2$, 1 uL at 50 ng of DNA, 0.25 mM of each dNTPs and 3.85 µL of milli-Q water, in a final reaction volume of 10 µL. Reaction conditions included a denaturation step at 94° for 5', followed by 30 cycles of 30" at 94°C, a locus-specific alignment gradient for 45" and eight additional cycles, distributed as follows: 30" at 94°C, 45" at 53°C (alignment temperature of the marked M13 tail), 45" at 72°C, and finally a final extension step at 72°C for 10'. PCR products were checked on a 2% agarose gel, stained with GelRed (Uniscience), and visualized with a UV light transilluminator (ETX-35.M, Vilmer Lourmat, Collégien France).

Table 1. Information on the microsatellite loci, which successfully amplified, used in this study. Presented for each locus: name of the primer, its sequences (Forward: F and Reverse: R), the fluorophore and the alignment temperature (Tº C) used in this study. Lchu-specific for *L. chrysomelas*; Leon-specific for *L. chrysopygus*.

	Loco	Primer sequences (5'-3')	Motif	Fluorophore	T°C
L. chrysomelas	*Lchu1*	F: GCTCAGGTGTTATTTATGTCCAAA R: GTTTCTTGCAACTATCTTGCATGTTCTGC	Tetra	NED	58°C
	Lchu3	F: AAGGCATGATGTATCTTGTTCTCA R: GTTTCTTATCTTTCTGTATGTGTCTCCCTGTCT	Tetra	NED	58°C
	Lchu4	F: TGACCAAAGAAAATGCAAAA R: GTTTCTTGCACAGGGTATTTAGCAGGA	Tetra	NED/PET	55°C
	Lchu5	F: TGATGCTAAAACAGAAGCATTT R: GTTTCTTGTCCTGATGTTCACAAAACCT	Tetra	FAM	55°C
	Lchu6	F: GCCTTAATTAGCACCAGAACC R: GTTTCTTACCACTCCAAGCCTTCAGTA	Di	FAM	55°C
	Lchu8	F: CACGGCAATGTGGGAATAA R: GTTTCTTTTCAGTAGTTGGGACTGGGATAA	Di	PET	58°C
	Lchu9	F: TTCATTGTAGCATTGTTGGTCAT R: GTTTCTTTTGCCTCCTCATAGTTCCTCAT	Di	PET	58°C
L. chrysopygus	*Leon2*	F: CTGCTTCTTGTTCCACTTCTTCTC R: GTTTGGGTGGTTGCCAAG	Di	FAM	55°C
	Leon21	F: CAGTTGAGGGAACAGGAATTA R: CACTGCACTGACAGAGCAAG	Di	VIC	60°C
	Leon27	F: AAGCGCAGATTTATTGATAGG R: TGCAGGTAAATGATGGTAATG	Di	NED	60°C
	Leon30	F: GGACCTGATTGAAGCAGTC R: TTCCCTGAGAATCTAATGGAG	Di	VIC	60°C

PCR products were genotyped on an automated sequencer 3730XL (Applied Biosystems, Waltham, Massachusetts, USA), and, subsequently, electropherograms were analysed in Geneious (KEARSE et al., 2012) for determination of alleles and genotypes. The occurrence of null alleles, dropout alleles, and stuttering was checked in the Micro-Checker program (VAN OOSTERHOUT et al., 2004). The polymorphic information content (PIC) of the loci was estimated with Cervus 3.0.3 (KALINOWSKI; TAPER; MARSHALL, 2007). Determination of allele number (A_N) and expected (H_E) and observed (H_O) heterozygosity was performed using GenAlex 6.3 (PEAKALL; SMOUSE, 2006). The allelic richness (A_R) and the inbreeding coefficients (F_{IS}) were determined using FSTAT 2.9.3.2 (GOUDET, 1995) and p-values for the excess (PL) and deficit (PS) of heterozygotes, and deviations from the Hardy-Weinberg Equilibrium between pairs of loci were tested with Genepop (RAYMOND; ROUSSET, 1995).

Structure analyses were performed in Structure 2.1 (PRITCHARD; STEPHENS; DONNELLY, 2000). To assess population structure, five replicates were considered for each run, for k=1-6, using the admixture model with 200,000 MCMC interactions and 40,000 burnin. The number of most likely genetic groups (k) was determined by Structure Haverst (EARL; VONHOLDT, 2012).

The Bottleneck software (PIRY; LUIKART; CORNUET, 1999a) was used to infer on bottleneck events by comparing the levels of excess of heterozygosity (He) related to expected equilibrium heterozygosity (Heq) (LUIKART; CORNUET, 1998; PIRY; LUIKART; CORNUET, 1999b). Since He is calculated from the allele frequency and the Heq from the allele number, it is expected that populations in recent bottleneck have an excess of heterozygosity (He>Heq), as in this process the allele number is reduced faster than heterozygosity (PIRY; LUIKART; CORNUET, 1999b). We considered three mutational equilibrium models: infinite allele model (IAM), two stepwise model (TPM), and stepwise mutation model (SMM), with 95% of mutation assumed for the TPM model and a variance of 12%. Significance was assessed from the Wilcoxon one-tailed rank test. The bottleneck event was only considered if both models, IAM and SMM, were significant. For corroboration, the L-shape graph was used considering 10 allele frequency classes (from 0.0–0.1 to 0.9–1.0), in which the category 0.0-0.1 has low frequency, 0.9-1.0 has high frequency, and the other categories has intermediate frequencies. Recent bottlenecks are considered if few proportions of alleles are in low

frequency allele class compared to one or more of the other classes, according to (LUIKART et al., 1998).

A predictive analysis for evaluating changes in A_N, H_E and H_O in the next 100 years were performed with Bottlesim 2.6 (KUO; JANZEN, 2003), using the allele frequencies and the calculated effective population size (Ne), and considering bottleneck scenarios for population reductions of 80%, 50% and 30%. The analyses were carried out with 1,000 iterations, considering complete overlapping of generations, dioecious reproduction, and sex ratio 1:1. We also assume that *L. chrysomelas* has a lifespan of 16 years (HOLST et al., 2006) and that it reaches sexual maturity at 2 years of age (CHAOUI; HASLER-GALLUSSER, 1999; RABOY; CANALE, 2008).

For hybridization inferences based on microsatellites we used the software EBhybrids (MOLTKE; STEPHENS, 2015). The dropout allele ratio obtained from MicroDrop (WANG; SCHROEDER; ROSENBERG, 2012) application in R (R Development Core Team 2020), and the identity probability obtained from the Structure software (PRITCHARD; STEPHENS; DONNELLY, 2000) were used as reference to check the following scenarios: (Pure1) offspring from *L. rosalia*, (Pure2) offspring from *L. chrysomelas*, (F1) offspring from *L. rosalia* and *L. chrysomelas*, (F2) offspring from F1's hybrids, (Bx1) offspring from backcross between *L. rosalia* and F1, and (Bx2) offspring from backcross between *L. chrysomelas* and F1.

3.4. Genomic analysis

3.4.1. Genotyping by Sequencing (GBS)

The preparation of genomic libraries was performed following the protocol suggested by (ELSHIRE et al., 2011). Briefly, 200 ng of each DNA sample were normalized to a concentration of 5ng/ul using a Qubit fluorometer (Life Technologies, Carlsbad, California, United State) and then digested with the enzyme Pstl (New England Biolabs, Ipswich, Massachusetts, United State). Subsequently, ligation reactions were carried out for binding of adapters to each DNA sample. Afterwards, a pool of the samples was purified with magnetic beads (Agencourt AMPure XP- BECKMAN COULTER) and amplified. After restriction and ligation reactions, libraries were selected for fragment

sizes between 200 to 450 bp. The library obtained was quantified in a real-time PCR using the KAPA Biosystems Quantification Kit (Illumina, San Diego, CA, USA). The quality of the libraries was evaluated on an Agilent 2100 BioAnalyzer (Agilent Technology, Santa Clara, CA, USA) using the High Sensitivity DNA Kit (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). Final quantification of the library was performed by another real-time PCR using the KAPA Biosystems Quantification Kit (Illumina, San Diego, CA, USA) and samples were diluted to 2pM. Finally, sequencing was performed, using an Illumina HiSeq2500 Platform (Illumina, San Diego, CA, USA).

3.4.2. Processing GBS data

Demultiplexing, quality filtering and clustering analyses were performed using the API (iPyRAD analysis tools) version of the iPyRAD 0.7.24 (EATON; OVERCAST, 2020) on a computational cluster of the "Laboratorio de Bioinformática y Genómica de la Biodiversidad", Universidad Nacional Mayor de San Marcos (UNMSM, Peru), following the seven main steps described as follows:

Step 1. Demultiplexing was performed to individualize the samples according to the combination of indexes (barcodes) used. After the individualization of each sample, it was possible to evaluate the quantity of reads generated per sample.

Step 2. Adapter and barcode sequences were trimmed, and reads were filtered for Phred quality Qscore<20. Only reads larger than 35 bp were retained.

Steps 3, 4, 5 and 6. The sequences were aligned with the reference genome of *L. chrysopygus* (unpublished data). Then, reads were pooled using a 90% sequence similarity threshold within and between samples to identify nucleotide polymorphisms (Step 3, 4 and 5) and orthologous loci (Step 6), with a minimum coverage of 6X reads per locus.

Step 7. The replication with the highest number of loci after final filtering was retained. Samples yielding over 200,000 raw sequencing reads and over 10X read depth were not removed. Missing data for 29.03% of the loci assembled in the samples were filtered out.

Finally, quality control and final filtering of the data was performed in the R package r2vcftools (https://github.com/nspope/r2vcftools). We searched for

heterozygosity excess based on deviations from Hardy-Weinberg equilibrium (HWE, $p < 0.0001$), an overall minor allele frequency (MAF) of 0.05 %, and linkage disequilibrium (LD, $r2 < 0.8$), conserving SNP loci found in at least one individual in all groups.

3.4.3. Detection of putative neutral and outliers SNPs and datasets

For the identification of putative neutral and outliers loci, we first performed a Principal Component Analysis (PCA), based on Mahalanobis distance with PCAdapt 4.3.3 (LUU; BAZIN; BLUM, 2017) in R (R Development Core Team 2020). Mahalanobis distance was calculated for each biallelic SNP. Scores that did not fall within the distribution of the bulk of the distance points were assigned as outliers (LUU; BAZIN; BLUM, 2017). To identify the best supported genetic clustering, 10 principal components (PC) were used, and the best PC number was identified using Cattell's graphical rule (LUU; BAZIN; BLUM, 2017). Outliers were determined according to false discovery rate (FDR) of 0.05, with the R package q-value (STOREY; TIBSHIRANI, 2003).

