Comparing iDNA from mosquitoes and flies to survey mammals in a semi-controlled area

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Abstract

Ingested-derived DNA (iDNA) from insects can represent a powerful tool for assessing vertebrate diversity because insects are easy to sample, have a varied diet, and are widely distributed. Despite these advantages, the use of iDNA for mammalian detection is still little explored, especially in the neotropical region. Here we aimed to compare the effectiveness of mosquitoes and flies to detect mammals in a semi-controlled area of a Zoo that houses native and non-native species. We evaluated the number of mammal species detected by the iDNA samplers and verified the distance range of each sampler group for detecting the mammal species. To capture mosquitoes and flies we used CDC (Center for Disease Control) and fish-baited plastic bottle traps, respectively, distributed in eight sampling points during five days. Using two mini-barcodes (12SrRNA and 16SrRNA) and the metabarcoding approach, we identified 45 Operational Taxonomic Units from 10 orders. There was no difference between the number of species recovered per individual insect, although the number of flies captured was higher, resulting in more mammal species recovered by this insect group. Eight species were recorded exclusively by mosquitoes and 20 by flies, suggesting that using both samplers allowed a more comprehensive screening of the biodiversity. The maximum distance recorded was 337 m for flies and 289 m for mosquitoes, but the average range distance did not differ between insect groups. Our essay proved to be quite efficient for the mammal detection, considering the high number of species detected with a reduced sampling effort. Thus, combining iDNA from different samplers and metabarcoding can be a powerful tool for mammal survey and monitoring in the neotropics.

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Keywords: biodiversity, biomonitoring, invertebrate-derived DNA, metabarcoding, vertebrate survey.

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Introduction

Environmental DNA (eDNA) has been proposed as an alternative for sampling several taxa, from microbes (e.g., Lauber et al., 2009) to vertebrates (e.g., amphibians, McKee et al., 2015; fish, Olds et al., 2016; reptiles, Kirtane et al., 2019; mammals, Leempoel et al., 2020), and consists of the species detection from genetic material spread in the environmental samples (e.g., water, soil, snow, etc.) (Bohmann et al., 2014; Cristescu & Hebert, 2018). Another complementary and more recent approach is the detection of vertebrate species using DNA obtained from the stomach or gut content of invertebrates that feed on vertebrates (invertebrate-derived DNA or ingested-derived DNA, iDNA) (Calvignac-Spencer et al., 2013; Rodgers et al, 2017). Generally, both eDNA and iDNA approaches have similar or more efficiency in detecting vertebrates than other conventional methods based on camera trapping, acoustic surveys, electrofishing, and visual surveys (Carvalho et al., 2021).

Using invertebrates, such as carrion-flies and mosquitoes, for sampling DNA of vertebrates has some advantages, considering these insects can be sampled easily, are cosmopolites, and can feed on all terrestrial vertebrates (Norris, 1965; Lynggard et al., 2019). In this context, the biomonitoring of mammal species can be benefited from iDNA approach, since many of the mammal species have elusive behavior and can be rare or present in low population densities, especially in disturbed habitats (Ripple et al., 2014). Also, biomonitoring in high biodiversity areas, such as the neotropics, can be a challenging task, notably in areas where a large part of the local biodiversity remains unknown to science. Thus, the iDNA may represent a powerful tool for such purpose. However, the use of this approach in the neotropics has been narrowly explored to date (Carvalho et al., 2021), and the comprehension of how iDNA can really represent an effective species

survey tool for this high biodiversity region is still little known (e.g., Lynggard et al., 2019; Massey et al., 2021; Rodgers et al., 2017).

Studies testing the eDNA approach in controlled areas (e.g., Clare et al., 2022; Lynggaard et al., 2022; Moyer et al., 2014) have been showing the importance of conducting previous experimental essays to support better field sampling practices. However, to our knowledge, there are no studies comparing insect groups as iDNA samplers in controlled areas, which could help answer questions about the sampling design, effort and efficiency of this emerging technique for assessing vertebrate biodiversity. Therefore, understanding the effectiveness of iDNA samplers for species surveys, including mammals, is important to guide best practices in sample collection.

