ENVIRONMENTAL DNA FOR BIODIVERSITY MONITORING IN BHUTAN:

RESULTS FROM A PILOT STUDY IN THE MANGDE CHHU RIVER BASIN

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Citation: DoFPS, WWF & ETH. 2024. eDNA for biodiversity monitoring in Bhutan: Results from a pilot study in the Mangde Chhu river basin. Thimphu, Bhutan

ISBN: 978-99980-782-6-0

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LIST OF ACRONYMS

asl: above sea level
CR: Critically Endangered
DNA: DeoxyriboNucleic Acid
DoFPS: Department of Forests and Park Services
eDNA: Environmental DeoxyriboNucleic Acid
EMBL: European Molecular Biology Laboratory
EN: Endangered
ENA: European Nucleotide Archive
ETH-Zurich: Federal Institute of Technology Zurich
GBIF: Global Biodiversity Information Facility
GNH: Gross National Happiness
HTML: HyperText Markup Language
IUCN: International Union for Conservation of Nature
L: Liter
MOTU: Molecular Operational Taxonomic Unit
NCBI: National Center for Biotechnology Information
NCD: Nature Conservation Division
NGOs: Non-Government Organizations
NT: Nearly Threatened
OTU: Operational Taxonomic Unit
PCR: Polymerase Chain Reaction
SD: Standard Deviation
VU: Vulnerable
WWF: World Wildlife Fund

FOREWORD

In the realm of biodiversity conservation, the power of innovation knows no bounds. It is with great excitement and anticipation that I introduce "Piloting Environmental DNA (eDNA) to Revolutionize Biodiversity Monitoring and Conservation Decision Making in Bhutan." This pioneering endeavor marks a significant leap forward in our collective efforts to safeguard the rich tapestry of life that thrives within Bhutan's borders.

Bhutan, with its breathtaking landscapes and unparalleled biodiversity, stands as a beacon of conservation excellence on the global stage. Yet, as we navigate the complexities of a rapidly changing world, the need for innovative approaches to biodiversity monitoring and conservation decision-making becomes ever more pressing.

Environmental DNA (eDNA) technology offers a transformative solution, harnessing the power of genetic material shed by organisms into their environment to provide invaluable insights into their presence and abundance. By tapping into this cutting-edge technology, Bhutan is poised to revolutionize its approach to biodiversity monitoring, offering a more efficient, cost-effective, and non-invasive means of assessing the health of its ecosystems.

This pioneering initiative embodies Bhutan's unwavering commitment to conservation leadership and sustainable development. By embracing innovation and fostering collaboration between government agencies, research institutions, and local communities, Bhutan is charting a course towards a future where biodiversity thrives and human well-being flourishes in harmony with nature.

As we embark on this journey together, let us draw inspiration from Bhutan's rich cultural heritage and reverence for the natural world. Let us seize this opportunity to pioneer new pathways towards a more sustainable and resilient future for all life on Earth.

I extend my heartfelt gratitude to all those who have contributed to this groundbreaking initiative and offer my wholehearted support for its continued success.

Lobzang Dorji
Director

ACKNOWLEDGEMENT

We would like to extend our heartfelt appreciation to all those who contributed to the completion of this technical report.

First and foremost, we express our gratitude to the Department of Forests and Park Services, Ministry of Energy and Natural Resources, Royal Government of Bhutan and WWF (US & Bhutan) for their invaluable guidance and support throughout the project. Their expertise and insights were instrumental in shaping the direction of the project.

We are also deeply thankful to the Project CAT supported by Discovery communications, Inc. for the funding support, which enabled us to conduct this study and generate meaningful findings.

Special thanks go to the field team members from Nature Conservation Division, Royal Manas National Park, Jigme Singye Wangchuck National Park, Zhemgang Forest Division and WWF-Bhutan whose dedication and hard work made sample collection and data gathering possible. Their commitment to quality ensured the integrity of our results.

Furthermore, we extend our appreciation to the laboratory technicians and analysts from SPYGEN and ETH Zurich whose thorough efforts in processing and analyzing eDNA samples contributed to the accuracy of our findings.

We are also grateful to the individuals and organizations who provided access to study sites and facilitated logistical arrangements, without which this research would not have been possible. We recognize and appreciate the collective effort of everyone involved in this endeavor. Your contributions have been indispensable in the successful completion of this eDNA technical report.

EXECUTIVE SUMMARY

Bhutan, with its rich biodiversity and commitment to conservation, stands ready to spearhead innovative environmental DNA (eDNA) technology for biodiversity monitoring. This pilot study establishes a groundbreaking initiative, exploring the potential of eDNA to enhance wildlife surveys and complement traditional methods, particularly concerning tiger populations and other high conservation value species.

This research pursued three objectives: (1) assessing eDNA's effectiveness for comprehensive biodiversity inventories of terrestrial vertebrates; (2) exploring its use in monitoring tiger and prey populations; (3) exploring its use for detecting and monitoring other high-value species, such as the Golden Mahseer. The pilot study focused on Royal Manas National Park and Zhemgang Forest Division. Water samples were collected from the main channel of the Mangde Chhu River, its tributaries, and stagnant water bodies throughout the area. DNA metabarcoding analysis used multiple primers to detect and identify vertebrate species across all classes (mammals, birds, reptiles, amphibians, and fish). However, due to an incomplete reference database, identifying many detected taxa at the species level remained challenging.

Despite reference database limitations, eDNA successfully detected a total of 201 unique vertebrate taxa with 134 identified to the species level, highlighting the technique's potential to reveal vast biodiversity, even in less well-studied regions. Main river samples yielded the highest species diversity, followed by tributaries. Stagnant water detection rates were significantly lower but vital for a comprehensive inventory. eDNA recovered a large proportion of carnivores and ungulates, with results partially aligning with concurrent camera trapping data, indicating promise for large-scale, cost-effective mammal monitoring.

Importantly, eDNA detected numerous IUCN-listed species, including the critically endangered white-bellied heron and the endangered golden mahseer, confirming its potential for monitoring rare and elusive wildlife species. Initial analysis also suggests a positive correlation between eDNA read counts (e.g., the quantity of eDNA in the sample) of certain species and their abundance in the area sampled, hinting at potential use of the technology for estimating relative animal abundance in future studies.

This pioneering study highlights the significant value of eDNA as a tool for biodiversity assessment. It demonstrates eDNA's ability to detect a wider range of species, including rare or elusive wildlife, making it particularly valuable for monitoring threatened species. The potential cost-effectiveness of eDNA and its ability to complement traditional survey methods offer further benefits. To fully realize eDNA's potential, strategic actions are recommended:

- Expanding Bhutan's DNA reference databases to improve species identification.
- Establishing a national eDNA framework for biodiversity monitoring.
- Investing in capacity building and technology.
- Refining eDNA protocols for specific species groups.
- Fostering community involvement in sampling and data collection.

By strategically integrating and refining eDNA technology for its own purpose, Bhutan can become a global leader in biodiversity conservation. A national eDNA framework would enable comprehensive biodiversity mapping and robust monitoring, transforming how Bhutan tracks trends and addresses critical ecological challenges. This aligns with the nation's commitments under the Kunming-Montreal Global Biodiversity Framework and Sustainable Development Goals (SDGs).

1 INTRODUCTION

1.1 BACKGROUND

Bhutan's strategic location at the ecotone of the northern Palearctic and southern Indo-Malayan biogeographic realm amidst the Eastern Himalayan global biodiversity hotspot makes it one of the most biologically diverse countries in the world (Myers et al. 2000). The great elevational gradient that the country possesses (from as low as 96 masl to 7600 masl) has enabled different climatic conditions and 6 vegetation zones (Wangda and Ohsawa 2006); tropical, subtropical, warm-temperate, cool-temperate, subarctic (cold temperate, subalpine), and arctic (alpine) zone. Today, 69.71% of the country is covered with relatively well-preserved forested areas (FMID 2023). Additionally, the country has significant inland water resources consisting of an extensive network of rivers, rivulets and streams arising from a high level of precipitation, glaciers and glacial lakes.

These well-preserved forests and freshwater ecosystems create unique habitats which harbor a rich biodiversity of over 11,200 species (NBC 2019). These include some 5,300 plant species under 220 families and 1,415 genera, almost 200 species of mammals, 800 to 900 species of butterfly and more than 120 freshwater fish species. The herpetological data recorded more than 160 species of amphibians and reptiles (NBC 2019). The country is also enormously rich in bird diversity with over 760 bird species recorded, 78% of which are resident and breeding, 7% migratory and 8% winter visitors (NEC 2014). Amongst all species, around 134 species are globally threatened and are of conservation importance. 21 species are Critically Endangered (CR), 43 are Endangered (EN), and 70 are Vulnerable (VU) (NBC 2019). A total of 513 species are protected by CITES against over-exploitation through international trade. This includes 40 species of fauna and three species of flora in Appendix I, and 56 species of fauna and 414 species of flora in Appendix II.

The great diversity of wildlife in Bhutan is attributed to commitment to conservation emerging from the rich culture, strong conservation policies and the leadership of our kings. The constitution of the kingdom of Bhutan mandates 60% of the country under forest cover for all the time to come and more than half of the country is within protected area networks encompassing national parks, wildlife sanctuaries, strict nature reserves and biological corridors. Moreover, environmental conservation is one of the pillars of "Gross National Happiness", the developmental philosophy envisioned by His Majesty the Fourth King, Jigme Singye Wangchuck, enabling a balanced approach towards socio-economic development and environmental conservation.

While the country is biologically diverse, most of the surveys and research to date have been limited to taxonomic groups such as mammals, birds, and plants. The most biodiverse groups by far are the invertebrate groups, including taxa such as molluscs, dragonflies and damselflies, beetles, bees and wasps, true flies, moths and butterflies. However, these groups remain largely unstudied (NBC 2019); therefore, the ecological significance and benefits accrued from this biodiversity are not completely understood. Moreover, most of the species documented are findings or incidental outcomes from major national surveys such as the National Forest Inventory, National Tiger Survey, National Snow Leopard Survey, National Elephant Survey, and others.

In order to strengthen baseline biodiversity information, assess biodiversity changes in the face of climate change and anthropogenic disturbances, and prioritize conservation actions, Bhutan has rolled out a biodiversity monitoring programme by designing taxa specific biodiversity monitoring protocols (DoFPS 2020). Biodiversity Monitoring Grids measuring 4km x 4km each were laid out across the protected areas and forest divisions and monitoring is planned for six broad taxa (mammals, birds, insects, aquatic biodiversity, herpetofauna, and plants). However, all these protocols use conventional monitoring methods to monitor them, and availability of the financial resources, technical expertise, and human resources has been a challenge.

In recent years, the use of environmental DNA (eDNA) sampling to generate information on species, populations, and communities has been gaining popularity. eDNA is the "genetic material obtained directly from environmental samples without any obvious signs of biological source material" (e.g., Thomsen and Willerslev 2015). eDNA sampling is based on the premise that DNA from higher organisms persist in the environment they live in and through eDNA sampling, environmental samples such as soil, water, snow or air, are collected for further laboratory extraction and analysis to ascertain the presence of the species. This technique has the potential to overcome some of the challenges associated with conventional biodiversity monitoring processes. For example, eDNA has the potential to complement most costly biodiversity measures, such as camera trapping, and even replace them in many situations (Lyet et al. 2021, Van Leeuwen and Michaux 2023). With the right investment, a national eDNA monitoring program for Bhutan could be possible in just a few years, providing a universal framework to assess changing wildlife populations and inform real-time management decisions.

Bhutan is piloting eDNA sampling techniques in the south-central part of the country to assess biodiversity and to re-evaluate the detection of threatened species such as tiger and its prey species, to explore ways towards its long-term population monitoring. The pilot study will also compare its results to those of the conventional biodiversity monitoring efforts and the simultaneous traditional tiger and prey surveys (using camera traps, sign surveys, and human reporting). The eDNA sampling will provide a unique opportunity to evaluate the robustness and cost-effectiveness of the tool later at large scale.

1.2 OBJECTIVES

The key objectives of the pilot study were as follows:
Objective 1: Evaluate eDNA's Potential for Comprehensive Biodiversity Inventories

- Assess the efficacy of eDNA in detecting a broad range of vertebrate species (mammals, birds, fish, amphibians, and reptiles) in Bhutan's diverse ecosystems.
- Quantify the increase in species detection using eDNA compared to standard methods.
- Explore the potential of eDNA to detect rare, cryptic, or elusive species that may be missed by traditional survey methods.

Objective 2: Pilot eDNA as a Complementary Tool for Tiger and Prey Monitoring

- Compare eDNA and camera trap data to determine the suitability of eDNA for detecting tiger presence and assessing relative prey species abundance.
- Investigate the correlation between tiger eDNA detection and prey species richness, providing insights into predator-prey co-occurrences.
- Evaluate the potential of eDNA to monitor range shifts or changes in prey base availability for tigers over time.

Objective 3: Identify Potential for Monitoring Other High-Value Species

- Assess the effectiveness of eDNA in detecting other species of conservation concern in Bhutan, such as the white-bellied heron, golden mahseer, red panda, or pangolins.
- Explore the potential to tailor eDNA protocols for the detection and monitoring of specific target species.
- Evaluate the feasibility of using eDNA for early detection of invasive species threats.

2 MATERIALS AND METHODS

2.1 STUDY AREA

A stretch of the Mangde Chhu spanning approximately 45 kilometers between Berti (upper stretch) and Pangkhar (lower stretch) was the main eDNA sampling site. The tributaries and ponds from both river-right and river-left on the catchment of the main river stretch also constituted the sampling sites. Six sampling sites were on the main channel of Mangde Chhu, eight on the tributaries and another six on stagnant water bodies. These sampling sites fall under Royal Manas National Park, Jigme Singye Wangchuck National Park and Zhemgang Forest Division in central Bhutan. The location was chosen mainly because of their high biological diversity and thorough databases of existing species. The availability of current species inventories is essential for comparing the results from eDNA sampling to conventional sampling methods. The lowest elevation sampled was in the Mangde Chhu basin (157 meters asl) and the highest elevation sampled was at Tali Pond in Zhemgang (1654 meters asl). The targeted species fauna group for this pilot included mammals, vertebrates, fishes and amphibians from the respective targeted sites.

