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Vertebrate environmental DNA from understory leaf swabs unravel the canopy fauna of tropical forests

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Abstract

The Amazon basin, one of the most biodiverse regions on Earth, remains underexplored despite its immense ecological importance. Arboreal species, which play critical ecological roles, are especially understudied due to the difficulties of accessing forest canopies. These knowledge gaps are particularly concerning as tropical ecosystems are experiencing unprecedented anthropogenic pressures. Addressing these challenges requires the development of efficient biomonitoring methods to uncover and preserve the Amazon's rich biodiversity.

I conducted an eDNA metabarcoding survey where I tested the viability of leaf swabbing, a new and innovative technique, for detecting arboreal and terrestrial vertebrates. Ninety leaves across five leaf types were swabbed across terra firme and várzea forests along the middle Juruá river, in the western Brazilian Amazon.

Twenty-eight species were detected from five taxonomic classes, with eight (28.6%) arboreal species detected. Várzea showed a significantly higher species detection rate per swab (14.68), compared to terra firme (7.64). However, the species turnover between the forests was extremely low (Jaccard dissimilarity index = 0.071). Forest type had a significant effect on read count, while leaf type and dimensions were non-significant.

While the leaf swabbing approach demonstrated its viability for detecting vertebrate terrestrial and arboreal taxa, it was limited by the low species richness detected in a highly biodiverse region. This limitation is likely attributed to incomplete reference databases and DNA degradation. These challenges reduce the method’s effectiveness in tropical forests. However, advancements in technology and expansion of reference databases could significantly improve this approach.

Keywords: Amazon; Arboreal; eDNA; Leaf swabs; Brazil; Rio Juruá

Table of contents

List of Abbreviations

eDNA	environmental DNA
TF	Terra firme
VZ	Várzea
PCR	Polymerase Chain Reaction
COI	cytochrome c oxidase subunit I
SD	Standard deviation
IQR	Interquartile range
CEABIO	Center of Advanced Studies in Biodiversity
UFPA	Federal University of Pará
NHM	Natural History Museum

Introduction

The Amazon spans approximately 6,8 million square kilometers, with 6,5 million square kilometers covered by forest (Vergara, 2022; Wittmann & Junk, 2016), representing the largest remaining continuous tropical forest on Earth (Phillips et al., 2008). It harbors ten percent of the world's species and contains high levels of endemism (Charity et al., 2016; Vergara, 2022). Although it is home to some of the richest animal and plant diversity on the planet (Jablonski et al., 2006; Meunier et al., 2024; Ritter et al., 2017; Vergara, 2022; Wittmann & Junk, 2016), much of its ecological complexity remains unknown (Carvalho et al., 2023; Collen et al., 2008).

The lowland forests of the Amazon basin, defined as forests below 400 meters above sea level, host a diverse mosaic of forest types. These forest types exhibit a substantial variation in soil quality, drainage, topography, climate, vegetation and successional stages (Campbell et al., 1986; Hoorn et al., 2010; Junk, 1997; Patton et al., 2000; Poorter et al., 2015; Ter Steege et al., 2013). The Amazon is broadly divided into unflooded and seasonally flooded forests (Junk, 1997).

Unflooded forests, known as terra firme, are rich in biodiversity and are found in areas above the maximum flood level of Amazonian rivers (Haugaasen & Peres, 2005; Hess et al., 2003; Junk, 1997). One of the main types of seasonally flooded forests, known as várzea, endure periodic floods from nutrient rich and sediment-laden white- waters rivers originating from the Andes (Junk, 1984; Junk et al., 2011), lasting up to six months (Wittmann et al., 2004).

Approximately 17% of the Amazon rainforest consists of wetlands, such as várzea (Hess et al., 2003). While várzea forests are less species rich than terra firme forests, they provide critical habitats for many species, including capybaras, manatees, river turtles and fish, as well as domestic animals (Bredin et al., 2020; Campbell et al., 1986; Junk et al., 2011; Wittmann et al., 2004). The hydrological and geological processes shaping várzea forests contribute to the unique composition of the flora and fauna (Assis et al., 2015; Bredin et al., 2020; Junk, 1984; Wittmann et al., 2013).

Biomonitoring is crucial for assessing the compositions and complexity of ecosystems, detecting environmental contamination, and identifying invasive species (Takahashi et al., 2023). According to the IUCN's red list, one quarter of all mammals are threatened (Sales et al., 2020), with human activities driving many terrestrial vertebrates—particularly in subtropical and tropical regions—toward extinction (Ceballos et al., 2020; Collen et al., 2008). Despite consistently high deforestation rates in

humid tropical rainforests, many areas remain unexplored (Carvalho et al., 2023; Fearnside, 1990; Kim et al., 2015; Voss & Emmons, 1996), largely due to the underfunding of conservation science in species-rich tropical regions (Gardner et al., 2008; Lawton et al., 1998; Pawar, 2003).

Efficient, large-scale biomonitoring methods are urgently needed to monitor species and ecosystems across temporal and spatial scales (Sales et al., 2020). Expanding our understanding of species composition, ecological niches, and geographic distributions in neotropical forests is critical for effective conservation and research prioritization (Oliveira et al., 2016; Voss & Emmons, 1996). However, despite decades of research, large gaps persist in our understanding of species distributions, ecological interactions, and habitat-specific biodiversity—even for well-studied groups like vertebrates (Oliveira et al., 2016). Addressing these gaps is essential to improving conservation strategies and biodiversity management.

Forest canopies have long eluded scientists because of logistical difficulties, costs and elusive behavior of arboreal fauna (Cannon et al., 2021; Kays & Allison, 2001; Lowman, 2004; Voss & Emmons, 1996). As a result, the arboreal vertebrate fauna is poorly understood (Kays & Allison, 2001; Nakamura et al., 2017; Voss & Emmons, 1996). A study by Kays and Allison (2001) suggests that three out of four terrestrial vertebrate species in the tropics are partially or fully arboreal. While detailed research has been established for many taxa, others, such as marsupials and small rodents lack detailed ecological data for many species (Kays & Allison, 2001). This continues to be relevant to long-term inventories as reported by Voss and Emmons (1996), where “missing” animals of the rainforest are often attributed to their elusive behavior or the difficulty for nonspecialists to identify them. Novel techniques and technology aim to address these issues by decreasing costs and making the canopy more accessible (Cannon et al., 2021). However, there are still huge knowledge gaps to fill.

In a conventional biodiversity survey of the Rio Juruá area, Patton et al. (2000) noted that certain taxa were likely overlooked due to limited sampling effort. Rapid survey techniques such as environmental DNA (eDNA) metabarcoding have emerged as efficient and economical alternatives of doing large scale biomonitoring of the world’s ecosystems (Goldberg et al., 2016; Ruppert et al., 2019; Sales et al., 2020). eDNA based approaches have the ability to detect populations with low densities, which is particularly useful in situations with limited sampling effort (Valentin et al., 2020).

eDNA can be defined as the total pool of DNA isolated from environmental samples (Pawlowski et al., 2020). This encompasses DNA released into the environment by organisms through various discharges, such as mucous secretions, feces, gametes, damaged tissue, skin cells, hair, bodily remains (Barnes & Turner, 2016; Furlan et al., 2016; Ushio et al., 2017).

The DNA of vertebrates, whether they are alive or deceased, persists in the environment, providing a valuable trace detectable through eDNA analysis. They leave their DNA both as airborne particles and on vegetation upon contact or when the airborne particles settle and accumulate in the surrounding environment (Lynggaard et al., 2023), including being washed down by rain (Macher et al., 2023). Polymerase chain reaction (PCR) allows small traces of DNA molecules to be amplified exponentially, making it a crucial technique for replicating DNA without the use of living organisms (Rahman et al., 2013).

eDNA based methods can reduce or even exclude the risk of negatively impacting species—especially for sensitive species—while collecting data (Goldberg et al., 2016; Thomsen & Willerslev, 2015). In contrast, traps can have physically harmful or even lethal consequences, and often capture non-target species (Harkins et al., 2019). Additionally, eDNA can enhance the accuracy of detecting morphologically similar species, particularly during early life stages such as eggs or larvae (Furlan et al., 2016).

Conventional survey techniques, such as visual monitoring, require significant expertise and often necessitate separate surveys for nocturnal and diurnal species (Voss & Emmons, 1996). Other conventional methods like acoustic monitoring is limited by the lack of comprehensive, expert-verified databases (Gibb et al., 2019) and its inability to detect non-vocal animals. Sampling eDNA offers a new and alternative approach that can reduce some of the limitations associated with these traditional survey methods (Allen et al., 2023). While the current cost of eDNA metabarcoding is comparable to conventional methods, it is anticipated to decrease with increased usage and technological advancements (Ruppert et al., 2019).

The usefulness of eDNA metabarcoding depends on the reliability of the reference DNA-sequence database (Mathon et al., 2021; Thomsen & Willerslev, 2015). Currently, there are still gaps between geographical and taxonomic coverage. Although, ongoing efforts to expand these databases are steadily closing those gaps (Thomsen & Willerslev, 2015). It is, however, expected that sequencing species at lower taxonomic levels will take longer to integrate into these databases (Thomsen & Willerslev, 2015).

eDNA metabarcoding based approaches based on analyzing DNA from rainwash (Macher et al., 2023), air (Clare et al., 2022; Lynggaard et al., 2022), wildflowers (Thomsen & Willerslev, 2015), soil, tree bark (Allen et al., 2023), and water samples (Carvalho et al., 2024), have proven to be great tools for providing information of species occurrence across different biomes (Goldberg et al., 2016). This enables the identification of different species composition and geographical distributions. This is particularly crucial as ecosystems worldwide – including tropical ecosystems – face unprecedented

anthropogenic pressures such as deforestation, mining, hunting, and climate change (Charity et al., 2016; Kim et al., 2015; Redford, 1992; Takahashi et al., 2023).

During rainfall events, eDNA from the upper forest layers trickles down towards the ground with the rainwater (Aucone et al., 2023; Macher et al., 2023; Valentin et al., 2021). Lynggaard et al. (2023) propose that leaf swabbing holds potential for detecting terrestrial vertebrate eDNA, presenting a promising opportunity to revolutionize biomonitoring efforts and fortify conservation initiatives. Theoretically, as eDNA trickles down from the canopy, it should be possible to detect canopy-dwelling species on ground-level vegetation using leaf swabbing. This sampling method is straightforward and adaptable, making it well-suited for large-scale biomonitoring endeavors, including citizen science initiatives. With its simplicity and cost-effectiveness, leaf swabbing could emerge as an invaluable tool for biomonitoring and tracking changes in ecosystem composition (Lynggaard et al., 2023).