Loci with overall minor allele frequency ($MAF < 0.05$) were excluded. PCAdapt outliers were scored based on *q-value-corrected p values*, with K set to 3. Results obtained from PCAdapt were used to identify outliers and neutral dataset, which was subsequently used for genetic diversity and population structuring analyses.

3.4.4. Population structure

Based on the PCAdapt results, we used one dataset of neutral SNPs for the genetic diversity and population structure analyses. To assess the population structure, we used the Adegenet package in R (R Development Core Team 2020), and performed a multivariate approach based on the Discriminant Analysis of Principal Components (DAPC) (JOMBART, 2008), without consider the sampling areas as priors for populations. To determine the best number of PCs to retain, we used the alpha score optimization considering PCs accounting for 80% of variance. The Bayesian Information Criterion (BIC) was used to determine the best number of discriminant components.

Subsequently, we estimated the individual ancestry and population clusters through a non-negative matrix factorization (SNMF) and the least squares optimization

procedure of the ancestry coefficients by using the function *snmf* in R package LEA (FRICHOT; FRANÇOIS, 2015). The function *snmf* was used with 100 repetitions per k values, that ranged from 1 to 10. To determine the best k value to explain the results, the cross-entropy criterion was adopted.

3.4.5. Genetic diversity

To estimate the genetic diversity for our genomic dataset, we used the R package (GOUDET, 2005), following the genetic structure assigned by the DAPC and SNMF analyses. We calculated the observed (H_O) and expected (H_E) heterozygosity, F_{IS} values and the overall F_{ST}, using the *basic.stats* function. To calculate the intervals of confidence for the population-specific F_{IS} we used the *boot.ppfis* function, with 1000 bootstrap replicates. The pairwise F_{ST} was calculated using the *genet.dist* function in the R package HIERFSTAT, following (WEIR; COCKERHAM, 1984).

To obtain the significance values we used the *pwfst* function of R package with 1,000 bootstrap replications (GRUBER et al., 2018). The effective population size (N_E) was estimated in NEESTIMATOR 2.1 (DO et al., 2014), using the heterozygosity excess method and the lowest allele frequency value of 0.0.

4. RESULTS

4.1. COI and SRY results

The PCRs showed success in the amplification of the COI and SRY regions, which generated fragments of approximately 577 bp and 180 bp, respectively, after sequencing and editing of the purified products. The alignment of the sequences showed that all Niteroi samples evaluated for the COI gene are from *L. chrysomelas*, indicating the maternal lineage contribution (Figure 2). However, the SRY gene evidenced monomorphism for the parental species (Figure 3), so it was not possible to identify the probable paternal lineage with this marker.

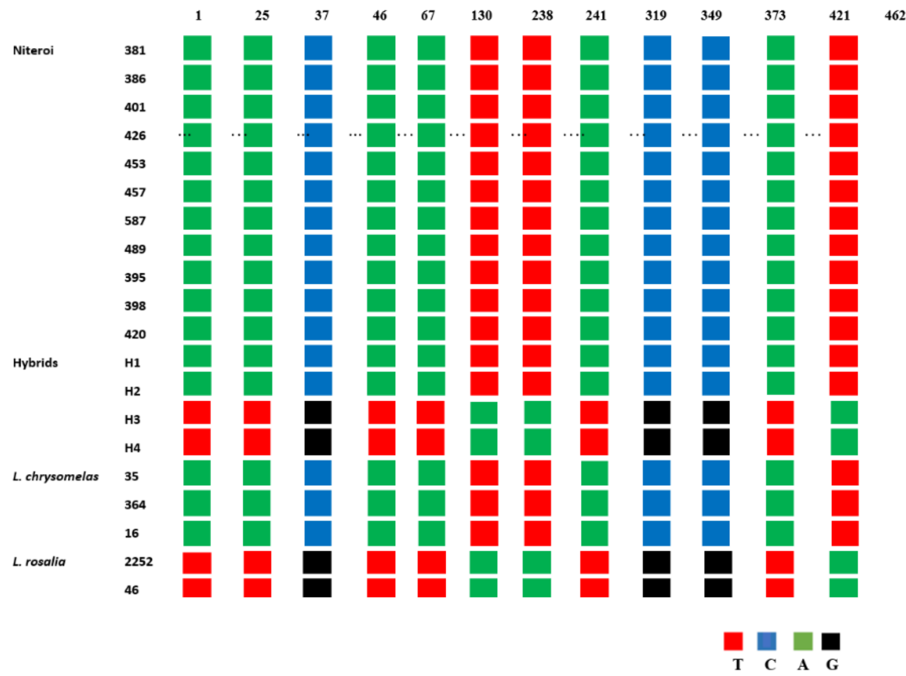

Figure 2. Mitochondrial COI gene alignment for three individuals of *Leontopithecus chrysomelas* (35, 364, 16), two of *Leontopithecus rosalia* (2252 and 46), 11 of Niteroi population (381, 386, 395, 398, 401, 420, 426, 453, 457, 489 and 587) and four of hybrids between the two pure species obtained from the Primatology Ceanter of Rio de Janeiro (H1, H2, H3, H4).

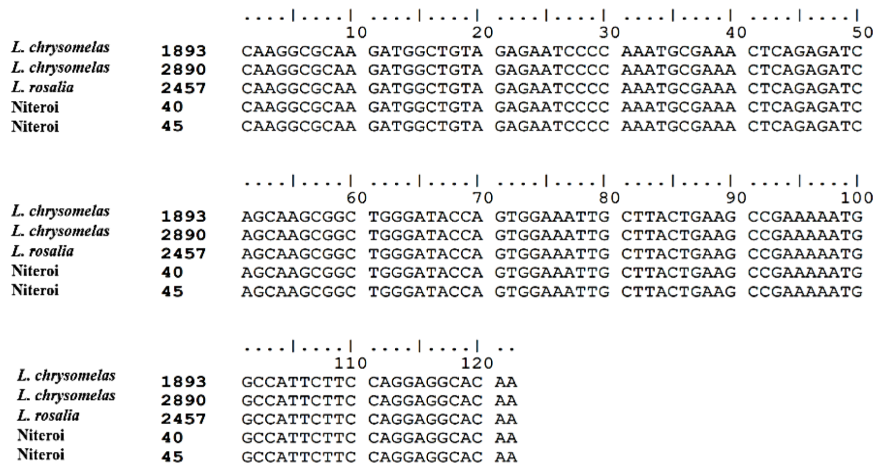

Figure 3. SRY gene alignment showing monomorphism for two individuals of *Leontopithecus chrysomelas* (1893 and 2890), one of *Leontopithecus rosalia* (2457), and two of the Niteroi population (40 and 45).

4.2. Results for microsatellite markers

For the microsatellite analyses we considered 74 individuals from Niteroi population (N=27), *L. chrysomelas* (N=26), *L. rosalia* (N=18), and captive hybrids (N=3). The PIC values ranged from 0.471 to 0.837, with a mean of 0.653, evidencing that all the loci used were highly informative. Null alleles due to excess homozygosity were detected at loci Lchu3, Lchu9 and Leon2, and stuttering was detected at Leon2 for the Niteroi population. Null alleles due to excess heterozygosity at loci Lchu1 for the *L.chrysomelas,* and null alleles due to excess heterozygosity at loci Lchu3 and Lchu6, and stuttering at loci Lchu3 and Leon27 for *L. rosalia* were also observed.

4.2.1. Structure and hybridization analyses

The structure analysis evidenced two main genetic groups (k=2) as the best result, according to Harverest Structure, grouping *L. chrysomelas* together with Niteroi (Figure 4A). For k=3 (Figure 4B), we observed three groups as follows: (1) Niteroi, (2) *L. chrysomelas* and (3) *L. rosalia.* When we evaluated *L. chrysomelas* and Niteroi only, the structure analysis evidenced two groups (Figure 5A). Also, the structure analysis within Niteroi population showed the existence of two distinct genetic groups (Figure 6).

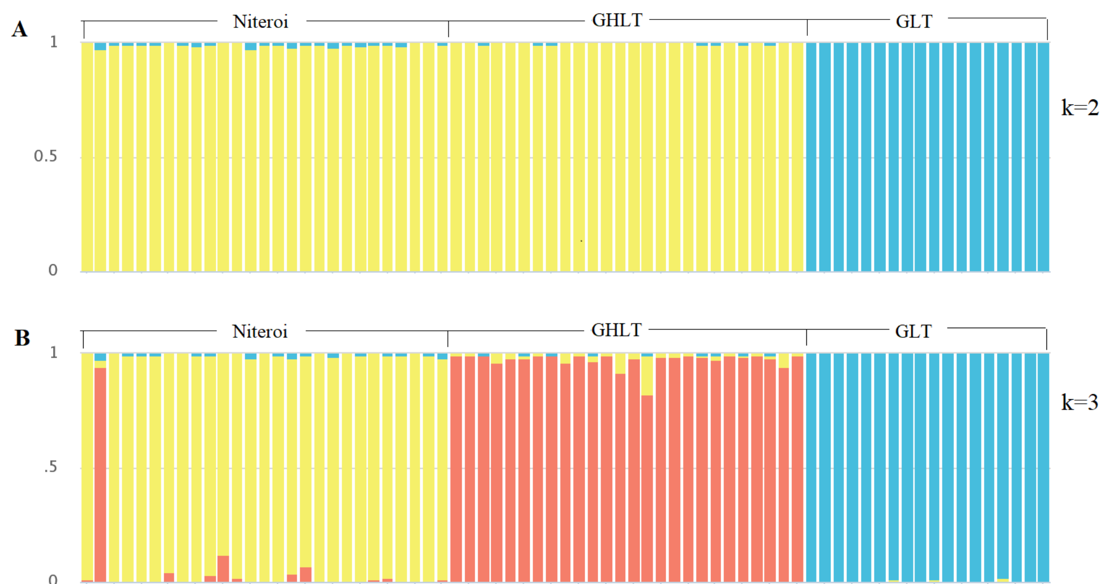

Figure 4. Structure results for individuals of the invasive population of Niteroi, of pure *Leontopithecus chrysomelas* (GHLT) and pure *Lentopithecus rosalia* (GLT), using microsatellites data, considering k=2 (A) and k=3 (B).