In this study, we aimed to assess the effectiveness of the iDNA approach using two groups of insects for surveying terrestrial mammals in a semi-controlled area that houses native and non-native vertebrate species. We evaluated the effectiveness of mosquitoes and flies as iDNA samplers by comparing (1) the number of mammal species detected, (2) the amount of sequence reads recovered; and (3) verifying the distance range of each sampler group by comparing the distance between the insect traps and the enclosed mammal locations. Our results were helpful to raise insights to guide further sampling design and effort for surveying mammals in high biodiversity areas, monitoring species in human-impacted areas, and supporting conservation strategies.

Materials and Methods

Study area and insect sampling

Insects were collected in the Parque Ecológico de São Carlos (PESC), Brazil (lat. -21.98784° and long. -47.87695º), a Zoo that houses about 35 enclosed native and non-

native mammal species, located next to a remnant of Cerrado (Brazilian Savanna). Free-living species may also visit the area occasionally. Mosquitoes and flies were collected using CDC (Center for Diseases Control) light traps and fish-baited plastic bottle traps, respectively. Insect collections were carried out during five consecutive days in the winter (June 2020), when the Zoo was closed to the public due to the coronavirus pandemic condition, in eight sampling points distributed across the PESC area (minimum distance of 100 m and maximum distance of 300 m among traps), totaling 40 insect-trap days (Fig. 1). At each sampling point, one CDC and three fish-baited plastic bottle traps were installed. The fish-baited plastic bottle traps for the flies collection were adapted from Calvignac-Spencer et al. (2013) and Rodgers et al. (2017). In both the CDC and fish-baited plastic bottle traps, a 50 ml sterilized plastic tube containing PA absolute ethanol was fitted at the bottom for immediate preservation of the collected insects (Fig. 1). Each tube was replaced every day and the collected insects were kept in PA absolute ethanol at -20ºC until their sorting and DNA extraction.

Morphological identification and sorting of insects

From the total of insects collected, only female blood-fed mosquitoes, and hematophagous and saprophagous flies were sorted out for DNA extraction. Mosquitoes were identified at genus level (*Aedes* spp., *Culex* spp., A*nopheles* spp.), while flies were identified at family level (Muscidae, Simuliidae, Psychodidae, Antjhopmyiidae, Calliphoridae, Fanniidae, Muscidae, Sarcophagidae) according to Forattini (2002) and Rafael et al. (2012). We separated the hematophagous and saprophagous flies according to their mouth morphology.

Molecular methods

DNA extraction was performed separately for each collected individual using a Chelex protocol, following Casquet et al. (2012), in an iDNA-dedicated laboratory. Then, mini-barcode sequences for the 12SrRNA and 16SrRNA mitochondrial (mtDNA) ribosomal genes were amplified using primers previously described for targeting vertebrate (12SV5F and 12SV5R; Riaz et al., 2011) and mammal (16Smam1 and 16Smam2; Taylor 1996) species, respectively. For the 12SV5F primer, we changed the first nucleotide to a degenerate base (5´ - YAGAACAGGCTCCTCTAG - 3´) to allow broader binding in more mammal species, as suggested by Kocher et al. (2017). The mini-barcode primers were designed to amplify approximately 135-139 bp (12SrRNA) and 130-134 bp (16SrRNA). Unique identifiers (*tags*) obtained from Axtner et al. (2019) were added to both forward (F) and reverse (R) primers to mark each sampler type from each sampling point (Supplementary Table S1), also reducing the sequencing cost. Polymerase Chain Reactions (PCRs) followed Rodgers et al. (2017) and were carried out within an UV-sterilized hood in an iDNA-dedicated PCR room. To check for contamination, PCR amplifications included a non-template sample as negative control. The amplified products were visualized on 1.5% agarose gels by electrophoresis. A second PCR was performed for samples that failed in the first one, as a new attempt for amplification. With these steps, we successfully amplified the iDNA from all sorted insects.