In this study, I performed swabbing of leaves in the understory of terra firme and várzea forest in the western Brazilian Amazon to assess whether this technique is a viable method to detect vertebrates in Neotropical forests. More specifically I asked these following questions: 1) Are there any differences in read count and species detections between terra firme, várzea and different leaf types and dimensions? 2) How well does this technique work for detecting arboreal species? 3) To what extent do the Leray and Riaz primers differ in sensitivity and specificity for detecting? Results are discussed in relation to previous studies, and I provide some directions for future work.

Materials and methods

2.1 Study area

The research was conducted in the Middle Juruà Region along Rio Juruá in the western Brazilian Amazon (Figure 1a). The leaf swabs were performed in October 2023, in a terra firme (4°29'16.9"S 66°42'17.4"W) and várzea forest (4°32'20.1"S 66°38'51.9"W). The Rio Juruá is fed by numerous smaller rivers, ranging from 5 to 10 meters in width and up to 60 km in length. Additionally, the Rio Juruá is accompanied by numerous larger floodplain lakes (Silvano et al., 2000). The fieldwork was conducted during the low water season, when the várzea forest is unflooded. The rainy season in western Amazonia is between November and March, with a dry season from May to October (Nobre et al., 2009). Medio Rio Juruá has an average annual air temperature of 24°C, receives an average yearly precipitation of approximately 2500 mm, and experiences 90% humidity for most of the year (de Vasconcelos et al., 2022).

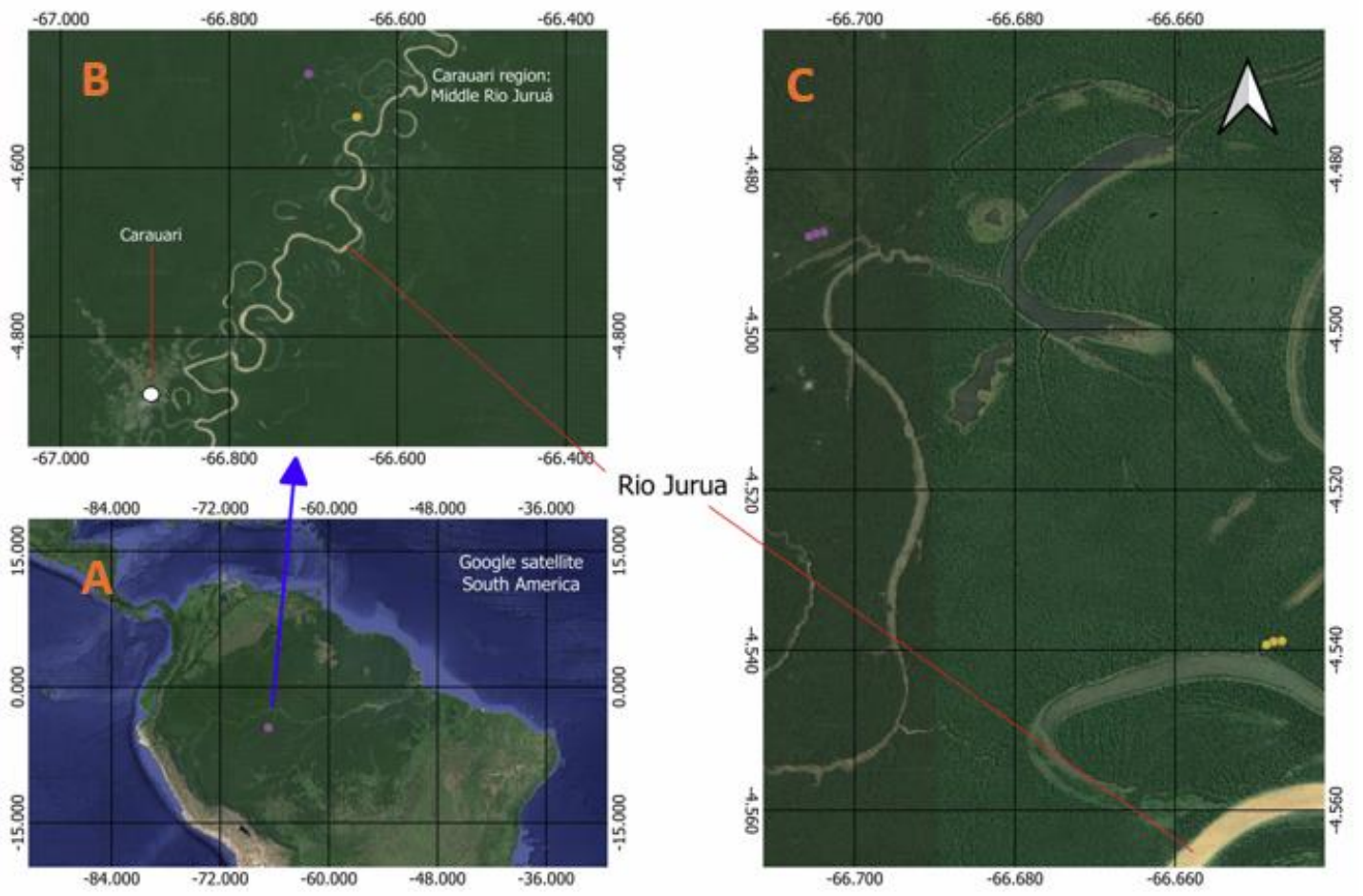

Figure 1 *A) Study area (purple dot) in the western Brazilian Amazon along the Rio Juruá. B) The location of the study sites in terra firme (purple dot) and várzea (yellow dot) north of Carauari town. C) The location of each site in terra firme (purple dots) and várzea (yellow dots).*

2.2 Data collection

A total of 90 leaf swabs were collected from the forest understory (Figure 3), evenly distributed with 45 collected in both terra firme and várzea. Within each forest type, leaves were swabbed at three different locations (Figure 1c). The study encompassed five distinct leaf types (Figure 2). To avoid contamination, disposable powderless nitril gloves were used when collecting data and replaced between each swab (Goldberg & Strickler, 2017; Vazquez et al., 2023). Each sterile cotton swab was dipped in ATL-buffer before the swabbing was performed on the upper surface of mature leaves. Each leaf was swabbed 10 times the entire length. All samples were immediately stored in Eppendorf tubes filled with 2 mL ATL-buffer. The samples were subsequently stored in air temperature, until they arrived in Belem where they were stored in freezers at -20° C.

2.3 Leaf Type

The classification of leaves was based on their morphology, including leaf type (simple or compound), leaf arrangement, shape, margin, venation, and base. Leaf Type A is characterized as a large, simple leaf with an elliptical shape and pinnate veins running along its surface (Figure 2A). The base of the leaf is uneven, and the margins are smooth and entire. Leaf Type B (Figure 2B) is a pinnately compound leaf with long, narrow leaflets arranged alternately along a rachis equipped with thorns. Each leaflet has parallel venation, a tapering base, and smooth, entire margins. Additionally, there are small thorns on the leaflets themselves. Leaf Type C (Figure 2C) consists of compound leaves with alternate leaflets that exhibit parallel veins. The leaflets have heart-shaped bases, a narrow and elongated shape, and smooth, entire margins. Leaf Type D (Figure 2D) is a pinnately compound leaf with alternate leaflets that have parallel venation, smooth and entire margins, tapering bases, and a linear shape. Lastly, Leaf Type E (Figure 2E) is a large, simple leaf with an oval shape, parallel venation, a heart-shaped base, and smooth, entire margins.

Figure 2 *Shows the different leaf types that was swabbed, A, B, C, D and E*

Figure 3 *Swabbing was performed using sterile cotton swabs on the upper surface of mature leaves. Nitril gloves were used to avoid contamination*

2.4 DNA extraction

The extraction of DNA from the leaf swabs took place at the Center of Advanced Studies in Biodiversity (CEABIO) at Federal University of Pará (UFPA). Before the extraction process took place, I minimized the risk of contamination by sterilizing all equipment in a 10% sodium hypochlorite solution for 10 minutes. This was followed by wiping the equipment with 70% alcohol and then rinsing with ultrapure water. Once dry, the equipment was placed under UV light for 15 minutes. Sterile single-use medical gloves were used and changed frequently. To extract the DNA, we used QIAGEN DNeasy Blood & Tissue Kit, and followed the manufacturers instruction (Blood & Kits, 2011; Qiagen, 2023). After extraction the samples were stored in refrigerators (4°C to 8°C) before they were sent to the Natural History Museum (NHM) at University of Oslo (UiO), where the samples were stored in freezer (-20C) before any processing was conducted.

2.5 PCR/Metabarcoding

The subsequent laboratory work, including PCR and metabarcoding was performed at the NHM. Here, I first prepared the sample plate containing the extracted DNA. Next, I prepared enough mastermix, consisting of AccuStart™ II PCR ToughMix® (MM2X) and H2O, to cover a full 96-well strip plate. To ensure there was enough mastermix, I added an extra 10% volume of both MM2X and H2O. This was mainly done to account for any volume loss during processes such as pipetting. A total of 16 µl of the mastermix was then added to each well of the 96-well PCR plate. Next, 2 µl of the selected primers were added to each well, and finally, 2 µl of DNA was added to complete the reaction mixture. Each strip (containing 8 wells) was then briefly centrifuged to ensure a uniform reaction mixture. This uniformity is essential for achieving accurate and consistent PCR results. It also ensures that all components are collected at the bottom of the wells and guarantees that the reagents are in full contact with each other and minimizes any loss of reagents.

Two different primer sets were used to target a broad range of vertebrate species, with each set run in 3 replicates to increase species detection and reduce the likelihood of false negatives (Ruppert et al., 2019). All strips were then placed into the PCR machine. Depending on the primers used, a specific program (Table 1) was selected to optimize the amplification conditions for each target sequence.

I used different sets of Leray and Riaz primers as both forward and reverse primers. For DNA amplification of the COI (cytochrome c oxidase subunit I) region, I used the following Leray primers: 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' and 5'- TANACYTCNGGRTGNCCRAARAAYCA-3'. DNA amplification of mitochondrial 12S rRNA was conducted using the following Riaz primers: 5'-ACTGGGATTAGATACCCC-3' and 5'- TAGAACAGGCTCCTCTAG-3'. Using two different primers sets helps to avoid any primer bias of amplifying some target sequences more than others (Ruppert et al., 2019). Leray primers target a 313 bp mitochondrial COI region, while Riaz 12S primers amplify a region of mitochondrial 12S ribosomal RNA genes, targeting fragments of approximately 106 bp (Leray et al., 2013; Riaz et al., 2011). Each well had their own unique set of forward and reverse primer tags to differentiate between the replicates (Table S1).

