Received: 24 February 2023 | Revised: 10 July 2023 | Accepted: 30 August 2023
DOI: 10.1002/edn3.474

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WILEY

ORIGINAL ARTICLE

Leech-derived iDNA complements traditional surveying methods, enhancing species detections for rapid biodiversity sampling in the tropics

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Funding information
Explorers Club, Grant/Award Number: Fjallraven Field Grant 2019

Abstract

Deforestation, exploitation, and other drivers of biodiversity loss in Madagascar leave its highly endangered and predominantly endemic wildlife at risk of extinction. Decreasing biodiversity threatens to compromise ecosystem functions and vital services provided to people. New, economical, and diverse methods of biodiversity monitoring can help to establish reliable baseline and long-term records of species richness. Metabarcoding with invertebrate-derived DNA (iDNA) has emerged as a promising new biosurveillance tool. An unexpected wet forest fragment tucked in the dry cliffs of Madagascar's southcentral plateau, the Ivohibory Protected Area (IPA), hosts a unique mosaic of species diversity, featuring both dry and wet forest species. Recently elevated to protected status, the IPA has been surveyed for flora and fauna with a range of inventory methods over the course of three years and six expeditions (2016, 2017, & 2019). We collected 1451 leeches over 12 days from the IPA to supplement known species richness and to compare results against current records. With iDNA, we pooled tissues, isolated, and amplified bloodmeal DNA with five sets of primers. We detected 20 species of which four are species of frogs previously undetected and three of which are previously unknown to exist in this region. iDNA surveys have the capacity to provide complementary data to traditional surveying methods like camera traps, line transects, and bioacoustic methods.

KEYWORDS
biodiversity, conservation genetics, DNA metabarcoding, iDNA, leeches, surveys

1 | INTRODUCTION

Scientists estimate 37% of biodiversity will be lost by the year 2100 if no further conservation measures are implemented, risking necessary ecosystem functions like the cycling of clean air and water, buffering the spread of infectious disease, and providing cultural and spiritual services (Ceballos et al., 2017; Harrison et al., 2014; Isbell et al., 2022). And because it is permanent, the loss of species is among the most pressing environmental issues today (Ceballos et al., 2020). Unparalleled rates of global biodiversity loss are attributed to detrimental human impacts such as logging, pollution, and climate change among others (Harfoot et al., 2021). The Convention on Biological Diversity drafts new targets to reduce the rate of extinction to under 20 species across all major groups (CBD, 2021), an action which is primarily evaluated in the context of biodiversity inventories. Such inventories are critical in monitoring and upholding these commitments by providing baseline data and measuring their impacts over time (Cristescu & Hebert, 2018).

The advent of environmental DNA (eDNA) and related metabarcoding methods have launched new possibilities for biodiversity monitoring. The bloodmeals of blood-feeding invertebrates have been targeted as sources of vertebrate host DNA for biodiversity surveys (Calvignac-Spencer et al., 2013; Gogarten et al., 2020; Kocher et al., 2017; Rodgers et al., 2017) in what is a subcategory of eDNA known as invertebrate-derived DNA (iDNA). Leeches have emerged as appealing iDNA options for their site fidelity (Tessler et al., 2018) slow rate of bloodmeal digestion (Schnell et al., 2012), and distribution across the Indo-Pacific, a region home to highly threatened ecosystems (Allan et al., 2019; Borda & Siddall, 2011; Brooks et al., 2002; Myers et al., 2000). Leech-derived iDNA has become increasingly implemented to inventory biodiversity (Drinkwater, Schnell, et al., 2019; Ji et al., 2022; Lynggaard, Oceguera-Figueroa, et al., 2022; Schnell et al., 2018) showing promise for its ability to provide complementary data when used alongside conventional survey methods like camera traps and field transects (Abrams et al., 2019; Weiskopf et al., 2018). And when paired with several biodiversity detection methods and species occupancy models, iDNA surveys have helped prioritize the protection of remote, degraded habitats (Tilker et al., 2020).

Madagascar's biodiversity is among the most imperiled in the world, where all taxa are highly susceptible to extinction due to human impacts (Harfoot et al., 2021). The rate of deforestation in Madagascar has more than doubled since 2010 (Vieilledent et al., 2018), making it simultaneously a hotspot of biodiversity and deforestation, globally (Harper et al., 2007; Hoang & Kanemoto, 2021; Myers et al., 2000). Furthermore, the loss of presently threatened Malagasy mammals is predicted to inflict more severe long-term impacts than all previous extinctions since humans arrived at the island (Michielsen et al., 2022), making conservation of extant taxa of highest conservation priority.

In 2016, a team from Stony Brook University and Centre ValBio research station was invited by the Malagasy community to survey a fragment of humid forest in the southcentral region previously unknown to researchers. Protected from routine anthropogenic burning by cliffs, the fragment has persisted in an otherwise hostile matrix (Frappier-Brinton & Lehman, 2022; Humbert, 1927). Preliminary surveys quickly revealed an atypical species assemblage, featuring several dry forest species known to exist exclusively in the western and southernmost regions (Machan, 2022; Otero Jimenez et al., 2023; P. Wright, personal communication). Subsequent inventories surveyed the IPA with a variety of traditional sampling methods: diurnal and nocturnal transect surveys, baited camera traps, mist nets, baited Sherman traps, audio surveys, and point counts for the detection of mammals, reptiles, amphibians, and birds. However, the biodiversity of the IPA has yet to be evaluated in the context of iDNA, which have been shown to detect taxa distinct from those detected by traditional means and which have been used to survey other wet forests in Madagascar (Fahmy et al., 2019). Comprehensive biodiversity inventories are needed to discern the species composition of this forest, which has recently been protected as a result of those preliminary inventories and is now known as the Ivohiboro Protected Area (IPA). These inventories will improve our understanding of species responses to habitat loss and fragmentation (Epley et al., 2020; Fahrig, 2017).

The extent of historical forest cover in Madagascar remains debated and some hold the island was once predominantly covered with rainforest (Harper et al., 2007), while others suggest pockets of humid forest have always existed among the grasslands of the central highland plateau (Dewar & Richard, 2007; Solofondranohatra et al., 2018). Detailed records of species richness will help elucidate the origins of the IPA and thus its role as either relict forest or refuge from frequent fire. Opportunities for research in IPA abound and will add context to the debate regarding degree of deforestation and historical forest cover across Madagascar.

Here, we compare iDNA detections against a thorough biodiversity survey of the IPA (Otero Jimenez et al., 2023) using pooled leech tissues from 1451 leeches, and a set of five primers optimized for the detection of vertebrates (vertebrate 12S, mammalian 16S, amphibian and osteichthid 16S, avian ND2, and reptilian COI). We assess the efficacy of sample pooling strategies, and we also compare our iDNA results to those conducted in neighboring Ranomafana National Park (Fahmy et al., 2019) and the forests of Andasibe to determine whether those fauna contribute taxonomically to the richness of this newly protected, unusual, and understudied site.

2 | MATERIALS AND METHODS

2.1 | Study sites

This study was conducted in the Ivohiboro Protected Area (IPA) located in the Ihorombe region of south-central Madagascar (Figure 1). The IPA stretches 3300 ha, of which 874 ha are classified as humid forest, ranging in elevation from 900to 1400 m and situated approximately 26 km southwest of Pic d'Ivohibe Special Reserve, the nearest protected area. It is surrounded by human-caused savannah and protected from annual burning by cliffs (Figure 2). The site is composed of two parcels, Ivohibory to the north and Analamary to the south. Four prior scientific expeditions to Ivohibory have been organized where a trail system has been established yet not heavily trafficked. Analamary is approximately two-thirds larger than Ivohibory and its diversity lesser known. This study represents the first scientific expedition to Analamary. The IPA does not receive tourists and to this point has been visited primarily by researchers and sporadically by the Malagasy community for cattle grazing.