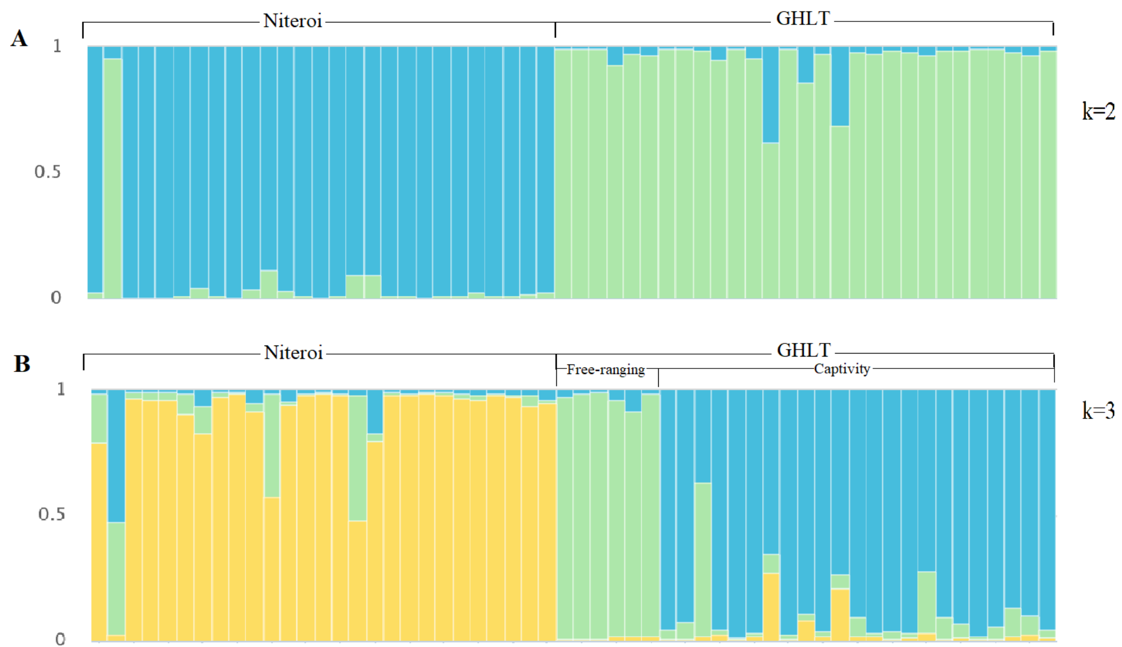

Figure 5. Structure results for individuals of the invasive population of Niteroi and of pure *Leontopithecus chrysomelas* (GHLT), using microsatellites data, considering k=2 (A) and k=3 (B).

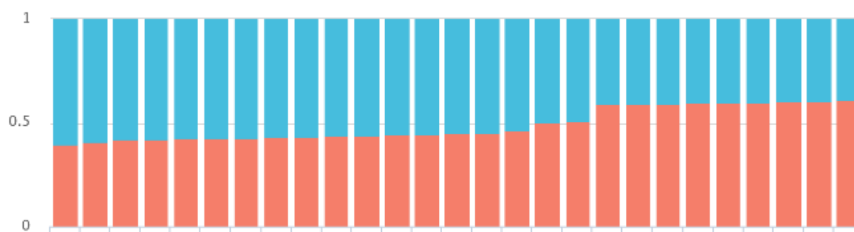

Figure 6. Structure results for lion tamarins of the invasive population of Niteroi, using microsatellite data, considering k=2

The F_{ST} value was 0.0900 for *L. chrysomelas* (GHLT) and Niteroi population, and 0.3427 for *L. rosalia* (GLT) and Niteroi (Figure 7). The F_{ST} value for the two genetic groups evidenced by the structure analyses in the Niteroi population was 0.0630, pointing out to a low genetic differentiation. The posterior probability analysis identified the individuals of the Niteroi population as pure *L. chrysomelas* (Figure 8, Supplementary Table 2).

	Niteroi	GHLT	GLT
GHLT	0.0990		
GLT	0.3427	0.2992	
Captive Hybrids	0.2196	0.1942	0.2199

Figure 7. F_{ST} value based on microsatellite data for individuals of the Niteroi population, *Leontopithecus chrysomelas* (GHLT), *Leontopithecus rosalia* (GLT), and captive hybrids.

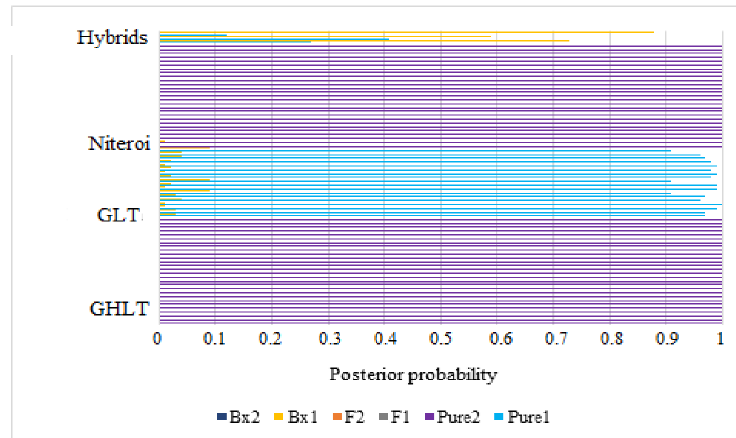

Figure 8. Posterior probability analysis based on microsatellite data for individuals of Niteroi (Niteroi), *Leontopithecus chrysomelas* (GHLT), *Leontopithecus rosalia* (GLT) and captive hybrids (Hybrids). Horizontal line represents each individual analysed. Pure1: offspring from *L. rosalia*, Pure2: offspring from *L. chrysomelas*, F1: offspring from *L. rosalia* and *L. chrysomelas*, F2: offspring from F1's hybrids, Bx1: offspring from backcross between *L. rosalia* and F1, and Bx2: offspring from backcross between *L. chrysomelas* and F1.

4.2.2. Genetic diversity for Niteroi population

The diversity analysis evidenced values for the allele number (A_N) ranged between 2 and 10 per locus, with the mean A_N equal to 4.364. The effective alleles number ($A_{N(E)}$) ranged between 1.117 and 6.227, and the mean $A_{N(E)}$ was 2.829. The allelic richness (A_R) ranged from 2 to 9.217, and the mean A_R was 4.0. The mean expected heterozygosity (H_E) was 0.525 and the mean observed heterozygosity (H_O) was 0.474. The endogamy coefficient (F_{IS}) was 0.118. Significant p-values for Hardy-Weinberg Equilibrium were observed in only three loci analyzed (Table 2). The bottleneck analyses showed significant p-value for heterozygosity excess only for the infinite allele model (IAM) (Table 3). The L-shape distribution evidenced a high proportion of alleles for the category of low frequency alleles (0.0-0.1) (Figure 10).

Table 2. Genetic diversity estimates based on microsatellite data for Niteroi population. N: sample number; A_N: number of alleles; $A_{N(E)}$: effective number of alleles, A_R: allelic richness; H_O: observed heterozygosity, H_E: expected heterozygosity, F_{IS}: Inbreeding coefficient due to deviation from Hardy-Weinberg, P_{DH}: p-values for the deficit of heterozygotes for the inbreeding coefficient $F_{IS;}$ P_{EH}: p-values for the excess of heterozygotes for the inbreeding coefficient F_{IS}, P_{HWE}: p-value for the Hardy Weinberg equilibrium; PIC: Polymorphic Information Content. *Statistically significant values.

Locus	N	A_N	$A_{N(E)}$	A_R	H_O	H_E	P_{HWE}	Fis	P_{DH}	P_{EH}	PIC
Lchu1	27	2,000	2,000	2.000	0,481	0,500	1,0000	0,056	0,5412	0,7487	0,478
Lchu3	24	7,000	6,227	6.986	0,625	0,839	0,0000*	0,275	0,0150	0,9850	0,828
Lchu4	20	2,000	1,600	2.000	0,400	0,375	1,0000	-0,041	0,7769	0,6820	0,471
Lchu5	25	4,000	2,189	3.720	0,560	0,543	0,0191	-0,011	0,4479	0,6397	0,778
Lchu6	27	3,000	2,976	3.000	0,778	0,664	0,1544	-0,153	0,9061	0,0972	0,691
Lchu8	26	4,000	2,805	3.692	0,731	0,643	0,6451	-0,116	0,7587	0,2692	0,707
Lchu9	21	2,000	1,208	2.000	0,000	0,172	0,0022*	1,000	0,0022*	1,0000	0,500
Leon2	25	7,000	4,153	6.421	0,480	0,759	0,0002*	0,385	0,0003*	1,0000	0,767
Leon21	21	5,000	1,581	4.712	0,381	0,367	0,1355	-0,013	0,6676	0,6968	0,654
Leon27	18	2,000	1,117	2.000	0,000	0,105	0,0287	1,000	0,0287	1,0000	0,473
Leon30	23	10,000	5,264	9.217	0,783	0,810	0,0372	0,056	0,2600	0,7513	0,837
	23,364	4,364	2,829	4	0,474	0,525		0,118			0,653

Table 3. Tests of within-population heterozygosity excess performed using BOTTLENECK. Probability values were determined using one-tailed Wilcoxon tests. IAM: Infinite alleles model, TPM: Two phase model, and SMM: Stepwise mutation model.

