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Drops of Diversity: Vertebrate rainwash eDNA metabarcoding in an Amazonian floodplain forest

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Abstract

Extensive research has been conducted along the Medio Juruá River in the western Brazilian Amazon, with vertebrate species being relatively well-documented. However, studies on biodiversity in this region has predominantly relied on traditional monitoring methods, and the potential use of environmental DNA (eDNA) based approaches remain largely unexplored. This study focused on eDNA metabarcoding, comparing active and passive rainwash filtering to detect vertebrate species, particularly arboreal taxa, on the Medio Juruá River floodplain during the low-water season. Three rainwash events were recorded. To amplify vertebrate eDNA, the Riaz 12S and Leray (COI) primer sets were used. The Leray (COI) primer found only two species, whereas Rias 12S detected a total of 45 species across four classes. The active filtering method found significantly more vertebrate species ($p = 0.00386$), but for exclusively arboreal species, no statistical significance between methods was found. Contrary to expectations, no amphibians and only one reptile was detected. Interestingly, this study detected the rare species bush dog (*Speothos venaticus*), highlighting the method's potential use for rare species detection. This study demonstrates the potential of rainwash eDNA in the Amazon floodplains as a minimally invasive biodiversity monitoring tool, but further refinements is required to improve detection rates.

Keywords: environmental DNA (eDNA); Metabarcoding; Amazonia; Medio Juruá River; Biodiversity monitoring; Arboreal vertebrates; Rainwash

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List of Abbreviations

eDNA	**e**nvironmental **DNA**
PCR	**P**olymerase **C**hain **R**eaction
VAES	**V**isual and **A**udio **E**ncounter **S**urvey
COI	**C**ytochrome c **o**xidase **s**ubunit I
RW	**R**ainwash **E**vent

Introduction

Biodiversity loss is one of the most pressing issues of our time (WWF, 2024). Increasing anthropogenic pressure on ecosystems threatens biodiversity across the globe (Ceballos et al., 2015; Gumbs et al., 2024; Hubbell et al., 2008; Pillay, Watson, et al., 2022), and a limited knowledge about biodiversity, distribution and populations trends slow conservation efforts (Berenguer et al., 2021; Pimm et al., 1995; Ribeiro et al., 2016). For example, Oliveira et al. (2016) demonstrated a significant bias in biodiversity monitoring, resulting in an apparent decline in biodiversity with increasing distance from access routes, despite biodiversity loss being linked to anthropogenic pressures (Barlow et al., 2016; Prist et al., 2012).

The Amazon rainforest has the largest uninterrupted tropical rainforest (Corlett & Primack, 2011, p. 4), and although it covers just 3.6% of Earth's terrestrial surface, this forest expanse is home to about 10% of known species (Lewinsohn & Prado, 2005; Maretti, 2014). In addition to the regional gaps in biodiversity knowledge, there are significant gaps in specific habitats within well-studied areas such as rainforest canopies (Nakamura et al., 2017). Rainforest canopies are structurally complex with intricate interactions and microclimates, providing many different niches promoting species diversity (de Souza Amorim et al., 2022; Thiel et al., 2021). Despite research on rainforest canopies has been conducted to some extent (Erwin, 2001; Kaizer et al., 2022), it remains to be a an underexplored area of research. Rainforest canopies are challenging to research and many different methods of assessing the animal biodiversity of canopies have been developed. Traditional, often labor intensive, costly, or invasive methods such as canopy walks (Inoue et al., 1995), camera traps (Whitworth et al., 2016), fogging (Potapov et al., 2024), and bioacoustics (Sethi et al., 2023) are used. There are multiple ways to address this biodiversity knowledge gap. Easily accessible regions often favor generalist species adapted to human-altered environments, underrepresenting less adaptable specialists (Filgueiras et al., 2021; Noble et al., 2023). Coupled with limited funding, developing tools to increase the cost-effectiveness of monitoring more remote regions could aid in conservation efforts in all regions of the Amazon.

In recent years, molecular methods of species detection has been developed (Ficetola et al., 2008), in which environmental DNA (eDNA) can be used to detect species using soil, water and even air samples (Allen et al., 2023; Serrao et al., 2021). eDNA has been shown to be a very useful method and is rapidly emerging to become a widely used technique for biodiversity

assessments (Beng & Corlett, 2020; Deiner et al., 2017). eDNA can be defined as "genetic material obtained directly from environmental samples (e.g. soil, sediment, water) without any obvious signs of biological source material" (Thomsen & Willerslev, 2014). This material can be substances shed by organisms such as urine, epidermal cells, hair, feces or intestinal cells. Environmental samples will therefore include DNA from species interacting with the environment, and depending on the type of research, eDNA samples can be analyzed using species-specific or non-targeted (metabarcoding) PCR primers (Jafar et al., 2024; Kitano et al., 2007).

Biodiversity surveys using eDNA based methods has the potential to be a cost-effective and less invasive than traditional methods as direct observation is not necessary (Zinger et al., 2020). Additionally, this method can also prove to be more sensitive than traditional methods of physically capturing and identifying organisms (Smart et al., 2015; Tucker et al., 2016). However, it is important to note that although eDNA is promising, it might not always be the most cost-effective tool. Tropical rainforests still largely lack a complete species DNA reference database (Zinger et al., 2020), further exacerbated by the fact that many species present in these forests remain undescribed, even for vertebrate taxa that are relatively well studied (Pillay, Venter, et al., 2022). Depending on the study design, traditional methods might be easier and less time consuming. Bálint et al. (2018) compared the cost-efficiency of eDNA sampling to visual and audio encounter survey (VAES) and found the cost-efficiency favors VAES in low-diversity regions, while for high-diversity regions eDNA was favored. However, a recent meta-analysis (Carvalho et al., 2022) could not conclude on the efficiency of eDNA in tropical regions, but the efficiency is expected to improve with further technical advancements (Burian et al., 2021).

When analyzing eDNA data, its crucial to consider potential false positives (type I error) or false negatives (type II error). Type I errors occur when species detected in a sample are not from the sample, but rather from contamination during collection, extraction or processing. Type II occurs when species are present in the ecosystem but not detected in the samples, suggesting that the species is not present even though it is. eDNA in aquatic environments break down at different rates depending on whether it's dissolved, particle absorbed, intracellular or intraorganellar, which in turn have different decay rates based on environmental conditions (Mauvisseau et al., 2022). Additionally, different organisms have different DNA shedding rates which could over- or underestimate select species providing a skewed picture

of the community composition (Allan et al., 2020). Moreover, DNA inhibition can also contribute to difficulties during processing and add to false negatives (Mauvisseau et al., 2022). Recognizing the potential pitfalls will aid in understanding and interpreting findings, which are crucial for information-based decision making. Despite the challenges outlined above, eDNA-based methods are powerful for rare or timid species detection as well as for recently introduced species (Lugg et al., 2018; Smart et al., 2015; Tucker et al., 2016). However, more comprehensive libraries are needed for this to hold true for tropical forests as well.

The present study is based on rainwash eDNA, which involves the collection of eDNA carried by rainfall as it washes over surfaces like tree canopies and soil, capturing genetic material from a wide range of organisms. Using rainwash eDNA to monitor biodiversity has the potential to capture samples from a large surface area over a short time span. A proof-of-principle study in the lower Rhine region of Germany showed the potential of using rain samplers to collect rainwash eDNA (Macher et al., 2022). This method was shown to be useful in closing the knowledge gap of canopy biodiversity at reduced costs. Additionally, it is a minimally invasive method while still providing comprehensive and accurate data.

However, rainwash eDNA for detecting arboreal fauna remains untested for tropical rainforest ecosystems. In the current study, I test this method in a rainforest setting in the western Brazilian Amazon. More specifically, I examine whether 1) rainwash eDNA metabarcoding can be utilized for detection of arboreal species in Amazonia, and 2) how active filtering such as direct water collection, and passive filtering such as letting water wash through a sponge, compare in this type of environment.