Leeches were also collected from Madagascar's northern wet forests from Andasibe's Analamazoatra Reserve, Torotorofotsy Ramsar Site, and Andasibe-Mantadia National Park. These leeches were collected to assess whether species from Andasibe contribute taxonomically to the richness of IPA and also to test pooling strategies for IPA samples. The Andasibe region experiences continued logging and much of the rainforest is secondary (Dolch et al., 2015). We also used leeches collected from Ranomafana National Park (as

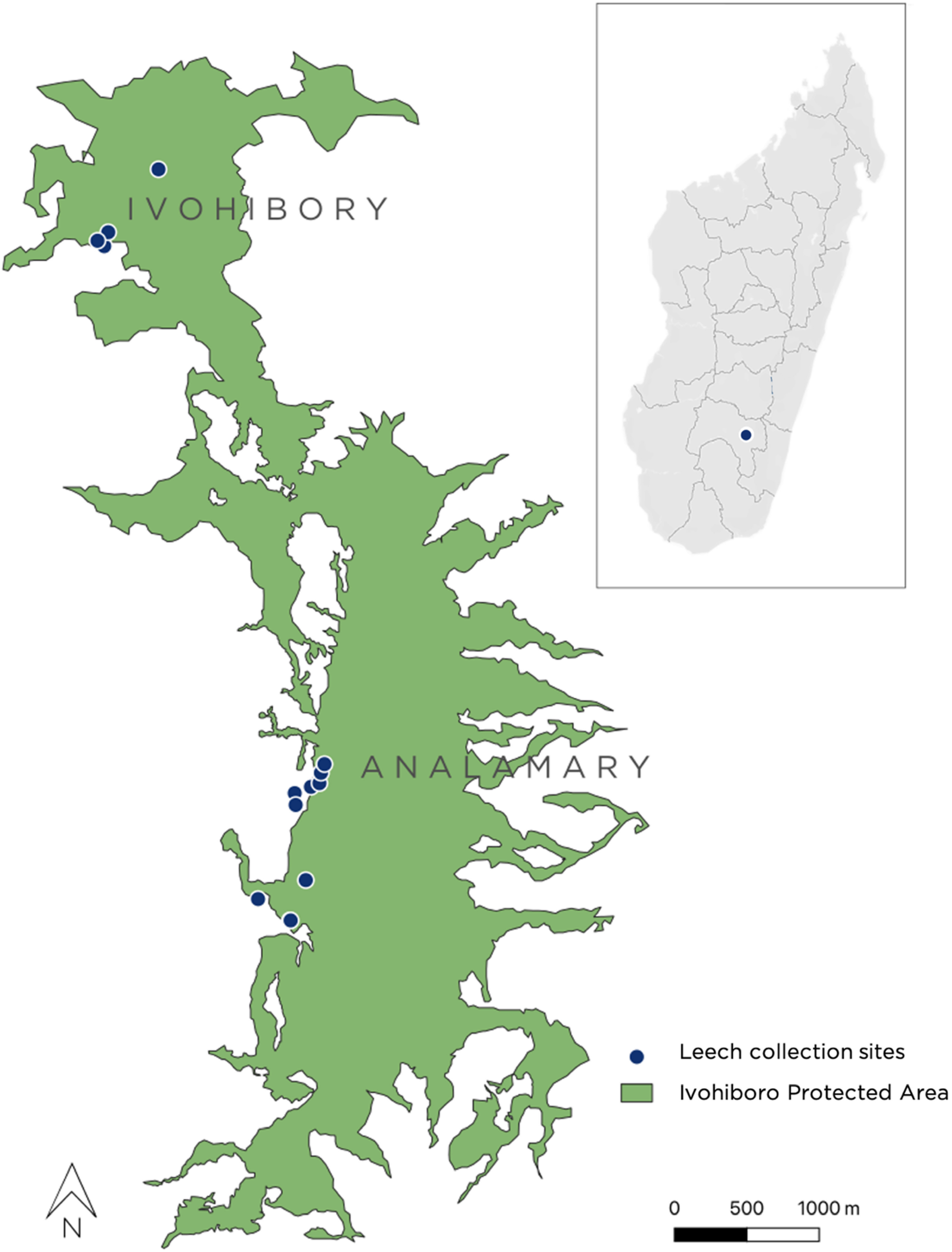


FIGURE 1 Map of study area and collection sites.

FIGURE 2 (a and b) Landscape of the Ivohibory Protected Area and surrounding grassland. (c) Forest interior within the Analamary parcel. (d) Terrestrial leeches (*Chtonobdella* spp.) collected by lead author. All photos credited to lead author (name to be inserted post peer review).

analyzed in Fahmy et al., 2019 and Fahmy et al., 2020) as a comparative site to IPA.

2.2 | Sample collection

A total of 1451 leeches (*Chtonobdella* spp.) were collected from IPA in November 2019. From Ivohibory, the northern parcel, 950 leeches were collected along 200 m transects on forest trails. These included along streams and at the highest elevations (~1100 m) of the forest. From Analamary, the southern parcel, 501 leeches were collected along 200 m transects both parallel and perpendicular to the primary stream in the forest. From the forests of Andasibe, where leeches were scarce, we collected only 66 leeches. Leeches were collected opportunistically along rivers and streams, where haemadipsids naturally congregate in times of drought (Drinkwater, Schnell, et al., 2019; Nesemann & Sharma, 2001). All leeches were collected as they approached researchers as part of their natural feeding and foraging behavior. Leeches observed to have latched or fed on humans were not collected. From Ranomafana National Park (RNP), leeches were collected along 200 m transects perpendicular to hiking trails (see Fahmy et al., 2019).

2.3 | Sample processing

In the field, individual leeches were sorted into groups by morphotype. We reported five morphotypes from Ivohibory and four from Analamary. All leeches were exported in line with Access and Benefit Sharing procedures for Madagascar as Party of the Nagoya Protocol (Permit numbers 290/19/MEDD/SG/DGEF/DGRNE, 033 N-EA02/MG20). All laboratory protocols were carried out at the Institute for Comparative Genomics at the American Museum of Natural History.

To prevent contamination, sample processing and DNA isolation were carried out in a PCR-free space, and we maintained a unidirectional workflow from pre-PCR to post PCR. All laboratory space was free from prior handling of fresh tissues of Malagasy fauna. All laboratory equipment was UV sterilized and all surfaces were sterilized with DNAway (ThermoFisher) and 70% ethanol between batches of pooled samples. We used filter pipette tips to limit the transfer of DNA aerosols across samples. From each leech, with a sterile, single-use blade, the caudal sucker was removed, and a bisection of the posterior third of the crop region was dissected for DNA isolation. A subsample of each morphotype was selected for COI sequencing to elucidate leech species identity prior to downstream analyses (Table S1). All laboratory procedures were performed by a single individual.

2.4 | DNA isolation and amplification

All 1451 leeches were dissected and organized into pools based first on leech species and then by collection locality. For more detail on pooling strategy, see Supplementary Information. We established 64 pools, each containing tissues from 6 to 38 ($\bar{x} = 20$) leeches and six negative extraction and PCR controls for a total of 70 pools. Each pool represents a single leech species and collection locality. Samples were agitated with 0.5 mm ceramic beads in a Fisherbrand Bead Mill 24 at 1.95 m/s for 1 min (Williams et al., 2020). 200 μL of liquid was removed from the agitated samples for overnight incubation with 720 μL ATL buffer and 80 μL proteinase K. DNA isolation was performed using Qiagen's DNeasy Blood and Tissue Kits and deviations from standard protocol included: Buffer AL and AW1 centrifugations at 5433 *g* instead of 4293 *g*, and Buffer AE incubation on the column membrane for 20 min instead of 1 min for the final elution. All other steps followed standard protocols.

