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Unraveling the dietary diversity of Neotropical top predators using scat DNA metabarcoding: A case study on the elusive Giant Otter

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Abstract

Large carnivores play a pivotal regulating role in maintaining healthy and balanced ecosystems; however, most of them are rare and elusive, and knowledge about their resource consumption is scarce. Traditional methods based on morphological identification of undigested remains are labor intensive and often not sufficiently accurate, leading to errors and biased ecological inferences. Here, we developed a multi-marker DNA metabarcoding approach to analyze the dietary diversity of giant otters (*Pteronura brasiliensis*) from fecal DNA while controlling predator species identity. We combined two mitochondrial markers, 12S rRNA and cytochrome c oxidase 1 (COI) gene, that target the full range of potential vertebrate and invertebrate prey. We compiled a local reference database of DNA barcodes for most potentially ingested fish, which were used to evaluate the specificity of the metabarcoding primers in silico. Most prey are identified at the species level (>90%) and the dietary profiles provided independently by the two markers are highly similar, whether in terms of list of prey or frequency of occurrences, hence validating the approach. We detected a higher number of rare fish prey with the 12S primers that amplified solely Teleost species while the degenerate COI primers revealed non-fish prey (e.g., amphibians, snakes, birds, and earthworms) and confirmed predator species identity. This study demonstrated that scat DNA metabarcoding is particularly useful to provide in-depth information on elusive carnivorous dietary profile. Our methodology opens up new opportunities to understand how top carnivores diet cope with the effects of anthropogenic alteration of ecosystems and identify conflicts with humans and livestock.

KEYWORDS
12S rRNA, COI, high-throughput sequencing, large aquatic carnivore, molecular diet, Neotropical wetland, prey detection, trophic interactions

1 | INTRODUCTION

Large predators have a major impact on the structure and function of ecosystems by limiting the populations of prey (Prugh et al., 2009; Ritchie et al., 2012) and structuring food webs through top-down trophic cascades (Paine, 1980). They therefore play a central role in a wide range of critical ecosystem processes (e.g., herbivory, predation, circulation of pathogens, flows of energy, and matter) and enhance biodiversity (Estes et al., 2011; Soulé et al., 2003; Wallach et al., 2015). Apex predators also represent the most imperiled species worldwide, primarily because their food requirements and wide-ranging behaviors bring them into conflicts with humans and livestock (Ripple et al., 2014). To define and set up relevant conservation and management practices, it is essential to have accurate knowledge on their resource consumption. Most carnivores are rare and elusive making observational studies logistically difficult (Long et al., 2007). Diet analyses thus traditionally rely upon the morphological identification of undigested remains in non-invasively collected feces. However, this approach is labor intensive, requires strong taxonomic skills, and is often not sufficiently accurate to discriminate related taxa, potentially leading to errors and biased ecological inferences (Morin et al., 2016; Weiskopf et al., 2016).

The development of high-throughput sequencing, DNA barcoding methods and databases, and the so-called “DNA metabarcoding” approach (Taberlet et al., 2012) now allows researchers to characterize the dietary range of hundreds of animals simultaneously from low quality/quantity eDNA in fecal or stomach contents (Alberdi et al., 2019; Ando et al., 2020). Recent studies illustrated that scat DNA metabarcoding (sDNA metabarcoding hereafter) is particularly relevant to carnivore research as it is much more cost- and time-efficient than conventional morphological approaches and offers a higher taxonomic resolution (Havmøller et al., 2020; Shehzad et al., 2012; Xiong et al., 2017). However, its use has remained marginal (<10% of the scat-based carnivore diet analyses, Monterroso et al., 2019) and it has rarely been applied to top predators in tropical ecosystems (but see Havmøller et al., 2020) despite promising results on herbivores (Alberdi et al., 2019; Hibert et al., 2011; Mallott et al., 2018; Quéméré et al., 2013), hematophagous arthropods (Rodgers et al., 2017), or bats (Bohmann et al., 2018). This lack of studies on top predators in tropical compared to temperate areas (Deagle et al., 2010 on little penguins; Shehzad et al., 2012 on leopard cats; Kumari et al., 2019 and Buglione et al., 2020 on Eurasian otters) may be partly attributed to specific methodological issues. First, the efficiency of sDNA metabarcoding is highly dependent on the accuracy and completeness of reference DNA barcode databases (Alberdi et al., 2019; Zinger et al., 2020). Tropical ecosystems house a considerable diversity of potential prey, of which only a part have been formally described and named, which makes the compilation of reference library particularly difficult (Quéméré et al., 2013). Second, sDNA yield depends on time since defecation and environmental conditions (Thuo et al., 2019). eDNA is much more prone to degradation in warm, tropical humid regions reducing the ability to detect prey (Ruppert et al., 2019).

Here, we investigate the dietary diversity of a giant otter population (*Pteronura brasiliensis*, Zimmerman, 1780) inhabiting a large wetland area in French Guiana in the northeast of the species range. The giant otter is one of the largest and most iconic river carnivores in South America. Once targeted by the pelt trade to near extinction, the species has been legally protected since 1973 and populations have since increased substantially (Duplaix et al., 2015). The species is still classified as “endangered” by the IUCN (2021) due to intensifying anthropogenic pressures including habitat loss and degradation, water pollution, ecotourism, and gold mining (Rosas-Ribeiro et al., 2012). There are very few studies on feeding habits of giant otter, and most have been carried out in the Amazon drainage (Cabral et al., 2010; Rosas-Ribeiro et al., 2012; Silva et al., 2014). Giant otters are primarily piscivorous but may opportunistically consume crustaceans, molluscs, and terrestrial vertebrates (Duplaix, 1980; Noonan et al., 2017). Detailed dietary profiles, ideally accurate to the species level, are necessary to identify favorite feeding resources, understand potential conflicts with fishermen, and better characterize the trophic role of giant otters in tropical freshwaters ecosystems.

