Title page

Amazonian mammal monitoring using aquatic environmental DNA

Short running title

Aquatic environmental DNA for monitoring mammals

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Abstract

Environmental DNA (eDNA) metabarcoding has emerged as one of the most efficient method to assess aquatic species presence. While the method could in theory be used to investigate non-aquatic fauna, its development for inventorying semi-aquatic and terrestrial fauna is still at its early stages. Here we aimed at investigating the reliability of aquatic eDNA metabarcoding for inventorying mammals in Neotropical environments, be they aquatic, semi-aquatic or terrestrial. We collected aquatic eDNA in 96 sites distributed along three Guianese watersheds and compared our inventories to expected species distributions and field observations derived from line transect samples. Species occurrences and emblematic mammals' richness patterns were consistent with the expected distribution of the fauna and our results revealed that aquatic eDNA metabarcoding brings additional data to line transect samples for diurnal non-aquatic (terrestrial and arboreal) species. eDNA also provided data on species not detectable in line transect surveys such as semi-aquatic, aquatic and nocturnal terrestrial and arboreal species. While wise application of the eDNA method to inventory mammals still needs some developments to optimize sampling efficiency, it can now be used as a complement to traditional surveys.

Keywords

Aquatic eDNA, metabarcoding, Amazonian mammals, monitoring

Introduction

Mammal biodiversity is currently impacted by various factors at an unprecedented rate (Bowyer, Boyce, Goheen, & Rachlow, 2019), and monitoring the state of biodiversity has thus become vital to assess trends and set priorities for conservation programs (Visconti et al., 2016). Among the methods used to inventory the fauna, environmental DNA has been

47 recently developed and is increasingly used (Taberlet, Bonin, Zinger, & Coissac, 2018).
48 eDNA consists in collecting DNA fragments from environmental samples (such as soil,
49 water, faeces, or air) to detect organisms (Taberlet et al., 2018). eDNA metabarcoding
50 surveys in aquatic environments are under active development because water acts as a
51 collector for DNA, allowing an integrative assessment of biodiversity from a locality
52 (Valentini et al., 2016; Cantera et al., 2020). However, if most of previous studies have
53 focused on assessing aquatic species or communities (Bylemans et al., 2018; Civade et al.,
54 2016; Fujii et al., 2019; Lopes et al., 2017; Tréguier et al., 2014), the approach could in
55 theory be used to investigate non-aquatic species (Rodgers & Mock, 2015). In fact, water also
56 collects DNA from non-aquatic organisms during bathing (Ushio et al., 2017), drinking
57 (Rodgers & Mock, 2015) swimming or when mammals defecate in the water (Harper et al.,
58 2019), but also potentially through soil drainage by the rain.

59 Several studies aimed at detecting mammals or other non-aquatic vertebrates with aquatic
60 eDNA. Early research focused on small water bodies that are expected to be intensively
61 visited by terrestrial animals. Rodgers and Mock (2015) successfully retrieved captive coyote
62 (*Canis latrans*) DNA in drinking water samples. Later on, Ushio et al. (2017) tested a
63 metabarcoding approach on eDNA collected from zoo drinking water and on small natural
64 ponds. They detected ten out of the 13 species present in the zoo enclosure, while they
65 retrieved from 15% to 89% of the mammalian species in the natural ponds. Similarly,
66 Klymus, Richter, Thompson, and Hinck (2017) surveyed vertebrate species in uranium mine
67 containment ponds and retrieved 18 terrestrial species including hard to observe taxa such as
68 the tiger salamander (*Ambystoma tigrinum*), and Egeter et al. (2018) detected four species out
69 of the ten expected species in drinking water bodies in Sahara desert. Waterholes left by the
70 African megafauna were also used as eDNA collectors, allowing the detection of 16 species
71 (Seeber et al., 2019). Given the demonstrated high potential of eDNA to detect terrestrial

fauna in small strategic water bodies that are expected to be more saturated in eDNA than larger water bodies (Harper et al., 2019), studies then focused on collecting eDNA from larger water bodies. Harper et al. (2019) evaluated eDNA metabarcoding of pond water as a tool for monitoring semi-aquatic, ground dwelling and arboreal mammals. They led a comparative study on how animal behaviour affects the release of eDNA in artificial versus natural environments. While mammal life habits and behaviour did not influence eDNA detection in artificial ponds, it played a major role in natural systems. Attempts to detect mammals in natural aquatic systems remain scarce and to date, only few studies explored the reliability of eDNA metabarcoding to detect non-aquatic species in rivers and streams. Among those studies, Sales et al. (2020a) retrieved 14 mammal families in the Amazon river and nine mammal families in the Brazilian Atlantic forest from aquatic eDNA. While this study highlights the potential of aquatic eDNA metabarcoding to detect non-aquatic species, the reliability of the method remained to be tested by investigating the spatial concordance between the species occurrences and their expected distribution. Indeed, one of the greatest challenges is that non-aquatic species are not in permanent contact with the water, potentially resulting in smaller amounts of DNA released in the water (Harper et al., 2019; Sales et al., 2020b). Consequently, false negatives (i.e. missing detections when species are present) may thus be more frequent than for the aquatic fauna, particularly in large water bodies (Harper et al., 2019; Seeber et al., 2019).

We here test the reliability of aquatic eDNA metabarcoding to detect aquatic, semi-aquatic, terrestrial and arboreal mammal fauna along three rivers of the Amazonian biome. To this purpose, we led a comparative analysis between aquatic eDNA metabarcoding and standardized visual faunistic inventories. We then discuss the spatial concordance between the observations of several emblematic Amazonian mammals retrieved with aquatic eDNA metabarcoding and their expected distribution.

Materials and methods

Study rivers

We collected aquatic eDNA in three large French Guianese rivers (Fig. 1): the Maroni river (612 km in length) which watershed extends over Suriname and French Guiana; the Oyapock river (404 km in length) which watershed extends over Brazil and French Guiana; and the Sinnamary river (262 km in length) situated within the territory of French Guiana. The three river basins are characterized by an equatorial climate with annual rainfall ranging from 3,600mm (north-east) to 2,000mm (south and west). These three rivers face different levels of anthropogenic pressures unevenly distributed along the watercourses as most people are concentrated in the coastal area. The Maroni river is the most inhabited with approximately 83 000 habitants (INSEE, 2017) unevenly distributed from Saint-Laurent-du-Maroni to Pidima village, which constitutes the most upstream human settlement on the Maroni river (Fig. 1). The Maroni river is the most affected by human activities, mainly illegal gold mining, which represented 8,058 ha of deforestation (0.37% of the catchment area in 2014) spanning from Saint-Laurent-du-Maroni to upstream of Maripasoula (Gallay et al., 2018). Only the most upstream part of the Maroni river (upstream from Pidima, Fig.1) is not impacted by human activities. The Oyapock river is more preserved with only three villages and approximately 6 000 habitants (INSEE, 2017). Gold mining is much less developed than on the Maroni drainage, and represented 1,547 ha of deforestation in 2014 (0.06% of the catchment area), mainly concentrated near the village of Camopi (Gallay et al. 2018). The Sinnamary river is not exploited for gold but the building of a large hydroelectric dam (Petit saut dam) in 1994-1995 has severely modified the landscape. 365 km^2 of primary rain forest were flooded, leaving hundreds of islands of various sizes covering a total area of 105 km^2 (Vié, 1999). Several human settlements are located downstream from the dam while the upstream part of the river remains free from human settlements, with only occasional

activities of recreational fishing. Hunting activities also occur along the watercourses, subsistence hunters being frequent in remote isolated areas. In small rural villages or gold mining camps, hunting for meat represents a non-negligible disturbance to the large vertebrate fauna (Richard-Hansen & Hansen, 2004). The hunting impact on wildlife populations is nevertheless concentrated on small superficies around human settlements and access paths (Richard-Hansen et al., 2019). The Sinnamary river being the less populated, it presents the weakest hunting pressure. Moreover, its upstream course belongs to the core area of the Guianese National Park (Parc Amazonien de Guyane) where access is restricted and hunting totally prohibited. Hunting is also prohibited in the Petit-Saut dam area. Contrastingly, hunting pressure is important along the Maroni course, and only the upstream areas remain hardly influenced by hunting because of their distance to human settlements. Along the Oyapock river, hunting pressure is lower than on the Maroni due to a lower human population density, but it expended all along the watercourse, because human settlements, although concentrated in three main villages, are dispersed all along the watercourse, including the most upstream areas (Fig. 1).