To visualize the amplified PCR products, 3 µl of the PCR product were mixed with 2 µl of loading dye and loaded onto a 1% agarose gel containing 4 µl of GelRed. The gel was run in 1× TAE buffer at 90V for 40 minutes alongside a 50-1500 bp ladder (FastRuler Low Range). The gel was then photographed under UV light to visualize the bands. ImageLab Software v6.0 was used to measure the amount and intensity of the DNA bands on the agarose gel.

To ensure that each amplicon (including negative controls) was represented equally, I normalized and combined all samples into two separate tubes. One for all Riaz samples and one for all Leray samples. PCR plates 1-3 (Leray primers) were normalized using an automated pipetting machine, whereas PCR plates 4-6 (Riaz primers) were normalized manually due to the smaller number of samples with amplified DNA. Specifically, PCR plate 1 was normalized to 100 ng, plate 2 to 55 ng, plate 3 to 80 ng, plate 4 to 25 ng, plate 5 to 8 ng and plate 6 to 5 ng.

After adjusting DNA concentrations, I used AMPure XP beads to purify and size-select DNA fragments. This process removes unwanted components such as primers, primer dimers, nucleotides, enzymes, and salts, ensuring that the DNA samples are free from contaminants. The purified samples were then run through BluePippin to separate DNA fragments and collect the desired size range. This step ensures that the DNA fragments are within the optimal size range before the samples are sent for sequencing, helping to reduce sequencing errors and improve the quality of the sequencing data.

Table 1 *PCR programs used for amplifying samples. The RIAZ_BR protocol (left) and the LERAY_BR protocol (right) both involve initial denaturation, followed by cycles of denaturation and annealing (with a 1°C decrease per cycle starting from 45°C), Both protocols conclude with a final extension at 72°C.*

RIAZ_BR		
Temperature	Time	Cycles
95C	5 minutes	1
95C	30 seconds	10
45C (1C/cycle	30 seconds	
72C	50 seconds	
95C	30 seconds	25
55C	30 seconds	
72C	50 seconds	
72C	2 minutes	1

LERAY_BR		
Temperature	Time	Cycles
94C	3 minutes	1
94C	10 seconds	5
45C (1C/cycle	20 seconds	
72C	30 seconds	
94C	10 seconds	30
54C	20 seconds	
72C	30 seconds	
72C	3 minutes	1

2.6 Bioinformatics

The samples were sent to the Norwegian Sequencing Centre (NSC) for sequencing. The Riaz samples were sequenced on a MiSeq Micro platform with 2x150 bp paired-end reads, yielding approximately 3 million paired-end reads. The Leray samples were sequenced on a MiSeq v2 2x250 bp paired-end reads, resulting in approximately 9,1 million reads.

The bioinformatic processes and filtering followed established methods (Raclariu-Manolică et al., 2023). Raw sequencing data were merged using PEAR (Paired-End reAd mergeR) to combine forward and reverse reads into contiguous sequences, optimizing read quality and length. Demultiplexing and initial data cleaning, including the removal of low-quality reads and adapter sequences, were

performed using OBITools. Preliminary quality filtering discarded reads shorter than 100 bp or longer than 150 bp for Riaz fragments, and shorter than 100 bp or longer than 350 bp for Leray fragments.

Modifications were made during the denoising and taxonomic assignment steps. Unique sequences were clustered at a 97% similarity threshold using USEARCH v11.0.54 to generate zero-radius operational taxonomic units (zOTUs), representing high-quality, unique sequences. A zOTU table was constructed to determine the abundance of each zOTU across the samples, excluding zOTUs with fewer than 10 reads across the dataset. Taxonomic assignments were performed using the k-mer-based approach SINTAX in VSEARCH v2.21.1 (Leray et al., 2022; Rognes et al., 2016), with a minimum similarity threshold of 90% for Riaz fragments and 95% for Leray fragments. Sequences were matched against the MIDORI2 reference database (Leray et al., 2022), and matches below these thresholds were excluded to ensure high confidence in taxonomic classification.

2.7 Filtering

Further filtering of the samples was performed to remove possible contaminants, false positives, and sequencing errors (as in Ribas et al., 2021). To account for contamination, I subtracted the maximum read detected in a negative control from all samples within each zOTU. Additionally, OTUs containing less than 10 reads were filtered out during the bioinformatic procedures, to reduce noise in the dataset and as an additional control of contamination. In total, 408 unique zOTUs were detected, corresponding to 167 unique species. In a final step, I removed any species unlikely to be found in the Amazon rainforest, resulting in a dataset of 35 unique zOTUs representing 28 “native” species. These “native” species were determined based on their known natural distribution, supplemented with sighting data from gbif.org and iNaturalist.org. Using this combination of sources, I identified which species could be considered “native” to the area, defined as occurring in the wild.

2.8 Data analysis

In Rstudio (R version 4.2.2), a Welch two-sample t-test was performed to compare the average number of unique species per swab between várzea and terra firme forests. This test was selected because it accounts for unequal variances and sample sizes, providing robust results when comparing means across the two forest types.

A two-way ANOVA was conducted with an alpha value of 0.05 to examine the effect of leaf type and forest type on the total number of reads. Additionally, the iNEXT package was used to perform an ordinary least squares (OLS) regression analysis, assessing the independent effects of leaf dimensions

(primary vein length and width) and forest type on the total number of reads (Hsieh et al., 2016). These two analyses complement each other, providing a more comprehensive understanding of how these factors influence read count.

The two-way ANOVA examines forest type and leaf type as categorical variables based on predetermined groupings and tests for potential interactions between these factors. In contrast, the OLS regression focuses on the independent effects of leaf dimensions, treating them as continuous variables. This allows the regression to capture subtle trends in how specific morphological traits influence read count, which might be overlooked when using categorical groupings in the ANOVA.

The vegan package in R was used to create a species accumulation curve with 1000 permutations (Dixon, 2003). These curves illustrate the relationship between sampling effort and species richness, providing an estimate of the completeness of sampling efforts in várzea and terra firme forests.

A Jaccard dissimilarity test was performed to compare species turnover between várzea and terra firme forests. This test quantified the compositional differences between the two forest types, revealing the extent of overlap or distinctiveness in species detected. Maps were made using QGIS (version 3.38.0).

Results

In total, the 90 leaf swabs identified the DNA sequences corresponding to 28 unique species (Table 2) from five different classes. Actinopteri was the most species rich class (Table S2), with 12 unique species (42.9%). This was followed by Mammalia with 6 species (21.4%), Aves (6 species, 21.4%), Insecta (3 species, 10,7%) and finally Amphibia with only 1 genus detected (3,6%). A full list of species is provided in Table 2.

Table 2 *List of species found in **terra firme** (TF) and **várzea** (VZ) forest, showing the total number of reads detected for each species (No. of reads), along with the number of samples (No. of Samples) in which each species was detected. Species exclusively detected in terra firme are marked with *, while species exclusively detected in várzea are marked with ***

Species	Common name	Forest Type	No. of reads		No. of samples	
			TF	VZ	TF	VZ
Anura						
Adenomera spp.	Tropical bullfrogs	TF/VZ	47	539	9	19
Blattodea						
Constrictotermes cavifrons	Amazonian nasute termite	VZ**	0	38	0	3
Characiformes						
Acestrorhynchus lacustris	Peixe-cachorro	TF/VZ	69	34311	17	29
Bryconops affinis	Orangefin tetra	TF/VZ	50	50653	11	23
Bryconops caudomaculatus	Tailspot tetra	TF/VZ	35	27185	11	30
Cyanocharax spp.	South American characins	TF/VZ	637	13117	27	42
Hoplias malabaricus	wolf fish	TF/VZ	7	53386	3	17
Mylossoma duriventre	silver mylossoma	TF/VZ	436	7031	21	39
Chiroptera						
Carollia brevicauda	Silky short-tailed bat	TF/VZ	126	24186	22	27
Cichliformes						
Crenicichla lepidota	Pike cichlid	TF/VZ	22	9526	6	27
Coleoptera						
Aspisoma sp.	Fireflies	TF*	22	0	1	0
Columbiformes						
Leptotila rufaxilla	Grey-fronted dove	TF/VZ	58	23232	11	18
Gymnotiformes						

Electrophorus electricus	Electric eel	TF/VZ	3177	6441	22	40
Gymnotus carapo	Banded knifefish	TF/VZ	10	7315	5	21
Sternopygus macrurus	Longtail knifefish	TF/VZ	39	10045	6	22
Hemiptera						
Liorhyssus hyalinus	Hyaline grass bug	TF/VZ	48	752	11	32
Passeriformes						
Campylorhamphus procurvoides	Curve-billed scythebill	TF/VZ	607	13103	25	43
Thamnophilus nigrocinereus	Blackish-grey antshrike	TF/VZ	4942	1159	15	38
Pelecaniformes						
Nycticorax nycticorax	Black-crowned night heron	TF/VZ	73	36437	10	20
Pilosa						
Choloepus didactylus	Linnaeus's two-toed sloth	TF/VZ	3430	7903	25	38
Primates						
Alouatta juara	Juruá red howler	TF/VZ	22	19307	7	18
Ateles belzebuth	White-bellied spider monkey	TF/VZ	1219	13816	14	17
Rodentia						
Coendou prehensilis	Brazilian porcupine	TF/VZ	8766	241	9	7
Hydrochoerus hydrochaeris	Capybara	TF/VZ	10	12411	6	25
Siluriformes						
Brachyplatystoma juruense	Zebra catfish	TF/VZ	4	13471	3	20
Pinirampus pirinampu	Flatwhiskered catfish	TF/VZ	336	29689	16	31
Trogoniformes						
Trogon curucui	Blue-crowned trogon	TF/VZ	42	17209	7	21
Trogon massena	Slaty-tailed trogon	TF/VZ	2779	83522	12	36

3.1 Effects of forest and leaf type

In total, 27 species were detected in both várzea and terra firme, and each forest type contained only one unique species not detected in the other (Table 2). Species turnover between forest types was therefore extremely low (Jaccard dissimilarity index = 0.071).

