Microsatellite markers for bearded capuchins (Sapajus libidinosus): transferability and characterization

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http://dx.doi.org/10.1590/0001-3765202120190802

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| Type | Article |
| URL | This version is available at: http://usir.salford.ac.uk/id/eprint/59481/ |
| Published Date | 2021 |

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INTRODUCTION

Capuchin monkeys are classified into two distinct clades: tufted or robust capuchins (*Sapajus*) and the non-tufted or gracile capuchins (*Cebus*) (Lynch-Alfaro et al. 2012, Lima et al. 2018). The clades are differentiated by morphological, ecological and molecular characteristics (Lynch-Alfaro et al. 2012). The tufted form has specialized cranial and dental structures for the exploitation of hard foods items, and possess forelegs, hands and feet that are shorter than those of the non-tufted clade (Silva 2001, Lynch-Alfaro et al. 2012). Additionally, the tufted capuchins make use of tools to obtain hard-to-access food items such as underground (e.g. roots) or encapsulated resources (e.g. palm nuts, cashew nuts) (Moraes et al. 2014). At present, eight species of tufted capuchin monkeys are recognized: *Sapajus apella*, *Sapajus cay*, *Sapajus flavius*, *Sapajus libidinosus*, *Sapajus macrocephalus*, *Sapajus nigritus*, *Sapajus robustus*, and *Sapajus xanthosternos* (IUCN 2019). *Sapajus libidinosus* are Neotropical primates, distributed in Brazil throughout Cerrado areas in the central, north and south-eastern regions, and in Caatinga areas in the north-eastern region (Rylands & Kierulff 2015). Although the *S. libidinosus* is not classified as a Threatened species (Rylands &
Kierulff 2015), it has been reclassified as “Near Threatened” by the Brazilian Red Book of Endangered species, owing to anthropogenic effects such as habitat loss and fragmentation (Fialho et al. 2015, Rylands & Kierulff 2015). Such impacts directly influence the viability of primate populations that rely on forest habitats to survive (Benchimol & Peres 2013, Liu et al. 2017). Nevertheless, the lack of studies on population genetics in *S. libidinosus* limits our knowledge of how habitat loss is genetically affecting the species. Polymorphic markers are not yet available to study their diversity and population structure.

Microsatellite markers have been developed and tested on other capuchin monkeys such as *Sapajus apella* (Escobar-Páramo 2000), *Cebus capucinus* (Muniz & Vigilant 2008) and *S. nigritus* (Tokuda et al. 2014). The characterization of genetic markers is a matter of great importance for population genetics studies, which are urgently required to assess gene flow and long-term viability of populations (Liu et al. 2017, León-Ortega & Gonzalez-Wangüemert 2015, Tian et al. 2017, Srbek-Araujo et al. 2018). Microsatellite markers may also be useful in behavioural studies (Tokuda et al. 2018, Yewers et al. 2019, Hoogland et al. 2019). Such studies contribute to effective management and conservation strategies (Ruiz Lopez et al. 2016, Storfer et al. 2018).

This study will provide molecular markers that can be used in future genetic studies on *Sapajus libidinosus* which need an urgent consideration in research and conservation agendas for the species and its habitat (e.g. Lynch-Alfaro et al. 2014). Using primers transferability has become a more economical and efficient way of obtaining genetic information from species when compared to developing new primers (Oliveira et al. 2006, Buzatti et al. 2016). Thus, here we aimed to test for amplification and characterize 14 microsatellite loci in *S. libidinosus*. These microsatellites were previously isolated from *Cebus capucinus* (Muniz & Vigilant 2008), which belongs to the same subfamily (i.e. Cebinae) of the *S. libidinosus* (Groves 2016). The molecular markers tested here will help to advance conservation actions and genetic management of *S. libidinosus* populations.