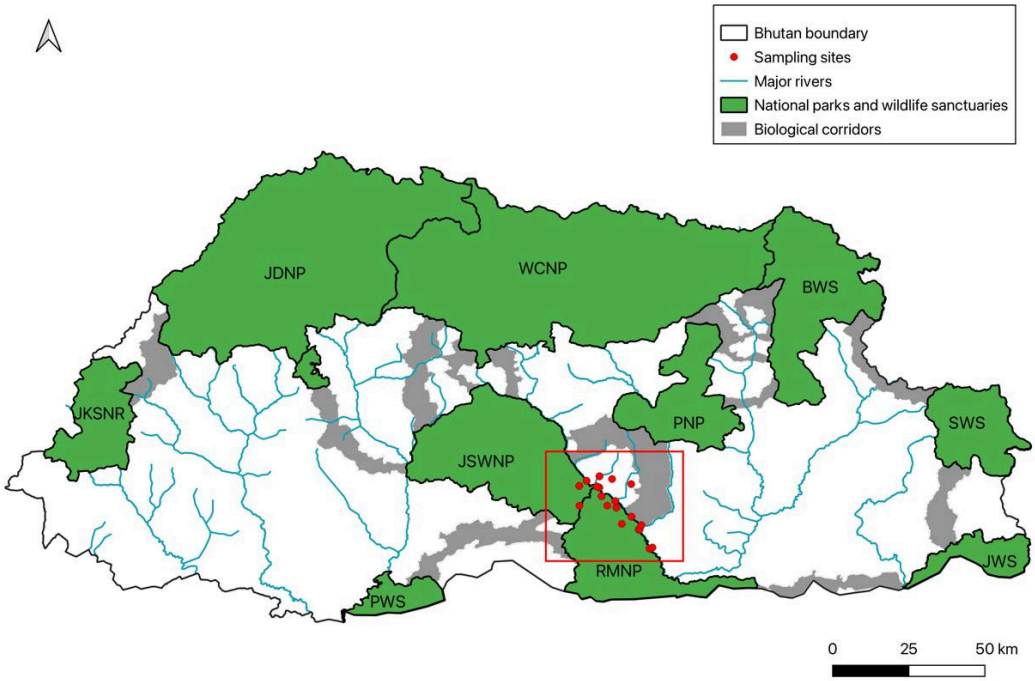

Figure 1: Location of the sampling site together with the national parks and biological corridors in Bhutan (JKSNR: Jigme Khesar Strict Nature Reserve, JDNP: Jigme Dorji National Park, WCNP: Wangchuck Centennial National Park, BWS: Bumdeling Wildlife Sanctuary, JSWNP: Jigme Singye Wangchuck National Park, PNP: Phrumsengla National Park, SWS: Sakten Wildlife Sanctuary, PWS: Phibsoo Wildlife Sanctuary, RMNP: Royal Manas National Park, JWS: Jumotsangkha Wildlife Sanctuary)

2.2 SAMPLING DESIGN AND METHODS

A total of 48 eDNA samples were collected from 20 sites from April 25, 2022 to May 3, 2022, using an Athena pump. Two different sampling protocols were implemented for the river sites (filtration of 30L and 60L water). A single protocol was implemented for the stagnant water sites (filtration of 2L water). A summary of the sampling procedure is included below. The filtration of 30L in 30 minutes was adopted from Cantera et al., 2019 and two replicates were done for each site on the running water, i.e., main channel and tributaries. Filtration of a minimum of 60L in a maximum of 3 hours (Lyet et al. 2021) was done for one replica. On the stagnant waters and ponds, one replica of 2L filtration was conducted. The sampling of a standard volume of water, which is pumped using a peristaltic pump, and passes through an eDNA filter that captures the DNA for analysis in the laboratory.

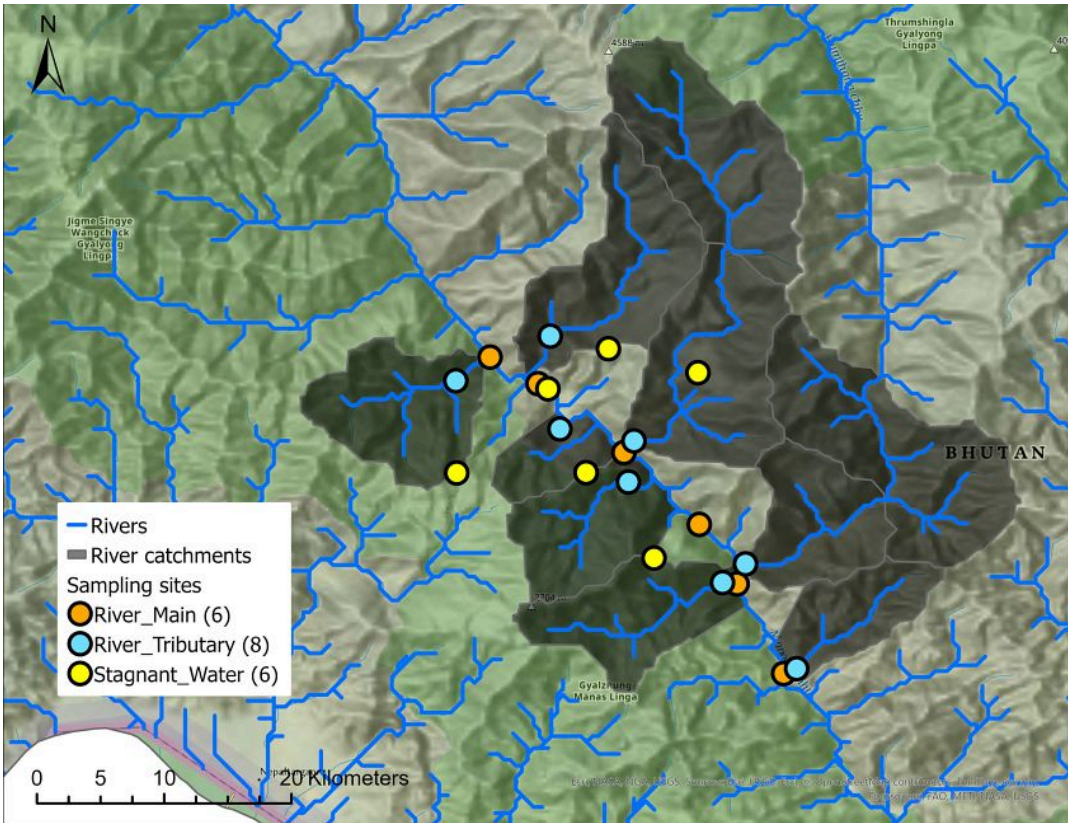

Figure 2: Sampling locations representing the three distinct sampling objects, main trunks of rivers, their tributaries and ponds (stagnant water)

The eDNA sampling protocols for the selected taxonomic group have been developed by SPYGEN and refined over the last ten years. These protocols have been optimized and standardized for high quality results.

Table 1: Description of the sampling effort and design.

		Number of replicates per site		
Sampling site	**No. of sites**	**Running water (30L)**	**Running water (60L)**	**Stagnant water (2L)**
River main channel	6	2	1	
River tributary	8	2	1	
Pond / Lake	6			1

2.3 LABORATORY ANALYSIS

The laboratory analysis was performed at SPYGEN (Le Bourget du Lac, France). DNA extractions were performed in a laboratory dedicated to process rare and degraded DNA following the protocol described in Pont et al. (2018). DNA amplifications were then conducted following Valentini et al. (2016) with universal primers for Fish (teleo, Valentini et al. 2016), Vertebrates (12S-V5, Riaz et al. 2011), Mammals (mamm01, Taberlet et al. 2018) and Amphibians (batra, Valentini et al. 2016). The PCR products were sequenced with a Next-Generation Sequencer. Negative controls were analyzed at each step of the protocol in order to control the purity of the reagents and detect potential cross contaminations during the experiment.

The sequence reads were analyzed through a bioinformatic pipeline allowing elimination of errors due to PCR and/or sequencer (via several controls) and comparison of each sequence read with the EMBL® databases for fish, vertebrates, mammals and amphibians. A list of species was obtained for each sample according to the NCBI nucleotide reference database, featuring the number of DNA sequences and the number of positive replicates associated with each species.


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2.4 DATA ANALYSIS

2.4.1 Reference databases coverage for each primer and taxonomic group

First, we aimed to understand the percentage of species present in Bhutan that were represented in the NCBI reference database. We also wanted to quantify the proportion of these species that could be identified to the species level. To evaluate the coverage of reference databases for each primer on each taxonomic group, we used the list of species present in Bhutan retrieved from the GBIF website (www.gbif.org). For each species, we checked the NCBI nucleotide reference database for any sequence of this species corresponding to the region of the mitochondrial genome targeted by the primer. We then reported the number of unique sequences present in the NCBI database and also recorded whether the sequence was unique to this species or shared with any other species. Following this method, we classified the species in four different categories:

1. **None:** No sequence of the species found in the NCBI database
2. **Shared:** One or several sequences of the species found in the NCBI database but all sequences are shared with one or several other species
3. **Mixed:** One or several sequences of the species found in the NCBI database but at least one sequence is shared with one or several other species and at least one sequence is only found for this species
4. **Unique:** Species present in the NCBI database and all sequences are unique and only found for this species

Species in the category "Unique" have a higher chance to be identified to the species level. Species in the "Mixed" category might be identified either to the species or to a higher taxonomic level depending on the sequence found in the sample. Species in the "Shared" and "None" categories can only be identified to the genus or higher taxonomic level.

This information is critical to understanding the results from an eDNA analysis. It gives an idea of the proportion of species that might be detected at the species level, and understand for instance, why some species might be absent from the results table despite being common in the study area. This can also inform strategies to improve the reference databases, as it provides a list of species that should be sequenced in priority. If some target species are missing or share one or all haplotypes with other species, then it is recommended to sequence several individuals of this species sampled from the region of interest.

2.4.2 Taxonomic assignment of sampled sequences

The first part of the bioinformatic analysis was performed using the programs in the OBITools package (http://metabarcoding.org/obitools, Boyer et al. 2016) by SPYGEN following Pont et al. (2018). The program ecotag was used for the taxonomic assignment of Molecular Operational Taxonomic Units (MOTUs or OTUs) with the sequences extracted from release 142 of European Nucleotide Archive (ENA) database (standard sequences) and a curated database, built from the previous one, by retrieving only the vertebrate species present in Bhutan (1146 species in total) following the GBIF database (www.gbif.org). All OTUs present in the negative controls were deleted from the database as suggested by Barba et al. (2014). We then applied an additional filtration step using the LULU tool (Frøslev et al. 2017). This step generated a contingency detection table (with number of reads) keeping OTUs corresponding to most likely true species variants (getting rid of the noise) thus providing a better idea of the total number of species detected including the ones not assigned to the species level. OTUs showing less than 90% similarity to the reference databases were removed. OTUs showing a match with sequences of a unique taxa were assigned to the species level. OTUs showing a match with more than one taxon in the reference database, were assigned to the genus or subfamily taxonomic level. Finally, considering the erroneous assignments of a few sequences to the wrong sample due to tag-jumps, all sequences with a frequency of occurrence below 0.001 per taxon and per library were discarded. After the bioinformatic filters, no reads were found in the extraction and PCR controls. Appendix 1 also displays a table with all the taxa recovered at the species level. The total number of positive detections are given for each primer in the last four columns of the table. Some taxa that were detected at the genus level were later assigned to the species level when this was the only known species of the genus present in Bhutan (see note OKSP that stands for Only Known Species Present in Bhutan).

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For each class of vertebrates and relevant primer, we presented the histogram illustrating the taxonomic resolution of all OTUs sampled split by order. We reported the results using mamm01 and vert05 primers for the mammal and bird class, results using mamm01, vert05 and amphibian primers for the amphibian class, and finally results using mamm01, vert05 and teleo primers for the fish class. The histograms show for each order the number of OTUs assigned to taxa at the order, family, genus and species level.

2.4.3 Species level analysis

A better understanding of the reference database coverage allows us to understand why some species are positively detected and why others are missed. It helps answer questions like “Are there any species present in the reference database that are missed completely?” or “What is the proportion of the species present in the reference database that are detected in each class or order for instance?”

Detection probability and power analysis

In order to estimate the performance of eDNA metabarcoding to detect certain species of interest, we estimated for certain species the detection probability per water sample. In this analysis, we assumed the likelihood of detecting a given species to be equivalent for a given marker across all sites or samples within a type of sampling location (main river, tributary, pond), but assumed that different markers might have different detection probability. We used a frequentist statistical approach to estimate the detection probabilities of each species with each primer and type of sampling location. All the analyses were performed using program *R v4.3.0* and package *unmarked*.

Using the estimated detection probability, we ran a power analysis to calculate the sampling effort required to guarantee the detection of a species given its imperfect detection. To do so, we built detection power curves that illustrate the probability of detecting the species at least one time for 1 to N samples collected and analyzed. These types of curves are very informative in conservation as they provide clues about the total effort that must be applied to guarantee a certain level of detection that is set arbitrarily. It is common practice to choose a threshold of 80% (known as the five-eighty convention, Walsh et al. 1999) which means that we want to know how many samples are required to have an 80% chance of detecting the species at least one time if present. Power curves or power thresholds are often used to adjust sampling effort in species focused monitoring programs.

Comparison between eDNA and camera trapping results

To explore the potential of eDNA as a complementary tool to camera traps, we compared our eDNA results with data from the 2021-22 national tiger camera trap survey (DoFPS 2023). For a fair comparison, we focused on camera traps located within the river catchments sampled by our eDNA collection in Royal Manas National Park and Zhemgang Forest Division. We selected a total of 40 camera traps for this analysis: 26 on the east bank of the Mangde Chhu River (Zhemgang side) and 14 on the west bank (Royal Manas side). To ensure a direct comparison of efficiency between eDNA and camera trapping methods, we excluded species absent from our reference database. This controlled for species that could not be identified during bioinformatic analysis, regardless of their presence in the environment or the eDNA samples.

We first compared the species detected by each method (eDNA and camera trapping) across the different eDNA sampling locations. Then, we assessed the correlation between the average number of eDNA reads per sample and the average number of camera trap detections for each species, focusing on both carnivores and ungulates. Since the camera trapping effort and sampling design differed slightly between the two surveys, we used this comparison primarily to identify broad patterns and potential relationships between eDNA and camera trap detection.

2.4.4 Taxonomic Biodiversity indicators

Description of the taxonomic biodiversity metrics

We propose a metric based on mammals given the vast amount of knowledge surrounding this taxon and the ease to communicate the importance of mammals for ecosystems and societies to the stakeholders and the public. While eDNA metabarcoding can recover a large number of taxa and would allow the metric to be expanded to many other groups, to be informative the metric should be accompanied with a large amount of side knowledge on the species ecology and potential distribution. Recognizing the potential limitation of eDNA metabarcoding to identify many sequences to species level taxa in Bhutan, we propose to apply this indicator to the genus level.

We first obtain the list of potential species at a site (specific to an eDNA metabarcoding marker), by aggregating all taxa detected across all samples and sites, which we can then compare with the realized metrics. Each mammal genus can be associated with a role in the ecosystem, which is, in turn, associated with some services provided. In addition, those categories are easily understood by the interested parties and the public, which allows efficient communication. To compute the metrics, we consider the following classes: Bats (pest control, seed dispersal, pollinators), Small herbivores (herbivory, seed dispersal), Large herbivores (herbivory, nutrient cycling), Small carnivores (insectivores, predation), Large carnivores (predation), Rodents (granivory, seed dispersal), and Primates (tree dwellings, seed dispersal) see Figure 3.