Pools served as template for amplification of loci corresponding to each of vertebrate 12S rDNA (Poinar et al., 1998), mammal 16S rDNA (Caragiulo et al., 2014), amphibian and osteichthyid 16S rDNA (Vences et al., 2016), avian ND2 (Payne & Sorenson, 2007), and reptile and amphibian COI (Nagy et al., 2012) designed for the identification of respective taxonomic groups (see Fahmy et al., 2020). Each pool represents a single leech species and collection locality. Each locus was amplified twice with 0.5 μL of each primer (10 μM), 22 μL of water, and 2 μL of pooled tissue DNA template for each of two versions of each primer pair using Amersham Hot Start Mix Ready-To-Go PCR beads (Cytiva) to account for varied amplification success based on primer pairing (Nichols et al., 2018). Each primer set was designed in two versions, one with a forward Illumina adapter (ACACTCTTTCCCTACACGACGCTCTTCCGATCT) and one with the reverse Illumina adapter (GACTGGAGTTCAGACGTGTGCTCTTCCGATCT). For each PCR reaction and for each locus, one reaction used the forward Illumina adaptor in combination with a reverse Illumina adaptor, and another with the forward primer with a reverse Illumina adaptor in combination with the reverse primer with a forward Illumina adaptor. Doing so corrects for potential read-quality bias in Illumina known from R1 and R2 paired-end sequencing (Tan et al., 2019; Williams et al., 2020). Thermocycling profiles were as follows: 94°C for 1 min, 40 cycles of 94°C for 15 s, annealing at 54°C (but 50°C for ND2 and COI) for 30 s, 70°C for 45 s, with a final cycle of 72°C for 2 min. Duplicate PCRs were combined by corresponding pool, purified using a 2:1 carboxylated bead-to-amplicon ratio of Agencourt AMPure (Beckman Coulter) and submitted for paired-end 250-bp sequencing on an Illumina MiSeq platform at GENEWIZ from Azenta Life Sciences, Inc. We targeted between 50,000 and 100,000 reads per sample as is standard (Bruce et al., 2021). We used the same set of primers and followed the same laboratory protocols as Fahmy et al., 2020. Raw sequencing reads are deposited in DRYAD (doi:10.5061/dryad.sxksn038h).

2.5 | Leech relationships

In the field, leeches were sorted by morphotype and grouped into pools first based on leech species then by collection locality. Of the 1451 leeches collected, a subsample of 51 leeches representing each morphotype was selected for amplification and sequencing of cytochrome c oxidase subunit 1 (COI), used to delimit leech species (Borda et al., 2008). These individual leeches were not included in iDNA analysis. We amplified COI with 500 μM of LCO1490 5′-GGTCAACAAATCAT AAAGATATTGG-3′, 500 μM of HHCO1 5′-GCTG CAAAAATRGCAAATACTGC-3′ (Folmer et al., 1994), 22 μL water, 2 μL template DNA, and Amersham Hot Start Mix Ready-To-Go PCR beads (Cytiva). The thermocycler profile was 94°C for 1 min, 35 cycles of 94°C for 45 s, 46°C for 30s, 68°C for 1 min, and a final cycle of 72°C for 7 min (Borda et al., 2008). PCR products were purified with a 2:1 ratio of Agencourt AMPure (Beckman Coulter) to amplified product. Purified amplicons were cycle sequenced and ethanol precipitated, and sequences were determined using an ABI 3730xl DNA Analyzer (Applied Biosystems). Resulting sequences had primers trimmed, were reconciled, and were manually edited for quality using CodonCode Aligner (CodonCode Corporation).

IPA leeches were analyzed phylogenetically in the context of candidate leeches endemic to Madagascar: *Chtonobdella meyeri*, *C. morsitans*, *C. vagans*, *C. fallax*, *C. niarchosorum*, and *C. mangevoensis* (Fahmy, 2023). We also included *C. seychellensis* as it constitutes a clade with all known Malagasy leeches (Tessler et al., 2016). *Haemadipsa picta* (Lai et al., 2011), *H. japonica* (Morishima & Aizawa, 2019), *C. tanae*, and *C. bilineata* served as outgroups. Sequences were aligned with MUSCLE (Edgar, 2004) and models for nucleotide evolution tested with JMODELTEST 2.1.4 (Darriba et al., 2012), selecting the TVM + I + G model. Maximum likelihood analysis was conducted with IQtree (Hoang et al., 2018; Minh et al., 2020; Nguyen et al., 2015; Trifinopoulos et al., 2016) on the CIPRES Scientific Gateway portal (Miller et al., 2011) with 1000 bootstrap replicates.

2.6 | Bioinformatics and taxonomic assignments

Raw Illumina reads had primers removed and were trimmed for quality (phred score 33, length at least 100 bp) with Trimmomatic (v. 0.38) (Bolger et al., 2014). Trimmed sequences representing nested substrings were dereplicated and clustered into operational taxonomic units (OTUs) at 98% sequence similarity with USEARCH v.5 (Edgar, 2010). OTUs were first queried with basic local alignment tool (BLAST; NCBI 2021) against a curated database of candidate whole mitochondrial genomes to filter non-vertebrate DNA contamination. Contaminants were discarded and remaining OTUs post filtering were then queried against NCBI's nr/nt databases on GenBank with the BLASTn function using high performance computer cluster (American Museum of Natural History). Parameters were set to retain only sequences with an e-value of at least e-30 and only the top 20 hits.

Taxonomic identification of hosts to the species level required a percent identity (PID) of at least 98%, query coverage of at least 80%, and a minimum 3% difference between the top BLASTn hit and the next best taxon. Remaining OTUs were evaluated at family level, requiring a conservative PID of 98, query coverage of at least 80%, and that the top two BLASTn hits belong to the same family. All OTUs for which the top 20 hits represent the same taxon were re-blasted for the top 100 hits. A determination to the species level was made if the top hit was assigned with at least 98PID and at least a 3% gap. If all 100 hits return a single taxon, we represent this taxon in our species-level assignments (Table S2). All OTUs which did not meet these criteria were discarded. All those that were assigned to *Homo sapiens* were also discarded. For each pool, for those assignments with multiple sequences, taxa were collapsed to reflect presence/absence.

2.7 | Statistical analysis

All inventorying methods are imperfect. Successful biodiversity monitoring with iDNA rests on a series of probabilities: that a leech fed on a host, that that leech was collected by the researcher, that the DNA retained in the leech is amplifiable, and that the host's DNA is sufficiently represented in the database (Cameron et al., 2007; Schnell et al., 2015). Thus, we expect iDNA to underestimate species richness and thereby use Chao2 estimators to measure alpha diversity as this metric accounts for potential under sampling (Hsieh et al., 2016).

Standard diversity indices such as Shannon entropy and the Simpson index do not proportionately reflect changes in species richness (Alberdi & Gilbert, 2019). In the context of iDNA studies, the Hill number statistical framework is advantageous to traditional indices of diversity because it simplifies the indices into more applicable equally abundant OTUs for metabarcoding (Alberdi & Gilbert, 2019; Hill, 1973).

Within the Hill number framework, changing q, the scaling parameter, adjusts the sensitivity to rare and abundant OTUs. Commonly used values, $q=0$, $q=1$, and $q=2$ correspond to species richness, the exponential of the Shannon index, and inverse of the Simpson index, respectively, and have been used in iDNA studies to measure the accumulation of diversity at sampling localities (Drinkwater et al., 2021).

We generated species accumulation curves for IPA biodiversity using the iNEXT package in R (Hsieh et al., 2016). See supplementary information for rarefaction curves representing all three sampled localities. We conducted a chi-squared test to discern host preferences across vertebrate taxonomic classes between leech species.

2.8 | Comparative data

We compare iDNA results to biodiversity inventory data from Stony Brook University's Centre ValBio (CVB) research station team. The CVB team conducted their inventory over the course of 3 years (2016, 2017, 2019) for a total of six expeditions to IPA, each between 10 and 20 days, on average with a team of 12 people. The CVB team used the following methods to survey vertebrate fauna: diurnal and nocturnal transects, baited Sherman traps, baited camera traps, bioacoustics surveys, and mist nets. For exhaustive results of their efforts, see Otero Jimenez et al. (2023).

3 | RESULTS

3.1 | Ivohiboro protected area

We sequenced ~5 million reads, ranging from 18,530 to 327,815 reads per pool, on average of 77,101 reads (±4556 SE). Reads were filtered for size (>100 bp), dereplicated, filtered for vertebrate DNA, and grouped into 62,582 unique OTUs of at least 98% similarity. These clusters yielded 9908 hits to *Homo sapiens* and were thus discarded. Negative controls were sequenced and revealed low quantities of domestic (cow, pig, dog, and chicken) and wild species. The majority of OTUs from our negative controls (85%) were hits to *Homo sapiens*. For each taxon detected in our negative controls, this number of reads (on average 5.6, ±1.18 SE) was removed from co-processed, field-collected samples. While domestic species often appear as laboratory contamination, they are extant in our survey area and have been documented by the CVB team; if they pass our above filtering criteria, we retain them among our species-level identifications. We examine our data both with and without domestic species. For all data integrating both wild and domestic species in our analyses, see Figures S2, S3.