**MATERIAL AND METHODS**

**Sampling**

We sampled individuals of *Sapajus libidinosus* from two localities in Sertânia, Pernambuco state, north-eastern Brazil: 1) Serra do Pinheiro: -37.20 w, -8.38 s; 2) and in Serra do Estrago: -37.40 w, -7.96 s (Figure 1). We collected 22 faecal samples in September of 2016 in the Serra do Estrago and 27 faecal samples in December 2016 in Serra dos Pinheiros. In each study area, we established food provision stations in order to attract the animals and consequently facilitate the collection of faeces. We monitored the provision stations using camera traps (model Bushnell 8MP) to confirm species identity as we did not habituate the animals (Figure 2). We also performed active searches in both areas to find the animals when they did not access the provision stations. We stored the faeces in Falcon tubes containing 50 ml of absolute alcohol in the field and then we froze them (-18°C) in the laboratory for up to 12 months prior to DNA extraction (Serra do Pinheiro samples were frozen for 12 months and Serra do Estrago samples for 9 months). Three blood samples from *S. libidinosus* individuals were used to ensure the right allele size in genotyping. We obtained two of those samples from free-living individuals from the municipality of Serra Talhada, Pernambuco, Brazil with the help of the National Center for Research and Conservation
of Brazilian Primates (CPB). We obtained the third sample from a captive individual located at the Dois Irmãos State Park Zoo in Recife, Pernambuco, during the routine health check-up of the individual. We kept the blood samples frozen before analysis.

**DNA extractions**

We extracted the DNA from blood samples using a QIAamp DNA Mini Kit (Qiagen), following the QIAamp® DNA Mini and Blood Mini Handbook protocol for DNA Purification from Blood or Body Fluids (Spin Protocol).

DNA from faecal samples was extracted using a QIAamp DNA stool kit (Qiagen), specifically developed for faecal DNA purification (Di Fiore 2003, Bradley et al. 2007). We used extraction protocols provided by Qiagen kits along with the adjusted protocols proposed by Di Fiore et al. (2009) and Tokuda (2012). Briefly, the changes we made in the protocols were: 1) the DNA was extracted from approximately 100mg of faeces; 2) faecal samples were incubated at room temperature in ASL buffer for 30 to 60 minutes, instead of 10 min; 3) the samples were incubated with proteinase K at 70 °C for 30 minutes, instead of 10 minutes; and 4) we added 100 µl of buffer AE and samples were incubated at room temperature for 20 to 30 minutes, instead of 200 µl for 1 minute. The modified protocol was used for the samples with a low concentration of *Sapajus libidinosus* DNA.

**Figure 1.** Location of the *S. libidinosus* populations sampled in the present study. The populations live in fragments of Caatinga Biome, in the state of Pernambuco, North-eastern Brazil. Serra do Pinheiro (SP): -37.20 w, -8.38 s, Serra do Estrago (SE): -37.40 w, -7.96 s. (States: CE-Ceará; RN- Rios Grande do Norte; PB- Paraíba; PE- Pernambuco; AL - Alagoas; SE- Sérojo; BA- Bahia).
Microsatellite amplification and analysis

We tested the transferability to *Sapajus libidinosus* of 14 microsatellites (Ceb01, Ceb02, Ceb04, Ceb07, Ceb08, Ceb09, Ceb10, Ceb105, Ceb115, Ceb119, Ceb120, Ceb121, Ceb127, Ceb130; Muniz & Vigilant (2008) developed for *Sapajus capucinus* and already tested for transferability on other Neotropical primate species (see Muniz & Vigilant 2008, Tokuda et al. 2014). The markers were tested in two steps. In the first, we used DNA extracts from the blood samples to test the amplification temperature gradient of each marker. The temperature gradient tested was between 54°C and 64°C. In each set of samples, we used negative controls to make sure the material was not contaminated. After identifying which markers amplified the blood DNA extracts, their annealing temperature and allele size, we tested these markers on stool DNA samples from *S. libidinosus* individuals from two localities. The Polymerase Chain Reactions (PCRs) mix contained 13 μL reaction volume and consisted for 2 μL DNA (5-63 ng/μL), 7.5 μL (2x) MyTaqMix (Bioline), 1.2 μL (6mg) bovine serum albumin, 0.3 μL (10μ/m) forward primer, 0.3 μL (10μ/m) reverse primer and 1.7 μL H₂O. We labelled each forward primer with the fluorochromes 6-FAM or HEX in the 5’ end. Amplifications were carried out under the following conditions: an initial denaturation step at 95°C for 1 min; 35 cycles at 95°C for 1 min, annealing at 56-64°C for 45 s, and extension at 72°C for 1 min; and a final extension for 10 min at 72°C. The difference between the PCR protocols of the blood and stool samples is that we used 1 μL of blood DNA extract and 2 μL for stool, adjusting only with the amount of water 2.7 μL and 1.7 μL, respectively. For both the blood and faeces samples, we used negative controls to make sure the material was not contaminated. For stool samples, we also used a positive control (i.e. blood DNA extract) to confirm the amplification of the marker. We...
visualised amplified fragments on 2% agarose gels. We genotyped PCR products through Source BionScience (ABI Prism 3730), and allele sizes were scored using the program Geneious version 11.1.4 (Kearse et al. 2012).

