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Environmental DNA metabarcoding as a useful tool for evaluating terrestrial mammal diversity in tropical forests

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Abstract. Innovative techniques, such as environmental DNA (eDNA) metabarcoding, are now promoting broader biodiversity monitoring at unprecedented scales, because of the reduction in time, presumably lower cost, and methodological efficiency. Our goal was to assess the efficiency of established inventory techniques (live-trapping grids, pitfall traps, camera trapping, mist netting) as well as eDNA for detecting Amazonian mammals. For terrestrial small mammals, we used 32 live-trapping grids based on Sherman and Tomahawk traps (total effort of 10,368 trap-nights); in addition to 16 pitfall traps (1,408 trap-nights). For bats, we used mist nets at 8 sites (4,800 net hours). For medium and large mammals, we used 72 camera trap stations (5,208 camera-days). We identified vertebrate and mammal taxa based on eDNA analysis (12S region, with V05 and Mamm01 markers) from water samples, including a total of 11 3-km transects for stagnant water sampling and seven small streams for running water sampling. A total of 106 mammal species were recorded. Building on sample-based rarefaction and extrapolation curves, both trapping grids and pitfall were successful, recording 91.16% and 82.1% of the expected species for these techniques (~22 and ~9 species), and 16.98% and 6.60% of the total recorded mammal species, respectively. Mist nets recorded 83.2% of the expected bat species (~48), and 34.91% of the total recorded species. Camera trapping recorded 99.2% of the predicted large- and medium-sized species (~31), and 33.02% of the total recorded species. eDNA recorded 75.4% of the expected mammal species for this technique (~68), and 47.0% of the total recorded species. eDNA resulted in a useful tool that saves on effort and reduces sampling costs. This study is among the first to show the large potential of eDNA metabarcoding for assessing Amazonian mammal communities, providing, in combination with conventional techniques, a rapid overview of mammal diversity with broad applications to monitoring, management and conservation. By including appropriate genetic markers and updated reference databases, eDNA metabarcoding method can be extended to the whole vertebrate community.

Key words: camera traps; environmental DNA; inventory techniques; live-trapping; mammals; Peru; pitfall trapping; Southwestern Amazon.

Introduction

Traditional mammal inventories use a variety of techniques and methods often specific to a particular taxonomic group (Voss and Emmons 1996). For example, those targeting small mammals commonly use conventional traps (e.g., Sherman, Tomahawk, Victor, and pitfall traps) arranged in line transects or grids, which are particularly useful for mouse opossums; short-tailed opossums; and terrestrial, semiaquatic, and arboreal rodents (Voss and Emmons 1996, Lim and Pacheco 2016). For medium and large terrestrial mammals, line transects, track and sign surveys and camera trapping are usually employed (Voss and Emmons 1996, Silveira et al. 2003, Tobler et al. 2008), whereas medium and

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large arboreal species are best surveyed with line transects and camera traps (Voss and Emmons 1996, Bowler et al. 2017). Bats are commonly surveyed with both mist-nets and acoustic monitoring (Flaquer et al. 2007, Larsen et al. 2007, Meyer et al. 2011, Frick 2013). However, to achieve a complete mammal inventory, all these methods need to be used in parallel, often requiring well-trained field staff for each method (Voss and Emmons 1996).

Species detection through environmental DNA (eDNA) has been proposed as a noninvasive and efficient method for biodiversity monitoring (Miya et al. 2015, Taberlet et al. 2018). eDNA was first used in the late 1980s to study microbes from sediments and have become more widely used since the mid-2000s, mostly in the field of microbiology (Taberlet et al. 2012). In recent years, the application of eDNA metabarcoding to detect the occurrence of macro-organisms, especially those living in aquatic ecosystems, has emerged as a useful tool for monitoring biodiversity (Valentini et al. 2016, Kristy et al. 2017, López-Bao et al. 2018). Moreover, the use of eDNA metabarcoding has also been effective for detecting terrestrial mammals based on 12S and 16S markers by using samples collected from natural saltlicks (Ishige et al. 2017) and soil (Leempoel et al. 2020). In addition, Ushio et al. (2017) showed that eDNA sampling based on water sources was promising to survey mammal diversity in cool temperate forests, and Harper et al. (2019) demonstrated that eDNA signals were stronger for semiaquatic than terrestrial mammals; both studies based on 12S markers. Thus, eDNA metabarcoding could complement conventional sampling techniques (Ishige et al. 2017, Leempoel et al. 2020), especially for detecting very rare or elusive species (Padgett-Stewart et al. 2016, Brozio et al. 2017, Lugg et al. 2017, Nevers et al. 2018).

Mammalian assemblages in the Neotropics have mainly been studied using conventional techniques. The use of eDNA in these ecosystems is still at a very early stage and its performance has yet to be evaluated. Here we compare the efficiency of more traditional methods (live trapping, pitfalls, camera trapping, and mist nets) and eDNA metabarcoding for detecting the presence of terrestrial mammals and bats in bamboo-dominated forests in the southwestern Peruvian Amazon. This study is the first to compare the eDNA method to more traditional sampling methods directly, in a Neotropical environment with high biodiversity in the southwestern Amazon.

Methods

Study area

The study was conducted in four Forest Stewardship Council (FSC) certified logging concessions located in the province of Tahuamanu in Madre de Dios, Peru (Fig. 1), in the southwestern Amazon bamboo forest (Josse et al. 2007). The study site is at an elevation between 160 and 380 m above sea level (a.s.l.), the average annual rainfall is 1,300–2,000 mm, with a rainy season from November until May, a dry season from June to October, and a mean annual temperature of 24°C (data provided by SENAMHI, https://www.senamhi.gob.pe/?&p=estaciones). The vegetation consists of a full or mixed *Guadua* forest with some emergent trees up to 40 m in height (e.g., *Dypteryx*). The dominant tree species in the study area are typical of southwestern Amazonian forest ecosystems with bamboo (Josse et al. 2007). Large- and medium-sized mammal richness in Tahuamanu forest concessions has been studied mainly with camera traps (Tobler et al. 2015, 2018), but there are no previous studies on small mammal assemblages. However, some small mammal surveys near the study area can be useful as reference (Abreu-Júnior et al. 2016, Carrasco-Rueda and Loiselle 2020).