For large-scale sequencing, we set four pooled-samples (two for 12SrRNA and two for 16SrRNA sequences), each with a final volume of 30 μl in which we pooled 16 tagged PCR products (Supplementary Table S1). The pooled-samples were cleaned using magnetic beads (Agencourt AMPure XP® – Beckman Coulter), quantified in a Qubit fluorimeter

(Thermo Fisher, Waltham, Massachusetts, USA), normalized to 50 ng/µl, and indexed using a Nextera Index kit® (Illumina, San Diego, California, USA). The paired-end metabarcoding sequencing was performed on an Illumina iSeq® platform, using an iSeq 100 v2 300 Cycle Reagent kit (2x150 bp), for a total of 70,000 reads/pooled-sample.

Sequence analysis and taxonomic assignment

The sequences obtained were first analyzed in the FastQC software (Andrews, 2010) to check the sequencing quality. The resulting sequences were demultiplexed using the *process_radtags* program in Stacks v2.59 (Catchen et al., 2013), in which the unique identifiers (*tags*) were used to trace back the information of each insect taxon and the sampling point locality (Supplementary Table S1). After that, following Rodgers et al. (2017), we merged the correspondent forward and reverse sequences and trimmed them to a minimum quality score threshold (-q) of 15, a minimum overlap (-v) of 100 bp and minimum length (-n) of 100 bp, using PEAR (Zhang et al., 2014). We discarded all singletons and obtained the OTUs (Operational Taxonomic Units) by clustering the reads with at least 97% of similarity, using USEARCH v.11.0.667 (Edgar, 2010). The obtained OTUs were compared with the sequences available in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for the species identification. For species definition, we used the criteria of high percentage of matches (98% – 100%), to retain species-level assignments. When a sequence had a match for two or more species, we defined the species according to the expected species occurrence for the studied area using IUCN and GBIF information. When a high percentage of matches was obtained, but the species does not occur in the area, we assigned the OTU to the species from the same genus

149 with natural occurrence in the region or enclosed at the zoo. This situation generally
150 happened when the mini-barcode sequence from the species was not available in the
151 GenBank, and it matched with other species from the same genus. For sequence matches
152 between 90 – 97.99%, we assumed the genus, family, or order assignment, and matches
153 less than 90% were removed. Finally, OTUs with relative abundance lower than 0.5 % (<8
154 reads) within each tagged amplification were also removed.
155
156 *Data Analysis*
157 We compared the total number of reads per detected mammal OTUs among sampler
158 groups (mosquito, saprophagous fly, hematophagous fly). We also compared the number of
159 mammal OTUs retrieved among sampler types (mosquitoes and flies). For standardizing
160 this comparison, we used as response variable a ratio between the number of OTUs
161 detected by the number of individuals used in each tagged pool (Supplementary Table S1).
162 The range of distance of each sampler group was evaluated by measuring the distance
163 between the insect trap where the insect was collected and the enclosed mammal location
164 for the identified species. For that, we mapped the mammal species enclosures within the
165 PESC and selected only the enclosed species (see ‡ in Table 1) without or with a low
166 probability of occurring in nature around PESC.
167 We compared the number of reads, the number of OTUs and the distance range
168 among sampler groups using a one-way ANOVA. We used the non-parametric Kruskal-
169 Wallis test when the data, even with log transformation, fit no parametric assumption of
170 normality and homogeneity of variance. All the data analyzes were conducted in the R
171 v.4.0.5 environment (R Core Team, 2021).
172

Results

We collected 21 (17 females and 4 males) mosquitoes and 46 flies in the total sampling period. The low number of mosquitoes and flies is likely associated with the collection season (winter), when dry and low temperatures reduce the number of these insects. From these, we used only 17 female blood-fed mosquitoes belonging to *Aedes* (1), *Culex* (13), and *Anopheles* (3) genera, and all 46 flies, separated by feeding habits into 11 hematophagous (Muscidae, Simuliidae, Psychodidae) and 35 saprophagous individuals (Anthomyidae, Calliphoridae, Fanniidae, Muscidae, Sarcophagidae). After next-gen sequencing and quality filtering, we obtained a total of 113,545 paired reads, of which 41,752 were of mammal species, discarding human, with mean number of reads equal to 386.9 ± 795.7 per OTUs for 12SrRNA (range: 16 – 3092) and 540.9 ± 924.3 for 16SrRNA (range: 8 – 3563) (Table 1). The mean number of reads retrieved per mammal species did not differ among samplers ($F_{2,70} = 0.078$, $p = 0.925$). We also retrieved 8,845 paired reads from other vertebrate species for the 12SrRNA gene (Supplementary Table S2).