We report a total of 20 OTUs assigned to the species-level from our IPA samples with a mean of 28 OTUs per sample (±12 SE), belonging to nine orders and 13 families. These represent seven amphibians, three birds, one fish, and ten mammals (Table S2). Among iDNA determinations from IPA, mammals contribute the greatest to both species richness (Figure 3) and relative number of detections across pools (57%), followed by birds (19%), amphibians (20%),

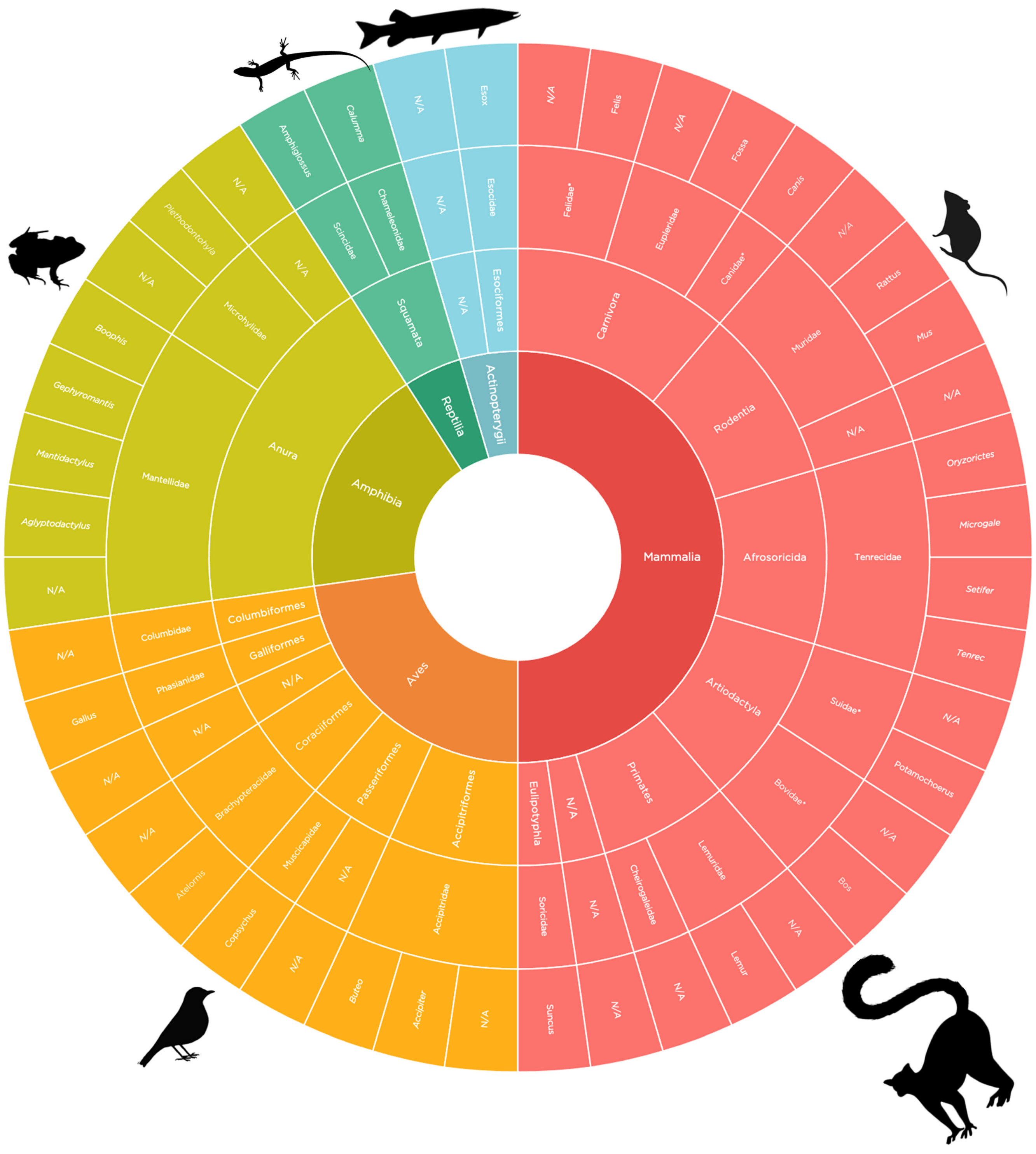


FIGURE 3 Classification of IPA taxa. N/A indicates a determination at that taxonomic level could not be made. Credit to Roberto Díaz Sibaja for the silhouette representing a lemur and to Karina Garcia for silhouette representing a bird. * = Domestic species.

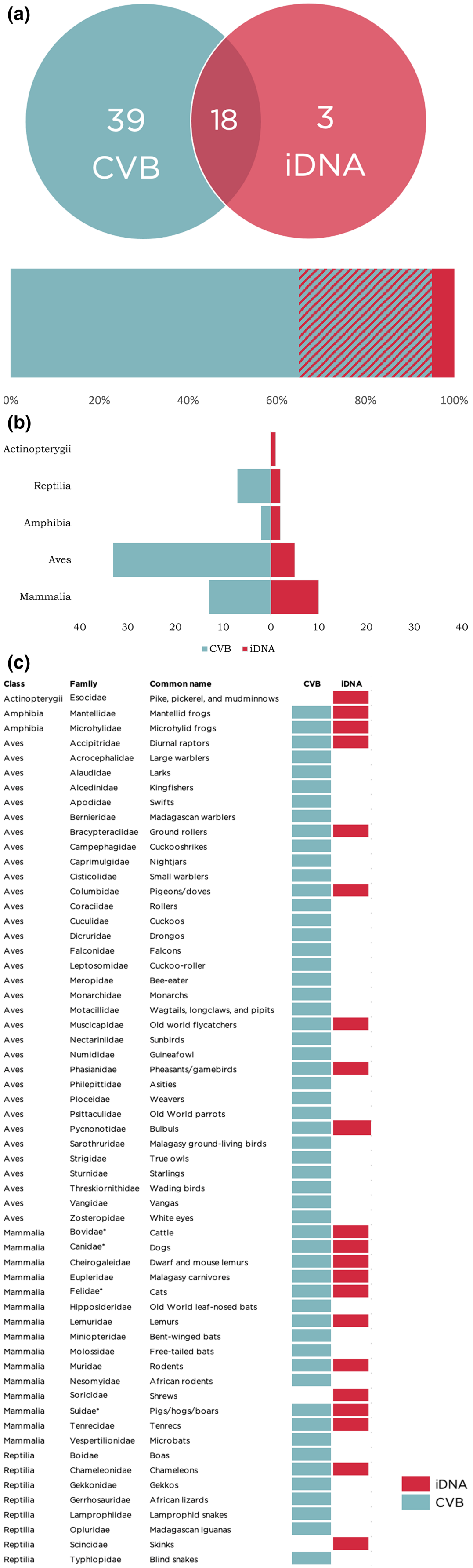
reptiles (1%) and fish (3%) (Figure S4). We report an additional six unique families not encompassed in our species level determinations, for a total of 21 family-level hits.

4647 reads returned results for which there is only one taxon in the top 20 hits from BLASTn. These are represented by *Boophis obscurus*, *Bos taurus*, *Canis lupus*, *Gallus gallus*, *Mantidactylus betsileanus*, *Mus musculus*, *Porcula salvinia*, *Rattus rattus*, and *Sus scrofa*. Five taxa represented species which were not already detected, and for which passed our determination criteria: *Boophis obscura*, *Mus musculus*, *Gallus gallus*, and *Porcula salvinia*. Clusters for each taxon for which PID was at least 98% and sequence length was at least 80% of the target locus were re-blasted for the top 100 hits. The next best taxon after *Boophis obscurus* is not sufficiently different (less than 3% difference between next best taxon) than other congenerics and we exclude *B. obscurus* from our species-level determinations. *Porcula salvinia* is not geographically represented in Madagascar and that determination was discarded. For *Mus musculus* and *Gallus gallus*, all 100 hits failed to return alternative hits and a species-level determination was made as observed in Table S2. All remaining reads, which were not classified to the species or family levels, were discarded.

DNA sequencing of the diagnostic COI region of a subsample of all leech morphotypes revealed a putative new clade unique to IPA (Figure 6). Based on our phylogenetic topology, we infer the majority of leeches analyzed for iDNA belong to *Chtonobdella fallax*. Our chi-squared test revealed no significant relationship between leech species and host class ($\chi^2 = 2.15$, $p = 0.54$, $\alpha = 0.05$).