Sampling methods and design

We sampled both logged and unlogged forest plots using camera traps, Sherman and Tomahawk traps, pitfall, mist nets, and eDNA sampling sites in order to record terrestrial mammals and bats (see Table 1).

Small terrestrial mammals (<1 kg)

We used 32 live-trapping grids spaced at least 200 m. Each grid consisted of three parallel 50-m transects set 20 m apart. Each transect contained five trap stations, spaced at 10-m intervals. This resulted in a total of 480 trap stations across the whole study area. We placed two Sherman traps (8 × 9 × 23 cm) at each trap station; one on the ground and the other on fallen logs, trees, vines, or lianas (ca. 2 m height). We also added a Tomahawk trap (41 × 13 × 13 cm) at the initial and final trap station of each transect. The traps were baited with a mixture of peanut butter, black raisins, rolled oats, and vanilla extract, a standardized procedure for small mammals (Voss and Emmons 1996). Trapping was conducted between September and November 2015 and traps on each grid were activated for nine consecutive nights, resulting in 135 station-nights (324 trap-nights) per grid, and a total of 4,320 station-nights across the whole study area (10,368 trap-nights).

In addition, we used pitfall trapping for small terrestrial mammals (marsupials and rodents). We installed 16 100-m-long lines of pitfall traps. Each pitfall line consisted of 11 20-L plastic buckets, spaced 10 m apart, with one bucket at each end. Drift fences, consisting of a continuous barrier running the total length of each line, were made of 80-cm-wide strips of hardware clear polyethylene clipped to vertical stakes hammered into the ground. The pitfall traps were operated for 8 d, resulting in 88 trap-nights per pitfall, and a total of 1,408 trap-nights for the whole area.

We used several external features and cranial morphometry to identify small mammals to the species level

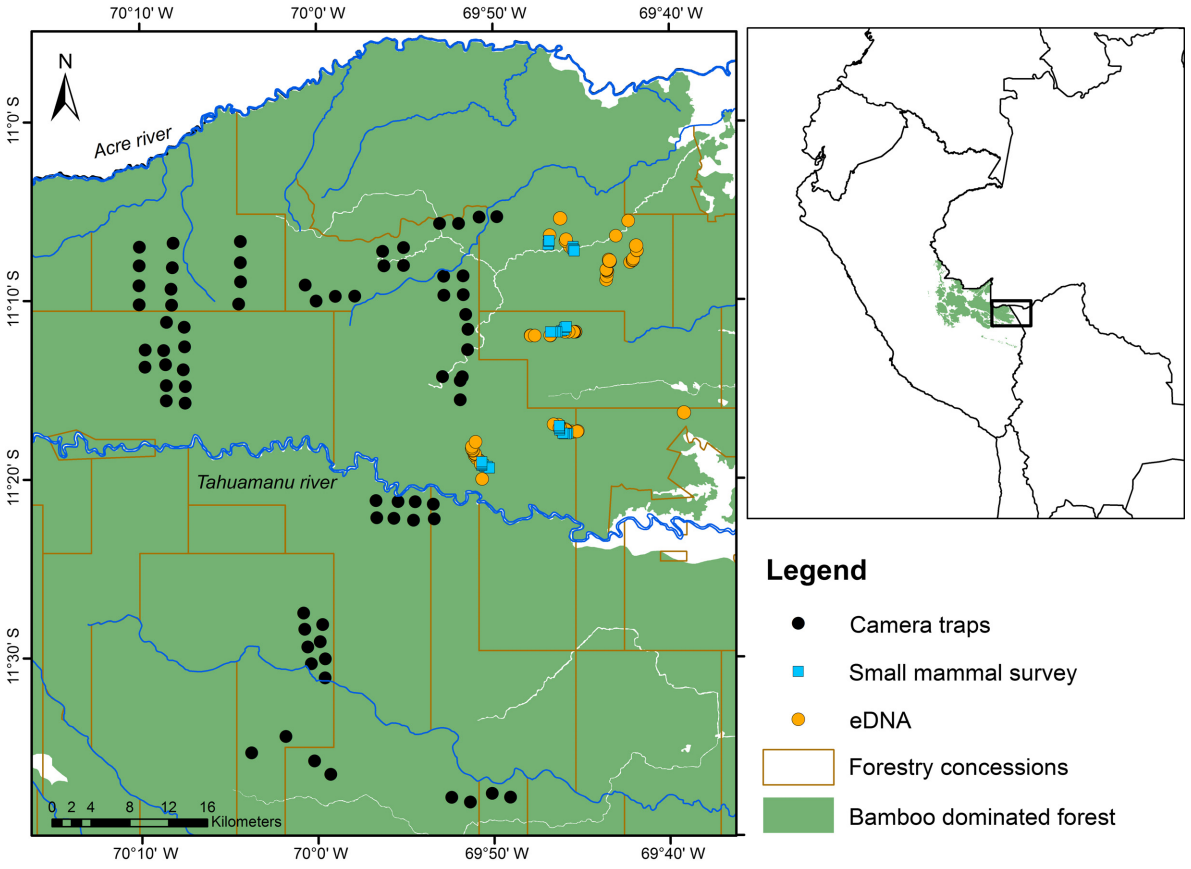

FIG. 1. The study area including the different methods used to estimate mammal diversity in bamboo-dominated forest in forestry concessions in Tahuamanu basin, Madre de Dios, Peru. Small mammal survey included mist nets, grids, and pitfall methods.

TABLE 1. Observed (N_{obs}) and estimated (N_{est}) species richness based on sample-based rarefaction (solid lines) and extrapolation (dashed lines) curves for each method.

Method	Sites	N_{obs}	N_{est}	Species coverage	Focal group
Grids	32	18	22.31 (15.66–28.95)	0.92	Small rodents and marsupials (both scansorial and terrestrial)
Pitfall	16	7	9.12 (4.12–14.11)	0.82	Small terrestrial rodents and marsupials
Mist nets	8	37	48.30 (37.58–59.02)	0.83	Bats
Camera traps	72	29	29.43 (25.87–32.99)	0.99	Medium- and large-sized terrestrial mammals
eDNA	18	50	68.45 (54.76–82.14)	0.75	All mammals (both arboreal and terrestrial)

Notes: N_{est} estimated based on doubling of the number of sites (Colwell et al. 2012, Chao et al. 2014). eDNA in MOTUs. Total recorded species was 106.