We here used a multi-marker DNA metabarcoding approach (Da Silva et al., 2019; da Silva et al., 2012) to assess the dietary diversity of giant otters from fecal DNA while controlling predator species identity (i.e., checking fecal samples really came from giant otters). To reliably identify fish prey, we combined two mitochondrial markers (12S rRNA and cytochrome c oxidase 1 [COI] gene) that partly overlap in the range of taxa potentially identified. For both markers, we compiled a local reference database of barcodes for most of the fish inhabiting the studied ecosystem and used these databases to evaluate the specificity of the metabarcoding primer in silico. We compared the diversity of prey retrieved by the two markers, discussed their strengths and limitations for tropical carnivorous diet analysis, and examined our results with regard to other published data on giant otter diet based on morphological identification of undigested hard parts. While 12S primers amplify solely teleost species (Valentini et al., 2016), the COI marker with degenerate primers was designed to amplify a large range of vertebrates and invertebrates (Tournayre et al., 2020). We therefore investigated its ability to identify potential macroinvertebrate prey despite many expected environmental contaminations.

2 | MATERIAL AND METHODS

2.1 | Study area and fecal collection

The study was conducted in the Kaw-Roura Marshes Natural National Reserve, a 94,700 ha reserve in the eastern coast of French Guiana (4°36′N, 52°07′W) in the Guiana Shield region (Figure S1). The reserve is mainly covered by seasonally flooded savannah with intermittent patches of palm swamp forest (Caut et al., 2019). The seasons in French Guiana are marked by a small wet season (Dec-Feb), a small dry season (Mar), a long wet season

(Apr-Jul), and a long dry season (Aug-Nov), which have a profound impact on the marshes around the Kaw river which fluctuate between dry savanna and flooded savanna. Members of giant otter groups defecate in communal latrines (Duplaix, 1980) in conspicuous places, usually located near otter campsites on riverbanks or small islands. Known latrines and other potential sites were regularly visited between January and March 2018 (48 days of sampling in total), spanning the change from the small wet season (Dec-Feb) and the small dry season (Mar). Spraints (i.e., the dungs of otters) were sampled from 12 communal latrines in three areas of the Kaw-Roura Reserve (Figure S1). A total of 59 spraints were collected using single-use gloves (Figure S2). Each spraint was placed in a sterile bag and stored at −20°C until DNA extraction. The research permit for sample collection was obtained from the DGTM Guyane.

2.2 | In silico evaluation of COI and 12S primer sets

To assess the dietary diversity of giant otters, we used two metabarcoding mitochondrial markers selected based on the literature: (a) The 12S rRNA region using the "12S-Teleo" primers described in Valentini et al., (2016) (teleo_F 5′-ACACCGCCCGTCACYCT-3′ and teleo_R 5′-CTTCCGGTAYACTTACCATG-3′). This marker specifically designed for fish DNA metabarcoding has a similar taxonomic coverage and resolution than the alternative primer pairs proposed by Miya et al., (2015) but amplified fragments of nearly half the size (*c.* 64 bp); (b) a 133 bp of the COI gene using degenerate primers based of an improved version of the Gillet et al., (2015) primers (MG2-LCO1490 5′-TCHACHAAYCAYAARGAYATYGG-3′ and MG2-univ-R 5′-ACYATRAARAARATYATDAYRAADGCRTG-3′; Tournayre et al., 2020). Carnivore scat misidentification is a common issue (e.g., one-fifth of all samples in diet studies in the review of Monterroso et al., 2019) and the molecular identification of the predator is highly recommended. This second minibarcode which targets a wide range of invertebrates and vertebrates in both temperate and tropical regions (Galan et al., 2018; Sow et al., 2019; Tournayre et al., 2020) was also used to control the identity of the predator (i.e., presence of *P. brasiliensis* DNA).

We evaluated the efficiency of the COI and 12S primer sets to amplify the 17 fish families present in the study area using the R package PrimerMiner v0.21 (Elbrecht & Leese, 2017a). All following analyses were run in R 3.4.3 (R Core Team, 2019). For each fish family, complete mitochondrial genomes were downloaded from NCBI (and BOLD for COI), using the *batch_download* function picking sequences from a maximum number of different genera including those inventoried in the study area when possible (1 to 117 genomes per fish family, 227 in total). Sequences were aligned separately using MAFFT v7.017 (Katoh et al., 2002) as implemented in GENEIOUS v8.1.7 (Kearse et al., 2012). Sequences upstream/downstream of the primer binding sites were trimmed and the alignments were visualized with PrimerMiner to check the specificity of primers. For each primer and fish family, we calculated a mean penalty score using the *evaluate_primer* function. This score is calculated as mismatch scoring that considers the adjacency, position, and type of mismatch between primers and template sequence by using the default tables for mismatch scoring. Primers that obtained a penalty score >120 were considered as inappropriate (Elbrecht et al., 2019; Elbrecht & Leese, 2017b).