Materials and methods

2.1 Study Area

During October 2023, fieldwork was conducted in the western Brazilian Amazon along the middle stretch of the Juruá river, 44km downstream of Carauari (Figure 1) during the dry season. A 450m transect line was set up (4.555851° S, 66.639305° W to 4.551965° S, 66.639032° W) between the Juruá river and an oxbow lake which was a part of the river that has been cut off from the meandering river (Constantine & Dunne, 2008). This is a várzea forest characterized by seasonal flooding, which deposits nutrient-rich sediments from whitewater (Irion, 1978). This remote fieldwork site was chosen to minimize anthropogenic disturbances and risk of contamination from human populations and livestock.

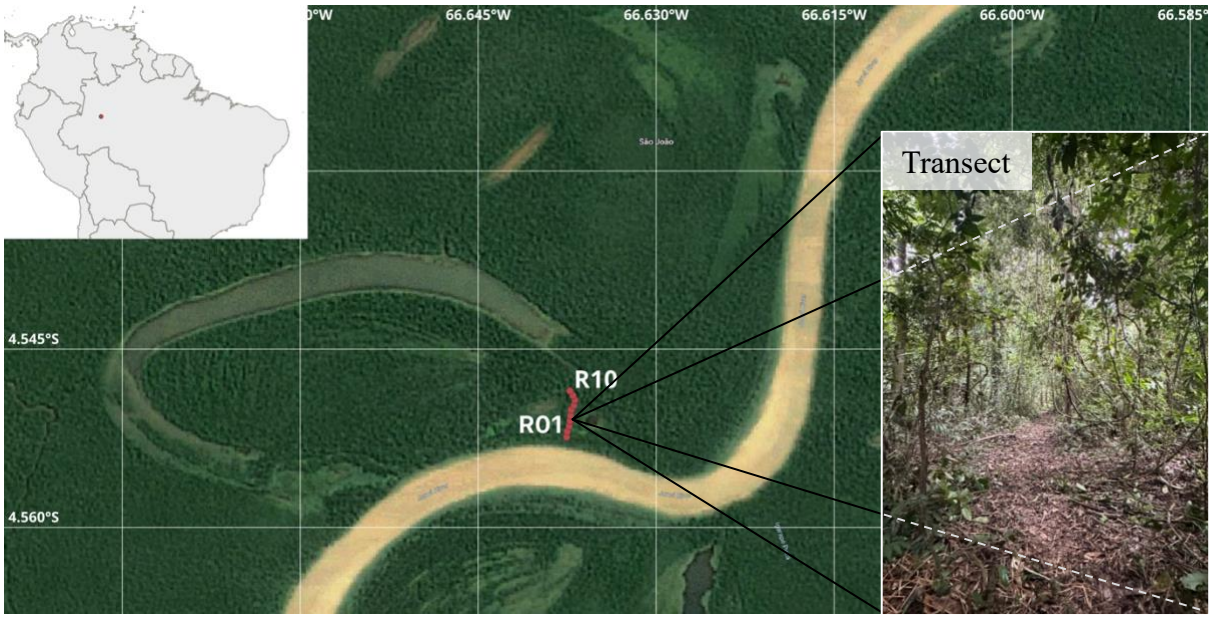

Figure 1. The study site in the western Brazilian Amazon (red dot, inset map) and the rainwash sampling locations in a floodplain forest along the Juruá river. "R01" marks the primary position of two out of 20 samplers, while "R10" marks the end point with the two final samplers.

2.2 Rain sampler design

Rain samplers were placed under the forest canopy to collect rainwash. Each sampler was constructed using a $1m^2$ polyethylene sheet, with 1.5m x 4mm nylon string attached to each corner. Two types were prepared: one having a bottle to collect water (Figure 2D) for subsequent active filtering (Figure 2F) and the other having a Whirl-Pak Speci-Sponge® for *in-situ*, passive, rainwash eDNA filtering (Figure 2C). A 30mm PVC union was attached in the center of each sampler (Figure 2B and 2E). Ten samplers were installed with a sponge (Figure 2B), and the other ten having the PVC union acting as a funnel for the collection bottle below

(Figure 2D and 2E). After each rainfall event, water from the collection bottles were poured to 2041ml Sterilized Whirl-Pak® standard bags, and the bottles were then sterilized for the next rainfall event. A runoff hole was made 20cm from the center to prevent structural failure in case of blockage during heavy rainfall. Each nylon string was attached to a bamboo stick at each corner carrying the sampler while directing the rainwash towards the center.

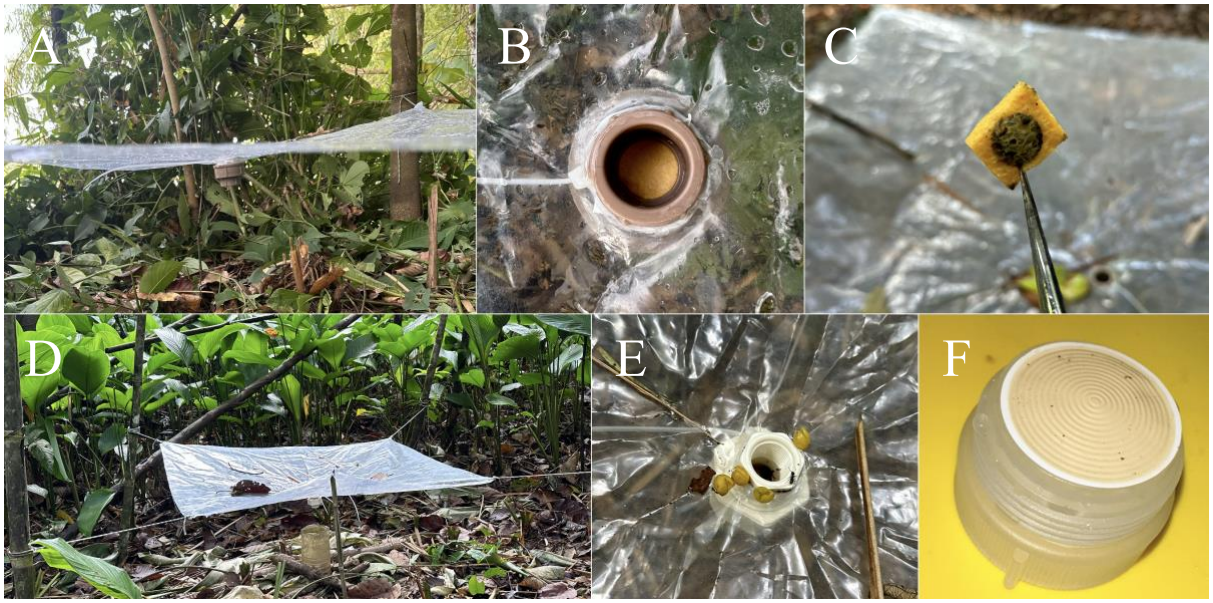

Figure 2. Rain sampler deployed in field showing sponge collection type (A, B, C), and filter type (D, E, F) after a rainfall event. Each rain sampler was held up by four bamboo sticks installed to fit the rain sampler in a good position (A, D), with the sponge fit inside the PVC union (B) for the sponge sampler. For the filter collection sampler, the PVC union functioned as a funnel to the bottle below (D), with the water collected for filtering ex-situ (F).

2.3 Rainwash sampling

Two rain samplers were set up every 50 meters along the transect. The samplers were initially sterilized with 10% bleach for 3 minutes, then washed off with 70% EtOH, followed by rinsing with distilled water before they were put in sterile bags for transportation. Latex gloves and face masks were used while deploying samplers and collection, and GPS coordinates were noted. Three rain events were recorded, and the samplers were cleaned after each event using the same protocol as described above.

After each subsequent rainfall event, the water collected in the Whirl-Pak® Standard Sterilized Sampling Bag was filtered through 0.8 µm pore size Whatman® nitrocellulose membrane (Figure 2F) using a 25mm Swinnex Filter Holder before being submerged in ATL-buffer in 2ml Eppendorf tubes. To lessen the thermal impact on the active filtering samples, they were stored in a cooled environment (ice box) immediately after arriving to base prior to processing. The passive filtering samples were submerged in 96% EtOH in Corning® 50 mL centrifuge tubes.

Negative controls were made both *in-situ* (field) as well as *ex-situ* (lab). For clarity, the active filtering samples and the passive filtering samples will hereafter be referred as "filter samples" and "sponge samples", respectively, throughout this thesis.