Then we apply the eDNA measurement and compare the percentage of detection of genera compared to the potential list for each of these categories. Finally, we produced the final metric which we called “integrative taxonomic diversity index”, by averaging values across all categories. A value of 1 indicates that all genera have been detected in each one of the categories.

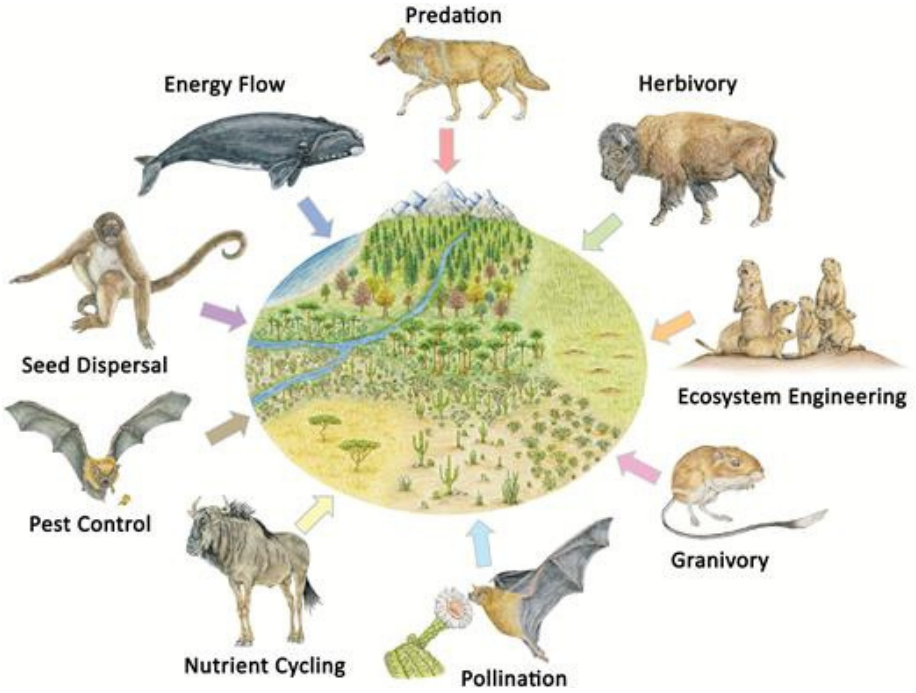

Figure 3: Mammals play important roles in all ecosystems performing a broad range of critical functions. The decline in abundance, local extirpation, or global extinction of mammals will have negative effects on ecosystem processes and will limit the number and amount of services these systems provide to human populations. (Lacher, T. E., & Davidson, A. D. (2019). The functional roles of mammals in ecosystems. Journal of Mammalogy, 100. © 2024 by Oxford University Press. Reprinted with permission).

Site comparison using indicators of cumulated biodiversity
We focus our comparison on the eDNA samples collected in the tributaries of the Mangde Chhu river only, which drain the Royal Manas National Park on the west basin, and Zhemgang Forest Division on the east basin.

To assess how the number of eDNA samples per site influenced our ability to detect differences in diversity between basins, we used a bootstrap resampling approach. To create 10 distinct datasets with increasing sample sizes, we randomly selected 1, 2, 3, ... 10 water samples from each basin, respectively. For each selection, we aggregated the lists of taxa identified on which we applied the metrics previously described, generating 10 sets of functional group-specific metrics and the integrative functional diversity index. To account for potential sampling bias and obtain more reliable estimates, we repeated the random selection and diversity calculation process 100 times for each sampling effort and site (2 basins times 10 sampling efforts). This bootstrapping procedure allowed us to generate mean and standard deviation values for both the individual functional group metrics and the integrative diversity index.

2.4.5 Exploring biological diversity beyond genus and species levels

Despite the lack of taxonomic resolution at the species level, it is still possible to get a very good sense of the total diversity captured by the eDNA sampling during the pilot study in Royal Manas National Park. To illustrate the large diversity of organisms detected across all classes of vertebrates, we have proposed a series of analyses that will help comprehend the incredible richness of the region selected for the pilot study.

Number of unique taxa detected at each taxonomic level across all primers
To illustrate the phylogenetic richness of the sequences detected in all the samples, we first calculated the total number of unique taxa detected at each taxonomic level (from class to species) for each primer (see Table 6 in section 3.4.1). However, these indicators are not representative of the entire species richness, which will be better illustrated in the next section.

Diversity of Operational Taxonomic Units (OTUs) detected for each primer
For each class of vertebrate (amphibians, birds, fish, and mammals), we used the total number of OTUs as a proxy for the species richness in our study area. Due to an incomplete reference database and, therefore, limited species-level resolution of the primers, we used OTUs instead of species richness for our analysis. We define OTU richness as the total number of OTUs in all ranks, including and under the taxonomic group of interest (amphibians, birds, fish, and mammals). In regions like Bhutan where reference databases are primarily incomplete, OTU richness provides a much more compelling picture of the biological diversity present as this indicator includes both species that were identified to the species level and the ones detected, but were assigned to a higher taxonomic level such as genus, family, order or class.

Accumulation curves of Operational Taxonomic Units (OTUs)

A species accumulation curve typically shows the cumulative number of species recorded in a particular environment as a function of the cumulative effort expended searching for them. It is used to estimate the number of additional taxa that can be found per added unit of sampling effort or a way to evaluate if the total sampling effort spent was sufficient to capture the entire diversity of the region (plateau of the curve). In order to evaluate the proportion of the total diversity sampled in our pilot study, we built OTU richness accumulation curves for each class of vertebrate and each primer across filtration replicates collected at all sampling locations using the R package vegan and its *specaccum* function (Oksanen et al. 2020). We generated 1000 accumulation curves using the 'random' method to generate the curves that describe the relationship between OTU richness and the number of eDNA samples.

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3 RESULTS AND DISCUSSION

3.1 COVERAGE OF REFERENCE DATABASE FOR EACH TAXONOMIC GROUP

3.1.1 Global coverage of the reference database for each class of vertebrate by primer

A comparison between the list of vertebrate species found in Bhutan (www.gbif.org) and the sequences available in Genbank for the 12S region of the mitochondrial genome has revealed notable deficiencies in the reference database, imposing limitations on our capacity to assign sequences to species level taxa (see Fig. 4). Among the classes represented, the mammal class stands out as the most comprehensive, showing 74% and 71% of the species listed in Genbank for the vertebrate and mammal primers. Amphibians rank as the second most well-represented group, boasting 46% coverage in Genbank for each primer able to detect organisms in this class. Fishes are slightly less represented, with 44%, 46%, and 46% of their species accounted for in Genbank for the vertebrate, mammal, and fish primers, respectively. In contrast, bird species exhibit a relatively poor presence in Genbank, with only 41% of their species covered by the Vertebrate primer and 36% by the Mammal primer. Lastly, the Reptile class lags significantly behind, with merely 37% and 12% of its species cataloged in the reference database for the vertebrate and mammal primer, respectively.

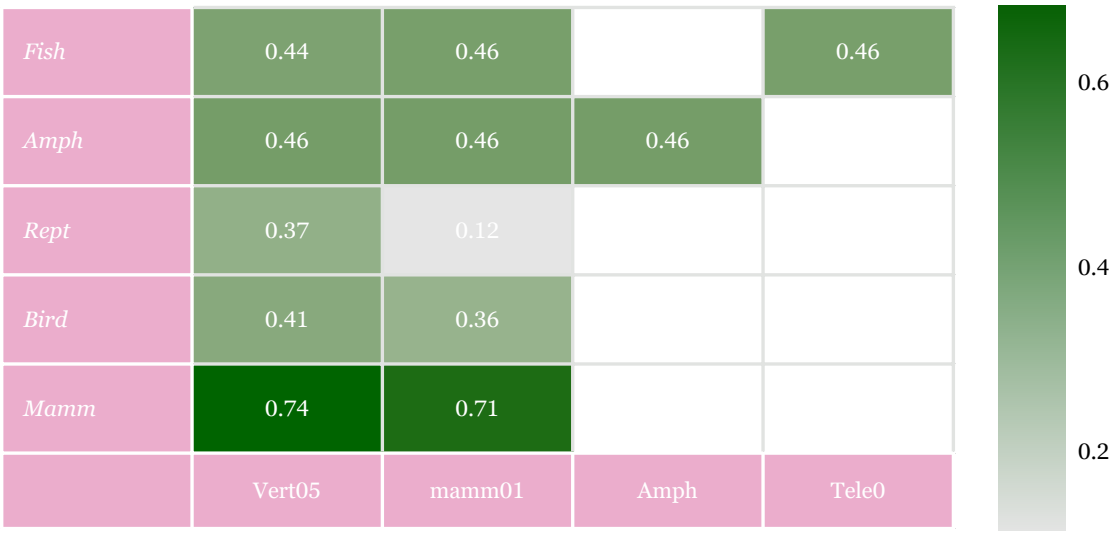

Figure 4: Reference database coverage by taxonomic group (Y-Axis) and primer (X-Axis). Numbers indicate the proportion of species represented in the database for each class. Color gradient represents coverage (light green = low, dark green = high).

3.1.2 Coverage of mammal species

The coverage for each mammal order on mamm01 and vert05 primers is presented in Table 2. These results illustrate the limited coverage of most orders on both primers. Carnivora is the most represented group with 30 species out of 35 present in Genbank. Rodentia stands out as the least represented order with 14 missing species for the mammal primer and 10 for the vertebrate primer.

		Sequences in Reference DB				Taxa Bhutan
Primer	**Order**	**None**	**Shared**	**Mixed**	**Unique**	**Total**
Mammal primer						
mamm01	Carnivora	5	0	2	28	35
mamm01	Cetartiodactyla	3	1	3	7	14
mamm01	Chiroptera	6	0	0	13	19
mamm01	Eulipotyphla	4	0	0	8	12
mamm01	Lagomorpha	5	0	0	2	7
mamm01	Perissodactyla	0	0	0	1	1
mamm01	Pholidota	0	0	0	1	1
mamm01	Primates	1	1	1	4	7
mamm01	Proboscidea	0	0	0	1	1
mamm01	Rodentia	14	0	0	18	32
Vertebrate primer						
vert05	Carnivora	5	0	7	23	35
vert05	Cetartiodactyla	3	1	3	7	14
vert05	Chiroptera	5	2	0	12	19
vert05	Eulipotyphla	4	0	0	8	12
vert05	Lagomorpha	4	0	0	3	7
vert05	Perissodactyla	0	0	0	1	1
vert05	Pholidota	0	0	0	1	1
vert05	Primates	1	1	1	4	7
vert05	Proboscidea	1	0	0	0	1
vert05	Rodentia	10	1	2	19	32

Table 2: Coverage of each mammal order for mamm01 and vert05 primers. The column Taxa Bhutan represents the total number of taxa present in the country. The four reference database categories are defined as follows: None = No sequence of the species found in NCBI; Shared = 1 or more sequences of the taxa in NCBI but all sequences are shared with 1 or more other taxa; Mixed = 1 or more sequences of the taxa in NCBI but at least 1 sequence is shared with 1 or more other taxa and at least 1 sequence is unique to this taxon; Unique = taxa present in NCBI and all sequences are unique and only found for this taxon.

The gaps identified in the comparison between Bhutan's vertebrate species and Genbank's 12S mitochondrial genome sequences pose significant challenges for species identification via metabarcoding. These limitations can result in inaccuracies in taxonomic assignment of sequences. To address this issue, collaborative efforts will be crucial for data sharing and targeted sequencing of mitochondrial genome of underrepresented taxonomic groups like reptiles, birds, and amphibians.

3.1.3 Summary of the Taxonomic Diversity Detected with Each Primer

In our analysis, we employed a range of primers to uncover the presence of organisms across various taxonomic levels. Table 3 provided below offers an insightful overview of the comprehensive taxonomic landscape revealed by each primer. It illustrates the total number of classes, orders, families, genera, and species detected, allowing us to paint a vivid picture of the taxonomic richness revealed in our eDNA samples. This multifaceted assessment enables a deeper understanding of the ecological complexity in regions with incomplete reference databases, highlighting the multitude of taxa that may coexist. It is important to emphasize that this approach not only underscores the importance of improving reference databases but also highlights the inherent diversity that might otherwise remain hidden. For all primers, we notice that the genus is the richest taxonomic level. This suggests that it is more relevant in such a situation to use the genus level than the species one to describe the biodiversity present and compare between sites.

Primer	Class	Order	Family	Genus	Species
mamm01	Mammalia	8	23	59	28
	Aves	14	33	61	22
	Amphibian	1	5	7	5
	Actinopteri	3	11	20	10
	All classes	26	72	147	65
vert05	Mammalia	8	22	62	38
	Aves	13	28	39	27
	Reptilia	2	2	1	0
	Amphibian	1	5	6	3
	Actinopteri	5	15	18	4
	All classes	29	72	126	72
amphibian	Mammalia	1	1	2	0
	Aves	6	6	8	3
	Amphibian	1	3	4	2
	Actinopteri	3	4	7	2
	All classes	11	14	21	7
teleost	Actinopteri	4	12	19	10
	All classes	4	12	19	10

Table 3: This table shows the total number of classes, orders, families, genus, and species detected by each primer.

3.2 SPECIES POSITIVELY DETECTED WITH EDNA

3.2.1 Overview

Despite the limitations posed by the incomplete reference database, we successfully identified numerous sequences at the species level by combining results from all samples and primers, as detailed in Appendix 1. This represents a total of 134 species, comprising 16 fishes (26% of species known in the study area), 7 amphibians (35%), 51 birds (7%), and 60 mammals (39%). None of the reptiles were detected at the species level. Notably, Appendix 1 includes not only sequences initially matched at the species level during the bioinformatic analysis but also those initially assigned to a genus level, which we were able to reassign to a unique species (as explained in the methods section). Such reassignment was feasible in cases where a single species from the assigned genus was known to exist in the study area. Whenever such reassignment occurred, it was accompanied by a corresponding note in the table. Furthermore, species in the table that are not documented as being present in Bhutan were highlighted. These instances may indicate either genuine new records of these species for Bhutan or the possibility that the sequence corresponds to a related species present in Bhutan but absent from the reference database. To reduce these uncertainties, it will be imperative to improve the completeness of the reference databases.