In total, we identified 45 OTUs of mammal species from 10 orders. We assigned 31 OTUs at the species level, while 14 we only identified at genus (6), family (5), or order (3) level (Table 1). The iDNA from insects recovered 44 % of the PESC enclosed mammal species, when considering the OTUs assignment to the species level. We also recovered domestic species and non-enclosed native mammal species free-living into or around the Zoo (Table 1). The number of detections for each OTU, considering both mini-barcodes and all iDNA samplers across the eight sampling points, was relatively low (mean ± SD: 3.9 ± 4.8 detections), but *Canis lupus familiaris*, *Coendou insidiosus*, and *Lycalopex vetulus* were detected 24, 16 and 15 times, respectively (Table 1). Regarding the two mini-barcodes, the 12SrRNA retrieved 24 OTUs, while the 16SrRNA retrieved 37, of which

eight and 21 OTUs were retrieved exclusively by 12SrRNA and 16SrRNA, respectively (Table 1). In general, mammal OTUs were more recovered by saprophagous flies (34) than mosquitoes (25) and hematophagous flies (14). However, when the number of OTUs retained by an insect individual was standardized by the number of insects used, we found no difference among samplers (Kruskal-Wallis= 0.14, df= 2, p= 0.934; mosquitoes: mean 3.6 ± 4.3 OTUs/mosquito, saprophagous flies: mean 2.7 ± 1.7 OTUs/fly, hematophagous flies: mean 2.8 ± 1.4 OTUs/fly). On the other hand, mosquitoes and flies retrieved exclusively eight and 20 species, respectively. For instance, hematophagous flies sampled two mammal species (*Lama* sp., and one species of Cervidae) not retrieved by the other samplers.

Considering the locality of the selected enclosed species in the PESC (see ‡ in Table 1), the distances between the sampling points and the enclosure location of the species detected ranged from 77 to 289 m for mosquitoes (mean 179.4 ± 79.5 m), 53 to 337 m for flies (mean 162.7 ± 73.6 m; 53 to 206 m (119.2 ± 67.1 m) for hematophagous, and 66 to 337 m (176.3 ± 72.1 m) for saprophagous). These distances did not differ among samplers ($F_{2,25} = 1.24$, p= 0.308) and reached areas outside the PESC (Fig. 1a).

Discussion

Our iDNA essay successfully assessed at least 44 % of the total enclosed mammal species existing at the studied Zoo, although this percentage can be higher taking into account that several OTUs were assigned only to the genus, family or order level. Other species that use freely the Zoo area or live in the Cerrado remnant around it were also recorded. These results were very auspicious considering the sampling effort, and the low number of insects collected and used for DNA sequencing. Our findings indicate that even

during a non-favorable season for insect capture (such as winter), it is possible to sample a large number of mammals by combining iDNA and metabarcoding approaches. Most of the enclosed species not detected were small mammals, represented mainly by small primates in the PESC, although the largest mammal housed at the Zoo, the non-native spectacled bear (*Tremarctos ornatus*), was not detected.

After sequence filtering, we retained about 81.1 % of the total paired reads sequenced from the four pooled-samples, and this result was very similar to the value of 82.3 % obtained by Rodgers et al. (2017) using the same primers to amplify both 12SrRNA and 16SrRNA genes from iDNA of carrion flies. In our study, approximately 63.2 % of the retained sequences, considering both genes, were from humans, likely due to the daily presence of the PESC staff, despite the Zoo was closed to the public. Indeed, Massey et al. (2021) found that 80 % of the total sequences obtained from mosquito iDNA were from human. Of the total paired reads obtained for both genes, 7.79 % detected bird or fish (a non-native salmon used to bait the fly traps), barely reducing the mammal coverage in the sequencing results. However, considering only the 12SrRNA amplification, 33.7 % were represented by bird and fish species, indicating that using only this mini-barcode region may decrease the success of mammal detection, despite the detection of other vertebrate groups. Even though the mammal specific 16SrRNA mini-barcode was more effective for the detection of mammals, both 12SrRNA and 16SrRNA showed complementary results concerning the total mammal species detection, suggesting that combining both may provide a better representativeness of the biodiversity.