	Heterozygosity excess p-values		
Mutacional modelo	**IAM**	**TPM**	**SMM**
Niteroi Population	0,00073*	0,25977	0,41553

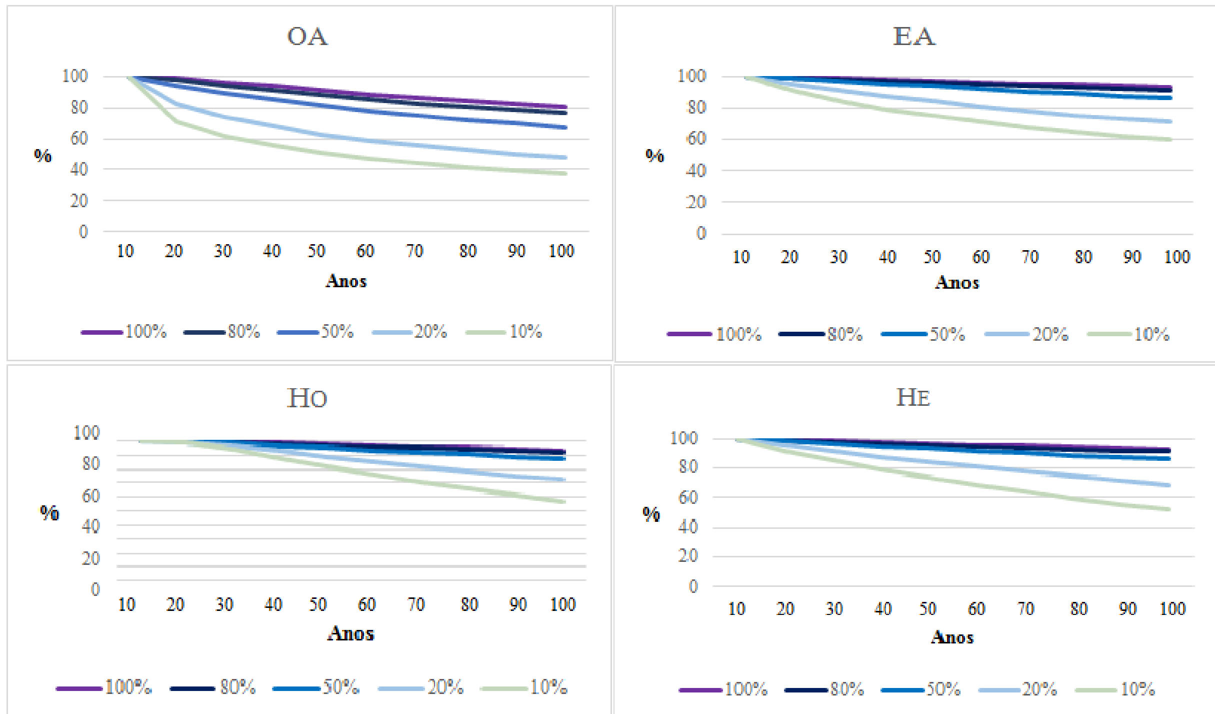

Figure 9. Prediction analysis using microsatellite data for estimatives of observed allele number (OA), effective allele number (EA), expected heterozygosity (H_E) and observed heterozigosity (H_O) reductions in Leontopithecus chrysomelas populations from Niteroi region over 100 years, using the 100%, 80%, 50% and 30% of effective population sizes (Ne=111).

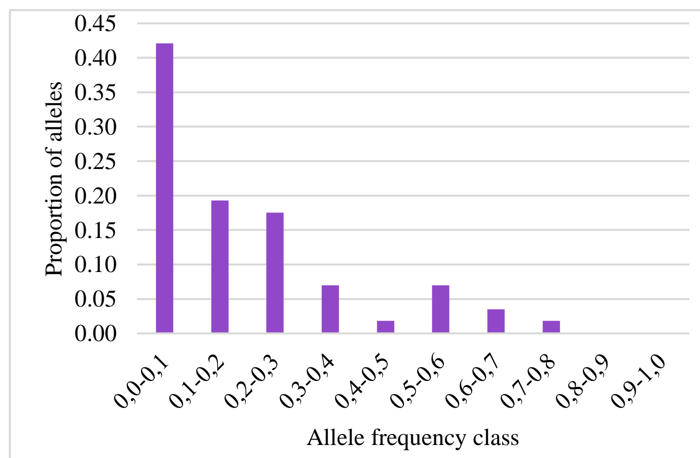

Figure 10. L-Shaped Distribution Graph used for detecting bottleneck based on microsatellite data for *Leontopithecus chrysomelas* from Niteroi.

4.3. Results from SNPs markers

The genomic library sequencing resulted in a total of 242.250.159 raw reads produced for 50 individuals, with 32 of the Niteroi population, 12 of captive and wild *L. rosalia*, three of captive *L. chrysomelas*, and three of captive hybrids between *L. rosalia* and *L. chrysomelas*. The raw data were mapped with the *L. chrysopygus* genome and filtered for low-quality data. After filtering, 40 samples (27 of Niteroi, seven of *L. rosalia*, three of *L. chrysomelas* and three of captive hybrids) were retained for further genomic analysis. A total of 16,9103 SNPs were identified for these samples. The quality pre-filtering procedure retained a total of 12,624 SNPs. After filtering, based on missing data (>70%), depth (6-1000), global minor allele frequency (MAF 0.8) and observed heterozygosity, we recovered 5,111 SNPs.

PCAdapt, according to the Cattell's graphical, showed that most variation was accounted for k=5, with the main proportion of variance explained for the two first PCs (Supplementary Figure 1). The distribution of the p-values was visualized with a Manhattan plot and a QQplot, and then these values were used to compute the q-values (Supplementary Figure 2). After FDR adjustment at 0.05, 857 SNPs were identified as outliers (Supplementary Figure 3). Thus, we considered four datasets for the subsequent genetic diversity and structure analyses.

The structure analysis using 4,254 neutral SNPs evidenced two main genetic groups (k=2), based on the entropy analysis, as follows: (1) *L. rosalia* (GLT) together with two confirmed hybrids with maternal contribution from *L. rosalia*, and (2) *L. chrysomelas* (GHLT) together with all individuals from Niteroi and one confirmed hybrid with maternal contribution from *L. chrysomelas* (Figure 11A). All analysed individuals from Niteroi showed a higher probability of belonging to the group of the pure *L. chrysomelas,* indicating that this population is not constituted by hybrids. The F_{ST} analysis confirmed a greater similarity between *L. chrysomelas* and individuals of the Niteroi population (F_{ST}=0.106), in addition to the higher differentiation between *L. rosalia* and individuals of the Niteroi population (F_{ST}=0.379). The captive hybrids showed moderate differentiation (F_{ST}=0.181) with *L. chrysomelas,* and higher differentiation (F_{ST}=0.451) with *L. rosalia* (Figure 13). For k=3, the structure evidenced Niteroi population as a single genetic cluster, showing little ancestry proportion from *L. chrysomelas* and *L. rosalia*; *L. rosalia* grouped to one confirmed hybrid with maternal contribution from *L. rosalia*; and

L. chrysomelas grouped to two confirmed hybrids, one of them with maternal contribution from *L. chrysomelas* and the other from *L. rosalia.*

When we analysed pure *L. chrysomelas* and Niteroi population samples only, the structure results evidenced population differentiation (Figure 14), since the k=2 was the most probable based on the entropy analysis. By analysing the population of Niteroi only, the entropy analysis also showed that the most probable k value is two, evidencing a sub-structuring in this population as well (Figure 15).

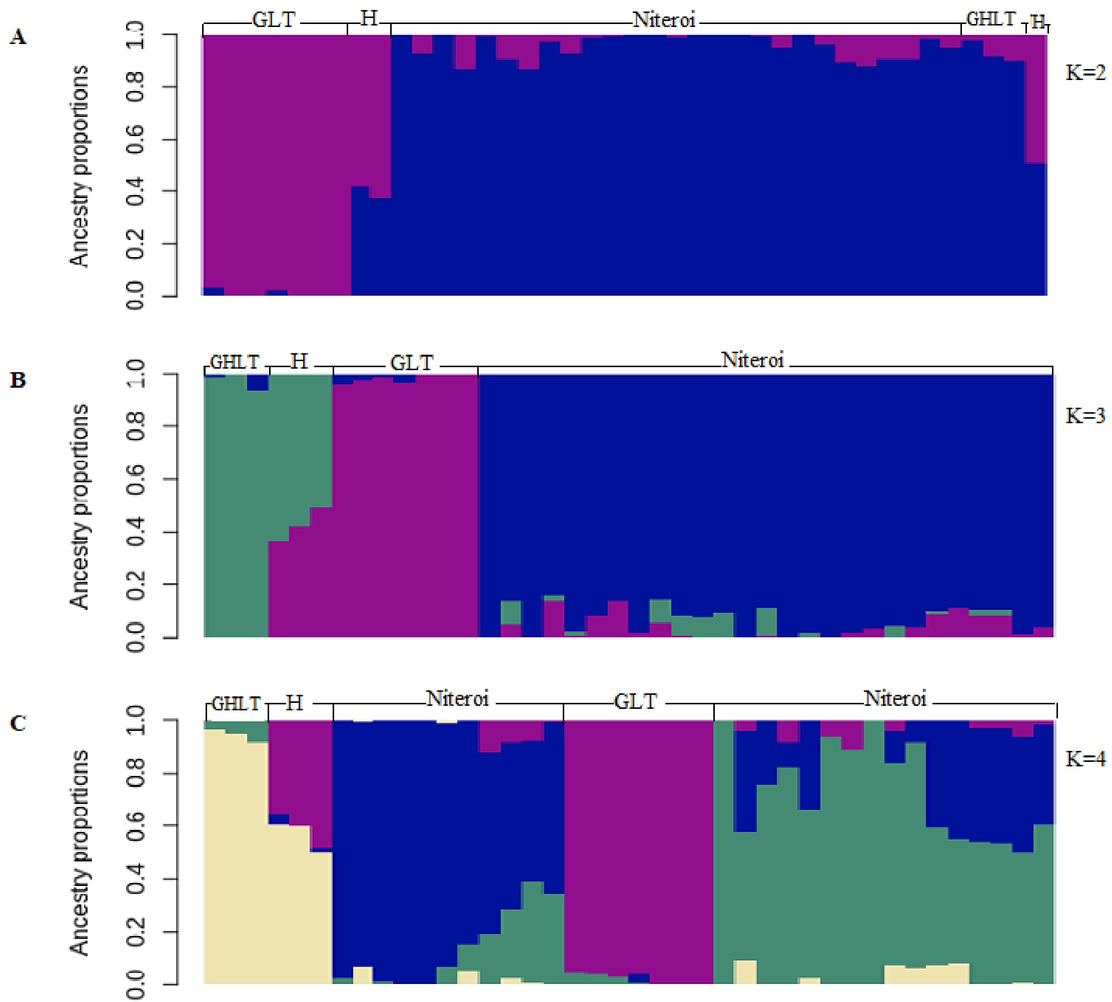

Figure 11. Structure analysis based on SNP data for *Leontopithecus chrysomelas*, *Leotopithecus rosalia*, captive hybrids from Primatology Center of Rio de Janeiro, and individuals from Niteroi population. GLT: *L. rosalia*, GHLT: *L. chrysomelas,* H: Hybrids from captivity, and Niteroi: individuals from Niteroi population.