Water collection and sampling

The eDNA sampling was conducted in November (dry season) 2017 for the Maroni river, November 2018 for the Oyapock river and November 2019 for the Sinnamary river. 96 sites were sampled using VigiDNA 0.45 µm filters (SPYGEN, le Bourget du Lac). Following Cantera et al. (2019), two samples were taken per site, with 34 litres of water filtered per sample during 30 minutes. A peristaltic pump (Vampire sampler, Burlke, Germany) and a single-use tube were used to pump the water through the encapsulated filtering cartridges. The input part of the tube was held few centimetres below the water surface and sampling was achieved in rapid hydromorphologic units to allow a better homogenisation of the DNA

146 in the water column. To avoid DNA contamination, the operators remained downstream from
147 the filtration either on the boat or on emerging rocks. After the filtration, the capsules were
148 emptied and filled with 80 mL of CL1 conservation buffer (SPYGEN) and stored in sterile
149 individual plastic bags in the dark. The samples were kept at room temperature until the DNA
150 extraction, performed within a month.

151 *eDNA laboratory and bioinformatics*

152 Each filtration cartridge was agitated for 15 min on an S50 shaker (cat
153 Ingenieurbüro™) at 800 rpm, emptied into a 50 mL tube and then centrifuged for 15 min at
154 15'000 g. The supernatant was then discarded with a sterile pipette leaving 15 mL of liquid at
155 the bottom of the tubes. After the addition of 33 mL of ethanol and 1.5 mL of 3 M sodium
156 acetate, the 50 mL tubes were stored at 20 °C during at least one night. The tubes were
157 subsequently centrifuged at 15'000 g for 15 min at 6°C and the supernatants were removed.
158 720 µl of ATL buffer from the DNeasy blood & Tissue Extraction Kit (Qiagen) were added
159 to the tubes. The tubes were vortexed and the supernatants were transferred to 2 mL tubes
160 with 20 µL of Proteinase K. The tubes were incubated at 56 °C during two hours. After this
161 step, the DNA extraction was led with the NucleoSpin® Soil (MACHEREY-NAGEL GmbH
162 & Co., Düren Germany) beginning at step six and following the manufacturer's instruction.
163 After the extraction step, the samples were tested for inhibition using qPCR following
164 the protocol of Biggs et al. (2015). The samples were diluted 5-fold before the amplification
165 if they were considered inhibited. DNA amplification was performed in a final volume of 25
166 µL including 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City,
167 CA), 10 mM of Tris-HCl, 50 mM of KCl, 2.5 mM of MgCl2, 0.2 mM of each dNTP, 0.2 µM
168 of 12S-V5 vertebrate marker (12S-V5 R 5'-TTAGATACCCCACTATGC-3' and 12S-V5 F
169 5'-TAGAACAGGCTCCTCTAG -3', Riaz et al. 2011) and 3 µL of DNA template. 4 mM of

human blocking primer for 12S-V5 (5'-CTATGCTTAGCCCTAAACCTCAACAGTTAAATCAACAAAACTGCT -C3 – 3' (De Barba et al., 2014) and 0.2 mg/mL of bovine serum albumin (BSA, Roche Diagnostic, Basel, Switzerland) were also added to the mixture. We performed 12 PCR replicates per field sample. In order to assign the sequences to the appropriate sample, the forward and reverse primers were 5'-labbeled with a unique eight-nucleotide tag for each PCR replicate. Both forward and reverse primers used an identical tag in order to minimize tag-switching issues (Schnell, Bohmann, & Gilbert, 2015). The PCR mixture was denatured at 95 °C for 10 min, followed by 50 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C and a final elongation step at 72 °C for 7 min. The amplification step was performed in a dedicated room with negative air pressure and physical separation from the DNA extraction rooms (with positive air pressure). The purified PCR products were then pooled in equal volumes to reach a sequencing depth of 500,000 reads per sample before the libraries preparation. Library preparation was performed at Fasteris facilities (Geneva, Switzerland) using Metafast protocol (www.fasteris.com/metafast). Two libraries were sequenced using an Illumina HiSeq 2500 (2x125 bp) (Illumina, San Diego, CA, USA) on a HiSeq Rapid Flow Cell v2 using the HiSeq Rapid SBS Kit v2 (Illumina, San Diego, CA, USA), three using a MiSeq (2x125 bp) (Illumina, San Diego, CA, USA) and the MiSeq Flow Cell Kit Version3 (Illumina, San Diego, CA, USA) and three using a NextSeq (2x150 bp+8) (Illumina, San Diego, CA, USA) and the NextSeq Mid kit (Illumina, San Diego, CA, USA). The libraries ran on the NextSeq were equally distributed in four lanes. The sequencing were performed at Fasteris (Geneva, Switzerland). Fourteen negative extraction controls and four negative PCR controls (ultrapure water, 12 replicates) were amplified per primer pair and sequenced in parallel to the samples to monitor possible contaminants.

The EMBL-EBI vertebrate database was downloaded from the European Nucleotide Archive (ENA) (http://ftp.ebi.ac.uk/pub/databases/embl/release/std/, release 134 for Maroni river sample, 138 for Oyapock and 140 for Sinnamary samples). The three releases were compared and the new mammalian species incremented in each new version did not belong to French Guiana. Our results were therefore uninfluenced by EMBL release number. We extracted from this database the relevant metabarcoding fragment using EcoPCR (Ficetola et al., 2010) and OBITools (Boyer et al., 2016). Our reference database thus includes the local database of French Guianese mammals (Kocher et al., 2017) which references 576 specimens of 164 species as well as all the vertebrate species available in EMBL.

The sequence reads were analysed using the functions of the OBITools package following the protocol described in Valentini et al. (2016). Briefly, forward and reverse reads were assembled using *illuminapairedend* program. Subsequently, the *ngsfilter* program was used to assign the sequences to each sample. A separate dataset was created for each sample by splitting the original dataset in several files using *obisplit*. Sequences shorter than 20 bp, or occurring less than 10 times per sample or labeled ‘‘internal’’ by the *obiclean* program, corresponding most likely to PCR errors, were discarded. The *ecotag* function was used for the taxonomic assignment of molecular operational taxonomic units (MOTUs). Taxonomic assignments from *ecotag* were also corrected to avoid over-confidence in assignments: species-level assignments were validated only for sequence identity with the reference database higher or equal than 98%. The MOTUs occurring with a frequency bellow 0.001 per library sample were considered as tag-jumps and discarded (Schnell, Bohmann, & Gilbert, 2015). These thresholds were empirically determined to clear all reads from the extraction and PCR negative controls included in our global data production procedure as suggested in De Barba et al. (2014). For the samples sequenced with the NextSeq, only species present in at least two lanes were retrieved.