3.2 | iDNA versus CVB

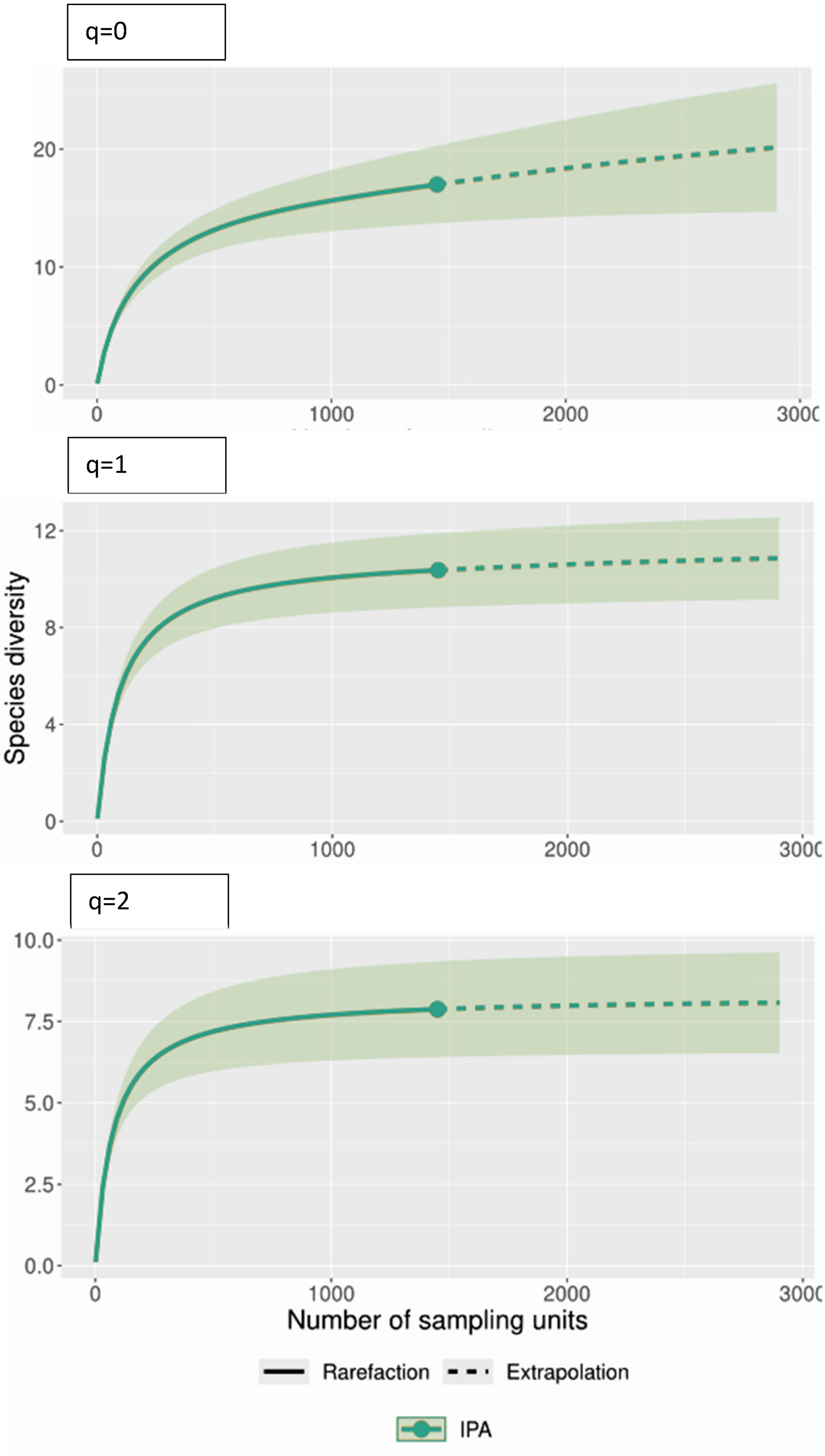
Over the course of six expeditions spanning approximately 90 days total, and with teams of on average 12 people, CVB's research team detected 110 vertebrate species (including domestics) belonging to 57 families in the IPA. With iDNA, in 12 days and a team of two people, we detected 20 species, of which three are domestic. iDNA surveys detected four species which the CVB team did not: *Mantidactylus ulcersosus*, *Boophis periegetes*, *Plethodontohyla inguinalis*, and *Mantidactylus femoralis*. iDNA surveys increased species-level detections by 3.63%. We identified 21 total families, including those for which species-level determinations could not be made. Of the 57 families identified by the CVB team, 18 were also detected by iDNA (Figure 4a), effectively 36% of known family-level richness in 78 fewer days and 20% of the people required. The CVB team detected more bird and reptile species than did iDNA (Figure 4b). Both iDNA and CVB surveys reveal that the IPA is predominately populated by small mammals. Among mammal results, our iDNA survey failed to detect bats (Chiroptera) and canopy dwelling species such as *Cryptoprocta ferox*, Madagascar's endemic carnivore. Both are documented by the CVB team. At the family-level, iDNA increased number of family detections by 0.5%. All three species accumulation curves representing IPA generated with iNEXT for $q = 0$, $q = 1$, and $q = 2$ reached an asymptote (Figure 5).



Class	Famliy	Common name	CVB	iDNA
Actinopterygii	Esocidae	Pike, pickerel, and mudminnows		🟥
Amphibia	Mantellidae	Mantellid frogs	🟦	🟥
Amphibia	Microhylidae	Microhylid frogs	🟦	🟥
Aves	Accipitridae	Diurnal raptors	🟦	🟥
Aves	Acrocephalidae	Large warblers	🟦	
Aves	Alaudidae	Larks	🟦	
Aves	Alcedinidae	Kingfishers	🟦	
Aves	Apodidae	Swifts	🟦	
Aves	Bernieridae	Madagascan warblers	🟦	
Aves	Bracypteraciidae	Ground rollers	🟦	🟥
Aves	Campephagidae	Cuckooshrikes	🟦	
Aves	Caprimulgidae	Nightjars	🟦	
Aves	Cisticolidae	Small warblers	🟦	
Aves	Columbidae	Pigeons/doves	🟦	🟥
Aves	Coraciidae	Rollers	🟦	
Aves	Cuculidae	Cuckoos	🟦	
Aves	Dicruridae	Drongos	🟦	
Aves	Falconidae	Falcons	🟦	
Aves	Leptosomidae	Cuckoo-roller	🟦	
Aves	Meropidae	Bee-eater	🟦	
Aves	Monarchidae	Monarchs	🟦	
Aves	Motacillidae	Wagtails, longclaws, and pipits	🟦	
Aves	Muscicapidae	Old world flycatchers	🟦	🟥
Aves	Nectariniidae	Sunbirds	🟦	
Aves	Numididae	Guineafowl	🟦	
Aves	Phasianidae	Pheasants/gamebirds	🟦	🟥
Aves	Philepittidae	Asities	🟦	
Aves	Ploceidae	Weavers	🟦	
Aves	Psittaculidae	Old World parrots	🟦	
Aves	Pycnonotidae	Bulbuls	🟦	🟥
Aves	Sarothruridae	Malagasy ground-living birds	🟦	
Aves	Strigidae	True owls	🟦	
Aves	Sturnidae	Starlings	🟦	
Aves	Threskiornithidae	Wading birds	🟦	
Aves	Vangidae	Vangas	🟦	
Aves	Zosteropidae	White eyes	🟦	
Mammalia	Bovidae*	Cattle	🟦	🟥
Mammalia	Canidae*	Dogs	🟦	🟥
Mammalia	Cheirogaleidae	Dwarf and mouse lemurs	🟦	🟥
Mammalia	Eupleridae	Malagasy carnivores	🟦	🟥
Mammalia	Felidae*	Cats	🟦	🟥
Mammalia	Hipposideridae	Old World leaf-nosed bats	🟦	
Mammalia	Lemuridae	Lemurs	🟦	🟥
Mammalia	Miniopteridae	Bent-winged bats	🟦	
Mammalia	Molossidae	Free-tailed bats	🟦	
Mammalia	Muridae	Rodents	🟦	🟥
Mammalia	Nesomyidae	African rodents	🟦	
Mammalia	Soricidae	Shrews		🟥
Mammalia	Suidae*	Pigs/hogs/boars	🟦	🟥
Mammalia	Tenrecidae	Tenrecs	🟦	🟥
Mammalia	Vespertilionidae	Microbats	🟦	
Reptilia	Boidae	Boas	🟦	
Reptilia	Chameleonidae	Chameleons	🟦	🟥
Reptilia	Gekkonidae	Gekkos	🟦	
Reptilia	Gerrhosauridae	African lizards	🟦	
Reptilia	Lamprophiidae	Lamprophid snakes	🟦	
Reptilia	Opluridae	Madagascan iguanas	🟦	
Reptilia	Scincidae	Skinks		🟥
Reptilia	Typhlopidae	Blind snakes	🟦	