When assessing the total number of species detected within each vertebrate class, our findings indicate a slightly higher number of species detected in the main river compared to the tributaries (Fig. 5 & 6). Specifically, samples from the main river revealed a greater diversity of fishes and mammals, whereas samples from the tributaries exhibited a higher detection of amphibian species. The number of bird species detected remained relatively consistent across both ecosystems. Moreover, our results unequivocally illustrate that samples collected in stagnant water habitats yielded significantly fewer species overall. In fact, these stagnant water samples yielded just a quarter of the species detected in the main river and tributaries, and this reduction was consistent across all vertebrate classes. This decreased efficiency may be attributed to the specific sampling protocol employed for stagnant water habitats, which involved filtering only 2 liters of water, in stark contrast to the 30 and 60 liters processed during sampling in running water habitats. These outcomes are also depicted in Figure 6, which presents a map illustrating the cumulative confirmed species count for each sampling location.

These findings suggest that prioritizing stagnant water sampling may not be the most cost-effective strategy for optimizing vertebrate species detection. Nevertheless, it is essential to highlight that a few fish, amphibian, and bird species were exclusively identified in the stagnant water samples. This suggests that if the aim is to compile the most comprehensive species inventory, incorporating this habitat into the sampling strategy remains essential.

Figure 5: Vertebrate diversity detected by eDNA across three types of aquatic habitats. The radar plot illustrates the relative diversity of vertebrate classes in stagnant water (gray), the main river channel (blue), and tributaries (orange). Vertebrate classes include fish (Actinopterygii), amphibians (Amphibia), reptiles (Reptilia), birds (Aves), and mammals (Mammalia). The further a point is from the center, the higher the detected diversity of that vertebrate class in a given habitat.

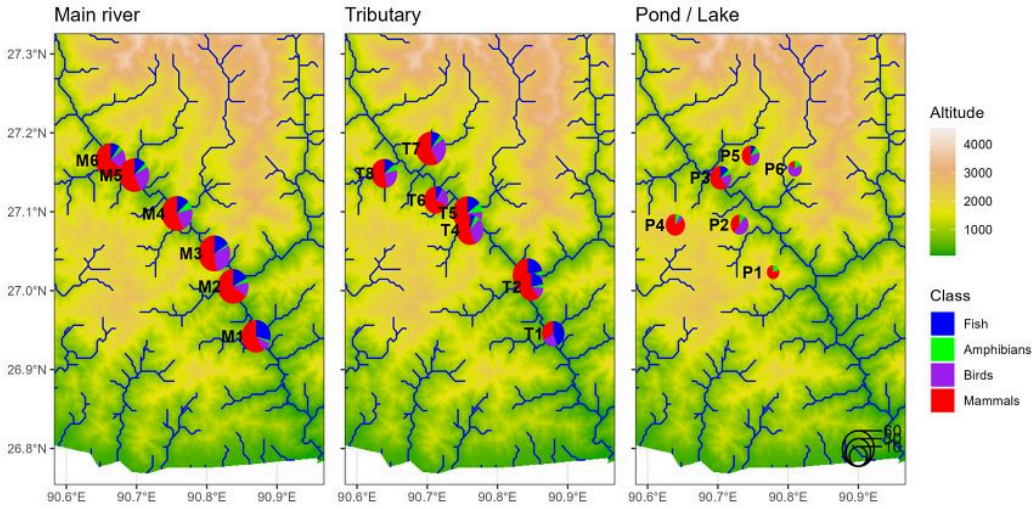

Figure 6: Map of total confirmed species per sampling location (all markers combined). The size of the circle is proportional to the total number of species detected. The sectors represent the proportion of species in each class of vertebrates. No reptile was detected at the species level. The figure is split in three panels to facilitate readability. The left panel shows the sampling locations on the main river, the middle panel shows the sampling locations on the tributaries and the right panel indicates the stagnant water sampling locations.

3.2.2 Mammal species with a focus on terrestrial carnivores and ungulates

Amongst the 134 species detected in our samples, we were able to identify 16 species of carnivores, 10 ungulates, 3 primates, 11 rodents and 15 bats. For the ungulates, *Capricornis sumatraensis* (36), *Muntiacus muntjac* (50), *Rusa unicolo*r (24), *Moschus fuscus* (27), and *Sus scrofa* (21) were the most frequently detected in our samples (see Appendix 1). These findings are largely in line with the outcomes of a concurrent camera trap survey carried out in Royal Manas National Park and the nearby Zhemgang Forest Division (Tab. 4, Fig. 8). Among carnivores, our eDNA data revealed the most frequently detected species in a descending order of prevalence were: *Paguma larvata* (54 detections), *Ursus thibetanus* (51), *Lutra lutra* (34), *Cuon alpinus* (26), *Lutrogale perspicillata* (25), *Prionailurus bengalensis* (20) and *Panthera tigris* (15). The detection of a large diversity of carnivores is very encouraging, particularly when considering the high detection probability of *Panthera tigris* on the mammal primer (0.75, haplotype shared with *P. pardus* on vertebrate primer). Indeed, while this group is usually recognized as quite difficult to detect using eDNA from water samples, our results suggest that eDNA could still be particularly useful for cost-efficiently sampling carnivores' diversity at large scales. It is also worth noting that several species, even some relatively abundant ones, were not detected with eDNA (Tab. 4). Amongst these we can mention two large carnivores: *Panthera pardus* and *Neofelis nebulosa*; two small carnivores: *Urva urva* and *Paradoxurus hermaphroditus*; and two ungulates: *Bos gaurus* and *Nemorhaedus goral*. Notably, eDNA detected four species missed by camera traps: *Melogale moschata, Martes foina, Budorcas taxicolor*, and *Axis porcinus*. The Indian hog deer detection is particularly significant, as it could represent a new species record for Zhemgang Forest Division.

Group	Species	CT	eDNA		
			Main River	Tributary	Pond
Large carnivores	Asiatic black bear *Ursus thibetanus*	1	1	1	1
Large carnivores	Dhole *Cuon alpinus*	1	1	1	
Large carnivores	Tiger *Panthera tigris*	1	1		
Large carnivores	Leopard *Panthera pardus*	1			
Large carnivores	Clouded leopard *Neofelis nebulosa*	1			
Small carnivores	Crab-eating mongoose *Urva urva*	1			
Small carnivores	Masked palm civet *Paguma larvata*	1	1	1	1
Small carnivores	Palm civet *Paradoxurus hermaphroditus*	1			
Small carnivores	Leopard cat *Prionailurus bengalensis*	1	1	1	1
Small carnivores	Chinese ferret-badger *Melogale moschata*		1	1	
Small carnivores	Yellow-throated marten *Martes flavigula*	1	1	1	1
Small carnivores	Red panda *Ailurus fulgens*	1	1		
Small carnivores	Marbled cat *Pardofelis marmorata*	1	1	1	
Small carnivores	Beech marten *Martes foina*		1	1	
Small carnivores	Binturong *Arctictis binturong*	1		1	
Small carnivores	Asian golden cat *Catopuma temminckii*	1	1		
Ungulates	Gaur *Bos gaurus*	1			
Ungulates	Himalayan goral *Nemorhaedus goral*	1			
Ungulates	Southern red muntjac *Muntiacus muntjak*	1	1	1	1
Ungulates	Sambar deer *Rusa unicolor*	1	1	1	1
Ungulates	Mainland serow *Capricornis sumatraensis*	1	1	1	
Ungulates	Black musk deer *Moschus fuscus*	1	1		
Ungulates	Wild pig *Sus scrofa*	1	1	1	1
Ungulates	Takin *Budorcas taxicolor*		1		
Ungulates	Indian hog deer *Axis porcinus*		1	1	

Table 4: Taxa detected with camera traps (CT) located within the catchment areas on each side of the Mangde Chhu covered by the eDNA sampling. A "1" in a cell indicates that the species was detected.

The maps presented in Figure 7 illustrate a significant contrast in the species diversity recovered with eDNA among the three types of habitats sampled, with a greater number of species detected in the main river than in the tributaries and stagnant water systems for both carnivores and ungulates.

While the available data may not provide a robust basis for comparison between eDNA and camera trapping methods, the comparison between our eDNA results and the results from the Zhemgang Forest Division camera trapping survey suggests a positive correlation between the average number of reads per eDNA sample and the average number of detections per camera for both carnivores and ungulates (see Fig. 8). Notably, this correlation appears to be more pronounced for ungulates. Similar relationships between eDNA detection and species relative abundance have been shown in Lyet et al. (2021). However, to validate this observation in the context of the Zhemgang Forest Division region, further analysis will be necessary, utilizing a more comprehensive camera trapping dataset. Nevertheless, these findings suggest that species with higher abundance tend to yield higher average eDNA read counts per sample, hinting at the potential utility of eDNA as a method for estimating broad categories of relative animal abundance.

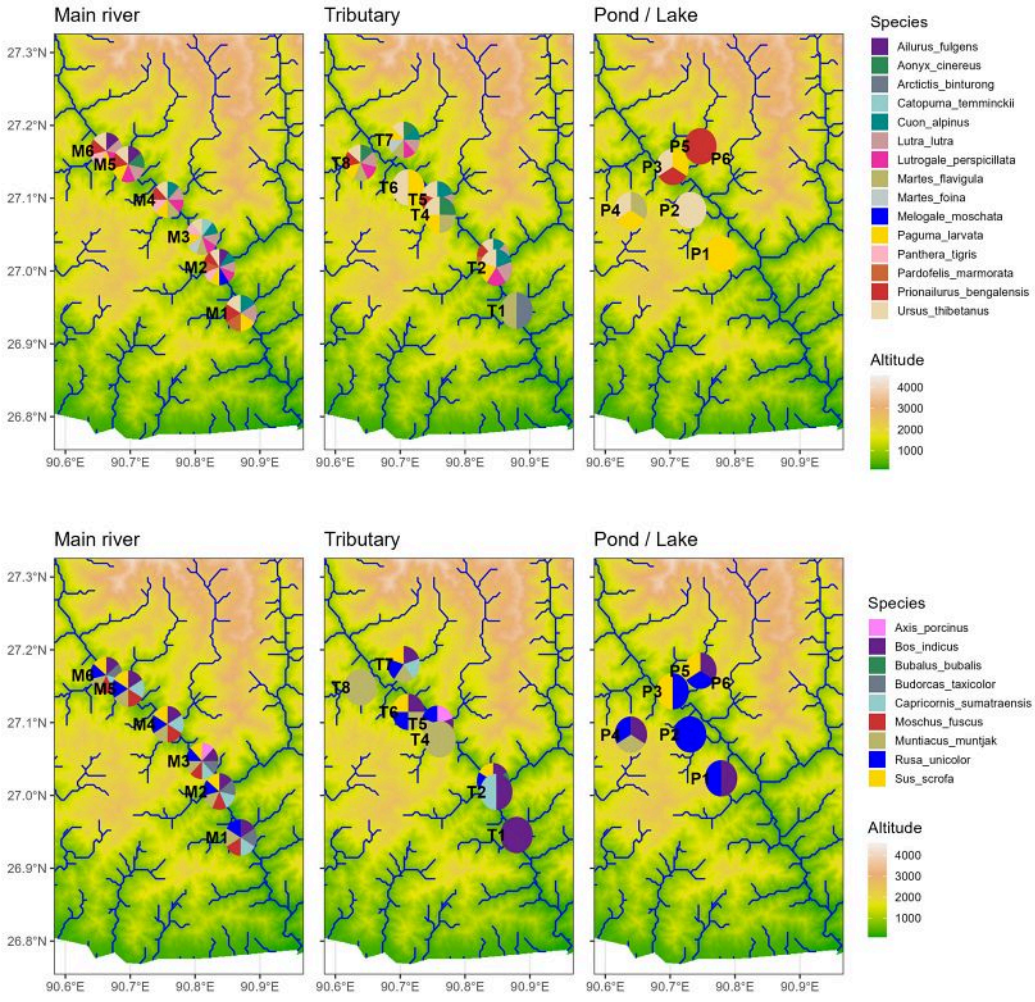

Figure 7. Map of total confirmed species of carnivore (upper panels) and ungulates (lower panels) detected at each sampling location (all markers combined). The size of the circle is proportional to the total number of reads per sample. Each sector represents a unique species. The left panel shows the sampling locations on the main river, the middle panel shows the sampling locations on the tributaries and the right panel indicates the stagnant water sampling locations.

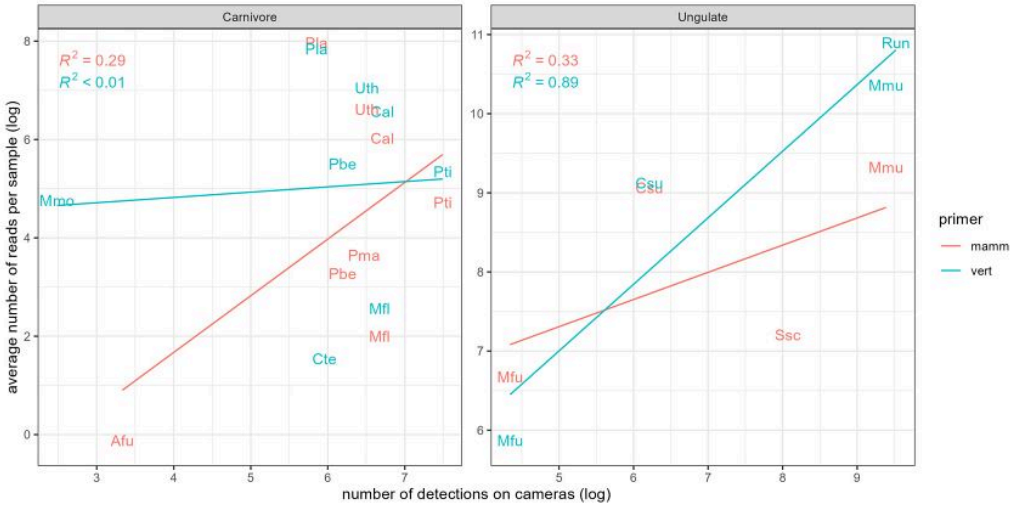

Figure 8. Comparison between the number of reads per eDNA sample and the number of detections on cameras from Zhemgang Forest Division.

3.2.3 Threatened species

Amongst the 134 species detected in our samples, 33 of them are listed in the IUCN red list of threatened species. In particular we found 1 Critically Endangered (CR) species of bird (*Ardea insignis*), 7 Endangered (EN) species including 1 fish (*Tor putitora*) and 6 mammals (*Axis porcinus, Moschus fuscus, Ailurus fulgens, Cuon alpinus, Panthera tigris, Tadarida latouchei*), 10 Vulnerable (VU) species comprising 3 fishes (*Cyprinion semiplotum, Aborichthys garoensis, Bagarius bagarius*), 1 bird (*Buceros bicornis*) and 6 mammals (*Budorcas taxicolor, Rusa unicolor, Aonyx cinereus, Lutrogale perspicillata, Ursus thibetanus, Arctictis binturong*), and finally 15 Nearly Threatened (NT) species including 2 fishes (*Anguilla bengalensis, Neolissochilus hexagonolepis), 2* amphibians *(Rhacophorus burmanus, Rhacophorus translineatus*), 2 birds (*Nisaetus nipalensis, Meiglyptes tukki*), and 9 mammals (*Capricornis sumatraensis, Catopuma temminckii, Pardofelis marmorata, Lutra lutra, Rousettus leschenaultia, Ia io, Myotis dasycneme, Macaca assamensis, Ratufa bicolor*).