Line transects data

86 line transects were realised between 1998 and 2018 (See Fig. S1). The line transect surveys were conducted as explained in de Thoisy, Brosse, and Dubois (2008) and in Richard-Hansen et al. (2015). Briefly, the line transect sampling consisted in visually recording the fauna by walking slowly (0.8–1.3 km/h) on linear forest tracks measuring 3-5 km, presenting the same forest structure (census Guitet, Pélissier, Brunaux, Jaouen, & Sabatier, 2015), but including various local habitats (i.e. hill, stream). Depending on the study, there was a single forest track (de Thoisy et al., 2008) or four tracks per site (Richard-Hansen et al., 2015). The surveys were repeated daily until a cumulated sampling distance of more than 100 km was reached in each site. Those inventories were conducted during the day (from 07:00 to 18:00), and hence, strictly nocturnal species were not observed.

Data analysis

Because we use the "12S-V5" vertebrate marker (Riaz et al., 2011) for the amplification, we obtained broad observations for various vertebrate taxonomic groups. Data were thus first sorted to only keep mammal taxa and MOTU assigned to the species level, thus retrieving 78 mammal species (see Table S1 and S2). Non-mammal species (amphibians, birds, reptiles) were discarded from this study because reference databases are still largely incomplete.

To make relevant comparisons between eDNA results and known spatial distribution of the species, we used the Faune-Guyane database (Faune-Guyane, 2020). It gathers citizen science data and observation data from scientific monitoring and constitutes the most detailed information on vertebrate distribution (excluding fishes) in French Guiana. We used the Faune-Guyane database to identify the "emblematic mammalian fauna" used to conduct the comparative analysis with line transects and to discuss the consistency of the observations

with the expected species distributions. The “emblematic mammalian fauna” included large (adult body mass > 1kg) mammals, but excluded the rarest species, those occurrences in French Guiana being not sufficient to draw a relevant distribution area. The “emblematic mammalian fauna” therefore excluded small mammals (adult body mass < 1 kg) including Chiroptera, Rodentia and Didelphimorphia which are not easily identifiable without animal capture as well as medium and large mammals (adult body mass > 1 kg) considered as very rare or rare (see Table S2 for species list). This index of rarity was based on the ratio between species observation number and total number of observations, and was adjusted by experts to consider species that are difficult to observe but not necessarily rare. After the exclusion of the less documented species (47 species excluded), we focussed on 31 fairly well studied species, hereafter referred to as the “emblematic mammalian fauna” to conduct the comparative analysis with line transects and to discuss the consistency of the observations with the expected distributions.

Line transects and eDNA metabarcoding survey methods are not directly comparable since they focus on different habitats/microhabitats, making site by site comparisons unrealistic. To estimate the reliability of eDNA metabarcoding, and provide a simple metric to compare the two survey methods, we used what we hereafter refer to as the *observation frequency*. Observation frequency represents the total number of sites where a species has been observed by a sampling method (line transects or eDNA metabarcoding) divided by the total number of sites. This metric differs from the species detection probability as it does not intend to define the probability of encountering at least one individual of a species present on a surveyed area (Boulinier, Nichols, Sauer, Hines, & Pollock, 1998), but it highlights the proportion of sites were a given species has been observed by aquatic eDNA metabarcoding or line transects. It permits to compare the two sampling methods, and therefore helps estimating the reliability of aquatic eDNA metabarcoding for observing non-aquatic species.

It also reveals if the observation frequency ranking is conserved between both methods. Irrespective of the sampling method, the observation frequency should be high for common and widely distributed species or species with high detection probabilities, while it should be low for rare species or species with low detection probabilities. Although the eDNA metabarcoding and the line transect sampling were not conducted at the same sites and on the same time, both samplings cover a substantial part of the Guianese territory and include most habitat types, levels of threats and anthropization, making relevant the broad comparison between eDNA metabarcoding and line transect sampling (see Fig.1 and Fig. S1).

The observation frequency metric was computed using species by site matrices from eDNA metabarcoding and line transect data (Table S3 and S4). The species observation frequencies were calculated for both survey methods and then compared with Signed-Rank Wilcoxon tests after species were classified as aquatic, terrestrial, arboreal, nocturnal and/or diurnal (Emmons & Feer, 1997; Hansen, Richard-Hansen, Dewynter, Pourcher, & Soissons-Tairraz, 2000) to determine the effect of mammal habitat and ecology on observation frequency. Simple linear regressions were then performed to test for a linear relationship between the species observation frequencies calculated for both survey methods. To estimate the spatial consistency of the species observations with their expected distributions in French Guiana, we displayed the species occurrence patterns of several emblematic mammals with fairly well known ecologies, as well as the species richness pattern of the 31 emblematic mammals considered in the study. All the analyses were computed in R software version 3.6.1 (2019-07-05) (R Core Team, 2019) and the maps were edited with arcGis software.

Results

A total of 152,645,960 sequences were obtained from the eDNA samples and 87,892,063 reads were kept after bioinformatics processing. Overall, we observed 78 mammal species across the three river sites resulting in 4,524,515 reads obtained after

293 bioinformatics analyses. The mammal species retrieved belonged to 72 genera, 33 families
294 and 11 orders (See Table S1).

295 Among the 78 species, five species classified as very rare were detected using eDNA:
296 the Emilia's gracile opossum *(Gracilinanus emiliae)*, the Guianan white-eared opossum
297 (*Didelphis imperfecta)*, the rufous mouse opossum (*Marmosa lepida)*, the white-faced spiny
298 tree rat (*Echimys chrysurus*) and, the bush dog (*Speothos venaticus*). Moreover, among the 31
299 species detected and referred to as the emblematic fauna, six are listed in the French-
300 Guianese IUCN red list (UICN France et al., 2017). The giant otter (*Pteronura brasiliensis*,
301 endangered) and West Indian manatee (*Trichechus manatus*, endangered), the lowland tapir
302 (*Tapirus terrestris*, vulnerable), and the jaguar (*Panthera onca*, nearly threatened), puma
303 (*Puma concolor*, nearly threatened) and white-lipped peccary (*Tayassu pecari*, nearly
304 threatened).

305 *Patterns of the emblematic fauna observation frequency*

306 Eight species were only observed with aquatic eDNA metabarcoding (observation
307 frequency in parentheses): the nocturnal kinkajou (*Potos flavus*, 52.08%), four-eyed opossum
308 (*Philander opossum*, 45.83%), lowland paca (*Cuniculus paca*, 29.17%), long-nosed armadillo
309 (*Dasypus kappleri*, 16.67%), Brazilian porcupine (*Coendou prehensilis*, 6.25%), the semi-
310 aquatic capybara (*Hydrochoerus hydrochaeris*, 48.96%), giant otter (17.71%), and the
311 aquatic West Indian manatee (4.17%) (Fig. 2).

312 The lowland tapir presented the highest observation frequency with aquatic eDNA
313 metabarcoding and was observed in 80.21% of the sites, while it was observed in only
314 30.23% of the line transect sites. Similarly, the southern tamandua (*Tamandua tetradactyla)*
315 and giant anteater (*Myrmecophaga tridactyla)* as well as the neotropical otter (*Lontra*
316 *laugicaudis*) were observed in 52.08%, 54.17%, and 28.13% of the sites with aquatic eDNA

317 metabarcoding while they were observed in 19.77%, 15.12%, and 4.65% of the sites with line
318 transect surveys, respectively. Finally, the jaguar was only slightly more observed with
319 eDNA metabarcoding (10.42%) than with line transects (9.30%) (Fig. 2).