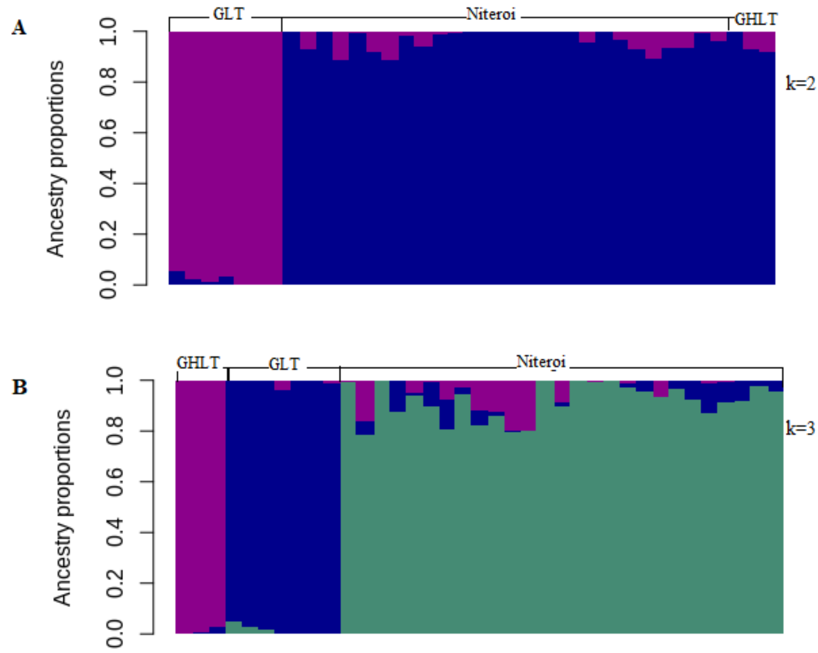

Figure 12. Structure analyses using SNP data obtained for pure *Leontopithecus chrysomelas*, *Leontopithecus rosalia*, and individuals from Niteroi population. GLT: *L. rosalia,* GHLT: *L. chrysomelas,* and Niteroi: individuals from Niteroi population.

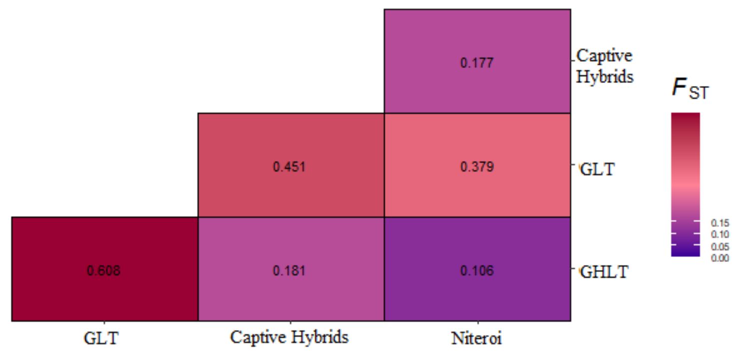

Figure 13. Fixation Index (F_{ST}) value based on SNP data for pure *Lentopithecus chrysomelas* (GHLT), pure *Leontopithecus rosalia* (GLT), individuals from Niteroi population (Niteroi), and captive hybrids.

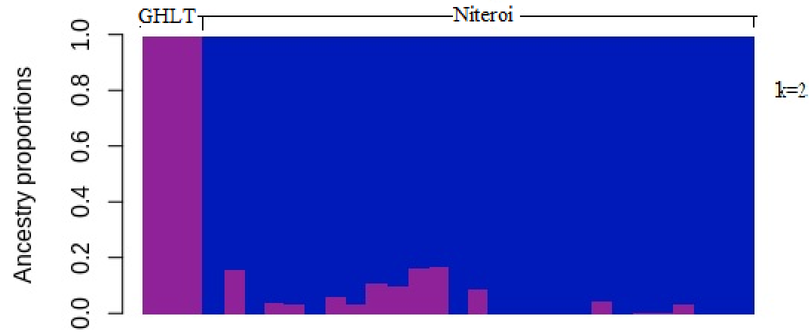

Figure 14. Structure analysis based on SNP data for *Leontopithecus chrysomelas* and individuals from Niteroi region showing the k=2. GHLT: *L. chrysomelas*, Niteroi: individuals from Niteroi.

Figure 15. Structure analysis based on SNP data for *Leontopithecus chrysomelas* from Niteroi population showing the k=2.

The genomic analysis for the SNPs identified in the invasive population of Niteroi evidenced values of observed heterozygosity and expected heterozygosity equal to 0.2497 and 0.2549, respectively. The total number of alleles was 8,760 and the allelic richness was 1,712. The effective population size (Ne) was 34 and the inbreeding coefficient (F_{IS}) was 0.0203.

5. DISCUSSION

Both genetic and genomic approaches employed in this study did not raise evidence of recent hybridization for the Niteroi's invasive population, based on the analyzed samples, suggesting that this population is composed by pure *L. chrysomelas*. The structure results for the pure species and individuals from Niteroi evidenced *L. chrysomelas* and Niteroi population in a single cluster different from the *L. rosalia* cluster. The fixation index obtained for both microsatellite (SSR) and SNP loci also pointed to a clear differentiation between *L. rosalia* and Niteroi population ($F_{ST\text{-}SSR}$=0.3427; $F_{ST\text{-}SNP}$=0.379), while for *L. chrysomelas* and Niteroi population the difference is pretty lower ($F_{ST\text{-}SSR}$=0.0900; $F_{ST\text{-}SNP}$=0.106), confirming the structure results. In addition, the posterior probability analysis performed using microsatellites indicated that all analyzed individuals from Niteroi have 100% of posterior probability of being *L. chrysomelas*, evidencing no signal of recent hybridization between *L. chrysomelas* and *L. rosalia.* Therefore, according to our analysis, we did not detect any hybrids or pure *L. rosalia*.

When we characterized the genetic structure for individuals from Niteroi and *L. chrysomelas* from captive and wild populations, we found a clear differentiation between *L. chrysomelas* from Niteroi and from other *L. chrysomelas* populations (see Figure 5 and 14). These results highlight population structure signs probably related to their respective distinct origins. In addition, we also detected a slight sub-structuring within the Niteroi population, based on both microsatellite and SNP analyses. Despite the unknown origin of the founder individuals of the invasive population, the initial founder effect and subsequent random events of genetic drift (BRAMBILLA et al., 2014; KRATZER et al., 2020; LE CORRE; KREMER, 1998) may have traced the current differentiation with other *L. chrysomelas* populations, but also within the population of Niteroi. It is well known that genetic differentiation depends on the degree of bottleneck events, the time elapsed since the population foundation (NEI; MARUYAMA; CHAKRABORTY, 1975), and the gene flow between populations (WRIGHT, 1931; LE CORRE; KREMER, 1998).

Regarding the absence of *L. rosalia*, it is difficult to infer about what happened in terms of viability and reproduction of the pure introduced GLTs. However, if the GLT couple left pure descendants, they are no longer in the region, as no animals with the *L. rosalia* phenotype were also observed or captured during the rescue of the lion tamarins

from Niteroi, that started in 2011 and in the subsequent years, nor during the monitoring of this invasive population to date. In this sense, *L. chrysomelas* may have replaced *L. rosalia* during the demographic expansion of the invasive population in that region. Such phenomenon has been commonly observed when native and/or alien species compete for resources and habitat (ALLENDORF; FLATHEAD; AITKEN, 2013). Of note, if there is pure GLTs in Niteroi they were not sampled at all. However, this is a very implausible scenario, since the monitoring of the invasive population has been careful and intensive over the years and our samples represented the different locations where the invasive *Leontopithecus* were found in Niteroi (KIERULFF, 2015; KIERULFF; INSTITUTO PRI-MATAS, 2012), and all of them had the *L. chrysomelas* phenotype.

Regarding the sub-structuring within the Niteroi population, this may be related to the founders and further population dynamic related to landscape aspects intrinsic to the Niteroi area. When we plot the individuals of the two genetic groups in the Niteroi map, according to their collection sites, we observe a much higher frequency of individuals belonging to just one genetic cluster (pink) in a geographic area located southwest of the RJ-108 road, since only one individual from the 12 collected on the right margin is likely to be assigned to the genetic group highlighted in yellow (Figure 16). On the other hand, we found both genetic subgroups on the left side. For lion tamarins, it is already reported that *L. rosalia* rarely crosses the width of a road (MICKELBERG, 2011). In addition, genetic and landscape studies showed that the genetic structure of *L. rosalia* populations is probably related to the landscape resistance (MORAES et al., 2018b). However, it is very difficult to explore putative scenarios for a plausible barrier to dispersion of *L. chrysomelas* from Niteroi since we do not have any information on the origin or genetic basis of the founders and on the places where the invasive species was first introduced. Nevertheless, it is noteworthy the F_{ST} value (0.0630) indicate low differentiation, suggesting an overall connectivity along the landscape of the Niteroi region, despite the presence of the RJ-108 road and the slight sub-structuring.

According to gene flow simulation studies in *Leontopithecus* spp. is expected that dispersion to be limited by barriers (DI FIORE; VALENCIA, 2014). Furthermore, the time elapsed since the founding of a population may still have been short to reach the equilibrium of the migratory drift (FULLER et al., 2020). Further, *L. rosalia* have demonstrated the ability to disperse approximately 8 km in a connected landscape (DI FIORE; VALENCIA, 2014; MORAES et al., 2018b).