2.4 Extraction

The collected DNA was extracted at the Center for Advanced Studies on Biodiversity (Ceabio) at the Federal University of Pará (UFPA) in Belém, using Dneasy® PowerSoil® Pro Kit with its following protocol (QIAGEN, 2023). Every step was done in a sterile lab by UV sterilization of flow hood, sterile benches, laboratory coat, face masks and latex gloves. Triplicates were made of the filter samples. After extraction, all samples were stored in a refrigerator before being sent to the Natural History Museum (NHM) at the University of Oslo (UiO).

2.5 PCR, gel electrophoresis and normalization

Samples were stored in a freezer at the Natural History Museum DNA lab, Oslo. As the DNA concentrations were very high, the samples were diluted 1:20. A mastermix (Supplementary Table 1) was prepared before loading it onto a primer plate. Triplicates of each sample were produced using both the Leray (cytochrome c oxidase subunit I (COI)) and Riaz (12S gene) primer before running through PCR (Supplementary Table 2). After the PCR step, the amplicons were loaded to gel electrophoresis using GelRed® Nucleic Acid Gel Stain and ran alongside a 50-1500 bp ladder (FastRuler Low Range) for visualization of DNA length and potential contamination to determine if further cleaning or dilution was necessary using ImageLab Software v6.0 under UV light. The subsequent step involved normalization to achieve a uniform DNA concentration across samples using an automatic pipetting machine prior to sequencing.

2.6 Bioinformatics

Bioinformatics processing, including merging, demultiplexing and data cleaning steps, was conducted as in Raclariu-Manolică et al. (2023) with a slight modification of denoising and the taxonomic assignment steps. The unique sequences were clustered at a 97% similarity threshold using USEARCH v11.054 to form ZOTUs (Zero Radius Operational Taxonomic Units). Subsequently, the taxonomy assignment was performed using the k-mer based approach SINTAX in VSEARCH v2.21.1 (Leray et al., 2022; Rognes et al., 2016), with clustered ZOTUs assigned at a minimum 90% similarity threshold using the MIDORI2 database. The fragments

from the Riaz primer were filtered to remove sequences shorter than 100bp and longer than 150bp. For the Leray primer, this range was between 100bp and 350bp. Negative controls were used to further clean the data. Ten read counts were removed from all reads to remove false positives, and only native species were included in the final dataset.

2.7 Statistical methods

2.7.1 Species composition

The phylogenetic tree was constructed using the *taxize* package in R querying the NCBI database to create a phylogenetic tree in Newick format using hierarchical relationships from the taxonomic data (Chamberlain & Szöcs, 2013). Visualization was performed using a combination of r packages: *ape* for reading and manipulating the tree structure, and *treeio*, *ggtree* and *ggtreeExtra* for visual representation (Wang et al., 2020; Xu et al., 2021; Xu et al., 2022). The creation of a stacked bar chart to visualize relative abundances across shared arboreal species count and the boxplot were carried out using the R package *ggplot2* (Wickham, 2016).

2.7.2 Diversity measurement

Species diversity was quantified using Hill numbers, which include species richness (q = 0), Shannon diversity (q = 1), and Simpson diversity (q = 2) (Chao et al., 2014). Calculations were performed using the *vegan* package in R, while visualization of the diversity metrics was conducted using the R package *iNEXT* (Hsieh et al., 2016).

Results

Three rainwash events were recorded. Rainwash event 1 (RW1) on 15/10/23, rainwash event 2 (RW2) on 20/10/23 and the last rainwash event (RW3) on 21/10/23. After the cleaning steps were made, there was a total of 382,748 and 311,177 reads for the filter and sponge samples, respectively, using the Riaz (12 S) primer. Despite the higher overall read count for filter samples, the average read count per species is lower for the filter samples compared to the sponge samples (Filter: 153.75 ± 36.49 SE; sponge: 260.98 ± 144.97 SE). The average read count of the filter samples was also comparatively lower for arboreal species (filter: 1080.68 ± 322.78 SE; sponge: 1581.79 ± 380.19 SE).The Leray (COI) primer did not provide satisfactory results, with only 21 and 471 reads across two species (*Ara severus* and *Saimiri sciureus*) for the filter and sponge methods, respectively. Therefore, results presented in this study are based exclusively on the Riaz primer.

3.1 Species composition

A total of 45 and 33 different vertebrate species were found across 43 and 32 genera and 34 and 25 families for the filter and sponge samples, respectively (Supplementary Table 6). These belong to four different classes: Actinopteri (total: 19 species, filter: 19, sponge: 13), Mammalia (total: 18 species, filter: 18, sponge: 15), Aves (total: 7 species, filter: 7, sponge: 4) and one from Reptilia (*Rhinoclemmys punctularia*) (Supplementary Table 6). A total of 20 arboreal species were detected (Filter: 20, sponge: 16). Of the 18 mammalian species detected, 13 are known to be arboreal (Figure 3A). The filter method detected one arboreal mammal (*Cacajao calvus)* not detected using the sponge method. Additionally, the filter method detected the arboreal bird species *Cephalopterus ornatus* in four samples, *Coragyps atratus* in one sample, and *Glaucidium brasilianum* in thirteen samples. Of the sixteen arboreal species detected by both methods, the proportion of reads varied with one method often contributing to a higher percentage of reads for specific species (Figure 3B).

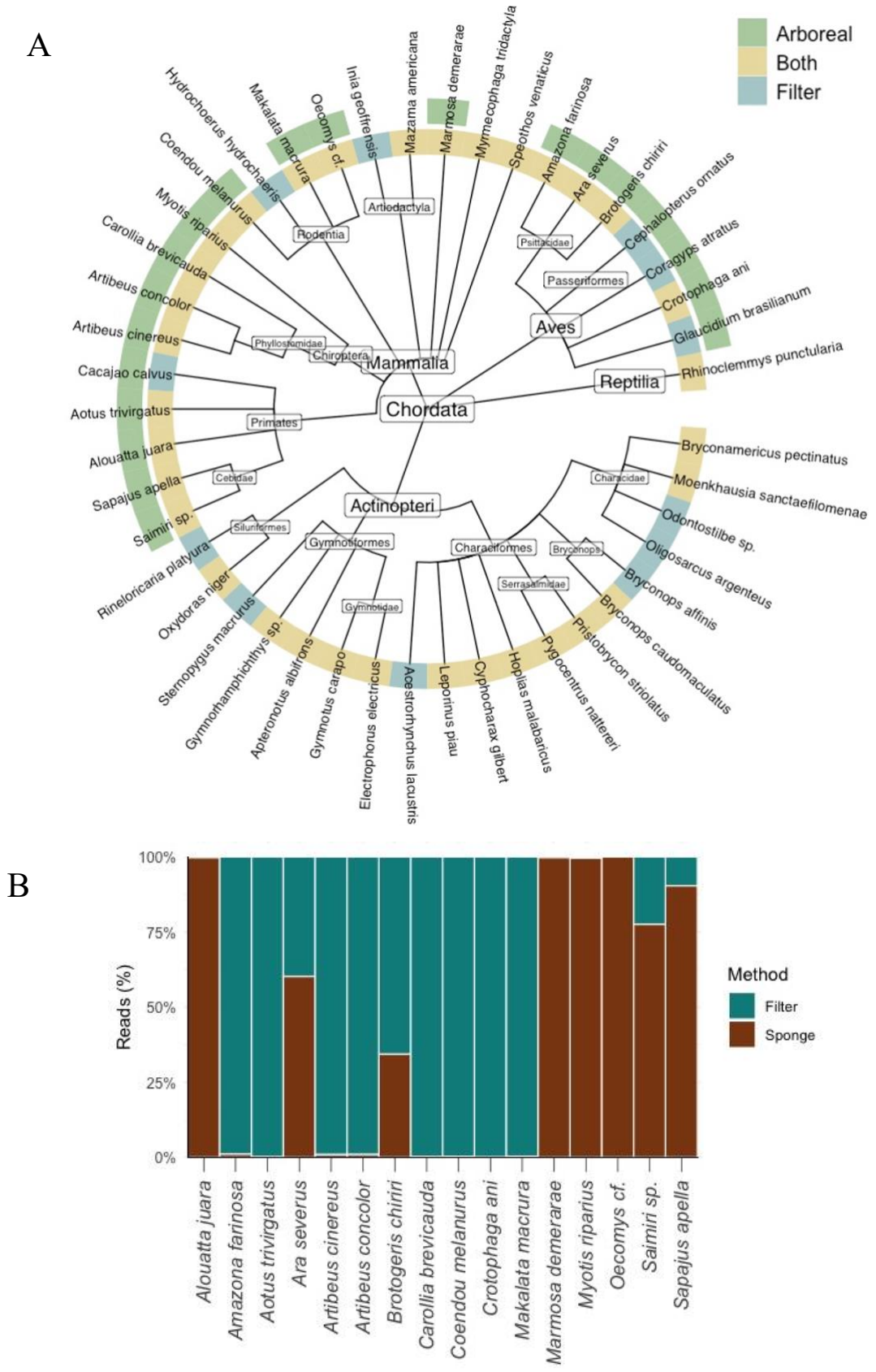

Figure 3. The phylogenetic tree (A) provides the taxonomic relationship of native species detected with the filter method (blue) and both methods (beige). Arboreal species are highlighted in green. The bar chart (B) shows the percentage of the total number of reads for arboreal species found in both the filter (Cyan) and sponge (brown) method.