We analysed the number of alleles per locus ($N_A$), observed heterozygosity ($H_o$), expected heterozygosity ($H_e$), probability of paternity exclusion ($Q$), identity index ($I$), polymorphic information content (PIC) and identity analysis, using CERVUS 3.0.7 software package (Kalinowski et al. 2007). We calculated the exact test of Hardy-Weinberg equilibrium using Arlequin v. 3.5.2.2 (Excoffier & Lischer 2010), and the fixation index ($F_{is}$) for each locus using the Fstat v. 2.9.3.8 (Goudet 2002), both analyses considering the Bonferroni correction of the confidence intervals (Rice 1989). To test for evidence of null alleles, we used the program MICRO-CHECKER (Van Oosterhout et al. 2004). We performed these analyses in two ways: the first, considering samples from each locality separately and the second, considering samples of both localities together.

RESULTS

We managed to amplify six of the 14 microsatellite markers used (Ceb 130, Ceb 120, Ceb 105, Ceb 10, Ceb 4, Ceb 7) using the blood samples (Table I). Subsequently, we tested the selected primers on the DNA extracted from the 49 faecal samples collected. We treated 11 faecal samples using the modified protocol for DNA extraction. Nine of those samples resulted in increased DNA concentration, demonstrating the effectiveness of the protocol. The remaining 38 samples were treated with the standard Qiagen protocol. Overall, we amplified the markers in 30 of the 49 samples collected, 15 in each locality. The mean proportion of amplified loci was 0.989 in Serra do Pinheiro, 0.956 in Serra do Estrago samples and 0.9722 considering the samples of both localities together. Ceb01, Ceb02, Ceb08, Ceb09, Ceb115, Ceb119, Ceb121, Ceb127 did not amplify in any sample. The number of alleles, the observed and the expected heterozygosities, the probability of paternity exclusion, the identity indexes and the polymorphic information contents are presented in Table I. No loci showed evidence of null alleles and the identity test showed that there are no repeated individuals in the sample. No locus showed a significant deviation from the Hardy-Weinberg equilibrium in Serra do Pinheiro, but in the Serra do Estrago, three loci presented a significant result Ceb130, Ceb120 and Ceb7 (Table I). When we analysed the samples of both localities together, the HWE was also significant for the Ceb130, Ceb120 and Ceb7 loci. The $F_{is}$ values showed negative and significant values ($p$ smaller) for some loci, indicating an excess of heterozygosity (Serra do Pinheiro: Ceb7; Serra do Estrago: Ceb120 and Ceb7; samples from both localities together: Ceb130, Ceb10 and Ceb4) (Table I).

DISCUSSION

We found that six of the 14 tested markers successfully amplified in the *Sapajus libidinosus* samples and thus, we can effectively use them when designing future population genetic studies for the species, considerably reducing the costs of microsatellite marker isolation. The absence of null alleles demonstrates that the genotyping performed in this study does not present errors such as non-amplified alleles, stutter peaks or short allele dominance (large allele dropout) (Van Oosterhout et al. 2004). All markers tested were tetranucleotides and markers of this type are considered more consistent for allele identification through automated techniques (Di
Table I. Genetic characterization, of the six microsatellite loci transferred to *Sapajus libidinosus* living in Serra do Pinheiro and Serra do Estrago in Sertânia municipality, Pernambuco State, north-eastern, Brazil. N = number of individuals tested, Ta = annealing temperature, (NA) Number of alleles, (Ho) observed heterozygosity, (He) expected heterozygosity, (PIC) polymorphic information content, (Q) probability of paternity exclusion, (I) identity index, (HWE) Hardy–Weinberg equilibrium (p = 0.0083 after Bonferroni correction) and (Fis) fixation index (p = 0.00341 for localities separately, and p= 0.0083 for single population after Bonferroni correction); * combined probability, *significant; ** p smaller significant, indicating excess heterozygosity)