Several studies have suggested that eDNA metabarcoding from stream water could be a cost-efficient method for detecting rare and threatened species (e.g., Evans et al. 2017, Deiner et al. 2017, Lyet et al. 2021, Thalinger et al. 2021). Our results strongly support this statement with the detection of exceptionally rare aquatic, semi aquatic and terrestrial species in four different classes of vertebrates: fish, amphibians, mammals, and birds. For many of these species, this is the first ever documentation of a positive detection using stream water samples and metabarcoding methods. For instance, *Ardea insignis*, known in the study area from only three individuals, was detected in three samples from three locations on the main river. Moreover, *Panthera tigris*, whose population in the area could count more than 30 individuals (DoFPS 2023), was detected in 15 samples from 4 locations on the main river. Another noteworthy result is the detection of two other Endangered carnivores, *Cuon alpinus* and *Ailurus fulgens*, as well as two Endangered ungulates, *Axis porcinus* and *Moschus fuscus*. Finally, *Tor putitora*

or Golden mahseer was detected in 23 samples from 5 locations on the main river and 2 tributaries. This species is a commercially important game fish with a very high table value. It is also known locally as Serngya and represents the symbol of good luck in the Bhutanese belief system. However, the natural stock in the country is declining due to illegal fishing and the deterioration of its natural habitat (NCD 2022).

The results of the occupancy and power analysis conducted for each species and primer highlighted above are shown in Table 5 and Figure 9. On the one hand, *Ardea insignis* was, without surprise, the most difficult species to detect with probabilities of 0.11 [0.03-0.35] on the mammal primer and 0.08 [0.01-0.41] on the vertebrate primer. Using the highest probability, we calculated that 14 samples would be required to reach an 80% detection power and 20 samples for 90% (see Fig. 9). On the other hand, for the five other species, the detection probabilities were remarkably high, with the highest probability observed for *T. putitora* on the fish primer and *M. fuscus* on the mammal primer. Although lower, the detection probabilities of the two carnivores (*P. tigris, C. alpinus*) and the other ungulate (*A. porcinus*) are also surprisingly high: 0.75 on the mammal primer for both carnivores and 0.63 on the vertebrate primer for the rare ungulate. Such high detection probabilities might seem natural for a fish but unheard of for terrestrial ungulates and carnivores. For these species, using detection probabilities of 0.94, 0.75, and 0.63, we calculated that 1, 2 and 3 samples, respectively, would be enough to reach a detection power of 90% (see Fig. 9).

Species	Primer	Detection probability			
		Estimate	SE	lower CI	upper CI
Ardea insignis	mamm	0.11	0.07	0.03	0.35
	vert	0.08	0.08	0.01	0.41
Panthera tigris	mamm	0.75	0.13	0.45	0.92
	vert	0.75	0.15	0.38	0.94
Cuon alpinus	mamm	0.75	0.09	0.54	0.88
	vert	0.50	0.13	0.27	0.73
Moschus fuscus	mamm	0.94	0.05	0.69	0.99
	vert	0.83	0.11	0.52	0.96
Axis porcinus	vert	0.67	0.31	0.11	0.97
Tor putitora	mamm	0.22	0.08	0.10	0.41
	tele	0.94	0.05	0.69	0.99

Table 5. Detection probability estimates and confidence intervals for six remarkable species. Detection probabilities were estimated for each species and primer using data from samples collected on the main river and tributaries. For each primer and species, we fitted a null occupancy model that considered a constant probability of detection and occupancy across samples.

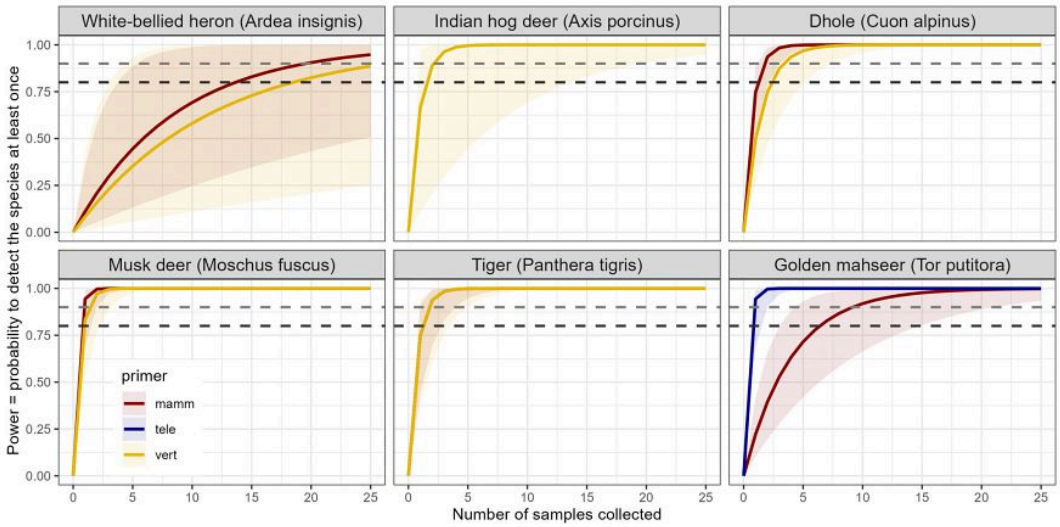

Figure 9. The detection power for six threatened species was calculated based on the detection probabilities presented in Table 5. In this context, detection power represents the likelihood of detecting a particular species at least once within a set of collected samples when that species is indeed present at the sampling site. This analysis involved the computation of detection power for each species and primer, considering a variable number of samples ranging from 1 to 25. Each individual species is showcased in its respective panel, with distinct colors used to represent different primers for clarity. Solid lines within the panels illustrate the detection power calculated based on the occupancy estimates, while the shaded regions represent the uncertainty surrounding the power, as determined by the lower and upper limits of the confidence intervals.

To conclude this section, we would like to address the quantitative aspect of our findings concerning the Golden mahseer, *Tor putitora*. While we acknowledge that establishing an accurate correlation between eDNA detection and species abundance, especially for terrestrial species, is challenging (Lyet et al. 2021), several studies have suggested that eDNA metabarcoding can reveal quantitative patterns of fish biodiversity in large rivers (Pont et al. 2018). Some studies have even proposed its potential use as a tool for assessing fish biomass (Rourke et al. 2022). The results obtained for the Golden mahseer show clear differences in the average number of reads detected per sample across sites, as shown in Figure 10. The highest number of reads, approximately 6,000, was observed at sites T1 and M1. This observation could indicate the presence of a core population of the threatened fish around this tributary or upstream in this tributary. Correlating these eDNA results with traditional population assessments along the river could help confirm any relationship between Golden mahseer fish biomass and DNA reads, therefore provide additional support for using eDNA as a cost-efficient monitoring tool for fish biomass.

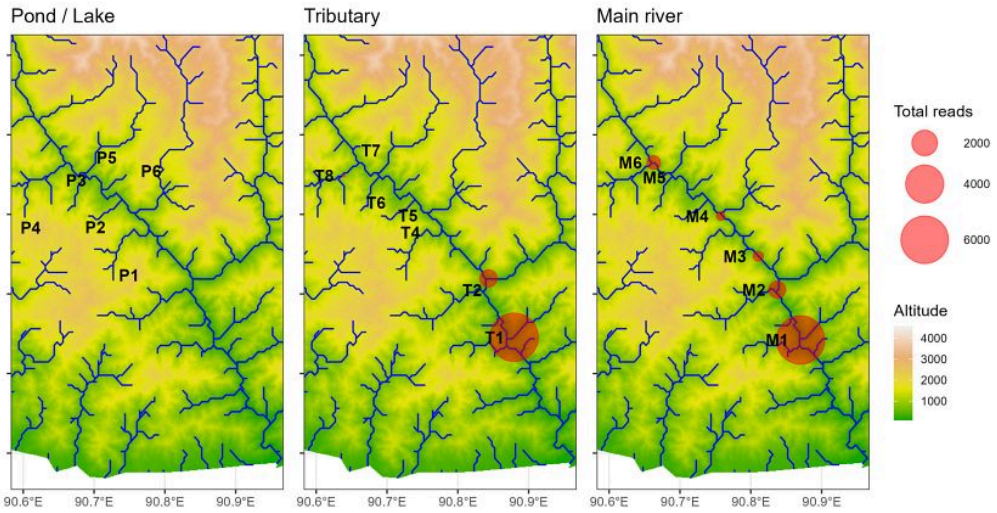

Figure 10. Presentation of the eDNA results for the Golden mahseer. For each site, the size of the red disk indicates the average number of reads found per sample. The number of reads in a sample correlates with the number of strains of DNA of the species contained in the original sample.

3.3 TAXONOMIC DIVERSITY INDICATORS

3.3.1 Venn Diagram

We created a Venn diagram to compare mammal diversity across different sites at the genus level. This graphic clearly showed how many genera were shared and unique to each site. Interestingly, while 32 genera were found in both Royal Manas National Park and Zhemgang Forest Division, Zhemgang hosted an additional 20, exceeding Royal Manas in diversity. This might seem surprising given the higher status of protection of Royal Manas National Park compared to Zhemgang Forest Division, however, upon closer examination, the explanation becomes clear. The tributaries of the Mangde Chhu define catchments that are significantly larger on the Zhemgang side than they are on the Royal Manas side, with one tributary in particular, the Chamkhar Chhu, boasting a vast upstream stretch spanning hundreds of kilometers. This translates to a significantly larger total catchment area in Zhemgang Forest Division compared to Royal Manas National Park, and research by Lyet et al. (2021) has established a positive correlation between catchment area and the number of taxa detected through eDNA samples. Thus, the apparent higher diversity in Zhemgang can most likely be attributed to the larger area actually sampled through eDNA.

This finding highlights the importance of considering catchment areas when interpreting eDNA-based diversity assessments.

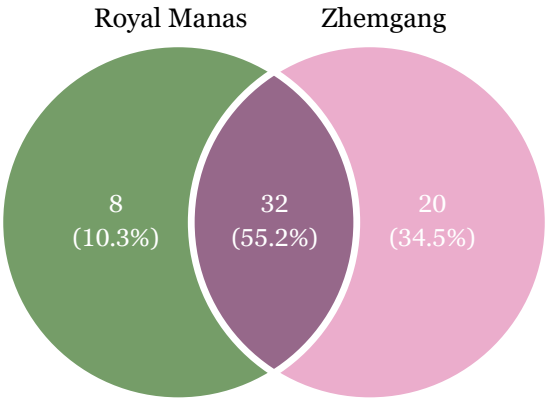

Figure 11. Venn Diagram showing the distribution of the taxa in Royal Manas National Park and Zhemgang Forest Division and the number of taxa in common between the two areas.

3.3.2 Cumulated diversity

To visualize the contribution of each taxonomic group to the overall diversity, we constructed radar diagrams using three sampling efforts: 1, 5 and 10 cumulated samples (Fig. 12). These diagrams show the mean percentages of the total diversity covered by each functional group. The reference line represents the maximum number of taxa per group detected across all sites and samples. The diagrams indicate that differences between Royal Manas National Park (green) and Zhemgang Forest Division (pink) are slightly visible with a single sample, and more pronounced with 10 samples. Overall, the cumulated diversity recovered from ten samples from Zhemgang covers more than 75% of the diversity in 5 of the six taxonomic groups, and globally. As previously observed on the Venn diagram, Zhemgang displays a higher diversity in four out of six mammal orders, with the largest difference shown in carnivores, ungulates and bats.

To further explore the relationship between sample size and the ability to detect differences between sites, we plotted the curves displaying the mean (±SD) integrative diversity index as a function of the number of eDNA samples used in the aggregation (Figure 13). Based on the shape and position of these curves, we can infer the minimum number of samples needed to capture the majority of the diversity present at each site and to statistically distinguish between sites. The curves suggest that only 3 samples may be sufficient to detect a difference in the mammal community between Royal Manas National Park and Zhemgang Forest Division.

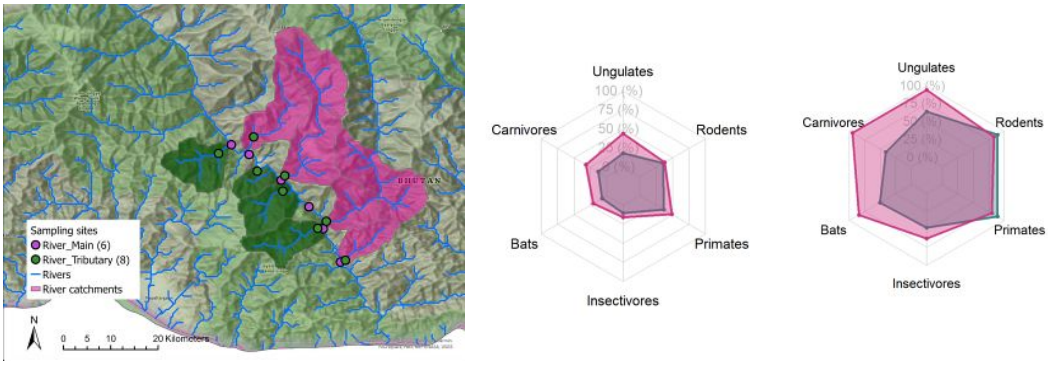

Figure 12: Radar diagrams showing the percentage of diversity covered per taxonomic group when cumulating the diversity from 1 and 10 samples. The outer line (100%) represents the total number of taxa detected across all sites and samples for each taxonomic group.

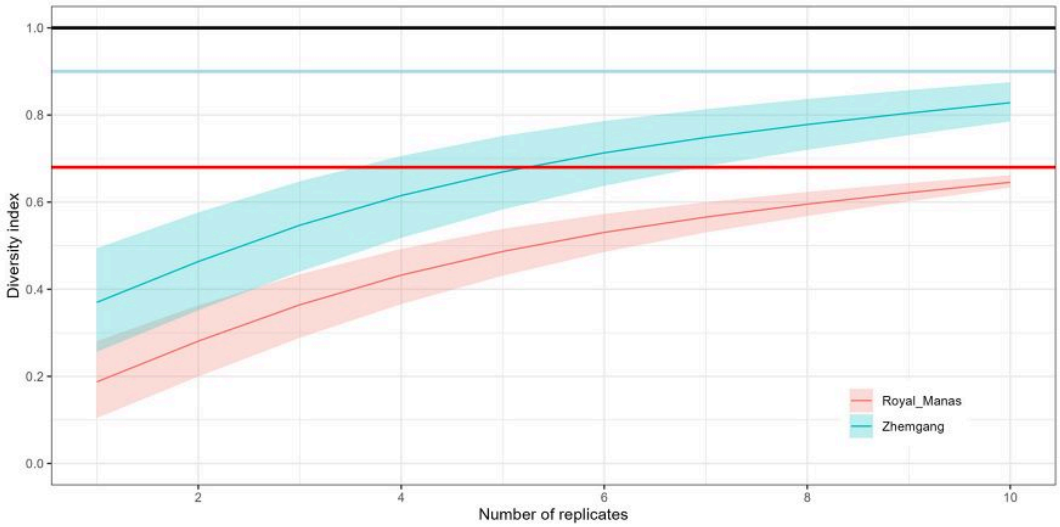

Figure 13: Accumulation curves showing the Integrative Diversity Index (±SD) for each site when considering the total diversity of mammals recovered from 1 to 10 eDNA water samples. The horizontal blue and red lines represent the total diversity recovered at each site when combining results from all samples collected at each site.