320 In contrast, the observation frequency of primates including the spider monkey (*Ateles*
321 *paniscus*), the wedge-capped capucin (*Cebus olivaceus*), the tufted capuchin (*Sapajus*
322 *apella*), the red-handed tamarin (*Saguinus midas*), the Guianan red howler (*Alouatta*
323 *macconnelli*), the white-faced saki (*Pithecia pithecia*), and the squirrel monkey (*Saimiri*
324 *sciureus*) did not exceed 41.67% with aquatic eDNA metabarcoding while it ranged from
325 34.88% to 98.84% with line transect surveys. Similarly, the observation frequency of the
326 common diurnal rodents including the red-rumped agouti (*Dasyprocta leporina*) and the red
327 acouchy (*Myoprocta acouchy*) as well as the ungulates collared pecari (*Pecari tajacu*), red
328 brocket (*Mazama americana*), and grey brocket (*Mazama nemorivaga*) and the tayra (*Eira*
329 *barbara*) ranged from 56.98% to 100% with line transects while it ranged from 2.08% to
330 45.83% with aquatic eDNA metabarcoding (Fig. 2).

331 Overall, the aquatic/semi-aquatic and the nocturnal fauna were significantly more
332 observed with eDNA metabarcoding than with line transects (Wilcoxon, $W = 193$, $p<0.001$, n
333 $= 15$). Observation frequency of the nocturnal and aquatic/semi-aquatic species ranged from
334 1.04 to 80.21% (median= 28.13) with aquatic eDNA metabarcoding while it ranged from 0 to
335 30.23% (median = 0) for line transects. Contrastingly, the diurnal non-aquatic fauna was
336 more observed with the line transect surveys than with aquatic eDNA metabarcoding
337 (Wilcoxon, $W = 26$, $p < 0.001$, $n = 17$). Observation frequency of the diurnal non-aquatic
338 species ranged from 2.08 to 45.83% (median = 21.87) for aquatic eDNA metabarcoding
339 while it ranged from 11.63 to 100% (median = 76.74) with line transects. The linear
340 regression revealed a marginally significant linear relationship between the observation
341 frequency of the two survey methods for aquatic/semi-aquatic and nocturnal species ($F_{(1,13)}$ =

342 4.20, p = 0.06, R = 0.43, slope = 1.23). Observation frequency of the aquatic/semi-aquatic
343 and nocturnal species was in average 4.7 times higher with aquatic eDNA metabarcoding
344 than with line transects (Fig. 2). On the contrary, there was a significant linear relationship
345 between the observation frequency of the diurnal non-aquatic species obtained with the two
346 survey methods ($F_{(1,14)}$ = 6.73 , p = 0.02, R = 0.53, slope = 0.24). Observation frequency of
347 the diurnal terrestrial and arboreal fauna was in average 3.4 times lower with aquatic eDNA
348 metabarcoding than with line transects (Fig. 2).

349 *Species occurrence patterns of emblematic species*

350 Mammals species with a restricted distribution area were retrieved in their known
351 habitat. The West Indian manatee was indeed observed in all three estuaries (sites M36, M37,
352 S22 and O37). Similarly, Cetacea, although not identified to the species or genera level were
353 observed in estuaries using eDNA metabarcoding (sites M36, M37 and O37; Fig. 3), which is
354 consistent with their known distribution (contrary to the nearby Amazon drainage, freshwater
355 dolphins do not occur in French Guiana).

356 More widespread species, that inhabit the entire Guianese territory were also retrieved
357 in a large part of the eDNA sites, or are clustered in the least anthropized areas for the species
358 known as sensitive to human disturbances. eDNA metabarcoding observations of the
359 capybara, the giant anteater, the kinkajou and the lowland tapir extended from the upstream
360 to the downstream of the three rivers (excepted for the sites located at the estuaries) (Fig. 3 &
361 4). In contrast, the giant otter, the neotropical otter and the spider monkey presented similar
362 spatial pattern of distribution and were mostly observed at the upstream of the rivers (Fig. 3
363 & 4). To the exception of site M5 and M7, the giant otter was observed in the six most
364 upstream sites of the Maroni river. On the Oyapock river, this species was observed in five
365 sites distributed all along the watercourse while it was retrieved in four sites located upstream

366 the dam, and in one site located downstream the dam of the Sinnamary river. The neotropical
367 otter was observed in two sites located at the upstream and in one site located near the
368 upstream human settlements on the Maroni river. On the Oyapock river, the species was also
369 observed in nine sites located all along the watercourse while the species was observed in 14
370 sites located in the upstream part of the Sinnamary river and in a single site located
371 downstream from the 'Petit Saut' dam (Fig. 3). Similarly, the spider monkey observations
372 occurred in four sites on the upstream of the Maroni river, in six sites distributed along the
373 Oyapock river and in nine sites located upstream the Sinnamary dam (Fig. 4). The jaguar
374 observations were scarce, notably on the Maroni river with only one observation at the
375 upstream and two observations at the upstream and the downstream of the Oyapock river. On
376 the Sinnamary, the species was observed in six upstream sites and in one site downstream the
377 dam (Fig. 4).

378 *Richness pattern of the emblematic fauna*

379 Out of the 31 emblematic mammals considered, 27, 28 and 31 mammal species were
380 observed using eDNA metabarcoding on the Maroni, the Oyapock and the Sinnamary rivers,
381 respectively. On the Maroni river, the site species richness ranged from 0 to 14 (median = 5)
382 while it ranged from 1 to 17 (median = 8) and from 2 to 20 (median = 14) for the Oyapock
383 and the Sinnamary river, respectively. The site species richness along the Maroni river was
384 heterogeneous with the most upstream sites being richer than the sites located downstream
385 from the Maripasoula village, to the exception of site M35. One site located at the
386 downstream of the Maroni river (M33) did not provide any emblematic mammal species
387 observation (Fig. 5). Along the Oyapock river, the site species richness was distributed more
388 homogeneously along the watercourse, the sites O9, O10, O11 and O30 being the richest
389 (from 14 to 17). The upstream of the Sinnamary river (S1 to S11, S15) presented the sites

with the highest species richness (14 – 20), which were concentrated at the upstream of the dam (Fig. 5).

Discussion

Although aquatic eDNA metabarcoding has widely been used to inventory aquatic fauna, the method is gaining new insights to inventory non-aquatic species. To date, the method remains exploratory as several challenges still need to be addressed. The reliability of this survey method has already been investigated by comparing the inventoried fauna to that obtained with other methods such as camera trapping (Sales et al., 2020a, 2020b). Here, comparing aquatic eDNA metabarcoding inventories to line transect observations over the Guianese territory revealed strong consistency between the expected species distributions and eDNA detections, making eDNA a promising tool to inventory both aquatic and terrestrial fauna.