The percentage of OTUs assigned to the species level (69 %) was higher or very similar to previous iDNA studies in neotropical regions (40 % and 45 %, Lynggard et al., 2019; 66 %, Massey et al., 2021). Rodgers et al. (2017), using iDNA to survey mammals in

a tropical island, obtained 60 % of the OTUs identified to the species level, although when the information about the species that occurred in the area was added, the assignment to the species level increased to 100 %. It suggests that the information on the species occurrence is important to assign a DNA sequence to a given species, as we also point out in our study. The main reason that impaired here the species-level identification was the lack of reference sequences. Particularly in the hyper-diverse neotropics, the lack of reference sequences in public database has been reported as a critical aspect that limits the use of metabarcoding (Kocher et al., 2017; Rodgers et al., 2017) and efforts to produce such sequences are still needed.

In general, most of the OTUs were detected in very low frequencies (Table 1), and this can be explained by the short sampling effort. Contrastingly, some outlier species (*C. familiaris*, *C. insidiosus*, and *L. vetulus*) were detected by both samplers (mosquitoes and flies) and in almost all sampling points. *C. familiaris* is a domestic species as it is *Equus caballus*, *Bos taurus*, *Sus scrofa* and *Cavia porcellus* also detected here. The three latter species are frequently present in meals offered to the enclosed animals. *L. vetulus* and *C. insidiosus* besides being enclosed at PESC they also inhabit the nearest areas (Cerrado and inland Atlantic Forest fragments).

Flies retrieved more OTUs than mosquitoes, but they were more sampled. We found no difference between samplers on the number of detected mammal species or the number of reads retrieved, when the number of individuals was equalized, despite the different feeding habits (hematophagous and saprophagous) of the insects used herein. In contrast, Massey et al. (2021) found that carryon flies retrieved higher vertebrate richness than mosquitoes and sandflies. Possibly, the increased number of mammal species retrieved per mosquito obtained here was due to the use of only female blood-fed individuals. It is

important to point out that even though flies can be easier collected (for instance, no need of lighting traps), flies and mosquitoes recovered here some different mammal species. Therefore, the combined use of both samplers was very important for the results obtained here.

The mean distance between the insect trap and the enclosed mammal sampled was similar between mosquitoes and flies, even though flies appeared to reach longer distances. However, these results can be biased by the sampling design since the distances traveled by the insects may cover areas beyond PESC (Fig. 1a), and the travel distance may be longer than obtained here. This putative large range can explain the increased detection of the native species (*L. vetulus* and *C. insidiosus*) which can be living around the PESC and the detection of domestic species, due to the proximity of the Zoo of natural Cerrado remnants and urban areas (Fig. 1a). This is the first study that assessed the distance reached for accessing genetic material from mammals using mosquitoes and flies. These results allow us to suggest that insect traps must be installed at least 660 m from each other to obtain independent sampling and indicate that a single trap station may cover at least 34 ha. A recent study using DNA from the air to access the biodiversity in a Zoo, detected the DNA until 245 m from the DNA source (a mammal species enclosure) (Clare et al., 2022), a shorter distance than that we found here for mosquitoes and flies.

In sum, the iDNA/metabarcoding methodology was efficient to detect mammals using a short-time sampling effort. Our results indicate no differences in the efficiency of mosquitoes and flies as iDNA samplers but highlighted that both must be used together for a broader representativeness of the mammal diversity, as well as, the use of the two mini-barcodes. These findings will be helpful to guide the sampling design and minimal effort to

survey mammals in high biodiversity areas, monitor species in human-impacted areas and support conservation strategies.