(Voss et al. 2004, Gardner 2007, Rossi et al. 2010, Patton et al. 2015). When possible, we also recorded the age (juvenile or adult) and weight of each animal before releasing it. For specimens collected, standard measurements were taken (total length, tail length, hindfoot length, and ear length). In cases of doubt on the taxonomic identification, we collected small tissue samples from the ears and preserved them in alcohol (90%) for complementary genetic analysis using the cyt-b gene (Appendix S1: Section S1) to confirm species identification. Additionally, some specimens were collected and identified in the laboratory for species groups that were difficult to identify in the field (e.g., *Proechimys*). Specimens and tissues were deposited at the Museum of Natural History Vera Alleman Haeghebaert (Universidad Ricardo Palma, Lima, Peru). We followed the guidelines

for the use of mammals in research as recommended by Sikes et al. (2016).

Bats

Bat inventories were conducted between September and November 2015 at a total of eight sites using ground-level mist netting technique (12 × 2.6 m). At each site, there were 10 mist nets set up at equal distance along a ~1-km transect. Sampling occurred during 10 consecutive nights, with the mist nets deployed from dusk until midnight (18:00–00:00). The nets were checked every ~20 min. Total trapping effort was 4,800 net hours. We followed the guidelines for the use of animals in research as recommended by the Guidelines of the American Society of Mammologists (Sikes et al. 2016). All captured bats were identified to species level using standard morphometric character references (Gardner 2007, Díaz et al. 2016). After processing, bats were released at the site of capture. Some individuals were collected as voucher specimens and were deposited in the Museum of Natural History Vera Alleman Haeghebaert (Universidad Ricardo Palma, Lima, Peru).

Medium and large mammals (>1 kg)

Between June and September 2017, we carried out a survey with 72 camera-trap stations spaced ~2 km apart to record observations of medium and large mammals (see Fig. 1). Camera traps (Bushnell® TrophyCam HD) were strapped to trees or stakes approximately 40 cm above ground and were active 24 h per day over an average of 72 d (3–86). Total trapping effort was 5,208 camera-days. All images and associated metadata were stored in Camera Base 1.7 (Tobler 2015).

eDNA sampling

Samples for the eDNA analysis were collected from stagnant and running water sources. A total of 11 3-km transects were surveyed for stagnant water sampling and seven small streams were selected for running water sampling. Along each transect, the presence of stagnant water bodies was recorded and marked with a GPS. For each stagnant body of water, 20 subsamples of 100 mL were collected following the protocol described in Miaud et al. (2019). The water was collected using a sterile sampling scoop with a handle, and each subsample was poured into a 2-L Whirl-Pak® bag. The bag was shaken to homogenize the sample and water was then filtered directly in the field through a VigiDNA® filter (SPYGEN) using a sterile 100-mL syringe. The quantity of water that could be filtered depended on the concentration of suspended sediment. In our study, we were able to filter between 120 and 600 mL of water per filter. A different strategy was used for running-water sampling: 8 L of running water was filtered using a syringe and VigiDNA® filter (SPYGEN). As much as possible, water was collected from the middle of the stream. Immediately after the filtration, all filters were then filled with 80 mL of CL1 preservative buffer (SPYGEN), labeled, and stored at room temperature.

eDNA lab analysis

The eDNA analysis was performed at SPYGEN (Le Bourget du Lac, France) following methods described in Pont et al. (2018) using vertebrate (V05) primers (Riaz et al. 2011) and mammal (Mamm01) primers (Taberlet et al. 2018), both located on the 12S region (Taberlet et al. 2018). DNA extraction was performed in a dedicated room for water DNA sample extraction, following the protocol described in Pont et al. (2018). This room was equipped with positive air pressure and UV treatment, and had frequent air renewal. Before entering the extraction room laboratory, personnel changed into full protective clothing comprising of a disposable body suit with hood, mask, laboratory shoes, overshoes, and gloves. All benches were decontaminated with 10% commercial bleach before and after each manipulation. For DNA extraction, each filtration capsule, containing the CL1 buffer, was agitated for 15 min on an S50 shaker (cat Ingenieurbüro™) at 800 rpm and then the buffer was emptied into a 50-mL tube before being centrifuged for 15 min at 15,000 × *g*. The supernatant was removed with a sterile pipette, leaving 15 mL of liquid at the bottom of the tube. Subsequently, 33 mL of ethanol and 1.5 mL of 3 M sodium acetate were added to each 50-mL tube and stored for at least one night at −20°C. The tubes were centrifuged at 15,000 × *g* for 15 min at 6°C, and the supernatants were discarded. After this step, 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) was added. The tubes were then vortexed, and the supernatants were transferred to 2-mL tubes containing 20 µL of Proteinase K. The tubes were finally incubated at 56°C for 2 h. Subsequently, DNA extraction was performed using NucleoSpin® Soil (MACHEREY-NAGEL GmbH & Co., Düren, Germany) starting from step 6 and following the manufacturer's instructions. The elution was performed by adding 100 µL of SE buffer twice. After the DNA extraction, the samples were tested for inhibition by quantitative polymerase chain reaction (qPCR) following the protocol in Biggs et al. (2015). If the sample was considered inhibited it was diluted fivefold before the amplification. DNA amplifications were performed in a final volume of 25 µL, using 3 µL of DNA extract as the template and either vertebrate primers (V05 primers, Riaz et al. 2011) or mammal primers (Mamm01 primers, Taberlet et al. 2018). The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA), 10 mmol/L Tris-HCl, 50 mmol/L KCl, 2.5 mmol/L MgCl2, 0.2 mmol/L each dNTP, 0.2 µmol/L of each primer pair, 4 µmol/L human blocking primer for the both primers (De Barba et al. 2013) and 0.2 µg/µL bovine

serum albumin (BSA, Roche Diagnostic, Basel, Switzerland). The primers were 5′-labeled with an eight-nucleotide tag unique to each sample (with at least three differences between any pair of tags), allowing the assignment of each sequence to the corresponding sample during sequence analysis. 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 (for vertebrate primers) or 57°C (for mammal primers), 1 min at 72°C, and a final elongation step at 72°C for 7 min in a room that is dedicated to amplified DNA and has negative air pressure and physical separation from the DNA extraction rooms (with positive air pressure). Twelve replicate PCRs were run per filtration capsule.