Observation frequencies between eDNA metabarcoding and line transects

Comparing eDNA metabarcoding observations to those of the traditional line transects revealed that nocturnal and aquatic species were observed more often in eDNA samples than in line transects, whereas diurnal terrestrial and arboreal species were more often observed using line transects. We nevertheless detected a marginally significant relationship between the observation frequency of nocturnal and semi-aquatic species and a significant relationship between the diurnal terrestrial and arboreal species from both methods indicating that eDNA metabarcoding retrieved a similar pattern of observation ranking than line transects. Although the observation frequency with aquatic eDNA metabarcoding is in average 3.4 times lower than using line transects for diurnal terrestrial and arboreal mammals, sampling eDNA in a site is achieved is less than an hour. We therefore believe that aquatic eDNA metabarcoding can constitute a useful complement to

414 line transect samples (or other sampling methods) for terrestrial and diurnal mammals given
415 that the eDNA collection by water filtrations can be rapidly achieved during survey
416 campaigns. A less stringent pattern was found for nocturnal and aquatic species, with giant
417 anteater and tamandua more frequently observed by both methods than the rare giant
418 armadillo (Carter, Superina, & Leslie, 2016; Catzeflis & Thoisy, 2012) or the elusive jaguar
419 which has a large individual home range and low population densities (Petit, Denis, Rux,
420 Richard-Hansen, & Berzins, 2018). For those species, observation frequency was fourfold
421 higher using aquatic eDNA metabarcoding than line transects. Together with the eight
422 species only observed with eDNA metabarcoding, this testifies for the capacity of this
423 method to detect nocturnal and aquatic species rarely or not observable in line transect
424 inventories.

425 These relationships remain however dependent on the species as the observation
426 frequency of some species can be biased by different parameters. Indeed, aquatic eDNA
427 metabarcoding may be sensitive to peculiar mammal's behaviour (Harper et al., 2019). For
428 instance, the tapir was observed in 80% of the eDNA sites regardless the proximity of
429 villages or the land use. Yet, the tapir is an appreciated game species due to its size and the
430 quality of its meat and is therefore under pressure in areas accessible to hunters (Richard-
431 Hansen et al., 2019; Tobler, Hibert, Debeir, & Richard-Hansen, 2014). This high observation
432 frequency was already observed by Sales et al. (2020b) and may be explained by the high
433 affinity of tapir for the water, combined to its habit of defecating in the water (Tobler,
434 Janovec, & Cornejo, 2010). Despite such species presenting particularities hindering fine-
435 scale observations, aquatic eDNA metabarcoding could constitute a valuable complement to
436 traditional samples, as it allows extending the range of species and habitats to be inventoried,
437 while saving time for biodiversity inventories.

438 *Species occurrence patterns*

439 Detailing the occurrences of the West Indian manatee illustrated the capacity of
440 aquatic eDNA metabarcoding to detect species only in their area of distribution. The West
441 Indian manatee was observed in all the three estuaries sampled and in no other site, estuaries
442 being the typical habitat of this species (de Thoisy et al., 2016). The Cetacea observations
443 were also exclusively retrieved at the estuarine sites. Those consistent observations to the
444 distribution area of the species thus constitute a proof of absence of false positives
445 (observation of the species outside their distribution area) for those species.

446 The occurrences of the mammals inhabiting all the territory showed that some species
447 were observed regardless of human proximity. Among them, the capybara and the kinkajou
448 were observed in half of the sites (50.52%). They are known as tolerant to human presence,
449 kinkajou being a discrete nocturnal and arboreal species disregarded by hunters; and capybara
450 being a generalist species not appreciated by hunters because of the strong taste of its meat
451 (hunting surveys show that they represent only 0.5 and 1.5% respectively out of 14,570
452 mammals hunted, Richard-Hansen et al. (2019)). Other species recognised to be negatively
453 impacted by anthropogenic activities such as the spider monkey or the neotropical and giant
454 otter (de Thoisy, Renoux, & Julliot, 2005; Rheingantz, de Menezes, & de Thoisy, 2014;
455 Richard-Hansen et al., 2019) were preferentially observed in the upstream part of the rivers,
456 which is free from dense human settlements or activities. For some observations, we cannot
457 nevertheless exclude that aquatic eDNA comes from the butchering of hunted animals
458 (animals are hunted far away, brought back and butchered in the villages), as the observations
459 of the spider monkey near the Trois-saut and Camopi villages are consistent with the hunting
460 habits of Wayapi and Teko people, heavily researching spider monkey for its meat (de
461 Thoisy, Richard-Hansen, & Peres, 2009; Richard-Hansen et al., 2019).

462 *Richness patterns*

463 Overall, inventories of the emblematic mammalians using aquatic eDNA metabarcoding
464 revealed strong species richness variations between the three rivers, with the Sinnamary river
465 presenting a high species richness in a large part of its course whereas the Maroni shows rich
466 assemblages only in restricted areas. This gradient is consistent with the human presence on
467 these rivers, the Maroni being the most inhabited and the Sinnamary river being much less
468 occupied by humans, with a human population density approximately 10-fold lower on the
469 Oyapock than on the Maroni (Gallay et al., 2018).

470 We also outlined a trend toward highest mammal richness in the upstream part of the
471 studied rivers, which are the least impacted by mining activities and the least densely
472 populated by humans (de Thoisy et al., 2010; Stach et al., 2009). Maximal species richness
473 values were indeed detected in the upstream part of the Sinnamary river, which is free from
474 any human settlement and integrally protected as part of the core area of the Parc Amazonien
475 de Guyane. Upstream Maroni is also free from human settlements, and traditional hunting
476 activities by local people remain limited due to the difficulty to access these areas. On the
477 contrary, the upstream part of the Oyapock river hosts around 1700 inhabitants which rely on
478 local fishing and hunting as sources of proteins (Richard-Hansen & Hansen, 2004).
479 Subsistence hunting and deforestation remains however scarce (only slash and burn
480 subsistence agriculture) and this is consistent with the upstream site species richness
481 remaining higher compared to the most downstream sites, despites hunted species
482 abundances were shown to be locally reduced by Richard-Hansen et al. (2019).

483 *Challenges and applications*

484 Although we globally retrieved consistent patterns of species distribution/richness that
485 are comparable to line transects, aquatic eDNA metabarcoding for assessing non-aquatic

species has some limitations. Among those limitations, false negative (i.e. missing observations of present species) is a common challenge encountered in most (if not all) survey methods (Tyre et al., 2003). While with aquatic communities such as fish, the species detectability may be conditioned by species relative abundance or species morphology and physiology (Hunter, Ferrante, Meigs-Friend, & Ulmer, 2019; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016b), false negatives may be more frequent when assessing non-aquatic fauna as those species are less (or not directly) in contact with the water. The heterogeneous liberation of DNA in the water is then dependent on species density, species morphological and physiological characteristics but also species behaviour and water affinity (Harper et al., 2019), probably influencing the detectability of species. Moreover, site characteristics and environmental conditions may also influence the quantity of eDNA retrieved and therefore impact the false negatives rate and the inventories (Lacoursière-Roussel, Côté, Leclerc, & Bernatchez, 2016a; Lacoursière-Roussel et al., 2016b; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Roussel, Paillisson, Tréguier, & Petit, 2015). In our study system, Cantera et al. (2019) demonstrated that for a same sampling effort, fish community inventories were significantly less exhaustive in large compared to small watercourses. We therefore cannot exclude such sampling effect between small and large watercourse.

Moreover, the spatial signal of eDNA (spatial extent of the downstream transport of eDNA) defining the spatial grain of the inventories may also be a determining parameter to consider when assessing the presence of species (Hauger, Hollis-Etter, Etter, Roloff, & Mahon. 2020). In our systems, Cantera et al. (2020) demonstrated that the downstream detection of eDNA was short (not exceeding few kilometres) but it might already be enough to observe vulnerable species in areas where the hunting pressure is concentrated on a small spatial extent (from 2 up to 5 km in the periphery of the river; Richard-Hansen et al., 2019).