3.4 PATTERNS OF GLOBAL VERTEBRATE DIVERSITY

Employing eDNA metabarcoding in areas with incomplete reference databases presents a significant challenge. In such cases, only a limited portion of the sequences retrieved from the samples can be confidently assigned to specific species. It is crucial to emphasize that the lack of taxonomic resolution should not be understood as a lack of diversity. One possible way of describing diversity with eDNA in places that are poorly referenced is to delineate the taxonomic diversity using a multifaceted approach. Below, we provide an overview of the taxonomic composition at each level, using Operational Taxonomic Units (OTUs). We build accumulation curves that inform how much of the total diversity present we have captured, and finally, we use sunburst diagrams to visualize this diversity.

3.4.1 Diversity of Operational Taxonomic Units (OTUs)

Exploring the vertebrate diversity of the Royal Manas National Park and Zhemgang Forest Division using OTUs reveals a large genetic diversity. While the challenge of incomplete reference databases restricts our ability to achieve species-level identifications, OTUs provide a robust method for estimating overall diversity. Our approach not only characterizes taxonomic units but also captures some intraspecific variation. Table 6 below, provide a comprehensive overview of OTUs identified at various taxonomic levels for every primer used in our study. For example, on the vertebrate primer, eDNA analysis detected a total of 671 OTUs, with 75 assigned to species level, 185 assigned to the genus level, 90 assigned to the family level, 46 assigned to the order level and 2 assigned to the class level (Table 6). As we look to the future, the expansion and enrichment of our reference databases emerges as a crucial task. This undertaking promises to significantly enhance species identifications, potentially mirroring the precision achieved in regions endowed with comprehensive reference datasets.


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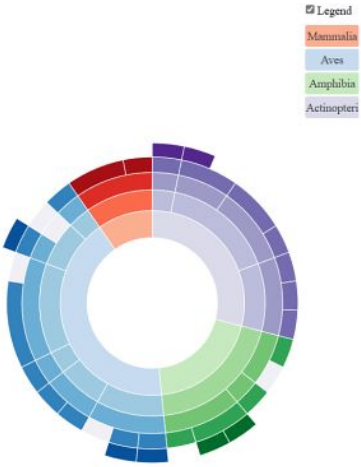
			Number of unique OTUs within each taxonomic level				
Primer	Class	OTUs	Class	Order	Family	Genus	Species
mamm01	Mammalia	183	0	10	46	94	33
	Aves	137	2	28	23	62	22
	Amphibian	28	0	3	6	14	5
	Actinopteri	45	0	5	15	15	10
	All classes	393	2	46	90	185	70
vert05	Mammalia	215	4	3	75	92	41
	Aves	126	4	34	23	38	27
	Reptilia	5	0	0	3	2	0
	Amphibian	42	0	3	16	20	3
	Actinopteri	283	96	48	86	49	4
	All classes	671	104	88	203	201	75
amphibian	Mammalia	3	0	0	0	3	0
	Aves	13	0	1	2	7	3
	Amphibian	6	0	0	1	3	2
	Actinopteri	9	0	0	0	7	2
	All classes	31	0	1	3	20	7
teleost	Actinopteri	80	0	9	30	31	10
	All classes	80	0	9	30	31	10

Table 6. Total number of unique sequences at each taxonomic level for each primer.

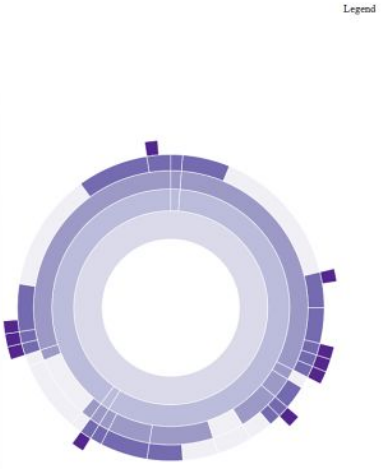
Below, we provide a graphical representation in the form of a sunburst diagram (Fig. 14) to explore the diversity of OTUs detected with our eDNA analyses for each primer. Sunburst diagrams are a nice way to represent the total diversity of OTUs at each taxonomic level. The lower ring indicates the class taxonomic level while the outer ring indicates the species taxonomic level. The proportion of the ring covered by each section at each taxonomic level is proportional to the number of OTUs found in this section. For instance, on the mammal primer sunburst graph, the number of mammals OTUs represents almost half of all OTUs detected.

Mammal primer interactive sunburst graph

Vertebrate primer interactive sunburst graph



Amphibian primer interactive sunburst graph



Teleost primer interactive sunburst graph

Figure 14. Sunburst graphs illustrating the diversity of OTUs found at each taxonomic level, for each primer. The link above each graph provides access to the interactive HTML figures to explore in detail.

3.4.2 Accumulation curves of Operational Taxonomic Units (OTUs)

While accumulation curves for all vertebrate classes and primers show progress in capturing OTU diversity, their lack of clear plateaus suggests further sampling is needed (Fig. 15). Notably, fish and amphibians seem closer to saturation than birds and mammals, likely requiring just a few additional samples to capture the full diversity. The vertebrate primer, however, showcases a steeper curve and higher noise levels, obscuring its plateau and requiring more focused investigation. Overall, the current sampling effort in the Royal Manas region seems insufficient to capture the full diversity of OTUs, especially for mammals and birds. Increased sampling effort can be prioritized in future studies to obtain a more accurate picture of the full OTU diversity within the study area. Moreover, a dedicated exploration of the vertebrate primer's noise will be necessary to better understand its suitability for wildlife inventories at large scale.

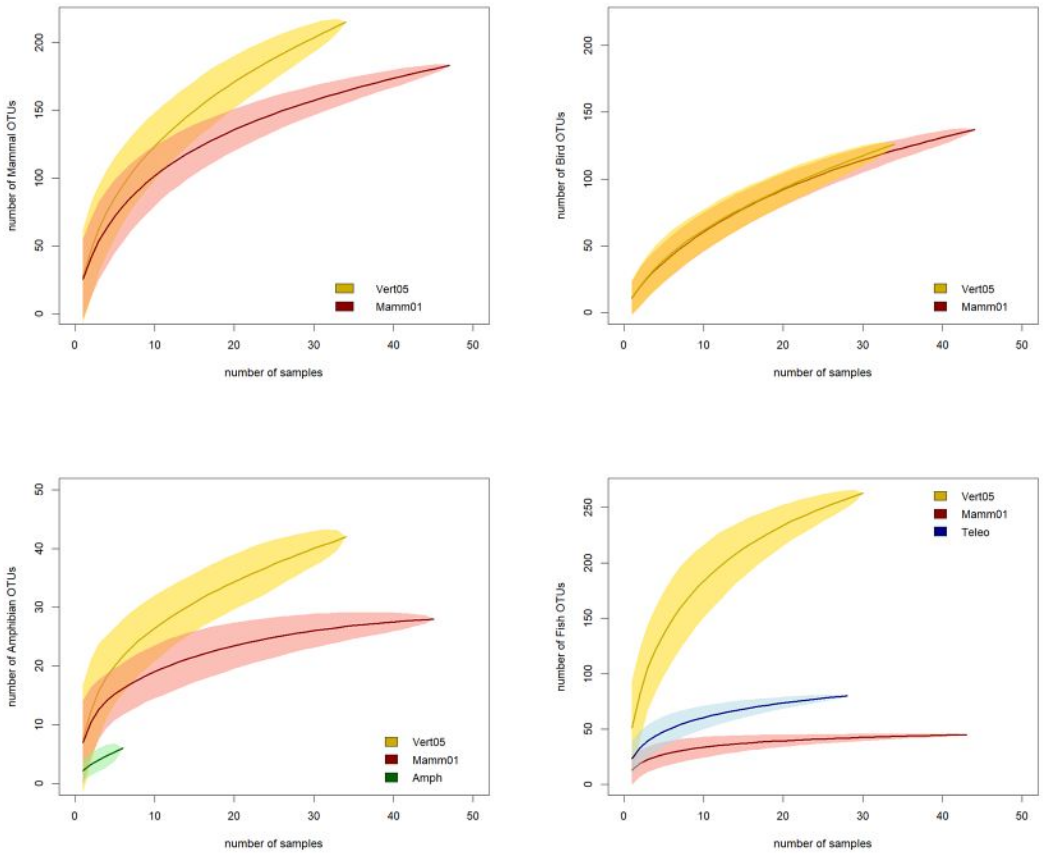


Figure 15. This figure presents accumulation curves for each class of vertebrates (mammals, birds, amphibians, and fish) amplified by four different primers. X-axis shows the cumulative sampling effort (number of samples) and Y-axis indicates the total number of Operational Taxonomic Units (OTUs). Each panel presents curves for all four vertebrate classes, distinguished by color and line style. Colors correspond to the unique primer used for each curve, with the legend displayed within each individual panel for clarity.

4 OPPORTUNITIES FOR BHUTAN

eDNA offers a groundbreaking approach for biodiversity assessment and monitoring in Bhutan. This pilot study demonstrates the potential of eDNA to detect a wide range of species, including rare and elusive ones, across diverse ecosystems. The non-invasive nature of the technology and its potential for cost efficiency are major advantages compared to traditional survey methods. While challenges like incomplete reference databases exist, these can be addressed through targeted research efforts and database expansion. The results of this study highlight the feasibility of scaling up eDNA across Bhutan, enabling comprehensive biodiversity mapping and the development of a national monitoring framework. This framework could revolutionize how Bhutan tracks biodiversity trends and offer a transformative solution to address Bhutan's pressing biodiversity monitoring challenges, enabling the nation to fulfill its commitments under the Kunming-Montreal Global Biodiversity Framework and relevant Sustainable Development Goals (SDGs). Below, we present how eDNA can also be directly applied to monitor tigers and their prey, assess fish populations, detect invasive species, and locate rare and elusive wildlife within Bhutan.

4.1 NATIONAL FRAMEWORK FOR BIODIVERSITY MONITORING

Mapping all biodiversity in Bhutan with eDNA

Concept description – The development of a national framework for the cost-efficient assessment and monitoring of biodiversity in Bhutan, encompassing forest, alpine, freshwater, and subterranean ecosystems, is vital for understanding the baseline and trends of biodiversity in the nation. Such a framework would facilitate the identification of biodiversity hotspots and the primary drivers of species diversity in Bhutan, as well as measure and predict the impact of changes such as climate change, changes in land use, and the introduction of invasive species. It could also inform the national Gross National Happiness (GNH) framework by contributing to the 'Ecological Diversity and Resilience' indicator and guide systematic conservation planning efforts. The use of eDNA technology has been identified as a promising tool for monitoring biodiversity on a large scale, with the potential to provide rapid, cost-effective, and non-invasive assessments of species diversity and distribution (e.g., Bohmann et al. 2014, Taberlet et al. 2018; Deiner et al. 2021). Incorporating eDNA into Bhutan's national biodiversity monitoring framework could enhance our understanding of the impacts of changes on Bhutanese species and ecosystems, inform conservation and management strategies aimed at mitigating these impacts, provide a baseline and cost-efficient tool for environmental impact assessment, and establish a foundation for data-driven, sustainable mechanisms for nature conservation.

Current issues and limitations – The primary limitations and knowledge gaps that need to be addressed revolve around enhancing the DNA reference database for all taxonomic groups in Bhutan, which encompasses a broad array of diverse habitats. This essential improvement will provide a solid basis for a more comprehensive and accurate assessment of biodiversity across the nation.

4.2 MONITORING TIGER AND PREY POPULATIONS WITH EDNA

Concept description – We propose to scale up eDNA for tiger and prey monitoring in Bhutan. This is motivated by the country's current tiger camera trapping survey at the national level, which provides a robust source of data to evaluate how the eDNA technology performs and its role in a more inclusive monitoring of Bhutan's incredible biodiversity. Critically, the simultaneous implementation of a traditional tiger and prey survey alongside a novel eDNA-based method also presents a unique opportunity to evaluate the robustness and cost-effectiveness of the latter at a large scale. In future, using eDNA at scale, in complement with camera trapping, would significantly reduce the costs of such ambitious monitoring programs. Further, it would improve our ability to assess the status of these wildlife populations and track changes over time with increased precision. This method is therefore well suited to evaluate the tiger presence in Bhutan and quantify the availability of prey at the landscape level. This project will allow us to address three main objectives: (1) map the tiger presence and relative abundance across all the areas sampled; (2) estimate the diversity and relative abundance of tiger prey; and (3) compare eDNA results with those from camera trapping to provide a solid benchmark for future conservation research on large carnivores and their prey.

4.3 EVALUATE FISH STOCK AND DETECT INVASIVE SPECIES

Concept description – eDNA has emerged as a promising non-lethal and non-invasive technique for evaluating fish populations and detecting invasive species within Bhutan's diverse freshwater ecosystems (Valentini et al., 2016; Sepulveda et al. 2020). Unlike traditional methods such as electrofishing, which can be difficult in Bhutan's rapid mountain streams and may disturb non-target species, eDNA requires only the collection and analysis of water samples to detect specific species. eDNA has proven effective in detecting a wide range of aquatic species, including both native and potentially invasive species (Pont et al. 2018, Coutant et al. 2021). For instance, it has been employed to detect the presence of invasive animal (e.g., zebra mussels, Treguier et al. 2014) and plant species (e.g., water hyacinth, Kuehne et al. 2020) that can cause significant damage to native ecosystems. eDNA has also been used for semi-quantitative estimation of fish biomass in a river system (Pont et al. 2018; Rourke et al. 2022). In summary, eDNA has the potential to enhance conservation planning in Bhutan and identify sustainable commercial opportunities, while informing management decisions and supporting sustainable freshwater resource use.

Current issues and limitations – The primary limitations and knowledge gaps that need to be addressed revolve around the completion of the reference database for fish and other aquatic species native to Bhutan and potential invasive organisms. Additionally, there is a need for a robust sampling and analysis method to understand the correlation between eDNA reads and fish biomass within Bhutan's unique aquatic ecosystems.