There was not a notable variability in arboreal species detection between sampling events, as reflected in the standard deviation of the detection rates (mean detection rate for filter: 4.03 ± 2.68 SD, sponge: 3.96 ± 2.69 SD, Figure 4). The poorest performance was observed during RW1 (filter: $\bar{x}$ = 2.60 ± 0.97 SD, sponge: $\bar{x}$ = 1.71 ± 0.49, Figure 4). In contrast, the best performance occurred during RW2 (filter: $\bar{x}$ = 6.10 ± 3.41 SD, sponge: $\bar{x}$ = 5.25 ± 3.28 SD, Figure 4), while RW3 demonstrated an intermediate performance (filter: $\bar{x}$ = 3.40 ± 1.78 SD, sponge: 4.56 ± 2.19 SD, Figure 4).

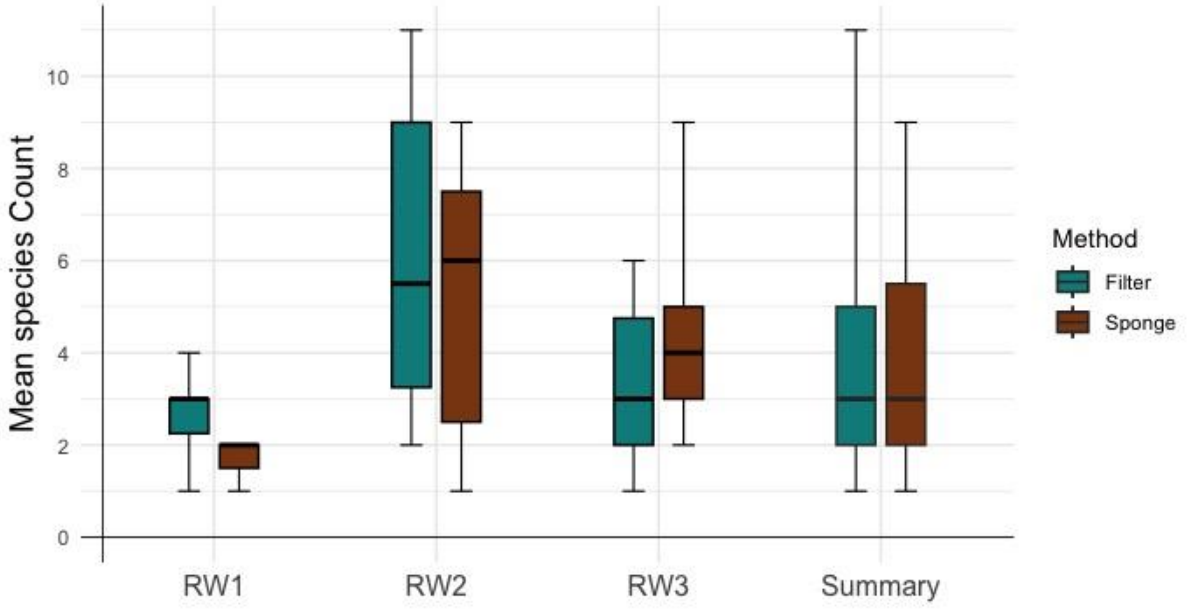

Figure 4. Mean arboreal species counts detected by filter (cyan) and sponge (brown) methods for three rainwash events (RW1, RW2, RW3) and pooled total (Summary). The boxes show the interquartile range (IQR) of species counts for each RW event, with the solid black line indicating the median. Whiskers represent the range of values within ± 1.5 times IQR.

The Generalized Linear Model (GLM) showed no significant difference in arboreal mammal species richness detected with the sponge and filter methods (Z = 0.000 ; p = 1.000, Table 1). Similarly, sampling date did not significantly influence species richness (RW2: Z = 1.543; p = 0.123, RW3: Z = 1.212; p = 0.226, Table 1) and the interaction between sampling method and rainwash events did not yield significant results (Sponge Method:RW2: Z = -0.714; p = 0.475; Sponge Method:RW3: Z = 0.115; p = 0.908, Table 1). A significant difference was found for GLM of total species richness between methods (p = 0. 00386, Supplementary Table 3).

Table 1. Results from the generalized linear model (GLM) of arboreal species richness, modeled using a Poisson distribution. Comparisons include methods (Filter vs Sponge), rainwash events (RW1, RW2, RW3) and their interactions. Estimates,

*standard errors, Z-values and p-values are reported for each predictor, with significant results denoted by asterisks: *** = p < 0.001.*

	Estimate	Std. Error	Z value	p-value	
(Intercept)	2.197	0.333	6.592	4.35e-11	***
Sponge Method	-2.997e-16	0.471	0.000	1.000	
RW2	6.360	0.412	1.543	0.123	
RW3	0.108	0.422	1.212	0.226	
Sponge Method:RW2	-4.353	0.610	-0.714	0.475	
Sponge Method:RW3	-0.069	0.600	0.115	0.908	

3.2 Species diversity

Total species richness (q = 0, Figure 5) was close to reaching an asymptote for the filter samples, while the sponge method was slightly further from reaching an asymptote (Filter: 45 observed, asymptotic diversity estimator = 47.00; Sponge: 33 observed, estimator = 35.71, Figure 5) implying additional sampling would uncover additional species. The curves for Shannon diversity (q = 1) for both methods almost reached an asymptote (Filter: 24.36 observed, estimator = 25.53; Sponge: 16.98 observed, estimator = 18.33, Figure 5). The Simpson diversity (q = 2) shows both methods reaching an asymptote (Filter: 14.78 observed, estimator = 15.18; Sponge: 11.20 observed, estimator = 11.67, Figure 5).

Arboreal species richness almost flattened out for both methods (Filter: 20 observed, estimator = 21.00; Sponge: 16 observations, estimator = 16.66, Figure 5). The curves for Shannon diversity (q = 1) reached an asymptote for both methods (Filter: 10.71 observed, estimator = 11.06; Sponge: 9.32 observed, estimator = 9.75, Figure 5). The Simpson diversity (q = 2) also shows both the methods reaching an asymptote (Filter: 7.06 observed, estimator = 7.20; Sponge: 6.88 observed, estimator = 7.09, Figure 5).