2.3 | Laboratory procedures

DNA was extracted from the 59 collected spraints using the QIAamp DNA Stool Mini Kit (QIAGEN) following instructions of the manufacturer with filter tips under a sterile hood in a PCR-free room. Each spraint was crushed and mixed in sterile water, and about 0.2 g of homogenized sample was used for DNA extraction. Samples were processed in batches of 24 including a negative control. Three independent amplicon sequencing libraries were built for each sample and marker using the two-step PCR strategy from Galan et al., (2018). We used a unique dual-indexing (Kircher et al., 2012) to reduce the index-hopping and make sure that libraries were sequenced and demultiplexed with the highest accuracy. Negative controls for extraction (NCext, 1 per DNA extraction session), PCR (1 NCpcr), and indexing (1 NCindex) were included on each 96-PCR microplates. PCR1 and PCR2 were performed using 2X QIAGEN Multiplex Kit Master Mix (QIAGEN). Sequences of PCR1 (gene-specific amplification) and PCR2 (indexing) primers and PCR conditions are detailed in Supplementary Methods S1. PCR products were checked by electrophoresis in 1.5% agarose gel before being pooled by volume (1 pool for each marker). Size selection was used to discard non-specific PCR products and primer dimers by excision on a low-melting agarose gel (1.25%) followed by a gel purification using the PCR Clean-up Gel Extraction Kit (Macherey-Nagel). The expected size of amplicons (including primers, indexes, and adaptors) is between 200 and 230 bp for 12S rRNA gene and 312 bp for COI. The two pools of libraries were quantified by qPCR using the KAPA library quantification kit (Roche), normalized at 4 nM and sequenced with a V2 500 cycle-kit reagent cartridge (Illumina) for 2 × 200 bp paired-end sequencing on an Illumina MiSeq platform (targeting about ~6600 reads per PCR replicate, ~20,000 per sample).

2.4 | Local reference database of barcodes

From the list of 113 freshwater fish species inventoried in the Kaw-Roura Marshes (Le Bail et al., 2012; Meunier et al., 2011), we compiled two local reference databases including 12S and COI DNA barcodes for 92 (81%) and 99 (87%) species, respectively (Table S1). For 12S, we used sequences compiled by Cilleros et al., (2018). COI sequences were obtained from BOLD (Ratnasingham & Hebert, 2007) through the ongoing project Gui-Bol (Barcoding Guianese fishes, access to fish identification through identification engine: https://www.boldsystems.org/index.php/IDS_OpenIdEngine).

2.5 | DNA sequence processing and denoising

The raw sequence reads were quality trimmed, and the adapter sequences removed using Cutadapt v2.9 (Martin, 2011). The remaining high-quality sequences were analyzed using the OBITOOLS package (http://metabarcoding.org/obitools; Boyer et al., 2016). The *illumina-pairend* program was used to assemble forward and reverse reads. Paired sequences were then assigned to each sample using *ngsfilter* and strictly identical sequences were clustered together using *obiuniq* (dereplication step). The sequences with total occurrence lower than 10 reads and shorter than 30 bp were removed using the *obigrep* command. To denoise the dataset, we first run the *obiclean* command with a maximum number of differences between variants (–d parameter) of 1. We kept only Molecular Operational Taxonomic Units (MOTUs) that were more often "head" or "singleton" than "internal" in the global dataset, "internal" reads being potential PCR substitutions or indel errors (Giguet-Covex et al., 2014). The *isBimeraDenovo* command from the dada2 package (Callahan et al., 2016) was also applied to identify and discard additional chimeric sequences. For COI, we kept only sequences between 130 and 139 bp. Lastly, to remove false-positive results, we discarded (a) not-shared occurrences among technical replicates (Robasky et al., 2014) (i.e., MOTUs observed in only one of the three replicates); and (b) MOTU occurrences with sequence counts below a MOTU-specific threshold corresponding to the maximum number of reads observed in a negative control for each MOTU. For each sample and MOTU, the remaining reads from the three technical replicates were summed in the abundance table.

2.6 | Taxonomic assignment

The taxonomic assignment of 12S and COI MOTUs was performed using the program *ecotag*, which assigned the query sequence to the last common ancestor in case the identification was ambiguous among sibling taxa (Boyer et al., 2016). Taxonomic assignment was first attempted based on the local "Kaw-Roura" reference databases of 12S or COI fish barcodes. In a second step, the remaining 12S unassigned sequences were taxonomically compared to a custom-made database built by performing in silico PCR (program *ecoPCR*) with the "12S-Teleo primers" (Ficetola et al., 2010) from all available 12S rRNA sequences in Genbank (release 141). For COI sequence analysis, we employed a two-step procedure: we first used the identification system (IDS) search algorithm in BOLD (Ratnasingham & Hebert, 2007). Then, for sequences with similarity results lower than 97% in BOLD, we tried to improve identification by matching sequences against an "ecoPCR" database extracted from Genbank built using the MG2-LCO1490/MG2-univ-R primers. MOTUs with best similarity score >97% with either the local, BOLD, or Genbank databases were assigned to a species or to a genus in cases of ambiguous identification at the species level (e.g., sibling taxa with identical barcodes). When the best match score is <97%, we applied different assignment rules for the two markers that have different rates of evolution: for 12S, when the best match score is <97% but >95%, sequences were assigned at the family level using the closest taxa while MOTUs <95%. For COI, MOTUs with similarity >90% and <97% were assigned at the Phylum or Class level (for Chordata), using the closest taxa. For both markers, MOTUs <90% were considered as unclassified taxa.

2.7 | Statistical analysis

Taxonomic dietary descriptions were summarized by frequency of occurrence at the family and species level. Statistical analyses were performed in R 3.4.3 (R Core Team, 2019) with the vegan 2.5–7 package (Oksanen et al., 2020). To evaluate the efficiency of our sampling effort and estimate the expected number of undetected MOTUs, dietary richness rarefaction curve was generated and extrapolated using the Chao method (Chao et al., 2014). To visualize patterns in dietary dissimilarity among sites ($N = 3$), sampling months (January, February, and March), and markers (COI vs. 12S), we performed non-metric multidimensional scaling (NMDS) ordinations based on a Jaccard coefficient matrix. We conduced permutational multivariate analysis of variance (PERMANOVA) with 1000 permutations to test for significant differences in fish prey composition among groups (Anderson, 2001).