4.4 MAPPING THE DISTRIBUTION OF RARE, CRYPTIC, AND HIGH VALUE SPECIES

Concept description – Effective detection of rare, cryptic, and high value species is crucial for conservation efforts. However, some species are so rare and elusive, they are almost impossible to detect with traditional methods, such as camera traps or aerial surveys. Finding a few isolated animals in vast landscapes necessitates significant investments of time and funds for conducting field surveys, which frequently conclude without providing definitive confirmation of the target species' presence or absence. To locate and save the last individuals of their kind, we urgently need to develop affordable and user-friendly solutions which can be deployed at a large scale and provide rapid results. eDNA has the potential to change the game. For instance, the method has been successful in detecting a variety of extremely rare species, including the critically endangered Mekong Giant catfish Pangasianodon gigas in freshwater (Bellemain et al. 2016), the smalltooth sawfish Pristis pectinata in coastal seawater (Lehman et al. 2022), and even more surprisingly, the Sumatran rhino, the red panda and the tiger on land (WWF, this report and other unpublished data). In this pilot study, we have successfully demonstrated that eDNA technology was capable of detecting the critically endangered white-bellied heron, a species with a known local population of just three individuals. The ability of eDNA to detect low abundance species makes it a valuable tool for conservation efforts, as it allows for the identification of areas where these species may be present and guides conservation actions. In addition, eDNA could be used to confirm the presence of species thought to be extinct in the wild. Overall, eDNA has the potential to greatly aid in the detection and protection of rare, cryptic, and high value species.

4.5 LIMITATIONS AND CHALLENGES

While eDNA offers substantial advantages over traditional methods for biodiversity monitoring in Bhutan, the deployment of the technology faces significant challenges and knowledge gaps that require attention for wider and successful implementation in the country.

DNA Reference Database – A comprehensive DNA reference database for Bhutanese species will be essential for more comprehensive and accurate assessment of biodiversity in the country. Gaps remain, particularly for certain taxonomic groups, which could lead to underreporting of biodiversity. Prioritized expansion of the reference database is needed.

Challenges in Biomass Estimation – While eDNA is a powerful tool for assessing the presence of organisms, determining the abundance of species from eDNA data remains a challenge. Robust sampling protocols and analysis methods are needed to better understand the correlation between eDNA reads and the biomass of terrestrial and aquatic vertebrates.

Accessibility to the technology – Currently, Bhutan lacks the infrastructure and resources to perform eDNA analysis independently. There are no specialized eDNA laboratories within the country, making Bhutan reliant on external facilities for sample processing and data analysis. This dependency creates significant logistical and financial challenges for Bhutanese researchers and conservation organizations.

Specialized Skills Requirement – In addition to technological barriers, the demand for specialized skills presents a significant challenge in harnessing the full potential of eDNA technology. Proficiency in data collection, molecular biology techniques, bioinformatics, and data analysis is crucial for accurate eDNA sampling, processing, and interpretation. However, the scarcity of professionals with expertise in these areas limits the capacity of organizations to effectively utilize eDNA technology for ecological monitoring and biodiversity conservation efforts.

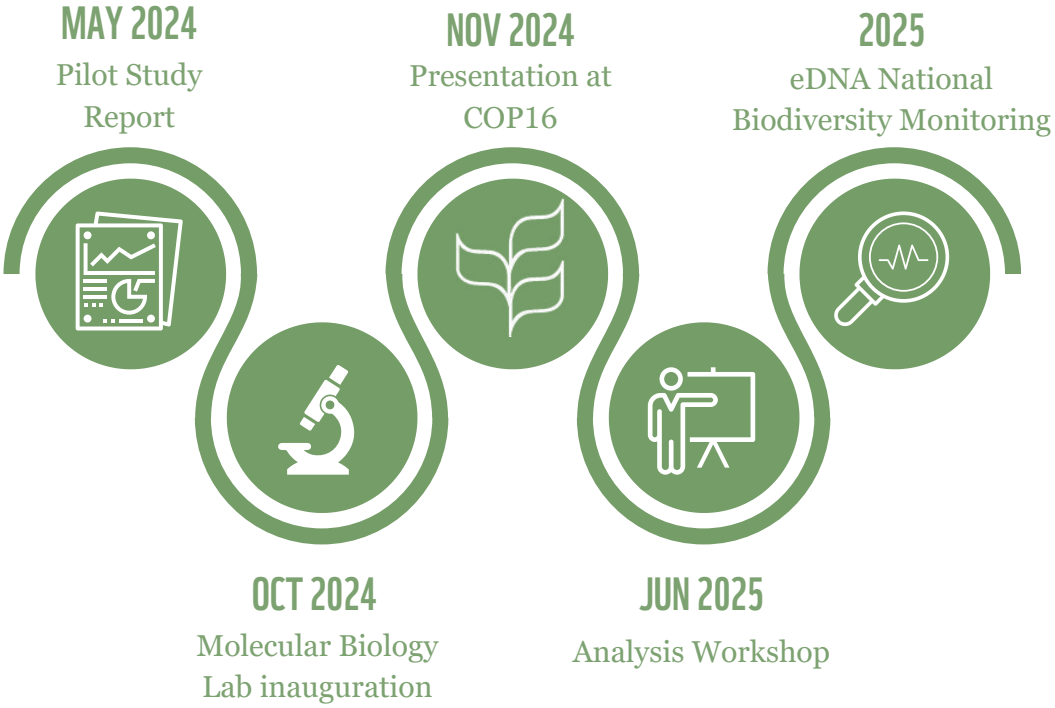
Addressing Limitations and Challenges – Acknowledging and addressing these limitations is crucial for maximizing the potential of eDNA technology for biodiversity assessment and conservation in Bhutan. Efforts required to address these limitations include:

- Establishment of specialized eDNA laboratories in the country.
- Dedicated training programs and workshops to build capacity in eDNA technology.
- Development of automated data analysis pipelines to simplify data interpretation.

4.6 NEXT STEPS

1. Proposal for a National inventory of biodiversity with eDNA (DoFPS, CNR, WWF, ETH-Zurich).
2. National Symposium to launch the Pilot Study report and present the strategy for scaling up eDNA technology at the national level (DoFPS, WWF, CNR).
3. Workshop to refine the eDNA sampling protocols for each taxonomic group to maximize detection probabilities and biodiversity distribution modeling at the national scale (WWF, DoFPS, ETH-Zurich).
4. Training session for eDNA sampling and data analysis (WWF, DoFPS, ETH-Zurich).
5. Samples collection at the national level (DoFPS, WWF).
6. Set up a molecular lab in Bhutan to process DNA samples locally (ETH-Zurich, CNR).
7. Improve the DNA reference database (ETH-Zurich, CNR).
8. Analysis of the eDNA samples and Biodiversity modeling (DoFPS, ETH-Zurich, CNR, WWF).
9. Present the initial results of the first Nation Wide eDNA Biodiversity Inventory at the 2024 United Nations Biodiversity Conference Of the Parties (COP16).

4.7 PROPOSED TIMELINE



5 REFERENCES

1. Eva, B., Harmony, P., Thomas, G., Francois, G., Alice, V., Claude, M. and Tony, D., 2016. Trails of river monsters: Detecting critically endangered Mekong giant catfish Pangasianodon gigas using environmental DNA. Global Ecology and Conservation, 7, pp.148-156.
2. Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., Douglas, W.Y. and De Bruyn, M., 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends in ecology & evolution, 29(6), pp.358-367.
3. Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P. and Coissac, E., 2016. obitools: A unix-inspired software package for DNA metabarcoding. Molecular ecology resources, 16(1), pp.176-182.
4. Cantera, I., Cilleros, K., Valentini, A., Cerdan, A., Dejean, T., Iribar, A., Taberlet, P., Vigouroux, R. and Brosse, S., 2019. Optimizing environmental DNA sampling effort for fish inventories in tropical streams and rivers. Scientific reports, 9(1), p.3085.
5. Coutant, O., Cantera, I., Cilleros, K., Dejean, T., Valentini, A., Murienne, J. and Brosse, S., 2021. Detecting fish assemblages with environmental DNA: Does protocol matter? Testing eDNA metabarcoding method robustness. Environmental DNA, 3(3), pp.619-630.
6. De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E. and Taberlet, P., 2014. DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. Molecular ecology resources, 14(2), pp.306-323.
7. Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., De Vere, N. and Pfrender, M.E., 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular ecology, 26(21), pp.5872-5895.
8. Deiner, K., Yamanaka, H. and Bernatchez, L., 2021. The future of biodiversity monitoring and conservation utilizing environmental DNA. Environmental DNA, 3(1), pp.3-7.
9. DoFPS 2020. Biodiversity Monitoring and Social Surveying Protocol of Bhutan, Department of Forests and Park Services, Ministry of Agriculture and Forests, Thimphu, Bhutan.
10. DoFPS 2023. Status of Tigers in Bhutan: The National Tiger Survey Report 2021–2022. Bhutan Tiger Center, Department of Forests and Park Services, Ministry of Energy and Natural Resources, Royal Government of Bhutan, Thimphu, Bhutan.
11. Evans, N.T., Shirey, P.D., Wieringa, J.G., Mahon, A.R. and Lamberti, G.A., 2017. Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. Fisheries, 42(2), pp.90-99.
12. Frøslev, T.G., Kjøller, R., Bruun, H.H., Ejrnæs, R., Brunbjerg, A.K., Pietroni, C. and Hansen, A.J., 2017. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. Nature communications, 8(1), p.1188.
13. FRMD, 2023. National Forest Inventory; Volume I: State of Forest Report. Forest Monitoring and Information Division, Department of Forests and Park Services.

14. Kuehne, L.M., Ostberg, C.O., Chase, D.M., Duda, J.J. and Olden, J.D., 2020. Use of environmental DNA to detect the invasive aquatic plants Myriophyllum spicatum and Egeria densa in lakes. Freshwater Science, 39(3), pp.521-533.
15. Lacher Jr, T. E., Davidson, A. D., Fleming, T. H., Gómez-Ruiz, E. P., McCracken, G. F., Owen-Smith, N., ... & Vander Wall, S. B. (2019). The functional roles of mammals in ecosystems. Journal of Mammalogy, 100(3), 942-964.
16. Lehman, R.N., Poulakis, G.R., Scharer, R.M., Hendon, J.M., Court, A.G., Wooley, A.K., Williams, A.M., Ajemian, M.J., Hadden, J.P., Beal, J.L. and McCallister, M.P., 2022. Environmental DNA evidence of the Critically Endangered smalltooth sawfish, Pristis pectinata, in historically occupied US waters. Aquatic Conservation: Marine and Freshwater Ecosystems, 32(1), pp.42-54.
17. Lyet, A., Pellissier, L., Valentini, A., Dejean, T., Hehmeyer, A. and Naidoo, R., 2021. eDNA sampled from stream networks correlates with camera trap detection rates of terrestrial mammals. Scientific Reports, 11(1), p.11362.
18. Myers, N., Mittermeier, R.A., Mittermeier, C.G., Da Fonseca, G.A. and Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature, 403(6772), pp.853-858.
National Biodiversity Centre (NBC), 2019. Biodiversity Statistics of Bhutan 2017 A
19. Preliminary Baseline. National Biodiversity Centre, Thimphu.
NEC. 2014. Fourth Report to the Convention on Biological Diversity Bhutan. Royal Government of Bhutan. Accessed from https://chm.cbd.int/database/record?documentID=201280
20. NCD 2022. Golden Mahseer Conservation Action Plan for Bhutan (2022-2032): Securing "Tigers of the Rivers". Nature Conservation Division, Department of Forests and Park Services, Ministry of Agriculture and Forests, Thimphu, Bhutan
21. Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J. and Suggests, M.A.S.S., 2007. The vegan package. Community ecology package, 10(631-637), p.719.
22. Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., Roset, N., Schabuss, M., Zornig, H., Dejean, T., 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Sci Reports 8, 10361.
23. Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P. and Coissac, E., 2011. ecoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. Nucleic Acids Resources. 39: e145.
24. Rourke, ML, Fowler, AM, Hughes, JM, et al. 2022. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environmental DNA 4: 9 – 33.
25. Sepulveda, A.J., Nelson, N.M., Jerde, C.L. and Luikart, G., 2020. Are environmental DNA methods ready for aquatic invasive species management?. Trends in ecology & evolution, 35(8), pp.668-678.
26. Taberlet, P., Bonin, A., Zinger, L. and Coissac, E., 2018. Environmental DNA: For biodiversity research and monitoring. Oxford University Press.

27. Thalinger, B., Deiner, K., Harper, L.R., Rees, H.C., Blackman, R.C., Sint, D., Traugott, M., Goldberg, C.S. and Bruce, K., 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA, 3(4), pp.823-836.
28. Thomsen, P.F. and Willerslev, E., 2015. Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. Biological conservation, 183, pp.4-18.
29. Tréguier, A., Paillisson, J.M., Dejean, T., Valentini, A., Schlaepfer, M.A. and Roussel, J.M., 2014. Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish Procambarus clarkii in freshwater ponds. Journal of Applied Ecology, 51(4), pp.871-879.
30. Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F. and Gaboriaud, C., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular ecology, 25(4), pp.929-942.
31. Van Leeuwen, P. and Michaux, J., 2023. Using eDNA for mammal inventories still needs naturalist expertise, a meta-analysis. Ecology and Evolution, 13(12), p.e10788.
Walsh, H.E., Kidd, M.G., Moum, T. and Friesen, V.L., 1999. Polytomies and the power of phylogenetic inference. Evolution, 53(3), pp.932-937.
32. Wangda, P. and Ohsawa, M., 2006. Gradational forest change along the climatically dry valley slopes of Bhutan in the midst of humid eastern Himalaya. Plant Ecology, 186, pp.109-128.

6 SUPPLEMENTARY MATERIALS

Appendix 1: Taxa detected at the species level. Total number of positive detections are given for each primer in the last four columns. Some taxa that were detected at the genus level were later assigned to the species level when this was the only known species of the genus present in Bhutan (see note OKSP that stands for Only Known Species Present in Bhutan). NKIB stands for "Species Not Known in Bhutan". NKIB-BC is not known in Bhutan but was observed in a neighboring country. ML for most likely species. L for likely species. EIB for Species Extinct in Bhutan).