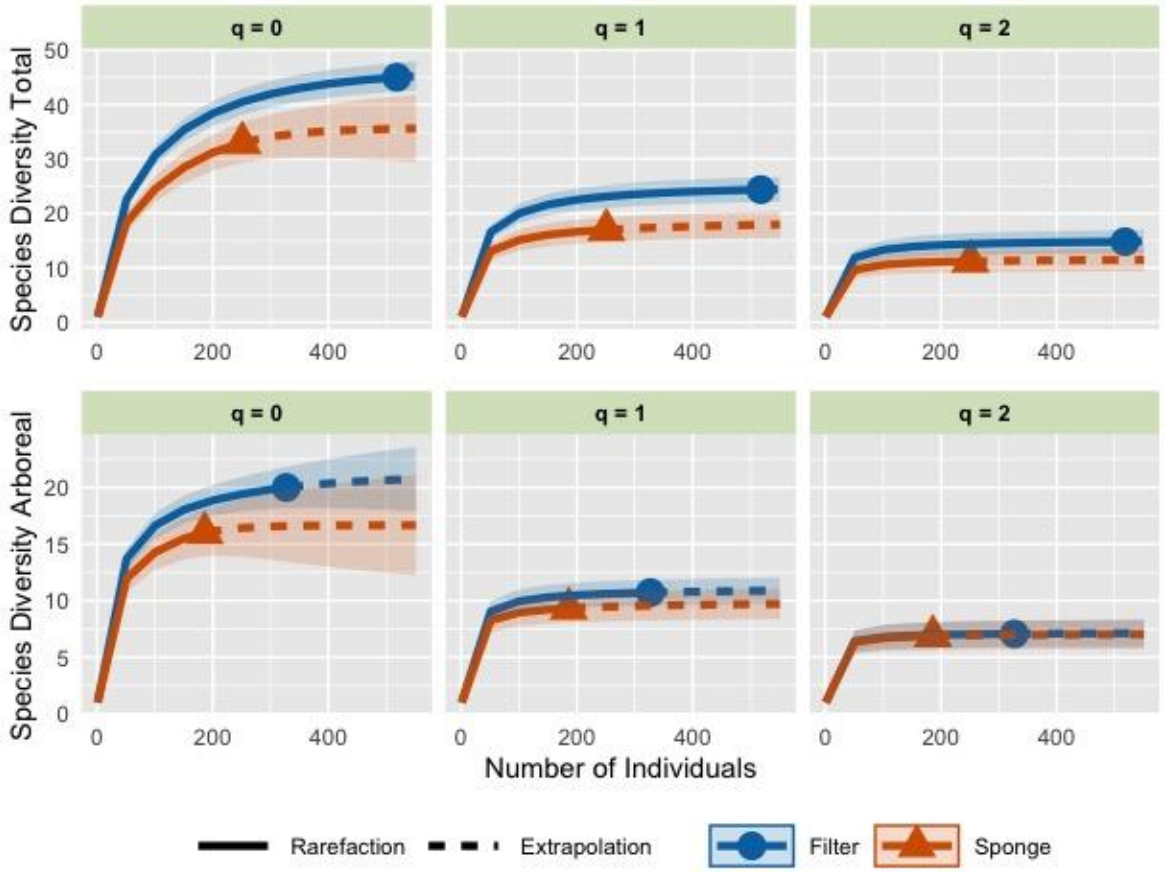

Figure 5. Individual-based species rarefaction (solid line) and extrapolation (dashed line) curves for all native species and arboreal based on Hill numbers (q = 0, 1, 2).

Discussion

4.1 Species detection

Rainwash eDNA sampling and analysis has been done before (Macher et al., 2022; Miwa et al., 2024). However, to my knowledge, this is the fist study to utilise this approach in a tropical forest setting and examine its use for detection of arboreal vertebrates. Both q = 1 (Shannon) and the Simpson (q = 2) accumulation curves (Figure 5) indicate that this method identifies the most common and dominant species within its detection range. However, a significant number of species in this region are left undetected (Supplementary Table 8). There are 62 well-documented non-aquatic mammal species occurring in the várzea region of the middle Juruá river (Supplementary Table 8). Contriary to expectations, the rainwash method only detected 17 of these (27% of the expected species). Notably, only seven of the 239 known avian species (Supplementary Table 8) were detected (2.9% of expected species), many of which are arboreal. A species richness of 45 (whereas 19 were fish (actinopteri), Supplementary Table 6) falls short with the 301 non-aquatic mammalian and avian species in this region (Supplementary Table 8). These findings raise questions about the methods' ability to capture the entire vertebrate community.

No amphibians and only one reptile was detected in this study, despite Vågen (2024) conducting field work in the same area and period identifying 27 different anuran species, many of which were tree frogs. These results highlight the varying detecatbility of certain taxonomic groups using eDNA based tools. The Riaz (12S) and Leray (COI) primers used in this thesis are broad spectrum primers, which might not be able to detect amphibians (Che et al., 2012; Vences et al., 2005). Here, a combination of primer sets could be useful (e.g. Amphibia 12S or 16S rDNA primer for amphibia (Evans et al., 2016; Vences et al., 2005) and RepCOI or 16S rDNA primer for reptila (Nagy et al., 2012; Vences et al., 2012)). Limited reference databases could also contribute to the lack of amphibians and reptililes detected. Although this study collected rainwash eDNA, more non-arboreal species than arboreal species were detected in both methods. Notable is the relatively high number of Actinopteri (fish) species detected: 19 species for the filter method and 13 in the sponge method. This is expected given the complicated food webs of this region (Hawes & Peres, 2014) with a lot of arboreal species (e.g. birds) feeding on aquatic animals, shedding their DNA through feces or remnants during consumption. Additionally, Kumar et al. (2022) showed that the Riaz (12S) primer are effective

for detecting fish species, in part explaining the relativly large detection of fish species in the current study.

Interestingly, the Amazonian River Dolphin (*Inia geoffrensis*) was detected by the filter method (Figure 3A). Its DNA could originate from a carcass consumed by a black vulture (*Coragyps atratus*), potentially carried to the forest canopy as Amazonia experienced a record drought during this period (Espinoza et al., 2024). DNA does not necessarily have to be directly from scavenging or prey, as previous studies have shown that DNA can travel by air (Clare et al., 2022). As *Inia geoffrensis* respires air, its DNA may have reached one of the samples as they were close to the riverbank. Additionally, this study area is a seasonally flooded forest where fluctuations in the river level can exceed 15 meters annually (de Vasconcelos et al., 2022). During periods of flooding lasting up to six months (Wittmann et al., 2004), the river overflows its banks inundating the forest understory. eDNA present in the floodwaters can stick to tree leaves submerged during flooding, potentially contributing to the DNA captured by the samples.

4.2 Method comparison

Despite Figure 4 suggesting an impact of consecutive days without rainfall (i.e., dry days), the statistical analysis did not find a significance between rainwash events (Table 1). The filter method detected fewer species with increasing dry days, while the sponge method detected more species up to a certain number of dry days. However, timing a rainfall event to a specific number of days without rainfall is challenging in tropical rainforests where rainfall is highly variable both spatially and temporarily.

The comparatively higher total species richness (Supplementary Table 6) detected using the filter method was significant (Supplementary Table 3), suggesting the filter method is able to capture a wider range of non-arboreal species. It could be influenced by the collection bottle's placement on the forest floor, potentially introducing additional DNA from soil or splashback. The positioning could contribute to a broader range of detected species, but could also dilute the DNA signal from rainwash-specific sources, resulting in the lower mean read count per species compared to the sponge method. The standing body of water for this method would also be subject to more microbiological activity which can affect DNA integrity and influence the read count across species. Additionally, the difference between methods could be attributed

to material degradation of the sponge, environmental factors (e.g. temperature, UV-B light irradiation, pH) and microbial activity affecting DNA over time (Mauvisseau et al., 2022). Sponges could gradually lose their capacity to capture and retain eDNA as they are exposed to the environment over time, whereas filters remain unaffected as they are only exposed during the filtering step. Both methods are affected by organic materials inhibiting PCR processing, potentially reducing the efficiency or reliability of eDNA detection. When focusing exclusively on arboreal species, the GLM results (Table 1) indicate that neither method nor temporal variation had a significant impact on arboreal species richness. The findings suggests that the filter method's design captures more diverse DNA at the cost of specificity and concentration. Despite the filter method having a higher sensitivity for non-arboreal species, the filter method is significantly more labor-intensive than the sponge method. The sponge method appears to be a better option if reducing fieldwork time and effort is a priority or when only investigating arboreal species. While the sponge method is logistically simpler and less labor-intensive, it may require a greater number of replicates or extended deployment periods to match the total species richness detected by the filter.

Further research should consider increasing spatial coverage, extending the timeframe to enhance the temporal coverage, or adjusting the study period closer to the wet season. A different approach, such as leaf swabbing for eDNA metabarcoding currently being explored by Johnsen (2024), could reveal a higher spiecies richness. A combination of eDNA metabarcoding from leaf swabbing and rainwash could be worth exploring as this would not require substantial additional fieldwork.