In várzea, the number of species detected per swab ranged from 3 to 26, while the swabs in terra firme detected between 0 and 19 unique species per swab (Figure 4). There was a significant difference in mean species counts per swab between the two forest types ($t(87.1) = 5.99$, $p < 0.0001$), with várzea having a higher mean (14.68) than terra firme (7.64).

The species identified across the highest number of individual swabs, excluding *Cyanocharax spp.* (as this could represent multiple species), was *Campylorhamphus procurvoides*. It was detected in 43 of 45 várzea swabs and 25 of 45 terra firme swabs. Additionally, of species detected in both forest types, only *Coendou prehensile* was detected in more swabs from terra firme than from várzea (Table 2).

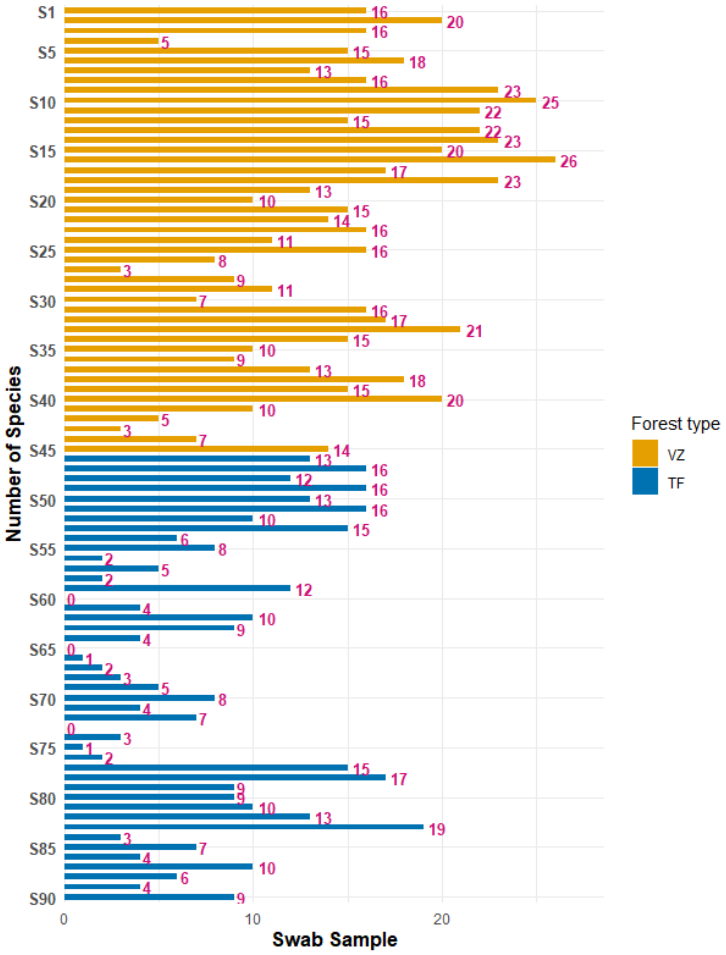

Figure 4 *displays the number of species detected per swab. Swab 1-45 marked in orange were collected in VZ, while swab 46-90 marked in blue were collected in TF.*

The richness of detected species increased rapidly with the first samples in both várzea and terra firme. Várzea reaches a plateau at 27 species after 37 samples (Figure 5, Table S3). Notably, beyond the first five swabs, additional sampling contributed minimally to the cumulative species richness. Similarly, in terra firme, species accumulation slowed significantly after fourteen swabs and reached a plateau of 27 species after 45 swabs (Figure 5, Table S3).

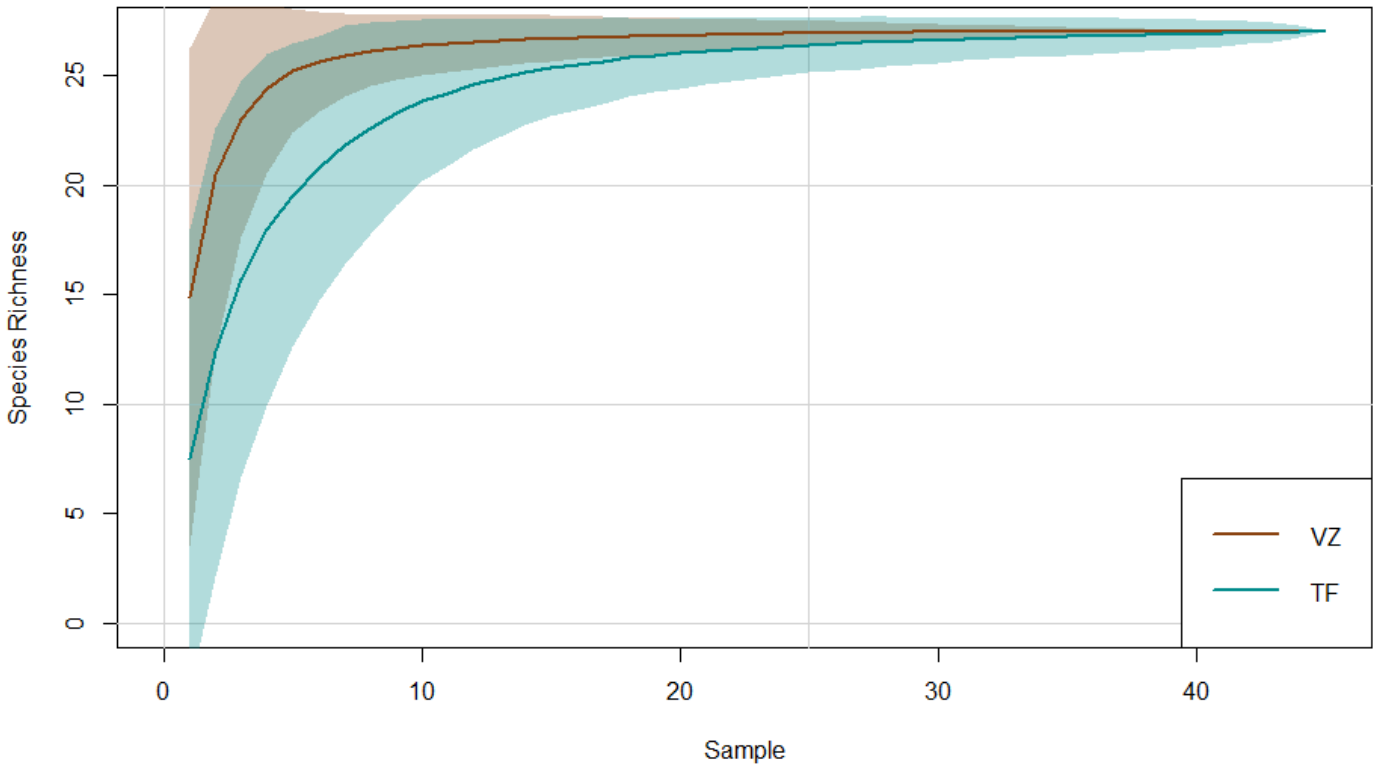

Figure 5 *species accumulation curve with species richness on the y axis and number of samples on the x axis. The accumulation curve is seen reaching a plateau and leveling out at 27 species for both forest*

The OLS regression model was a significant for the effect of forest type on number of reads ($t(80) = 5.01$, $p < 0.001$), where várzea was associated with 11.240 more reads per leaf than terra firme. The model explained 22.45% of the overall read count variability ($R^2 = 0.2245$). Várzea swabs have a high variability in the number of reads across samples compared to terra firme (Figure 6). The variability in the number of reads per species is also a lot more variable in várzea than terra firme (Figure 7). The number of reads for species such as *Trogon massena*, *Alouatta juara* and *Bryconops caudomaculatus* were particularly more variable among swabs in várzea compared to terra firme.

Trogon massena had the highest total number of reads (15.89%, Table S4) across both forest types. This was followed by *Hoplias malabaricus* (9.83%) and *Bryconops affinis* (9.34%). Species such as *Hydrochoerus hydrochaeris*, *Brachyplatystoma juruense*, *Hoplias malabaricus* exhibited some of the largest differences in number of reads between the two forest types (Figure 7, Table 2). Of the species found in both forest types, only *Coendou prehensilis* and *Thamnophilus nigrocinereus* had a higher number of reads in terra firme (Figure 7, Table 2).

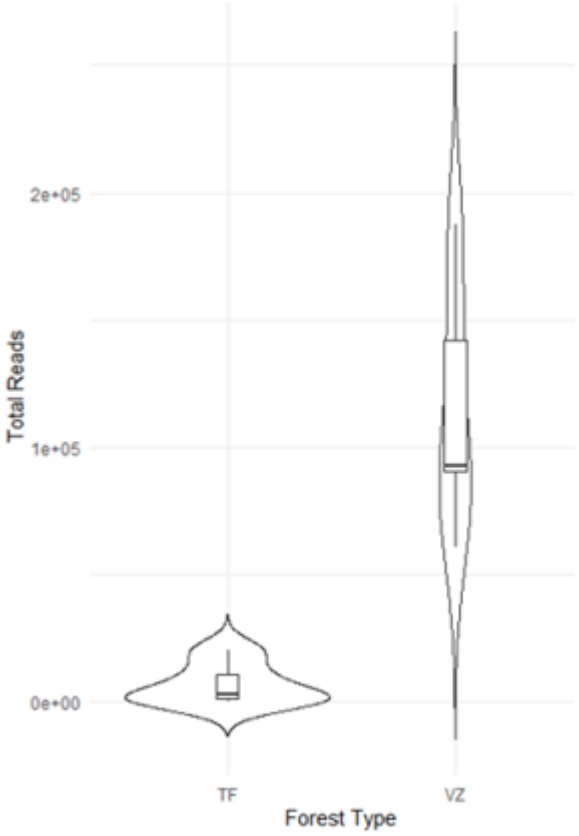

Figure 6 *violin plots illustrating the distribution of total reads count based on and forest type (TF or VZ). The violin plots highlight the density and variation in read counts, with narrower sections indicating fewer reads and wider sections representing higher density. Embedded within each violin plot are boxplots that provide additional details on the median (black horizontal line within each box) and the interquartile range (box limits).*