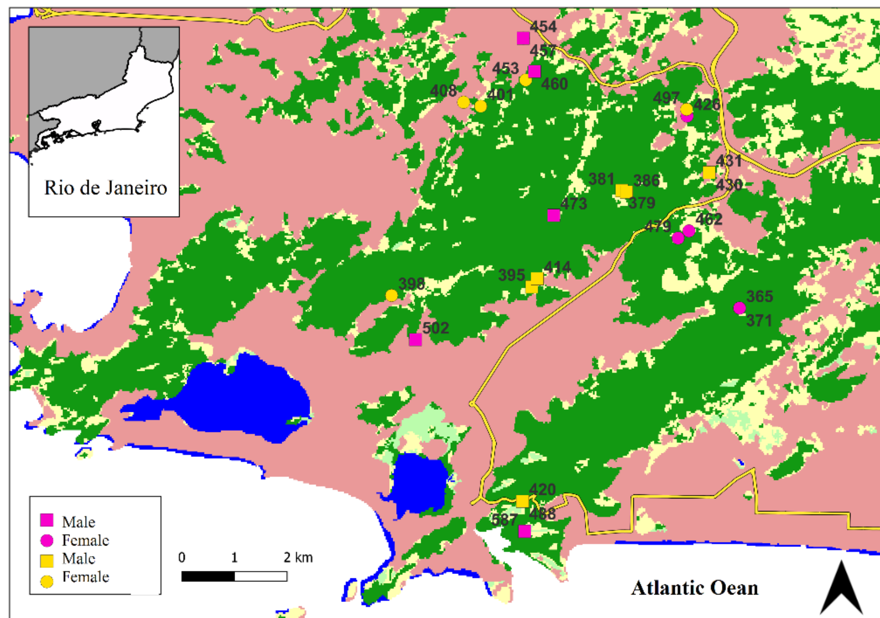

Figure 16. Geographic location of the municipality of Niteroi in the state of Rio de Janeiro indicating the collection sites of *Leontopithecus chrysomelas* considering the results for the structure analysis (k=2), that evidenced two genetic groups highlighted in yellow and pink.

Sub-structuring with moderate dispersion and a potential gene flow may favour an increase in heterozygosity and genetic diversity levels through new allele influxes (LACY, 1987). Indeed, the genetic diversity parameters estimated based on a highly informative microsatellite set (PIC = 0,653), showed values of H_O and H_E for the Niteroi invasive population of *L. chrysomelas* similar to other wild populations of *L. chrysomelas* (MORAES, et al., 2018a) and also of *L. rosalia* (GRATIVOL; BALLOU; FLEISCHER, 2001). In addition, despite founder events may lead to heterozygosity reductions, since the number of founders is usually small, as is the case for the *L. chrysomelas* population of Niteroi, the subsequent population growth rate is relevant to increase the level of diversity, due to new mutations that are expected to increase the mean heterozygosity (NEI; MARUYAMA; CHAKRABORTY, 1975).

Indeed, the foundation of the invasive Niteroi population came from only two males and two females of *L. chrysomelas* since our results did not evidence signs of hybridization with *L. rosalia.* In addition, the historical records did not report any new introduction in the area (KIERULFF C., personal communication). Therefore, the origin of the Niteroi population starts from a very narrow genetic base, in which theoretically

the negative consequences of the strong bottleneck are inherent, promoting genetic diversity loss and allele frequency changes (ALLENDORF; FLATHEAD; AITKEN, 2013). However, the rapid growth of a small population can help to minimize the loss of genetic diversity (ALLENDORF; FLATHEAD; AITKEN, 2013; BLANCHONG; SORIN; SCRIBNER, 2013) and enable adaptive change (TEMPLETON, 2008), as have been reported for introduced elk populations (BLANCHONG; SORIN; SCRIBNER, 2013; CONARD et al., 2010; WILLIAMS et al., 2002). A founder population can give rise to a variety of alleles from one generation to another and the frequency of deleterious alleles can be highlighted (ALLENDORF; FLATHEAD; AITKEN, 2013), and then present purifying positive effects (CVIJOVIĆ; GOOD; DESAI, 2018; KHAN et al., 2021). According to the population genetic theory studies, in inbred populations with no gene flow, the purifying selection can purge deleterious alleles decreasing the inbreeding depression (GARCÍA-DORADO, 2012; HEDRICK; GARCIA-DORADO, 2016).

Members of the Callitrichidae family have in general the highest reproductive taxa among primates (TARDIF; JAQUISH, 1997), a relevant biological characteristic that probably contributes to the rapid population growth. Added to this, the probable availability of resources in the Niteroi region and the absence or low competitiveness are also relevant aspects that possibly contributed to a better adaptation and rapid growth of the population. Therefore, despite the Niteroi invasive population has a founder population size really small, that in general contributes to the loss of rare alleles (ALLENDORF, 1986; FUERST; MARUYAMA, 1986) and of allelic richness (BLANCHONG; SORIN; SCRIBNER, 2013), both these values are comparable to those reported for Brazilian ex situ populations of *L. chrysomelas* (ALIAGA-SAMANEZ et al., 2022, in prep.) and are higher than those found for wild *L. chrysomelas* populations (MORAES et al., 2018a). In addition, the values of H_E and H_O are also comparable to those from captive (ALIAGA-SAMANEZ et al., 2022, in prep.) and wild populations of *L. chrysomelas* (MORAES., 2018a) and *L. rosalia* (GRATIVOL; BALLOU; FLEISCHER, 2001) (Table 4).

Table 4. Genetic diversity data for Niteroi population studied herein and for populations of *L. rosalia* and *L. chrysomelas* previously studied using microsatellite markers. N: sample number; A_N: number of alleles; A_R: allelic richness; H_O: observed heterozygosity and H_E: expected heterozygosity.

Species	Origin	Population	N	A_N	A_R	H_O	H_E	Reference
L. chrysomelas	Wild	Niteroi	23	4.364	4	0.474	0.525	Present study
	Wild	RPPN-Ararauna	84	5.3	1.6	0.5	0.6	(MORAES, et al., 2018a)
		Ilheús	17	3.5	1.5	0.5	0.5	
		Teimoso	7.0	2.3	1.4	0.4	0.4	
		Barro Branco	6.2	3.5	1.7	0.6	0.7	
	Captive	CPRJ	55	5.091	4.857	0.654	0.637	ALIAGA-SAMANEZ *et al.,* 2022 (in preparation)
		FPZSP	49	4.182	4.091	0.552	0.560	
L. rosalia	Wild	Poço das Antas	27	3.8	-	0.65	0.66	(GRATIVOL; BALLOU; FLEISCHER, 2001)
		SJ	16	3.0	-	0.55	0.56	
		LB	8	2.3	-	0.34	0.53	
		Bauen	6	2.0	-	0.43	0.42	

The inbreeding coefficient (F_{IS}) was 0.118 for microsatellites and 0.0203 for SNPs; and both values evidence inbreeding and heterozygosity deficiency. Such results are similar to that found for wild populations of *L. chrysomelas* from RPPN-Ararauna and Barro Branco fragments in Bahia (MORAES et al., 2018a). It is interesting that a heterozygosity excess suggests recent bottleneck events, since bottlenecked populations usually evidence higher observed heterozygosity than expected heterozygosity (LUIKART; CORNUET, 1998). Based on this premise, surprisingly, Niteroi population has no signals of recent bottleneck. Indeed, our Bottleneck analyses based on the Wilcoxon test, that is the most appropriate test when less than 20 loci are considered (PIRY; LUIKART; CORNUET, 1999b), showed no evidence of bottleneck in the Niteroi population, except for the IAM model, that can indicate false-positive results, since an excess of heterozygosity in populations that do not exhibit a bottleneck can be mistakenly detected when microsatellites are used. In addition, microsatellites tend to evolve following a SMM-like pattern, that is considered the most suitable model for analyses using this type of locus (LUIKART; CORNUET, 1998).

We also detected a high proportion of alleles for the category of low frequency alleles (0.0-0.1) by the L-Shaped Distribution Graph, suggesting no evidence of recent bottleneck as well. In a recent bottleneck is expected that few alleles be found in the low

frequency alleles class. In the mutation-drift equilibrium the rarest allele class (<0.1%) is expected to be much more frequent giving the L-shape (CRISTESCU et al., 2010). As after a bottleneck the rarest alleles (the low frequency alleles class) are quickly lost and is expected that fewer alleles be found in this category, so the L-shape no longer appears (LUIKART; CORNUET, 1998). Therefore, none of our results evidenced a recent bottleneck in the Niteroi population, suggesting only a population expansion. Thus, although historical records indicate that the population was originated from a very narrow genetic base, its rapid population growth probably helped to minimize the negative impacts of genetic drift and founder effects, and thus, avoid the loss of genetic variation. Even so, inbreeding was observed in this population.

The Ne calculated for the microsatellite markers was 111 individuals, which indicates the best Ne to conserve 90% of the genetic diversity over the next 100 years. According to the theoretical model the ideal Ne=475/L, where L is the generational time in years (FRANKHAM, ; BALLOU; BRISCOE, 2008), and assuming that L is four years, since sexual maturity is reached at 2 years of age (CHAOUI; HASLER-GALLUSSER, 1999; RABOY; CANALE, 2008), the Ne calculated by the equation is 119 individuals that is really near to the previously one (Ne=111). Moreover, the prediction analysis without bottleneck reductions showed that the population will retain by 90% of genetic diversity (GD) in the next 100 years. Even more, a bottleneck of 50% will maintain more than 80% of the effective allele number, and the expected and the observed heterozygosity. Such data suggest an ideal situation for population persistence based on theoretical assumptions, since for a population with 1,000 individuals, that is the case of the invasive population studied herein, 10% of the total population size is expected as Ne, and this effective population size is capable of retaining genetic diversity and evolutionary potential over generations, and thus avoid local extinctions (FRANKHAM; BALLOU; BRISCOE, 2008).

Overall, our findings raise insights on the resilience of the *L. chrysomelas* species and its capacity to adapt to a new environment and restore genetic diversity. Several studies have been demonstrating the island effect on the genetic consequences of different species (apud TEMPLETON, 2006, 2008). Here we highlight a study-case in an endangered primate species that has been suffering the negative impacts of anthropogenic actions, such as fragmentation and habitat loss (KIERULFF et al., 2008c), and surprisingly demonstrate an enormous capacity to survive and persist over the years when

is introduced in an allochthonous habitat, even in a non-ideal scenario of population expansion based only on four founders. Such results also shed light on the history of the wild population restoring of the lion tamarins, which includes species that had or still have total population sizes very reduced (e.g. *L. caissara*, 300 individuals), and even the species *L. chrysopygus*, that was already considered extinct from nature during approximately 65 years and nowadays presents genetic diversity levels comparable to those from other wild populations of *Leonthopitecus* spp. (AYALA-BURBANO et al., 2017; GRATIVOL; BALLOU; FLEISCHER, 2001; MARTINS; GALETTI JUNIOR, 2010; MORAES et al., 2018a).