These limits interrogate to which extent should aquatic eDNA metabarcoding be used for biodiversity monitoring and particularly species of concern including invasive, pathogenic, threatened, endangered and other vulnerable species. In our study, incidental detections (unanticipated detection of species of concerns) may be precious to improve knowledge on species distributions, but the lack of regularity and exhaustivity of the method may represent a risk if used as the sole method to assess the presence of such species or to monitor the state of biodiversity (See Darling et al. (2020) for a review on this aspect).

Despite those limits, we believe aquatic eDNA metabarcoding provides an efficient way to complement and extend traditional inventories with common, rare, and endangered species as illustrated by the six species of UICN concern and the fiv species classified as very rare observed. Moreover, aquatic eDNA metabarcoding provides presence data for species not detectable in traditional surveys, be they aquatic or nocturnal. For instance, the widespread distribution of kinkajou revealed by eDNA strikingly contrasts with the rarity of visual observations, but cope with local camera trap experiments revealing its local commonness (Coutant, 2019). Aquatic eDNA metabarcoding therefore offers a way to extend our knowledge on mammal occurrences. Despite a lower observational frequency than the traditional line transect method for diurnal and terrestrial fauna, the sampling effort needed to collect an eDNA sample (no more than a couple of hours for a single person) makes it easily implementable together with line transect or other surveys to complement and extend inventories.

Conclusion

The present study demonstrated that aquatic eDNA metabarcoding represents a promising fast and efficient method to inventory both aquatic and terrestrial mammal fauna in Neotropical species rich environments. In a global context of accelerated biodiversity

erosion, the development of efficient methods to assess biodiversity is a prerequisite for conservation. We therefore believe that aquatic eDNA metabarcoding provides a unique opportunity to extend rapidly our biodiversity knowledge, and even if some challenges still need to be addressed, it can already be used as a complementary method to existing mammal inventory techniques without investigating considerable fieldwork efforts.

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Conflicts of interest

AV and TD are research scientists in a private company specialized on the use of eDNA for biodiversity monitoring (SPYGEN).

Data accessibility

The reference database sequences and all Illumina raw sequence data will be available on Dryad.

Supporting Information

Additional Supporting Information may be found in the online version of this article

Author contributions

O.C., S.B., J.M., C.R.H., and B.d.T conceived the ideas and designed methodology; O.C., S.B., J.M., J.B.D., and R.V. collected the data; A.V. and T.D. conducted the laboratory work; A.V. and T.D. conducted bioinformatic analyses; O.C. analysed the data; O.C., S.B., J.M., C.R.H., and B.d.T led the writing of the manuscript. All authors contributed critically to the drafts.

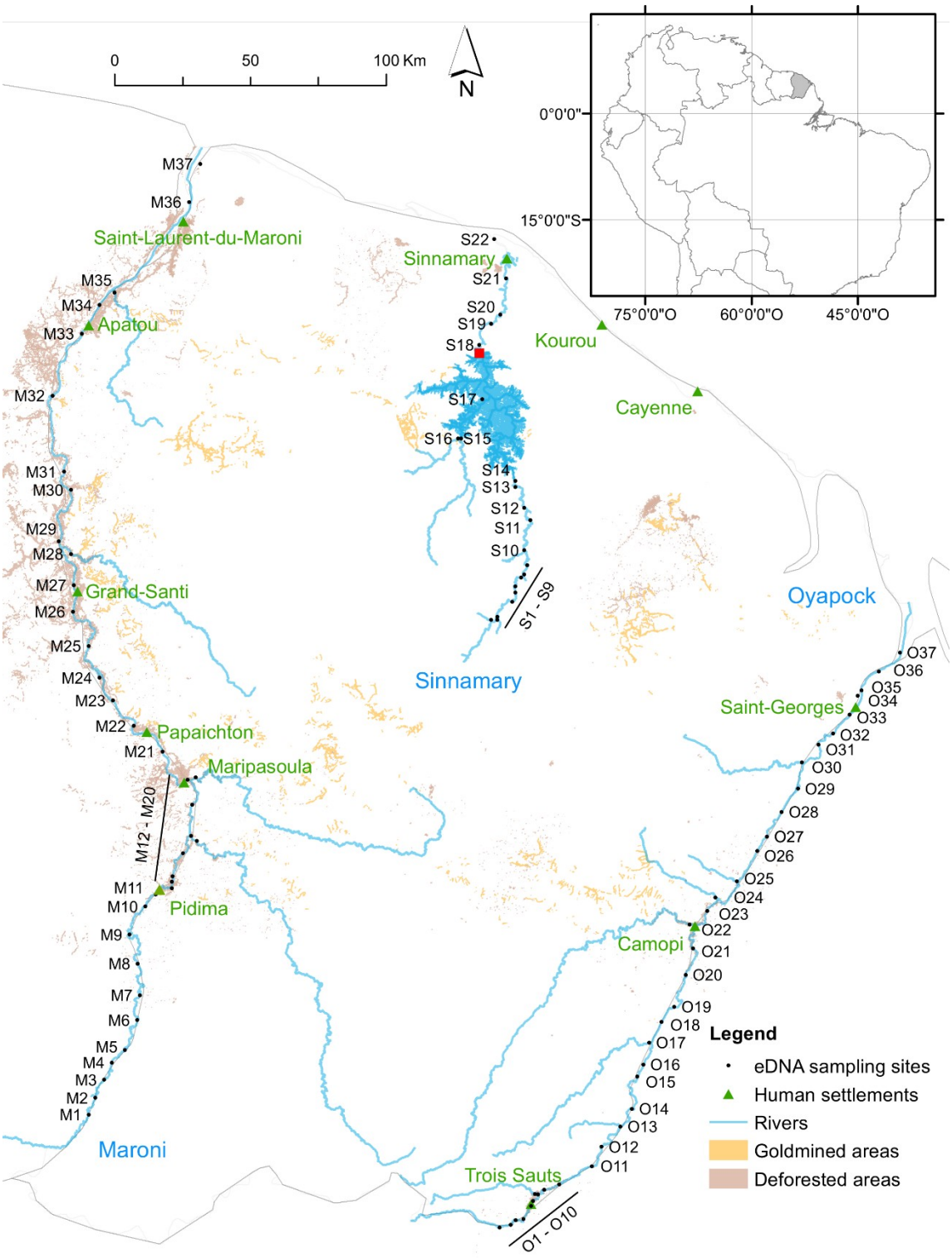

Figure 1. eDNA sampling sites. M1 to M37 indicate the sites sampled on the Maroni river, S1 to S22 the sites sampled on the Sinnamary river and O1 to O37 those sampled on the Oyapock river. Information about gold-mined surfaces was compiled by the WWF using Landsat satellite images of deforestation due to gold-mining in 2015 (WWF. 2016). This dataset represents the most recent information available on gold-mining over the Guianese territory. Forest loss surfaces were extracted using the Global Forest Change dataset (Hansen et al., 2013). This dataset identifies the areas deforested from 2001 to 2017 using global Landsat satellite image at 30 meters spatial scale. The red rectangle on the Sinnamary river represents the dam location. Inset map indicate the location of French Guiana in South America.