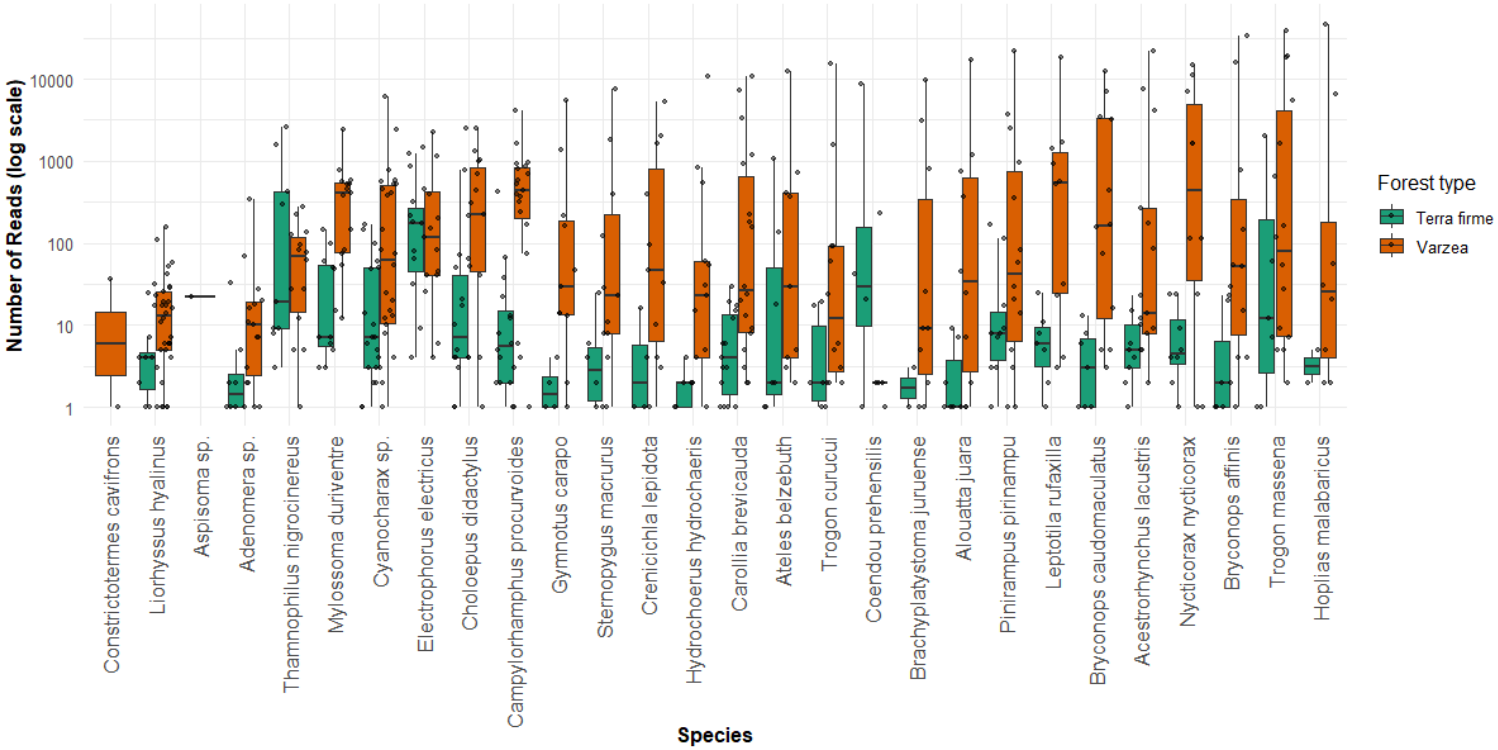

Figure 7 *shows the distribution of reads counts for species detected in terra firme (green) and várzea (orange). The figure displays the number of reads on a logarithmic scale on the y axis and species on the x axis. The median (black line) is displayed within each boxplot, as well as any outliers in the data (dots).*

Leaf dimensions did not significantly affect overall read counts (t(80) = 0.39, p = 0.697). In addition, there was no significant difference in the number of reads between leaf types (two-way ANOVA: $F_{4,4}$ = 1.08, p = 0.4699), although leaf type B clearly has a higher variability in the number of reads across leaves than the other leaf types (Figure 8).

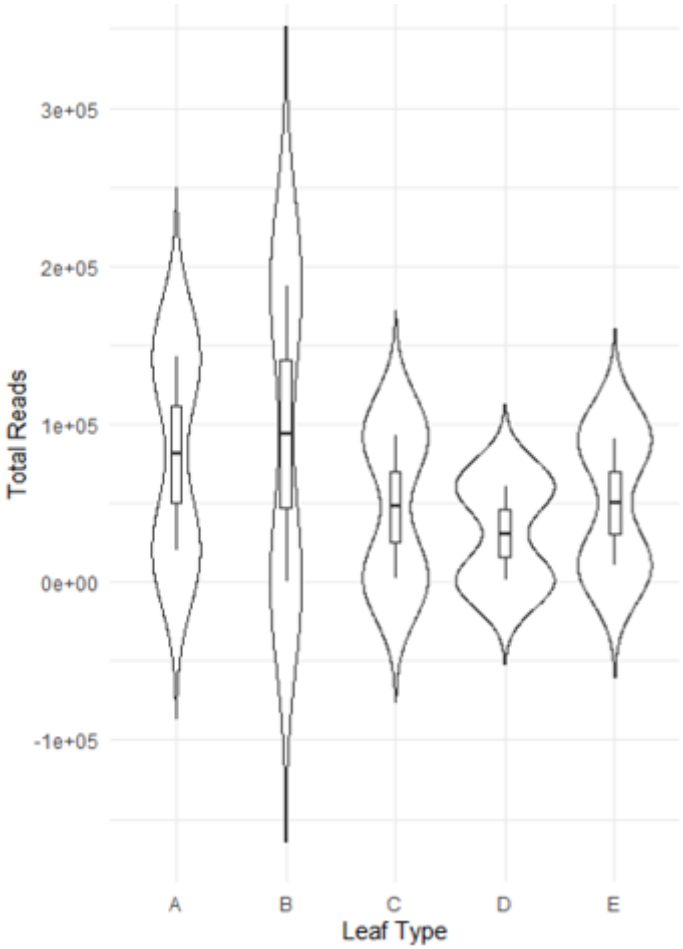

Figure 8 *violin plots displaying the distribution of total reads based on leaf type (A-E). The shape of each violin represents the probability density of the data at different levels of total reads, while embedded boxplots highlight the median (black horizontal line), interquartile range (box limit)*

3.2 Arboreal species detection

A total of eight arboreal species (8/28 = 28.6%) were detected (Figure 9). All eight species were detected across all sites (Table S5). Site 5 had the highest proportion of arboreal reads relative to total reads per site, whereas site 6 had the lowest (Figure S1). *Trogon massena* had the highest read detection percentage in várzea (16.19%, Table S6) and across all várzea study sites (Figure 9, Table S5). *Coendou prehensilis* had the highest detection percentage of reads across terra firme (32.45%, Table S6), whereas it had the lowest detection percentage of reads across várzea (0.05%, Table S6) and lowest at each várzea study site (Table S5). *Alouatta juara* had the lowest read detection in terra firme (0.08% Table S6), but not across all terra firme study sites (Table S5).

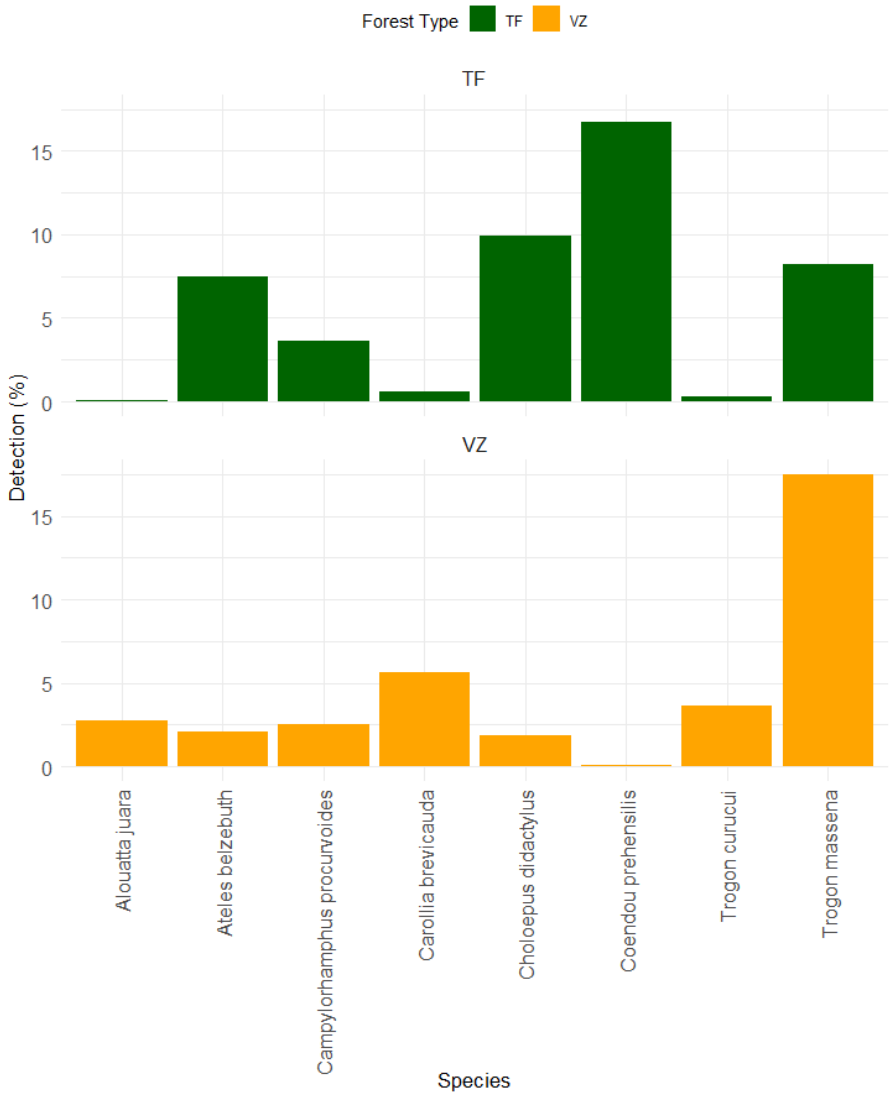

Figure 9 *displays arboreal species detected across TF and VZ. The reads are displayed as a proportion of the total reads detected per forest type.*

3.3 Primers and species detection

The swabs generated 2,998,999 reads using the Riaz primer and 9,134,066 reads with the Leray primer. The Leray primer detected 28 native species, including one species found exclusively in várzea (*Constrictotermes cavifrons*) and another exclusively in terra firme (*Aspisoma sp.*; Figure 10). In contrast, the Riaz primer detected 24 native species (Figure 11), all of which were detected in várzea, while 6 species were detected in terra firme. The Riaz primer detected at least one species in 14 of the 45 terra firme swabs, compared to 40 of the 45 swabs in várzea. The Leray primer detected at least one species in 42 of the 45 terra firme swabs and in all 45 várzea swabs. On average, the Leray primer identified 11.8 species per swab, compared to 1.1 species per swab for the Riaz primer.