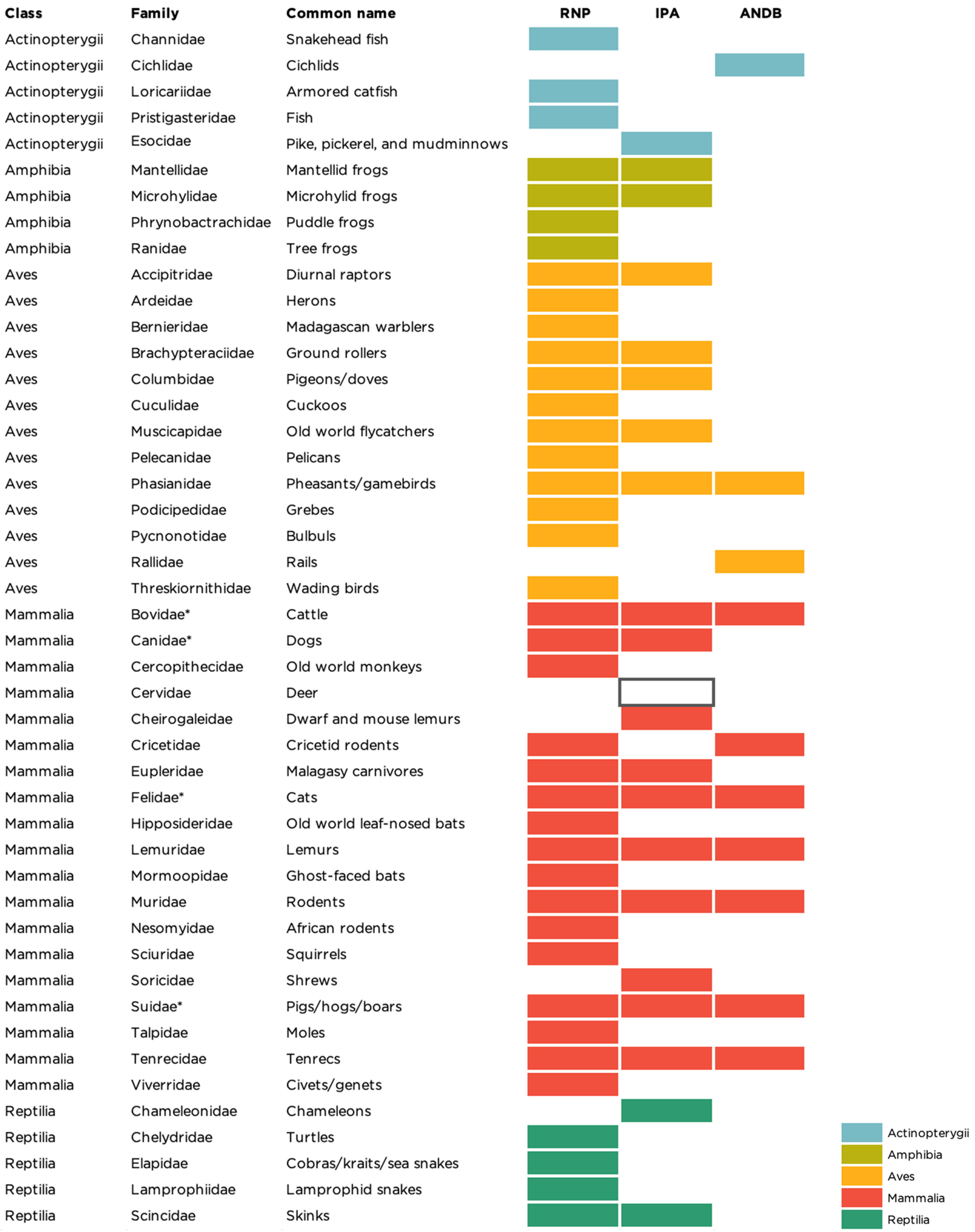
FIGURE 4 Family-level detections comparing iDNA and Centre ValBio (CVB) results. The CVB team carried out a series of inventories with various techniques, including camera traps, bioacoustics surveys, and baited Sherman traps over the course of three years and six expeditions. The results of their efforts are shown here. (a) Number of families in common as detected by both iDNA and CVB's surveys of the IPA. Overlap of family detections from IPA with iDNA and surveys deployed by Centre ValBio. (b) Proportion of families categorized by taxonomic class detected with iDNA or by CVB's surveys. (c) Family-level resolution of shared and distinct detections of CVB and iDNA surveys. * = Domestic species.

FIGURE 5 Sample-size-based rarefaction and extrapolation sampling curves. Incidence-based. Scaling parameters (q) for Hill number framework shown above each curve. $Q=0$ corresponds to species richness, $q=1$ to the exponential of the Shannon index, and $q=2$ to the inverse of the Simpson index. Endpoint setting: 2902, Number of knots = 100, number of bootstraps = 100, confidence interval = 0.95. Where number of individuals is equal to number of leeches analyzed. Species diversity reflects family-level diversity. Domestic species not included in this analysis.



3.3 | IPA versus RNP versus ANDB

Pools from Andasibe returned three species-level hits and 10 family-level identifications (Figure 6, Table S2). Aside from *Felis catus*, species detected with iDNA from Andasibe were not represented in IPA (Table S2). At family-level resolution, RNP and IPA share Mantellidae, Microhylidae, Accipitridae, Brachypteraciidae, Columbidae, Muscicapidae, Phasianidae, Bovidae, Eupleridae, Felidae, Lemuridae, Muridae, Suidae, Tenrecidae and Scincidae. All three sites share common diversity at the family level: Phasianidae, Bovidae, Felidae, Lemuridae, Muridae, Suidae, and Tenrecidae (Figure 6).



Class	Family	Common name	RNP	IPA	ANDB
Actinopterygii	Channidae	Snakehead fish	🟦		
Actinopterygii	Cichlidae	Cichlids			🟦
Actinopterygii	Loricariidae	Armored catfish	🟦		
Actinopterygii	Pristigasteridae	Fish	🟦		
Actinopterygii	Esocidae	Pike, pickerel, and mudminnows		🟦	
Amphibia	Mantellidae	Mantellid frogs	🟨	🟨	
Amphibia	Microhylidae	Microhylid frogs	🟨	🟨	
Amphibia	Phrynobactrachidae	Puddle frogs	🟨		
Amphibia	Ranidae	Tree frogs	🟨		
Aves	Accipitridae	Diurnal raptors	🟧	🟧	
Aves	Ardeidae	Herons	🟧		
Aves	Bernieridae	Madagascan warblers	🟧		
Aves	Brachypteraciidae	Ground rollers	🟧	🟧	
Aves	Columbidae	Pigeons/doves	🟧	🟧	
Aves	Cuculidae	Cuckoos	🟧		
Aves	Muscicapidae	Old world flycatchers	🟧	🟧	
Aves	Pelecanidae	Pelicans	🟧		
Aves	Phasianidae	Pheasants/gamebirds	🟧	🟧	🟧
Aves	Podicipedidae	Grebes	🟧		
Aves	Pycnonotidae	Bulbuls	🟧		
Aves	Rallidae	Rails			🟧
Aves	Threskiornithidae	Wading birds	🟧		
Mammalia	Bovidae*	Cattle	🟥	🟥	🟥
Mammalia	Canidae*	Dogs	🟥	🟥	
Mammalia	Cercopithecidae	Old world monkeys	🟥		
Mammalia	Cervidae	Deer		□	
Mammalia	Cheirogaleidae	Dwarf and mouse lemurs		🟥	
Mammalia	Cricetidae	Cricetid rodents	🟥		🟥
Mammalia	Eupleridae	Malagasy carnivores	🟥	🟥	
Mammalia	Felidae*	Cats	🟥	🟥	🟥
Mammalia	Hipposideridae	Old world leaf-nosed bats	🟥		
Mammalia	Lemuridae	Lemurs	🟥	🟥	🟥
Mammalia	Mormoopidae	Ghost-faced bats	🟥		
Mammalia	Muridae	Rodents	🟥	🟥	🟥
Mammalia	Nesomyidae	African rodents	🟥		
Mammalia	Sciuridae	Squirrels	🟥		
Mammalia	Soricidae	Shrews		🟥	
Mammalia	Suidae*	Pigs/hogs/boars	🟥	🟥	🟥
Mammalia	Talpidae	Moles	🟥		
Mammalia	Tenrecidae	Tenrecs	🟥	🟥	🟥
Mammalia	Viverridae	Civets/genets	🟥		
Reptilia	Chameleonidae	Chameleons		🟩	
Reptilia	Chelydridae	Turtles	🟩		
Reptilia	Elapidae	Cobras/kraits/sea snakes	🟩		
Reptilia	Lamprophiidae	Lamprophid snakes	🟩		
Reptilia	Scincidae	Skinks	🟩	🟩	