3 | RESULTS

3.1 | In silico evaluation of 12S and COI primer sets

In silico evaluation of the potential fish family prey (227 mitochondrial genomes from the 17 fish families surveyed in the study area) showed mostly little or zero penalty values for the degenerate COI (mean = 2.5, ranging from 0 to 37.6) and 12S primers (mean = 7.39, ranging from 0 to 90.9, Table S2). For both markers, sequence alignments revealed very few mismatches between primers and template-binding sites (Figure 1).

3.2 | Sequence data analysis

The MiSeq sequencing of the 192 PCR products (3 replicates for 59 samples +5 negative controls) generated 1,419,319 reads for the 12S (24,056 reads/sample in average) and 1,871,773 reads for the COI (31,724 reads/sample). After the successive filtering and denoising steps, 1,118,638 reads (78%) corresponding to 31 unique sequences (MOTUs) were retrieved for 12S and 1,152,649 reads (70%) corresponding to 2726 MOTUs for COI. All except one fecal sample (i.e., 58 out of the 59 samples) included giant otter DNA and were therefore kept for further analysis. For 12S, negative controls included only human DNA with a maximum number of 782 reads. For COI, MOTU-specific thresholds (i.e., maximum number of reads observed in negative controls for each MOTU)

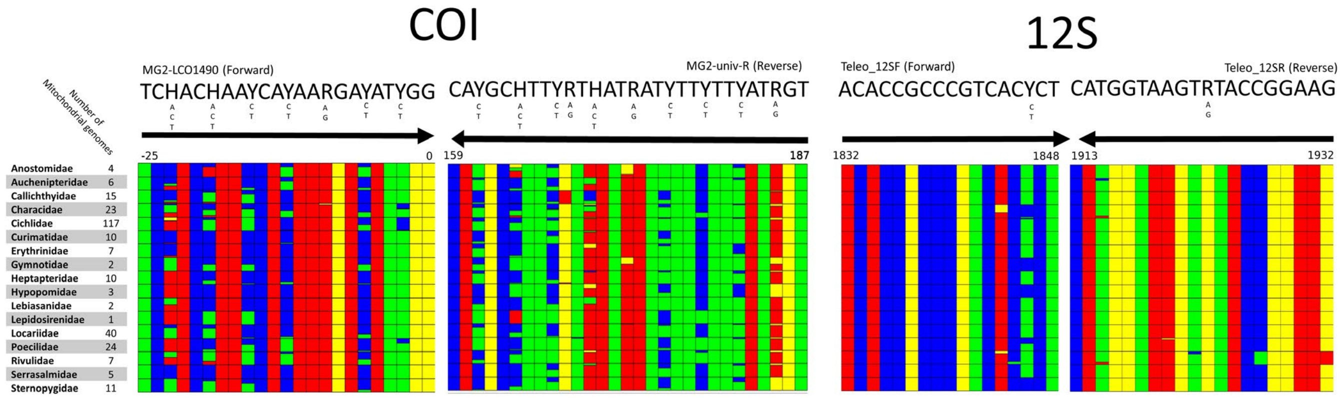

FIGURE 1 In silico evaluation of COI and 12S primers. Base composition plots (green = thymine, blue = cytosine, red = adenine, yellow = guanine) generated with PrimerMiner for the MG2-COI and 12S-Teleo primer binding sequences. For each fish family present in the study area, sequences were extracted from complete mitochondrial genomes downloaded from NCBI (and BOLD for COI). The COI and 12S primer positions are relative to the Folmer region (Folmer et al., 1994) and the *Cyprinus Carpio* mitochondrial genome (Valentini et al., 2016), respectively