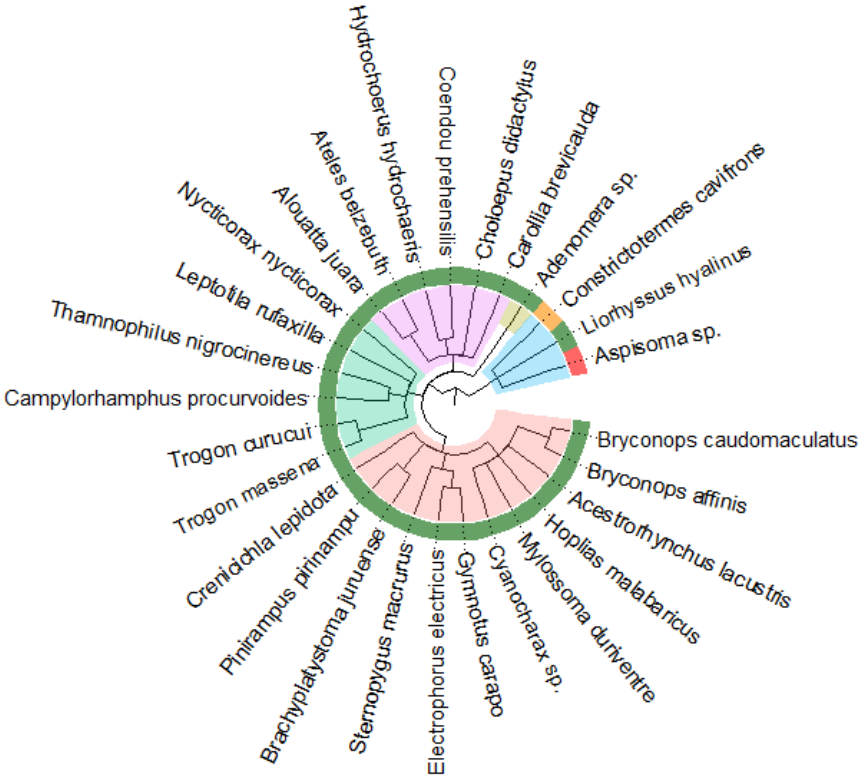

Figure 10 *phylogenetic tree based on species detected with Leray primer. The color of the outer ring represents the habitat of each species: Green = species detected in both TF and VZ. Yellow = species only found in VZ Red = species only found in TF. The tree shows general taxonomic groupings marked with colors, with mammals (mammalia, purple), birds (Aves, light green), amphibians (amphibia, yellow), insects (Insecta, blue) and ray-finned fishes (Actinopteri, red) forming separate clades. Branch lengths do not represent the true evolutionary relationships, as the tree structure is based on taxonomic data in the species list.*

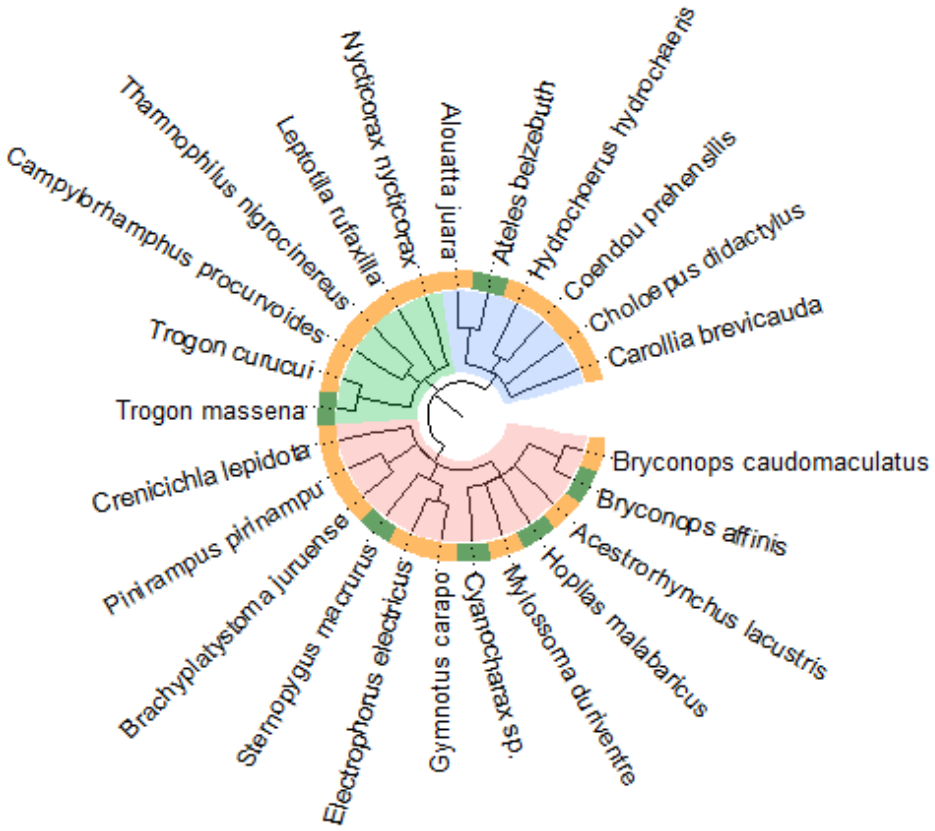

Figure 11 p*hylogenetic tree of species detected with the Riaz 12S primer. The color of the outer ring represents the habitat of each species: Species marked in green are detected in both TF and VZ, while species marked in yellow are only detected in VZ. The tree shows general taxonomic groupings marked with colors, with mammals (purple), birds (Aves, red), and ray-finned fishes (Actinopteri, light green) forming separate clades. Branch lengths on the tree do not represent evolutionary relationships, as the tree structure is based on taxonomic data in the species list.*

Discussion

4.1 Várzea and terra firme species

The number of species detected by leaf swabs in this study was very low, with 27 species found in each forest type. This result is surprising given that more than 300 vertebrate species have been documented along the Rio Juruá (Costa et al., 2018; Del-Rio et al., 2021; Hawes & Peres, 2014; Patton et al., 2000; Peres, 1997; Scabin & Peres, 2021; Vågen, 2024).

Since many species in an assemblage are rare, it is common to overlook species during sampling (Chao et al., 2014). This leads many biodiversity studies to underestimate the true species richness (Gotelli & Colwell, 2001), which seems to be the case of this study. Most of the swabs included the same few species and very rarely did any of the swabs contain any new species as demonstrated by the species accumulation curve, which rises rapidly in species richness within the first few swabs (Figure 5, Table S4). The accumulation curve reaches as asymptote of only 27, indicating that there are few rare species in the dataset. This is unexpected as tropical rainforests contain many rare species (Grenyer et al., 2006). The low species richness found in this study likely stems from limitations in reference database and DNA degradation.

The number of species detected in terra firme and várzea was equal, which is surprising given that terra firme forests generally harbor higher species richness than várzea along the Rio Juruá (Costa et al., 2018; Gascon et al., 2000; Hawes & Peres, 2014; Malcolm et al., 2005; Peres, 1997). Additionally, várzea exhibited significantly higher DNA read counts and species detections per swab. This could be attributed to the higher community density and biomass of arboreal folivores in várzea (Peres, 1997), likely drawn to the emergence of new leaves, which offer high nutritional value and lower concentrations of chemical defenses (Haugaasen & Peres, 2007; Peres, 1997).

In addition, várzea forests are known to produce more fruit and experience shorter periods of food scarcity (Peres, 1997). During the dry season, várzea provides an abundant supply of fruits, seeds, and nutrient-rich foliage, attracting terrestrial frugivores and other large vertebrates as water levels recede (Costa et al., 2018; Haugaasen & Peres, 2007; Hawes & Peres, 2016). These species remain in várzea until dry land disappears again during the wet season (Haugaasen & Peres, 2007; Hawes & Peres, 2014). Haugaasen and Peres (2007) found that both forest types bear high levels of immature fruits during the transition from dry to wet season in October, with várzea showing substantially higher levels. These

fruits mature at the onset of the rainy season, and their abundance is significantly and positively related to the monthly numbers of terrestrial vertebrates in várzea. However, it is worth noting that this study was conducted near Rio Purus, where hydrological and fruiting patterns may differ from those along Rio Juruá. The overlap of dry and wet seasons, with the availability of both immature and mature fruits as well as nutrient-rich foliage, likely contributes to the higher DNA read counts (Figure 5) and species detection rates per swab observed in várzea (Figure 6).

While few species detected in this study are primarily frugivorous (e.g., *Trogon curucui*, *Trogon massena*, *Ateles belzebuth*, and *Carollia brevicauda* (Di Fiore et al., 2008; Fleming, 1991; Remsen Jr et al., 1993), they still contribute to the observed differences. These species exhibited higher read counts and detection rates in várzea compared to terra firme (Table 2). Other species, such as the Juruá red howler monkey, Linnaeus's two-toed sloth, and Brazilian porcupine are primarily folivorous (Adam, 1999; Julliot & Sabatier, 1993; Moreau et al., 2003), but incorporate fruit into their diets, further highlight the importance of várzea as a seasonal resource hub.

Forty percent of the species detected in this study were fish (*Actinopteri*), with both forest types yielding similar numbers (Table 2). The surprisingly high detection of fish species in terra firme is intriguing, given its distance from aquatic habitats. One plausible explanation is the secondary dispersal of fish DNA by birds that prey on fishes. Birds may transport fish remains into the forest through discarded carcasses or deposit fish DNA via their droppings after consuming their prey.