795 **Figures**

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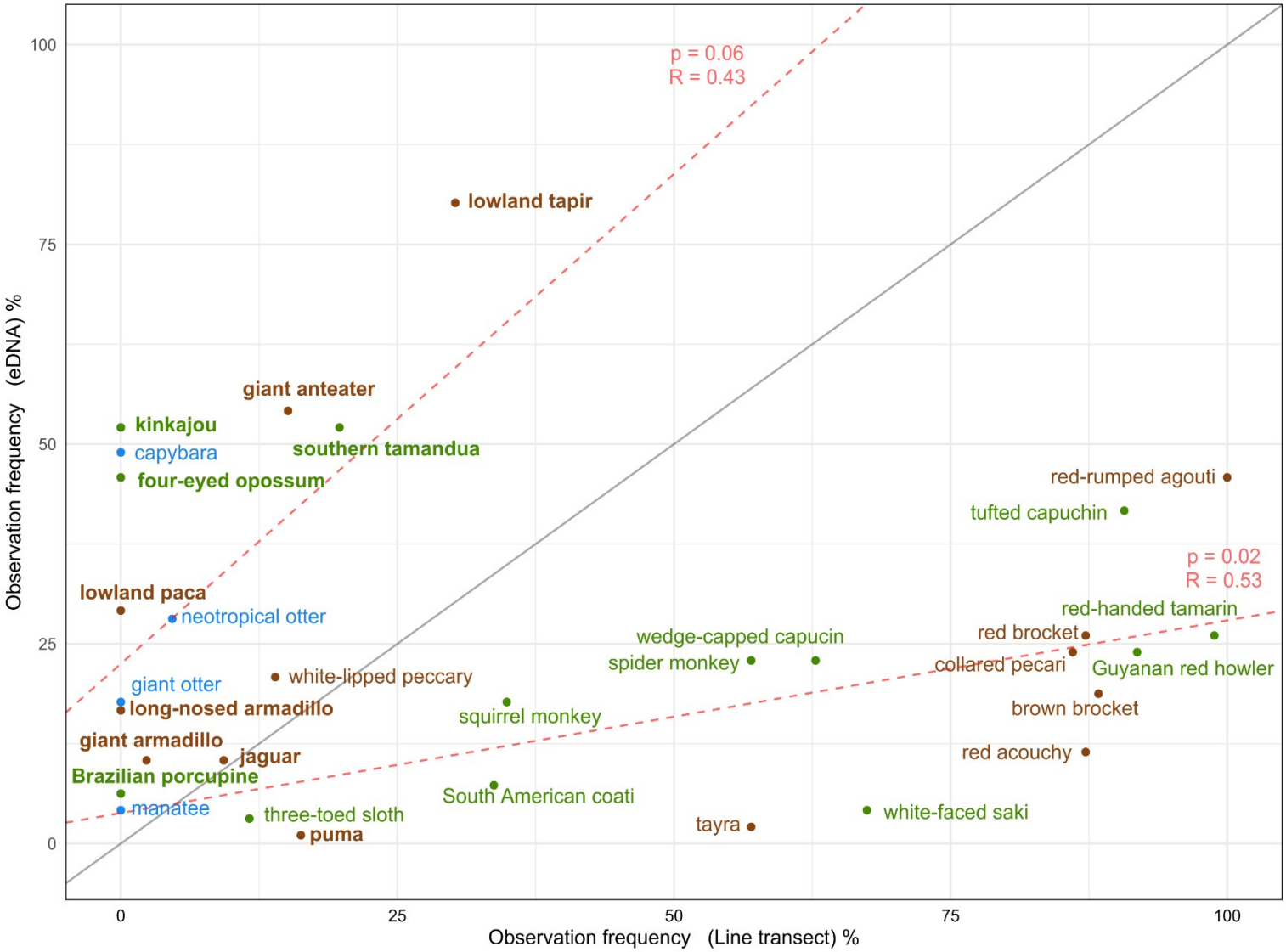

Figure 2. Observation frequency (%) of the emblematic mammalian fauna obtained with eDNA metabarcoding and with line transects. Species in blue are aquatic or semi-aquatic, species in green are arboreal and species in brown are terrestrial. Bold species names refer to nocturnal species while regular font corresponds to diurnal species. The dotted lines correspond to the linear regressions with the pvalue (p) and correlation coefficient (R) indicated in red.

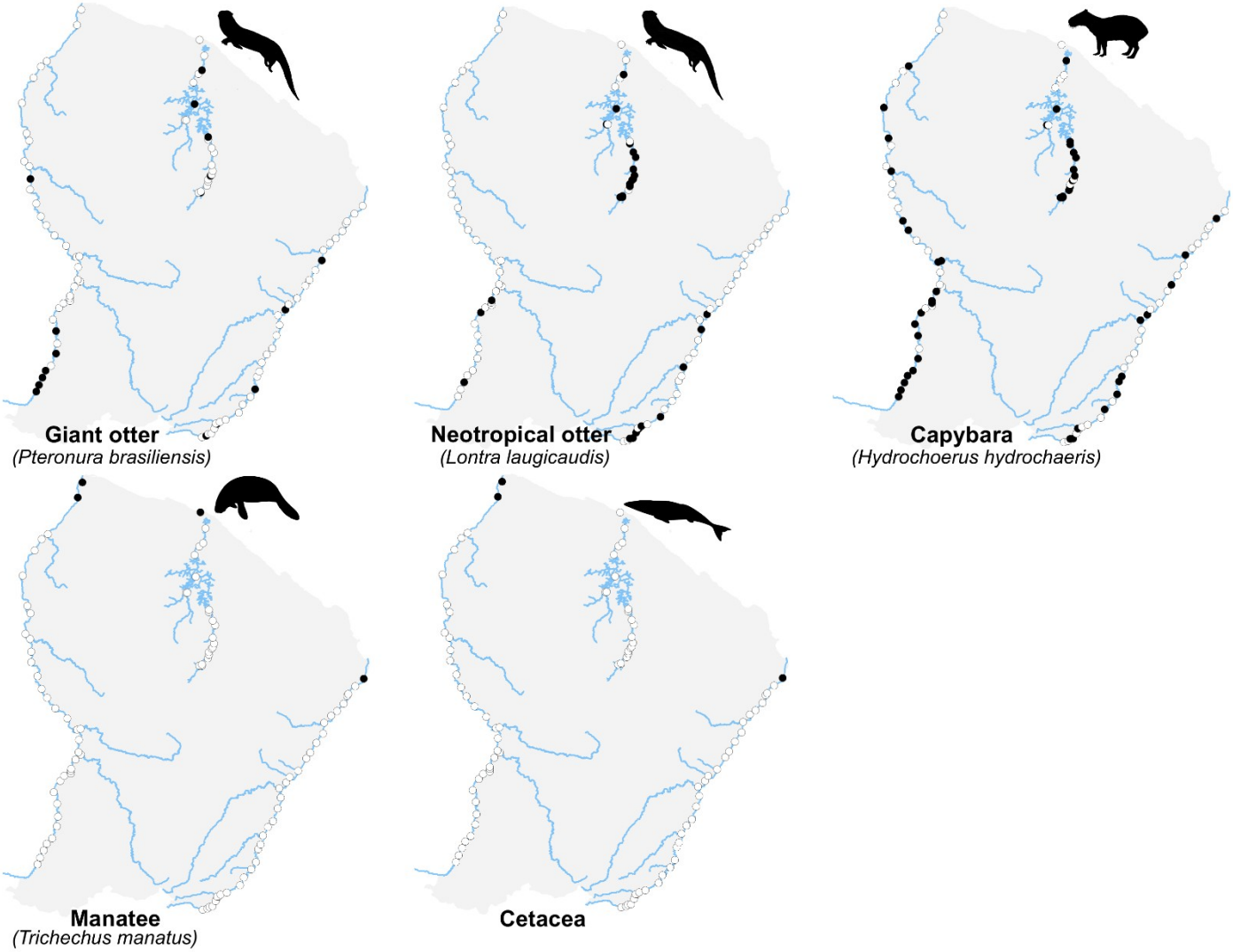

Figure 3. Species occurrences of several aquatic and semi-aquatic species. Presence (black dots) or absence of observations (white dots) in eDNA sampling sites are indicated on the maps.