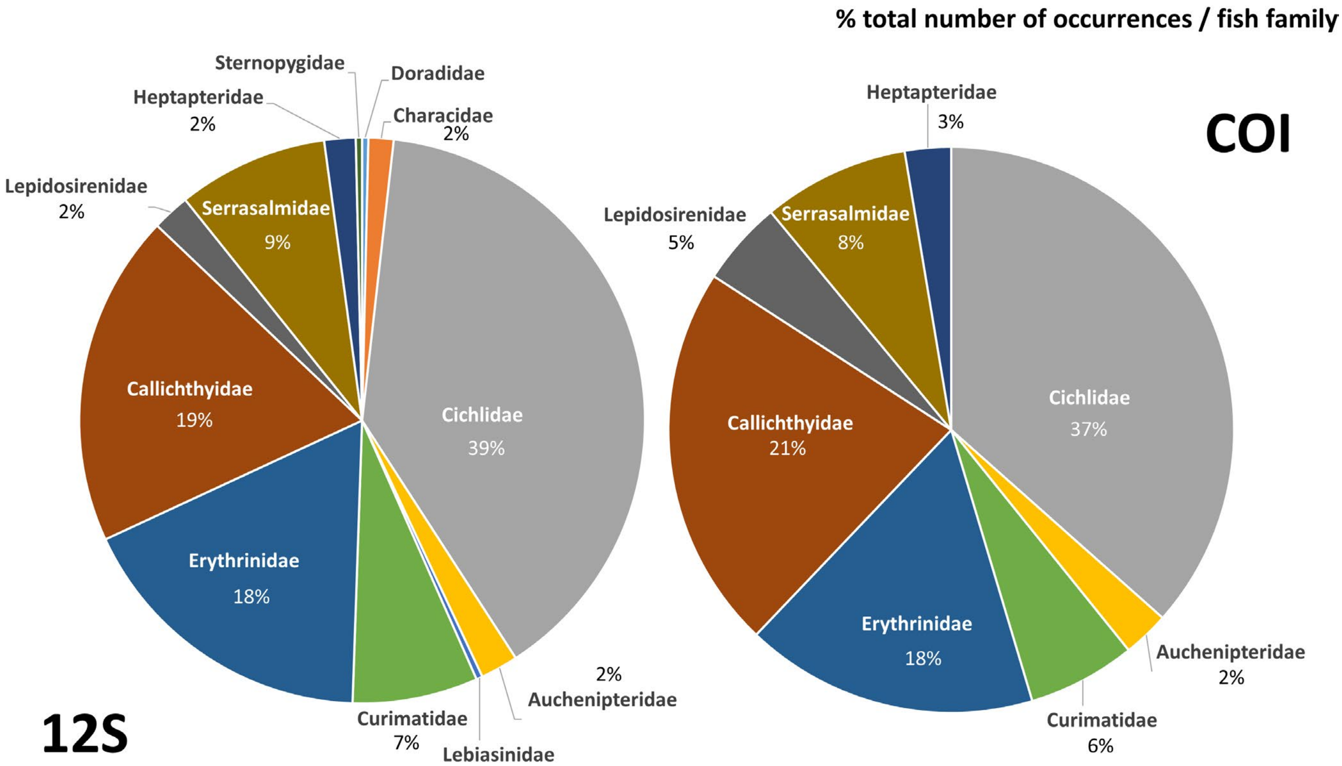

FIGURE 2 Proportion of occurrences of each fish prey family (FO_T) for 12S rRNA and COI. FO_T was estimated based on the 316 (for 12S) and 245 (for COI) occurrences found in the 58 feces samples

varied from 24 to 2249 reads, assigned to human or bacteria/unassigned taxa DNA. None of these contaminations corresponded to potential prey.

3.3 | Prey taxonomic identification and diversity

3.3.1 | 12S

The 12S rRNA marker revealed 31 fish prey taxa from 25 different genera and 12 families (Table S3). Most prey, 28 MOTUs out 31, were identified at the species level for a total of 25 species. In some rare cases, several MOTUs with sequence identify >97%, and most often occurring in different samples, were assigned to the same species (three MOTUs to *Crenicichla saxatilis* and two MOTUs to *Hoplias malabaricus*). The number of 12S MOTUs per spraint varied between 1 and 12 (mean = 5.44, SD = 2.76) totaling 316 taxa occurrences. The most frequent prey belonged to the Cichlidae (FO_T = 39%, FO_T is the frequency of occurrence across the total of 316 taxa occurrences), followed by Callichthyidae (19%), Erythrinidae (18%), Serrasalmidae (9%), and Curimatidae (7%) (Figure 2). *Hoplosternum littorale* (Callichthyidae) was the most frequently found prey species in the giant otter spraints with a frequency of occurrence (FO_S, here FO_S is the frequency of prey species across spraints) of 84%, followed by *Hoplias malabaricus* (Erythrinidae, 69%, considering the two variants), and *Chaetobranchus flavescens* (Cichlidae, 60%) (Figure 3, Tables S3). We noted that the average FO_S is relatively low (19.10%, SD = 23.51%) with a substantial part of the prey (i.e., 14 out of the 24 identified species) found in five or less samples (FO_S < 10%). Chao2 asymptotic richness estimate (mean = 50.69 ± 23.21) suggested the presence of few undetected prey taxa compared to actual number prey detected (Figure S3).

3.3.2 | Cytochrome c oxidase 1

For COI, only 387 out of the 2726 MOTUs (14%) were assigned to a taxonomic group, of which less than half had an identity score >95% (165 MOTUs). Nevertheless, these 165 MOTUs represent more than half of the sequence reads (596,145 reads, i.e., 52%). Predator (Giant otter) DNA corresponded to only 8% of the assigned reads (3% of the sequencing effort). The vast majority of the 387 MOTUs were affiliated to invertebrates taxa including numerous arthropods (230 MOTUs) and rotifers (60 MOTUs) (Figure S4a,b). Arthropod MOTUs mostly corresponded to flies (129 MOTUs), small arachnids and springtail taxa that likely colonized the fecal samples after defecation (i.e., environmental contamination) or small aquatic crustaceans (e.g., copepods, branchiopods, and ostracods) likely resulting from secondary predation. We identified 27 potential prey taxa including 23 fish, 1 snake (*Eunectes sp.* most likely the green anaconda, 1 occ.), 1 amphibian (*Pipa pipa*, *1* occ.), 1 bird (*Cairina moschata*, two occ.), and 1 earthworm (*Pontoscolex corethrurus*, 6 occ.). Among the 23 fish taxa, we identified 19 species, corresponding to 19 genera and 9 families, with three MOTUs assigned to the same species (*Crenicichla saxatilis*) (Figure 3, Table S4). All species except *Trachelyopterus coriaceus* were also revealed with the 12S rRNA barcode. The number of COI prey items per spraint varied between 1 and 16 (mean = 4.31, SD = 2.54) totaling 245 occurrences. Chao2 asymptotic richness estimate (mean ± SE = 28.49 ± 0.85) is very close to actual number of prey taxa detected (Figure S3).

3.3.3 | Comparison of the fish diet between markers, sites, and sampling months

Frequencies of occurrence of fish prey taxa (FO_S, Figure 2) and fish family (FO_T, Figure 1) obtained using 12S and COI were remarkably similar. The NMDS ordination showed a strong dietary niche overlap between 12S and COI (Figure 4) with no significant difference in fish prey composition ($F_{1,114}$ = 0.59, r^2 = 0.005, p = 0.83). By contrast, fish diet varied significantly among sites ($F_{2,53}$ = 4.16, r^2 = 13.15, p = 0.001, Figure S5) but did not differ among sampling months ($F_{2,53}$ = 4.16, r^2 = 0.98, p = 0.48, Figure S6).