Fishes in várzea displayed a much higher read count compared to terra firme (Tabel 2). This is not surprising, as many fishes in the Neotropics inhabit várzea forests when it is flooded, feeding on huge quantities of seeds from terrestrial plants, lianas and trees, as well as fruits, other fishes or terrestrial and aquatic invertebrates (Barletta et al., 2010; Horn et al., 2011; Rejas, 2018). Numerous plants have their fruit phenology synchronized with the flooding and drop large amounts of seeds and fruits into the water, which over 150 frugivore fishes in the Neotropics take advantage of (Horn et al., 2011). The swabs detected species such as *Mylossoma duriventre,* which feed on terrestrial plants, fruits and seeds (Rejas, 2018), and *Hoplias malabaricus* that uses flooded forests as breeding grounds (Barletta et al., 2010).

The timing since the last flood pulse in várzea likely affects eDNA read counts. Robson et al. (2016) suggest that fishes experiencing thermal stress may shed DNA at higher rates, which could partially explain the high detection rate and the elevated read counts in várzea. This is particularly relevant as the Amazon Basin experienced record-breaking heat events between July 2023 and February 2024 (Espinoza et al., 2024; Meunier et al., 2024; Taylor, 2024). If fish experienced thermal induced stress during the period leading up to the dry season and the swabbing, more DNA would be accumulated in the water and deposited on leaves in várzea when the water receded.

However, as várzea progresses further into the dry season, the input of fish DNA from floodwaters decreases, while eDNA degrades. This degradation could lead to an increased number of false negatives over time (Goldberg et al., 2016). The combined effects of reduced eDNA input and rapid degradation likely diminish detection rates and could produce false negatives as time passes. The interplay between heat-induced DNA shedding, flood pulse timing, and DNA degradation thus likely creates complex temporal patterns in eDNA detectability.

Forest type was a significant predictor of read count, whereas leaf type and dimensions were not. Interestingly, leaf type B, which was equipped with thorns, exhibited a much larger range of total reads. In contrast, none of the other leaf types were equipped with thorns. This suggests that the presence of thorns in leaf type B may contribute to the observed variability in read counts. This is likely due to thorns "ripping" of DNA from the fur or skin cells of animals that brush against the leaf as they walk past. Thorns may also "catch" fur that floats in the air. This morphological trait may also help these leaves to retain more DNA on their surface compared to leaves with smooth edges.

4.2 Arboreal species

Of the 28 detected species, eight (28,6%) utilize the forest canopy. All eight species were detected in both forest types and across all sites (Figure 8, Tabel S2 and S3). Although this proves that the leaf swabbing approach holds the capacity to detect arboreal species, it detected very few compared to the assemblages along Rio Juruá. For example, (Peres, 1997) recorded up to 14 sympatric primate species in terra firme and 7 in várzea along Rio Juruá, yet my swabs only detected two species. Another study utilizing camera traps and line-transect censuses registered 33 arboreal vertebrate species in terra firme (Scabin & Peres, 2021).

A plausible explanation of the low arboreal species detection could stem from the timing of the last rainfall and time of the leaf swabbing. During rainfall, eDNA from the canopy trickles down with the rainwater (Aucone et al., 2023; Macher et al., 2023; Valentin et al., 2021). In the days leading up to the swabbing, heavy rainfall occurred 3 and 5 days prior in várzea and 4 and 6 days prior in terra firme. However, a study by Valentin et al. (2021) reported that even small amounts of rainfall could remove eDNA from the surfaces of leaves. This suggests that during heavy rainfall, eDNA might be wiped clean not only from the canopy but also from leaves in the understory and on ground vegetation. At the same time, as the forest dries, eDNA from the canopy could be deposited on understory leaves as water evaporates from their surfaces rather than running off completely.

If all preexisting eDNA on leaves is completely removed during rainfall events, it may create an opportunity to collect "snapshots" of recent animal activity. Newly deposited eDNA would begin to

accumulate, and swabbing this fresh eDNA could reduce the risk of misreads from older, degraded DNA while capturing the recent presence of animals (Coissac et al., 2012; Valentin et al., 2021). The dynamics of rainwater runoff and eDNA removal remain poorly understood, highlighting the need for further investigation in future studies.

4.3 eDNA and primers

The Leray primer outperformed the Riaz primer by detecting more species, more species per swab and generating a higher number of reads. The bioinformatics and filtering process completed in the study is well considered and follows that of other eDNA studies, yet the low number of species detected is unexpected. Despite the potential of eDNA metabarcoding methods to revolutionize biodiversity assessments, there are several limitations that must be considered.

A significant pitfall of eDNA is the risk of contamination, which could happen in any of the steps of the eDNA collection and lab processing (Thomsen & Willerslev, 2015). Several of the zOTUs identified in my study came from species in the Indo-Malayan region or from Madagascar. However, it is unlikely that these zOTUs are caused by contamination from either the extraction process performed at CEABIO at UFPA or from the amplification process completed at NHM in Oslo. Neither of the labs had any projects from these areas within the last years nor had any contamination from these areas in other projects. However, during the filtration process, negative controls with positive reads were not further assessed to see which species were detected.

Regardless of this, I deem it more likely that the zOTU's detected from other regions than the Amazon is likely because of the amplified short DNA fragments, mismatches or poor reference database for species present in the area (Leray et al., 2022). DNA degradation is possibility accelerated in warm and humid terrestrial environments, such as tropical regions (Goldberg et al., 2018; Sirois & Buckley, 2019; Valentin et al., 2021). The breakdown of DNA into small, fragmented sections of genetic material can impede barcode recovery (Coissac et al., 2012). This degradation can lead to false negative which occur when a species is present but remains undetected due to the time lag between its activity and the sampling effort, which reduces the quality and quantity of detectable DNA (Goldberg et al., 2016). This can complicate the effectiveness and reliability of eDNA as a tool for accurate biodiversity assessment, as the degradation could limit species detection and identification (Ruppert et al., 2019).

Although the swabbing process was completed in two days, it is essential to consider the temperature and humidity over an extended period, as DNA accumulates and degrades gradually in the environment (Coissac et al., 2012; Klepke et al., 2022; Lynggaard et al., 2023). In the 15–20 days leading up to sample collection, temperatures in the várzea forest averaged 27.7°C, with frequent peaks in the high thirties

and occasional surpassing 40°C (Taylor, 2024, see also Espinoza et al., 2024; Meunier et al., 2024). This period also had an average humidity of 88.5%, often reaching full saturation (100%) (Taylor, 2024, see also Espinoza et al., 2024; Meunier et al., 2024). Although, recent heavy rainfall events potentially renewed the eDNA accumulated on the leaves only a few days prior to the swabbing. The extreme conditions could have degraded the eDNA at my study sites, reducing the ability of the leaf swabs to gather high quality eDNA.

The success of eDNA metabarcoding based approaches is heavily reliant on comprehensive and well-curated reference databases (Ruppert et al., 2019), which are lacking for the Amazon (Carvalho et al., 2023). Furthermore, the effectiveness is also heavily reliant on the choice of primer sets and target loci, as these factors are crucial for the successful detection and accurate identification of studied taxa (Leray et al., 2013; Riaz et al., 2011; Ruppert et al., 2019). As this study demonstrates, the Leray primer is much more efficient than Riaz primer at detecting species in both terra firme and várzea forests and in generating reads – at least in the part of the Amazon where the current study took place.

Additional biases include the detection of DNA from dead organisms, the persistence of extracellular DNA in the environment, and the potential for false readings caused by taxonomic selectivity—where primers amplify some species more effectively than others (Ruppert et al., 2019; Thomsen & Willerslev, 2015). Over-amplification of certain species can distort the representation of their read counts, disproportionately influencing the detection percentage relative to the total read count (Coissac et al., 2012). Another risk is wrong taxonomic assignments or when two or more species have identical sequences that are amplified, which can create a false positive for one or more species (Mathon et al., 2021). Both *Cyanocharax spp*. and *Mylossoma duriventre* shared one zOTU, the latter was only discovered by this shared zOTU. However, *Cyanocharax spp*. were found in two zOTUs. This means that if *Mylossoma duriventre* is a false positive, the shared zOTU should not have been detected at all, leaving *Cyanocharax spp*. overrepresented in the dataset. Another possibility is that *Mylossoma duriventre* is a false negative and simply because of its shared zOTU it is misidentified, which would mean that *Cyanocharax spp*. is represented accurately in the dataset.

To achieve more accurate results, one could produce more replicates of the samples and use a broader primer combined with more specific primers. However, this would heavily increase the equipment, laboratory time, and label costs. This would also increase the time to get the dataset and increase the time and effort needed to work on the datasets.

Although there are limitations of eDNA, it offers an innovative approach to species detection. One of the key benefits of eDNA metabarcoding is its ability to detect multiple species simultaneously in a relatively short timeframe (Fonseca, 2018), making it particularly valuable for detecting cryptic or elusive species. Conventional methods often require extensive, habitat-specific expertise in species

composition and ecology. In contrast, the collection of eDNA samples is relatively straightforward and does not require extensive ecological knowledge.

The simplicity and adaptability of eDNA based approaches could enable citizen science (Lynggaard et al., 2023), where local people could be sent to study sites, collect samples, and send them to a laboratory for DNA analysis—all within a matter of days. This limits the need for intense sampling effort to gather the initial datasets (Coissac et al., 2012). By comparison, a traditional field survey could take months, if not years, to complete—especially when searching for cryptic or elusive species—even with the aid of highly skilled local people who possess extensive knowledge of species composition and distribution.

However, while collecting eDNA samples is fast and straightforward, the subsequent steps in the workflow—such as DNA extraction, amplification, sequencing, and bioinformatics—require a high level of expertise across several disciplines, including molecular biology and bioinformatics. These steps are essential for producing the complete dataset and may take weeks if not months to complete, depending on the workload of the laboratories involved. The advantage, however, is that these specialized skills are widely available across global research facilities, whereas the habitat-specific knowledge required for conventional surveys is often limited to a smaller group of specialists.

Conclusion

eDNA-based methods, such as leaf swabbing, offer a simple and straightforward approach to data collection. This study provides insights into the effectiveness of this method in a Neotropical region, examining the influence of forest types, leaf characteristics, and primer selection. The Leray primer outperformed the Riaz primer, detecting more species and generating higher read counts, underscoring the critical role of primer selection in eDNA metabarcoding.

Although leaf swabbing successfully detected both terrestrial and arboreal fauna, it identified only 28 species in total, including 8 arboreal species—despite the region's rich terrestrial and arboreal vertebrate biodiversity. This low detection rate is likely influenced by poor reference databases and DNA degradation. Furthermore, the complex interplay between rainfall, eDNA runoff, and the deposition of new eDNA remains poorly understood and warrants further studies.