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Author contributions

BHS, KGRC, CSC and PMGJr conceptualized and performed the study design. BHS, KGRC, CSC, SCE and CCG performed the laboratory activities and data analysis. The first draft of the manuscript was written by BHS and PMGJr, and all authors contributed to discussing the results and editing the manuscript.

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Data accessibility

Additional information is available in the electronic supplementary material.

FIGURE CAPTIONS

Fig1. Study area, sampling points and insect traps to the study of iDNA for sampling mammals in the Parque Ecológico de São Carlos. a) PESC limit, sampling points and species enclosures chosen to estimate the distance range of samplers; b) fish-baited plastic bottle traps for fly collection; c) CDC (Center for Diseases Control) light traps for mosquito collection. *Buffer limit with the estimated distance reached by insects, highlighting the outside areas that we may have sampled in our experimental essay.

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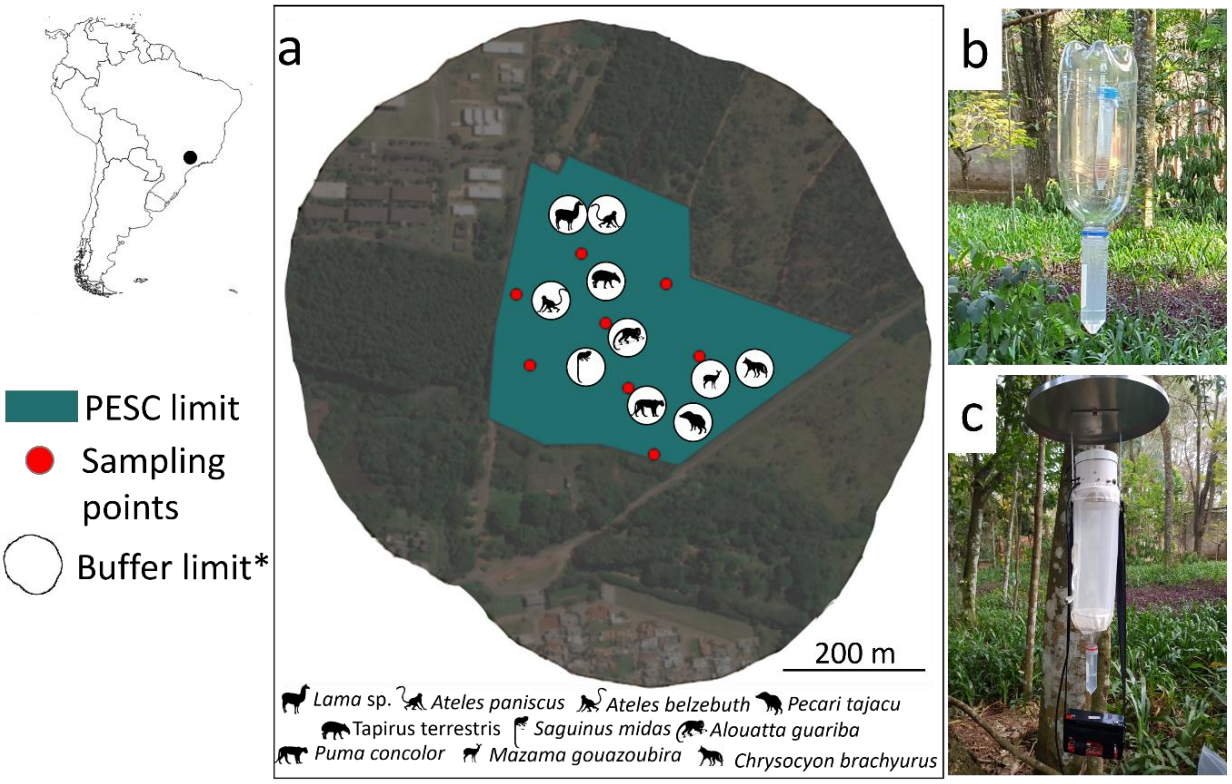

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Table 1. Frequency of mammal species detection. In parentheses, the number of reads recovered from the iDNA from mosquitos and flies using 12SrRNA an16SrRNA genes. ‡Selected enclosure species within the PESC for the distance measures; [1]only identified at family level; [2]only identified at the order level. †Species with a high match percentage that were first assigned to a species with non-natural occurrence in the study area, and therefore we assumed a co-generic species with natural occurrence in the region.