🟦 Actinopterygii
🟨 Amphibia
🟧 Aves
🟥 Mammalia
🟩 Reptilia

FIGURE 6 Family-level classifications of iDNA results from Ranomafana National Park (RNP) (Fahmy et al., 2020), the Ivohibory Protected Area (IPA) and Andasibe protected areas (ANDB). * = Domestic species. Cervidae represents a family detected in IPA but which is not known to exist in Madagascar.

4 | DISCUSSION

Our iDNA survey increases species detections in the IPA when used as a complementary technique to more traditional methods such as camera traps, baited traps and line transect surveys. By pooling leech tissues prior to DNA isolation and amplifying host DNA with five primer sets, we reveal 20 species-level determinations and 21 taxonomic families. All mutual iDNA species-level determinations are corroborated by CVB's survey. Birds and mammals constitute the most diverse groups detected with iDNA (Figure 3), whereas birds and reptiles represent CVB's most diverse groups (Figure 4b, c). Excluding domestic species, we identify ambiguous taxa belonging to the following families, Esocidae, Soricidae, and Scincidae, detected exclusively with iDNA and all of which are small, ground dwelling or aquatic species which are often well camouflaged and difficult to detect with cameras, even positioned at ground level (Abrams et al., 2019; Tilker et al., 2019; Weiskopf et al., 2018) (Figure 4c).

We report four amphibian species exclusively detected with iDNA, resulting in an increase of 3.63% of species-level detections in IPA. While minimal, an increase at all is surprising given the years of extensive, thorough inventorying effort deployed by the CVB team. Our results expand the distribution of *Mantidactylus ulcerosus*, *Plethodontohyla inguinalis*, and *Boophis pereigetes* based on their current records of geographic distribution from the International Union for the Conservation of Nature (IUCN). This points to the complementarity of iDNA to conventional inventory methods, especially in detecting amphibians (Rocha et al., 2012). Similar results have been observed in both iDNA and eDNA contexts (Coutant et al., 2021; Weiskopf et al., 2018), demonstrating the utility of eDNA to detect those species unaccounted for in traditional surveys, further lending credibility to the value of iDNA and eDNA more broadly in biosurveillance (Fediajevaite et al., 2021).

All taxa represent geographically plausible hits, including those to the family Cervidae, which may represent a population of deer introduced from Europe (Saggiomo et al., 2020; Tattersall, 2005). CVB surveys have not observed deer in the IPA, which would have been likely for such large-bodied fauna and so we tentatively represent this result only in Figure 6 as more research is needed. Furthermore, it is yet unclear whether this population of deer persists to the present day as some argue this population has been extirpated from Madagascar (Russell et al., 2016).

We performed two PCR replicates per pool to balance sequencing costs, but we recommend at least three replicates to allow for the detection of taxonomic outliers (Shirazi et al. 2021). 9.3% of samples (6/64) returned less than 50,000 reads per sample, with the lowest read number being 18,530. Because alpha diversity increases with sequencing depth (Shirazi et al. 2021), such low sequencing depth often leaves a higher proportion of undetected species. This could be a result of low levels of host DNA (Pereira-Marques et al., 2019) and can result in false negatives.

While iDNA does not inform species abundance, it successfully detects the presence of rare and endangered species. Here, we identify several species classified as threatened with extinction according to the International Union for the Conservation of Nature: *Boophis periegetes* (NT), *Fossa fossana* and *Gephyromantis spinifer* (VU), and *Lemur catta* (EN). We present the first species-level identifications of lemurs with iDNA, both in the case of *Hapalemur griseus* in Andasibe and *Lemur catta* in the IPA, emphasizing the utility of iDNA in tracking and monitoring what are among the world's most endangered mammals (Lafleur et al., 2016) and Madagascar's most emblematic species.

Domesticated species are frequently detected with iDNA and may represent standard laboratory contamination. However, we include these taxa here on the basis that their presence is in fact known in Madagascar and in the IPA region. Furthermore, we visually identified a bushpig deep in our forested survey area and Malagasy villagers continue to use both the surrounding savannah and humid forest to graze zebu, the local cattle. In a recently protected fragment like the IPA, presence of domesticated species should be monitored as the presence of dogs poses threats to endemic wildlife (Guedes et al., 2021). Addressing the impacts of domestics on the survival of wildlife should be prioritized, especially in a fragment where their coexistence is observed.

Our results improve our understanding of the natural foraging behavior of haemadipsids. Based on our iDNA results and personal observation, we posit that Malagasy haemadipsids ascend at least to the forest understory while questing for hosts. Despite evidence of geophagy in both lemur species detected, *H. griseus* and *L. catta* (Pebsworth et al., 2019), they are largely arboreal lemurs and spend most of their lives above ground. At the family level, Esocidae, or pike/pickerel/mudminnow fishes represent a family which has been introduced to Madagascar (Bertoli & Pallavicini, 2016), whereas cichlids constitute an endemic group (Matschiner et al., 2020); both supporting evidence of terrestrial leeches feeding on fish (Fahmy et al., 2020). Although they cannot swim, when dropped in water, haemadipsids sink and crawl out (Phillips et al., 2020), introducing the potential for seeking aquatic hosts. Our fish host determinations assert that the terrestrial leeches of Madagascar feed opportunistically even on freshwater fish, perhaps suggesting an amphibious proclivity for Malagasy haemadipsids. And because we find no significant difference between leech species and frequency of hosts detected, we maintain that Malagasy haemadipsids feed indiscriminately on vertebrate hosts. Further insights into the behavior, life history, and species diversity of Malagasy haemadipsids are needed to inform best practices of iDNA inventories and to document the life history of this understudied group.

Haemadipsid species are often cryptic, and morphology alone is insufficient in delimiting species. Of our subsample ($n=51$) of all leeches collected from IPA and based on sequencing of the COI region, we find all but two morphotypes ($n=45$) are assigned to *Chtonobdella fallax*. We also find evidence for a putative new clade, represented by six leeches and two morphotypes (Figure 7). Further genetic analysis and morphological assessment are needed to describe this new taxon.

All species accumulation curves for all scaling parameters reach an asymptote reflecting sufficient sampling with iDNA from IPA.

FIGURE 7 Relationships of IPA leeches represented in green based on maximum likelihood analysis of COI with *H. picta*, *H. japonica*, *C. tanae*, and *C. bilineata* and as outgroups. Branches supported with bootstrap values. Scale bar represents substitutions per site.