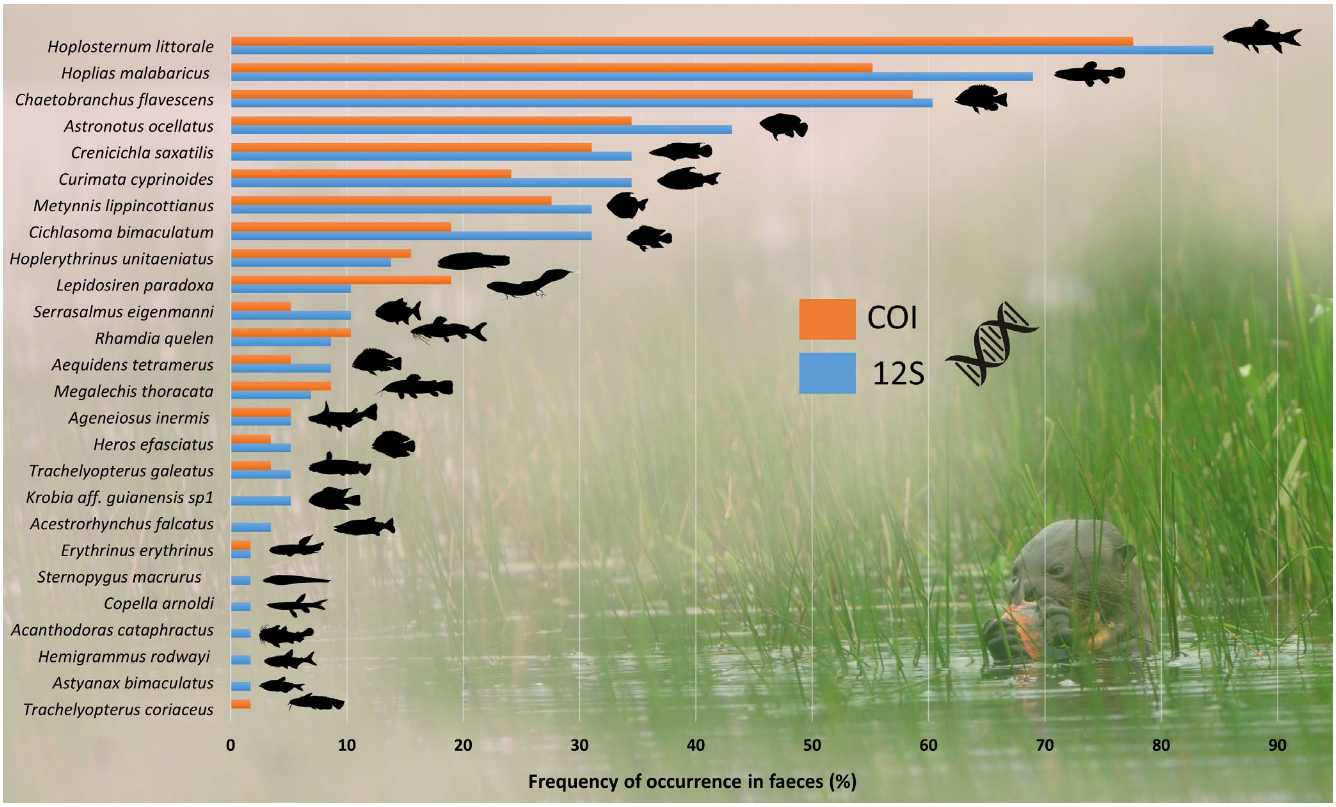

FIGURE 3 Frequency of occurrences (FO_S) of each fish prey species for COI and 12S rRNA. Occurrences from different MOTUs assigned to a same species were grouped together

4 | DISCUSSION

Apex predators play a pivotal regulating role in maintaining healthy and balanced ecosystems (Estes et al., 2011); therefore, we need accurate and complete knowledge on their trophic ecology. Here, we used a multi-marker scat DNA metabarcoding approach to examine the dietary range of giant otters inhabiting the Kaw-Roura seasonally flooded savannahs in French Guiana. The two major outcomes are (a) the high accuracy of taxonomic assignments with >90% of prey taxa assigned at the species level and (b) the remarkable similarity between the dietary profiles provided by the two genetic markers (12S and COI), whether in terms of list of fish prey, or number of occurrences per fish species and family, which provides a strong validation of the method. Below, we first review the strength and limitations of the metabarcoding approach for tropical carnivore diet analysis compared to traditional methods and provide some recommendations. Then, we discuss what the results reveal about the dietary ecology of giant otters and potential conflicts with fishermen.

4.1 | Powers and limitation of the DNA metabarcoding approach for tropical carnivorous diet analysis

4.1.1 | A significant gain of taxonomic resolution

The combined use of highly discriminant markers and of local reference databases of DNA barcodes offered a significant gain of resolution and sensitivity compared to the few previous studies on the giant otter diet based on direct observations or morphological identification of undigested remains (Cabral et al., 2010; Rosas et al., 1999; Silva et al., 2014). The great majority of prey taxa were identified at the species level (85% for 12S and 91% for COI) while previous work only provided identification at the family or genus level (Cabral et al., 2010; Silva et al., 2014). Our level of taxonomic resolution is comparable and even higher than previous metabarcoding studies on carnivores in temperate or polar regions where prey diversity is much smaller (Kumari et al., 2019 on Eurasian otters; Shehzad et al., 2012 on leopard cats; Deagle et al., 2010 on little penguins; Deagle et al. 2009 on fur seals). Such high accuracy can be largely explained by the presence of a nearly exhaustive local reference database of fish barcodes, itself based on a large and regularly updated inventory of the ichthyofauna of the study area by taxonomists (Le Bail et al., 2012; Meunier et al., 2011). Although a taxonomic expertise is not mandatory for the metabarcoding analysis itself, it is essential for the compilation, quality control, and regular curation of barcode reference databases (Santos & Branco, 2012; de Sousa et al., 2019). Particular attention should be paid to obtaining representative databases at least at the family level to limit misidentifications or unassigned sequences.