Taxon ID	Occurrence of the OTU/taxon in the study area	Mosquitoes (N=17)		Saprophagous Flies (N=35)		Hematophagous Flies (N=11)	
		12SrRNA	**16S**rRNA	**12S**rRNA	**16S**rRNA	**12S**rRNA	**16S**rRNA
Mammal							
Artiodactyla							
Cervidae[1]	-					1 (46)	1 (22)
Bos taurus	Domestic	2 (479)	1 (365)	4 (233)	3 (416)	2 (668)	1 (365)
Equus caballus	Domestic	1 (127)	1 (328)				
Lama sp.‡	Enclosed					1 (2482)	1 (1654)
Mazama gouazoubira‡	Enclosed/free living			1 (16)			
Pecari tajacu‡	Enclosed/free living	1 (147)	1 (114)	2 (74)	1 (9)		
Sus scrofa	Domestic/Meal	2 (39)	1 (151)	3 (1205)	3 (1446)		
Carnivora							
Canidae[1]	-		1 (334)	1 (8)	1 (36)	1 (47)	1 (202)
Canis lupus familiaris	Domestic	2 (408)	3 (545)	7 (2302)	9 (2698)	1 (382)	2 (47)
Chrysocyon brachyurus‡	Enclosed/free-living				1 (19)		
Lycalopex vettulus	Enclosed/free-living		5 (2699)		6 (683)		4 (181)
Nasua nasua†	Free-living		1 (44)				
Procyon cancrivorus†	Free-living		1 (48)				
Puma concolor‡	Enclosed/free-living		3 (261)		2 (119)		1 (111)
Chiroptera							
Chiroptera[2]	-		1 (21)				
Cingulata							
Cingulata[2]	-				2 (213)		
Dasypus novemcinctus	Free-living			1 (72)	2 (41)		
Euphractus sexcinctus	Free-living			3 (517)	1 (8)	1 (33)	
Euphractus sp.	Free-living			3 (74)		1 (26)	
Didelphimorphia							
Didelphis albiventris	Free-living				2 (121)		
Didelphis sp.	Free-living		1 (119)				1 (48)
Lagomorpha							
Sylvilagus brasiliensis†	Free-living		1 (207)		1 (64)		
Perissodactyla							
Tapirus terrestris‡	Enclosed			1 (2645)	1 (1550)		1 (56)
Pilosa							

Bradypus variegatus	Free-living				1 (421)		
Tamandua tetradactyla	Enclosed/ free living	1 (27)			1 (273)		
Primates							
Primates[2]	-			1 (45)			
Callithrichidae[1]	-				1 (21)		
Pitheciidae_OTU1[1]	-		3 (925)		2 (736)		1 (16)
Pitheciidae_OTU2[1]	-		1 (19)				
Alouatta guariba‡	Enclosed		1 (16)		2 (139)		
Ateles belzebuth‡	Enclosed		1 (109)	1 (529)	2 (186)		2 (303)
Ateles paniscus‡	Enclosed			1 (301)			
Callicebus nigrifrons†	Free-living	2 (1231)		2 (268)	1 (197)		
Callithrix penicillata					1 (118)		
Callithrix sp.	-			1 (42)			
Saguinus midas‡	Enclosed	1 (99)					
Rodentia							
Cavia porcellus	Meal				1 (37)		
Coendou insidiosus	Enclosed/free-living		5 (536)		8 (1814)		3 (260)
Cuniculus paca	Free-living			1 (12)			
Hydrochoerus hydrochaeris	Free-living	2 (2276)	3 (2139)		2 (481)		
Myocastor coypus	Free-living				1 (35)		
Rattus norvegicus	Free-living/ Meal	1 (466)					
Rattus rattus	Free-living/ Meal			1 (86)	1 (91)		
Rattus sp.	Free-living/ Meal		1 (9)				
Trinomys sp.	Free-living		1 (8)		1 (106)		
Total OTUs		10	21	17	28	7	12
Total Reads		5299	8997	8429	12078	3684	3265

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