After amplification, the samples were titrated using capillary electrophoresis (QIAxcel; Qiagen GmbH, Hilden, Germany) and purified using the MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was titrated again using capillary electrophoresis. The purified PCR products were pooled in equal volumes to achieve a theoretical sequencing depth of 300,000 reads per sample. PCR and PCR purification were performed in a room that is dedicated to amplified DNA analysis, has negative air pressure, and is physically separated from the eDNA extraction room. All benches were decontaminated with 10% commercial bleach before and after each manipulation. Library preparation and sequencing were performed at Fasteris (Geneva, Switzerland). The library was prepared using the MetaFast protocol (Fasteris, https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis), and paired-end sequencing (2 × 125 base pairs [bp]) was carried out on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, California, USA) with the HiSeq SBS Kit v4 (Illumina) following the manufacturer's instructions. One negative extraction control and one negative PCR control (ultrapure water, 12 replicates) were amplified and sequenced in parallel to the samples to monitor possible contaminants. After the filtering pipeline, the extraction and PCR negative controls no sequence reads remained in those samples.

Sequence reads were analyzed using programs implemented in the OBITools package (Boyer et al. 2015) following the protocol described in Valentini et al. (2016). The forward and reverse reads were assembled using the *illuminapairedend* program and assigned to each sample using the *ngsfilter* program. A separate data set was created for each sample by splitting the original data set into several files using *obisplit*. For each sample, sequences shorter than 20 bp occurring less than 10 times per PCR replicate or labeled "internal" by the *obiclean* program, most likely corresponding to PCR/sequencing errors, were discarded. Reference databases for vertebrate and mammal sequences were built using the ecoPCR program (Bellemain et al. 2010, Ficetola et al. 2010) using the same vertebrate and mammal primers, on all the sequences present in the *embl* release 127. Taxonomic assignment of the molecular operational taxonomic unit (MOTUs) was performed using those reference databases and the program *ecotag*. MOTUs showing less than 98% similarity to the reference database were removed and records from domestic animals were discarded from eDNA results, that is, *Canis lupus familaris* in two sites and the genus *Sus* in one site. All taxa with a frequency of occurrence below 0.0003 per library in each sample, were considered as tag-jumps (Schnell et al. 2015*a*) and discharged. The results were analyzed as presence/absence in each eDNA sampling site (both transect and small streams, see Species richness analysis).

Species richness analysis

Because the main objective of this study was to compare the efficiency of the different sampling techniques for detecting Amazonian terrestrial mammals, we did not conduct any comparisons between logged and unlogged forest blocks. We used all grid, pitfall trap, and mist net sites as sampling units for small mammals and all camera stations for medium and large mammals. All stagnant and running water samples were combined for a total of 18 eDNA sampling units. We recorded the species richness (MOTUs) for each sampling unit (see Table 1). In order to assess the completeness of each sampling technique, we estimated sample coverage as a function of the extrapolated, incidence-based species richness, based on a doubling of the number of sites as recommended in (Colwell et al. 2012, Chao et al. 2014), which were obtained by iNEXT in R (Hsieh et al. 2015). In addition, we used bootstrap methods to construct confidence intervals generated by 1,000 bootstrap resampling, for species richness of any rarefied or extrapolated sample. Thus, we followed the approach provided by Chao and Jost (2012), where sample coverage is defined as a measure of completeness or the proportion of species in the community, which is represented in the sample (i.e., the recorded species).

Sampling cost

To compare costs between techniques, we considered four components: logistics, field staff, equipment, and lab work. Logistics includes all transportation of field teams, supplies, and food. Staff includes permanent researchers and field assistants required for the inventory. Equipment can be divided into reusable (traps and mist nests) and single-use items (eDNA kits). Finally, lab work includes all activities that cannot be conducted in the field, such as eDNA analysis, genetic analysis for species identification, and postprocessing of camera trap data. Costs of equipment by grid sampling were based on Sherman and Tomahawk traps. Lab costs for eDNA were estimated by kit, and include lab analyses and bioinformatics. Lab costs also increased the total costs for small mammals (i.e., determination of specimens based on morphometry or DNA) and for camera traps

TABLE 2. Costs of inventory techniques for terrestrial mammals based on survey duration (US$).

Sampling	Units	Unit cost	Equipment cost	Labor and lab cost	Transportation	Total cost	Cost by species
Small mammals							
Sherman	240	31.25	7,500.00				
Tomahawk	48	39.95	1,917.60				
Mist nets	20	20.00	400.00				
			9,817.60	19,200.00	6,600.00	35,617.60	574.47
Medium- and large-sized mammals							
Camera trapping	72	170.16	12,251.52	16,000.00	6,000.00	34,251.52	951.43
All mammals							
eDNA†	18	400.00	–	9,720.00‡	4,650.00	14,370.00	287.40

Notes: Team for both small mammals and medium- and large-sized mammals included six people for 48 and 40 d, respectively, and two for eDNA (18 d). By each team, we include one specialist for small mammals (US$100/d), one for eDNA survey (US$100/d), and one for camera trapping (US$100/d). Local assistant by day is US$40.00. Food cost is US$10·d^{-1}·person^{-1}.

†eDNA in MOTUs.

‡Includes eDNA kits.

(i.e., camera trap image processing). The estimated costs were based on total labor days for each technique: 48 d for small mammal's survey (i.e., terrestrial small mammals and bats), 40 d for camera traps, and 18 d for eDNA (see Table 2). The equation for eDNA cost was

$$\text{eDNACost} = n(K + L) + T + d(f \times F + s \times S),$$

where K is the cost per eDNA kit (US$400), L is the labor cost per kit (US$140), s is the number of field specialists, S is the cost per specialist (US$100), f is the number of field assistants, F is the cost per field assistant per day (US$40), d is the total number of days that is required to collect samples (~18 d), T is the cost for transportation, and n is the number of sites that was sampled (18). The equation for each traditional technique cost was

$$\text{Traditional}_{\text{Cost}} = \text{Equipment} + T + d(f \times F + s \times S),$$

where Equipment is the cost for each technique (see Table 2), s is the number of field specialists, S is the cost per specialist (US$100), f is the number of field assistants, F is the cost for field assistant per day (US$50), d is the total number of days that is required for field work, and T is the cost for transportation.