### Serra do Pinheiro forest fragment

| Locus | GenBank ID | Ta (°C) | N | Allele size range (pb) | Na | Ho | He | PIC | Q | I | HWE | Fis |
|-------|------------|---------|---|-----------------------|----|----|----|-----|---|---|-----|-----|
| Ceb130 | EU019215   | 64      | 15 | 210 - 282             | 7  | 0.933 | 0.869 | 0.820 | 0.679 | 0.046 | 0.23354 | -0.077 |
| Ceb120 | EU019211   | 64      | 14 | 248 – 278             | 7  | 0.643 | 0.815 | 0.759 | 0.596 | 0.073 | 0.35481 | 0.217 |
| Ceb105 | EU019208   | 64      | 15 | 228 - 240             | 4  | 0.733 | 0.697 | 0.620 | 0.419 | 0.160 | 0.62538 | -0.055 |
| Ceb10  | EU019203   | 59      | 15 | 238 – 250             | 4  | 0.800 | 0.605 | 0.543 | 0.355 | 0.215 | 0.47371 | 0.217 |
| Ceb7   | EU019200   | 64      | 15 | 123 – 175             | 7  | 1.000 | 0.768 | 0.713 | 0.541 | 0.096 | 0.10861 | -0.317* |
| Ceb4   | EU019199   | 54      | 15 | 166 – 214             | 5  | 0.867 | 0.602 | 0.519 | 0.327 | 0.238 | 0.14529 | -0.339 |

### Serra do Estrago forest fragment

| Locus | GenBank ID | Ta (°C) | N | Allele size range (pb) | Na | Ho | He | PIC | Q | I | HWE | Fis |
|-------|------------|---------|---|-----------------------|----|----|----|-----|---|---|-----|-----|
| Ceb130 | EU019215   | 64      | 15 | 262 – 278             | 4  | 1.000 | 0.724 | 0.645 | 0.442 | 0.145 | 0.00009* | -0.077 |
| Ceb120 | EU019211   | 64      | 15 | 250 – 274             | 4  | 1.000 | 0.701 | 0.619 | 0.413 | 0.163 | 0.0165* | -0.448** |
| Ceb105 | EU019208   | 64      | 15 | 224 – 272             | 5  | 0.667 | 0.653 | 0.580 | 0.384 | 0.188 | 0.23531 | -0.022 |
| Ceb10  | EU019203   | 59      | 15 | 238 – 278             | 4  | 0.933 | 0.717 | 0.650 | 0.459 | 0.137 | 0.68687 | -0.315 |
| Ceb7   | EU019200   | 64      | 13 | 127 – 167             | 4  | 0.769 | 0.588 | 0.521 | 0.334 | 0.233 | 0.61242 | -0.066 |
| Ceb4   | EU019199   | 54      | 13 | 166 – 178             | 4  | 0.894 | 0.791 | 0.583 | 0.9501 | 0.19439 | 0.19439 | -0.360** |

### Serra do Pinheiro + Serra do Estrago

| Locus | GenBank ID | Ta (°C) | N | Allele size range (pb) | Na | Ho | He | PIC | Q | I | HWE | Fis |
|-------|------------|---------|---|-----------------------|----|----|----|-----|---|---|-----|-----|
| Ceb130 | EU019215   | 64      | 30 | 262 – 278             | 7  | 0.967 | 0.810 | 0.768 | 0.604 | 0.070 | 0.00163* | -0.197** |
| Ceb120 | EU019211   | 64      | 29 | 250 – 274             | 9  | 0.828 | 0.775 | 0.728 | 0.557 | 0.090 | 0.00708* | -0.069 |
| Ceb105 | EU019208   | 64      | 30 | 224 – 272             | 6  | 0.700 | 0.668 | 0.606 | 0.41  | 0.169 | 0.03228 | -0.049 |
| Ceb10  | EU019203   | 59      | 30 | 238 – 258             | 5  | 0.867 | 0.666 | 0.618 | 0.433 | 0.156 | 0.19439 | -0.308** |
| Ceb7   | EU019200   | 64      | 28 | 127 – 167             | 8  | 1.000 | 0.819 | 0.777 | 0.618 | 0.065 | 0.00000* | -0.371 |
| Ceb4   | EU019199   | 54      | 28 | 166 – 178             | 6  | 0.821 | 0.603 | 0.549 | 0.364† | 0.209 | 0.25641 | -0.226** |