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SUPPLEMENTAL MATERIAL

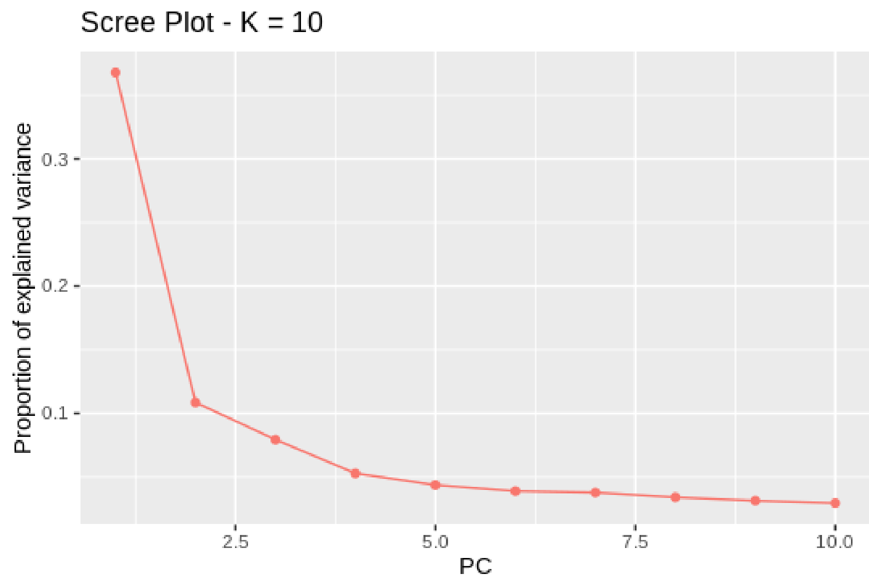

Figure S 1. Scree plot produced in PCAdapt showing the percentage of explained variance for each PC and the selection of K=5 populations.

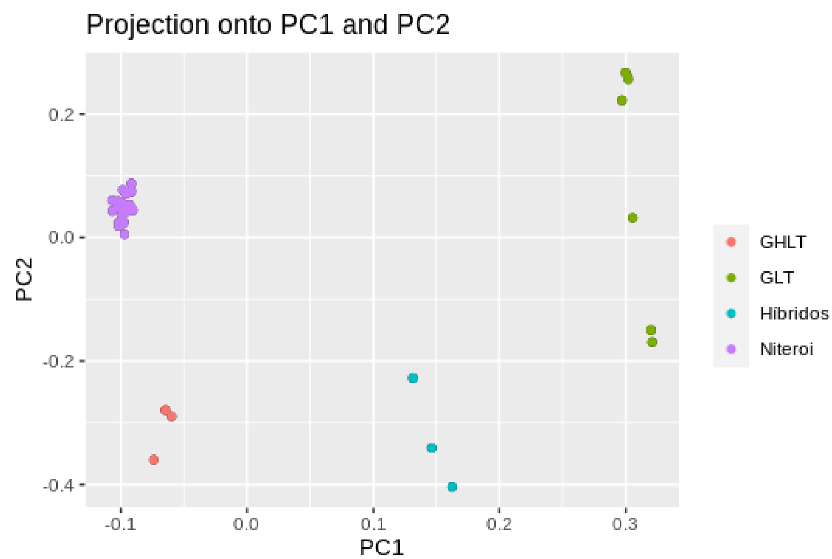

Figure S 2. Principal coordinate analysis (PCA) showing the scores on the first and second principal coordinates for the all-genomic dataset.

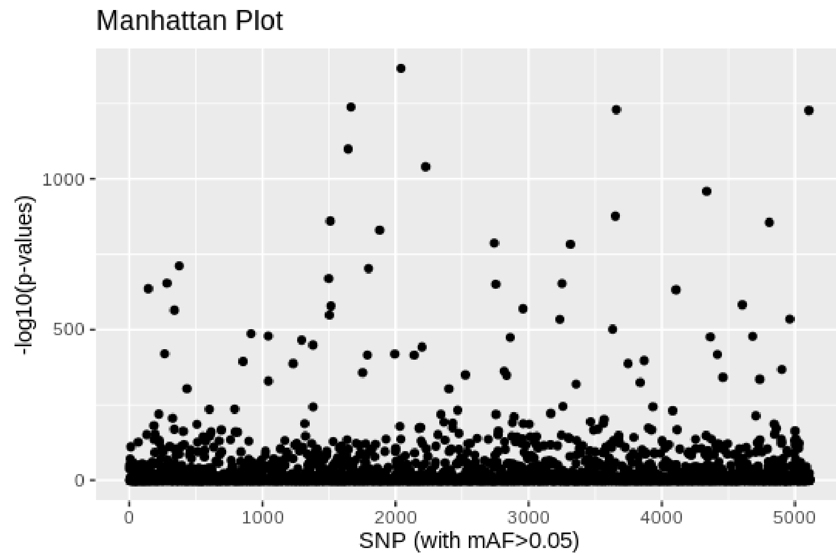

Figure S 3. Distribution of the empirical p-values obtained by PCAdapt visualized through a Manhattan plot.

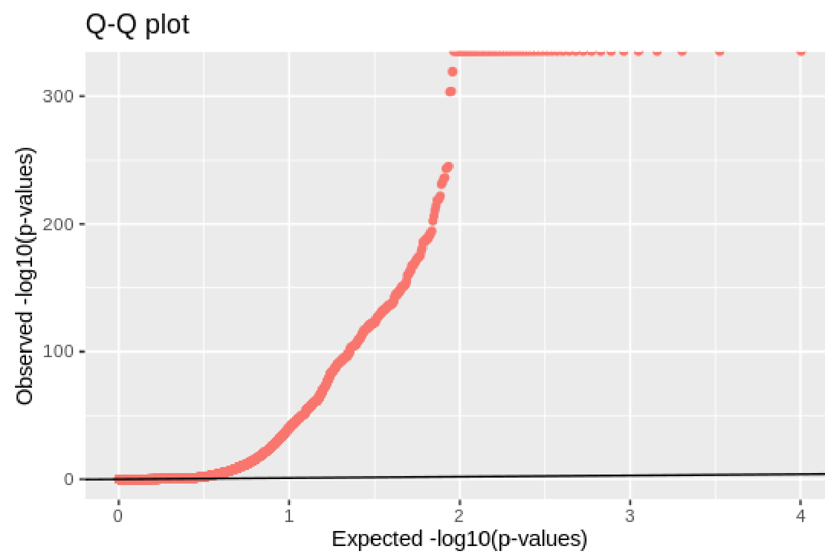

Figure S 4. QQ-plot (below) showing the cut off of 0.05%.

Table S 1. Summary of data for *Leontopithecus* samples analyzed, considering individuals from Niterói, four hybrids between the species kept at the Centro de Primatologia do Rio de Janeiro (CPRJ, RJ), and pure specimens of *Leontopithecus rosalia* and *Leontopithecus chrysomelas.* X: Analyses performed from genotyping by sequencing (GBS), amplification and sequencing of the mitochondrial gene Cytochrome Oxidase I (COI) and of the sex determination gene of the Y chromosome (SRY), and amplification and genotyping of microsatellite loci or short repeated sequences (SSR).

Sample	**ID Colection**	**Specie**	**Sex**	**Origin**	**Capture place**	**GBS**	**COI**	**SRY**	**SSR**	**Type of sample**
40	MAM-253	*Leontopithecus sp.*	Male	Free-ranging	Itaipuacu (Niteroi)	-	-	X	-	Tissue
45	MAM-258	*Leontopithecus sp.*	Female	Free-ranging	Itaipu (Niteroi)	-	-	X	-	Tissue
365	MAM-0220	*Leontopithecus sp.*	Female	Free-ranging	Itaipu (Niteroi)	X	-	-	X	Tissue
454	MAM-0227	*Leontopithecus sp.*	Female	Free-ranging	Itaipu (Niteroi)	X	-	-	X	Tissue
371	MAM-0215	*Leontopithecus sp.*	Female	Free-ranging	Verdejante (Niteroi)	X	-	-	X	Tissue
381	MAM-0216	*Leontopithecus sp.*	Male	Free-ranging	Verdejante (Niteroi)	X	X	-	X	Tissue
395	MAM-0221	*Leontopithecus sp.*	Male	Free-ranging	Jacaré (Niteroi)	X	X	-	X	Tissue
430	MAM-0229	*Leontopithecus sp.*	Female	Free-ranging	Rio Ouro (Niteroi)	X		-	X	Tissue
401	MAM-0224	*Leontopithecus sp.*	Female	Free-ranging	Jardim América (Niteroi)	X	X	-	X	Tissue
453	MAM-0223	*Leontopithecus sp.*	Female	Free-ranging	Jardim América (Niteroi)	X	X	-	X	Tissue
420	MAM-0222	*Leontopithecus sp.*	Male	Free-ranging	Jardim América (Niteroi)	X	X	-	X	Tissue
408	MAM-0244	*Leontopithecus sp.*	Female	Free-ranging	Muriqui pequeno (Niteroi)	X	-	-	X	Tissue
421	MAM-0239	*Leontopithecus sp.*	Male	Free-ranging	Muriqui pequeno (Niteroi)	X	-	-	X	Tissue
449	MAM-0231	*Leontopithecus sp.*	Female	Free-ranging	Várzea das Moças (Niteroi)	X	-	-	X	Tissue
426	MAM-0234	*Leontopithecus sp.*	Female	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	-	Tissue
398	MAM-0218	*Leontopithecus sp.*	Female	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	X	Tissue
414	MAM-0230	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X		-	X	Tissue
386	MAM-0217	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	X	Tissue
431	MAM-0243	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	-	-	X	Tissue
379	MAM-0219	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	-	-	X	Tissue
457	MAM-0232	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	X	Tissue
466	MAM-0225	*Leontopithecus sp.*	Female	Free-ranging	Jararacoçu (Niteroi)	X			X	Tissue
462	MAM-0242	*Leontopithecus sp.*	Female	Free-ranging	Muriqui pequeno (Niteroi)	X			X	Tissue
460	MAM-0228	*Leontopithecus sp.*	Male	Free-ranging	Itaipu (Niteroi)	X			X	Tissue
471	MAM-236	*Leontopithecus sp.*	Male	Free-ranging	Muriqui pequeno (Niteroi)	X			X	Tissue
473	MAM-241	*Leontopithecus sp.*	Male	Free-ranging	Salvador (Niteroir)	X			X	Tissue
479	MAM-238	*Leontopithecus sp.*	Male	Free-ranging	Muriqui pequeno (Niteroi)	X			X	Tissue
488	MAM-235	*Leontopithecus sp.*	Female	Free-ranging	Jacaré (Niteroi)	X			X	Tissue
587	MAM-0289	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	X	Tissue
489	MAM-0233	*Leontopithecus sp.*	Male	Free-ranging	Várzea das Moças (Niteroi)	X	X	-	X	Tissue
Hib72	MAM-0172	Confirmed hybrid	Female	Captivity	CPRJ (RJ)	-	X	-	-	Blood
Hib73	MAM-0173	Confirmed hybrid	Female	Captivity	CPRJ (RJ)	X	X	-	-	Blood
Hib06	MAM-0169	Confirmed hybrid	Male	Captivity	CPRJ (RJ)	X	X	-	-	Blood
Hib07	MAM-0171	Confirmed hybrid	Male	Captivity	CPRJ (RJ)	X	X	-	-	Blood
2252	MAM-0167	*L. rosalia*	Female	Captivity	CPRJ (RJ)	X	X	-	X	Blood
2457	MAM-0166	*L. rosalia*	Male	Captivity	CPRJ (RJ)	X	-	X	X	Blood
FA8	FA8	*L. rosalia*	Male	Free-ranging	Rio de Janeiro	X	-	-	X	-
AF22	AF22	*L. rosalia*	Female	Free-ranging	Rio de Janeiro	X	-	-	X	-
GLT200	MAM-0622	*L. rosalia*	-	Traffic	FPZSP	-	-	-	X	Hair
GLT206	MAM-0623	*L. rosalia*	-	Traffic	FPZSP	-	-	-	X	Hair