RESULTS

A total of 106 mammal species were detected with all methods combined: 26 species of small terrestrial mammals, 41 species of medium and large terrestrial mammals (including primates), and 39 species of bats (Appendix S1: Tables S1, S2). We captured a total of 117 small terrestrial mammals, with 99 captures (representing 18 species) in live trapping grids, and 18 captures (7 species) in pitfall traps. Sherman and Tomahawk traps had a capture rate (captures per trap night) of 0.95%, and pitfall traps 0.70%. Eight small mammal species were exclusively recorded within live trapping grids, two scansorial marsupials (*Marmosa regina* and *Marmosops bishopi*) and six terrestrial rodents (*Euryoryzomys nitidus*, *Hylaeamys perenensis*, *Oligoryzomys microtis*, *Proechimys pattoni*, *Proechimys simonsi*, and *Proechimy steerei*). Only three species were exclusively captured by pitfall traps, two terrestrial marsupials (*Monodelphis emiliae* and *Monodelphis peruviana*) and one terrestrial rodent (*Neacomys musseri*). The DNA analysis using cyt-b gene from three young specimens collected and four individuals released confirmed the presence of three species of marsupials: *Marmosa demerarae*, *Marmosops ocellatus* with 98% of identity with *M. ocellatus* "Jurua group" and *Marmosops noctivagus* with 96% of identity with samples of *M. noctivagus* "group D" following Díaz-Nieto et al. (2016). Likewise, based on cyt-b, we confirmed the presence of *P. pattoni* and *Proechimys brevicauda* (Appendix S1: Table S1). eDNA recorded seven species of small terrestrial mammal including three species (*Caluromys*, *Marmosa lepida*, *Dactylomys boliviensis*, and *Microsciurus* sp.) not detected with the other methods.

Using mist nets and eDNA, we recorded 39 species: 32 species of Phyllostomidae, three Molossidae, one Thyropteridae, and three Vespertilionidae (Appendix S1: Table S1). Mist nets detected 37 species, of which 30 were only detected by this method, and eDNA sampling detected 9 bat species, which included 2 species (*Chiroderma salvini* and *Thyroptera lavali*) that were only detected by this method (Appendix S1: Table S2).

Our camera traps recorded 29 species of medium- and large-sized terrestrial mammals, with 19 species exclusively detected by this method (Appendix S1: Table S1). Most Carnivora species were exclusively detected with camera traps, except for *Panthera onca* and *Procyon*, which were also detected with eDNA. eDNA detected 20 species of medium and large mammals (including primates; Appendix S1: Table S1). The primates *Aotus* and *Ateles*, and the capybara (*Hydrochaeris hydrochaeris*) were exclusively detected with eDNA.

The sample coverage differed by sampling method (Fig. 2 and Table 1). Camera trapping was the most