4.3 Future applications

Traditional methods rely on direct observation or costly equipment, such as camera trapping. However, camera traps are a one-time purchase while molecular approaches have a high cost associated to lab materials and labor for each individual study. Studies have shown that eDNA based methods can be more cost-efficient, particularly in species rich regions and with increasing sampling efforts (Bálint et al., 2018; Leasi et al., 2018; Lyet et al., 2021). Future advances in molecular eDNA based methods are expected to increase the cost-efficiency of these methods (Fu et al., 2021; Smart et al., 2016). Allen et al. (2023) emphasizes that eDNA metabarcoding can be especially valuable where field conditions pose safety hazards as it can reduce field time due to fewer visits needed to obtain comparable results.

Although eDNA metabarcoding plays an increasingly important role in biodiversity research, there remain significant shortcomings that needs to be addressed. This paper underlines the need of proper tools (e.g. fitting primers) and DNA metabarcoding reference databases. eDNA based methods can be especially effective when addressing rare species. de Oliveira et al. (2018) estimated the relative abundance of the bush dog (*Speothos venaticus*) to be 0.060 – 0.185 individuals per 100 trap-days, while in this thesis the bush dog appeared in all three rainwash events (Supplementary table 7) across 16 samples. Allen et al. (2023) demonstrated that a qPCR-based approach was able to detect specific species to a higher degree, indicating eDNA could be a useful tool for assessing rare or newly introduced species. Coupled with advancements in robotics (e.g. drones Aucone et al. (2023)), it could significantly reduce fieldwork and reduce biases towards biodiversity research in easily accessible regions.

Conclusion

This study demonstrates the potential of rainwash eDNA metabarcoding as a minimally invasive tool for biodiversity monitoring in várzea forests along the Juruá River. By comparing active (filter) and passive (sponge) eDNA collection methods, this study highlights the strengths and limitations of each approach in detecting both arboreal and non-arboreal vertebrate species. The findings confirm that although rainwash eDNA can detect a diverse array of species, the results are varying. While the filter method detected a higher species richness overall, the sponge method proved logistically simpler and more consistent for detecting arboreal species.

Furthermore, this study emphasizes the inherent challenges of eDNA-based monitoring in tropical regions, such as environmental and microbial degradation of DNA. Additionally, challenges remain particularly in detecting amphibians and reptiles which were underrepresented in the results. These gaps stress the need for expanded reference libraries and primer optimization to enhance taxonomic coverage. However, both methods successfully detected the rare species *Speothos venaticus*, highlighting its potential use for rare species detection. While rainwash eDNA holds promise as a complementary method to traditional biodiversity surveys, its application requires further refinement to enhance its reliability and effectiveness as a biodiversity monitoring tool.

Future research should aim to address these limitations by extending sampling efforts, using a greater diversity or specificity of primer sets, and exploring combinations with emerging technologies such as drone-assisted sampling. With continued advancements, eDNA metabarcoding has the potential to revolutionize biodiversity monitoring in challenging and biodiverse ecosystems like the Amazon.

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Appendix

Table S1. PCR Mastermix, DNA and Primer volumes. Note that DNA and Primers were added to individual cells after the PCR Mastermix was added.

PCR Mastermix, DNA and Primer volumes	
Ingredient	**µl**
AccuStart™ II PCR ToughMix®	7.5
MilliQ H_2O	4.5
DNA sample	1.0
Primer	2.0
Total	**15**

Table S2. Riaz (RIAZ_BR, left) and Leray (LERAY_BR, right) protocol for PCR amplification. Both begin with an inital denaturation step, followed by cycles of denaturation and annealing during which the annealing step decreases by 1°C per cycle starting at 45°C.

RIAZ_BR			**LERAY_BR**		
Temperature	Time	Cycles	Temperature	Time	Cycles
95°C	5 minutes	1	94°C	3 minutes	1
95°C	30 seconds	10	94°C	10 seconds	5
45°C (1°C/cycle)	30 seconds		45°C (1°C/cycle)	20 seconds	
72°C	50 seconds		72°C	30 seconds	
95°C	30 seconds	25	94°C	10 seconds	30
55°C	30 seconds		54°C	20 seconds	
72°C	50 seconds		72°C	30 seconds	
72°C	2 minutes	1	72°C	3 minutes	1

*Table S3. Results from the generalized linear model (GLM) of total species richness, modeled using a Poisson distribution. Comparisons include methods (Filter vs Sponge) and rainwash events (RW1, RW2, RW3). Estimates, standard errors, Z-values and p-values are reported for each predictor, with significant results denoted as: "***" = $p \leq 0.001$, "**" = $p \leq 0.01$.*

	Estimate	**Std. Error**	**Z value**	**Pr(>F)**	
(Intercept)	3.3424	0.1601	20.878	< 2e-16	***
Sponge Method	-0.4680	0.1620	-2.890	0. 00386	**
RW2	0.2822	0.1953	1.445	0.14837	

RW3	0.1603	0.2006	0.799	0.42421

Table S4. Forward (F) and reverse (R) primer of Leray (COI) and Riaz (12S) used.

	Primer F seq	**Primer R seq**
Leray	GGWACWGGWTGAACWGTWTAYCCYCC	TANACYTCNGGRTGNCCRAARAAYCA
Riaz	ACTGGGATTAGATACCCC	TAGAACAGGCTCCTCTAG

*Table S5. Leray and Riaz primer F and R sequences. **W** can be either base A or T, **Y** are either pyrimidine, **N** are any base, **R** are either purines.*

Index (F/R)	**Sequence 5' – 3'**
1	CCTAAACTACGG
2	TGCAGATCCAAC
3	CCATCACATAGG
4	GTGGTATGGGAG
5	ACTTTAAGGGTG
6	GAGCAACATCCT
7	TGTTGCGTTTCT
8	ATGTCCGACCAA
9	AGGTACGCAATT
10	ACAGCCACCCAT
11	TGTCTCGCAAGC
12	GAGGAGTAAAGC
13	GTTACGTGGTTG
14	TACCGCCTCGGA
15	CGTAAGATGCCT
16	TACCGGCTTGCA
17	ATCTAGTGGCAA
18	CCAGGGACTTCT
19	CACCTTACCTTA
20	ATAGTTAGGGCT

21	GCACTTCATTTC
22	TTAACTGGAAGC
23	CGCGGTTACTAA
24	GAGACTATATGC

Table S6. Number of native species grouped by Family, Class, Genus, Species, Arboreal, Arboreal Mammalian and Non-arboreal. The /unique is the number not found in the other method.

	Filter/unique	**Sponge/unique**	**Total**
Class	**4/0**	**4/0**	**4**
Actinopteri	19/6	13/0	19
Aves	7/3	4/0	7
Mammalia	18/3	15/0	18
Reptilia	1/0	1/0	1
Family	**34/9**	**25/0**	**34**
Genus	**43/11**	**32/0**	**43**
Species	**45/12**	**33/0**	**45**
Arboreal	20/4	16/0	20
Arboreal Mammalian	13/1	12/0	13
Non-arboreal	25/8	17/0	25

Table S7. Native species detected separated by class, showing which method detected: F = Filter method, S = Sponge method. All dates are days in the month of October 2023.