All loci: Combined probability, *significant; **p smaller significant, indicating excess heterozygosity*
Fiore 2003, Liu et al. 2008). Tokuda et al. (2014) tested eight (i.e. Ceb3, Ceb8, Ceb9, Ceb11, Ceb119, Ceb120, Ceb121, and Ceb130) of the primers developed for *Cebus capucinus* in 21 *S. nigerius* and only Ceb3, Ceb11 and Ceb130 amplified successfully. Despite amplifying successfully in *S. nigerius*, Ceb3 and Ceb11 loci showed only two alleles (Tokuda et al. 2014). On the other hand, Ceb130 showed a greater polymorphism with eight alleles for *S. nigerius* (Tokuda et al. 2014). Comparing the parameters of the Ceb130 locus, common to the three species tested, *C. capucinus* (Muniz & Vigilant 2008), *S. nigerius* (Tokuda et al. 2014) and *S. libidinosus* (present study), we found differences in annealing temperature and size of the alleles. The annealing temperature ranged from 59°C in *C. capucinus* (Muniz & Vigilant 2008), to 60°C in *S. nigerius* (Tokuda et al. 2014) and 64°C in *S. libidinosus*. The size of alleles ranged from 182-218 in *C. capucinus* (Muniz & Vigilant 2008) and 210-282 in *S. libidinosus*, and this information was not available for *S. nigerius*. Muniz & Vigilant (2008) developed primers for *C. capucinus* and tested the transferability of these primers to 23 Neotropical primate species. However, the number of individuals tested was very low in each species (only three species were tested using 10 individuals). Despite the low number of individuals, the species tested were polymorphic in at least two loci and species from the same family of *S. libidinosus* (i.e. *S. apella* n = 10 individuals, *C. olivaceus* n = 3 and *S. xanthosternos* n = 3) amplified the largest number of loci (Muniz and Vigilant, 2008), probably due to their phylogenetic proximity.