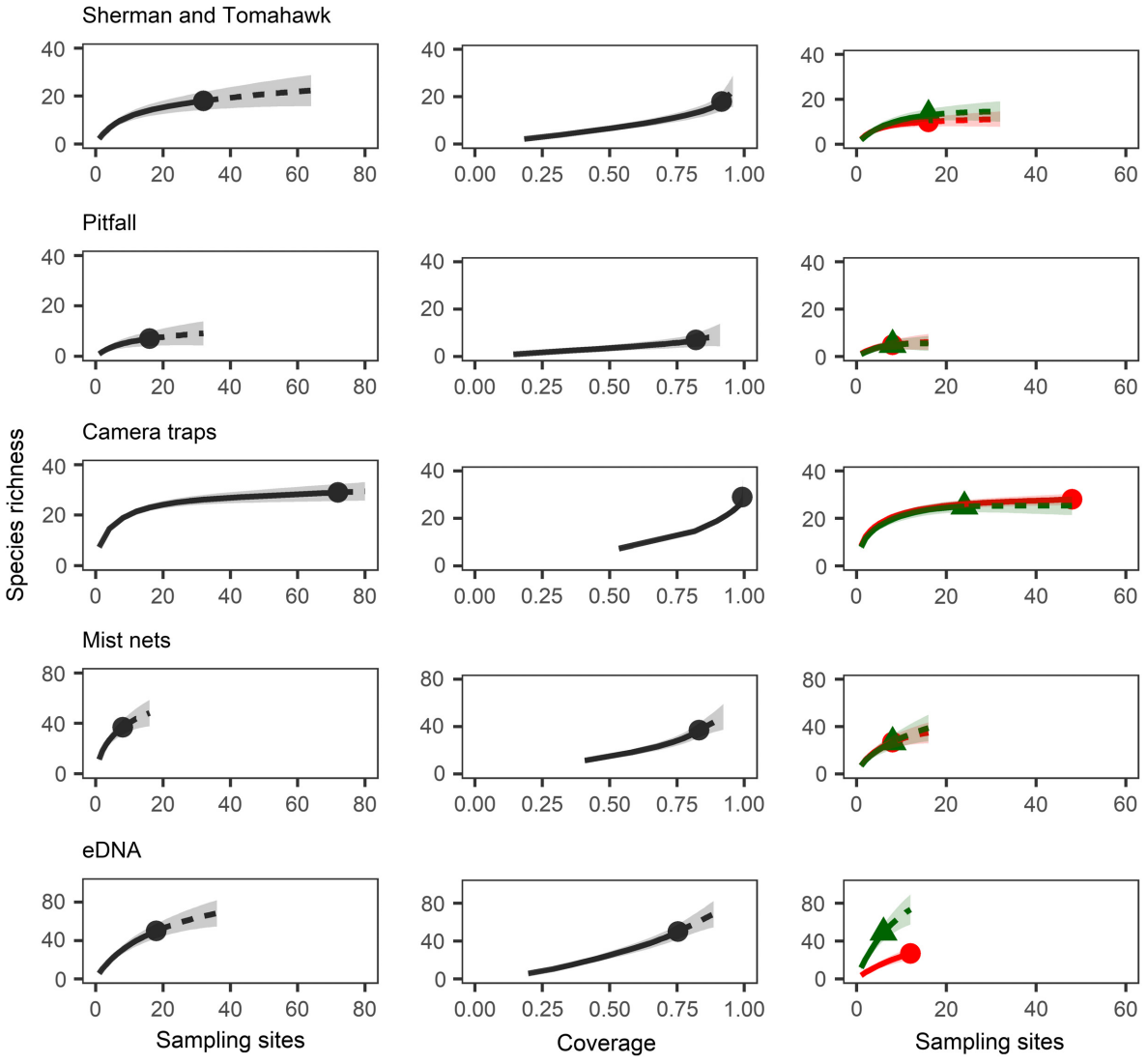

FIG. 2. Species accumulation curves for each method (Sherman + Tomahawks traps, pitfall trapping, mist nets, camera traps, and eDNA (as MOTUs). Left: sample-based rarefaction (solid lines) and extrapolation (dashed lines) curves for species richness for each method. The 95% confidence intervals (gray-shaded regions) were obtained by a bootstrap method based on 200 replications (see Chao et al. 2014). Right: coverage-based rarefaction (solid line) and extrapolation (dashed line) plots with 95% confidence intervals. In both cases, total samples are denoted by solid dots, and up to double the reference sample size (dashed lines), except by camera traps. Total recorded species was 106. Third column shows the performance of each technique for both logged (red) and unlogged forests (green).

successful, with a 99.2% (98.6–99.9%) of sample coverage, but recorded only 33.02% of the total recorded species ($N = 106$), followed by grids with 91.6% (87.0–96.2%), mist nets with 83.2% (75.4–91.0%), and pitfall trapping with 82.1% (60.0–100.0%), which recorded 16.98%, 34.91%, and 6.60% of the total recorded species ($N = 106$), respectively. eDNA sample coverage was 75.4% (67.1–83.6%), suggesting that more taxa can be recorded with this method. In addition, there were no differences in species richness between logged and unlogged sites based on traditional techniques. However, based on eDNA we found that unlogged sites were more diverse than logged sites (Fig. 2). Moreover, eDNA metabarcoding recorded 47% of the total recorded species; thus, it recorded more taxa than any of the other methods and with less effort and cost (Figs. 3, 4, Table 2).

In terms of field effort, traditional methods required more than twice (40–48 d) the time used in the eDNA survey (18 d). Similarly, traditional methods require more people (six) compared to molecular techniques (two; Table 2). Also, although traditional methods for small mammals give a cost per species of US$574.53, and medium- and large-sized mammals of US$979.13,

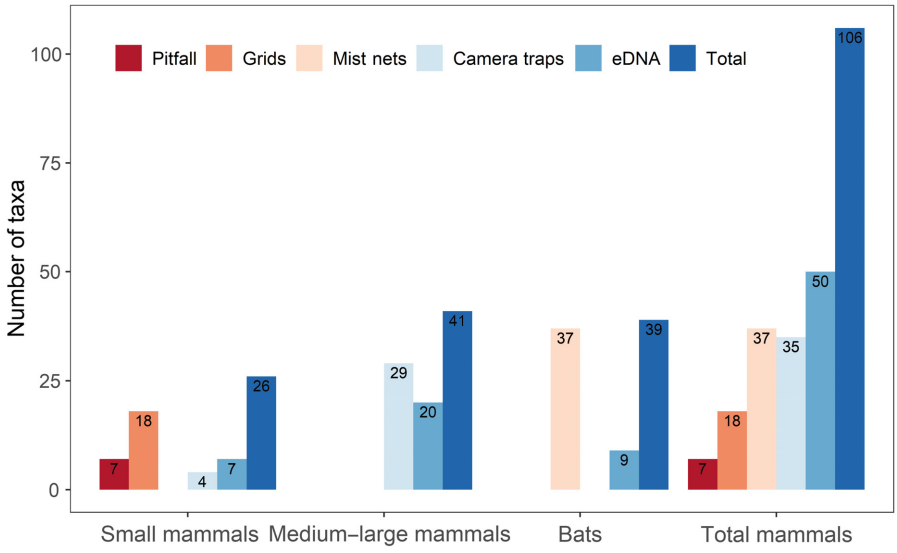

FIG. 3. Number of species recorded for each survey method. eDNA in MOTUs.

eDNA costs turn out to be cheaper by about the half of the small mammal's assessment (US$284.18).

DISCUSSION

We conducted the most comprehensive inventory of mammals in bamboo-dominated forests in the southwestern Peruvian Amazon by combining traditional methods (live trapping, pitfalls, camera trapping, and mist nets) and eDNA metabarcoding. In this study, we showed that a combination of traditional and modern methods could improve our understanding of mammal diversity with rapid inventories. As we explained, each technique provides a partial overview of the total mammal diversity in the study area (6.6–34.89%), so that they ideally should be combined in any rapid inventory. However, eDNA provides a big picture of the total diversity (~50%), including records provided by the other techniques.

Mammal detection

Our results confirm that a combination of methods is required to detect a large proportion of the entire mammal community in Neotropical rainforests. For small mammals multiple trap types and placement options give the best results (Voss and Emmons 1996, Hice and Velazco 2012, dos Santos et al. 2015, Lim and Pacheco 2016, Ardente et al. 2017) and several studies showed that the addition of pitfall traps led to greatly increased estimates of species richness and abundance of Neotropical small mammals (Hice and Velazco 2013, Bovendorp et al. 2017). Pitfall traps typically capture species thought to be rare or within different subsets of the small mammal community, especially terrestrial marsupials of the genus *Monodelphis* and semiaquatic rodents like the genus *Neusticomys* (Umetsu et al. 2006, Hice and Velazco 2012, dos Santos et al. 2015, Palmeirim et al. 2019). In the same way, Tomahawk traps are recommended to capture large marsupials and rodents such as those in the genus *Proechimys* spp. (Hice and Velazco 2013, dos Santos et al. 2015). Using both pitfall and live traps together increases the efficiency of sampling in both marsupials and rodents (Hice and Schmidly 2002, Hice and Velazco 2012, Bovendorp et al. 2017). So, adding more complementary methods to the study, such as eDNA, guarantee a better understanding of the current mammal diversity. The same applies to bat inventory and to medium- and large-sized mammals, as we detailed above. In addition, eDNA allows detection of species that were not detected by any of the traditional methods.