GLT3673	-	*L. rosalia*	-	Free-ranging	Rio de Janeiro	-	-	-	X	Blood
GLT3794	-	*L. rosalia*	-	Free-ranging	Rio de Janeiro	-	-	-	X	Blood
GLT3863	-	*L. rosalia*	-	Free-ranging	Rio de Janeiro	-	-	-	X	Blood
GLT3903	MAM-0618	*L. rosalia*	Male	Traffic	CPRJ	-	-	-	X	Blood
GLT3904	MAM-0619	*L. rosalia*	Male	Traffic	CPRJ	-	-	-	X	Blood
GLT3905	MAM-0620	*L. rosalia*	Female	Traffic	CPRJ	-	-	-	X	Blood
GLT3906	MAM-0621	*L. rosalia*	Female	Traffic	CPRJ	-	-	-	X	Blood
MLD01	MAM-0556	*L. rosalia*	-	Free-ranging	Serra (RJ)	-	-	-	X	Blood
MLD02	MAM-0557	*L. rosalia*	-	Free-ranging	Serra (RJ)	-	-	-	X	Blood
MLD05	MAM-0560	*L. rosalia*	-	Free-ranging	Serra (RJ)	-	-	-	X	Blood
MLD06	MAM-0561	*L. rosalia*	-	Free-ranging	Serra (RJ)	-	-	-	X	Blood
MLD11	MAM-0566	*L. rosalia*	-	Free-ranging	Serra (RJ)	-	-	-	X	Blood
46	46	*L. rosalia*	-	-	Rio de Janeiro	-	X	-	-	-
L.01	MAM_0678	*L. chrysomelas*	Female	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
L.02	MAM_0679	*L. chrysomelas*	Male	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
L.03	MAM_0680	*L. chrysomelas*	Male	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
L.04	MAM_0681	*L. chrysomelas*	Male	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
L.05	MAM_0682	*L. chrysomelas*	Male	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
L.06	MAM_0683	*L. chrysomelas*	Male	Free-ranging	Ilhéus (Bahía)	-	-	-	X	Blood
35	2560	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	X	-	-	Blood/Hair
16	2711	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	X	-	-	Blood/Hair
2890	MAM-0159	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	-	X	-	Blood
2152	MAM-0104	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	X	-	-	X	Blood/Hair
2364	MAM-0112	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	X	X	-	X	Blood/Hair
1894	MAM-0139	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	X	-	-	X	Blood/Hair
1893	MAM-0145	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	-	X	-	Hair
Gi02lc	MAM-0095	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi03lc	MAM-0096	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi46lc	MAM-0129	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi47lc	MAM-0130	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi50lc	MAM-0131	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi52lc	MAM-0132	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi54lc	MAM-0133	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi57lc	MAM-0136	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi58lc	MAM-137	*L. chrysomelas*	Female	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi60lc	MAM-0138	*L. chrysomelas*	Male	Captivity	CPRJ (RJ)	-	-	-	X	Blood/Hair
Gi24SP	MAM-0591	*L. chrysomelas*	Male	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi25SP	MAM-0592	*L. chrysomelas*	Male	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi26SP	MAM-0599	*L. chrysomelas*	Female	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi27SP	MAM-0579	*L. chrysomelas*	Female	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi39SP	MAM-0575	*L. chrysomelas*	Female	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi40SP	MAM-0585	*L. chrysomelas*	Female	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi41SP	MAM-0600	*L. chrysomelas*	Male	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi42SP	MAM-0107	*L. chrysomelas*	Female	Captivity	FPZSP (SP)	-	-	-	X	Blood/Hair
Gi43SP	2538	*L. chrysomelas*	-	Captivity	FPZSP (SP)	-	-	-	X	Hair
Gi44SP	MAM-0577	*L. chrysomelas*	Male	Captivity	FPZSP (SP)	-	-	-	X	Hair

Table S 2. Posterior probability analysis for Niteroi population, *L. chrysomelas, L. rosalia* and confirmed hybrids from captivity. Pure1: L. rosalia specie, Pure2: L. chrysomelas specie, F1: offspring of *a L. rosalia* and a *L. chrysomelas*, F2: offspring of two F1s, Bx1: backcross between *L. rosalia* and an F1 and Bx2: backcross between *L. chrysomelas* and a F1.

	Samplen ID	Pure1	Pure2	F1	F2	Bx1	Bx2
L. chrysomelas	GHLT678	0	**1**	0	0	0	0
	GHLT679	0	**1**	0	0	0	0
	GHLT680	0	**1**	0	0	0	0
	GHLT681	0	**1**	0	0	0	0
	GHLT682	0	**1**	0	0	0	0
	GHLT683	0	**1**	0	0	0	0
	GHLT1894	0	**1**	0	0	0	0
	GHLT2152	0	**1**	0	0	0	0
	GHLT24SP	0	**1**	0	0	0	0
	GHLT25SP	0	**1**	0	0	0	0
	GHLT26SP	0	**1**	0	0	0	0
	GHLT27SP	0	**1**	0	0	0	0
	GHLT39SP	0	**1**	0	0	0	0
	GHLT40SP	0	**1**	0	0	0	0
	GHLT41SP	0	**1**	0	0	0	0
	GHLT42SP	0	**1**	0	0	0	0
	GHLT43SP	0	**1**	0	0	0	0
	GHLT44SP	0	**1**	0	0	0	0
	GHLT02RJ	0	**1**	0	0	0	0
	GHLT03RJ	0	**1**	0	0	0	0
	GHLT46RJ	0	**1**	0	0	0	0
	GHLT47RJ	0	**1**	0	0	0	0
	GHLT50RJ	0	**1**	0	0	0	0
	GHLT52RJ	0	**1**	0	0	0	0
	GHLT54RJ	0	**1**	0	0	0	0
	GHLT57RJ	0	**1**	0	0	0	0
	GHLT58RJ	0	**1**	0	0	0	0
	GHLT60RJ	0	**1**	0	0	0	0
L. rosalia	GLT200	**0,97**	0	0	0	0,03	0
	GLT206	**0,97**	0	0	0	0,03	0
	GLT2252	**0,99**	0	0	0	0,01	0
	GLT2457	**1**	0	0	0	0,01	0
	GLT3673	**0,96**	0	0	0	0,04	0
	GLT3794	**0,97**	0	0	0	0,03	0
	GLT3863	**0,91**	0	0	0	0,09	0
	GLT3903	**0,99**	0	0	0	0,01	0
	GLT3904	**0,99**	0	0	0	0,02	0
	GLT3905	**0,91**	0	0	0	0,09	0
	GLT3906	**0,98**	0	0	0	0,02	0
	GLTAF8	**0,99**	0	0	0	0,01	0
	GLTAF22	**0,98**	0	0	0	0,02	0
	MLD01	**0,99**	0	0	0	0,01	0
	MLD02	**0,98**	0	0	0	0,02	0
	MLD05	**0,97**	0	0	0	0,04	0
	MLD06	**0,96**	0	0	0	0,04	0
	MLD11	**0,91**	0	0	0	0,09	0
Niteroi Population	365N	0	**1**	0	0	0	0
	371N	0	**1**	0	0	0,01	0
	379N	0	**1**	0	0	0	0
	381N	0	**1**	0	0	0	0
	386N	0	**1**	0	0	0	0

	395N	0	**1**	0	0	0	0
	398N	0	**1**	0	0	0	0
	401N	0	**1**	0	0	0	0
	408N	0	**1**	0	0	0	0
	414N	0	**1**	0	0	0	0
	420N	0	**1**	0	0	0	0
	421N	0	**1**	0	0	0	0
	430N	0	**1**	0	0	0	0
	431N	0	**1**	0	0	0	0
	449N	0	**1**	0	0	0	0
	453N	0	**1**	0	0	0	0
	454N	0	**1**	0	0	0	0
	457N	0	**1**	0	0	0	0
	460N	0	**1**	0	0	0	0
	462N	0	**1**	0	0	0	0
	466N	0	**1**	0	0	0	0
	471N	0	**1**	0	0	0	0
	473N	0	**1**	0	0	0	0
	479N	0	**1**	0	0	0	0
	488N	0	**1**	0	0	0	0
	489N	0	**1**	0	0	0	0
	587N	0	**1**	0	0	0	0
Hybrids	Hib06	0,27	0	0	0	0,73	0
	Hib07	0,41	0	0	0	0,59	0
	Hib73	0,12	0	0	0	0,88	0