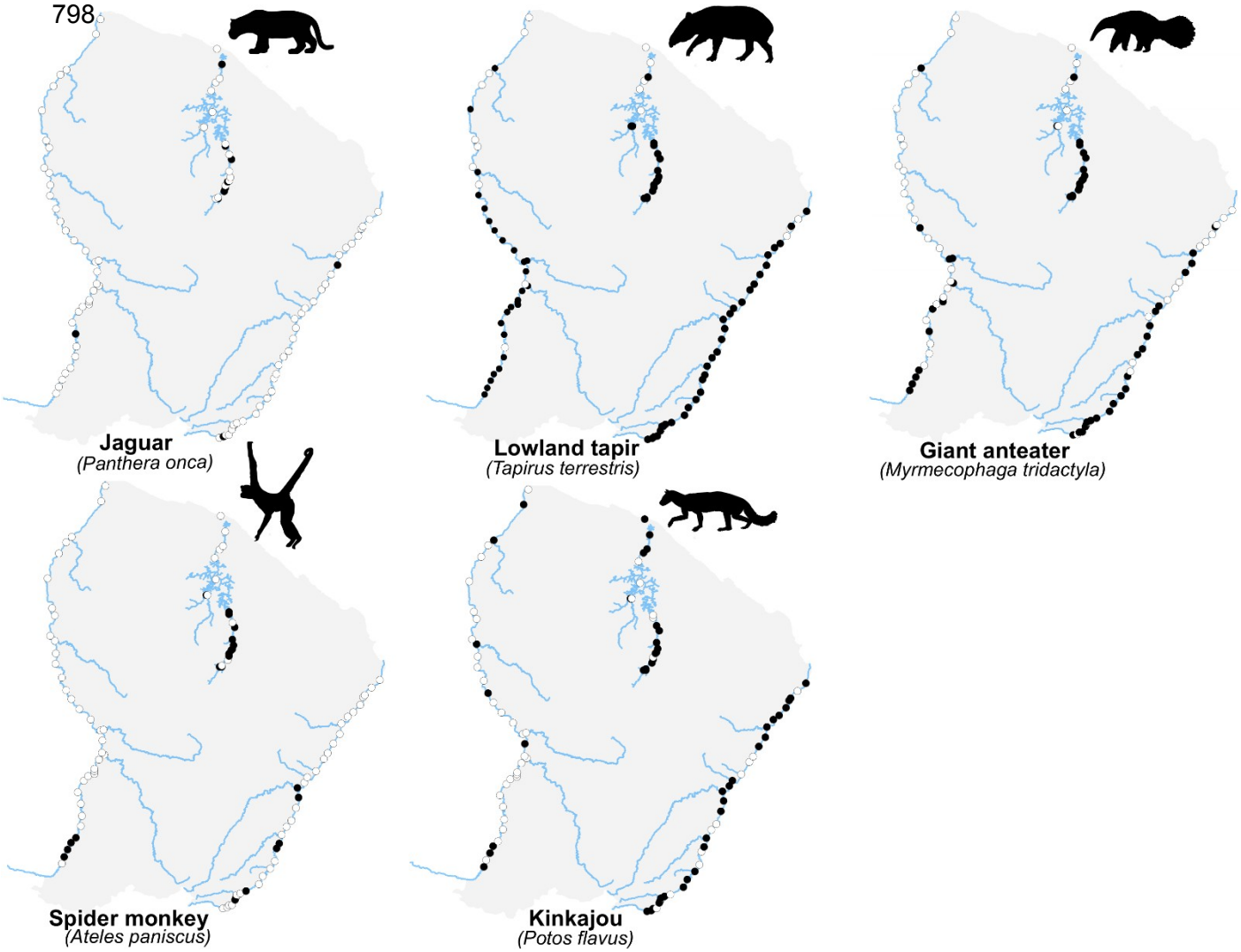

Figure 4. Species occurrences of several terrestrial or arboreal species. Presence (black dots) or absence of observations (white dots) in eDNA sampling sites are indicated on the maps.

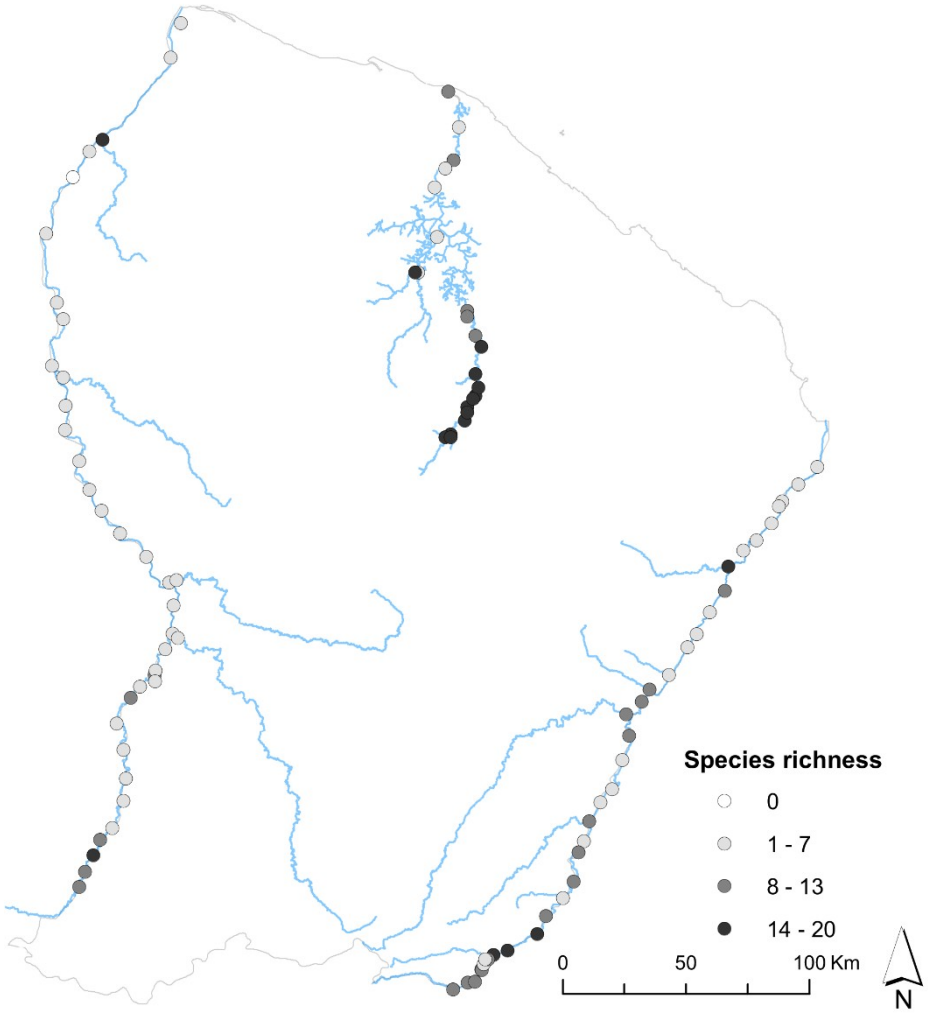

Figure 5. Emblematic mammal's species richness observed at each eDNA sampling site