In terms of large- and medium-sized terrestrial mammals, camera traps are extremely effective for inventorying this assemblage and have become the standard method (Tobler et al. 2008, O'Connell et al. 2011, Mena et al. 2020*b*) providing useful information on species richness, occupancy, activity patterns, and density (O'Connell et al. 2011, Burton et al. 2015). Indeed, our inventory with camera traps was nearly complete (see sampling coverage), providing a reliable description of the expected large- and medium-sized mammals of the study area, similar to other nearby forestry concessions (Tobler et al. 2015, 2018). Therefore, we can assume that our camera trapping effort set a high standard for comparison to eDNA metabarcoding. It turns out that eDNA was not very efficient in recording species from this group. Although it successfully recorded some of the most common species such as paca (*Cuniculus paca*), red brocket deer (*Mazama americana*), collared peccary (*Pecari tajacu*), and lowland tapir (*Tapirus terrestris*), it missed other common species such as the brown agouti

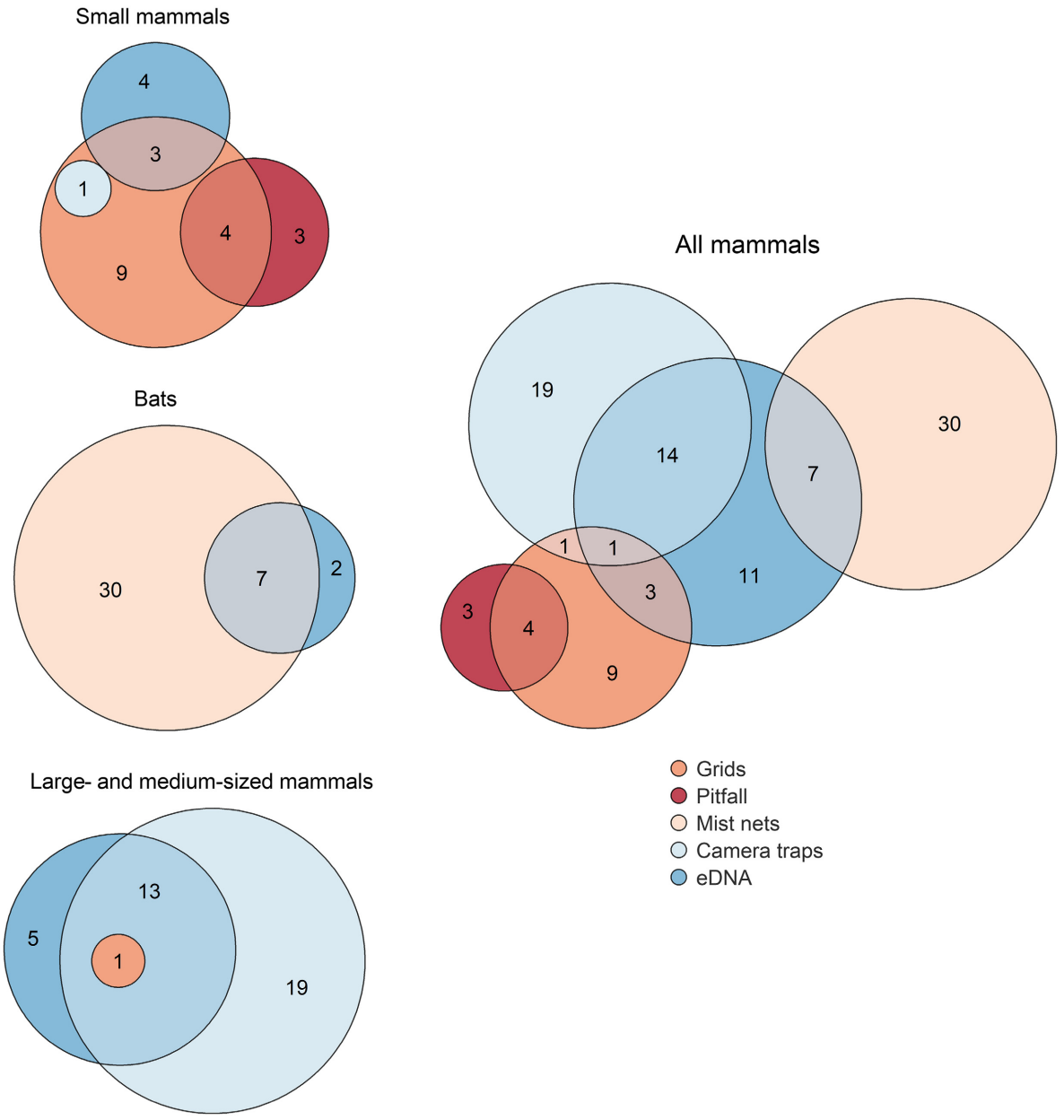

FIG. 4. Number of species recorded for each survey method by mammal group. eDNA based on Appendix S1: Table S1.

(*Dasyprocta variegata*), the common opossum (*Didelphis marsupialis*), and most of the carnivore species. Nevertheless, eDNA metabarcoding recorded some species not recorded with camera traps (e.g., *Lontra longicaudis*). Also, primates and other arboreal mammals, only detected by the laborious arboreal camera trapping (Tremaine et al. 2014, Bowler et al. 2017) or line transects not used in this study, were detected by eDNA. Those included one arboreal marsupial (*Caluromys lanatus*) and two arboreal rodents (*Dactylomys boliviensis* and *Microsciurus* sp.). Ishige et al. (2017) reported some differences between the results of the detections of large- and medium-sized mammals based on eDNA, and those from camera traps, in natural attractants such as salt-licks, in Sabah, Borneo. However, Leempoel et al. (2020) found that eDNA from soil samples mirrored species occurrence from camera traps in California, USA, but the number of large- and medium-sized mammal species there was much lower than those typically found in tropical forests such as Peru and Borneo. It appears that detection depends on the rarity of the species and how the species releases DNA in the environment. For

instance, in the study of Cantera et al. (2019), where 10 DNA replicates were collected in six Guianese sites to study fish diversity with a similar protocol that in our survey, they found that both common and rare species were detected in all the replicates, but most of the species that were detected in few eDNA replicates were indeed rare species, based on traditional sampling.