4.1.2 | Toward a more comprehensive overview of the dietary diversity and plasticity of carnivores

Because it is far less labor intensive than traditional approaches, the DNA metabarcoding approach is particularly suitable for carnivore diet analysis for which a large number or fecal samples are often required to have a comprehensive overview of the dietary range (Monterroso et al., 2019). In our case, the number of prey taxa per spraint was relatively low (five on average) likely because of the short transit time of giant otters (*c.* 3 hr, Carter et al., 1999). More than half of the prey had low frequency of occurrence (FO <10%)

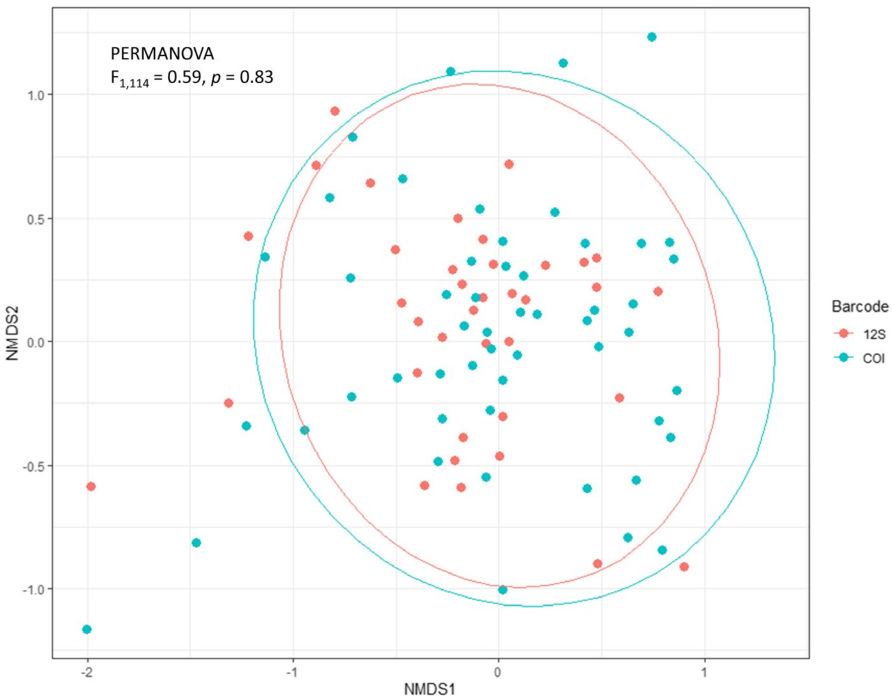

FIGURE 4 Nonmetric multidimensional scaling (NMDS) ordination of fish prey compositions according to markers (12S rRNA and COI). Ellipses represent the standard deviation of each group. *F*-score and *p*-value of the PERMANOVA (1000 permutations) are specified in the upper-left corner

and more than 50 samples were necessary to capture the dietary diversity of the local giant otter population as illustrated by the prey accumulation curves (Figure S1). The possibility to process hundreds or even thousands of samples in relatively short time paves the way toward more ambitious sampling designs (Alberdi et al., 2019) to investigate dietary shifts along environmental gradients and assess how large carnivores adjust their dietary requirements (i.e., dietary plasticity) in response to global change (de Sousa et al., 2019; Wong & Candolin, 2015).

4.1.3 | Using multi-marker metabarcoding data in dietary analysis of trophic generalists

Because there is no ideal metabarcode, it is increasingly recognized that molecular dietary studies of trophic generalists required a mix of markers that amplify the full diversity of prey ingested (Alberdi et al., 2019; De Barba et al., 2014; Taberlet et al., 2018 but see Elbrecht et al., 2019). Here, we combined two markers (12S and COI) that together target the full range of potential vertebrate and invertebrate prey of giant otters including fish, macroinvertebrates, reptiles, and small mammals (Duplaix, 1980). The two markers greatly overlapped in the range of prey amplified: 70% of the fish prey were identified by both 12S and COI (100% for the top 15) with very similar frequency of occurrences (Figure 3). Fish prey composition did not vary significantly between markers (Figure 4), therefore increasing confidence in the results.

The two barcodes also have their own strengths and limitations. The 12S-Teleo primer pairs (Valentini et al., 2016) are extremely robust, with highly conserved priming sites providing highly reliable DNA amplifications and sequencing. This limits PCR amplification bias and misidentification risks when analyzing samples with a mixture of phylogenetically distant fish prey with different starting amount of template DNA. Also, they amplify solely fish DNA without co-amplifying host, bacterial, and fungal DNA so more sequencing coverage can be harnessed to detect rare fish prey (i.e., represented by a small amount of DNA in the fecal samples). Another strength is the small size of the amplified fragments (<80 bp), which make the 12S-Teleo barcode particularly suitable to amplify fish DNA from fecal samples collected in the tropical realm where fecal DNA is rapidly degraded. It should be noted that in our case, the small size of the amplified fragments has no detrimental impact on the taxonomic resolution since most of the fish prey have been identified at the species level.