Class	**Family**	**Species**	**Method**	**Dates**
Actinopteri	Acestrorhynchidae	Acestrorhynchus lacustris	F	15, 20
	Anostomidae	Leporinus piau	F/S	15, 20, 21
	Apteronotidae	Apteronotus albifrons	F/S	15, 20, 21
	Characidae	Bryconamericus pectinatus	F/S	15, 20, 21
	Characidae	Moenkhausia sanctaefilomenae	F/S	15, 20, 21
	Characidae	Odontostilbe sp.	F	20
	Characidae	Oligosarcus argenteus	F	15, 20, 21
	Curimatidae	Cyphocharax gilbert	F/S	15, 20, 21
	Doradidae	Oxydoras niger	F/S	15, 20, 21
	Erythrinidae	Hoplias malabaricus	F/S	15, 20, 21

	Gymnotidae	Electrophorus electricus	F/S	15, 20, 21
	Gymnotidae	Gymnotus carapo	F/S	15, 20, 21
	Iguanodectidae	Bryconops affinis	F	15, 20
	Iguanodectidae	Bryconops caudomaculatus	F/S	15, 20, 21
	Loricariidae	Rineloricaria platyura	F	20
	Rhamphichthyidae	Gymnorhamphichthys sp.	F/S	15, 20, 21
	Serrasalmidae	Pristobrycon striolatus	F/S	15, 20, 21
	Serrasalmidae	Pygocentrus nattereri	F/S	15, 20
	Sternopygidae	Sternopygus macrurus	F	15, 20
Aves	Cathartidae	Coragyps atratus	F	15
	Cotingidae	Cephalopterus ornatus	F	20, 21
	Crotophagidae	Crotophaga ani	F/S	15, 20, 21
	Psittacidae	Amazona farinosa	F/S	15, 20, 21
	Psittacidae	Ara severus	F/S	15, 20, 21
	Psittacidae	Brotogeris chiriri	F/S	15, 20, 21
	Strigidae	Glaucidium brasilianum	F	20, 21
Mammalia	Aotidae	Aotus trivirgatus	F/S	21
	Atelidae	Alouatta juara	F/S	15, 20, 21
	Canidae	Speothos venaticus	F/S	15, 20, 21
	Cebidae	Saimiri sp.	F/S	15, 20, 21
	Cebidae	Sapajus apella	F/S	20, 21
	Cervidae	Mazama americana	F/S	15, 20, 21
	Cricetidae	Oecomys cf.	F/S	15, 20
	Didelphidae	Marmosa demerarae	F/S	15, 20, 21
	Echimyidae	Makalata macrura	F/S	20, 21
	Erethizontidae	Coendou melanurus	F/S	15, 20, 21
	Hydrochaeridae	Hydrochoerus hydrochaeris	F	15, 20, 21
	Iniidae	Inia geoffrensis	F	15, 20, 21
	Myrmecophagidae	Myrmecophaga tridactyla	F/S	20, 21
	Phyllostomidae	Artibeus cinereus	F/S	15, 20, 21
	Phyllostomidae	Artibeus concolor	F/S	20, 21
	Phyllostomidae	Carollia brevicauda	F/S	20
	Pitheciidae	Cacajao calvus	F	15

	Vespertilionidae	Myotis riparius	F/S	15, 20, 21
Reptilia	Geoemydidae	Rhinoclemmys punctularia	F/S	15, 20, 21

Table S8. Potential species of várzea in the Medio Juruá region of Amazonia. Under the "stratum" column, Mammalia and Reptilia are grouped in arboreal (A) or terrestrial (T). The number in parenthesis after class highlights the number of species in this class are listed.

Family	*Species*	Stratum
Mammalia (62)		
Primates		
Atelidae	*Alouatta seniculus*[a]	A
	Lagothrix cana[b]	A
	Ateles chamek[b]	A
Cebidae	*Saguins mystax*[a]	A
	Saimiri boliviensis[b]	A
	Saimiri macrodon[b]	A
	Saimiri sciureus[a]	A
	Saguinus fuscicollis[a]	A
	Cebus albifrons[a]	A
	Cebus apella[a]	A
	Cebus unicolor[b]	A
	Sapajus macrocephalus[b]	A
Pitheciidae	*Pithecia monachus*[b]	A
	Pithecia albicans[b]	A
	Aotus nigriceps[a]	A
	Cacajao calvus[a]	A
	Callicebus cupreus[b]	A
	Callicebus torquatus[b]	A
Callitrichidae	*Cebuella pygmaea*[b]	A
Pilosa		
Myrmecophagidae	*Tamandua tetradactyla*[b]	A
	Myrmecophaga tridactyla[b]	T
Cyclopedidae	*Cyclopes didactylus*[b]	A
Choloepodidae	*Choloepus didactylus*[b]	A
Artiodactyla		
Cervidae	*Mazama americana*[a]	T
	Mazama nemorivaga[b]	T
	Mazama gouazoupira[c]	A
Tayassuidae	*Tayassu pecari*[a]	T

	Pecari tajacu[a]	T
Rodentia		
Dasyproctidae	*Dasyprocta fuliginosa*[a]	T
	Myoprocta spp.[b]	T
Cuniculidae	*Cuniculus paca*[b]	T
Echimyidae	*Echimys spp.*[a]	A
	Isothrix spp.[a]	A
	Proechimys spp.[b]	T
Erethizontidae	*Coendou sp.*[b]	A
Cricetidae	*Rhipidomys sp.*[b]	A
Sciuridae	*Guerlinguetus ignitus*[b]	A
	Urosciurus spadiceus[b]	A
	Sciurus spadiceus[c]	A
Carnivora		
Mustelidae	*Eira barbara*[a]	T
	Galictis vittata[b]	T
	Pteronura brasiliensis[b]	T
Procyonidae	*Potos flavus*[b]	A
	Procyon cancrivorus[b]	T
	Nasua nasua[a]	T
Felinae	*Panthera onca*[b]	T
	Leopardus wiedii[b]	A
	Leopardus pardalis[b]	T
	Puma concolor[b]	T
	Puma yagouaroundi[b]	T
Canidae	*Atelocynus microtis*[b]	T
	Speothos venaticus[b]	T
Cingulata		
Chlamyphoridae	*Priodontes maximus*[b]	T
Dasypodidae	*Dasypus kappleri*[b]	T
	Dasypus novemcinctus[b]	T
Didelphimorphia		
Didelphidae	*Caluromys lanatus*[b]	A
	Didelphis marsupialis[b]	T
	Glironia venusta[b]	A
	Metachirus nudicaudatus[b]	T
	Philander sp.[b]	T
Perissodactyla		
Tapiridae	*Tapirus terrestris*[b]	T

Reptilia (1)		
Testudinidae	*Geochelone spp.*[b]	T
Aves (239)		
Gruiformes		
Psophiidae	*Psophia leucoptera*[a]	
Aramidae	*Aramus guarauna*[d]	
Rallidae	*Aramides cajaneus*[d]	
Tinamiformes		
Tinamidae	*Crypturellus spp.*[a]	
	Tinamus guttatus[d]	
	Tinamus major[d]	
Anseriformes		
Anatidae	*Dendrocygna autumnalis*[d]	
	Neochen jubata[b]	
Anhimidae	*Anhima cornuta*[d]	
Galliformes		
Cracidae	*Ortalis guttata*[a]	
	Crax globulosa[a]	
	Mitu tuberosum[b]	
	Penelope jacquacu[b]	
Suliformes		
Phalacrocoracidae	*Phalacrocorax brasilianus*[d]	
Anhingidae	*Anhinga anhinga*[d]	
Ciconiiformes		
Ciconiidae	*Mycteria americana*[d]	
Pelecaniformes		
Ardeidae	*Tigrisoma lineatum*[d]	
	Ardea cocoi[d]	
	Pilherodius pileatus[d]	
	Nycticorax nycticorax[d]	
	Agamia agami[d]	
Threskiornithidae	*Mesembrinibis cayennensis*[d]	
Accipitriformes		
Cathartidae	*Cathartes aura*[d]	
	Coragyps atratus[d]	
Pandionidae	*Pandion haliaetus*[d]	
Accipitridae	*Rostrhamus sociabilis*[d]	
	Harpagus bidentatus[d]	

	Ictinia plumbea[d]
	Geranospiza caerulescens[d]
	Buteogallus schistaceus[d]
	Rupornis magnirostris[d]
	Harpagus diodon[d]
Falconiformes	
Falconidae	*Daptrius ater*[d]
	Ibycter americanus[a]
	Milvago chimachima[d]
	Falco rufigularis[d]
Charadriiformes	
Charadriidae	*Hoploxypterus cayanus*[d]
	Charadrius collaris[d]
Scolopacidae	*Actitis macularius*[d]
	Calidris fuscicollis[d]
	Calidris melanotos[d]
Jacanidae	*Jacana jacana*[d]
Laridae	*Sternula superciliaris*[d]
	Phaetusa simplex[d]
Rynchopidae	*Rynchops niger*[d]
Columbiformes	
Columbidae	*Leptotila rufaxilla*[d]
	Geotrygon montana[d]
	Columbina talpacoti[d]
	Patagioenas cayennensis[d]
	Patagioenas plumbea[d]
	Patagioenas subvinacea[d]
	Leptotila rufaxilla[d]
	Geotrygon montana[d]
Psittaciformes	
Psittacidae	*Ara macao*[d]
	Ara severus[d]
	Aratinga spp.[a]
	Psittacara leucophthalmus[d]
	Brotogeris sanctithomae[d]
	Pionites leucogaster[d]
	Graydidascalus brachyurus[d]
	Pionus menstruus[d]