Class	N	Order	Family	Species	Note	IUCN	amph	fish	vert	mamm
Actinopteri	1	Anguilliformes	Anguillidae	*Anguilla bengalensis*	OKSP	NT	0	19	20	25
Actinopteri	2	Centrarchiformes	Centrarchidae	*Lepomis gibbosus*	NKIB	LC	2	0	0	0
Actinopteri	3	Cypriniformes	Cyprinidae	*Crossocheilus latius*		LC	0	8	7	5
Actinopteri	4	Cypriniformes	Cyprinidae	*Cyprinion semiplotum*		VU	0	4	4	4
Actinopteri	5	Cypriniformes	Cyprinidae	*Neolissochilus hexagonolepis*		NT	0	23	0	32
Actinopteri	6	Cypriniformes	Cyprinidae	*Tor putitora*		EN	0	17	0	6
Actinopteri	7	Cypriniformes	Danionidae	*Barilius bendelisis*		LC	0	3	5	2
Actinopteri	8	Cypriniformes	Danionidae	*Devario aequipinnatus*	OKSP	LC	0	2	5	1
Actinopteri	9	Cypriniformes	Gobionidae	*Pseudorasbora parva*		LC	1	0	0	0
Actinopteri	10	Cypriniformes	Leuciscidae	*Alburnus alburnus*		LC	0	0	0	1
Actinopteri	11	Cypriniformes	Nemacheilidae	*Aborichthys garoensis*	OKSP	VU	0	0	8	0
Actinopteri	12	Salmoniformes	Salmonidae	*Oncorhynchus clarkii*	NKIB	NE	0	1	0	0
Actinopteri	13	Siluriformes	Sisoridae	*Bagarius bagarius*		VU	0	3	3	2
Actinopteri	14	Siluriformes	Sisoridae	*Creteuchiloglanis kamengensis*		DD	0	18	0	26
Actinopteri	15	Siluriformes	Sisoridae	*Glyptothorax annandalei*		LC	0	24	0	30
Actinopteri	16	Siluriformes	Sisoridae	*Pseudecheneis sulcata*		LC	0	24	18	35

Amphibia	17	Anura	Bufonidae	*Duttaphrynus himalayanus*		LC	0	0	13	3
Amphibia	18	Anura	Bufonidae	*Duttaphrynus stuarti*		DD	0	0	0	16
Amphibia	19	Anura	Megophryidae	*Megophrys maosonensis*		NE	0	0	1	0
Amphibia	20	Anura	Rhacophoridae	*Polypedates braueri*		NE	0	0	3	0
Amphibia	21	Anura	Rhacophoridae	*Polypedates megacephalus*		LC	0	0	0	2
Amphibia	22	Anura	Rhacophoridae	*Rhacophorus burmanus*		NT	1	0	0	1
Amphibia	23	Anura	Rhacophoridae	*Rhacophorus translineatus*		NT	4	0	0	3
Aves	24	Accipitriformes	Accipitridae	*Accipiter gularis*	NKIB	LC	0	0	0	1
Aves	25	Accipitriformes	Accipitridae	*Accipiter nisus*		LC	0	0	1	1
Aves	26	Accipitriformes	Accipitridae	*Nisaetus nipalensis*		NT	0	0	0	1
Aves	27	Accipitriformes	Accipitridae	*Spilornis cheela*	ML	LC	0	0	0	1
Aves	28	Anseriformes	Atidae	*Branta canadensis*	NKIB	LC	2	0	0	0
Aves	29	Anseriformes	Atidae	*Cygnus olor*	NKIB	LC	1	0	0	0
Aves	30	Apodiformes	Apodidae	*Aerodramus brevirostris*	OKSP	LC	0	0	0	2
Aves	31	Bucerotiformes	Bucerotidae	*Buceros bicornis*		VU	0	0	7	0
Aves	32	Columbiformes	Columbidae	*Chalcophaps indica*		LC	0	0	1	1
Aves	33	Columbiformes	Columbidae	*Columba hodgsonii*		LC	0	0	1	1
Aves	34	Columbiformes	Columbidae	*Columba livia*		LC	0	0	0	2
Aves	35	Columbiformes	Columbidae	*Macropygia unchall*	OKSP	LC	0	0	0	3
Aves	36	Columbiformes	Columbidae	*Streptopelia orientalis*		LC	0	0	0	1
Aves	37	Columbiformes	Columbidae	*Treron sieboldii*	NKIB	LC	0	0	1	0
Aves	38	Coraciiformes	Alcedinidae	*Alcedo atthis*		LC	0	0	2	3
Aves	39	Coraciiformes	Cerylidae	*Ceryle rudis*		LC	0	0	4	3

Aves	40	Galliformes	Phasianidae	*Arborophila torqueola*		LC	0	0	3	3
Aves	41	Galliformes	Phasianidae	*Gallus gallus*	OKSP	LC	1	0	18	2
Aves	42	Galliformes	Phasianidae	*Ithaginis cruentus*		LC	0	0	2	1
Aves	43	Galliformes	Phasianidae	*Lophophorus impejanus*		LC	0	0	4	0
Aves	44	Galliformes	Phasianidae	*Meleagris gallopavo*	NKIB	LC	0	0	0	4
Aves	45	Gruiformes	Rallidae	*Amaurornis phoenicurus*		LC	0	0	2	2
Aves	46	Gruiformes	Rallidae	*Fulica atra*	OKSP	LC	2	0	0	0
Aves	47	Passeriformes	Corvidae	*Garrulus glandarius*		LC	0	0	0	1
Aves	48	Passeriformes	Corvidae	*Nucifraga caryocatactes*	OKSP	LC	0	0	0	1
Aves	49	Passeriformes	Corvidae	*Urocissa flavirostris*	OKSP	LC	0	0	0	1
Aves	50	Passeriformes	Estrildidae	*Lonchura striata*	OKSP	LC	0	0	0	3
Aves	51	Passeriformes	Fringillidae	*Chlorodrepanis virens*	Hawai i	LC	0	0	0	1
Aves	52	Passeriformes	Fringillidae	*Coccothraustes coccothraustes*		LC	1	0	0	0
Aves	53	Passeriformes	Irenidae	*Chloropsis hardwickii*		LC	0	0	3	0
Aves	54	Passeriformes	Leiothrichidae	*Garrulus lanceolatus*		LC	0	0	6	0
Aves	55	Passeriformes	Leiothrichidae	*Leiothrix argentauris*		LC	0	0	0	2
Aves	56	Passeriformes	Leiothrichidae	*Liocichla phoenicea*	OKSP	LC	0	0	0	1
Aves	57	Passeriformes	Leiothrichidae	*Minla ignotincta*		LC	0	0	0	1
Aves	58	Passeriformes	Muscicapidae	*Muscicapa griseisticta*	NKIB-BC	LC	0	0	1	0
Aves	59	Passeriformes	Muscicapidae	*Phoenicurus frontalis*		LC	0	0	1	0
Aves	60	Passeriformes	Muscicapidae	*Saxicola maurus*		NE	0	0	17	0
Aves	61	Passeriformes	Passeridae	*Prunella himalayana*		LC	0	0	1	0
Aves	62	Passeriformes	Pycnonotidae	*Hypsipetes leucocephalus*	OKSP	LC	0	0	0	9
Aves	63	Passeriformes	Pycnonotidae	*Ixos mcclellandii*		LC	0	0	2	0
Aves	64	Passeriformes	Stenostiridae	*Culicicapa ceylonensis*		LC	0	0	2	0
Aves	65	Passeriformes	Timaliidae	*Erpornis zantholeuca*		LC	0	0	1	1

Aves	66	Passeriformes	Timaliidae	*Erythrogenys gravivox*	NKIB	LC	0	0	4	0
Aves	67	Passeriformes	Timaliidae	*Pomatorhinus ochraceiceps*	NKIB-BC	LC	0	0	6	0
Aves	68	Passeriformes	Timaliidae	*Yuhi nigrimenta*		LC	0	0	6	2
Aves	69	Passeriformes	Turdidae	*Monticola gularis*	NKIB	LC	0	0	8	0
Aves	70	Passeriformes	Zosteropidae	*Zosterops palpebrosus*	OKSP	LC	0	0	0	4
Aves	71	Pelecaniformes	Ardeidae	*Ardea insignis*		CR	0	0	1	2
Aves	72	Pelecaniformes	Phalacrocoracidae	*Phalacrocorax carbo*		LC	0	0	1	1
Aves	73	Piciformes	Picidae	*Meiglyptes tukki*	NKIB	NT	0	0	1	0
Aves	74	Piciformes	Picidae	*Sasia ochracea*		LC	0	0	0	1
Mammalia	75	Artiodactyla	Bovidae	*Bos indicus*		DO	0	0	26	0
Mammalia	76	Artiodactyla	Bovidae	*Bos mutus*	EIB	VU	0	0	3	8
Mammalia	77	Artiodactyla	Bovidae	*Bubalus bubalis*		DO	0	0	1	0
Mammalia	78	Artiodactyla	Bovidae	*Budorcas taxicolor*		VU	0	0	1	6
Mammalia	79	Artiodactyla	Bovidae	*Capricornis sumatraensis*	OKSP	NT	0	0	14	22
Mammalia	80	Artiodactyla	Cervidae	*Axis porcinus*	L	EN	0	0	3	0
Mammalia	81	Artiodactyla	Cervidae	*Muntiacus muntjak*	OKSP	LC	0	0	20	30
Mammalia	82	Artiodactyla	Cervidae	*Rusa unicolor*		VU	0	0	24	0
Mammalia	83	Artiodactyla	Moschidae	*Moschus fuscus*	ML	EN	0	0	10	17
Mammalia	84	Artiodactyla	Suidae	*Sus scrofa*		LC	0	0	1	20
Mammalia	85	Carnivora	Ailuridae	*Ailurus fulgens*		EN	0	0	0	3
Mammalia	86	Carnivora	Canidae	*Canis lupus*		DO	0	0	7	2
Mammalia	87	Carnivora	Canidae	*Cuon alpinus*		EN	0	0	8	18
Mammalia	88	Carnivora	Felidae	*Catopuma temminckii*		NT	0	0	1	0
Mammalia	89	Carnivora	Felidae	*Panthera tigris*		EN	0	0	6	9
Mammalia	90	Carnivora	Felidae	*Pardofelis marmorata*		NT	0	0	0	3

Mammalia	91	Carnivora	Felidae	*Prionailurus bengalensis*	OKSP	LC	0	0	7	13
Mammalia	92	Carnivora	Mustelidae	*Aonyx cinereus*		VU	0	0	5	0
Mammalia	93	Carnivora	Mustelidae	*Lutra lutra*		NT	0	0	12	22
Mammalia	94	Carnivora	Mustelidae	*Lutrogale perspicillata*	OKSP	VU	0	0	9	16
Mammalia	95	Carnivora	Mustelidae	*Martes flavigula*		LC	0	0	3	5
Mammalia	96	Carnivora	Mustelidae	*Martes foina*	L	LC	0	0	1	3
Mammalia	97	Carnivora	Mustelidae	*Melogale moschata*		LC	0	0	3	0
Mammalia	98	Carnivora	Ursidae	*Ursus thibetanus*		VU	0	0	20	31
Mammalia	99	Carnivora	Viverridae	*Arctictis binturong*		VU	0	0	0	1
Mammalia	100	Carnivora	Viverridae	*Paguma larvata*	OKSP	LC	0	0	24	30
Mammalia	101	Chiroptera	Molossidae	*Tadarida latouchei*	NKIB	EN	0	0	1	8
Mammalia	102	Chiroptera	Pteropodidae	*Cynopterus sphinx*		LC	0	0	1	0
Mammalia	103	Chiroptera	Pteropodidae	*Eonycteris spelaea*		LC	0	0	5	10
Mammalia	104	Chiroptera	Pteropodidae	*Megaerops niphanae*		LC	0	0	0	1
Mammalia	105	Chiroptera	Pteropodidae	*Rousettus leschenaultii*		NT	0	0	9	21
Mammalia	106	Chiroptera	Pteropodidae	*Sphaerias blanfordi*		LC	0	0	3	6
Mammalia	107	Chiroptera	Rhinolophidae	*Rhinolophus luctus*		LC	0	0	0	1
Mammalia	108	Chiroptera	Vespertilionidae	*Harpiocephalus harpia*	OKSP	LC	0	0	9	13
Mammalia	109	Chiroptera	Vespertilionidae	*Hypsugo cadornae*		LC	0	0	0	3
Mammalia	110	Chiroptera	Vespertilionidae	*Ia io*		NT	0	0	1	0
Mammalia	111	Chiroptera	Vespertilionidae	*Miniopterus fuliginosus*		NE	0	0	0	1
Mammalia	112	Chiroptera	Vespertilionidae	*Myotis dasycneme*		NT	0	0	2	0
Mammalia	113	Chiroptera	Vespertilionidae	*Pipistrellus coromandra*		LC	0	0	0	1
Mammalia	114	Chiroptera	Vespertilionidae	*Pipistrellus tenuis*		LC	0	0	3	0
Mammalia	115	Chiroptera	Vespertilionidae	*Scotomanes ornatus*		LC	0	0	1	0
Mammalia	116	Eulipotyphla	Soricidae	*Chimarrogale himalayica*	OKSP	LC	0	0	3	8

Mammalia	117	Eulipotyphla	Soricidae	*Episoriculus leucops*		LC	0	0	0	2
Mammalia	118	Eulipotyphla	Soricidae	*Nectogale elegans*	OKSP	LC	0	0	17	0
Mammalia	119	Eulipotyphla	Soricidae	*Sorex minutus*	OKSP	LC	0	0	0	1
Mammalia	120	Eulipotyphla	Soricidae	*Suncus murinus*		LC	0	0	3	0
Mammalia	121	Primates	Cercopithecidae	*Macaca assamensis*		NT	0	0	0	38
Mammalia	122	Primates	Cercopithecidae	*Macaca mulatta*		LC	0	0	2	6
Mammalia	123	Primates	Cercopithecidae	*Semnopithecus schistaceus*	OKSP	LC	0	0	1	0
Mammalia	124	Rodentia	Hystricidae	*Atherurus macrourus*		LC	0	0	9	0
Mammalia	125	Rodentia	Hystricidae	*Hystrix brachyura*		LC	0	0	12	21
Mammalia	126	Rodentia	Muridae	*Niviventer eha*		LC	0	0	5	0
Mammalia	127	Rodentia	Muridae	*Rattus andamanensis*		LC	0	0	2	0
Mammalia	128	Rodentia	Muridae	*Rattus nitidus*		LC	0	0	9	9
Mammalia	129	Rodentia	Muridae	*Rattus tanezumi*		LC	0	0	0	2
Mammalia	130	Rodentia	Sciuridae	*Callosciurus pygerythrus*		LC	0	0	21	0
Mammalia	131	Rodentia	Sciuridae	*Hylopetes alboniger*	OKSP	LC	0	0	11	10
Mammalia	132	Rodentia	Sciuridae	*Petaurista yunanensis*		NE	0	0	14	0
Mammalia	133	Rodentia	Sciuridae	*Ratufa bicolor*	OKSP	NT	0	0	6	8
Mammalia	134	Rodentia	Sciuridae	*Tamiops mcclellandii*		LC	0	0	3	0

A joint publication of the Department of Forests and Park Services, Ministry of Energy and Natural Resources, World Wildlife Fund and the Swiss Federal Institute of Technology in Zurich.