The COI minibarcode identified few vertebrate and invertebrate prey not revealed by the 12S but also missed several rare fish prey species ($N = 7$ species for total of seven occurrences). The sequences of the undetected species are present in both 12S and COI reference databases, and we checked the absence of mismatches between COI primers and template sequences (Figure 1). The most likely reason is amplification biases due to the hybridization of primers to unspecific DNA targets resulting in an insufficient sequencing coverage, hampering the detection of rare prey. Although the sequencing effort was similar for the two barcodes, the COI primers preferentially amplified arthropods, bacteria, protozoa, and unidentified taxonomic groups consuming >60% or the reads. Contrary to 12S, the degenerated COI primers used here hybridized in DNA conserved regions over large taxonomic range. This may be an advantage when studying the diet of a generalist species but, conversely, potential prey corresponded to only 12% of the identified MOTUs and less than 20% of the initial number of reads. It must be emphasized that predator DNA corresponded to only 8% of the assigned reads (Figure S4) so custom-designed blocking primers to inhibit giant otter DNA amplification (see Vestheim & Jarman, 2008) were not necessary in our case. Another hypothesis for the missing prey is the size of the COI barcode which is two times longer than for 12S (although among the smallest COI mini-barcodes proposed in the literature; Elbrecht et al., 2019).

4.1.4 | Giant otter dietary diversity and range

By combining the results of the two markers, we identified at least 35 prey taxa, of which, the vast majority are fish, with other food items (e.g., amphibians, reptiles, birds and earthworms) being consumed sporadically (i.e., less than 10 occurrences in total). Comparison with previous dietary analysis on populations from the central and western Amazonian basin (Cabral et al., 2010; Rosas-Ribeiro et al., 2012; Silva et al., 2014) is limited by the low taxonomic resolution of morphological identifications of prey remains in the feces, as well as by strong regional variation in environmental conditions and prey availability (hydroelectric reservoir *vs* large rivers vs. shallow marshes) (Duplaix et al., 2015). Among-site variation in prey composition was even observed at small scale within the Kaw-Roura reserve potentially reflecting local micro-variation in fish community composition. Consistent with previous work, a large proportion of the giant otter's prey was slow-moving benthic fish from the Cichlidae and the Erythrinidae families, with the highly sedentary and abundant erythrinid *Hoplias malabaricus* (Erythrinidae) found in >60% of the fecal samples (Table S5, Figure S7). In contrast to Amazon basin populations (Cabral et al., 2010; Duplaix, 1980; Rosas et al., 1999), the giant otter diet at Kaw-Roura is dominated by a high proportion of Siluriform catfish prey (Callichthyidae), taxa specific to marsh ecosystems (Hypopomidae, Lepidosirendidae, Curimatidae) and relatively few Characidae. Characidae are abundant and diverse in the Kaw-Roura area (>20 species) but most species are small sized and rarely exceed 5–6 cm (except *Astyanax bimaculatus* which was found as prey), while our results showed that giant otter preferentially feed on fish larger than 10 cm. The only exceptions are *Hemigrammus rodwayi* and *Copella arnoldi* that were marginally detected as prey with less than 5% frequency of occurrence and likely represent secondary predation (i.e., DNA from gut contents of ingested prey). The most consumed prey is the armored catfish *Hoplosternum littorale* which was observed in >80% of spraints. This species, locally named “atipa bosko,” is a popular food fish and a valuable resource for local human populations. However, before invoking a potential conflict of interest, more detailed data on prey abundance and human fishing pressure are needed to evaluate whether giant otter opportunistically or selectively feed on *Hoplosternum littorale* (Rosas-Ribeiro et al., 2012).

Moreover, it should be pointed out that giant otters feed heavily on *Hoplias malabaricus* that are itself a major predator of armored catfishes (Mol, 1996). Testing the potential key role of giant otter in regulating the Kaw-Roura food web would therefore be useful, since the closely related sea otter (*Enhydra lutris*) is a famous keystone species playing a crucial role in maintaining coastal North American marine biodiversity (Estes & Palmisano, 1974). This illustrates the importance of acquiring a detailed knowledge of the trophic network as a whole to better evaluate the impact of giant otters on the fish community, and its potential negative (through predation) or positive (through top-down control on fish predators) effect on *Hoplosternum littorale*. Therefore, giant otter could either act as a competitor or as an auxiliary to local fishermen. Such knowledge would be necessary to set conservation policies that will profoundly differ according to the role of giant otters.

5 | CONCLUSION AND PERSPECTIVE

Our study demonstrated that scat DNA metabarcoding is a particularly powerful tool to provide in-depth information on elusive carnivorous dietary profile in tropical aquatic ecosystems. We showed that a multi-marker approach can be used to confidently identify a broad range of vertebrate prey with an unprecedented high taxonomic resolution while controlling predator identity. Putting aside the fecal sampling step, our approach is robust, far more efficient than conventional morphological methods and easy to implement. A critical issue is the reliability and level of completeness of barcoding reference database that rely on the inventory and identification efforts of taxonomists. Large carnivores face enormous threats that have caused massive declines in their populations and geographic ranges, including habitat loss and degradation, and depletion of prey (Ripple et al., 2014). DNA metabarcoding opens up new opportunities to understand how neotropical top carnivores cope with the effects of anthropogenic-driven alteration of ecosystems and identify conflicts with humans and livestock (Ripple et al., 2014).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

EQ, MA, and MG designed the study; MA did the sampling under JO supervision. MA and VT carried out the DNA extraction and quality controls under NT supervision; SB, JM, RC, and PYLB built the reference database of barcodes. MG did the PCRs and sequencing. EQ, MA, and MG performed the analyses and interpreted the data; EQ and MG led the writing and all authors contributed to the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Supplementary data deposited in ZENODO (https://doi.org/10.5281/zenodo.4607927) include the following: (i) raw sequence data (FASTQ files), (ii) raw abundance tables, (ii) filtered abundance tables including taxonomic affiliations, (iv) final prey occurrence tables, and (v) unix scripts used to produce the data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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