In our study, despite the sampling effort deployed, bat diversity was surprisingly low compared to other studies conducted in the Amazon, even considering only understory mist-netting (Sampaio et al. 2003). The most likely explanation for this result is the exclusion of complementary field methods for bat inventories such as canopy mist netting, harp traps, acoustic monitoring, and collecting at roosting sites (Voss and Emmons 1996). In fact, detection of bat species is affected by the number of surveys, the season, and of course by sampling methods (Meyer et al. 2011). Another explanation could be habitat due to bamboo dynamics and how diversity could be affected, previously pointed out by Silman et al. (2003). In our study, eDNA was a complementary technique to mist netting and provided two additional species to the bat inventory. Several species detected by eDNA were only identifiable to the level of genus or were misidentified because of an incomplete reference library.

eDNA efficiency

Our study confirms that eDNA sampling can efficiently identify terrestrial mammals through DNA collection from ponds or streams (Harper et al. 2019). The performance of this methods depends, among other factors, on the use of proper genetic markers. Both V05 and Mamm01 markers amplified two different regions of the 12S, and because most of the sequences of mammal species present in the *embl* database cover only one of these regions of the 12S, the use of both markers increased the species detection. In our case, only 36% of the species were detected with both markers. Of the remaining 64%, 36% of the species were only detected with V05 marker and 28% only with Mamm01 (Appendix S1: Table S2). Likewise, the eDNA results outperformed results from all other field methods in terms of both number of taxa identified and richness (Figs. 2, 3), and species accumulation curves showed that increasing sampling effort would result in more species detections. As a result, eDNA can provide a preliminary overall biodiversity overview. Moreover, the efficiency of the eDNA approach may be increased by improving the reference sequence databases, as suggested in Valentini et al. (2016). In fact, only about 58% of total species recorded were present in the reference database (i.e., 62 of the 106 species; Appendix S1: Table S1).

Our results show that eDNA can be a powerful tool for terrestrial mammal and bat inventories, especially in terms of effort and cost. In addition, despite our sample size for eDNA being moderate, we recorded differences between logged and unlogged sites in terms of mammal diversity. Indeed, eDNA efficiently detected species with habitat preferences that were not targeted for traditional techniques, mainly arboreal species at unlogged sites (e.g., *Caluromys lanatus*, *Ateles chamek*, *Saimiri boliviensis*, *Dactylomys boliviensis*, and *Microsciurus* sp.). Furthermore, although traditional methods are very useful for detecting cryptic species, updating distributional ranges, or even discovering new species, these methods require a significantly larger investment of effort and resources than using a method such as eDNA (see Table 2). Other studies have already shown that this method is more cost efficient for amphibians and mammals (Smart et al. 2016, Lugg et al. 2017, Abrams et al. 2018, Bálint et al. 2018). The costs and processing times of molecular techniques have been decreasing in recent decades as their precision has improved, so we believe there will be a trend in the use of molecular techniques for environmental evaluations and monitoring.

eDNA obstacles to be solved

In this study, the efficiency of the eDNA method was dependent on available water sources and reliable reference databases. In order to improve the potential of eDNA, it is important to enhance the reference databases for more accurate and efficient assignment of the examined metabarcodes to taxonomic names (Taberlet et al. 2018). In our survey, we found issues with the reference sequences in the database for the genus *Proechimys* (and others). Based on live trapping we recorded four species of *Proechimys*: *P. brevicauda*, *P. pattoni*, *P. simonsi*, and *P. steerei* (Patton et al. 2015), whereas the eDNA method revealed the presence of other two species, *Proechimys cuvieri* and *Proechimys quadruplicatus.* Nevertheless, the occurrence of *P. cuvieri* and *P. quadruplicatus* is questionable because their typical distribution ranges are far away from the study area; *P. quadruplicatus* occurs north of the Marañon river (Peru) and throughout the northwestern Amazon Basin, while the southernmost record of *P. cuvieri* is Sarayacu, Ucayali River (Peru; Patton and Leite 2015). Moreover, considering that the four species recorded with grids were not present in the embl database (r127), the best match for the obtained eDNA sequences of this genus were related species. Other questionable identifications caused by a lack of reference sequences in the databases were for *Procyon lotor*, *Artibeus jamaicensis*, *Ateles belzebuth*, and *Saimiri sciureus* (Appendix S1: Table S2), and again in all these cases a closely related species of the same genus was identified. Other related issues could be misidentifications of species in embl or outdated names (i.e., for primates). Because of these issues, it is important that someone with taxonomic expertise and a good knowledge of the fauna in the study area review the species list generated from eDNA and remove or correct species that cannot possibly occur there. However, the lack of complete reference databases can and should be overcome in the short term (Grey et al. 2018).

Conclusion

We are living a time in which technology is fundamental for the study of biodiversity, providing us with the opportunity to improve the scale of data collection while reducing effort and investment of resources. Camera traps are an example of this; over the last 20 yr they have become a standard tool for wildlife studies around the globe (O'Connell et al. 2011, Burton et al. 2015). eDNA is one of the next emerging technologies for species inventories and has already demonstrated its efficiency, especially in freshwater ecosystems (Padgett-Stewart et al. 2016, Nevers et al. 2018). As an innovative methodology, eDNA confirmed that it can provide a rapid overview of mammal diversity in the Amazon. However, to enhance this technique it is necessary to continue increasing and updating the information in public DNA databases, as the best match for eDNA sequences depends on updated and uploaded reference data. Our eDNA design was limited in terms of sampling units and, as species accumulation curves showed, its performance will likely improve with additional effort. Other approaches based on invertebrate-derived DNA, through sampling of blow flies or leeches for example, have proven useful to identify vertebrates in tropical regions (Calvignac-Spencer et al. 2013, Schnell et al. 2015*b*, Gogarten et al. 2020) and could be used in future studies to further improve biodiversity assessment. To conclude, eDNA techniques can be useful for biodiversity monitoring and can complement traditional techniques in providing data for effective management and conservation initiatives.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/eap.2335/full

Data Availability

The Illumina raw sequence data (Mena et al. 2020*a*) are available on Figshare: https://doi.org/10.6084/m9.figshare.12646550.v2