The obtained heterozygosity values indicate that the study localities of *Sapajus libidinosus* have a high genetic variation. The values of the paternity exclusion index (Q) in our study show the reliability of the loci for paternity test use (Wang et al. 2015). Studies previously conducted for other primates consider indexes around 0.9660 and 0.9999 as being powerful for paternity testing (Chambers et al. 2004, Stevanovic et al. 2010, Wang et al. 2015). In our study, these values varied among localities and showed good power of exclusion. The identity index indicates that the probability of identifying two different individuals as being of the same genotype is low, demonstrating the reliability of the studied sites for this type of estimation. Values between 0.01-0.0001 are sufficiently low and indicate that the chosen loci are effective to distinguish between individuals (Waits et al. 2001). One can also access genetic variability among genotypes by calculating PIC values for each of the six loci. The PIC value can range from 0 - 1.0, the closer to one, the greater the allele variability in a tested locality (Guo & Elston 1999). This value is calculated by considering the number of known alleles and their frequency of distribution (Botstein et al. 1980, Guo & Elston 1999). The PIC values indicated a high variability for the markers analysed here, in both our study sites. We found that the Ceb130 marker would be the most polymorphic marker followed by Ceb120, Ceb10 and Ceb4. Polymorphic markers are more useful for distinguishing individuals and understanding the relationships between them (Guo & Elston 1999, Li et al. 2014). In the Serra do Pinheiro, there was no deviation from the HWE. However, three loci deviated from the HWE in the Serra do Estrago (Ceb130, Ceb120 and Ceb7) and also when analysing the samples from both localities together. Deviation from HWE can be an outcome from heterozygosity excesses (Cornuet & Luikartt 1996), which was the case in our study. The species social system may have contributed to this result. The mating system of the study species is considered to be polygamous (Fialho et al. 2015), increasing the possibility of mating between the individuals of the group. They present a hierarchy of dominance maintained by males, but males disperse while females remain in the group (Verderane 2010).
The study locations presented approximately 25 individuals, a common size for groups of capuchin monkeys. The standard size of *S. libidinosus* groups is 6 to 20 individuals (Rylands & Kierulff 2015), although groups of 40 to 53 individuals have previously been recorded (Ferreira et al. 2009, Moraes et al. 2014). It is worth pointing out that the DNA extracted from the faecal samples was “impure” primate DNA and despite the low concentrations (i.e. between 5 and 63 ng/µl), it was still possible to evaluate the efficiency of the primers tested. Some researchers recommend the multiple-tube approach in order to minimise the allelic dropout errors that may occur in low-concentration DNA from non-invasive samples (Gerloff et al. 1999, Kohn et al. 1999). However, due to few amounts of biological material and DNA we did not use the multiple-tube approach. Also, according to Morin et al. (2001) faecal DNA extracts with values higher than 201pg (0.201 ng) already guarantee high confidence (99% of certainty) and amplification precision of these extracts, being able to present allelic dropout in only 5.2% of samples. Due to the absence of null alleles and the observed heterozygosity higher than expected heterozygosity, we believe that genotyping error due to allelic dropout (genotype a heterozygous as a homozygous) did not occur in the sample set. The low concentration of DNA in our samples could also be a result of the storage time of the samples. For logistical reasons, we had to store samples from Serra do Estrago for one year, and samples from Serra do Pinheiro for nine months before the DNA extractions. The type and time of storage of faecal samples are thought to influence the success of DNA extraction (Piggott & Taylor 2003). There is no consensus on the best technique to be used, because often the type of storage will influence the choice of DNA extraction technique (Waits & Paetkau 2005, Vallet et al. 2007). Other factors also need to be taken into account in order to obtain a good sample, such as the type of diet of the species and the site where it was collected (Broquet et al. 2007). Herbivorous animals present secondary metabolites that may inhibit DNA amplification (Broquet et al. 2007, Vallet et al. 2007). Good quality DNA samples (from blood and tissue) are difficult to collect for species that are threatened, elusive, rare, occupy inaccessible areas or occur at low densities (Salgado-Lynn et al. 2016). Thus, testing and using samples collected in a non-invasive manner became a relevant alternative for conservation genetics studies (Di Fiore 2003, Broquet et al. 2007, Salgado-Lynn et al. 2016). The microsatellite markers transferred to *Sapajus libidinosus* can be widely used in different fields in the future such as population genetics, molecular ecology, population structure, gene flow and landscape connectivity studies. Thus, this validation of such molecular tools will contribute to the development of effective conservation strategies for capuchin monkeys and other primates.

**Acknowledgments**

The study complies with Brazilian legislation (ICMBio, SISBIO license: 52404-1, CITES license: 17BR023734/DF). We are grateful to Valdir Balbino from University Federal of Pernambuco, Robert Jehle and Marian Denson from the University of Salford for fruitful discussions in the early stages of the study. We are also grateful to Felipe França, José Silva, Silvanete Silva, Antônio Alencar Sampaio, Guga Pinheiro, João Silva and Yuri Marinho for assistance in the field. We also thank logistical support from Agência Estadual de Meio Ambiente at Pimenteira State Park, Parnamirim Irrigated Agriculture Station and Programa de Pós-graduação em Biologia Animal. BLCM was funded by scholarships from FACEPE (Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco, IBPG-1013-2.04/14) and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) Finance Codes 001 and PDSE - 88881134891/2016-01. DBB was funded by scholarships from CAPES, PDSE - Finance Code 88881132672/2016-01. JPSA is supported by CAPES (527091) and FACEPE (BCT-0025-2.05/17). The study was supported by grants from FACEPE (APQ-1534-2.04/10; APQ-0143-2.04/14) and...
Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNpq, Universal 445071/2014-1) to BB. BB is a CNpq productivity grantee (309256/2019-4).

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How to cite

MORAES BLC, BORGES DB, SOUZA-ALVES JP, BOUBLI JP & BEZERRA B. 2021. Microsatellite Markers for Bearded Capuchins (Sapajus libidinosus): Transferability and Characterization. An Acad Bras Cienc 93: e20190802. DOI 10.1590/0001-3765202120190802.

Manuscript received on July 22, 2019; accepted for publication on November 7, 2019.

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BLCM and BB conceived and designed the study; BLCM conducted the field work; BLCM, JPSA and DBB conducted laboratory experiments; BLCM and DBB analysed the data; BLCM, BB and JPB wrote the manuscript, all authors read and approved the final version.