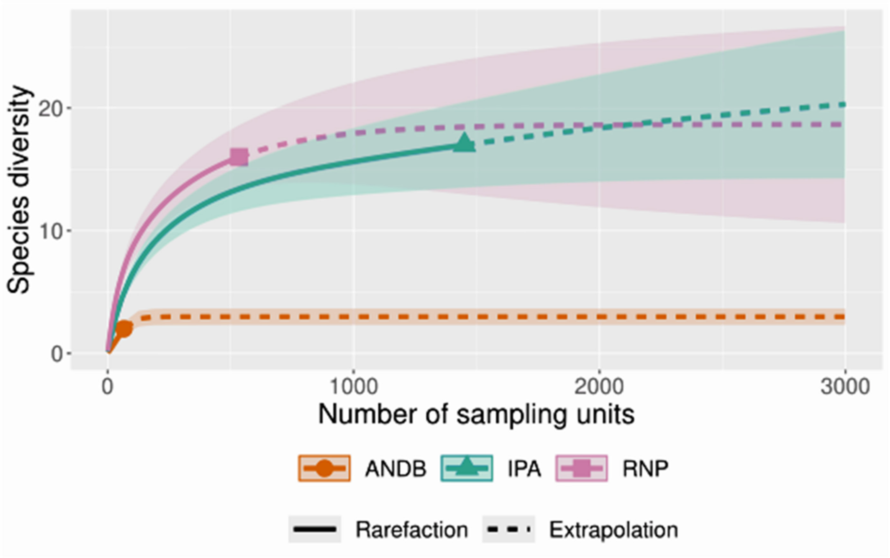


FIGURE 8 Species accumulation curves for each of Andasibe (ANDB), Ranomafana National Park (RNP), and Ivohiboro Protected Area (IPA). Species diversity reflects family-level determinations with iDNA across all three sites. Domestic species not included in this analysis.

We determine about 2000 leeches are required to capture most species richness from the IPA, as species richness plateaus thereafter. Leech sampling effort from RNP and Andasibe were not sufficient to capture total species richness of those sites (Figure 8). Based on extrapolated values, approximately 1300 leeches and 500 leeches would need to be collected to reflect the species richness of RNP and Andasibe, respectively, with iDNA (Figure 8). False negatives in iDNA surveys reflect several conditions which must be met for host DNA to be detectable from leech crops (see above); and in the case of Andasibe, false negatives also reflect sampling effort (Figure 8). Leeches were scarce in the forests of Andasibe despite our collection during the rainy season. We collected only 66 leeches over the course of seven days, whereas in RNP and IPA, we collected >500 leeches with equal effort. Leech scarcity in Andasibe, our most degraded site, supports the hypothesis that leeches serve as indicators of forest quality (Drinkwater, Williamson, et al., 2019). Degraded forests, those without intact canopies, inherently lack suitable moist habitat thus leeches become more scarce and less active.

Family-level richness across our three sites represent taxa which are either domesticated or are endemic and widespread across Madagascar. We observe greatest species richness from RNP, followed by IPA, and then ANDB. Low species richness in ANDB can be attributed to sampling effort and forest quality. While we collected over two times the number of leeches in IPA than in RNP, RNP harbors greater diversity than IPA, both as determined by iDNA and traditional surveys. RNP was inaugurated in 1991 and has benefitted from decades of protection from resource extraction and poaching. The area of RNP (43,500 ha) is also over 12 times that of IPA (3300 ha), which may also contribute to greater species richness, reflecting long standing support for species-area relationships in ecology (Lomolino & Weiser, 2001; Losos & Schluter, 2000; MacArthur & Wilson, 1963; Schoener, 1976; Triantis et al., 2012; Warren et al., 2015).

Despite its small size and isolation, IPA supports a rich ecological community, with taxa representing every trophic level, including Madagascar's apex predator, *Cryptoprocta ferox* (Otero Jimenez et al., 2023). The results of the CVB inventory show that mammals constitute the greatest proportion of dry forest species, while reptiles constitute the second greatest proportion of dry forest species, followed by amphibians and birds. *Lemur catta* in IPA represent a population new to science, one unique for its adaptation to arboreality in humid forest. *L. catta* was also the only dry forest species detected with iDNA (Table S2). Although they are opportunistic feeders and habitat generalists, these lemurs are predominantly terrestrial, found on dry, rocky outcrops (Fardi et al., 2018). Even neighboring populations found in the Ambositra-Vondrozo Corridor in nearby Pic d'Ivohibe and Andringitra National Park live exclusively on cliffs above the treeline, exposed to the most extreme climate on the island, historically the only location to receive snowfall (Goodman & Langrand, 1996).

Most mammal and bird species from IPA are habitat generalists and are found in both humid and dry forests (Figure S5A). Amphibians are not known to disperse distances greater than 15 km, their maximum migratory range (Smith & Green, 2005), yet the nearest tract of rainforest is over 24 km to the east. Satellite imagery suggests IPA was once part of the continuous humid forest that forms the Ambositra-Vondrozo Corridor (Ramiadantsoa et al., 2015), which may explain their presence in IPA. Conversely, the CVB team locate *Gephyromantis corvus* in IPA, classified as endangered and found exclusively in the dry forests of the southwest. This taxon is classified as endangered and was previously found exclusively in the dry forests of the southwest. Analysis of population structure and gene flow are needed to assess biogeographic origins of these unexpected taxa. And if current populations are below minimum viable size, the unique ecological community and relative species richness may ultimately represent a time-lagged extirpation as a response to habitat fragmentation (Broekman et al., 2022; Isbell et al., 2022).

The IPA is an area of conservation priority not only for its unique community assemblage but also for its potential to shed light on the historical forest cover of Madagascar (Humbert, 1927; Federman et al., 2015; Solofondranohatra et al., 2020). This newly protected forest will elucidate ecological and evolutionary responses to deforestation and serve as a living laboratory to test hypotheses regarding biogeography and habitat fragmentation: Have species expanded their known ranges into the IPA in search of forest refugia? Or does the composition of the IPA reflect remnant communities of older, intact forests? Because of its relative proximity to the COFAV and differentially sized northern and southern parcels, the IPA presents an opportunity to assess the relationships among dispersal, fragment size, and species richness (Saura, 2021). Future studies should aim to survey neighboring Pic d'Ivohibe and Andringitra National Park with iDNA and other methods to document patterns and viability of dispersal across taxonomic groups. Biogeographic divergence age analysis of IPA taxa is needed to investigate ecological and evolutionary responses to deforestation and to test hypotheses related to vicariance and dispersal (Yoder & Nowak, 2006; Otero Jimenez et al., 2023). The potential contributions to our understanding of ecology, biogeography, and adaptation cannot be overstated. For these reasons, long term monitoring and rigorous reforestation efforts are needed to secure the future of these small, isolated populations.

The applications of iDNA and eDNA continue to grow and expand, featuring various invertebrates (Gogarten et al., 2020; Massey et al., 2022) and environmental mediums (Allen et al., 2021; Leempoel et al., 2020; Lyet et al., 2021; Lynggaard, Bertelsen, et al., 2022) as sources of residual DNA. Yet, to our knowledge, many eDNA inventories of terrestrial ecosystems remain limited to the sampling of mammals (Abrams et al., 2019; Drinkwater et al., 2021; Tilker et al., 2019). Few studies assess diversity across taxonomic groups (Ji et al., 2022) and we encourage researchers to expand genetic loci and target taxa to increase identifications and improve taxonomic resolution. Future iDNA studies should also conduct systematic, concurrent inventories with conventional biodiversity sampling techniques to account for temporal variation in species richness.

AUTHOR CONTRIBUTIONS

Designed research: MF, PCW, and EH. Collected data and organized expedition: MF, DA, and PCW. Analyzed data: MF. Wrote the paper: MF and EH.

ACKNOWLEDGMENTS

We would like to warmly thank the town of Ihosy and the village of Analamary for their hospitality and for permission to enter the forest, without which this study would not have been possible. We thank Centre ValBio research staff for their thorough biodiversity inventories, with special thanks to Zakamanana Francois. We thank the

Malagasy Institute for the Conservation of Tropical Environments (MICET) for their technical and logistical support. We thank Rainforest Trust rangers for their efforts in sample collection. We thank Madagascar's Ministry of Environment for research and export permits. We would like to thank the American Museum of Natural History and Fordham University for their collaboration on this project.

FUNDING INFORMATION

We thank Fjallraven and The Explorers Club for funding field expenses. Permit number: 290/19/MEDD/SG/DGEF/DGRNE.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequencing reads are deposited in DRYAD (doi:10.5061/dryad.sxksn038h).

BENEFIT SHARING

Benefits Generated: We conducted our study in partnership with the local Malagasy community. Our Malagasy collaborators included villagers, rangers, rangers in training, and research technicians, and those with direct contributions to this study are cited as coauthors and in the Acknowledgements.

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SUPPORTING INFORMATION

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How to cite this article: Fahmy, M., Andrianoely, D., Wright, P. C., & Hekkala, E. (2023). Leech-derived iDNA complements traditional surveying methods, enhancing species detections for rapid biodiversity sampling in the tropics. *Environmental DNA*, *5*, 1557–1573. https://doi.org/10.1002/edn3.474