	Othopsittaca spp.[a]
	Pyrrhura spp. [a]
	Pionities spp. [a]
	Pionopsitta spp.[a]
	Amazona festiva[d]
	Amazona amazonica[d]
Cuculiformes	
Cuculidae	*Piaya cayana*[d]
	Crotophaga major[d]
	Crotophaga ani[d]
	Coccyzus melacoryphus[d]
Strigiformes	
Strigidae	*Glaucidium brasilianum*[d]
Caprimulgiformes	
Caprimulgidae	*Nyctiphrynus ocellatus*[d]
	Hydropsalis climacocerca[d]
Apodiformes	
Apodidae	*Chaetura brachyura*[d]
Trochilidae	*Glaucis hirsutus*[d]
	Phaethornis ruber[d]
	Phaethornis hispidus[d]
	Phaethornis philippii[d]
	Campylopterus largipennis[d]
	Heliothryx auritus[d]
Trogoniformes	
Trogonidae	*Trogon collaris*[d]
	Trogon ramonianus[d]
	Trogon melanurus[d]
	Pharomachrus pavoninus[d]
Coraciiformes	
Alcedinidae	*Megaceryle torquata*[d]
	Chloroceryle amazona[d]
	Chloroceryle americana[d]
	Chloroceryle inda[d]
	Chloroceryle aenea[d]
Momotidae	*Momotus momota*[d]
Piciformes	
Galbulidae	*Galbalcyrhynchus purusianus*[d]

	Galbula cyanescens[d]
	Jacamerops aureus[d]
Bucconidae	*Bucco macrodactylus*[d]
	Monasa nigrifrons[d]
	Chelidoptera tenebrosa[d]
	Capito aurovirens[d]
	Eubucco richardsoni[d]
Ramphastidae	*Ramphastos tucanus cuvieri*[d]
	Pteroglossus azara mariae[d]
	Pteroglossus beauharnaesii[d]
	Pteroglossus castanotis[d]
	Pteroglossus inscriptus[d]
	Ramphastos tucanus cuvieri[d]
	Selenidera reinwardtii[b]
Picidae	*Melanerpes cruentatus*[d]
	Colaptes punctigula[d]
	Celeus flavus[d]
	Dryocopus lineatus[d]
	Campephilus melanoleucos[d]
Passeriformes	
Thamnophilidae	*Taraba major melanurus*[d]
	Thamnophilus doliatus radiatus[d]
	Megastictus margaritatus[d]
	Thamnomanes saturninus huallagae[d]
	Pygiptila stellaris purusiana[d]
	Myrmotherula brachyura[d]
	Myrmotherula multostriata[d]
	Myrmotherula assimilis assimilis[d]
	Dichrozona cincta[d]
	Cercomacra cinerascens sclateri[d]
	Cercomacroides fuscicauda[d]
	Cercomacroides serva[d]
	Myrmoborus leucophrys leucophrys[d]
	Myrmoborus myotherinus[d]
	Hypocnemis peruviana[d]
	Hypocnemoides maculicauda[d]
	Myrmelastes hyperythrus[d]
	Hafferia fortis fortis[d]

	Hylophylax punctulatus[d]
	Willisornis poecilinotus griseiventris[d]
	Phlegopsis nigromaculata nigromaculata[d]
	Phlegopsis erythroptera ustulata[d]
Formicariidae	*Formicarius colma*[d]
	Formicarius analis[d]
Scleruridae	*Sclerurus rufigularis*[d]
Dendrocolaptidae	*Dendrocincla fuliginosa*[d]
	Certhiasomus stictolaemus[d]
	Glyphorynchus spirurus[d]
	Nasica longirostris[d]
	Dendrexetastes rufigula[d]
	Dendrocolaptes certhia[d]
	Dendroplex picus[d]
	Xiphorhynchus elegans juruanus[d]
	Xiphorhynchus obsoletus[d]
	Xiphorhynchus guttatus[d]
Furnariidae	*Furnarius leucopus*[d]
	Synallaxis albigularis[d]
	Synallaxis gujanensis[d]
	Certhiaxis cinnamomeus[d]
	Certhiaxis mustelinus[d]
	Automolus subulatus[d]
	Automolus ochrolaemus[d]
	Automolus rufipileatus[d]
	Xenops tenuirostris[d]
Tyrannidae	*Mionectes oleagineus*[d]
	Lophotriccus vitiosus[d]
	Poecilotriccus latirostris[d]
	Todirostrum maculatum[d]
	Tyrannulus elatus[d]
	Myiopagis gaimardii[d]
	Tolmomyias sulphurescens insignis[d]
	Tolmomyias poliocephalus[d]
	Tolmomyias flaviventris[d]
	Myiobius barbatus[d]
	Lathrotriccus euleri[d]
	Pyrocephalus rubinus[d]

	Ochthornis littoralis[d]
	Legatus leucophaius[d]
	Myiozetetes similis[d]
	Pitangus sulphuratus[d]
	Philohydor lictor[d]
	Griseotyrannus aurantioatrocristatus[d]
	Megarynchus pitangua[d]
	Tyrannus melancholicus[d]
	Tyrannus savana[d]
	Myiarchus tuberculifer[d]
	Myiarchus swainsoni[d]
	Myiarchus ferox[d]
	Attila cinnamomeus[d]
	Attila citriniventris[d]
	Attila bolivianus[d]
	Attila spadiceus[d]
	Knipolegus poecilocercus[d]
	Cotinga maynana[d]
	Querula purpurata[d]
	Cephalopterus ornatus[d]
Pipridae	*Pipra filicauda*[d]
	Ceratopipra rubrocapilla[d]
Tityridae	*Schiffornis major*[d]
	Tityra semifasciata[d]
	Pachyramphus rufus[d]
	Pachyramphus polychopterus[d]
	Cyclarhis gujanensis[d]
	Vireo olivaceus[d]
	Hylophilus thoracicus[d]
Hirundinidae	*Tachycineta albiventer*[d]
	Progne tapera[d]
	Progne chalybea[d]
	Stelgidopteryx ruficollis[d]
Troglodytidae	*Microcerculus marginatus*[d]
	Troglodytes aedon musculus[d]
	Pheugopedius genibarbis[d]
	Cantorchilus leucotis[d]
	Cyphorhinus arada modulator[d]

Donacobiidae	*Donacobius atricapilla*[d]
Turdidae	*Turdus hauxwelli*[d]
	Turdus amaurochalinus[d]
Thraupidae	*Eucometis penicillata*[d]
	Nemosia pileata[d]
	Tachyphonus surinamus[d]
	Tachyphonus luctuosus[d]
	Ramphocelus nigrogularis[d]
	Ramphocelus carbo[d]
	Thraupis episcopus[d]
	Thraupis palmarum[d]
	Tangara mexicana[d]
	Tangara chilensis[d]
	Tangara schrankii[d]
	Tangara callophrys[d]
	Dacnis flaviventer[d]
	Dacnis lineata[d]
	Hemithraupis guira[d]
	Thlypopsis sordida[d]
	Volatinia jacarina[d]
	Sporophila americana[d]
	Sporophila castaneiventris[d]
	Sporophila lineola[d]
	Paroaria gularis[d]
Passerellidae	*Ammodramus aurifrons*[d]
Icteridae	*Psarocolius spp.*[d]
	Cacicus solitarius[d]
	Cacicus cela[d]
	Icterus croconotus[d]
	Lampropsar tanagrinus[d]
	Chrysomus icterocephalus[d]
Polioptilidae	*Polioptila plumbea*[d]
	Ramphocaenus melanurus[d]
Fringillidae	*Euphonia laniirostris*[d]

[a]Hawes and Peres (2014). [b]Scabin and Peres (2021). [c]Patton et al. (2000). [d]Del-Rio et al. (2021).

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