Additionally, this study highlights the potential influence of leaf morphology on eDNA detectability. Leaves with thorns, such as leaf type B, exhibited much higher variability in read counts compared to other leaf types, suggesting that morphological traits may enhance DNA retention. Further studies are recommended to explore this relationship.

In its current state, eDNA metabarcoding in tropical regions is best used as a complementary tool to traditional methods. Combining these approaches can enhance biodiversity assessments and provide more accurate insights into species composition and population trends.

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Supplementary

Table S1 shows the sequence of forward and reverse primers and tags.

Forward Index	Forward index sequence	Reverse Index	Reverse Index sequence
F1	CCTAAACTACGG	R1	CCTAAACTACGG
F2	TGCAGATCCAAC	R2	TGCAGATCCAAC
F3	CCATCACATAGG	R3	CCATCACATAGG
F4	GTGGTATGGGAG	R4	GTGGTATGGGAG
F5	ACTTTAAGGGTG	R5	ACTTTAAGGGTG
F6	GAGCAACATCCT	R6	GAGCAACATCCT
F7	TGTTGCGTTTCT	R7	TGTTGCGTTTCT
F8	ATGTCCGACCAA	R8	ATGTCCGACCAA
F9	AGGTACGCAATT	R9	AGGTACGCAATT
F10	ACAGCCACCCAT	R10	ACAGCCACCCAT
F11	TGTCTCGCAAGC	R11	TGTCTCGCAAGC
F12	GAGGAGTAAAGC	R12	GAGGAGTAAAGC
F13	GTTACGTGGTTG	R13	GTTACGTGGTTG
F14	TACCGCCTCGGA	R14	TACCGCCTCGGA
F15	CGTAAGATGCCT	R15	CGTAAGATGCCT
F16	TACCGGCTTGCA	R16	TACCGGCTTGCA
F17	ATCTAGTGGCAA	R17	ATCTAGTGGCAA
F18	CCAGGGACTTCT	R18	CCAGGGACTTCT
F19	CACCTTACCTTA	R19	CACCTTACCTTA
F20	ATAGTTAGGGCT	R20	ATAGTTAGGGCT
F21	GCACTTCATTTC	R21	GCACTTCATTTC
F22	TTAACTGGAAGC	R22	TTAACTGGAAGC
F23	CGCGGTTACTAA	R23	CGCGGTTACTAA
F24	GAGACTATATGC	R24	GAGACTATATGC

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

Table S2 the number of unique species found per class and the total number of times these species were detected across swabs.

Class	Unique Species Count	Total Species Count
Actinopteri	12	476
Amphibia	1	29
Aves	6	247
Insecta	3	47
Mammalia	6	206

Table S3 Shows the cumulative species richness and standard deviation (SD) of both terra firme and varseà.

VZ Samples	Richness	SD	TF Samples	Richness	SD
1	14.88	5.70	1	7.51	5.22
2	20.45	4.02	2	12.36	5.11
3	23.02	2.69	3	15.69	4.51
4	24.40	1.93	4	17.96	4.01
5	25.18	1.41	5	19.52	3.46
6	25.62	1.13	6	20.77	3.02
7	25.92	0.94	7	21.82	2.73
8	26.13	0.81	8	22.60	2.42
9	26.27	0.73	9	23.30	2.10
10	26.37	0.69	10	23.84	1.84
11	26.45	0.65	11	24.20	1.67
12	26.51	0.62	12	24.57	1.49
13	26.58	0.57	13	24.88	1.36
14	26.63	0.55	14	25.13	1.22
15	26.66	0.53	15	25.33	1.11
16	26.70	0.49	16	25.49	1.03
17	26.73	0.46	17	25.65	0.97
18	26.77	0.43	18	25.80	0.89
19	26.80	0.41	19	25.91	0.83
20	26.82	0.39	20	26.00	0.80
21	26.85	0.36	21	26.09	0.77
22	26.87	0.34	22	26.16	0.73
23	26.89	0.32	23	26.25	0.69
24	26.90	0.30	24	26.31	0.65
25	26.91	0.28	25	26.38	0.62
26	26.92	0.27	26	26.44	0.61
27	26.94	0.24	27	26.48	0.59
28	26.95	0.21	28	26.53	0.56
29	26.96	0.19	29	26.57	0.54
30	26.97	0.18	30	26.61	0.52
31	26.98	0.15	31	26.64	0.50
32	26.98	0.14	32	26.68	0.48
33	26.98	0.13	33	26.71	0.46
34	26.99	0.10	34	26.74	0.45
35	26.99	0.10	35	26.76	0.43
36	26.99	0.08	36	26.79	0.41
37	27.00	0.07	37	26.82	0.39
38	27.00	0.05	38	26.84	0.36
39	27.00	0.03	39	26.86	0.35

40	27.00	0.03	40	26.88	0.32
41	27.00	0.00	41	26.91	0.29
42	27.00	0.00	42	26.93	0.26
43	27.00	0.00	43	26.95	0.22
44	27.00	0.00	44	26.98	0.13
45	27.00	0.00	45	27.00	0.00

Table S4: Total species reads across both forest types combined (TF and VZ). The total number of reads per species across all swabs from both forest types have been added. The total percentage of reads per species has been calculated.

Species	Total Species Reads	Total Forest Reads	Percentage Detection
Trogon massena	86301	543038	15.89
Hoplias malabaricus	53393	543038	9.83
Bryconops affinis	50703	543038	9.34
Nycticorax nycticorax	36510	543038	6.72
Acestrorhynchus lacustris	34380	543038	6.33
Pinirampus pirinampu	30025	543038	5.53
Bryconops caudomaculatus	27220	543038	5.01
Carollia brevicauda	24312	543038	4.48
Leptotila rufaxilla	23290	543038	4.29
Alouatta juara	19329	543038	3.56
Trogon curucui	17251	543038	3.18
Ateles belzebuth	15035	543038	2.77
Cyanocharax sp.	13754	543038	2.53
Campylorhamphus procurvoides	13710	543038	2.52
Brachyplatystoma juruense	13475	543038	2.48
Hydrochoerus hydrochaeris	12421	543038	2.29
Choloepus didactylus	11333	543038	2.09
Sternopygus macrurus	10084	543038	1.86
Electrophorus electricus	9618	543038	1.77
Crenicichla lepidota	9548	543038	1.76
Coendou prehensilis	9007	543038	1.66
Mylossoma duriventre	7467	543038	1.38
Gymnotus carapo	7325	543038	1.35
Thamnophilus nigrocinereus	6101	543038	1.12
Liorhyssus hyalinus	800	543038	0.15
Adenomera sp.	586	543038	0.11
Constrictotermes cavifrons	38	543038	0.01
Aspisoma sp.	22	543038	0.00

Table S5: This table displays the distribution of arboreal species reads across individual sites.

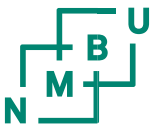
Site	Species	Arboreal Reads	Total Reads Per Site	Detection Percentage
1	Alouatta juara	18137	248128	7.31
1	Ateles belzebuth	12287	248128	4.95
1	Campylorhamphus procurvoides	7592	248128	3.06
1	Carollia brevicauda	10798	248128	4.35
1	Choloepus didactylus	1567	248128	0.63
1	Coendou prehensilis	2	248128	0.00
1	Trogon curucui	210	248128	0.08
1	Trogon massena	39149	248128	15.78
2	Alouatta juara	801	99758	0.80
2	Ateles belzebuth	1116	99758	1.12
2	Campylorhamphus procurvoides	3178	99758	3.19
2	Carollia brevicauda	11225	99758	11.25
2	Choloepus didactylus	2752	99758	2.76
2	Coendou prehensilis	236	99758	0.24
2	Trogon curucui	1660	99758	1.66
2	Trogon massena	25348	99758	25.41
3	Alouatta juara	369	168139	0.22
3	Ateles belzebuth	413	168139	0.25
3	Campylorhamphus procurvoides	2333	168139	1.39
3	Carollia brevicauda	2163	168139	1.29
3	Choloepus didactylus	3584	168139	2.13
3	Coendou prehensilis	3	168139	0.00
3	Trogon curucui	15339	168139	9.12
3	Trogon massena	19025	168139	11.32
4	Alouatta juara	12	5896	0.20
4	Ateles belzebuth	1060	5896	17.98
4	Campylorhamphus procurvoides	454	5896	7.70
4	Carollia brevicauda	48	5896	0.81
4	Choloepus didactylus	849	5896	14.4
4	Coendou prehensilis	43	5896	0.73
4	Trogon curucui	18	5896	0.31
4	Trogon massena	649	5896	11.01
5	Alouatta juara	8	17825	0.04
5	Ateles belzebuth	18	17825	0.10
5	Campylorhamphus procurvoides	63	17825	0.35
5	Carollia brevicauda	57	17825	0.32
5	Choloepus didactylus	2548	17825	14.29
5	Coendou prehensilis	8702	17825	48.82
5	Trogon curucui	5	17825	0.03

5	Trogon massena	2064	17825	11.58
6	Alouatta juara	2	3292	0.06
6	Ateles belzebuth	141	3292	4.28
6	Campylorhamphus procurvoides	90	3292	2.73
6	Carollia brevicauda	21	3292	0.64
6	Choloepus didactylus	33	3292	1.00
6	Coendou prehensilis	21	3292	0.64
6	Trogon curucui	19	3292	0.58
6	Trogon massena	66	3292	2.00

Table S6. Total read percentage of arboreal species per forest type.

Species	Forest Type	Total Reads Species	Total Reads Forest	Percentage Detection
Alouatta juara	TF	22	27013	0.08
Alouatta juara	VZ	19307	516025	3.74
Ateles belzebuth	TF	1219	27013	4.51
Ateles belzebuth	VZ	13816	516025	2.68
Campylorhamphus procurvoides	TF	607	27013	2.25
Campylorhamphus procurvoides	VZ	13103	516025	2.54
Carollia brevicauda	TF	126	27013	0.47
Carollia brevicauda	VZ	24186	516025	4.69
Choloepus didactylus	TF	3430	27013	12.7
Choloepus didactylus	VZ	7903	516025	1.53
Coendou prehensilis	TF	8766	27013	32.45
Coendou prehensilis	VZ	241	516025	0.05
Trogon curucui	TF	42	27013	0.16
Trogon curucui	VZ	17209	516025	3.33
Trogon massena	TF	2779	27013	10.29
Trogon massena	VZ	83522	516025	16.19

Figure S1 shows the percentage of total arboreal reads against non-arboreal reads per site.



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